DUDDINGTONIA FLAGRANS: CHLAMYDOSPORE PRODUCTION DURING DEHYDRATION, CAPTURE EFFICIENCY FOR CYATHOSTOMINS VS *PANAGRELLUS REDIVIVUS*, AND THE EFFECTS OF $NaHSO_4$ ON POULTRY LITTER MICROBIOME DIVERSITY AND VIABILITY

by

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ABSTRACT

Duddingtonia flagrans is a nematode trapping-fungus that has shown promising results as a tool to combat parasitic nematode infections in livestock. The fungus interrupts the parasitic lifecycle by trapping and killing larval stages on pasture to prevent re-infection of animals. One barrier to the fungus' commercial use is scaling up production of the fungus, and specifically of chlamydospores, which survive the digestive tract to grow in fecal pats on pasture, thus have potential as a feed through anthelmintic. The purpose of this study was to evaluate the effect of dehydration on sporulation of the fungus. Disks of Duddingtonia flagrans type strain (ATCC® 13423™) were grown on 17% cornmeal agar for 26 days at 30° C, then split into three groups; dried quickly at 38° C and 37% humidity over 48 hours, dried more slowly at 24° C and 55% humidity over 10 days, or kept at 30° C and sealed with parafilm to prevent loss of moisture (control). Half of each dried culture was resuspended in water, then half of each culture was heated to liquify, homogenized through vortexing. Spores were then counted in a Neubauer hematocytometer. Both the 2 day and 10 day drying techniques yielded significantly more spores than the control (Welch Two Sample t-test p-values of .0359 and .0411, respectively). The difference in number of spores was insignificant between the two drying techniques, although a visual representation of the data shows less variability in the measurements of the slower 10 day drying.

Biocontrol fungus Duddingtonia flagrans may have differing efficacy on parasitic nematodes of different species, and in the presence of free-living soil nematodes. D. flagrans was cultured on three petri dishes for each trial set, which were randomly assigned to have equine parasitic cyathostomin larvae, free-living

Panagrellus redivivus, or a mixture of both nematodes added. After 24 hours of interaction the plates were observed and each nematode was categorized as trapped or not trapped by the fungus. This was repeated for three trial sets. Trapped and not trapped counts from the trial sets were aggregated for statistical analysis. Chi-squared and fisher's exact tests were both conducted to compare the number of cyathostomin larvae captured at the 24 hour mark in mixed plates to the number captured at 24 hours in monocultured plates. A one sample proportions test was conducted on the P. redivivus and on the cyathostomin free/captured ratios, respectively, to determine if the samples of each group were more or less likely to be captured at that time point, and to establish a 95% confidence interval of the true population percentage captured at 24 hours under these conditions. The monoculture cyathostomin capture rate was 53%, and in the mixed cyathostomin and P. redivivus cultures the parasitic cyathostomin larvae had a capture rate of 56%. Chi-squared and fisher's exact tests comparing the two cyathostomin conditions both yielded p-values of 1; no discernable difference in means. The total capture rate for P. redivivus was 66.14%, and the total capture rate for cyathostomin larvae was 54.55%. P. redivivus are significantly more likely to be trapped than untrapped at 24 hours of interaction with the fungus ($p<.001$), whereas the cyathostomin larvae are not. D. flagrans does trap different nematode species at different rates. This should be taken into consideration when selecting it as a biological control agent for specific parasitic species to be managed. D. flagrans does not appear to trap parasitic species at differing rates in the presence of free living nematodes; the pasture nematode content does not need to be taken into consideration.

The purpose of the project was to measure abundance of live/dead populations of Enterococcus cecorum, Clostridium perfringens, and Staphylococcus aureus in

poultry litter following treatment with sodium bisulfate (SB). Sodium bisulfate is a litter treatment that lowers the litter pH and ammonia. This experiment measured secondary effects on the poultry litter microbiome including commensal and pathogenic organisms. Broiler litter from a treatment and a control house was sampled at -1, 2, 24, and 27 days. The treatment house received SB at a rate of 100 lb/1000 ft2 on days -1 and 24. Samples were mixed with sterile phosphate-buffered saline (PBS) to suspend bacterial cells, and then washed by centrifugation and resuspended in PBS. Supernatants (500 ml aliquots) were collected and treated with either PMAxx™ or sterile PBS buffer (control), or autoclaved and PMA treated (heat-killed control) to monitor PMA binding efficiency. The samples were dark/light incubated for PMA activation. These treated solutions were centrifuged, DNA was extracted from the pelleted cells using QIAmp Powerfecal kit. Differential qPCR (40 cycles) comparing PMA-treated and untreated DNA with species specific primers to target bacteria of interest was used to differentiate between live/dead cells. DNA from a composite sample from each house at each timepoint was sequenced using Illumina MiSeq for comparison. Data from the averages of each time point's PMA treated and untreated cycle threshold (Ct) values were compared to create a ΔCt to represent relative dead levels of bacteria, the PMA treated and PMA treated heat killed data was used to create a ΔCt to help determine PMA binding efficiency for each target organism. MiSeq data was normalized to absolute abundance before comparison. The treatment house had consistently slightly higher levels of dead bacteria overall. S. aureus was not found in either house. C. perfringens may require further investigation into PMA binding efficiency. E. cecorum shows a consistent increase in levels of dead bacteria in the treatment house demonstrating it may be reduced by SB. Some bacterial

families present in the survey data for the control house were found to be eliminated in the treatment house.

BACKGROUND

Small strongyle, also known as cyathostome, infections in the horse can present a number of clinical symptoms; as the nematodes feed on intestinal mucosa, and intact cysts that they form can reduce nutrient absorption, some weight loss, lethargy, reduction of coat quality, colitis and other symptoms associated with nutrient deficiency and intestinal perturbation can be observed. The most serious disease syndrome that can be caused by these infections is known as acute larval cyathostomosis, characterized clinically by weight loss and sudden onset diarrhea (Murphy 1997*)*. Weight loss associated with acute larval cyathostomosis can be significant, is more common in young horses and can be fatal, Figure 1 shows an example of this weight loss. Acute larval cyathostomosis occurs more frequently in the spring at the onset of warmer weather as this triggers multiple small strongyle cysts to

erupt simultaneously within the gut (Reid 1995).

Figure 1 A horse suffering from weight loss associated with cyathostome infection (Murphy 1997)

At the turn of the century small strongyle infections became “the principal pathogenic parasite” of the horse, since anthelmintics had been widely successful in eliminating the presence of the previously more common large strongyles (Love 1999). Anthelmintics initially were not as successful at treating small strongyle infections due to a unique aspect of their life cycle; L3 or third stage larvae that pass through the digestive tract after being picked up from pasture then burrow into the mucosal layer of the intestinal wall and form a cyst. While encysted they remain in a state of arrested development for a period of up to two years, and in this state they have limited susceptibility to drug treatments (Church 1986). A diagram of

the full lifecycle of the small strongyle can be seen in Figure 2. Partially due to this life cycle attribute, small strongyles have become the most important gastrointestinal helminth to infect horses, often comprising 95-100% of the total worm burden (Nielsen 2012).

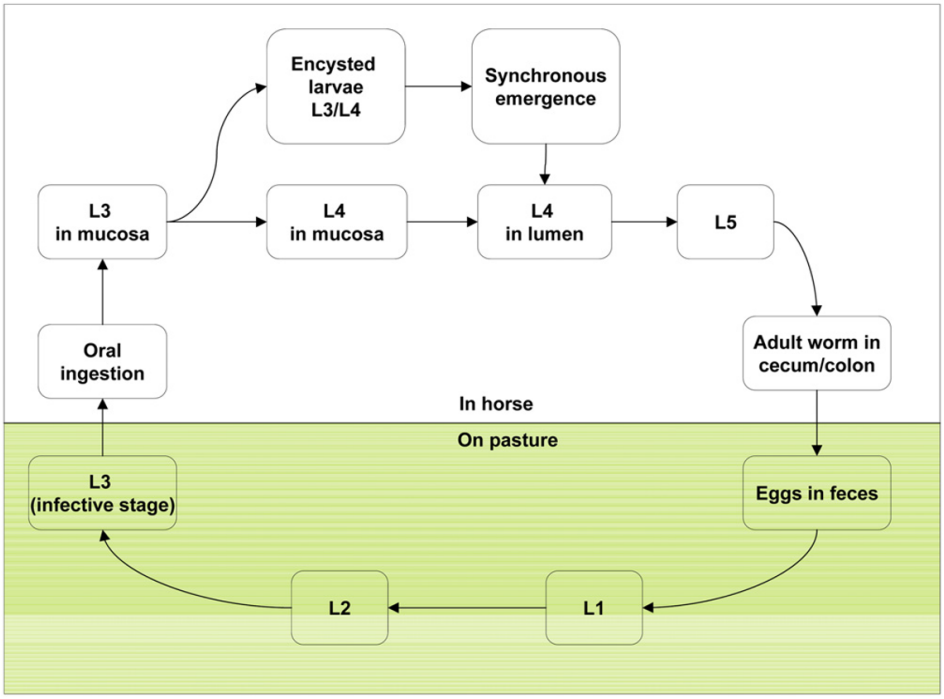


Figure 2 Shows the life cycle of small strongyles, with hatching and initial three larval stages occurring on pasture, fourth larval stage in the intestine either immediately in the lumen or post encystment, then fifth adult worm stage in the lumen producing eggs. (Corning 2009)

To compound this issue of their life cycle stage preventing their effective treatment, there have since been numerous reports of drug resistance in small strongyle populations around the world, some exhibiting resistance across the board to all common anthelmintics used. A study done in 2014 in the Mid-Atlantic United States found that the newest anthelmintic drug class being used moxidectin and

ivermectin still had effective percentages of 100% and 98.84% respectively, but that the drugs pyrantel pamoate, oxibendazole, and fenbendazole only had 43.33%, 20.83%, and 6.14% effective percentages (based on each anthelmintic's ability to provide 90% reduction of fecal egg count) (Smith 2015). In Argentina more than 80% of farms studied showed helminth populations with resistance to benzimidazoles (Ardusso 2016) and studies have shown a quickening recovery of helminth populations after moxidectin and ivermectin treatments in Argentina, Belgium, Italy, and The Netherlands (Arduso 2016, Geurden 2014). This increase in the prevalence of resistance to anthelmintic drugs and faster recovery times of helminth populations have led to the need for re-examining of current drug treatment practices, as well as an importance for discovery of alternative methods of control.

One promising control method is the use of nematophagous fungi. There are several types of nematophagous fungi, they can be classified as endoparasites that infect nematodes using their spores, predatory that form trap structures to entangle and consume larvae and adults, and opportunistic fungi that parasitize eggs, cysts and female nematodes (Herrera-Estrella 2016). Predatory trap forming are among the most studied and widely used as biological control agents, and *Duddingtonia flagrans* is of particular interest in livestock parasitic control due to the fungal chlamydospore's ability to survive the digestive tract of both ruminants and non-ruminants and form hyphal networks in their feces (Faedo 1997).

Measuring the efficacy of fungal feed additives can be difficult for this control method when used on a target organism such as small strongyles in equine infections because the fungi specifically effect the nematodes in stages of their life cycle outside of their host. This means that the traditional method of fecal egg count reduction used

to measure efficacy of drug treatments is not useful, at least for measuring the immediate effects, since the fungi prevent re-infection of the horse by controlling infectivity of pasture rather than reducing the amount that the horse is actively shedding. One method used in a study in tropical southeastern Brazil was to use the presence of small strongyle larvae in herbage at distances of 0-20 cm and 20-40 cm from fecal pats of infected horses that were treated with fungal spores. An overall reduction in number of larvae present per kg of dry matter collected was found from start to finish of the trial, although some treatment months saw an increase – it can be difficult to control for environmental factors. The same study did also find a reduction in fecal egg counts over a period of six months (Braga 2009). An additional consideration when determining treatment efficacy is that 'small strongyles' encompasses a wide range of about fifty species of nematodes, with ten to fourteen abundant species (Verkaaik 2016). These species are typically treated as one infection as a horse often carries multiple species and the eggs and larvae are not morphologically differentiable. (Although a method of differentiating the larvae based on body length with sheath, body width, distance to nerve ring, esophagus length, and intestine length was developed, it only was able to correctly identify larvae 63% of the time)(Kornás 2010). Twenty-one species can now be identified by solely eggs and larvae using reverse line blot assay (Cwiklinski 2012). Although this is not typically done in the industry as the treatment method is the same across species and it would be an unnecessary expense, it should be a consideration as the fungus may have different effects across different small strongyle species.

D. flagrans has been shown to react differently to different nematode species; a Brazilian isolate of the fungi was found to have formed specialized trap structures and

fully entrapped, pierced the cuticle, and caused death of the free-living soil nematodes *Panagrellus* sp. after 4 hours of contact with the fungi, however L3 larvae of the sheep parasites trichostrongylides (predominately *Haemonchus* sp.) that were added to the fungal culture simultaneously were still motile after 10 hours, and were not observed to have been penetrated until 15 hours (Cruz 2011). Another study found that just *Haemonchus contortus* added alone to a fungal culture did not have its cuticle pierced until a 24-36 hour period (Campos 2008). A Chinese strain of *D. flagrans* was shown to penetrate the cuticle of trichostrongylide L3 at 14 hours (Wang 2015). Since the control mechanism of the fungus when used to treat equine cyathostomin infections is to entrap and kill larval stages of the nematodes while they are in fecal pats before they are able to move out onto the pasture, it follows that the fungi's ability to work effectively is a function of the time it takes to recognize that the nematode species is present in its surroundings, form trapping structures, and efficiently trap and neutralize the parasites.

Chapter 1

STIMULATING *DUDDINGTONIA FLAGRANS* CHLAMYDOSPORE PRODUCTION THROUGH DEHYDRATION

Introduction

As anthelminthic drug resistance continues to increase in parasitic nematode populations around the globe, alternative treatment methods and control regimens continue to gain importance. *Duddingtonia flagrans* is a predatory fungus that forms trapping structures in the presence of nematodes, capturing and digesting them. The ability of *D. flagrans* to grow in fecal pats, persist on pasture, then capture and kill larval stages of nematodes makes it an attractive biological control agent. It targets parasites in the environmental life-cycle stage, thereby preventing infection. In addition, *D. flagrans* forms chlamydospores that are capable of surviving the digestive tract of host animals (Larsen et al. 1992, 1999; Grønvold et al. 1993, Faedo et al. 1997, Ojeda-Robertos et al. 2009), delivering the fungus directly to the fecal pats where the larvae will hatch. This allows it to be used as a feed additive, which is a more convenient and efficient treatment method than broadcast spreading over pasture. The fungus has a minimal environmental impact and a broad range of potential targets in both ruminant and non-ruminant host species (Braga 2014).

Even with the fungus's ability to be used as a direct feed additive, large scale production of the fungus has been one of the major limiting factors restricting its use commercially (Santurio et al. 2009). Previous studies have examined the effects of growing the fungus in different solid and liquid culture mediums, such as measuring

the growth rates in shake flask media types (Garner K et al. 2000), use of a two-step liquid/solid technique using sterile grain (Santurio et al. 2009), and measuring the spore production rates using different growth inducing media additives and temperatures (Sagüés et al. 2012), all in an effort to discover how to optimize fugus and fungal chlamydospore production. Chlamydospores are an adaptation that evolved to survive harsh conditions, where nutrients are shunted to a thick-walled specialized hyphal segment. They are also sometimes referred to as resting spores, and are thought to be the portion of the fungus that survives digestion (Ojeda-Robertos et al. 2009).

Although studies have been performed on optimizing growth of chlamydospores during fungal hyphal growth, examination of the effects of altering environmental conditions on existing cultures is understudied. The objective of this experiment was to quantify the effect of fast drying the fungus (high temperature and low humidity) compared with slow drying (low temperature and high humidity) on the production of chlamydospores compared to cultures that were continuously left in moist conditions.

Materials and Methods

Fungal strain

For this study the strain used was *D. flagrans* (ATCC® 13423™), strain designation CBS 565.50 [IMI 101314], originally isolated from vegetable compost in England (Duddington CL 1949). Initial culture material was ordered from the

American Type Culture Collection and reconstituted then maintained by continuous culture transfer. *D. flagrans* identity was confirmed using MO BIO PowerFecal DNA extraction kit, followed by PCR amplification of the ITS2 genomic region using the primers ITS86F – GTGAATCATCGAATCTTTGAA and ITS4 – TCCTCCGCTTATTGATATGC (Vancov T 2009). PCR product was sequenced using Sanger sequencing at the University of Delaware DNA Sequencing & Genotyping center at the Delaware Biotechnology Institute, sequence was trimmed and blasted against the NCBI database using Geneious® software.

Fungal cultures

D. flagrans cultures were grown on BBL™ Corn Meal Agar (BD Biosciences, Sparks, MD) 17g/L with no media additives or supplements. Two incubation chambers were used to maintain 30° C and 38° C temperatures, and 24° C was ambient room temperature. A mobile AcuRite® (Chaney Instrument Co., Lakek Geneva, WI) humidity detector was used to monitor humidity for the two unsealed culture groups.

A total of 12 cultures were grown in sealed plates at 30° C for 26 days, inoculated by media transfer from the same established growth plate. These were randomly sorted in to 3 groups of 4 each. One group was uncovered and dehydrated at 38° C and 37% humidity. The second group was uncovered and dehydrated at 24° C

and 55% humidity. The third group was kept sealed and left at 30° C until collection and spore counting as a control.

The control cultures weighed on average 2.4g. Half of each control culture was collected (average 1.16g) and added to a 2mL Eppendorf tube and heated to 95° C for 90 minutes vortexing for 20 seconds every 30 minutes to liquify and homogenize the solution. The heated solution was sampled twice for each culture, and quickly loaded into a Hemocytometer and observed in phase contrast for spore counting.

The fully dried cultures had an average weight of .1g. Half of each dry culture was collected (~0.05g) and added to a 2mL Eppendorf tube along with 1200 uL of Milli-Q purified water to equalize the weight/volume of the control samples, heated for 90 minutes at 95° C with vortexing to homogenize (as described above), then quickly loaded into a Hemocytometer for spore counting, two samplings from each culture. Counts were verified following 12 hours of heating at 95°C (to fully denature the agar) with vortexing as described above. The spore counts were conducted using all 9 counting grids in the hemocytometer and converted to spores per milliliter by averaging and then dividing by the known volume of each grid (0.0001mL) and rounded to the nearest whole spore.

30 days after the conclusion of the study a subsample of each culture was added to a sterile cornmeal agar plate and observed to confirm viability of the spores. 2 mL of the liquified media used for spore counting was also plated and observed to determine if any spores had remained viable after continued exposure to extreme heat (95° C).

Statistical analysis

One tailed Welch Two Sample t-test were performed in R (R Core Team 2016) using R Studio (RStudio Team 2016) to compare the population means of numbers of spores per ml for each culture group to controls, taking into account the unequal variances in the sample groups. Data visualizations were also produced in R (R Core Team 2016) using R Studio (RStudio Team 2016).

Results

The fungal DNA extracted and sequence to confirm the identity of the fungus had a post-quality trimmed read length of 318 bp and 100% match to Duddingtonia flagrans partial sequence, E Value of $3.43x10^{-164}$. The cultures that were dehydrated at 38° C and 37% humidity appeared completely dehydrated after 48 hrs, and the cultures dehydrated at 24° C and 55% humidity appeared completely dehydrated after 10 days. Figure 3 shows a visual example of the difference between a wet and a dry culture, although these images are not quantitative and the difference in volume should be taken into consideration between the two images with a 92% reduction in volume from the wet sample to the dry sample. It is also evident that the normal hyphal growth has been extremely dehydrated to the point of destruction in the dry sample, but the relative size of the chlamydospores remains similar as they are resistant to dehydration.

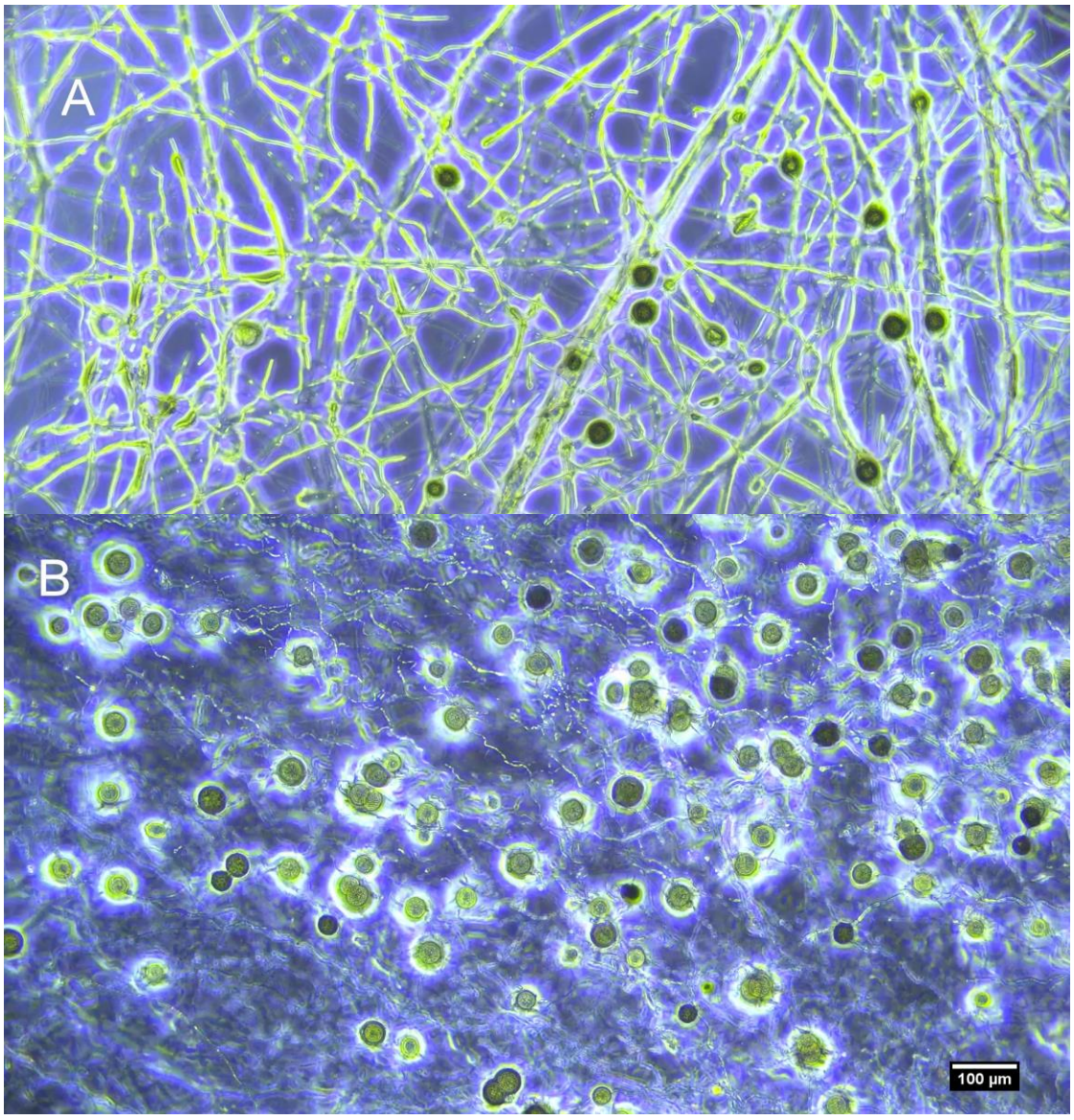


Figure 3 A: Sample of *Duddingtonia flagrans* kept in wet conditions on 17% cornmeal agar. B: Sample of *Duddingtonia flagrans* grown on 17% cornmeal agar and subsequently dehydrated. The difference in media volume is approx. 92% reduction between A and B.

Wet sample number 2 in the control group had a crack in the parafilm seal and dried to a weight of .25g, compared to the other Wet samples at an approximate weight of 2.5g. This culture was still sampled but the data was excluded from analysis because of its unique conditions. The two sampling methods used were sampling immediately after heating and vortexing for the first two samplings from each group, and then sampling after 12hrs of 95°C and vortexing for the third sampling in each group. A Welch Two Sample t-test between the third sampling and each of the first two samplings for the respective treatment groups did not show a significant difference so all three samplings were used in data analysis. Weights of the culture material used before the addition of water to normalize the volumes can be seen in Table 1.

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
Wet	1.23g	0.12g	0.99g	1.27g
Air	0.06g	0.05g	0.05g	0.06g
Inc	0.06g	0.06g	0.08g	0.05g

Table 1 Weight of each culture collected for counting after drying treatment, Wet corresponds to control cultures, Air to 24° C drying group, Inc to 38° C drying group.

Both of the dried cultures had significantly more spores ($p < .05$) than the control culture with averages of 42,780 and 39,260 spores/mL for the quickly and

slowly dried cultures, respectively, and an average of 21,480 spores/mL for the moist cultures. The spore counts can be seen in Table 2. A Welch Two Sample t-test shows the difference in means between the two drying methods is insignificant ($p = .80$). The slower drying method had a lower mean number of spores, and lower maximum and minimum spores measured, but produced more consistent measurements than the quicker drying method as seen in the violin density plot Figure 4.

	Count 1	Count 2	Count 3
Wet 1	37,778	37,778	24,444
Wet 2	33,333	30,000	53,333
Wet 3	11,111	16,667	28,889
Wet 4	10,000	22,222	15,556
Air 1	22,222	50,000	115,556
Air 2	15,556	10,000	33,333
Air 3	20,000	108,889	16,667
Air 4	18,888	75,556	26,667
Inc 1	24,444	22,222	123,333
Inc 2	28,889	63,333	31,111
Inc 3	22,222	24,444	27,778
Inc 4	46,667	30,000	26,667

Table 2 Number of spores per mL after conversion from Neubauer hemocytometer counting chamber, with Wet corresponding to control cultures, Air to 24° C drying group, Inc to 38° C drying group.

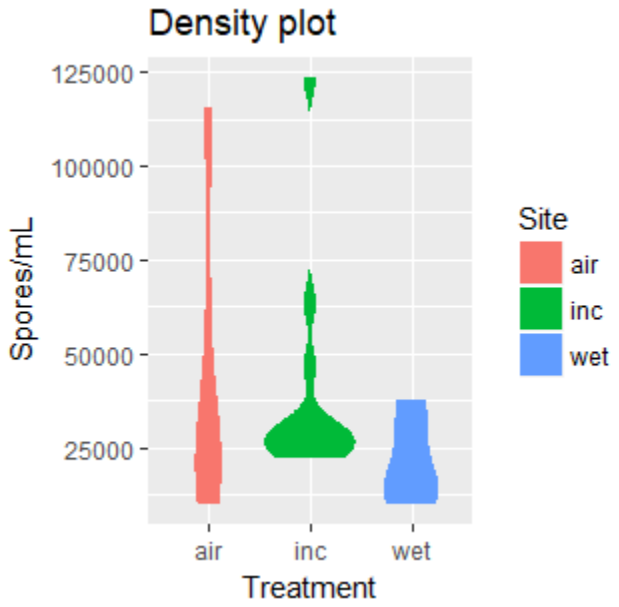


Figure 4 Violin density plot of number of spores per milliliter, with Air corresponding to 24° C drying group, Inc to 38° C drying group, and Wet to control cultures that were kept moist.

All cultures successfully grew when a portion (<0.025g) was re-plated one month after the conclusion of the experiment; both the 24° C and 38° C treatment groups remained viable with visible hyphal growth when plated. No growth was seen when the liquified samples used for spore counting were plated, spores remained intact and visible but were sterilized at 24hrs of 95° C.

Discussion

While chlamydospore production can be increased through optimization of growth media and supplementation (Sagüés et al 2012), further processing of fungal cultures such as challenging with dry conditions explored in this study can increase the number of chlamydospores produced, increasing *Duddingtonia flagran's* commercialization potential. An added benefit to this technique for industrial application is its low cost. With the 24° C and 38° C treatments yielding similar results, incubation chambers would be optional, allowing a producer to focus on time-to-dry vs energy requirements for cost benefit analysis.

The increase in spores during drying should be taken into consideration when determining the effective dosage in chlamydospores per gram if the final stage of the feed additive is a dry supplement. Studies have shown a reduction in spore viability when passing through the digestive track of ruminants as high as 89.7% (Ojeda-Robertos et. al. 2009). It is necessary to have a sufficient number of spores present in the feed to create the desired growth of *D. flagrans* in feces. Effective dosage has been observed in a variety of parasitic nematode host species such as goats (5×10^5 chlamydospores/kg body weight (BW)) (Parauda 2005), sheep (10^5 chlamydospores/kg BW) (Peña M.T. 2002), horses (2×10^6 chlamydospores/kg BW) (Baudena M.A. 2000) and even exotics such as giraffes and antelope (3×10^4 chlamydospores/kg BW) (Young 2018) often measured in chlamydospores per kg bodyweight. If the number of chlamydospores being added to feed was measured in

moist culture before a process of drying and being added to feed, the actual number of spores being fed and so actual effective dosage could be much higher than the measured amount.

Chlamydospore production may also be promoted using media supplements such as meso-inositol 0.5 % to Sabouraud glucose agar or Tween20 for increased mycelium growth (Sagüés, Fusé, Iglesias, Moreno and Saumell (2012). Combined with post-growth drying, these measures suggest a comprehensive strategy to maximize spore production.

A follow-up of general spore viability was done to demonstrate the drying did not render the spores non-viable, the result of all cultures from the 24° C, 38° C, and 30° C groups remaining viable is not surprising since the spores survive that approximate temperature range when passing through a digestive tract and that *D. flagrans* has been shown to survive drying for as long as 7 years (Braga 2011). While the viability of the dried culture spores demonstrate the robustness of this method, our dried samples (8% moisture) were maintained at 37% and 55% humidity environments and were not exposed to extreme desiccation. Lower viability may result from vacuum desiccation or more extreme heat treatment. Additionally, specific spore percentage viability was not measured, only that <0.025g of the dried media (averaging approx. 16,000 spores) created visible mycelial growth when plated; percentage viability may be a consideration in future experimentation.

In conclusion, the drying of *Duddingtonia flagrans* cultures can significantly increase production of chlamydospores solving a limiting factor in the design of

production pipelines for commercial use of this species. The moisture content should also be taken into consideration in experimental design when measuring number of spores per gram in any feed additive as a fluctuation in this moisture level could change the number of spores being administered.

Chapter 2

PREDATORY FUNGUS CAPTURE EFFICIENCY FOR EQUINE CYATHOSTOMIN LARVAE IN THE PRESENCE AND ABSENCE OF FREE-LIVING SOIL NEMATODES

Introduction

Cyathostomin infections are near ubiquitous in equine populations globally and are becoming increasingly resistant to drug treatments (Nielsen 2018). These parasitic intestinal nematodes have proven virtually impossible to eradicate due to their duel internal and external life-cycle, combined with their ability to encyst in the mucosal wall of the intestine, rendering drug treatments less effective. The emerging increased drug resistance has caused the need for alternative treatment methods to these intestinal parasites in order to contain them to subclinical worm burdens. One of the promising alternatives is use of nematophagous fungi as a feed additive intended to grow in fecal pats and on pasture to interrupt the life-cycle and prevent re-infection of grazing animals. One such fungus, *Duddingtonia flagrans*, has shown promising results and its use has been tested in several species such as goats (Parauda 2005), sheep (Peña M.T. 2002), horses (Baudena M.A. 2000) and even exotics such as giraffes and antelope (Young 2018).

When using a biological control agent such as *Duddingtonia flagrans* there are several environmental factors to consider, including species affinity and trap formation. The fungus can grow in the absence of nematodes, and starts trap formation when they enter its environment. It also has been shown to trap different species of

nematodes more quickly than others. SDH 035 isolate was recorded capturing *Trichostrongylus spp.* of parasitic nematodes after 8 hours of interaction with the fungus, and killing them after 48 hours of interaction (Wang 2015). Isolate CG 768 captured free living nematodes *Panagrellus redivivus* after 70 minutes of interaction, and killed in 5 hours (Cruz 2011). The same isolate then was shown to capture *Trichostrongylus spp.* after 4 hours of interaction and killed in 15 hours when in the presence of *P. redivivus* in the same culture (Cruz 2011). It is not clear from these studies alone if the reduced trap time of the parasitic larvae was due to strain difference, presence of *P. redivivus* in the culture or coincidence. This study was designed to isolate and quantify the effect of the presence of free-living nematodes *P. redivivus* on the trapping time of *D. flagrans* when trapping equine cyathostomin third stage larvae, and to identify if *P. redivivus* is consistently trapped more quickly.

Methods

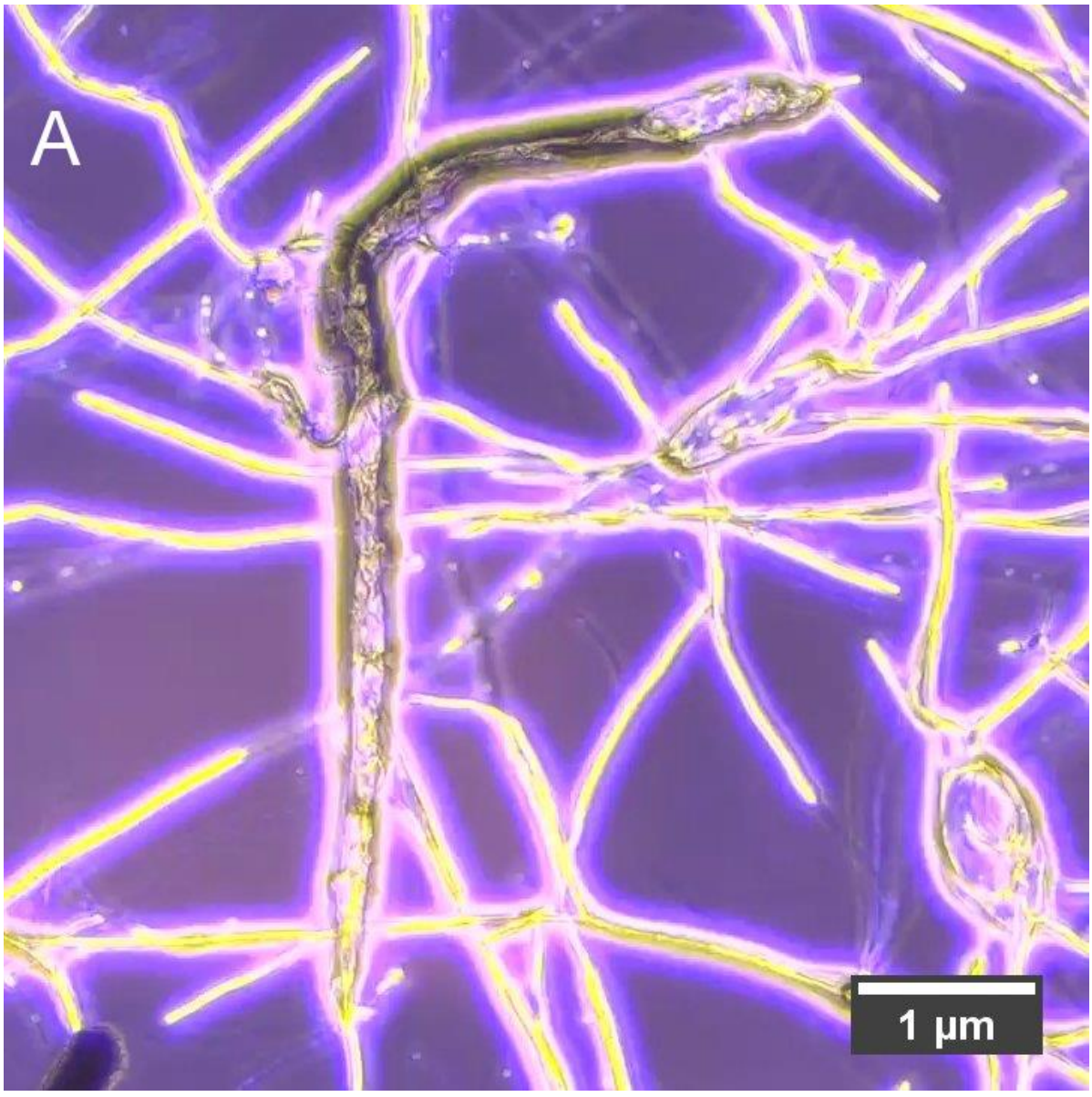
D. flagrans (ATCC® 13423™), strain designation CBS 565.50 [IMI 101314], originally isolated from vegetable compost in England (Duddington CL 1949) was ordered from the American Type Culture Collection (ATCC). Culture was reconstituted and maintained by continuous culture transfer. The Identity of the fungal strain was verified with MO BIO PowerFecal DNA extraction kit, followed by PCR amplification of the ITS2 genomic region using the primers ITS86F – GTGAATCATCGAATCTTTGAA and ITS4 – TCCTCCGCTTATTGATATGC

(Vancov T 2009). A Sanger sequencing at the University of Delaware DNA Sequencing & Genotyping center at the Delaware Biotechnology Institute was used to sequence the PCR product, the resulting sequence was trimmed and blasted against the NCBI database using Geneious® software.

Three Petri dishes of *Duddingtonia flagrans* type strain ATC 13423 were cultured on 17% cornmeal agar at 28° C for 30 days for each trial. Equine manure was incubated for 8 days at 27° C to allow for cyathostomin larvae to hatch and mature, then the incubated feces was transferred to Baermann apparatuses for larval collection. Baermann apparatuses were harvested after 48 hours and L3 larvae were morphologically identified before use. *Panagrellus redivivus* bread stock culture was ordered from Carolina Biological Supply Co. (Burlington, NC) and maintained on damp potato meal. *P. redivivus* were collected from the potato meal culture by pipetting liquid portions of the culture into an Eppendorf tube and diluting with Milli Q purified water.

The *D. flagrans* cultured dishes were randomly assigned to have exclusively cyathostomin larvae added, exclusively *P. redivivus* added, or a mixture of both added. Nematodes were then added to their respective cultures, then the plates were sealed with parafilm to prevent dehydration and left to interact for 24 hours. After 24 hours of interaction each plate was observed under phase light microscopy 10X magnification and each observed nematode was recorded as either trapped or free along with its species and the type of plate it was on; monoculture or mixed. On the mixed nematode culture plates the type of nematode was identified morphologically,

L3 cyathostomin larvae have long whip like tails and less variable size than *P. redivivus*. Images were captured of some of the interactions (Figure 5). This was repeated for three trials and the results were combined for statistical analysis. Counts of free and captured nematodes of each type can be seen in Table 3.



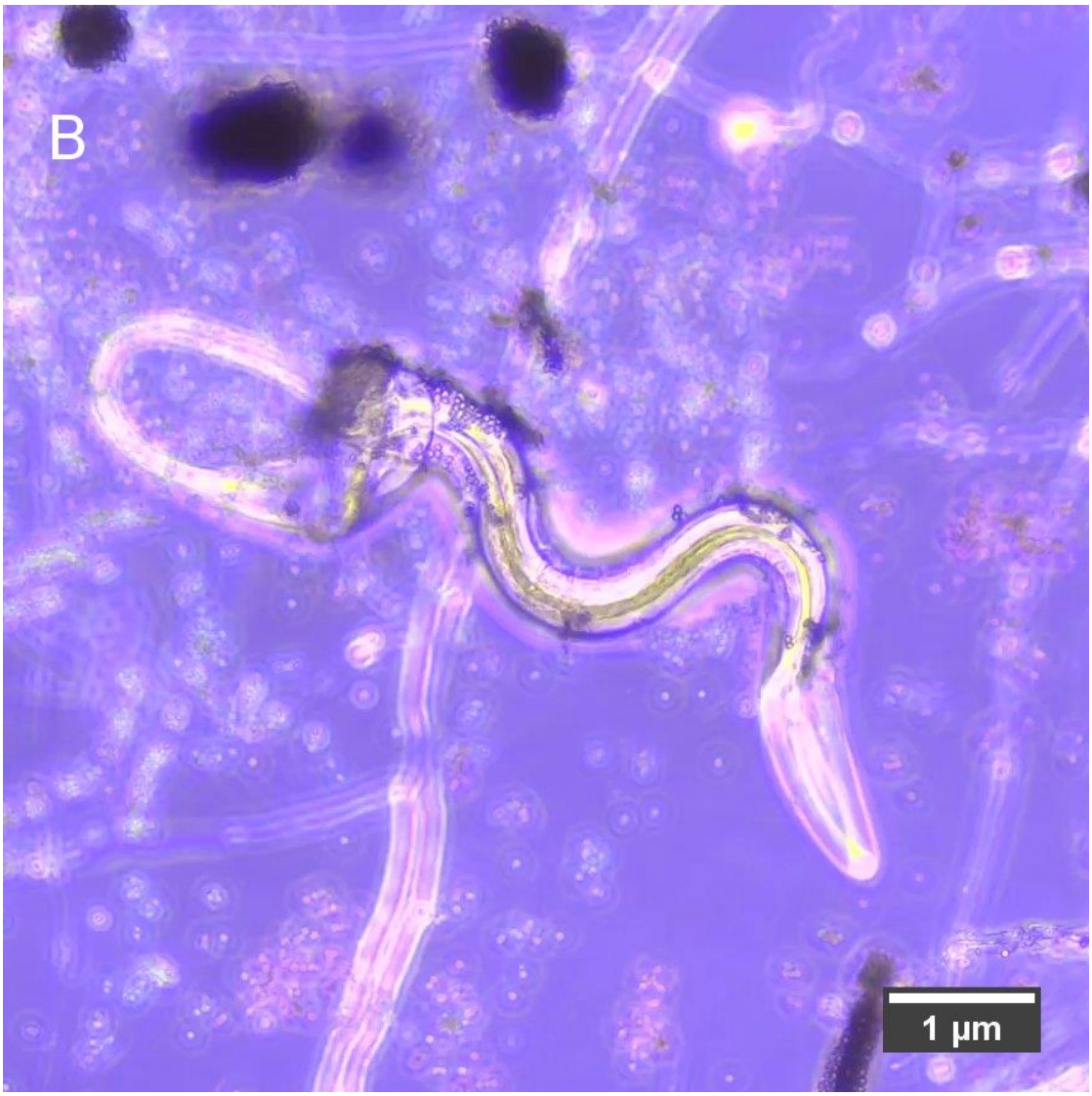


Figure 5 A: Phase contrast image of *Panagrellus redivivus*trapped and colonized at 24 hrs of interaction. B: Phase contrast image of Cyathostomin L3 larvae trapped at 24 hours of interaction with infection bulb in its tail region.

Trial 1	Cyathostomin	P. redivivus	Cyathostomin mixed	P. redivivus mixed
free	2	4	2	1
captured	3	8	6	3

Trial 2	Cyathostomin	P. redivivus	Cyathostomin mixed	P. redivivus mixed
free	6	4	8	4
captured	6	9	7	11

Trial 3	Cyathostomin	P. redivivus	Cyathostomin mixed	P. redivivus mixed
free	6	15	6	15
captured	7	28	7	25

Totals	Cyathostomin	P. redivivus	Cyathostomin mixed	P. redivivus mixed
free	14	23	16	20
captured	16	45	20	39

Table 3 Counts of captured nematodes and of free nematodes observed on each monoculture and mixed culture plate at for each trial set.

Trapped vs free worm totals were calculated and graphics produced in Microsoft Excel. Chi-squared and fisher's exact tests were both conducted to compare the number of cyathostomin larvae captured at the 24 hour mark in mixed plates to the number captured at 24 hours in monocultured plates. A one sample proportions test was conducted on the *P. redivivus* and on the cyathostomin free/captured ratios, respectively, to determine if the samples of each group were more or less likely to be captured at that time point, and to establish a 95% confidence interval of the true population percentage captured at 24 hours under these conditions. Statistical tests were performed in R (R Core Team 2016) using R Studio (RStudio Team 2016).

Results

Confirming the identity of the *D. flagrans* used, the DNA extracted from the fungus had a post-quality trimmed read length of 318 bp and 100% match to *Duddingtonia flagrans* partial sequence, E Value of $3.43x10^{-164}$.

There was no significant difference between the number of cyathostomin L3 larvae captured at 24 hours when added to *D. flagrans* cultures alone when compared to cyathostomin L3 captured in the presence of *P. redivivus*. The monoculture cyathostomin capture rate was 53%, and in the mixed cyathostomin and *P. redivivus* cultures, the parasitic cyathostomin larvae had a capture rate of 56%. Chi-squared and fisher's exact tests comparing the two cyathostomin conditions both yielded p-values of 1; no discernable difference in means. The total free and captured nematodes for each condition can be seen in Figure 6.

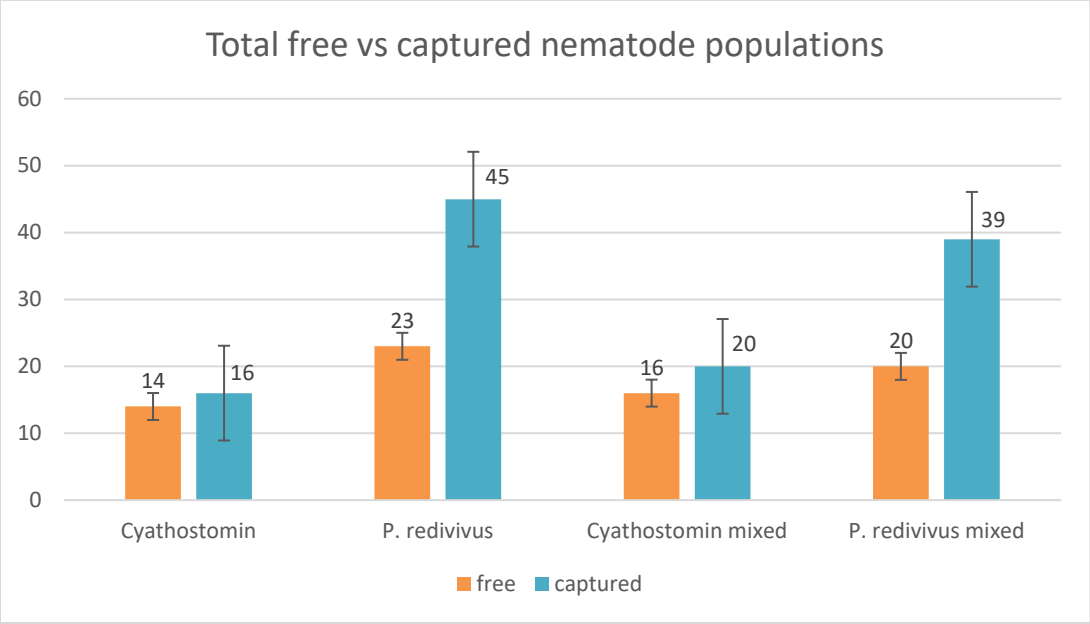


Figure 6 Captured nematodes by species by plate type, displayed with SE.

Due to the lack of difference in capture rate between the separately added and joint-added nematode samples, for further statistical analyses comparing the cyathostomin and *P. redivivus* capture rates, the sample totals were combined (Table 4). This allows for a stronger understanding of the probable true population mean for nematodes of each type captured under these conditions at 24 hours of interaction. The total capture rate for *P. redivivus* was 66.14%, and the total capture rate for cyathostomin L3 was 54.55% (Figure 7). A one sample proportions test for the free-living *P. redivivus* yields a p-value of .0004, showing they are significantly more likely to be trapped than untrapped at 24 hours of interaction with the fungus in these conditions. The 95% confidence interval for the true population mean is between 57% - 74% of nematodes of this species captured at this timepoint. In contrast the

cyathostomin larvae are not more or less likely to be captured at 24 hours of interaction, with their true population mean predicted to be between 42% - 67% captured at this timepoint (95% CI).

	All Cyathostomin	All P. redivivus
free	30	43
captured	36	84

Table 4 Combined totals for each nematode type, across mixed and monoculture plates.

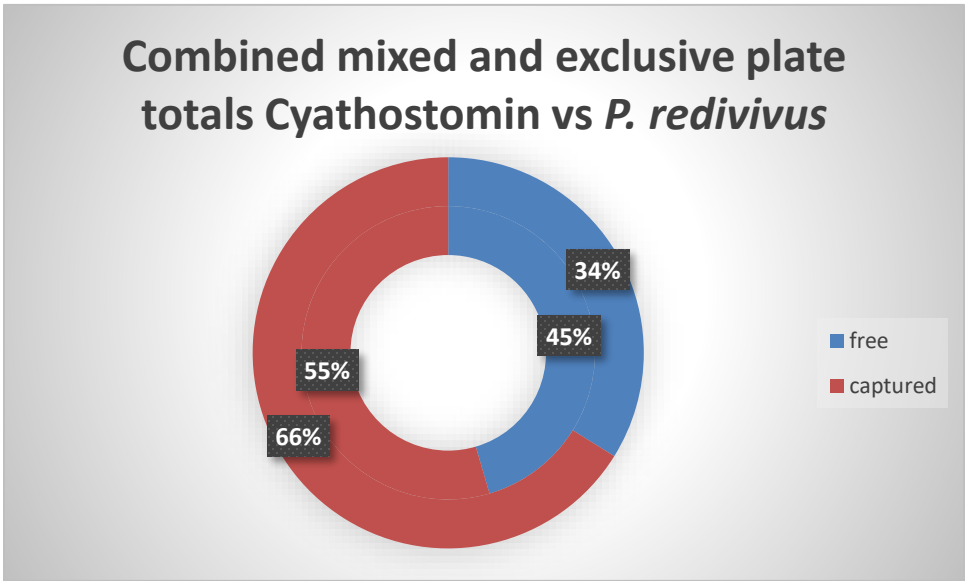


Figure 7 Nematode percentages captured at 24 hrs of interaction from all trials and plate types, outer ring represents P. redivivus, inner represents cyathostomin L3.

Discussion

This study was effective in both identifying a differential effect of the fungus *D. flagrans* on differing species of nematodes and exploring the effect of multiple species concurrently introduced to the fungal environment. Previous research that had identified differences in trapping time between *P. redivivus* and infective larvae of Trichostrongylides, and differences between other studies had noted that: "These variations may be due to the species and inoculum of nematode species, isolate of the fungi, or methodology." (Cruz 2011). This experiment was designed specifically to focus on the variations in nematode species, and to quantify the effect of nematode species on trapping efficiency beyond just qualitative observation of a single nematode's trap time. An observation time of 24 hours was chosen based on an optimization trial that found significant numbers of both *P. redivivus* and equine cyathostomin L3 larvae captured at this time point.

A metric specifically of interest is whether the parasitic nematode larvae are captured at a different rate in the presence of free-living nematodes, as that would directly impact *D. flagrans'* use as a biological control agent. If the naturally occurring nematode populations in pasture significantly impacted the fungus's trapping of parasitic larvae, then pastures would need to be surveyed before *D. flagrans* was selected as a treatment method, to determine its expected efficacy. Additionally, any studies performed on pasture regarding its efficacy would need to take into account environmental populations and further study would be required on specific free-living nematode species common in each region. The findings of this study suggest that will not be necessary, and that specifically the presence of *P. redivivus*, which was shown

here to be trapped significantly faster than parasitic cyathostomin L3s, does not influence the capture time of cyathostomin L3 larvae.

The findings regarding the difference in capture rate between *P. redivivus* and cyathostomin L3 larvae at 24 hours of interaction are also important to *D. flagrans* use as a biological control agent. With a clear majority of *P. redivivus* captured at 24 hours, but closer to half of cyathostomin L3 captured at that time, the fungus has demonstrated an ability to trap one type of nematode more quickly than another. It will require further study to determine the underlying mechanism, whether it be nematode mobility, cuticle texture, chemical signature, or something else that causes enhanced capture rate of specific nematode species. Based on the outcome of this study, researchers and commercial outfits exploring *D. flagrans* as a biological control for infective parasitic larvae should consider it may have varying efficacy on various species of target parasites.

Chapter 3

THE EFFECTS OF SODIUM BISULFATE ON POULTRY LITTER MICROBIOME DIVERSITY AND PATHOGEN VIABILITY

Introduction

Propidium monoazide (PMA) is a photoreactive dye that has a high affinity for DNA. Once attached to DNA strands and exposed to high levels of visible light, it forms covalent linkages preventing the strands from being unwound and replicated. Since PMA does not permeate live bacterial cell membranes, but can cross through dead cells, it can be used in combination with RT-qPCR to assess the viability of bacterial cells (Emerson et al., 2017). Prior to DNA extraction, samples are incubated in liquid medium with PMA in a light-tight environment for a period of time to allow the PMA to saturate the dead cells, then exposed to light to form the linkages.

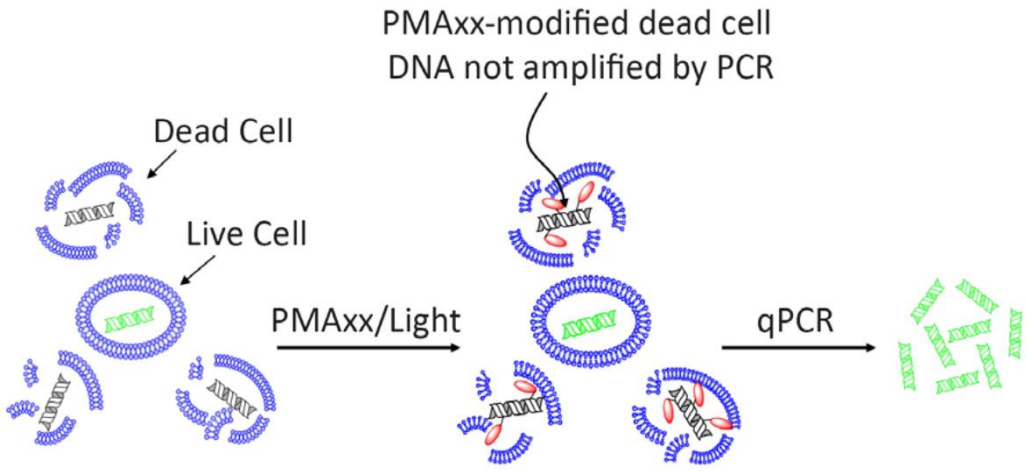


Figure 8 Illustration of PMA mechanism from Biotium.

Since linked DNA has more difficulty passing through filtration steps in DNA extraction leading to less DNA from dead cells in solution post-extraction, and also inhibits replication of the bound DNA in the PCR reaction (skewing dead cell representation in a qPCR reaction), cell culture concentrations were matched in PMA treated and untreated sample groups before treatment, and then volumes were matched for pPCR experimentation in all post-PMA treatment steps. This accounts for the reduction in DNA extraction yield from PMA treatment, whereas matching DNA concentrations for qPCR would negate that effect.

Methods

UD broiler research facility with two identical houses was used for this study. One house (H1) was designated as the treatment house with PLT application (100 lb/1000 ft2) on -1 and 24 days of age while the other house (H2) was used as control and not treated with PLT. The two houses had same temperature set points, but different ammonia concentration level. The ammonia concentration in the treatment house was kept below 25 ppm with PLT while the ammonia concentration in the control house was set to two times of the concentration in the treatment house by controlling fan runtime and ventilation rate. Litter samples (5 g) were collected from 18 points inside each house on four sampling dates including -1, 2, 24, and 27 day of age (labeled as T0, T1, T3, and T4). The litter samples from a same array were mixed and composite samples were collected. There were two composite samples from each house on -1 day and three composite samples on other dates from each house. Samples were shaken to homogenize, then 5 grams were taken and added to 50 mL of sterile phosphate-buffered saline (PBS) to create a 10% slurry for PMAxx™ treatment, and another 5 grams were taken to create an identical slurry for untreated testing. Slurries

were homogenized by vortexing, then allowed to stand briefly for large particles to settle, supernatant was transferred to a new 50 mL falcon tube and centrifuged for 15 minutes at 3500 rpm to pack cells. Supernatant was removed and discarded, then cells were resuspended in 15 mL sterile PBS (33% solution) by vortexting. 500 uL was collected from each re-suspended cell culture and added to a clear Eppendorf tube, 5 uL of 2.5 nM working solution of PMAxx™ was added to the treatment sample group, 5 uL of sterile PBS buffer was added to the untreated sample group. Samples were incubated in complete darkness for 10 minutes with occasional shaking, then incubated in bright light for 15 minutes with occasional flicking to mix. Tubes were centrifuged at 5000 x g for 10 minutes to pellet, DNA was extracted from the pelleted cells using Qiagen soil/fecal extraction kit. Another 500 uL from the untreated 33% PBS cell culture solution was collected and autoclaved, then 5 uL of 2.5 nM solution of PMAxx™ was added, and the dark/light incubation steps were repeated and DNA extracted to create a heat-killed control, for a total of 3 sample groups; untreated, PMAxx™ treated, and heat killed PMAxx™ treated.

The DNA extracted was quantified using Qubit and qualified using NanoDrop. Specific primers for general bacteria, Clostridium perfringens, Enterococcus cecorum, Staphylococcus aureus, general Lactobacillus spp., and Lactobacillus brevis were ordered from IDT for species specific live/dead cell examination. For overall bacteria forward primer CGGYCCAGACTCCTACGGG and reverse primer TTACCGCGGCTGCTGGCAC were used to target a 200bp region of 16S DNA (Lee 1996). For C. perfringens forward primer ATGCAAGTCGAGCGAKG and reverse primer TATGCGGTATTAATCTYCCTTT were used to target a 120bp region of 16S specific to Clostridium Cluster 1 (Rinttilä 2004). For E. cecorum forward primer

TATTTCAGCTATTGTTGACTCTA and reverse primer GCATCGGCAATATAGTTAC were used to target the chaperonin-60 gene (Dumonceaux 2006). For S. aureus forward primer GCGATTGATGGTGATACGGTT and reverse primer AGCCAAGCCTTGACGAACTAAAGC were used to target a 279bp region of the NucA gene (Brakstad 1992). For genus level Lactobacillus spp. primers CTCAAAACTAAACAAAGTTTC and CTTGTACACACCGCCCGTCA were used to target a 250bp region of the 16S and flanking region of 23S genes (Dubernet 2002). For Lactobacillus brevis specifically, forward primer ATCCGGCGGTGGCAAATCA and reverse primer AATCGCCAATCGTTGGCG were used to target a 335bp region of the horA gene (Ma 2017).

DNA extractions were performed on cultures of C. perfringens and S. aureus to confirm the validity of those primers, DNA from samples of the equine microbiome that were previously sequenced were used to test the other primers. 5 uL of PowerUp SYBR Green Master Mix, 1 uL of each forward and reverse primer, 1.5 uL of DNA template and 1.5 uL of PCR grade purified water were combined for a total of 10uL to be run on an Applied Biosystems™ 7900HT Real-Time PCR system. The qPCR was run at an initial temperature of 50° C for 2 minutes, followed by 95° C for 10 minutes, and then 40 cycles of 95° C for 15 seconds and 60° C for 1 minute with ramp rates of 100% between temperatures.

ABI Prism® 7900HT Sequence Detection System 2.4 was used to analyze the resulting fluorescence data and produce Ct values for each sample in the three sample groups, using each primer set based on the system's detection of background fluorescence and an automatically determined threshold. The Ct values for the

multiple sampling points for each house and time point were averaged to produce an untreated Ct, a PMAxx™ treated ΔCt, and a heat killed PMAxx™ treated Ct average for each time point. The averages were then subtracted to create a ΔCt value between untreated/PMAxx™ and PMAxx™/heat killed.

DNA was also sent to be sequenced on an Illumina MiSeq system using 16S targeting to assess overall bacterial abundance levels. A composite sample was created for each sampling date and treatment house by homogenizing the 33% solutions suspended in PBS and drawing 125 uL from each sampling to combine. The samples underwent the same PMA treatment as the 500uL samples used in the qPCR trials. The composite samples were then centrifuged at 5000 x g for 10 minutes to pellet cells, and DNA was extracted from the pelleted cells using Qiagen soil/fecal extraction kit and sent for sequencing.

Results

The Ct value corresponds to the number of thermocycles in the PCR reaction before the amount of DNA amplification crossed the background threshold; the smaller the number the more replicable DNA (more of the target bacteria) is present. Ct value averages can be seen in Tables 5 through 10. The untreated average can be thought of as total dead and alive bacteria, the PMA treated as the alive bacteria, and the heat killed PMA treated as a control on the PMA treatment. The (PMA Ct minus untreated Ct average) shows a relative value for amount of dead bacteria, (heat killed Ct minus PMA Ct) can show a relative value for amount of alive bacteria taking into account limits of the PMA in binding dead bacterial DNA in the sample. These comparisons are displayed in Figures 9 and 10.

All Bacteria			
4.9 H1 avg	9.799286	4.9 H2 avg	9.437311
4.9 H1 PMA avg	12.17265	4.9 H2 PMA avg	11.60287
4.9 H1 HEAT avg	17.60191	4.9 H2 HEAT avg	16.51174
4.12 H1 avg	8.830666	4.12 H2 avg	11.17537
4.12 H1 PMA avg	12.27773	4.12 H2 PMA avg	14.22946
4.12 H1 HEAT avg	17.63574	4.12 H2 HEAT avg	17.58859
5.4 H1 avg	8.857297	5.4 H2 avg	9.399943
5.4 H1 PMA avg	11.32125	5.4 H2 PMA avg	11.77365
5.4 H1 HEAT avg	16.46962	5.4 H2 HEAT avg	15.74265
5.7 H1 avg	9.594367	5.7 H2 avg	8.94679
5.7 H1 PMA avg	12.40931	5.7 H2 PMA avg	11.63984
5.7 H1 HEAT avg	17.19618	5.7 H2 HEAT avg	16.35319

Table 5 Ct value averages for all sampling times and treatment groups H1 and H2 for all bacteria

Clostridium perfringens			
4.9 H1 avg	24.97759	4.9 H2 avg	25.62106
4.9 H1 PMA avg	28.10612	4.9 H2 PMA avg	28.60212
4.9 H1 HEAT avg	25.00049	4.9 H2 HEAT avg	31.90586
4.12 H1 avg	23.49946	4.12 H2 avg	24.26598
4.12 H1 PMA avg	24.67482	4.12 H2 PMA avg	27.78302
4.12 H1 HEAT avg	33.52536	4.12 H2 HEAT avg	31.22758
5.4 H1 avg	24.03483	5.4 H2 avg	23.66002
5.4 H1 PMA avg	26.21657	5.4 H2 PMA avg	28.91669
5.4 H1 HEAT avg	25.75559	5.4 H2 HEAT avg	26.22941
5.7 H1 avg	22.03801	5.7 H2 avg	23.33436
5.7 H1 PMA avg	27.16822	5.7 H2 PMA avg	28.10719
5.7 H1 HEAT avg	30.19422	5.7 H2 HEAT avg	29.27519

Table 6 Ct value averages for all sampling periods and treatment groups H1 and H2 for *Clostridium perfringens*

Enterococcus cecorum

4.9 H1 avg	28.17872	4.9 H2 avg	25.78165
4.9 H1 PMA avg	29.73642	4.9 H2 PMA avg	30.65509
4.9 H1 HEAT avg	34.01508	4.9 H2 HEAT avg	33.08286
4.12 H1 avg	24.77	4.12 H2 avg	23.59501
4.12 H1 PMA avg	27.22262	4.12 H2 PMA avg	29.58608
4.12 H1 HEAT avg	33.22407	4.12 H2 HEAT avg	32.95569
5.4 H1 avg	26.06977	5.4 H2 avg	23.40623
5.4 H1 PMA avg	29.86592	5.4 H2 PMA avg	27.44839
5.4 H1 HEAT avg	31.90119	5.4 H2 HEAT avg	31.30552
5.7 H1 avg	23.50641	5.7 H2 avg	23.54459
5.7 H1 PMA avg	27.80326	5.7 H2 PMA avg	26.42056
5.7 H1 HEAT avg	32.41342	5.7 H2 HEAT avg	31.81089

Table 7 Ct value averages for all sampling periods and treatment groups H1 and H2 for *Enterococcus cecorum*

Lactobacillus general

4.9 H1 avg	32.11901	4.9 H2 avg	27.06079
4.9 H1 PMA avg	32.00356	4.9 H2 PMA avg	35.0488
4.9 H1 HEAT avg	undeterm	4.9 H2 HEAT avg	35.75542
4.12 H1 avg	24.37443	4.12 H2 avg	24.7974
4.12 H1 PMA avg	30.22482	4.12 H2 PMA avg	35.75575
4.12 H1 HEAT avg	undeterm	4.12 H2 HEAT avg	undeterm
5.4 H1 avg	23.77844	5.4 H2 avg	22.61329
5.4 H1 PMA avg	29.85319	5.4 H2 PMA avg	27.8863
5.4 H1 HEAT avg	35.29172	5.4 H2 HEAT avg	32.75552
5.7 H1 avg	20.2359	5.7 H2 avg	21.33705
5.7 H1 PMA avg	28.83397	5.7 H2 PMA avg	26.24792
5.7 H1 HEAT avg	undeterm	5.7 H2 HEAT avg	undeterm

Table 8 Ct value averages for all sampling periods and treatment groups H1 and H2 for *Lactobacillus* general

Lactobacillus brevis

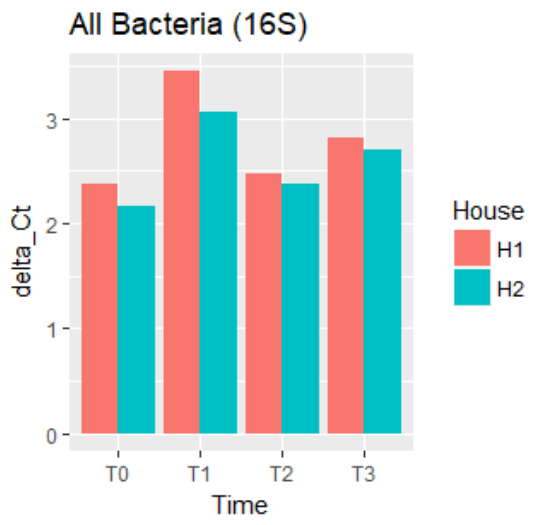
4.9 H1 avg	35.05673	4.9 H2 avg	32.40968
4.9 H1 PMA avg	34.97372	4.9 H2 PMA avg	35.84951
4.9 H1 HEAT avg	35.44718	4.9 H2 HEAT avg	34.41018
4.12 H1 avg	32.95506	4.12 H2 avg	33.15626
4.12 H1 PMA avg	35.23432	4.12 H2 PMA avg	35.29869
4.12 H1 HEAT avg	undeterm	4.12 H2 HEAT avg	35.72694
5.4 H1 avg	34.76044	5.4 H2 avg	32.79336
5.4 H1 PMA avg	33.41395	5.4 H2 PMA avg	34.10852
5.4 H1 HEAT avg	34.23479	5.4 H2 HEAT avg	32.42322
5.7 H1 avg	32.88123	5.7 H2 avg	34.23414
5.7 H1 PMA avg	34.71139	5.7 H2 PMA avg	35.1686
5.7 H1 HEAT avg	33.96847	5.7 H2 HEAT avg	37.05385

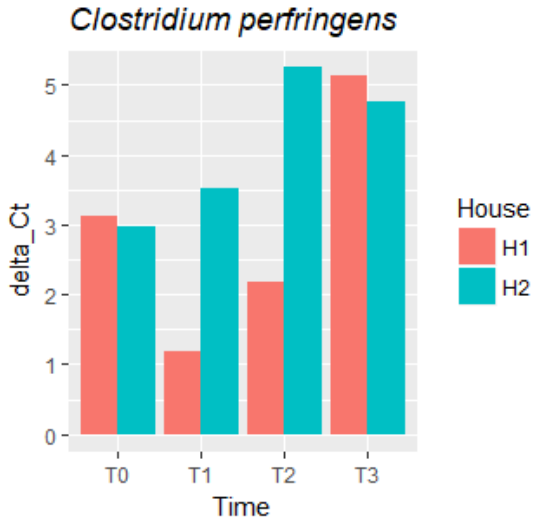
Table 9 Ct value averages for all sampling periods and treatment groups H1 and H2 for *Lactobacillus brevis*

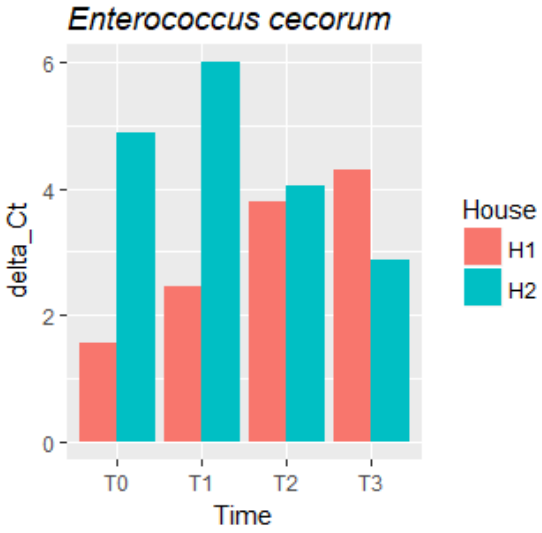
Staphylococcus aureus

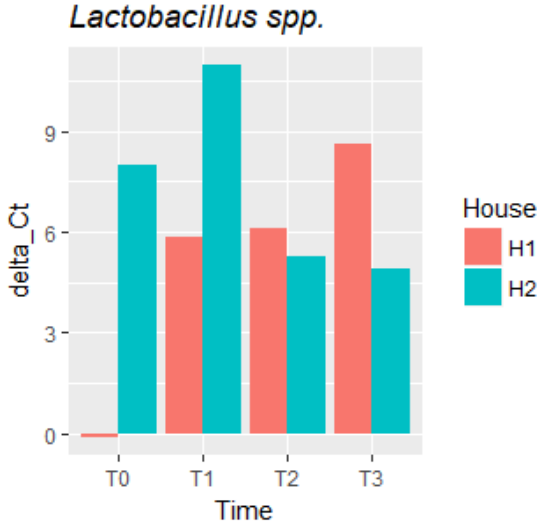
4.9 H1 avg	33.0342	4.9 H2 avg	34.28942
4.9 H1 PMA avg	33.33016	4.9 H2 PMA avg	37.27713
4.9 H1 HEAT avg	34.15488	4.9 H2 HEAT avg	36.39218
4.12 H1 avg	32.44206	4.12 H2 avg	35.72258
4.12 H1 PMA avg	33.65415	4.12 H2 PMA avg	undeterm
4.12 H1 HEAT avg	35.82618	4.12 H2 HEAT avg	36.80483
5.4 H1 avg	32.17291	5.4 H2 avg	32.39111
5.4 H1 PMA avg	33.4663	5.4 H2 PMA avg	32.52345
5.4 H1 HEAT avg	34.07096	5.4 H2 HEAT avg	33.12805
5.7 H1 avg	32.92019	5.7 H2 avg	31.75245
5.7 H1 PMA avg	35.29913	5.7 H2 PMA avg	undeterm
5.7 H1 HEAT avg	34.92532	5.7 H2 HEAT avg	36.49134

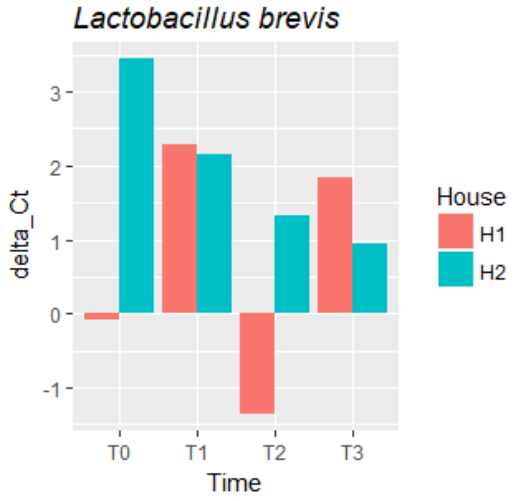
Table 10 Ct value averages for all sampling periods and treatment groups H1 and H2 for *Staphylococcus aureus*


All Bacteria (16S)
delta_Ct
0
1
2
3
T0
T1
T2
T3
Time
House
H1
H2


Clostridium perfringens
delta_Ct
0
1
2
3
4
5
T0
T1
T2
T3
Time
House
H1
H2

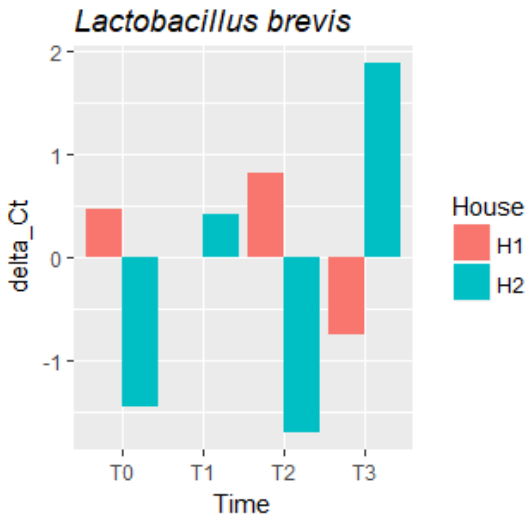

Enterococcus cecorum
delta_Ct
0
2
4
6
T0
T1
T2
T3
Time
House
H1
H2

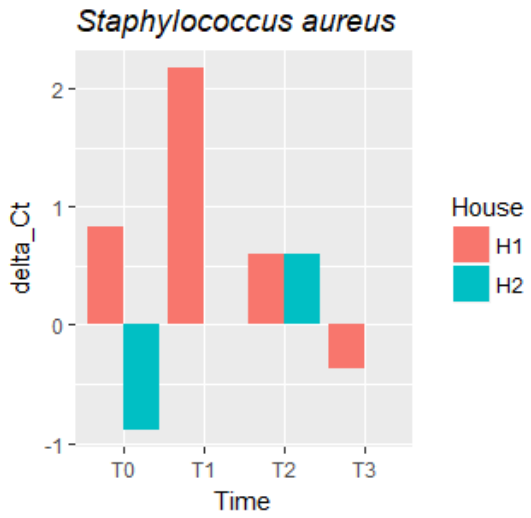

Lactobacillus spp.
delta_Ct
0
3
6
9
T0
T1
T2
T3
Time
House
H1
H2

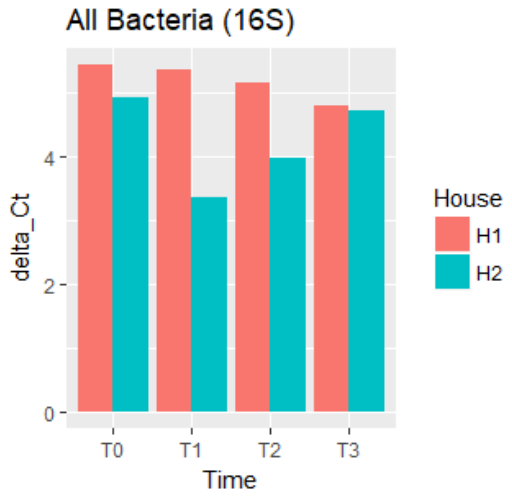

Lactobacillus brevis
delta_Ct
-1
0
1
2
3
T0
T1
T2
T3
Time
House
H1
H2

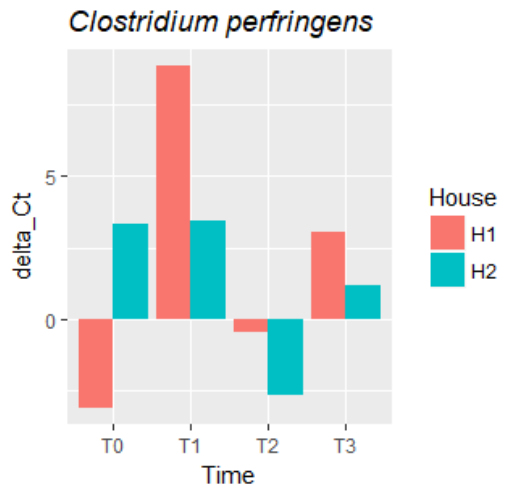

Staphylococcus aureus
delta_Ct
0
1
2
3
T0
T1
T2
T3
Time
House
H1
H2

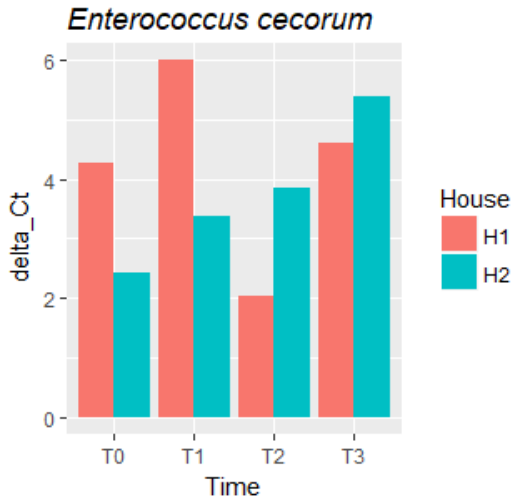
Figure 9 Change in PMA Ct average and untreated Ct average, **representing relative levels of dead bacteria**: H1 is the treatment house, H2 is control.


Lactobacillus brevis
delta_Ct
2
1
0
-1
T0
T1
T2
T3
Time
House
H1
H2


Staphylococcus aureus
delta_Ct
2
1
0
-1
T0
T1
T2
T3
Time
House
H1
H2


All Bacteria (16S)
delta_Ct
4
2
0
T0
T1
T2
T3
Time
House
H1
H2


Clostridium perfringens
delta_Ct
5
0
T0
T1
T2
T3
Time
House
H1
H2


Enterococcus cecorum
delta_Ct
6
4
2
0
T0
T1
T2
T3
Time
House
H1
H2

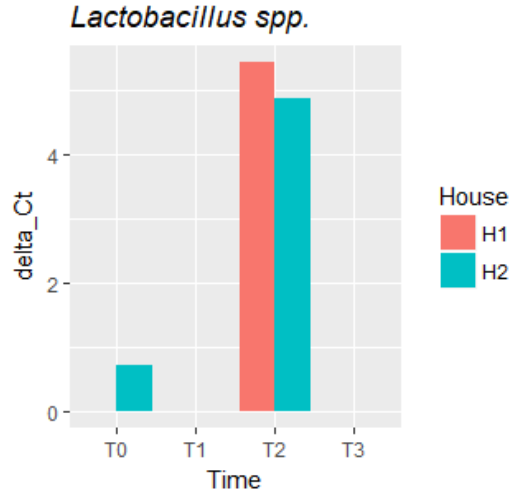

Lactobacillus spp.
delta_Ct
4
2
0
T0
T1
T2
T3
Time
House
H1
H2

Figure 10 Change in Heat killed PMA treated average Ct and PMA treated average Ct: **representing relative live levels controlling for PMA binding efficiency**, H1 is treatment, H2 is control.

For the data obtained from Illumina sequencing, OTUs were selected at a 99% match threshold and counts were normalized to absolute abundance using bacterial kingdom total counts for each sample. Bacterial families that had a presence in more than 50 percent of non-PMA treated samples were then selected for comparison. The community abundance makeup was compared between samples that were not PMA treated to show overall abundance levels, and also between PMA treated samples to potentially show live bacterial community make up. This comparison can be seen in Figures 11 and 12.

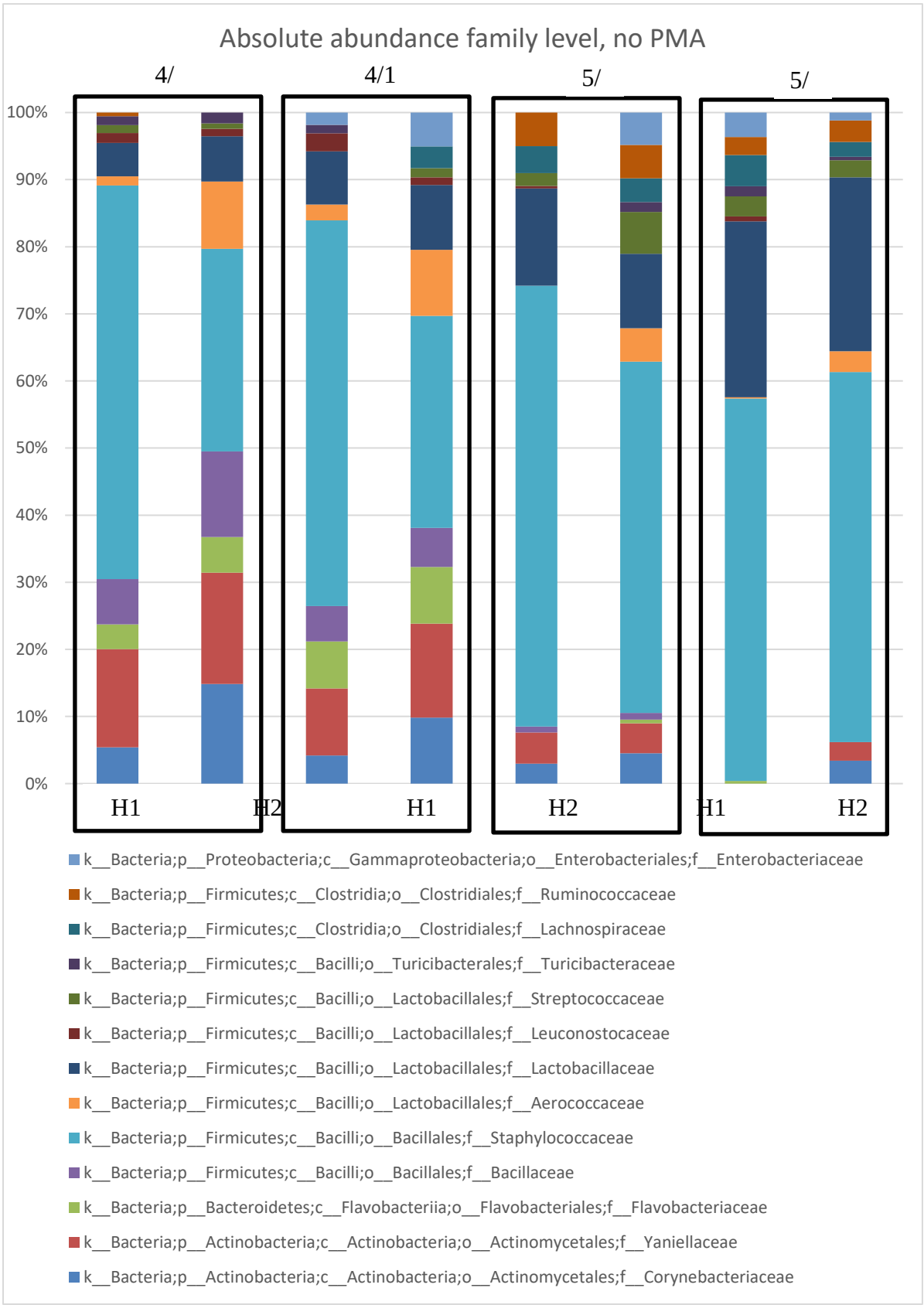

Absolute abundance family level, no PMA
4/
4/1
5/
5/
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
H1
H2
H1
H2
H1
H2
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Yaniellaceae
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae

Figure 11 Comparison of absolute abundance of bacterial families that had a presence in more than 50 percent of non-PMA treated samples

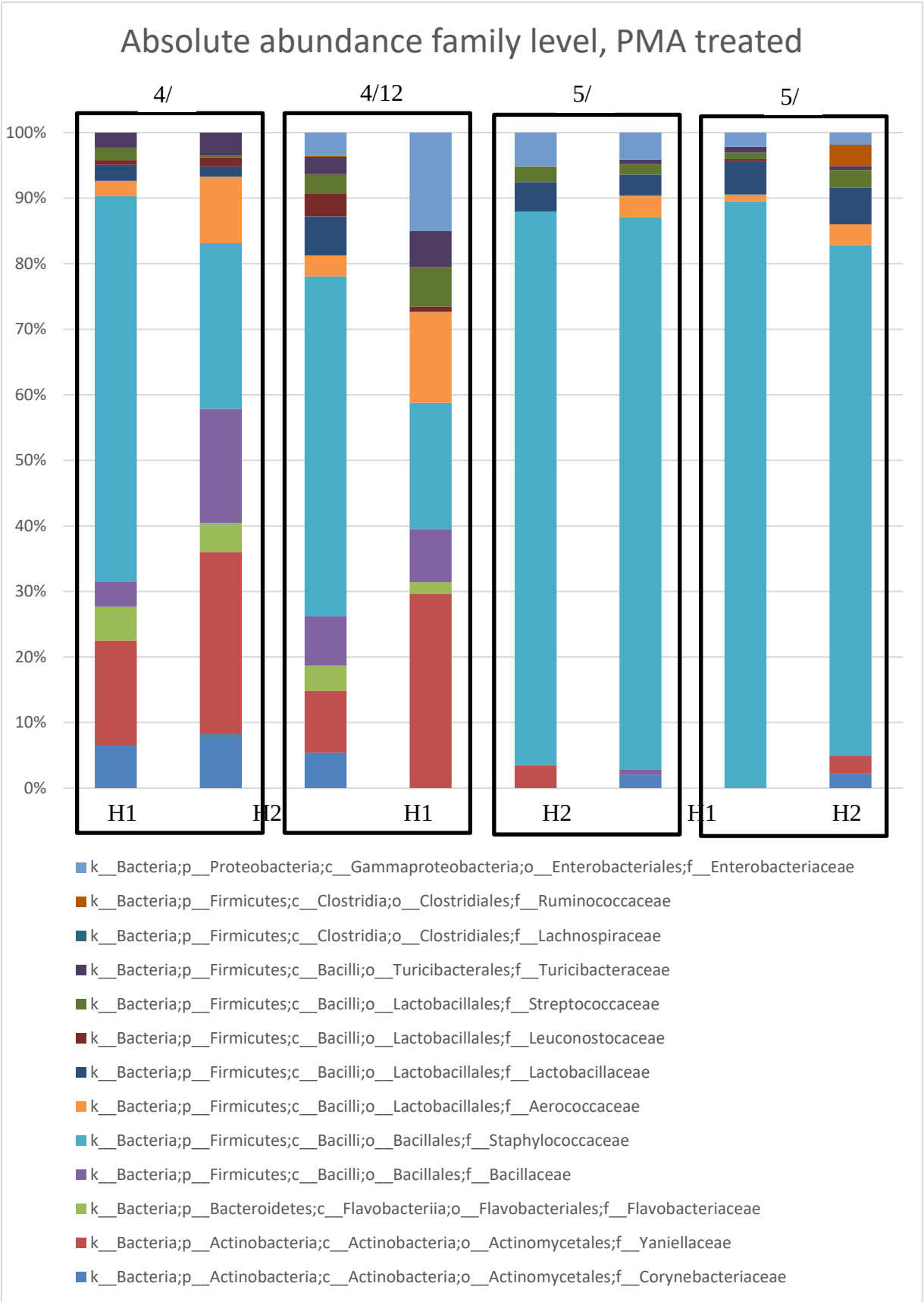

Absolute abundance family level, PMA treated
4/
4/12
5/
5/
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
H1
H2
H1
H2
H1
H2
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Yaniellaceae
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae

Figure 12 Comparison of absolute abundance of bacterial families that had a presence in more than 50 percent of PMA treated samples

Discussion

Amplifiable bacterial 16S quantity overall followed similar trends between the treatment and control houses in the qPCR results, with slightly higher relative dead percentage in the treatment house. Of the five specific bacteria tested for, the *Lactobacillus brevis* and *Staphylococcus aureus* species did not see any amplification above threshold until over 30 cycles, even in the untreated samples. This signifies extremely little to no genetic material in the samples and so no conclusions can be drawn about live/dead rates for these species, only the fact that they are relatively absent in the litter in both houses.

The general *Lactobacillus spp.* started out relatively absent at day 0 but consistently grew in presence in both the control and the treatment houses at each timepoint. Several of the heat killed PMA treated samples for *Lactobacillus spp.* never reached a detectable amplification over the threshold during the 40 thermocycles and so had no Ct value, this is not a surprising result; in fact, ideally the PMA is able to bind all available DNA in a sterilized sample and prevent all amplification. Over 60% of the heat killed PMA treated *Lactobacillus spp.* samples showed no detectable amplification, which is unique among the species tested, and may signify a particularly susceptible membrane to PMA penetration in dead cells of this genus. The total levels of *Lactobacillus spp.* grew faster than the alive levels and both remained

similar between the treatment and the control houses, although the percentage of dead bacteria relative to the total increased over time in the treatment house and decreased in the control house.

Enterococcus cecorum had a presence in both houses, with an increase after -1 day. This species had the most consistent trend of an increasing percentage of dead bacteria relative to the total over time in the treatment house, even more pronounced than the *Lactobacillus spp.*, and the opposite trend for the control house. This combined with the fact that when controlling for PMA binding efficiency the control house levels of live *E. cecorum* had a very consistent increase over time that was not seen in the treatment house, provides the most compelling evidence that of the specific species examined, this species may have its viability reduced by the litter treatment under these conditions. Since *E. cecorum* is considered an emerging pathogen in poultry and can cause considerable losses in broiler flocks (Jung and Rautenschlein, 2014), this is an exciting preliminary result. Further experimentation would be necessary to confirm the treatment's effect and explore its mode of action, for example whether the treatment directly effects the viability of *E. cecorum* or alters the community to encourage growth of some bacterial resource competitor.

Clostridium perfringens was present at overall similar levels in both treatment and control houses. In looking at the comparison of relative dead percentages it would appear that they increase in both houses over time and that they are higher in the control house than the treatment house; however, for this species the heat killed PMA treated group and potential PMA binding efficiency needs to be taken into account

when interpreting those results. Several of the heat killed PMA treated samples yielded lower Ct values than the just PMA treated samples, meaning there was more un-bound available DNA to replicate in the heat killed samples somehow. One possible explanation would be a sampling error that caused a significantly higher amount of DNA to be present in the heat killed sample, overwhelming the available PMA and preventing as much DNA from being bound, but since *C. perfringens* is the only species with significant levels present that this occurred in and identical sampling techniques were used for each this is unlikely. Another possibility would be if the killing technique was ineffective, and although *C. perfringens* is known to produce spores that are heat resistant, 20 minutes at 121° C in the autoclave should easily be enough to kill the spores (Talukdar et al., 2016) so that is also unlikely. A third possibility is that the bacterial membrane or cell contents have some sort of inhibitory effect on PMA binding that prevents DNA from being permanently linked in the PMA incubation and light treatment process, yielding inconsistent results in dampening PCR replication. The PMA treated heat killed and unheated samples both had reduced amplification compared to the raw samples for this species but without more consistent results between the PMA treatments it is not possible to draw conclusions on the live/dead percentage of *C. perfringens* from this experiment, only that overall levels (dead plus alive) remain consistent throughout the trial in both treatment and control groups.

From the sequencing data at the family level the overall bacterial makeup of the samples not treated with PMA had relatively similar trends between the PLT

treated and control houses. Both saw a decline in *Aerococcaceae*, *Bacillaceae* and *Yaniellaceae* over time. Although at first glance it may appear that the lactic acid producing family *Aerococcaceae* appeared in higher abundance in the control house, considering the starting population was much higher in the control house, the reduction from time point to time point is similar and the difference between houses is not likely an indication that the PTL treatment had an effect. Both houses saw a sharp drop of *Flavobacteriaceae* between the 2-day and 24-day time points, and *Staphylococcaceae* remained the dominant family in both groups throughout. Notably within the *Staphylococcaceae* family and the *Staphylococcus* genus, *Staphylococcus aureus* specifically was not found in quantifiable levels, which mirrors the qPCR results. The *Streptococcaceae* increased in absolute abundance levels in both houses. Some inferences can be drawn about the oxygen content of the litter in both houses with the disappearance of *Flavobacteriaceae* which are mostly aerobic (Bernardet and Nakagawa, 2006) and the appearance of *Ruminococcaceae* which are all obligate anaerobes (Bayer et al., 2006) at that same time indicates a significant shift towards an anaerobic environment, which is expected as the same litter remains used throughout the lifetime of the birds being raised on it and it continues to be colonized.

In the PMA treated samples family level community make ups similar to those seen in the non-PMA treated samples were found for the -1-day time point but then had a greater deviation over time. Trends such as reduction in *Bacillaceae* and *Yaniellaceae* over time, and disappearance of *Flavobacteriaceae* between the 2-day and 24-day sampling periods remained the same, but *Staphylococcaceae* dominated

the community make up in both houses at later sampling times in the PMA treated samples. This may be caused partially by the sample treatment method, the PMA treatment technique involving multiple mixing and incubating steps in the presence of oxygen could kill obligate anaerobes present in the samples, thus allowing PMA to bind and inhibit their ability to be sequenced - artificially reducing their abundance levels and so reducing community variation when looking at absolute abundance levels at later time points.

One immediate change seen after the first PLT treatment (between -1-day and 2-day) in the community MiSeq absolute abundance non-PMA data is that in the control group H2 saw the appearance of *Lachnospiraceae* and the treatment group H1 did not. Since all members of the *Lachnospiraceae* family are anerobic (Vos et al., 2009) this could indicate that either the litter treatment or treatment method caused an aeration of the litter creating this change in microbiome makeup. This is a family common to digestive tracts so is expected to be introduced to the litter, the family contains butyric acid producing bacteria so a downstream effect may be a reduction in the rate of acidification of the litter.

Directly after the second PLT treatment (24-day to 27-day) in the non-PMA MiSeq absolute abundance data there is a disappearance of *Bacillaceae, Yaniellaceae* and *Corynebacteriaceae* families in the treatment group H1, where as the control group H2 only sees the disappearance of *Bacillaceae*. The change in levels of *Corynebacteriaceae* in the treatment group is interesting because this family represents a diverse range of species including both commensal bacteria and

opportunistic pathogens. A few of the pathogens this family contains include *C. pseudotuberculosis, C. tenuis, C. striatum, C. minutissimum*, but most notably is the bacteria responsible for causing diphtheria in humans *Corynebacterium diphtheriae*. *C. diphtheriae* is a zoonotic pathogen that has been shown to infect both humans and broiler chickens (Enurah L.U et al., 2016). When taken to the species level the primary *Corynebacteriaceae* found in this study was *Corynebacterium stationis* (data not shown), which has been isolated clinically from human blood samples (Kim R., Reboli A. C., 2015) and is considered a medically relevant species (Bernard K., 2012) so in this case the reduction in the treatment house could be viewed as beneficial.

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Appendix

Ct VALUES FOR ALL POULTRY LITTER RT qPCR

Column1	Column2	Column3	Column4	Column6
SDS 2.4	AQ Results	1.0		
Filename	full_Cperf_run1			
PlateID				
Assay Type	Absolute Quantification			
Run DateTime	10/3/18 7:31:31 PM			
Operator				
ThermalCycleParams				
Sample Information				
Well	Sample Name	Detector Name	Reporter	Ct
25	4.12H1-1	SYBR Master Mix	SYBR	24.166735
27	4.12H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
26	4.12H1-1PMA	SYBR Master Mix	SYBR	28.020155
28	4.12H1-2	SYBR Master Mix	SYBR	23.448477
30	4.12H1-2HEAT	SYBR Master Mix	SYBR	35.982254
29	4.12H1-2PMA	SYBR Master Mix	SYBR	22.994154
31	4.12H1-3	SYBR Master Mix	SYBR	22.250746
33	4.12H1-3HEAT	SYBR Master Mix	SYBR	31.06846
32	4.12H1-3PMA	SYBR Master Mix	SYBR	23.01015
37	4.12H2-1	SYBR Master Mix	SYBR	24.131884
39	4.12H2-1HEAT	SYBR Master Mix	SYBR	29.853607
38	4.12H2-1PMA	SYBR Master Mix	SYBR	27.363052

40	4.12H2-2	SYBR Master Mix	SYBR	24.556402
42	4.12H2-2HEAT	SYBR Master Mix	SYBR	32.06121
41	4.12H2-2PMA	SYBR Master Mix	SYBR	28.012468
43	4.12H2-3	SYBR Master Mix	SYBR	24.10965
45	4.12H2-3HEAT	SYBR Master Mix	SYBR	31.76793
44	4.12H2-3PMA	SYBR Master Mix	SYBR	27.97355
1	4.9H1-1	SYBR Master Mix	SYBR	24.837631
3	4.9H1-1HEAT	SYBR Master Mix	SYBR	32.070667
2	4.9H1-1PMA	SYBR Master Mix	SYBR	27.181011
4	4.9H1-2	SYBR Master Mix	SYBR	26.41241
6	4.9H1-2HEAT	SYBR Master Mix	SYBR	34.179817
5	4.9H1-2PMA	SYBR Master Mix	SYBR	27.051931
7	4.9H1-3	SYBR Master Mix	SYBR	24.42841
9	4.9H1-3HEAT	SYBR Master Mix	SYBR	33.751484
8	4.9H1-3PMA	SYBR Master Mix	SYBR	27.323708
10	4.9H1-4	SYBR Master Mix	SYBR	24.231894
12	4.9H1-4HEAT	SYBR Master Mix	SYBR	34.695072
11	4.9H1-4PMA	SYBR Master Mix	SYBR	30.867813
13	4.9H2-1	SYBR Master Mix	SYBR	25.444626
15	4.9H2-1HEAT	SYBR Master Mix	SYBR	29.84899
14	4.9H2-1PMA	SYBR Master Mix	SYBR	27.460188

16	4.9H2-2	SYBR Master Mix	SYBR	25.017632
18	4.9H2-2HEAT	SYBR Master Mix	SYBR	31.625828
17	4.9H2-2PMA	SYBR Master Mix	SYBR	28.995758
19	4.9H2-3	SYBR Master Mix	SYBR	26.231142
21	4.9H2-3HEAT	SYBR Master Mix	SYBR	33.65375
20	4.9H2-3PMA	SYBR Master Mix	SYBR	29.734203
22	4.9H2-4	SYBR Master Mix	SYBR	25.790846
24	4.9H2-4HEAT	SYBR Master Mix	SYBR	32.494877
23	4.9H2-4PMA	SYBR Master Mix	SYBR	28.218315
49	5.4H1-1	SYBR Master Mix	SYBR	23.564604
51	5.4H1-1HEAT	SYBR Master Mix	SYBR	21.973392
50	5.4H1-1PMA	SYBR Master Mix	SYBR	25.6991
52	5.4H1-2	SYBR Master Mix	SYBR	24.736929
54	5.4H1-2HEAT	SYBR Master Mix	SYBR	23.070404
53	5.4H1-2PMA	SYBR Master Mix	SYBR	26.916735
55	5.4H1-3	SYBR Master Mix	SYBR	23.802948
57	5.4H1-3HEAT	SYBR Master Mix	SYBR	32.22296
56	5.4H1-3PMA	SYBR Master Mix	SYBR	26.03387
61	5.4H2-1	SYBR Master Mix	SYBR	22.766453
63	5.4H2-1HEAT	SYBR Master Mix	SYBR	25.027847
62	5.4H2-1PMA	SYBR Master Mix	SYBR	26.646074

64	5.4H2-2	SYBR Master Mix	SYBR	23.281546
66	5.4H2-2HEAT	SYBR Master Mix	SYBR	30.792812
65	5.4H2-2PMA	SYBR Master Mix	SYBR	31.9196
67	5.4H2-3	SYBR Master Mix	SYBR	24.932049
69	5.4H2-3HEAT	SYBR Master Mix	SYBR	22.86757
68	5.4H2-3PMA	SYBR Master Mix	SYBR	28.184397
73	5.7H1-1	SYBR Master Mix	SYBR	23.158491
75	5.7H1-1HEAT	SYBR Master Mix	SYBR	32.110027
74	5.7H1-1PMA	SYBR Master Mix	SYBR	28.3191
76	5.7H1-2	SYBR Master Mix	SYBR	21.13012
78	5.7H1-2HEAT	SYBR Master Mix	SYBR	29.625914
77	5.7H1-2PMA	SYBR Master Mix	SYBR	25.893675
79	5.7H1-3	SYBR Master Mix	SYBR	21.825417
81	5.7H1-3HEAT	SYBR Master Mix	SYBR	28.846722
80	5.7H1-3PMA	SYBR Master Mix	SYBR	27.29189
85	5.7H2-1	SYBR Master Mix	SYBR	24.993608
87	5.7H2-1HEAT	SYBR Master Mix	SYBR	29.4547
86	5.7H2-1PMA	SYBR Master Mix	SYBR	29.306198
88	5.7H2-2	SYBR Master Mix	SYBR	22.282106
90	5.7H2-2HEAT	SYBR Master Mix	SYBR	29.178442
89	5.7H2-2PMA	SYBR Master Mix	SYBR	25.70434

91	5.7H2-3	SYBR Master Mix	SYBR	22.727362
93	5.7H2-3HEAT	SYBR Master Mix	SYBR	29.192427
92	5.7H2-3PMA	SYBR Master Mix	SYBR	29.311024

Table 11 Ct values for all *C. perfringins* qPCR runs

Column1	Column2	Column3	Column4	Column6
SDS 2.4	AQ Results	1.0		
Filename	full_Ecec_run1			
PlateID				
Assay Type	Absolute Quantification			
Run DateTime	10/29/18 3:36:43 PM			
Operator				
ThermalCycleParams				
Sample Information				
Well	Sample Name	Detector Name	Reporter	Ct
25	4.12H1-1	SYBR Master Mix	SYBR	24.997444
27	4.12H1-1HEAT	SYBR Master Mix	SYBR	33.757023
26	4.12H1-1PMA	SYBR Master Mix	SYBR	29.911707
28	4.12H1-2	SYBR Master Mix	SYBR	24.668861
30	4.12H1-2HEAT	SYBR Master Mix	SYBR	34.095985
29	4.12H1-2PMA	SYBR Master Mix	SYBR	26.314922
31	4.12H1-3	SYBR Master Mix	SYBR	24.64368
33	4.12H1-3HEAT	SYBR Master Mix	SYBR	31.819199

32	4.12H1-3PMA	SYBR Master Mix	SYBR	25.441221
37	4.12H2-1	SYBR Master Mix	SYBR	24.11566
39	4.12H2-1HEAT	SYBR Master Mix	SYBR	31.808798
38	4.12H2-1PMA	SYBR Master Mix	SYBR	29.658512
40	4.12H2-2	SYBR Master Mix	SYBR	22.034552
42	4.12H2-2HEAT	SYBR Master Mix	SYBR	33.49624
41	4.12H2-2PMA	SYBR Master Mix	SYBR	30.937656
43	4.12H2-3	SYBR Master Mix	SYBR	24.63482
45	4.12H2-3HEAT	SYBR Master Mix	SYBR	33.562042
44	4.12H2-3PMA	SYBR Master Mix	SYBR	28.16208
1	4.9H1-1	SYBR Master Mix	SYBR	28.059317
3	4.9H1-1HEAT	SYBR Master Mix	SYBR	33.372894
2	4.9H1-1PMA	SYBR Master Mix	SYBR	30.498451
4	4.9H1-2	SYBR Master Mix	SYBR	27.633125
6	4.9H1-2HEAT	SYBR Master Mix	SYBR	35.192112
5	4.9H1-2PMA	SYBR Master Mix	SYBR	29.33356
7	4.9H1-3	SYBR Master Mix	SYBR	27.07238
9	4.9H1-3HEAT	SYBR Master Mix	SYBR	33.48023
8	4.9H1-3PMA	SYBR Master Mix	SYBR	28.053602
10	4.9H1-4	SYBR Master Mix	SYBR	29.950047
12	4.9H1-4HEAT	SYBR Master Mix	SYBR	Undetermined

11	4.9H1-4PMA	SYBR Master Mix	SYBR	31.060078
13	4.9H2-1	SYBR Master Mix	SYBR	25.467266
15	4.9H2-1HEAT	SYBR Master Mix	SYBR	30.37774
14	4.9H2-1PMA	SYBR Master Mix	SYBR	31.12683
16	4.9H2-2	SYBR Master Mix	SYBR	25.306084
18	4.9H2-2HEAT	SYBR Master Mix	SYBR	34.179962
19	4.9H2-3	SYBR Master Mix	SYBR	26.491888
21	4.9H2-3HEAT	SYBR Master Mix	SYBR	33.253906
20	4.9H2-3PMA	SYBR Master Mix	SYBR	33.16345
22	4.9H2-4	SYBR Master Mix	SYBR	25.861368
24	4.9H2-4HEAT	SYBR Master Mix	SYBR	34.519833
23	4.9H2-4PMA	SYBR Master Mix	SYBR	27.881464
17	4.9H2-2PMA	SYBR Master Mix	SYBR	30.44862
49	5.4H1-1	SYBR Master Mix	SYBR	25.250183
51	5.4H1-1HEAT	SYBR Master Mix	SYBR	31.543125
50	5.4H1-1PMA	SYBR Master Mix	SYBR	28.44912
52	5.4H1-2	SYBR Master Mix	SYBR	27.121826
54	5.4H1-2HEAT	SYBR Master Mix	SYBR	31.508604
53	5.4H1-2PMA	SYBR Master Mix	SYBR	32.037872
55	5.4H1-3	SYBR Master Mix	SYBR	25.8373
57	5.4H1-3HEAT	SYBR Master Mix	SYBR	32.651844

56	5.4H1-3PMA	SYBR Master Mix	SYBR	29.110764
61	5.4H2-1	SYBR Master Mix	SYBR	23.635004
63	5.4H2-1HEAT	SYBR Master Mix	SYBR	31.085001
62	5.4H2-1PMA	SYBR Master Mix	SYBR	Undetermined
64	5.4H2-2	SYBR Master Mix	SYBR	22.828823
66	5.4H2-2HEAT	SYBR Master Mix	SYBR	30.979353
65	5.4H2-2PMA	SYBR Master Mix	SYBR	27.448387
67	5.4H2-3	SYBR Master Mix	SYBR	23.754858
69	5.4H2-3HEAT	SYBR Master Mix	SYBR	31.852213
68	5.4H2-3PMA	SYBR Master Mix	SYBR	Undetermined
73	5.7H1-1	SYBR Master Mix	SYBR	23.370485
75	5.7H1-1HEAT	SYBR Master Mix	SYBR	31.270382
74	5.7H1-1PMA	SYBR Master Mix	SYBR	25.305033
76	5.7H1-2	SYBR Master Mix	SYBR	23.661695
78	5.7H1-2HEAT	SYBR Master Mix	SYBR	33.378124
77	5.7H1-2PMA	SYBR Master Mix	SYBR	27.975988
79	5.7H1-3	SYBR Master Mix	SYBR	23.487047
81	5.7H1-3HEAT	SYBR Master Mix	SYBR	32.59174
80	5.7H1-3PMA	SYBR Master Mix	SYBR	30.128767
85	5.7H2-1	SYBR Master Mix	SYBR	24.102869
87	5.7H2-1HEAT	SYBR Master Mix	SYBR	31.290373

86	5.7H2-1PMA	SYBR Master Mix	SYBR	25.948824
88	5.7H2-2	SYBR Master Mix	SYBR	23.097683
90	5.7H2-2HEAT	SYBR Master Mix	SYBR	33.142376
89	5.7H2-2PMA	SYBR Master Mix	SYBR	26.274794
91	5.7H2-3	SYBR Master Mix	SYBR	23.433214
93	5.7H2-3HEAT	SYBR Master Mix	SYBR	30.999914
92	5.7H2-3PMA	SYBR Master Mix	SYBR	27.038054

Table 12 Ct values for all *E. cecorum* qPCR runs

Column1	Column2	Column3	Column4	Column6
SDS 2.4	AQ Results	1.0		
Filename	full_LactGen_run1			
PlateID				
Assay Type	Absolute Quantification			
Run DateTime	10/10/18 5:27:26 PM			
Operator				
ThermalCycleParams				
Sample Information				
Well	Sample Name	Detector Name	Reporter	Ct
25	4.12H1-1	SYBR Master Mix	SYBR	23.407654
27	4.12H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
26	4.12H1-1PMA	SYBR Master Mix	SYBR	34.4905
28	4.12H1-2	SYBR Master Mix	SYBR	23.7973

30	4.12H1-2HEAT	SYBR Master Mix	SYBR	Undetermined
29	4.12H1-2PMA	SYBR Master Mix	SYBR	28.692984
31	4.12H1-3	SYBR Master Mix	SYBR	25.918346
33	4.12H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
32	4.12H1-3PMA	SYBR Master Mix	SYBR	27.490988
37	4.12H2-1	SYBR Master Mix	SYBR	22.464235
39	4.12H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
38	4.12H2-1PMA	SYBR Master Mix	SYBR	33.99197
40	4.12H2-2	SYBR Master Mix	SYBR	24.339725
42	4.12H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
41	4.12H2-2PMA	SYBR Master Mix	SYBR	37.519535
43	4.12H2-3	SYBR Master Mix	SYBR	27.588236
45	4.12H2-3HEAT	SYBR Master Mix	SYBR	Undetermined
44	4.12H2-3PMA	SYBR Master Mix	SYBR	Undetermined
1	4.9H1-1	SYBR Master Mix	SYBR	32.76319
3	4.9H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
2	4.9H1-1PMA	SYBR Master Mix	SYBR	31.558954
4	4.9H1-2	SYBR Master Mix	SYBR	Undetermined
6	4.9H1-2HEAT	SYBR Master Mix	SYBR	Undetermined
5	4.9H1-2PMA	SYBR Master Mix	SYBR	32.448162
7	4.9H1-3	SYBR Master Mix	SYBR	30.91535

9	4.9H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
8	4.9H1-3PMA	SYBR Master Mix	SYBR	Undetermined
10	4.9H1-4	SYBR Master Mix	SYBR	32.6785
12	4.9H1-4HEAT	SYBR Master Mix	SYBR	Undetermined
11	4.9H1-4PMA	SYBR Master Mix	SYBR	Undetermined
13	4.9H2-1	SYBR Master Mix	SYBR	23.135527
15	4.9H2-1HEAT	SYBR Master Mix	SYBR	35.75542
14	4.9H2-1PMA	SYBR Master Mix	SYBR	11.939942
16	4.9H2-2	SYBR Master Mix	SYBR	26.612848
18	4.9H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
17	4.9H2-2PMA	SYBR Master Mix	SYBR	Undetermined
19	4.9H2-3	SYBR Master Mix	SYBR	30.11823
21	4.9H2-3HEAT	SYBR Master Mix	SYBR	Undetermined
20	4.9H2-3PMA	SYBR Master Mix	SYBR	Undetermined
22	4.9H2-4	SYBR Master Mix	SYBR	28.37655
24	4.9H2-4HEAT	SYBR Master Mix	SYBR	Undetermined
23	4.9H2-4PMA	SYBR Master Mix	SYBR	35.0488
49	5.4H1-1	SYBR Master Mix	SYBR	22.892006
51	5.4H1-1HEAT	SYBR Master Mix	SYBR	34.586437
50	5.4H1-1PMA	SYBR Master Mix	SYBR	24.996315
52	5.4H1-2	SYBR Master Mix	SYBR	24.906649

54	5.4H1-2HEAT	SYBR Master Mix	SYBR	35.99701
53	5.4H1-2PMA	SYBR Master Mix	SYBR	31.18841
55	5.4H1-3	SYBR Master Mix	SYBR	23.53666
57	5.4H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
56	5.4H1-3PMA	SYBR Master Mix	SYBR	33.374836
61	5.4H2-1	SYBR Master Mix	SYBR	21.99575
63	5.4H2-1HEAT	SYBR Master Mix	SYBR	32.75552
62	5.4H2-1PMA	SYBR Master Mix	SYBR	25.924114
64	5.4H2-2	SYBR Master Mix	SYBR	23.08226
66	5.4H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
65	5.4H2-2PMA	SYBR Master Mix	SYBR	27.891302
67	5.4H2-3	SYBR Master Mix	SYBR	22.761845
69	5.4H2-3HEAT	SYBR Master Mix	SYBR	Undetermined
68	5.4H2-3PMA	SYBR Master Mix	SYBR	29.843496
73	5.7H1-1	SYBR Master Mix	SYBR	18.48294
75	5.7H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
74	5.7H1-1PMA	SYBR Master Mix	SYBR	25.080654
76	5.7H1-2	SYBR Master Mix	SYBR	21.078045
78	5.7H1-2HEAT	SYBR Master Mix	SYBR	Undetermined
77	5.7H1-2PMA	SYBR Master Mix	SYBR	28.116789
79	5.7H1-3	SYBR Master Mix	SYBR	21.146723

81	5.7H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
80	5.7H1-3PMA	SYBR Master Mix	SYBR	33.304466
85	5.7H2-1	SYBR Master Mix	SYBR	21.509283
87	5.7H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
86	5.7H2-1PMA	SYBR Master Mix	SYBR	25.83788
88	5.7H2-2	SYBR Master Mix	SYBR	21.02241
90	5.7H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
89	5.7H2-2PMA	SYBR Master Mix	SYBR	26.619034
91	5.7H2-3	SYBR Master Mix	SYBR	21.47946
93	5.7H2-3HEAT	SYBR Master Mix	SYBR	Undetermined
92	5.7H2-3PMA	SYBR Master Mix	SYBR	26.286842

Table 13 Ct values for all *Lactobacillus* genus level qPCR runs

Column1	Column2	Column3	Column4	Column6
SDS 2.4	AQ Results	1.0		
Filename	full_Lbrevis_run1			
PlateID				
Assay Type	Absolute Quantification			
Run DateTime	10/23/18 3:14:15 PM			
Operator				
ThermalCycleParams				
Sample Information				
Well	Sample Name	Detector Name	Reporter	Ct

25	4.12H1-1	SYBR Master Mix	SYBR	Undetermined
27	4.12H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
26	4.12H1-1PMA	SYBR Master Mix	SYBR	33.599514
28	4.12H1-2	SYBR Master Mix	SYBR	33.143234
30	4.12H1-2HEAT	SYBR Master Mix	SYBR	Undetermined
29	4.12H1-2PMA	SYBR Master Mix	SYBR	36.869125
31	4.12H1-3	SYBR Master Mix	SYBR	32.766895
33	4.12H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
32	4.12H1-3PMA	SYBR Master Mix	SYBR	Undetermined
37	4.12H2-1	SYBR Master Mix	SYBR	32.195545
39	4.12H2-1HEAT	SYBR Master Mix	SYBR	34.986603
38	4.12H2-1PMA	SYBR Master Mix	SYBR	35.298687
40	4.12H2-2	SYBR Master Mix	SYBR	33.700886
42	4.12H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
41	4.12H2-2PMA	SYBR Master Mix	SYBR	Undetermined
43	4.12H2-3	SYBR Master Mix	SYBR	33.57234
45	4.12H2-3HEAT	SYBR Master Mix	SYBR	36.46727
44	4.12H2-3PMA	SYBR Master Mix	SYBR	Undetermined
1	4.9H1-1	SYBR Master Mix	SYBR	37.50578
3	4.9H1-1HEAT	SYBR Master Mix	SYBR	34.72058
2	4.9H1-1PMA	SYBR Master Mix	SYBR	35.61808

4	4.9H1-2	SYBR Master Mix	SYBR	32.159565
6	4.9H1-2HEAT	SYBR Master Mix	SYBR	35.96974
5	4.9H1-2PMA	SYBR Master Mix	SYBR	Undetermined
7	4.9H1-3	SYBR Master Mix	SYBR	33.39173
9	4.9H1-3HEAT	SYBR Master Mix	SYBR	35.65122
8	4.9H1-3PMA	SYBR Master Mix	SYBR	34.329365
10	4.9H1-4	SYBR Master Mix	SYBR	37.16985
12	4.9H1-4HEAT	SYBR Master Mix	SYBR	23.177378
11	4.9H1-4PMA	SYBR Master Mix	SYBR	Undetermined
13	4.9H2-1	SYBR Master Mix	SYBR	Undetermined
15	4.9H2-1HEAT	SYBR Master Mix	SYBR	34.430767
14	4.9H2-1PMA	SYBR Master Mix	SYBR	36.855774
16	4.9H2-2	SYBR Master Mix	SYBR	32.746113
18	4.9H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
17	4.9H2-2PMA	SYBR Master Mix	SYBR	Undetermined
19	4.9H2-3	SYBR Master Mix	SYBR	Undetermined
21	4.9H2-3HEAT	SYBR Master Mix	SYBR	34.3896
20	4.9H2-3PMA	SYBR Master Mix	SYBR	35.0782
22	4.9H2-4	SYBR Master Mix	SYBR	32.07325
24	4.9H2-4HEAT	SYBR Master Mix	SYBR	Undetermined
23	4.9H2-4PMA	SYBR Master Mix	SYBR	35.614567

49	5.4H1-1	SYBR Master Mix	SYBR	36.922497
51	5.4H1-1HEAT	SYBR Master Mix	SYBR	34.02138
50	5.4H1-1PMA	SYBR Master Mix	SYBR	32.609043
52	5.4H1-2	SYBR Master Mix	SYBR	34.722366
54	5.4H1-2HEAT	SYBR Master Mix	SYBR	34.448208
53	5.4H1-2PMA	SYBR Master Mix	SYBR	34.218853
55	5.4H1-3	SYBR Master Mix	SYBR	32.63647
57	5.4H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
56	5.4H1-3PMA	SYBR Master Mix	SYBR	Undetermined
61	5.4H2-1	SYBR Master Mix	SYBR	Undetermined
63	5.4H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
62	5.4H2-1PMA	SYBR Master Mix	SYBR	34.02272
64	5.4H2-2	SYBR Master Mix	SYBR	33.449768
66	5.4H2-2HEAT	SYBR Master Mix	SYBR	30.55152
65	5.4H2-2PMA	SYBR Master Mix	SYBR	34.905228
67	5.4H2-3	SYBR Master Mix	SYBR	32.13696
69	5.4H2-3HEAT	SYBR Master Mix	SYBR	34.294918
68	5.4H2-3PMA	SYBR Master Mix	SYBR	33.39762
73	5.7H1-1	SYBR Master Mix	SYBR	33.127014
75	5.7H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
74	5.7H1-1PMA	SYBR Master Mix	SYBR	Undetermined

76	5.7H1-2	SYBR Master Mix	SYBR	31.46669
78	5.7H1-2HEAT	SYBR Master Mix	SYBR	33.96847
77	5.7H1-2PMA	SYBR Master Mix	SYBR	33.8117
79	5.7H1-3	SYBR Master Mix	SYBR	34.049988
80	5.7H1-3PMA	SYBR Master Mix	SYBR	35.61107
81	5.7H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
85	5.7H2-1	SYBR Master Mix	SYBR	38.108955
87	5.7H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
86	5.7H2-1PMA	SYBR Master Mix	SYBR	Undetermined
88	5.7H2-2	SYBR Master Mix	SYBR	31.358467
90	5.7H2-2HEAT	SYBR Master Mix	SYBR	36.274563
89	5.7H2-2PMA	SYBR Master Mix	SYBR	36.469143
91	5.7H2-3	SYBR Master Mix	SYBR	33.234993
93	5.7H2-3HEAT	SYBR Master Mix	SYBR	37.833145
92	5.7H2-3PMA	SYBR Master Mix	SYBR	33.868065

Table 14 Ct values for all *L. brevis* qPCR runs

Column1	Column2	Column3	Column4	Column6
SDS 2.4	AQ Results	1.0		
Filename	full_Saur_run1			
PlateID				
Assay Type	Absolute Quantification			
Run DateTime	9/25/18 5:38:18 PM			
Operator				

ThermalCycleParams				
Sample Information				
Well	Sample Name	Detector Name	Reporter	Ct
25	4.12H1-1	SYBR Master Mix	SYBR	30.072319
27	4.12H1-1HEAT	SYBR Master Mix	SYBR	Undetermined
26	4.12H1-1PMA	SYBR Master Mix	SYBR	Undetermined
28	4.12H1-2	SYBR Master Mix	SYBR	Undetermined
30	4.12H1-2HEAT	SYBR Master Mix	SYBR	35.826176
29	4.12H1-2PMA	SYBR Master Mix	SYBR	Undetermined
31	4.12H1-3	SYBR Master Mix	SYBR	34.8118
33	4.12H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
32	4.12H1-3PMA	SYBR Master Mix	SYBR	33.65415
37	4.12H2-1	SYBR Master Mix	SYBR	Undetermined
39	4.12H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
38	4.12H2-1PMA	SYBR Master Mix	SYBR	Undetermined
40	4.12H2-2	SYBR Master Mix	SYBR	35.72258
42	4.12H2-2HEAT	SYBR Master Mix	SYBR	36.1786
41	4.12H2-2PMA	SYBR Master Mix	SYBR	Undetermined
43	4.12H2-3	SYBR Master Mix	SYBR	Undetermined
45	4.12H2-3HEAT	SYBR Master Mix	SYBR	37.431053
44	4.12H2-3PMA	SYBR Master Mix	SYBR	Undetermined

1	4.9H1-1	SYBR Master Mix	SYBR	33.41118
3	4.9H1-1HEAT	SYBR Master Mix	SYBR	34.154884
2	4.9H1-1PMA	SYBR Master Mix	SYBR	33.33016
4	4.9H1-2	SYBR Master Mix	SYBR	31.4787
6	4.9H1-2HEAT	SYBR Master Mix	SYBR	Undetermined
5	4.9H1-2PMA	SYBR Master Mix	SYBR	Undetermined
7	4.9H1-3	SYBR Master Mix	SYBR	32.83658
9	4.9H1-3HEAT	SYBR Master Mix	SYBR	Undetermined
8	4.9H1-3PMA	SYBR Master Mix	SYBR	Undetermined
10	4.9H1-4	SYBR Master Mix	SYBR	34.410355
12	4.9H1-4HEAT	SYBR Master Mix	SYBR	Undetermined
11	4.9H1-4PMA	SYBR Master Mix	SYBR	Undetermined
13	4.9H2-1	SYBR Master Mix	SYBR	33.342773
15	4.9H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
14	4.9H2-1PMA	SYBR Master Mix	SYBR	Undetermined
16	4.9H2-2	SYBR Master Mix	SYBR	35.025642
18	4.9H2-2HEAT	SYBR Master Mix	SYBR	Undetermined
17	4.9H2-2PMA	SYBR Master Mix	SYBR	Undetermined
19	4.9H2-3	SYBR Master Mix	SYBR	Undetermined
21	4.9H2-3HEAT	SYBR Master Mix	SYBR	36.39218
20	4.9H2-3PMA	SYBR Master Mix	SYBR	37.27713

22	4.9H2-4	SYBR Master Mix	SYBR	34.499847
24	4.9H2-4HEAT	SYBR Master Mix	SYBR	Undetermined
23	4.9H2-4PMA	SYBR Master Mix	SYBR	Undetermined
49	5.4H1-1	SYBR Master Mix	SYBR	31.392859
51	5.4H1-1HEAT	SYBR Master Mix	SYBR	35.142548
50	5.4H1-1PMA	SYBR Master Mix	SYBR	33.4663
52	5.4H1-2	SYBR Master Mix	SYBR	32.497204
54	5.4H1-2HEAT	SYBR Master Mix	SYBR	33.81879
53	5.4H1-2PMA	SYBR Master Mix	SYBR	Undetermined
55	5.4H1-3	SYBR Master Mix	SYBR	32.628654
57	5.4H1-3HEAT	SYBR Master Mix	SYBR	33.251534
56	5.4H1-3PMA	SYBR Master Mix	SYBR	Undetermined
61	5.4H2-1	SYBR Master Mix	SYBR	Undetermined
63	5.4H2-1HEAT	SYBR Master Mix	SYBR	32.512985
62	5.4H2-1PMA	SYBR Master Mix	SYBR	32.523895
64	5.4H2-2	SYBR Master Mix	SYBR	32.62343
66	5.4H2-2HEAT	SYBR Master Mix	SYBR	32.562416
65	5.4H2-2PMA	SYBR Master Mix	SYBR	32.52301
67	5.4H2-3	SYBR Master Mix	SYBR	32.15879
69	5.4H2-3HEAT	SYBR Master Mix	SYBR	34.308754
68	5.4H2-3PMA	SYBR Master Mix	SYBR	Undetermined

73	5.7H1-1	SYBR Master Mix	SYBR	Undetermined
75	5.7H1-1HEAT	SYBR Master Mix	SYBR	36.675034
74	5.7H1-1PMA	SYBR Master Mix	SYBR	33.890522
76	5.7H1-2	SYBR Master Mix	SYBR	34.037247
78	5.7H1-2HEAT	SYBR Master Mix	SYBR	34.33268
77	5.7H1-2PMA	SYBR Master Mix	SYBR	35.4578
79	5.7H1-3	SYBR Master Mix	SYBR	31.803135
81	5.7H1-3HEAT	SYBR Master Mix	SYBR	33.768253
80	5.7H1-3PMA	SYBR Master Mix	SYBR	36.54906
85	5.7H2-1	SYBR Master Mix	SYBR	34.975388
87	5.7H2-1HEAT	SYBR Master Mix	SYBR	Undetermined
86	5.7H2-1PMA	SYBR Master Mix	SYBR	Undetermined
88	5.7H2-2	SYBR Master Mix	SYBR	30.544342
90	5.7H2-2HEAT	SYBR Master Mix	SYBR	36.491344
89	5.7H2-2PMA	SYBR Master Mix	SYBR	Undetermined
91	5.7H2-3	SYBR Master Mix	SYBR	29.737633
93	5.7H2-3HEAT	SYBR Master Mix	SYBR	Undetermined
92	5.7H2-3PMA	SYBR Master Mix	SYBR	Undetermined
34	C10	SYBR Master Mix	SYBR	23.059565
35	C11	SYBR Master Mix	SYBR	Undetermined
36	C12	SYBR Master Mix	SYBR	Undetermined

46	D10	SYBR Master Mix	SYBR	Undetermined
47	D11	SYBR Master Mix	SYBR	Undetermined
48	D12	SYBR Master Mix	SYBR	Undetermined
58	E10	SYBR Master Mix	SYBR	Undetermined
59	E11	SYBR Master Mix	SYBR	5.5917697
60	E12	SYBR Master Mix	SYBR	Undetermined
70	F10	SYBR Master Mix	SYBR	Undetermined
71	F11	SYBR Master Mix	SYBR	5.5500855
72	F12	SYBR Master Mix	SYBR	Undetermined
82	G10	SYBR Master Mix	SYBR	6.0528727
83	G11	SYBR Master Mix	SYBR	7.1014404
84	G12	SYBR Master Mix	SYBR	Undetermined
94	H10	SYBR Master Mix	SYBR	Undetermined
95	H11	SYBR Master Mix	SYBR	Undetermined
96	H12	SYBR Master Mix	SYBR	Undetermined

Table 15 Ct values for all *S. aureus* qPCR runs

Column1	Column2	Column3	Column4	Column6
SDS 2.4	AQ Results	1.0		
Filename	full_AllBact_run1			
PlateID				
Assay Type	Absolute Quantification			
Run DateTime	9/13/18 8:01:05 PM			
Operator				

ThermalCycleParams				
Sample Information				
Well	Sample Name	Detector Name	Reporter	Ct
25	4.12H1-1	SYBR Master Mix	SYBR	9.108909
27	4.12H1-1HEAT	SYBR Master Mix	SYBR	18.135227
26	4.12H1-1PMA	SYBR Master Mix	SYBR	15.798597
28	4.12H1-2	SYBR Master Mix	SYBR	8.806827
30	4.12H1-2HEAT	SYBR Master Mix	SYBR	17.854073
29	4.12H1-2PMA	SYBR Master Mix	SYBR	10.849995
31	4.12H1-3	SYBR Master Mix	SYBR	8.5762615
33	4.12H1-3HEAT	SYBR Master Mix	SYBR	16.917929
32	4.12H1-3PMA	SYBR Master Mix	SYBR	10.184611
37	4.12H2-1	SYBR Master Mix	SYBR	10.8200445
39	4.12H2-1HEAT	SYBR Master Mix	SYBR	16.948048
38	4.12H2-1PMA	SYBR Master Mix	SYBR	13.890139
40	4.12H2-2	SYBR Master Mix	SYBR	14.027044
42	4.12H2-2HEAT	SYBR Master Mix	SYBR	17.676285
41	4.12H2-2PMA	SYBR Master Mix	SYBR	15.727611
43	4.12H2-3	SYBR Master Mix	SYBR	8.679019
45	4.12H2-3HEAT	SYBR Master Mix	SYBR	18.141443
44	4.12H2-3PMA	SYBR Master Mix	SYBR	13.070644

1	4.9H1-1	SYBR Master Mix	SYBR	10.031151
3	4.9H1-1HEAT	SYBR Master Mix	SYBR	17.67226
2	4.9H1-1PMA	SYBR Master Mix	SYBR	12.504623
4	4.9H1-2	SYBR Master Mix	SYBR	9.306549
6	4.9H1-2HEAT	SYBR Master Mix	SYBR	18.753418
5	4.9H1-2PMA	SYBR Master Mix	SYBR	11.573882
7	4.9H1-3	SYBR Master Mix	SYBR	9.791849
9	4.9H1-3HEAT	SYBR Master Mix	SYBR	16.585678
8	4.9H1-3PMA	SYBR Master Mix	SYBR	12.277863
10	4.9H1-4	SYBR Master Mix	SYBR	10.067596
12	4.9H1-4HEAT	SYBR Master Mix	SYBR	17.396273
11	4.9H1-4PMA	SYBR Master Mix	SYBR	12.334222
13	4.9H2-1	SYBR Master Mix	SYBR	9.578787
15	4.9H2-1HEAT	SYBR Master Mix	SYBR	15.101679
14	4.9H2-1PMA	SYBR Master Mix	SYBR	13.028106
16	4.9H2-2	SYBR Master Mix	SYBR	9.76826
18	4.9H2-2HEAT	SYBR Master Mix	SYBR	16.370623
17	4.9H2-2PMA	SYBR Master Mix	SYBR	11.470055
19	4.9H2-3	SYBR Master Mix	SYBR	8.974521
21	4.9H2-3HEAT	SYBR Master Mix	SYBR	18.075924
20	4.9H2-3PMA	SYBR Master Mix	SYBR	12.564141

22	4.9H2-4	SYBR Master Mix	SYBR	9.427676
24	4.9H2-4HEAT	SYBR Master Mix	SYBR	16.498716
23	4.9H2-4PMA	SYBR Master Mix	SYBR	9.349171
49	5.4H1-1	SYBR Master Mix	SYBR	8.55069
51	5.4H1-1HEAT	SYBR Master Mix	SYBR	13.142514
50	5.4H1-1PMA	SYBR Master Mix	SYBR	10.39991
52	5.4H1-2	SYBR Master Mix	SYBR	9.787896
54	5.4H1-2HEAT	SYBR Master Mix	SYBR	Undetermined
53	5.4H1-2PMA	SYBR Master Mix	SYBR	12.215379
55	5.4H1-3	SYBR Master Mix	SYBR	8.233305
57	5.4H1-3HEAT	SYBR Master Mix	SYBR	19.796728
56	5.4H1-3PMA	SYBR Master Mix	SYBR	11.348471
61	5.4H2-1	SYBR Master Mix	SYBR	9.55442
63	5.4H2-1HEAT	SYBR Master Mix	SYBR	13.121004
62	5.4H2-1PMA	SYBR Master Mix	SYBR	10.485287
64	5.4H2-2	SYBR Master Mix	SYBR	9.49493
66	5.4H2-2HEAT	SYBR Master Mix	SYBR	20.042606
65	5.4H2-2PMA	SYBR Master Mix	SYBR	11.934351
67	5.4H2-3	SYBR Master Mix	SYBR	9.150478
69	5.4H2-3HEAT	SYBR Master Mix	SYBR	14.064354
68	5.4H2-3PMA	SYBR Master Mix	SYBR	12.901304

74	5.7H-1PMA	SYBR Master Mix	SYBR	12.3831835
73	5.7H1-1	SYBR Master Mix	SYBR	9.774669
75	5.7H1-1HEAT	SYBR Master Mix	SYBR	20.036318
76	5.7H1-2	SYBR Master Mix	SYBR	9.521665
78	5.7H1-2HEAT	SYBR Master Mix	SYBR	16.923782
77	5.7H1-2PMA	SYBR Master Mix	SYBR	11.455049
79	5.7H1-3	SYBR Master Mix	SYBR	9.486768
81	5.7H1-3HEAT	SYBR Master Mix	SYBR	14.628429
80	5.7H1-3PMA	SYBR Master Mix	SYBR	13.3897085
85	5.7H2-1	SYBR Master Mix	SYBR	8.82258
87	5.7H2-1HEAT	SYBR Master Mix	SYBR	16.196287
86	5.7H2-1PMA	SYBR Master Mix	SYBR	11.841925
88	5.7H2-2	SYBR Master Mix	SYBR	8.61409
90	5.7H2-2HEAT	SYBR Master Mix	SYBR	16.485937
89	5.7H2-2PMA	SYBR Master Mix	SYBR	10.995623
91	5.7H2-3	SYBR Master Mix	SYBR	9.403701
93	5.7H2-3HEAT	SYBR Master Mix	SYBR	16.37736
92	5.7H2-3PMA	SYBR Master Mix	SYBR	12.081977
34	C10	SYBR Master Mix	SYBR	Undetermined
35	C11	SYBR Master Mix	SYBR	Undetermined
36	C12	SYBR Master Mix	SYBR	Undetermined

46	D10	SYBR Master Mix	SYBR	Undetermined
47	D11	SYBR Master Mix	SYBR	37.09686
48	D12	SYBR Master Mix	SYBR	Undetermined
58	E10	SYBR Master Mix	SYBR	Undetermined
59	E11	SYBR Master Mix	SYBR	Undetermined
60	E12	SYBR Master Mix	SYBR	35.03646
70	F10	SYBR Master Mix	SYBR	Undetermined
71	F11	SYBR Master Mix	SYBR	4.175103
72	F12	SYBR Master Mix	SYBR	Undetermined
82	G10	SYBR Master Mix	SYBR	Undetermined
83	G11	SYBR Master Mix	SYBR	Undetermined
84	G12	SYBR Master Mix	SYBR	2.5095913
94	H10	SYBR Master Mix	SYBR	29.25601
95	H11	SYBR Master Mix	SYBR	Undetermined
96	H12	SYBR Master Mix	SYBR	11.542554

Table 16 Ct values for all 16S all-bacteria qPCR runs