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Testing the Effect of Grapefruit Seed Extract (GFSE) on the Mastitsis-Causing Alga, Prototheca

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TESTING THE EFFECT OF GRAPEFRUIT SEED EXTRACT (GFSE) ON THE MASTITIS-CAUSING ALGA, PROTOTHECA

by

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ABSTRACT

Mastitis is an inflammation of the mammary gland that costs the United States dairy industry between $1.7 and $2 billion annually [9]. Approximately 97% of mastitis infections are caused by bacteria, which can be treated with antibiotics [2]. However, mastitis infections can be caused by the alga, Prototheca. Specifically, it is *P. zopfii* (genotype 2) and *P. blaschkeae* that have been found in cows with mastitis [7]. There are currently no approved treatments for protothecal mastitis. The following experiments a tested the effects of grapefruit seed extract (GFSE) on *P. zopfii* (genotype 2) using spectrophotometry and plating techniques. GFSE was chosen because previous research has shown it has antimicrobial properties, it is water soluble, inexpensive, and has the potential to be a legal treatment. Prototheca isolation medium (PIM) broth was inoculated with *P. zopfii* colonies to 47% transmittance (T). Serial dilutions of GFSE were made 1:1 with distilled water (DW) starting at 4,000ug/mL down to 125ug/mL. These dilutions were mixed 1:1 with 47% PIM and incubated for 72 hours. Absorbance readings were taken at 0, 24, 48, and 72 hours, and the samples plated on blood agar to evaluate the sterility of the samples.It was found that not even the highest concentration of GFSE was effective at inhibiting the *P. zopfii* (G2) colonies. However, this study does not preclude that GFSE may be effective against *P. zopfii* growth under different conditions.

DEDICATION

I would like to dedicate this thesis to my grandfather, Charles MacDonald Jr. He was my biggest fan during my youth, and had tremendous faith in my abilities to be something great. He inspired me to follow my dreams towards veterinary school, and for that I am forever grateful. You will always be loved and missed, Papa.

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Firstly, I would like to thank Dr. Anne Lichtenwalner for advising me throughout the entire process of my Senior Capstone and my Honors Thesis. She gave me a greater appreciation for lab work and research that I never thought I would have. I really never expected to enjoy working on my project as much as I did, and I thank her for this opportunity.

Nirajan Adhikari deserves tremendous thanks for being so instrumental in experimental planning, performing the experiments, and assisting in the data analysis. He was very willing to take time away from his own graduate studies to help me in my academic career, and I am tremendously thankful for that.

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INTRODUCTION

Mastitis is an inflammation of the mammary gland that results in scarring of the mammary tissue, reduced milk production, and sometimes systemic illness [1]. Mastitis is seen most commonly in dairy cattle, and can be caused by physical trauma (about 1% of cases), or by an infection [1]. The organisms that cause this infection usually enter the udder through the teat canal [2]. The organisms kill the milk-secreting epithelial cells within the udder, which then slough off and are replaced with scar or connective tissue, thus leading to reduced milk production. Mastitis is separated in to four categories: peracute, acute, subacute, and subclinical [1]. Peracute mastitis presents as a swollen, hot, and red udder followed by systemic illness that includes fever, depression, shivering, weight loss, and sometimes death. Acute mastitis also includes severe udder inflammation, but less severe or no systemic signs. Subacute mastitis presents as a mildly infected udder, with no systemic illness. The least severe, subclinical, is when the mammary gland and cow show no outward signs, but when the milk is cultured and a somatic cell count (SCC) performed, the milk shows the presence of infectious organisms and an increase in SCC. Mastitis infections can lower production rates on dairy farms by as much as 15-20% per infected animal [2], and some farms can have infection rates of up to 70% of the cattle.

Decreased milk production, increased cost of treatments, and increased culling due to mastitis result in a dramatic decrease of profit for farmers. The biggest economic loss to farmers is from reduction in total milk produced. Consider a 500-cow herd that is averaging 50 lbs. milk/cow/day for 365 days per year. If they experience a 20% milk loss at a price of $16/100 lbs. of milk, in one year the farm would lose $58,400 due to mastitis

[2]. Mastitis incidence rates on well-managed farms are generally less than 20%, but some farms may have as many as 70% of the cattle with mastitis in at least one quarter of the udder at any one time.

The largest portions of mastitis infections, approximately 95%, are caused by bacteria such as *Streptococcus agalactiae, Staphylococcus aureus, Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, and coagulase-negative Staphylococci [2]*. Treatment options for bacterial mastitis infections include intra-mammary infusions and systemic antibiotic treatments. Antibiotics that are approved for lactating dairy cows include amoxicillin, ampicillin, ceftiofur, cepharin, cloxacillin, erythromycin, hetacillin, novobiocin, penicillin G and pirlimycin[15]. While these antibiotics are being used and for usually four to five days afterwards [15], the milk cannot be sold for human consumption. If caught selling contaminated milk the farm is rendered a severe financial penalty and must pay for the entire truck of contaminated milk. If a farm is found to violate residue laws three times within a year, the milker's permit is revoked [16]. The top reasons cited for residue violation are that the milker was too rushed, there was a new milker working that day, the treated cow just rejoined the milking herd, or there was a failure to mark the treated cows [15]. The costs of treatment, the money lost from milk withholding, and any accidental fines make treating bacterial mastitis still costly to the farmer.

The other 5% of mastitis infections are caused by other organisms, including Prototheca. Prototheca is a colorless alga that persists in wet environments containing decaying organic matter such as feces or plants [10]. There are five different species of Prototheca, but only *P. zopfii* (gentoype 2) and *P. blaschkeae* have been found within the

milk secretions of dairy cows with mastitis [7]. There is currently no treatment for protothecal mastitis, and due to its negative effect on milk quality and production, culling is usually suggested for infected cows [10]. Culling results in decreases in profits for dairy farmers, due to the cost of replacing the cow.

While the number of Prototheca-caused mastitis cases is not extremely high [10], the alga is resistant to current mastitis treatments and can be resistant to current pasteurization methods [12]. This study tests whether a naturally occurring fungicide can effectively kill common strains of Prototheca, *in vitro.* If so, steps could be made towards creating a viable treatment of protothecal mastitis, and thus Maine dairy farmers may eventually benefit by using an inexpensive treatment to avoid culling infected cattle.

Studies of many different algaecides, including amphotericin B, nystatin, polymyxin B, gentamicin and neomycin, have been conducted on Prototheca [12]. These algaecides were all tested *in vitro* and most were successful at inhibiting the Prototheca species to some degree. However, farmers cannot legally use these algaecides in dairy cattle. Farmers do not wish to lose profit by throwing away milk, or risk receiving a large fine for sending milk containing antibiotics or other chemicals into the processing plant. Instead of treating a cow with protothecal mastitis, they would either milk the cow last to avoid spreading the infection to rest of the herd via milking equipment, or they would cull the infected animal. Use of good hygiene and culling infected animals is the best route to avoid the spread of infection. However, culling the infected animals can be costly to the farmer. Therefore, finding an inexpensive way to treat a prototheca infection that allows the milk to be sold soon after treatment would be the best way to return profits to Maine dairy farmers.

Effective methods of treating Prototheca should be based on the principles of action of antimicrobials known to be effective against related organisms. Prototheca is closely related to yeast, which is a fungus containing ergosterol in its cell membrane. Ergosterol is a sterol similar in composition to cholesterol [4], providing support to the cell membrane. Compounds, such as amphotericin B [5], kill yeast by attaching to ergosterol and creating ion channels, a process called channel-mediated membrane permeabilization [5]. These channels allow ions to leak out of the cell, causing death of the cell via osmosis. The cell membrane of Prototheca is 4% ergosterol [11], suggesting that agents that kill yeast via ergosterol binding may also kill Prototheca species.

A "natural" remedy suggested to treat yeast infections in humans is grapefruit seed extract (GFSE) [3]. Grapefruit seed extract (GFSE) has antibacterial properties when diluted as much as 1:512 with only 15 minutes contact time [6] against both gram negative and gram positive organisms. It has also been suggested to be effective against 770 different types of bacteria and 93 different fungi [8]. The mechanism for GFSE activity against yeast is unknown, and some suggest it is not the GFSE that is effective against yeast, but possibly the preservative in commercially prepared GFSE [13]. Therefore, obtaining pure GFSE for experimentation purposes is ideal. The GFSE used in this experiment was purchased online, and while the package stated it was "Free of: sugar, soy, dairy, yeast, gluten, corn, and additives" there is no guarantee from the company that the GFSE is free of any chemicals from the processing.

Given that GFSE has been reported to be antifungal, it may be effective against Prototheca growth. Ideally, any anti-protothecal mastitis treatment would be water soluble, which would allow it to spread throughout the mammary system after an

intramammary infusion. GFSE is at least slightly water soluble [6], and relatively inexpensive, making it a good candidate to test in the search for an anti-protothecal treatment.

If GFSE is effective against *P. zopfii* growth during an *in vitro* trial, perhaps future trials can show positive *in vivo* effects. Ultimately, one would have to ensure that milk produced by a cow receiving GFSE treatment still follows the guidelines of the Pasteurized Milk Ordinance (PMO) set forth by the United States Public Health Service (USPHS).The ultimate goal of a practical Prototheca treatment is to effectively inhibit Prototheca growth, to have no impact on the milk quality, and to be inexpensive.

The objective was to test the efficacy of GFSE to prevent the growth of *P.zopfii, in vitro*. Our hypothesis was that GFSE will successfully inhibit *P.zopfii (*G2*)* growth because it appears to be effective against yeast, a close relative to Prototheca.

MATERIALS AND METHODS

Experiment 1

The first step to the experiment was determining what concentration of GFSE would dissolve fully in water. We could then determine the starting point for the serial dilutions of GFSE treatments. Using a graduated cylinder, we measured out 100mL of water into a beaker. The GFSE powder used was purchased online from PureBulk, Inc. (Roseburg, Oregon). The lab analysis of the GFSE can be found in Appendix 1. We started by adding 1 gram of GFSE, and stirred well with a glass rod. We then centrifuged the solution for 5 minutes at 2500rpm. We found that a pellet formed at the bottom, and thus the GFSE did not fully dissolve in the water. We repeated the process with less GFSE until we found that we could dissolve 0.4grams of GFSE in the 100mL of water

without pellet formation. Thus, we decided that we should start with a stock 4000ug/mL GFSE solution for the serial dilutions.

Experiment 2

Stock GFSE dilutions of 4000ug/mL, 2000ug/mL, 1000ug/mL, 500 ug/mL, 250ug/mL and 125ug/mL were prepared using 1:1 serial dilutions in distilled water (DW). We started with 50mL of DW and added 0.2grams of GFSE, yielding a stock solution of 4,000ug/mL. We used a heating plate and a glass stirring rod to help dissolve the GFSE fully. The GFSE was heated to boiling temperature, or around 100^{oF}. From this beaker we measured 25mL of the 4,000ug/mL solution into a graduated cylinder, and poured it into the next container. We added 25mL of DW, giving a new 2,000ug/mL solution. The container was inverted and shaken to ensure proper mixing. This dilution processed continued until we had 125ug/mL. The GFSE dilutions were then autoclaved to ensure sterility.

A *P. zopfii* (genotype 2) sample was obtained from the American Type Culture Collection (ATCC). The isolate was stored on cryobeads at -80^{o}C at The University of Maine. The *P. zopfii* was cultured on a Sabouraud dextrose agar (SDA) plate for 48hours at 28^{o}C. A colony was examined under a microscope using a wet-mount to visually conclude that *P. zopfii* cultures were indeed the organisms growing on the plate. If the organisms appeared colorless, spherical, and having multiple endospores like those seen in Figure 1, we concluded they were *P. zopfii.* After confirming we had *P. zopfii* colonies, a single colony was picked and streaked onto another SDA plate for 96 hours at 28^{o}C.

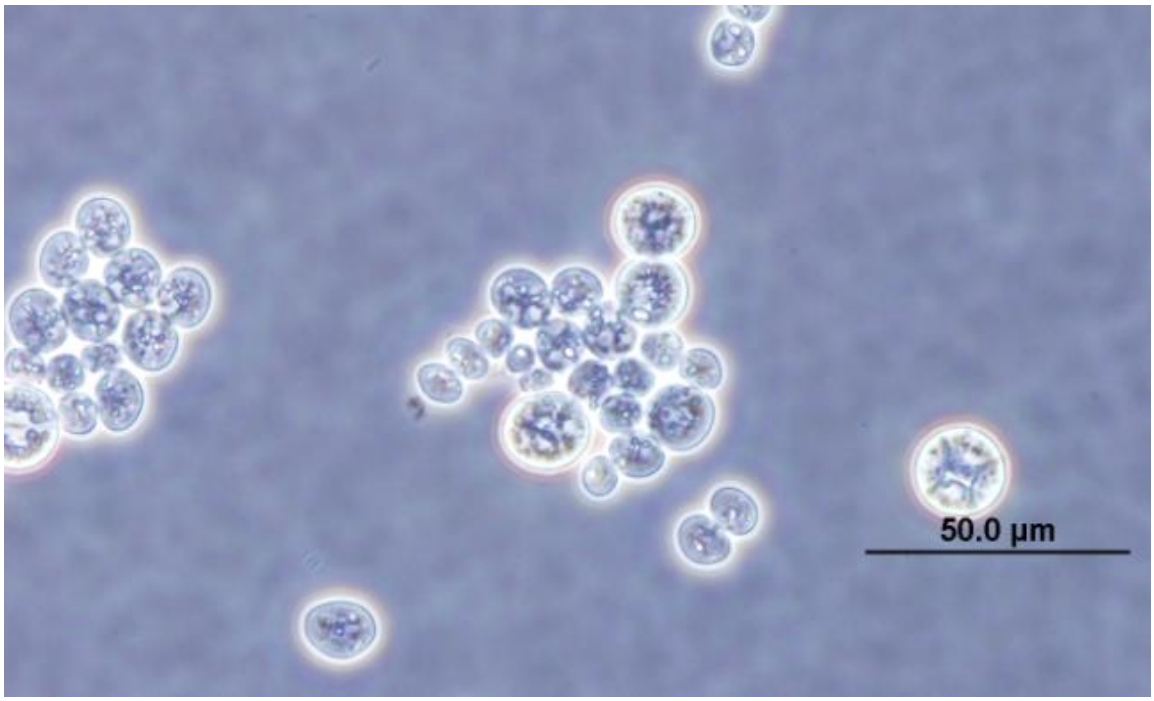

Fig.1. *P. zopfii* colonies cultured and photographed in the University of Maine Animal Health Lab in Hitchner Hall at the University of Maine.

	GSE Dilutions					
Treatments	2,000ug/mL	1,000ug/mL	500ug/mL	250ug/mL	125ug/mL	62.5ug/mL
Treatment 1	A1	B1	C1	D1	E1	F1
Treatment 2	A2	B2	C2	D2	E2	F2
Controls						
PIM 47%	Ia	Ib	Ic	Id	Ie	If
PIM 47% + Distilled Water	X1	X2	X3	X4	X5	X6
PIM ONLY	Pa	Pb	Pb	Pd	Pe	Pf
GSE ONLY	G1	G2	G3	G4	G5	G6
GSE+PIM ONLY	GP1	GP2	GP3	GP4	GP5	GP6
Distilled Water	DW	DW	DW	DW	DW	DW

Fig.2. Labeling of the glass tubes during the experiment. Tubes were covered in aluminum foil and autoclaved to ensure sterility. There were two trials of treatments performed, labeled Treatments 1 & 2. The PIM 47% and PIM 47%+Distilled Water tubes were positive controls (contained Prototheca). The PIM ONLY, GFSE ONLY, GFSE+PIM ONLY, and Distilled Water tubes were all negative controls (did not contain Prototheca).

Autoclaved tubes were set up in racks and labeled according to Figure 2 (above). We had four negative controls (Pa-Pf, G1-G6, GP1-GP6, and the 6 DW tubes). There were 6mLs of autoclaved Prototheca Isolation Media (PIM) broth pipetted into each tube

Pa-Pf (36mLs total). The PIM broth was created for another experiment in 2012, and had been refrigerated since. The ingredient list can be found in Appendix 2. We expected the PIM ONLY tubes to remain at 0 absorbance after incubation when blanked with PIM, indicating no colony growth and therefore that the PIM broth was sterile. For the next negative control, there were 3mLs of each GFSE dilution pipetted into tubes G1-G6 (18mLs total). These also served as negative controls, to ensure the sterility of the GFSE concentrations. For the third negative control, there were 3mLs each of GFSE dilution and 3mLs of autoclaved PIM broth pipetted into tubes GP1-6. These also serve as blanks in the spectrophotometer for the treatment tubes (A1A2-F1F2), because with the exception of *P. zopfii* colonies, the treatment tubes are the same as these negative controls. In the final negative control, 3mLs of autoclaved DW were pipetted into each DW tube (18mLs total), and used as a negative control to show the DW used throughout the experiment was sterile.

We also used 2 positive controls for this experiment. There were6mLs of PIM broth e pipetted in to tubes Ia-If. Using sterile loops, the PIM broth was inoculated with *P. zopfii* from the SDA plate. Using a PIM broth blank, the %Transmittance (%T) levels were read using a Bausch & Lomb Spectronic 20 spectrophotometer. All readings for this experiment were done at 570nm, and every tube that entered the spectrophotometer was wiped clean with a KimWipe® to ensure there were no contaminants on the outside of the tube that may affect the readings. The tubes were stirred with the loop, then vortexed thoroughly using a Fisher Scientific Standard Vortex Mixer at speed 6 for approximately 30seconds-1minute. The goal was to create a solution of roughly 47% T. The reason behind choosing 47% T was based on previous work done in the University

of Maine Animal Health Lab (UMAHL). The research showed that 47% T is equivalent to approximately $1x10^6$ colony forming units (CFUs), which would have been useful if we decided to do any plate counts. These 47% PIM tubes arethe positive controls, to ensure that the *P. zopfii* will grow in PIM broth after incubation.

The other positive control was tubes X1-X6, which contained half 47% PIM broth and half DW. We needed this second positive control for two reasons. The first was to show that the broth and water combination used in the treatments could indeed grow *P. zopfii*. We suspect the *P. zopfii* will still grow in this diluted PIM broth solution because the *P. zopfii* is an alga with a thick cell wall, and thus will not be susceptible to such a change in osmolarity. The second reason for this positive control is it will provide appropriate absorbance readings to compare the treatments tubes to (which are half 47% Prototheca and half DW+GFSE), because we are unsure whether the *P. zopfii* would continue to proliferate well in a more hypotonic solution than the PIM broth.

PIM broth (7mL) was then pipetted into six tubes (not pictured in Figure 1). Using a sterile loop, the PIM broth was inoculated with *P. zopfii*. Using a PIM blank, the T levels were read. All six tubes had exactly 47% T, or 0.33 absorbance (A). The first tube of 7mL was vortexed and 3mL were pipetted into treatment tube A1, and 3mL more into tube A2. This was continued with all six tubes until all treatment tubes (A1A2-F1F2) contained 3mL of 47%T PIM. Then, 3mL of each corresponding GFSE dilution was pipetted into the treatment tubes and then vortexed thoroughly. The absorbance levels were read, using the corresponding GFSE+PIM control tubes (GP1-GP6) as blanks in the spectrophotometer.

All tubes were incubated at 28°C for 24 hours. The tubes were then vortexed and absorbance levels were read and recorded for all treatment tubes (A1A2-F1F2), as well as the 47% PIM controls (Ia-If), and the 47% PIM + DW (X1-X6). The samples then continued to incubate and absorbance readings were read and recording in the same fashion at 48 and 72 hours.

At 72 hours the GFSE+PIM dilution blanks (GP1-GP6), one of each treatment tube (A1-D1,E2,F1), and three of the PIM47% controls (Ia-Ic) were plated on blood agar plates, as diagrammed in Figure 2. We used 1uL loops to streak each 1/3 of the plates. These plates were then incubated at 34°C, and observed for colony forming units (CFU) at 24 and 48 hours. We expected to see no growth on the negative controls (GP1-GP6), solely *P. zopfii* growth on the positive controls (Ia-Ic), and if the GFSE had effectively killed the *P. zopfii* colonies, and no growth on the treatments (A1-D1, E2, F1) would indicate the GFSE had effectively killed the *P. zopfii*. At 72 hours we used wet-mounts under a microscope to observe any colony growth.

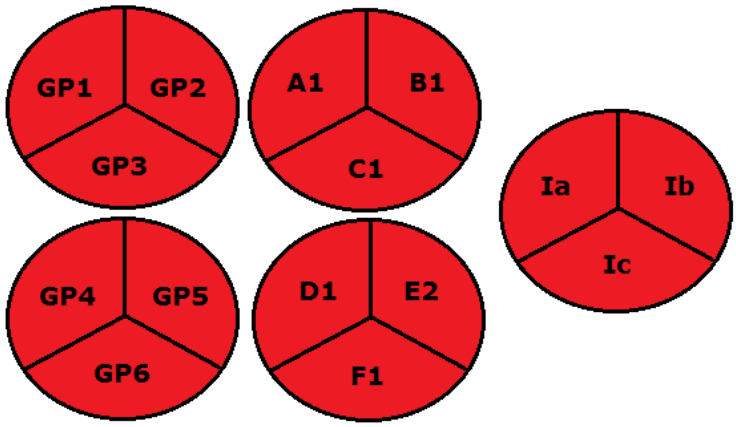

Fig. 3. This is a diagram of the blood agar plates. We used sterile 1uL loops to streak each 1/3 of the plates. The plates were observed at 24, 48, and 72 hours for abnormal colony growth, which would indicate the solutions harbored organisms other than *P. zopfii*. At 72 hours any abnormal colonies were observed using a wet-mount under a microscope, and the assumed *P. zopfii* colonies from A1-E2, F1 and Ia-Ic were also checked under the microscope to ensure they were in fact *P. zopfii* colonies.

Experiment 3

This experiment was performed using the exact same procedures as Experiment 2, except the GFSE solutions were not autoclaved. Subjecting the GFSE to the extreme temperatures in the autoclave may have altered the components in the GFSE, therefore we used filtration to sterilize the GFSE rather than autoclaving it. The same serial dilutions of GFSE were made in DW as in Experiment 2. The solutions were then run through Corning Incorporated 0.2um sterile syringe filters into sterile tubes. A single *P. zopfii* colony from the previously used cultures was streaked onto a fresh SDA plate and incubated for 48hours at 28^{o}C.

Once again, 2 sets of 6 sterile treatment tubes were set up in a rack (A3A4-F3F4). We created 6 more tubes with 7mL of 47% PIM broth from the colonies on the new SDA plate. We used the inoculating and spectrophotometry techniques from Experiment 2 to create a prototheca concentration of 47% T. From these 6 tubes, we pipetted 3mL of 47% PIM into each of the 12 treatment tubes. We then pipetted 3mL of each filtered GFSE dilution into corresponding tubes. The tubes were then vortexed thoroughly and incubated for 72 hours at 28^{o}C.

As was done in Experiment 2, we then created individual blanks for the treatment tubes. We set up 6 tubes for blanks (GP1-GP6). We pipetted 3mL of sterilized PIM broth into them. Next we pipetted 3mL of the sterilized GFSE dilution into the blank tubes. We used these blanks for each corresponding GFSE treatment. Each tube placed in the spectrophotometer was wiped clean with a KimWipe®, and read at 570nm. We recorded the absorbance readings for the treatments using these blanks.

We also used the same positive and negative controls for this experiment as in Experiment 2. The positive controls to ensure sterility were once again 6 tubes of 6mLs of 47% PIM broth (Ia-If), and 6 tubes of 3mLs of 47%PIM : 3mL of DW (X1-X6). The negative controls were 6 tubes of 6mLs of sterilized PIM broth (Pa-Pf), and the 6 tubes of 3mL of GFSE dilutions and 3mL of PIM broth (the blanks for the treatments).All samples were incubated at 28^{o}C for 72 hours, and both and absorbance readings were done at 24 hour intervals.

At 72 hours the GFSE serial dilutions (4,000ug/mL – 125ug/mL), one of each treatment tube (A3, B4, C3, D4, E3, F4), and three of the PIM47% controls (Ia-Ic) were plated on blood agar plates, as diagrammed in Figure 4. We used 1uL loops to streak each 1/3 of the plates. These plates were then incubated at 34^{o}C, and observed for colony forming units (CFU) at 24 and 48 hours. We expected to see no growth on the negative controls (GP1-GP6), solely *P. zopfii* growth on the positive controls (Ia-Ic), and if the GFSE had effectively killed the *P. zopfii* colonies, there would also be no growth on the treatments (A3B4, etc). At 72 hours we used wet-mounts under a microscope to observe any colony growth.

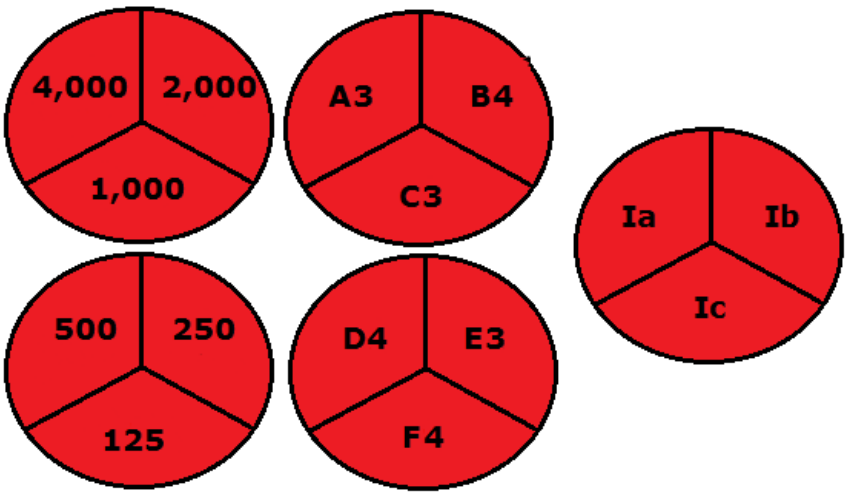

Fig. 4. This is a diagram of the blood agar plates. We used sterile 1uL loops to streak each 1/3 of the plates. The plates were observed at 24, 48, and 72 hours for abnormal colony growth, which would indicate the solutions harbored organisms other than *P. zopfii*. At 72 hours any abnormal colonies were observed using a wet-mount under a microscope, and the assumed *P. zopfii* colonies from A1-E2, F1 and Ia-Ic were also checked under the microscope to ensure they were in fact *P. zopfii* colonies.

RESULTS

Experiment 2

Figure 5 shows that the absorbance of the first positive control, the 47% PIM, continued to increase linearly over the course of incubation.

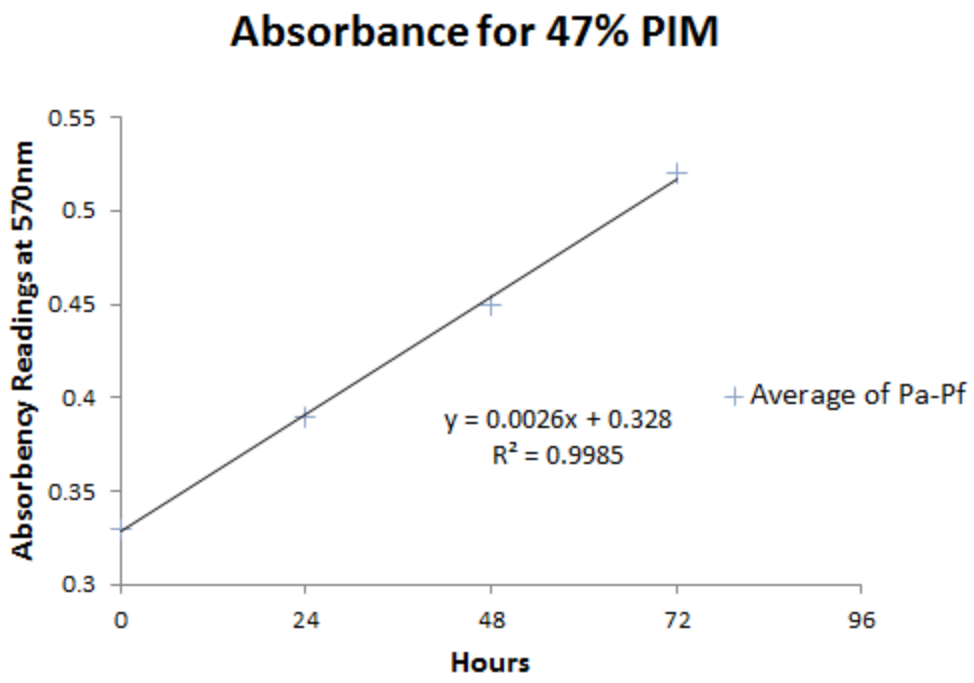

Fig. 5. The absorbance of the positive control, 47% PIM, continued to increase over the 72 hours of incubation. Only the average of the six controls (Pa-Pf) has been graphed, and a linear best-fit line was applied.

The other positive control used in this experiment was 47% PIM + DW (X1-X6) in a 1:1 ratio. Similar to the previous positive control, only the average of the absorbance readings was graphed. As seen in Figure 6, the absorbance readings increased in a linear fashion, with an R^2 value of 0.999 and a slope of 0.00224 A/hr.

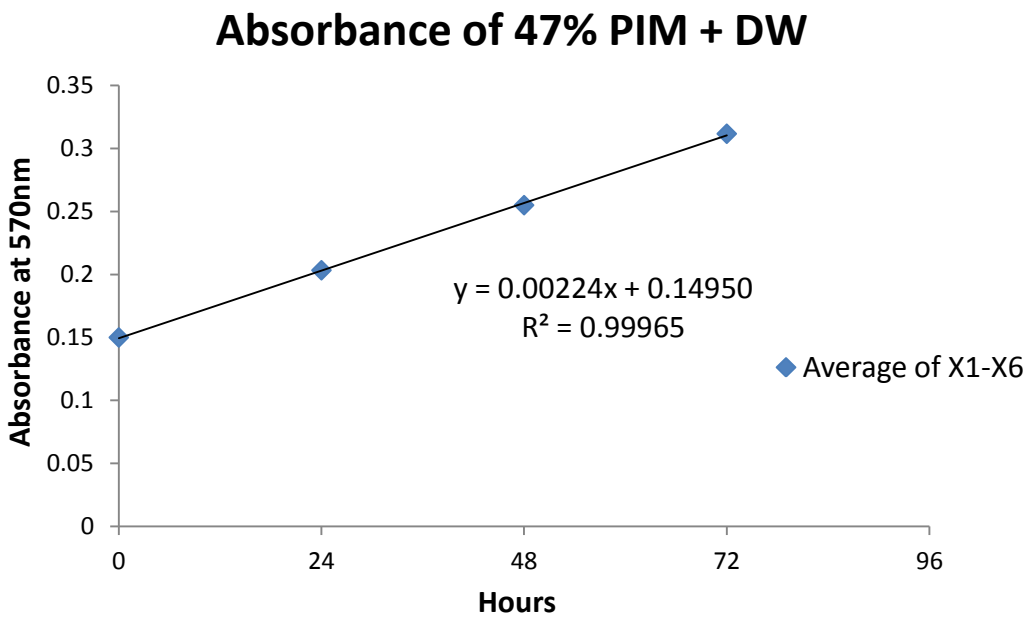

Fig. 6. The average absorbance readings for the positive controls (X1-X6) increased in a linear fashion over the 72 hours of incubation. The linear best-fit line has an R2 value of 0.999 and an equation of y=0.00224x + 0.1495. The slope of these absorbance readings will be compared to the slopes of the treatment tubes (A1A2-F1F2).

As seen in Figure 7, the absorbance of the GFSE treatments increased during incubation. The absorbances of the two trials of each treatment concentration were averaged before being graphed. Linear best-fit lines using Microsoft Excel were applied to each GFSE treatment absorbance reading (not pictured). The 47% positive control was also graphed. The slope values of each GFSE treatment were compared to the slope values of the 47% PIM + DW positive control in Table 1.

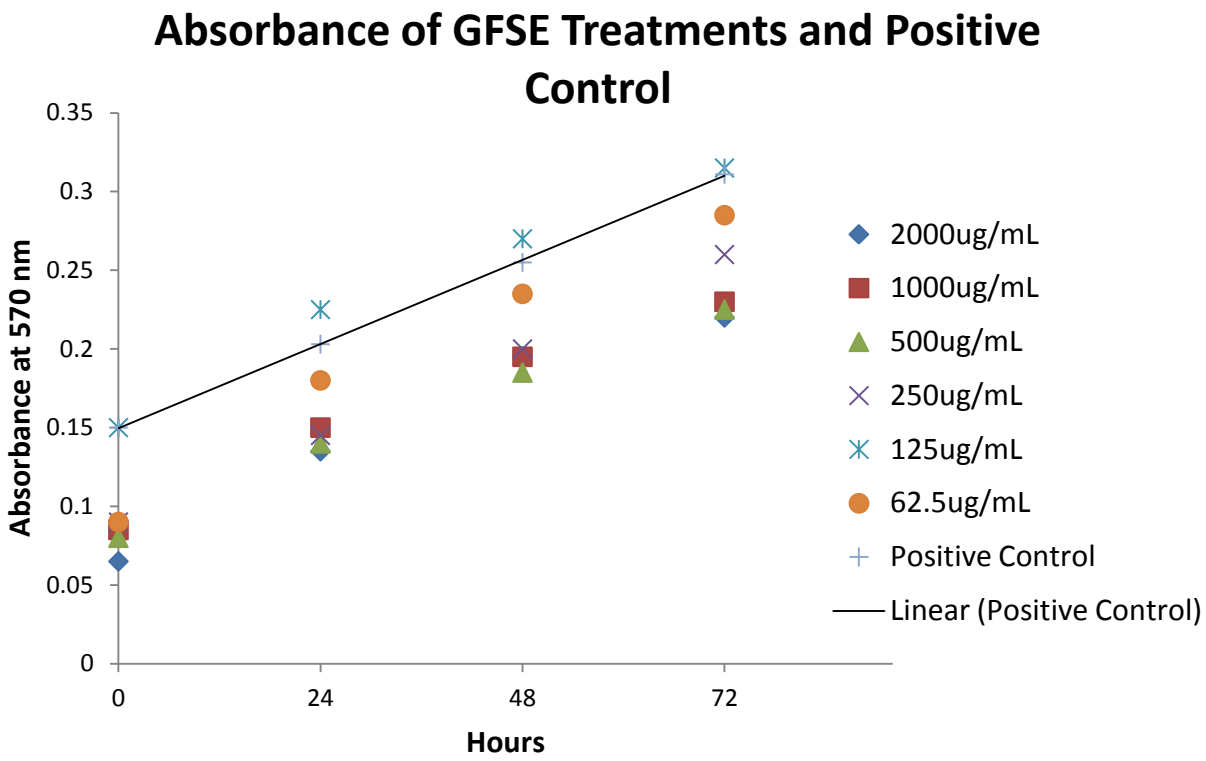

Fig. 7. The absorbance readings for two trials of each treatment were averaged and then graphed. The GFSE treatment absorbances increased linearly during incubation. The positive control is graphed for comparison, and the best-fit linear line is shown to emphasize the general slope. The slopes of the best-fit lines are compared to that of the positive control in Table 1.

Table 1. Comparing the slopes of the absorbance readings of the treatments to the slopes of the absorbance readings of the 47% PIM + DW controls.

47% PIM + DW Controls	Slope (A/hr)	#STD Away from Mean	Treatments	Slope (A/hr)	# STD Away from Mean	Average
X1	0.00208	-1.468	2000ug/mL	0.00200	-0.106	0.615
X2	0.00221	-0.266	2000ug/mL	0.00238	1.337	
X3	0.00238	1.337	1000ug/mL	0.00192	-3.071	-2.269
X4	0.00233	0.936	1000ug/mL	0.00208	-1.468	
X5	0.00221	-0.266	500ug/mL	0.00179	-4.272	-2.269
X6	0.00221	-0.266	500ug/mL	0.00221	-0.266	
Average	0.002236		250ug/mL	0.00196	-2.670	-0.066
Standard Deviation	0.000104		250ug/mL	0.00250	2.538	
			125ug/mL	0.00217	-0.667	0.135
			125 ug/mL	0.00233	0.936	
			62.5ug/mL	0.00233	0.936	0.935897
			62.5 ug/mL	0.00233	0.936	

The slope values for 47%PIM + DW treatments were calculated using Excel. The average and standard deviation were found. The slopes of the treatments were then calculated in a similar manner, and compared to the standard deviation of the control. The numbers of standard deviations from the mean of the two similar treatments were then averaged.

The % T and A readings of the negative control, PIM ONLY broth, (Pa-Pf) showed no change after incubation.

The blood agar plate with Ia-Ic showed growth in all three thirds. All the colonies were uniform (small, greyish-clear colonies), and upon examination under a microscope using a wet mount, they appeared to be *P. zopfii* as they appeared similar to those shown in Figure 1. There was a single yellowish colony in Ic, that was considered to be a contaminant from the plating process, as it was not widespread throughout the plate.

The blood agar plates with GP1-GP6 showed no growth, except another small yellowish colony in GP2. This also was considered to be a contaminant from the plating process as well, as there was just a single colony.

The final blood agar plates, containing A1-D1, E2, and F1, showed what appeared to be solely *P. zopfii* growth (small, greyish clear colonies). Upon examination under the microscope using a wet-mount they appeared to be *P. zopfii*.

Experiment 3

Comparable to Experiment 2, the absorbance readings of the positive control, 47% PIM, increased linearly over time in Figure 8. The exponential trend line did not seem to fit as accurately as in Experiment 2 (see Figure 4), as indicated by the R^2 value.

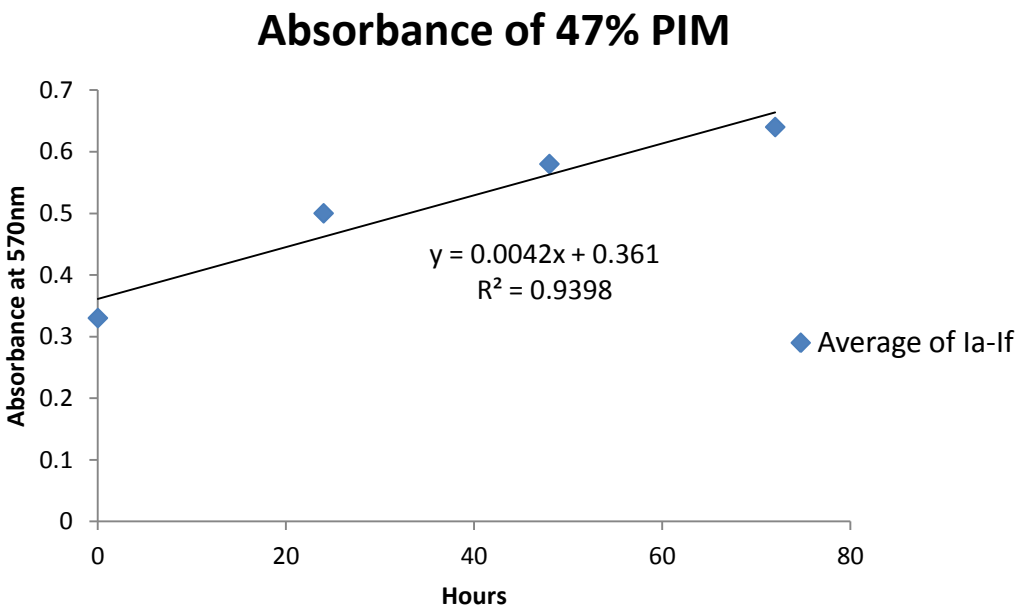

Fig. 8. The absorbance for the positive controls in Experiment 3 also continued to increase over time. The linear trend-line did not fit as well, as indicated by the R^2 value, and the data suggests the *P. zopfii* colonies grew extremely fast during the initial 24 hour incubation period.

Figure 9 shows the absorbance readings of the 47% PIM + DW increasing linearly, as in Experiment 2. Again, a best-fit linear line was applied, and the slope value was compared to that of the GFSE treatments.

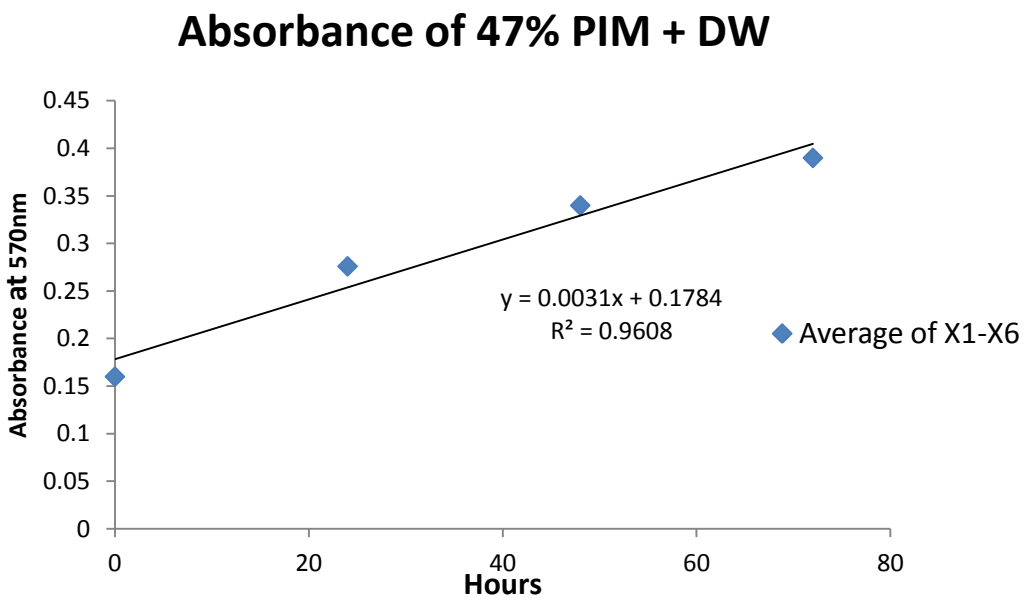

Fig. 9. The absorbance readings for 47% PIM + DW increased in a linear fashion during incubation. This slope is used in comparison with the treatments from Experiment 3.

Figure 10 contains the absorbance readings from the GFSE treatments. The slopes of the linear best-fit lines from Figure 10 are compared to the positive control slope (also pictured). These comparisons are seen in Table 2.

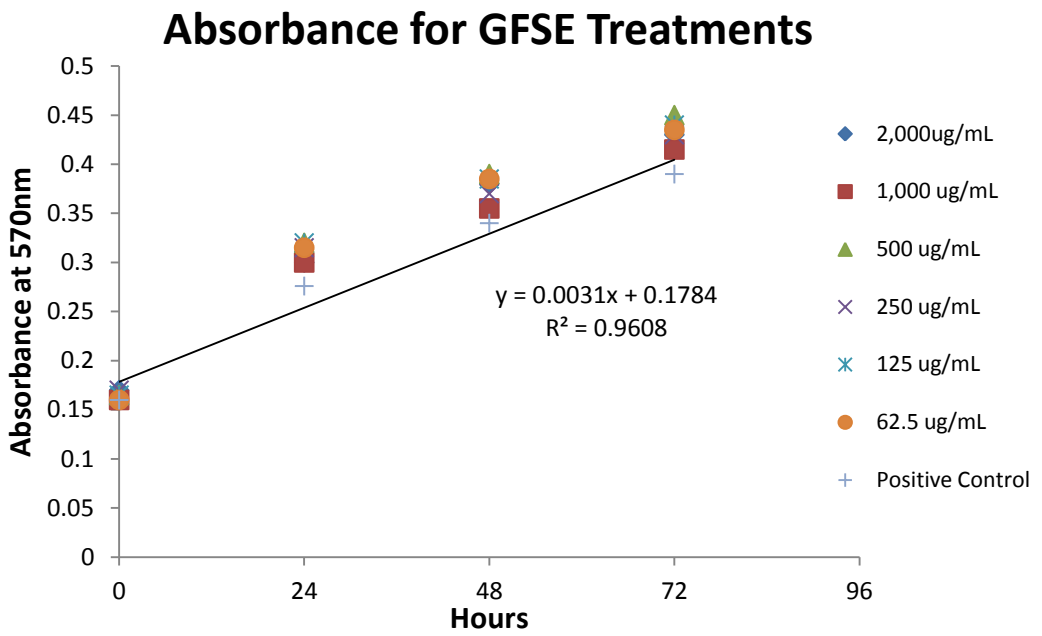

Fig. 10. The absorbance readings of the GFSE treatments increase linearly over the incubation period. The slopes of the best-fit lines are compared to the slope of the best-fit line of the positive control, 47% PIM + DW. This is included on the graph, and a best-fit line has been applied.

Table 2.

47% PIM + DW Controls	Slope (A/hr)	#STD Away from Mean	Treatments	Slope (A/hr)	# STD Away from Mean	Average
X1	0.00317	-1.693	2000ug/mL	0.003583	-0.1722	-0.1722
X2	0.00366	0.14	2000ug/mL	0.003583	-0.1722	
X3	0.00356	-0.2419	1000ug/mL	0.003333	-1.0879	-0.7821
X4	0.004	1.362	1000ug/mL	0.0035	-0.4762	
X5	0.00363	-0.01273	500ug/mL	0.004083	1.65934	0.82051
X6	0.00375	0.4456	500ug/mL	0.003625	-0.0183	
			250ug/mL	0.0035	-0.4762	-0.5531
Average	0.00363		250ug/mL	0.003458	-0.63	
Standard Deviation	0.000273		125ug/mL	0.0035	-0.4762	0.28755
			125 ug/mL	0.003917	1.05128	
			62.5ug/mL	0.003667	0.13553	0.36447
			62.5 ug/mL	0.003792	0.59341	

The slope values for 47%PIM + DW treatments were calculated using Excel. The average and standard deviation were found. The slopes of the treatments were then calculated in a similar manner, and compared to the standard deviation of the control. The numbers of standard deviations from the mean of the two similar treatments were then averaged.

The PIM ONLY tubes from Experiment 3 showed no change in absorbance, and thus were considered sterile.

The blood agar plate with Ia-Ic showed what appeared to be a lawn of *P. zopfii* colonies after 48 hours of incubation. There were no other colonies other than the small, white greyish ones associated with *P. zopfii*. The blood agar plates with the standard stock solution showed no colony growth. The final blood agar plates, containing the treatments (A3B4, etc) also showed a lawn of *P. zopfii*-like growth, and no other colonies.

DISCUSSION AND CONCLUSION

Experiment 2

Figure 5 and theblood agar plate results illustrate that PIM broth facilitates the growth of *P. zopfii*. The increasing absorbance suggests the *P. zopfii* continued to increase in turbidity, thus increasing in numbers after incubation. Also, once Ia-Ic were plated on blood agar, all the colonies appeared to be *P. zopfii* colonies, and the absorbance readings were not due to the growth of other colonies. The other positive control, the 47% PIM + DW also showed an increase in absorbance levels after incubation, as shown in Figure 6. This indicates the *P. zopfii* was able to proliferate in a more hypotonic solution than just PIM broth. When the slopes of the 47%T PIM and 47%T PIM + DW are compared, there is very little difference, although it appears the additional water may have slowed the *P. zopfii* growth slightly. Thus, we can conclude the *P. zopfii* was able to proliferate when the 47% PIM was combined in a 1:1 ratio with DW, and we now have absorbance readings to compare the GFSE treatments to.

The PIM ONLY tubes, one of the negative controls, showed no change in absorbance, indicating sterility. Another negative control, the GFSE+PIM broth (GP1-GP6), once plated on blood agar, proved to be sterile (except for the single contaminant assumed to be from plating). The blood agar plates were used instead of SDA or PIM plates because blood agar plates facilitate the growth of most all organisms. Therefore, they would be a better indicator of contamination over SDA or PIM plates that only facilitate growth of certain organisms. This means the GFSE treatments were all sterile, and that GP1-GP6 were appropriate blanks for the GFSE treatment tubes (A1A2-F1F2). We can also infer the GFSE negative controls (G1-G6) were sterile (since they came from the same stock GFSE solutions as the GP1-GP6 tubes), so we did not plate them on blood agar.

Figure 7 indicates that *P. zopfii* continued to proliferate in PIM broth regardless of the presence of GFSE. When compared to the positive control, it appears the slope of the GFSE treatments is similar to that of the control, even though the y-intercepts are quite different. We believe this was due to improper mixing of the 47% PIM before it was pipetted into the treatment tubes. Also, it can be inferred from the plating of the GFSE treatment tubes that the organisms that were continuing to grow were indeed *P. zopfii*, and not some contaminant. Table 1 allows us to infer that the presence of the GFSE, in any of the tested concentrations, had no effect on the *P. zopfii* growth.

Table 1 shows the calculated best-fit slopes of the positive control, 47% PIM +DW. The average of these slopes was found, as well as the standard deviation. The slopes of the treatment tubes were also found, and compared to the average slope of the controls by using the standard deviations. Typically, anything within 2 standard

deviations is considered within the "normal" limits. The slope of the highest concentration of GFSE has an average # of standard deviations of less than 1from the mean, meaning it is not different from that of the control. This means the growth rate of the *P. zopfii* subjected to the highest level of GFSE is not different from the growth rate of the *P. zopfii* control. The 1,000ug/mL and 500ug/mL treatments have an average # of standard deviations from the mean of over 2. This would make us assume the growth rate of the *P. zopfii* in these treatments is different from that of the control. However, because the higher concentration did not seem to inhibit *P. zopfii* growth, it seems unusual for a lower concentration to slow the growth rate. We believe these results are due to the fact we only have 2 trials of the treatments, and it is likely that with more trials the average slope would be within 2 standard deviations from the slope of the controls. The lower concentration treatments are all also within 1 standard deviation of the control, suggesting the growth rate of the *P. zopfii* in the treatments are similar to the growth rate of the *P. zopfii* the control. However, with our limited number of trials we do not have enough data to accurately perform a statistical analysis.

Experiment 3

The difference between Experiment 2 and Experiment 3 was that the GFSE concentrations were sterilized with a syringe filter instead of being autoclaved. Figure 7 and the blood agar plates illustrate the PIM broth supported the growth of the *P. zopfii.* The absorbance readings increased linearly during incubation, and once Ia-Ic were plated on blood agar, it was shown the colonies were all *P. zopfii*. Theother positive control, the 47% PIM + DW also showed increasing absorbance levels after incubation, as shown in Figure 8. Thus, we can conclude the *P. zopfii* was able to proliferate when the 47% PIM

was combined in a 1:1 ratio with DW, and we had absorbance readings to compare the GFSE treatments to.

The PIM ONLY tubes, one of the negative controls, did not show any change in absorbance after incubation. This means the PIM media was sterile, and the filtration did indeed sterilize the GFSE solutions. Once we plated the serial dilutions of GFSE on blood agar, they showed no culture growth. This means the filters also worked to sterilize the GFSE treatments.

The information in Figure 10 confirmed *P. zopfii* continued to proliferate in the PIM broth regardless of the presence of GFSE because the absorbance readings continued to increase after incubation. Also, it can be inferred from the plating of the GFSE treatment tubes that the organisms that were continuing to grow were indeed *P. zopfii*, and not some contaminant. The absorbance rates were increasing in a nearly identical fashion for all treatments, thus suggesting that the presence of the GFSE did not affect the growth rate. Table 2 allows us to infer that the presence of the GFSE, in any of the tested concentrations, had no effect on the *P. zopfii* growth.

Table 2 shows the calculated best-fit slopes of the positive control, 47% PIM +DW. The average of these slopes was found, as well as the standard deviation. The slopes of the treatment tubes were also found, and compared to the average slope of the controls by using the standard deviations. Once again, anything within 2 standard deviations is considered within the "normal" limits. The highest concentration of GFSE has an average # of standard deviations of less than 1, meaning it considered similar to the control. This means the growth rate of the *P. zopfii* subjected to the highest level of GFSE is not different from the growth rate of the *P. zopfii* control. In fact, if you look at

slopes all of the treatments, they are all within 1 standard deviation of the average of the controls. Thus, it is safe to say, the growth rate of the *P. zopfii* in all the treatment tubes was equivalent to that of the growth rate in the positive control 47% PIM + DW, and thus the GFSE concentrations had no effect on the growth rate of the *P. zopfii*.

Experiment 3 was run because we realized we may have rendered the antimicrobial compounds in the GFSE inactive when we autoclaved it in Experiment 2. We autoclaved the GFSE because we did not know if it was sterile or not, and did not want other organisms to grow in the PIM broth and effect the spectroscopy results. However, we neglected to realize that by heating the GFSE solutions to 212^{o}F, we were possibly inactivating compounds within the GFSE. Thus, while there was technically GFSE in with the *P. zopfii*, there is a possibility the GFSE was no longer capable of killing the *P. zopfii*. Instead of autoclaving the GFSE solutions, we decided to redo the experiment, and to run the GFSE dilutions through a 0.2micron filter. The filter would "catch" any bacteria, and thus sterilize the GFSE without denaturing any compounds within it. However, it was simply a hypothesis that we rendered the GFSE ineffective by autoclaving it.

Realistically, there is a chance the GFSE compounds were still intact during Experiment 2, and we realized that we did not have a control to prove whether the GFSE was rendered ineffective. However, it was noted that the color of the GFSE after being filtered was much lighter than the autoclaved GFSE. We are unsure of whether this is because autoclaving the GFSE darkened the color (thus suggesting the compounds may have been affected by autoclaving), or if perhaps the filters were catching enough of the GFSE to cause a reduction in color. The filters did indeed prevent a significant amount of

GFSE from passing through, as indicated by the difficulty in pushing down the plunger of the syringe, and by the filter turning slightly reddish in color. Whether the filters were catching the "active ingredients" in the GFSE is unknown, thus the concentrations of GFSE in Experiment 3are not guaranteed accurate due to the filtering process. However, the colors indicated there was indeed more GFSE in the highest concentration treatment, and we can say the growth rate of the *P. zopfii* did not appear different from that of the treatment in Experiment 3.

Overall, the data does not support our hypothesis that GFSE inhibits the growth of *P. zopfii.* There are three main reasons why we may have obtained the data we did. Firstly, perhaps GFSE does not have any antimicrobial properties at all. Many researchers have suggested that it is the chemicals used to extract the GFSE that can cause it to be antimicrobial against other bacteria and fungi [13], not the actual compounds found in the GFSE. To determine this we should have run the experiment on bacteria that are easier to kill than the algae *P. zopfii*, such as *E. coli*. If we had done this during Experiment 2 with the autoclaved GFSE, and the GFSE inhibited *E. coli* growth, then we would have known the GFSE does have some form of antimicrobial properties. We also would have known the GFSE was still activated after it was autoclaved and thus would not have had to run Experiment 3.

If the GFSE was effective against the *E. coli* and not the *P. zopfii*, we would know that the concentrations of GFSE used were simply ineffective against the wall or some other aspect of the *P. zopfii* cells. This would be the second reason why we obtained the results we did.

Another possibility is that perhaps we were not using a high enough concentration of GFSE to effectively kill the *P. zopfii*. We attempted to use the highest concentration that would fully dissolve into the DW. However, we also could have used a different solvent to increase the concentration of the GFSE. This tactic may have resulted in a solution that is not a viable treatment for intramammary infusion for dairy cattle mastitis, because the treatment would not disperse well through the water (milk) in the cow's udder. Thus, we did not explore this experimental route. Alternatively, we could have altered the pH of the solution to increase the amount of GFSE that would dissolve in solution. Because the pH of the GFSE solution was between 4-7, that means the GFSE is slightly acidic. Had we added a base to the DW, we could have increased the amount of GFSE that could be dissolved in the DW and thus increased the concentration. However, we would have wanted to keep the pH between 5 and 7, because this is the optimum pH for *P. zopfii* growth.

Future research would require ensuring the GFSE does indeed have some antimicrobial properties, by testing it against some bacteria such as *E. coli*. If the GFSE is found to possess antimicrobial properties, we could attempt to increase the GFSE concentration using buffers. Once the highest concentration is reached and tested against the *P. zopfii*, we could attempt to use a different solvent to increase the concentration even more to sufficiently support or disprove the if GFSE is effective against Prototheca growth. As mentioned, the only problem with this is the solvent may not be sufficient for intramammary injection.

DATA TABLES

Experiment 2

Table 3. Absorbance (A) of GFSE treatments

Treatments	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
A1	0.06	0.12	0.18	0.2
A2	0.07	0.15	0.21	0.24
B1	0.07	0.13	0.17	0.21
B2	0.1	0.17	0.22	0.25
C1	0.08	0.13	0.17	0.21
C2	0.08	0.15	0.2	0.24
D1	0.08	0.12	0.17	0.22
D2	0.1	0.17	0.23	0.28
E1	0.11	0.19	0.23	0.27
E2	0.19	0.26	0.31	0.36
F1	0.08	0.16	0.21	0.25
F2	0.1	0.21	0.26	0.32

The spectrophotometer was blanked with GP1-GP6 for each corresponding dilution of GFSE (A1,A2-F1,F2). The spectrophotometer was set to 570nm. Based on the continual decrease of T, it can be concluded that the prototheca continued to grow, and thus the different concentrations of GFSE had no effect on protothecal growth.

Table 4. Absorbance of 47% PIM

47% PIM	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
Ia	0.34	0.35	0.44	0.48
Ib	0.33	0.37	0.45	0.5
Ic	0.335	0.4	0.46	0.51
Id	0.33	0.42	0.45	0.54
Ie	0.34	0.43	0.45	0.55
If	0.33	0.39	0.45	0.5

The spectrophotometer was blanked with PIM broth and was set to 570nm. Based on the continual increase of A, it can be concluded that the prototheca continued to grow, and thus was a successful positive control.

Table 5. Absorbance of 47% PIM + Distilled Water

47% PIM + DW	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
X1	0.15	0.2	0.25	0.3
X2	0.15	0.2	0.25	0.31
X3	0.15	0.21	0.27	0.32
X4	0.15	0.21	0.26	0.32
X5	0.15	0.2	0.25	0.31
X6	0.15	0.2	0.25	0.31

The spectrophotometer was blanked with 3mL of PIM broth and 3mL of distilled water and was set to 570nm. Based on the continual increase of A, it can be concluded that the prototheca continued to grow, and thus was a successful positive control.

Table 6. Absorbance of PIM ONLY

PIM ONLY	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
Pa	1	N/A	1	N/A
Pb	1	N/A	1	N/A
Pc	1	N/A	1	N/A
Pd	1	N/A	1	N/A
Pe	1	N/A	1	N/A
Pf	1	N/A	1	N/A

The spectrophotometer was blanked with PIM broth and was set to 570nm. Based on the consistent absorbency readings, it can be inferred that: there was no growth within the PIM broth, and thus was a successful positive control.

Experiment 3

Table 7. Absorbance (A) of GFSE treatments

Treatments	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
A1	0.17	0.3	0.38	0.43
A2	0.17	0.3	0.38	0.43
B1	0.16	0.3	0.35	0.41
B2	0.16	0.3	0.36	0.42
C1	0.16	0.32	0.4	0.46
C2	0.17	0.32	0.38	0.44
D1	0.17	0.31	0.37	0.43
D2	0.17	0.32	0.37	0.43
E1	0.16	0.31	0.37	0.42
E2	0.17	0.33	0.4	0.46
F1	0.16	0.31	0.28	0.43
F2	0.16	0.32	0.39	0.44

The spectrophotometer was blanked with GP1-GP6 for each corresponding dilution of GFSE (A1,A2-F1,F2). The spectrophotometer was set to 570nm. Based on the continual decrease of T, it can be concluded that the prototheca continued to grow, and thus the different concentrations of GFSE had no effect on prototheca growth.

Table 8. Absorbance of 47% PIM

47% PIM	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
Ia	0.33	0.5	0.6	0.66
Ib	0.33	0.5	0.61	0.64
Ic	0.33	0.5	0.59	0.64
Id	0.33	0.5	0.56	0.62
Ie	0.33	0.5	0.58	0.64
If	0.33	0.5	0.56	0.64

The spectrophotometer was blanked with PIM broth and was set to 570nm. Based on the continual increase of A, it can be concluded that the prototheca continued to grow, and thus was a successful positive control.

Table 9. Absorbance of 47% PIM + Distilled Water

47% PIM + DW	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
X1	0.16	0.28	0.35	0.4
X2	0.16	0.28	0.35	0.4
X3	0.15	0.27	0.33	0.38
X4	0.15	0.28	0.34	0.4
X5	0.16	0.28	0.34	0.4
X6	0.16	0.27	0.33	0.37

The spectrophotometer was blanked with 3mL of PIM broth and 3mL of distilled water and was set to 570nm. Based on the continual increase of A, it can be concluded that the prototheca continued to grow, and thus was a successful positive control.

Table 10. Absorbance of PIM ONLY

PIM ONLY	A at 0 hours	A at 24 hours	A at 48 hours	A at 72 hours
Pa	1	N/A	N/A	1
Pb	1	N/A	N/A	1
Pc	1	N/A	N/A	1
Pd	1	N/A	N/A	1
Pe	1	N/A	N/A	1
Pf	1	N/A	N/A	1

The spectrophotometer was blanked with PIM broth and was set to 570nm. Based on the consistent absorbency readings, it can be inferred that: there was no growth within the PIM broth, and thus was a successful positive control.

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APPENDIX 1

Certificate of Analysis

Product Name:	**Grapefruit Seed Extract 10:1**
Botanical Name:	***Citrus Grandus***
Country of Origin:	**China**
Batch Number:	**20110610**
Manufacture Date:	**Aug. 25, 2011**
PureBulk Lot Number:	**20120323, 20120705**
Test Date:	**Mar. 12, 2012**
Retest Date:	**Aug. 24, 2014**

Test Results Obtained by PureBulk

ITEMS	SPECIFICATIONS	RESULTS
Identification (TLC)	Positive ID	Confirmed
Identification (ATR FT-IR)	Positive ID	Confirmed

The following information is an indirect translation of information provided from the Manufacturer's Certificate o Analysis, and should not be used solely as an instrument for strict quality control.

ITEMS	SPECIFICATIONS	RESULTS
Color	Reddish	Conforms
Odor	Characteristic	Conforms
Appearance	Fine Powder	Conforms
Ratio Extract	NLT 10:1	Conforms
Mesh Size	90% through 80 mesh	Conforms
Loss on Drying	NMT 10%	8%
Bulk Density	40~0g/100ml	Conforms
Residue of Solvents	NMT 5,000ppm	Conforms
Heavy Metals	NMT 20.0ppm	< 20.0ppm
Lead (Pb)	NMT 2.0ppm	< 2.0ppm
Arsenic (As)	NMT 2.0ppm	< 2.0ppm
Cadmium (Cd)	NMT 1.0ppm	< 1.0ppm
Mercury (Hg)	NMT 1.0ppm	< 1.0ppm
Pesticide Residues	Meet USP <561>	Conforms
Total Plate Count	NMT 10,000cfu/g	Conforms
Yeast & Mold	NMT 1,000cfu/g	Conforms
E.Coli	Negative	Conforms
Salmonella	Negative	Conforms
Staphylococcus	Negative	Conforms
Conclusion:	This product conforms to enterprise standards.	

Storage Conditions: **Keep in sealed container and under 25°C.**
Keep away from strong light, heat and moisture.

Purebulk.Inc verifies that the information contained herein is true and correct to the best of our knowledge.

Verified By: Kaitlyn Lindorfer / D. Gilbert

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APPENDIX 2

ATCC medium: 1371 *Prototheca* isolation medium (PIM)

Potassium hydrogen phthalate.....10.0 g
NaOH.....0.9 g
MgSO4 .....0.1 g
KH2PO4 .....0.2 g
NH4Cl .....0.3 g
Glucose.....10.0 g
Thiamine . HCl.....0.001 g
Agar.....20.0 g
5-Fluorocytosine*.....0.25 g
Distilled water to.....1.0 L
Adjust pH to 5.1 +/- 0.1 and autoclave at 121C for 15-20 minutes.

*May be left out of medium for routine maintenance.

AUTHOR'S BIOGRAPHY

Kendra MacDonald was born in Portsmouth, NH on March 29, 1991. She was raised in Wells, ME her entire life, and went through the Wells-Oqunquit school system. In 2009 Kendra graduated as Valedictorian of Wells High School, and proceed to go to the University of Maine. She graduated with a B.S. in Animal & Veterinary Sciences with a concentration in Pre-Veterinary Medicine, and a minor in Neuroscience. She was also President of the Maine Animal Club, and Vice-President of the Pre-Vet Club at the University of Maine.

Upon graduation, Kendra will move to Rochester, NH. She will continue her summer job as a waitress in Ogunquit, ME. She intends to re-apply to veterinary schools in the fall, while taking veterinary technician classes, and hopes to one day specialize in equine medicine.