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Tackling Stress in Sheep through the Addition of Natural Feed Supplements and the Monitoring of Fecal Bacterial Levels

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TABLE OF CONTENTS

ABSTRACT

Farmers are being challenged to elevate their animals' standard of living due to consumer demands, public health concerns and a developing understanding of the physiological effects of stress. In order to mitigate the negative effects of stress, we need a way to identify and address them. The first objective of this project is to investigate means to lower stress levels in sheep (*Ovis aries)* using a cost effective/natural product. A treatment group of sheep was supplemented with *Matricaria chamomilla,* commonly known as chamomile, and then run through a stress test. After initial analysis, chamomile fed sheep showed both lower average (139 bpm vs. 162 bpm) and max heart rates (184 bpm vs. 195 bpm) during a stress test. Chamomile fed sheep also had lower salivary cortisol both prior to and after exposer to stress stimuli. The second objective of this project is to develop a non-invasive approach to monitoring stress in sheep. Fluctuations in the microbiome have previously been linked to changes in stress; therefore, the relative abundance of fecal bacterial species may act as a proxy for animal health and relative stress. If the specific microbial profiles to an animal's stress level are correlated, monitoring changes in the microbiome may provide a new way of gauging stress in sheep. This knowledge can be used to develop better animal husbandry techniques and improve welfare among livestock.

LITERATURE REVIEW

The journey from farm to table is complicated with numerous factors affecting the welfare of livestock destined for human consumption. Many factors such as nutrition and preventive medical care are easily managed, but others, such as stress, can prove more challenging. Stress can be tricky to define and even harder to control. It can range from short-term fear to long-term physical degradation due to poor living conditions. The effects of stress can be as insignificant as a small spike in serum cortisol levels to irreversible muscle damage. The magnitude of a stressful event on an animal typically depends on the type, duration, and intensity of the stressor along with the susceptibly of the animal to those factors (Ferguson *et al*., 2008). An area currently being explored within the realm of stress research involves looking at the effects of acute and chronic stress on meat quality and there collective implications on public health. These include increased bruising and an inadequate drop in meat pH, which can be measured quantitatively. The increasing numbers of antibiotic resistant viruses occurring due to widespread use of medicated feed, however, are harder to quantify (Liverani *et al*., 2013).

How does this relate back to stress? A growing demand for meat has led to higher stocking densities and increased transportation distances, both of which are known to reduce meat quality. More often than not, reduction in meat quality is linked with increased stress. Too much stress leads to weakened immune systems and increased incidences of sickness. This increase in sickness has led to increased antibiotic practices. Widespread use of antibiotics at sub-therapeutic levels aid in

decreasing the risk of spreading existing zoonotic diseases, but encourages the emergence of new antibiotic resistant bacteria (Liverani *et al*., 2013). Antibiotic resistance produced within animals has been shown to be transferable to human pathogens (Marshall and Levy, 2011). This provides a serious challenge to the field of public health. However, through better control of stress and an improved understanding of its implications on animal wellbeing, the possibility of developing new zoonoses and the transfer of antibiotic resistant pathogens can be reduced despite the growing meat market.

The Effects of Stress on the Body

First coined in the 1930's by Hans Selye, stress described how people adjust to the constant changes in and around themselves (Szabo *et al.,* 2012). Selye believed that regardless of stimuli, the body would react in the same physiological way in order to maintain homeostasis (Carroll and Forsberg, 2007). While our physiological understanding of stress has increased dramatically since Selye, its definition has changed little. Today, stress is understood to encompass the physiological and behavioral changes in an animal brought about by physical, interoceptive (from stimuli within the body), or physiological threats or stressors (von Borell, 2001). The physiological or behavioral changes brought about by stressors are responses to maintain or reestablish homeostasis within an organism's body. Animals can display a variety of reactions to various stressors, but the primary path to restoring homeostasis is done through the coordinated

response of the central nervous system, endocrine system, and immune system (von Borell, 2001). The process begins when internal or external stimuli are perceived by cognitive brain centers, such as the cerebral cortex. Through nerve signals, corticotrophin-releasing hormone (CRH) and vasopressin (VP) neurons are activated releasing CRH and VP by means of axon terminals. CRH and VP are transported to the anterior pituitary gland where they activate the hypothalamic-pituitary-adrenocortical (HPA) axis encouraging the production and release of adrenocorticotropic hormone (ACTH; Borell, 2001; Carroll and Forsberg, 2007). An increase in ACTH stimulates the release of glucocorticoids, such as cortisol, from the adrenal cortex, which affects a wide range of biological functions within the body, such as carbohydrate metabolism and immune function. Further, glucocorticoids are known to enhance the synthesis of catecholamines, which help control heart rate, pupil dilation, vasoconstriction in the skin and gut, vasodilatation in leg muscles, and increased glucose production in the liver (Carroll and Forsberg, 2007).

Catecholamines are produced primary in the adrenal medulla, which is located in the center of the adrenal gland. The main catecholamines are norepinephrine and epinephrine (noradrenaline and adrenaline). The physiological responses catecholamines control play essential roles in an animal's short-term reaction to stress. Commonly referred to as the flight-fight syndrome, the sympathetic nervous system causes animals to be more proactive ("fight") while the parasympathetic nervous system makes them more reactive ("flight"). Animals that have a higher sympathetic to parasympathetic nervous system ratio tend to be in a higher state of physical or physiological arousal. This is due to the fact that the

parasympathetic nervous system acts to bring an organism back to homeostasis after a stressful event (von Borell, 2001).

The behaviors that stressed animals exhibit, whether it is a reaction to threatening stimuli or induced by a biochemical change, can vary widely. There are an array of behaviors currently recognized to be indicative of stress including increased immobility, increased locomotion, decreased sleep or resting, increased alertness, decreased eating or drinking, increased vocalization, and increased elimination (Cockram, 2004). Deciphering what a behavior means can be challenging because both extremes within a behavior are often observed. Locomotion is a good example. Both an increase and decrease in locomotion can be indicative of fear. An increase could be due to multiple escape attempts, but it could also be due to increased exploration because of natural curiosity (Gougoulis *et al*., 2010). Immobilization can be caused by the natural docile nature of an animal or it could indicate a high level of fear and nervousness (Cockram, 2004). Interestingly, changes in outward behavior are not always accompanied by changes on the biochemical level. Hastings *et al*. (1992) found that deer whom had been hand-tamed struggled just as much as free-ranging deer when restrained, but the hand-tamed deer exhibited lower cortisol levels than their free-ranging counterparts. Analyzing behavior in the context of identifying stressors can be very challenging. As previously shown, behavior does not always line up with quantitative data, such as cortisol levels, which researchers have come to rely on indicating the need for further research.

How do we measure stress?

Animals under stress can experience a myriad of physiological and behavior changes, as previously mentioned. These changes help them cope with stressors and influence their ability to maintain or return to homeostasis. To understand how various factors influence an animal's wellbeing, being able to measure stress is key. Researchers have devised an array of methods to measure the behavioral and physiological changes that accompany stress, all of which have been met with varying degrees of success. Currently, almost all stress-measuring methods fall under the categories of biochemical or behavior, but researchers are not required to use one system over another, which can make comparing studies difficult (Hopster *et al*., 1999).

One of first indicators researchers investigate when measuring stress is cortisol level. Cortisol is released from the adrenal cortex by hormonal activation of ACTH and is one of the main glucocorticoids associated with stress. It can be measured in a variety of ways, but blood concentration appears to be the most popular collection method due to its reliability and accuracy. Referred to as blood plasma or serum cortisol concentration, samples are predominantly drawn from the jugular vein and analyzed using radioimmunoassay procedures (Miller *et al.*, 1989, Yates *et al.*, 2010, Stott, 1981). Drawing blood from the jugular is very involved and normally requires the animal to be confined or restrained in some way. This handling of the animal can be stressful by itself and has been shown to

unintentionally effect results (Hopster *et al*., 1999). This relationship is recognized; therefore, researchers have developed other various ways to measure cortisol levels.

Some studies make use of remote blood sampling devices, while others utilize other non-invasive sampling procedures. Mediums such as urine, saliva, milk, hair and feces have all been considered (Fisher *et al*., 2010, Peric *et al*., 2013, Miller *et al*., 1989, Gygax *et al*., 2006). Saliva and urine collection still pose an issue when it comes to minimizing the amount of handling an animal is exposed. Saliva has shown fairly accurate in indicating serum cortisol levels. In two separate studies, Yates *et al*. (2010a and 2010b) found that salivary samples were indicative of serum samples in ewe lambs and yearlings. Analyzing cortisol levels from hair has also been done, but it has not proven helpful when looking at acute stress. In a study done by Peric *et al*. (2013), the activity of the hypothalamic-pituitary-adrenal (HPA) axis in heifers was analyzed using cortisol gathered from hair samples. The researchers pointed out the hair is good for detecting long-term cortisol concentrations, as it is indicative of at least one month of cortisol activity. Analyzing cortisol levels in milk has also been done in multiple studies with varying degrees of accuracy in relation to blood serum levels (Gygax *et al*. 2006). It is somewhat limiting, though, as it can only be used with lactating animals and still requires a high degree of contact, depending on the system. Measuring cortisol levels in feces currently seems to hold the most promise in terms identifying stress in a non-invasive manner. Fecal samples are easily collected without stressing the animal and can be easily analyzed (Mostl and Palme, 2002).

While measuring cortisol levels is one of the most accepted ways to gauge stress, its use is cautiously advised. Like many other hormones, cortisol has a circadian rhythm, fluctuating as the day progresses (Mostle and Palme, 2002). It also has very little value when considered out of context. Other activities such as sexual intercourse or episodic releases of hormones can cause cortisol to rise even though an animal may not be encountering any stressors (von Borell, 2001). The natural circadian rhythm of cortisol release can also be disrupted when an animal is under a prolonged period of extreme stress making it difficult to find a normal, unstressed baseline. Other physiological responses researchers use to gauge stress include heart rate and respiration rate. Both have been tied to increased stress but again need to be analyzed within context. Also, both can increase with increased movement and may not necessarily indicate the presence of a stressor (von Borell, 2001) .

When considered in context, analyzing cortisol levels can be a useful tool in gauging stress. However, due to its obvious pitfalls, researchers have devised other behavioral and physiological methods. One of the most basic approaches in livestock is to analyze production and reproduction. Animals physiologically prioritize energy needs (maintenance before growth or reproduction). An animal under stress will produce less milk or lay fewer eggs (Abidin and Khatoon, 2013; Sevi and Caroprese, 2012). Using statistical techniques and data on an animal's environment, researchers can determine how various environmental factors influence stress based on an animals production rate or reproductive success (Stott, 1981). Researchers can also identify stressors through behavioral analysis. Behavior can be

studied non-invasively and provide good insight into how an animal may perceive a situation (Gougoulis *et al*., 2010). Previous studies have explored a wide range of behavioral responses including movement (time spent standing vs. lying down, kicking, struggling), sleeping/resting patterns, alertness, eating/drinking patterns (feeding, grazing, sucking), and vocalization (Gougoulis *et al*., 2010 and Destrez *et al*., 2012). Behavior is typically recorded and analyzed later by individuals trained to use a behavioral analysis system. Another behavioral response researchers study is aversion, which is measured by the degree of force it takes to move an animal from one place to another. If an animal experienced something it considers stressful, it is less likely to go back to the place the event happened. Cows will walk through an isle into a squeeze chute without issue as long as nothing aversive happens along the way. After one noxious treatment in the squeeze shoot, cattle are most likely to balk and turn away from the entrance to the race (Grandin, 1997).

Types of Stress from Farm to Table

As defined earlier, stress encompasses the physiological and behavioral changes in an animal brought about by physical, interoceptive, or physiological stressors (von Borell, 2001). Each of these stressors affect the animal in some way or another; some have permanent damage while others have little documented effect. The level at which a stressor negatively affects an animal is based upon the type, duration, and intensity of the stressor in terms of the susceptibly of the animal to them (Ferguson *et al*., 2001). Animals destined for consumption are exposed to a

wide array of stressors that can potentially impact their quality. These occur at different points and can prove challenging to control. Physical stressors can include hunger, thirst, fatigue, or thermal extremes (Grandin, 1997). Interoceptive stressors stem from the body and include toxemia and systemic infections (Rinaman, 1999). Physiological stressors vary widely but include restraint, handling, or exposure to novel situations (Grandin, 1997). When an animal is exposed to a stressor, they have an initial acute reaction, which activates the HPA axis. The HPA axis, in turn, activates the release of glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla. These chemicals enact a variety of responses in the animal mentioned earlier. Exactly how an animal responds, however, depends on the stressor with which they are being faced (Mostl and Palme, 2002; Gougoulis *et al.,* 2010).

Heat stress, a type of physical stress, occurs when animals are exposed to a mix of high environmental temperatures and high levels of humidity. These conditions do not allow for proper dissipation of heat given off during metabolic processes and prevents the animal from maintaining homeostasis (Webster, 1983). Heat stress is chronic when animals are exposed to high temperatures for long periods of time. It is considered acute when animals are exposed to short and sudden intervals of extremely high temperatures (Emery, 2004). In chickens, heat stress is known to lower feed intake, lower immunity, decrease nutrient absorption, decrease fertility, increase mortality, and decrease egg quality and production (Abidin and Khatoon, 2013). In sheep, heat stress has been shown to similarly decrease body weight, decrease growth weight, and increase respiration rate (Marai

et al., 2006). Udder quality may also be impacted. Sevi and Caroprese (2012) emphasize that heat stress reduces natural mammary defense leading to increased bacterial colonization in the udder. This increase in bacterial load increases the somatic cell count in milk, lowering the quality of the milk. Animals destined for consumption may experience heat stress while being raised, during transportation to a processing facility, or while being held at a processing facility (Abidin and Khatoon, 2013, Srikandakumar *et al*., 2003, Ferguson and Warner, 2008).

Nutritional stress is another form of physical stress animals can undergo before slaughter. For most livestock, nutritional stress comes in the form of dehydration, hunger, or improper feeding. The most obvious reaction to nutritional stress is weight loss. Bray *et al*. (1989) found that lambs fed a low nutrition diet lost weight compared to lambs fed a high-quality diet. In cows and sheep, weight loss is most extreme during the first 12 hours of feed and water deprivation. A portion of the weight loss is due to the lack of feed and water intake but catabolic processes also play a major role. As the animal's supply of metabolites runs low, its ability to regulate homeostasis declines. For animals sent to slaughter, a period of food and water deprivation is highly likely. Feed is typically withheld for two reasons. The first is to reduce the amount of digesta in the gut, which helps reduce the amount of waste excreted on other animals, on trucks, and on the roads. A reduction in digesta also helps prevent carcass contamination. The second main reason feed is withheld is to permit a more accurate carcass weight in situations where animals are sold by weight (Hogan *et al*., 2007). In most cases, food is withheld for less than 24 hours. However, in some cases, this period can extend up to 72 hours. The most significant

weight loss occurs within the first 24 to 48 hours with the weight consisting mostly of feces and urine. As that time frame stretches beyond 48 hours however, tissue catabolism and dehydration become the major contributors to weight loss. These losses negatively affect the health of the animal and decrease the true carcass weight by over 7% leading to losses for the producer (Ferguson and Warner, 2008).

Animals may also experience physiological stress in the form of fear. Examples of situations that may create fear stress include shearing (in sheep), sorting, drenching, transporting, and housing in novel environments. Ruiz-de-la-Torre *et al*. (2001) studied the effects of movement on stress responses in sheep and found that animals exposed to a rough journey had higher cortisol levels and higher heart rates than those exposed to a smooth journey. Hargreaves and Huston (1989) analyzed the stress responses of sheep during routine husbandry procedures. Using 30 castrated merinos, they monitored responses to shearing, crutching, drenching, dipping, and drafting. Based on measurements of plasma cortisol concentration and hematocrit detection, sheep reacted to shearing, crutching, and drafting as more stressful events than drenching and dipping. Interestingly, the heart rate of sheep being blood sampled was highest when the handler approached the animal. Blood was collected with a permanent catheter so it was unlikely that sheep were afraid of the pain associated with the human but rather, they were fearful of the actual human (Hargreaves and Huston, 1989).

Animals bound for slaughter experience fear stress in a variety of ways. They often undergo increased handling and human contact, transportation, novel

environments, and changes in social structure due to separation from their herd and the mixing together of groups at auctions and slaughter plants. A fear stressor will activate the HPA axis allowing the release of glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla. The release of catecholamines has a strong effect on energy metabolism especially in glycogenolysis and gluconeogenesis. Depletion of muscle glycogen at slaughter has a major impact on the final meat quality (Ferguson and Warner, 2008). A decline in quality can lead to lower profits, which is something the meat industry prefers to avoid.

The Relationship Between Meat Quality, Producers and Public Health

When discussing meat quality, we need to approach it from the sides of both the consumer and the producer. The producer is looking to make as much profit as possible while still aligning their products with the consumers' wants. They are mainly concerned with the yield and quality of saleable meat (Warriss, 1990). Factors that influence these include the amount of bruises on a carcass, the occurrence of dark, firm dry meat (DFD), and the ultimate pH of meat (Belk *et al*., 2002; Bray, 1988). Dark, firm, dry meat also known as dark-cutting beef (DCB) in cattle has a firm and dry appearance, as well as a purple coloring. A pH higher than 6 is most often to blame for DCB. In addition, pH can affect flavor, tenderness, water-holding capacity and storage life (Bray, 1988). Consumers generally allow color and tenderness to influence their meat buying habits (Warriss, 1990). Gauging tenderness can prove challenging to producers as gauging tenderness on a carcass is

nearly impossible. Tenderness is very subjective and creating a scale that aligns with consumers' taste and a mechanical measurement is nearly impossible (Devine *et al*., 1993).

While quantifying tenderness is very difficult, measuring other factors that affect meat quality, such as pH, are simple. Post-slaughter pH is greatly influenced by pre-slaughter muscle glycogen stores (Warriss, 1993). When an animal is exposed to a fear stressor, activation of the HPA axis occurs. The activation allows the release of glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla. Catecholamines cause significant changes in energy metabolism, such as gluconeogenesis and glycogenolysis within muscles (Kuchel, 1991). Both of these processes raise the mobilization of energy, increasing glucose delivery to skeletal muscle and the brain. An increase in glucose delivery leads to a depletion of glycogen stored within the muscles. If this depletion is severe enough pre-slaughter, it can negatively affect meat quality of the animal (Ferguson and Warner, 2008). Normal muscle glycogen levels within sheep and cows range from 75 to 120 mmol/kg with a critical threshold for meat quality fall between 45-57 mmol/kg (Immonen *et al*., 2000; Tarrant, 1989). Should the muscle glycogen levels fall below that, the ultimate desired pH cannot be attained. After an animal dies, the glycogen left stored in the muscles is converted to lactic acid. As lactic acid increases, meat pH drops. Ideally, the lactic acid will decrease the pH from around 7.0 to approximately 5.5. In cattle, this change normally takes 24 to 48 hours. Should the muscle glycogen be below the critical thresholds at slaughter due to some form of stress, acidification is limited and the desired pH drop is unable to occur (Hogan *et al.,* 2007).

Work has been done to specifically show the consequences of various meat pHs. Meat with a pH ranging from 5.8 to 6.0 has an abnormal color and an increased risk of spoilage (Warriss, 1990). This spoilage is often due to not enough glucose available for bacteria to break down. Instead, amino acids are metabolized, which leads to the production of ammonia and other "off-odors" (Shaw and Dainty, 1980). A pH above 6 produces DFD meat making it hard to market. The meat takes on the dry, dark appearance because many of the muscle pigments remain undenatured due to the lack in pH drop (Warriss, 1990). While muscle glycogen can be restored in production animals, it often takes a few days and in the United States, animals are typically slaughtered the day they arrive at the plant (Warriss, 1990; Ferguson and Warner, 2008).

Decreasing stress in animals destined for slaughter has been shown in numerous studies to improve final meat quality. Jeremiah *et al*. (1988) showed that cattle trucked a shorter distance and slaughtered within four hours had more acceptable tenderness, flavor, and juiciness as per a consumer panel than a group trucked over long distance and left in the feedlot for up to 24 hours. Ruiz-de-la-Torre *et al*. (2001) also found a similar trend in sheep trucked over a long period of time verses a short period of time.

How does all of this relate back to stopping the spread of new antibiotic resistant pathogens? Production companies are looking to make a profit. Increased stress within their animals causes monetary losses. In 1992, it was determined that if every defect from a steer or heifer raised for slaughter could be eliminated,

producers would increase profits by $27.26 per head (Smith *et al*., 1992). Two years later, it was determined that if every defect could be avoided in cull cows and bulls, profits would increase by $38.43 a head (Smith *et al*., 1994). Producers are taking steps to reduce stress within their animals because it increases meat quality and decreases profits losses. Further, it is well-known that stressed steers and heifers are more likely to shed bacterial pathogens such as *Salmonella* and *E. coli* in their feces, which has the potential to infect other cows, the workers handling the cows, and the meat produced by the cows (Belk *et al*., 2002). In order to keep workers safe, increase profits, and minimize the chance of spreading bacterial pathogens to consumers, production companies are willing to take steps in order to reduce the amount of stress their animals' experience.

Meat producers are truly the first lines of defense when it comes to stopping the spread of pathogens related to meat consumption. Cortesi (1994) states this relationship well, "Animal welfare will probably be maximized if economical, ethical, and qualitative considerations coincide." An increase in animal welfare translates to better conditions over the course of the animal's life, which includes a decrease in stress. There is also a growing concern over the widespread use of sub-therapeutic antibiotics and the welfare of animals during the slaughter process. Again, producers are willing to reduce their use of antibiotics because consumers desire products that have had little to no antibiotic use. They are also willing to elevate standards of living for meat animals to satisfy the desires of their customers. As the meat industry continues to grow, changes to the farm to table process are bound to occur. While there are many negative side effects to the direction the meat industry

is currently taking, consumers do have the ability to demand products of certain quality. If their voice is loud enough, the welfare of production animals can only improve which in turn can reduce stress and help prevent the spread of antibiotic resistant pathogens.

EXPERIMENTAL INTRODUCTION

As the disconnect between people and the land grows larger and larger, livestock producers need to meet the consumers growing want for inexpensive, humanely raised, quality meat (Back, 2013). We already know that stress is a major factor that currently stands in the way of farmers increasing production because it impacts animal health drastically. It has been shown to decrease productivity, quality and profitability within livestock, as well as general welfare (Smith and Grandin, 1998).

An area of interest relating to stress not widely studied is how fear stress affects the microbiome of ruminant animals, such as cattle and sheep. Previous studies have found that heat and nutritional stress can dramatically affect the environment within the rumen (Galyean *et al*, 1999, Hogan *et al*., 2007, Tajimaa *et al*., 2007). Hogan *et al*. (2007) stated that sudden feed cessation to the rumen will cause a change in the composition and size of rumen bacterial communities. When feed is withheld for even short periods of time, ruminal pH drops and rumen osmotic plasma pressure increases. Bacteria that derive nutrients from carbohydrates are the first to die as those substrates ferment the fastest (Hogan *et al*., 2007). However, some non-cellulolytic bacteria, such as *Selenomonas ruminantium*, are able to survive on cellobiose, a disaccharide made from cellulose, created by cellulolytic bacteria, such as *Fibrobacter succinogens*. Methane production is also known to decrease to about 10% of pre-fasting levels within 48

hours of feed deprivation indicating that methane producing bacteria, such as *Methanobrevibacter ruminantium*, pass out of the rumen (Hogan *et al*., 2007). While it is not known how large of an impact acute fear stress has on rumen bacterial colonies, it is believed to have a greater impact than water and feed deprivation (Galyean *et al*, 1999). Heat stress may be more similar to fear stress as ruminal changes in both cases are indirectly due to larger physiological changes within the animal (Tajimaa *et al*., 2007).

Researchers have attempted to define and address these larger physiological changes. Destrez *et al*. (2012) designed an isolation/fearfulness test to examine how sheep respond to stressful situations. It was hypothesized that by reducing fearfulness with the administration of Diazepam (commonly known as Valium), pessimistic-like behaviors could be reduced. The results did show a reduction in fearfulness, but Diazepam cannot be used within the meat industry due to its effects on meat (chemical residues, long withholding period, etc.; TroyLab, 2008). In addition to Diazepam's effects on meat, its high cost and the fact that it is a controlled substance make it impractical for livestock producers to use. Other drugs such as Xylazine (a common sedative), opioids, and barbiturates have also been shown to reduce stress in livestock during various stressful events, but they have the same drawbacks as Diazepam: dangerous chemical residues, high costs, and controlled availability (Ali and Al-Qarawi, 2002).

In order to get around the issue of drug residues, cost, and limited availability, all natural additives are being explored. Studies involving natural

additives and ruminant animals typically focus on how the additives affect the rumen on a microbial and chemical level, but there are a handful of studies that explore the additives impact on stress (Ali and Al-Qarawi, 2002; Lima de Souza Reis *et al*., 2006). Yang *et al*. (2010) tested the effects of cinnamon oil on dry matter intake, growth performance, carcass characteristics, and blood metabolites in beef cows. Unfortunately, cinnamon oil had little effect and is not useful in reducing stress within feedlot beef cows. This is supported by Chaves *et al*. (2008) on the meat quality of slaughter lambs; therefore cinnamon oil seems to have little promise in terms of reducing stress. Tea catechins and rosemary extract have also been examined and while both have shown to improve meat longevity when added post slaughter, feed supplementation pre-slaughter has had very little effect on meat quality (O'Grady *et al*. 2006). The addition of vitamin E to pre-slaughter diets in pigs was shown to lower heart rates and produce a calmer pig during vibration stress tests, but had no effect on meat quality. Supplementation of Vitamin C, tryptophan or magnesium were believed to have the potential to lower stress in pigs exposed to a stress test, but had little to no effect on meat quality (Peeters *et al*. 2006).

In a unique study done by Lima de Souza Reis *et al*. (2006), Nelore calves were *fed Matricaria chamomilla,* commonly known as chamomile (CH_{12}) for 30 days and then stressed through constraint. *M. chamomilla* is an all-natural anxiolytic or calming agent used in humans and animals (Awad *et al*. 2007; McKay *et al*. 2006). It resulted in an observed decrease in the levels of stress in the calves (Lima de Souza Reis *et al*. 2006). While the biochemical mechanisms of how chamomile may work are unknown, it might inhibit the production of cortisol. This study shows promise

for chamomile to be used as a feed additive to reduce pre-slaughter stress in animals. In order to expand on the results found, my research aims to expand on Lima de Souza Reis *et al.* (2006) study by using a slightly different approach.

While decreasing stress in livestock is a major part in increasing production and improving welfare in animals, decreasing stress also plays a key role in public health. The increased demand for livestock has led to higher stocking densities, an increase in animals fed a high concentrate diet, and increased transportation distance (Liverani *et al*., 2013). These actions lead to higher stress levels within the livestock. Increased stress leads to a weakened immune system and an increased incidence of sickness (Liverani *et al*., 2013; Silbergeld *et al*., 2009). High concentrate diets and high stocking densities also create ideal environments for various pathogenic bacteria, such as *Escherichia coli* and *Salmonella,* causing an increase in the use of antibiotics (Hogan *et al*., 2007). Widespread use of antibiotics leads to the development of antibiotic resistant bacteria, which increases the likelihood of the development of an antibiotic resistant zoonotic disease (Liverani *et al*., 2013; Koock *et al*., 2013). My research also aims to explore the development of a diagnostic tool that will aid in the identification of factors that indirectly influence the development of antibiotic resistant zoonotic diseases.

Researchers have also devised various methods to quantify the adjustments livestock make to stress but many techniques actually induce further stress (Hopster *et al*., 1999). To help alleviate this issue, my study aims to quantify the relative abundance of fecal bacterial species, before and after a stress-inducing

situation. If specific microbial profiles correlate to an animal's stress level, monitoring changes in the microbiome may provide a non-invasive way of gauging stress in ruminal livestock. Assuming fear stress has a similar effect to nutritional stress on rumen microbial populations, I hypothesize that there will be a rise in the relative concentrations of the six bacterial species post-stress test.

Additionally, this project will investigate means to lower stress levels in sheep using a product free of damaging residues. Chamomile will be supplemented because of its previously proven ability to reduce stress and its high natural availability in the area of the study (Awad *et al*., 2007; McKay and Blumberg, 2006). It could provide an affordable, easily accessible supplement available to farmers to help reduce stress in pre-slaughter sheep. When compared to a control group, I hypothesized that the addition of Chamomile to a sheep's diet will help reduce the amount of anxiety experienced in novel situations thus keeping the effects of stress and fearfulness to a minimum.

MATERIALS AND METHODS

Animals and diets

The study was carried out on Branch Brook Farm in Nicholson, PA from October 2013 to January 2014. The farm holds more than 300 sheep year-round with the population reaching close to 400 during lambing seasons. The flocks are worked with humans and border collies. The farm approaches management from a humane angle; around 75% of lambs go to humane-co op markets in and around New York City while the rest of the flock is retained or sold for breeding stock.

Animal treatment and experimental protocols were approved by the Animal Care and Use Committee of Alfred University (PRN 2013-001). The subjects included a mix of sheep breeds including Border Cheviot, Clun Forest and Blue Face Leicester. All were dewormed with Valbezan (fenbendezol) at about two months of age. Some were reworked throughout the summer with Ivermectin when needed. Lambs and ewes were included in the study for different purposes

The basal diet was a premade mix composed of 41% gluten, 30% cracked corn, 23% soybean hulls, 3% molasses, 2% calcium carbonate, and 1% mineral mix, Ovine Plus. Beyond the addition of chamomile to the treatment group's feed, the diet of the ewes did not change in composition or amount so an adaption period was deemed unnecessary. The ewes had unlimited access to hay composed of Timothy, June Grass, Brome, Clover and Alfalfa and fresh water at night and during the day. This part of the study was conducted during October therefore there was still plenty of good quality grass so hay consumption during the day was low.

The basal diet for the lambs was the same as the ewes. Both groups were used to being fed in long troughs and this practice was maintained through out the study. They also had access to grass during the day but as testing took place during January in northeast Pennsylvania, grass quantity was limited.

Chamomile administration

The treatment group (CHAM) in experiment 1 had ¼ pound of organic powdered dried chamomile flowers mixed in with each feeding (2 per day split between 15 ewes) with each ewe receiving approximately 0.53 ounces of powder per day. The CHAM group in experiment 2 had ¼ pound of organic powdered dried chamomile flowers mixed in with each feeding (2 per day split between 5 lambs) with each lamb receiving approximately 3.6 ounces of powder per day. It was determined that the concentration of chamomile in the first experiment was too low and was increased for experiment 2.

Experiment 1

Figure 1a.

Figure 1b.

Fig.1a. Two blue dots indicated fecal collection ewe.

Fig.1b. Purple dots on back of head indicate CHAM sheep.

In October 2013, an initial run of the stress test was performed so unforeseeable issues could be solved with four mature ewes. The experiment, to test the impact of stress on fecal bacterial species, was an improved version of this initial trial run. Thirty ewes were split into two groups: a control group and a chamomile group whose diet was supplemented with chamomile. Two sheep from each group were randomly selected for fecal sample collection. A week before the study, the ewes were run through a sorting chute with every other sheep being assigned to either the control (CON) or chamomile (CHAM) group. The CHAM group was identified by a large purple dot spray painted with scourable livestock paint on

the back of the head. Fecal sample collection sheep were further identified by two blue dots placed above their tails. Initially, each group had 32 ewes, but two from each group were eliminated due to the appearance of lameness during the week leading up to the stress test. To keep the environmental conditions as similar as possible, the ewes were housed together in one flock. They went out to a large field during the day and stayed in a barn at night. At feeding times, the ewes were run through the sorting chute and the CHAM group was sorted out and fed in a separate area from the CON group.

Experiment 2

One day before the study, a group of 15 six-month old lambs, along with the larger group they were being housed with, was run through a sorting chute. These lambs were randomly assigned to three groups: a control group (CON), which was not put through a stress test, a group put through the stress test (NON-CHAM), and a group fed chamomile and put through the stress test (CHAM).

The lambs within each group were not separated from each other during feeding to limit isolation-induced stress as well as to prevent acclimation to isolation as the stress test had an isolation component.

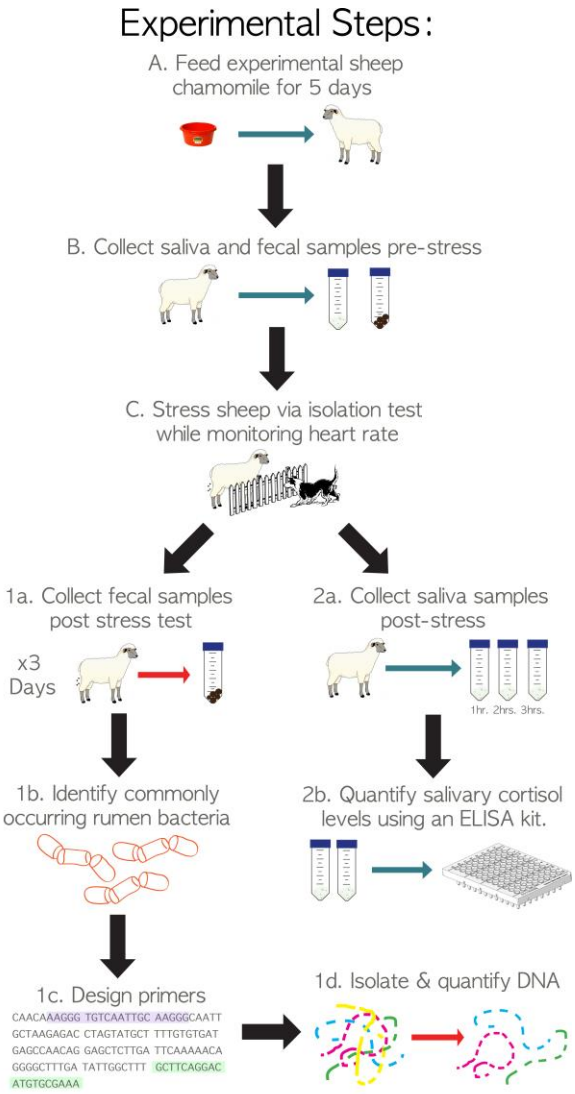

Figure 2. Flow chart showing the steps taken in Experiment 2 from start to finish.

Stress induction and heart rate recording

Heart rate data was collected using a Polar H7 Bluetooth Smart Heart Rate Sensor with a modified chest strap allowing it to be clipped around the sheep easily and efficiently. The readings from the monitor were sent wirelessly to Polar Beat, an iPad app, designed specifically for the monitor (Polar Electro Oy 2010). The stress tests for the two experiments varied slightly. The CON group of lambs was not put through the stress test as they were being used to establish a normal cortisol cycle. When they entered the middle area of the chute, they were allowed to pass through to the holding pen.

The samples for experiment 1 were collected in October from 4 adult ewes. An isolation area was built next to the sorting chute with two solid sides and a grated sliding door caddy corner to second gate that swung open. Sheep entered one by one into the isolation area from the grated sliding door and exited *via* the gate to

a second holding pen. The initial plan was to strap the heart rate sensor to the sheep before they entered into the isolation area. However, due to the large size of the ewes and the narrowness of the chute, it was impossible to fit our arms around the sheep. Because of this, the ewes had to be let into the isolation area and then cornered with a panel to allow placement of the heart rate sensor. Using moveable panels to corner sheep is a common catching technique within sheep husbandry. Once one person cornered the ewe with a panel, another strapped on the monitor. Both people would then exit the isolation area and the stress test would begin. While the idea of the stress test was to stress the sheep, all of the handling before hand most likely added to effects of the isolation test. To further increase stress, two border collies were positioned directly outside the isolation area. The ewe being stressed could see and smell the border collies throughout the duration of the stress test, which lasted two minutes and thirty seconds. The ewe was allowed to exit the isolation area after the heart rate monitor was removed.

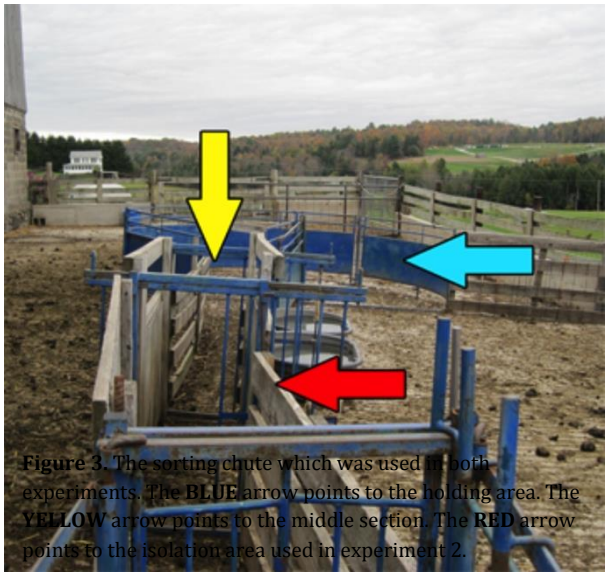

Figure 3. The sorting chute which was used in both experiments. The **BLUE** arrow points to the holding area. The **YELLOW** arrow points to the middle section. The **RED** arrow points to the isolation area used in experiment 2.

Three days before experiment 2, 10 of the 15 lambs had a strip of wool removed from their foreflank and belly area. The area was shaved to provide good contact for the heart rate monitor. Instead of building an isolation area, I utilized an already existing chute. The chute can be divided into three sections by two sliding doors located at two different

locations on the chute. The lambs were loaded into the end of the chute and both doors were closed to block off the front half of the chute. One by one, the lambs entered the middle section of the chute and had the heart rate monitor attached. The lambs were about half the size of the ewes used initially, which made them much easier to work with. It was fairly easy to reach your arms around the lambs in the chute and put the monitor on them. In order to increase the connection between the lamb's skin and the heart rate monitor, electrode jelly was smeared onto the electrodes of the monitor before being strapped to the lambs. Once hooked up to the heart rate monitor, the lambs entered the last part of the chute, which acted as the isolation area. The door to the middle section was also left closed during this time so the lamb being stressed could not see any other sheep. Again, two border collies were positioned on either side of the chute. Both dogs sat and stared directly at the lamb being stressed moving little throughout the course of the day. Heart rate was recorded for two minutes and thirty seconds. Once time was up, the heart rate monitor was removed and the lamb was allowed to exit the chute via a sliding door into a holding pen.

Saliva collection and analysis

Saliva in both experiments was collected to analyze the changes in salivary cortisol levels. In the first experiment, a sample was taken prior to the stress test (PRE) and one-hour post stress test (PST1). The saliva was collected using a 5 mL syringe without a needle. The syringe was placed in the corner of the ewe's mouth

and the plunger drawn back. This turned out to be a very inefficient collection method as it could take up to 10 minutes to get enough saliva for testing. It also made the sheep nervous to the point where they refused to continue entering the shoot after the first sample collection. In order to correct this, they had to be led through the chute a number of times with a bucket of corn the second day of testing. The saliva was frozen at -20°C for two days and then stored at -80°C until analysis.

Figure 4. Rope placement for saliva collection.

In the second run of the trial with lambs, a new method of collection was adopted. Saliva samples were taken more frequently and an unbleached all natural cotton rope was used for collection. A sample was taken from all three groups prior to the stress test (PRE), one hour after (PST1), two hours after (PST2) and three hours after (PST3). The rope was placed in the lambs' mouths somewhat like a horse bit. The rope was pulled up and tied behind the lambs' ears. It was tied tight enough to stay in the mouth, but not tight enough to gag the lambs. The ropes sat in their mouths for seven to ten minutes and were then cut off and put into labeled conical tubes. The tubes were stored at -20°C until analysis.

The sheep's cortisol levels were processed using a Cortisol ELISA kit from Arbor Assays (product #: K003-H5). Saliva sample ropes were thawed completely and centrifuged to pull the saliva out of the cotton rope. To accomplish this, the caps

and bottom tips of 1.5 mL microcentrifuge tubes were cut off and the cut tubes were placed inside 15 mL conical tubes. The ropes were transferred to these tubes and then centrifuged at 2,000 rpm for six minutes. 50 µL of each saliva sample was pipetted into the appropriate wells on the microtitre plate coated with goat anti-mouse IgG. Each sample was run three times. 50 µL of the six cortisol standard reagents and one cortisol control were added to the appropriate wells as well. Each column also had a non-specific binding (NSB) well at the top, which only contained the assay buffer. 25 µL of cortisol conjugate was added to all wells followed by cortisol antibody, which was added to all wells except the NSB wells. The plates were then covered and allowed to sit at room temperature on a rocker for one hour. After sitting, the plates were aspirated and washed four times with wash buffer. 100 µL of TMB substrate was added to all wells and the plates sat for half an hour at room temperature. 50 µL of stop solution was then added to all wells and the plates were read with a Vmax Kinetics Microplate Reader at a wavelength of 450 nm. The cortisol samples were calculated based upon optical density.

Fecal collection

The second aim of this study is to quantify the relative abundance of six separate bacteria species found in the fecal material of sheep. The fecal material used for this was collected during the stress test of ewes. After pre-stress saliva collection, pre-stress fecal samples were collected from four separate sheep: two from the CON group and two from the CHAM group. The ewes were separated off from their group and held in four separate 6x8 pens. Once fecal samples were

collected, the ewes were put through the isolation stress test described earlier. Due to the slow rate at which the microbiome of the rumen is known to change, fecal samples were collected from the four same ewes for three days post-stress testing. The sheep grazed out on pasture during the day and stayed in pens overnight. Fresh fecal samples were collected from each ewe in the morning and then frozen until analysis.

Table 1. Steps taken to purify and isolate fecal bacteria.

I. Cell lysis:

1. 1. An entire pellet (≈0.50 grams) of fecal material is thawed and suspended in TE buffer (4 mg per 1 mL).
2. The mixture is left to sit overnight on a shaker.
3. The tube is centrifuged for 15 minutes at 100 x g.
4. Aliquot the supernatant into 3 separate 1.5 mL micro-centrifuge tubes and discard pellet.
5. Centrifuge the obtained supernatant at 13 000 x g at 4°C for 10 minutes.
6. Keep pellet. Wash the pelleut 3x by suspending it in 1.5 mL of acetone. Centrifuge each preparation at 13 000 g for 10 minutes at 4°C.

II. Purification:

7. Resuspend pellet in 500 µl of CTAB buffer and incubated at 60°C for 1 hour.
8. Add an equal volume of Phenol:Chloroform:IA
9. Centrifuge for 10 minutes at 14 000 x g at 4°C.
10. Collect upper aqueous layer.

III. DNA precipitation:

11. Add 0.67 volumes of isopropyl alcohol and allow to sit overnight at -20°C.
12. Centrifuge for 10 minutes at 14 000 x g at 4°C
13. Remove the supernatant carefully, then wash the pellet once or twice with cold EtOH.
14. Spin for 15 minutes at max speed at 4°C.
15. 1Remove supernatant and dry the pellet by leaving tube open @ room temp
16. Resuspend pellet in sterile H_2O or TE & store at -20°C

Extracting DNA from fecal material proved quite challenging. Low DNA yields and DNA free of inhibitory substances present the major obstacles (Yu and Morrison 2004). For many years, the only approach to analyzing the microbiota within the rumen has been conventional cultural techniques (Faubladier *et al*. 2013). These

techniques are limiting as many of the bacteria within the rumen die once in contact with air. In addition, only certain bacteria will grow on certain medias. By using a set media when culturing a rumen, one is already selecting against certain bacterium, which are unable to grow in that environment. Therefore, I chose to develop my own protocol for analysis (Table 1). By using PCR and by analyzing bacterium that are already dead, we hoped to avoid the selectivity posed by traditional methods.

Fecal Analysis

The relative concentration of each bacteria type will be measured using a quantitative polymerase chain reaction (qPCR). The amount of the bacterial DNA amplified is linked to the fluorescence intensity of a fluorescent reporter molecule. The qPCR machine will analyze and interpret the differences in fluorescent intensity and generate a graph. The relative microbe abundance will be calculated by comparing the distance between the two curves. The data will be normalized against the relative abundance of the Sheep DNA.

Bacterial Identification & Primer Design

Six bacterial species were quantified: *Treponema bryantii, Fibrobacter succinogenes, Ruminococcus albus, Megasphaera elsdenii, Selenomonas ruminantium, and Methanobrevibacter ruminantium M1*. These bacteria were chosen because they are

commonly found in the rumen and have also been detected in fecal samples (Dowd *et al*. 2008). Each bacterium plays a slightly different role; Table 2 states the functions and products of the six focus species. In order to identify the bacterium within the fecal samples, specific primers were designed that would amplify a coding region unique to each bacteria.

Table 2. Chosen bacterial species and their primers.

Bacteria/DNA	Target	Primers (forward & reverse)
Fibrobacter succinogenes	DNA	F: GGTATGGGATGAGCTTGC R:GCCTGCCCCTGAACTATC
Ruminococcus albus	DNA	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA
Treponema bryantii	DNA	F: AGTCGAGCGGTAAGATTG R: CAAAGCGTTTCTCTCACT
Megasphaera elsdenii	DNA	F: TTTTCCGCCTTATGGATGCG R: TGTATGAAACGCTGGAAGCC
Selenomonas ruminantium	DNA	F: ATTCCCGCTGGTCTTTATCCTG R: ATTCACTGAAAGGCGGGAAC
Methanobrevibacter ruminantium	DNA	F: TCTTGGTGGTTCTCCTGATGAG R: TACGTCATGCTTTCCATCGC
E. Coli_2	rRNA	F: TTCGTGTTTGCACAGTGCTG R: AGAAGGCACGCTGATATGTAGG
E. Coli_3	rRNA	F: TGTCAGCATTCGCACTTCTG R: TAAACCATGCACCGAAGCTG
Sheep (*Ovis aries*) DNA	mtDNA	F: AGCAGAAACAAACCGAGCAC R: AATGGTCCGGCAGCATATTC

Statistical Analysis

An analysis was preformed using Minitab 16.0 for Windows software (2007). All tests conducted used a 95% confidence interval and significant level of α=0.05. Due to data not being normally distributed and a small sample size, non-parametric

tests were used for analysis. The data was analyzed to ascertain stress levels and heart rate changes similarly across treatment groups. Salivary Cortisol levels were analyzed using the Kruskal-Wallis test (H), a one-way analysis of variance using ranks for non-parametric data. Heart rate was analyzed with the Mann-Whitney test (*u*), a two sample rank test for non-parametric data. Similarities between heart rate and cortisol levels will be analyzed using the Pearson's Correlation test. Behavior video analysis results were compared using a paired T-test.

RESULTS

Experiment 1

To date, I have not been able to quantify the relative abundance of any of the bacterium isolated from the feces and am still refining the bacterial extraction procedures. DNA is present and clean enough to run down a gel when CTAB is used as a purifying agent (Figure 5).

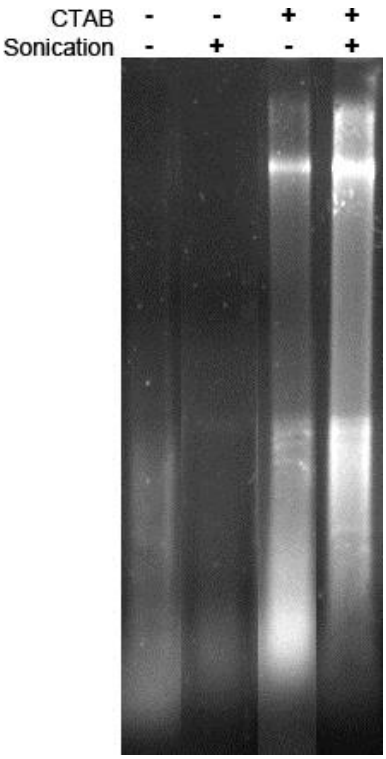

Figure 5. DNA Gel showing DNA run with and without CTAB and sonication.

I expect to see the greatest concentrations of *Treponema bryantii*, *Megasphaera elsdenii*, and *Selenomonas ruminantium* in fecal material 24 hours post-stress testing, as these are non-cellulolytic bacteria. Larger concentrations would be expected 36 to 48 hours post-stress testing because they may have established a symbiotic relationship with *Fibrobacter succinogenes* or *Ruminococcus albus*, which are both cellulolytic bacteria (Dowd *et*

al., 2008). If bacteria collection continues for four days post-testing, I expect to see higher than normal levels of *F. succinogenes* and *R. albus*. In addition, large quantities of *Methanobrevibacter ruminantium* would be expected to be excreted within 48 hours post-stress testing.

Experiment 2

Salivary Cortisol Levels

Across the four saliva-sampling times, salivary cortisol concentrations tended to decrease from pre-stress to post-stress testing in all three groups (Figure 6). The groups of sheep (CON, CHAM, NON) differed the most before the stress test (H=5.54; P=0.063). However, Kruskal-Wallis test will always give a P-value above 0.05 if there are less than seven samples in a group (n=5)(Cheung and Klotz, 1997). Overall, the CHAM group had the lowest salivary cortisol concentration (1.554 ng/mL) while the NON group had the highest (3.797 ng/mL).

The next salivary cortisol sampling (PST1) took place one hour post-stress test. The concentrations for salivary cortisol varied across the three groups, but not significantly (H=3.66; P=0.160). Overall, the CON group had the lowest salivary cortisol concentration (0.790 ± 0.652 ng/mL) while the NON group had the highest (2.355 ± 1.384 ng/mL). All three groups had a exhibited a drop in concentration from the previous sampling.

The next samples were taken two hours post stress test (PST2). Again, the salivary cortisol concentrations varied across the groups but not significantly (H=3.62; P=0.164). Overall, both CHAM and NON cortisol levels decreased while CON levels increased (0.790 ± 0.652 ug/mL → 1.230 ± 1.221 ug/mL). CHAM had the lowest concentration (0.999 ± 0.487 ng/uL) while NON exhibited the highest (1.927 ± 1.102 ng/mL).

The final salivary cortisol samples were taken three hours post stress test (PST3). The values varied across the groups but not significantly (H=2.88; P=0.237). Across the three groups, all of the cortisol concentrations dropped from the previous sampling. The NON group had the lowest concentration (0.663 ± 0.473 ng/mL) while the CHAM group had the highest concentration (0.879 ± 0.563).

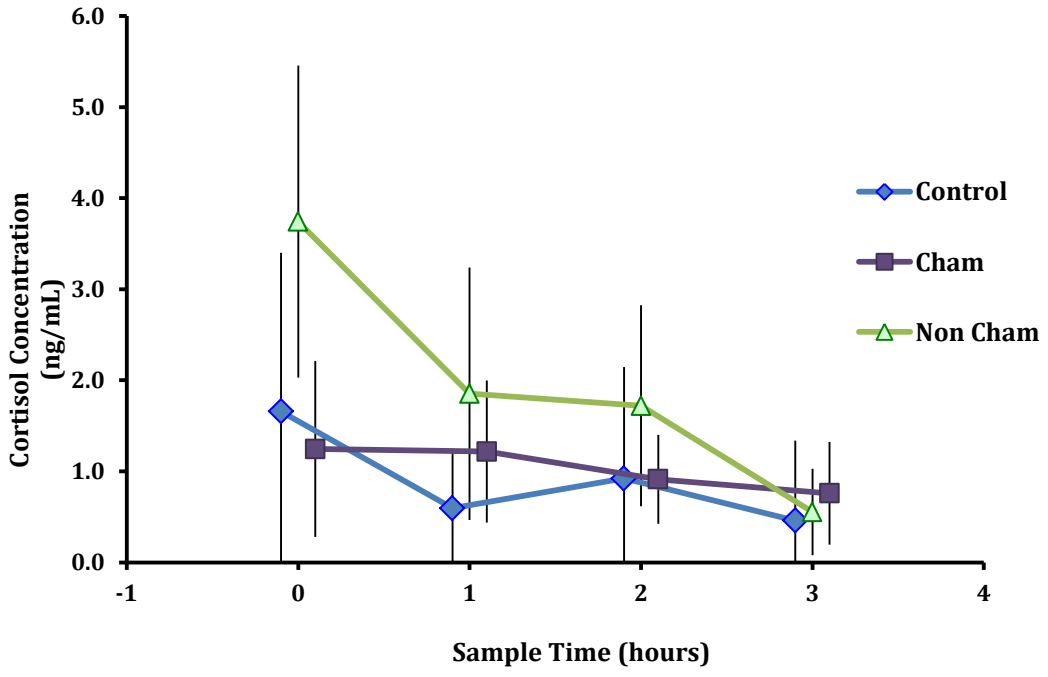

Figure 6: Salivary cortisol measurements taken from lambs supplemented with chamomile (CHAM), without chamomile (NON), and lambs not run through the stress test (CON). 0 indicates 1 hour pre-stress test and 1-3 indicates x amount of hours post stress test.

Stress Test Heart Rates

The mean max heart rate for the CHAM group was lower (183.6 ± 24.6 bpm) than the NON group (195.4 ± 8.65 bpm). CHAM and NON lambs exhibited similar heart rates (P= 0.2996; Figure 7) The mean average heart rate for the CHAM group (139.2 ± 20.6 bpm) was lower than the average heart rate for the NON group (162 ± 16.0 bpm). Although not significant (P=0.069), these groups were exhibiting contrasting stress levels (Figure 7).

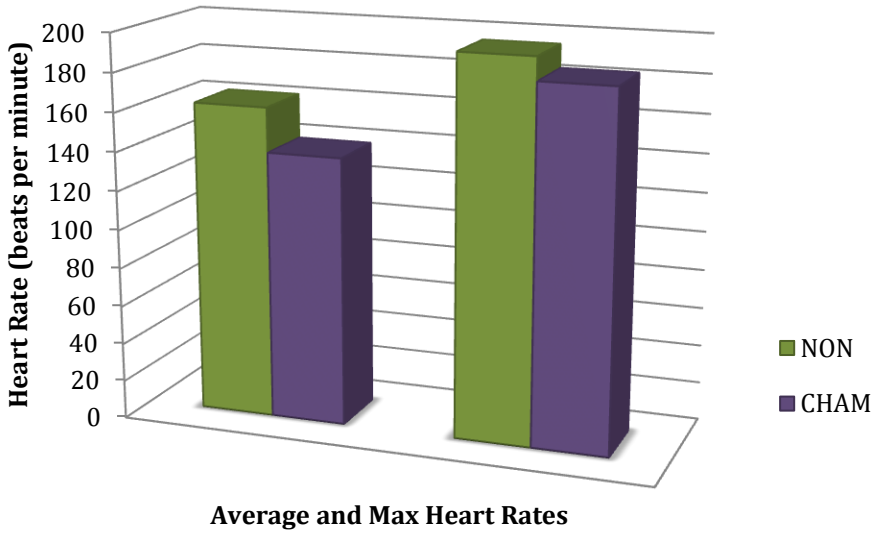

Figure 7. Average and max heart rates during stress test.

Video Behavior Analysis

Behavior of the sheep was recorded during the stress test and analyzed using a system adopted from Destrez *et al.* (2012). Table 3 is an ethogram describing each

behavior recorded. The only behavior that varied significantly between the two groups was vigilance with the CHAM group being more vigilant (t=-2.63, P=0.047; Table 4).

Table 3. Description of activities observed during stress test

Activity	Description
Crossed Zones (*n*)*	Number of times body crossed middle divider of isolation area
Climbed Wall (*n*)	Number of attempts to climb sides of isolation area
Stomped Foot (*n*)	Number of a hoof was deliberately picked up and stomped down
Vigilance (s)**	Time spent without locomotion, head still with ears still or moving back and forth
Vocalizations ((*n*)	Number of bleats and snorts made
Startle Responses (*n*)	Number of reactions where several muscles of the body contracted
Canine Acknowledgement (*n*)*	Number of times head turned to stare directly at dogs on either side

* n=number of times **s=time spent in seconds

Table 4. Two-Sample T-Test results for the two groups.

Activity	CHAM (*n*=5)	NON (*n*=5)	T-Value	P-Value
Crossed Zones	0.2 ± 0.45	2 ± 2.55	1.55	0.195
Climbed Wall	0.0 ± 0	1 ± 2.24		
Stomped Foot	1.2 ± 1.30	0.0 ± 0		
Vigilance	56.6 ± 9.53	29.34 ± 21.07	-2.63	0.047
Vocalizations	0.4 ± 0.55	0.8 ± 1.10	0.73	0.498
Startle Responses	1.2 ± 1.10	2.2 ± 2.39	0.85	0.433
Canine Acknowledgement	6.8 ± 1.30	9.2 ± 3.11	1.59	0.173

Discussion:

The main goal of this study was to address the issue of stress in sheep from two different angles. With the first experiment, the aim was to analyze the relative

abundances of six bacterial species and determine if they fluctuate with acute stress and/or with the addition of chamomile to a sheep's diet. While this area of study has yet to be completed, the development of a reliable DNA purification and isolation protocol is a major step in achieving this project goal. The second experiment in the study aimed to determine if the addition of chamomile to a sheep's diet can reduce stress when exposed to a stressful situation. The amount of stress the sheep were experiencing was gauged by analyzing heart rate, salivary cortisol concentrations, and observed behaviors during the stress test.

Lima de Souza Rei *et al.* (2006) found that chamomile can reduce the physiological and behavioral signs of stress produced by the sheep. The cortisol samples obtained in this experiment are within range of concentration values found by other studies looking at salivary cortisol levels in sheep (Lima de Souza Rei *et al.,* 2006; Yates *et al*., 2010a; Yates *et al.,* 2010b; *Yates et al*., 2009). Cortisol samples taken one hour before the stress test (PRE) varied significantly between the CHAM and NON lambs. The NON and CON lambs also varied though not significantly. This is interesting because up until the actual stress test, the treatment of the NON and CON groups was exactly the same thus the cortisol concentrations should also be the same. The process of moving the lambs from the barn and into the shoot could be perceived as stressful, which could explain the difference in cortisol concentrations between the CHAM and NON lambs if the chamomile was having the desired effect. The difference could also indicate that chamomile lowers the normal daily salivary cortisol concentrations in the lambs regardless of stressors.

When looking at the cortisol levels across the different groups and sampling periods, there is a general decrease in cortisol in all three groups with a few minor exceptions. Ideally, there should have been a spike in the cortisol samples taken in the CHAM and NON groups one hour post stress test (1PST), but there was not. This could have a few different implications. There was also a spike in cortisol in the CHAM group two hours post stress test (PST2), which is hard to explain. Three hours post sampling (3PST), both the NON and CON lambs had lower salivary cortisol levels than the CHAM lambs although not significantly.

Heart rate was monitored in the CHAM and NON lambs during the stress test. Both the average and max heart rates were lower in CHAM lambs than NON lambs although not significantly. Heart rates varied from 139.2 bpm to 195.4 bpm, which is similar to heart rates observed by Lima de Souza Rei *et al*. (150 bpm to 175 bpm). Normal heart rate for sheep is around 70 to 80 beats per minute (Canadian Sheep Foundation, 2013). Heart rate and cortisol levels were not directly comparable because it is impossible to pinpoint at what time point cortisol released during the stress test would show up in the saliva samples. If a drastic rise in salivary cortisol had been observed, a rational conclusion would be to assume that the spike was due to the stress test and heart rates could be compared directly to those values. No discernable rise in cortisol can be seen in this data, which could have a few different explanations.

The video analysis looked at seven separate behaviors. The only behavior that varied significantly between the CHAM and NON group was vigilance ($P=0.047$).

The CHAM lambs spent more time being vigilant than the NON lambs, which contradicts Lima de Souza Rei *et al. (*2006). Diazapam treated lambs were less vigilant (less fearful) than control lambs suggesting that there is a negative relationship between time spent in vigilance and fearfulness. If this is true, my data suggests that from a behavioral standpoint, the CHAM lambs were more fearful and, therefore, more stressed during the stress test than the NON lambs. Based upon the heart rate and cortisol data collected, my data suggests otherwise but locomotion can be difficult to analyze in sheep as it presents in both extremes (standing completely still vs. actively trying to escape; Gougoulis *et al*., 2010). The CHAM lambs also displayed increased foot stomping, while the NON lambs displayed increase in crossed zones, wall climbing, startled response, and canine acknowledgement.

Interestingly, while the CHAM acknowledged the dogs less than the NON lambs (6.8 ± 1.30 vs. 9.2 ± 3.11), when they displayed stopping behavior it was done while staring at the dogs. Stomping in sheep is seen as an anti-predator behavior and this finding could be interpreted in two ways (Berger, 1978). One interpretation could be that the CHAM lambs were less fearful of the dogs than the NON lambs and were less hesitant to address the threat of the dogs. A second interpretation of this behavior could be that the CHAM lambs felt more threatened than NON lambs and felt the need to display the behavior to protect themselves. Being however that the CHAM lambs looked at the dogs less than the NON lambs, the first interpretation seems more likely.

One of the largest discrepancies in this study is that there was not rise in cortisol following the stress test. When an animal is stressed, their salivary and/or serum cortisol concentrations rise (Mostle and Palme, 2002; Hargreaves and Hutson, 1990; Ruiz-de-la-Torre *et al.,* 2001; Lima de Souza Rei *et al.*, 2006). There are several reasons as to why a spike in cortisol was not observed. The first is that the stress were not stressed enough. It is possible that while the lambs appeared stressed due to elevated heart rate, they were not stressed enough for cortisol concentrations to rise. A more likely explanation, however, is that the rise was simply missed due to the sampling schedule. The post stress collection times were chosen somewhat arbitrarily. However, it salivary cortisol concentrations can match serum cortisol concentrations as quickly as six minutes in sheep (Yates et al., 2010) and up to thirty minutes in humans (Eriksson et al., 1998). This was a major oversight and should be addressed in future studies. The change in cortisol levels that was observed is most likely due to the daily cycle of cortisol. Fulkerson and Tang (1979) found that cortisol levels in ewes peaked just after midnight and continued to fall till late afternoon. My study was conducted over the course of a day with the first samples being taken between 9 and 10 am and the last being taken between 2 and 3 pm in the afternoon. Thus, the pattern observed in this set of data fits the pattern of the circadian rhythm of cortisol secretion.

Even though the cortisol levels of all three treatment groups fell over the course of the four sampling periods, the CHAM lambs had the smallest range of values (CHAM: 0.991 ng/mL; CON: 1.322 ng/mL; NON: 3.134 ng/mL). This result could have some larger implications. While many of the differences were statically

insignificant, this trend warrants further research. It appears that chamomile does have an effect on cortisol levels in sheep. It is possible that I did not feed enough chamomile to have the effect wanted. There was also some variation within the groups and this could largely be due to the set up of the study. Due to the environment in which the experiment was carried out in, controlling for various factors proved challenging. The lambs were not fed individually so it is very possible that some lambs received more chamomile than others. It is also possible that others received more grain than others, which could have impacted their metabolic state at the time of testing. The NON and CON groups were housed with a larger flock of sheep for the five days leading up to the trial while the CHAM lambs were sorted out of the same group a day before the study began. Changing herd structure within a flock has been shown to impact stress levels (Freestone and Lyte, 2010). Since the CHAM sheep were taken from their herd and then mixed back in with it for testing, I would have expected their cortisol levels to be higher at the start of the study assuming the chamomile was not having its desired effect and that the sheep had not acclimated to their environment.

In the future, a more controlled environment would be ideal for carrying out the study. There was also no control for maximum cortisol levels. Every flock of sheep is different genetically, environmentally, and mentally, thus taking data from other studies may causes errors. In the future, taking a sheep from the same herd and stressing it out to a maximum capacity would be helpful when analyzing data. It would also be good to run a test to determine the circadian cortisol rhythm of the flock being studied. Further, changing cortisol sampling times would also be

beneficial. Ideally, the samplings would increase and start immediately after the stress test. In analyzing the data, some of the numbers may be misleading due to the small sample sizes of each group. Each treatment group had only five lambs due to the limitations in the environment the study was carried out in. Increasing the sample size to at least thirty sheep would be ideal.

My study found some indication that chamomile may have an anxiolytic-like effect on sheep with CHAM sheep being less stressed than NON lambs. During the stress test, the CHAM sheep had lower max and average heart rates and displayed behaviors indicative of a less fearful animal. In addition, salivary cortisol levels were lower at the start of the study and varied the least in CHAM sheep when compared to NON and CON lambs. The use of chamomile to reduce stress in sheep requires further research, but this study provides a good starting point for those wishing to pursue the area further.

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