5-2023

Effect of Increasing Levels of Gossypol and Fatty Acids Coming From Whole Cottonseed on Rumen Fermentation, Nutrient Digestibility and Microbial Community Composition in Continuous Culture Fermenters

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EFFECT OF INCREASING LEVELS OF GOSSYPOL AND FATTY ACIDS COMING FROM WHOLE COTTONSEED ON RUMEN FERMENTATION, NUTRIENT DIGESTIBILITY AND MICROBIAL COMMUNITY COMPOSITION IN CONTINUOUS CULTURE FERMENTERS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal, Dairy, and Veterinary Sciences

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UTAH STATE UNIVERSITY
Logan, Utah

2023

ABSTRACT

Effect of increasing levels of gossypol and fatty acids coming from whole cottonseed on rumen fermentation, nutrient digestibility and microbial community composition in continuous culture fermenters

by

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In the dairy industry, whole cottonseed (WCS) has been effectively used as a feedstuff, as it contains adequate levels of fiber, protein and fat. However, including supplemental fats, such as WCS, in ruminant diets have been shown to negatively impact fiber digestion. In this study we determined the impact of increasing dietary WCS relative to rumen fermentation, nutrient digestibility, and microbial community composition. Two experiments were conducted, each as a replicated 4×4 Latin square, using continuous culture fermenters (n=8). Treatments included a control diet without WCS, or the control diet plus 5, 10, or 15% (dry matter) WCS. The control diet was a 50:50 orchardgrass hay:concentrate mixture fed twice daily. In the second experiment, soybean meal and cottonseed meal (CSM) were added to ensure the diets were balanced for fiber and protein.

In both experiments, the prokaryotic community within the rumen fluid was characterized by sequencing the V4 region of the 16S rRNA gene, and protozoa were counted. Data were analyzed using a mixed model including the fixed effect of treatment and the random effects of period and fermenter. Linear, quadratic and cubic contrasts were tested. In the first experiment, no treatment effect ($P \geq 0.23$) was observed for NDF or starch digestibility, or for acetate concentration. Butyrate and ammoniacal N concentrations increased (Quadratic, $P \leq 0.05$), while propionate concentration tended to decrease (Quadratic, $P=0.08$) as WCS was added. Increasing WCS in the diet decreased (Linear, $P \leq 0.01$) protozoa count and relative abundance of archaea ($P=0.03$). Analysis of phyla abundance showed that six phyla were affected by treatments. The most abundant phyla across treatments were the Firmicutes and Bacteriodetes. Firmicutes abundance increased (Linear, $P=0.02$) whereas Bacteriodetes decreased (Linear, $P \leq 0.05$) with the addition of CS. Lachnospiraceae and Prevotellaceae were the most abundant bacteria families in all the samples. No treatment effect ($P \geq 0.30$) was observed for Lachnospiraceae, while Prevotellaceae abundance tended to decrease (Linear, $P=0.07$) as CS was added in the diet. The two most abundant genera across samples were Prevotella and Pseudobutyribrio, and the addition of WCS decreased the abundance of both (Linear, $P \leq 0.05$). In the second experiment, inclusion of CSM did not alter NDF digestibility (Linear, $P=0.21$) or ammonia production (Linear, $P=0.21$). No effects were seen relative to acetate (Linear, $P=0.32$), butyrate (Quadratic, $P=0.16$) or propionate (Linear, $P=0.12$) concentrations with the addition of WCS. Bacteria population linearly increased ($P \leq 0.02$) with inclusion of WCS while the opposite was observed for archaea (Linear, $P \leq 0.03$). Our results indicate that increasing levels of WCS up to 15% (DM) in the diet do not negatively impact fiber or

starch digestion in the rumen. Furthermore, inclusion of WCS positively affected microbial community composition at different levels.

(68 pages)

PUBLIC ABSTRACT

Impact of increasing dietary cottonseed on rumen fermentation, nutrient digestibility, and microbial community composition in continuous culture fermenters

Camila Castro Veloz

In this study we determined the impact of increasing dietary whole cottonseed (WCS) on rumen fermentation, nutrient digestibility, and microbial community composition. This study contributes novel information to the dairy community deepening the understanding of how including different levels of WCS can affect the rumen environment. This research was conducted in continuous culture fermenters. Treatments included a control diet without WCS, or the control diet plus 5, 10, or 15% (dry matter) WCS. The control diet was a 50:50 orchardgrass hay:concentrate mixture fed twice daily. In the second experiment, soybean meal and cottonseed meal (CSM) were included, and rations were balanced for fiber and protein. For the first experiment, no treatment effect was observed for neutral detergent fiber (NDF) or starch digestibility, or for acetate concentration. Butyrate and ammoniacal nitrogen concentrations increased, while propionate concentration tended to decrease as WCS was added. Increasing WCS in the diet decreased protozoa count and relative abundance of archaea. Firmicutes abundance increased whereas Bacteriodetes decreased with the addition of WCS. Lachnospiraceae and Prevotellaceae were the most abundant bacteria families in all the samples. The two most abundant genera across samples were Prevotella and Pseudobutyribrio, and the addition of WCS decreased the abundance of both. For the second experiment, with diets adjusted for fiber and protein, inclusion of

WCS did not affect NDF digestibility or ammonia production. Acetate and propionate decreased with the addition of WCS while no treatment effect was observed for butyrate production. Bacteria population increased with inclusion of WCS while the opposite was observed for archaea. Our results indicate that increasing levels of WCS up to 15% (dry matter) diet do not negatively impact fiber and starch digestion in the rumen. Furthermore, inclusion of WCS at affected the microbial community composition at different levels. In conclusion, supplementation of WCS had an overall positive impact on the rumen as it did not affect rumen fermentation or nutrient digestibility, and it increased bacteria population and decreased archaea concentrations.

DEDICATION

This I dedicate to my grandfather: wherever you are, I know you are tremendously proud.

ACKNOWLEDGMENTS

First of all, a big “thank you” to my major professor Dr. Fernanda Batistel for her invaluable help and support throughout the entire process, including carrying out this research during a pandemic, with all its implications. I will forever be grateful for the enormous opportunity she has given me. Thanks to the members of the USU ruminant nutrition lab; without your assistance, this research wouldn’t have been possible.

I would also like to thank the members of my committee. Dr. Thornton- Kurth, today I thank you for always saying yes and for always showing will to help and teach me whenever it’s been necessary. It has been a pleasure being your student and an honor to have you as a committee member during this project. Dr. Young, I’m grateful for your friendship, mentorship and for believing in me even when I didn’t. Even though most of our interactions were online, you have always been very present and wanting nothing but the best for me.

Of course, thanks to family, my pillar. I would like to thank my dad, Eusebio, for always supporting every single one of my decisions. Thanks to my mother, Nathalie, my role model and best friend. Your help and love mean the world to me and kept me going even when I thought things were too difficult. You made distance seem so small.

I thank Cotton Inc. for providing financial support for this research.

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CHAPTER I

LITERATURE REVIEW

Dairy cattle are the most efficient of all farm livestock, providing a rich food source available throughout most of the year (Hodgson, 1979). Dairy cattle are a critical part of the global food system and play an essential role in the sustainability of rural areas. A ration must contain all the necessary nutrients in order to maximize efficiency, production and economic viability within a dairy. One of the coproducts of the textile industry utilized as a feed in the dairy industry is cottonseed. Whole cottonseed (WCS) is a feedstuff that is commonly included in dairy rations across the United States.

The usage of WCS in dairy rations has increased over the years due to its relatively high digestibility (53.4%) (Pena et al., 1986) as well as fiber (~24%), protein (~23%) and fat (~20%) levels (Cooke and Bernard, 2005). Previous research shows that cows that receive 15% WCS (dry matter (DM)) in the diet do not exhibit any alterations in dry matter intake (DMI), but have 3.5% more fat corrected milk (FCM; +2.8 kg/day) compared with cows receiving a control diet without any WCS (Mena et al., 2001b). The control ration had 3.4% fat versus 6.0% fat in the treatment ration. These effects are presumably caused by the high fat concentration in those diets and the resultant increased energy content, which is known to improve milk yield and milk composition in dairy cows (Wu and Huber, 1994). Although including WCS in the ration has resulted in improved production, there are two factors, the concentration of polyunsaturated fatty acids and gossypol, that can limit the inclusion of WCS in the diet of dairy cows.

It is well established that high dietary concentrations of polyunsaturated fatty acids negatively affect fiber digestion in the rumen due to the negative effects of these fatty acids on microbial populations (Enjalbert et al., 1994). Free long chain fatty acids reduce the activity of cellulolytic microorganisms and also physically coat the feed particles limiting the access of the rumen microbes (Fuquay et al., 2011). Furthermore, gossypol, a phenolic component used by the cotton plants as a defense mechanism against insects, has also been shown to negatively affect rumen microbes, decreasing their numbers at first exposure (Wang et al., 2020). However, the gossypol content in WCS varies depending on the species of the cotton plant (Pons et al., 1953). For example, the cottonseed variety Pima contains a higher gossypol content compared to the Upland variety (Sullivan et al., 1993). Gossypol concentrations of WCS variety can go from 0.02 to 6.64%. The Upland kind, used in the present studies, contains 0.65% of total gossypol. Despite these negative attributes of WCS, it is still an attractive feedstuff for its high fiber content, protein, and energy. However, the impacts of WCS on the rumen microbial populations warrants further research.

The Gastrointestinal Tract of Ruminants

Ruminants are herbivores that possess a distinctive digestive anatomy that allows them to digest fibrous materials. The digestive system includes the mouth, tongue, salivary glands, esophagus, four-compartment stomach, pancreas, gall bladder, and small and large intestines. In the first portion of the ruminant digestive system, prehensile organs are present and essential for food intake. Lips, tongue, lower incisor teeth, and the dental pad (i.e., the rigid pad formed by the lack of upper incisors) are used for getting food into the mouth. The esophagus functions bidirectionally in ruminants, allowing them to regurgitate their cud for further chewing in order to reduce plant particle size which, in turn, enables ruminal microbes to gain access to structural

carbohydrates and other feed components. Ruminants secrete large quantities of saliva from different types of salivary glands. These secretions lubricate and moisten the feed, facilitating the processes of mastication and swallowing. Additional functions of the saliva include antifoaming properties and rumen pH stabilization (Weinberg and Sheffner, 1976).

The next portion of the ruminant digestive system consists of the four chamber stomach: the rumen, reticulum, omasum, and abomasum. The rumen and reticulum contain many different types of microorganisms that function to ferment feed, ultimately providing energy in the form of volatile fatty acids (VFA) for both the host and the microbes. Muscular contractions in the rumen and reticulum mix the digesta, allow for regurgitation of the feed bolus for re-mastication, and also support eructation of gaseous fermentation end products (i.e., carbon dioxide (CO_2)and methane (CH_4) (Weiss, 1953). The solid portion left behind in the rumen typically remains for up to 48 hours, depending on the diet, and forms a dense mat in the rumen where microbes can use the fibrous feedstuffs to make precursors for acetate, propionate and butyrate (Parish and Rhinehart, 2008). The third compartment of the stomach in a ruminant is the omasum. It has many leaves (lamellae), which allow particles to be retained for a longer time, increase surface area, and help to filter feed particles by size (Prins et al., 1972). The omasum also absorbs water, ammonia, and VFA. Lastly, comes the abomasum, which functions similar to a stomach in non-ruminant animals with acidic and protease secretions (Dijkstra et al., 2005). Digesta then flows into the small intestine where continued digestion and absorption of nutrients occurs.

The small intestine consists of the duodenum, jejunum, and ileum (Niwińska, 2012). Once digesta reaches the small intestine, it mixes with pancreatic and biliary secretions. These secretions maintain adequate pH levels of the digesta and also contain enzymes that digest protein, carbohydrates, and fats (Umphrey and Staples, 1992). Contractions starting at the

gastroduodenal junction cause digesta to pass from the duodenum through the small intestine (Dijkstra et al., 2005).The jejunum, the longest part of the small intestine, is located between the duodenum and the ileum and is where degradation products from carbohydrates and other organic products are absorbed. The jejunum is followed by the ileum and its primary purpose is to absorb vitamins and other nutrients that were not absorbed by the jejunum (Church, 1993).

The final steps of the digestive process take place in the large intestine. The large intestine consists of the cecum, colon, and rectum. Fermentation of any remaining feedstuffs occurs in the large intestine. Water, water soluble vitamins and VFA are absorbed in the large intestine. Unprocessed and unabsorbed feedstuffs are then eliminated in the form of feces (Mao et al., 2012) .

Rumen Fermentation

The rumenoreticulum holds microorganisms including bacteria, protozoa, archaea, and fungi that interact with one another and the host, forming a symbiotic relationship and playing a vital role in fermentation and feed digestion (Choudhury et al., 2012). The microbes ferment feed to produce VFA, which are used by the cow as energy for both maintenance and milk production. Fermentation also results in the supply of microbial protein that is also available to the host (Krause et al., 2003). The primary VFA that are produced are acetate, propionate and butyrate (Van Houtert, 1993). Dietary carbohydrates, like cellulose, hemicellulose, pectin, starch and sugars, are the main fermentation substrates. They are degraded to hexoses and pentoses before being fermented to VFA via the intermediate pyruvate (France and Dijkstra, 2005). Understanding rumen fermentation provides substantial benefits, as rumen dynamics are responsible for supplying nutrients to the animal (Krehbiel, 2014). Without the rumen microbes,

numerous adverse effects can occur relative to the health of the animal and its ability to digest and utilize feedstuffs, ultimately impacting productivity (Dehority, 2003).

Volatile Fatty Acids

The fermentation of fibrous carbohydrates in the rumen produces VFA, which can provide approximately 70-80% of a ruminant's energy needs (Van Houtert, 1993). Almost all VFA produced in the rumen are absorbed across the rumen wall by simple diffusion. Once they are absorbed, they will undergo different levels of metabolism. A small proportion (10-20%) will go to the omasum and/or abomasum (Hogan and Weston, 1969). Acetate, propionate, and butyrate are the predominant VFA produced from fermentation, while valerate, isobutyrate, and isovalerate are produced in lesser amounts, relatively. Acetate is used to provide energy and for the synthesis of short and medium chain fatty acids in the mammary and adipose tissue glands. Propionate is the primary precursor for glucose synthesis in the liver (Aiello et al., 1989) and is necessary to support glucose demands (Seal and Reynolds, 1993). The rumen epithelium primarily uses butyrate for energy (Bergman, 1990). In addition, fermentation provides energy in the form of ATP to promote microbial growth, which, in turn, provides the animal with protein in the form of microbial protein.

Bacteria

Bacteria play important roles in the biological degradation of plant fiber due to their large biomass and activity level. Under optimum conditions, doubling times of bacteria range from 15 mins. to 16 hours depending on the type of bacteria. Bacteria are acquired by the animal by direct contact with other cattle or by indirect contact with contaminated elements, such as food or drinking water. Bacteria can be classified based on the substrates that they use and their final

products of fermentation. However, it is important to keep in mind that the same bacterium can fulfill more than one metabolic function.

According to their function, rumen bacteria can be divided into primary and secondary bacteria. The two main primary bacteria are cellulolytic and amylolytic. Cellulolytic bacteria ferment structural carbohydrates (cellulose, hemicellulose, pectins). Important cellulolytic species include *Bacteroides succinogenes, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium loch headii* and *Cillobacterium cellulosolvens*. Healthy rumen pH can range from 5.8 to 7.0. The optimal pH for cellulolytic bacteria is 6.0-6.8.

Amylolytic bacteria are an essential group of microorganisms in the rumen of cows on concentrate diets (Maroune and Bartos, 1987). Optimal pH for this type of bacteria is 5.5-6.0. High grain diets result in an increase in the amount of starch in the rumen. *Streptococcus bovis*, an amylolytic bacterium, is normally present in low numbers in cows fed high forage diets (Matthews et al., 2019). *Bacteroides amylophilus, Succinomona amylolítica, Butyrividrio fibrisolvens, Lachnospira multíparus* and *Bacteroides ruminícola* are considered important amylolytic bacteria species.

Fibrobacter succinogenes, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Eubacterium cellulosolven*s and *Eubacterium ruminantium* are some examples of fibrolytic bacterial species (Stewart et al., 1997). Due to their ability to digest cellulose being much higher than that of other cellulolytic ruminal species, *F. succinogenes, R. flavefaciens* and *R. albus* have been considered the primary representative cellulolytic bacterial species in the rumen (Koike and Kobayashi, 2009).

Bacteria that utilize intermediate rumen acids, carry out a secondary fermentation of the end products of other rumen bacteria. These acids include lactate and succinate. Bacteria such as

Megasphaera elsdenii and *Selenomonas ruminantium* can ferment lactate into acetate, propionate or long-chain fatty acids. Succinate is the main end-product of many rumen bacteria, including the cellulolytic ones, it is converted in propionate and Co2 by *S. Ruminantium, Veillonella alcalescens, Anaerovibrio lipolytica* and *Propionibacteria.*

Archaea

The bacteria are most abundant followed by archaea (Wang et al., 2017). One of the natural by-products of ruminal fermentation, CH_4 is produced by the archaea by utilizing CO_2 and hydrogen (**H_2**). This H_2 comes from fiber degrading bacteria, protozoa and fungi. Archaea in the rumen use H_2 to reduce CO_2 to produce CH_4. This process keeps levels of H_2 low, directing fermentation towards production of other end products. H_2 accumulation allows more feed being fermented in the rumen (Kumar et al., 2013). Ruminant livestock can produce 250 to 500 L of methane (CH_4)per day (Johnson and Johnson, 1995). CH_4 is a greenhouse gas with global warming effects. CH_4 emission from enteric fermentation has been targeted as an important source of greenhouse gas (Moraes et al., 2014). Ruminal CH_4 production also represents a loss of energy for the animal (Johnson and Johnson, 1995), which could otherwise be available for animal growth or milk production. As such, it is necessary to improve understanding of methane-producing archaea and its role in the process of ruminal fermentation.

Protozoa

In addition to the bacteria in the rumen, there are larger organisms which have been designated protozoa (Williams and Coleman, 1997). The protozoal population in the rumen is composed of holotrichs and entodiniomorphs, the latter being by far the more numerous (Jouany, 1996). The protozoa composition in the rumen can vary with the dietary composition (Nakamura and Kanegasaki, 1969). Holotrich protozoa are very important in utilizing soluble sugars.

Holotrichs help to control the rate of carbohydrate fermentation when large quantities of soluble carbohydrates are present in the diet. The entodiniomorphs are responsible for controlling starch digestion by engulfing whole starch granules (Rode, 2000). This restricts bacterial access to the starch and slows down the fermentation process, Also, entodiniomorphs predate on rumen bacteria and engulf and digest them (Rode, 2000). However, despite contributing up to 50% of the bio-mass in the rumen and having a major impact on the rumen ecosystem as well as on the ruminant's welfare, the role of protozoa in rumen microbial ecosystem remains unclear. However, protozoa are an important component of the microbiome and are commonly used as an indicator of rumen health.

Fungi

Fungi are primary colonizers of fibrous plant materials in the rumen and are able to mechanically break up lignin-containing plant cell walls (Bauchop, 1979). Fungi possess fibrolytic enzymes including cellulases and hemicellulases (Joblin et al., 1989), which produce a rhizoidal system that can penetrate plant tissues, allowing access to fermentable carbohydrates that are not accessible to other rumen microorganisms (Bauchop, 1981). Even though fungi mostly colonize the lignocellulosic tissues, their ability to degrade and utilize lignin is not clear and their overall contribution in the rumen ecosystem has yet to be identified (Bauchop, 1979). Compared to bacteria, fungi have a much slower generation time (20-60 mins. vs. 5 hours). Additionally, some bacteria such as *Ruminococcus spp* can suppress fungal growth causing fungi not to be the predominant microorganisms in the rumen.

Fiber Digestion in the Rumen

Fiber is the predominant component of the plant cell wall and is primarily comprised of carbohydrates, including cellulose and hemicellulose, and the phenolic compound lignin. Fiber

cannot be digested by mammalian enzymes, but it is digested by enzymes produced by microorganisms in the rumen (Parish and Rhinehart, 2008). Cellulose is the most abundant fiber constituent (Van Soest, 1967), and it is composed of numerous glucose units linked by β-bonds. Starch, a carbohydrate source present in all feeds but found in increased concentrations in grains, is also comprised of glucose molecules, linked by α-bonds. Hemicellulose, the second most abundant fiber component, is composed of simple sugars and uronic acid. Although similar to cellulose, hemicellulose is less resistant to chemical agents (Van Soest, 1967). Lignin has an important role in plants in the formation of cell walls, but reduces the value of feed in cattle as it is largely indigestible by rumen microbes (Cunha and Miller, 2012). Lignin can prevent other nutrients from being attacked by rumen microbes (Dehority et al. 1962).

Cellulolytic (e.g. *Fibrobacter succinogenes)* and noncellulolytic (e.g. *Selenomonas ruminantium*) microorganisms in the rumen play a critical role in fiber digestion (Flint and Forsberg, 1994). Bacteria, protozoa, and fungi are the predominant microorganisms involved in this process. Microorganisms also need to penetrate additional layers of recalcitrant material present in other compounds like lignin, silica, and tannins, which are contained in roughage feed particles. Other factors such as plant structure, population density of fiber-digesting microorganisms and other animal-related factors such as mastication and salivation will also have an impact on ruminant fiber digestion (Cheng et al. 1991).

Bacteria play an important role in the biological degradation of plant fiber. They make up a large proportion of the rumen biomass and have higher activity than other types of microorganisms present in the rumen (Cholewińska et al., 2021). *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* are the predominant cellulolytic bacterial species in the rumen due to their greater ability to digest cellulose compared to other ruminal

bacterial species (Koike and Kobayashi, 2009). Lactating dairy cows have requirements for fiber in order to maintain normal rumination, chewing and saliva production, and normal ruminal function. Understanding the process of fiber digestion helps to create a diet for the cow that will improve both production and health of the animal.

Protein Digestion in the Rumen

Ruminants are able to use both true protein and non-protein nitrogen (NPN) sources of protein due to the microbial population in the rumen. Furthermore, in ruminant nutrition, dietary protein can be classified as rumen-degradable protein (RDP) or rumen-undegradable protein (RUP). The RDP, as the name suggests, is the fraction of dietary nitrogen (NPN and part of the true protein) that will be degraded and used by the rumen microbes, while the RUP is the portion of dietary protein that escapes the fermentation process in the rumen and can be digested and absorbed in other sections of the gastrointestinal tract. Microbial protein is eventually used by the animal and is often a source of high-quality protein.

During the fermentation process in the rumen, microbes release proteases and break down RDP into peptides, amino acids and ammonia (NH_3), which will be further utilized for the synthesis of microbial protein. The excess protein leaving the rumen is absorbed in the small intestine. NPN are compounds that contain nitrogen, but are not a protein, and can be converted into protein by the rumen microbes. This nitrogen can be utilized by the microbes for growth and synthesis of microbial protein. Unneeded ammonia diffuses across the rumen wall, goes to the portal vein, and is taken to the liver where it is detoxified into urea. This process of urea synthesis is laborious and energetically expensive (Hristov et al., 2019). After detoxification of ammonia in the liver, urea can be recycled back into the rumen through the saliva, or is eliminated via the kidneys through urine or milk (Bach et al., 2005).

Microbial protein arriving at the small intestine in the form of protozoa or microbes is typically the major source of protein for ruminants (Storm and Ørskov, 1983). RUP enters the abomasum and small intestine for digestion and absorption. The amino acid needs of the animal are met by both RUP and microbial proteins. Any proteins, in the form of both RUP and microbial protein, that are not absorbed in the small intestine will be excreted in the form of waste. Understanding protein digestion in a ruminant is critical to improve both the health and the productivity of the animal, and better understand composition of waste products that are produced.

Lipid Digestion in the Rumen

Lipids are present in forages, and are predominantly present in the leaf, which contains 6-7% of the dry weight of leaf tissue (Harfoot, 1981). Additionally, lipids can be provided in the diet as supplemental fat sources from either animal or plant sources. Fat is a concentrated energy source and can increase energetic density of the ration in animals that have a high production need, such as lactating dairy cows. The majority of fats in animal diets come in the form of triglycerides, a three-carbon molecule made of a glycerol backbone attached to three fatty acids (Drackley, 2007). These fatty acids that compose triglycerides can be unsaturated which have no double bonds, or saturated containing double bonds (Vander Wal, 1964).

In ruminant animals, dietary lipids begin being metabolized in the reticulorumen (Harfoot, 1981). The first step of lipid metabolism in the rumen is lipolysis, or the hydrolysis of fat. Hydrolysis of triacylglycerol is done predominantly by microbial lipases, produced mainly by the *Anaerovibrio lipolytica* bacterial species (Henderson and Hodgkiss, 1973). This process occurs rapidly in ruminal digesta. The rate of lipolysis increases with an increasing amount of unsaturation of fatty acids (Jenkins, 1993). This process releases saturated fatty acids, monounsaturated and polyunsaturated, and must be carried out prior to biohydrogenation

(Castillo et al., 2013). Biohydrogenation is a biological process done by the microbial ecosystem in which unsaturated fatty acids are turned into saturated end products (Mosley et al., 2002). The main role of ruminal biohydrogenation is to reduce the toxic effects of unsaturated fatty acids on bacterial growth (Palmquist et al., 2005).

Increased fat content in feeds can lower fiber digestion in the rumen, presumably caused by the presence of long-chain fatty acids inhibiting rumen bacteria (Palmquist et al., 1986). Rumen protected lipid supplements can be a way to increase dietary lipid intake, leading to an increase in energy consumption, without negatively impacting the microbial population (Wiseman, 1984). Additionally, the formation of the short chain (C2-C5) fatty acids represent an enormous contribution to lipid metabolism after absorption from the rumen and indirectly through bacterial and protozoal lipid synthesis (Noble, 1981). Nutrients are only beneficial to the cow if they can be digested and absorbed; otherwise, they pass through the digestive tract and are excreted. Understanding how lipids are digested will help us determine better ways to incorporate these in the diet of high-producing animals such as dairy cows without harming the ruminal ecosystem.

Cottonseed

The cotton plant belongs to the genus Gossypium of the family Malvaceae. The plant has a fruit that opens when maturity is reached and contains cottonseeds inside. The balance of nutrients contained in WCS has made it an ingredient commonly used in rations for lactating dairy cows. In 2005, it was ranked as one of the top five feedstuffs used in the midwest, southeast, and southwest regions of the United States (Bertrand et al., 2005). Levels as high as 25% (DM basis) of the ration are fed to dairy cows and primarily have a positive impact on production (Coppock et al., 1985). Some of these positive outcomes include an increase in milk production along with a boost in butter fat (DePeters et al., 1985a). Previous research analyzed

the effects of feeding different amounts of gossypol from WCS (7.5% and 15% of DM), a feed that has relatively high amounts of gossypol and cottonseed meal (3.5% and 7.5% of DM), a feed that has very little, if any gossypol(Mena et al., 2001a) . This study was done in lactating dairy cows and found that milk yield for cows fed diets with increased WCS content had increased milk production (up to 5 kg/d higher) compared to those receiving diets with lower WCS and gossypol concentrations (Mena et al., 2001a). These effects are presumably caused by the high fat concentration in those diets (6%) and increased energy content, which is known to improve milk yield in dairy cows (Wu and Huber, 1994).

There are some factors that limit the use of including WCS in a ration. One of these factors is the high concentration of fat which can ultimately lead to negative effects on fiber digestion. Fiber digestibility may be reduced due to the negative effects of the free oil on microbial population in the rumen. When relatively large amounts (25% DM) of WCS are fed to dairy cattle, free oil physically coats fibrous material, and this reduces the access of cellulolytic microorganisms to the fiber (Fuquay et al., 2011). Another significant factor is gossypol, an anti-nutrient contained by cotton plants which defends the plant from insect pests (Chan et al., 1978).

There are two existing types of commercial WCS: low-lint (Pima seed) and high-lint (whole linted Upland). On a dry matter basis, Pima WCS contains 50 to 100 g/kg less fiber, with higher fat and protein content (Arieli, 1998). Also, the Pima cottonseed has a larger gossypol content compared to whole linted Upland and is often processed prior to being fed to cattle by cracking or grinding to improve nutrient utilization and performance (Sullivan et al., 1993).

Gossypol

Gossypol is a yellow, polyphenolic compound found primarily in the pigment glands of the cotton plant, and it exists in both free and bound forms (Santos et al., 2003). The gossypol

content will vary depending on the species of the cotton plant (Pons et al., 1953). Gossypol can be found in concentrations reaching up to 1.7% and occasionally more than 6% of the seed's dry weight (Margalith, 1967). In the whole seed, gossypol is mostly found as free gossypol and it is believed that free gossypol in WCS is less toxic because it has a longer ruminal retention time and is therefore more subject to the detoxifying activity of microorganisms (Chain et al., 2017). When WCS is processed, gossypol binds to proteins (Calhoun et al., 1995) making it unavailable for intestinal absorption and increasing the animals' tolerance to the feed containing WCS (Rathore et al., 2020). Gossypol can cause impairment of male reproduction in ruminants (Gadelha et al., 2014). Concentrations as high as 400 ppm free gossypol are considered toxic, and 800 ppm free gossypol results in some mortalities of growing bull calves (Risco et al., 1992). Other negative effects of gossypol may include respiratory distress, anorexia, weakness, and even death (Gadelha et al., 2014). The immune system can also be negatively affected (Gadelha et al., 2014).

The rumen microbial community contains some gossypol-degrading bacteria (Russell and Rychlik, 2001). However, it is possible that feeding excessive amounts of gossypol in the free form may exceed this protective mechanism and impair animal performance. The soluble and easily degraded components in feeds are always utilized by rumen microbes first, followed by the insoluble, but potentially digestible components. In addition, previous studies have shown that the activity of gossypol in animals that were fed WCS products, might lead to a substantial change in the equilibrium of the microbial composition of the gastrointestinal tract of these animals (Margalith, 1967). It is not known for sure what is the ruminal capacity for the detoxification of gossypol, and undoubtedly there are risks if WCS is included at high levels in the diet.

Unsaturated Fatty Acids

The most common way to increase energy of ruminant diets is to provide them with fats. Most of the fat fed to ruminants is unsaturated, and in many high producing ruminant diets, fat represents around 5% of the total DM, 6% being the acceptable upper limit. Oilseeds such as flax seeds, rapeseed, soybeans and cottonseed can be an important source of fat in ruminant diets as they are rich in unsaturated fatty acids (Doreau and Ferlay, 2015). These may include oleic acid, linoleic acid and alpha-linolenic acid. A limit to the fat supplementation of ruminant diets is the negative effect on ruminal digestibility, especially when high levels of unsaturated fatty acids are present (Brooks et al., 1954).

Table 1.1 Fatty acid content of whole cottonseed (WCS) (%)

Fatty acid	(Dowd et al., 2010)	(Lukonge et al., 2007)	(Nergiz et al., 1997)	(Yunusova et al., 1991)
C14:0	0.56-1.4	0.62-0.93	0.67-1.08	0.2-0.6
C16:0	19.6-27.6	20.6-25.1	20.1-26.8	20.7-43.2
C16:1	0.43-0.79	0.41-0.59	0.82-1.23	0.8-3.2
C18:0	2.0-3.2	0.22-2.79	1.87-2.37	0.9-4.4
C18:1	12.8-22.2	15.2-18.5	14.0-17.6	11.3-26.9
C18:2	44.0-59.3	52.0-57.2	51.2-59.2	26.1-58.8
C18:3	0.15-0.25	0.17	0.51	-
C20:0	0.20-0.45	0.22-0.33	0.16	-
C22:0	0.08-0.20	0.11-0.18	-	-
C24:0	0.08-0.20	0.23	-	-

Summary

Earlier research suggests that including WCS in diets for lactating cows can improve milk production as it is a rich source of fat, protein and fiber for dairy cows. However, the use of WCS and the presence of gossypol pose a threat to important microorganisms that break down feed to produce VFA, which are used by the cow as energy for maintenance and milk production. As such, more research needs to be conducted to determine the impacts of including WCS in the

ration of dairy cows relative to its effects on rumen fermentation. The objective of this study was to identify the effects of increasing levels of WCS relative to rumen fermentation, while also characterizing populations of microorganisms. The hypothesis was that inclusion levels as high as 15% (DM) would negatively affect nutrient digestion and microbial community composition while inclusion levels of 5% or 10% would be considered safe to feed without negatively impacting fermentation, nutrient digestion and microbial community composition.

CHAPTER II

EFFECT OF INCREASING LEVELS OF GOSSYPOL AND FATTY ACIDS COMING FROM WHOLE COTTONSEED ON RUMEN FERMENTATION, NUTRIENT DIGESTIBILITY AND MICROBIAL COMMUNITY COMPOSITION IN CONTINUOUS CULTURE FERMENTERS

INTRODUCTION

As a major producer and exporter of cotton, the US plays a vital role in the global cotton market. In 2019, the US produced nearly 20 million bales of cotton worth about $7 billion (USDA, 2021). The lint is removed from the plant for use in many different industries, while the leftover cottonseeds can be used as animal feed. Whole cottonseed (WCS) is generally treated as solid waste by the textile industry (Moreira et al., 2004). Thus, utilization of WCS as a feedstuff for livestock not only proposes a cheap feedstuff for farmers, but is also beneficial for the environment (Bhat, 2021).

WCS is commonly used in rations for lactating dairy cows as it serves as a source of energy, fiber, and protein. The relatively high oil content makes the WCS an attractive option for animals with high energy requirements, such as lactating dairy cows (Cooke et al., 2007a). In an

experiment with cows fed diets containing either 0% or 20% WCS (DM), results showed that WCS supplementation increased milk yield by 2 kg/day (Belibasakis and Tsirgogianni, 1995). Another study in which cows were fed diets with 0, 10, 15, or 20% WCS (DM), showed percentages of total milk solids increased from 11.87% to 12.17% when including WCS at 20% versus the control diet that did not contain any WCS (DePeters et al., 1985b). In the same experiment, 4% fat-corrected milk and milk fat were increased with the addition of WCS (DePeters et al., 1985b). Overall, WCS is a good source of essential nutrients, and its use in rations of lactating dairy cows shows a positive effect on animal performance. However, gossypol and relatively high levels of unsaturated fatty acids are considered detrimental factors when determining the amount of WCS that should be included in a diet.

The high content (12.75%) of unsaturated fatty acids in WCS can lead to milk fat depression and decrease fiber digestion (Conte et al., 2018). Unsaturated fatty acids are biohydrogenated and are converted into *trans* fatty acids in the rumen. *Trans* isomers play a role in causing milk fat depression (Chilliard et al., 2000) and have a significant impact on dairy economics because the majority of producers are paid for milk solids. Gossypol is a polyphenolic compound present in WCS and can have detrimental effects on the rumen microbiome and overall health and production of the animal. For example, gossypol acetate decreased *Fibrobacter succinogenes* and *Ruminococcus albus*, which are two important fiber-digesting bacteria (Kothari et al., 2018). In another study, PCR analysis showed that addition of 5, 10, and 15 mg/mL gossypol acetate solution decreased the populations of *Fibrobacter succinogenes, Ruminococcus albus, Butyrivibrio fibrisolvens, Prevotella ruminicola, Selenomonas ruminantium*, and increased *Ruminococcus flavefaciens*, protozoa, and total bacteria in cultured rumen fluids in comparison with the control (Wang et al., 2021). Although WCS can have

positive impacts on dairy cow production, the presence of gossypol and unsaturated fatty acids can have deleterious effects on fiber digestion. However, it is currently unknown what percentage of WCS can be included in the diet without negatively affecting rumen fermentation.

As such, the goal of the present research was to analyze different, increasing levels of WCS in order to determine how much WCS should be added to the diet of milking cows. To test this, two different experiments were conducted. The objective for experiment one was to determine the effects of increasing levels of WCS on rumen fermentation, nutrient digestibility, and microbial community composition. In experiment two, increasing concentrations of WCS were tested as well, but all diets were balanced for protein and NDF content. The objective of experiment two was to test the effect of increasing levels of WCS, independent of protein and NDF content in the diet, relative to rumen fermentation, nutrient digestibility and microbial community composition. For the first experiment, we hypothesized that high levels of cottonseed (20% DM) would negatively impact rumen fermentation, microbial population and nutrient and fiber digestibility compared to the control. Similarly, for the second experiment, it was hypothesized that with high levels of WCS, parameters such as rumen fermentation, microbial population (including protozoa count), and nutrient and fiber digestibility would be negatively impacted.

MATERIALS AND METHODS

Two experiments were performed in continuous culture fermenters to evaluate the hypotheses. Both experiments were similarly performed, except for the dietary treatments.

Continuous Culture Operation

Eight continuous culture fermenters, designed according to Teather and Sauer (1988) were used in a 4 × 4 Latin square design with 4 periods of 10 days each; 6 days for adaptation

and the last 4 days for sampling. Rumen content was collected from a lactating Holstein cow fitted with a rumen cannula fed a diet of approximately 54% forage and 46% concentrate mix. The rumen contents were squeeze filtered through double layered, grade 60 cheese cloth into pre-warmed 39°C containers. Filtered rumen content was inoculated 1:1 with 39°C artificial saliva prepared as previously described and transferred into the fermenters (Weller and Pilgrim (1974)). Continuous stirring of fermenter contents was achieved by a central paddle set at a speed of 50 rpm, CO_2 was administered at a fixed rate of 20 mL/min to displace O_2 and a circulating water bath maintained the temperature of the fermenters at 39°C. Artificial saliva was delivered continuously using a peristaltic pump to maintain a 10%/hour fractional dilution rate. The fermenters pH was maintained at a range from 5.8 to 6.6.

Experimental diets – Experiments 1 and 2

The diets for both experiments (Tables 2.1 and 2.2) consisted of a 50:50 orchard grass hay and concentrate mixture (40 g DM/day) fed twice daily (0800 and 1600). The treatment diets for Experiment 1 were: 1) control diet without WCS; 2) control diet plus 5% WCS; 3) control diet plus 10% WCS; and 4) control diet plus 15% WCS. The treatment diets for Experiment 2 were the same as experiment 1, except in the second experiment, diets were balanced for fiber and protein.

Data and Sample Collection and Analysis

D 7, 8, 9, and 10 of each period, outflow was collected and kept on ice to prevent further fermentation. Subsamples of the effluent were collected as described below for analyses. A subsample of 500 mL of effluent from each fermenter was frozen at -20°C and then freeze dried (FreeZone 12, Labconco, Kansas City, MO) for digestibility analyses. Dried effluent was composed by period and treatment and ground through a 1mm screen with a Wiley mill (Arthur

H. Thomas, Philadelphia, PA) and analyzed for NDF (Van Soest et al., 1991) using the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY). In starch analysis, D-glucose, corn starch, and a reagent blank were run with each set of test samples (Hall, 2009). Starch samples (90-100 mg) were weighed and then sodium acetate buffer (pH 5.0) and 0.1 mL heat-stable -amylase were added. After mixing, the tube was incubated for 1 h at 100°C and the tub was mixed using a vortex mixer at 10, 30, and 50 min of incubation. Amyloglucosidase (1 mL) solution was added, and the tube was mixed and incubated again. Distilled water (20 mL) was added to the tube and mixed. The weight of the tube was recorded, and 1.5 mL sample solution was transferred to a 2 mL microcentrifuge tube and centrifuged at 12,000 x g for 10 min. Dilutions were prepared by weight of sample solutions so that they fell within the standard curve. For ash analysis, feed materials were tested following the conditions recommended by AOAC at an ignition at 600°C for 2 h (Thiex et al., 2019). The thoroughness of the ignition was determined by the color of the ash residue and a corresponding reduction in residue weight.

VFA composition of the samples were analyzed through gas chromatography as previously described (Eun and Beauchemin, 2007). A subsample of 20 mL of effluent form each fermenter was added to a bottle containing 1 mL of 6 N HCl to stop fermentation and then frozen at -40°C for VFA analysis. Stock solutions of valeric acid, propionic acid, acetic acid, and butyric acid were prepared by dissolving each VFA in diethyl ether. For the sample preparation, micro-centrifuge tubes were filled and spun at 16128 x g for 2 mins. Then, the supernatant was transferred to another micro-centrifuge tube with 200 µl of crotonic acid solution and spun at 12000 RPM for 10 mins. The supernatant was removed and later transferred to auto sampler vials. Auto sampler vials with samples were set in the gas chromatographer. An inlet liner was

used to clean “dirty’’ samples. Helium, nitrogen and hydrogen were used as carrier gases during gas chromatography.

To measure microbial growth, on d 3 buffer and fermenters were enriched with 10% 15N at 25 mg/L. Background effluent sample was taken and fermenters and dosed with 15N, a microbial marker. A sample of effluent was taken prior to the primed, continuous infusion for background 15N analysis. One daily sample of the effluent was taken on d 7, 8, 9, and 10 and composited by fermenter for analysis. All samples were immediately frozen at −20°C. Sub-samples of effluent to be used for 15N analysis were raised to a pH of approximately 9 using 25% NaOH in order to volatilize ammonia from the sample. Bacterial and effluent samples were analyzed for 15N diffusion as previously described (Wenner et al., 2017). A total of 9.3 mL of sample solution was added to a 15 mL Falcon tube. Then 700 µl of diluted trichloracetic acid (TCA) was added and incubated on ice for 30 mins. The tubes were centrifuged at 28000 x g for 20 mins. and the supernatant was pipetted into different specimen cups with paper filter disks. Filter disks were pipetted with sulfuric acid (H_2SO_4), making sure that the sample and the paper disk would not make contact. Using a syringe, specimen tubes were injected with NaOH. The containers were left for 6 days at room temperature. Lastly. filter disks and a beaker containing 100 mL of H_2SO_4 were put in a desiccator and left overnight. After drying, the paper disks were transferred to tin capsules to later be analyzed as previously described (Hristov et al., 2001).

DNA extraction

16S rRNA gene sequencing, or 16S amplicon sequencing, was performed to determine the relative abundance of bacterial communities. Samples from each period were collected directly from the fermenters and frozen at −80°C. Total genomic DNA was extracted from the thawed composite samples using the bead beating method as previously described (Yu and

Mohn, 1999) followed by purification of the DNA using a QIAamp column (Qiagen, Valencia, CA). The DNA samples were used in PCR amplification in which DNA went through denaturation, then annealing, where DNA molecules bind to the target DNA. Lastly, DNA polymerase was extended along the template strands. Sample concentrations were measured using Qubit™ fluorometer (Invitrogen). PCR was performed using universal primers flanking the variable 4 (V4) region of the 16S rRNA (Kozich et al., 2013). Samples were quantified with a Qubit fluorometer, pooled on an equimolar basis, and sequenced with MiSeq v3 kit (2×300 cycles, Illumina) according to the manufacturer's protocol. All sequences were demultiplexed on the Illumina MiSeq system. Further, sequence processing was performed using mothur v1.45.1 (Schloss et al., 2009) following a protocol described previously (Kozich et al., 2013). Briefly, paired-end sequences were combined into contigs, and poor-quality sequences were removed. Bacterial sequences were aligned and classified using the SILVA 16S rRNA database (Pruesse et al., 2007). All sequences were grouped into 97% operational taxonomic units (OTU) by uncorrected pairwise distances and furthest neighbor clustering. Prokaryotic communities were normalized to equal sequence counts near the lowest sample, and these normalized OTU tables were used in all further analyses.

For ruminal protozoa counting, 1:1 dilution of ruminal fluid to formalin solution was made and mixed. The mixture was stained with methyl green and allowed to stand for at least 4 h before counting. Protozoa were counted with the Sedgewick-Rafter Cell chamber. The slide was set for 10 mins. to allow settling of protozoa. 50 microscopic fields were counted per sample, using a 10X objective (Dehority, 1984).

Statistical Analysis

The data from both experiments were analyzed using the MIXED procedure of SAS v.9.4 (SAS Institute, Inc. Cary, NC) according to the following model:

$$Y_{ijk} = \mu + p_i + f_j + T_k + e_{ijk,}$$

Where Y_{ijk} = variable of interest, μ = overall mean, p_i = random effect of period (i = 1 to 4), f_j = random effect of fermenter (j = 1 to 8), T_k = fixed effect of treatment (k = Control, 5% cotton seed, 10% cotton seed, and 15% cotton seed), and e_{ijk} = residual error.

RESULTS

Experiment 1

Different levels of WCS did not affect NDF or starch digestibility when compared with control ($P \geq 0.22$; **Table 2.3**). Ammonia levels showed a linear increase with WCS inclusion rate (Linear, $P < 0.01$). Acetate concentration was not affected by the different treatments ($P \geq 0.23$). For butyrate concentrations, an increase was shown with the addition of WCS at the level of 5% relative to the control (Quadratic, $P \leq 0.05$). However, propionate production showed a tendency to decrease at 5% WCS when compared to the control (Quadratic, $P = 0.08$). Isobutyrate and valerate concentrations each increased as WCS was added in the diet (Linear, $P < 0.01$).

Rumen alpha diversity (Table 2.4) showed no treatment effect when analyzed via n seqs ($P \geq 0.41$). Chao-index was used to calculate richness of bacterial communities during the experiment. Microbial community diversity and an evenness of abundance among the species by was calculated using the inverse-Simpson index. Similarly, Sobs, Coverage, Ace and Chao index did not show a treatment effect ($P \geq 0.20$). Diversity measured by the Shannon index showed a linear increase with the addition of WCS (P= 0.05). Simpson exhibited a linear decrease with higher levels of WCS (P = 0.02). Inverse Simpson results showed a linear effect of increasing with the addition of WCS ($P < 0.01$).

Total microbial community structure (Bray-Curtis) and composition (Jaccard) were calculated (Table 2.5). The PERMANOVA was run to determine the differences in community structure and composition between the treatments. In this case, no effects were observed ($P > 0.94$). A linear effect was seen when measuring kingdom relative abundance (Table 2.6) for both bacteria and archaea ($P = 0.01$). In response to the increasing amounts of WCS, bacteria increased its abundance while archaea decreased its abundance as the amounts of WCS increased.

The relative abundances of bacterial phyla from different treatment groups are displayed in Table 2.7. A total of nine phyla were identified within the ruminal bacterial population from 16S rRNA sequencing. The most abundant phyla across treatments were the *Firmicutes* and *Bacteroidetes*. *Euryarchaeota, Thermoplasmatota, Desulfobacterota, Patescibacteria, Spirochaetota*, and *Verrucomicrobiota* were observed as well. *Verrucomicrobiota* did not show any interaction effects (Linear, $P \geq 0.39$). Relative abundance of *Thermoplasmatota* and *Actinobacteriota* were not influenced by the addition of WCS (Cubic, $P \geq 0.39$). *Firmicutes* abundance increased with the addition of WCS (Linear, $P = 0.02$).

The 16S rRNA sequencing of ruminal bacteria DNA samples from continuous culture fermeneters detected 19 families (Table 2.8) and 41 bacterial genera (Table 2.9). *Lachnospiraceae* and *Prevotellaceae* were the most abundant bacteria families in all the samples. No treatment effect was ($P > 0.30$) observed for *Lachnospiraceae*, while *Prevotellaceae* abundance tended to decrease as WCS was added to the treatment ration (Linear, $P = 0.07$). *Methanobacteriaceae, Clostridiaceae, Corynebacteriaceae, Desulfovibrionaceae, F082, Planococcaceae, Rikenellaceae, Prevotellaceae, Ruminococcaceae, Veillonellaceae and Spirochaetaceae* decreased their abundance with the increased percentage of cottonseed (Linear,

$P \geq 0.01$). As WCS was added, *Anaerovoracaceae, Oscillospiraceae, Bifidobacteriaceae,* and *Christensenellaceae* abundance increased as well (Linear, $P < 0.01$). *Bacillaceae* decreased with WCS at 10% (Cubic*,* $P = 0.07$*).*

Among the 42 identified genera (Table 2.9)*, Ruminococcus, Saccharofermentans, Lactobacillus, Oribacterium, NK4A214_group, Shuttleworthia, Bifidobacterium, Moryella, Monoglobus, Flexilinea* and *Pseudoscardovia* exhibited an increase in abundance with the addition of WCS (Linear, $P \leq 0.05$) while *Pseudobutyrivibrio, Rikenellaceae_RC9_gut_group, Kurthia, Methanobrevibacter, Desulfovibrio, Methanosphaera, Empedobacte, Absconditabacteriales_*and *Lachnospiraceae_FCS020_group* decreased their abundance with the addition of WCS (Linear, $P \leq 0.05$). Genera *Prevotella, Butyrivibrio, F082_ge, Treponema* showed a tendency for decrease ($P \leq 0.10$) while *CAG-352 (*$P = 0.08$*), Marvinbryantia (*$P = 0.07$*)* and *Lachnospiraceae_UCG-002* ($P \leq 0.10$) each exhibited a tendency to increase.

Experiment 2

No treatment effects were observed when measuring NDF digestibility or ammonia ($P \geq 0.21$) production in the continuous culture fermenters (Table 2.10). Starch digestibility decreased linearly across the different treatments, including WCS (5%-15%) when the treatments were balanced for protein and NDF ($P = 0.02$). As the percentage of WCS increased, proportions of acetate were not affected (Linear, $P \geq 0.32$). Similar effects were seen in the production of propionate (Linear, $P \geq 0.12$). Butyrate concentrations were not affected by including any level of WCS (Linear, $P \geq 0.22$). The structure of the microbiomes evaluated by alpha diversity measures **(Table 2.11)**, exhibited effects between control and 5% ($P \leq 0.05$) WCS as compared to 10% and 15%, which indicates the average proportional abundance of bacterial species

showed significant responses suggesting that the diet containing 5% WCS in continuous culture fermenters was richer and more diverse.

Looking at the microbial community composition, there was an increasing linear effect for bacteria population ($P \leq 0.02$). Archaea, on the other hand, showed a linear decrease with the increasing levels of WCS (Linear, $P \leq 0.03$). Bray-Curtis dissimilarity and Jaccard distance (Table 2.12) measuring microbial similarity in response to increasing WCS did not show a treatment effect ($P \geq 0.92$).

After being fixed and stained, rumen protozoa counting results (Table 2.13) revealed that as the amount of WCS was increased in each treatment, the number of protozoa decreased (Linear, $P \leq 0.01$). Microbial community composition was not examined past the kingdom level in experiment 2.

DISCUSSION

This study measured the impact of increasing levels of WCS on rumen fermentation, fiber digestibility and microbial community composition in continuous culture fermenters. Being that WCS is a rich source of nutrients, it is commonly included as a component of rations for lactating dairy cows. However, WCS contains gossypol, a compound that can cause a decrease in the level of rumen microorganisms, affecting fiber degradation (Acamovic and Stewart, 1993). It is currently thought that a high tolerance to gossypol by adult ruminants could be attributed to microbial detoxification via binding to soluble proteins and microbial degradation in the rumen (Reiser and Fu, 1962). Furthermore, it is believed that the use of fats in diets of dairy cows can negatively impact fiber digestibility when high amounts of unsaturated fats are present (Brooks et al., 1954, Jenkins, 1993). Thus, more research is needed to determine how different inclusion levels of WCS affect rumen fermentation.

Experiment 1

In experiment 1, the present study determined how including WCS at different levels (5, 10 or 15%) impacted rumen fermentation and microbial communities. NDF or starch digestibility did not differ across treatment diets. Previous research has shown decreased NDF digestibility in sheep as the proportions (150, 300 or 500 g of WCS) in the diet increased, but this may be attributed to the low nitrogen content of the grass hay utilized in the different treatments (Ismartoyo, 2017). However, our results agree with previous studies including levels as high as 15% WCS in which authors did not see significant effects of WCS on digestibility of fiber components in dairy cows (Smith et al., 1981). Taken together, these results demonstrate that including WCS at levels up to 15% of the diet does not negatively impact fiber digestibility.

Acetate, propionate, butyrate, ammonia, isobutyrate, valerate and isovalerate concentrations were analyzed. Acetate concentration was not affected by the different treatments in the present study. Propionate exhibited a tendency for a quadratic decrease while butyrate concentration showed a quadratic effect as it increased. This contradicts previous research, in which diets with 5%, 15% and 30% cottonseed were fed to Holstein cows and an increase was seen on acetate concentrations, however, the same study saw a decrease in proprionate and butyrate but no effects in valerate, isovalerate or isobutyrate (Horner et al., 1988). Decreased molar proportions of butyrate have been reported as dietary concentrations of free fatty acids increase (Avila et al., 2000). Ammonia, butyrate, isobutyrate, valerate and isovalerate proportions increased along with the WCS levels. Noftsger et al. (2003) reported increased concentrations of isobutyrate and valerate in fermenters when supplemented with 2- hydroxy-4 butanoic acid. Authors suggested that the increase was due to reduced use of these volatile fatty acids for synthesis of the corresponding amino acids because ammonia concentrations were similar across treatments. However, propionate decreased by 1.5% at 5% WCS in the treatment

ration. In the current study, there were no visible treatment effects in the production of caproic acid. When feeding 13.96% DM WCS and comparing WCS with normal concentrations of free fatty acids (FFA) (6.8%) vs. WCS with high FFA (24.1%, 22.3%), Cooke et al. (2007b) saw no alterations on ruminal fermentation other than an increase in isobutyrate for diets with high concentrations of FFA in WCS.

Different kinds of analyses of the microbiome showed significant changes to alpha diversity occurred with the administration of 5% WCS. The structure of the microbiomes as evaluated by alpha diversity measures, exhibited effects between control and 5% WCS as compared to 10% and 15% WCS. Simpson index, which measures the degree of concentration, comparison of diversity measured by the Shannon index, and Inverse Simpson, which indicates the average proportional abundance of bacterial species showed significant responses suggesting that the diet containing 5% WCS in continuous culture fermenters was richer and more diverse. Diversity and development of rumen microbiota is critical for ruminant livestock production. These results confirm that diets with inclusion of WCS can positively impact the rumen microbial community.

At the kingdom level, it was observed that bacteria and archaea were both altered by treatments such that archaea concentration decreased as the levels of WCS increased, while bacteria increased. This is consistent with what was found in a previous study in which supplementation of WCS in the diet of dairy cows resulted in decreased methane (CH_4) emissions (Grainger et al., 2010). CH_4 is a greenhouse gas that is produced by the microbes that break down the feedstuffs consumed by livestock. The reduction of livestock emissions, without affecting animal production, is one of the most desirable goals in the industry. It has been found that oilseeds have CH_4-reducing effects in ruminants (Martin et al., 2010). Previous research

reported that *Moringa oleifera*, containing a significant amount of oil (up to 40%) altered the composition and diversity of methanogens, hence mitigating CH_4 emissions in dairy cows (Dong et al., 2019). However, more research of the CH_4-lowering effect of WCS needs to be done in order the to understand this in more detail.

The present study observed that *Firmicutes* and *Bacteroidota* were the dominant species when analyzing for phylum relative abundance across fermenters. Compared to other research, similar results have been shown (Mao et al., 2015; Dai et al., 2017). In the present experiment, *Firmicutes* exhibited an increase as WCS was supplemented while *Bacteroidota* decreased with the addition of WCS. Previous research suggests that the chemical composition of WCS could potentially inhibit *Bacteroidota*, and promote the activity of bacteria in *Firmicutes* phylum (Evans et al., 2011; Wang et al., 2021) For this study, *Firmicutes* bacteria activity was promoted, suggesting that the amount of free gossypol present in WCS could inhibit *Bacteroidota* and promote *Firmicutes* abundance. *Firmicutes* is considered one of the main fiber degraders in the rumen, while *Bacteroidota* is considered a main degrader of non-fibrous carbohydrates in the rumen. Earlier research showed that gossypol had the ability to inhibit the population of *Bacteroidota* bacteria while promoting abundance of *Firmicutes* (Wang et al., 2021). It's been demonstrated that the *Firmicutes*-*Bacteroidota* ratio affects energy use and body fat in mammals suggesting that the composition of the bacterial community has a strong influence on the animals' physiological parameters (Xiao and Kang, 2020). Research has shown that the *Firmicutes*-*Bacteroidota* ratio is strongly correlated with milk fat yield, increasing fat in blood and tissue (Jami et al., 2014). Understanding the role that different bacteria in the rumen possess will allow us to regulate the microbiome for a better milk yield and utilization of resources.

Lachnospiraceae and *Prevotellaceae* were the most abundant bacteria families in all the samples. The *Prevotellaceae* family comprises common rumen bacteria such as *Prevotella ruminicola. Prevotella* has been reported as the most abundant family in the rumen of adult dairy cattle, and it is also associated with ruminal carbohydrate and protein fermentation (Chiquette et al., 2008). In this study, treatments did not cause any effects on *Lachnospiraceae* relative abundance. *Prevotellaceae* showed a tendency for decrease as WCS was supplemented. *Lachnospiraceae* and its family members are known for their cellulolytic activity, and are also related to butyrate production (Vacca et al., 2020). This could justify the increase in butyrate concentrations. It's also been found that *Lachnospiraceae* species are associated with feed efficiency in Nellore steers (Lopes et al., 2021). These findings suggest that the addition of WCS to the diet may negatively impact protein metabolism in the rumen.

Supplementation of WCS had an effect on 26 out of the 42 genera that were found. Genera relative abundance of *Ruminococcus, Saccharofermentans, Lactobacillus, Oribacterium, NK4A214_group, Shuttleworthia, Bifidobacterium, Moryella, Monoglobus, Flexilinea* and *Pseudoscardovia* increased with increasing WCS. Abundance of *Pseudobutyrivibrio, Rikenellaceae_RC9_gut_group, Kurthia, Methanobrevibacter, Desulfovibrio, Methanosphaera, Empedobacter, Absconditabacteriales_*and *Lachnospiraceae_FCS020_group* decreased with WCS. It is possible that dietary changes may have led to a shift in cellulolytic bacterial microbiome. In the past, no other research has measured the effect of WCS on specific genera of the rumen microbiome. Overall, these genera are part of the bovine digestive tract and their presence is indicative of proper physiological development of the rumen.

Experiment 2

The second experiment measured the effect of increasing levels of WCS when diets are balanced for protein and NDF on rumen fermentation, fiber digestibility and rumen microbial communities.

When compared to the control treatment, NDF digestibility increased with the addition of WCS when all treatment diets are balanced for fiber and protein. These finding are in agreement with Nogueira et al. (2019) who found that feeding WCS (with and without vitamin E) increased NDF by 123% when comparing it to a control diet. Jorge et al. (2008) didn't see any effects on NDF digestibility when adding 150g/kg of WCS to the diet of Holstein steers. Similarly, Smith et al. (1981) found that there were no significant effects of WCS at 5, 15, or 25% on digestibility of fiber components, although there was a trend toward decreased cellulose digestibility. All these findings differ from previous studies by Ismartoyo (2017), in which NDF digestibility was reduced with the inclusion of 150, 300 or 500 g/d of WCS. The author speculates that this could be due to the detoxification process done to free gossypol by the rumen microbes. Whole oilseeds decrease the digestion problems by the encapsulation of fatty acids in the hard seed coat (Jenkins and Lundy, 2001). Other authors have concluded that fat supplementation in the form of oilseeds has less of a negative effect on fiber digestibility when compared to oil supplementation (Patra, 2013). These results indicate that the use of WCS in the diet does not cause any negative effects on rumen fermentation and can be used safely at the levels analyzed in the present study.

In the present study, no differences in concentration of the main three VFA (acetate, propionate, and butyrate) nor ammonia were observed. Similar findings were detected by Anderson et al. (1979), when 9.5% WCS was fed to lactating cows. When utilizing Upland WCS to feed fermenting bottles, Wang et al. (2022) saw a lower production of total VFA compared to when cottonseed meal was fed. This is presumably because of the lower gossypol content present

in cottonseed meal. In previous studies feeding 12% DM WCS, VFA production was not altered and ammonia concentration results exhibited that they were in the range of values indicated by earlier research, and no relevant differences were shown (Harrison et al., 1995). WCS at 16.2% did not show marked changes in VFA production in the rumen of dairy cows(Sklan et al., 1992). These findings suggest that the fat contained in WCS is slowly released in the rumen. Along with the results from previous research, these results suggest that supplementation of WCS does not alter VFA production in the rumen.

When looking at the kingdom relative abundance, bacteria increased along with the WCS levels and archaea decreased linearly in the present study. When testing gossypol's detrimental effects on ruminal fermentation characteristics, previous researchers saw that when exposed to different levels of gossypol, some fibrolytic bacteria were reduced but others increased their population (Wang et al., 2021). Still there was an obvious detrimental effect of the gossypol addition on rumen fermentation by decreasing microbial activity when the gossypol inclusion exceeded 0.5 mg/g. In a study done in sheep, gossypol effects on CH_4 emissions were measured. No significant relation between gossypol addition and CH_4 emissions was found, suggesting that gossypol does not affect rumen methanogenesis (Lima et al., 2014).

Protozoa are an important component of the rumen microbiome and their presence is indicative of overall rumen health. In the present study ruminal protozoa count showed a linear decrease with the addition of WCS. When measuring the efficacy of WCS to limit protozoal population, other authors Dayani et al. (2007) saw a decreased total protozoal population in sheep. In the same study, Holotrich and cellulolytic protozoa disappeared, while *Entodinium* sp. was the only one persisting. A study done by Ismartoyo (2015) showed that feeding up to 500g/d of WCS significantly depressed rumen protozoa in sheep. Rumen defaunation in cattle has been

shown to increase bacterial population and increase their efficacy on bacterial protein synthesis (Nguyen et al., 2020). Defaunation and severely affected protozoal population are potentially due to gossypol's high concentration. It has been demonstrated that high concentrations of gossypol have strong inhibitory effects on bacteria and antimicrobial properties(Vadehra et al., 1985). Even though defaunation can show potentially positive effects on the ruminal efficiency, a safe and effective method is yet to be found.

CONCLUSIONS

The first study showed that increasing levels of WCS have increasing effects relative to ammonia concentrations, along with isobutyrate, valerate and isovalerate. Digestibilities of starch and NDF were not affected. Additionally, bacteria population increased while archaea decreased. This indicates that the diets used during this experiment provide an environment in which growth and bacterial activity of the rumen microbes are maximized. In the second study, in which diets were adjusted for fiber and protein, similar to the first experiment, WCS levels did not affect primary VFA or ammonia concentrations. Bacteria populations showed an increase. However, the results of this experiment demonstrate that addition of WCS did negatively impact archaea and protozoal population, meaning that the use of whole cottonseed could help mitigate CH_4 potentially through defaunation of rumen protozoa.

TABLES AND FIGURES

Table 2.1. Ingredient composition of treatment diets containing different levels of whole cottonseed (WCS) delivered to continuous culture fermenters daily in experiment 1.

	Treatments			
Ingredient, % DM	Control	5% WCS	10% WCS	15% WCS
Whole cottonseed	-	5	10	15
Ground corn	16	16	16	16
Canola meal expelled	14.6	14.6	14.6	14.6
Beet pulp	4.61	4.61	4.61	4.61
Robot pellet	14.73	14.73	14.73	14.73
Lactating cow mineral	1.52	1.52	1.52	1.52
Orchardgrass Hay	48.52	48.52	48.52	48.52

Table 2.2. Ingredient composition of treatment diets containing different levels of whole cottonseed (WCS) delivered to continuous culture fermenters daily in experiment 2

	Treatments			
Ingredient, % DM	Control	5% WCS	10% WCS	15% WCS
Whole Cottonseed	-	5	10	15
Soybean meal	6.73	4.48	2.1	-
Cotton hulls	8.3	5.5	2.8	-
Ground corn	13.22	13.22	13.22	13.22
Canola meal expelled	12.7	12.7	12.7	12.7
Beet pulp	3.81	3.81	3.81	3.81
Robot pellet	11.42	11.42	11.42	11.42
Lactating cow mineral	1.34	1.34	1.34	1.34
Orchardgrass Hay	42.6	42.6	42.6	42.6

Table 2.3. Rumen fermentation and digestibility in response to 5%, 10% and 15% whole cottonseed (WCS) in continuous culture fermenters in experiment 1.

Item	Control	5% WCS	10% WCS	15% WCS	SEM	*P-value Contrasts*		
						Linear	Quadratic	Cubic
NDF digestibility, %	63.3	62.74	67.0	65.1	2.75	0.34	0.73	0.22
Starch digestibility, %	80.9	81.3	81.9	80.8	2.86	0.97	0.58	0.74
Bacterial C flow, g/d	5.49	5.34	5.28	4.90	0.60	0.23	0.49	0.71
Bacterial N, g/kg of NDF digestibility	112.7	110.6	105.6	100.2	23.8	0.34	0.41	0.55
N flow, g/d	1.94	1.88	2.03	2.06	0.22	0.46	0.65	0.76
Ammonia, mg/dL	7.53	9.08	9.21	9.4	0.36	<0.01	0.05	0.13
Acetate, mmol/mol	51.6	51.6	52.7	52.1	0.62	0.27	0.62	0.23
Propionate, mmol/mol	25.9	24.4	24.5	24.9	0.64	0.28	0.08	0.62
Butyrate, mmol/mol	15.9	17.9	17.2	17.1	0.59	0.15	0.02	0.09
Isobutyrate, mmol/mol	0.32	0.33	0.34	0.34	0.02	<0.01	0.4	0.7
Valerate, mmol/mol	2.44	2.66	2.76	2.94	0.15	<0.01	0.85	0.64
Isovalerate, mmol/mol	2.06	2.35	2.44	2.52	0.13	0.01	0.39	0.70
Caproic, mmol/mol	0.74	0.73	0.7	0.76	0.07	0.87	0.39	0.64

Table 2.4. Rumen alpha diversity in response to 5%, 10% and 15% of whole cottonseed (WCS) in continuous culture fermenters in experiment 1.

Item	Control	5% WCS	10% WCS	15% WCS	SEM	*P value Contrasts*		
						Linear	Quadratic	Cubic
n seqs	88485	88554	88452	88543	97	0.87	0.91	0.41
Sobs	2060	2253	2151	2288	133	0.20	0.77	0.24
Coverage	0.996	0.996	0.996	0.996	0.001	0.87	0.77	0.61
Ace	2470	2530	2379	2560	214	0.87	0.71	0.45
Chao index	2851	3008	2741	3041	361	0.82	0.81	0.47
Shannon	5.80	6.04	6.01	6.17	0.12	0.02	0.69	0.26
Simpson	0.013	0.008	0.010	0.007	0.002	0.05	0.73	0.12
Inverse Simpson	98	129	131	155	17.6	0.01	0.81	0.41

Table 2.5. P values associated with Permanova analysis of microbial similarity in response to increasing levels of whole cottonseed in continuous culture fermenters in experiment 1.

		Index	
Items	**Test**	**Bray-Curtis**	**Jaccard**
Treatment	PERMANOVA	0.94	0.95

Table 2.6. Kingdom relative abundance in response to 5%, 10% and 15% of whole cottonseed (WCS) in continuous culture fermenters in experiment 1.

Item	Control	5% WCS	10% WCS	15% WCS	SEM	*P value Contrasts*		
						Linear	Quadratic	Cubic
Bacteria	97.27	97.52	97.85	98.64	0.42	0.01	0.39	0.78
Archaea	2.73	2.48	2.15	1.36	0.42	0.01	0.39	0.78

Table 2.7. Phylum relative abundance in response to 5%, 10% or 15% whole cottonseed (WCS) in continuous culture fermenters in experiment 1.

Item	Control	5% WCS	10% WCS	15% WCS	SEM	*P value Contrasts*		
						Linear	Quadratic	Cubic
Euryarchaeota	2.72	2.47	2.15	1.35	0.420	0.01	0.40	0.77
Thermoplasmatota	0.011	0.007	0.011	0.006	0.005	0.59	0.99	0.39
Actinobacteriota	5.66	5.22	6.04	4.94	1.658	0.83	0.81	0.60
Bacteroidota	24.0	15.7	12.8	11.5	6.455	0.05	0.43	0.85
Desulfobacterota	0.52	0.35	0.34	0.33	0.062	<0.01	0.03	0.29
Firmicutes	66.0	74.9	76.7	78.8	5.968	0.02	0.35	0.64
Patescibacteria	0.18	0.10	0.06	0.07	0.049	0.02	0.20	0.95
Spirochaetota	0.89	0.32	0.27	0.30	0.258	0.07	0.18	0.64
Verrucomicrobiota	0.62	0.53	0.43	0.48	0.185	0.39	0.63	0.80

Table 2.8. Family relative abundance in response to 5%, 10% or 15% whole cottonseed (WCS) in continuous culture fermenters in experiment 1.

Item	Control	5% WCS	10% WCS	15% WCS	SEM	*P value Contrasts*		
						Linear	Quadratic	Cubic
Methanobacteriaceae	2.72	2.47	2.15	1.35	0.420	0.01	0.40	0.77
Anaerovoracaceae	0.07	0.10	0.12	0.12	0.017	0.02	0.44	0.89
Bacillaceae	0.16	0.35	0.16	0.14	0.077	0.41	0.12	0.07
Bifidobacteriaceae	0.06	0.72	0.86	1.03	0.155	<0.01	0.10	0.37
Christensenellaceae	5.12	5.60	5.93	6.41	0.920	0.22	1.00	0.93
Clostridiaceae	1.47	0.95	0.18	0.13	0.640	0.04	0.63	0.64
Corynebacteriaceae	4.24	3.30	3.96	2.47	1.520	0.39	0.82	0.49
Desulfovibrionaceae	0.50	0.34	0.33	0.32	0.056	<0.01	0.03	0.34
F082	4.51	2.86	3.10	1.62	1.255	0.08	0.94	0.44
Fibrobacteraceae	0.24	0.10	0.08	0.13	0.078	0.30	0.24	0.87
Lachnospiraceae	41.3	45.1	41.5	46.1	4.620	0.47	0.91	0.30
Oscillospiraceae	4.34	5.03	6.51	5.66	0.556	0.01	0.07	0.10
Planococcaceae	1.25	1.10	0.79	0.88	0.321	0.24	0.66	0.63
Prevotellaceae	15.2	9.80	7.37	8.13	4.216	0.07	0.27	0.98
Rikenellaceae	2.94	1.90	1.59	1.12	0.826	0.03	0.60	0.72
Ruminococcaceae	6.29	8.00	12.70	9.81	2.290	0.03	0.14	0.13
Spirochaetaceae	0.88	0.30	0.26	0.28	0.256	0.07	0.18	0.63
Thermoactinomycetaceae	0.014	0.016	0.011	0.015	0.003	0.88	0.86	0.20
Veillonellaceae	0.0003	0.005	0.003	0.004	0.002	0.12	0.14	0.12

Table 2.9. Genera relative abundance in response to increasing levels 5%, 10% or 15% whole cottonseed (WCS) in continuous culture fermenters in experiment 1.

Item	Control	5% WCS	10% WCS	15% WCS	SEM	*P value Contrasts*		
						Linear	Quadratic	Cubic
Lachnospiraceae_unclassified	14.3	18.6	17.3	18.9	1.433	0.04	0.31	0.15
Prevotella	13.5	8.67	6.56	6.82	3.750	0.06	0.31	0.97
Christensenellaceae_R-7_group	5.07	5.55	5.87	6.36	0.913	0.21	0.99	0.93
Acetitomaculum	5.34	5.95	5.35	5.92	1.830	0.82	0.99	0.63
Pseudobutyrivibrio	6.10	4.71	4.09	3.66	1.001	0.05	0.57	0.88
Butyrivibrio	5.60	4.23	3.53	4.06	0.792	0.08	0.16	0.85
Ruminococcus	1.99	3.08	4.34	4.66	0.788	0.01	0.63	0.75
NK4A214_group	2.90	3.40	3.83	4.02	0.464	0.04	0.68	0.92
Corynebacterium	4.16	3.24	3.88	2.42	1.490	0.39	0.82	0.49
F082_ge	4.51	2.86	3.10	1.62	1.255	0.08	0.94	0.44
Saccharofermentans	1.43	1.86	1.99	2.49	0.162	<0.01	0.82	0.32
Methanobrevibacter	2.38	2.15	1.87	1.16	0.364	<0.01	0.38	0.73
Rikenellaceae_RC9_gut_group	2.90	1.87	1.55	1.10	0.811	0.03	0.60	0.73
CAG-352	0.94	1.93	2.17	2.16	0.563	0.08	0.29	0.80
Lactobacillus	0.09	1.74	1.48	1.85	0.528	<0.01	0.08	0.12
Oribacterium	0.51	0.81	0.77	1.57	0.204	<0.01	0.15	0.14
Shuttleworthia	0.53	0.78	0.75	0.86	0.211	0.05	0.51	0.37
Lachnobacterium	0.61	0.58	0.51	0.55	0.139	0.59	0.75	0.71
Bifidobacterium	0.05	0.64	0.67	0.77	0.123	<0.01	0.07	0.26
Treponema	0.87	0.30	0.25	0.27	0.252	0.07	0.18	0.62
Moryella	0.24	0.40	0.43	0.51	0.112	0.03	0.59	0.65
Desulfovibrio	0.48	0.32	0.31	0.30	0.051	<0.01	0.04	0.34
Kurthia	0.62	0.25	0.28	0.18	0.146	0.02	0.26	0.33
Anaerosporobacter	0.25	0.23	0.28	0.35	0.081	0.18	0.50	0.87
Methanosphaera	0.34	0.32	0.27	0.19	0.054	0.03	0.57	0.98
Empedobacter	0.40	0.33	0.21	0.17	0.102	0.03	0.83	0.70
Lachnospira	0.35	0.25	0.22	0.27	0.088	0.41	0.28	1.00
Coprococcus	0.22	0.24	0.22	0.23	0.055	0.97	0.81	0.60
Marvinbryantia	0.15	0.23	0.22	0.27	0.054	0.07	0.71	0.44
Flexilinea	0.11	0.17	0.19	0.19	0.021	0.01	0.15	0.87
Syntrophococcus	0.14	0.14	0.15	0.23	0.045	0.17	0.42	0.75
Fibrobacter	0.24	0.10	0.08	0.13	0.078	0.30	0.23	0.87
Howardella	0.14	0.13	0.14	0.13	0.038	0.86	0.97	0.71
Monoglobus	0.07	0.10	0.11	0.16	0.021	0.01	0.52	0.56
Pseudoscardovia	0.002	0.056	0.149	0.212	0.075	<0.01	0.93	0.75
Absconditabacteriales_	0.18	0.10	0.06	0.07	0.048	0.02	0.19	0.93
Lachnospiraceae_UCG-002	0.08	0.10	0.10	0.11	0.017	0.10	0.58	0.66

Lachnospiraceae_FCS020_group	0.15	0.08	0.06	0.04	0.025	<0.01	0.29	0.69
Arcobacter	0.01	0.06	0.18	0.07	0.079	0.37	0.29	0.34
Papillibacter	0.05	0.07	0.07	0.06	0.018	0.44	0.29	0.96
Megasphaera	0.0003	0.0049	0.0024	0.0033	0.001	0.24	0.15	0.07

Table 2.10. Rumen fermentation and digestibility in responses to increasing levels of gossypol and fatty acids coming from whole cottonseed (WCS) in continuous culture fermenters in experiment 2.

		5%	10%	15%		*P value Contrasts*		
	Control	WCS	WCS	WCS	SEM	Linear	Quadratic	Cubic
NDF digestibility, %	58.5	62.9	63.1	63.3	3.37	0.21	0.42	0.71
Starch digestibility, %	83.9	84.1	83.2	82.9	0.45	0.02	0.48	0.32
N-NH_3 mg/dL	10.2	10.6	10.3	11.2	0.53	0.21	0.75	0.69
Acetate, mmol/mol	55.6	53.9	53.0	50.0	0.66	0.32	0.76	0.32
Propionate, mmol/mol	29.6	28.0	27.6	26.8	0.71	0.12	0.25	0.054
Butyrate, mmol/mol	16.7	16.1	17.0	17.3	0.45	0.22	0.16	0.25

Table 2.11. Rumen alpha diversity in response to increasing levels of gossypol and fatty acids coming from whole cottonseed (WCS) in continuous culture fermenters in experiment 2.

						P-value Contrasts		
Item	Control	5% WCS	10% WCS	15% WCS	SEM	Linear	Quadratic	Cubic
n seqs	88675	88765	88657	88654	100	0.91	0.87	0.711
Sobs	2060	2253	2151	2288	136	0.24	0.67	0.55
Coverage	0.996	0.996	0.996	0.996	0.00	0.92	0.77	0.61
Ace	2470	2530	2379	2560	218	0.81	0.71	0.45
Chao index	2851	3008	2741	3041	369	0.80	0.81	0.47
Shannon	5.80	6.04	6.01	6.17	0.15	0.04	0.65	0.46
Simpson	0.016	0.009	0.08	0.006	0.03	0.05	0.73	0.25
Inverse Simpson	98	115	125	146	19.0	0.01	0.81	0.59

Table 2.12. P values associated with Permanova analysis of microbial similarity in response to increasing levels of gossypol and fatty acids coming from whole cottonseed in continuous culture fermenters in experiment 2.

		Index	
Items[1]	**Test**	**Bray-Curtis**	**Jaccard**
Treatment	PERMANOVA	0.92	0.96

Table 2.13. Kingdom relative abundance in response to increasing levels of gossypol and fatty acids coming from whole cottonseed (WCS) in continuous culture fermenters in experiment 2.

						P-value Contrasts		
Item	Control	5% WCS	10% WCS	15% WCS	SEM	Linear	Quadratic	Cubic
Bacteria	96.34	96.5	97.01	98.3	0.46	0.02	0.45	0.65
Archaea	2.65	2.51	2.47	2.01	0.42	0.03	0.47	0.71
Protozoa count, cell 10^3/mL	8.74	6.15	6.34	4.83	0.45	<0.01	0.11	0.63

REFERENCES

Acamovic, T., and C. Stewart. 1993. The effect of gossypol on the rumen microbial degradation of grass hay under consecutive batch culture (CBC). Proceedings of the British Society of Animal Production (1972) 1993:171-171.

Aiello, R., L. Armentano, S. Bertics, and A. Murphy. 1989. Volatile fatty acid uptake and propionate metabolism in ruminant hepatocytes. J. Dairy Sci. 72(4):942-949.

Anderson, M., D. Adams, R. Lamb, and J. Walters. 1979. Feeding whole cottonseed to lactating dairy cows. J. Dairy Sci. 62(7):1098-1103.

Arieli, A. 1998. Whole cottonseed in dairy cattle feeding: a review. Animal Feed Science and Technology 72(1-2):97-110.

Avila, C., E. DePeters, H. Perez-Monti, S. Taylor, and R. Zinn. 2000. Influences of saturation ratio of supplemental dietary fat on digestion and milk yield in dairy cows. J. Dairy Sci. 83(7):1505-1519.

Bach, A., S. Calsamiglia, and M. Stern. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9-E21.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. Applied and Environmental Microbiology 38(1):148-158.

Bauchop, T. 1981. The anaerobic fungi in rumen fibre digestion. Agriculture and Environment 6(2-3):339-348.

Belibasakis, N., and D. Tsirgogianni. 1995. Effects of whole cottonseeds on milk yield, milk composition, and blood components of dairy cows in hot weather. Animal Feed Science and Technology 52(3-4):227-235.

Bergman, E. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological reviews 70(2):567-590.

Bertrand, J., T. Sudduth, A. Condon, T. Jenkins, and M. Calhoun. 2005. Nutrient content of whole cottonseed. J. Dairy Sci. 88(4):1470-1477.

Bhat, R. 2021. Valorization of Agri-Food Wastes and By-Products: Recent Trends, Innovations and Sustainability Challenges.

Brooks, C. C., G. B. Garner, C. W. Gehrke, M. E. Muhrer, and W. H. Pfander. 1954. The Effect of Added Fat on the Digestion of Cellulose and Protein by Ovine Rumen Microorganisms. J. Anim. Sci. 13(4):758-764. doi: 10.2527/jas1954.134758x

Calhoun, M., S. Kuhlmann, and B. Baldwin. 1995. Assessing the gossypol status of cattle fed cotton feed products. In: Proceedings Pacific Northwest Nutrition Conference

Castillo, J., M. Olivera, and J. Carulla. 2013. Description of the biochemistry mechanism of polyunsaturated fatty acid ruminal biohydrogenation: A review. Revista UDCA Actualidad & Divulgación Científica 16(2):459-468.

Chain, E. P. o. C. i. t. F., H. K. Knutsen, L. Barregård, M. Bignami, B. Brüschweiler, S. Ceccatelli, M. Dinovi, L. Edler, B. Grasl-Kraupp, and C. Hogstrand. 2017. Presence of free gossypol in whole cottonseed. EFSA J 15(7):e04850.

Chan, B., A. Waiss Jr, R. Binder, and C. Elliger. 1978. Inhibition of lepidopterous larval growth by cotton constituents. Entomologia Experimentalis et Applicata 24(3):294-300.

Chilliard, Y., A. Ferlay, R. M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. In: Annales de zootechnie. p 181-205.

Chiquette, J., M. Allison, and M. Rasmussen. 2008. Prevotella bryantii 25A used as a probiotic in early-lactation dairy cows: effect on ruminal fermentation characteristics, milk production, and milk composition. J. Dairy Sci. 91(9):3536-3543.

Cholewińska, P., W. Górniak, and K. Wojnarowski. 2021. Impact of selected environmental factors on microbiome of the digestive tract of ruminants. BMC Veterinary Research 17(1):1-10.

Choudhury, P. K., S. K. Sirohi, A. K. Puniya, and P. P. Chaudhary. 2012. Harnessing the diversity of rumen microbes using molecular approaches. Livestock Green House Gases: emission and options for mitigation. Satish Serial Publishing House, Delhi, Section-B:65-82.

Church, D. C. 1993. The Ruminant Animal: Digestive Physiology and Nutrition. Waveland Press.

Conte, G., C. Dimauro, A. Serra, N. P. P. Macciotta, and M. Mele. 2018. A canonical discriminant analysis to study the association between milk fatty acids of ruminal origin and milk fat depression in dairy cows. J. Dairy Sci. 101(7):6497-6510.

Cooke, K., J. Bernard, and J. West. 2007a. Performance of lactating dairy cows fed whole cottonseed coated with gelatinized starch plus urea or yeast culture. J. Dairy Sci. 90(1):360-364.

Cooke, K., J. Bernard, C. Wildman, J. West, and A. Parks. 2007b. Performance and ruminal fermentation of dairy cows fed whole cottonseed with elevated concentrations of free fatty acids in the oil. J. Dairy Sci. 90(5):2329-2334.

Cooke, K. M., and J. K. Bernard. 2005. Effects of feeding whole cottonseed coated with starch, urea, or yeast on performance of lactating dairy cows. J. Dairy Sci. 88(Suppl 1):394.

Coppock, C., J. Moya, J. West, D. Nave, J. Labore, and C. Gates. 1985. Effect of lint on whole cottonseed passage and digestibility and diet choice on intake of whole cottonseed by Holstein cows. J. Dairy Sci. 68(5):1198-1206.

Dai, X., P. J. Weimer, K. A. Dill-McFarland, V. L. Brandao, G. Suen, and A. P. Faciola. 2017. Camelina seed supplementation at two dietary fat levels change ruminal bacterial community composition in a dual-flow continuous culture system. Frontiers in microbiology 8:2147.

Dayani, O., G. Ghorbani, M. Alikhani, H. Rahmani, and P. Mir. 2007. Effects of dietary whole cottonseed and crude protein level on rumen protozoal population and fermentation parameters. Small Ruminant Research 69(1-3):36-45.

Dehority, B. A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. Applied and Environmental Microbiology 48(1):182-185.

DePeters, E., S. Taylor, and A. Aguirre. 1985a. Whole cottonseed increases milk fat, decreases milk protein. California Agriculture 39(5):23-24.

DePeters, E., S. Taylor, A. Franke, and A. Aguirre. 1985b. Effects of feeding whole cottonseed on composition of milk. J. Dairy Sci. 68(4):897-902.

Dijkstra, J., J. M. Forbes, and J. France. 2005. Quantitative aspects of ruminant digestion and metabolism. CABI Pub. Cambridge.

Dong, L., T. Zhang, and Q. Diao. 2019. Effect of dietary supplementation of Moringa oleifera on the production performance and fecal methanogenic community of lactating dairy cows. Animals 9(5):262.

Doreau, M., and A. Ferlay. 2015. Linseed: a valuable feedstuff for ruminants. OCL Oilseeds and fats crops and lipids 22(6):9 p.

Dowd, M. K., D. L. Boykin, W. R. Meredith Jr, B. T. Campbell, F. M. Bourland, J. R. Gannaway, K. M. Glass, and J. Zhang. 2010. Fatty acid profiles of cottonseed genotypes from the national cotton variety trials.

Drackley, J. K. 2007. Overview of fat digestion and metabolism in dairy cows. University of Illinois: Urbana, IL, USA:1-9.

Enjalbert, F., R. Moncoulon, M. Vernay, and D. Griess. 1994. Effects of different forms of polyunsaturated fatty acids on rumen fermentation and total nutrient digestibility of sheep fed prairie hay based diets. Small Ruminant Research 14(2):127-135.

Eun, J.-S., and K. Beauchemin. 2007. Enhancing in vitro degradation of alfalfa hay and corn silage using feed enzymes. J. Dairy Sci. 90(6):2839-2851.

Evans, N. J., J. M. Brown, R. D. Murray, B. Getty, R. J. Birtles, C. A. Hart, and S. D. Carter. 2011. Characterization of novel bovine gastrointestinal tract Treponema isolates and comparison with bovine digital dermatitis treponemes. Applied and Environmental Microbiology 77(1):138-147.

Fuquay, J. W., P. L. McSweeney, and P. F. Fox. 2011. Encyclopedia of dairy sciences. Academic Press.

Gadelha, I. C. N., N. B. S. Fonseca, S. C. S. Oloris, M. M. Melo, and B. Soto-Blanco. 2014. Gossypol toxicity from cottonseed products. Sci. World J. 2014

Grainger, C., R. Williams, T. Clarke, A.-D. Wright, and R. Eckard. 2010. Supplementation with whole cottonseed causes long-term reduction of methane emissions from lactating dairy cows offered a forage and cereal grain diet. J. Dairy Sci. 93(6):2612-2619.

Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. of AOAC Int. 92(1):42-49.

Harfoot, C. 1981. Lipid metabolism in the rumen, Lipid metabolism in ruminant animals. Elsevier. p. 21-55.

Harrison, J., R. Kincaid, J. McNamara, S. Waltner, K. Loney, R. Riley, and J. Cronrath. 1995. Effect of whole cottonseeds and calcium salts of long-chain fatty acids on performance of lactating dairy cows. J. Dairy Sci. 78(1):181-193.

Henderson, C., and W. Hodgkiss. 1973. An electron microscopic study of Anaerovibrio lipolytica (strain 5S) and its lipolytic enzyme. Microbiology 76(2):389-393.

Hodgson, H. J. 1979. Role of the dairy cow in world food production. J. Dairy Sci. 62(2):343-351.

Horner, J., L. Windle, C. Coppock, J. Labore, J. Lanham, and D. Nave. 1988. Effects of whole cottonseed, niacin, and niacinamide on in vitro rumen fermentation and on lactating Holstein cows. J. Dairy Sci. 71(12):3334-3344.

Hristov, A. N., A. Bannink, L. A. Crompton, P. Huhtanen, M. Kreuzer, M. McGee, P. Nozière, C. K. Reynolds, A. R. Bayat, and D. R. Yáñez-Ruiz. 2019. Invited review: Nitrogen in ruminant nutrition: A review of measurement techniques. J. Dairy Sci. 102(7):5811-5852.

Hristov, A. N., P. Huhtanen, L. M. Rode, S. Acharya, and T. A. McAllister. 2001. Comparison of the ruminal metabolism of nitrogen from 15N-labeled alfalfa preserved as hay or as silage. J. Dairy Sci. 84(12):2738-2750.

Ismartoyo, I. 2015. The Effects of Feeding Whole Cottonseed as a Supplement for Sheep Fed a Basal Diet Grass Hay on Feed Digestibility and the Rumen Fermentation. Jurnal Ilmu dan Teknologi Peternakan 4(2):50-55.

Ismartoyo, M. 2017. Effect of feeding whole cottonseed as a supplement on digestibility and rumen fermentation characteristics of sheep. Bangladesh J. of Ani. Sci. 46(4):239-243.

Jami, E., B. A. White, and I. Mizrahi. 2014. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PloS one 9(1):e85423.

Jenkins, T. 1993. Lipid metabolism in the rumen. J. Dairy Sci. 76(12):3851-3863.

Jenkins, T., and F. Lundy. 2001. Feeding various fat sources to lactating dairy cows and their effects on milk quality.

Joblin, K., G. P. Campbell, A. J. Richardson, and C. Stewart. 1989. Fermentation of barley straw by anaerobic rumen bacteria and fungi in axenic culture and in co-culture with methanogens. Letters in applied microbiology 9(5):195-197.

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73(8):2483-2492.

Jorge, J., L. Zeoula, I. Prado, R. Silva, R. Andrade, J. Prado, and E. Bublitz. 2008. Lipids in diets for Holstein steers: apparent digestibility. Revista Brasileira de Saúde e Produção Animal 9:743-753.

Jouany, J.-P. 1996. Effect of rumen protozoa on nitrogen utilization by ruminants. J Nutr

126(suppl_4):1335S-1346S.

Koike, S., and Y. Kobayashi. 2009. Fibrolytic rumen bacteria: their ecology and functions. Asian-Australasian J. Ani. Sci. 22(1):131-138.

Kothari, R. K., N. M. Nathani, C. Mootapally, J. K. Rank, H. B. Gosai, B. P. Dave, and C. G. Joshi. 2018. Comprehensive exploration of the rumen microbial ecosystem with advancements in metagenomics, Metagenomics. Elsevier. p. 215-229.

Krause, D. O., S. E. Denman, R. I. Mackie, M. Morrison, A. L. Rae, G. T. Attwood, and C. S. McSweeney. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. FEMS microbiology reviews 27(5):663-693.

Kumar, S., S. S. Dagar, A. K. Puniya, and R. C. Upadhyay. 2013. Changes in methane emission, rumen fermentation in response to diet and microbial interactions. Research in Veterinary Science 94(2):263-268.

Lima, P. d. M. T., P. B. Oliveira, A. Campeche, G. D. Moreira, T. d. P. Paim, C. McManus, A. L. Abdalla, A. M. M. Dantas, J. R. de Souza, and H. Louvandini. 2014. Methane emission of Santa Inês sheep fed cottonseed by-products containing different levels of gossypol. Tropical animal health and production 46(1):285-288.

Lopes, D. R. G., M. de Souza Duarte, A. J. La Reau, I. Z. Chaves, T. A. de Oliveira Mendes, E. Detmann, C. B. P. Bento, M. E. Z. Mercadante, S. F. M. Bonilha, and G. Suen. 2021. Assessing the relationship between the rumen microbiota and feed efficiency in Nellore steers. J.Anim. Sci. Biotechnol.

12(1):1-17.

Lukonge, E., M. T. Labuschagne, and A. Hugo. 2007. The evaluation of oil and fatty acid composition in seed of cotton accessions from various countries. J. Sci. Food Agric.

87(2):340-347.

Mao, S., M. Zhang, J. Liu, and W. Zhu. 2015. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. Scientific reports 5(1):1-14.

Mao, S., R. Zhang, D. Wang, and W. Zhu. 2012. The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. BMC veterinary research 8(1):1-13.

Margalith, P. 1967. Inhibitory effect of gossypol on microorganisms. Applied microbiology 15(4):952-953.

- Maroune, M., and S. Bartos. 1987. Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. J Appl Bacteriol 63(3):233-238.

Martin, C., D. P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4(3):351-365.

Matthews, C., F. Crispie, E. Lewis, M. Reid, P. W. O'Toole, and P. D. Cotter. 2019. The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. Gut microbes 10(2):115-132.

Mena, H., J. Santos, J. Huber, J. Simas, M. Tarazon, and M. Calhoun. 2001a. The effects of feeding varying amounts of gossypol from whole cottonseed and cottonseed meal in lactating dairy cows. J. Dairy Sci. 84(10):2231-2239.

Mena, H., J. E. Santos, J. T. Huber, J. M. Simas, M. Tarazon, and M. C. Calhoun. 2001b. The effects of feeding varying amounts of gossypol from whole cottonseed and cottonseed

meal in lactating dairy cows. J. Dairy Sci 84(10):2231-2239. doi: 10.3168/jds.S0022-0302(01)74670-X

Moraes, L. E., A. B. Strathe, J. G. Fadel, D. P. Casper, and E. Kebreab. 2014. Prediction of enteric methane emissions from cattle. Global change biology 20(7):2140-2148.

Moreira, V., L. Satter, and B. Harding. 2004. Comparison of conventional linted cottonseed and mechanically delinted cottonseed in diets for dairy cows. J. Dairy Sci. 87(1):131-138.

Mosley, E. E., G. L. Powell, M. B. Riley, and T. C. Jenkins. 2002. Microbial biohydrogenation of oleic acid to trans isomers in vitro. J. Lipid Res. 43(2):290-296.

Nakamura, K., and S. Kanegasaki. 1969. Densities of ruminal protozoa of sheep established under different dietary conditions. J. Dairy Sci. 52(2):250-255.

Nergiz, C., H. Yalçin, and H. Yildiz. 1997. Some analytical characters of cottonseed varieties grown in Turkey. Grasas y Aceites 48(6):411-414.

Nguyen, S. H., H. D. T. Nguyen, and R. S. Hegarty. 2020. Defaunation and its impacts on ruminal fermentation, enteric methane production and animal productivity. Livestock Research for Rural Development 32:4.

Niwińska, B. 2012. Digestion in ruminants. Carbohydrates-Comprehensive Studies on Glycobiology and Glycotechnology. InTech, DOI 10(51574):245-258.

Noftsger, S., N. St-Pierre, S. Karnati, and J. Firkins. 2003. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) on microbial growth in continuous culture. J. Dairy Sci. 86(8):2629-2636.

Nogueira, R. G. S., F. Perna, A. S. C. Pereira, and P. H. M. Rodrigues. 2019. Nutrient digestibility and changes in feeding behavior of cattle fed cottonseed and vitamin E. Scientia Agricola 76:112-122.

Palmquist, D., T. Jenkins, and A. Joyner Jr. 1986. Effect of dietary fat and calcium source on insoluble soap formation in the rumen. J. Dairy Sci. 69(4):1020-1025.

Palmquist, D. L., A. L. Lock, K. J. Shingfield, and D. E. Bauman. 2005. Biosynthesis of conjugated linoleic acid in ruminants and humans. Advances in food and nutrition research 50:179-217.

Parish, J. A., and J. D. Rhinehart. 2008. Fiber in beef cattle diets.

Patra, A. K. 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. Livestock science 155(2-3):244-254.

Pena, F., H. Tagari, and L. Satter. 1986. The effect of heat treatment of whole cottonseed on site and extent of protein digestion in dairy cows. J. Anim. Sci. 62(5):1423-1433.

Pons, W., C. Hoffpauir, and T. Hopper. 1953. Gossypol in Cottonseed, Influence of Variety of Cottonseed and Environment. J. Agric. Food Chem. 1(18):1115-1118.

Prins, R., R. Hungate, and E. Prast. 1972. Function of the omasum in several ruminant species. Comparative Biochemistry and Physiology Part A: Physiology 43(1):155-163.

Rathore, K. S., D. Pandeya, L. M. Campbell, T. C. Wedegaertner, L. Puckhaber, R. D. Stipanovic, J. S. Thenell, S. Hague, and K. Hake. 2020. Ultra-low gossypol cottonseed: Selective gene silencing opens up a vast resource of plant-based protein to improve human nutrition. Critical Reviews in Plant Sciences 39(1):1-29.

Reiser, R., and H. C. Fu. 1962. The mechanism of gossypol detoxification by ruminant animals. J. Nutr. 76(2):215-218.

Risco, C. A., C. A. Holmberg, and A. Kutches. 1992. Effect of graded concentrations of gossypol on calf performance: toxicological and pathological considerations. J. Dairy Sci. 75(10):2787-2798.

Rode, L. M. 2000. Maintaining a healthy Rumen–An overview. Advances in Dairy Technology 12:101-108.

Russell, J. B., and J. L. Rychlik. 2001. Factors that alter rumen microbial ecology. Science 292(5519):1119-1122.

Santos, J., M. Villasenor, P. Robinson, E. DePeters, and C. Holmberg. 2003. Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. J. Dairy Sci. 86(3):892-905.

Seal, C., and C. Reynolds. 1993. Nutritional implications of gastrointestinal and liver metabolism in ruminants. Nutrition research reviews 6(1):185-208.

Sklan, D., R. Ashkenazi, A. Braun, A. Devorin, and K. Tabori. 1992. Fatty acids, calcium soaps of fatty acids, and cottonseeds fed to high yielding cows. J. Dairy Sci. 75(9):2463-2472.

Smith, N., L. Collar, D. Bath, W.-L. Dunkley, and A. Franke. 1981. Digestibility and effects of whole cottonseed fed to lactating cows. J. Dairy Sci. 64(11):2209-2215.

Storm, E., and E. Ørskov. 1983. The nutritive value of rumen micro-organisms in ruminants: 1. Large-scale isolation and chemical composition of rumen micro-organisms. BJN 50(2):463-470.

Sullivan, J. L., J. T. Huber, and J. M. Harper. 1993. Performance of dairy cows fed short staple, Pima, and cracked Pima cottonseed and feed characteristics. J. Dairy Sci. 76(11):3555-3561. doi: 10.3168/jds.S0022-0302(93)77694-8

Teather, R., and F. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. J. Dairy Sci. 71(3):666-673.

Thiex, N., L. Novotny, and A. Crawford. 2019. Determination of Ash in Animal Feed: AOAC Official Method 942.05 Revisited. J. AOAC Int. 95(5):1392-1397. doi: 10.5740/jaoacint.12-129

Umphrey, J. E., and C. Staples. 1992. General anatomy of the ruminant digestive system. University of Florida Cooperative Extension Service, Institute of Food and ….

USDA. 2021. Cotton.

Vacca, M., G. Celano, F. M. Calabrese, P. Portincasa, M. Gobbetti, and M. De Angelis. 2020. The controversial role of human gut lachnospiraceae. Microorganisms 8(4):573.

Vadehra, D., N. Kalla, M. Saxena, R. Hashia, P. Kaur, and L. Gupta. 1985. Antimicrobial activity of gossypol acetic-acid. IRCS MEDICAL SCIENCE-BIOCHEMISTRY 13(1):10-11.

Van Houtert, M. 1993. The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. Animal Feed Science and Technology 43(3-4):189-225.

Van Soest, P. 1967. Development of a comprehensive system of feed analyses and its application to forages. J. Anim, Sci. 26(1):119-128.

Van Soest, P. v., J. Robertson, and B. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74(10):3583-3597.

Vander Wal, R. 1964. Triglyceride structure. Advances in lipid research 2:1-16.

Wang, C., Y. Li, T. MaiTiSaiYiDi, H. Yang, and K. Yang. 2020. Effect of dietary gossypol supplement on fermentation characteristics and bacterial diversity in the rumen of sheep. PloS one 15(6):e0234378.

Wang, S., K. Giller, M. Kreuzer, S. E. Ulbrich, U. Braun, and A. Schwarm. 2017. Contribution of ruminal fungi, archaea, protozoa, and bacteria to the methane suppression caused by oilseed supplemented diets. Frontiers in microbiology 8:1864.

Wang, W., Q. Wu, W. Li, Y. Wang, F. Zhang, L. Lv, S. Li, and H. Yang. 2022. High-Gossypol Whole Cottonseed Exhibited Mediocre Rumen Degradability and Less Microbial Fermentation Efficiency than Cottonseed Hull and Cottonseed Meal with an In Vitro Gas Production Technique. Fermentation 8(3):103.

Wang, W.-K., Y.-L. Wang, W.-J. Li, Q.-C. Wu, S.-L. Li, and H.-J. Yang. 2021. Gossypol exhibited higher detrimental effect on ruminal fermentation characteristics of low-forage in comparison with high-forage mixed feeds. Toxics 9(3):51.

Weinberg, M. S., and A. L. Sheffner. 1976. Buffers in ruminant physiology and metabolism.

Weiss, K. 1953. Physiological studies on eructation in ruminants.

Weller, R., and A. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. BJN 32(2):341-351.

Wenner, B., J. de Souza, F. Batistel, T. Hackmann, Z. Yu, and J. Firkins. 2017. Association of aqueous hydrogen concentration with methane production in continuous cultures modulated to vary pH and solids passage rate. J. Dairy Sci. 100(7):5378-5389.

Williams, A. G., and G. S. Coleman. 1997. The rumen protozoa. In: P. N. Hobson and C. S. Stewart, editors, The Rumen Microbial Ecosystem. Springer Netherlands, Dordrecht. p. 73-139.

Wu, Z., and J. Huber. 1994. Relationship between dietary fat supplementation and milk protein concentration in lactating cows: a review. Livestock Production Science 39(2):141-155.

Xiao, H., and S. Kang. 2020. The role of the gut microbiome in energy balance with a focus on the gut-adipose tissue axis. Frontiers in genetics 11:297.

Yu, Z., and W. W. Mohn. 1999. Killing two birds with one stone: simultaneous extraction of DNA and RNA from activated sludge biomass. Can. J. Microbiol. 45(3):269-272.

Yunusova, S., S. Gusakova, A. Glushenkova, U. Nadzhimov, S. Turabekov, and S. Musaev. 1991. A comparative investigation of the fatty acid compositions of the seeds of a number of lines of a genetic collection of Gossypium hirsutum. Chemistry of Natural Compounds 27(2):147-150.