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Evaluation of Dietary Phytochemicals as Rumen Modifiers in Lactating Dairy Cows

Sonia Rodriguez Jimenez
South Dakota State University

EVALUATION OF DIETARY PHYTOCHEMICALS AS RUMEN MODIFIERS IN LACTATING DAIRY COWS

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SONIA RODRIGUEZ JIMENEZ

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree in Biological Sciences and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Johan S. Osorio, Ph.D. Date
Thesis Advisor

Vikram V. Mistry, Ph.D. Date
Head, Dairy and Food Science Department

Dean, Graduate School Date

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LIST OF ABBREVIATIONS

AA	Amino Acid
ADF	Acid Detergent Fiber
ADIA	Acid Detergent Insoluble Ash
AP	Absorbed protein
Arg	Arginine
ATP	Adenosine triphosphate
BCS	Body Condition Score
BW	Body Weight
CNCPS	Cornell Net Carbohydrate Protein System
CON	Control Diet (no tannins)
CP	Crude Protein
CT	Condensed Tannins
Cys	Cysteine
d	Days
DHIA	Dairy Herd Improvement Association
DIM	Days in Milk
DM	Dry Matter
DMI	Dry Matter Intake
EAA	Essential amino acids
ECM	Energy-Corrected Milk
ECP	Endogenous crude protein
FA	Fatty Acid

FAO	Food and Agriculture Organization of the United Nations
FCE	Feed Conversion Efficiency
His	Histidine
HT	Hydrolyzable Tannins
Leu	Leucine
Lys	Lysine
MCP	Microbial crude protein
ME	Metabolizable energy
Met	Methionine
MP	Metabolizable protein
MUN	Milk Urea Nitrogen
N	Nitrogen
NDF	Neutral Detergent Fiber
NE_L	Net Energy of Lactation
NFC	Non Fiber Carbohydrate
NH_3-N	Ammonia N
NPN	Non-Protein Nitrogen
NRC	National Research Council
NUE	Nitrogen Use Efficiency
OM	Organic Matter
peNDF	Physically Effective Neutral Detergent Fiber
PUN	Plasma Urea Nitrogen
RDP	Rumen Degradable Protein

RUP	Rumen Undegradable Protein
SBM	Soybean meal
SCC	Somatic Cell Count
SNF	Solids-Non-Fat
SOD	Superoxide dismutase
TMR	Total Mixed Ration
TRT	Treatment diet (with phytochemicals)
TTD	Total Tract Digestibility
USDA	United States Department of Agriculture
VFA	Volatile Fatty Acid

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ABSTRACT

EVALUATION OF DIETARY PHYTOCHEMICALS AS RUMEN MODIFIERS IN LACTATING DAIRY COWS

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Phytochemicals such as tannins included in dairy cow nutritional programs, as a natural feed additive, promise to improve rumen bypass protein and consequently reduce the feeding cost by decreasing the amount of CP needed in the ration. Additionally, if tannins are fed along with high quality protein sources, this could improve the AA profile reaching the small intestine and potentially improving health and performance of dairy cows. The black pepper, specifically its alkaloid piperine, is a nontoxic, natural dietary compound with a broad range of physiological activity. Lactating dairy cows ingesting these phytochemicals (tannin extracts and black pepper) will potentially benefit by increasing feed efficiency, protein reaching the duodenum, oxidative stress, and immunity. Objectives of this study were to evaluate the effects of phytochemicals in the ration of lactating dairy cows by assessing feed intake and efficiency, rumen fermentation, milk yield and composition, and blood metabolites and biomarkers. We hypothesized that diets containing phytochemicals would increase milk and component yields, benefit rumen fermentation, and improve feed efficiency and nutrient utilization as well as prevent oxidative stress. Sixteen Holstein cows (14 multiparous and 2 primiparous; DIM =114 ± 20) were used in a crossover design experiment with an adaptation period. Cows were

randomly assigned to a treatment sequence according to DIM, lactation number, prior milk yield averages, and body weight. Treatments included: 1) basal diet with soybean meal pellets at 3.37% of DM (CON), 2) basal diet with soybean meal pellets fed at 3.37% of DM containing phytochemicals at 4.4% concentration (TRT). The experiment lasted a total of 56 d and consisted of a 14-d adaptation (covariate) period and two 21-d treatment periods. All milk weights were recorded daily, and milk samples were collected during the last 3 d of each period in both milkings. Rumen fluid, body condition scores (BCS), and body weights were collected on day 19 and 20 of each period. Blood samples were collected from the coccygeal vein on d 13 and 20 during the adaptation and treatment periods, respectively. Data were analyzed using MIXED procedures of SAS. Dry matter intake was similar among treatments. Milk yield as well as fat and protein yield decreased in TRT cows in comparison to CON. Propionate proportion in VFA was lower in TRT cows. Greater apparent total-tract digestibility was observed in DM, OM, and CP when feeding TRT diets. Similarly, glucose concentrations were lower in TRT cows in comparison to CON. Albumin and the antioxidant activity measured by SOD was increased in TRT cows. The concomitant decrease in propionate proportion and blood glucose could partially explain the decrease in milk yield in TRT cows. Although the effects observed in VFA proportions and apparent total-tract digestibility of nutrients are indicatives that these phytochemicals act as rumen modifiers, further research is needed to optimize their dosage for an effective response in the rumen.

Keywords: Phytochemicals, Lactation Performance, Dairy Cow

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Food consumption tendencies in developing countries are changing as incomes rise. The demand for animal protein sources such as meat, milk, and dairy products is rising (Bruinsma, 2003). Additionally, this increasing demand is predicted to grow faster than production, resulting in a deficit to supply this demand. In meat products, this deficit will rise to 5.9 million ton per year in 2030, while in the case of milk and dairy products, the deficit will be around 39 million ton per year in 2030. Therefore, increase efficiency in the production of animal protein will be fundamental for food security in the future, where increased animal protein production will be done with less farmland, and a lower environmental impact (Pimental, 1997).

Worldwide the demand of protein of animal origin is projected to increase in global population and with it the requirements for human nutrition, especially in the emerging economies. Protein consumption has been emphasized not only as a major factor to maintain satisfactory growth population but also to promote adequate childhood development USDA (2016). In fact, there is a strong relationship between protein intake of animal source and an improvement in cognitive function, growth, and physical activity of children as well as healthier pregnancies (Neumann et al., 2003). The recommended daily dietary protein intake for adult men and women is 0.80 g good quality protein/kg of body weight (BW) per day (USDA, 2016).

Researchers have reported that is not only important to consume enough amount of protein to fill the daily requirements but also the protein quality has a significant impact on human health. The latter has been more evident between protein from animal vs. plant

origin, which have different amino acids (AA) profile. Animal protein sources provide a complete source of high-quality protein by encompassing several essential amino acids (EAA), whereas plant sources generally lack one or more of the EAA (Hoffman, 2004). The AA profile in protein from animal origin are more readily available to be metabolized and synthesize new proteins (Campbell, 2013). Therefore, it has been observed that relatively AA deficiency has been corrected when transferring from plant proteins to animal proteins based diets, and the underlying factor is associated with the different AA profile of these protein sources (Campbell, 2013).

The enrichment in EAA find in protein from animal origin confers this protein source its high quality for human nutrition, as well as the by-products of animal origin such as milk and derivates, where such protein source will be a key element to sustain the ever-growing world population (Van Hooijdonk and Hettinga, 2015). It has been predicted that by 2030, beef will be the greater meat import of developing countries and milk will have more than doubled as a net export of the developed world (Narrod et al., 2011).

The dairy industry faces many challenges in the future such as increased feed prices and awareness on the environmental impact of feeding N in excess (FAO, 2009). These challenges can only be overcome with more effective nutritional programs built upon refined nutritional models that can account for an optimal protein and AA utilization when feeding low CP diets, and consequently improving feed efficiency while decreasing the environmental impact of dairy farms.

Feed efficiency in dairy cows

Dairy cows are a valuable asset in the supply of animal protein around the world and an essential element in the dairy industry business model. In last decades, the dairy industry has increased the demand for improved feed efficiency (FCE; kg milk/kg DMI)(Berry and Crowley, 2013). The FCE in dairy cattle is complementary measured as well by the nitrogen (N) use efficiency (NUE; N in milk/100g N intake) in order to control the utilization of N in lactating dairy cows (Arndt et al., 2015). The NUE has been associated with a high variability between 10 and 40%, with an average of 25%, which has implications for lactating dairy cattle performance and the environment (Calsamiglia et al., 2010).

Over the years, genetic selection for high milk production cows (USDA, 2013) and reduction of maintenance requirements also called 'dilution of maintenance' by improving the metabolizable energy (ME) efficiency use for milk production (Bauman et al., 1985) have contributed to the improvement of FCE. However, genetic selection for greater milk production will no longer lead to significant increments in FCE, partially due to higher rate of passage in cows with high DMI and milk production that ends in a lack of digestible energy (NRC, 2001).

Therefore, major improvements in dairy cattle nutrition in the past years have been achieved through a deep understanding of the different physiological changes along with changes in nutrient partitioning that occur during lactation (Bauman et al., 1985) as well as closing gaps of knowledge in nutrient digestion by ruminants (Ranathunga et al., 2010) However, there still much to learn in order to improve FCE in dairy cows. A previous study reported that cows with greater FCE consumed 21% more DMI, had an increase of 98% in fat- and protein-corrected milk, and excreted lower amounts of manure per kg of fat- and

protein-corrected milk, but delivered the same amount of gases (CH_4 and CO_2) than low FCE cows (Arndt et al., 2015). The same study reported that high FCE cows had lower energy losses in terms of feces, urine, and heat than low FCE cows, which could be related to an increased in available metabolic energy for milk production. The current challenge is to refine the nutritional models obtain higher FCE while emitting less noxious gasses (Asner G. P., 2010) and N (Galloway et al., 2010) to the environment.

Currently, nutritionists focus on balancing diets for optimum performance of the animal with less energy and N losses and at a low cost. Protein has become an expensive nutrient to include into the dairy cattle ration. Under typical conditions, the most effective strategy to reduce N losses when feeding dairy cows is to manipulate the level of crude protein offered, or the type of protein offered with respect to its rumen degradability and consequently affecting the efficiency of N use in the rumen (Tamminga, 1996). Therefore, protein efficiency and N utilization while maintaining optimal productivity can considerably reduce feed costs per unit of tissue gain or milk protein produced (NRC, 2001).

Crude protein nutrient for dairy cows

The structure of the proteins is based on the linear polymers formed with monomer units or building blocks of AA, linked together by peptide bonds (Berg J.M., 2002). The AA are small organic compounds that consist of a central carboxyl group linked to an amino group, along with a variable side chain component which is specific for each AA (Whitford, 2005). Since AA are the core component of the CP fed to cows, the key role of

AA in proteins synthesis and subsequently maintenance, growth, and performance of dairy cows cannot be overstated (NRC, 2001).

Proteins per se are formed by linear chains of AA (linear structure) that rotate and fold when some AA link each other to lead to more complex structures (secondary, tertiary and quaternary) (Berg J.M., 2002) and the protein structure is a key factor in determining its susceptibility to microbial proteases and, thus, its degradability (Bach et al., 2005). Therefore, differences among proteins depend on its AA sequence, size, shape, function, solubility, function, digestibility, and some of these characteristics will likely affect its fermentation rates in the rumen (Assoumani et al., 1992; Romagnolo et al., 1994; NRC, 2001).

The concomitant increased in milk protein value, higher feed prices, and increased awareness on the environmental impact of feeding excess N to dairy cows has motivated the dairy industry to look for formulating and feeding diets with less CP through a deeper understanding of AA digestion, absorption, and utilization by dairy cattle (Lean et al., 2018). Crude protein definition encompasses true protein or metabolizable protein (MP), ammonia (NH_3-N), nitrates, amines, amides, free AA and peptides, non-protein nitrogen (NPN), and nucleic acid nitrogen (NRC, 2001). According to the Cornell Net Carbohydrate Protein System (CNCPS), crude protein can be classified into fractions A, B, and C, depending on their rate and degradability level in the rumen (Sniffen et al., 1992; Licitra G. et al., 1996). The NPN (i.e., ammonia, peptides, amino acids) is fraction A, considered completely soluble; fraction B is differentiated into B_1, B_2, and B_3, and embrace progressively decreasing degradability in the rumen. Fraction C is unavailable true protein, and likely escape the rumen fermentation (Tylutki and Fox, 2005). These CP fractions are

broadly categorized into rumen degradable protein (RDP) and rumen undegradable protein (RUP).

Dietary CP reaching the rumen is degraded by enzymes such as proteases, peptidases, and deaminases produced by ruminal microorganisms causing delivery of peptides, AA and NH_3-N into the rumen (Bach et al., 2005). In ruminants, the N has a recycling pattern used by the animal when the efficiency of conversion of digested N to AA is low. This is an energy-dependent process occurs for anabolic use of N to form NH_3-N, mainly in the rumen but also, in the small intestine. Also, in the liver, a rate of 40-80% of urea is synthesized from NH_3-N and then returned to the rumen (Harmeyer and Martens, 1980), and out of this urea, 35-55% is converted into microbial protein in both cow and sheep. The N recycling process into the rumen, in the presence of high populations of proteolytic bacteria and protozoa, causes N loss; if these microorganisms are removed or inhibited, dietary protein flow to the duodenum may be improved (Lapierre and Lobley, 2001).

Increasing RUP results on greater proportion of absorbed N as AA in the small intestine and this led to lower urea synthesis and greater milk protein output (Blouin, 1997). Therefore, the RUP and RDP fraction in CP can have a significant impact on overall N absorption and utilization as well as liver ureagenesis. Recycling of urea synthesized in the liver can provide a substantial contribution to available N for the rumen (Lapierre and Lobley, 2001). Reintroduced urea in the rumen is captured by the microorganisms and utilized as sustenance producing microbial protein (MCP) (McDonald et al., 1988; Van Soest, 1994). Peptides and AA escaping rumen fermentation are considered RUP, and along with MCP leaving the rumen are the major source for MP, and after reaching the

small intestine are absorbed to meet the animal protein requirement (McDonald et al., 1988).

Rumen Degradable Protein (RDP)

In recent years, nutritional goals in dairy cow nutrition have been to supply adequate amounts of RDP for optimal ruminal microbial efficiency, in order to obtain the maximum animal productivity with low-cost dietary CP feed sources (NRC, 2001). The key of providing adequate amounts CP in the form of RDP leads for optimal ruminal fermentation as the major source of protein nourishment for rumen microbes that, in turn, they will convert to MCP (McDonald et al., 1988). Therefore, adequate amounts of MCP are important to obtain the desired animal performance (Stern et al., 1994). However, if RDP exceeds the amount required by ruminal microorganisms, the protein is degraded to NH_3-N, absorbed through the rumen wall into the bloodstream, metabolized to urea in the liver, and excreted in the urine (Broderick, 2003). In an experiment conducted by Reynal and Broderick (2005), cows were fed 4 diets with varying amount of RDP. Then, as CP and RDP decreased in the diet, urinary N excretion decreased about 60g/cow/d, but also the percentage of protein in milk decreased. They concluded that 11.7% RDP (DM basis) is the best result in both profitability and environmental quality. Similarly, NRC (2001) suggested for maximum milk yield and milk protein yield 12.2% RDP of the diet DM.

The main factors that affect protein degradation in the rumen are the protein type and interactions with other nutrients such as carbohydrates, the ruminal pH, passage rate, and the predominant microbial population, which is dependent on diet composition (Bach et al., 2005). Proteins solubility is determined by their susceptibility to microbial proteases,

thus, the rate of protein degradability (Romagnolo et al., 1994). Also, protein structure and non-covalent interactions will determine the protein degradability in the rumen (Schwingel and Bates, 1996) which is inversely related to the passage rate (Ørskov and McDonald, 1979).

Another factor affecting RDP digestibility is the rumen pH, which is fundamental in the maintenance of the equilibrium of the microbial populations and the ruminal fermentation. Kopecny and Wallace (1982) reported that the optimal rumen pH for proteolytic enzymes range from 5.5 to 7, thus, the pH will also condition RDP degradability in the rumen. Rumen pH under 6 is associated with high concentrate diets and consequent reduction of fiber and CP digestibility, and VFA production (Cardozo et al., 2000; Cardozo et al., 2002).

Microbial protein accounts for the majority of MP absorbed in the small intestine in ruminants, in turn, MCP is mainly derived from RDP entering the rumen, and this accounts most of the CP in the diet. Therefore, replacing true protein in RDP for a low-cost RDP source such as NPN as urea could be a reasonable strategy to increase the profits in a dairy farm operation. However, Broderick and Reynal (2009) observed that by modifying the composition of RDP from soybean meal as true protein to urea as NPN reduced milk and milk components, primarily by depressing MCP formation in the rumen. Low RDP or degradability in the rumen, will lead to insufficient MCP synthesis, and the deficit has to be covered by recycling N (Satter and Slyter, 1974). This compensation mechanism allows cows to tolerate low RDP diets by supplementing NPN in limiting N diets which Satter and Slyter (1974) suggested may promote microbial growth to increase linearly when NH_3-N concentration is below 5 mg/dL. However, rumen microbial growth

has been observed to be maximal when NH_3-N in the rumen ranges from 8-10mg/dl (Hume D., 1970).

Degradation of RDP in the rumen occurs by proteolytic and non-proteolytic enzymes, and the combination of microbial and enzymatic activities maximize protein degradation (Endres and Stern, 1993). Some nutrients, as starch, interfere with protein degradation, and Assoumani et al. (1992) reported that addition of amylase increased ruminal protein degradation of cereal grains up to 20 %. Several studies also suggested an increase in protein degradation when adding cellulases into in vitro digestions (Kohn and Allen, 1995; Abdelgadir et al., 1996). Low RDP diets fed to dairy cows can decrease total-tract digestibility of DM (Lee et al., 2012) and fiber (Schwab et al., 2005).

Reduction of the variation and the factors affecting ruminal protein degradation will require adequate predictions models for RDP supply and degradation rates are necessary to maximize MCP, which is the main source for MP, while minimizing losses of excess N.

Rumen Undegradable Protein (RUP)

A widely used protein system in North America called absorbed protein (AP) model of NRC (NRC, 1985; 1989), introduced the concept of RDP and RUP, and with it the differentiation in the proportion of dietary protein escaping rumen fermentation or RUP. Therefore, this system is useful to estimate the amount of CP being utilized by the rumen microbes as RDP and absorbed in the small intestine as RUP, with the aim to optimize these fractions for high producing dairy cows.

Nutritionists and dairy producers have also been looking for the alternative ways to replace RDP sources by supplying RUP in order to reduce costs of the ration. Therefore,

in terms of milk production, when substituting a high RDP with a high RUP source of high-quality protein, the yield is expected to increase (Santos et al., 1998a). Supplements with high RUP of high-quality protein in dairy diets often have been used to increase MP flow and a better AA profile in MP reaching the small intestine. Recommendations of MP for early and mid-lactation dairy cows is 11.6% and 10.2% DM basis respectively (Kahn and Line, 2010).

Ipharraguerre et al. (2005) summarized data showing that adding up to 10.2 % RUP fed in the form of expeller soybean meal, heated, xylose-treated soybean meal, or whole roasted soybeans in the diet at the expense of soybean meal, had little effect on MP supply, overall because they were limited in EAA such as methionine (Met) and lysine (Lys). A limited supply of EAA in RUP and MCP reaching the small intestine in dairy cows, particularly of Met and Lys will likely be partly responsible for promoting low cow performance (Bremmer et al., 1997; Xu et al., 1998). Santos et al. (1998a) reported that the most consistent RUP sources providing beneficial effects on lactation performance are fish meal and treated soybean meal, mainly due to their high content of EAA such as Met and Lys in fish meal, and His, Phe and Arg in soybean meal.

Various physical and chemical methods such as formaldehyde and heat treatment have been applied grains and cereals in order to increase their RUP fraction (Church, 1988). Increasing the RUP fraction without an adequate balancing of the RDP fraction required for the microbes may depress MCP flow to the duodenum. The latter could be associated with substantial reduction in the N substrate and may limit MCP production in the rumen (Santos et al., 1998a).

The MCP synthesis is more likely to be insufficient in diets fed to high producing dairy cows (9000kg to 14000kg milk a year), thus RUP supplementation may alleviate to correct this insufficient protein supply (Santos et al., 1998b). Commonly, SBM is replaced by high RUP sources, however, some have observed no response in milk yield (Santos et al., 1998a). This effect may be explained by a lower MCP synthesis (Clark et al., 1992; Schwab, 1994; Schingoethe, 1991), a poor EAA profile in the RUP source implemented (Chandler, 1991; Schwab, 1994; Schingoethe, 1991), a sufficient supply of RUP in the control diet (NRC, 1985; 1989), or a decreased digestibility of the RUP sources in the small intestine (Schwab, 1994; Schingoethe, 1991). Improvement in milk performance has been suggested when the RUP sources have a complementary AA profile to the MCP profile (Clark et al., 1992; Chen et al., 1993; Schwab, 1994; Schingoethe, 1991).

Increasing RUP supply and an adequate profile of AA to the small intestine, while reducing the RDP, will likely increase the margin of profit of the ration. Thus, by adjusting RUP and an adequate profile of AA in the ration, an increase in milk production is expected.

Microbial crude protein

Protein utilization in the rumen is the result of metabolic activity of ruminal microorganisms determined by susceptibility of the protein to be degraded by microbial proteases (Bach et al., 2005), peptidases and deaminases (Wallace, 1996), where NH_3-N is the major end product of ruminal protein degradation (McDonald, 1952).

Ruminal MCP synthesis depends mainly on the availability of carbohydrates and N in the rumen (Hoover and Stokes, 1991). A simultaneous release of fast degradable

starch (i.e., energy) and protein sources (e.g., NH_3-N) stimulates greater efficiency in MCP synthesis (Herrera-Saldana et al., 1990; Aldrich et al., 1993).

Microbes found in the rumen such as bacteria (over 200 species), protozoa (more than 20 species) and fungi (at least 12 species) and their interactions have an essential role in ruminal MCP synthesis, which are also an important contribution to the AA supply of the cow (Schwab and Broderick, 2017). Clark et al. (1992) reported that AA accounted for 54.9 to 86.7% of the total N. Sok et al. (2017) determined that 82.4% of CP in bacteria in the total AA composition, composition of protozoa and bacteria differed in 5 out of 10 EAA, and bacteria resulted in 42% lower in Lys concentration than protozoa. Bacteria capture the majority of NH_3-N released in the rumen from AA deamination and hydrolysis of NPN compounds, but under specific dietary conditions, NH_3-N can be released over the rate of absorption by the rumen wall or uptake by ruminal bacteria. The latter scenario will likely occur under an excess of RDP or insufficient energy supply to the rumen (Maeng et al., 1997).

Although MCP is primarily driven by the constant supply of dietary protein, NPN, recycled N as NH_3-N, the availability of energy in the form of ATP can have a significant impact on microbial growth (Church, 1988). Also, ATP uncoupling can happen if NH_3-N and other nutrients are deficient and then, fermentation continues but ATP produced is not used for microbial growth. Bacteria and protozoa obtain their energy needs from fermentable substrates primarily starch in the ration, and the byproduct of this fermentation are VFA, which are the major energy source for the cow (Church, 1988). Thus, MCP synthesis is regulated by energy and protein availability into the rumen (Church, 1988).

Related to MCP synthesis, researchers have observed that basal NH_3-N concentration will maintain MCP synthesis in the rumen (Schwab and Broderick, 2017), but NH_3-N concentrations exceeding 5 mg of N/dL have been reported to be not beneficial for MCP synthesis (Satter and Slyter, 1974). Erdman et al. (1986) carried out an experiment applying different NH_3-N concentrations on in situ digestion of various feedstuffs and concluded that the minimum ruminal NH_3-N concentration needed to maximize in situ digestion depended on the feedstuff fermentability, and it was greater as higher fermentability of the feed. In a review by Schwab et al. (2005), it was indicated that the optimum concentration of ruminal NH_3-N appeared to be diet-dependent and influenced by factors such as the type of N source and carbohydrate fermentability as well as factors affecting passage rate such as DMI. Since optimal MCP is influenced by N and carbohydrate availability in the rumen, greater ruminal NH_3-N concentrations may be needed when feeding highly fermentable carbohydrates (Annison, 1956). Also, a range of 5-11mM of ruminal NH_3-N concentrations was needed to maximize microbial N flows from the rumen, but it was conditioned to the diet type and fermentation conditions (Schwab et al., 2005).

Optimal MCP synthesis evaluated through in vitro fermentation studies revealed a quadratic effect describing MCP synthesis during ideal conditions, with maximum efficiency of bacteria utilization of N of a 69% at MCP synthesis efficiency of 29 g bacterial N/kg OM fermented (Bach et al., 2005). Thus, adequate amounts of N available in the rumen are necessary to optimize rumen function and maximize synthesis of microbial protein (Schwab and Broderick, 2017).

The essential contribution of the microbes in the rumen to the MP in the form of AA required by the animal has been shown in studies supplying urea and ammonium salts to the ruminants as the only CP source (Oltjen, 1969). Under these conditions, FCE, growth, and N retention were reduced around 35% compared with supplying the same CP equivalent in the diet with isolated soy protein sources. Peptides and free-AA in the rumen product of true protein degradation stimulate MCP synthesis in the rumen (Russell et al., 1992). Lactating dairy cows fed all NPN as CP source diets had EAA concentrations depressed in blood plasma (Virtanen, 1966).

Microbial protein synthesized in the rumen along with the RUP fraction in the diet, pass to the abomasum where they are digested and then absorbed as small peptides and AA in the small intestine, serving as a source of MP for the ruminant (NRC, 1985). The microbial mass that flows from the rumen into the lower gastrointestinal tract supplies an overall CP of 20 to 60% DM basis (Church, 1988) and from 50 to 80% of the MP (Storm and Orskov, 1983). Stern et al. (1994) documented the importance of maximizing MCP synthesis efficiency to support high levels of milk production.

In terms of volume, MCP synthesized by rumen microbes represents the major supply of AA in the MP utilized by the cow, and it is regulated by energy and N availability into the rumen. Therefore, maximizing MCP synthesis should be at the core of any nutritional program in dairy cows with the aim to utilize a low-cost source of AA in MCP, and complement this with RUP sources.

***Metabolizable protein* (MP)**

The metabolizable protein is the true digestible and absorbed protein for the animal comprised of MCP and RUP. The complexity of estimating MP in dairy cows cannot be overstated, encompassing the rumen microbial dynamics, degradation rates for RDP, passage rate, AA profile in the RUP fraction, etc. Because these factors coupled with the fundamental importance of MP in dairy cow nutrition, several nutritional models have been built to estimate MP in dairy cow rations including NRC (2001), Spartan Dairy Ration Evaluator (Michigan State University, East Lansing), and Cornell Net Carbohydrate Protein System (Cornell University, Ithaca).

The MCP, RUP, and to a lesser extent but still important, endogenous CP (ECP) contribute to MP being absorbed in the small intestine, as the true protein digested post-ruminally and AA absorbed by the intestine (NRC, 2001). In the rumen, endogenous protein in saliva, sloughed epithelial cells and the remains of lysed ruminal microorganisms comprised the ECP (NRC, 2001). The proportion of MP supplied by MCP has been estimated in the CNCPS around 60% (Russell et al., 1992), assuming that 15% N is in the form of nucleic acids and the remaining 25% is located in the cellular wall so it would not be available.

The most limiting AA in dairy cows diets are Lys and Met (Noftsger and St-Pierre, 2003; Socha et al., 2005). Requirements of these AA have been described around 7.2-7.3% and 2.4-2.5% of MP for Lys and Met, respectively (Rulquin et al., 1993; NRC, 2001; Doepel et al., 2004). The importance of these AA as limiting factors in dairy cow rations has consistently observed and documented. (Patton, 2010) A meta-analysis by Lean et al. (2018) using the CNCPS model to estimate the effect of Met and leucine (Leu) in MP in

performance parameters, observed that these AA increased milk protein yield and only Met was associated with an effect on milk protein percentage. The importance of an optimal balance or profile of AA in MP reaching the small intestine relies on an evident limitation for performance of the dairy cows.

Strategies to optimize RUP and MP

The Association of American Feed Control Officials defines "rumen-protected" nutrient/s fed in such a form that provides an increase in that specific nutrient/s flow, not been ruminal fermented and then, being available to absorption in the small intestine (Noel, 2000; NRC, 2001). Thus, rumen-protected proteins are feeds that have been treated or processed in ways to decrease ruminal protein degradability and increase the content of digestible RUP. The main reason is that it is highly expensive to cover the MP needs by adding protein as RDP to the ration instead to increase the RUP fraction by supplementing protein protected of the ruminal degradation.

Methods previously evaluated to decrease protein degradation in the rumen include heat, chemical agents, or a combination of heat and chemical agents (Satter, 1986; Broderick et al., 1991; Schwab, 1995) but the real challenge is to identify treatments that increase digestible RUP to the extent that justifies the cost of the treatment. Although these treatments (e.g., heat and formaldehyde) allow increasing RUP with minimal loss of AA, if excessive treatment is applied, it may reduce post-ruminal protein digestibility and absorption and consequently, the decreasing the potential MP available for the animal (Merchen et al., 1997).

In North America the most common method to decrease rumen protein degradability is heat processing, however, excessive heat treatment can denature proteins and results on Maillard or protein-carbohydrate reactions, and cross-links protein-protein. Alternative methods include cooker-expeller processing of oilseeds, heat treatment added of solvent extracted oilseed meals, roasting, extrusion, pressure toasting, and legume seeds micronization, and also cereal grains and protein supplements expander treatment (NRC, 2001). The content of digestible RUP may be optimized by strict control of heating conditions (Schwab, 1995). In situ studies evaluating the effect of heat processed feedstuffs on ruminal degradation of protein indicate a decrease in fraction A, while increasing fractions B and C, but with a decrease in the fractional degradation rates of the B fraction (Goelema et al., 1999). The aim of heat processing of feedstuffs rich in protein under optimal conditions is to significantly reduce ruminal protein degradability without adverse effects on post-ruminal digestion and absorption of MP.

The chemical treatment method is the second most used method to increase the RUP fraction in feedstuffs, and have different categories depending on the treatment: binding to proteins but with little or no alteration of protein structure (e.g., tannins), by denaturation altering protein structure, or combining and introducing crosslinks in proteins (Broderick et al., 1991; Schwab, 1995). Chemical treatment methods alone have not received wide acceptance in the dairy industry, and rather combinations of chemical agents with heat treatments protocols seem a more effective approach (NRC, 2001). The amino acid profile contained in high RUP proteins and intestinal digestibility of the RUP fraction are the key for effective protein use.

Protein from animal origin such as fish meal, meat, and blood meal along with other feedstuffs such as gluten meal, heat treated soy flour, and casein treated with formaldehyde are also good sources of RUP (NRC., 1996). Lastly, there are also some forages that possess certain compounds called tannins that reduces protein degradability in the rumen and increases AA's availability for the cow (Broderick, 1995).

Tannins in dairy cow nutrition

Tannins are natural plant phenolic compounds that precipitate proteins and with a known ability to reduce proteolysis. Plant phenolics have been implicated in the resistance of plants against bird depredation, insect attack, preharvest seed germination, and diseases caused by fungi, bacteria, and viruses. Also, certain tannins have been indicated to have antibacterial, antioxidant, and flavor-inducting effects (Singleton, 1981).

Interactions between tannins and proteins have been reported to be both tannin and protein specific (Asquith and Butler, 1986). For this reason, tannins have been considered as a good dietary source for optimal protein use by ruminants, however they have low palatability and decrease in feeding value, DMI, and protein digestibility (Donnelly, 1969).

As suggested by Freudenberg (1960), the most acceptable division of tannins derived from plant origin is condensed (CT) and hydrolyzable (HT) tannins based on structural types. The principal difference between these two groups arises from their mode of action. The HT are made up of a carbohydrate core whose hydroxyl groups are esterified with phenolic acids (mainly gallic and hexahydroxydiphenic acid) (Frutos et al., 2004). In the other hand, the CT do not easily break down with acid, and they undergo progressive polymerization under the action of acids to yield the amorphous phlobaphenes or tannin

reds. (Haslam, 1966). Condensed tannins are the major polyphenols of commonly consumed foods, widespread in fruits, vegetables, forage plants, cocoa, red wine, and certain food grains including sorghum, finger millet, and legumes (Guesnel, 1968).

Tannins in the presence of proteins can increase the insoluble fraction of such proteins, primarily by association with the plant cell wall (NRC, 2001). However, elevated concentrations of tannins in the diet reduce voluntary intake and nutrient digestibility (Frutos et al., 2004). This is due to a reduction in palatability by the reaction of tannins when in contact with mucoproteins of saliva that neutralize the action of tannins causing and astringent sensation (McLeod, 1974). And, tannins can be detrimental to digestibility primarily by modifying ruminal fermentation patterns, along with changes in intestinal digestibility (Frutos et al., 2004).

Tannins originate from different plant species and, therefore, have different physical and chemical properties (Mangan, 1988), and, in turn, this confers them a rich and diverse variety of biological properties (Zucker W. V., 1983). The essential property of tannins is to be combined with proteins and other polymers such as cellulose, hemicellulose, and pectin, to form stable complexes (Mangan, 1988). Therefore, ingestion of small amounts of CT by ruminants can prevent bloat, improve nitrogen retention, and reduce excretion of urea, a precursor of ammonia and the greenhouse gas nitrous oxide. (Kronberg et al., 2007).

The tannin-protein complexes form in the rumen are normally dissociate at low pH < 3.5, for that reason this dissociation will happen in the abomasum (McNabb et al., 1998) and the duodenum (pH ~ 2.5) and proteins may become available for digestion in the lower

sections of the small intestine (Jones and Magan, 1977). This change in pH also may cause that some of the complexes will reform impeding digestion of the proteins.

Tannins also have been considered harmful in high dosage scenarios. Mitjavila et al. (1977) suggested that tannins may have a destructive action on the mucosal lining of the digestive tract, as observed in rats fed tannic acid included in the diets, with subsequently increased excretion of mucoproteins, sialic acid, and glucosamine in feces. Several studies have reported HT tannins with a toxic effect, but, in contrast, there are a few studies reporting toxicity of CT tannins. McLeod (1974) suggested toxicity of tannins depend on their molecular size as tannins with high molecular weights cannot be absorbed through the small intestine. Therefore, high toxicity tannins are those with low molecular weight. Intoxications by tannins can cause anorexia, depression, ruminal atony, hepatic and renal failure, ulcers in the digestive tract and severe gastroenteritis (Spier et al., 1987; Zhu et al., 1992). Therefore, tannins fed in sufficient amounts may cause hypersecretion of gastric and duodenal mucus and cause epithelial edema, irritation and tissue breakdown of the digestive tract. Thus, the chronic ingestion of large amount of tannins may damage the gastrointestinal surface, and in those conditions, they might be absorbed and produce harmful effects (Salunkhe et al., 1990). Parenteral administration of tannins can cause significant toxicity, but acute toxicity via oral administration is low. Rectal toxicity of tannic acid is about twice its oral toxicity (Boyd et al., 1965,). Some studies have reported the liver and kidneys to be subjected to severe damage from tannins ingestion and injection (Vohra et al., 1966; Price and Butler, 1980). The injection of tannic acid at biochemical and cellular levels may cause liver fibrosis, necrosis, polyribosome disaggregation, inhibition of the microsomal enzyme, and synthesis of nucleic acid and protein (Bodway

et al., 1969; Oler et al., 1976). However, CT appear to have beneficial effects for cattle by preventing bloating in the rumen, which is a condition of high rumen pressure and fatal, if not treated immediately, where CT can prevent this by precipitating proteins (Jones et al., 1973). Beneficial effects of tannins in N metabolism of ruminants have been reported by protecting the dietary protein from deamination by rumen bacteria (Hatfield, 1970). Improving protein efficiency and N metabolism use by the cow while striving for optimal productivity is a matter of environmental and profitable concern in any responsible dairy farm operation (Salunkhe et al., 1990; NRC, 2001). A study carried out by (Driedger and Hatfield, 1972) shown a 90% reduction in dietary CP degradation in the rumen by treating and feeding soybean meal with 10% extra tannic acid to lambs. Also, it was observed that lambs consuming the soybean meal with tannic acid improved daily gain and N balance.

The overall effect of tannins in ruminant nutrition may depend on the CP availability and the dynamics involving tannin-protein complexes. Dissociation of tannin-protein complexes will liberate tannins, and the latter may cause damage to the intestinal tract or form new complexes with endogenous proteins. In case the complex does not dissociate in the abomasum or the intestine, there will be no benefit to the animal from the protein being protected in the rumen (Price and Butler, 1980).

Black pepper

The increased consumer awareness on the potential overuse of antibiotics in animal production has developed a focus on using natural additives to decreasing the use of antibiotics while preventing diseases and maintaining adequate in animal performance (Kirubakaran et al., 2016). Plant extracts as herbs and spices have become more often used

as appetite and digestion stimulants as well as stimulants of physiological functions, prevention, and treatment of certain pathologies and as antioxidants (Korošec et al., 2009). Several studies have shown beneficial effects of herbs and spices on feed intake, immune functions and health, rumen fermentation and productivity of the animals (Greathead, 2003; Cardozo et al., 2006). The effectiveness of active compounds in herbs and spices largely depends on the dosage, where high doses may be toxic (Korošec et al., 2009).

Dietary black pepper, for instance, is a spice known to increase the bioavailability of drugs and other phytochemicals, which may be attributed to increased absorption, resulting from alteration in membrane lipid dynamics and change in the conformation of enzymes in the intestine (Srinivasan, 2007; Srinivasan, 2009). Also, piperine, the bioactive constituent of black pepper has been reported to promote β-carotene absorption in the intestine (Veda and Srinivasan, 2009) as well as reducing cholesterol uptake by internalizing the cholesterol transporter proteins (Duangjai et al., 2013).

Currently, there is a lack of data on the effects of black pepper fed to dairy cows, but if those beneficial effects observed in monogastrics can be extrapolated to ruminants, it could represent a new tool for dairy farmers to optimize dairy cow rations. There is still a need to clarify the phytochemical composition and the mechanisms of action for many herbs, spices, and their extracts and furthermore, to assess the appropriate dosage that should be safely used in dairy cows.

Summary of Literature and Research Justification

The ability of tannins to form complexes (i.e., reversible and irreversible) with proteins, and alter ruminal fermentation by enhancing feed N efficiency and consequently

milk production (Powell et al., 2011a) has increased the interest for including them in the diet of lactating dairy cows. There are two major groups of tannins: hydrolyzable (HT) and condensed (CT) (Freudenberg, 1960) and depending on the type of tannin, the amount fed, its molecular weight and structure, it may have an adverse or beneficial effect in animals (Frutos et al., 2004). Originally, tannins were thought only to have detrimental effects to ruminants, but over time their effects have resulted in reducing protein degradation in the rumen and thus, significantly decrease the excretion of urea, urine and fecal N (Aguerre et al., 2010a) and reduce environmental wastes of N (Powell et al., 2011a; b).

Tannins help to increase the RUP fraction in the diet, and this results on greater proportion of N absorbed as MP in the lower gut, which leads to lower urea synthesis and greater milk protein output (Blouin, 1997). Therefore, feeding less CP by adding tannin extracts to dairy cows may enhance feed N use and milk production, abate N excretion as well as reduced the price of the ration for dairy cows.

This experiment aimed to evaluate dietary phytochemicals in the form of tannins extracts and alkaloid piperine from black pepper in diets fed to lactating dairy cows compared to a control diet. This will be evaluated by examining effects on milk yield and composition, feed intake and efficiency, blood metabolites, rumen fermentation concentrations, and total tract digestibility. It was hypothesized that diets containing a tannin supplement would increase milk and component yields, improve feed efficiency, and nutrient utilization, and benefit rumen fermentation to yield a more productive and efficient dairy cow.

CONCLUSIONS

Current demand for protein from animal origin is predicted to increase in the next years in order to feed human population, especially in the emerging economies. Thus, it is important to maintain protein efficiency, N utilization, and optimal performance to considerably reduce feed costs in a dairy farm. This aim can be reached by 1) adequate prediction models for RDP supply and digestion rates to maximize MCP, which is the main source for MP, while minimizing losses of excess N, and 2) increase RUP supply while decreasing the amounts of RDP and ensuring an optimal profile of AA contained in the final MP.

Based on the above, alternative methods that can increase the RUP supply to the small intestine, such as tannins, can positively affect the protein efficiency in ruminants. Additional research on herbs and spices such as black pepper will allow us to understand how their bioactive compounds (e.g., piperine) can modify the rumen fermentation dynamics and potentially improve protein efficiency in dairy cows.

Chapter 2: EVALUATION OF DIETARY PHYTOCHEMICALS AS RUMEN MODIFIERS IN LACTATING DAIRY COWS

INTRODUCTION

The dairy industry has increased the input of N into the N pool of the average dairy farm operation resulting in ecosystem disruption and environmental degradation (Galloway et al., 2003; Galloway et al., 2008). Included in the N pool are N contained in feed and manure, organic and inorganic soil N, N fixed by legume crops and inorganic fertilizer N and all biological systems including livestock are limited incorporating N into products (Powell et al., 2011a). Ammonia volatilization is a major pathway of agricultural N loss and environmental concern as atmospheric haze and potentially human health hazard (Bobbink, 2010; Dise et al., 2010). Dairy cows excrete N mostly as urea that increases NH_3-N emissions when an excess of CP is included in the ration (Powell et al., 2011a). A recent goal in dairy cow nutrition is to feed less CP in order to reduce N emissions, therefore, tannins extracts have been used as feed additives to improve FCE in dairy cows (Benchaar et al., 2008) by reducing urea N excretion and ammonia (NH_3-N) loss (Aguerre et al., 2010b; Powell et al., 2011a) while reducing methane (CH_4) gas production (Baert et al., 2016).

Tannins capacity to form complexes with different feed nutrients, especially proteins, becomes very important in dairy cow nutrition, since such complexes may decrease or escape rumen fermentation (Mole and Waterman, 1986; Hagerman et al., 1992). This characteristic is due to the tannins binding their numerous phenolic groups with the carbonyl groups of peptides (McLeod, 1974; Hagerman et al., 1992). In dairy cow rations, an increase in RUP without compromising RDP for rumen microbes is desirable

to promote greater proportion of absorbed N as AA, and this led to lower urea synthesis and greater milk protein output (Blouin, 1997). Therefore, tannins may allow decreasing the dietary CP, by reducing RDP and increasing RUP, which in turn can improve the profit of a dairy operation by reducing feed costs per cow without compromising milk production (Rotz et al., 1999). Tannins from plant origin are classified as condensed (CT) and hydrolyzable (HT) tannins based on structural types (Freudenberg, 1960). The use of HT tannins during in vitro rumen fermentation models, resulted in a reduction of NH_3-N and VFA (Baert et al., 2016). Thus, the addition of tannin extracts into the diet of dairy cows may improve the dietary N efficiency, by promoting N being secreted as milk protein instead of urea in urine (Aguerre et al., 2016). Although tannins have been associated with protein binders, recent studies have introduced potential additional effects such as antispasmodic, reduction of rumen starch degradation, modulation of the local immune response (Díaz, 2017). Additionally, tannins have been associated with modulation of the gastrointestinal microbiota, by favoring the proliferation of bacteria that improve energy efficiency and reduction of greenhouse gases emission (Powell et al., 2011a; b).

The use of alternative herbs and spices such as black pepper has received less attention than tannins in dairy cattle nutrition research. However, beneficial effects observed in monogastrics and humans could be extrapolated to ruminants. Among the many effects observed in monogastrics associated with black pepper or piperine (i.e., the active compound) are anti-inflammatory action, secretion of saliva, and stimulate bile acids synthesis in the liver and consequently lipid digestion and absorption in the small intestine (Frankič et al., 2009). Unfortunately, there is lack of knowledge on whether the effects can be translated into ruminants.

The objective of this experiment was to evaluate the effects of supplementing phytochemicals in the form of tannins and piperine (as black pepper) in the ration of lactating dairy cows. This will be evaluated by examining effects on milk yield and composition, feed intake and efficiency, blood metabolites and the antioxidant enzyme superoxide dismutase (SOD), rumen fermentation concentrations, and total tract digestibility. Our general hypothesis was that diets containing phytochemicals, and mainly based prior data on tannins extracts will increase milk yield and components, improve feed efficiency, improve the antioxidant status, and benefit rumen fermentation in order to enhance dairy cow performance.

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All experimental procedures were approved by Institutional Animal Care and Use Committee (IACUC17-010A) at the South Dakota State University. Sixteen mid-lactation Holstein dairy cows (14 multiparous and 2 primiparous), with 111 ± 20 days in milk (DIM) at South Dakota State University Dairy Research and Training Facility (DRTF) were used in a crossover design with an adaptation period. Cows were randomly assigned to a treatment sequence (n=8/treatment) according to DIM, lactation number, prior milk yield averages (kg/d), and body weight (BW). The experiment lasted a total of 56 d and consisted of a 14-d adaptation (covariate) period and two 21-d treatment periods. Treatment diets were a basal diet supplemented with soybean meal pellets fed at 3.37% of DM (CON), and a basal diet supplemented with soybean meal pellets fed at 3.37% of DM containing phytochemicals (i.e., tannins extracts and black pepper; TRT) at 4.4% concentration.

During the adaptation period, cows were fed the control diet, and baseline values were obtained for all variables. During the first treatment period, half of the cows were fed the CON diet with no phytochemicals, whereas the remaining cows were fed the TRT diet supplemented with the phytochemicals at 40g/d. Diets were group switched for the second treatment period. The ingredient and nutrient composition of the diets fed as TMR are described in Table 1. Diets were formulated to meet the requirements of the average cow in the group according to the NRC (2001).

Animals Management

The experiment was conducted at the DRTF at South Dakota State University from February to April 2017. Cows enrolled in the study were housed in a naturally ventilated barn with access to mattress-bedded freestalls. Cows were fed individually once daily at 0730 h using an individual Calan gate system (American Calan Inc., Northwood, NH). Individual refusals were weighed daily, and feed offered was adjusted daily to achieve 5 to 10% refusals.

Individual BCS and BW were recorded at the start of the study and at the end of each period on 2 consecutive days. Cows were scored for BCS (scale 1 = thin to 5 = obese, with quarter-point increments) by three individuals and the average score was used for statistical analysis (Wildman et al., 1982).

Feed Samples and Analysis

Dry matters (DM) of the ingredients were obtained weekly, and the diet was adjusted accordingly to maintain DM ratios of ingredients in the TMR. Weekly samples

of ingredients and TMR were collected and stored at -20˚C until further analysis. Additional TMR samples were collected once per week to determine particle size and using the Penn State Particle Separator (Kononoff et al., 2003).

All the ingredients used in the diet (alfalfa hay, alfalfa haylage, corn silage, cotton seeds, grain mix, straw, soybean meal pellets, tannins pellets) were individually composited at the end of the study by ingredient along the experiment, to create eight total samples per ingredient for nutrient analysis. Each TMR sample (CON and TRT) were composited by period (Covariate (CON), P2 and P3 (both CON and TRT) and orts were composited by cow and period. All the fecal samples were composited by cow and period. An equal volume was taken from each individual timepoint fecal sample by cow and period to end up with a representative fecal sample.

Ingredients and TMR were then sent for nutrients composition (CP, NDF, ADF) analysis (Dairy One Lab., Ithaca, NY). Fecal and orts samples were sent to the same lab for nutrient composition (CP, NDF, ADF, Ash, and ADIA as an internal digestibility marker). For N content, samples were analyzed by combustion using a CN628 Carbon/Nitrogen Determinator (Form 203-821-392, 09/10Rev0., Leco Corporation, St. Joseph,MI). Then, N content was multiplied by 6.25 to calculate crude protein (CP). Neutral detergent fiber (NDF) (Van Soest et al., 1991) was sequentially analyzed using the Ankom 200 fiber analysis system (Ankom Technology, Macedon, NY). Specifically, it was applied the Ankom technology method 6. First, α-amylase and sodium sulfite were added at the start of the digestion and then, a neutral detergent solution was used. Acid detergent fiber (ADF) (AOAC 1977, Method 973.18) was analyzed using Ankom technology method

5 with an acid detergent solution and analyzed sequentially using the Ankom 200 fiber analysis system (Ankom Technology, Macedon, NY).

Acid detergent insoluble ash (**ADIA**) was used as an internal marker and analysis was conducted on all TMR and Orts samples. The method for ADIA analysis consists of analyzing the sample for ADF content (Robertson and Van Soest, 1981) and then determining the ash content using a modified procedure of the AOAC 17th ed., method 935.29 (2002). Digestibility calculations were determined according to Merchen (1988).

Milk Samples and Analysis

Milk samples were collected from both milkings during the last three days of each period and stored at -20˚C until further analysis.

Milk samples were composed by day and period into vials (Thermo Scientific™ 90mL Capitol Vial for Milk Sampling, Fisher Scientific Waltham, MA) based on the weighted volume of each sampling day's milking. Then, composed milk samples were sent to Heart of America DHIA Laboratory (Kansas City, MO) for milk composition analysis. Fat, protein, and lactose were analyzed by mid-infrared spectroscopy (AOAC, 2006; Bentley 2000 Infrared Milk Analyzer, Bentley Instruments, Chaska, MN). Laser technology (Soma Count 500, Bentley Instruments, Chaska, MN) was used to analyze the somatic cell count (**SCC**) of the milk samples, and milk urea nitrogen (**MUN**) was determined using a modified Berthelot reaction (ChemSpec 150 Analyzer, Bentley Instruments, Chaska, MN).

Blood Samples and Biomarker Analyses

Blood samples were collected from the coccygeal vein on d 13 and 20 during the adaptation and treatment periods, respectively, for blood metabolites. Blood was drawn into 10-mL vacutainer tubes containing either clot activator (BD Vacutainer; Cat# 02-685A, BD and Co., Franklin Lakes, NJ) or lithium heparin for serum and plasma, respectively separator tube (BD Vacutainer; Cat# 02-685-3B, BD and Co., Franklin Lakes, NJ.). After blood collection, tubes with lithium heparin were placed on ice and tubes with clot activator were kept at room temperature (21˚C) until centrifugation (~30 min; CR412 centrifuge; Jouan Inc., Winchester, VA). Serum samples were centrifuged at 1300 × *g* for 15 min at 22˚C, while plasma samples at 1300 × *g* for 15 min at 4˚C. Aliquots of serum and plasma were stored at -80˚C for further analysis.

One aliquot by cow by period was prepared and sent to an external laboratory (Veterinary & Biomedical Sciences Department Lab, Brookings, SD) to analyze NEFA (NEFA-HR ACS-ACOD method; Cat# 999-34691, Wako Life Sciences, Inc., Mountain View, CA) and BHB (BHB reagent set; Pointe Scientific, Inc. Research Drive, Canton, MI) concentrations. For the rest of the analysis, once samples were thawed and vortexed, PUN, glucose, albumin and SOD concentrations were analyzed with commercially available enzymatic or colorimetric assay kits on a microplate spectrophotometer (Cary 50, Varian Inc., Walnut Creek, CA). Bromocresol green was used to analyze albumin (procedure No.0285, Stanbio Laboratory, Borene, TX) and diacetyl monoxime was used to analyze PUN (procedure 0580, Stanbio Laboratory, Boerne, TX). Glucose was determined by the glucose oxidase reaction (Trinder, 1969), using a glucose kit (Code No.997-03001, Wako Life Sciences, Inc., Mountain View, CA). Superoxide Dismutase

(SOD) was determined using a SOD assay kit (ESOD-100, EnzyChrom^{TM}, BioAssay Systems, Hayward, CA).

Rumen Fluid Collection and Analysis

Rumen fluid was collected via esophageal tubing on 19d and 20d of each period, and 4 h after feeding. After discarding 200-300 mL of fluid to minimize saliva contamination, approximately 60 mL of rumen fluid were collected. Samples were immediately measured for pH using a handheld pH meter (pH tester, Cat# 13-200-263, Oakton Instruments, Vernon Hills, IL). Then, rumen fluid (10 mL) was transferred into 2 vials (Cat# 03-337-4, Fisher Scientific Waltham, MA), previously acidified with either 200 µL of 50% (vol/vol) sulfuric acid or 2 mL of 25% (wt/vol) metaphosphoric acid and stored at -20˚C until later analyses of ammonia N (**NH_3-N**) and VFA.

Stored rumen fluid samples containing sulfuric acid were thawed and centrifuged for 10 min at 10,000 × rpm and 10°C (Eppendorf 5403 Centrifuge, Eppendorf North America, Hauppauge, NY), and according to Chaney and Marbach (1962) ammonia N of the samples was analyzed with a colorimetric assay on a micro-plate spectrophotometer (Cary 50, Varian Inc., Walnut Creek, CA). The preserved rumen fluid samples were thawed and centrifuged for 10 min at 10,000 rpm and 10°C, and VFA concentrations were analyzed. An automated GC (Model 6890; Hewlett-Packard Co., Palo Alto, CA) was used to measure concentrations of those VFA by a flame-ionization detector. Separation of VFA was performed on a capillary column (15 m × 0.25 mm i.d.; Nukol, 17926-01C; Supelco Inc., Bellefonte, PA) and using an internal standard, 2-ethylbutyrate. The injector port had

a split ratio of 100:1 and it was set at 250°C and flow rate of 1.3mL/min of helium. The column was maintained at 140°C and the detector at 250°C.

Apparent Total-Tract Digestibility

Fecal samples were collected during days 20 and 21 of each period. Acid detergent insoluble ash (ADIA) was used as an internal digestibility marker. Orts were collected individually per cow prior to morning feeding during the last 3 days of each period. From the last day of orts collection, fecal grab samples were collected every 6 h over a 48 h period and were stored at -20˚C until further processing and analysis.

Feed samples were dried for 48 h at 55˚C in a Despatch oven (Style V-23, Despatch Oven Co., Minneapolis, MN) for DM determination, and were grounded to a 4 mm particle size using a Wiley Mill (model 3; Arthur H. Thomas Co., Philadelphia, PA). Further grinding to a 1 mm particle size was done using an ultracentrifuge mill (Brinkman Instruments Co., Westbury, NY).

The **ADIA** was used as an internal marker by using the ash of the ADF fraction of fecal and orts samples. The ash from TMR and the ADF fraction were analyzed at SDSU dairy science department laboratory. The method for ADIA analysis consists of analyzing the sample for ADF content (Robertson and Van Soest, 1981) and then determining the ash content using a modified procedure of the method 935.29 of AOAC (2002). Digestibility calculations were determined according to Merchen (1988). Sample ash content was determined by incinerating a 1g sample overnight at 450˚C in a controlled temperature furnace preheated (AOAC Official Method 942.05). The organic matter (**OM**) was estimated by OM = (100 – % Ash).

Statistical analysis

Data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) with period and dietary treatment as fixed effects and cow nested within sequence as the random effect. The crossover design in this study was evaluated using the following model:

$$Y_{ijkl}=\mu+\delta_k+\pi_{l(k)+}\alpha_i+\beta_j+\varepsilon_{ijkl}$$

Where Y_{ijkl} is the dependent, continuous variable; μ is the overall mean; δ_k is the fixed effect of the *k*th sequence (*k* = 1 and 2); $\pi_{l(k)}$ is the random effect of the *l*th cow within the *k*th sequence ($l = 1, \ldots, n_{l(k)}$); α_i is the fixed effect of the *i*th dietary treatment (*i* = 1 and 2); β_j is the fixed effect of the *j*th period (*j* = 1 and 2); and ε_{ijkl} is the residual error (Lyman and Longnecker, 2001).

Energy-corrected milk (ECM) was calculated by using the following equation: ECM = [(0.327 x kg milk) + (12.95 x kg fat) + (7.2 x kg protein)] (Orth, 1992). Feed efficiency was calculated by ECM/DMI. Data are presented as least square means with the highest standard error of the mean (SEM) among the values. Statistical differences were declared significant at $P \leq 0.05$ and tendencies $P \leq 0.15$.

RESULTS

Feed Analysis, Particle Size, and Apparent Total-Tract Digestibility

Diet formulation and nutrient composition for CON and TRT diets are presented in Table 1. The CON diet contained 0.7% more CP than the TRT diet. Both diets had comparable amounts of DM, OM, ashes, and NDF. There was a 2.43% more of

ADF in the CON diet. Diets were formulated to meet the nutritional requirements set by the NRC (2001). Individual ingredients nutrient compositions (i.e., DM, CP, NDF, ADF, NFC and TDN in %DM basis) are presented in Table 2.

The TMR particle size was measured with the Penn State Particle Separator, and it is presented in Table 3. Percentage of particles retained in the upper sieve (19 mm) was 14.2%. The sieves where most of the particles were retained on were the middle sieve (8 mm) (32.9%) and the bottom pan (37.1%).

Total-tract digestibility results are presented in Table 4. There was a greater ($P \leq 0.03$) digestibility for DM, OM, and CP in cows fed the TRT diet in comparison to CON. Digestibility of ADF was greater ($P = 0.01$) in CON treatment cows than TRT. The NDF digestibility was not affected ($P = 0.13$) by dietary treatments.

Lactation Performance Parameters

Main effects for DMI, BW, BCS, milk production and composition parameters as well as feed efficiency are presented in Table 5. The lactation parameters of milk yield, protein yield, lactose yield, ECM, FE were greater ($P \leq 0.03$) in cows fed CON diet in comparison to TRT. Similarly, BW was greater ($P = 0.03$) in cows fed the CON diet than TRT. A trend for greater ($P = 0.10$) fat yield in CON cows than TRT was observed. All other lactation performance parameters were not affected by dietary treatments.

Metabolic Profile

The main effects for blood metabolites are presented in Table 6. The concentration of glucose was lower ($P = 0.02$) in cows fed the TRT diet in comparison to CON. In contrast

to glucose, greater (P = 0.05) concentration of BHB and albumin was observed in TRT cows than CON. Similarly to BHB and albumin, there was a trend (P = 0.06) for greater SOD in TRT cows than CON. The concentrations of PUN and NEFA were not affected ($P \geq 0.13$) by dietary treatments.

Rumen Fermentation

Main effects for ruminal fermentation parameters are presented in Table 7. The proportion of acetate, as well as the ratio acetate:propionate (A:P), were greater ($P \leq 0.04$) in TRT cows than CON. In contrast, the proportion of propionate was lower (P = 0.05) in cows fed TRT diet than CON. Similar to propionate, a trend (P = 0.09) was observed for a lower proportion of valerate in cows fed TRT diet than CON. The pH, NH_3-N, total VFA concentrations as well as other VFA proportions were not affected ($P \geq 0.42$) by dietary treatments (Table 7).

DISCUSSION

Feed Analysis, Particle Size, and Apparent Total-Tract Digestibility

The lower CP % in the TRT diet in comparison to CON could be associated with the lower CP % (49.1 % vs. 51.2 %) observed in pellets containing the phytochemical pellets in contrast to the soybean meal pellets fed in the CON diet (Table 2).

Neutral detergent fiber concentration was elevated but still within the range recommended by the NRC (2001). The percentage of particles retained in the upper sieve (19 mm) was greater (14.25%) than the Penn State Particle Separator guidelines of 2-8% for the upper sieve (Kononoff and Heinrichs, 2003). Percentage of particles retained on the

middle sieve (8 mm) (32.91%) and the bottom pan (37.1%) were within the recommended ranges. Physically effective NDF (peNDF) stimulates rumination and salivation (Mertens, 1997)as those particles size pass through the rumen at a slower rate. The peNDF is determined by measuring the percentage of dietary particles greater than 1.18 mm and multiply the dietary NDF. Since the NDF was similar between treatments diets (29.5 % vs. 28.5 %; Table 1), we assumed this parameter did not influence the current results. And, the calculated peNDF for the diets combined was 18.2 peNDF, which was lower than recommendations (Mertens, 1997; Kononoff et al., 2003). However, this lower peNDF was not reflected in a rumen pH below 6 (Table 7), suggesting that for cows in this experiment an 18.2 peNDF was enough to maintain an adequate rumen environment.

McSweeney et al. (1988) suggested that condensed tannins may increase digestibility of OM. But, others have reported that tannins may have a negative effect on nutrient absorption (Driedger and Hatfield, 1972; McNabb et al., 1998). This may be due to the persistence of tannin-protein complexes in the intestine, or to the formation of new tannin complexes. Also, intestinal absorption may experience modifications by the interaction of tannins with intestinal mucosa (Mitjavila et al., 1977).

Aguerre et al. (2016) reported tannins supplementation in progressively increased dosage decreased apparent digestibility of nutrients. Overall digestibility values for both treatments were low compared to other published literature using a variable amount of expeller soybean meal (Olmos Colmenero and Broderick, 2006c) and tannins extracts (Aguerre et al., 2016). Frutos et al. (2004) observed that when adding soybean meal in a range from 10–250 g/kg of quebracho tannins, there was a decreased of *in vitro* intestinal digestibility of protein. However, our results showed cows fed tannins digested 8.24 %

more CP than CON cows. Since DMI and other parameters associated with the rumen such as pH and NH_3-N were not affected by dietary treatments, further research is needed to determine if this increased in digestibility could be associated with the inclusion of tannins extracts or black pepper in the diet.

In agreement with our results, several studies have shown that fiber degradation in the rumen can be drastically reduced in animals that consume tannin-rich feeds (McSweeney et al., 2001; Hervás et al., 2003).

Lactation Performance

Crude protein concentrations in the CON diet were slightly greater than does recommend for lactating dairy cows (NRC, 2001). Olmos Colmenero and Broderick (2006c) reported that increasing dietary CP content from 16.6 % to 17.6 % did not affect milk production and decrease N efficiency while increasing urinary N excretion. Olmos Colmenero and Broderick (2006b) fed diets ranging from 13.5 % to 19.4 % CP and observed no effect on milk yield and milk protein yield when feeding diets with more than 16.5 % CP, which was associated with a linear increase in urinary N excretion and consequently a sharp decline in N efficiency. Other studies also reported that an increase in dietary CP did not have a significant effect on milk production (Leonardi et al., 2003; Olmos Colmenero and Broderick, 2006a). In contrast, other studies reported that milk yield of early lactation cows was increased by 1.4 and 2.1 kg/d when dietary CP was increased from 16.7 to 18.3 (Dinn et al., 1998) and from 17.0 to 19.0% of DM (Grummer et al., 1996), respectively.

Although no differences in DMI were observed in the current study, others have observed that tannins may decrease voluntary feed intake (Barry and Duncan, 1984; Barry and Manley, 1984). Also, Hervas et al. (2003) fed tannins intraruminal at different dosages and reported no effects on DMI. Also, Salunkhe et al. (1990) observed that since tannins are strongly astringent, they may depress feed intake as well as decrease animal productivity.

Lower milk fat and protein yields, as well as ECM in cows, fed the TRT diet could be primarily associated with the overall decrease milk yield in this group and to a lesser extent on the rumen fermentation patterns, where TRT cows had lower propionate proportion (Table 7). Since propionate is the main substrate for gluconeogenesis in dairy cows, the latter effect agrees with a lower blood glucose concentration in TRT cows than CON (Table 6).

Increased milk production has been observed when small ruminants were supplemented with condensed tannins in a grazing system (Wang et al., 1996a). Such effect was not observed in the current study, which could be associated with physiological differences between dairy cows and ewes, feeding systems (grazing vs. confinement), and level of tannins supplementation. Decreased feed conversion efficiency (FCE) in TRT cows was expected due to lower milk yield than in CON cows. The FCE is not a constant across herds or within a herd throughout the year because the dairy cow has the ability, through the neuroendocrine system, to partition nutrients to meet nutritional demands in order to maintain normal body functions (Shirley, 2006).

Plasma metabolites

The greater glucose concentrations in CON cows could be partially explained by a greater proportion of propionate VFA in the rumen, a precursor for glucose synthesis as an energy supply in ruminants (Church, 1988). Cows fed the TRT diet had numerically lower PUN concentrations (Table 6). Some studies have reported that tannins may increase efficiency in nitrogen recycling (Powell et al., 2011b) to the rumen resulting in lower rumen NH_3-N concentration and consequently with lower PUN. Larger amounts of N are recycled because tannins stimulate increased saliva production (Frutos et al., 2004). Lower PUN means lower potential waste as urinary N excretion. However, in the current study, this effect could also be associated with the greater CP % (18.1 % vs. 16.8%) in the CON diet.

The significance of greater BHB in TRT cows remains to be elucidated. Since this ketone body is a product from the partial oxidation of NEFA, it is a reliable indicator of an energy imbalance (Contreras et al., 1996). However, in the current study NEFA concentration was not affected by dietary treatments, hence, the difference in BHB cannot be attributed to NEFA or an increase in fatty acid oxidation in the liver. Also, BHB may result from the metabolization of butyrate VFA in the rumen wall, but neither butyrate concentration or proportion in the rumen was affected by the diet. Although BHB was increased in TRT cows, both groups had a BHB concentration under the pathological threshold of 10mg/dL.

Albumin is commonly associated with liver function and inflammation, primarily during stress periods in dairy cows (Bertoni et al., 2008). Since albumin is one of the major proteins synthesized in the liver, therefore, is quite puzzling to observe a lower albumin

concentration in CON cows fed a diet with 18.1 % CP. Further research on phytochemicals such as tannins or piperine (i.e., active compound in black pepper) fed to dairy cows could help understand if in fact, they can improve liver function during stress periods such as the transition period. Regardless of the effects observed in albumin, both groups had albumin concentrations associated with a normal liver function (Bertoni et al., 2008).

The imbalance between production (i.e., oxidants) and neutralization (i.e., antioxidants) of reactive oxygen species (ROS) results in the occurrence of oxidative stress. Elevated concentrations of ROS are harmful to cell structures and cause lipid and protein peroxidation and the products resulting from this reaction may accumulate being the reason of aging and also some diseases (Giergiel and Kankofer, 2015). In the case of tannins, they have been directly associated with antioxidant properties by binding free radicals with their aromatic rings or hydroxyl groups and forming resonance-stabilized phenoxyl radicals (Rice-Evans et al., 1996). Indirectly, tannins have been associated with selectively induce antioxidant enzyme gene expression, likely through activation of nuclear factor E2-related factor 2 (*NRF2*). The *NRF2*, in turn, is a master gene regulator that has been observed to upregulate the expression of key genes encoding for antioxidant enzymes such as SOD and glutathione peroxidase (GSH-Px), when feeding tannins to rodents (Yeh and Yen, 2006). Similar results have been observed in transition dairy cows fed chestnut tannins with a resulting increase in SOD, GSH-Px, and overall antioxidant capacity (Liu et al., 2013). Our results agree with those observed by Liu et al. (2013), with a trend ($P = 0.06$) for greater SOD concentration in TRT cows (Table 6). These results further confirm the antioxidant effect of tannins in dairy cow rations.

Rumen Fermentation

Both treatment groups had a mean pH in the rumen within the normal physiological range of 6.1- 6.8 (Van Soest, 1994). Studies, where tannins have been fed to dairy cows, have reported an inconsistent increased (Ben Salem et al., 2000) or decreased (Bhatta et al., 2007) ruminal pH. Also, Yildiz et al. (2005) suggested tannins may not produce an effect on ruminal pH, as it occurred in our study.

Several studies have shown that feeding specific tannins from quebracho (Frutos et al., 2004; Getachew et al., 2008) and chestnut (Sliwinski et al., 2004) reduced NH_3-N concentration in ruminal fluid as a result of a lower ruminal protein degradation likely due to the formation of tannin-protein complexes. Aguerre et al. (2016) reported that tannin supplementation did not influence ruminal pH but was effective in decreasing ruminal NH_3-N concentration. Also, the results in Aguerre et al. (2016) study showed that total VFA concentration, molar proportions of acetate, propionate, butyrate, and A:P were not affected by the tannins, but the proportions of isobutyrate and isovalerate were greater in cows that did not consume tannins. Beauchemin et al. (2007) observed a trend for a decrease in total VFA concentration and the A:P ratio when supplementing quebracho tannins at 2% of DMI, while Dschaak et al. (2011) observed a decrease in total VFA concentration only when supplementing quebracho tannins at 3% of DMI. However, Benchaar et al. (2008) reported that total concentrations of VFA and individual molar proportions of VFA were not affected by feeding 0.64% of diet DM of quebracho tannin extracts. Total VFA concentration and patterns results may be associated with tannin supplementation level, tannin sources, and rumen microbes adaptation time to tannins (Makkar, 2003).

The contrasting effect of greater acetate and lower propionate proportions in TRT cows suggests that rumen fermentation dynamics were affected by this diet including tannins and piperine (from black pepper). However, these results to some extent are confounded with the lower CP % in the TRT diet, but the unchanged NH_3-N do not allow us to confirm this. Certainly, any biological significance on the lower propionate proportion that could lead to lower glucose in TRT cows, in turn, these effects could partially explain a lower substrate availability for lactose in the mammary, and consequently the lower milk yield in TRT cows.

CONCLUSION

Our research findings described the effects of supplementation of phytochemicals (quebracho bark, chestnut leaves, and black pepper) into the lactating dairy cows ration. Our results describe a potential adverse effect of feeding these phytochemicals at 40 g/d, primarily based on the lower milk yield, but this effect is confounded with the lower CP observed in the TRT diet. But, the lack of effect on parameters related to N efficiency such as NH_3-N, PUN, MUN, and milk protein % do not allow us to conclude that the lower CP in TRT diet causes a significant effect. The unchanged DMI between treatments suggests that these phytochemicals at this inclusion rate in the diet did not affect palatability. And, in fact, the greater albumin and SOD in TRT cows are indicative of potential beneficial effects of these phytochemicals on liver function and oxidative stress, that should be further evaluated during stress period such as the transition period of dairy cows. The concomitant decrease in propionate proportion and blood glucose could partially explain the decrease in milk yield in TRT cows. Although the effects observed in VFA proportions and apparent

total-tract digestibility of nutrients indicates that these phytochemicals act as rumen modifiers, further research is needed to optimize their dosage and predictable effect in the rumen.

Table 1. Ingredient composition for the CON and TRT treatment diets fed to lactating dairy cows and analyzed nutrient composition of the total mixed rations (CON and TRT) fed.

	Treatment[1]			
Components	CON	SE[6]	TRT	SE
Ingredient, % DM[2]				
Corn silage	29.97		29.97	
Alfalfa haylage	10.55		10.55	
Alfalfa hay	7.94		7.94	
Whole cottonseed	6.32		6.32	
Straw	3.22		3.37	
Soybean meal (47%) pellets	3.37		-	
Tannins extracts pellets	-		3.54	
QLF Dairy Sugar 38	2.89		2.89	
Calcium phosphate	0.10		0.10	
Magnesium Oxide	0.17		0.17	
Sodium Bicarbonate	0.85		0.85	
Limestone Ca	0.85		0.85	
Salt	0.36		0.36	
Corn Fine	25.06		25.06	
Distillers grains dry	2.28		2.28	
Soybean meal	4.80		4.80	
Rumen-inert fat[3]	0.68		0.68	
Binder	0.03		0.03	
Urea 281% CP	0.27		0.27	
Vitamin E	0.03		0.03	
JPW Dairy Vitamin Premix[4]	0.09		0.09	
JPW Dairy TM Premix[5]	0.09		0.09	
Yeast	0.07		0.07	
Biotin 1%	0.01		0.01	
Chemical analysis				
DM, %	55.77	1.15	56.53	1.01
Ash, %	8.31	0.21	7.54	0.12
OM, %	91.69	0.21	92.46	0.12
CP, %	18.10	0.84	17.4	0.55
NDF, %	29.50	0.30	28.50	1.10
ADF, %	21.83	0.48	19.40	1.10
NE_L, Mcal/ kg DM	1.70	<0.01	1.71	0.01

[1]Control diet no phytochemicals (**CON**); CON diet plus phytochemicals (**TRT**).
[2]Ingredients included in the ration formulated by using Spartan Dairy Ration Evaluator 3.0
[3]Energy Booster 100 (MSC, Carpentersville, IL)
[4]Contained: 25.8 % Ca (DM basis) 1,545 IU/kg Vitamin A, 387 IU/kg Vitamin D, and 4,826 IU/kg Vitamin E (JPW Nutrition, Sioux Falls, SD).
[5]Contained: 11.7 % Ca (DM basis), 1.96 % S, 10,527 mg/kg Fe, 63,158 mg/kg Zn, 12,632 mg/kg Cu, 63,158 mg/kg Mn, 325 mg/kg Se, 632 mg/kg Co, and 1,053 mg/kg I (JPW Nutrition, Sioux Falls, SD).
[6]Standard Error.

Table 2. Analyzed nutrient composition of major ingredients used in the CON and TRT diets.

Item, % DM[1]	DM, %	CP	NDF[2]	ADF	NFC	TDN
Corn silage	40.4	9.2	36.6	22.2	45.7	71
Alfalfa haylage	34.6	28.3	34.6	26.7	24.0	62
Alfalfa hay	84.6	23.1	43.7	36.2	21.2	59
Whole cottonseed	90.9	26.9	44.7	30.7	2.5	78
Straw	85.3	5.0	80.2	54.9	5.9	49
Soybean meal (47%) pellets	87.6	51.2	11.4	7.2	29.2	79
Tannins extracts pellets	88.1	49.1	11.8	6.3	-	84
Grain mix	86.4	15.0	13.4	6.5	-	83

[1]Nutrition composition expressed as % DM, unless otherwise indicated.
[2]NFC = 100 - (% NDF + % CP + % EE + % Ash) (NRC, 2001).

Table 3. Particle distribution and physically effective fiber using the Penn State Particle Separator of the basal total mixed ration.

Sieve[1]	% retained on each sieve[2]	SE[3]
Upper (19 mm)	14.2	1.0
Middle (8 mm)	32.9	1.27
Lower (1.18 mm)	15.7	0.8
Bottom Pan	37.1	1.8

[1]Particle size distribution of the TMR was measured using the Penn State Particle Separator (Kononoff and Heinrichs, 2003).
[2]TMR = total mixed ration of the CON diet; 55.8% DM, 8.6% Ash, 18.1% CP, 29.5 % NDF and 21.8 % ADF.
[3]Standard error.

Table 4. Apparent total-tract digestibility of nutrients in cows fed a CON or TRT diet.

	Treatment[1]			*P*-value[2]		
Item, % digested	CON	TRT	SEM[3]	TRT	Period	Seq
DM	57.75	66.06	2.69	0.03	0.14	0.04
OM	58.95	67.27	2.63	0.03	0.11	0.03
CP	55.32	63.56	2.31	0.01	0.60	0.02
NDF	51.04	39.26	6.19	0.13	0.10	0.39
ADF	54.65	32.99	5.60	0.01	0.13	0.20

[1]Control diet no phytochemicals (**CON**); CON diet plus phytochemicals (**TRT**).
[2]TRT = Treatment and Seq=Sequence.
[3]Largest standard error of the mean is shown

Table 5. Cows performance based on the different treatment diets. Dry matter intake, milk yield and composition, efficiency calculations, and body characteristics for cows fed a CON or TRT diet.

Item	Treatment[1]			*P*-value[2]		
	CON	TRT	SEM[3]	TRT	P	Seq
DMI, kg/d	23.99	23.29	1.25	0.40	0.95	0.93
Milk, kg/d	32.06	28.75	2.31	<0.01	0.01	0.82
Fat, %	3.72	3.83	0.16	0.48	0.83	0.77
Fat, kg/d	1.17	1.09	0.05	0.10	0.08	0.61
Protein, %	3.18	3.15	0.04	0.23	0.90	0.73
Protein, kg/d	1.02	0.89	0.06	<0.01	<0.01	0.99
Lactose, %	4.76	4.73	0.06	0.53	0.39	0.86
Lactose, kg/d	1.53	1.37	0.12	<0.01	0.01	0.79
SNF, %	8.77	8.73	0.05	0.42	0.67	0.84
MUN, mg/dL	12.32	12.52	0.25	0.33	<0.01	0.03
SCC,[4] (1000/mL)	1.73	1.83	0.08	0.30	0.26	0.15
ECM,[5] kg/d	32.94	29.99	2.24	<0.01	0.01	0.80
Feed conversion efficiency[6]	1.38	1.27	0.07	0.03	0.11	0.67
Body weight, kg	719.6	711.0	11.14	0.03	0.02	0.47
BCS[7]	2.55	2.62	0.05	0.25	0.15	0.26

[1] Control diet no phytochemicals (**CON**); CON diet plus phytochemicals (**TRT**).
[2]Trt = Treatment; P = Period, Seq=Sequence.
[3] Largest standard error of the mean is shown.
[4]Data were log-transformed data before statistics.
[5]ECM = [(0.327 x kg milk) + (12.95 x kg fat) + (7.2 x kg protein)] (Orth, 1992).
[6]Feed conversion efficiency = ECM/DMI.
[7]Body condition score with 1 = emaciated and 5 = obese (Wildman et al., 1982).

Table 6. Plasma metabolite concentrations of cows fed CON and TRT diets.

	Treatment[1]			*P*-value[2]		
Plasma metabolite	CON	TRT	SEM[3]	TRT	P	Seq
Glucose, mg/dL	73.40	69.50	1.36	0.02	0.02	0.11
PUN[4], mg/dL	16.34	14.41	0.89	0.13	0.00	0.66
NEFA[5], mmol/L	0.09	0.08	0.06	0.57	0.02	0.49
BHB, mg/dL	4.49	5.36	0.30	0.05	0.18	0.21
Albumin, g/dL	3.33	3.49	0.06	0.05	0.05	0.44
SOD, U/ml	0.71	0.95	0.09	0.06	0.95	0.11

[1] Control diet no phytochemicals (**CON**); CON diet plus phytochemicals (**TRT**).
[2]Trt = Treatment; P = Period; Seq=Sequence.
[3]Largest standard error of the mean is shown
[4]Plasma urea nitrogen.
[5]Data were log-transformed data before statistics.

Table 7. Ruminal pH, NH_3-N, and VFA concentrations of cows fed CON and TRT diets.

	Treatment[1]			*P*-value[2]		
Rumen measure	CON	TRT	SEM	TRT	P	Seq
pH	6.75	6.70	0.05	0.43	0.29	0.75
NH_3-N, mg/dL	4.63	4.83	0.51	0.77	0.10	0.54
Acetate, m*M*	57.23	60.46	3.80	0.55	0.01	0.80
Propionate, m*M*	33.58	31.33	1.96	0.42	0.03	0.70
Butyrate, m*M*	9.74	9.76	0.50	0.98	<0.01	0.80
Isovalerate, m*M*	2.23	2.24	0.06	0.92	<0.01	0.10
Valerate, m*M*	2.05	2.02	0.12	0.87	<0.01	0.33
Total VFA, m*M*	104.94	105.76	5.68	0.92	<0.01	0.78
Acetate[3]	54.44	56.60	1.13	0.03	0.27	0.95
Propionate[3]	32.03	29.92	1.09	0.05	0.18	0.79
Butyrate[3]	9.31	9.33	0.29	0.99	0.47	0.53
Isovalerate[3]	2.10	2.10	0.10	0.97	0.44	0.49
Valerate[3]	2.06	1.85	0.10	0.09	0.05	0.45
Acetate:Propionate	1.75	1.94	0.10	0.04	0.34	0.70

[1]Control diet no phytochemicals (**CON**); CON diet plus phytochemicals (**TRT**).
[2]Trt = Treatment; P = Period; Seq=Sequence.
[3]m*M*/100 m*M*.

REFERENCES

Abdelgadir, I. E. O., J. L. Morril, and J. J. Higgins. 1996. Ruminal availabilities ofprotein and starch: Effects on growth and ruminal and plasma metabolites of dairy calves. J. Dairy Sci. 79:283–290.

Aguerre, M. J., M. C. Capozzolo, P. Lencioni, C. Cabral, and M. A. Wattiaux. 2016. Effect of quebracho-chestnut tannin extracts at 2 dietary crude protein levels on performance, rumen fermentation, and nitrogen partitioning in dairy cows. J Dairy Sci. 99:4476-4486.

Aguerre, M. J., M. A. Wattiaux, M. C. Capozzolo, P. Lencioni, and C. Cabral. 2010a. Effect of quebracho-chestnut tannin extracts at two dietary crude protein levels on nitrogen partitioning in lactating dairy cows. J. Dairy Sci. 93 (E-Suppl. 1):446 (Abstr.).

Aguerre, M. J., M. A. Wattiaux, T. Hunt, and B. R. Larget. 2010b. Effect of dietary crude protein on ammonia-N emission measured by herd nitrogen mass balance in a freestall dairy barn managed under farm-like conditions. Animal. 4:1390-1400.

Aldrich, J. M., L. D. Muller, G. A. Varga, and L. C. J. Griel. 1993. Non structural carbohydrate and protein effects on rumen fermentation, nutrient flow, and performance of dairy cows. J. Dairy Sci. 76:1091– 1105.

Annison, E. F. 1956. Nitrogen metabolism in the sheep. Protein digestion in the rumen. Biochem. J. 64:705–714.

AOAC 1977. Method 973.18. Fiber (acid detergent) and lignin (H2SO4) in animal feed.

AOAC, I. 2002. Official Methods of Analysis. 17th ed. Association of Official Analytical Chemists. Gaithersburg, MD.

Arndt, C., J. M. Powell, M. J. Aguerre, P. M. Crump, and M. A. Wattiaux. 2015. Feed conversion efficiency in dairy cows: Repeatability, variation in digestion and metabolism of energy and nitrogen, and ruminal methanogens. J Dairy Sci. 98:3938-3950.

Asner G. P., A. S. R. 2010. Livestock and the global carbon cycle. Pages 69–82 in Livestock in a Changing Landscape: Drivers, Consequences, and Responses. Vol. 1. H. Steinfeld, H. A. Mooney, F. Schneider, and L. E. Neville, ed. Island Press, Washington, DC.

Asquith, T. N. and L. G. Butler. 1986. Interaction of condensed tannins with selected proteins. Phytochemistry, 25 1591.

Assoumani, M. B., V. F., J. L., and S. C. J. 1992. Refinement ofan enzymatic method for estimating the theoretical degradability of proteins in feedstuffs for ruminants. Anim. Feed Sci. Technol. 39:357–368.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. J Dairy Sci. 88 Suppl 1:E9-21.

Baert, N., F. W. Pellikaan, M. Karonen, and J.-P. Salminen. 2016. A study of the structure-activity relationship of oligomeric ellagitannins on ruminal fermentation in vitro. J. Dairy Sci. . 99:8041–8052.

Barry, T. N. and S. J. Duncan. 1984. The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep. 1. Voluntary intake. Br J Nutr. 51:485-491.

Barry, T. N. and T. R. Manley. 1984. The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep. 2. Quantitative digestion of carbohydrates and proteins. Br J Nutr. 51:493-504.

Bauman, D. E., S. N. McCutcheon, W. D. Steinhour, P. J. Eppard, and S. J. Sechen. 1985. Sources of variation and prospects for improvement of productive efficiency in the dairy cow: a review. J Anim Sci. 60:583-592.

Beauchemin, K. A., S. M. McGinn, T. F. Martinez, and T. A. McAllister. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85:1990–1996.

Ben Salem, H., A. Nefzaou, L. Ben Salem, and J. L. Tisserand. 2000. Deactivation of condensed tannin in Acacia cyanophylla Lindl. foliage by polyethylene glycolin feed blocks. Effect on feed intake, diet digestibility, nitrogen balance, microbial synthesis and growth by sheep. Livest. Prod. Sci. 64, 51–60.

Benchaar, C., T. A. McAllister, and P. Y. Chouinard. 2008. Digestion, ruminal fermentation, ciliate protozoal populations, and milk production from dairy cows fed cinnamaldehyde, quebracho condensed tannin, or Yucca schidigera saponin extracts. J Dairy Sci. 91:4765-4777.

Berg J.M., T. J. L., Stryer L. 2002. Protein Structure and Function (Chapter 3). Biochemistry. 5th edition. New York: W H Freeman.

Berry, D. P. and J. J. Crowley. 2013. Cell Biology Symposium: genetics of feed efficiency in dairy and beef cattle. J Anim Sci. 91:1594-1613.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci. 91:3300-3310.

Bhatta, R., S. Vaithiyanathan, N. P. Singh, and D. L. Verma. 2007. Effect of feeding complete diets containing graded levels of Prosopis cineraria leaves on feed intake,

nutrient utilization and rumen fermentation in lambs and kids. Small Rumin. Res. 67, 75–83.

Blouin, J.-P. 1997. Effet de la dégradabilité protéique sur les métabolismes splanchnique et protéqiue de la vache laitière. Mémoire de maîtrise. Université Laval. Quebec, Canada.

Bobbink, R. 2010. Impacts of atmospheric nitrogen deposition on semi-natural ecosystems: A synthesis. p. 12. In Proc. of the 5th International Nitrogen Conference. 3–7 Dec. 2010. New Delhi, India.

Bodway, A. A. B., A. E. White, and G. H. Lathe. 1969. The effect of tannic acid on the synthesis of protein and nucleic acid by rat liver. Biochem. J. 113,307.

Boyd, E. M., K. Bereczky, and I. Godi. 1965,. The acute toxicity of tannic acid administered intragastrically. Canadian Medical Association journal. Vol.92, pp.1292-7.

Bremmer, D. R., T. R. Overton, and J. H. Clark. 1997. Production and composition of milk from Jersey cows administered bovine somatotropin and fed ruminally protected amino acids. J. Dairy Sci. 80:1374–1380.

Broderick, G. A. 1995. Desirable characteristics of forage legumes for improving protein utilization in ruminants. J Anim Sci. 73:2760-2773.

Broderick, G. A. 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. J. Dairy Sci. 86:1370–1381.

Broderick, G. A. and S. M. Reynal. 2009. Effect of source of rumen-degraded protein on production and ruminal metabolism in lactating dairy cows. J Dairy Sci. 92:2822-2834.

Broderick, G. A., R. J. Wallace, and E. R. Ørskov. 1991. Control of rate and extent of protein degradation. . Pages 541– 592 in Physiological Aspects of Digestion and Metabolism in Ruminants. T.Tsuda,Y.Sasaki, and R. Kawashima (eds.) Academic Press, Orlando, FL.

Bruinsma, J. 2003. World agriculture: towards 2015/2030. An FAO perspective. Rome, Food and Agriculture Organization of the United Nations/London, Earthscan.

Calsamiglia, S., A. Ferret, C. K. Reynolds, N. B. Kristensen, and A. M. van Vuuren. 2010. Strategies for optimizing nitrogen use by ruminants. Animal. 4:1184-1196.

Campbell, T. C. 2013. Animal vs. Plant Protein. T. Colin Campbell Center for Nutrition Studies. http://nutritionstudies.org/animal-vs-plant-protein/

Cardozo, P., S. Calsamiglia, and F. A. 2002. Effects of pH on nutrient digestion and microbial fermentation in a dual flow continuous culture system fed a high concentrate diet. J. Dairy Sci. 85(Suppl. 1):182.

Cardozo, P., S. Calsamiglia, and A. Ferret. 2000. Effect of pH on microbial fermentation and nutrient flow in a dual flow continuous culture system. J. Dairy Sci. 83(Suppl. 1):265.

Cardozo, P. W., S. Calsamiglia, A. Ferret, and C. Kamel. 2006. Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. Journal of Animal Science, 84: 2801–2808

Chandler, P. T. 1991. Quantitative and qualitative characteristics of protein sources and interrelationships with energy. Virginia Dairyman 12:10, 12.

Chaney, A. L. and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin Chem. 8:130-132.

Chen, K. H., J. T. Huber, C. B. Theurer, D. V. Armstrong, R. C. Wanderley, J. M. Simas, S. C. Chan, and J. L. Sullivan. 1993. Effect of protein quality and evaporative cooling on lactational performance of Holstein cows in hot weather. J. Dairy Sci. 76: 819–825.

Church, D. C. 1988. The Ruminant Animal, Digestive Physiology and Nutrition. Ed.Prentice. Alan, Englewood Cliffs, N.J

Clark, J. H., T. H. Klusmeyer, and M. A. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75:2304–2323.

Contreras, P. A., L. Valenzuela, F. Wittwer, and H. Böhmwald. 1996. Metabolic nutritional imbalances in smallholders dairy herds in Valdivia, X Region, Chile. Vol. 28.

Díaz, C. J. M. C., C.; Martín, R.L.; Pin, V.N.D.; Colombatto, D.; Farber, M.D.; Fernández, M.M.E.;. 2017. Impact of Chestnut and Quebracho Tannins on Rumen Microbiota of Bovines. BioMed Research International. 2017:11.

Dinn, N. E., J. A. Shelford, and L. J. Fisher. 1998. Use of the Cornell Net Carbohydrate and Protein System and rumen-protected lysine and methionine to reduce nitrogen excretion from lactating dairy cows. J. Dairy Sci. 81:229–237.

Dise, N. B., M. Ashmore, S. Belyazid, A. Bleeker, R. Bobbink, W. deVries, J. W. Erisman, T. Spranger, C. Stevens, and L. van den Berg. 2010. Nitrogen deposition as a threat to European terrestrial biodiversity. p. 19. In Proc. of the 5th Int. Nitrogen Conf.. 3–7 Dec. 2010. New Delhi, India.

Doepel, L., D. Pacheco, J. J. Kennelly, M. D. Hanigan, I. F. Lopez, and H. Lapierre. 2004. Milk protein synthesis as a function of amino acid supply. J. Dairy Sci. 87:1279–1297.

Donnelly, E. D., andAnthony, W.B. . 1969. Relationship of tannin, dry matter digestibility and crude protein in sericea lespedeza. Crop Sci. . 9:361–362.

Driedger, A. and E. E. Hatfield. 1972. Influence of tannins on the nutritive value of soybean meal for ruminants. Journal of animal science. 34:465.

Dschaak, C. M., C. M. Williams, M. S. Holt, J.-S. Eun, A. J. Young, and B. R. Min. 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. J. Dairy Sci. 94:2508–2519.

Duangjai, A., K. Ingkaninan, S. Praputbut, and N. Limpeanchob. 2013. Black pepper and piperine reduce cholesterol uptake and enhance translocation of cholesterol transporter proteins. J Nat Med. 67:303-310.

Endres, M. I. and M. D. Stern. 1993. Effects of pH and diets containing various levels of lignosulfonate-treated soybean meal on microbial fermentation in continuous culture. J. Dairy Sci. 76 (Suppl. 1) :177.

Erdman, R. A., G. H. Proctor, and J. H. Vandersall. 1986. Effect of rumen ammonia concentration on in situ rate and extent of digestion of feedstuffs. J. Dairy Sci. 69:2312–2320.

FAO. 2009. How to feed the world 2050; High-level expert forum. Accessed April 24, 2014. http://www.fao.org/wsfs/forum2050/wsfsbackground-documents/hlef-issues-briefs/en/.

Frankič, T., M. Voljč, J. Salobir, and V. Rezar. 2009. Use of herbs and spices and their extracts in animal nutrition. Acta Agriculturae Slovenica. 94:95-102.

Freudenberg, K. 1960. [Catechins and hydroxyflavandiols as components of tannins]. Experientia. 16:101-105.

Frutos, P., G. Hervás, F. J. Giráldez, and A. R. Mantecón. 2004. Review.Tannins and ruminant nutrition. Spanish Journal of Agricultural Research 2 (2), 191-202.

Galloway, J., F. Dentener, M. Burke, A. F. Bouwman, R. A. Kohn, H. A. Mooney, S. Seitzinger, and C. Kroeze. 2010. The impact of animal production systems on the nitrogen cycle. Pages 83–95. Vol 1. in Livestock in a Changing Landscape: Drivers, Consequences, and Responses. H. Steinfeld, H. A. Mooney, F. Schneider, and L. E. Neville, ed. Island Press, Washington, DC: .

Galloway, J. N., J. D. Aber, J. W. Erisman, S. P. Seitzinger, R. W. Howarth, E. B. Cowling, and B. J. Cosby. 2003. The nitrogen cascade. BioScience 53:341–356. doi:10.1641/0006-3568(2003)053[0341:TNC]2.0.CO;2.

Galloway, J. N., A. R. Townsend, J. W. Erisman, M. Bekunda, Z. Cai, J. F. Freney, L. A. Martinelli, S. P. Seitzinger, and M. A. Sutton. 2008. Transformations of the nitrogen cycle: Recent trends, questions, and potential solutions. Science 320:889–892. doi:10.1126/science.1136674.

Getachew, G., W. Pittroff, D. H. Putnam, A. Dandekar, S. Goyal, and E. J. DePeters. 2008. The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on in vitro rumen fermentation and microbial protein synthesis. Anim. Feed Sci. Technol. 140:444–461.

Giergiel, M. and M. Kankofer. 2015. Age and sex-related changes in superoxide dismutase activity in bovine tissues. Czech J. Anim. Sci. 60 (8): 367–374

Goelema, J. O., A. Smits, L. M. Vaessen, and A. Wemmers. 1999. Effects of pressure toasting, expander treatment and pelleting on in vitro and in situ parameters of protein and starch in a mixture of broken peas, lupins and faba beans. Anim. Feed Sci. Technol. 78:109– 126.

Greathead, H. 2003. Plants and plants extracts for improving animal productivity. Proceedings of the nutrition Society, 62: 279–290.

Grummer, R. R., K. Slark, B. S. J., M. L. Luck, and J. A. Barmore. 1996. Soybeans versus animal sources of rumen-undegradable protein and fat for early lactation dairy cows. J. Dairy Sci. 79:1809–1816.

Guesnel, V. C. 1968. Fractionation and properties of the polymeric leucocyanidin of the seeds of Theobroma cacao. pHYTOCHEMIstry, 7, 1583.

Hagerman, A. E., C. T. Robbins, Y. Weerasuriya, T. C. Wilson, and C. McArthur. 1992. Tannin chemistry in relation to digestion. J. Range Manage. 45: 57-62.

Harmeyer, J. and H. Martens. 1980. Aspects of urea metabolism in ruminants with reference to the goat. J. Dairy Sci. 63:1707-1728.

Haslam, E. 1966. Chemistry of vegetable tannins. Academic Press, London, New York,.

Hatfield, E. E. 1970. Achieving the potential of high quality dietary proteins of ruminants. Feedstuffs, 42, 23.

Herrera-Saldana, R., R. Gomez-Alarcaon, M. Tobrabi, and T. Huber. 1990. Influence ofsynchronizing protein and starchdegradation in the rumen on nutrient utilization and microbial protein synthesis. J. Dairy Sci. 73:142– 148.

Hervás, G., P. Frutos, F. J. Giráldez, A. R. Mantecón, and Á. d. P. M.C. 2003. Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Anim Feed Sci Tech 109, 65-78.

Hervas, G., V. Perez, F. J. Giraldez, A. R. Mantecon, M. M. Almar, and P. Frutos. 2003. Intoxication of sheep with quebracho tannin extract. J Comp Pathol. 129:44-54.

Hoffman, J. R. F. M. J. 2004. Protein – Which is Best? J Sports Sci Med.

Hoover, W. H. and S. R. Stokes. 1991. Balancing carbohydrates and protein for optimum rumen microbial yield. J.DairySci.74:3630– 3644.

Hume D., M. R. J., Somers M. 1970. Synthesis of microbial protein in the rumen. I. Influence of the level of nitrogen intake. Australian Journal of Agricultural Research. 21, 283-304.

Ipharraguerre, I. R., J. H. Clark, and D. E. Freeman. 2005. Rumen fermentation and intestinal supply of nutrients in dairy cows fed rumen-protected soy products. J Dairy Sci. 88:2879-2892.

Jones, W. T., L. B. Anderson, and M. D. Ross. 1973. Bloat in cattle, detection of protein precipitants (flavolans) in legumes. N.Z.J.Agric.Res., 16, 441.

Jones, W. T. and J. L. Magan. 1977. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia scop.*) with fraction I leaf protein and with submaxillary mucoprotein and their reversal by polythylene glycol and pH. J. Sci. Food Agric., 28, 126.

Kahn, C. M. and S. Line. 2010. The Merck veterinary manual. 10th Ed. Merck & Co.Whitehouse Station, N.J.

Kirubakaran, A., M. Moorthy, R. Chitra, and G. Prabakar. 2016. Influence of combinations of fenugreek, garlic, and black pepper powder on production traits of the broilers. Veterinary World. 9:470-474.

Kohn, R. A. and M. S. Allen. 1995. In vitro protein degradation of feeds using concentrated enzymes extracted from rumen contents. Anim. Feed Sci. Technol. 52:15–28.

Kononoff, P. J. and A. J. Heinrichs. 2003. The effect of corn silage particle size and cottonseed hulls on cows in early lactation. J Dairy Sci. 86:2438-2451.

Kononoff, P. J., A. J. Heinrichs, and D. R. Buckmaster. 2003. Modification of the Penn State forage and total mixed ration particle separator and the effects of moisture content on its measurements. J Dairy Sci. 86:1858-1863.

Kopecny, J. and R. J. Wallace. 1982. Cellular location and some properties of proteolytic enzymes of rumen bacteria. Appl. Environ. Microbiol. 43:1026–1033.

Korošec, T., M. Voljč, J. Salobir, and V. Rezar. 2009. Use of herbs and spices and their extracts in animal nutrition. Vol. 94.

Kronberg, S. L., E. J. Scholljegerdes, G. Barcelo-Coblijn, and E. J. Murphy. 2007. Flaxseed treatments to reduce biohydrogenation of alpha-linolenic acid by rumen microbes in cattle. Lipids. 42:1105-1111.

Lapierre, H. and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84(E. Suppl.):E223–E236.

Lean, I. J., M. B. de Ondarza, C. J. Sniffen, J. E. P. Santos, and K. E. Griswold. 2018. Meta-analysis to predict the effects of metabolizable amino acids on dairy cattle performance. Journal of Dairy Science. 101:340-364.

Lee, C., A. N. Hristov, T. W. Cassidy, K. S. Heyler, G. A. H. Lapierre, M. J. d. V. Varga, and P. R. A. 2012. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 95:6042–6056.

Leonardi, C., M. Stevenson, and L. E. Armentano. 2003. Effect of two levels of crude protein and methionine supplementation on performance of dairy cows. J. Dairy Sci. 86:4033–4042.

Licitra G., Hernandez T.M., and Van Soest P.J. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Animal Feed Science and Technology. 57, 347–358.

Liu, H. W., D. W. Zhou, and K. Li. 2013. Effects of chestnut tannins on performance and antioxidative status of transition dairy cows. J Dairy Sci. 96:5901-5907.

Lyman, R. O. and M. Longnecker. 2001. Introduction to statistical methods and data analysis. Boston, MA : Cengage Learning, [2016] ©2016.

Maeng, W. J., H. Park, and H. J. Kim. 1997. The role of carbohydrate supplementation in microbial protein synthesis in the rumen. In Rumen Microbes and Digestive Physiology in Ruminants (Onodera et al, eds.), pp. 107– 119. Japan Sci. Soc. Press, Tokyo/S. Karger, Basel.

Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin. Res. 49:241–256.

Mangan, J. L. 1988. Nutritional effects of tannins in animal feeds. Nutr Res Rev. 1:209-231.

McDonald, C. L., S. P. Gittins, and J. B. Rowe. 1988. Effect of time of year and prior feeding experience on feeding behavior of sheep as if for live export. Proc. Aust. Soc. Anim. Prod., 17, pp. 226-229.

McDonald, I. W. 1952. The role of ammonia in ruminal digestion of protein. Biochem. J. 51:86–90.

McLeod, M. N. 1974. Plant tannins - Their role in forage quality. Nutr Abst Rev 44, 803-812.

McNabb, W. C., J. S. Peters, L. Y. Foo, G. C. Waghorn, and S. J. Jackson. 1998. Effect of condensed tannins prepared from several forages on the in vitro precipitation of ribulose-1,5-bisphospathe carboxilase (rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1). J Sci Food Agric 77, 201-212.

McSweeney, C. S., P. M. Kennedy, and A. John. 1988. Effect of ingestion of hydrolyzable tannins in Terminalia oblongata on digestion in sheep fed Stylosanthes hamata. Aust J Agr Res 39, 235-244.

McSweeney, C. S., B. Palmer, D. M. McNeill, and D. O. Krause. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim Feed Sci Tech 91, 83-93.

Merchen, N. R. 1988. Digestion, Absorption and Excretion in Ruminants. Pages 182-189 in The Ruminant Animal: Digestive Physiology and Nutrition. D. C. Church, ed. Prentice Hall Inc., New Jersey.

Merchen, N. R., J. C. Elizalde, and J. K. Drackley. 1997. Current perspective on assessing site of digestion in ruminants. J Anim Sci. 75:2223-2234.

Mertens, D. R. 1997. Creating a system for meeting the fiber requirements of dairy cattle. J. Dairy Sci. 80:1463–1482.

Mitjavila, S., C. Lacombe, G. Carrera, and R. Derache. 1977. Tannic acid and oxidized tannic acid on the functional state of rat intestinal epithelium. The Journal of nutrition. Vol.107(12), pp.2113-21.

Mole, S. and P. G. Waterman. 1986. Tannic acid and proteolytic enzymes: Enzyme inhibition or substrate deprivation? Phytochemistry. 26:Pages 99-102.

Narrod, C., M. Tiongco, and R. Scott. 2011. Current and predicted trends in the production, consumption and trade of live animals and their products. Rev Sci Tech. 30:31-49.

Neumann, C. G., N. O. Bwibo, S. P. Murphy, M. Sigman, S. Whaley, L. H. Allen, D. Guthrie, R. E. Weiss, and M. W. Demment. 2003. Animal source foods improve dietary quality, micronutrient status, growth and cognitive function in Kenyan school children: background, study design and baseline findings. J Nutr. 133:3941S-3949S.

Noel, R. J. 2000. Official feed terms. Pages 187– 200 In Association of American Feed Control Officials, Official Publication 2000.

Noftsger, S. and N. R. St-Pierre. 2003. Supplementation of methionine and selection of highly digestible rumen undegradable protein to improve nitrogen efficiency for milk production. J. Dairy Sci. 86:958–969.

NRC. 1985. Ruminant nitrogen usage. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

NRC. 1989. Nutrient Requirements of Dairy Cattle. 6th rev. ed. Natl. Acad. Sci., Washington, DC.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

NRC. 1996. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

Oler, A., M. W. Neal, and C. K. Mitchell. 1976. Tannic acid acute hepatotoxicity following administration by feeding tube. Food Cosmet. Toxiciol., 14, 565.

Olmos Colmenero, J. J. and G. A. Broderick. 2006a. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. J Dairy Sci. 89:1704-1712.

Olmos Colmenero, J. J. and G. A. Broderick. 2006b. Effect of dietary crude protein concentration on ruminal nitrogen metabolism in lactating dairy cows. J Dairy Sci. 89:1694-1703.

Olmos Colmenero, J. J. and G. A. Broderick. 2006c. Effect of amount and ruminal degradability of soybean meal protein on performance of lactating dairy cows. J Dairy Sci. 89:1635-1643.

Oltjen, R. R. 1969. Effects of feeding ruminants non-protein nitrogen as the only nitrogen source. J. Anim. Sci. 28:673–682.

Ørskov, E. R. and L. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. 92:499–503.

Orth, R. 1992. Sample day and lactation report, DHIA 200 Fact Sheet A-2, Mid-States, DRPC, Ames, IA.

Patton, R. A. 2010. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: A meta-analysis. J. Dairy Sci. 93:2105–2118.

Pimental, D. 1997. Water resources: agriculture, the environment and society. Bioscience, 47:97-106.

Powell, J. M., M. J. Aguerre, and M. A. Wattiaux. 2011a. Dietary crude protein and tannin impact dairy manure chemistry and ammonia emissions from incubated soils. J Environ Qual. 40:1767-1774.

Powell, J. M., M. J. Aguerre, and M. A. Wattiaux. 2011b. Tannin extracts abate ammonia emissions from simulated dairy barn floors. J Environ Qual. 40:907-914.

Price, M. L. and L. G. Butler. 1980. Tannins and Nutrition, Bull. No.272 Agric. Exp. Stn., Purdue University, West Lafayette, IN, 47907.

Ranathunga, S. D., K. F. Kalscheur, A. R. Hippen, and D. J. Schingoethe. 2010. Replacement of starch from corn with nonforage fiber from distillers grains and soyhulls in diets of lactating dairy cows. J Dairy Sci. 93:1086-1097.

Reynal, S. M. and G. A. Broderick. 2005. Effect of dietary level of rumen degraded protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88:4045-4064.

Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 20:933-956.

Robertson, J. B. and P. J. Van Soest. 1981. The detergent system of analysis and its application to human foods. Pages 123-158 in The Analysis of Dietary Fiber in Food. W.P.T. James and O. Theander, eds. Marcel Dekker Inc., New York, NY.

Romagnolo, D., C. E. Polan, and W. E. Barbeau. 1994. Electrophoretic analysis of ruminal degradability of corn proteins. J Dairy Sci. 77:1093-1099.

Rotz, C. A., L. D. Satter, D. R. Mertens, and R. E. Muck. 1999. Feeding strategy, nitrogen cycling, and profitability of dairy farms. J Dairy Sci. 82:2841-2855.

Rulquin, H., P. M. Pisulewski, R. Verite, and J. Guinard. 1993. Milk production and composition as a function of postruminal lysine and methionine supply: A nutrient-response approach. Livest. Prod. Sci. 37:69–90.

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J Anim Sci. 70:3551-3561.

Salunkhe, D. K., J. K. Chavan, and S. S. Kadam. 1990. Dietary tannins: consequences and remedies. CRC Press, Inc., Boca Raton, Florida. ISBN: 0-8493-6811-1. .

Santos, F. A., J. E. Santos, C. B. Theurer, and J. T. Huber. 1998a. Effects of rumen-undegradable protein on dairy cow performance: a 12-year literature review. J Dairy Sci. 81:3182-3213.

Santos, F. A. P., J. T. Huber, C. B. Theurer, R. S. Swingle, J. M. Simas, K. H. Chen, and P. Yu. 1998b. Milk yield and composition of lactating cows fed steam-flaked sorghum and graded levels of ruminally degradable protein. J. Dairy Sci. 81: 215–220.

Satter, L. D. 1986. Protein supply from undegraded dietary protein. J. Dairy Sci. 69:2734–2749.

Satter, L. D. and L. L. Slyter. 1974. Effect of ammonia concentration of rumen microbial protein production in vitro. Br J Nutr. 32:199-208.

Schingoethe, D. J. 1991. Protein quality and amino acid supplementation in dairy cattle. Pages 101–106. Proc. Southwest Nutr. Manage. Conf., Tempe, AZ. Dep. Anim. Sci., Univ. Arizona, Tucson.

Schwab, C. G. 1994. Optimizing amino acid nutrition for optimum yields of milk and milk protein. Pages 114–129 in Proc. Southwest Nutr. Manage. Conf., Phoenix, AZ. Dep. Anim. Sci., Univ. Arizona, Tucson.

Schwab, C. G. 1995. Protected proteins and amino acids for ruminants. Pages 115–141 in R.J. Wallace and A. Chesson, eds. Biotechnology in Animal Feeds and Animal Feeding. V.C.H.Press,Weinheim,Germany.

Schwab, C. G. and G. A. Broderick. 2017. A 100-Year Review: Protein and amino acid nutrition in dairy cows. J Dairy Sci. 100:10094-10112.

Schwab, C. G., P. Huhtanen, C. Hunt, and T. Hvelplund. 2005. Nitrogen requirements of cattle. Pages 13–70 in Nitrogen and Phosphorus Nutrition of Cattle. E. Pfeffer and A. Hristov, ed. CABI Publishing, Wallingford, UK.

Schwingel, W. R. and D. B. Bates. 1996. Use of sodium dodecyl sulfate polycramide gel electrophoresis to measure degradation of soluble soybean proteins by Prevotella rumininocola GA33 or mixed ruminal microbes in vitro. J. Anim. Sci. 74:475–482.

Shirley, J. E. 2006 Feed Efficiency Is an Important Management Tool for Dairy Producers. High Plains Dairy Conference. Industry presentation.

Singleton, V. L. 1981. Naturally occuring food toxicants: phenolic substances of plant origin common in foods. Adv. Food. Res., 27, 149.

Sliwinski, B. J., M. Kreuzer, F. Sutter, A. Machmuller, and H. R. Weststein. 2004. Performance, body nitrogen conversion and nitrogen emission from manure of

dairy cows fed diets supplemented with different plant extracts. J. Anim. Feed Sci. 13:73–91.

Sniffen, C. J., J. D. O'Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J Anim Sci. 70:3562-3577.

Socha, M. T., D. E. Putnam, B. D. Garthwaite, N. L. Whitehouse, N. A. Kierstead, C. G. Schwab, G. A. Ducharme, and J. C. Robert. 2005. Improving intestinal amino acid supply of pre- and postpartum dairy cows with rumen-protected methionine and lysine. J. Dairy Sci. 88:1113–1126.

Sok, M., D. R. Ouellet, J. L. Firkins, D. Pellerin, and H. Lapierre. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. J. Dairy Sci. 100:5241–5249.

Spier, S. J., B. P. Smith, A. A. Seawright, B. B. Norman, S. R. Ostrowski, and M. N. Oliver. 1987. Oak toxicosis in cattle in northern California: clinical and pathologic findings. J Am Vet Med Assoc 191, 958-964.

Srinivasan, K. 2007. Black pepper and its pungent principle – Piperine: A review of diverse physiological effects. Critical Reviews in Food Science and Nutrition, 47, 735–748.

Srinivasan, K. 2009. Black pepper (Piper nigrum) and its bioactive compound piperine. In B. B. Aggarwal & A. B. Kunnumakkara (Eds.), Molecular targets and therapeutic uses of spices: Modern uses for ancient medicine (pp. 25–64). Ames, USA: World Scientific Publishing Co. Inc.

Stern, M. D., G. A. Varga, J. H. Clark, J. L. Firkins, J. T. Huber, and D. L. Palmquist. 1994. Evaluation of chemical and physical properties of feeds that affect protein metabolism in the rumen. J. Dairy Sci. 77:2762–2786.

Storm, E. and E. R. Orskov. 1983. The nutritive value of rumen micro-organisms in ruminants. 1. Large-scale isolation and chemical composition of rumen micro-organisms. Br J Nutr. 50:463-470.

Tamminga, S. 1996. A review on environmental impacts of nutritional strategies in ruminants. J Anim Sci. 74:3112-3124.

Trinder, P. 1969. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol. 22:158-161.

Tylutki, T. and D. Fox. 2005. The Cornell net carbohydrate and protein system: An evolving model. Journal Of Animal Science, 83, 392.

USDA. 2013. National Agricultural Statistics Service. Vol (Nov. 16). Retrieved from http://www.nass.usda.gov.

USDA. 2016. DRI Nutrient Reports. Vol (July 11). Retrieved from http://www.fnic.nal.usda.gov/dietary-guidance/dietary-reference-intakes/dri-nutrient-reports.

Van Hooijdonk, T. and K. Hettinga. 2015. Dairy in a sustainable diet: a question of balance. Nutr Rev. 73 Suppl 1:48-54.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2nd Edition, Cornell University Press, Ithaca, 476.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science. 74:3583-3597.

Veda, S. and K. Srinivasan. 2009. Influence of dietary spices – Black pepper, red pepper and ginger on the uptake of β-carotene by rat intestines. Journal of Functional Foods. 1:394-398.

Virtanen, A. I. 1966. Milk production of cows on protein-free feed. Science 153:1603–1614.

Vohra, P., F. H. Kratzer, and M. A. Joslyn. 1966. The growth depressing and toxic effects of tannin to chicks Poutr. Sci., 45, 135.

Wallace, R. J. 1996. Ruminalmicrobialmetabolismofpeptidesandamino acids. J. Nutr. 126:1326S–1334S.

Wang, Y., G. B. Douglas, W. G.C., B. T.N., and F. A.G. 1996a. Effect of condensed tannins in Lotus corniculatus upon lactation performance in ewes. J Agr Sci 126, 353-362.

Whitford, D. 2005. Proteins: Structure and Function. Chapter 2. Amino acids: the building blocks of proteins. ed. John Wiley & Sons Ltd.

Wildman, E. E., G. M. Jones, P. E. Wagner, R. L. Boman, J. H. F. Troutt, and T. N. Lesch. 1982. A Dairy Cow Body Condition Scoring System and Its Relationship to Selected Production Characteristics. Journal of Dairy Science. 65 495-501.

Xu, S., J. H. Harrison, W. Chalupa, C. Sniffen, W. Julien, H. Sato, T. Fujieda, H. Watanabe, T. Ueda, and H. Suzuki. 1998. The effect of ruminal bypass lysine and methionine on milk yield and composition of lactating cows. J. Dairy Sci. 81:1062–1077.

Yeh, C. T. and G. C. Yen. 2006. Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance-associated protein 3 mRNA expression. J Nutr. 136:11-15.

Yildiz, S., I. Kaya, Y. Unal, D. Aksu Elmali, S. Kaya, M. Censiz, M. Kaya, and A. Oncuer. 2005. Digestion and body weight change in Tuj lambs receiving Oak (Quercus hartwissiana) leaves with and without PEG. Anim. Feed Sci. Technol. 122, 159–172.

Zhu, J., L. J. Filippich, and M. T. Alsalami. 1992. Tannic acid intoxication in sheep and mice. Res Vet Sci 53, 280-292.

Zucker W. V. 1983. Tannins: does structure determine function? An ecological perspective. Am Nat. 121, 335-365