

ABSTRACT

DAVES, MEREDITH G. Effects of protected fat when fed to dairy cattle and the interaction between supplemental fat and antibiotics in mixed cultures.

Fat is added to diets of lactating dairy cattle as an affordable method of increasing the amount of energy available to the animals. Unsaturated fatty acids are desirable end-products of milk production. However, their presence in the rumen can be toxic to ruminal bacteria, which will then decrease digestion. Unsaturated fatty acids are biohydrogenated into saturated fatty acids by specific bacteria in the rumen, which will decrease positive benefits associated with their consumption. Our first study evaluates the efficiency of calcium salts VALFEED 100M and VALFEED 200 in protecting unsaturated fatty acids from biohydrogenation in the rumen and also observes the effects of these “protected fats” on lactation performance.

Twenty-eight Holstein cows in early lactation were divided equally into four different treatment groups: 1) Control (with no supplemental fat); 2) Prilled fat; 3) Calcium salt of VALFEED 100M; 4) Calcium salt of VALFEED 200. Fat supplements were added to diets in place of corn in the concentrate mix at 3.2% of total dry matter intake. Cows were housed in free stalls equipped with Calan gates. The cows were fed twice daily, at 0800 and 1500 in amounts to allow ad libitum consumption. Weekly feed samples were taken and dried in order to calculate daily dry matter intake. Milk production was measured daily, and milk fat and protein content were analyzed at days 30 and 90. Body weights were taken at the beginning (day 0) and the end (day 90) of the trial.

Prilled and VALFEED 100M fat supplements decreased ($P < 0.05$) dry matter intake. Body weights were higher ($P < 0.05$) in cows fed the control diet than those fed the prilled and VALFEED 100M diets. VALFEED 200-treated cows had similar ($P > 0.10$) body weights when compared to all treatment groups. Milk yield was highest in cows fed VALFEED 200 (37.1

kg/d), and cows fed prilled and VALFEED 100M diets had lower ($P < 0.05$) milk yields than those fed the control diet (32.0 kg/d, 32.8 kg/d, and 35.1 kg/d, respectively). Feeding VALFEED 100M significantly decreased ($P < 0.05$) milk fat percentage when compared to the control and prilled diets. VALFEED 200 feeding did not significantly alter milk fat percentage when compared to prilled and control diets. Both VALFEED 100 and VALFEED 200 decreased ($P < 0.05$) milk protein percentage. Feeding VALFEED 100M and VALFEED 200 resulted in an increase ($P < 0.05$) in the *cis*- and *trans*- isomers of oleic acid (C 18:1) content of milk when compared to the control and prilled treatments. Both VALFEED 100M and VALFEED 200 also increased ($P < 0.05$) linoleic acid (C 18:2) content in milk fat. The addition of VALFEED 100 to dietary rations depresses milk fat percentage ($P < 0.05$) and increases ($P < 0.05$) the percentage of trans-fatty acids in the milk when compared to the addition of VALFEED 200, suggesting that VALFEED 100 is less inert in the rumen than VALFEED 200.

As previously mentioned, fat can be used as a feed additive to provide additional energy for the diet, but it also affects ruminal fermentation by decreasing waste loss and increasing feed efficiency. Ionophores, such as monensin, are drugs that alter ion transport and concentration gradients in specific ruminal bacteria. In doing so, they are capable of altering rumen fermentation and improving feed efficiency. Bacitracin is a non-ionophore antibiotic that affects similar bacteria as monensin but has a different mode of action. It also increases feed efficiency in ways similar to monensin. But, when ionophores are added to diets supplemented with fat, ionophore efficiency decreases. This interaction stimulated interest in conducting our second study. The inclusion of fat and ionophore-antibiotics to experimental diets and the sequence of their addition were investigated.

Rumen fluid collected from a dry, fistulated cow was incubated in 8 fermentors for a total of eight days. The first two days of the trial represent a stabilization period. On day three, two fermentors received monensin (50 ppm), two received bacitracin (50 ppm), and the other four received fat (4.4% of DMI). On day 6, one of the fermentors receiving monensin and one receiving bacitracin got fat. The other two continued receiving either only monensin or only bacitracin. Of the remaining four fermentors that were receiving fat, one received monensin, one received bacitracin and the other two continued to only receive fat. Methane and pH were recorded several times daily. Culture samples were taken on days 2, 5, and 8 for analysis of SCFA, LCFA, NH_3 , and PCR.

There were no statistical differences ($P > 0.10$) in acetate and propionate production (mM) among treatments. However, the concentration of butyrate was higher ($P < 0.01$) in cultures treated with monensin when compared with cultures treated with bacitracin. The proportion of C 18:2 in rumen bacteria increased ($P < 0.05$) when monensin was added prior to fat in comparison to when fat was added prior to monensin. Preliminary results indicate that the sequence of fat and antibiotic addition effects the growth of Gram- and Gram+ bacteria. The results from this study show that the sequence in which fat and ionophore-antibiotics are added to mixed rumen cultures can alter the response of bacteria to the additives.

**EFFECTS OF PROTECTED FAT WHEN FED TO DAIRY CATTLE AND THE
INTERACTION BETWEEN SUPPLEMENTAL FAT AND ANTIBIOTICS IN
MIXED CULTURES.**

by

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A Thesis presented to the Graduate School and the
Animal Science Department in partial fulfillment
of the requirements for the degree of
Master of Science

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DEDICATION

This thesis is dedicated in loving memory to my godmother, Joan Marie Rich, my role model; the most dedicated, selfless, caring person who completely devoted her life to her friends, her family, and to God. I can only strive to become someone like her; may she rest in peace.

BIOGRAPHY

Meredith Gail Daves was born in Charlotte, North Carolina on September 13, 1979, to parents John and Sharon Daves. She was raised there with her two older brothers, Brad and Matthew. Her family moved to Gastonia, North Carolina, in 1987. She attended and was well involved in high school athletics and extracurricular activities at Ashbrook High School, where she was voted “Most likely to succeed”. She received several service awards, including the Naomi Cunningham Award for commitment to the Heart Society of Gaston County. She graduated with honors in the top 5% of her class from Ashbrook High School in June of 1997.

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INTRODUCTION

Lactating dairy cattle expend more energy during peak milk production than what is consumed through their diets, creating a negative energy balance in the animal. Consuming an energy-dense diet is necessary in order for high-producing dairy cattle to maintain adequate milk output. Fat is added to the diet to increase energy density without decreasing fiber content. Concentrates are typically added to the diet as an energy source and substitute for forage content of the diet, which will decrease the fiber content of the diet, negatively affecting rumen bacteria (Andrew et al., 1991).

It is, however, necessary to monitor the amount and type of fat supplement added to the diet. Fats added to the diet in amounts exceeding 5% of dry matter intake can reduce digestion in the rumen. Long-chain fatty acids interact with microorganisms in the rumen, interfering with their digestive actions, and creating toxic effects within the rumen (Chalupa et al., 1986). Unsaturated fatty acids tend to be more toxic to the ruminal bacteria than saturated fatty acids. However, unsaturated fatty acids, when present in milk, can offer anti-atherogenic and possibly anti-carcinogenic effects to humans when consumed, therefore representing desirable components of milk (Parodi, 1997).

When unsaturated fatty acids (UFAs) enter the rumen, specific species of bacteria attack the terminal carboxyl group of the UFAs, add hydrogen atoms to this end, and biohydrogenate the fatty acids. These fatty acids are now “saturated” with hydrogen atoms and form saturated fatty acids. Saturated fatty acids can generate negative health effects on the human body when consumed in high quantities, such as increasing the risk for atherosclerosis, hypertension, and heart disease.

If the free carboxyl end of unsaturated fatty acids is protected from bacterial enzymatic attack in the rumen, then biohydrogenation cannot occur. Calcium is a divalent cation, and, when added to the diet, can bind to the terminal carboxyl group of the fatty acid, forming a calcium salt, or soap. This calcium salt is insoluble within the ruminal environment; therefore, its formation can protect the unsaturated fatty acids from biohydrogenation, and allow unsaturated fatty acids to remain intact in milk. By binding the unsaturated fatty acids, the calcium also protects ruminal microbes from any toxic effects due to exposure to the fatty acids, and will prevent decreases in digestion. Therefore, if calcium salt formation is effective, the combined calcium salt will escape degradation in the rumen, increasing the proportion of unsaturated fatty acids present in milk (Palmquist and Jenkins, 1980).

Ionophores, such as monensin, are antibiotics that are added to diets of dairy cattle to decrease waste loss, increase digestibility, and increase feed efficiency in cattle by reducing methane levels, increasing the production of propionate, and decreasing the production of ammonia (Russell and Strobel, 1989). Ionophores are lipophilic substances that are capable of binding to the lipid bilayer of bacterial cell membranes. The interior of the ionophore is hydrophilic and capable of binding cations. Charged ions, which are “lipophobic” cannot normally pass through this lipid bilayer, but the binding of cations to ionophores neutralizes their charges and facilitates their movement through the membrane and into the bacterial cell (Pressman, 1976).

Monensin selectively binds sodium (Na^+) and potassium (K^+) ions, which increases their subsequent transport rates. The concentration of Na^+ is greater outside the cell than inside the cell. This concentration gradient is maintained by the sodium-potassium pump, which requires ATP to pump three molecules of sodium out of the cell and two molecules of potassium into the

cell, redistributing concentration and charge of the cell. Diffusion of sodium down its electrochemical gradient into the cell fuels the active transport of sugars and amino acids into the cell to provide the bacteria with nourishment. Monensin stimulates the influx of sodium coupled with the efflux of hydrogen, which increases the concentration of sodium within the cell (Russell et al., 1988). Thus, monensin eliminates the sodium gradient and decreases solute transport.

Monensin also stimulates the efflux of potassium coupled with the influx of hydrogen ions. Hydrogen ions are naturally pumped out of the cell, establishing a chemical and electrical gradient, or the proton motive force, which is used to drive ATP synthesis (Mitchell, 1961). When hydrogen ions are pumped into the cell as a result of monensin action, this proton motive force is diminished, decreasing ATP synthesis. The accumulation of free hydrogen ions in the cell also creates acidic conditions toxic to the bacterial cell. The bacteria then deplete cellular ATP stores in an attempt to reduce acidity and re-establish the proton motive force. These combined actions of monensin result in cellular death (Dawson and Boling, 1987).

Bacitracin is a polypeptide antibiotic effective against gram-positive bacteria with similar effects as monensin on fermentation. However, bacitracin has a different mode of action and affects gram-positive bacteria by altering its peptidoglycan layer of the bacterial cell wall. Peptidoglycan is a strong structural component of the cell wall, and its alteration results in decreased cellular control. Bacitracin also inhibits the dephosphorylation of phospholipid carriers in the membrane, therefore disrupting cellular transport (Stewart and Strominger, 1967).

Fat, as previously mentioned, can also be used as a feed additive, increasing energy density of the diet. Fat also influences fermentation in similar ways as ionophores and bacitracin in increasing feed efficiency. However, when fat is added in combination with ionophores to

diets, ionophores efficiency is reduced. According to Clary et al. (1993), ionophores added to diets consisting of supplemental fat did not increase average daily gain or feed efficiency.

Ionophores and fat appear to be negatively associated due to alterations in rumen fermentation. Ionophores can inhibit biohydrogenation in the rumen, increasing levels of unsaturated fatty acids and decreasing levels of saturated fatty acids (Fellner et al., 1997). Linoleic acid (C 18:2) has been shown to be incorporated into the cell membrane preferentially as phosphatidylcholine, with the amount of lipid incorporated into the membrane greater than the amount of lipid synthesized de novo from SCFA precursors (Demeyer et al., 1978).

When ionophores are added to phospholipid vesicle membranes, they become associated with the polar phosphate head groups of the lipid, decreasing membrane fluidity. The binding of ionophores to lipid groups in the membrane decreases rates of cation transport, which indicates impairment of ionophore efficiency (Kolber and Haynes, 1981). Ionophores, when associated with phospholipids in the membrane, restrict rotational freedom of lipid moieties in the membrane. The ionophores, bound to the phosphate head groups, are prevented from completely inserting into the membrane. The binding of ionophores to lipid groups may interfere with their binding to cations, which would explain decreased rate transports associated with binding to membrane phospholipids (Sankaram et al., 1987).

Research conducted in areas regarding the efficiency of protection of supplemental fat within the rumen, the supplementation of diets with different antibiotics and fat, and the sequence of fat and antibiotic addition will provide evidence for the benefits of such dietary additions.

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Literature Review

Fat

Merit of Fat Addition

Ruminants depend on lipids as an energy source in order to sustain high milk output while maintaining body weight during high production. Research conducted by Maynard, Loosli, and associates dating back to the 1920's set the pace for fat supplementation to the diets of dairy cattle. These scientists observed that cows fed diets comprised of 4-7% fat experienced a 2-10% increase in milk production when compared to those cows fed diets comprised of only 1-3% fat (Palmquist and Jenkins, 1980).

During peak lactation, dairy cattle experience a negative energy balance, and they cannot consume enough energy to compensate for increased milk production and subsequent energy loss. If the energy density of these diets is not increased, then the lactating cattle will begin to lose weight and will not be capable of producing high quantities of milk. When grains and starches are added to diets to increase energy density, the level of forage present in the diet must be reduced, which will also reduce the amount of fiber present in the diet. Supplementing the diet with fat can increase energy density of the diet without lowering the total fiber content of the diet (Andrew et al., 1991). When the amount of roughage present in the diet is lowered and the amount of starch is increased, rumen lipolysis and biohydrogenation will decrease, also increasing the proportion of unsaturated fatty acids in the milk and adipose tissue of cattle (Hungate, 1966).

Toxicity theories

Fat included in the diet in amounts greater than 5% of dry matter have been shown to depress milk fat and milk yield by interfering with microbes in the rumen (Chalupa et al., 1986).

When fats are added to the diet in concentrations greater than 5%, the capacity of microbial bacteria to absorb the fatty acids is exceeded, and bacterial metabolism will consequently be impeded (Demeyer and Van Nevel, 1995). High amounts of added fat have been shown to depress fiber digestion in dairy cattle. This effect may occur due to the physical coating of fibrous feed matter with fat, preventing contact of the feed with microorganisms in the rumen and microbial digestion. Fat may also inhibit the action of many microorganisms in the rumen by adhering to the surface of their cell membranes and prohibiting enzymatic activity usually characteristic of the respective microorganism. Fat also appears to have a toxic effect on protozoa, decreasing microbial competition and allowing more bacteria to survive in the rumen (Palmquist and Jenkins, 1980).

Unsaturated fatty acids are much more toxic to bacteria in the rumen than are saturated fatty acids. This may be explained by the fact that saturated fatty acids, which are solid at room temperature and have a higher melting point than unsaturated fatty acids, will be more insoluble in the rumen and less likely to adhere to either fibrous feed particles or microbes, therefore exerting less inhibition on digestion and fermentation in the rumen (Chalupa et al., 1986).

Unsaturated fatty acids may also adhere to and alter the fluidity of the cytoplasmic membranes of bacteria, which may decrease the permeability of the bacterial membrane (Jenkins, 1993).

Biohydrogenation and its role in Lipid Metabolism

Microorganisms living in the rumen environment have evolved and developed a process to protect themselves from the toxic effects of unsaturated fatty acids present in the diet (Kemp and Lander, 1984). Biohydrogenation is a metabolic pathway that completes the conversion of unsaturated fatty acids into saturated fatty acids. The major unsaturated fatty acids that enter the rumen consist of linolenic acid (C18:3, cis-9, cis-12, cis-15), which is prevalent in the diets of

grazing cattle, and linoleic acid (C18:2, cis-9, cis-12), which mostly occurs in diets supplemented with fat. These unsaturated fatty acids cannot be converted to saturated fatty acids unless they first experience lipolysis, which produces a fatty acid with a free terminal carboxyl group. This carboxyl group must be present in order for the isomerase enzyme to act on the fatty acid and complete the first step of biohydrogenation (Kepler et al., 1970 and Hazlewood et al., 1976).

During this first step, the electronegative end of the isomerase enzyme binds to the carboxyl group of the fatty acid, allowing its active site to bind the pi electrons of the double bond, creating a conformation that allows electron transfer to take place. Two molecules of alpha-tocopherolquinol (TQH₂) are oxidized to the semiquinone form of TQH, providing the hydrogen atoms necessary for reduction of the double bonds in hydrogenation. The double bond present at the twelfth carbon in the fatty acid is thus converted into its trans-11 isomer, which forms the cis, trans conjugated intermediate that serves as a prerequisite for further hydrogenation. Subsequent steps occur with the transfer of two hydrogen atoms to each double bond until the unsaturated fatty acid has become a saturated fatty acid with no double bonds, or stearic acid (C18:0) (Kepler et al., 1971).

When unsaturated fatty acids are added to the diet and biohydrogenated, they can reduce methanogenesis, or the formation of methane within the rumen. Methane (CH₄) is formed by the reduction of carbon dioxide with hydrogen, and it will not be produced unless there is an excess of hydrogen atoms present in the rumen environment. The presence of hydrogen acceptors other than carbon dioxide in the rumen will compete with the available carbon dioxide for hydrogen atoms and therefore decrease overall methane production. When unsaturated fatty acids are reduced with hydrogen atoms, they form end products such as stearic acid, which is better

absorbed and more usable than methane, which is lost as a waste product. Stearic acid also provides more calories per unit than any unsaturated fatty acids for the animal. Therefore, adding unsaturated fatty acids can improve feed efficiency by decreasing substrate availability for methane production. Furthermore, because the unsaturated fatty acids affect methane production only by competing for the usage of hydrogen atoms, they only interfere with methanogenesis without having adverse effects on cellulolytic or amylolytic bacteria function (Czerkawski et al., 1966).

The extent of biohydrogenation can be affected by the form of fatty acid that enters the rumen. If free fatty acids, in the absence of feed, enter the rumen, the process may not complete or result in formation of stearic acid. However, when fat enters the rumen in conjunction with feed particles, the feed particles theoretically compete with bacteria for absorption sites for the fatty acids, which will prevent the bacteria from becoming completely coated with lipid and toxic unsaturated fatty acids to the point of impeding metabolism. In this case, biohydrogenation is able to continue to completion (Henderson, 1973).

Bacterial populations in the rumen differ in chemical composition and metabolic function and can be divided based on their associations with digesta in the rumen. Liquid associated bacteria (LAB) is found floating freely in the liquid portion of the rumen, while solid associated bacteria (SAB) is found attached to solid feed particles in the rumen. The concentration of SAB in the rumen is controlled by diet and is often increased with concentration of structural carbohydrate, which allows the SAB to produce a “bacterial slime” to facilitate its attachment to the feed (Cheng et al., 1977). Therefore, in theory, fat present in the diet would coat feed particles in the rumen and decrease attachment of SAB (Devendra and Lewis, 1974).

Legay-Carmier and Bauchart (1989) conducted a study analyzing the effects of adding soya-bean oil to diets of dairy cattle on the fatty acid concentrations of the separate fractions of bacteria in the rumen. SAB, when analyzed after fat supplementation, had three times the concentration of fatty acids than did LAB. These results may be explained by the fact that dietary fat easily binds to feed particles, and the SAB attached to these feed particles passively absorbs the dietary fat and incorporates it into the cell. Bacteria may incorporate fats in order to decrease energy expenditure necessary for de novo fatty acid synthesis, which can be avoided if fat is obtained from dietary sources.

O'Kelly and Spiers (1991) also conducted a study to analyze effects of supplementing fat (in the form of safflower oil) to diets of cattle. When LAB and SAB were analyzed, each type of bacteria experienced increased levels of unsaturated fatty acids with slight decreases in saturated fatty acids. All classes of fatty acids present in the bacteria were higher in SAB than LAB except for polyunsaturated fatty acids (PUFAs). It is apparent from this experiment that the rumen bacteria take up long chain fatty acids from the environment and incorporate them into more complex forms of lipid. The amount of fatty acids that are incorporated into the lipid membranes of rumen bacteria is greater than the amount synthesized from short chain fatty acid precursors in the bacterial cell (Demeyer et al., 1978). After incorporating lipid into bacterial membranes, the bacteria are able to store energy for the synthesis of cellular components. The uptake of unsaturated fatty acids can protect unsaturated fatty acids and reduce levels of biohydrogenation in the rumen (O'Kelly and Spiers, 1991).

Human Health Concerns

Biohydrogenation, which results in the conversion of unsaturated fats into saturated fats, poses a concern for many humans. Inclusion of unsaturated fats in the diet provides anti-

atherogenic properties and is associated with positive health benefits, while the consumption of high amounts of saturated fats is correlated with many health problems. Conjugated linoleic acid (CLA), which is actually an intermediate formed during the biohydrogenation of linoleic acid (C18:2) can contribute many significant benefits to human health if consumed and not converted to a saturated fatty acid in the rumen, therefore being secreted into cow's milk.

CLA has many isomers, but is mostly found in milk as either *cis*-9, *trans*-11 CLA, which is the principal dietary form, or *trans*-10, *cis*-12 CLA, which is the isomer most prevalent in adipocytes. A double bond is described as “*trans*” if the two hydrogen atoms attached to the double bond are positioned on opposite sides of the double bond. When a *trans* double bond is formed, it produces a “kink” in the carbon chain, which will slightly change the structure of the fatty acid into a more solid form. If the double bond is oriented in the “*cis*” conformation, the two hydrogen atoms attached to it are positioned on the same side of the double bond, which will not change the structure of the carbon chain. These two CLA isomers act synergistically to decrease fat gain and inhibit carcinogenesis (Pariza et al., 2001).

Park, et al. (1997) were the first to conduct a study comparing the effects of a control diet to one that contained CLA at 0.5% of dry matter in six week-old mice. The diet containing CLA showed a significant decrease in body fat and increase in lean tissue gain. Also coinciding with these effects was an increase in levels of carnitine palmitoyltransferase (CPT) in skeletal muscle, which is the rate-limiting enzyme in beta-oxidation. Lipoprotein lipase levels in the blood of the rats treated with CLA was significantly reduced, which would result in decreased fatty acid uptake in the tissues, decreasing fatty acid gain.

Tsuboyama-Kasaoka et al. (2000) confirmed findings that indicated that a diet supplemented with 1% CLA resulted in apoptosis, or cell death, of adipocytes in the adipose

tissue of mice. The mechanism behind this phenomenon is unclear, but it may be related to the ability of CLA to inhibit differentiation of preadipocytes.

CLA also has a profound effect in stimulating the immune system. Supplemental CLA has shown inhibitory effects on chemically-induced carcinogenesis, specifically, skin cancer, in rodent and chicken models (Ha et al., 2001). Although the exact role of CLA in cancer inhibition is unknown, its isomers may act synergistically to alter the metabolism of linoleic acid and vitamin A to somehow alter cancer growth. These isomers have also worked to increase levels of tumor necrosis factor-alpha (TNF-alpha) and interleukin-1, two cytokines that stimulate immune function (Parodi, 1999). Dietary CLA has been shown to further increase synthesis of immunoglobulin in rat lymphocytes (Yamasaki et al., 2000), therefore increasing the immune response to other pathogenic agents. Considering the rodent model can be used to represent human response to dietary supplementation, there is evidence to indicate that increased quantities of CLA available for human consumption in cow's milk can help decrease fat gain while decreasing cancer growth and increasing immunity.

Conjugated linoleic acid offers another benefit when present in cow's milk. Baumgard et al. (2000) proved that feeding 50 g/day of CLA abomasally (to escape biohydrogenation in the rumen) increased the content of all CLA isomers in the cow's milk while also depressing the percentage of fat present in the milk. According to the NRC (National Research Council, 1989), energy costs associated with the secretion of fat into milk represents approximately 50% of the energy present in milk. Therefore, if CLA decreases milk fat percentage, or increases MFD (milk fat depression), it can help reduce energy costs of high-producing, lactating dairy cattle.

Hanson et al. (1998) also demonstrated a way in which CLA can be included in the diet without being converted into a saturated fatty acid by rumen microbes so that it could be present

in the end milk product. CLA, when converted into the form of a calcium salt, increased CLA concentrations in the milk by 61.5% while decreasing milk fat by 34%. Giesy et al. (2002) then discovered that increasing the dose of CLA fed as a calcium salt will increase the CLA content of milk linearly while also inhibiting the synthesis of fatty acids C8:0 (caprylic acid), C10:0 (capric acid), and C12:0 (lauric acid). Increasing the content of C18:2 in the diet can decrease the extent of formation of saturated fatty acids, or C18:0 (stearic acid), while increasing the amount of C18:1(oleic acid) produced in the rumen. Therefore, increasing the amount of linoleic acid in the diet can decrease biohydrogenation in the rumen (Fellner et al., 1995).

Formation of Calcium Salts

The formation of calcium salts and their interactions with unsaturated fatty acids permits passage of unsaturated fatty acids such as CLA through the rumen without automatic conversion into saturated fatty acids by biohydrogenation. These salts act to protect the beneficial qualities associated with unsaturated fatty acids present in milk. The inhibitory effects of fatty acids on microorganisms in the rumen can also be reversed when these fatty acids form insoluble complexes with metal cations. In 1954, Brooks et al. discovered that alfalfa ash, when added to the diet, would reverse the negative effects of corn oil, an unsaturated fatty acid, on fiber digestibility. Later, in 1958, it was realized that the calcium present in the alfalfa ash was the factor that resulted in decreased negative effects. Also, in 1972, El Hag and Miller discovered that adding calcium chloride to malt distiller's grain (which is made up of high amounts of lipid) increased digestion of both hemicellulose and cellulose fractions present in the feed. Calcium, which is a divalent cation, binds to the terminal fatty acid group (which is normally attacked during biohydrogenation) when added to an unsaturated fatty acid, forming an insoluble calcium

salt, or soap. This soap formation protects the carboxyl group of the unsaturated fatty acid and prevents its association with rumen microbes (Palmquist and Jenkins, 1980).

Calcium is added to feed primarily in the form of dicalcium phosphate or calcium chloride. When calcium is added as calcium chloride, the formation of insoluble soaps with fatty acids occurs more readily than with the addition of calcium as dicalcium phosphate. Therefore, calcium chloride is more efficient in increasing fiber digestibility. This difference is most likely due to the fact that calcium chloride is more soluble in water and active in the rumen environment. Differences in fatty acid properties also affect the efficiency of salt formation. Longer, more saturated fatty acids form insoluble complexes with calcium more readily than unsaturated fatty acids. *Trans*-fatty acid isomers tended to form calcium salts more readily than *cis*-fatty acid isoforms. The extent of calcium salt formation can affect digestibility and efficiency of rumen fermentation (Jenkins and Palmquist, 1982).

Andrew et al. concluded that animals fed supplemental fat in the form of calcium salts produced more milk when compared to cattle consuming the same amount of feed and energy. The diets differed only in the feed source used to provide energy to the lactating cattle. Those receiving concentrate as an energy source did not produce increased amounts of milk as did those cattle receiving “protected fat” as their energy source. Increased energy density can be provided from the supplemental fat without decreasing digestion. Any negative influence that supplemental fat exerts on microbial fermentation, therefore limiting production, is controlled when the unsaturated fatty acids are bound to the calcium salt and unable to contact rumen bacteria (1991).

Ionophores

Effects on fermentation in the rumen

Monensin, an ionophore antibiotic originally utilized in the poultry industry as a coccidiostat, was later discovered as an intermediate in increasing energy efficiency in ruminants. Ionophores are toxic to many bacteria, protozoa, and fungi, and are therefore classified as antibiotics. The Food and Drug Administration approved its use in ruminant animals in the 1970's, allowing farmers and producers to benefit from its diverse effects on fermentation in the rumen (Richardson et al., 1976). Bacitracin is a polypeptide antibiotic that is mostly effective against gram-positive bacteria (Merck, 1979). It, like monensin, can increase feed efficiency and average daily gain in cattle, but its effects on rumen fermentation has not been studied extensively as monensin has (Church, 1977).

Methane (CH_4) is the “hydrogen sink” of the rumen environment in that it is produced in order to dispose of unnecessary hydrogen atoms present in the rumen. Cattle can produce up to 12 liters of methane per hour, or an approximate 12% loss of feed energy. Inclusion of ionophores in a ruminant diet can decrease methane loss by 30% (Schelling, 1984). Acetate (volatile fatty acid produced as an end-product of fermentation) levels also tend to decrease in response to ionophore addition in the diet. Hydrogen atoms are also necessary and used for the formation of propionate in the rumen, and hydrogen is produced as a by-product of acetate production. Therefore, while acetate production decreases, propionate concentration increases, decreasing the acetate:propionate ratio within the rumen (Wallace et al., 1980). However, it has been proven that monensin is not toxic to methanogenic bacteria. When Van Nevel and Demeyer (1977) provided a constant flow of hydrogen gas to a rumen environment, methane production increased linearly, indicating that ionophores do not effect the actual production of

methane. Decreases in methane and acetate levels must be due to decreased levels of substrate, or hydrogen in the rumen.

Protein that enters the ruminal environment is mostly converted to ammonia and volatile fatty acids by bacteria in the rumen. Ammonia, which can be converted to less toxic forms by ammonia-utilizing bacteria, can be produced in amounts that exceed the processing capacity of these bacteria. In these cases, ammonia floats freely in the rumen and is absorbed through the rumen wall and transported to the liver, where it is converted to urea. Some of the urea is recycled back to the rumen, but the majority of it is lost in the urine as waste. The addition of monensin to ruminant diets decreased the production of ammonia. Monensin has been shown to inhibit the growth of proteolytic bacteria, but most likely inhibits deamination rather than proteolysis. The inhibition of these processes increases protein efficiency in the rumen due to decreased production of ammonia, requiring less energy for its conversion to urea and excretion (Russell and Martin, 1984).

Cellulose, a complex plant starch that is not digestible by mammals, can be digested by ruminants. However, when monensin is added to the diet, cellulose digestion tends to decrease, which will result in decreased feed intake due to the presence of undigested “bulk” present in the rumen. Fiber intake does not decrease, but when feed intake decreases, the passage rate of the feed will decrease, allowing more time for digestion of feed (Russell and Strobel, 1989). When more time is allotted for digestion of feed, less material passes into the large intestine undigested and prepared for excretion. More feed is digested and nutrients utilized, while less material is lost as feces, resulting in increased feed efficiency (Wedegaertner and Johnson, 1983).

When cattle are fed diets high in concentrates in order to increase the energy density of the diet, bacteria in the rumen digest the concentrate and produce lactic acid as a by-product.

Increased levels of lactic acid in the diet will decrease pH levels, resulting in a more acidic environment than the near-neutral rumen environment that exists when fed forage-based diets. This condition, also known as rumen acidosis, results in indigestion, and in severe cases, founder and death. However, when monensin is added in conjunction with concentrate to the diet, lactic acid production decreases, increasing the pH to a more neutral value (Dennis et al., 1981).

Mode of Action

Increases in feed efficiency and benefits associated with monensin supplementation result from the interaction of monensin with ruminal bacteria. The bacteria in the rumen are able to survive by using transport mechanisms to acquire nutrients from feed particles in the rumen. Hydrophobic compounds such as ammonia and acetate can cross the bacterial membrane by passive diffusion, traveling down their respective concentration gradients and using no ATP. However, hydrophilic or lipophobic compounds cannot cross the membrane and enter the bacterial cell unless transported by carrier proteins by facilitated diffusion.

Active transport is the transport mechanism which is necessary in order to accumulate a substance that must travel into the bacterial cell against its concentration gradient. In this case, ATP must be hydrolyzed to provide the energy for this process to occur (primary active transport), or ion gradients may be utilized to couple solute transport (secondary active transport). Mitchell (1961) developed the chemiosmotic theory, which explains the method in which bacteria couple electron transport to ATP synthesis. When protons are expelled from the bacterial cell, a protonmotive force (PMF) is created, which consists of both chemical ($Z\Delta pH$) and electrical ($\Delta\Psi$) gradients. Energy stored in the PMF can be used to synthesize ATP for transport of solutes (Kashket, 1985).

The electrical gradient established by the translocation of protons outside the cell leaves the inside of the cell more negative. Therefore, positively charged solutes can be drawn in by the electrical gradient to enter the cell. However, neutral or negatively charged solutes can only enter the cell if they are transported in conjunction with positively charged ions. Bacteria use symport (transport of different ions in the same direction) to couple the entrance of either hydrogen or sodium ions with an anion. The majority of bacteria use sodium as a coupling agent for ion transport. It is present in high concentrations in bacterial environments, and it can be transported in and out of the cell without disturbing the near-neutral pH of bacterial cells (Shulachev, 1987).

Symport can occur because sodium binds to a catalytic site on a carrier protein to enter the cell, and the negatively charged ion binds to another site on the protein, due to allosteric binding. Allosteric binding occurs when the binding of one ion facilitates the binding of another by altering the conformation of the binding protein (Strobel et al., 1989). The two ions are then capable of crossing the membrane by traveling down the sodium concentration gradient into the cell. For example, amino acids serine, threonine, and alanine are each taken up by a sodium-transport mechanism in the bacterium *S.bovis* (Russell, et al., 1988).

Feed additives affect bacteria and their ability to grow and reproduce by interrupting their transport mechanisms. Ionophores can inhibit bacterial transport of nutrients by disrupting the bacterial ion gradients (Russell and Strobel, 1989). Ionophores are lipophilic, which means they can easily traverse the lipid-rich bacterial membrane. The outside of the ionophore is hydrophobic, and the interior is hydrophilic, and charged, allowing it to bind cations. Ionophores are classified as uniporters if they bind a single cation, and antiporters if they can bind cations and protons (Pressman, 1976). Once the ionophore binds the cation, the ion loses its charge, and

it is capable of crossing the lipid bilayer and entering the bacterial cell. Ionophores tend to be more selective for specific ions, but the translocation of ions is dependent on the concentration gradient of the particular ion (Russell et al., 1990).

Monensin is an antiporter and is highly selective for the sodium ion, but can also work to expel potassium from the cell when present in high concentrations. When monensin is present in the rumen, it expels hydrogen ions from the cell in exchange for the transport of sodium into the cell, which diminishes the sodium gradient. This mechanism becomes detrimental to bacteria, which use the entrance of sodium into the cell down its concentration gradient to drive the entrance of sugars and amino acids into the cell against their electrochemical gradients.

Monensin inverts the pH gradient of the bacteria and makes the interior of the bacterial cell more acidic than the exterior. Monensin stimulates the efflux of potassium coupled with the influx of free hydrogen ions. The build-up of hydrogen ions within the cell creates acidic conditions which are toxic to rumen bacteria. The high concentration of hydrogen ions within the cell also works to decrease the proton-motive force, decreasing the production of ATP for the cell (Russell, 1987).

S. bovis, a gram-positive bacteria, produces lactic acid as an end product of fermentation of high-concentrate diets. When *S. bovis* was treated with monensin, the sodium-dependent transport of amino acids such as serine, was almost completely terminated (Russell, 1987). The treatment of monensin then decreased the production of lactic acid, increased rumen pH levels, and prevented rumen acidosis in those dairy cattle consuming high-concentrate diets (Chow and Russell, 1990).

Bacitracin is not an ionophore and has a different mechanism of action than monensin within the bacterial cell. Peptidoglycan is a polysaccharide-containing substance that forms a

structural layer surrounding both gram-positive and gram-negative bacteria, but present in greater proportions in gram-positive bacteria, which lack an outer membrane present in gram-negative bacteria. This layer allows bacteria to maintain a strong osmotic gradient between the extracellular environment and the cell, preventing the loss of salts and solutes from within the cell. However, bacitracin stimulates the accumulation of uridine 5'diphosphate-acetylmuramyl-pentapeptide on the peptidoglycan layer, altering its protective characteristics. This accumulation inhibits the dephosphorylation of a phospholipids carrier in the membrane, which will prevent entrance of nutrients into the cell (Stewart and Strominger, 1967).

Therefore, the survival of bacteria, which is dependent upon the transport of solutes into the cells for nourishment, can be demolished by the addition of ionophores, particularly monensin, or other antibiotics, such as bacitracin. The method in which monensin disrupts ion transport in bacteria can explain its effects on hydrogen-producing bacteria. Decreased availability of hydrogen in the rumen resulting from monensin treatment will reduce the formation of methane and acetate, increasing feed efficiency in the rumen.

Ionophore and Fat Interactions

The addition of ionophores and unsaturated fats to diets of cattle produces similar effects in decreasing methane production, increasing propionate production, and increasing feed efficiency. Zinn (1988) evaluated the feeding of commercial feed-fat and determined how effects may be altered in combination with ionophore supplementation. Steers were fed either control diets of mostly hay and barley, control diet with fat, control diet with ionophore, or control diet with fat and ionophore. Control diets with added fat increased levels of stearic acid production while lowering oleic acid production, but diets with added ionophore decreased

stearic acid levels while increasing levels of oleic acid. When fat and ionophore were added together, the effects of monensin on fatty acid levels were not significant. Also, when fat and monensin were added in conjunction, methane levels decreased while propionate levels and feed efficiency increased, but additive improvements were not significantly greater than individual effects of either fat or ionophore.

Clary et al. (1993) also compared the effects of ionophore with no supplemental fat with the effects of ionophore and supplemental fat added to dairy cattle diets on ruminal fermentation. When ionophore was added with no supplemental fat, the molar proportion of acetate decreased while the molar proportion of propionate increased. However, when fat was added, molar proportions of acetate and propionate did not differ among control diets (no added fat or ionophore). Increases in feed efficiency that occur when ionophores are added without supplemental fat did not take place when ionophores were added to diets in conjunction with supplemental fat (1993).

Fellner et al. (1997) studied the effects of different ionophores on the level of biohydrogenation within the rumen due to the limited amount of research conducted that relates ionophore action to lipid metabolism. Many theories, such as that by Chen and Wolin (1979), state that ionophores work to decrease methane production by decreasing growth of Gram-positive bacteria that produce hydrogen. Therefore, it can be speculated that the addition of ionophores to the ruminant diet could interfere with biohydrogenation by reducing the availability of hydrogen atoms for unsaturated fatty acid reduction. This study evaluated the effect of ionophore supplementation on rumen fermentation and fatty acid biohydrogenation by bacteria in the rumen. Ionophores were added to continuous culture for two days before adding linoleic acid (C18:2_{n-6}). When ionophores were added to the cultures alone, concentrations of

stearic acid (C18:0) decreased from 47.5% to 36% of total fatty acids. When linoleic acid was added to the cultures alone, C18:0 levels decreased slightly (by 3%), but decreased more (by 15%) when added in conjunction with ionophore. The ionophore and linoleic addition also increased levels of oleic acid (C18:1), with greater effects resulting from the addition of the ionophore. Supplemental C18:2 had no effect on levels of C18:2 concentrations in rumen culture, but the addition of ionophore increased the concentrations of C18:2 in rumen culture by two or three times the original amount present with the control diet.

Ionophores added to the rumen cultures in this study were effective in inhibiting biohydrogenation by decreasing the production of saturated stearic acid, while increasing levels of oleic acid, and allowing higher levels of linoleic acid to remain intact in the rumen. The theory presented by Chen and Wolin in 1979 could be challenged due to the fact that Gram-positive, hydrogen producing bacteria convert linoleic acid to oleic acid but not to stearic acid. Gram-negative bacteria convert oleic acid to stearic acid. When ionophores are added to rumen culture, they cannot be inhibiting Gram-positive bacteria while increasing oleic acid production and decreasing stearic acid production.

The addition of ionophores in this study also showed increases in the total concentration of linoleic acid in ruminal cultures. This increase is mainly due to increases in the cis-9, trans-11 C18:2 isomer, or conjugated linoleic acid (CLA). If this increased response is indicative of the levels of CLA present in the milk of the cattle, then the positive health benefits associated with CLA can be made more available with the addition of ionophores to the diet of cattle (Fellner et al., 1996).

Antibiotic Resistance

History

Antibiotics such as ionophores offer many benefits to animals and humans both medically and economically. For example, between the years of 1935 and 1955, the introduction of penicillin and other important antibiotics increased the average lifespan of humans by eight years. Antibiotics are prescribed to humans in order to inhibit the growth of or to kill bacteria and microbes. Antibiotics are implemented in animal diets for similar reasons, but they can also be administered to animals in order to promote efficient growth. Unfortunately, when these miracle drugs are overused and over-prescribed, resistant strains of bacteria can develop and decrease the overall effectiveness of the drugs (Jackson and Vogt, 2001)

According to the National Institute of Medicine, over 145 million courses of antibiotics are prescribed annually in community settings. As bacteria become continuously exposed to the same drug, they eventually develop resistance against that drug in order to survive. This can occur by either random mutation of bacterial DNA that happens to resist a specific antibiotic, or by genetic transfer between bacterial species. If one strain of bacteria randomly mutates into a form that is resistant to an antibiotic, it can then “spread” its resistance in three major ways. In the process of transformation, one strain of bacteria can take up DNA fragments into its cellular environment from other bacteria that are dying. Viruses can also transfer any DNA that they may have coding for resistance to bacteria through the process of transduction. Finally, the process of conjugation involves cell to cell contact between bacteria in which the bacteria transfer their DNA structure via plasmids, which carries separate DNA from bacterial chromosomes. Through these mechanisms, a population of bacteria can transfer DNA and double its size within fifteen minutes (Jackson and Vogt, 2001).

The most controversial issue concerning antibiotic resistance involves the supplemental feeding of antibiotics to commodity animals in either subtherapeutic or therapeutic doses. Subtherapeutic doses of antibiotics are low doses of medication administered for extended periods of time. These low doses are used to reduce the incidence of disease and death while also enhancing animal performance by increasing growth rates. Conversely, therapeutic doses are given in high doses for a shorter period of time in order to treat existing disease. The use of subtherapeutic doses has been associated with prolonged infection, treatment failure, and the emergence of resistant organisms (Ahmed et al., 1984).

There are some species of bacteria that are inherently resistant to certain antibiotics. In this case, low doses of these antibiotics will not affect the resistant bacteria, but will inhibit bacteria sensitive to the drug. The resistant bacteria will then have less competition in the animal's system and will be more able to multiply and occupy a larger portion of the animal. Therapeutic doses will exhibit a larger inhibitory effect on sensitive bacteria, killing more bacteria than subtherapeutic doses, and leaving more room for antibiotic-resistant bacteria to thrive (Gordon et al., 1959; Kobland et al., 1987).

Health Concerns

When antibiotic-sensitive bacteria are inhibited by treatments, the ability of these bacteria to suppress the resistant bacteria through competitive inhibition is eliminated. These resistant bacteria may be more virulent than sensitive bacteria. When bacteria are treated with antibiotics, they are excreted in high proportions from the body for extended periods of time, increasing the possibility for animal exposure via soil, bodies of water, and untreated sewage. Also, any animals that have developed the resistant bacteria may retain these bacteria in their meat, and may infect humans if meat is consumed undercooked. The use of many antibiotics added to the

feed of commodity animals has been banned or investigated (Aserkoff and Bennett, 1969).

However, because antibiotic resistance occurs in conjunction with increased weight gain and feed efficiency, in many cases, the resistant bacteria present no harm.

Development of Resistance

According to Russell and Strobel (1989), there are three main mechanisms by which bacteria resist the effects of antibiotics. Bacteria can synthesize enzymes such as *beta*-lactamases that are capable of degrading the antibiotic and preventing its action on the bacteria. They could also alter the cellular target of the antibiotic. Finally, they could somehow alter their cell membranes in some manner in order to prevent the entry of the antibiotic into the cell.

The resistance of bacteria to ionophores has been related to the cell wall structure of the bacteria. Gram-negative bacteria are usually ionophore-resistant due to the presence of the strong outer membrane that is impermeable to larger macromolecules and some solute movement. Gram-positive bacteria, however, lack the protective outer membrane present on Gram-negative bacteria and are usually sensitive to ionophore action (Nikaido and Nakae, 1979). Because the action of ionophores is related to ion movement, anything that may affect the movement of ions across the cell membrane may increase resistance to ionophores (Stouthammer, 1973).

Bacterial Membrane Permeability

The outer membrane present in gram-negative bacteria (in addition to the peptidoglycan layer found in gram-positive bacteria) serves as a protective barrier to many antibiotics that affect gram-positive bacteria. If these membranes do not completely inhibit the entry of antibiotics into the cell, then they at least slow the amount of antibiotic that enters the cell,

making it easier for the bacteria to attack the amount that invades and to establish resistance against the antibiotic (Richmond and Curtis, 1974).

This outer membrane is made up of unique lipid moieties known as lipopolysaccharides (LPS). The LPS are made up of 6-7 fatty acid chains, which are all saturated, instead of the 2-3 fatty acid chains that make up phospholipids, which are usually unsaturated. The membrane also consists of lipoproteins covalently bound to the peptidoglycan layer by an amino group. The outer membrane also contains several different types of proteins, and the most important type is the porin protein. Porins form pores or channels that allow the passage of small, hydrophilic molecules. These molecules cannot exceed 1.2nm, which estimates the average size of the porin channels. Porin-deficient mutants that have been studied allow the entrance of antibiotics and other inhibitors into the bacterial cell (Nakae, 1976).

Antibiotic resistance has been identified in mutant gram-negative bacteria that are deficient in LPS. Normally, LPS interacts with the porin proteins in such a manner that their presence is necessary in order to keep the porin channels open. If the bacteria are void of LPS, the channels close, which will prevent the passage of antibiotics across the membrane (Schindler and Rosenbush, 1978). When LPS are not present in the membrane, the concentration of phospholipids within the membrane will increase from approximately 30 to 50%. LPS are not permeable to hydrophobic substances, therefore, their presence decreases permeability to hydrophobic molecules. When the phospholipid content of the membrane increases, the passage rate of hydrophobic solutes within the cell increases linearly (Nikaido, 1976).

Kolber and Haynes (1981) prepared fluorescent ionophores in order to study the binding of these antibiotics to the membranes of ruminal bacteria. These ionophores were added to membranes containing phosphatidylcholine derivatives. The fluorescent ionophores bound to

the polar head groups of the outer layer of the cell membrane. This interaction interfered with interactions between the outer and inner layers of the membrane. The rate constant of ion transport, which is normally increased due to ionophore binding to the membrane, decreased when ions were transported across these lipid vesicles. When lipid is added to the outer membrane of the bacteria, the fluidity of the membrane will decrease, which also decreases ion transport, or efficiency of the ionophore.

Few studies have been conducted in order to determine the interaction between ionophores and biological membranes, other than those involved in measuring ion transport. Sankaram, et al. (1987) inserted multilamellar lipid vesicles (MLVs) and unilamellar lipid vesicles (ULVs) into bacterial membrane bilayers and then added ionophores to these membranes. Nuclear magnetic resonance (NMR) was used to measure the effect of ionophores on the orientation of lipid moieties in the membrane. Control media, containing no ionophores, exhibited symmetry within the lipid bilayer. However, when ionophores were added, the orientation of the bilayer was altered.

The addition of ionophores has an effect of “broadening” the membrane without affecting melting temperature. This “broadening” effect can be explained by the interaction of ionophores with the phospholipid head groups of the lipid bilayer. The ionophores insert into the membrane between lipid head groups and interfere with the interactions between the phospholipid head groups and the choline present in the bilayer. The ionophores are not able to completely insert into the membrane because they remain associated with membrane head groups (Sankaram et al., 1987). Ionophores have terminal carboxyl groups that form an electrostatic interaction with the polar head groups and the choline tetramethyl-ammonium groups of the lipid chains in the membrane, disrupting interactions within the membrane. Due to these interactions, ionophores

are not free to bind and facilitate entrance of cations into the cell. By doing so, the ionophores “broaden” the lipid chains within the membrane, restrict the motional freedom of the bilayer, and decrease the fluidity of the membrane (Sankaram et al., 1987).

Juneja and Davidson (1993) studied the effects of changing the fatty acid composition of the gram-negative bacteria *Listeria monocytogenes* on resistance to antimicrobial compounds. The bacteria were grown in the presence of exogenous C14:0, C18:0, or C18:1 for one day. The bacteria were then grown in mediums containing different antimicrobial agents, and their subsequent susceptibility to these agents were measured and recorded. Cells grown in the presence of C14:0 and C18:0 exhibited increased resistance when compared to those cells grown in the presence of C18:1. Saturated fatty acids have higher melting points and are more solid than unsaturated fatty acids such as C18:1. The more saturated fatty acids, when incorporated into the bacterial membrane, decreased the fluidity of the membrane, while the unsaturated fatty acids decreased membrane fluidity. The less fluid membrane offered more protection to the bacterial membrane by making it more difficult for antimicrobial agents, or antibiotics to become more soluble and pass through the membrane, increasing resistance to these compounds.

As previously stated, gram-positive bacteria are more sensitive to ionophore action, and are often eliminated from the rumen when exposed to ionophores. Gram-negative bacteria, although not as sensitive to ionophores as gram-positive bacteria due to their protective outer membrane, are physiologically modified in response to continuous exposure to ionophores. These bacteria can grow in the presence of ionophores, but only after a “lag phase” during which no growth is possible (Chen and Wolin, 1979). For example, *Bacteriodes ruminicola*, gram-negative, peptide- and sugar-degrading bacteria that constitute 60% of the rumen population (Stewart and Bryant, 1988), is initially inhibited when exposed to ionophore. However, as *B.*

ruminicola is grown in increasing concentrations of ionophore, it becomes increasingly resistant to the ionophore, somehow adapting to its effects (Dawson and Boling, 1984).

Newbold, Wallace, and Watt (1992) grew a parent strain of *B. ruminicola* without exposing it to ionophore and compared it to *B. ruminicola* grown in the presence of tetroneasin (an ionophore similar to monensin). Several significant differences were observed. The *B. ruminicola* grown with tetroneasin developed resistance to the ionophore. Later, the two different strains were subcultured and grown in a tetroneasin-free medium. The parent strain grew more quickly than the tetroneasin-resistant strain, but both strains were able to grow. However, when the two strains were subcultured and grown in a medium containing tetroneasin, the growth of the parent strain was completely inhibited, while the resistant strain grew at a rate identical to that when grown without the ionophore. These results indicate that the tetroneasin-resistant bacteria physiologically adapted to the ionophore in such a way that would decrease its growth in antibiotic-free media but allow growth in the presence of ionophore (1992).

Tetroneasin was radioactively labeled in order to observe its binding to the two strains of bacteria within the rumen culture. The ionophore bound to the parent strain and accumulated on it two times as much as that which accumulated on the resistant strain, representing increased association of the ionophore with the parent strain. When the tetroneasin-resistant bacteria was washed with EDTA (ethylenediaminetetraacetic acid), which disrupts the outer membrane of gram-negative bacteria, sensitizing them to ionophores (Booth et al., 1979), the differences in ionophore binding between the two strains was eliminated. These results suggest that adaptive differences in the resistant bacteria result from changes in the outer membrane, altering permeability of the bacterial cell (Newbold et al., 1992). The barrier of the outer membrane

excludes the entrance of substances greater than 600 Da, and the molecular weights of tetroneasin and monensin are 628 and 670 Da, respectively (Nikaido & Nakae, 1979).

Because *B. ruminicola* is a peptide-hydrolyzing bacteria, two different-sized peptides were added to the cultures containing the bacteria which measured 607 Da and 460 Da (Newbold et al., 1992). Both strains of bacteria completely hydrolyzed the smaller peptide, but the parent strain degraded the larger peptide 45% more than did the resistant strain. Once again, when the tetroneasin-resistant bacteria were washed with EDTA and the outer membrane surrounding the bacteria was removed, the resistant strain was equally capable of degrading the larger peptide as the parent strain.

Therefore, decreased binding of the large molecular weight-ionophores to the outer membrane of the resistant bacteria, and the decreased hydrolysis of large peptides by the tetroneasin-resistant bacteria can be attributed to altered characteristics of the outer membrane. The outer membrane of the resistant *B. ruminicola* exhibits decreased permeability when compared to the parent strain of *B. ruminicola*, suggesting that adaptation to the ionophore tetroneasin altered the composition and porosity of the protective outer membrane (Newbold et al., 1992). Monensin resistance seems to be related to the existence of outer membrane cellular envelope compounds that are composed of cytoplasmic membrane lipids, which will prevent the entrance of the lipophilic ionophores and their effects on the bacterial cell (Russell and Strobel, 1988).

Because monensin can catalyze both sodium-proton antiport and the exchange of protons into the cell with the efflux of potassium from the cell, measuring ion transport can be used to indicate sensitivity to the ionophore. Monensin is most commonly associated with catalyzing sodium influx, but because the concentration of sodium outside the cell is so high, the movement

of sodium into the cell cannot be distinguished between passive diffusion and that catalyzed by monensin action. Therefore, potassium efflux, or “potassium depletion”, can be measured within a bacterial cell to approximate monensin sensitivity (Lana and Russell, 1996).

Callaway and Russell (1999) studied the effects of both wild-type and monensin-resistant *Prevotella bryantii* after further exposure to monensin in rumen cultures. Potassium depletion was used to measure sensitivity to the ionophore in each type of *P. bryantii*, which is a gram-negative bacteria that can become resistant to monensin after exposure, but is initially sensitive to the ionophore. Each type of bacteria bound to monensin in equal proportions, but the wild-type *P. bryantii* was more difficult to wash away from the bound monensin than was the resistant bacteria, suggesting that monensin bound more tightly to the wild-type than to the resistant strain. Monensin-resistant *P. bryantii* grew more quickly and without decline than the wild-type when grown in media containing monensin, and it also grew as quickly as the wild-type in monesin-free media. However, the resistant strain was more susceptible to energy starvation than the wild-type, indicating that a more protective outer membrane, although protecting against the antibiotic, may also restrict the entrance of nutrients into the cell.

The action of ionophores resulting in increased levels of potassium depletion provides evidence that ionophores can alter rumen bacteria in some way. These bacteria are not killed, but when continuously exposed to ionophore, a higher concentration of ionophore is necessary to evoke the same level of potassium depletion, indicating a change in membrane physiology. Nearly half of ruminal bacteria never exposed to ionophore was resistant to monensin, and this number continued to grow, becoming capable of replacing those bacteria inhibited by the ionophore. The fact that bacteria that have not been exposed to ionophore can be resistant to the

antibiotic suggests that resistance may be an aspect of growth and evolution rather than an inherited genetic trait (Lana and Russell, 1996).

Three additional gram-negative bacteria species, including *Prevotella ruminicola*, *Veillonella parvula*, and *Fibrobacter succinogenes*, were studied and found to increase resistance to ionophores when grown in increasing concentrations of the antibiotics (Newbold et al., 1993). As the resistance of these bacteria increased when they adapted to grow in the presence of one type of ionophore, such as monensin, its resistance to other ionophores such as tetracycline and lasalocid also increased. Even though each type of ionophore exerts distinct effects on the bacteria, a common mechanism of defense must develop in the bacteria, such as decreased porosity. The existence of cross-resistance to different ionophores/antibiotics diminishes the idea that alternating different types of ionophores will prevent adaptation to the antibiotics and decrease antibiotic resistance among animals and humans (Newbold et al., 1993).

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Experiment 1

EFFECTS OF FEEDING PROTECTED FAT SUPPLEMENTS TO LACTATING DAIRY COWS ON FEED INTAKE, MILK YIELD, AND MILK COMPOSITION.

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ABSTRACT

This study evaluates the efficiency of calcium salts VALFEED 100M and VALFEED 200 in protecting unsaturated fatty acids from biohydrogenation in the rumen and observes their effects on lactation performance.

Twenty-eight Holstein cows in early lactation were divided equally into four different treatment groups: 1) control (with no supplemental fat); 2) prilled fat; 3) calcium salt of VALFEED 100M; 4) calcium salt of VALFEED 200. Fat supplements were added to diets in place of corn in the concentrate mix at 3.2% of total dry matter intake. Cows were housed in free stalls equipped with Calan gates. The cows were fed twice daily, at 0800 and 1500 in amounts to allow ad libitum consumption. Milk production was measured daily, and milk fat and protein content were analyzed at days 30 and 90. Body weights were taken at the beginning (day 0) and the end (day 90) of the trial.

Prilled and VALFEED 100M fat supplements decreased ($P < 0.05$) dry matter intake. Body weights were higher ($P < 0.05$) in cows fed the control diet than those fed the prilled and VALFEED 100M diets. Cows fed VALFEED 200 had similar ($P > 0.10$) body weights when compared to all treatment groups. Milk yield was highest in cows fed VALFEED 200 (37.1 kg/d), and cows fed prilled and VALFEED 100M diets had lower ($P < 0.05$) milk yields than those fed the control diet (32.0 kg/d, 32.8 kg/d, and 35.1 kg/d, respectively). Feeding VALFEED 100M significantly decreased ($P < 0.05$) milk fat percentage when compared to the control and prilled diets. Feeding VALFEED 200 did not significantly alter milk fat percentage when compared to prilled and control diets. Both VALFEED 100 and VALFEED 200 decreased ($P < 0.05$) milk protein percentage. Feeding VALFEED 100M and VALFEED 200 resulted in an increase ($P < 0.05$) in the *cis*- and *trans*- isomers of oleic acid (C 18:1) content of milk when

compared to the control and prilled treatments. Both VALFEED 100M and VALFEED 200 also increased ($P < 0.05$) linoleic acid (C 18:2) content in milk fat. The addition of VALFEED 100 to dietary rations depresses milk fat percentage ($P < 0.05$) and increases ($P < 0.05$) the percentage of trans-fatty acids in the milk when compared to the addition of VALFEED 200, suggesting that VALFEED 100 is less inert in the rumen than VALFEED 200.

INTRODUCTION

Lactating dairy cattle expend and secrete more energy during milk production than what is consumed through their diets, creating a negative energy balance in the animal (Sklan et al., 1992). Consuming an energy-dense diet is necessary in order for high-producing dairy cattle to maintain adequate milk output. Concentrates are typically added to the diet as an energy source and substitute for forage content of the diet, which will decrease the fiber content of the diet, negatively affecting ruminal bacteria. Fat is added to the diet at the expense of concentrates to increase energy density without decreasing fiber content (Andrew et al., 1991).

It is, however, necessary to monitor the amount and type of fat supplement added to the diet. Fats added to the diet in amounts exceeding 5% of dry matter intake can reduce digestion in the rumen. Long-chain fatty acids interact with microorganisms in the rumen, interfering with their digestive action, and creating toxic effects within the rumen (Chalupa et al., 1986). Unsaturated fatty acids tend to be more toxic to the ruminal bacteria than saturated fatty acids. However, unsaturated fatty acids, when present in milk, can offer anti-atherogenic and possibly anti-carcinogenic effects to humans when consumed, therefore, representing desirable components of milk (Parodi, 1997).

When unsaturated fatty acids (UFA) enter the rumen, specific bacteria attack the terminal carboxyl group of the UFA, add hydrogen atoms to this end, and biohydrogenate the fatty acids. These fatty acids are now “saturated” with hydrogen atoms and constitute the saturated fatty acids. Saturated fatty acids can generate negative health effects on the human body when consumed in high quantities, such as increasing the risk for atherosclerosis, hypertension, and heart disease.

If the free carboxyl end of unsaturated fatty acids is protected from bacterial enzymatic attack in the rumen, then biohydrogenation cannot occur. Calcium is a divalent cation, and, when added to the diet, can bind to the terminal carboxyl group of the fatty acid, forming a calcium salt, or soap. Calcium salts are insoluble in the ruminal environment and can protect the unsaturated fatty acids from biohydrogenation, allowing unsaturated fatty acids to remain intact in the rumen. By binding to the unsaturated fatty acids, the calcium also protects ruminal microbes from the toxic effects of the fatty acids, minimizing any detrimental effects on digestion. Therefore, feeding calcium salts would have a less negative effect on ruminal fermentation and result in increased passage of free fatty acids present in milk (Palmquist and Jenkins, 1980).

The objective of our study was to evaluate the effects of calcium salt fat supplements VALFEED 100 and VALFEED 200 on dietary intakes, body weights, and lactation performance.

MATERIALS AND METHODS

Animals and Diets

Twenty-eight Holstein cattle in early lactation were blocked by milk production, days in milk, and parity into four different treatment groups (Table 1). Each group of seven cows was then randomly assigned to one of four treatments as follows: 1) control diet (containing no supplemental fat); 2) diet + prilled fat source; 3) diet + calcium salt VALFEED100; 4) diet + calcium salt VALFEED 200. The fat replaced corn in the concentrate mix of the basal diet and was included in amounts equal to 3.2% of total dietary dry matter. Fat supplements VALFEED 100 and VALFEED 200 were provided by Valley Products, Inc and consisted primarily of vegetable oil. Both supplements were similar in their fatty acid profiles and contained mostly C 18:2 and C 18:1. Total fat percentage of VALFEED 100 and VALFEED 200 were 38 and 60%,

respectively. Total fat percentage of the prilled fat source was greater than 99% and it contained mostly saturated fatty acids C 16:0 and C 18:0.

The basal diet consisted of: corn silage, alfalfa haylage, whole cottonseed, nutrimax bypass protein, and concentrate mix, and was fed as a total mixed ration. Each dietary ingredient was weighed individually and blended in a drum-type mixer (Data Ranger®; American Calan, Inc., Northwood, NH). The ingredient composition of the basal diet and concentrate mix is included in Tables 2 and 3, respectively.

All cows were housed in free stalls equipped with Calan gates. Each cow was allowed two weeks prior to initiation of the study to adapt to the Calan gates. The cows were then fed their respective experimental diets for a period of 90 days. Feed was offered twice daily at 0800 and 1500 in amounts to allow ad libitum feed consumption.

Sample Collection and Analytical Analysis

Individual feed intakes were measured daily. Weekly feed composite samples were then ground through a Wiley mill with a 1 mm screen and stored for subsequent chemical analysis. Samples were analyzed for dry matter and Kjeldahl nitrogen content using AOAC (1984, 1999) procedures. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an Ankom 200 fiber extractor (Ankom Technologies, Fairport, NY) according to the method of Van Soest et al. (1991). Fatty acid concentration and lipid profiles of the fat sources were measured according to the method of Fellner et al. (1995).

Cows were milked twice daily, and milk production was measured and recorded daily. Milk samples were taken at days 30 and 90 of the trial. One aliquot of 10 mL was preserved with 2-bromo-2-nitropropane-1,3 diol and then shipped to the United DHIA Laboratory (Blacksburg, VA) where they were analyzed for fat, protein, and milk urea nitrogen (MUN) by

infrared spectroscopy (Multispec Mark I®; Foss Food Technology, Eden Plains, MN). Another aliquot of the milk samples was frozen at -70°C for analysis of milk lipid profile. Lipids from these samples were extracted by adding 10 mL of chloroform:methanol:ammonium hydroxide (12:12:1, vol/vol). The procedure developed by Kramer et al. (1997) was used for methylation of the milk lipids and analysis of fatty acid methyl esters using gas chromatography (model CP-3800; Varian, Walnut Creek, CA).

Calculations and Statistical Analysis

Fat-corrected milk (FCM; 4%) (Tyrrell and Reid, 1965) was calculated as follows:

$$4\% \text{ FCM (kg/d)} = (16.22 \times \text{milk fat, kg/d}) + (0.43 \times \text{milk yield, kg/d})$$

Data for intakes, body weights, and lactation performance were analyzed as a randomized complete block design using the general linear models (GLM) procedure of SAS (1999).

Significance was set at $P < 0.05$, and a tendency toward significance was set at $P < 0.10$.

RESULTS

Throughout the duration of the fat supplementation trial, three batches of the VALFEED 100M calcium salt and two batches of the VALFEED 200 calcium salt were received for supplementation of the experimental diets. Upon arrival of each batch, a grab sample was taken of the fat supplements and it was immediately dried and analyzed for dry matter and fat content (Table 5). The VALFEED 100 fat source had dry matter percentages of 68.8, 69.7, and 69.6 from batches 1, 2, and 3 respectively. The ether extract as a percent of dry matter was 43.0, 35.7, and 34.0 from batches 1, 2, and 3, respectively. The VALFEED 200 fat source had dry matter percentage values of 82.0 and 86.2, with ether extract values as a percent of dry matter amounting to 56.0 and 63.5 from batches 1 and 2, respectively. The prilled fat source was

purchased commercially in one batch, with a dry matter percentage of 99.8 and ether extract as a percent of dry matter of 99.0.

Dry matter intake was highest in cows fed the control diet without any fat supplementation (Table 6). Cows consuming prilled and VALFEED 100 supplemented diets consumed significantly less than the control group, while cows fed VALFEED 200 supplemented diets had dry matter intakes similar ($P > 0.10$) to the other three diets.

Increases in body weight among treatment groups were consistent with increases in dry matter intake (Table 6). Cows fed the control diet had the highest ($P < 0.05$) body weight gain when compared to cows consuming prilled and VALFEED 100 diets. VALFEED 200 supplemented diets did not significantly increase body weights when compared to the other three treatments.

Milk production (kg/d) was highest ($P < 0.05$) in cows fed the VALFEED 200 diet (Table 6). VALFEED 100 supplemented diets had similar ($P > 0.10$) effects on milk production as the prilled diet, which was significantly lower than the control diet. Fat-corrected milk yields (4%, in kg/d) were similar ($P > 0.10$) among control, prilled, and VALFEED 200 treatments, and VALFEED 100 treatments yielded the lowest 4% FCM yield (Table 5). Cows fed the control and prilled diets produced milk with a higher ($P < 0.05$) milk fat percentage than cows fed VALFEED 100, but milk fat percentage in milk from VALFEED 200 supplemented cows did not differ ($P > 0.10$) when compared to all treatment groups (Table 6). Milk protein percentage was highest in cattle fed the control diet and decreased significantly in cattle fed the VALFEED 100 and VALFEED 200 supplemented diets (Table 5). Milk protein percentage in milk from the prilled supplemented group did not significantly differ from the other three treatment groups. Control, prilled, and VALFEED 200 treatment groups did not significantly differ in milk fat

production (kg/d) (Table 6), but the VALFEED 100 treatment group produced significantly lower amounts of milk fat. Milk protein production (kg/d) did not differ ($P > 0.10$) across treatments. Milk urea nitrogen (MUN) also did not differ ($P > 0.10$) across treatments (Table 6).

Fatty acid composition of milk produced by the different treatment groups is reported (Table 7). Feeding VALFEED 100 produced the greatest effect in decreasing C 8:0, C 10:0, and C 12:0 levels, followed by feeding VALFEED 200. Feeding control diets with no fat supplementation resulted in the highest ($P < 0.05$) levels of C 8:0, C 10:0, and C 12:0, while feeding prilled diets produced similar ($P > 0.10$) effects to the control and VALFEED 200 diets. Supplementing diets with VALFEED 100 and VALFEED 200 significantly reduced C 14:0 levels when compared to both control and prilled diets. Fat supplementation had no significant effects across treatments in production of C 15:0, C 17:0, and C 18:0. VALFEED 100 and VALFEED 200 supplementation also decreased ($P < 0.05$) levels of C 16:0 when compared to control and prilled diets.

Addition of VALFEED 100 and VALFEED 200 increased ($P < 0.05$) total oleic acid (C 18:1) content when compared to the control and prilled diets. The increase was due to an increase ($P < 0.05$) in both the *cis*- and *trans*-isomers of C 18:1 (Table 7). The increase in C 18:1 *trans*-isomers was greater ($P < 0.05$) with the addition of VALFEED 100 than VALFEED 200. The C 18:1 to C 16:0 ratio in milk fat was 0.91, 0.98, 1.44, and 1.35 from the control, prilled, VALFEED 100, and VALFEED 200 treatment groups, respectively.

Both VALFEED 100 and VALFEED 200 significantly increased the linoleic acid (C 18:2) content of the milk when compared to the control and prilled diets (Table 7), more than doubling the amount of linoleic acid found in milk from cows fed control and prilled diets. Feeding VALFEED 100 and VALFEED 200 also significantly increased the conjugated linoleic

acid (CLA) content of milk (Table 6). Levels of the *cis*-9, *trans*-11 CLA isomer were highest ($P < 0.05$) when cows were fed VALFEED 100; feeding VALFEED 200 increased the *cis*-9, *trans*-11 isomer of CLA, which was similar ($P > 0.10$) to the effects of the prilled fat. Levels of the *trans*-10, *cis*-12 CLA isomer in milk were highest when cows were fed VALFEED 200; VALFEED 200 also increased *trans*-10, *cis*-12 CLA when compared to the control.

DISCUSSION

Dry matter intakes (DMI) of cows fed the control diet were higher than those of cows fed the prilled and VALFEED 100-supplemented diets. VALFEED 200-fed cows did not have significantly lower intakes. Although the VALFEED 100 and VALFEED 200 fat sources had higher moisture content and stronger odors than the prilled fat source, the trial cows did not experience any observable problems with palatability of the calcium salts.

Studies conducted with feeding supplemental calcium salts have reported variable effects on feed intake (Andrew et al., 1991, and Chalups et al., 1986). According to Andrew et al., the addition of long-chain fatty acids as calcium salts depressed dry matter intake (1991). However, variation in response to the diets can be dependent on the type and amount of fat added. Oilseeds and liquid fats can only be added to the diet at 6% of total dietary dry matter without decreasing fiber digestion, but rumen-inert fats can be added to the diet up to 8% of total dietary dry matter without negatively affecting digestion (Chalupa et al., 1986).

Supplementation of diets with VALFEED 200 increased DMI and milk yield when compared to supplementation with VALFEED 100. Milk efficiency, expressed as kg of FCM per kg of dry matter intake, was significantly higher with feeding of the VALFEED 200 fat source. Milk production was significantly lower in cows fed the prilled and VALFEED 100

diets, which had lower dry matter intakes. Milk fat percentage and milk fat yield was similar among cows treated with the control, prilled, and VALFEED 200 diets, but significantly lower when the VALFEED 100 diet was fed. Feeding supplemental fat produces variable responses in milk fat synthesis. Diets high in fat often exert negative effects on fiber-digesting bacteria in the rumen. Decreased levels of fiber digestion can decrease acetate:propionate ratios, and, therefore, decrease de novo fatty acid synthesis in the mammary gland. The addition of unsaturated fatty acids, especially from plant oils, to diets, will also increase biohydrogenation and production of trans-fatty acids in the rumen (Palmquist and Jenkins, 1982).

Reductions in milk fat yield can be coupled to an increase in *trans*-C 18:1 fatty acids produced in cows fed a high concentrate/low roughage diet (Gaynor et al., 1995). If a fat source is completely protected and inert in the rumen, it will not affect fiber digestion or trans-fatty acid production (Bauman and Griinari, 2001). Feeding VALFEED 100 resulted in increased levels of *trans*-C 18:1 fatty acids and a reduction in milk fat production when compared to feeding VALFEED 200, which suggests that VALFEED 200 remains more inert in the rumen.

Milk protein percentage decreased with the addition of VALFEED 100 and VALFEED 200 to the diet when compared to the control diet. Other studies have also shown decreases in milk protein percentage with the inclusion of supplemental fat in the diet (Andrew et al., 1991). Palmquist and Moser explained the phenomenon by the increase in insulin resistance as a result of dietary fat, which in turn decreases insulin-dependent uptake of amino acids by the mammary gland (1981). However, the mechanism behind milk protein depression with fat supplementation remains unknown. Fat is added to diets at the expense of concentrate concentration. Fat is not fermented in the rumen, but grains and starch are fermented into volatile fatty acids in the rumen. These VFAs, in conjunction with ammonian, combine to form amino acids, which are used to

synthesize microbial crude protein in the rumen. Therefore, the addition of fat decreases the amount of substrate availability for microbial protein synthesis, possibly contributing to a decrease in overall milk protein percentage (Firkins, 1996).

Feeding VALFEED 100 resulted in a depression of saturated fatty acids C 8:0 through C 12:0. VALFEED 200 treatment reduced these saturated fatty acids when compared to the control, but not to the extent of VALFEED 100 treatment. Both VALFEED 100 and VALFEED 200 had similar effects in reducing levels of fatty acids C 14:0, C 12:0, C 10:0, and C 8:0. The production of fatty acids with 14 carbons and less, and 50% of C 16:0 formation is a result of de novo fatty acid synthesis in the mammary gland. The depression of these fatty acids can be explained by inhibitory roles of supplemental fat on fiber digestion and de novo fatty acid synthesis (Gaynor et al., 1995).

VALFEED 100 and VALFEED 200 supplementation increased levels of oleic acid (C 18:1), linoleic acid (C 18:2), and conjugated linoleic acid (CLA) levels. Increased levels of unsaturated fatty acids in the milk can only occur when unsaturated fatty acids present in feed or supplements escape microbial action in the rumen. Biohydrogenation normally converts dietary unsaturated fatty acids into saturated fatty acids through the addition of hydrogen atoms, which would increase saturated fatty acids and decrease unsaturated fatty acid levels in milk. Feeding VALFEED 100 and VALFEED 200 increased production of *cis*- and *trans*- C 18:1 when compared to the control and prilled diets, but supplementation of VALFEED 100 increased *trans*-C 18:1 levels significantly more than did VALFEED 200. *Cis*- and *trans*- isomers are intermediaries of biohydrogenation. The presence of unsaturated fatty acids in milk from cows treated with calcium salts VALFEED 100 and VALFEED 200 proves that the fat sources must

remain at least partially inert in the rumen. However, it appears that VALFEED 200 is more stable in the rumen than VALFEED 100.

SUMMARY AND CONCLUSIONS

Fat supplements were added to experimental diets at 3.2% of total dietary dry matter, and fat levels in the diet from concentrate sources amounted to 5% of total dry matter. Therefore, combined fat was equal to 8.2% of dietary dry matter, a level that showed not to be detrimental to rumen microbes and fiber digestion if the fat sources were inert in the rumen. Consumption of the prilled and VALFEED 100 fat sources decreased dry matter intakes when included at this level. However, the VALFEED 200 fat source did not depress dry matter intake. Milk production increased when cows were fed the VALFEED 200 fat source. The supplementation of the VALFEED 200 fat source, along with controlled and prilled diets, also increased milk efficiency expressed in terms of kg of fat corrected milk per kg of dry matter intake when compared to VALFEED 100. VALFEED 100 and VALFEED 200 supplementation stimulated an increase in the levels of unsaturated fatty acids in milk, and, therefore, remained partially inert in the rumen. However, VALFEED 100 supplementation tended to also increase trans fatty acid levels, especially *trans*-C 18:1 fatty acids, contributing to milk fat depression (MFD).

VALFEED 200 can be utilized as an efficient fat source in addition to diets consisting of basal fat levels without decreasing dry matter intake. This fat source is also efficient in increasing milk yield and unsaturated fatty acid content of milk by decreasing rumen biohydrogenation, which will contribute to increased health benefits associated with human milk consumption.

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Table 1. Average milk yield, days in milk and parity for cows across the four treatments prior to the start of the experiment.

Variable	N	Mean	SD
ValFeed 100			
Milk	7	66.4	23.9
DIM	7	46.9	33.1
Parity	7	1.3	0.5
ValFeed 200			
Milk	7	65.1	13.1
DIM	7	38.4	29.2
Parity	7	1.3	0.5
Control			
Milk	7	66.0	19.7
DIM	7	47.6	34.5
Parity	7	1.3	0.5
Prilled			
Milk	7	64.4	15.3
DIM	7	46.4	26.3
Parity	7	1.3	0.5

Table 2. Ingredient composition of experimental diets (% of dry matter intake)

Ingredient	Control	Prilled	VF 100	VF 200
Corn Silage	33.8	33.8	33.8	33.8
Alfalfa Silage	15.0	15.0	15.0	15.0
Whole Cottonseed	16.8	16.8	16.8	16.8
Nutrimax Bypass	3.7	3.7	3.7	3.7
Concentrate Mix	30.7	30.7	30.7	30.7

Table 3. Ingredient composition of concentrate mix (% of dry matter)

Item	Control	Prilled	VF 100	VF 200
Corn grain ground	22.3	18.4	18.4	18.4
Soybean Meal	5.4	6.1	6.1	6.1
Supplemental fat	0.0	3.2	3.2	3.2
Limestone	0.8	0.8	0.8	0.8
Salt	0.4	0.4	0.4	0.4
MgO	0.09	0.09	0.09	0.09
NaHCO ₃	0.68	0.68	0.68	0.68
Bentonite	0.83	0.83	0.83	0.83
Dyna-Mate	0.06	0.06	0.06	0.06
McNess 1401	0.12	0.12	0.12	0.12

Table 4. Chemical composition of experimental diets.

Item	Control	SD	Prilled	SD	VF 100	SD	VF 200	SD
Dry Matter, %	50.0	0.76	48.6	1.16	48.4	1.37	48.0	2.29
Crude Protein, %	16.9	0.68	16.3	1.14	16.3	0.98	16.1	0.20
Neutral Detergent Fiber, %	47.2	1.27	47.4	0.28	48.9	0.62	50.5	1.63
Acid Detergent Fiber, %	26.7	0.53	25.9	0.40	26.4	1.12	27.2	0.49

Table 5. Ether extract content of supplemental fat sources.

<u>Fat Source</u>	<u>DM, %</u>	<u>EE, % of DM</u>
VF 100 - Batch 1	68.8	43.0
VF 100 - Batch 2	69.7	35.7
VF 100 - Batch 3	69.6	34.0
VF 200 - Batch 1	82.0	56.0
VF 200 - Batch 2	86.2	63.5
Prilled	99.8	99.0

Table 6. Dry matter intake, milk production and composition by cows fed experimental diets.

Item	Diets				SE
	Control	Prilled	VF 100	VF 200	
DM intake, kg/d	27.0 ^a	20.0 ^b	21.2 ^b	22.1 ^{ab}	1.90
BW ¹ , Kg	548.4 ^a	524.9 ^b	521.2 ^b	534.2 ^{ab}	16.87
Milk, kg/d	35.1 ^b	32.0 ^c	32.8 ^c	37.1 ^a	1.07
4% FCM, kg/d	31.1 ^a	29.2 ^{ab}	24.7 ^b	30.5 ^a	1.58
Milk/DMI, kg/kg	1.5	1.6	1.5	1.7	0.07
FCM/DMI, kg/kg	1.3 ^b	1.5 ^a	1.2 ^c	1.4 ^{ab}	0.04
Milk fat, %	3.3 ^a	3.4 ^a	2.4 ^b	2.9 ^{ab}	0.19
Milk protein, %	2.9 ^a	2.7 ^{ab}	2.6 ^b	2.6 ^b	0.07
Milk fat, kg/d	1.1 ^a	1.1 ^a	0.8 ^b	1.1 ^a	0.06
Milk protein, kg/d	1.0	0.9	0.9	1.0	0.06
MUN, mg/100ml	14.8	14.2	13.6	13.7	0.55

¹Initial body weights were used as a covariate.

^{a,b,c}Means with different superscripts differ ($P < 0.05$)

Table 7. Fatty acid composition of milk from cows fed experimental diets.

Fatty Acid (mg/g total FA)	Diets				SE
	Control	Prilled	VF 100	VF 200	
C8:0	0.6 ^a	0.4 ^{ab}	0.3 ^b	0.4 ^{ab}	0.09
C10:0	1.3 ^a	1.0 ^{ab}	0.6 ^b	0.9 ^a	0.17
C12:0	1.7 ^a	1.3 ^{ab}	0.9 ^b	1.0 ^{ab}	0.14
C14:0	7.8 ^a	6.6 ^b	5.0 ^c	5.4 ^c	0.36
C15:0	0.8	0.7	0.8	0.6	0.03
C16:0	31.8 ^a	31.5 ^a	25.5 ^b	25.8 ^b	0.79
C17:0	0.5	0.6	0.5	0.5	0.01
C18:0	18.8	19.1	17.2	18.3	1.04
C18:1 _{cis}	26.6 ^b	28.1 ^{ab}	29.1 ^a	29.1 ^a	0.78
C18:1 _{trans}	2.2 ^c	2.8 ^c	7.6 ^a	5.6 ^b	0.43
C18:2	2.2 ^b	2.3 ^b	4.5 ^a	4.9 ^a	0.45
C18:3	0.3	0.3	0.3	0.2	0.03
<i>c9t11</i>	0.4 ^c	0.7 ^b	1.0 ^a	0.8 ^{ab}	0.08
<i>t10c12</i>	0.03 ^c	0.04 ^{bc}	0.08 ^{ab}	0.12 ^a	0.01

^{a,b,c}Means with different superscripts differ ($P < 0.05$)

Experiment 2

EFFECT OF THE SEQUENCE OF FAT AND ANTIBIOTIC-IONOPHORES ON RUMINAL FERMENTATION AND MICROBIAL LIPIDS.

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ABSTRACT

When ionophores are added to diets supplemented with fat, ionophore effects decrease. This interaction stimulated interest in conducting this study. The inclusion of fat and ionophore-antibiotics and the sequence of their addition was investigated.

Ruminal fluid collected from a dry, fistulated cow was incubated in 8 fermentors for a total of eight days. The first two days of the trial represented a stabilization period. On day three, two fermentors received monensin (50 ppm), two received bacitracin (50 ppm), and the other four received fat (4.4% of DMI). On day 6, one of the fermentors receiving monensin and one receiving bacitracin got fat. The other two continued receiving either only monensin or only bacitracin. Of the remaining four fermentors that were receiving fat, one received monensin, one received bacitracin and the other two continued to only receive fat. Methane and pH were recorded several times daily. Culture samples were taken on days 2, 5, and 8 for analysis of SCFA, LCFA, NH_3 , and PCR.

There were no statistical differences ($P > 0.10$) in acetate and propionate production (mM) among treatments. However, the concentration of butyrate was higher ($P < 0.01$) in cultures treated with monensin when compared with cultures treated with bacitracin. The proportion of C 18:2 in rumen bacteria increased ($P < 0.05$) when monensin was added prior to fat in comparison to when fat was added prior to monensin. Preliminary results indicate that the sequence of fat and antibiotic addition effects the growth of Gram- and Gram+ bacteria. The results from this study show that the sequence in which fat and ionophore-antibiotics are added to mixed rumen cultures can alter the response of bacteria to the additives.

INTRODUCTION

Ionophores, such as monensin, are antibiotics that are added to diets of dairy cattle to reduce methane levels, increase production of propionate, decrease production of ammonia, and increase overall feed efficiency by decreasing waste production and increasing digestibility (Russell and Strobel, 1988). Ionophores are lipophilic substances that are capable of binding to the lipid bilayer of bacterial cell membranes. The interior of the ionophore is hydrophilic and capable of binding cations. Charged ions (“lipophobic”) cannot normally pass through the lipid bilayer, but the binding of cations to ionophores neutralizes their charges and facilitates their movement through the membrane and into the bacterial cell (Pressman, 1976).

Monensin selectively binds sodium (Na^+) and potassium (K^+) ions, which increases their subsequent transport rates. The concentration of Na^+ is greater outside the cell than inside the cell. This concentration gradient is maintained by the sodium-potassium pump, which requires ATP to pump three molecules of sodium out of the cell and two molecules of potassium into the cell, redistributing concentration and charge of the cell. Diffusion of sodium down its electrochemical gradient into the cell fuels the active transport of sugars and amino acids into the cell to provide the bacteria with nourishment. Monensin stimulates the influx of sodium coupled with the efflux of hydrogen, which increases the concentration of sodium within the cell (Russell et al., 1988). Thus, monensin eliminates the sodium gradient and decreases solute transport.

Monensin also stimulates the efflux of potassium coupled with the influx of hydrogen ions. Hydrogen ions are naturally pumped out of the cell, establishing a chemical and electrical gradient, or the proton motive force, which is used to drive ATP synthesis (Mitchell, 1961). When hydrogen ions are pumped into the cell as a result of monensin action, this proton motive force is diminished, decreasing ATP synthesis. The accumulation of free hydrogen ions in the

cell also creates acidic conditions toxic to the bacterial cell. The bacteria then deplete cellular ATP stores in attempt to reduce acidity and re-establish the proton motive force. These combined actions of monensin result in apoptosis (Dawson and Boling, 1987).

Bacitracin is a polypeptide antibiotic effective against gram-positive bacteria with similar effects as monensin on fermentation. However, bacitracin has a different mode of action and affects gram-positive bacteria by altering its peptidoglycan layer of the bacterial cell wall. Peptidoglycan is a strong structural component of the cell wall, and its alteration results in decreased cellular control. Bacitracin also inhibits the dephosphorylation of phospholipid carriers in the membrane, therefore disrupting cellular transport (Stewart and Strominger, 1967).

Fat can also be used as a feed additive, increasing energy density of the diet. Fat influences fermentation in similar ways as ionophores and bacitracin in increasing feed efficiency. However, when fat is added in combination with ionophores in diets, ionophores have reduced effects. According to Clary et al. (1993), ionophores added to diets containing supplemental fat did not increase average daily gain or feed efficiency, which are effects consistent when ionophores are added in the absence of fat.

Ionophores and fat appear to be negatively associated due to alterations in rumen fermentation. Ionophores can inhibit biohydrogenation in the rumen, increasing levels of unsaturated fatty acids and decreasing levels of saturated fatty acids (Fellner et al., 1997). Linoleic acid (C 18:2) has been shown to be incorporated into the cell membrane preferentially as phosphatidylcholine, with the amount of lipid incorporated into the membrane greater than the amount of lipid synthesized de novo from SCFA precursors (Demeyer et al., 1978).

When ionophores are added to phospholipid vesicle membranes, they associate with the polar phosphate head groups of the lipid, decreasing membrane fluidity. The binding of

ionophores to lipid groups in the membrane decreases rates of cation transport, which indicates impairment of ionophore efficiency (Kolber and Haynes, 1981). Ionophores, when associated with phospholipids in the membrane, restrict rotational freedom of lipid moieties in the membrane. The ionophores, bound to the phosphate head groups, are prevented from completely inserting into the membrane. The binding of ionophores to lipid groups may interfere with their binding to cations, which would explain decreased rate transports associated with binding to membrane phospholipids (Sankaram et al., 1987).

Therefore, the interaction between lipid moieties and ionophore-antibiotics can possibly alter the mechanistic properties of the additives. When lipids are present in the membrane (added previously in the diet), ionophores tend to have reduced effects. Therefore, the sequence of addition of fat and ionophore-antibiotics may affect the response of bacteria to the antibiotic feed additives.

The objective of our study was to evaluate the effect of adding fat to mixed cultures prior to and following the addition of ionophore-antibiotics on fermentation and microbial populations.

MATERIALS AND METHODS

Rumen Fluid collection and Fermentor set-up

Six liters of ruminal fluid was collected from a non-lactating, fistulated cow fed a predominately forage diet at the North Carolina State University Dairy Educational Unit. This fluid was transported to the laboratory in vacuum-sealed containers. In the laboratory, the fluid was then filtered through a double layer of cheesecloth into a wide-mouth flask and mixed thoroughly. Approximately 700 mL of ruminal fluid was distributed equally among eight fermentors. A few hours prior to addition of ruminal fluid, the fermentors were flooded with

carbon dioxide (CO₂) gas to displace any oxygen present and to stabilize an anaerobic environment similar to that in the rumen. CO₂ gas continued to flow through the fermentors at a fixed rate of 20 mL/min. A hot water bath was circulated around each fermentor, maintaining a temperature of 39°C, creating a warm environment in which ruminal bacteria are able to thrive. Artificial saliva was prepared in the lab as described by Slyter et al. (1966) and delivered to fermentors at a constant rate of .73mL/min, resulting in a fractional dilution rate of 6.8%/h. Automatic stirrers suspended in the center of each fermentor mixed cultures continuously at 10 rpm.

Dietary Treatments

The basal diet was the same across treatments, consisting of 100% forage. Alfalfa pellets were fed as the forage source at 90% dry matter. Therefore, 15 g of alfalfa as fed, or 13.5 g of dry matter was fed to each fermentor per day, divided equally between two feedings, throughout the entire eight days of the trial. Anaerobic solutions of monensin and bacitracin were added to diets throughout the trial. Monensin and bacitracin were dissolved in ethanol at .15g per 100 mL of ethanol, resulting in 225µL of antibiotic per dose. Therefore, monensin and bacitracin were administered at 50 ppm. The fat source used as a feed additive in experimental diets was vegetable oil, which mostly consisted of linoleic acid (C 18:2). Fat was fed at 4.4% of total dietary dry matter, which was .3mL or 300µL per dose.

The first two days of fermentor incubation represented a control period, allowing for stabilization of fermentor contents. During this time, alfalfa was fed twice daily, but no antibiotics or fat was administered. On day 3, monensin (500µL/d) was added to fermentors 1 and 3, bacitracin (500µL/d) was added to fermentors 5 and 7, and fat (600µL/d) was added to fermentors 2, 4, 6, and 8. This treatment continued through day 5 of the trial. On day 6, two of

the cultures receiving fat also began to receive antibiotic; fermentor 4 received fat and monensin, and fermentor 8 received fat and bacitracin. Fermentor 3 continued to receive monensin, but then also began to receive fat, and fermentor 7 continued to receive bacitracin, and also began to receive fat. Fermentors 2 and 6 continued to only receive fat, fermentor 1 continued to only receive monensin, and fermentor 5 continued to receive only bacitracin. This treatment continued through day 8, the final day of the trial.

Sample collection and analytical procedures

Methane and VFA

Ten μL of headspace gas samples from each fermentor were drawn into a gas-tight syringe (Hamilton Co., Reno, NV) and analyzed for methane (CH_4) using gas chromatography (model CP-3800; Varian, Walnut Creek, CA). These CH_4 samples were taken several times daily; before and after feedings. The pH of rumen contents in each fermentor was monitored continuously by pH probes inserted into the rumen fluid and recorded when CH_4 samples were taken, several times per day. Two mL of mixed rumen fluid was taken from each fermentor on day 3 (at the end of the control period) and day 8 (at the end of the trial) prior to AM feedings and analyzed for volatile fatty acid (VFA) content by gas chromatography (model CP-3800; Varian, Walnut Creek, CA) and also for ammonia-nitrogen ($\text{NH}_3\text{-N}$) using a colorimetric assay (Beechner and Whitten, 1970).

Lipid Content of Rumen, Solid and Liquid Adherent Bacteria

Five mL of ruminal fluid was also taken from each fermentor prior to AM feedings on day 3 and day 8 of the trial and frozen at -70°C for future analysis of ruminal lipid content. Frozen samples were thawed, methylated (Kramer et al., 1997), and analyzed for fatty acid composition by GLC (model CP-3380; Varian, Walnut Creek, CA). On the last day of the trial,

day 8, the remaining culture fluid, approximately 500 mL, were frozen at -20°C for subsequent analysis. Samples were thawed and then strained through cheesecloth and the strained fluid was used to isolate solid-adherent bacteria (SAB) and liquid-adherent bacteria (LAB) (Legay-Carmier and Bauchart, 1989). Lipids present in the SAB and LAB were extracted according to the procedure developed by Kates (1991) with some modifications. Six mL of the LAB/SAB bacteria pellet solution was added to 14 mL of methanol-chloroform (2:1). The mixture was shaken and left at room temperature for 1-2 hours, while shaking intermittently. The mixture was then centrifuged at 1000 rpm for 5 minutes. The supernatant extract was decanted into a 50 mL conical tube. The residue was re-suspended in 5 mL of methanol-chloroform-water (2:1:0.8). The mixture was shaken and centrifuged at 2000 rpm for 5 minutes. The supernatant extracts were combined and added to 5 mL of both chloroform and water. The mixture was then centrifuged for 2000 rpm for 5 minutes. The bottom chloroform phase was then carefully removed and placed into a tube to dry completely. The dried phase was then methylated (Kramer et al., 1997) and analyzed for fatty acids by gas chromatography (model CP-3800; Varian, Walnut Creek, CA).

Bacterial DNA and Real-Time PCR

On day 3, approximately 6 mL of ruminal fluid were taken from each of 8 fermentors and combined (totaling approximately 50 mL of total fluid), and on day 8, 50 mL of rumen fluid were taken from each of 8 fermentors and frozen at -70°C for subsequent DNA extraction. Bacteria in the culture contents were isolated using differential centrifugation (Legay-Carmier and Bauchart, 1989; Weisbjerg et al., 1991).

Extraction of bacterial DNA from the isolated pellet and real-time PCR were conducted using previously published procedures (Tajima et al., 2000; Tajima et al., 2001 and Whitford et

al., 1998). Extraction of bacterial DNA was accomplished with the following modifications. Five hundred μL of rumen fluid bacterial suspension was mixed with 500 μL of phenol equilibrated with 10mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.5g of 0.1 mm glass beads in a 2 mL conical, screw-cap tube. Then, 20 μL of 20% SDS was added, and the tubes were shaken 3 times for 2 minutes each, using a mini bead-beater with 3 minute intervals on ice. The tubes were then centrifuged for 5 minutes at 13,200 rpm, and the supernatant was transferred to a fresh tube. Then, 0.6 mL of phenol equilibrated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA was added and mixed by inverting. This was then centrifuged at 13,200 rpm for 15 minutes, and the top layer was transferred to a clean tube. Two volumes (600 μL) of isopropanol was added, and DNA was allowed to precipitate overnight. Then, the tubes were once again centrifuged at 13,200 rpm for 15 minutes. Isopropanol was poured off and the mixture dried. DNA was then re-suspended in 50 μL 1X RNase One ribonuclease buffer and treated with 5 μL RNase one ribonuclease, then it was placed in a 37°C water bath for 45 minutes. Then, 50 μL of 1X TE (pH 8.0) was added and mixed gently by inverting, then 500 μL of buffered phenol was added and mixed by inverting, and centrifuged for 15 minutes at 13,200 rpm. The aqueous layer was pipetted to a new tube. Two volumes of isopropanol (200 μL) was added and left to precipitate overnight at 4°C, then centrifuged at 13,200 rpm for 15 minutes. The isopropanol was then poured off and the pellet left to dry. DNA was then re-suspended in 100 μL 1X TE buffer at pH 8.0.

Real-time PCR on the isolated ruminal fluid DNA was performed according to methods described by Tajima et al. (2001). The original procedure could not be duplicated and it was necessary to improvise the method for repeatability and accuracy of data. The procedure that we used in our lab is as follows. The “master mix” was made, consisting of QuantiTect SYBR

Green PCR master mix 1 (Qiagen) (10 μ L), a forward primer (Tajima et al), 1 μ L, a reverse primer (Tajima et al), 1 μ L, and 7 μ L of SdH_2O , totaling 19 μ L of master mix. Then, 1 μ L of DNA and 19 μ L of master mix is added to each reaction tube. The plate was then sealed with an adhesive film and run in the Opticon thermo cycler (MJ research) at 94°C for 15 minutes, 94°C for 1 minute, annealing temperature for 30 seconds, 72°C for 1 minute, and then the plate was read. The plate was heated at 94°C for 1 minute and annealed, and at 72°C for 1 minute to be read forty more times. Then, it was heated again at 72°C for 10 minutes. The melting curve from 50°C to 90°C was read every 1°C and is held 2 seconds between reads. It was then cooled to 4°C, marking the end of the procedure.

Statistical Analysis

Data were analyzed according to a randomized complete block design using the Proc Mixed procedure of SAS (1999). Significance was set at $P < 0.05$, and a tendency toward significance was set at $P < 0.10$.

RESULTS

Total short-chain fatty acid (SCFA) production was higher ($P < 0.05$) in cultures treated with bacitracin when compared to those treated with monensin. Bacitracin tended ($P < 0.10$) to increase acetate production when compared to monensin, but acetate production was not significantly different when cultures were treated with either monensin or fat. Propionate production was 11.7 mM, 10.9 mM, and 10.3 mM when cultures were treated with monensin, bacitracin, and fat, respectively, but were not significantly different. Butyrate production ranged from 3.49 mM to 4.0 mM and 4.0 mM when treated with monensin, bacitracin, and fat, respectively, but did not significantly differ. When cultures were treated with monensin, valerate

production tended to be higher ($P < 0.10$) than when treated with fat. Isobutyrate levels were lower ($P < 0.01$) when cultures were treated with monensin when compared to cultures treated with fat. When cultures were treated with *monensin* isovalerate levels were higher ($P < 0.01$) than when cultures were treated with fat. However, cultures treated with bacitracin produced higher ($P < 0.01$) levels of isovalerate than cultures treated with monensin.

The effects that monensin, bacitracin, and fat had on molar proportions of SCFA production (molar %) in the rumen do not differ substantially (Table 1). The production of acetate in the cultures did not differ significantly when monensin, bacitracin, or fat was added. The addition of monensin increased ($P < 0.01$) ruminal propionate when compared to bacitracin but was similar ($P > 0.10$) to cultures receiving fat. The addition of fat tended to increase ($P < 0.10$) butyrate proportion compared to the addition of monensin. Treatment of cultures with monensin increased ($P < 0.05$) molar proportion of valerate and isovalerate when compared to treatment with fat. The addition of fat to cultures increased ($P < 0.01$) isobutyrate molar proportion when compared to monensin. The acetate to propionate (A:P) ratio was lower ($P < 0.01$) when monensin was added compared to that when bacitracin was added, but not significantly lower than the ratio produced when fat was added.

The effect of the sequence of the addition of fat and antibiotics on the molar proportions of short-chain fatty acids is shown in Table 2. Total production of SCFA was higher ($P < 0.05$) when fat was added to cultures prior to monensin when compared to adding monensin prior to fat. Adding fat prior to bacitracin also tended to increase ($P < 0.10$) total SCFA production when compared to adding bacitracin prior to fat. Acetate production (mM) was higher ($P < 0.05$) when fat was added prior to monensin when compared to fat added after monensin. There were no significant differences in acetate production when fat was added before or after bacitracin.

Propionate production was higher ($P < 0.05$) in cultures receiving fat prior to bacitracin when compared to cultures receiving bacitracin prior to fat. Propionate production did not differ according to sequence of monensin and fat addition. However, butyrate production was significantly higher ($P < 0.01$) when fat was added prior to monensin when compared to cultures receiving monensin prior to fat. Adding fat prior to bacitracin tended to increase ($P < 0.10$) valerate production when compared to adding bacitracin prior to fat. Isovalerate production increased ($P < 0.01$) when fat was added to cultures prior to monensin compared to when monensin was added prior to fat.

No statistical differences in acetate, propionate, and butyrate molar proportions were observed when fat was added before either monensin or bacitracin compared to when either monensin or bacitracin was added prior to fat. Molar proportion of valerate increased ($P < 0.05$) when monensin was added to cultures prior to fat compared to cultures that received fat prior to monensin. Isobutyrate molar proportion was significantly higher when bacitracin was added prior to fat than when fat was added prior to bacitracin. The A:P ratio did not differ due to the sequence in which fat and antibiotic were added to the cultures.

The concentration of methane (nmol/mL) was altered by the addition of the additives (Figure 1). Monensin significantly ($P < 0.05$) reduced methane concentration when compared with cultures receiving either bacitracin or fat. Sequence of fat and antibiotic addition to cultures also affected methane production (Figure 2). Cultures that received monensin prior to fat had a lower ($P < 0.05$) concentration of methane compared with cultures that received fat prior to monensin. However, the addition of bacitracin either before or after the addition of fat, to ruminal cultures, did not have a significant effect on methane production.

The concentration of long-chain fatty acids in ruminal cultures (mg/g of total fatty acids) that received monensin, bacitracin, or fat is reported in Table 3. Palmitic acid (C 16:0) concentrations were higher in cultures that received monensin or bacitracin compared with those that received fat. Concentration of C 18:0 increased significantly when fat was added to cultures. Both monensin and bacitracin resulted in a lower ($P < 0.01$) C 18:0 concentration. Monensin, bacitracin, or fat had no significant effect on total C 18:1 or trans-C 18:1 concentration. However, production of *cis*-C 18:1 increased ($P < 0.05$) when monensin was added to cultures above levels present when either fat or bacitracin was added to cultures. Levels of linoleic acid (C 18:2) were significantly higher in cultures receiving fat than in cultures receiving monensin or bacitracin.

The effect of the sequence of antibiotic and fat addition on the concentration of long-chain fatty acids in the rumen is shown in Table 4. There were no effects ($P > 0.10$) in concentration of C 16:0 (in mg/g of total fatty acid) when fat was added either before or after monensin or bacitracin. C 18:0 concentration was 16.5 mg/g of total FA and 23.2 mg/g total FA when monensin was added prior to and after fat was added, respectively, but concentration did not significantly differ. Total C 18:1 concentration was virtually identical with varying sequence of antibiotic and fat addition. *Trans*-C 18:1 concentration was 16.7 mg/g total FA and 19.4 mg/g total FA when monensin was added prior to and following fat addition, respectively, but no significant differences in concentration were reported. Levels of *cis*-C 18:1 increased ($P < 0.05$) when monensin was added prior to fat when compared with levels produced when fat was added prior to monensin. The sequence of fat and bacitracin addition had no effect on *cis*-C 18:1 production. The production of C 18:2 also increased ($P < 0.1$) when monensin was added prior

to fat compared to levels produced when fat was added prior to monensin. Sequence of bacitracin and fat addition had no effect on C 18:2 in cultures.

The fatty acid profile of liquid-associated bacteria (LAB) in the rumen cultures is shown in Table 5. The addition of monensin resulted in increased ($P < 0.01$) concentration (mg/g of total FA) of C 16:0 in LAB when compared to the addition of fat. C 18:0 concentration varied (23.1, 26.4, and 30.6) when monensin, fat, and bacitracin were added, respectively, but did not significantly differ. No significant effects were seen in concentration of total C 18:1 when cultures were treated with monensin, bacitracin, or fat. *Trans*-C 18:1 concentration was 12.8, 16.4, and 21.2 when treated with bacitracin, monensin, and fat, respectively, but were not significantly different. *Cis*-C 18:1 concentrations ranged from 12.5 to 14.1 and 14.6 when treated with monensin, fat, and bacitracin, respectively, but were not different ($P > 0.10$). C 18:2 concentration was 1.2, 3.5, and 3.7 when cultures were treated with bacitracin, monensin, and fat, respectively, but were not significantly different.

The effect of the sequence of fat and antibiotic addition to cultures on LAB lipid content (in mg/g of total fatty acid) is shown in Table 6. When monensin was added to cultures prior to fat, C 16:0 levels were higher than in cultures that received fat prior to monensin. Adding bacitracin to cultures before fat increased ($P < 0.05$) total C 18:1 and *trans*-C 18:1 concentration compared with the addition of fat prior to bacitracin. There were no significant differences in C 18:0, *cis*-C 18:1, or C 18:2 due to the sequence of fat and antibiotic addition.

The effects of adding monensin, bacitracin, and fat to cultures on the lipid content (in mg/g of total fatty acid) of solid-associated bacteria (SAB) is shown in Table 7. Addition of monensin tended to increase ($P < 0.10$) C 16:0 concentration more than the addition of fat. There were no statistical differences among treatments in the concentration of C 18:0, C 18:1 (total and

trans-), or C 18:2 in cultures receiving the additives. The addition of bacitracin to cultures tended to increase ($P < 0.10$) *cis*-C 18:1 concentration when compared to cultures receiving monensin. Concentration of the *trans*-10, *cis*-12 CLA isomer was higher ($P < 0.05$) in cultures receiving fat compared to cultures receiving monensin.

The effects of the sequence of fat and antibiotic addition on lipid content (in mg/g of total fatty acid) of SAB are shown in Table 8. There were no statistical differences in production of C 16:0 and C 18:1 (total, *cis*-, and *trans*-) due to sequence of additive addition. The addition of bacitracin prior to the addition of fat to cultures tended to increase ($P < 0.10$) C 18:0 concentration when compared to cultures receiving fat prior to bacitracin. There were no significant differences in concentration of C 18:0 according to sequence of fat and monensin addition to cultures. There were also differences in C 18:2 concentrations due to sequence of additive addition. When monensin was added prior to fat, C 18:2 levels were significantly higher than levels of C 18:2 produced when fat was added prior to monensin. Sequence of addition of bacitracin and fat did not effect ($P > 0.10$) C 18:2 concentration.

The effects of ionophore-antibiotics, fat, and the sequence of their addition to ruminal cultures on relative changes in *Prevotella ruminicola* is shown in Figure 3. The x-axis of the figure represents a percent increase in *P. ruminicola* populations with the different treatments above those populations present prior to treatment. The y-axis illustrates the three different additives followed by the different sequences of additive addition. Cultures receiving monensin had the greatest increase (~600%) in the population of *P. ruminicola* compared to cultures that received bacitracin (26% increase) or fat (212% increase) alone. When monensin was added to culture contents prior to fat, the population of *P. ruminicola* tended to increase (~900%) more

than populations that received fat prior to monensin (500% increase). Sequence of fat and bacitracin addition to cultures did not affect populations of *P. ruminicola* in the rumen fluid.

Treponema Bryantii, a gram-positive bacterial species also thriving in the rumen, was measured in cultures across treatments (Figure 4). Addition of fat to cultures resulted in a 100% increase in populations of *T. bryantii* when compared to populations present when cultures received no additive. Addition of monensin and bacitracin increased populations by 50% and 40%, respectively, above pre-treatment populations. The sequence of monensin and fat addition to cultures did not have a large effect on the percentage of *T. bryantii* present in cultures. However, cultures that received fat prior to bacitracin seem to have increased growth in populations of *T. bryantii* when compared to cultures that received bacitracin prior to fat.

DISCUSSION

The treatment of ruminal cultures with monensin, bacitracin, and had similar effects on concentrations of acetate and propionate in rumen cultures. Monensin significantly increased propionate concentration when compared to bacitracin. These results are consistent with studies comparing the effects of monensin and bacitracin on ruminal fermentation (Russell et al., 1988, and Russell and Strobel, 1989). Monensin and bacitracin have been shown to decrease acetate while significantly increasing propionate due primarily to their altering fermentation patterns by targeting mainly gram-positive bacteria (Russell and Strobel, 1988). The addition of long-chain fatty acids to diets has also been studied, and it has been documented that supplemental fat can reduce acetate:propionate (A:P) ratios by 50-60% (Chalupa et al., 1986), which is consistent with decreased acetate and increased propionate production that occurred in our study in response to added fat.

Although monensin and fat had similar effects on altering concentrations of acetate and propionate in rumen cultures, these two additives had different effects on isoacids valerate, isovalerate, and isobutyrate. Isoacids are formed during the breakdown of protein. Cellulolytic bacteria lack transport systems to absorb protein and more complex amino acids but can absorb the more simple-structured isoacids. Therefore, isoacids are essential growth factors for cellulolytic bacteria (Russell and Sniffen, 1984). Cellulolytic bacteria are extremely sensitive to monensin (Russell and Strobel, 1989). Valerate and isovalerate concentrations increase in response to treatment with monensin compared to being treated with fat. Because certain cellulolytic bacterial populations decline in response to monensin, their growth factors, the isoacids, are not utilized. Therefore, the concentrations of valerate and isovalerate may increase in response to the decline in cellulolytic bacteria caused by monensin exposure. Fat can also have a toxic effect on cellulolytic bacteria (Demeyer and Van Nevel, 1995) but may specifically target different bacteria than monensin. This may explain how fat increases isoacid isobutyrate concentrations when compared to monensin while other isoacids increase when exposed to monensin when compared to fat.

The sequence of ionophore-antibiotics and fat addition did not significantly alter molar proportions of acetate, propionate, or butyrate in rumen cultures. Fat and the ionophore-antibiotics individually had similar effects on concentrations of acetate, propionate, and butyrate in cultures, but monensin effects were increased in magnitude. Therefore, because the additives had similar effects, the sequence in which these additives are added to cultures should not alter these effects. However, molar proportions of valerate were altered due to sequence of monensin and fat addition to cultures. . When monensin was added to cultures prior to fat, it increased valerate above levels present in cultures when fat was added prior to monensin.

Free long-chain fatty acids, especially unsaturated fatty acids (which were added in this study), exert a toxic effect on methanogenic bacteria, therefore decreasing formation of methane in the rumen (Demeyer and Van Nevel, 1995). Bacitracin also acts against gram-positive methanogenic bacteria, reducing methanogenesis (Church, 1977). However, monensin, an ionophore, consistently has the strongest effect in decreasing the production of methane by selecting against formate and hydrogen-producing bacteria, which normally provide substrates necessary for methanogenesis (Van Nevel and Demeyer, 1977). Monensin had the greatest effect in lowering ruminal methane when compared with either bacitracin or fat.

When monensin was added to cultures prior to the addition of fat, methane levels were lower than levels in cultures that received fat prior to monensin. Monensin is a lipophilic ionophore with a mode of action dependent on its binding to bacterial cell membranes. Monensin affects bacteria by binding to the membrane and facilitating the movement of specific cations into and out of the cell, disrupting ion gradients, solute transport, and ATP synthesis (Russell et al, 1988; Dawson and Boling, 1987). When fat (especially linoleic acid, or C 18:2) is added to the diet, it is incorporated into the membrane as phosphatidylcholine (Demeyer et al., 1978). When ionophore is added to phosphatidylcholine-containing vesicles in the bacterial cell membrane, the ionophore is incapable of completely inserting into the membrane due to its interaction with the polar head groups of the lipid compound. In response, ion transport rates normally accelerated by ionophore action in the membrane are decreased, representing a decrease in ionophore efficiency (Kolber and Haynes, 1981).

Therefore, when the supplemental fat source (mostly C 18:2) was added to diets prior to the addition of monensin, the ionophore did not decrease methane production to the extent that methane production decreased when monensin was added to cultures prior to fat. Efficiency of

monensin decreased in response to fat added to the diet and to the bacterial membrane. The addition of bacitracin before and after the addition of fat did not result in the production of significantly different levels of methane. Bacitracin, a polypeptide antibiotic which is not an ionophore, has a different mode of action than monensin in inhibiting methanogenesis. The insertion of bacitracin into the membrane is not required for bacitracin to alter characteristics of methanogenic bacteria; therefore, the addition of fat prior to the addition of bacitracin does not alter its efficiency (Stewart and Strominger, 1967).

Addition of fat to cultures increased the concentration of stearic acid (C 18:0) when compared to cultures receiving monensin and bacitracin. Cultures receiving monensin and bacitracin received no supplemental C 18:2 and were offered forage-based diets, providing very little substrate for biohydrogenation. Increases in stearic acid (the end product of biohydrogenation) with fat supplementation are consistent with rapid biohydrogenation of linoleic acid by ruminal microorganisms. (Kepler et al., 1971). Ionophores can inhibit biohydrogenation in the rumen, increasing levels of unsaturated fatty acids, especially oleic acid (C 18:1), while decreasing levels of saturated fatty acids, such as C 18:0 (Fellner et al, 1997). In this case, the addition of monensin decreased C 18:0 levels below that of cultures receiving fat while increasing levels of *cis*-C 18:1 above that of cultures receiving either bacitracin or fat. Levels of C 18:2 were significantly higher in cultures receiving supplemental fat, but, these cultures were receiving supplemental C 18:2 while cultures treated with monensin and bacitracin received none.

The sequence of antibiotic and fat addition did have an effect on ruminal lipid metabolism. When monensin was added prior to fat, *cis*-C 18:1 and C 18:2 production was higher than that produced when fat was added prior to monensin. According to Fellner et al,

monensin can inhibit biohydrogenation due to its effects on specific hydrogenating bacteria (1997). However, when C 18:2 is added first and possibly incorporated into the bacterial membrane, the efficiency of monensin may be reduced, and cis-C 18:1 and C 18:2 levels are not increased to the extent that they are when monensin is added to cultures before addition of fat. In contrast; the sequence of bacitracin addition did not affect biohydrogenation, which may be explained by the difference between the mode of action of monensin and bacitracin.

Solid associated bacteria are adherent to feed particles in the rumen while LAB are free-floating bacteria and are associated with the liquid phase in the rumen. Solid associated bacteria, that are more predominant than LAB, have approximately three times the amount of fatty acid content than does LAB, due to preferential absorption of dietary fatty acids from attached feed particles (Legay-Carmier and Bauchart, 1989).

When monensin was added to cultures, C 16:0 levels present in LAB was significantly higher than levels present in LAB in cultures treated with bacitracin or fat. C 16:0 is a product of de novo lipid synthesis and is not related to biohydrogenation. Previous studies indicate that ionophores can increase microbial lipid synthesis (O'Kelly and Spiers, 1990), which is consistent with our data. When monensin was added prior to fat, levels of C 16:0 were greater than levels present in LAB when fat was added to cultures prior to monensin, which is consistent with the effects of fat addition on altering the bacterial membrane, which in turn can alter ionophore binding and efficiency.

When the lipid composition of SAB was evaluated, there were no statistical differences in LCFA concentration among treatments with monensin, bacitracin, and fat. There was a trend in increased C 16:0 concentration when monensin was added to cultures compared to those receiving bacitracin or fat. These changes in lipid composition are consistent with findings by

O'Kelly and Spiers (1990). When monensin was added prior to fat, levels of C 18:2 present in SAB were higher than those levels present when fat was added to cultures prior to monensin. When fat was added to diets first, the fat was apparently incorporated into the SAB, preventing the complete insertion and action of monensin, resulting in decreased levels of C 18:2 when compared to cultures receiving monensin first, avoiding any problems with interactions with fat in the membrane.

Prevotella ruminicola is a gram-negative, sugar-digesting, protein metabolizing bacterial species that is abundant in the rumen, constituting up to 60% of all ruminal bacteria.

P.ruminicola populations appeared to increase more when treated with monensin than when treated with either fat or bacitracin. The growth of *P.ruminicola* is normally depressed upon initial exposure to monensin, but then the species is notorious for developing resistance to monensin, thus increasing its growth rate in monensin-containing media (Dawson and Boling, 1984). Our results appear to indicate that *P.ruminicola* populations increase above pre-treatment populations more when treated with monensin when compared to cultures treated with bacitracin and fat. Sequence of monensin and fat addition also appear to impact growth of *P.ruminicola*. When fat was added prior to monensin, the fat could have been taken up by the bacteria, possibly preventing the complete insertion and exposure of monensin to *P.ruminicola*. This limited exposure to the ionophore may prevent *P.ruminicola* from developing resistance to monensin, which results in decreased levels of the bacterial species when fat was added prior to monensin.

Treponema bryantii is a gram-positive bacterial species that works in conjunction with other bacteria in the digestion of cellulose (Kudo et al., 1986). The addition of fat to diets seemed to increase the growth of *T.bryantii* when compared to growth in cultures receiving monensin and bacitracin. Monensin and bacitracin are known to have detrimental effects on

gram-positive bacteria to the lack of protection from the antibiotics provided by their bacterial membranes (Russell and Strobel, 1988). In this case, the addition of fat prior to the addition of bacitracin appeared to increase the population of *T.bryantii* when compared to the population in cultures receiving bacitracin prior to fat. The addition of fat to diets can result in the coating of fat on either feed particles or microorganisms (Devendra and Lewis, 1974). This “coating effect” may protect *T.bryantii* from the harmful effects of bacitracin to the unprotected gram-positive bacteria.

SUMMARY AND CONCLUSIONS

The addition of monensin to ruminal cultures produced expected responses in altering short-chain fatty acid and methane concentrations. Monensin addition also inhibited biohydrogenation by decreasing C 18:0 formation and thereby resulting in greater amounts of C 18:2 remaining intact. Bacitracin produced effects similar to those of monensin but was less effective. Adding fat to the cultures also produced similar effects on ruminal fermentation as antibiotic addition but to a lesser extent.

Methane production was influenced by the sequence of monensin and fat addition, but was not affected by the sequence of bacitracin and fat addition. The sequence of fat addition to cultures receiving monensin altered the biohydrogenation of rumen lipids. Linoleic acid was preferentially incorporated into the bacterial membrane, and, in doing so, impeded complete insertion of monensin into the membrane, and decreased its efficiency. The production of C 18:2 in ruminal lipids and solid-adherent bacterial lipids was altered by the sequence of fat addition to diets supplemented with monensin.

Finally, the sequence of fat addition to cultures receiving monensin and bacitracin appeared to shift the growth patterns of *Prevotella ruminicola* and *Treponema bryantii*.

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Table 1. The effect of monensin (M), bacitracin (B) and fat (F) addition on short chain fatty acids (SCFA) in mixed cultures of ruminal microorganisms.

Item	Additive			SE	Contrast	
	M	B	F		M vs F	M vs B
	mM					
SCFA, Total	48.9	58.6	46.3	4.06	NS	0.05
Individual SCFA						
Acetate (A)	29.8	35.2	29.9	2.99	NS	0.10
Propionate (P)	11.7	10.9	10.3	.87	NS	NS
Butyrate	3.49	4.00	4.00	.38	NS	NS
Valerate	1.10	1.19	0.92	.08	0.08	NS
Isobutyrate	0.28	0.30	0.40	.04	0.01	NS
Isovalerate	2.58	3.40	0.97	.16	0.0001	0.001
	Molar %					
Acetate (A)	60.9	60.6	64.3	2.19	NS	NS
Propionate (P)	23.8	18.8	22.4	1.09	NS	0.01
Butyrate	7.17	6.95	8.65	0.60	0.07	NS
Valerate	2.31	2.07	2.01	0.16	0.03	NS
Isobutyrate	0.58	0.52	0.87	0.08	0.003	NS
Isovalerate	5.26	5.93	2.10	0.34	0.0001	NS
A:P	2.57	3.30	2.93	0.19	NS	0.01

Table 2. The effect of the sequence in which monensin (M), bacitracin (B) and fat (F) are added on short chain fatty acids (SCFA) in mixed cultures of ruminal microorganisms.

Item	Sequence of Additive				SE	Contrast	
	MF	FM	BF	FB		MF vs FM	BF vs FB
	mM						
SCFA, Total	47.9	57.9	44.3	52.7	4.30	0.04	0.08
Individual SCFA							
Acetate (A)	28.2	35.5	27.8	33.0	3.15	0.03	NS
Propionate (P)	12.0	12.8	8.28	10.9	0.94	NS	0.03
Butyrate	3.76	5.04	3.97	4.45	0.38	0.01	NS
Valerate	1.11	1.13	1.03	1.23	0.09	NS	0.10
Isobutyrate	0.30	0.35	0.38	0.34	0.04	NS	NS
Isovalerate	2.54	3.17	2.81	2.99	0.18	0.01	NS
	Molar %						
Acetate (A)	58.9	61.2	62.7	62.0	2.40	NS	NS
Propionate (P)	25.1	24.6	18.6	20.5	1.19	NS	NS
Butyrate	7.89	8.70	8.97	8.68	0.66	NS	NS
Valerate	2.33	1.97	2.33	2.32	0.16	0.03	NS
Isobutyrate	0.64	0.61	0.87	0.67	0.08	NS	0.06
Isovalerate	5.31	5.56	6.55	5.82	0.38	NS	NS
A:P	2.38	2.79	3.38	3.08	0.21	NS	NS

Table 3. The effect of monensin (M), bacitracin (B) and fat (F) on ruminal culture long chain fatty acids.

FA	Additive				Contrast	
	M	B	F	SE	M vs F	M vs B
	mg/g of total FA					
C16:0	21.4	21.6	17.6	0.94	0.003	NS
C18:0	23.0	26.9	36.4	3.35	0.004	NS
C18:1	37.7	36.5	33.1	3.51	NS	NS
Trans	21.4	19.5	20.6	2.60	NS	NS
Cis	17.8	14.9	13.9	1.54	0.05	NS
C18:2	3.42	4.62	5.97	1.07	0.04	NS

Table 4. The effect of the sequence in which monensin (M), bacitracin (B), and fat (F) are added on ruminal culture long chain fatty acids.

FA	Additive				SE	Contrast	
	MF	FM	BF	FB		MF vs FM	BF vs FB
	mg/g of total FA						
C16:0	18.4	18.0	18.2	16.4	1.73	NS	NS
C18:0	16.5	23.2	26.6	26.0	3.70	NS	NS
C18:1	37.6	35.9	33.1	36.2	3.78	NS	NS
Trans	16.7	19.4	16.8	17.1	3.20	NS	NS
Cis	20.9	16.4	16.2	19.1	1.76	0.05	NS
C18:2	16.9	12.8	16.1	15.6	1.17	0.004	NS
CLA							
c9t11	0.44	0.35	0.34	0.28	0.12	NS	NS
t10c12	0.16	0.22	0.14	0.08	0.05	NS	NS

Table 5. The effect of monensin (M), bacitracin (B), and fat (F) on long chain fatty acids in liquid adherent bacteria.

FA	Additive			SE	Contrast	
	M	B	F		M vs F	M vs B
	mg/g of total FA					
C16:0	26.2	26.2	19.8	1.33	0.002	NS
C18:0	23.1	30.6	26.4	7.09	NS	NS
C18:1	28.9	27.4	35.2	5.55	NS	NS
Trans	16.4	12.8	21.2	3.81	NS	NS
Cis	12.5	14.6	14.1	3.73	NS	NS
C18:2	3.5	1.2	3.7	1.05	NS	0.16
CLA						
c9t11	0.46	0.40	0.73	0.21	0.18	NS
t10c12	0.17	0.49	0.50	0.17	0.14	NS

Table 6. The effect of the sequence in which monensin (M), bacitracin (B) and fat (F) are added on long chain fatty acids in liquid adherent bacteria.

FA	Additive				SE	Contrast	
	MF	FM	BF	FB		MF vs FM	BF vs FB
	mg/g of total FA						
C16:0	23.4	20.0	21.6	21.3	1.33	0.07	NS
C18:0	15.4	15.8	27.6	18.8	7.09	NS	NS
C18:1	39.2	44.3	59.0	39.5	5.55	NS	0.02
Trans	26.2	27.9	44.6	23.8	3.81	NS	0.005
Cis	13.1	16.4	14.4	15.7	3.73	NS	NS
C18:2	5.5	5.5	4.6	5.7	1.05	NS	NS
CLA							
c9t11	0.82	0.54	0.67	1.38	0.21	NS	0.011
t10c12	0.26	0.44	0.24	0.66	0.17	NS	0.11

Table 7. The effect of monensin (M), bacitracin (B) and fat (F) on long chain fatty acids in solid adherent bacteria.

FA	Additive			SE	Contrast	
	M	B	F		M vs F	M vs B
	mg/g of total FA					
C16:0	22.6	20.6	17.6	2.08	0.08	NS
C18:0	25.8	28.4	28.9	5.30	NS	NS
C18:1	34.7	35.6	38.8	6.53	NS	NS
Trans	19.6	13.7	21.1	3.72	NS	NS
Cis	15.1	25.1	14.5	5.22	NS	0.10
C18:2	2.7	1.1	2.9	1.00	NS	NS
CLA						
c9t11	0.67	0.98	1.15	0.28	NS	NS
t10c12	0.27	0.21	0.42	0.05	0.04	NS

Table 8. The effect of the sequence of monensin (M), bacitracin (B) and fat (F) addition on long chain fatty acids in solid adherent bacteria.

FA	Additive				SE	Contrast	
	MF	FM	BF	FB		MF vs FM	BF vs FB
	mg/g of total FA						
C16:0	18.8	19.0	20.4	17.3	2.08	NS	NS
C18:0	15.7	21.6	31.4	19.9	5.3	NS	0.07
C18:1	40.5	41.2	34.1	40.2	6.53	NS	NS
Trans	21.9	26.3	21.8	21.4	3.72	NS	NS
Cis	18.6	14.9	12.3	18.9	5.22	NS	NS
C18:2	10.4	6.5	3.0	4.8	1.00	0.03	NS
CLA							
c9t11	1.7	1.2	0.7	1.8	0.28	NS	0.02
t10c12	0.46	0.63	0.44	0.68	0.05	0.04	0.09

Figure 1. The effect of antibiotics and fat on methane concentration

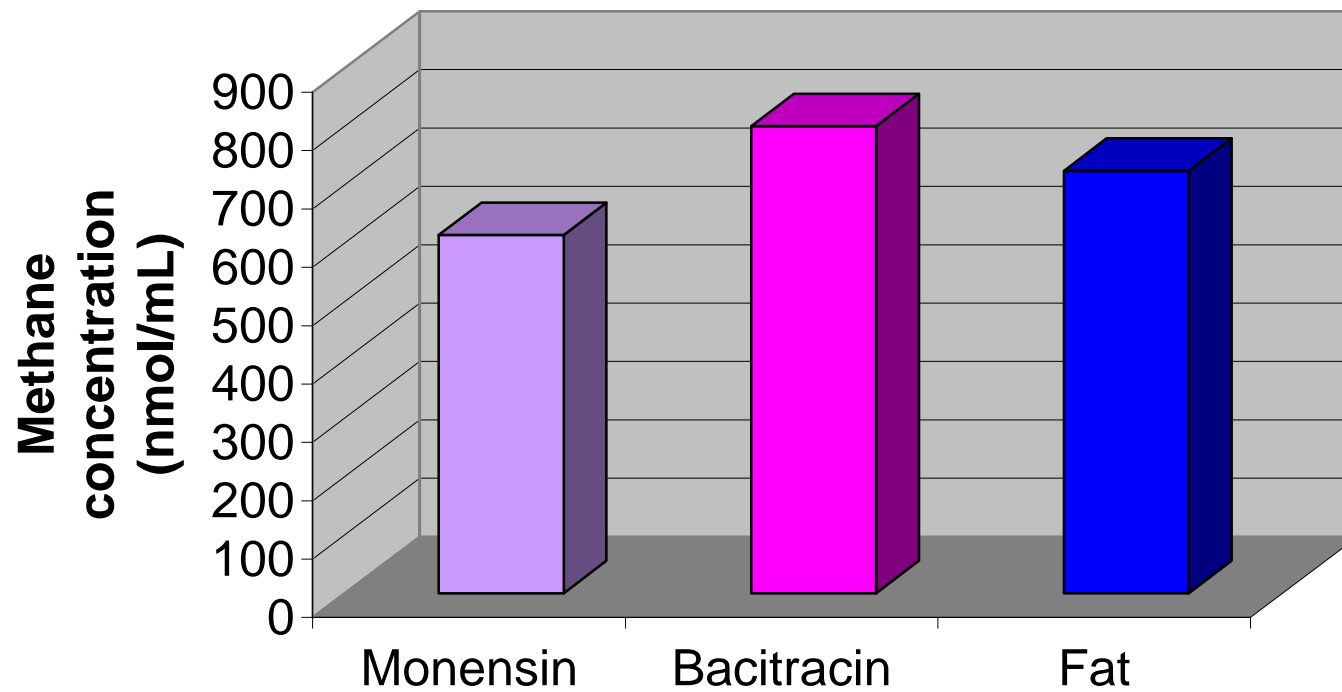


Figure 2. The effect of sequence of fat and antibiotic addition on methane concentration

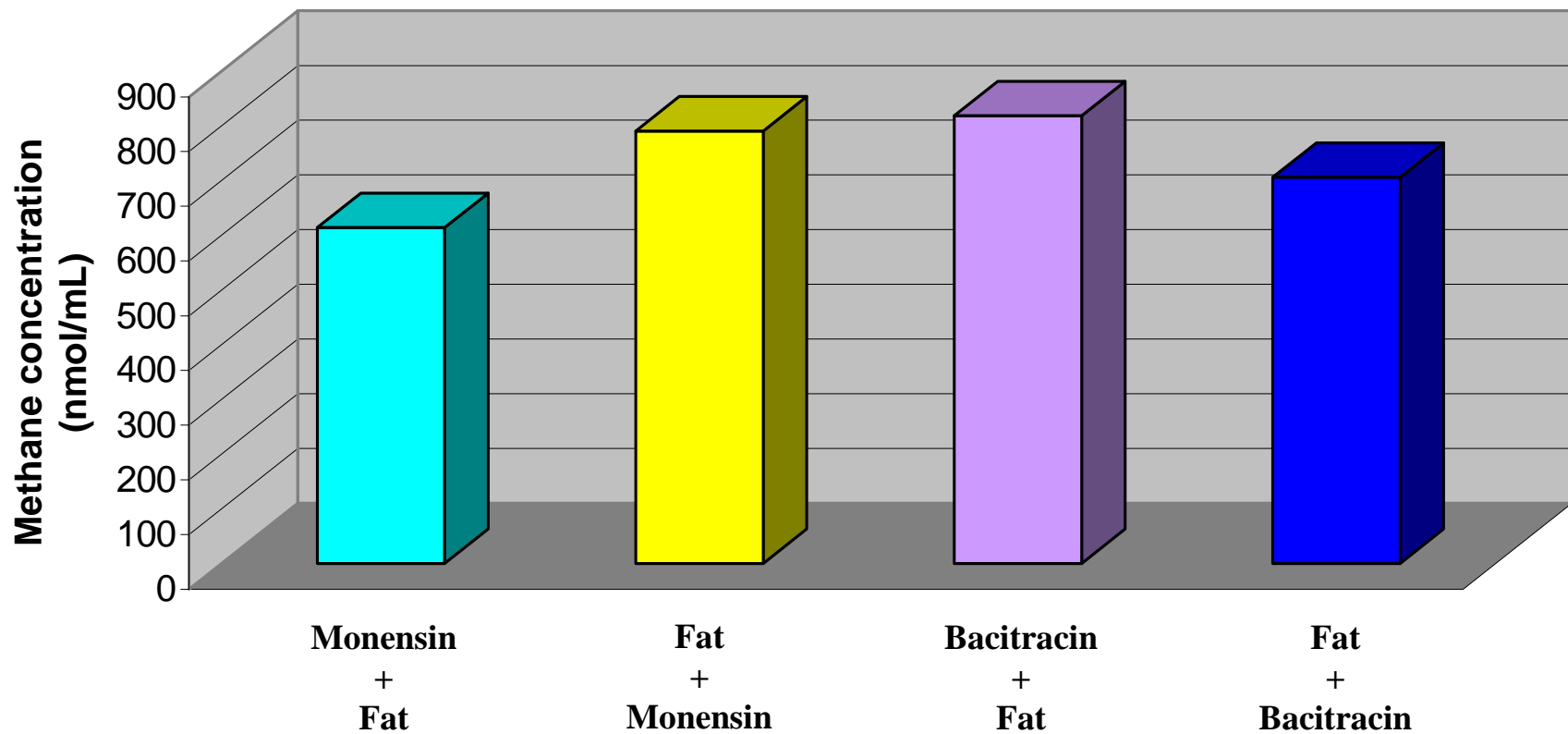


Figure 3. The effect of antibiotics, fat and sequence on the relative shifts in the populations of *Prevotella ruminicola*

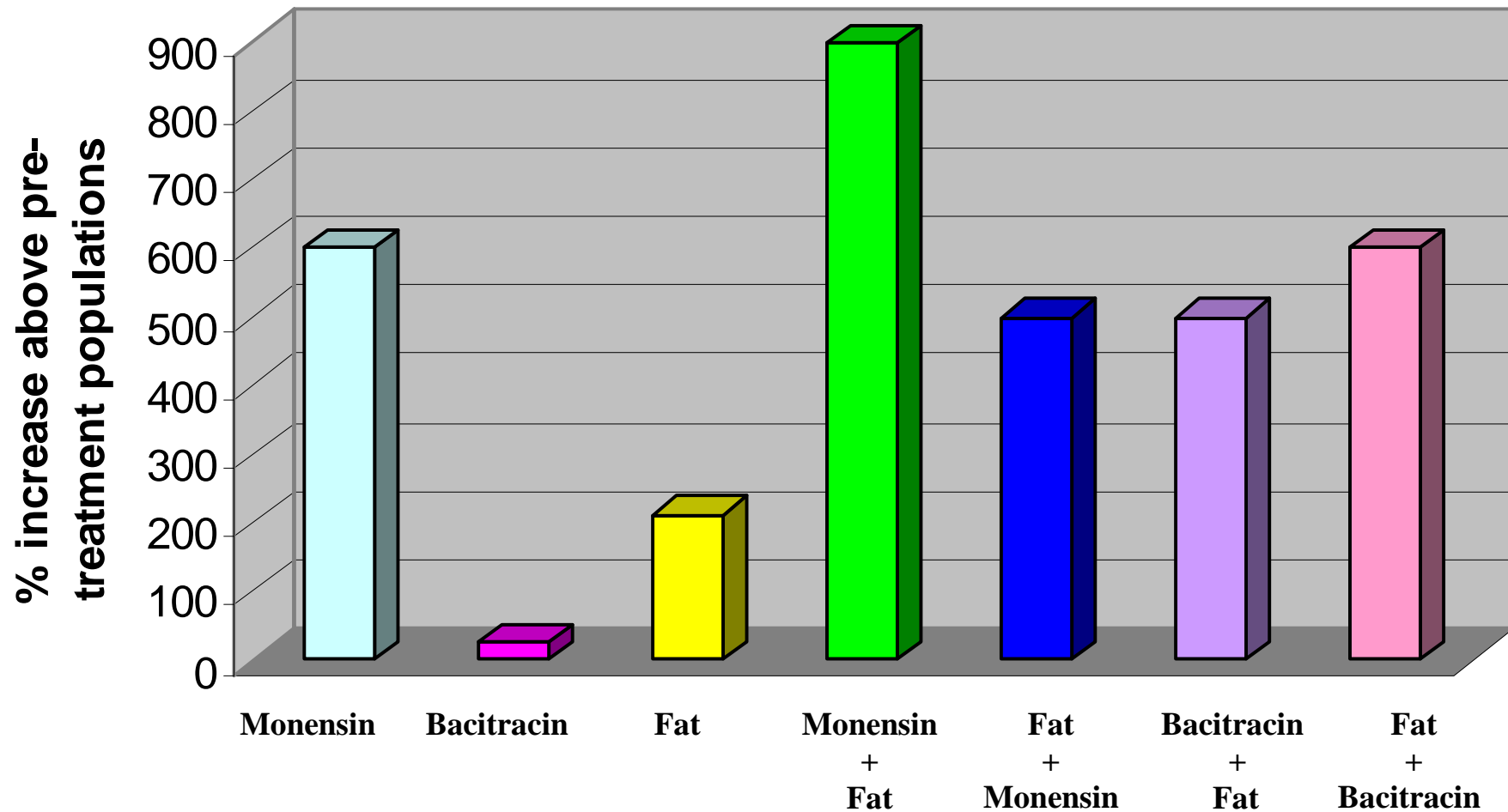


Figure 4. The effect of antibiotics, fat and sequence on the relative shifts in the populations of *Treponema bryantii*

