HEATHER ANN LYNCH
The Effect of Ensiling on Malate Content of Alfalfa, Corn, and Wheat
(Under Direction of SCOTT A. MARTIN)

Based on our understanding of ruminal fermentation, nutritionists and microbiologists try to formulate diets or develop feed supplements to manipulate the fermentation and increase the efficiency of production. Over the years, various techniques have been explored including dietary supplementation with ionophores, direct fed microbial products (yeast and fungi), or organic acids. Two of these approaches were further explored in my thesis 1) an investigation of the concentration of malate in a variety of ensiled forages and 2) the effects of *Saccharomyces cerevisiae* live cell yeast and yeast culture on in, alfalfa hay, and Coastal bermudagrass hay.

INDEX WORDS: Silage, Rumen, Microorganism, *Saccharomyces cerevisiae*, Malate, Alfalfa, Corn, Wheat, Anaerobic, Fermentation

THE EFFECT OF ENSILING ON MALATE CONTENT OF ALFALFA, CORN, AND WHEAT

by

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DEDICATION

To my family, friends, mentors, and colleagues who have supported my career goals throughout my life and during this program. I especially would like to dedicate this thesis to my nephew Tommy, a scientist for the next generation.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

History has shown that, both humans and animals can benefit from anaerobic microorganisms. One example includes anaerobic microbial food preservation methods to produce food for human consumption (i.e., yogurt, sauerkraut, and alcoholic beverages). Similarly, anaerobic methodology is used to produce silage for dairy and beef cattle. Silage is produced by anaerobic fermentation and preserves forages for animal consumption. In addition, ruminant animals have a unique symbiotic relationship with anaerobic microorganisms for the digestion of low quality feedstuffs. The microbial populations involved in food preservation (i.e., silage) and feedstuff digestion are different but equally complex. In order to exist in these complicated ecosystems, the different microorganisms have evolved into their own niches and developed unique survival strategies. It is hoped that by understanding the details of both silage fermentation and ruminal fermentation that the efficiency of production by domestic ruminants can be improved.

Silage

Silage can be defined as any type of plant material that undergoes a fermentation process that is usually stored in a silo (Whittenbury, 1968; McCullough, 1978; Ball, 1996). According to Van Soest (1994), "Silage, or more correctly ensilage, means the composting of fresh forage in an anaerobic system and its preservation by means of an acidic fermentation of the sugars present in the forage". It preserves most of the nutrients

in vegetative forage so that it may be stored and used later as a stable feed for the daily operation of a dairy, during winter months, dry periods, and/or as needed (Whittenbury, 1968; McCullough, 1978; Ball, 1996). However, utilization of nutrients during the ensiling process has led investigators to study the production of a complete fermentation with the least amount of nutrient loss.

"Manipulation of the process of ensilage to achieve adequate preservation, minimal losses, and a high nutritive value which is acceptable to the animal, requires a knowledge of the basic processes involved" (Edwards and McDonald, 1978). The ensiled forage passes through a series of steps during the fermentation including:

1) oxygen utilization until plant cell death, continuous proteolysis by enzymes, oxygen exhausted, and exchange of microbial population from aerobic to anaerobic.
2) homolactic and heterolactic fermentation of plant cell nutrients (soluble carbohydrates) and the anaerobic lactic acid producing bacteria begins to proliferate and decrease the pH.
3) increase microbial activity of acid-tolerant species and acid production.
4) an approximate pH value of 4.0 (depending on the moisture content) inhibits the further growth of the lactic acid bacteria and the silage reaches a stable or preserved state. The stable phase is when acid production is greatly reduced and the secondary anaerobic microorganisms equalize acid production with acid consumption (Zimmer, 1969; Edwards and McDonald, 1978).

At the appropriate plant maturity the crop is harvested and prepared for ensiling. There are various factors that affect silage quality and subsequent animal production, including

plant composition, weather at the time of ensiling, fermentation losses, silage nutrients, and animal acceptance. The primary determinant for the production of quality silage lies within the microbial fermentation. Therefore, the major acid producing and acid utilizing silage microorganisms will be discussed.

Silage Microorganisms

Enterobacteriacae

The Enterobacteriacae, also known as the coliform bacteria and acetic acid bacteria, are active in the first phases of the silage fermentation (Breirem and Ulvesli, 1960; Langston and Conner, 1962; Beck, 1978). They are Gram-negative, non-sporeforming, rod-shaped, aerobic to facultative anaerobic, often motile, non-pathogenic, catalase positive, nitrate-reducing microorganisms (Beck, 1978; Woolford, 1984). The enterobacteriacae ferment carbohydrates, including glucose, and lactose, and alcohol (Beck, 1978; Woolford, 1984). They have weak proteolytic activity; however, they can deaminate and decarboxylate amino acids (Beck, 1978). Beck (1978) explains that, "[f]ermentation products were found to be mainly acetic acid, CO_2 and lactic acid and to a less degree ethanol, 2,3-butanediol and H_2, the amount being dependent upon various factors, especially the pH and temperature." The Enterobacteriacae are not desirable for silage fermentation because they compete for fermentable sugars with lactic acid microorganisms, which are more efficient at reducing pH (Breirem and Ulvesli, 1960; Langston and Conner, 1962; Beck, 1978).

Lactic Acid Producing Microorganisms

The lactic acid producing microorganisms are microaerophilic, Gram-positive, non-spore-forming, often non-motile, rod or cocci shaped, and lack catalase (Beck, 1978;

Woolford, 1984). They are further divided into groups based on their type of fermentation (homofermentative or heterofermentative), stoichiometric properties of lactic acid produced, temperature growth range, and metabolic catalase (Beck, 1978; Woolford, 1984; Schleifer and Ludwig, 1992). "Four genera of lactic acid bacteria are associated with silage: *Lactobacillus, Streptococcus, Leuconostoc,* and *Pediococcus*" (Woolford, 1984). Distribution of bacteria associated with silage depends on the maturity of the silage.

The lactic acid producing bacteria use one of two biochemical pathways during their fermentation. "The fate of the sugars during ensilage depends upon whether the homofermentative or heterofermentative lactic acid producing bacteria predominate after the establishment of anaerobiosis" (Edwards and McDonald, 1978). Homolactic fermentation involves the glycolytic pathway, while the heterolactic fermentation follows the hexose monophosphate pathway. These two pathways have different products and efficiency values. Homolactic fermentation of glucose and fructose yields two moles of lactate per mole of sugar fermented, complete dry matter recovery, and 0.7% loss of energy (Edwards and McDonald, 1978). "The heterolactic fermentation of glucose yields one mole of lactate, one mole of ethanol, one mole of carbon dioxide [24% loss of dry matter and 1.7% loss of energy]" (Edwards and McDonald 1978). "Heterolactic fermentation of fructose yields one mole of lactate, one mole of acetate, one mole of carbon dioxide, two moles of mannitol [5% loss of dry matter and 1% loss of energy] for each [three] moles of fructose fermented" (Edwards and McDonald, 1978). Both types of bacteria ferment pentoses by using the same pathway, which yields one mole of lactate and acetate per mole of pentose (Edwards and McDonald, 1978). Heterolactic

fermentation is responsible for the accumulation of acetic acid, which is less desirable in silage (Beck, 1978). Ideally, homolactic fermentation will predominate during the ensiling process for the most efficient preservation. "In view of the preponderance of [lactic acid producers] on herbage, the nature of the carbohydrate in the material to be ensiled is of considerable importance" (Edwards and McDonald, 1978). However, other forage components are also important. "McDonald and Whittenbury (1973) reported that [the] organic acids, citrate and malate, are fermented by a number of pathways resulting in the formation of several products, including lactate, acetate, formate, ethanol, 2,3-butanediol and acetoin" (Beck, 1978). Others have also reported the breakdown of malate and citrate in silage (Keddie, 1959; Wood and Holzapfel, 1992). Keddie (1959) suggested, "that [the dissimilation of malate] is a usual feature of the low temperature homofermentive group [(*lactobacilli*)]."

Clostridia

Clostridia or butyric acid bacteria are very important in influencing silage quality because they cause silage spoiling (Gibson, 1965; Beck, 1978; Woolford, 1984). Even though they are not usual inhabitants of the forage, they may inoculate the silage during harvest from the soil but this remains unclear (Gibson, 1965; Woolford ,1984). These bacteria can grow during any stage of the silage fermentation, but they generally grow during the later stages of fermentation (Woolford, 1984). Clostridia are strict anaerobes, Gram-positive, spore forming, motile, rod-shaped, and without catalase activity (Bryant, 1956; Beck 1978; Woolford, 1984). They ferment sugars, proteins and organic acids (Beck, 1978; Woolford, 1984). Woolford (1984) describes that silage clostridia can be broken down into two groups, saccharolytic and proteolytic. "The saccharolytic species

include *C. butyricum, C. paraputrificum, C. sphenoides, C. tyrobutyricum,* and *C. scatol.* The proteolytic species are *C. bifermentans* and *C. sporogenes* as well as the combined saccharolytic/proteolytic species *C. perfringens* [which infrequently occurs in silage]"(Woolford, 1984). The saccharolytic type can ferment lactate and are low pH tolerant, while the proteolytic type ferment proteins and amino acids (Bryant, 1956; Gibson, 1965; Beck 1978; Woolford, 1984). Clostridial activity in silage can lower the concentration of the essential amino acids lysine, threonine, and serine (Beck, 1978). Both the saccharolytic type and proteolytic type can ferment two moles of lactic acid in the presence of acetate to produce one mole of butyrate, CO_2 , and H_2, which increases the pH (Beck 1978). Reduction in essential amino acid content and increased pH negatively affects the quality of the silage.

Bacillus

Bacillus are Gram-positive, aerobic to facultative, spore-forming, catalase producing, rod-shaped microorganisms. Under anaerobic conditions, they are able to reduce nitrogen (Beck, 1978; Woolford, 1984; Wood and Holzapfel, 1992). Bacillus are often found in deteriorating silage after the fermentation process is complete and the silo is opened (Beck, 1978). Therefore, these microorganisms are not considered to be significant to silage fermentation (Woolford, 1984). They are rarely isolated from good quality silage (Beck, 1978).

Yeast

Yeast also contribute to the instability of silage. They are oval or elliptical, and budding, spore and pseudomycelia forming microorganisms. Their abilities to oxidize or ferment a variety of sugars are used for classification purposes. There are two

physiological groups of yeast involved in silage. The first are bottom-growing (sedimentary) yeasts that ferment sugars, but not lactic acid. They are members of the genus *Torulopsis*. The second group is the top growing (pellicle) yeast that primarily breakdown lactic acid (Bryant, 1955; Gibson, 1965; Beck, 1978; Woolford, 1984). "Isolates of the latter group were members of the following genera: *Candida, Hansenula,* and *Pichia*, and occasionally *Saccharomyces*" (Woolford, 1984). "The accumulation of high amounts of ethanol in aerated silage (Laube, 1967) indicates the possibility of ethanol fermentation caused by yeasts" (Beck, 1978).

Fungi

Fungi and propionic bacteria have also been isolated from silage, but are not significant in the silage fermentation process. Fungi are acid tolerant and it is thought that they appear after the silo is opened from germination of spores which survived the silage fermentation (Woolford, 1984). The propionic bacteria use lactic acid to form propionic acid in silage (Woolford, 1984). They also produce CO_2, acetic acid, butyric acid, and formic acid (Woolford, 1984).

Silage Microbiology Manipulation

Preservation of vegetative forages for feeding at a desired time after storage is made possible by the elaborate relationship of the above discussed microorganisms. Recent research has focused on the manipulation of this process by pre-treating the forage prior to ensiling. Some pre-treatments include wilting, inoculating with lactic acid bacteria (Kung et al., 1990; Froetschel et al., 1991; Nadeau et al., 2000), adding enzymes (Jaakkola et al., 1990; Pitt, 1990; Jacobs and McAllen, 1991; Jacobs et al., 1991; Nadeau et al., 2000), or adding acids to quickly reduce the pH (Beck, 1978; McHan, 1986;

Choung and Chamberlain, 1992; Ridla, 1993; Nadeau, 1996; Shepard and Kung, 1996; Ridla and Uchida, 1997; Nadeau et al., 2000). There are numerous factors affecting silage quality and more research is needed to explore additional management techniques to help the producer have more control over the fermentation process.

The Ruminant

Random House Webster's College Dictionary defines a ruminant as, "any of a group of four-footed, even toed, and cud chewing mammals, as the cattle, buffalo, bison, goat, deer, antelope, camel, giraffe, llama, etc. which have a stomach consisting of four divisions or chambers, the rumen, reticulum, omasum, and abomasum; the grass, etc. that they eat is swallowed, unchewed, and passes into the rumen or reticulum from which it is regurgitated, chewed and mixed with saliva, again swallowed, and then passed through the reticulum and omasum into the abomasum, where it is acted on by the gastric juice." This definition has one major oversight, it does not mention the anaerobic fermentation by ruminal microorganisms, which is the animal's primary source of digestion. Ruminant animals have developed a symbiotic relationship with microorganisms (Van Soest, 1994; Russell and Wilson, 1996). The ruminant animal provides a suitable environment for the microorganisms to inhabit and grow, and the microorganisms provide the ruminant animal nutrients for growth via their metabolic products and as microbial protein.

According to Van Soest (1994), the ruminant animal has one stomach with several compartments depending on the species. For example, cattle, sheep, deer, and antelope have four compartments, while llamas and camels have three compartments (Van Soest, 1994). The stomach occupies three-quarters of the ruminant's abdominal

cavity, with the rumen being the largest compartment. Ruminants are pre-gastric fermenters. Therefore, the anaerobic fermentation occurs before the digesta reaches the abomasum (true gastric stomach). The food enters the reticulum or rumen based on its size and weight. The honeycomb-shape of the reticulum assists in additional breakdown of larger material, and distributes smaller roughage and large particulate matter to the rumen, and liquids and finely ground material to the omasum. The omasum regulates the flow from the reticulo-rumen to the abomasum. It also acts as a filter for larger particles, which will re-enter the reticulo-rumen. "Although particles of 5 cm may pass through the reticulo-omasal orifice, most particles leaving the rumen are smaller than 1 mm" (Welch, 1986). Arguably, neither of the first three chambers have mammalian enzymes or mucosa (Jouany and Ushida, 1999). The rumen is responsible for most of the mixing of digesta and is the major site of nutrient absorption and fermentation. The rumen supports up to 10^9-10^{10} cells of bacteria per ml, 10^6 protozoa per ml, and $10^3 - 10^5$ fungi per ml and can contain 50 to 70 liters of liquid and digesta (Van Soest, 1994; Weimer, 1996; Jouany and Ushida, 1999; White et al., 1999). "The microbes that inhabit the gastrointestinal tracts of herbivorous vertebrates are the main agents for the digestion of complex carbohydrates in ingested plant material", (Van Soest, 1994). The major characteristics and functions of these microorganisms will be discussed in the following sections.

The Ruminal Microorganisms

The rumen environment provides several unique factors that allow anaerobic microorganisms to flourish. Although it is a continuous system with a constant turnover of liquid and particulate matter (Hungate, 1966; Van Soest, 1994; Wells and Russell, 1996; Weimer, 1996; Van Kessel and Russell, 1996), the predominant ruminal

microorganisms have a generation time that is less than the retention time of the rumen (Van Soest, 1994). The ruminant provides an anaerobic, isothermal environment (38˚ - 41°C), with an oxidation-reduction potential of –3.0 V (Van Soest, 1994). Although there is a constant flow of water and ingesta out of the rumen, and production of volatile fatty acids (VFA), the pH remains relatively constant between 6.5 to 6.8 (Hungate, 1966; Van Soest, 1994). The pH stability is maintained by several factors. The high concentration of bicarbonate in ruminant saliva, microbial production of VFA, as well as absorption of VFA through the rumen wall can contribute to maintaining a stable pH (Hungate, 1966; Van Soest, 1994).

Although the environment is relatively stable, there is tremendous competition among the ruminal bacteria, protozoa and fungi. Each microbial species has evolved in several ways to increase their survivability. "Species may be considered in terms of the substrate used, products formed, or growth requirements" (Van Soest. 1996). The ruminal microorganisms can be differentiated according to the substrate that they prefer to attack (Hungate, 1966). The cellulolytic, amylolytic, and other substrate digesters (including pectin, lactate, glycerol, methanol, and hydrogen), and protozoa and fungi will be briefly discussed.

Cellulolytic Bacteria

"Plant structural carbohydrates are major contributors to the energy requirements of the ruminant" (Weimer, 1998). The structural carbohydrate, cellulose, is the most abundant polymer in the world and the major component of forages (Weimer, 1996; Russell and Wilson, 1996). It is a linear homopolymer of glucose with a β 1-4 linkage between the glucose monomers. Cellulose sheets arrange in a manner similar to bricks.

This makes the compound extremely resistant to hydrolysis and provides the plant its strength and physical structure components. Fortunately, ruminants have cellulolytic bacteria with the ability to break these β 1-4 bonds into smaller units, for example cellobiose and cellodextrins. These smaller units can also be utilized by the bacteria. However, "[f]orage intake and digestibility are limited by forage cell walls, which are only partially digested by ruminal microorganisms" (Nadeau et al., 1996).

The primary cellulolytic bacteria are *Ruminococcus flavefaciens, Ruminococcus albus,* and *Fibrobacter succinogenes* (Russell and Wilson, 1996; Weimer, 1996; Shi et al., 1997; Weimer, 1998). Although limited, some strains of *Butyrivibrio fibrisolvens* are cellulolytic (Weimer, 1996; Russell and Wilson, 1996). All cellulolytic bacteria are also able to digest hemicellulose (Hungate, 1966). The cellulolytic bacteria attach themselves to the surface of the fiber using a polysaccharide secretion (Shi et al., 1997; Weimer, 1998). Shi et al. (1997) conducted batch culture and continuous culture experiments and found that, "[m]ore than 70% of the total extent of adherence that was observed for each strain occurred during the first minute of incubation." *R. flavfaciens, R. albus,* and *B. fibriosolvens* attach to the surface with a capsule. Hungate (1966) described *F. succinogenes* as having "mucoid extracellular material" or a "bleb", which acts similarly to a capsule. A bleb secretes a polysaccharide-protein mix to attach to the particulate surface. The direct attachment via a capsule or bleb has several advantages. For example, attachment creates its own micro-environment where the enzymes and products from hydrolysis are localized near the cell surface (Weimer, 1998). Close association to the fiber surface decreases proteolysis of cellulase and predation by the larger protozoa (Weimer, 1998).

The two *Ruminococcus* species are considered the most active plant fiber degraders in the rumen and *R. albus* outnumbers *R. flaveflaciens* (Stewart et al., 1997). This can be attributed to a more limited range of substrate fermentation by *R. flaveflaciens* compared to *R. albus*. *R. flaveflaciens* and *R. albus* require branched-chain VFA (BCVFA), vitamins, and ammonia for growth. *Megasphaera elsdenii*, which will be discussed later, provides these cellulolytic bacteria with BCVFA in the rumen by deaminating branched chain amino acids (Allison, 1978, Stewart et al., 1997).

"*F. succinogenes* is one of the most widespread bacteria of the rumen (Van Gylswyk and Vander Toorn, 1986; Varel and Dehority, 1989)" (Stewart et al., 1997). This bacterium can compete with the *Ruminococci* by degrading some cellulose that is not used by *R. flavefaciens*. In addition, *F. succinogenes* has a greater ability of adhering to more kinds of feed particles (Shi et al., 1997), and is resistant to feed antibiotics (Stewart et al., 1997). However, *R. flaveflaciens* can compete because it adheres to food particles faster than the other predominant cellulolytic species (Weimer, 1996; Shi et al., 1997). Finally, *R. albus* is competitive because it can adapt to rapid growth on low concentrations of cellobiose, uses hemicellulose and pentoses, and produces bacterocins which inhibit other cellulolytic ruminal bacteria (Shi et al., 1997; Stewart et al., 1997).

Amylolytic Bacteria

The predominant starch-digesting bacteria are *Prevotella ruminicola, Ruminobacter amylophillus, Selenomonas ruminantium, Streptococcus bovis* and *Succinomonas amylophillus*. "[Prevotella sp.] is one of the most numerous groups of ruminal bacteria and is found in ruminants fed on various diets. In silage-fed cattle from Sweden, these bacteria accounted for up to 60% of all isolates (Van Gylswyk, 1990)"

(Stewart et al., 1997). *P. ruminicola* makes up to 19% of total bacterial counts, which, "is partially explained by its ability to use a variety of carbon and nitrogen sources and its highly efficient energy metabolism" (Lou et al., 1997). *P. ruminicola* also degrades protein and peptides (Stewart et al., 1997; Griswold et al., 1999). Even though they do not degrade cellulose, many of the amylolytic bacteria depend on the crossfeeding of end products by the cellulolytic bacteria for growth (Stewart et al., 1997).

Another bacterium, *R. amylophillus*, appears to be the predominant starch digester, even though it cannot utilize glucose (Hungate, 1966; Stewart et al., 1997). As seen with cellulolytic bacteria, *R. amylophillus* attaches to the surface of the starch grain during digestion (Hungate, 1966). It has been estimated that 15% of the isolates recovered from a grain mixture fed diet was *R. amylophillus* (Stewart et al., 1997).

S. ruminantium is numerous when the ruminant is fed cereal grains and can account for between 22 and 51% of the total cell count (Stewart et. al., 1997). Similar to *Prevotella* sp., *S. ruminantium* ferments a wide range of substrates, including lactate. *S. ruminantium* can decarboxylate succinate to form propionate (Stewart et al., 1997) and is able to store glycogen for later use if other substrates are not available (Lou et al, 1997). *S. ruminantium* has been identified as an important ruminal bacterium against acute acidosis. Slyter defines acute acidosis as "a result of excessive consumption of fermentable carbohydrates which causes a non-physiological decrease in pH and the production of toxic factors" (1976). Acidosis is a result of lactate accumulation in the rumen and decline of total VFA production (Slyter, 1976). *S. ruminantium* can ferment lactate into acetate and propionate using the randomizing pathway at low glucose concentrations. (Stewart et al., 1997). In addition, *S. ruminantium* has a higher tolerance

for low pH than most other ruminal bacteria. These adaptations are important when high concentrations of starch are fed to ruminants because ruminal pH drops with increased lactate accumulation. *S. ruminantium* decreases the concentration of lactate in the rumen, and contributes to total VFA production, which can be advantageous to maintaining the normal rumen environmental conditions.

S. bovis is a common species on most diets, but it is rarely present at high numbers unless large amounts of starch or soluble sugars are fed. *S. bovis* has both extracellular and intracellular amylase activity, and it is the most rapidly active amylolytic species on cereal grains which allows *S. bovis* to play a major role in the development of rumen acidosis when cattle are fed high concentrations of starch (Stewart et al., 1997). Rumen turnover rate increases when high grain diets are fed, but because *S. bovis* is capable of very fast growth rates (Russell et al., 1981; Stewart et al., 1997) it can easily compete with other ruminal bacteria under these conditions. Because *S. bovis* is a homolactic fermenter, the main fermentation product is lactate (more than 50% of fermentation products). Compared to most ruminal bacteria, *S. bovis* is very tolerant of low pH in the rumen (i.e., pH 4.55). It is believed that this bacterium is more resistant to low extracellular pH because it allows internal pH to fall and is tolerant of intracellular acidic conditions (Stewart et al., 1997).

Other Ruminal Bacteria

There are several other ruminal bacteria that are competitive in the rumen by using less preferred substrates. Their numbers are smaller in the rumen than the others that I have discussed; however, their roles are important in maintaining the ecosystem. As mentioned in several sections, crossfeeding among ruminal bacteria is necessary for

the ecosystem because the ruminant diet does not provide all of the nutritional requirements for ruminal bacteria. The following species use unique substrates and/or provide requirements to other ruminal bacteria already discussed.

Lachnospira multiparous has the ability to degrade pectins and is found in the rumen in high number when cattle are fed legumes (Hungate, 1966; Stewart et al., 1997). They make up to 1 to 3 % of total rumen counts when cattle are fed forage diets. "*L. multiparus* penetrate the cut edges of the clover leaflets and caused extensive and rapid maceration of the tissues" (Stewart et al., 1997). Grass tissues are not as extensively macerated. *L. multiparous* have both endo- and exo acting enzymes, and most of the *Lachnospira* colonies are found in the solid fraction of rumen digesta (Hungate, 1966).

The first microorganism isolated from the bovine rumen (1959) was *Succinovibrio dextrinosolvens* (Stewart et al., 1997). *S. dextrinosolvens* use the dextrins produced by starch degraders for carbon sources, in addition to attacking pectin and a few sugars (Hungate, 1966). "Many strains are ureolytic", (Stewart et al., 1997). Because they are not proteolytic, *S. dextrinosolvens* require amino acids and NH_3 for nitrogen sources. "The EMP glycolytic enzymes have been demonstrated in this species, as well as a Co^{++}-dependent enzyme which catalyzes the carboxylation of phosphoenolpyruvate to oxalacetate, ATP being formed" (Hungate, 1966).

Another bacterium that is found in ruminants fed high concentrations of grain is *Megasphaera* sp. *M. elsdenii* can use glucose, fructose, and lactate. The fermentation end-products depend on the substrate. Glucose is often fermented to caproate and formate, and lactate is fermented using the acrylate pathway to butyrate, propionate, isobutyrate, valerate, CO_2, and H_2 (Stewart et al., 1997). The BCVFA are required by

cellulolytic ruminal bacteria for growth. This is another example of crossfeeding. "Since *M. elsdenii* is not subject to catabolite repression by glucose or maltose, its contribution to lactate catabolism is thought to increase after the feeding of soluble carbohydrates which repress lactate fermentation by *Selenomonas* and other lactate-using bacteria" (Stewart et al., 1997). *M. elsdenii* can help against ruminal acidosis in the rumen because of its ability to use lactate as a substrate. However, *M. elsdenii* is more sensitive to low pH than *S. bovis.* Therefore, *M. elsdenii* will wash out of the rumen (pH = 4.9) before *S. bovis* (pH = 4.55). Similar to *S. ruminantium* and *F. succinogenes*, *M. elsdenii* can store glycogen (Lou et. al. 1997).

Anaerovibrio lypolytica can hydrolyze lipids, use lactate, and have membranous blebs, which release extracellular lipase (Stewart et al., 1997). This bacterium has not been studied to the same extent as other ruminal bacteria species. Therefore, more research is needed with this ruminal bacteria.

The predominant methane producer in the rumen is *Methanobacterium ruminantium* (Hungate, 1966). The ruminal, "[m]ethanogens provide mixed anaerobic microbial populations in the gut, sediments and anaerobic engineered ecosystems such as waste digesters with a route for disposal of hydrogen, and thus provide an important means by which reduced cofactors may be oxidized" (Stewart et al., 1997). These unique microorganisms resemble eukaryotes more than bacteria. *M. ruminantium* uses H_2 and CO_2 as substrates that yield methane and water (Hungate 1966). Additionally, formate, acetate, methylamine and methanol can act as substrates (Stewart et al., 1997). Methanogens have a close relationship with ciliate ruminal protozoa and can be seen attached to protozoa in electron micrographs.

Protozoa

Protozoa are less numerous than bacteria in the rumen, but they can represent up to 50% of the total microbial biomass (Jouany and Ushida, 1999). "The number of protozoa or their biomass is related to the energy content of the diet" (Jouany and Ushida, 1999). For example, protozoa concentration increases with the amount of starch available in the diet.

There are two general types of ruminal protozoa, flagellates and ciliates. The flagellates are smaller and much less abundant than the cilliates in the adult rumen. However, *Momocercomonas ruminantium* and *Callimastix fromtalis* are found in large numbers in calves before the larger ciliates develop (Hungate, 1966). The ciliates are divided into two orders, Trichostomatida with the family Isotrichidae and Entodinomorphida with the family Ophryoscolecidae. The Isotrich ciliates are the primary starch degraders, while the ophryoscolecidae use a variety of substrates (Jouany and Ushia, 1999). Most ophryoscolecidae are able to use plant cell wall carbohydrates (Jouany and Ushia, 1999) and all protozoa have high deaminase activity. It has been suggested that more than 63% of dietary starch in the rumen is either engulfed or metabolized through the protozoa pool. Therefore, amylolytic bacteria are challenged by increased competition for substrate and threat of predation by the much larger protozoa. Often, protozoa engulf amylolytic bacteria because they are attached to the starch granules. "[P]rotozoa contribute to slow down the rumen digestion of starch and regulate the release of end products of fermentation as well as the pH (Ushida et al., 1991) and the osmotic pressure (Mendoza et al., 1993)" (Jouany and Ushia, 1999). The ammonia they form is secreted into the rumen medium, which can be used by bacteria. This is another

example of crossfeeding. Ruminal bacteria do not depend on the protozoa as seen by defaunation of the rumen and subsequent successful survival of the ruminal bacteria.

Fungi

The slow growing ruminal fungi were originally classified as protozoa. In the 1970s, Orpin discovered the presence of chitin and reproductive methods similar to fungi, and they were reclassified (Oprin, 1977). Although found in low numbers in the rumen, the fungi are important in fiber digestion. All rumen fungi digest fiber and degrade cell wall carbohydrates (Ho and Abdullah, 1999). "Rumen fungi are among the few fungi which can degrade crystalline cellulose" (Ho and Abdullah, 1999) and they have the enzymes necessary to digest hemicellulose, xylans, and phenolic acid ester bonds (Ho and Abdullah, 1999). Although some believe the fungi to be as important as ruminal bacteria, they have received limited research attention.

Manipulation of the Rumen

Based on our understanding of ruminal fermentation, nutritionists and microbiologists try to formulate diets or develop feed supplements to manipulate the fermentation and increase the efficiency of production. Over the years, various techniques have been explored including dietary supplementation with ionophores (Dennis et al., 1981; Henderson et al., 1981; Wampler et al., 1998; Domescik and Martin, 1999), direct fed microbial products (yeast and fungi) (Martin and Nisbet, 1992; Callaway and Martin, 1997; Martin et al., 1999; Sullivan and Martin, 1999), or organic acids (Russell et al., 1980; Martin and Streeter, 1995; Callaway and Martin, 1996; Martin, 1998; Martin et al., 1999; Martin et al., 2000). Two of these approaches were further explored in my thesis 1) an investigation of the concentration of malate in a

variety of ensiled forages and 2) the effects of *Saccharomyces cerevisiae* live cell yeast and yeast culture on in vitro mixed ruminal microorganism fermentation.

Addition of malate to the ruminant diet has been explored as another alternative to antimicrobial feed additives. Malate has been shown to increase lactate uptake and utilization by *S. ruminantium* (Nisbet and Martin, 1994; Callaway et al., 1997; Martin 1998; Martin et al., 1999; Martin et al., 2000) and reduces the drop in pH observed in ruminal acidosis cases (Martin and Streeter, 1995; Callaway et al., 1997; Martin, 1998; Martin et al., 1999). The addition of DL-malate to feedlot diets increased gain efficiency and average daily gain by 21 and 22%, respectively (Martin et al., 1999). However, at the concentration used in these feedlot studies, malate supplementation is estimated to cost between $0.09 and $0.18 per head per day (Martin et al., 1999). Therefore, in an attempt to look at alternative malate sources Callaway and Martin (1997) surveyed the malate levels in several varieties of alfalfa and bermudagrass. They found that both forages would theoretically provide an adequate concentration of ruminal malate if cattle were fed 6.0 kg of forage per day each. However, recent in situ research showed that malate disappeared from ground alfalfa and bermudagrass hays within 30 min of exposure to the ruminal environment (Martin et al., 2000). In addition to fresh forages and hays, there is much interest in the ruminant feed industry regarding malate content of ensiled forages. Therefore, the first objective of my thesis was to evaluate the effect of ensiling two forages (alfalfa, wheat) and corn on malate content.

Recently, with the public's growing concern of antibiotics being added to the feed of production animals, interest has developed to explore alternative forms of gastrointestinal microflora manipulation (Martin et al., 1999). However, compared with

the efforts to detail the effects of antimicrobial compounds on ruminal fermentation, little research has been conducted to evaluate alternatives to antimicrobial compounds. Although direct-fed microbial (DFM) products have been added to feed as a supplement for many years, they have only been examined as an alternative to antimicrobial compounds in the past 10 years or so (Martin and Nisbet, 1992; Sullivan and Martin, 1999). Favorable results have been reported on altering ruminal fermentation and improving animal performance; however, these effects have been variable and inconsistent (Martin and Nisbet, 1992). For example, Callaway and Martin (1997) demonstrated that a filter-sterilized filtrate of Diamond V XP *Saccharomyces cerevisiae* culture stimulated growth of pure cultures of ruminal bacterium on both lactate and cellobiose medium. However, Sullivan and Martin (1999) showed that the effect of *S. cerevisiae* culture on mixed ruminal microorganism fermentation with various substrates had little effect on final pH or fermentation products; meaning growth of ruminal microorganisms was not stimulated.

Most of the in vivo and in vitro research with *Sacc cerevisiae* has focused on *Sacc cerevisiae* culture that includes *Sacc cerevisiae* yeast plus the media on which it was grown. Over the past few years there has been increasing interest in comparing the effects of *Sacc cerevisiae* live cell yeast products to *Sacc cerevisiae* culture products on the ruminal fermentation. It is important to be able to determine what effects are due to the culture medium versus the *Sacc cerevisiae*. Therefore, the second objective of my thesis was to compare the effects of a *Sacc cerevisiae* live cell yeast product to a *Sacc cerevisiae* culture product on the in vitro mixed ruminal microorganism fermentation of ground corn, soluble starch, alfalfa hay, and Coastal bermudagrass hay.

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CHAPTER II

EFFECT OF ENSILING ON MALATE CONTENT OF ALFALFA, CORN, AND WHEAT[1]

[1]Lynch, H. A., and S. A. Martin. To be submitted to the Journal of Dairy Science

ABSTRACT

The objective of this study was to evaluate the effect of ensiling alfalfa, wheat, and corn on malate content. Samples were grown, collected, and ensiled in 7.5 L plastic buckets with airtight lids. All samples were either ensiled immediately after cutting, or wilted for at least one h between treatments. Sources of corn either were grown in drought conditions or irrigated conditions. No other forages were irrigated during growth. Alfalfa was ensiled at two different cutting lengths. The experimental silos were kept for 6 weeks at 25°C and then opened for analysis. The organic and volatile fatty acids, percent dry matter, and pH were measured. Because soluble nutrient losses are associated with the ensiling process, concentrations of malate were expected to be lower in all silage treatments. Silage samples of all varieties had decreased malate and pH values and increased lactate, acetate, butyrate, propionate, and total VFA concentrations. However, the loss in malate during the ensiling was less than in hay. No common trends based on percent dry matter treatment were observed. Alfalfa had the lowest concentration of malate and corn (drought-stressed) had the highest concentration of malate among all of the plant varieties. Increased dietary concentrations of malate might help reduce problems associated with subclinical acidosis by stimulating lactate utilization by *S. ruminantium*. Malate concentrations in silage may be sufficient to improve ruminal pH of lactating dairy cattle.

INTRODUCTION

The dicarboxylic acid, malate, stimulates lactate uptake as much as 10-fold by the predominant ruminal bacterium *Selenomonas ruminantium* (Nisbet and Martin, 1990; Nisbet and Martin, 1991; Nisbet and Martin, 1993; Nisbet and Martin, 1994; Strobel and Russell, 1991). Malate concentrations between 0.03 and 10 m*M* increased lactate uptake in a dose-response manner (Nisbet and Martin, 1991). Based on our observation that concentrations of organic acids (i.e., malate) other than VFA are low in ruminal fluid, they potentially limit the growth of *S. ruminantium*. When mixed ruminal microorganisms were incubated in medium that contained cracked corn or soluble starch, malate treatment decreased lactate concentrations and increased final pH (Callaway and Martin, 1996; Martin and Streeter, 1995). Furthermore, infusing different concentrations of DL-malate into the rumen of steers fed an 80% rolled grain diet increased ruminal pH that was consistently greater than 6.0 in the presence of 12 m*M* DL-malate over the 12 h sampling period (Martin et al., 1999). These results suggested that increasing dietary concentrations of malate might help to reduce problems associated with subclinical acidosis by stimulating lactate utilization by *S. ruminantium*.

Because the cost of supplementing diets with DL-malate is estimated to range between $0.09 to $0.19/head daily under feedlot conditions (Martin et al., 1999), inclusion of malate as a feed additive in ruminant diets may not be economically feasible. Intermediates of the citric acid cycle accumulate in plant tissue and malate can comprise up to 1.5% of the DM of mature grasses (Bohman, 1983). Therefore, forages that are high in organic acids might provide a vehicle for the inclusion of malate in ruminant diets. To address the possibility of using forages as a source of malate, a study was

conducted to determine the concentrations of malate present in five alfalfa varieties (Alfagraze, Apollo Supreme, Cimarron, Crockett, and Magnum III) and three bermudagrass hay (Coastal, Tifton-78, and Tifton-85) varieties at different stages of maturity (Callaway et al., 1997). Malate concentrations ranged between 2.9 and 4.5% of alfalfa DM at 42 d of maturity, while malate concentrations were between 1.9 and 2.3% of bermudagrass DM at 41 d of maturity (Callaway et al., 1997). When in situ experiments were conducted with early and late harvested alfalfa, Coastal bermudagrass, and Tifton 85 bermudagrass hays, malate was solubilized and utilized in these ground forage samples within 30 min after reaching the rumen (Martin et al., 2000).

Even though malate is present in forages commonly fed to dairy cattle (Callaway et al., 1997), the availability of this dicarboxylic acid once the ingested forage reaches the rumen is unknown. In vitro studies have shown that 7.5 m*M* malate is completely fermented within 10 to 24 h by mixed ruminal microorganisms (Callaway and Martin, 1997; Russell and Van Soest, 1984). McDonald and Whittenbury (1973) reported that citrate and malate in silage are fermented by a number of pathways resulting in the formation of several end products. Others have also reported the breakdown of malate and citrate in silage (Keddie, 1959; Wood and Holzapfel, 1992). Keddie (1959) suggested that the dissimilation of malate is a usual feature of the homofermentive group of bacteria (i.e., lactobacilli) found in silage. Because little information is available regarding malate content in modern silage, the objective of this study was to examine the effects of ensiling alfalfa, corn and wheat on malate content.

MATERIALS AND METHODS

Samples of three different forages, corn (*Zea mays*), alfalfa (*Medicago sativa*), and wheat (*Triticum aestivum*), were collected and ensiled in 7.5 L plastic buckets with airtight lids. The lid was equipped with a one-way valve to release fermentation gases. These laboratory silos have been reported to provide ensiling conditions that are similar to farm silos (Stirling, 1951). The Gore wheat was planted (November 1, 1999) and harvested (April 25, 2000 and May 1, 2000) at the University of Georgia Dairy Cattle Center (Athens, GA) with and average temperature of 12.68°C. The wheat crop was fertilized at planting and in February with 34% NH_3-N at an application rate of 336.4 kg/hectare and had a water balance of 2.85 cm. Drought-stressed corn was also grown at the University of Georgia Dairy Cattle Center (Athens, GA). The corn (Dekalb 687RR®) was fertilized (18:6:12 N-P-K, 538.5 kg/hectare; 34% NH_3-N, 560.7 kg/hectare) when planted (April 12, 2000) and had an average temperature of 24.32°C. The corn was harvested on July 12, 2000 and had a water balance of –27.20 cm. The irrigated corn and alfalfa were grown at the University of Georgia Plant Sciences Farm (Watkinsville, GA). The irrigated corn (Dekalb 662RR®) was fertilized (14:7:14 N-P-K, 785.0 kg/hectare; 34% N, 336.4 kg/hectare) when planted (April 10, 2000) and irrigated throughout its growth with 23.62 cm of water. The total water balance for the irrigated corn was –5.54 cm after accounting for applied irrigation. Therefore, the corn was not water stressed. The irrigated corn was harvested on July 13, 2001 and had an average temperature of 24.32°C. The alfalfa (multiple varieties) was irrigated at planting (October 1, 1998) and harvested at 1/10 bloom stage (May 25, 2000). The average temperature during this growth period was 15.65°C. The alfalfa was fertilized (0:10:20 N-P-K, 560.7 kg/hectare)

and had a water balance of –11.23 cm. All crops were harvested and cut to 1.27 cm (Stirling, 1951) except the corn (irrigated) and the alfalfa (cut for haylage). The irrigated corn was hand cut with a sickle and fed through a forage chopper (1.27cm), while the alfalfa (cut for haylage) was harvested with a flail mower (45.72 cm).

All samples were either ensiled immediately after cutting, or wilted for at least one h between treatments. Duplicate samples were uniformly packed into the experimental silo using a hydraulic press. The experimental silos were kept at room temperature (25°C) for 6 weeks and then opened for analysis. Once experimental silos were opened, they were hand-mixed to ensure a representative sample of all silage. Organic acids were extracted by blending 25 g of sample with 100 mL of distilled water for 2 min on high speed (Proctor-Silex, USA). The blended mixture was then filtered through four layers of cheesecloth. The filtrate pH was measured with a pH meter and then centrifuged (10,000 x g, 10 min, 25°C) and filtered through a 0.45-:m membrane filter. Malate was quantified by HPLC (Shimadzu LC-10AS liquid chromatograph, RID-6A refractive index detector, SCL-10A system controller, SIL-10A auto-sampler, C-R5A integrator, 50-:l loop; Shimadzu Scientific Instruments, Columbia, MD) at 50°C using a Bio-Rad HPX-87H organic acid column (Bio-Rad Laboratories, Hercules, CA) (Martin and Park 1996). Samples were eluded from the column with 0.013N H_2SO_4 at a flow rate of 0.6 mL/min. Dry matter (DM) was determined by drying 100 g of sample at 65°C for 5 days in a drying oven (Grieve Corp., SA-350 Drying oven, Roundlake, IL). All remaining unanalyzed green chop and silage samples were stored at -20°C.

All silage fermentations were conducted in duplicate (n = 2) from the same harvest at the same time. Data were analyzed by a general linear model procedure (SAS).

The model contained % DM, malate, lactate, acetate, propionate, butyrate, total volatile fatty acid (VFA), and pH for each forage type (before and after ensiling) and level of % DM. Least square means for concentrations of % DM, organic acids, and pH are reported and significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Photosynthesis utilizes citric acid cycle activity in plants to fix carbon dioxide into biomass. Growing plants accumulate intermediate metabolites of the citric acid cycle, and tissues associated with photosynthesis, such as leaves, tend to accumulate the highest concentrations of malate (Vickery and Pucher, 1940; Dikjshoorn, 1973). Silage is a preservation method, which stores the nutrients in vegetative forage so that it may be used as feed a stable feed for daily dairy cattle operations, during winter months, dry periods, and/or as needed (Whittenbury, 1968; McCullough 1978; Ball, 1996). However, recognition of the altering of nutrients during the ensiling process led investigators to study silage fermentation in an attempt to minimize the amount of nutrient loss. One method to obtain a more complete fermentation is to wilt the harvest (Gordon et al., 1964), thereby increasing the green chop %DM before ensiling. Low %DM forages contain more water in the silage, which can dilute the strength of the lactic acid, and increase the time needed to reach the stable state. In this study, the ensiled forages were wilted to achieve a significant difference in %DM between at least one treatment ($P < 0.05$). To our knowledge, concentrations of malate in modern cultivars of alfalfa, corn, and wheat have not been examined, but because of the positive effect that malate has on the utilization of lactate by the predominant ruminal bacterium *S. ruminantium* (Nisbet

and Martin, 1990; Nisbet and Martin, 1991; Nisbet and Martin, 1993; Nisbet and Martin, 1994), perhaps malate content in silage influences high producing dairy cows.

Because soluble nutrient losses are associated with the ensiling process, concentrations of malate were expected to be lower in all silage treatments. Silage samples of all varieties had decreased malate and pH values and increased lactate, acetate, butyrate, propionate, and total VFA concentrations. All silage visually appeared ensiled. These conclusions were supported by the following tables and results.

Although alfalfa is leafy, which is the portion of the plant that contains most of the concentration of malate, it had the lowest concentration of malate among all of the plant species (Table 2.1). The % DM was significantly different ($P < 0.05$) between the low and medium %DM and the high %DM for silage samples. Furthermore, the low green chop %DM sample significantly increased ($P < 0.05$) in dry matter during the ensiling process. The concentration of malate was significantly lower ($P < 0.05$) in green chop as the %DM increased. This effect may have been caused by the continued plant respiration during alfalfa wilting. There was no difference among the silage samples between treatments for malate, and all the silage samples were significantly lower in malate content than the low and medium %DM green chop samples. There was no difference in the malate concentration between the green chop high %DM sample and all of the silage samples. Also, there was no difference between the green chop measurements for lactate, acetate, butyrate, total VFA, or pH. A significant increase ($P < 0.05$) in lactate, acetate, and total VFA concentration was found with all silage samples, however the low and medium %DM treatments were significantly higher than the high %DM silage treatment. Our results agree with Teller et al. (1989) and Gordon, (1964),

who found that acetic acid concentrations were considerably higher for direct cut silage than in wilted silage and that the lowest %DM had the most acetic acid, respectively. The low and medium %DM silage samples were higher ($P< 0.05$) in propionate than the high %DM silage sample and the medium and high %DM green chop treatments. There is no difference in propionate among the low %DM green chop sample and all other treatments. The green chop medium and high %DM were significantly lower than the propionate concentration in the low and medium %DM silage samples. The low %DM silage sample was significantly higher than the high %DM silage sample for total VFA concentrations. Furthermore, the medium %DM was not significantly different from either the low %DM or high %DM silage total VFA concentrations. The pH values correspond to the lactate concentration values. The pH values decrease relative to an increase in lactate. There was no pH difference between the green chop samples, although the green chop samples were significantly higher ($P < 0.05$) than all silage pH samples. Both the low and medium %DM silage samples were significantly ($P < 0.05$) lower in pH than the high %DM silage sample. This is related to the lactate concentrations among treatments. The pH values are higher than what is seen in normally preserved silage, however, the silage visually appeared preserved. These high pH values could be due to the larger particle size of the silage material and continued aerobic degradation during the initial stages of ensiling. This would delay the time an anaerobic state is created and thus lead to a decrease in available nutrients for anaerobic microorganisms to ferment, a higher pH, and restricted fermentation.

Examining the alfalfa (cut for silage) data supports these conclusions (Table 2.2). This alfalfa was harvested from the same crop, however it was additionally chopped

(1.27 cm) for ensiling after its initial harvest when it was cut down for haylage purposes. The green chop low and medium %DM samples were significantly ($P<0.05$) lower in pH from the high %DM green chop sample, but were not different from each other. The low %DM green chop significantly ($P < 0.05$) decreased in %DM during the ensiling process, while the high %DM green chop increased in % DM. The low %DM green chop was not different ($P < 0.05$) from the medium %DM silage sample. The low and high %DM silage samples and the high %DM green chop samples were significantly ($P < 0.05$) different from each other and the rest of the treatments. There was no difference in malate content among the green chop or silage samples. All malate concentrations significantly ($P < 0.05$) decreased during the ensiling process. This is consistent with Keddie (1959), and Wood and Holzapfel (1992) observations that malate is broken down during the ensiling process. An inverse trend is seen with the lactate concentrations. Again, there are no differences ($P < 0.05$) among the silage and green chop treatment groups, however lactate concentration significantly increases during the ensiling process. Acetate, butyrate, propionate, and total VFA concentrations showed no effects among the treatments. The pH values better represent a more complete fermentation and are lower than the alfalfa cut for haylage data. There was no difference ($P < 0.05$) in pH among the green chop % DM treatments. The pH decreased ($P < 0.05$) during ensiling and the low and medium % DM silage samples were significantly lower ($P < 0.05$) then the high %DM silage sample.

Wheat (*Triticum aestivum*) is a cool season grass (Ball et al. 1996). The %DM between low and medium %DM for both green chop and silage samples were not different among themselves, but were significantly ($P < 0.05$) lower than the high %DM

for both green chop and silage (Table 2.5). The malate concentrations were numerically higher than alfalfa. The malate concentrations for the low %DM sample was significantly ($P< 0.05$) higher than all other treatments expect the medium %DM green chop sample. The medium %DM green chop sample was only significantly higher than the low %DM silage sample and not different among all other treatments. The medium %DM green chop samples was not significantly ($P < 0.05$) different than either the low %DM green chop sample or the high %DM green chop sample. Malate concentration for high %DM green chop was not significantly ($P < 0.05$) different from the medium and high %DM silage samples. This effect may be due to wilting. There was no difference ($P < 0.05$) observed among silage samples for malate. The lactate concentration was not different among the green chop samples. Lactate significantly increased ($P < 0.05$) during ensiling among all silage samples, but greater results were observed for the low and medium %DM silage samples. There was no difference among the green chop samples for acetate. The high %DM silage sample was significantly higher ($P < 0.05$) than all green chop samples, and it was significantly lower than the low %DM silage sample. The medium %DM silage sample was not different from either silage sample for acetate. Our results agree with Teller et al. (1989) and Gordon, (1964), who found that acetic acid concentrations were considerably higher for direct cut silage than in wilted silage and that the lowest %DM had the most acetic acid, respectively. Butyrate and total VFA concentrations were not different among the green chop samples. The low and medium %DM samples were significantly higher ($P < 0.05$) than all the green chop samples and the high %DM silage for butyrate and total VFA concentrations. Furthermore, there was no difference between the high %DM silage sample and the green chop samples. No

effects were observed among treatments for propionate. The pH for all green chop samples were not significantly different. All the silage samples had significantly lower ($P < 0.05$) pH values than the green chop samples. The low and medium % DM silage samples were significantly lower ($P < 0.05$) than the high %DM silage sample. The pH values were representative of preserved silage.

The low and medium %DM silage and low %DM green chop sample were significantly different ($P < 0.05$) from the high %DM green chop and silage samples for the irrigated corn crop (Table 2.4). There was no difference between the high %DM green chop and silage samples. The medium %DM green chop sample was not significantly ($P > 0.05$) different from any of the treatments. The low %DM green chop sample was only significantly ($P>0.05$) different from the high %DM green chop sample. No significance was observed with malate or propionate concentrations. However, the malate concentrations were numerically higher than the alfalfa and wheat. The lactate, acetate, butyrate, and total VFA concentrations did not differ among the green chop samples. During ensiling the lactate concentrations significantly ($P < 0.05$) increased for all silage samples. The low %DM silage sample is significantly ($P > 0.05$) higher than the high %DM silage sample for lactate concentration, however the medium %DM sample is not significantly ($P > 0.05$) different from either of these samples. The silage samples were significantly higher ($P < 0.05$) than the green chop samples for acetate and total VFA concentrations. The butyrate concentration for low and high %DM silage samples significantly increased ($P < 0.05$) during ensiling, although there was no difference among the silage concentrations. No difference was observed between the medium %DM silage sample and all other treatments for butyrate. The green chop pH

measurements were increased significantly as green chop %DM decreased. During ensiling all the samples significantly decreased ($P < 0.05$) in pH, although there was no statistical difference among silage samples.

The drought stressed corn %DM was significantly different ($P < 0.05$) between the low and medium %DM silage and green chop samples from the high %DM silage and green chop samples (Table 2.3). When comparing the %DM of green chop of the drought stressed and irrigated corn, it was observed that the drought-stressed corn had a lower %DM. The irrigated corn received 23.62 cm of water during its grow period. The corn stalks were cut at the base by hand and transported to the University of Georgia Dairy (Athens, GA) for chopping. Perhaps, some of the moisture of the crop was lost during transport. There was no significance in malate, acetate, or propionate among treatments. Drought stressed corn had the highest concentration of malate in the silage among all forage varieties examined. Lactate significantly increased ($P < 0.05$) in all silage samples during ensiling. No difference was noticed among silage or green chop lactate values. Butyrate, total VFA, and pH values were not different among the green chop samples. The medium % DM silage butyrate and total VFA was significantly ($P < 0.05$) higher than all the other samples. There was no difference between the low and high %DM silage sample and the green chop samples for butyrate and total VFA concentrations. During ensiling, all silage samples pH values significantly decreased ($P < 0.05$). The decrease pH values correspond with the increase in lactate, and represent a complete fermentation.

Although, forages grown in the Southeast are often associated with low quality, many dairies feed high concentrations of cereal grains to high producing cows.

Saccharolytic rumen bacteria ferment starch or sugars into lactate. Accumulation of lactate causes ruminal acidosis, which decreases feed efficiency because of reduced intake and other physiological problems (Slyter, 1976). Acidosis is a problem in the dairy industry, especially in high producing dairy cows fed high grain diets (Hinders, 1995). Therefore, a reduction in the accumulation of lactate in the rumen of these high-producing cows through enhanced utilization of lactate by *S. ruminantium* may possibly improve production efficiency. Ruminal lactate can be fermented by *S. ruminantium* to propionate (Gottschalk, 1986), and the uptake of lactate by this predominant ruminal bacterium is stimulated in the presence of malate (Nisbet and Martin, 1994; Callaway et al., 1997; Martin, 1998; Martin et al., 1999; Martin et al., 2000). Furthermore, recent research (Martin and Streeter, 1995) showed that, when mixed ruminal microorganisms fermented cracked corn or soluble starch, malate treatment decreased concentrations of lactate and CH_4 as well as the ratio of acetate to propionate in a manner analogous to ionophore treatment.

Martin et al. (2000) suggested that malate was solubilized and utilized within 30 min after reaching the rumen. The malate concentrations in alfalfa and Bermuda grass hay, used in the in situ study, were between 3.8 and 32.9 mg/g of DM (Martin et al, 2000). In our study, all of the silage samples, except for the medium and high %DM alfalfa (silage cut) silage, were (Table 2.2) above 3.8 mg/g of DM. In fact, most of the silage samples in our study were greater than the reported 32.9 mg/g of DM (Tables 2.3 to 2.5). The higher value can be contributed to less aerobic respiration of the forage before preservation. All malate concentrations decreased numerically during ensiling. Since, malate can be used as a carbon source by silage microorganisms and is broken

down (Keddie, 1959; McDonald and Whittenbury, 1973; Wood and Holzapfel, 1992). However, the loss in malate during the ensiling was less than in hay. No common trends based on %DM treatment were observed.

Martin et al. (2000) also concluded that once the studied forages were placed within 50- to 100-L rumen the concentration of malate would be diluted. Therefore, taking into account the dilution factor plus the fact that organic acids can be fermented quickly, (Russell and Van Soest, 1984; Callaway and Martin, 1997), significant malate concentrations in ruminal fluid would likely not be detected (Martin et al., 2000). Similar conclusions may apply to our study. However, since the concentrations were twice as high in some cases, silage may provide positive malate effects to dairy cattle. Ruminal malate concentrations between 4 and 12 m*M* were effective at reducing subclinical acidosis in steers fed a high-grain diet (Martin et al. 1999). In situ studies with silage samples are recommended to determine the effects of ruminal fermentation on the available malate in silage.

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Table 2.1. End Products of Alfalfa (cut for haylage) silage and green chop.

	Alfalfa (cut for haylage)							
Fermentation Products	Silage				Green Chop			
	Low	Med	High	SEM	Low	Med	High	SEM
%DM	41.9[b]	42.6[b]	56.5[a]	2.2	23.9[c]	37.3[bc]	61.0[a]	3.1
Malate[d]	3.9[c]	7.4[c]	9.4[c]	1.4	33.1[a]	20.7[b]	9.6[c]	2.0.
Lactate[d]	16.8[a]	16.9[a]	10.1[b]	1.1	2.3[c]	0.6[c]	0.8[c]	1.6
Acetate[d]	11.5[a]	10.9[a]	7.7[b]	0.7	0.5[c]	0.3[c]	0.2[c]	0.9
Butyrate[d]	0.9	0.9	0.6	0.3	0.00	0.00	0.00	0.4
Propionate[d]	2.6[a]	2.4[a]	1.1[b]	0.2	1.5[ab]	1.2[b]	0.5[b]	0.3
Total VFA[d]	15.1[a]	14.1[ab]	9.4[b]	1.1	2.0[c]	1.5[c]	0.7[c]	1.5
pH	5.15[c]	5.27[c]	5.58[b]	0.04	6.34[a]	6.30[a]	6.37[a]	0.05

[a,b,c] Means within a row with no common superscripts differ (P<0.05).
[d] Expressed as mg/g of DM

Table 2.2. End Products of Alfalfa (cut for silage) silage and green chop.

	Alfalfa (cut for silage)							
Fermentation Products	Silage				Green Chop			
	Low	Med	High	SEM	Low	Med	High	SEM
%DM	37.0[e]	38.8[d]	47.5[a]	0.4	39.5[cd]	41.0[c]	44.9[b]	0.5
Malate[f]	5.2[b]	0.1[b]	0.1[b]	2.8	35.9[a]	26.3[a]	23.1[a]	3.9
Lactate[f]	24.0[a]	24.3[a]	22.9[a]	3.1	2.5[b]	2.3[b]	2.1[b]	4.4
Acetate[f]	13.0	14.4	12.6	2.9	2.4	1.9	1.8	4.1
Butyrate[f]	0.5	0.6	0.3	0.2	0.0	0.0	0.0	0.2
Propionate[f]	0.3	0.7	0.6	0.3	1.0	0.9	0.8	0.5
Total VFA[f]	13.8	15.7	13.4	3.2	3.3	2.9	2.5	4.6
pH	4.52[c]	4.54[c]	4.60[b]	0.01	6.31[a]	6.32[a]	6.37[a]	0.01

[a,b,c,d,e] Means within a row with no common superscripts differ (P<0.05).

[f] Expressed as mg/g of DM

Table 2.3. End Products of Corn (drought stressed) silage and green chop.

	Corn (drought stressed)							
Fermentation Products	Silage				Green Chop			
	Low	Med	High	SEM	Low	Med	High	SEM
%DM	24.2[b]	24.4[b]	29.7[a]	0.8	22.7[b]	24.2[b]	30.2[a]	1.2
Malate[c]	92.5	35.9	93.2	11.2	109.8	132.3	115.1	15.9
Lactate[c]	27.7[a]	22.1[a]	22.9[a]	2.8	0.4[b]	0.4[b]	0.4[b]	4.0
Acetate[c]	12.2	13.7	8.3	1.7	0.7	1.1	1.0	2.4
Butyrate[c]	1.9[b]	22.4[a]	2.2[b]	1.6	0.0[b]	0.1[b]	0.1[b]	2.3
Propionate[c]	0.7	4.0	1.0	0.8	0.0	0.5	0.3	1.1
Total VFA[c]	14.8[b]	40.1[a]	11.5[b]	3.9	0.7[b]	1.7[b]	1.4[b]	5.6
pH	3.75[b]	4.10[b]	3.83[b]	0.12	5.74[a]	5.60[a]	5.82[a]	0.17

[a,b] Means within a row with no common superscripts differ ($P<0.05$).
[c] Expressed as mg/g of DM

Table 2.4. End Products of Corn (irrigated) silage and green chop.

	Corn (irrigated)							
Fermentation Products	Silage				Green Chop			
	Low	Med	High	SEM	Low	Med	High	SEM
%DM	23.9^{cd}	24.7^{cd}	28.9^{abc}	0.6	26.0^{bcd}	26.9^{bcd}	30.00^{ab}	0.8
Malate[f]	43.9	83.6	35.8	9.1	98.9	104.5	98.6	12.9
Lactate[f]	25.8^{a}	33.0^{ab}	20.8^{b}	2.3	0.5^{c}	0.4^{c}	0.4^{c}	3.2
Acetate[f]	9.2^{a}	12.7^{a}	10.9^{a}	1.2	0.0^{b}	0.4^{b}	0.4^{b}	1.7
Butyrate[f]	13.9^{a}	8.3^{ab}	11.4^{a}	1.7	0.0^{b}	0.1^{b}	0.0^{b}	2.5
Propionate[f]	0.6	1.0	0.8	0.2	0.0	0.0	0.1	0.2
Total VFA[f]	23.6^{a}	21.9^{a}	23.1^{a}	0.6	0.0^{b}	0.6^{b}	0.6^{b}	0.9
pH	3.70^{d}	3.70^{d}	3.78^{d}	0.02	5.63^{a}	5.37^{b}	4.89^{c}	0.03

^{a,b,c,d,e} Means within a row with no common superscripts differ (P<0.05).

[f] Expressed as mg/g of DM

Table 2.5. End Products of Wheat silage and green chop.

	Wheat							
Fermentation Products	Silage				Green Chop			
	Low	Med	High	SEM	Low	Med	High	SEM
%DM	31.7[b]	32.3[b]	51.8[a]	1.0	31.6[b]	34.8[b]	55.4[a]	1.4
Malate[f]	57.1[c]	58.2[bc]	61.0[bc]	3.3	88.9[ab]	73.9[abc]	66.4[bc]	4.7
Lactate[f]	23.8[a]	23.8[a]	12.1[b]	1.8	0.4[c]	0.3[c]	0.3[c]	2.6
Acetate[f]	10.2[a]	9.5[ab]	6.1[b]	1.0	1.2[c]	0.6[c]	0.9[c]	1.4
Butyrate[f]	10.2[a]	10.8[a]	2.3[b]	1.3	0.2[b]	0.0[b]	0.0[b]	1.8
Propionate[f]	1.8	2.0	1.0	0.3	0.0	0.0	0.3	0.5
Total VFA[f]	22.2[a]	22.3[a]	9.4[b]	2.5	1.4[b]	0.6[b]	1.2[b]	3.5
pH	3.97[c]	3.95[c]	4.29[b]	0.03	6.12[a]	6.06[a]	6.10[a]	0.04

[a,b,c,d,e] Means within a row with no common superscripts differ (P<0.05).

[f] Expressed as mg/g of DM

CHAPTER III

EFFECTS OF *SACCHAROMYCES CEREVISIAE* CULTURE AND *SACCHAROMYCES CEREVISIAE* LIVE CELLS ON IN VITRO MIXED RUMINAL MICROORGANISM FERMENTATION[2]

[2] Lynch, H. A., and S. A. Martin. To be submitted to the Journal of Dairy Science

ABSTRACT

The objective of this study was to compare the effects of a *Saccharomyces cerevisiae* live cell yeast product to a *Saccharomyces cerevisiae* culture product on the in vitro mixed ruminal microorganism fermentation of ground corn, soluble starch, alfalfa hay, and Coastal bermudagrass hay. In the presence of ground corn, neither concentration (0.35 or 0.73 g/L) of *Sacc. cerevisiae* culture or *Sacc. cerevisiae* live cells had any effect on final pH, H_2, CH_4, propionate, or butyrate. *Sacc. cerevisiae* culture had no effect on acetate, but both concentrations of *Sacc. cerevisiae* live cells decreased acetate and the acetate:propionate ratio. When soluble starch was the substrate, both concentrations of *Sacc. cerevisiae* live cells and 0.73 g/L of *Sacc. cerevisiae* culture decreased the acetate:propionate ratio. Even though the treatment effects were not statistically significant, both concentrations of *Sacc. cerevisiae* live cells and 0.73 g/L of *Sacc. cerevisiae* culture numerically decreased lactate concentrations compared to the control incubations. When alfalfa hay was the substrate, *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells had no effect on H_2, propionate, butyrate, or the acetate:propionate ratio. Both concentrations of *Sacc. cerevisiae* culture decreased final pH and in vitro dry matter disappearance and the 0.73 g/L treatment decreased acetate. In comparison, both treatments of *Sacc. cerevisiae* live cells increased final pH and decreased acetate and in vitro dry matter disappearance. Neither yeast treatment had much effect on the Coastal bermudagrass hay fermentations.

INTRODUCTION

Based on growing concern regarding the use of antibiotics in animal production, there is much interest in exploring alternatives to antimicrobial feed additives (Martin et al., 1999). *Saccharomyces cerevisiae* feed additives have been used as an alternative to antimicrobial feed additives for the past 10+ yr. Some of the benefits associated with *Sacc. cerevisiae* include increased DM and NDF digestion (Carro et al., 1992), increased initial rates of fiber digestion (Williams et al., 1991), and increased milk production in dairy cattle (Harris and Webb, 1990; Kung et al., 1997; Piva et al, 1993; Williams et al., 1991). In vitro experiments have also reported, in some cases, *Sacc. cerevisiae* culture favorably altered the mixed ruminal microorganism fermentation and stimulated lactate uptake and cellulose digestion by pure cultures of predominant ruminal bacteria (Callaway and Martin, 1997, Martin and Nisbet, 1992; Nisbet and Martin, 1991). Even though the effects of *Sacc. cerevisiae* are not always consistent (Martin and Nisbet, 1992), several models have been proposed regarding the stimulatory effects of yeast culture on the ruminal fermentation (Dawson, 1990;. Lyons et al., 1993; Wallace, 1994).

Most of the in vivo and in vitro research with *Sacc. cerevisiae* has focused on *Sacc. cerevisiae* culture that includes *Sacc. cerevisiae* yeast plus the media on which it was grown. Over the past few years there has been increasing interest in comparing the effects of *Sacc. cerevisiae* live cell yeast products to *Sacc. cerevisiae* culture products on the ruminal fermentation. It is important to be able to determine what effects are due to the culture medium versus the *Sacc. cerevisiae*. Therefore, the objective of this study was to compare the effects of a *Sacc. cerevisiae* live cell yeast product to a *Sacc.*

cerevisiae culture product on the in vitro mixed ruminal microorganism fermentation of ground corn, soluble starch, alfalfa hay, and Coastal bermudagrass hay.

MATERIALS AND METHODS

Ruminal contents were collected from a 800-kg ruminally fistulated Hereford steer that was maintained on pasture and fed concentrate supplement (60% corn gluten meal, 30% soy bean meal, and 10% ground corn) once daily. Ruminal contents were obtained in the morning and squeezed through four layers of cheesecloth into a 1000 mL Erlenmeyer flask with an O_2-free CO_2 headspace. The flask was then placed in a 39°C water bath and remained undisturbed for 30 min, allowing the feed particles to rise to the top of the flask. Particle-free fluid from the flask was anaerobically transferred (20% vol/vol) to a medium (pH 6.5) containing 292 mg of K_2HPO_4, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, and 600 mg of cysteine hydrochloride per liter. Particle-free fluid and medium were mixed, and 40 mL was transferred anaerobically to 160-mL serum bottles that contained either no substrate, 0.4 g of ground corn, 0.4 g of soluble starch (Difco Laboratories, Detroit, MI), 0.4 g of alfalfa hay, or 0.4 g of Coastal bermudagrass hay. Diamond V XP yeast culture (Diamond V Mills, Inc, Cedar Rapids, IA) and PMX70SBK live cell yeast (Bioproducts, Fairlawn, OH) were added to achieve final concentrations of 0.35 and 0.73 g/L. These concentrations are consistent with current recommended feeding levels. Incubations containing only *Sacc. cerevisiae* culture or *Sacc. cerevisiae* live cells were also run. The bottles were sealed (CO_2 atmosphere) with butyl rubber stoppers and aluminum caps to contain gas pressure, and placed in a 39°C

waterbath for either 24 h (ground corn, soluble starch) or 48 h (alfalfa hay, bermudagrass hay) and periodically mixed.

After 24 h (no substrate, ground corn, and soluble starch) or 48 h (no substrate, alfalfa, and bermudagrass) of incubation, a gas sample (0.5 mL) was removed from each bottle and analyzed for hydrogen (H_2) and methane (CH_4) on a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a Porapak Q column (60°C, 20 mL/min of N_2 carrier gas). The bottles were then uncapped, and the pH was measured immediately with a pH meter. Bottles were then emptied into centrifuge tubes, centrifuged (10,000 x g, 4°C, 15 min) and the cell free supernatant fluids were stored at -20°C.

To examine the effects of *Sacc. cerevisiae* culture or *Sacc. cerevisiae* live cells on forage fiber digestion by mixed ruminal microorganisms, alfalfa hay and Coastal bermudagrass hay incubations were performed. Serum bottles were prepared as described above and incubated for 48 h. After 48 h, bottles were uncapped and poured into centrifuge tubes and centrifuged (10,000 x *g*, 4°C, 15 min). Pellets were resuspended in deionized water and poured back into the original serum bottles and stored (4°C). Undigested residue was collected on a pre-weighed oven-dried Whatman no. 1 filter (Whatman Lab Sales, Inc., Hillsboro, OR) by vacuum filtration. The filter and undigested residues were then oven-dried at 105°C for 24 h to remove excess moisture and weighed. In vitro dry matter disappearance (**IVDMD**) was calculated as original dry sample weight minus dry residue weight divided by the original sample weight. This value was then multiplied by 100 to derive IVDMD percentage.

Acetate, propionate, butyrate, and lactate concentrations were measured by HPLC using an organic acid column (Callaway and Martin, 1997). All fermentations were performed on duplicate days with two replicates per day (n = 4). Data were analyzed by a general linear model procedure (SAS). All incubations were analyzed by fitting a model that contained *Sacc. cerevisiae* culture or *Sacc. cerevisiae* live cell dosage (0.0, 0.35, and 0.73 g/L). Least squares means for all treatments were reported and significance was tested at $P < 0.05$.

RESULTS AND DISCUSSION

In the absence of added substrates, *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells had no effect on H_2 or acetate (Tables 3.1 and 3.2). However, in the 24 h incubations 0.73 g/L of *Sacc. cerevisiae* culture decreased ($P < 0.05$) final pH, while 0.35 g/L of *Sacc. cerevisiae* live cells increased ($P < 0.05$) final pH (Table 3.1). Both concentrations of *Sacc. cerevisiae* culture decreased ($P < 0.05$) final pH in the 48 h incubations, while *Sacc. cerevisiae* live cell treatment increased ($P < 0.05$) final pH (Table 3.2). Both concentrations of *Sacc. cerevisiae* culture increased ($P < 0.05$) CH_4 concentrations and 0.73 g/L of *Sacc. cerevisiae* live cells increased ($P < 0.05$) CH_4 after 24 h, whereas all yeast treatments increased ($P < 0.05$) CH_4 in the 48 h incubations. Neither yeast treatment had any effect on acetate, propionate, butyrate, or the acetate:propionate ratio after 24 h (Table 3.1), but in the 48 h incubations both yeast culture treatments increased ($P < 0.05$) propionate and butyrate and decreased ($P < 0.05$) the acetate:propionate ratio (Table 3.2). Addition of 0.73 g/L of *Sacc. cerevisiae* live cells increased ($P < 0.05$) propionate and butyrate and both concentrations of *Sacc. cerevisiae* live cells decreased ($P < 0.05$) the acetate:propionate ratio. Previous research

showed that a filtrate of *Sacc. cerevisiae* culture (Diamond V XP, Diamond V Mills, Inc., Cedar Rapids, IA) contained glucose, lactate, malate, formate, succinate, and aspartate (Callaway and Martin, 1997). Therefore, it is likely that fermentation of these carbon and energy sources by mixed ruminal microorganisms account for the observed increased concentrations of fermentation products in the absence of added substrates. Similar results have been observed upon in vitro incubation of other *S. cerevisiae* cultures with mixed ruminal microorganisms in the absence of added carbon and energy sources (Martin et al., 1990; Sullivan and Martin, 1999).

To determine the effects of *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells on fermentation of corn, mixed ruminal microorganisms were incubated with ground corn (0.4 g/40 mL of media = 10 g/L) for 24 h (Table 3.3). As expected, final pH was lower and the concentrations of most fermentation products were much higher than the concentrations observed in the absence of carbohydrates (Table 3.3 vs Table 3.1). Neither *Sacc. cerevisiae* culture or *Sacc. cerevisiae* live cells had any effect on final pH, H_2, CH_4, propionate, or butyrate (Table 3.3). *Sacc. cerevisiae* culture had no effect on acetate, but both concentrations of *Sacc. cerevisiae* live cells decreased ($P < 0.05$) acetate and the acetate:propionate ratio. Even though the concentrations were low lactate was increased ($P < 0.05$) when 0.73 g/L of yeast culture was added to the incubations, whereas 0.73 g/L of live cells decreased ($P < 0.05$) lactate. Previous studies have reported that other *Sacc. cerevisiae* cultures decrease ruminal lactate concentrations (Koul et al., 1997; Williams et al., 1991).

To evaluate the effect of yeast treatment under conditions that induced very high lactate concentrations, mixed ruminal microorganism fermentations of soluble starch

were conducted (Table 3.4). As expected, final pH was lower and lactate concentrations were higher in these incubations compared to the ground corn fermentations (Table 3.4 vs Table 3.3). Neither *Sacc. cerevisiae* culture or *Sacc. cerevisiae* live cells had any effect on final pH, CH_4, acetate, propionate, or butyrate. A small increase ($P < 0.05$) in H_2 occurred in the presence of 0.35 g/L *Sacc. cerevisiae* culture. Similar results were observed in a previous in vitro study (Sullivan and Martin, 1999). *Sacc. cerevisiae* live cell treatment (0.73 g/L) decreased ($P < 0.05$) H_2. These changes in H_2 associated with yeast treatment are most likely of little physiological significance. Both concentrations of *Sacc. cerevisiae* live cells and 0.73 g/L of *Sacc. cerevisiae* culture decreased ($P < 0.05$) the acetate:propionate ratio. Even though the treatment effects were not statistically significant, both concentrations of *Sacc. cerevisiae* live cells and 0.73 g/L of *Sacc. cerevisiae* culture numerically decreased lactate concentrations compared to the control incubations.

The effects of *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells on the mixed ruminal microorganism fermentation of alfalfa hay and Coastal bermudagrass hay were determined (Tables 3.5 and 3.6). As expected, final pH remained above 6.0 and acetate concentrations were increased in the forage incubations compared to the ground corn and soluble starch incubations (Tables 3.5 and 3.6 vs Tables 3.3 and 3.4). When alfalfa hay was the substrate, *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells had no effect on H_2, propionate, butyrate, or the acetate:propionate ratio (Table 3.5). Both concentrations of *Sacc. cerevisiae* culture decreased ($P < 0.05$) final pH and IVDMD and the 0.73 g/L treatment decreased ($P < 0.05$) acetate. In comparison, both treatments of *Sacc. cerevisiae* live cells increased ($P < 0.05$) final pH and decreased ($P < 0.05$) acetate

and IVDMD. There was also a small decrease ($P < 0.05$) in CH_4 in the presence of 0.35 g/L of *Sacc. cerevisiae* live cells. In the case of Coastal bermudagrass hay, *Sacc. cerevisiae* culture decreased ($P < 0.05$) final pH (Table 3.6). However, no treatment effects were observed for the fermentation end products or IVDMD and *Sacc. cerevisiae* live cells had no effect on final pH, IVDMD, or fermentation end products. Previous research reported that *Sacc. cerevisiae* culture did not significantly affect IVDMD of either alfalfa hay or Coastal bermudagrass hay (Sullivan and Martin, 1999). In addition, *Sacc. cerevisiae* culture had little effect on the rate or extent of digestion of both forages by mixed ruminal microorganisms (Sullivan and Martin, 1999). Our results are consistent with these previous observations.

Even though there has been increasing interest by ruminant nutritionists regarding the difference(s) between *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells as feed supplements, few studies have been conducted to evaluate the effects of *Sacc. cerevisiae* live cells on the mixed ruminal microorganism fermentation. In our experiments, we did detect some differences between these two types of direct fed microbial products on the mixed ruminal microorganism fermentation. In the case of ground corn and alfalfa hay, both concentrations of *Sacc. cerevisiae* live cells decreased ($P < 0.05$) acetate more so than did *Sacc. cerevisiae* culture (Tables 3.3 and 3.5). In addition, *Sacc. cerevisiae* live cells treatment had a tendency to increase final pH in several fermentations, while *Sacc. cerevisiae* culture tended to reduce final pH (Tables 3.1, 3.2, and 3.5).

Several studies have suggested that *Sacc. cerevisiae* culture moderates ruminal pH by increasing lactate utilization by ruminal lactate-utilizing bacteria (Koul et al., 1997; Martin and Nisbet, 1992; Nisbet and Martin, 1991, Williams et al., 1991). When

ground corn was fermented by mixed ruminal microorganisms, low concentrations of lactate were detected (Table 3.3). However, *Sacc. cerevisiae* culture treatment increased ($P < 0.05$) lactate concentration, while 0.73 g/L *Sacc. cerevisiae* live cells decreased ($P < 0.05$) lactate. In the case of soluble starch fermentations, higher concentrations of lactate were produced, but neither *Sacc. cerevisiae* treatment had any significant effect on lactate (Table 3.4). Therefore, dietary supplementation with either forms of *Sacc. cerevisiae* may be beneficial in reducing ruminal lactate concentrations on diets that do not induce high ruminal lactate concentrations.

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TABLE 3.1. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on the 24 h in vitro mixed ruminal microorganism fermentation in absence of added substrate.

Fermentation		Yeast culture, g/L		Live cells, g/L		
product	Control	0.35	0.73	0.35	0.73	SEM
pH	6.46[a]	6.46[a]	6.43[b]	6.49[c]	6.47[ac]	0.01
H_2, m*M*	0.01	0.02	0.02	0.01	0.02	0.01
CH_4, m*M*	1.02[a]	1.53[b]	2.36[c]	1.16[a]	1.75[b]	0.10
Acetate, (A) m*M*	10.6	11.0	9.9	9.4	10.7	0.60
Propionate, (P) m*M*	2.2	2.2	2.0	1.9	2.2	0.15
Butyrate, m*M*	1.5	1.7	1.6	1.4	1.6	0.11
A:P ratio	4.97	5.19	5.10	5.44	5.07	0.13

[a,b,c] Means within a row lacking a common superscript letter differ ($P < 0.05$).

TABLE 3.2. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on the 48 h in vitro mixed ruminal microorganism fermentation in absence of added substrate.

Fermentation		Yeast culture, g/L		Live cells, g/L		
product	Control	0.35	0.73	0.35	0.73	SEM
pH	6.49[a]	6.46[b]	6.47[b]	6.52[c]	6.51[ac]	0.01
H_2, m*M*	0.00	0.00	0.01	0.02	0.00	0.00
CH_4, m*M*	1.16[a]	2.03[b]	2.56[c]	2.01[b]	2.54[c]	0.02
Acetate, (A) m*M*	14.2	16.0	15.8	14.6	16.5	0.01
Propionate, (P) m*M*	2.6[a]	3.1[b]	3.1[b]	2.7[a]	3.1[b]	0.01
Butyrate, m*M*	1.7[a]	2.0[b]	2.0[b]	1.8[ab]	2.1[b]	0.10
A:P ratio	5.65[a]	5.27[b]	5.08[c]	5.49[d]	5.42[d]	0.03

[a,b,c,d]Means within a row lacking a common superscript letter differ ($P < 0.05$).

Table 3.3. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on in vitro mixed ruminal microorganism fermentation of ground corn.

Fermentation		Yeast Culture, g/L		Live cells, g/L		
product	Control	0.35	0.73	0.35	0.73	SEM
pH	5.89	5.95	5.85	5.95	5.92	0.02
H_2, m*M*	0.28	0.16	0.21	0.22	0.16	0.04
CH_4, m*M*	11.5	11.8	10.6	11.0	11.8	0.58
Lactate, m*M*	0.10[a]	0.14[a]	0.27[b]	0.10[a]	0.02[c]	0.02
Acetate, (A) m*M*	24.0[a]	22.0[ab]	22.9[a]	19.0[bc]	19.2[c]	1.14
Propionate, (P) m*M*	11.5	10.3	11.4	9.8	9.8	0.59
Butyrate, m*M*	7.8	6.9	8.1	6.7	6.7	0.41
A:P ratio	2.22[abc]	2.35[ab]	2.12[ac]	2.06[c]	2.03[ac]	0.06

[a,b,c]Means within a row lacking a common superscript letter differ ($P < 0.05$).

Table 3.4. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on in vitro mixed ruminal microorganism fermentation of soluble starch.

Fermentation		Yeast culture, g/L		Live cells, g/L		
product	Control	0.35	0.73	0.35	0.73	SEM
pH	5.26	5.21	5.34	5.25	5.26	0.04
H_2, m*M*	0.18[ac]	0.26[bc]	0.23[abc]	0.16[a]	0.09	0.02
CH_4, m*M*	7.1	6.9	7.9	7.1	6.2	0.39
Lactate, m*M*	20.9	21.4	17.2	19.6	19.1	1.16
Acetate, (A) m*M*	18.2	17.3	15.1	17.4	17.2	1.58
Propionate, (P) m*M*	7.7	7.6	7.0	9.1	9.3	0.87
Butyrate, m*M*	3.9	3.6	3.2	3.6	3.6	0.39
A:P ratio	2.47[a]	2.4[a]	2.2[b]	1.91[c]	1.87[c]	0.05

[a,b,c]Means within a row lacking a common superscript letter differ ($P < 0.05$).

Table 3.5. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on in vitro mixed ruminal microorganism fermentation of alfalfa hay.

Fermentation		Yeast culture, g/L		Live cells, g/L		
product	Control	0.35	0.73	0.35	0.73	SEM
pH	6.29[a]	6.28[b]	6.27[b]	6.32[c]	6.32[c]	0.01
H_2, m*M*	0.03	0.05[a]	0.03	0.03	0.03	0.00
CH_4, m*M*	13.8	13.9	13.9	11.0[a]	13.5	0.37
Acetate, (A) m*M*	43.8[a]	43.1[a]	37.4[c]	35.3[b]	32.2[b]	2.57
Propionate, (P) m*M*	11.6	11.3	10.1	9.2	8.5	0.74
Butyrate, m*M*	4.1	4.2	3.8	3.4	3.1	0.27
IVDMD, %	59.4[a]	56.9[b]	56.9 [b]	53.8[c]	54.4[c]	0.68
A:P ratio	3.79	3.80	3.72	3.81	3.77	0.04

[a,b,c]Means within a row lacking a common superscript letter differ ($P < 0.05$).

Table 3.6. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on in vitro mixed ruminal microorganism fermentation of Coastal bermudagrass hay.

Fermentation		Yeast culture, g/L		Live cells, g/L		
product	0.00	0.35	0.73	0.35	0.73	SEM
pH	6.27[d]	6.24[abc]	6.24[abc]	6.26[bd]	6.27[d]	0.01
H_2, m*M*	0.06	0.06	0.04	0.06	0.03	0.01
CH_4, m*M*	13.0	14.2	13.9	13.7	14.6	0.05
Acetate, (A) m*M*	34.5	34.9	33.4	33.8	33.7	0.01
Propionate, (P) m*M*	8.9	9.1	9.1	8.8	8.9	0.02
Butyrate, m*M*	3.8	4.1	4.0	3.9	3.9	0.15
IVDMD, %	58.1	56.9	66.3	52.5	51.3	4.05
A:P ratio	3.88	3.82	3.65	3.83	3.81	0.06

[a,b,c,d]Means within a row lacking a common superscript letter differ ($P < 0.05$).

CHAPTER IV

CONCLUSIONS

Because soluble nutrient losses are associated with the ensiling process, concentrations of malate were expected to be lower in all silage treatments. Silage samples of all varieties had decreased malate and pH values and increased lactate, acetate, butyrate, propionate, and total VFA concentrations. All silage visually appeared ensiled.

Martin et al. (2000) also concluded that once the studied forages were placed within 50- to 100-L rumen the concentration of malate would be diluted. Therefore, taking into account the dilution factor plus the fact that organic acids can be fermented quickly, (Russell and Van Soest, 1984; Callaway and Martin, 1997), significant malate concentrations in ruminal fluid would likely not be detected (Martin et al., 2000). Similar conclusions may apply to our study. However, since the concentrations were twice as high in some cases, silage may provide the positive malate effects to dairy cattle. Ruminal malate concentrations between 4 and 12 m*M* were effective at reducing subclinical acidosis in steers fed a high-grain diet (Martin et al. 1999). In situ studies with high malate concentrations silage samples is recommended to determine the effects of ruminal fermentation on the available malate.

Even though there has been increasing interest by ruminant nutritionists regarding the difference(s) between *Sacc. cerevisiae* culture and *Sacc. cerevisiae* live cells as feed supplements, few studies have been conducted to evaluate the effects of *Sacc. cerevisiae* live cells on the mixed ruminal microorganism fermentation. In our

experiments, we did detect some differences between these two types of direct fed microbial products on the mixed ruminal microorganism fermentation. In the case of ground corn and alfalfa hay, both concentrations of *Sacc. cerevisiae* live cells decreased ($P < 0.05$) acetate more so than did *Sacc. cerevisiae* culture (Tables 3.3 and 3.5). In addition, *Sacc. cerevisiae* live cells treatment had a tendency to increase final pH in several fermentations, while *Sacc. cerevisiae* culture tended to reduce final pH (Tables 3.1, 3.2, and 3.5).

Several studies have suggested that *Sacc. cerevisiae* culture moderates ruminal pH by increasing lactate utilization by ruminal lactate-utilizing bacteria (Koul et al., 1997; Martin and Nisbet, 1992; Nisbet and Martin, 1991, Williams et al., 1991). When ground corn was fermented by mixed ruminal microorganisms, low concentrations of lactate were detected (Table 3.3). However, *Sacc. cerevisiae* culture treatment increased ($P < 0.05$) lactate concentration, while 0.73 g/L *Sacc. cerevisiae* live cells decreased ($P < 0.05$) lactate. In the case of soluble starch fermentations, higher concentrations of lactate were produced, but neither *Sacc. cerevisiae* treatment had any significant effect on lactate (Table 3.4). Therefore, dietary supplementation with either forms of *Sacc. cerevisiae* may be beneficial in reducing ruminal lactate concentrations on diets that do not induce high ruminal lactate concentrations.