EFFECT OF A SILAGE MICROBIAL INOCULANT ON ANIMAL PERFORMANCE AND SILAGE DIGESTIBILITY

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ABSTRACT

EFFECT OF A SILAGE MICROBIAL INOCULANT ON ANIMAL PERFORMANCE AND SILAGE DIGESTIBILITY

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Alfalfa forage was ensiled in two concrete stave silos. One silo served as a control (C), while the other was inoculated (I) with Lactobacillus plantarum. Silages in combination with slowly degradable (SD) and rapidly degradable (RD) protein sources were fed to lactating Holsteins and beef heifers. Silage digestion was evaluated by a feeding trial with Holstein steers and in vitro dry matter digestibility (IVDMD). Digestion of fresh alfalfa leaves by rumen cellulolytic species alone or in combination with L. plantarum in vitro were viewed with scanning electron microscopy.

Lactic acid bacteria (LAB) counts were greater in I than C by d 3. Lactic acid was greater ($p<.05$) and ammonia-N was lower ($p<.01$) in I than C during feedout. Fat corrected milk, protein and fat was greater ($p<.05$) for cows fed ISD than CSD. Steers fed CRD had the greatest feed efficiency, and lowest ($p<.10$) average daily gain. No differences were observed in digestibility.

To my mother and father, Alice and Jim, whose encouragement, love and support throughout the years has made all things possible.

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1.0 INTRODUCTION

The ensiling of forage crops, such as alfalfa has increased in popularity over the past few decades. The ability to harvest a high quality feedstuff without nutrient losses associated with poor drying conditions and the ability to store this material for long periods of time, makes ensiling economical. Successful fermentation of forages, however, relies on the quality of the ensiled material and fermentation rate (McCullough, 1978). Forage material must contain a sufficient amount of plant sugars to be used as substrate by epiphytic lactic acid bacteria (LAB), as well as sufficient numbers of these bacteria to convert sugars into lactic acid (Weinberg, et al., 1988). The rate and efficiency of acid production by epiphytic LAB are important factors in efficient silage making. A low pH inhibits other microbial activity thereby, restricting the breakdown of plant proteins into a highly soluble form, which is inefficiently utilized by the cow (Chamberlain, et al., 1986). Growth of clostridia resulting from plant protein degradation can cause high ammonia and butyric acid concentrations, as well as support a poor preservation and lower dry matter intakes.

In order to improve fermentation, a suitable LAB inoculant should be added to forage material at the time of ensilement. An inoculation containing sufficient homofermentative LAB would ensure rapid and efficient utilization of soluble carbohydrates and a faster decline in pH. A more rapid fermentation could

increase dry matter recovery and may preserve plant proteins, producing a higher quality silage.

Several studies using microbial inoculants have reported variable results. Many of these (Ohyama et al., 1975; Carpintero et al., 1979; Lindgren et al., 1983; Rooke et al., 1985) have reported advantages with the addition of a microbial inoculant while others (Throne, 1981; Ely et al., 1982; Buchanan-Smith and Yao, 1981; and Moon et al., 1981) have had negative effects with inoculation. The conditions under which these inoculants are effective have not been defined. Crop characteristics such as dry matter (DM) content, water soluble carbohydrate (WCS) content, buffering capacity and initial pH all affect the ensiling process and thus could affect inoculation (Pitt and Leibensperger, 1987).

The concept of inoculating forages has been widely accepted as common practice throughout many parts of the world. Although several studies have indicated changes in fermentation which occur with inoculation, little attention has been directed toward the effect of specific inoculants on the nutritional quality of silage.

The objectives of the following studies were designed to measure fermentation effects as well as the nutritional value of inoculated alfalfa silage through livestock production trials, in vitro laboratory experiments and electron microscopy.

2.0 REVIEW OF LITERATURE

2.1 Fermentation of Alfalfa Forage

2.1.1 The Fermentation Process

Silage fermentation consists of biochemical changes which occur in fresh plant material during ensilement. Activity is initiated during wilting when epiphytic bacteria use soluble plant sugars as a substrate and multiply. Once ensiled, plant enzymes use glucose, fructose, sucrose and fructans along with trapped oxygen to produce water, carbon dioxide and energy. The energy which is produced cannot escape the forage matter, thus is liberated as heat, increasing the temperature of the plant material. These reactions continue as long as oxygen and sugars are available, and can continue through feedout. Such an event could result in large amounts of nutrients broken down into carbon dioxide and water, leading to a considerable amount of DM loss (Woolford, 1984). Control of this activity is a question of chop length, rate of ensiling, silo design, sealing and general management (McDonald, 1979).

The majority of organisms found on growing crops are aerobes. The number of LAB is generally low (Stirling, 1953; Keddie, 1959; Stirling and Whittenbury, 1963). However, counts usually rise significantly by the time the herbage reaches the silo. The microbial increase is due to inoculation of forage by farm machinery (Gibson, et al., 1961; Henderson, et al., 1972), and liberated sap made available as

substrate during the chopping and laceration of fodder (Greenhill, 1964).

Multiplication of these microbes can continue until sugar substrates are depleted. Forage species, DM content, substrate availability and buffering capacity are all factors which affect fermentation. In addition, the number and species of anaerobic bacteria can play a major role in the quality of fermentation (Carr, et al., 1984; Ely, et al., 1982; Ely, et al., 1981; Kung, Jr., et al., 1984; McDonald and Henderson, 1962; Moon, et al., 1981). The greater the number of homofermentative LAB, the more lactic acid is produced and the quicker the drop in pH (Muck, 1989). A decrease in the pH of the ensiled mass needs to be low enough to inhibit undesirable microbial activity and endogenous plant catabolic processes (Shockey, 1988). It is also important that the pH declines at a rapid rate to prevent proteolysis, thus preserving the maximum amount of nitrogen (N) as protein N (McDonald, 1981).

2.1.2 Effect of Lactic Acid Producing Bacteria During Fermentation

The principle of microbial inoculation was first adopted in 1909 by Bouillant and Crolbois, when they applied lactic acid inoculants to beet pulp to improve fermentation (Watson and Nash, 1960). Later, in 1930, Ruschmann and Koch and in 1934, Rushmann and Meyer (Fenton, 1987) documented that the rate of acidification during silage fermentation is dependent on epiphytic bacteria found on fodder plants.

There are numerous microorganisms found on growing plants (Woolford, 1984), with the number tending to increase with plant maturity and advancement of the season (Kroulick, et al., 1955). The majority of these are Gram negative aerobes, which will not thrive in the anaerobic environment of the silo. Thus their

enzymatic processes contribute little to silage preservation. However, the Gram positive lactic acid producing bacteria, are facultative anaerobes, which enables them to utilize soluble sugars to carry out metabolic functions aerobically on the plant or anaerobically in the silo. The number of LAB on growing alfalfa is generally low, usually less than 100 cfu/g and reduced further during wilting (Keddie, 1959; Stirling and Whittenbury, 1963). However, counts of lactobacilli usually rise significantly by the time they reach the silo. This is partly due to inoculation of microorganisms from farm machinery (Henderson, et al., 1972; Gibson, et al., 1961).

Until 1978, there was little known about the composition of microflora during silage fermentation. However, Beck (1978) studied the qualitative changes in LAB during the fermentation of grass and red clover with high and low DM contents. He reported that fermentation in wilted and unwilted silage was initiated by homofermentative LAB being 5% of total lactobacilli present by day 4. However, after 142 d of fermentation, 75% of all lactobacilli in the silage with the low DM and 98% of the lactobacilli in the silage with high DM were heterofermentative. Beck suggested that bacteriologic shift could be due to a greater acetate tolerance in heterofermentative bacteria. Table 1 shows the bacterial species commonly found in silage (McDonald, 1981). The dominant organisms in silage according to Langston and Bouma (1960) are L. plantarum, L. brevis and Pediococcus sp.

Gibson, et al., (1958) reported that L. plantarum and L. acidophilus were the dominant homofermentative bacteria in fermentation. While others (Langston, et al., 1962; Moon, 1981, and Moon, et al., 1981) revealed evidence that streptococci and leuconostocs initiate fermentation and are superceded by species of Lactobacilli and Pediococci.

TABLE 1. Classification of Lactic Acid Bacteria Important in Silage

(A) Heterofermentative

Coccus

Leuconostoc mesenteroides
Leuconostoc dextranicum
Leuconostoc cremoris

Rod

Lactobacillus brevis
Lactobacillus fermentum
Lactobacillus buchneri
Lactobacillus viridesceno

(B) Homofermentative

Coccus

Streptococcus faecalis
Streptococcus faecium
Pediococcus acidilactici
Pediococcus cerevisiae
Pediococcus pentosaceus

Rod

Lactobacillus plantarum
Lactobacillus curvatus
Lactobacillus casei
Lactobacillus coryniformis subsp. coryniformis

McDonald, P. 1981

Table 2 illustrates the products of an anaerobic sugar fermentation by LAB described by Whittenbury and coworkers (1967). Glucose and fructose are the most common soluble sugars utilized by LAB, however LAB can also ferment pentoses, xylose and arabinose, which are formed from the degradation of hemicellulose (Dewar, et al, 1963) and amino acids (Rodwell, 1953).

2.1.3 Plant Proteolysis

The deamination of protein in silage is another process resulting from plant enzyme activity. The breakdown of fresh plant material can be caused by plant proteases (Bergen, et al., 1974; Ohshima and McDonald, 1978), however, most proteolytic activity is a result of aerobic conditions inside the silo.

Figure 1 illustrates post-harvest nitrogen metabolism in ensiled plant material from hay and cereal crops (Bergen, 1974). Fresh forage material contains 70-90% of the total nitrogen in the form of protein while the remaining 10-30% is non-protein nitrogen consisting of free amino acids, amides and small concentrations of urides, amines, nucleotides, chlorophyll, low molecular weight peptides and amino acids bound in non-protein form (Hegarty and Peterson, 1973). It is not uncommon for 50-60% of the true protein nitrogen to be broken down into simpler non-protein nitrogenous compounds in preserved forage (Whittenbury, 1967).

Amino acids resulting from proteolysis can be metabolized into ammonia (deamination), amines (decarboxylation) and unidentified nitrogenous compounds (Bergen, et al., 1974; Ohshima and McDonald, 1978). A good quality silage is characterized by low concentrations of ammonia-N, amines and other compounds produced from the break down of amino acids (Bergen, 1984). If aerobic conditions remain in the silo it creates an environment which allows yeast and mold to

TABLE 2. Anaerobic Pathways of Sugar Metabolism by Lactic Acid Bacteria

Homofermentative
1 glucose--------> 2 Lactic acid
1 fructose--------> 2 Lactic acid
1 pentose---------> 1 Lactic acid + 1 Acetic acid
Heterofermentative
1 glucose---------> 1 Lactic acid + 1 Ethanol + 1 Carbon dioxide
3 fructose--------> 1 Lactic acid + 2 Mannitol + 1 Acetic acid
1 Pentose---------> 1 Lactic acid + 1 Acetic acid

Whittenbury, et al., 1967

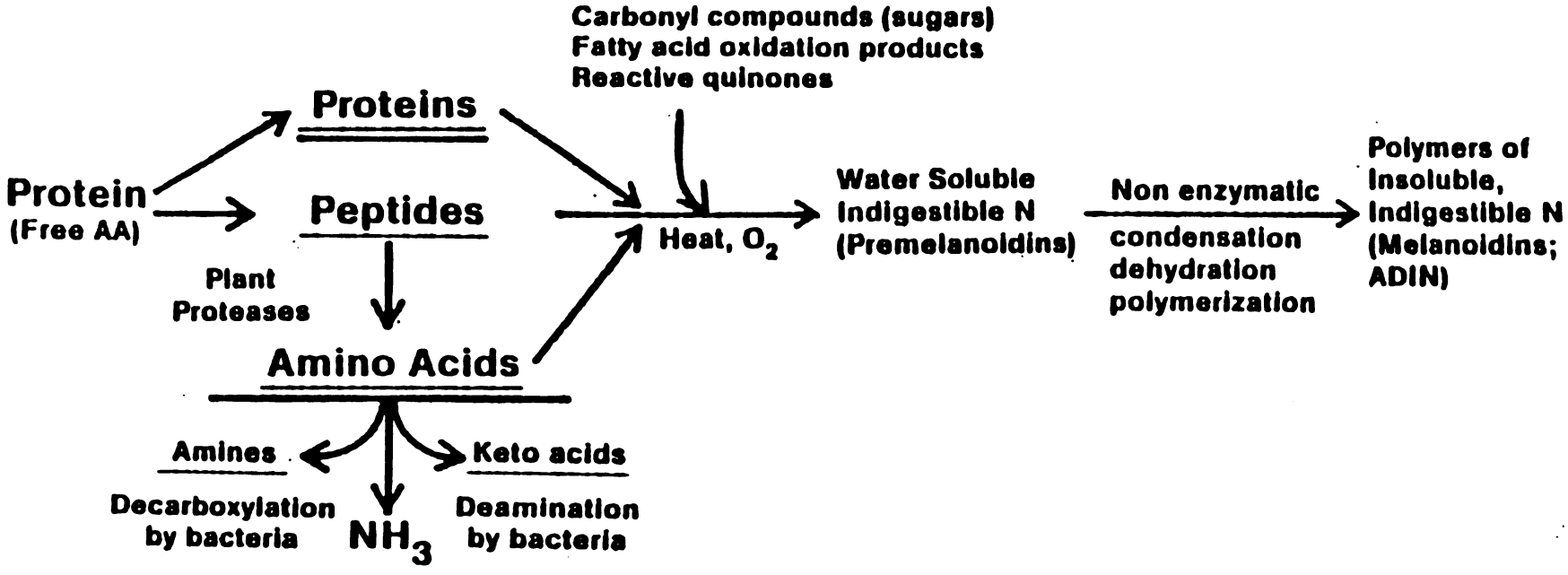

Figure 1. Transformation of N constituents during ensiling.

multiply and increase the silage temperature (Bergen, 1984). Clostridial fermentation is associated with ammonia, butyric acid and a higher pH than that found with lactic acid bacteria. This results in an unstable and often unpalatable silage. Butyric acid produced by sacchrolytic organisms which metabolize lactate and sugars, (Table 3) often serves as an indicator of clostridial activity. The result of this type of fermentation occurs at a high DM or a low pH (Whittenbury, et al., 1967). Woolford (1984) suggested that clostridial activity is suppressed at a dry matter above 31% and/or a pH below 4.5. Under ideal conditions, sufficient numbers of lactic acid producing bacteria occurring naturally, would produce a drop in pH during day 2-5 of ensilement. Bergen and coworkers (1974) suggested that DM of forage material at the time of ensilement is the most decisive factor influencing the amount of protein degradation which will occur during fermentation. The lower the DM, the larger the amount of plant protein escaping proteolysis. Thus, DM at the time of ensiling and rate at which the pH falls during fermentation are factors one must consider during silage preservation.

2.1.4 Substrate Utilization During Silage Fermentation

The major water soluble carbohydrates (WSC) found in forage material are glucose, fructose, sucrose and fructosans. The most available sugars for microbial substrate are glucose and fructose, due to the continual hydrolysis of sucrose and fructosans to glucose and fructose monomers (Whittenbury, et al., 1967).

The WSC content as well as the fructose/glucose ratio of green fodder plants varies depending on species, weather conditions, stage of growth, time of day, wilting conditions and fertilizer application (Woolford et. al., 1982).

Soluble carbohydrates present in forage material after aerobic metabolism are

TABLE 3. Biological Reactions Associated with Clostridial Fermentation

Organic Acids

2 Lactic acid----------> 1 Butyric acid + $2CO_2$ + $2H_2$

Amino Acids

(A) Coupled oxidation-reduction reactions

1 Alanine + 2 Glycine----->3 Acetic acid + $3NH_3$ + $1CO_2$

(B) De-amination

3 Alanine--------> 2 Propionic acid + 1 Acetic acid + $3NH_3$ + $1CO_2$

1 Valine---------> 1 Isobutyric acid + 1 NH_3 + 1 CO_2

1 Leucine--------> 1 Isovaleric acid + 1 NH_3 + 1 CO_2U

(C) Decarboxylation

Histidine--------> Histamine

Lysine----------> Cadaverine

Arginine---------> Ornithine-------->Putrescine

Tryptophan-------> Tryptamine

Tyrosine---------> Tyramine

fermented by a variety of microorganisms, however, under ideal conditions LAB ferment sugars and produce an intolerable acidic environment for other microorganisms (Whittenbury, et al., 1967). Lactic acid bacteria utilize soluble sugars through two fermentable pathways to produce lactate (Table 2, Whittenbury, et al., 1967), as previously described. Homofermentative LAB are the most desirable for they are more efficient in producing lactate than heterofermentative LAB (producing 2 moles of lactic acid versus one mole), and more efficient in conservation of DM (McDonald, et al., 1973). One cannot predict a final ratio of fermentation products, for it is possible to have 100% variation occur in the amount of lactic acid produced under two similar circumstances.

In addition to phosphate, several organic acids also are commonly found in fresh herbage and silage. These acids include malate, citrate, and glycerate (McDonald, 1979). Organic acids in combination with their salts comprise a buffering system in plants (Playne and McDonald, 1966). Legumes contain higher amounts of acid (0.6 to 0.8% of DM) than grasses (0.2 to 0.6% of DM), as well as higher protein and more cations which contribute to a much greater buffering system.

Considerable interest has been given to those organic acids in silage which buffer within the pH range of 4-6. Early stages of fermentation are characterized by the dissimilation of organic acids by LAB (Edwards and McDonald, 1978). The main products of citrate and malate fermentation by LAB are shown in Table 4 (Whittenbury, et al.,1967). Products from these reactions include formation of organic salts (lactate, acetate), neutral products (ethanol, acetone and 2,3 butanediol) and alkaline released cations (Whittenbury, et al., 1967). Other substrates which can be fermented by LAB include amino acids (Rodwell, 1953).

TABLE 4. Fermentation of Organic Acids as Substrates by Lactic Acid Bacteria

A. 1 Citric acid--------> 2 Acetic acid + 1 formic acid + 1 carbon dioxide

or

2 Citric acid--------> 2 Acetic acid + 1 acetone (or 2,3 butanediol) + 4 carbon dioxide.

or

2 Citric acid--------> 3 Acetic acid + 1 lactic acid + 3 carbon dioxide

B. 1 Malic acid---------> 1 Acetone (or 2,3 butanediol) + 4 carbon dioxide

or

2 Malic acid---------> 1 Acetone (or 2,3 butanediol) + 4 carbon dioxide

or

1 Malic acid---------> 1 Acetic acid (or ethanol) + 1 formic acid + 1 carbon dioxide

Whittenbury et al., 1967

Brady (1966) demonstrated that L. plantarum and L. brevis can deaminate serine, arginine, glutamine and aspargine.

2.1.5 Aerobic Stability of Silage

The most important factor in achieving high quality silage is rapid occurrence of anaerobiosis in the silo. Other factors influencing aerobic deterioration include quantity of substrate, DM of the ensiled crop, botanic origin and ambient temperature (Woolford, 1990).

Aerobic deterioration of silage ultimately results in complete mineralization of easily oxidized nutrients which are broken down into CO_2 and H_2O, generating heat and resulting in DM losses (Woolford, 1984). Studies have shown that DM losses over a period of 5-15 days can be as great as 32%. Once the process of aerobic deterioration commences, it is practically impossible to stop (Honig and Woolford, 1979).

An ingress of air as small as 100 to 150 mg O_2/kg DM is adequate to make silage highly susceptible to aerobic deterioration (Woolford, et al. 1979). Upon exposure to oxygen, conditions become favorable for proliferation of aerobic bacteria, yeasts and fungi (Moon et al., 1980 and Woolford et al., 1982). In most silages, yeasts have the ability to increase in numbers from $<10^2$ to 10^{12} cfu/g DM by day 3 of aerobic exposure (Beck 1963, as cited by Woolford, 1990).

Yeasts involved in aerobic deterioration have been classified as acid-utilizers comprised of Candida, Endomycopsis, Hansenula and Pichia sp. and sugar-utilizers which are Torulopsis sp. (Gross and Beck, 1970, as cited by Woolford, 1990; Moon and Ely 1979; Johnsson and Pahlow, 1984). A high population of yeasts does not necessarily mean a silage will deteriorate. Instead, quantity of

lactate-utilizing yeasts decides whether a silage will deteriorate or not upon aerobic exposure (Johnnson and Pahlow, 1984).

Thermophilic filamentous fungi are also found in deteriorating silage, however their growth is generally slower and thus have little affect on silage as a feed.

Woolford and Cook (1978) treated silage material with antibiotics that had antibacterial and antifungal properties. Their studies revealed the involvement of proteolytic bacteria from the genus Bacillus. Bacteria appear to initiate deterioration in maize silages, followed by yeasts (Woolford et al., 1978). Deterioration in cereal crops and grass silages on the other hand, begins with yeasts (Woolford et al., 1979). However, Woolford (1984) concluded that this inconsistency concerning the identity of microbial groups responsible for the onset of aerobic deterioration lies in the properties of ensiled material, specifically DM content rather than botanic origin.

Primary substrates of aerobic deterioration have been described as nitrogen free extracts which included water soluble carbohydrates and organic acids (Honig and Woolford, 1979). Woolford (1990) suggests that the organisms involved in aerobic deterioration will use a wide range of substrates which include those found in the original crop and others which are produced by fermentation. Regardless of the substrate utilized, deterioration in forage crops is always accompanied by a loss of residual sugars and the evolution of ammonia and carbon dioxide. The latter can be directly equated to DM loss and its measurement can be used to monitor the progress of deterioration (Woolford, 1990). Fermentation acids (such as acetic and lactic acids), amino acids and proteins are all used as substrates (Woolford, 1984). The pH increases with acid depletion and tends to be greatest at the silage surface where exposure to oxygen is greatest (Woolford, 1978).

Aerobic deterioration occurs in all silages to some varying degree, except for those undergoing an extensive secondary fermentation. This deterioration depreciates conservation efficiency, causes nutritional losses and can even pose a potential health hazard to livestock. Such management practices as rapid silo filling, special cutting equipment for forage removal, resealing between feed-outs and use of an effective inoculant at the proper application rate can minimize aerobic deterioration.

2.1.6 Silage Inoculants

At the present time, there are several silage inoculants on the market. They have been reported to influence the rate and extent of silage fermentation. Typical ingredients found in inoculant may include enzymes, bacteria, molds, micronutrients for microorganisms or a mixtures of all these to influence forage respiration and fermentation rate (Parker, 1979). Bolsen (1978) has described silage inoculants as "those products that supply lactic acid producing microorganisms and enzymes and/or microorganisms that increase the availability of carbohydrates and other nutrients to lactic acid producing microorganisms".

Commercially available inoculants not only vary in ingredients but in type of preparation (dried, liquid, freeze-dried) and packaging (bottles, vacuum packs and paper sacks).

Whittenbury (as cited by Beck, 1978) described the requirements of a quality silage microorganism as follows:

1. It must be fast growing and able to compete with and dominate other microorganisms present in silage.

2. It must be homofermentative.

3. It must be acid tolerant down to a silage pH of 4.0.

4. It must possess the ability to ferment glucose, fructose, sucrose, and preferably fructosans and pentosans.

5. It should have no action on organic acids.

And in 1975, McCullough described the following as requirements of a cost effective quality inoculant:

1. The cost of the additive must be less than the silage lost without the additive.

2. Addition of the additive must result in a more efficient fermentation than occurs naturally.

3. The additive should produce a silage with a greater digestibility energy and/or protein than untreated silage.

Several workers have shown varying results from inoculation, including advantageous results (Rooke et al., 1985; Ohyama et al., 1975 and Owens, 1977) and non-significant results (Ely et al., 1982; Moon et al., 1981 and Buchanan-Smith and Yao, 1981).

2.2 Rumen Cellulolytic Bacteria and Their Role in Fiber Digestion

2.2.1 Rumen Microbial Fermentation and Digestion

The rumen is an ideal fermentation site. It makes up one-seventh of the total mass of a ruminant's body weight (Russell and Hespell, 1981). The rumen remains at a constant temperature of 39°C and is well buffered by salivary secretions. The microflora inhabiting the rumen is dense containing approximately 10^{10} to 10^{11} bacterial and 10^{5} protozoal cells per milliliter of rumen contents. There is an extensive diversity and synergism in the ecosystem which contains more than 200 species of bacteria and over 20 species of protozoa (Bryant and Robinson, 1962).

During ruminal fermentation, feedstuffs are broken down and fermented into short chain fatty acids through microbial metabolism and are used as the ruminant's energy source, while the animal relies heavily on the microbial mass as a protein source. Methane, heat, and ammonia are formed as well, representing a loss of energy and nitrogen to the animal. The balance of fermentation products determines the efficiency of nutrient utilization in ruminants. In turn, this balance is ultimately controlled by the various microorganisms found in the rumen.

2.2.2 Plant Cell Wall Constituent

In ruminants the plant cell wall is extensively degraded and utilized as an energy source by the rumen microflora. Plant cell walls are indigestible by animal enzymes, however, gastrointestinal microflora partially degrade cell wall material.

The cell wall of plants is made up of an organic matrix of cellulose, hemicellulose, lignin and other small fractions of pectins, gums mucilages, cutin, tannin, bound cell wall protein and cell wall minerals.

Cellulose

First recognized by Payen in 1939 (Whistler and Smart, 1953), cellulose is the most abundant carbohydrate in the world. Its recycling is dependent on microbial activity which produces carbon dioxide during degradation. An enormous amount of energy lies in these cellulosic carbohydrates, making them an excellent food source for herbivores. Cellulose is the largest component of plant cell walls, thus serving as a primary structural element. Linked at the C-1 and C-4 position through glycosidic linkages, individual anhydrous glucose molecules make up the linear polymer in a beta configuration. Glucan chains consist of 100 to 10,000 or more units of glucose (Ott and Tennent, 1954), and are held together by tight hydrogen bonds (Albersheim, 1975) between the hydroxyl group of a sugar on one chain and an oxygen atom of another. Chains are also held together by VanderWaals forces.

Hemicellulose

Hemicellulose is the second largest constituent found in plant material (Phillips, 1940). First named in 1891 by Schultz (Whistler and Richards, 1970), hemicellulose has been defined as the polysaccharide in plant tissues other than cellulose which is extracted with alkali and hydrolyzed in acid (Collings, 1979). Hemicellulose is a complex mixture of polysaccharides which constitute much of the cell wall matrix (Bailey and Gaillard, 1965). It is a polybeta 1-4 D-

xylanopyranose based on a backbone of xylose residues, with branches of arabinose, glucose and/or galactopyranosides (Akin and Barton, 1983).

Lignin

Lignin is a polymer of phenylpropanoid units intimately associated with structural carbohydrates (Himmelsbach and Barton, 1980), and plays a major role in reducing microbial attack on cell walls (Akin and Barton, 1983). Phenolic acids such as p-coumaric acid and ferulic acid which are precursors of lignin can bind to structural carbohydrates which inhibits carbohydrate degradation (Hartley et al., 1974).

Other Constituents

Pectin is comprised of chains of galacturonic acid, galactans and arabinans (Aspinall, 1973). Pectins are not pure polysaccharides, but mixed and branched, forming complex polysaccharide structures. It is found in intracellular spaces in the cell wall and is associated with cellulose in other cell layers (Esau, 1965). Hemicellulose, pectin and lignin play an important role as matrix substances for the cell wall.

Cowling (1976) demonstrated that crystallinity and lignification are the most important factors in determining the susceptibility of cellulose to enzyme degradation. It has been shown that specific enzymes which attack glucan bonds in cellulose chains are incapable of attacking an intact plant fiber (Albersheim, 1975). Thus accessibility of cellulose to microbial enzymes and chemical reagents depends on the arrangement of cellulose within the cell wall (Collings, 1979).

Although some plant material is accessible and easily digested, the degradation

of fiber material in the rumen is a result of complex microbial processes (Cheng et al., 1980). These processes include the digestion of plant cell walls, to yield microbial cell growth and fatty acids end products. As with any ecological system, the microorganism should be attracted to its nutrient substrate. It has been demonstrated that plant material undergoing colonization and digestion by rumen microorganisms includes the adherence of bacteria, protozoa and fungi, however, bacteria are responsible for the majority of the digestion which takes place in the rumen (Hungate, 1966). Akin and Barton (1983) found through the use of the scanning electron microscope (SEM) that plant cell wall digestion did not occur unless rumen bacteria were closely associated with or completely adhered to the cell walls.

2.2.3 Rumen Cellulolytic Species

Based on relative numbers in the rumen of domestic ruminants and their ability to attack various forms of cellulose in pure cultures, the major rumen cellulolytic bacteria are Ruminococcus flavefaciens (Sijpestein, 1951), Ruminococcus albus (Hungate, 1957), and Bacteroides fibrobacter succinogenes (Hungate, 1950). These are the three major species which obtain their energy for growth solely through cellulose fermentation (Bryant, 1973). B. fibrisolvens will digest cellulose to a lesser extent (Bryant, 1973; Hungate, 1966). Each of these species except B. succinogenes are capable of utilizing hemicellulose-type components from forage (Dehority and Scott, 1967). B. succinogenes is the most active cellulolytic, bacterium digesting the more resistent cellulose such as cotton fibers and mature hay to a greater extent than Ruminococcus, which are active,

but show much more variation between strains in ability to degrade more resistant cellulose (Bryant, 1973).

Minato and coworkers (1966), noted that both Ruminococcus and B. succinogenes adhere to fiber during digestion, however, B. succinogenes was firmly attached to the cell wall. A few other cellulolytic species of the genus Clostridium (Hungate, 1957; Shane et al., 1969) and Eubacterium cellulosolvens (Bryant et al., 1958; Van Gylswyck and Hoffman, 1971) have been found in the rumen occasionally.

The largest numbers of cellulolytic bacteria are found when the ruminant is fed a high roughage diet, however in ruminants fed cellulose as the total feed source, cellulolytic bacteria only comprise 25% of the total rumen microbial population (Slyter et al., 1971). Many non-cellulolytic bacteria found in the rumen are responsible for the degradation of pectins and xylans. Numerous synergistic interactions between cellulolytics and noncellulolytic species occur and has been shown to enhance cellulose degradation (Dehority and Scott, 1967).

Rumen cellulolytics produce cellulose enzymes which hydrolyze insoluble cellulose into soluble cellulodextrins or sugars, some of which they can absorb and ferment to obtain energy for growth (Schaefer and King, 1965; Sheth and Alexander, 1969).

End products of cellulose degradation include acetate, propionate, butyrate, CO_2 methane, and microbial cells. This includes interacting populations of 1) rumen cellulolytic bacteria, 2) carbohydrate fermenting species which can use products hydrolyzed from cellulose, 3) species which will degrade succinate, formate and any lactate produced from microbes in 2 and 4) methanogenic bacteria which will reduce CO_2 using H_2 or formate as an electron donor (Hungate, 1950).

All rumen cellulolytic bacteria require one or more B-vitamins for growth. Biotin is the most common vitamin required by the cellulolytics. However, some strains of R. albus also require pyridoxine. A few strains of R. albus may require folic acid, riboflavin or thiamine (Bryant, 1973). The vitamins required by R. flavefaciens strains are similar to those required by R. albus (Bryant and Robinson, 1961; Gill and King, 1958; Scott and Dehority, 1965), with some strains requiring pyridoxine and cobalamine which in some cases can be replaced by methionine (Scott and Dehority, 1965). B. Succinogenes requires biotin, using this as its primary B-vitamin. P-aminobenzoic acid has been shown to stimulate the growth in some strains of R. albus (Bryant and Robinson, 1961; Scott and Dehority, 1956). B. succinogenes has a requirement for Na^+ and a great demand for Ca^{2+} (Bryant et al., 1959). The other cellulolytics have a lower demand for K^+, Na^+, and Ca^{2+}. Ferrous iron and Zn^{2+} has been found to stimulate microbial activities even further (Matturi, 1972).

All of the rumen cellulolytics have a requirement for sulfur. B. succinogenes utilizes cysteine or sulfide, but not sulfate (Bryant et al, 1959). The Ruminococcus grow well in media containing sulfide or sulfate (Bryant, 1973).

The main nitrogen source for cellulolytic bacteria is ammonia (Bryant and Robinson, 1961; Bryant et al., 1959, Dehority, 1963). The ammonia is a product of non-cellulolytic bacteria metabolism. This is just another example of co-existence and cooperation between rumen species. Cellulolytic bacteria lack the ability to use organic nitrogen sources for growth and though not established, it appears that they probably lack the mechanism for transporting amino acids or peptides into the cell (Pittmann et. al, 1967). Although Ruminococcus bacteria cannot use amino acids if present, B. succinogenes will utilize the amide nitrogen from glutamine

and asparagine for growth and function (Bryant and Robinson, 1961).

Many strains of rumen cellulolytic bacteria require a carbon source beyond that of the energy source. The source commonly used by these bacteria is CO_2 or bicarbonate. B. succinogenes and R. flavefaciens require large amounts of CO_2, which is fixed into pyruvate during glycolysis (Caldwell et al., 1969). Without CO_2, these bacteria are unable to obtain energy in the form of carbon, for growth (Bryant, 1973). They also use CO_2 for biosynthetic purposes (Allison, 1969; Allison 1970). R. albus does not require large amounts of CO_2 for growth, but requires small amounts for optimal growth and for biosynthetic processes (Bryant, 1973).

Short chain fatty acids, better known as volatile fatty acids are essential for growth of the three major rumen cellulolytics at 0.5-0.3mM in batch cultures (Dehority and Scott, 1967). Carbon skeletons from these fatty acids are not degraded, but incorporated into certain cellular constituents (Bryant, 1973). Bacteroides succinogenes is the only cellulolytic that requires the straight chain valeric acid, which can be replaced by longer chain acids (Wegner and Foster, 1963). The cellulolytic bacteria utilize the various branched chain fatty acids, such as C_{14} and C_{16} from isobutyric, C_{15} and C_{17} from isovaleric, and anteisa C_{15} and C_{17} from 2-methyl-butyrate (Allison, et al., 1962; Wegner and Foster, 1963). These branched chain fatty acids are also precursors for fatty aldehydes in these bacteria. One or more of the above fatty acids are used for the biosynthesis of amino acids: valine, leucine, and isoleucine respectively (Allison et al., 1962; Robinson and Allison, 1969; Allison, 1970) via reductive carboxylation reactions (Bryant and Robinson, 1961; Allison, 1969).

2.2.4 Cellular Attachment and Digestion of Plant Material

There are many factors which influence the rate and extent of forage cell wall digestion. Feeds containing fractions of cellulose and hemicellulose are relatively insoluble in the rumen and are degraded slowly (Dehority, 1973; Van Soest, 1973). Degradation is highly influenced by structural factors. Such factors would include the close association of lignin with cellulosic materials, acting as a barrier against bacterial cellulases (Russell and Hespell, 1981). Crystallinity also effects digestion (Bryant and Robinson, 1962). Russell and coworker (1981), showed that high crystalline fibers were readily degraded by cellulases from certain cellulolytic bacteria while fiber digestion was much slower for other cellulolytic species. Those who have made extensive observations (Akin and Amos, 1975; Akin et al., 1974) of mixed cultures of rumen bacteria have observed that many rumen bacteria appear to adhere to plant cell walls by means of thin fibrous capsules. In many of these observations, it has been noted that the bacteria digest plant cell wall material and infiltrate the resultant cavities.

Cheng and coworkers (1977) found that bacteria in the rumen of cows fed corn silage versus other forage based diets had the least bacterial slime formation, but every bacterial cell showed some extracellular structure. Although some plant material is accessible and easily digested, the process is long and sequential (Akin and Amos, 1975). Digestion begins with penetration through the stomata (Baker and Harris 1947) and colonization on fiber macerations produced from mastication. Dinsdale et al., (1978) in an <u>in vitro</u> study demonstrated that mixed populations of rumen bacteria released 12 to 36% of the dry matter of damaged cells in legume leaves. These organic nutrients are used to support enormous proliferations of bacteria in intracellular space and at the leaf surface. Subsequently, plant cell

walls are ruptured by certain species of bacteria who digest cellulose in grasses and cellulose and pectins in legumes (Dinsdale et al., 1978). Plant protoplasm which remains to be digested supports a further proliferation of bacteria until bacterial microcolonies fill plant cell wall compartments, while refractory cells remain uncolonized (Akin and Amos, 1975).

3.0 FERMENTATION CHARACTERISTICS AND NUTRITIVE VALUE OF ALFALFA FORAGE ENSILED WITH AND WITHOUT ADDITION OF A BACTERIAL INOCULANT

3.1 Introduction

Preservation of forage crops as silage has increased in popularity over the past years due to excellent conservation of nutrients and the ability to obtain a higher quality roughage. The success of ensiling forage relies on the presence of adequate numbers of microorganisms, soluble sugars for use as substrates and an anaerobic environment. Fulfillment of these conditions will allow a lactic acid fermentation to predominate (Whittenbury, et al., 1967). Kroulik, et al., (1955) reported that there was a considerable variation in the numbers of bacteria found on green plants and cut forages. Bacterial populations varied with the type of plant, anatomical location, season, weather conditions and plant maturity.

Bacteria responsible for a rapid fermentation and production of a quality forage are predominately lactic acid producers (Kempton and Clement, 1959; Langston and Bouma, 1960). The addition of Lactobacillus sp. to fresh forages has been recommended for control of silage fermentation (Lesens and Shultz, 1968; McDonald, et al., 1964). Previous efforts (Bolsen, 1978; Thomas, 1978) to utilize microbial additions to silage have varied from no response to increased DM and protein recovery.

As milk production increases, the requirement for total N for the lactating cow

increases. The intake of ruminally degradable N often exceeds the amount which is converted into microbial protein. Consequently, protein nitrogen supply to the small intestine may be limiting. Efficiency of N utilization is improved as more rumen undegradable protein is fed (Waldo and Glenn, 1984). Titgemeyer, et al., (1989) evaluated amino acid disappearance from the small intestine with four dietary protein supplements. In their study, each protein supplement was inadequate in at least one of the essential amino acids, thus suggesting that amino acid requirements of ruminants should be supplied by a combination of protein supplements.

The objectives of this study were to examine the ensiling characteristics of alfalfa forage treated with or without the addition of a bacterial inoculant and to evaluate the response of lactating Holsteins and crossbred beef heifers fed the silage in combination with a slow or rapidly degradable rumen protein source.

3.2 Materials and Methods

3.2.1 Silo Filling and Sampling

Two hundred and sixty tons of 1/10th bloom first cutting alfalfa forage was wilted to 45% dry matter (DM), chopped to .6 cm length and ensiled in two top unloading upright concrete stave silos (4.3 x 18.3 M). One silo served as a control silo, containing uninoculated forage material (C), while the other was inoculated (I) with a commercial inoculant (Ecosyl, CIL Inc., Ontario, Canada N6A 4L6). The inoculant contained a strain of Lactobacilli plantarum and was applied in liquid form at the blower to provide 2.5×10^6 colony forming units cfu/g of chopped forage. Each silo was filled in an alternate load sequence. Incoming loads of forage were sampled for DM determination and composited based on whether they were harvested in the AM or PM of each filling day. Samples were frozen (-10 °C) for later laboratory analyses. Thermocouples positioned at the center and outer perimeter of the silos. Two were placed at four elevations (1.5, 5.3, 9.1 and 12.9 m) in each silo. Temperature changes were monitored over a 45 d post ensilement period. Three nylon bags were buried near the thermocouples at each of the four elevations in each silo. Upon retrieval, bags were emptied and the contents were frozen for later laboratory analyses. Differences in DM weights in each bag before and after ensiling were used to estimate DM recovery. Samples of fermented silage were taken with a Pennsylvania State Forage Sampler (Nasco, Fort Atkinson, WI 53538) from ports in a door 1.5 m from the bottom of the silo on d 0, 1, 2, 3, 5, 7, 10, 13 and 45 post ensiling for LAB enumeration and chemical analyses. During feedout, samples of silage were taken twice weekly from each

silo. Dry matter was determined, and samples were composited and frozen (-10 °C) for later laboratory analyses.

3.2.2 Lactic Acid Bacteria Enumeration

One hundred g of forage material were diluted with 900 ml of sterilized, distilled water, placed in a Waring blender (Waring Products Inc., New York, NY), and agitated for 30 s. The homogenate was strained through 2 layers of cheesecloth. Serial dilutions (1:10 ml) were prepared using a 0.1% peptone (Difco, Detroit, MI) medium. Microbial enumeration was determined on LBS (BBL, Cockeysville, MD) agar plates inoculated with .2 ml of appropriate dilutions, using a micropipetter. Plates were incubated aerobically for 45 hrs and colony forming units were counted presumptively as lactic acid producing bacteria.

3.2.3 Aerobic Stability

Aerobic stability of inoculated and uninoculated forage was studied eight months post-ensiling to determine the quality of the silage upon exposure to air. Approximately 1.3 kg of alfalfa silage from each silo was placed into each of 16 styrofoam containers (1600 cm^3) and stored at room temperature (23 °C). Temperature was monitored on a daily basis for 14 d. Duplicate containers were emptied and subsamples obtained for both treatments on d 0, 1, 3, 5, 7, 10, and 14 of air exposure. One hundred g of silage were collected by mixing the entire contents of each container and taking random subsamples. These samples were frozen (-10 °C) for future laboratory analyses.

Temperature, pH, DM, total N, lactic acid, ammonia N, soluble carbohydrate and VFA's served as indices of silage stability.

3.2.4 Preparation of Forage Samples

Fresh and fermented samples were removed from the freezer and minced through a Hobart macerator. Approximately 100 g of material were placed in a convection oven (60 °C) for 48 hrs, to determine DM (AOAC, 1984). Dried samples were ground through a Cyclotec sample mill (Tecator Inc., Herndon, VA), for further analyses. Dried plant material was ashed in a muffle furnace (600 °C) overnight to determine ash content (AOAC, 1984). Gross energy was determined on the wet minced samples using an Automatic Adiabatic Bomb Calorimeter (Parr Instrument Co., Moline, IL).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined according to the procedures of Goering and Van Soest (1970).

A 10% homogenate was prepared by mixing 20 g of fresh or fermented forage material with 180 g of distilled water and blended in a Sorvall Omnimixer (Ivan Sorvall Inc., Newton, CT). The homogenate was strained through two layers of cheesecloth, and allowed to stand for 15 min. before pH determinations were made.

Total N concentrations of fresh and fermented plant material was determined by semi-micro Kjeldahl digestion followed by colorimetric N analysis (AOAC, 1984) using a Technicon Autoanalyzer II (Technicon, Terryton, NY). The difference between total N and N content after protein precipitation with 50% sulfosalicylilic acid (SSA), 1 part SSA to 10 parts of 10% homogenate, and centrifuged at 15,000 x g for 20 min., was used to represent soluble N. Acid detergent insoluble nitrogen

was determined by Kjeldahl nitrogen analysis on the ADF residue. Ammonia-N concentration was determined on 10% homogenates using the Technicon Autoanalyzer II.

Lactic acid concentration was determined using appropriate aliquots of water soluble extract according to the procedure of Barker and Summerson (1941).

Soluble carbohydrate analysis (Dubois et al., 1956) was performed on the 10% plant homogenates.

Volatile fatty acid concentrations in fresh and fermented plant tissues were determined by gas chromatography. Twenty ml of 10% homogenate was diluted with 4 ml of 25% metaphosphoric acid and centrifuged at 15,000 x g for 20 min. Two ul of supernatant were injected into a Hewlett-Packard Gas Chromatograph (5840A, Hewlett-Packard, Farmington Hills, MI 48024) with flame ionization detector equipped with a 1.8 m x .2 mm stainless steel column (Supelco MR56559) packed with 10% SP-1200 and 1% H_3PO_4 on chromosorb WAW (80/100--Supelco Inc., Bellefonte, PA).

3.2.5 Lactation Trial

Thirty-two Holstein cows were blocked according to calving date and parity. At initiation of the trial, cows averaged 59 d post-partum. Cows were fed a 40:60 alfalfa silage:concentrate total mixed ration ad libitum, along with five pounds of alfalfa hay per day. At the end of the 21 d preliminary period, cows began a 56 d experimental period and were fed a ration comprised of 50% alfalfa silage and 50% concentrate in sufficient quantities to allow a 10% refusal. A 2 x 2 factorial arrangement of treatments was utilized to differentiate differences in milk

TABLE 5. Diet Ingredients Fed to Holstein Cows During Lactation Trial

Ingredients	Rumen Degradability	
	Slow (SD)	Rapid (RD)
	% DM Basis	
Alfalfa silage	50.00	50.00
High moisture corn	41.03	41.80
Soybean meal	2.05	8.20
Corn gluten meal	3.77	0.00
Blood and meat meal	2.05	0.00
Mono-dicalcium phosphate	0.00	0.41
Trace Mineral Salt	0.33	0.35

enzymatic processes contribute little to silage preservation. However, the Gram positive lactic acid producing bacteria, are facultative anaerobes, which enables them to utilize soluble sugars to carry out metabolic functions aerobically on the plant or anaerobically in the silo. The number of LAB on growing alfalfa is generally low, usually less than 100 cfu/g and reduced further during wilting (Keddie, 1959; Stirling and Whittenbury, 1963). However, counts of lactobacilli usually rise significantly by the time they reach the silo. This is partly due to inoculation of microorganisms from farm machinery (Henderson, et al., 1972; Gibson, et al., 1961).

Until 1978, there was little known about the composition of microflora during silage fermentation. However, Beck (1978) studied the qualitative changes in LAB during the fermentation of grass and red clover with high and low DM contents. He reported that fermentation in wilted and unwilted silage was initiated by homofermentative LAB being 5% of total lactobacilli present by day 4. However, after 142 d of fermentation, 75% of all lactobacilli in the silage with the low DM and 98% of the lactobacilli in the silage with high DM were heterofermentative. Beck suggested that bacteriologic shift could be due to a greater acetate tolerance in heterofermentative bacteria. Table 1 shows the bacterial species commonly found in silage (McDonald, 1981). The dominant organisms in silage according to Langston and Bouma (1960) are L. plantarum, L. brevis and Pediococcus sp.

Gibson, et al., (1958) reported that L. plantarum and L. acidophilus were the dominant homofermentative bacteria in fermentation. While others (Langston, et al., 1962; Moon, 1981, and Moon, et al., 1981) revealed evidence that streptococci and leuconostocs initiate fermentation and are superceded by species of Lactobacilli and Pediococci.

TABLE 1. Classification of Lactic Acid Bacteria Important in Silage

(A) Heterofermentative

Coccus

Leuconostoc mesenteroides
Leuconostoc dextranicum
Leuconostoc cremoris

Rod

Lactobacillus brevis
Lactobacillus fermentum
Lactobacillus buchneri
Lactobacillus viridesceno

(B) Homofermentative

Coccus

Streptococcus faecalis
Streptococcus faecium
Pediococcus acidilactici
Pediococcus cerevisiae
Pediococcus pentosaceus

Rod

Lactobacillus plantarum
Lactobacillus curvatus
Lactobacillus casei
Lactobacillus coryniformis subsp. coryniformis

McDonald, P. 1981

Table 2 illustrates the products of an anaerobic sugar fermentation by LAB described by Whittenbury and coworkers (1967). Glucose and fructose are the most common soluble sugars utilized by LAB, however LAB can also ferment pentoses, xylose and arabinose, which are formed from the degradation of hemicellulose (Dewar, et al, 1963) and amino acids (Rodwell, 1953).

2.1.3 Plant Proteolysis

The deamination of protein in silage is another process resulting from plant enzyme activity. The breakdown of fresh plant material can be caused by plant proteases (Bergen, et al., 1974; Ohshima and McDonald, 1978), however, most proteolytic activity is a result of aerobic conditions inside the silo.

Figure 1 illustrates post-harvest nitrogen metabolism in ensiled plant material from hay and cereal crops (Bergen, 1974). Fresh forage material contains 70-90% of the total nitrogen in the form of protein while the remaining 10-30% is non-protein nitrogen consisting of free amino acids, amides and small concentrations of urides, amines, nucleotides, chlorophyll, low molecular weight peptides and amino acids bound in non-protein form (Hegarty and Peterson, 1973). It is not uncommon for 50-60% of the true protein nitrogen to be broken down into simpler non-protein nitrogenous compounds in preserved forage (Whittenbury, 1967).

Amino acids resulting from proteolysis can be metabolized into ammonia (deamination), amines (decarboxylation) and unidentified nitrogenous compounds (Bergen, et al., 1974; Ohshima and McDonald, 1978). A good quality silage is characterized by low concentrations of ammonia-N, amines and other compounds produced from the break down of amino acids (Bergen, 1984). If aerobic conditions remain in the silo it creates an environment which allows yeast and mold to

TABLE 2. Anaerobic Pathways of Sugar Metabolism by Lactic Acid Bacteria

Homofermentative

1 glucose---------> 2 Lactic acid

1 fructose--------> 2 Lactic acid

1 pentose---------> 1 Lactic acid + 1 Acetic acid

Heterofermentative

1 glucose---------> 1 Lactic acid + 1 Ethanol + 1 Carbon dioxide

3 fructose--------> 1 Lactic acid + 2 Mannitol + 1 Acetic acid

1 Pentose---------> 1 Lactic acid + 1 Acetic acid

Whittenbury, et al., 1967

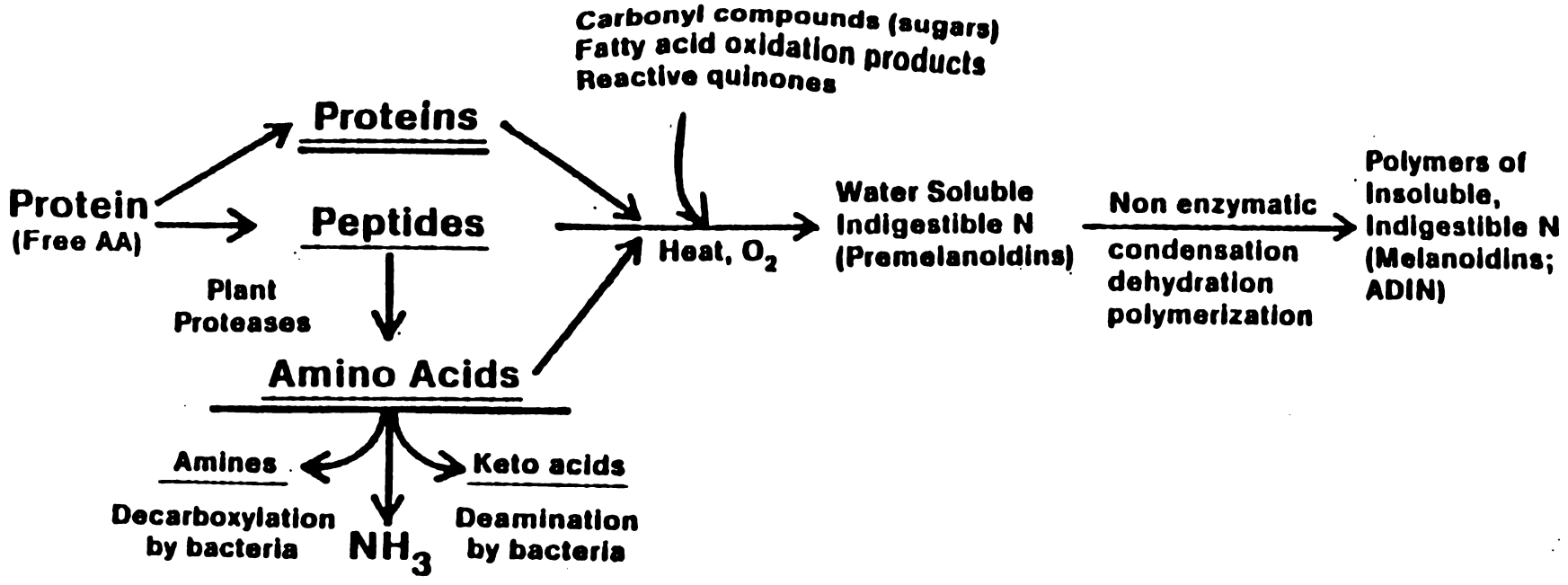

Figure 1. Transformation of N constituents during ensiling.

multiply and increase the silage temperature (Bergen, 1984). Clostridial fermentation is associated with ammonia, butyric acid and a higher pH than that found with lactic acid bacteria. This results in an unstable and often unpalatable silage. Butyric acid produced by sacchrolytic organisms which metabolize lactate and sugars, (Table 3) often serves as an indicator of clostridial activity. The result of this type of fermentation occurs at a high DM or a low pH (Whittenbury, et al., 1967). Woolford (1984) suggested that clostridial activity is suppressed at a dry matter above 31% and/or a pH below 4.5. Under ideal conditions, sufficient numbers of lactic acid producing bacteria occurring naturally, would produce a drop in pH during day 2-5 of ensilement. Bergen and coworkers (1974) suggested that DM of forage material at the time of ensilement is the most decisive factor influencing the amount of protein degradation which will occur during fermentation. The lower the DM, the larger the amount of plant protein escaping proteolysis. Thus, DM at the time of ensiling and rate at which the pH falls during fermentation are factors one must consider during silage preservation.

2.1.4 Substrate Utilization During Silage Fermentation

The major water soluble carbohydrates (WSC) found in forage material are glucose, fructose, sucrose and fructosans. The most available sugars for microbial substrate are glucose and fructose, due to the continual hydrolysis of sucrose and fructosans to glucose and fructose monomers (Whittenbury, et al., 1967).

The WSC content as well as the fructose/glucose ratio of green fodder plants varies depending on species, weather conditions, stage of growth, time of day, wilting conditions and fertilizer application (Woolford et. al., 1982).

Soluble carbohydrates present in forage material after aerobic metabolism are

TABLE 3. Biological Reactions Associated with Clostridial Fermentation

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Organic Acids

2 Lactic acid----------> 1 Butyric acid + $2CO_2$ + $2H_2$

Amino Acids

(A) Coupled oxidation-reduction reactions

1 Alanine + 2 Glycine----->3 Acetic acid + $3NH_3$ + $1CO_2$

(B) De-amination

3 Alanine--------> 2 Propionic acid + 1 Acetic acid + $3NH_3$ + $1CO_2$

1 Valine---------> 1 Isobutyric acid + 1 NH_3 + 1 CO_2

1 Leucine--------> 1 Isovaleric acid + 1 NH_3 + 1 CO_2U

(C) Decarboxylation

Histidine--------> Histamine

Lysine----------> Cadaverine

Arginine---------> Ornithine-------->Putrescine

Tryptophan-------> Tryptamine

Tyrosine---------> Tyramine

---

fermented by a variety of microorganisms, however, under ideal conditions LAB ferment sugars and produce an intolerable acidic environment for other microorganisms (Whittenbury, et al., 1967). Lactic acid bacteria utilize soluble sugars through two fermentable pathways to produce lactate (Table 2, Whittenbury, et al., 1967), as previously described. Homofermentative LAB are the most desirable for they are more efficient in producing lactate than heterofermentative LAB (producing 2 moles of lactic acid versus one mole), and more efficient in conservation of DM (McDonald, et al., 1973). One cannot predict a final ratio of fermentation products, for it is possible to have 100% variation occur in the amount of lactic acid produced under two similar circumstances.

In addition to phosphate, several organic acids also are commonly found in fresh herbage and silage. These acids include malate, citrate, and glycerate (McDonald, 1979). Organic acids in combination with their salts comprise a buffering system in plants (Playne and McDonald, 1966). Legumes contain higher amounts of acid (0.6 to 0.8% of DM) than grasses (0.2 to 0.6% of DM), as well as higher protein and more cations which contribute to a much greater buffering system.

Considerable interest has been given to those organic acids in silage which buffer within the pH range of 4-6. Early stages of fermentation are characterized by the dissimilation of organic acids by LAB (Edwards and McDonald, 1978). The main products of citrate and malate fermentation by LAB are shown in Table 4 (Whittenbury, et al.,1967). Products from these reactions include formation of organic salts (lactate, acetate), neutral products (ethanol, acetone and 2,3 butanediol) and alkaline released cations (Whittenbury, et al., 1967). Other substrates which can be fermented by LAB include amino acids (Rodwell, 1953).

TABLE 4. Fermentation of Organic Acids as Substrates by Lactic Acid Bacteria

A. 1 Citric acid--------> 2 Acetic acid + 1 formic acid + 1 carbon dioxide

or

2 Citric acid--------> 2 Acetic acid + 1 acetone (or 2,3 butanediol) + 4 carbon dioxide.

or

2 Citric acid--------> 3 Acetic acid + 1 lactic acid + 3 carbon dioxide

B. 1 Malic acid---------> 1 Acetone (or 2,3 butanediol) + 4 carbon dioxide

or

2 Malic acid---------> 1 Acetone (or 2,3 butanediol) + 4 carbon dioxide

or

1 Malic acid---------> 1 Acetic acid (or ethanol) + 1 formic acid + 1 carbon dioxide

Whittenbury et al., 1967

Brady (1966) demonstrated that L. plantarum and L. brevis can deaminate serine, arginine, glutamine and aspargine.

2.1.5 Aerobic Stability of Silage

The most important factor in achieving high quality silage is rapid occurrence of anaerobiosis in the silo. Other factors influencing aerobic deterioration include quantity of substrate, DM of the ensiled crop, botanic origin and ambient temperature (Woolford, 1990).

Aerobic deterioration of silage ultimately results in complete mineralization of easily oxidized nutrients which are broken down into CO_2 and H_2O, generating heat and resulting in DM losses (Woolford, 1984). Studies have shown that DM losses over a period of 5-15 days can be as great as 32%. Once the process of aerobic deterioration commences, it is practically impossible to stop (Honig and Woolford, 1979).

An ingress of air as small as 100 to 150 mg O_2/kg DM is adequate to make silage highly susceptible to aerobic deterioration (Woolford, et al. 1979). Upon exposure to oxygen, conditions become favorable for proliferation of aerobic bacteria, yeasts and fungi (Moon et al., 1980 and Woolford et al., 1982). In most silages, yeasts have the ability to increase in numbers from $<10^2$ to 10^{12} cfu/g DM by day 3 of aerobic exposure (Beck 1963, as cited by Woolford, 1990).

Yeasts involved in aerobic deterioration have been classified as acid-utilizers comprised of Candida, Endomycopsis, Hansenula and Pichia sp. and sugar-utilizers which are Torulopsis sp. (Gross and Beck, 1970, as cited by Woolford, 1990; Moon and Ely 1979; Johnsson and Pahlow, 1984). A high population of yeasts does not necessarily mean a silage will deteriorate. Instead, quantity of

lactate-utilizing yeasts decides whether a silage will deteriorate or not upon aerobic exposure (Johnnson and Pahlow, 1984).

Thermophilic filamentous fungi are also found in deteriorating silage, however their growth is generally slower and thus have little affect on silage as a feed.

Woolford and Cook (1978) treated silage material with antibiotics that had antibacterial and antifungal properties. Their studies revealed the involvement of proteolytic bacteria from the genus Bacillus. Bacteria appear to initiate deterioration in maize silages, followed by yeasts (Woolford et al., 1978). Deterioration in cereal crops and grass silages on the other hand, begins with yeasts (Woolford et al., 1979). However, Woolford (1984) concluded that this inconsistency concerning the identity of microbial groups responsible for the onset of aerobic deterioration lies in the properties of ensiled material, specifically DM content rather than botanic origin.

Primary substrates of aerobic deterioration have been described as nitrogen free extracts which included water soluble carbohydrates and organic acids (Honig and Woolford, 1979). Woolford (1990) suggests that the organisms involved in aerobic deterioration will use a wide range of substrates which include those found in the original crop and others which are produced by fermentation. Regardless of the substrate utilized, deterioration in forage crops is always accompanied by a loss of residual sugars and the evolution of ammonia and carbon dioxide. The latter can be directly equated to DM loss and its measurement can be used to monitor the progress of deterioration (Woolford, 1990). Fermentation acids (such as acetic and lactic acids), amino acids and proteins are all used as substrates (Woolford, 1984). The pH increases with acid depletion and tends to be greatest at the silage surface where exposure to oxygen is greatest (Woolford, 1978).

Aerobic deterioration occurs in all silages to some varying degree, except for those undergoing an extensive secondary fermentation. This deterioration depreciates conservation efficiency, causes nutritional losses and can even pose a potential health hazard to livestock. Such management practices as rapid silo filling, special cutting equipment for forage removal, resealing between feed-outs and use of an effective inoculant at the proper application rate can minimize aerobic deterioration.

2.1.6 Silage Inoculants

At the present time, there are several silage inoculants on the market. They have been reported to influence the rate and extent of silage fermentation. Typical ingredients found in inoculant may include enzymes, bacteria, molds, micronutrients for microorganisms or a mixtures of all these to influence forage respiration and fermentation rate (Parker, 1979). Bolsen (1978) has described silage inoculants as "those products that supply lactic acid producing microorganisms and enzymes and/or microorganisms that increase the availability of carbohydrates and other nutrients to lactic acid producing microorganisms".

Commercially available inoculants not only vary in ingredients but in type of preparation (dried, liquid, freeze-dried) and packaging (bottles, vacuum packs and paper sacks).

Whittenbury (as cited by Beck, 1978) described the requirements of a quality silage microorganism as follows:

1. It must be fast growing and able to compete with and dominate other microorganisms present in silage.

2. It must be homofermentative.

3. It must be acid tolerant down to a silage pH of 4.0.

4. It must possess the ability to ferment glucose, fructose, sucrose, and preferably fructosans and pentosans.

5. It should have no action on organic acids.

And in 1975, McCullough described the following as requirements of a cost effective quality inoculant:

1. The cost of the additive must be less than the silage lost without the additive.

2. Addition of the additive must result in a more efficient fermentation than occurs naturally.

3. The additive should produce a silage with a greater digestibility energy and/or protein than untreated silage.

Several workers have shown varying results from inoculation, including advantageous results (Rooke et al., 1985; Ohyama et al., 1975 and Owens, 1977) and non-significant results (Ely et al., 1982; Moon et al., 1981 and Buchanan-Smith and Yao, 1981).

2.2 Rumen Cellulolytic Bacteria and Their Role in Fiber Digestion

2.2.1 Rumen Microbial Fermentation and Digestion

The rumen is an ideal fermentation site. It makes up one-seventh of the total mass of a ruminant's body weight (Russell and Hespell, 1981). The rumen remains at a constant temperature of 39°C and is well buffered by salivary secretions. The microflora inhabiting the rumen is dense containing approximately 10^{10} to 10^{11} bacterial and 10^{5} protozoal cells per milliliter of rumen contents. There is an extensive diversity and synergism in the ecosystem which contains more than 200 species of bacteria and over 20 species of protozoa (Bryant and Robinson, 1962).

During ruminal fermentation, feedstuffs are broken down and fermented into short chain fatty acids through microbial metabolism and are used as the ruminant's energy source, while the animal relies heavily on the microbial mass as a protein source. Methane, heat, and ammonia are formed as well, representing a loss of energy and nitrogen to the animal. The balance of fermentation products determines the efficiency of nutrient utilization in ruminants. In turn, this balance is ultimately controlled by the various microorganisms found in the rumen.

2.2.2 Plant Cell Wall Constituent

In ruminants the plant cell wall is extensively degraded and utilized as an energy source by the rumen microflora. Plant cell walls are indigestible by animal enzymes, however, gastrointestinal microflora partially degrade cell wall material.

The cell wall of plants is made up of an organic matrix of cellulose, hemicellulose, lignin and other small fractions of pectins, gums mucilages, cutin, tannin, bound cell wall protein and cell wall minerals.

Cellulose

First recognized by Payen in 1939 (Whistler and Smart, 1953), cellulose is the most abundant carbohydrate in the world. Its recycling is dependent on microbial activity which produces carbon dioxide during degradation. An enormous amount of energy lies in these cellulosic carbohydrates, making them an excellent food source for herbivores. Cellulose is the largest component of plant cell walls, thus serving as a primary structural element. Linked at the C-1 and C-4 position through glycosidic linkages, individual anhydrous glucose molecules make up the linear polymer in a beta configuration. Glucan chains consist of 100 to 10,000 or more units of glucose (Ott and Tennent, 1954), and are held together by tight hydrogen bonds (Albersheim, 1975) between the hydroxyl group of a sugar on one chain and an oxygen atom of another. Chains are also held together by VanderWaals forces.

Hemicellulose

Hemicellulose is the second largest constituent found in plant material (Phillips, 1940). First named in 1891 by Schultz (Whistler and Richards, 1970), hemicellulose has been defined as the polysaccharide in plant tissues other than cellulose which is extracted with alkali and hydrolyzed in acid (Collings, 1979). Hemicellulose is a complex mixture of polysaccharides which constitute much of the cell wall matrix (Bailey and Gaillard, 1965). It is a polybeta 1-4 D-

xylanopyranose based on a backbone of xylose residues, with branches of arabinose, glucose and/or galactopyranosides (Akin and Barton, 1983).

Lignin

Lignin is a polymer of phenylpropanoid units intimately associated with structural carbohydrates (Himmelsbach and Barton, 1980), and plays a major role in reducing microbial attack on cell walls (Akin and Barton, 1983). Phenolic acids such as p-coumaric acid and ferulic acid which are precursors of lignin can bind to structural carbohydrates which inhibits carbohydrate degradation (Hartley et al., 1974).

Other Constituents

Pectin is comprised of chains of galacturonic acid, galactans and arabinans (Aspinall, 1973). Pectins are not pure polysaccharides, but mixed and branched, forming complex polysaccharide structures. It is found in intracellular spaces in the cell wall and is associated with cellulose in other cell layers (Esau, 1965). Hemicellulose, pectin and lignin play an important role as matrix substances for the cell wall.

Cowling (1976) demonstrated that crystallinity and lignification are the most important factors in determining the susceptibility of cellulose to enzyme degradation. It has been shown that specific enzymes which attack glucan bonds in cellulose chains are incapable of attacking an intact plant fiber (Albersheim, 1975). Thus accessibility of cellulose to microbial enzymes and chemical reagents depends on the arrangement of cellulose within the cell wall (Collings, 1979).

Although some plant material is accessible and easily digested, the degradation

of fiber material in the rumen is a result of complex microbial processes (Cheng et al., 1980). These processes include the digestion of plant cell walls, to yield microbial cell growth and fatty acids end products. As with any ecological system, the microorganism should be attracted to its nutrient substrate. It has been demonstrated that plant material undergoing colonization and digestion by rumen microorganisms includes the adherence of bacteria, protozoa and fungi, however, bacteria are responsible for the majority of the digestion which takes place in the rumen (Hungate, 1966). Akin and Barton (1983) found through the use of the scanning electron microscope (SEM) that plant cell wall digestion did not occur unless rumen bacteria were closely associated with or completely adhered to the cell walls.

2.2.3 Rumen Cellulolytic Species

Based on relative numbers in the rumen of domestic ruminants and their ability to attack various forms of cellulose in pure cultures, the major rumen cellulolytic bacteria are Ruminococcus flavefaciens (Sijpestein, 1951), Ruminococcus albus (Hungate, 1957), and Bacteroides fibrobacter succinogenes (Hungate, 1950). These are the three major species which obtain their energy for growth solely through cellulose fermentation (Bryant, 1973). B. fibrisolvens will digest cellulose to a lesser extent (Bryant, 1973; Hungate, 1966). Each of these species except B. succinogenes are capable of utilizing hemicellulose-type components from forage (Dehority and Scott, 1967). B. succinogenes is the most active cellulolytic, bacterium digesting the more resistent cellulose such as cotton fibers and mature hay to a greater extent than Ruminococcus, which are active,

but show much more variation between strains in ability to degrade more resistant cellulose (Bryant, 1973).

Minato and coworkers (1966), noted that both Ruminococcus and B. succinogenes adhere to fiber during digestion, however, B. succinogenes was firmly attached to the cell wall. A few other cellulolytic species of the genus Clostridium (Hungate, 1957; Shane et al., 1969) and Eubacterium cellulosolvens (Bryant et al., 1958; Van Gylswyck and Hoffman, 1971) have been found in the rumen occasionally.

The largest numbers of cellulolytic bacteria are found when the ruminant is fed a high roughage diet, however in ruminants fed cellulose as the total feed source, cellulolytic bacteria only comprise 25% of the total rumen microbial population (Slyter et al., 1971). Many non-cellulolytic bacteria found in the rumen are responsible for the degradation of pectins and xylans. Numerous synergistic interactions between cellulolytics and noncellulolytic species occur and has been shown to enhance cellulose degradation (Dehority and Scott, 1967).

Rumen cellulolytics produce cellulose enzymes which hydrolyze insoluble cellulose into soluble cellulodextrins or sugars, some of which they can absorb and ferment to obtain energy for growth (Schaefer and King, 1965; Sheth and Alexander, 1969).

End products of cellulose degradation include acetate, propionate, butyrate, CO_2 methane, and microbial cells. This includes interacting populations of 1) rumen cellulolytic bacteria, 2) carbohydrate fermenting species which can use products hydrolyzed from cellulose, 3) species which will degrade succinate, formate and any lactate produced from microbes in 2 and 4) methanogenic bacteria which will reduce CO_2 using H_2 or formate as an electron donor (Hungate, 1950).

All rumen cellulolytic bacteria require one or more B-vitamins for growth. Biotin is the most common vitamin required by the cellulolytics. However, some strains of R. albus also require pyridoxine. A few strains of R. albus may require folic acid, riboflavin or thiamine (Bryant, 1973). The vitamins required by R. flavefaciens strains are similar to those required by R. albus (Bryant and Robinson, 1961; Gill and King, 1958; Scott and Dehority, 1965), with some strains requiring pyridoxine and cobalamine which in some cases can be replaced by methionine (Scott and Dehority, 1965). B. Succinogenes requires biotin, using this as its primary B-vitamin. P-aminobenzoic acid has been shown to stimulate the growth in some strains of R. albus (Bryant and Robinson, 1961; Scott and Dehority, 1956). B. succinogenes has a requirement for Na^+ and a great demand for Ca^{2+} (Bryant et al., 1959). The other cellulolytics have a lower demand for K^+, Na^+, and Ca^{2+}. Ferrous iron and Zn^{2+} has been found to stimulate microbial activities even further (Matturi, 1972).

All of the rumen cellulolytics have a requirement for sulfur. B. succinogenes utilizes cysteine or sulfide, but not sulfate (Bryant et al, 1959). The Ruminococcus grow well in media containing sulfide or sulfate (Bryant, 1973).

The main nitrogen source for cellulolytic bacteria is ammonia (Bryant and Robinson, 1961; Bryant et al., 1959, Dehority, 1963). The ammonia is a product of non-cellulolytic bacteria metabolism. This is just another example of co-existence and cooperation between rumen species. Cellulolytic bacteria lack the ability to use organic nitrogen sources for growth and though not established, it appears that they probably lack the mechanism for transporting amino acids or peptides into the cell (Pittmann et. al, 1967). Although Ruminococcus bacteria cannot use amino acids if present, B. succinogenes will utilize the amide nitrogen from glutamine

and asparagine for growth and function (Bryant and Robinson, 1961).

Many strains of rumen cellulolytic bacteria require a carbon source beyond that of the energy source. The source commonly used by these bacteria is CO_2 or bicarbonate. B. succinogenes and R. flavefaciens require large amounts of CO_2, which is fixed into pyruvate during glycolysis (Caldwell et al., 1969). Without CO_2, these bacteria are unable to obtain energy in the form of carbon, for growth (Bryant, 1973). They also use CO_2 for biosynthetic purposes (Allison, 1969; Allison 1970). R. albus does not require large amounts of CO_2 for growth, but requires small amounts for optimal growth and for biosynthetic processes (Bryant, 1973).

Short chain fatty acids, better known as volatile fatty acids are essential for growth of the three major rumen cellulolytics at 0.5-0.3mM in batch cultures (Dehority and Scott, 1967). Carbon skeletons from these fatty acids are not degraded, but incorporated into certain cellular constituents (Bryant, 1973). Bacteroides succinogenes is the only cellulolytic that requires the straight chain valeric acid, which can be replaced by longer chain acids (Wegner and Foster, 1963). The cellulolytic bacteria utilize the various branched chain fatty acids, such as C_{14} and C_{16} from isobutyric, C_{15} and C_{17} from isovaleric, and anteisa C_{15} and C_{17} from 2-methyl-butyrate (Allison, et al., 1962; Wegner and Foster, 1963). These branched chain fatty acids are also precursors for fatty aldehydes in these bacteria. One or more of the above fatty acids are used for the biosynthesis of amino acids: valine, leucine, and isoleucine respectively (Allison et al., 1962; Robinson and Allison, 1969; Allison, 1970) via reductive carboxylation reactions (Bryant and Robinson, 1961; Allison, 1969).

2.2.4 Cellular Attachment and Digestion of Plant Material

There are many factors which influence the rate and extent of forage cell wall digestion. Feeds containing fractions of cellulose and hemicellulose are relatively insoluble in the rumen and are degraded slowly (Dehority, 1973; Van Soest, 1973). Degradation is highly influenced by structural factors. Such factors would include the close association of lignin with cellulosic materials, acting as a barrier against bacterial cellulases (Russell and Hespell, 1981). Crystallinity also effects digestion (Bryant and Robinson, 1962). Russell and coworker (1981), showed that high crystalline fibers were readily degraded by cellulases from certain cellulolytic bacteria while fiber digestion was much slower for other cellulolytic species. Those who have made extensive observations (Akin and Amos, 1975; Akin et al., 1974) of mixed cultures of rumen bacteria have observed that many rumen bacteria appear to adhere to plant cell walls by means of thin fibrous capsules. In many of these observations, it has been noted that the bacteria digest plant cell wall material and infiltrate the resultant cavities.

Cheng and coworkers (1977) found that bacteria in the rumen of cows fed corn silage versus other forage based diets had the least bacterial slime formation, but every bacterial cell showed some extracellular structure. Although some plant material is accessible and easily digested, the process is long and sequential (Akin and Amos, 1975). Digestion begins with penetration through the stomata (Baker and Harris 1947) and colonization on fiber macerations produced from mastication. Dinsdale et al., (1978) in an <u>in vitro</u> study demonstrated that mixed populations of rumen bacteria released 12 to 36% of the dry matter of damaged cells in legume leaves. These organic nutrients are used to support enormous proliferations of bacteria in intracellular space and at the leaf surface. Subsequently, plant cell

walls are ruptured by certain species of bacteria who digest cellulose in grasses and cellulose and pectins in legumes (Dinsdale et al., 1978). Plant protoplasm which remains to be digested supports a further proliferation of bacteria until bacterial microcolonies fill plant cell wall compartments, while refractory cells remain uncolonized (Akin and Amos, 1975).

3.0 FERMENTATION CHARACTERISTICS AND NUTRITIVE VALUE OF ALFALFA FORAGE ENSILED WITH AND WITHOUT ADDITION OF A BACTERIAL INOCULANT

3.1 Introduction

Preservation of forage crops as silage has increased in popularity over the past years due to excellent conservation of nutrients and the ability to obtain a higher quality roughage. The success of ensiling forage relies on the presence of adequate numbers of microorganisms, soluble sugars for use as substrates and an anaerobic environment. Fulfillment of these conditions will allow a lactic acid fermentation to predominate (Whittenbury, et al., 1967). Kroulik, et al., (1955) reported that there was a considerable variation in the numbers of bacteria found on green plants and cut forages. Bacterial populations varied with the type of plant, anatomical location, season, weather conditions and plant maturity.

Bacteria responsible for a rapid fermentation and production of a quality forage are predominately lactic acid producers (Kempton and Clement, 1959; Langston and Bouma, 1960). The addition of Lactobacillus sp. to fresh forages has been recommended for control of silage fermentation (Lesens and Shultz, 1968; McDonald, et al., 1964). Previous efforts (Bolsen, 1978; Thomas, 1978) to utilize microbial additions to silage have varied from no response to increased DM and protein recovery.

As milk production increases, the requirement for total N for the lactating cow

increases. The intake of ruminally degradable N often exceeds the amount which is converted into microbial protein. Consequently, protein nitrogen supply to the small intestine may be limiting. Efficiency of N utilization is improved as more rumen undegradable protein is fed (Waldo and Glenn, 1984). Titgemeyer, et al., (1989) evaluated amino acid disappearance from the small intestine with four dietary protein supplements. In their study, each protein supplement was inadequate in at least one of the essential amino acids, thus suggesting that amino acid requirements of ruminants should be supplied by a combination of protein supplements.

The objectives of this study were to examine the ensiling characteristics of alfalfa forage treated with or without the addition of a bacterial inoculant and to evaluate the response of lactating Holsteins and crossbred beef heifers fed the silage in combination with a slow or rapidly degradable rumen protein source.

3.2 Materials and Methods

3.2.1 Silo Filling and Sampling

Two hundred and sixty tons of 1/10th bloom first cutting alfalfa forage was wilted to 45% dry matter (DM), chopped to .6 cm length and ensiled in two top unloading upright concrete stave silos (4.3 x 18.3 M). One silo served as a control silo, containing uninoculated forage material (C), while the other was inoculated (I) with a commercial inoculant (Ecosyl, CIL Inc., Ontario, Canada N6A 4L6). The inoculant contained a strain of Lactobacilli plantarum and was applied in liquid form at the blower to provide 2.5 x 10^6 colony forming units cfu/g of chopped forage. Each silo was filled in an alternate load sequence. Incoming loads of forage were sampled for DM determination and composited based on whether they were harvested in the AM or PM of each filling day. Samples were frozen (-10 ^{0}C) for later laboratory analyses. Thermocouples positioned at the center and outer perimeter of the silos. Two were placed at four elevations (1.5, 5.3, 9.1 and 12.9 m) in each silo. Temperature changes were monitored over a 45 d post ensilement period. Three nylon bags were buried near the thermocouples at each of the four elevations in each silo. Upon retrieval, bags were emptied and the contents were frozen for later laboratory analyses. Differences in DM weights in each bag before and after ensiling were used to estimate DM recovery. Samples of fermented silage were taken with a Pennsylvania State Forage Sampler (Nasco, Fort Atkinson, WI 53538) from ports in a door 1.5 m from the bottom of the silo on d 0, 1, 2, 3, 5, 7, 10, 13 and 45 post ensiling for LAB enumeration and chemical analyses. During feedout, samples of silage were taken twice weekly from each

silo. Dry matter was determined, and samples were composited and frozen (-10 °C) for later laboratory analyses.

3.2.2 Lactic Acid Bacteria Enumeration

One hundred g of forage material were diluted with 900 ml of sterilized, distilled water, placed in a Waring blender (Waring Products Inc., New York, NY), and agitated for 30 s. The homogenate was strained through 2 layers of cheesecloth. Serial dilutions (1:10 ml) were prepared using a 0.1% peptone (Difco, Detroit, MI) medium. Microbial enumeration was determined on LBS (BBL, Cockeysville, MD) agar plates inoculated with .2 ml of appropriate dilutions, using a micropipetter. Plates were incubated aerobically for 45 hrs and colony forming units were counted presumptively as lactic acid producing bacteria.

3.2.3 Aerobic Stability

Aerobic stability of inoculated and uninoculated forage was studied eight months post-ensiling to determine the quality of the silage upon exposure to air. Approximately 1.3 kg of alfalfa silage from each silo was placed into each of 16 styrofoam containers (1600 cm^3) and stored at room temperature (23 °C). Temperature was monitored on a daily basis for 14 d. Duplicate containers were emptied and subsamples obtained for both treatments on d 0, 1, 3, 5, 7, 10, and 14 of air exposure. One hundred g of silage were collected by mixing the entire contents of each container and taking random subsamples. These samples were frozen (-10 °C) for future laboratory analyses.

Temperature, pH, DM, total N, lactic acid, ammonia N, soluble carbohydrate and VFA's served as indices of silage stability.

3.2.4 Preparation of Forage Samples

Fresh and fermented samples were removed from the freezer and minced through a Hobart macerator. Approximately 100 g of material were placed in a convection oven (60 °C) for 48 hrs, to determine DM (AOAC, 1984). Dried samples were ground through a Cyclotec sample mill (Tecator Inc., Herndon, VA), for further analyses. Dried plant material was ashed in a muffle furnace (600 °C) overnight to determine ash content (AOAC, 1984). Gross energy was determined on the wet minced samples using an Automatic Adiabatic Bomb Calorimeter (Parr Instrument Co., Moline, IL).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) was determined according to the procedures of Goering and Van Soest (1970).

A 10% homogenate was prepared by mixing 20 g of fresh or fermented forage material with 180 g of distilled water and blended in a Sorvall Omnimixer (Ivan Sorvall Inc., Newton, CT). The homogenate was strained through two layers of cheesecloth, and allowed to stand for 15 min. before pH determinations were made.

Total N concentrations of fresh and fermented plant material was determined by semi-micro Kjeldahl digestion followed by colorimetric N analysis (AOAC, 1984) using a Technicon Autoanalyzer II (Technicon, Terryton, NY). The difference between total N and N content after protein precipitation with 50% sulfosalicylilic acid (SSA), 1 part SSA to 10 parts of 10% homogenate, and centrifuged at 15,000 x g for 20 min., was used to represent soluble N. Acid detergent insoluble nitrogen

was determined by Kjeldahl nitrogen analysis on the ADF residue. Ammonia-N concentration was determined on 10% homogenates using the Technicon Autoanalyzer II.

Lactic acid concentration was determined using appropriate aliquots of water soluble extract according to the procedure of Barker and Summerson (1941).

Soluble carbohydrate analysis (Dubois et al., 1956) was performed on the 10% plant homogenates.

Volatile fatty acid concentrations in fresh and fermented plant tissues were determined by gas chromatography. Twenty ml of 10% homogenate was diluted with 4 ml of 25% metaphosphoric acid and centrifuged at 15,000 x g for 20 min. Two ul of supernatant were injected into a Hewlett-Packard Gas Chromatograph (5840A, Hewlett-Packard, Farmington Hills, MI 48024) with flame ionization detector equipped with a 1.8 m x .2 mm stainless steel column (Supelco MR56559) packed with 10% SP-1200 and 1% H_3PO_4 on chromosorb WAW (80/100--Supelco Inc., Bellefonte, PA).

3.2.5 Lactation Trial

Thirty-two Holstein cows were blocked according to calving date and parity. At initiation of the trial, cows averaged 59 d post-partum. Cows were fed a 40:60 alfalfa silage:concentrate total mixed ration ad libitum, along with five pounds of alfalfa hay per day. At the end of the 21 d preliminary period, cows began a 56 d experimental period and were fed a ration comprised of 50% alfalfa silage and 50% concentrate in sufficient quantities to allow a 10% refusal. A 2 x 2 factorial arrangement of treatments was utilized to differentiate differences in milk

TABLE 5. Diet Ingredients Fed to Holstein Cows During Lactation Trial

Ingredients	Rumen Degradability	
	Slow (SD)	Rapid (RD)
	% DM Basis	
Alfalfa silage	50.00	50.00
High moisture corn	41.03	41.80
Soybean meal	2.05	8.20
Corn gluten meal	3.77	0.00
Blood and meat meal	2.05	0.00
Mono-dicalcium phosphate	0.00	0.41
Trace Mineral Salt	0.33	0.35

production by feeding one of two protein supplements containing different levels of rumen degradable protein with each alfalfa silage (Table 5). The protein supplement with rapid rumen degradability (RD) contained primarily soybean meal, whereas the second protein supplement contained a blend of 50% corn gluten meal, 25% blood and meat meal and 25% soybean meal, which represented a slowly degraded rumen protein source (SD). Total mixed rations were sampled once a week for DM determination. Samples were composited and sent to a commercial laboratory (Ohio Agr. and Dev. Center, Wooster, OH) for nutritional analyses. All four diets were balanced for 17.5% crude protein and ranged from 17.5 to 18.5% throughout the experimental period. Feed intake and milk yields were recorded daily. Milk was sampled on two consecutive milkings each week, composited and taken to the Michigan Dairy Herd Improvement Association (DHIA) Laboratory (East Lansing, MI 48823) for determination of total protein and fat. Cows were weighed weekly.

3.2.6 Growth Trial

Seventy-one Hereford x Angus heifers (226 kg) were randomly assigned to eight pens of nine head each with the exception of one pen containing eight head. Animals were weighed on two consecutive d at 28 d intervals. Heifers were fed once each day, with intakes and orts measured daily. A one week adjustment period was utilized to familiarize heifers with the 50:50 alfalfa:corn silage diet.

Following the preliminary period, each pen was randomly assigned to one of four treatments (Table 6) which included control or inoculated alfalfa silage and corn silage fed with one of two protein supplements used in the lactation trial. Diets were formulated to contain 14.0% crude protein and fed for 104 d.

TABLE 6. Diet[a] Ingredients Fed to Beef Heifers During Growth Trial

Ingredient	Rumen Degradability	
	Slow (SD)	Rapid (RD)
	% DM Basis	
Corn silage	51.60	51.60
Alfalfa silage	45.20	45.20
Corn gluten meal	1.54	0.00
Soybean meal	.77	3.07
Blood and meat meal	.77	0.00

[a]Formulated to contain 30,000 IU vitamin A/hd/d; 150 mg/hd/d monensin; .25% T.M. salt; 1 ppm/hd/d Se; .6% K; .5% Ca; .3% P.

3.3 Statistical Analyses

Statistical analysis of fermentation parameters and the growth trial were conducted with the General Linear Models Subroutine in SAS (SAS Institute, 1987). Least square means were generated to compare treatments. Mean comparisons were made with Bonferroni's T-test, as described by Gill (1978). Initial weight of beef heifers at the beginning of the trial was used as a covariate in the analysis. Results of the lactation trial were analyzed as a repeat measurement design with cows blocked according to calving date and parity. Milk production during the 21 d preliminary period was used as a covariate in the analysis of the experimental period.

3.4 Results and Discussion

3.4.1 Silage Composition

Materials entering silos were similar in DM, pH and lactic acid content, while forage entering the inoculated silo had a greater ammonia N and water soluble carbohydrate (WSC) content than that entering the control silo (Table 7). Water soluble nitrogen (WSN) was greater for the material entering the control silo versus the inoculated silo.

The pH of ensiled forage material is presented in Figure 2. A decline in pH started immediately after ensilement and continued throughout 45 d post-ensilement, with the lowest pH around d 5. This pattern reflects the changes in lactobacilli population for control and inoculated silage (Table 8). Initial population size was similar on d 0, however inoculation caused a 3-fold increase in lactate producing organisms within 24 hours. Lactobacilli numbers in the control silage were still increasing on day 13, but were still less than the number of organisms present in the inoculated silo on day 3.

Inoculated silage had a greater overall average temperature by d 2 and remained greater ($p<.05$) throughout the 45 d post-ensilement period (37.6 vs. 36.2 ^{0}C; Figure 3). This supports Woodford and Satters findings in which inoculation increased silage temperature an average of .64 ^{0}C over a 14 d post-ensilement period. Silage temperatures were significantly different ($p<.01$) at the various elevations within the silos (Table 9). Temperature means for the four elevations were 36.75, 40.43, 37.79 and 32.51 ^{0}C for 1.5, 5.3, 9.1 and 12.9 m, respectively.

Temperature at the various elevations in the two silos are illustrated in Table 10. As one would expect temperatures were greatest in the middle of the silos with the inoculated silage having a greater temperature at all locations, except 12.9 m

TABLE 7. Composition of Forage Material Placed Into the Silos

	Control	Inoculated
DM (%)	46.40	45.20
pH	6.20	6.20
Lactic Acid[a]	0.08	0.05
Ammonia-N[b]	2.50	5.10
Water Soluble N[b]	34.00	30.60
Water Soluble Carbohydrates[c]	8.80	10.10

[a]Expressed as g/100 g DM.
[b]Expressed as % of Total N.
[c]Expressed as % of DM.

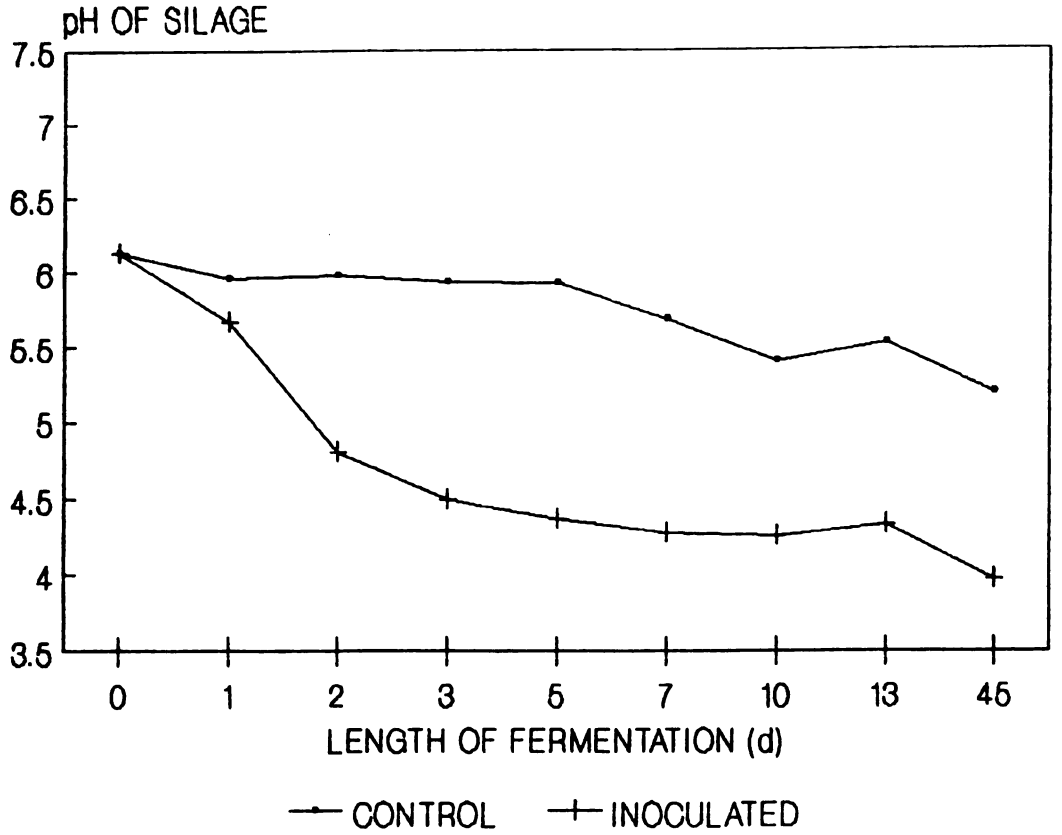

Figure 2. Average pH of the Control and Inoculated Silages During First 45 d Post-ensiling.

TABLE 8. Lactobacilli Numbers in Silage Material Post-Ensiling[a]

Day	Control	Inoculated
0	1.6×10^6	1.7×10^{6b}
1	2.6×10^6	1.1×10^9
3	3.5×10^7	3.2×10^9
5	7.8×10^7	1.8×10^8
13	2.8×10^8	9.6×10^7
45	---	---

[a]Expressed as cfu/g DM.
[b]Fresh material entering silo before inoculation

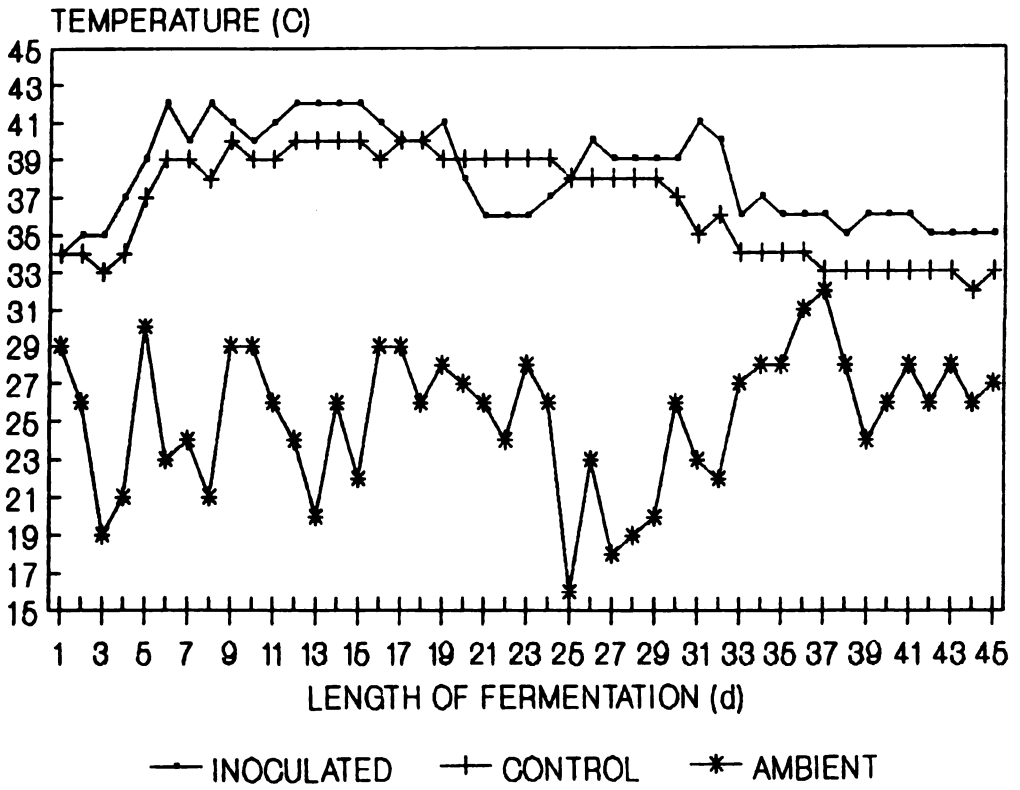

Figure 3. Average Temperature of the Control and Inoculated Silages During First 45 d Post-ensiling.

TABLE 9. Mean Temperatures at the Various Elevations Within Each Silo

Location (m)	Temperature (C)
1.5	36.75[a]
5.3	40.43[b]
9.1	37.79[a]
12.9	32.51[c]

[a,b,c]Values within columns with unlike superscripts differ ($p<.05$)

TABLE 10. Effect of Elevation and Treatment on Silo Temperatures (C°)

	ELEVATION (Meters)			
	1.5	5.3	9.1	12.9
Control	35.4	39.4	37.2	32.5
Inoculated	38.1	41.9	38.5	32.2

where the control silage had a slightly higher temperature.

Fermentation characteristics from ensilage in buried bags were similar for both silos except for DM and gross energy (Table 11). The inoculated silage was significantly lower ($p<.05$) in DM content and significantly higher ($p<.05$) in energy.

Fermentation characteristics of silage post-ensilement are shown in Table 12. Barnett (1954) subdivided silage fermentation into four phases; 1) plant respiration; 2) acetic acid production by aerobic bacteria; 3) lactic acid and acetic acid production by lactobacilli and streptococci and 4) a relatively stable period providing sufficient fermentation has occurred. Post-ensilement parameters are presented as phase 1-3 (1-4 d), phase 4 (5-21 d) and feedout (>100 d). Inoculated silage supported a more active microbial population during the first three phases of fermentation, which coincided with the faster rate of temperature increase. Similar results have been reported by Kung et al., (1981) which demonstrated inoculation increased microbial populations and lactic acid concentrations prior to d 7 in laboratory silos. Total LAB counts were significantly greater ($p<.01$) for the inoculated silage during the first three phases. As a result, lactic acid content was greater ($p<.01$) for the inoculated silage through d 21 and during feedout ($p<.05$) as compared to the control. Control silage required more than 21 d to accumulate similar concentrations of lactic acid as the inoculated material possessed by d 4. Moon, et al. (1981) previously demonstrated the increased extent and rate of lactic acid accumulation that occurs with inoculation. The pH of both silages declined over time however, the inoculated silage declined at a faster rate and had a lower pH ($p<.01$) throughout the first 21 d as compared to the control silage. As lactic acid accumulated, pH decreased. Ammonia-N concentrations were lower ($p<.01$)

TABLE 11. Fermentation Characteristics of Alfalfa Silage in Buried Bags

	Control	SD	Inoculated	SD
DM	44.0[c]	6.06	38.8[d]	5.40
pH	4.5	0.08	4.45	0.19
LA[a]	3.1	1.00	3.40	1.30
WSC[a]	4.77	1.80	4.18	1.24
TN[a]	3.00	0.38	2.86	0.21
WSN[a] (% of TN)	66.20	2.48	64.6	6.60
NH_3-N (% of TN)	12.17	2.89	11.04	2.69
NDF[a]	45.20	4.35	47.10	2.64
ADF[a]	34.50	0.82	34.8	2.22
ADIN[a] (% of TN)	7.40	1.60	6.00	0.90
ASH[a]	8.85	1.00	8.45	0.43
Energy[b]	9.89[d]	1.52	10.89[c]	2.19
DM Recovery (%)	93.55	4.75	93.41	3.42

[a] All values are %'s expressed on DM basis except DM and pH.
[b] Energy is expressed as Kcal/g DM.
[c,d] Values within rows with unlike superscripts differ (P<.05).

TABLE 12. Characteristics of Fermentation During Ensiling for Inoculated (I) and Control (C) Silage

	Phase 1-3 d 1-4			Phase 4 d 5-21			Feedout		
	C	I	SEM	C	I	SEM	C	I	SEM
LAB[a]	6.87[e]	9.26[f]	0.22	7.96	8.30	0.19	---	---	---
LA[b]	0.19[e]	2.45[f]	0.45	1.73[e]	5.32[f]	0.39	2.45[g]	3.04[h]	.16
WSC[c]	8.54	6.45	1.20	7.24[e]	3.00[f]	1.04	5.95[e]	6.04[f]	.44
pH	5.96[e]	4.99[f]	0.23	5.64[f]	4.31[e]	0.20	4.60	4.49	.08
NH_3-N[d]	3.43[e]	1.89[f]	1.71	2.71[e]	1.08[f]	1.48	12.08[e]	8.86[f]	.63

[a]LAB = Lactic acid bacteria, Log CFU/g wet forage.
[b]LA = Lactic acid, g/100 g DM.
[c]WSC = Water soluble carbohydrate as % DM.
[d]NH_3-N= Ammonia nitrogen as % total N.
[e,f]Means within a phase with unlike superscripts differ ($p<.01$).
[g,h]Means within a phase with unlike superscripts differ ($p<.05$).

throughout ensiling for the inoculated silage.

During feedout, control silage had greater ($p<.01$) DM content and less ($p<.05$) gross energy (Table 13). The greater gross energy concentration in the inoculated silage would indicate less carbon loss occurred than with the control silage. The other chemical indices measured were similar ($p<.10$) for both silage treatments. Dry matter recovery estimates calculated from 12 buried bags were 93.55 and 93.41% for control and inoculated silage treatments, respectively. The estimates of recovery from buried bags was greater than recoveries from the entire silos (93.5 vs. 81.0%). The 12% percentage unit difference may be attributed to more aerobic losses on the exposed silage surfaces or weighing errors which would not have occurred with the buried bags. The large percentage difference in the DM recovery between the silos is unknown. DM percentages did differ between forage entering the silos, however, this difference was also seen in the silage removed from the silos.

3.4.2 Silage Aerobic Stability

Temperature and DM losses were similar for the control and inoculated silage (Figure 4) throughout the first 9 d of aerobic exposure. However, on d 9 the temperature began to increase in the inoculated silage, followed by an increase beginning on d 10 for the control silage. By d 14, both silages had achieved similar temperatures. Dry matter losses were evident by d 1 and continued at an equal rate for both silages until d 10, at which time the rate of deterioration increased for the inoculated silage. Dry matter losses occurred during the first nine d without major increases in temperature.

Dry Matter, N, and pH all increased, while ammonia-N, lactic acid and acetate

TABLE 13. Chemical Indices of Fermented Forage During Feedout[a]

	Control	Inoculated	SE
Dry matter, %	43.60[b]	42.40[c]	.28
Total nitrogen, %	2.91	2.92	.03
Water soluble nitrogen, %	64.20	63.27	7.55
NDF, %	45.20	44.60	1.56
ADF, %	34.60	34.80	.33
ADIN, %	6.88	7.48	.43
Ash, %	9.05	9.08	.39
Gross energy, kcal/g DM	9.96[d]	10.22[e]	.06
Acetate, g/kg DM	29.855	28.755	1.657
Proprionate g/kg DM	1.184	1.834	0.452
Isobutyrate g/kg DM	0.110	0.026	0.043
Butyrate g/kg DM	1.291	0.661	0.394
Isovalerate g/kg DM	0.1277	0.046	0.045
Valerate g/kg DM	0.0l0	.002	0.005
Dry matter recovery, %	93.55	81.0	1.42

[a]All means are expressed on a DM basis with the exception of DM, pH, and DM recovery.
[b,c]Means with unlike superscripts differ ($p<.01$).
[d,e]Means with unlike superscripts differ ($p<.05$)

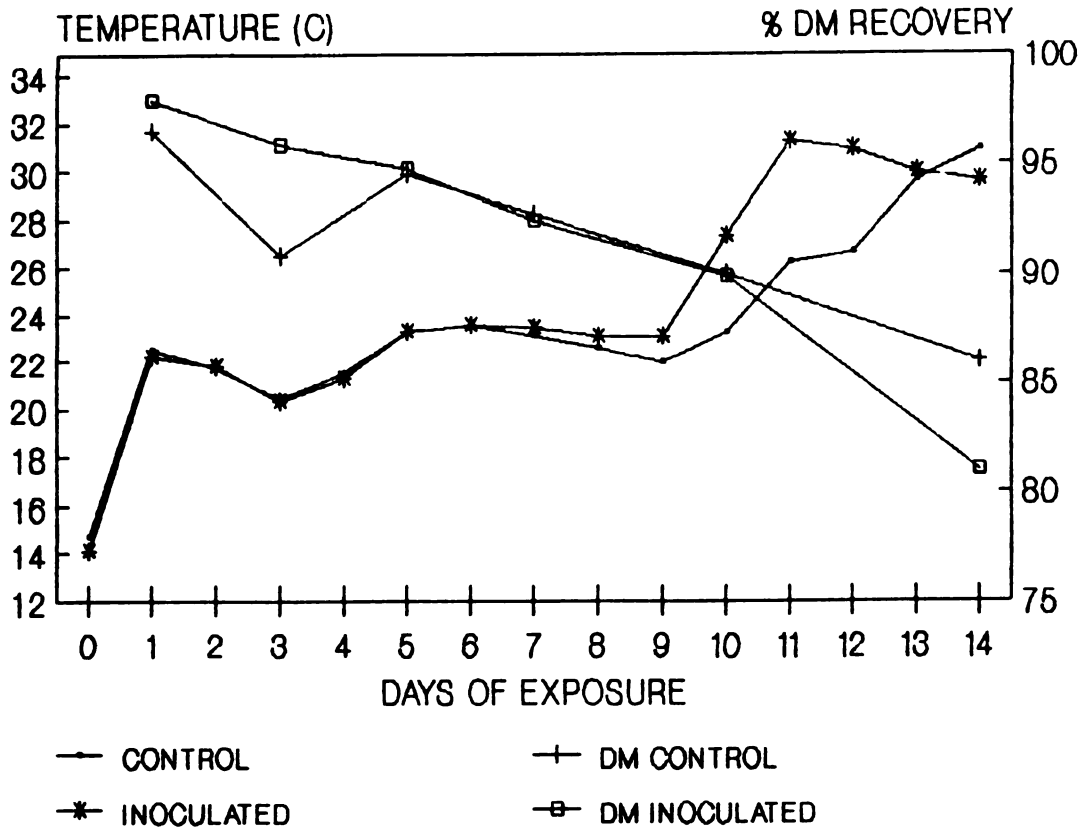

Figure 4. Temperature and Dry Matter Recovery for Control and Inoculated Silage During Aerobic Exposure.

decreased as length of exposure increased (P<.001). Inoculated silage had a significantly lower (p<.001) concentration of ammonia-N, WSC, acetate, isobutyrate (p<.05) and isovalerate (p<.1) than the control silage. However, lactic acid (p<.001) and propionate (p<.05) concentrations were significantly greater for inoculated silage throughout aerobic exposure. No differences were observed for DM, N, pH, ammonia-N, WSC, propionate, butyrate and isovalerate between treatments on any particular day (Table 14). Lactic acid content was significantly greater (p<.05) on day 0, 1, 3 (p<.10), 7 and 10 for inoculated silage.

3.4.3 Lactation Trial

Milk production of lactating dairy cows fed control or inoculated alfalfa silage supplemented with different degradable proteins is presented in Table 15. Catt;e fed the control silage supplemented with the more slowly degradable protein had the lowest dry matter intake thus having the least weight gain throughout the trial period. The largest weight gain was observed in cattle fed the inoculated silage supplemented with the slow degradable protein source. This weight gain can be attributed to the large dry matter intakes observed in this treatment group.

There was a significant interaction between silage treatment and protein supplement. Cattle fed the more slowly degraded (SD) protein source with the inoculated silage had an increase in 3.5% fat corrected milk (FCM) production by 2.1 kg/d (p<.05), as compared to SD added to the control silage, likewise they had increased daily yields of fat and protein. This increased production of FCM appeared to be the result of a 3.7 kg/d additional dry matter intake (DMI). Cows produced similar milk yields with RD supplemented to either silage treatment. Within the rapidly degraded protein supplement, FCM production was similar for

TABLE 14. Silage Characteristics Throughout Aerobic Exposure of 14 Days[a]

	LENGTH OF EXPOSURE (Days)																				
	0			1			3			5			7			10			14		
	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM
DM (%)	47.1	48.7	1.24	51.7	52.4	1.75	54.4	52.4	1.75	49.3	52.1	1.10	53.1	54.3	1.75	57.7	56.0	1.11	56.0	59.4	1.11
TN (%)	3.23	3.25	.09	3.18	3.21	.12	3.12	3.26	.12	3.15	3.15	.08	3.11	3.24	.12	3.49	3.33	.08	3.48	3.59	.08
pH	4.54	4.23	.57	4.73	4.40	.81	4.65	4.35	.81	4.68	4.37	.51	4.68	4.28	.81	6.74	6.65	.51	7.06	7.67	.51
LA (%)	6.39[b]	8.98[c]	.80	4.74[b]	8.93[c]	1.14	5.99[b]	8.77[c]	1.41	6.33	5.30	.72	4.50[d]	7.28[e]	1.14	2.74[d]	4.28[e]	.72	3.06	3.02	.72
NH_3-N (%) of TN)	12.16	8.97	.92	12.36	9.75	1.29	12.39	9.75	1.29	12.72	9.81	.82	12.56	9.66	1.29	7.49	8.67	.82	8.98	7.53	.82
WSC (%)	5.23	6.00	.47	6.25	5.75	.67	6.23	5.68	.67	4.84	5.95	.42	6.34	5.64	.67	4.17	3.61	.42	3.39	2.92	.72

[a]All values are on a DM basis except pH and DM.

[b,c]Values within days and within rows with unlike superscripts differ ($p<.10$).

[d,e]Values within days and within rows with unlike superscripts differ ($p<.05$).

TABLE (Con't.). Silage Characteristics Throughout Aerobic Exposure of 14 Days[a]

	0			1			3			5			7			10			14		
	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM	C	I	SEM
Acetate	13.699	8.454	.631	8.018	7.948	.901	7.763	7.673	.901	7.197	5.771	.557	5.418	1.698	.901	2.195	1.499	.558	1.579	1.437	.558
Proprionate	0.015	0.016	.012	0.008	0.033	.017	0.013	0.048	.017	0.003	0.019	.010	0.018	0.028	.017	0.013	0.031	.010	0.025	0.031	.010
Isobutyrate	0.209	0.035	.028	0.081	0.081	.039	0.161	0.091	.039	0.134	0.016	.025	0.091	0.051	.039	0.049	0.119	.024	0.059	0.074	.024
Butyrate	0.098	0.016	.024	0.048	0.033	.034	0.088	0.088	.034	0.040	0.012	.021	0.028	0.018	.034	ND	0.014	.021	0.010	0.036	.021
Isovalerate	0.050	ND[b]	.012	0.032	0.027	.017	0.077	0.017	.017	0.012	ND	.011	0.032	0.027	.017	0.035	0.057	.010	0.377	0.039	0.10
Valerate	ND	0.006	.003	0.029	0.009	.032	0.165	0.009	.032	0.024	0.008	.020	0.035	0.029	.032	ND	0.042	.020	0.024	0.062	.020

[a]Values expressed as g/kg DM.

[b]ND = not detected

TABLE 15. Response of Holstein Cows Fed Alfalfa Silage With or Without the Addition of a Microbial Inoculant

	Slowly Degradable		Rapidly Degradable		
	C	I	C	I	SED
DM intake, kg/d	17.80	21.50	20.70	20.90	7.60
Weight change, kg/d	6.20	23.50	20.70	17.20	21.40
Milk production, kg/d	27.00[b]	31.80[c]	29.80[b,c]	29.90[b,c]	4.33
3.5% FCM[a], kg/d	27.50[b]	31.90[c]	29.50[b,c]	29.80[b,c]	3.46
Fat, kg/d	0.97[b]	1.12[c]	1.03[b,c]	1.04[b,c]	.12
Protein, kg/d	0.87[b]	0.98[b,c]	0.95[b,c],	0.98,[b,c]	.12
Lactose, kg/d	1.41	1.53	1.54	1.54	.12
Solids, kg/d	3.44	4.04	3.37	3.77	2.22

[a] Fat corrected milk.
[b,c]Means within rows with different superscripts differ ($p<.05$).

cows fed either silage. Similar results were reported by Gordon (1989) in which lactating animals fed inoculated silage showed a 7% increase (P<.05) in FCM. Grant and Colenbrander (1986) also reported an increase in FCM production with inoculated alfalfa silage as compared to control silage. While Grant and Colenbrander did not suggest a reason for an increase in milk production, Gordon suggested the animal production response was consistent with the increase in the metabolizable energy (ME) intake. Lactating cattle in this study showed a slight increase in consumption of inoculated silages which seemed to follow FCM production. These DM intake responses along with change in weight over the study period were not significantly different. It has been demonstrated (NRC, 1985) that lactating dairy cattle fed alfalfa silage based diets supplemented with slowly degradable protein in the rumen will produce more milk. This increased FCM with supplementation of slowly degradable protein may not exist with all alfalfa silages.

The value of a protein source in producing an increase in performance is determined by its ability to 1) supply limiting amino acids (AA) to the small intestine and 2) to supply N available for use by rumen microorganisms. Titgemeyer and coworkers (1989) demonstrated that blood meal and corn gluten meal supplied larger amounts of total AA and AA nitrogen to the duodenum than soybean meal and feather meal. The addition of blood meal significantly (p<.05) increased lysine, histidine, arginine and valine concentrations in the duodenum, while corn gluten meal increased (p<.05) methionine, isoleucine, leucine and tyrosine concentrations.

Perhaps this increase in AA to the lower gut with the small increase in energy exhibited in the inoculated silage is responsible for the positive milk production

response seen in cattle fed the inoculated silage with slow degradable protein.

Cows in all treatment groups showed similar concentrations of lactose and milk solids.

3.4.4 Growth Trial

Results of the beef heifer growth trial are shown in Table 16. Dry matter intake was similar for all treatment groups. This is in agreement with the findings of Kennedy and coworkers (1989) who showed no increase in DM intake for finishing steers fed inoculated grass silage as compared to the control treatment.

The heifers fed SD supplemented control silage gained more weight ($P<.07$) than RD supplemented cattle fed control silage. Weight gains were similar for both protein supplementation regimes with inoculated silage. Expression of average daily gain (ADG) per unit of metabolic body size indicated that heifers fed control silage with a slow degradable protein source gained faster than the other three treatments. These results do not support data compiled from a six trial summary in which Bolsen and Hinds (1984) found no significant differences in performance between animals fed control or inoculated silage.

There are no explanations as to why similar results were not observed in both animal production trials. Perhaps the higher nutrient demand and AA requirements of lactating cows as compared to a growing heifer may explain the different results between the two trials.

TABLE 16. Performance of Crossbred Heifers Fed Alfalfa Silage With and Without the Addition of a Microbial Inoculant

	CONTROL		INOCULATED		
	RD	SD	RD	SD	SEM
No. of Animals	17	18	18	18	---
Initial Weight, Kg	207.40	208.30	244.10	235.20	
Final Weight, Kg	295.90[c]	309.80[d]	343.40[e]	346.90[e]	6.40
DM Intake, Kg/d	5.81	6.71	7.22	7.56	.51
DM Intake, Kg/wt$^{.75}$/d	.092	.104	.102	.106	.072
ADG, Kg/d	.84[a]	.97[b]	.97[b]	1.01[b]	.028
ADG, Kg/wt$^{.75}$/d	.013[c]	.015[d]	.014[c,d]	.014[c,d]	.0006
Gain/Feed	.147	.146	.134	.134	.008

[a,b] Values within rows with unlike superscripts differ ($p<.07$).
[c,d,e]Values within rows with unlike superscripts differ ($p<.10$)

3.5 CONCLUSION

In summary, inoculation of alfalfa silage with a microbial inoculant resulted in a three-fold increase in lactobacilli numbers within 24 hours. Lactic acid content of the inoculated alfalfa silage was greater and the pH lower throughout the first three weeks of ensiling resulting in a fermentation with less protein degradation and less gross energy loss. The favorable shift in fermentation pattern with inoculation did not result in greater DM recovery.

Fat corrected milk production increased with SD supplementation of inoculated silage, however a similar response was not evident in the control silage treatment.

Both silages tended to be stable under aerobic conditions through d 9. Inoculation of alfalfa increased the rate of fermentation, however, DM recovery and aerobic stability were not positively influenced. Cows fed the inoculated silage did respond to slowly degradable protein supplementation. Inoculation appeared to reduce proteolysis and energy losses which the high producing dairy cow was able to utilize for greater milk production. The higher gross energy concentration of the inoculated silage would be advantageous for high producing dairy cows since DM intake generally limits production. The ability of the cows to respond to rumen undegradable protein in this trial may be a result of the added energy provided by the alfalfa silage.

4.0 EFFECT OF A BACTERIAL SILAGE INOCULANT ON FIBER DIGESTION AND RUMEN CELLULOLYTIC SPECIES

4.1 Introduction

Currently, several commercial microbial inoculants are available for use on ensiled forages. Most are marketed on the premise that epiphytic lactobacilli populations are often too low to support a rapid fermentation, and consist either of a single Lactobacillus strain or a mixture of selected Lactobacilli, Streptococci and Pediococci strains. Recently, scientists have reported that silage inoculation improves dry matter (DM) and acid detergent fiber (ADF) digestibility of ensiled forage material in ruminants (Harrison, 1989; Hooper, 1989; Harrison et.al., 1989). These experiments were not designed to evaluate the mechanism of the observed increase in digestibility. Further research is needed to determine the chemical or physical change in the inoculated silage and its effect on rumen microbes during digestion.

Whether the effects of microbial inoculants on fiber degradation are direct or indirect is unknown. A direct effect might include inoculant bacteria having the capacity to degrade or utilize fiber components released from alfalfa degradation, such as dextrins, pectins, cellobiose, xylose, arabinose and oligomeric fragments. Such species of lactobacilli have been isolated from the rumen. Sharpe et al. (1973) isolated a species of lactobacillus from the bovine rumen characterized as being able to use cellobiose with similar morphological characteristics as Lactobacillus plantarum. The organism was named Lactobacillus ruminus. A continual consumption of end products from cellulolytic digestion could stimulate a

higher rate of fiber digestion by these bacteria. A second direct effect could involve lactobacilli interacting with cellulolytics in colonization of alfalfa particles, providing a sticky matrix for an immediate attachment to alfalfa which would facilitate attachment of cellulolytic bacteria to alfalfa.

An obvious indirect effect of inoculant stimulation of fiber digestion by rumen cellulolytic bacteria involves the production of some major growth factor within the treated silage material which is required by the microbes. Thus the lactobacilli inoculant itself would not be involved directly in increasing the fiber degradation but supply the growth factor. Interactions between rumen cellulolytic species and epiphytic bacteria or silage inoculant bacteria have not been investigated as of the present time.

The objectives of these studies were: (1) To determine if selected microbial inoculants and isolated epiphytic strains improve the digestibility of forages and (2) To determine if their effect is directly on forage degradation as scavengers or indirectly by their metabolic interactions with cellulolytic species.

4.2 Materials and Methods

4.2.1 Digestion Trial

Seven Holstein steers (269 kg) were utilized in a crossover design to determine the digestibility of control (C) and inoculated (I) silages. Steers were housed in individual metabolism pens with slotted floors and fed a total alfalfa silage diet for a one week adaptation period. After the adaptation period, steers were randomly assigned to one of two diets. Diets included C and I silage fed at 2.5% of body weight on a dry matter (DM) basis (Table 17) Minerals and vitamins were supplemented according to recommendations of the National Research Council for beef cattle (NRC, 1984). Cross-over periods were 14 d long, with steers receiving chromium oxide Cr_2O_3 at .5% of body weight. Chromium oxide was used as a digestion marker to measure the percent digestibility of CP, ADF and NDF. It was administered orally in a gelatin capsule at the same time each day, beginning on d 1 of the study. On d 9-14 of each period, fecal samples were collected from the rectum of each steer four times daily (6 h intervals) and composited.

Fecal material was analyzed for DM, ADF, NDF and CP as previously described in Chapter 3 of this manuscript. Chromium content was determined by the procedure of Fenton and Fenton (1979) with modifications. Fecal samples were dried in a convection air oven at 60°C for 48 h and ground through a Wiley mill equipped with a 1 mm screen. Approximately .5 g of ground dry feces was digested using 40 ml of nitric acid and 7 ml of 70% perchloric acid. Samples were heated until oxidation was complete and diluted to a volume of 100 ml with deionized distilled water. Chromium content of the diluted sample was measured on a

TABLE 17. Characteristics of Alfala Silage Fed to Holstein Steers During The Digestibility Trial

	Control	Inoculated
DM (%)	50.30	47.25
pH	4.38	4.38
Lactic Acid[a]	3.50	4.30
WSC[a]	5.90	5.48
TN[a]	3.23	3.07
WSN (% of TN)	61.90	67.15
Ammonia-N (% of TN)	8.13	5.35
NDF[a]	45.15	45.25
ADF[a]	34.15	33.90
ADIN (% of TN)	4.95	5.15
ASH[a]	8.30	8.40
Energy[b]	8.28	8.91

[a]Values expressed as a percentage of DM.
[b]Energy is expressed as Kcal/g.

Leeman Ion Coupled Plasma Spectrophotometer 40 (ICP) using the National Institute of Safety and Health (NIOSH) method 7300.

Percent digestibility of NDF, ADF and CP was calculated using the method described by Church (1983) as shown in Figure 5.

Feed intake and orts were measured on a daily basis and silage samples were taken weekly, composited and analyzed for DM, pH, lactic acid, water soluble carbohydrates (WSC), water soluble nitrogen (WSN), total nitrogen (N), ammonia-N, ash, energy, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent insoluble nitrogen (ADIN).

4.2.2 Electron Microscopy

Cultures of two major rumen cellulolytic bacteria Ruminococcus albus 7 and Ruminococcus flavefaciens FD-1, were individually grown to mid-exponential phase in media shown in Table 24. Rather than glucose, alfalfa was used as a energy substrate so that after cultures were mixed with L. plantarum there would be no transfer of glucose for use as a substrate by the lactobacilli. L. plantarum was isolated from a commercial silage inoculant (#1174) manufactured by Pioneer Hibred International (Des Moines, Iowa), and grown to mid-log phase in LBS medium (Table 24). Then .1 ml of each cellulolytic and .1 ml of lactobacilli were mixed in co-culture in 9.8 ml of media shown in Table 21. Each strain of bacteria was also transferred individually into 9.9 ml of this media. In each tube small transverse sections of fresh alfalfa leaves (2 mm x 5 mm) were placed and saturated 2 h before inoculation. Three tubes of each bacterial treatment were placed in an incubator at 39°C for 36 h. Tubes were shaken every 6 hours. All media was anaerobic and remained so until leaves were removed.

FIGURE 5. Calculation for Digestibility Using Accumulation of Chromium Concentration in Feces.

$$\text{Fecal output (g nutrient/d)} = \frac{\text{Indicator consumed (g/d)}}{\text{Indicator conc. in feces (g/g nutrient)}}$$

$$\text{Digestibility} = 100 - 100 * \frac{\text{\% Indicator in feed}}{\text{\% Indicator in feces}} * \frac{\text{\% Nutrient in feces}}{\text{\% Nutrient in feed}}$$

Church, D.C., 1983.

Alfalfa leaves were removed and were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer on ice for 2 h. Following fixation, samples were washed with 0.1 M phosphate buffer and were dehydrated in a graded ethanol series (25%, 50%, 75%, 95% and 100%). Samples were then critical point dried and adhered to 10 mm aluminum stubs with double sided tape. A small line of graphite was drawn from the sample to the outer perimeter of the stub. Each specimen was then gold coated in a Film Vac sputter coater and viewed at 15 kilovolts in a JEOL, JSM-35 CF scanning electron microscope at Michigan State University's Center for Electron Optics.

4.2.3 Growth Enhancements

Inoculated and control silages were obtained from the appropriate silos and 500 g of each silage along with 1000 ml of water were blended using a Waring blender. Contents were strained through cheesecloth and filtered through a sterile millipore filter. This filtrate was autoclaved and 2.7 ml was added to sterile test tubes containing 6.3 ml of sterile GCS-RF media (Table 29). Each tube was inoculated with .1 ml of R. albus, B. succinogenes, and R. flavefaciens. Cellulolytic cultures were also inoculated at .1 ml to sterile test tubes containing 9 ml GCS-RF media. Mono cultures of individual cellulolytics acted as a control for comparison of growth curves. Cultures O.D.'s were read hourly at 600 nm.

4.2.4 In Vitro Digestion

Samples of C and I silages were obtained from the appropriate silos were ground with dry ice through a Wiley mill using a 3 mm screen. This silage material along with rumen fluid from a fistulated Holstein cow maintained on a

alfalfa hay diet was use to determine in vitro dry matter digestibility (IVDMD) according to the two stage method described by Tilly and Terry (1963). Silage was not dried prior to digestion due to concern for altering any factors which may increase silage digestibility, thus .5 g was used instead of .25 g as a sample weight. Twenty-eight tubes were used for each treatment, with 4 being emptied at each of 0, 4, 8, 16, 24, 36 and 48 h. Two tubes were also prepared as blanks, containing no silage material and were emptied at similar time endpoints.

4.3 Statistical Analysis

Statistical analysis of the digestion trial were conducted using the General Linear Models Subroutine in SAS (SAS Institute, 1987). Least square means were generated to compare digestibility of the two treatments. Mean comparisons were made with Bonferroni's T-test as described by Gill (1978).

One animal was eliminated from the data set because of extreme illness and injury which required antibiotic treatment.

In vitro dry matter digestibility data was analyzed using the General Linear Models Subroutine in SAS. Least square means were generated for silage treatments for each hour and LSD was used for mean comparisons.

4.4 Results and Discussion

4.4.1 Digestion Trial

There were no statistical differences observed in initial weight, final weight, ADG and feed efficiency (Table 18). The Holstein steers tended to gain more weight while being fed the inoculated silage as compared to the control, thus having a better gain to feed ratio. However, this was not significant.

Table 19 illustrates the digestibility of the alfalfa silage. Crude protein content, NDF and ADF digestibility of the two silages were very similar. The inoculated silage had a numerically lower CP digestibility and a slightly greater NDF and ADF digestibility. These differences however were not statistically different. This data does not support the work of Harrison et. al. (1989). Percentage of ADF and DM in their study was significantly increased with inoculation. The bacterial inoculant applied in their study however contained other strains of microbes including pediococci and streptococci. These microbes could possibly have affected the digestibility of the inoculated material. Perhaps the one strain of lactobacilli contained in the inoculant used in this study does not alter the physical or chemical properties needed to cause the increase seen by Harrison et.al., (1989) and Hooper (1989). The climatic conditions during the time of this trial could also have exhibited an effect on digestibility. The weather was extremely hot and humid. The air ventilation system in the metabolism room at The Beef Cattle Research Center did not maintain adequate air flow. This in turn could have been a reason for the one animal becoming sick and eliminated from the trial.

TABLE 18. Performance of Holstein Steers fed Alfalfa Silage With and Without The Addition of a Microbial Inoculant

	Control	Inoculated	SEM
No. of Animals	6	6	
Initial Wt., kg	279.26	275.85	11.53
Final Wt., kg	280.40	282.67	10.94
DM intake, kg/d	7.164	7.47	.243
ADG, kg/d	0.08	0.405	.220
Gain/Feed	0.0117	0.0553	.0298

TABLE 19. Digestibility of Alfalfa Silage With and Without the Addition of a Microbial Inoculant

	Control	Inoculant	SEM
Crude protein[a]	52.2	51.64	2.13
Neutral deterg. fiber[a]	36.2	38.26	2.44
Acid detergent fiber[a]	33.3	34.69	2.37

[a]Percentages on a DM basis.

4.4.2 Electron Microscopy

Figure 6 and 7 illustrate what a normal alfalfa leaf looks like when observed under a scanning electron microscope before and after digestion by rumen cellulolytic microbes. These micrographs have been included to exhibit the difference in alfalfa leaves before and after digestion.

The leaf exhibited in Figure 6 through scanning electron microscopy (SEM) reveals large bundles of mesophyll and parenchyma bundle sheath undigested, thus leaving large pits where nutrient solubles appear throughout the leaf. A fresh transverse section of an alfalfa leaf undigested is shown in Figure 7. Open cells revealing inside nutrients are exposed for rumen cellulolytic colonization and digestion.

Figure 8 shows R. albus in monoculture attached to an alfalfa leaf after 24 h of digestion. R. albus tended to form large clusters around the leaf solubles. Although R. albus will adhere to its nutrient substrate, it did not produce clear defined zones of erosion in the leaf, however it appears to degrade the readily available inter-cell nutrients. Extracellular enzymes have been isolated and defined in R. albus as well as studies revealing that anywhere from 0 to 49% of the strains will attach to their nutrient substrate. R. albus in this culture which was strain 7, appeared to have little problem in attaching and degrading part of the alfalfa leaf. Figure 9 and 10, however, reveal R. albus in co-culture with L. plantarum. L. plantarum is not present in these micrographs because none of these organisms attached to the alfalfa leaf. R. albus tended to gather around the stomata (Figure 9) of the leaf. This supports the findings of Baker and Harriss (1947), who suggested that digestion begins with penetration through the stomata. Several clusters consisting of 6 to 20 cocci of R. albus were observed around leaf

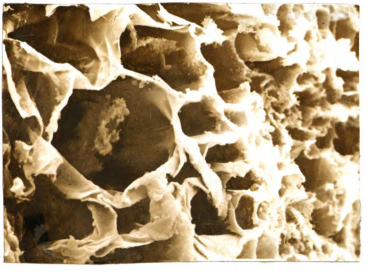

Figure 6. Digested Alfalfa leaf in rumen fluid. 24h. 1800X.

Figure 7. Undigested Alfalfa Leaf. Cut surface of transverse leaf section exposed. 2000X.

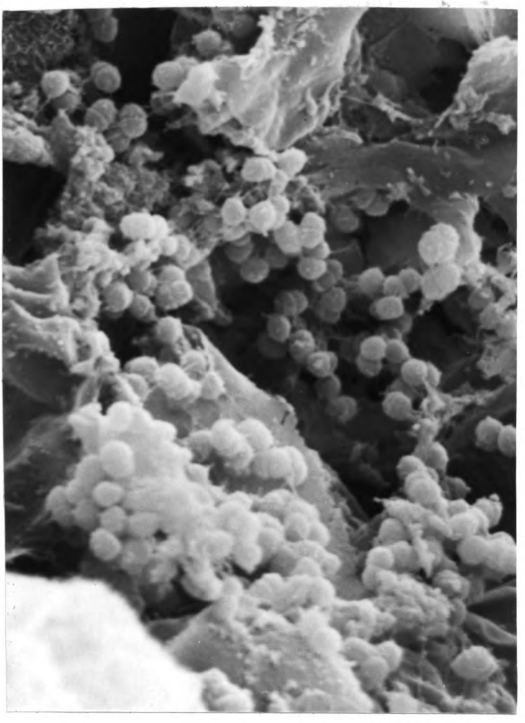

Figure 8. Ruminococcus albus in monoculture, attached to a degraded alfalfa leaf. 24h. 3000X.

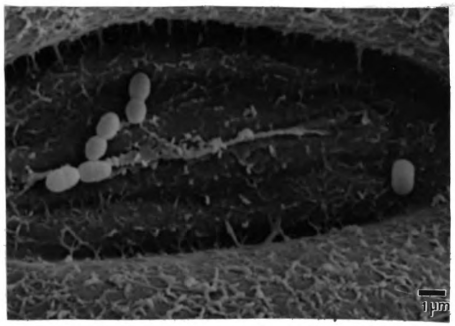

Figure 9. R. albus attached to an alfalfa leaf stomata while in co-culture with L. plantarum. 24h. 4000X.

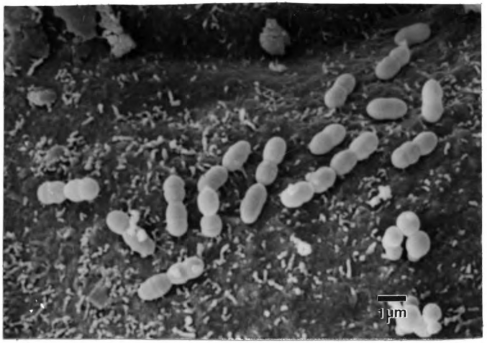

Figure 10. R. albus in co-culture with L. plantarum (not present) attached to an alfalfa leaf. 24h. 7000X.

stomates. There was no penetration through the waxy cuticle on the leaf's surface or pitting, as often observed during cellulolytic fiber digestion (Akin, 1980).

The lack of attachment of L. plantarum and large numbers of R. albus when placed in co-culture could result from L. plantarum using plant sugars as a substrate and driving the pH down with large amounts of lactic acid being produced as its primary end product. Stewart (1977) and Stewart et al. (1979) have shown that the reduction of rumen pH from 7.0 to 6.0 has a profound effect on the activity of cellulolytic bacteria, specifically affecting its attachment to cell wall materials.

When alfalfa leaves that had been exposed in monoculture and co-culture with R. flavefaciens and L. plantarum were viewed by SEM (Figures 11 and 12), they revealed many of the same observations seen with R. albus. R. flavefaciens has been known to exhibit a pronounced capsule (Akin and Rigsby, 1985), however, on the heavily colonized leaf (Figure 12) this physical feature was not observed. Collings (1979) reported string like projections on R. flavefaciens during cell wall digestion, observed under SEM. These features failed to be present on R. flavefaciens both in monoculture and co-culture with L. plantarum. R. flavefaciens did readily digest parts of the plant cell wall and its components when in a pure monoculture. L. plantarum failed in attaching itself to any part of the alfalfa leaf and not one bacterium was located using SEM (Figure 11). R. flavefaciens formed long chains when in co-culture versus clumping in monoculture. Perhaps this clumping of bacterium could result or contribute to the pit formation often seen in digestion of plant material by Ruminococcus sp. (Cheng et al., 1983).

Often synergistic effects among species appear to influence fiber digestion (Miura et al., 1983). In this experiment the effect tended to be negative.

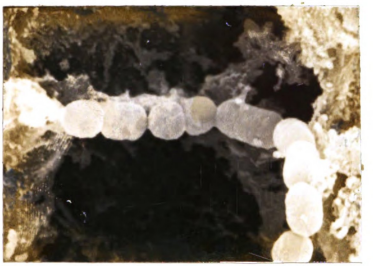

Figure 11. R. flavefaciens in co-culture with L. plantarum (not shown) attached to an alfalfa leaf. 24h. 16000X.

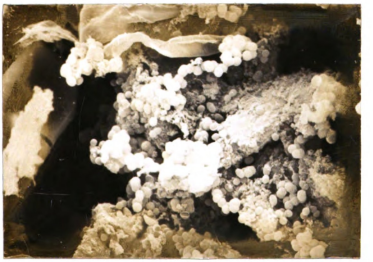

Figure 12. R. flavefaciens in mono-culture attached to an alfalfa leaf. 24h. 3000X.

An SEM study done by Brazle and Harbers (1977) on the digestion of hay revealed that the leaf cuticle and epidermis were sloughed after 24 h of digestion, causing extensive mesophyll degradation, with only the cuticle, abaxial hairs and partially hydrolyzed vascular tissues remaining. The 24 h digestion period in vitro should have been sufficient time to allow considerable digestion of alfalfa leaves. This proved to be true when cellulolytics were in monoculture however when in co-culture, the pH may have had a chance to rise when available substrate for L. plantarum was depleted and lactic acid was no longer produced. A greater number of cellulolytics might have attached to the alfalfa leaves, however, the possibility of L. plantarum becoming directly associated with the leaf is very unlikely.

4.4.3 Growth Enhancements

Rumen cellulolytic growth curves with and without the addition of extract obtained from inoculated silage is shown in Figures 13 through 15. R. albus (Figure 13) and R. flavefaciens (Figure 14) tended to grow faster throughout the entire exponential phase without the addition of the silage extract in the media. However, B. succinogenes' growth rate (Figure 15) was stimulated with the addition of silage extract. This increase was observed beginning 1 h post-transfer of the culture and continued throughout most of the exponential growth phase. This suggests that a growth factor provided by the silage extract is stimulating the growth of B. succinogenes. It has been demonstrated that B. succinogenes is the most active rumen cellulolytic species, digesting the more resistant cellulose (Bryant, 1973), as well as attaching itself more firmly to fiber particles than the Ruminococcus sp. (Minato et.al., 1966). B. succinogenes is the only cellulolytic which has a requirement for valeric acid. An elevated concentration of valeric acid

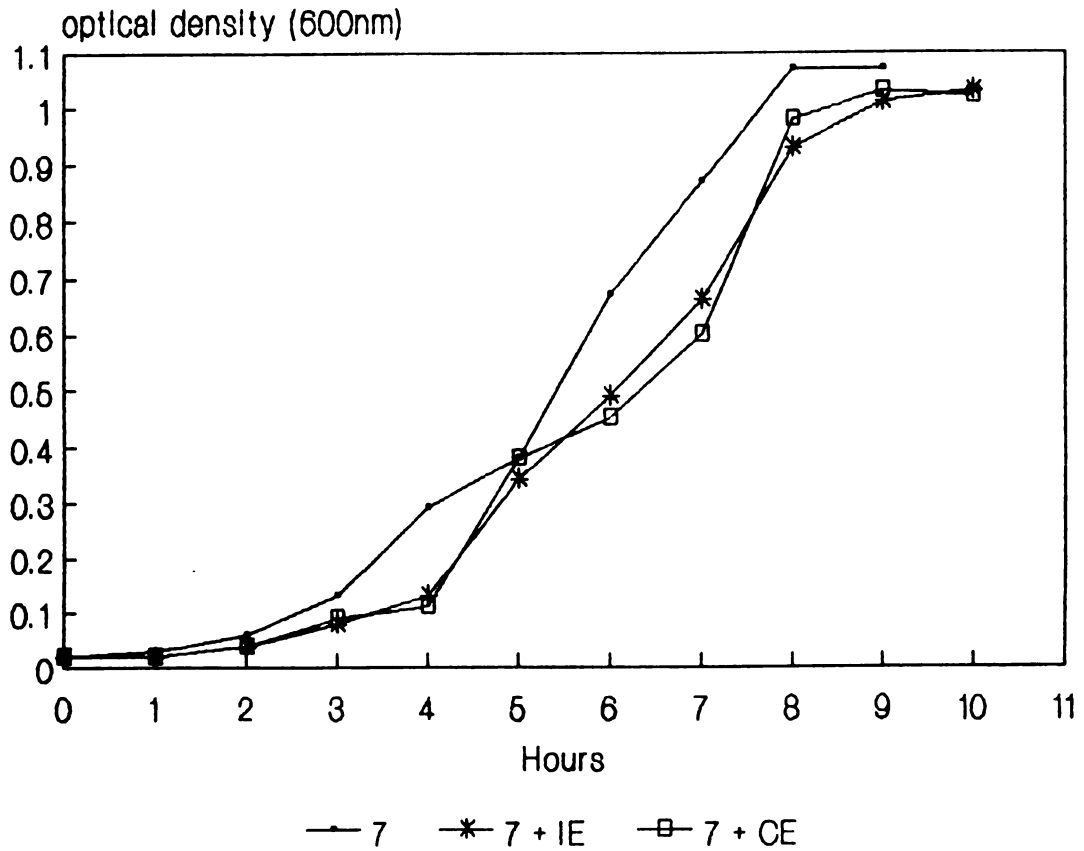

Figure 13. Growth of Ruminococcus albus 7 With and Without Silage Extract.

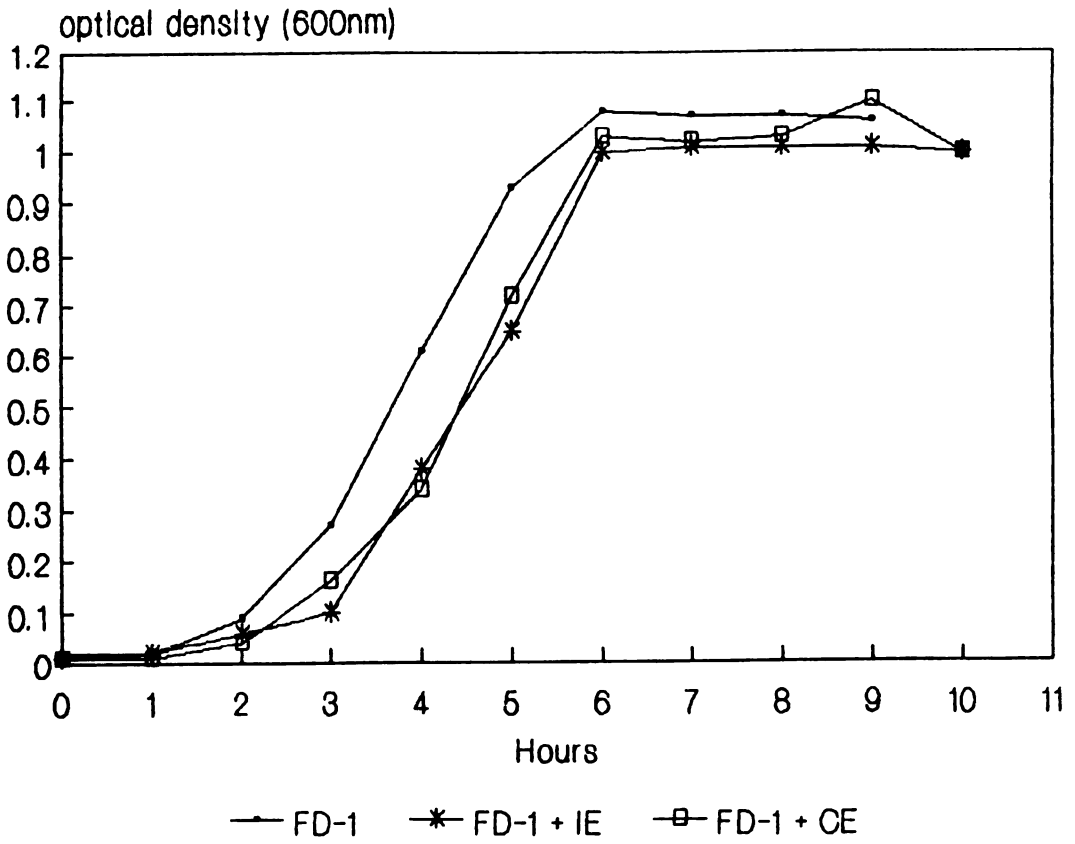

Figure 14. Growth of Ruminococcus flavefaciens FD-1 With and Without Silage Extract.

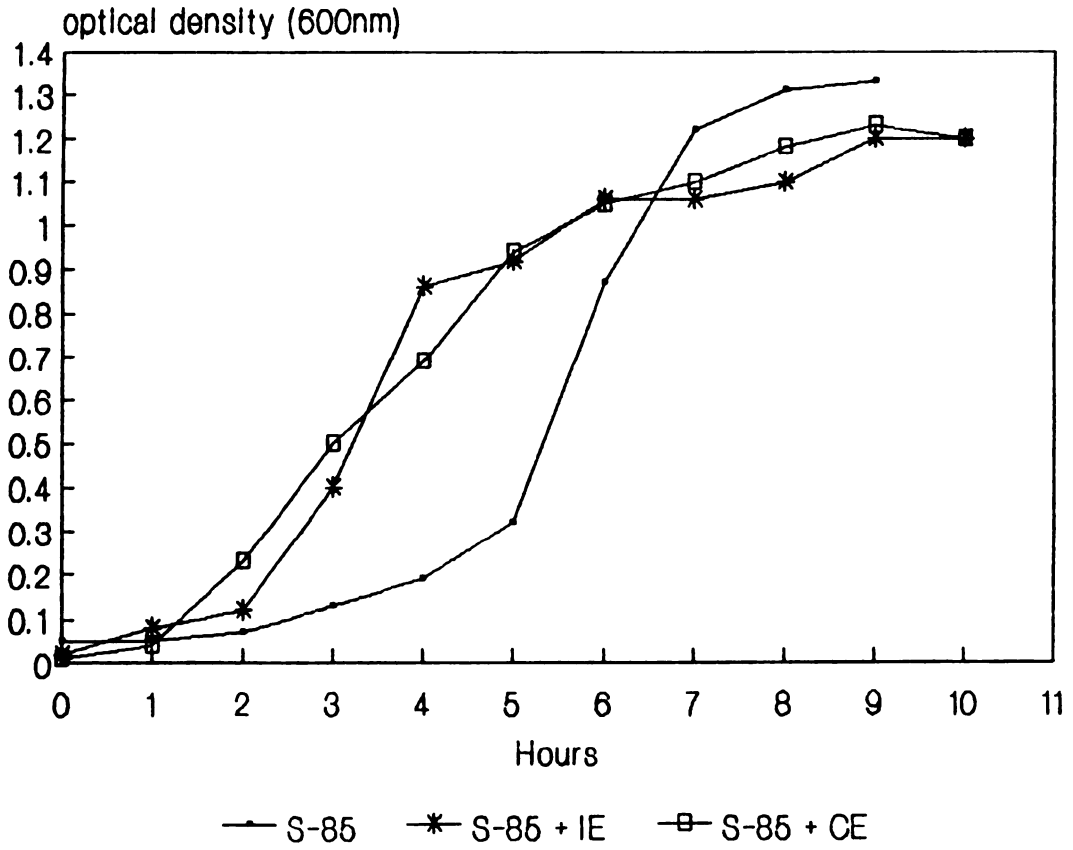

Figure 15. Growth of Bacteroides succinogenes S-85 With and Without Silage Extract.

in the inoculant supernatant could cause this increase in growth response.

Unfortunately at the time the electron microscope was being used, a clean culture of B. succinogenes was not available in our lab. The interaction of these bacterium with L. plantarum might have been detected.

4.4.4 In Vitro Digestibility

There were no significant differences observed in in vitro DM digestibility between the two silages (Table 20). The percentage of DM disappearance is very similar for both silage treatments following a similar pattern throughout the 48 hour period (Figure 16.). The inoculated silage exhibited a greater amount of DM digestibility at 8 and 16 h, however this difference was very small. This supports the data from the Holstein steer experiment reported earlier in this chapter, in which CP, ADF and NDF digestibility did not differ between treatments. Digestibility of DM peaked at 24 h and remained elevated through 48 h.

TABLE 20. In Vitro Digestibility of Dry Matter (IVDMD) of Control and Inoculated Silage

	Treatment		
Hour	Control	Inoculated	SEM
0	0.00	0.00	1.5
4	29.35	29.65	
8	49.83	55.25	
16	57.48	61.03	
24	69.80	69.20	
36	69.85	68.73	
48	69.20	70.10	

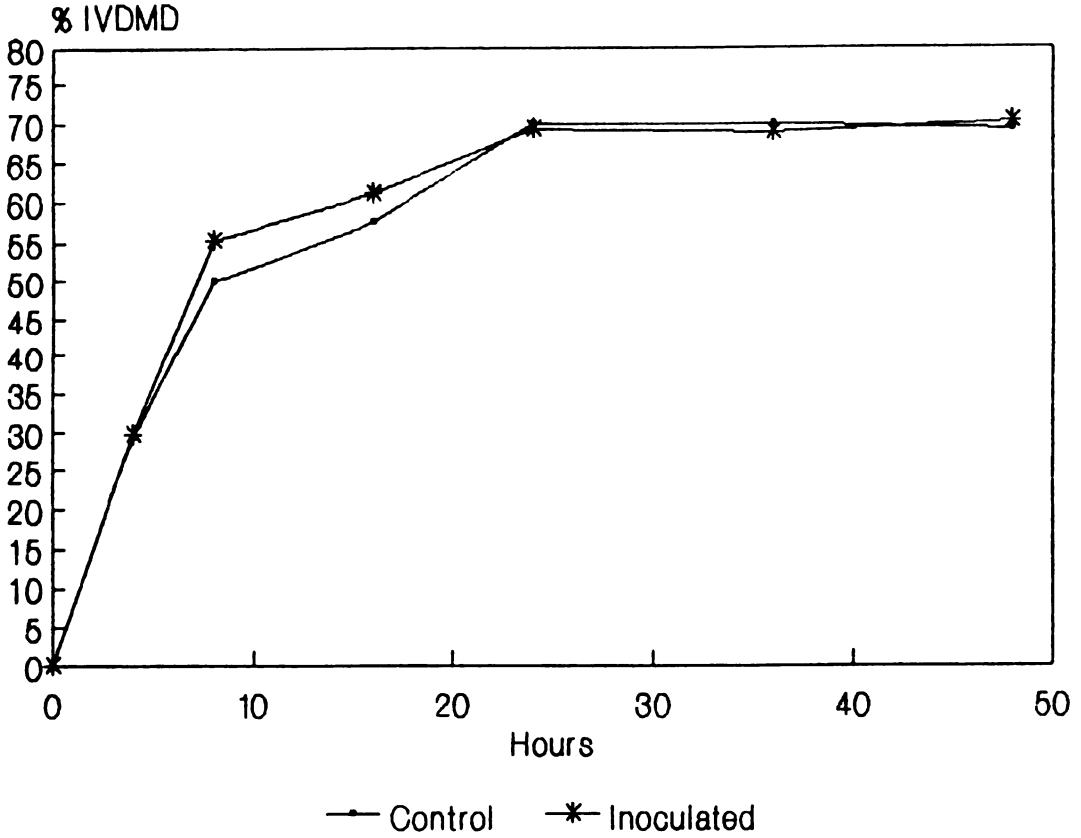

Figure 16. In Vitro Digestibility of Dry Matter (IVDMD) of C and I Silages.

4.5 Conclusion

The result of this study indicated that there are no differences in the percentage of crude protein, ADF and NDF digestibilities in inoculated versus control silage. The conclusion drawn from the 48 h IVDMD study is similar to the in vitro animal digestibility trial with no differences detected. The differences exhibited in other studies resulting in an increase in digestibility could be due to the fact that the silage inoculant contained more than one strain of microorganism. Perhaps a synergistic effect amongst these microbes in the silage itself could cause an increase in digestion, or a combination of metabolic products produced by them during fermentation.

Electron microscopy revealed no direct interactions between Ruminococcus species and L. plantarum. The interaction had a negative effect with fewer numbers of cellulolytic organisms attaching to alfalfa leaf particles in the presence of L. plantarum. A more efficient and perhaps effective way to reveal these interactions would be to run this experiment in vivo.

There were no indirect effects of microbial interactions observed in the growth enhancement study. Growth of R. albus and R. flavefaciens was not enhanced with the addition of silage extract from either the control or inoculated silage, however B. succinogenese did exhibit a greater growth rate when grown in media containing inoculant supernatant.

More studies are needed to identify the mechanism causing this increase in digestibility as well as more studies providing data supporting the hypothesis that silage inoculation increases the digestibility of the silage. Studies in the future should involve singular as well as multi-species bacterial inoculants.

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APPENDIX

TABLE 21 Composition of alfalfa forage entering silos before treatment[a].

	DM	pH	LA[b]	TN	WSN	NH_3N	WSC	E[c]	ASH	NDF	ADF	ADIN
Control:												
	43.8	5.55	0.0	3.14	38.27	1.86	7.63	9.54	9.30	47.0	30.9	3.65
	51.3	5.45	0.0	3.17	37.88	1.71	8.11	8.11	9.20	44.8	31.2	4.04
	46.4	5.50	0.0	2.78	28.74	4.48	9.89	9.05	9.00	46.4	32.3	4.99
	42.9	5.65	0.0	2.69	37.20	0.90	9.45	9.63	9.10	50.0	32.6	3.52
	45.6	5.45	0.0	3.30	33.37	7.62	9.67	9.14	9.30	42.8	33.3	3.80
	48.9	5.50	0.0	2.78	28.74	0.23	7.94	8.49	9.10	50.8	34.8	4.13
$\bar{X}$	46.4	5.51	0.0	2.97	34.03	2.80	8.78	8.99	9.16	46.9	32.5	4.01
Inoculated:												
	49.9	5.30	0.0	3.20	40.63	6.68	8.98	8.28	9.5	43.2	28.5	3.65
	47.3	5.70	0.0	2.99	36.76	7.41	9.63	8.71	9.0	48.3	30.4	3.86
	44.0	5.35	0.0	2.56	23.44	3.99	11.05	9.32	8.4	45.2	31.4	4.78
	46.8	5.35	0.0	2.90	27.62	5.24	10.24	8.89	8.8	49.4	31.6	3.06
	35.4	5.80	0.0	3.34	26.91	5.62	8.65	11.86	8.8	47.1	31.8	3.52
	47.9	5.35	0.0	3.18	28.27	1.86	12.22	8.68	9.5	45.2	31.8	3.60
$\bar{X}$	45.2	5.47	0.0	3.02	30.60	5.13	10.12	9.29	9.0	46.4	30.9	3.74

[a] All values are expressed as a percent of dry matter, except pH, DM and energy.
[b] Lactic acid is expressed as g/100g DM.
[c] Energy is expressed as Kcal/gDM.

TABLE 22 Composition of samples bored from ports 1.5 m from bottom of each silo[a].

	DM		pH		LA[b]		WSC		TN		WSN	
Day	C	I	C	I	C	I	C	I	C	I	C	I
0	47.0	44.9	6.13	6.13	0.00	0.00	10.20	11.80	3.22	3.15	34.16	31.75
1	56.6	50.4	5.96	5.67	0.58	1.46	8.78	8.03	3.33	3.47	42.04	46.11
2	55.0	46.9	5.98	4.81	0.56	2.38	8.97	7.09	3.31	3.12	45.32	41.67
3	56.6	48.2	5.94	4.50	0.61	3.64	7.88	4.23	3.33	3.30	45.05	51.52
5	55.0	40.4	5.93	4.37	0.66	4.52	7.13	2.77	2.98	2.96	50.34	60.81
7	53.3	50.8	5.69	4.28	0.85	5.47	7.47	4.05	3.55	3.23	47.89	55.73
10	47.0	45.6	5.41	4.26	2.71	5.90	6.61	2.85	3.30	3.46	57.58	54.91
13	53.2	40.8	5.54	4.34	3.18	5.42	7.47	2.34	3.34	3.42	50.90	58.48
45	50.4	46.5	5.21	3.97	4.35	4.87	4.26	5.16	3.09	3.06	64.72	62.09

	Ammonia N		Energy[c]		ASH		NDF		ADF		ADIN	
	C	I	C	I	C	I	C	I	C	I	C	I
0	7.02	7.56	8.89	9.22	8.7	9.0	43.6	47.5	31.3	31.4	3.60	3.79
1	7.43	8.27	7.72	8.63	9.4	9.2	44.5	44.4	28.7	29.8	3.71	4.04
2	8.04	8.01	7.58	8.84	9.2	8.9	44.8	46.5	30.3	32.6	3.39	3.97
3	7.18	6.83	7.34	8.46	9.3	9.7	41.5	44.4	29.8	33.2	4.92	4.63
5	10.16	6.70	7.56	10.30	9.0	8.6	40.4	46.5	28.3	32.9	4.56	4.45
7	8.13	7.01	8.08	8.66	9.3	9.6	44.9	44.9	28.7	29.9	3.07	3.43
10	11.94	6.41	9.14	9.47	9.1	8.8	44.8	45.0	29.6	30.3	3.59	3.21
13	9.88	7.58	7.81	9.97	9.4	9.3	40.2	47.9	28.3	32.5	4.32	2.85
45	12.71	8.34	8.17	8.90	9.6	9.3	40.1	42.0	30.6	32.1	4.56	4.51

[a] All values are expressed as a percent of dry matter, except DM, pH and E.
[b] Lactic acid is expressed as g/100gDM.
[c] Energy is expressed as Kcal/gDM.

TABLE 23 Composition of silage during feedout[a].

	DM		pH		LA[b]		WSC		TN		WSN		Ammonia N	
WEEK	C	I	C	I	C	I	C	I	C	I	C	I	C	I
1	48.2	49.4	4.85	6.60	1.40	1.32	2.50	5.12	2.67	3.10	56.18	51.61	15.33	6.29
2	44.4	45.5	4.85	5.50	2.05	2.11	4.43	5.71	2.94	3.22	54.42	59.01	13.68	14.76
3	46.1	41.2	4.85	4.40	1.49	2.59	6.62	6.44	3.36	2.98	62.50	70.47	11.67	12.56
4	46.9	46.8	4.75	4.20	1.50	3.93	9.28	6.30	3.36	3.09	65.48	66.01	13.69	10.20
5	46.1	47.4	4.55	4.20	3.21	2.21	7.09	10.29	3.36	3.36	62.50	65.48	13.88	9.51
6	37.5	39.0	4.40	4.40	2.70	3.75	7.90	13.76	3.36	3.02	68.45	76.16	14.01	11.97
7	38.3	37.2	4.45	4.40	2.96	3.19	8.81	12.34	3.25	3.12	67.69	70.51	14.20	11.16
8	30.7	30.7	4.90	4.40	2.18	2.29	8.76	3.05	3.01	2.98	63.12	63.76	16.56	15.11
9	30.3	30.3	4.90	4.80	1.48	2.50	3.09	4.28	2.77	2.88	64.98	59.03	18.77	17.26
10	44.7	42.5	4.65	4.55	2.45	2.94	5.37	3.87	2.40	2.72	66.67	62.50	12.47	7.02
11	42.7	42.3	4.65	4.15	2.05	2.95	5.16	2.96	2.53	2.70	75.10	62.96	13.36	8.44
12	44.4	43.6	4.50	4.35	2.43	2.69	5.61	5.70	2.45	2.75	69.39	61.82	11.19	9.70
13	43.9	40.8	4.65	4.30	2.96	2.99	6.33	5.18	2.50	2.66	72.00	60.15	12.35	8.69
14	44.1	40.9	4.60	4.15	2.34	3.25	5.90	4.34	2.53	2.51	67.19	63.75	11.23	7.34
15	42.6	41.3	4.35	4.30	2.38	3.29	4.38	5.21	2.54	2.56	66.93	61.84	10.97	6.32
16	46.0	42.0	4.50	4.10	2.68	2.75	5.16	6.36	2.93	2.82	58.02	60.28	9.99	5.14
17	44.0	42.0	4.60	4.25	2.34	3.42	4.68	5.43	2.75	2.85	61.82	59.65	7.72	5.31
18	41.0	41.0	4.50	4.35	2.44	2.82	6.26	5.63	2.99	2.98	60.20	57.05	10.69	5.20
19	43.0	43.0	4.45	4.30	2.58	2.98	5.39	5.15	2.74	2.91	62.04	58.42	9.05	5.63
20	54.4	52.3	4.45	4.25	3.30	4.30	6.34	4.96	3.18	3.07	64.00	67.20	8.60	6.70
21	51.3	48.1	4.35	4.30	3.10	4.20	5.60	5.18	3.28	3.07	62.20	66.90	8.70	5.30
22	49.3	46.4	4.40	4.45	3.90	4.40	6.19	5.77	3.17	3.06	61.60	67.40	7.56	5.39
$\bar{X}$	43.6	42.4	4.59	4.48	2.45	3.03	5.95	6.05	2.91	2.92	64.20	63.27	12.07	8.86

[a] All values are expressed as a percent of dry matter, except DM and pH.
[b] Lactic acid is expressed as g/100gDM.

TABLE 23 (con't.) Comparison of silage during feedout[a].

WEEK	ASH		E[b]		NDF		ADF		ADIN	
	C	I	C	I	C	I	C	I	C	I
1	8.7	11.3	8.91	8.52	50.0	51.4	35.8	42.5	6.44	17.14
2	9.3	11.2	9.61	9.36	48.6	48.5	38.0	40.2	8.01	14.98
3	10.0	10.0	9.45	10.40	39.8	41.6	33.0	34.6	8.35	9.87
4	9.5	9.6	9.17	8.97	38.2	49.0	33.4	38.3	7.55	7.81
5	9.5	9.3	9.40	9.12	41.6	37.8	32.0	31.2	8.29	8.17
6	9.6	9.4	11.50	10.98	37.4	39.4	33.3	32.7	7.04	8.45
7	9.5	9.0	11.09	11.45	47.8	43.6	33.7	34.4	7.36	6.84
8	9.5	9.2	14.03	13.47	47.0	45.9	36.5	36.1	7.28	6.78
9	9.5	9.2	14.35	14.34	52.3	48.3	36.1	36.9	7.82	6.79
10	8.8	9.0	9.60	10.10	50.8	45.5	35.6	33.3	7.57	6.00
11	9.1	9.2	9.84	10.00	46.7	47.0	35.2	35.3	6.96	7.45
12	8.8	9.3	9.64	9.83	49.8	46.3	35.6	32.9	7.41	6.34
13	9.0	8.0	9.66	10.46	47.7	47.7	36.0	34.8	7.06	6.02
14	9.0	8.9	9.65	10.62	48.3	46.3	35.2	34.8	7.37	6.65
15	8.8	8.3	9.76	10.37	44.6	43.5	34.9	34.4	7.42	6.58
16	9.1	8.3	9.26	10.48	43.4	42.7	34.1	33.4	5.59	6.51
17	9.9	8.5	9.74	10.12	41.9	41.1	3.32	33.0	6.64	5.44
18	8.7	8.4	10.36	10.46	41.1	39.2	33.9	31.9	6.24	5.35
19	9.0	8.5	9.78	9.95	44.1	41.6	34.3	33.7	6.01	5.79
20	8.1	8.5	7.86	8.08	44.3	44.4	34.4	34.6	5.10	5.20
21	8.5	8.3	8.16	8.72	43.6	45.0	33.3	33.7	4.90	4.90
22	8.1	8.5	8.39	9.10	46.7	45.5	35.0	34.1	5.00	5.40
X	9.05	9.08	9.96	10.22	45.2	44.6	34.6	34.8	6.88	7.48

[a] All values are on expressed as a percent of dry matter, except DM, pH and energy.
[b] Energy is expressed as Kcal/gDM.

TABLE 24. Medium Used to Grow Rumen Cellulolytic Bacteria to Mid-Exponential Phase

Ingredient	Amount per 300 ml
Ground alfalfa	1.5 g
Starch	.2 g
Yeast Extract	.6 g
Trypticase	1.5 g
Rumen fluid	60.0 ml
Mineral #1[a]	11.2 ml
Mineral #2[a]	11.2 ml
Resazurin	0.3 ml
$NaCO_3$	15.0 ml
Cysteine-HCl	6.0 ml
Distilled H_2O	193.6 ml

[a]Composition of mineral mixes are in Table 25.

TABLE 25. Mineral Mixes Used in Rumen Cellulolytic and Digestion Media[a]

Mineral #1

Ingredient	Amount
K_2PO_4	.6%
Distilled H_2O	1000 ml

Mineral #2

Ingredient	Amount
KH_2PO_4	.6%
$(NH_4)_2SO_4$	.6%
NaCl	1.2%
$MgSO_4$ $7H_2O$	.245%
$CuCl_2$ $2H^2O$	.159%
Distilled H_2O	1000 ml

[a]Ingredients were dissolved in H_2O and media is autoclaved at 15 psi for 20 minutes.

TABLE 26. Lactobacilli (LBS) Medium

Ingredient	Amount per 100 ml
Trypticase	1 g
Yeast Extract	.5 g
Dextrose	.6 g
Monopotassium phosphate	.2 g
Ammonium citrate	2 g
Tween 80	.1 g
Sodium Acetate	2.5 g
Magnesium Sulfate ($MgSO_4$)	.0575 g
Manganese Sulfate ($MnSO_4$)	.012 g
Ferric Sulfate ($FeSO_4$)	.0
$NaCO_3$	5.0 ml
Cysteine HCl	2.0 ml

TABLE 27. Medium Used In Digestion of Alfalfa Leaf With Individual and Co-cultures

Ingredient	Amount Per 100 ml
Trypticase	0.3 g
Yeast extract	0.2 g
Rezasurin	0.1 ml
Mineral #1[a]	7.5 ml
Mineral #2[a]	7.5 ml
VFA[b]	0.3 ml
$FeSO_4$ $7H_2O$	1.0 ml
$CoCl_2$ $6H_2O$	1.0 ml
Cysteine-HCl (2.5%)	2.0 ml
Na_2CO_3 (8.0%)	5.0 ml

[a]Composition of mineral mixes are shown in Table 25.
[b]Composition of VFA mixture shown in Table 28.

TABLE 28. Volatile Fatty Acid Mixture Used for Digestibility Medium

Ingredient	Amount
Acetic acid	17 ml
Propionic acid	6 ml
N-butyric acid	4 ml
Isobutyric acid	1 ml
DL-d-Methyl N butyric acid	1 ml
N-valeric acid	1 ml
Isovaleric acid	1 ml
Phenylacetic acid	1 g

TABLE 29. GCS-RF Medium

Ingredient	Amount per 300 ml
Glucose	0.2 g
Cellobiose	0.2 g
Starch	0.2 g
Yeast Extract	0.6 g
Trypticase	1.5 g
Rumen fluid	60.0 ml
Mineral #1[a]	11.2 ml
Mineral #2[a]	11.2 ml
Resazurin	0.3 ml
Distilled H_2O	193.6 ml
Cysteine-HCl	6.0 ml
$NaCO_3$	15.0 ml

[a]Composition of mineral mixtures are shown in Table 25.