Waris MEHMOOD

THE EFFECT OF MOLASSES, UREA AND EPIPHYTIC FERMENTATIVE MIXED BACTERIAL CULTURE AS ADDITIVES ON LABORATORY SCALE GRASS SILAGE FERMENTATION PROCESS AND SILAGE QUALITY ANALAYSIS

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FATİH UNIVERSITY

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Master of Science in
Genetics and Bioengineering

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APPROVAL PAGE

This is to certify that I have read this thesis written by Waris MEHMOOD and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics and Bioengineering.

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M.Sc Thesis –Genetics and Bioengineering
June 2013

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ABSTRACT

Silage is considered as alternative feed stuff for the ruminants animals during the season of fresh forage scarcity. The goal of making silage is to preserve as much as the original nutritive value of the crop as possible so that it can be fed throughout the year. Many factors affect the ensiling process such as moisture, dry matter, crude protein, organic matter content of the plant and ensiling conditions such as temperature, pH and anaerobiosis Poor fermentations can lead to excessive run off, loss of nutrients, and production of spoiled silage that is no longer fit for feeding and must be disposed of. Understanding the fermentation process and how it interacts with management factors such as silo packing speed, silage pack density, type of additive used, chop length, silo management during storage, and silo management during feed-out should help us to minimize nutritive losses during fermentation. In this study, the influence of additives to improve silage quality was investigated. For this purpose, molasses, urea and lactic acid bacteria inoculants were tested as representative additives. 3 sets of lab-scale experiments to test those additives were planned having each 3 replicates versus control. Results indicated that applying different additives like molasses, feed grade urea and fermented juice of epiphytic lactic acid bacteria can significantly increase the quality in terms of pH, dry matter content, moisture content, crude protein degradability and organic acids production.

Keywords: Silage, solid phase fermentation, grass, additives.

KATKI MADDESI OLARAK MELAS, ÜRE VE EPIFIK FERMENTATIF BAKTERIYEL KARIŞIK KÜLTÜRÜN LABORATUVAR ÖLÇEKLI OT SILAJ FERMENTASYON PROSESI ÜZERINE ETKISI VE SILAJ KALITESININ ANALIZI

Waris MEHMOOD

Yüksek Lisans Tezi – Genetik ve Biyomühendislik
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ÖZ

Silaj taze yem kıtlığı olduğu dönemlerde ruminantlar hayvanlar için alternatif yem malzemesi olarak kullanılabilmektedir. Silaj yapmanın amacı taze yem malzemesinin yılın düğer dönemlerinde de bozulmadan besin değerini korumaktır. Silaj prosesi işlenilen bitkinin nem içeriği, kuru madde, protein ve organik madde muhtevası gibi özellikleri yanında pH, sıcaklık ve anaerobik koşullar da etkiler. Kötü fermentasyon koşulları fazla süzüntü suyu oluşumuna ve bu şekilde ürünün besin değerinin kaybolmasına ve hayvan besini olarak kullanılamayacak imha edilmesi gereken bir atık oluşumuna sebep olabilir. Bu sebeple ürünün silaj olarak üretilmek üzere işlenmesi için fermentasyon prosesine etki eden katkı maddeleri, tanecik büyüklüğü gibi faktörler yanında, depolama, deponun yönetimi, paketleme gibi işletme özelliklerinin de iyi anlaşılması gerekir. Bu şekilde elde edilen ürünün besin değeri korunabilir. Bu çalışmada silaj kalitesini arttırmak amacıyla katkı maddelerinin etkileri araştırılmıştır. Bu amaçla melas, üre ve laktik asit bakteri kültürü temsil edici katkı maddesi olarak test edilmiştir. Her birinin kontrol setine karşılık 3 replikası olan 3 farklı sette söz konusu katkı maddeleri için laboratuar ölçekli deney sistemleri oluşturulmuştur. Deney setlerinden elde edilen bulgular melas, üre ve laktik asit bakteri kültürünün katkı maddesi olarak kullanılması durumunda silaj kalitesinin pH, kuru madde, nem, protein ve organik asit içeriği bakımından iyileştiğimi göstermiştir

Anahtar Kelimeler: Silaj, katı faz fermentasyonu, Çim, katkı maddeleri.

I lovingly dedicate this thesis to my parents (Late)

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SYMBOL/ABBREVIATION	
AA	Acetic acid
Avg	Average
CP	Crude Protein
COD	Chemical Oxygen Demand
D	Day
DM	Dry Matter
DMI	Dry Matter Intake
FM	Fresh Matter
FJLB	Fermented Juice of Lactic Acid Bacteria
G	Grams
H	Hours
Kg	Kilograms
L	Liter
LA	Lactic Acid
LAB	Lactic Acid Bacteria
N	Nitrogen
NDF	Neutral Detergent Fibre
NPN	Non Protein Nitrogen
OM	Organic Matter
SSF	Solid State Fermentation
TKN	Total Kjeldahl Nitrogen
VFA	Volatile Fatty acids
WSC	Water Soluble Carbohydrates

CHAPTER 1

INTRODUCTION

Silage is a animal feed stuff produced by anaerobic digestion by microorganisms, as a result of that acids are produced which lower the pH, in this way forage is preserved for future use. Plant material is digested in the ruminants in the same way as occurs in the silage making process. Plenty of micro organisms live in the rumen of ruminants and there is a symbiotic relationship between them. By the process of anaerobic fermentation plant material is broken down in the silos, in the same way occurs in the rumen. By knowledge of rumen fermentation and silage fermentation leads further development of efficient preservation of forages for optimization of ruminants.

Most of the feed stuffs used for preservation are forages and those play significant role in feeding of ruminants both in terms of nutrition physiology and economics. These storage methods should ensure minimum losses of DM and energy (Muck, 1988). By using some additives, the natural fermentation ability of forage will be enhanced. The process of ensiling dependent on the storage of high moisture grasses, early stage grass with high leaves to stem ratio. During the process of anaerobic fermentation organic acids are produced particularly lactic acid is produced by the fermentation of water soluble carbohydrates present in the plants. pH is lowered by these acids, that leads to the inhibition of growth of putrefactive microorganism like clostridia and bacilli. Increase in the production of Clostridia and bacilli can lead the more utilization of lactic acids and also degrade the plant proteins and amino acids to produce such end products like butyric acid, ammonia and amines that have little nutritive value.

With the increase in the meat and dairy industries, as well as the cost of concentrates increases, so there is demand for alternative quality feed. In this regard silage making ensures the year round availability of forage. Ensiling of legumes and grass makes the farmers self sufficient in the period of green fodder scarcity.

Ensiling is a method that has been known for hundreds of years and been used inworld since 18th century. Ensiling is a better way to preserve forage than hay making, because the method is not as rain sensitive. Lactic Acid Bacteria (LAB) are the organisms responsible for the preservation, they ferment Water Soluble Carbohydrates (WSC) under anaerobic conditions and produce lactic acid, which lowers the pH. These conditions inhibit growth of other harmful microorganisms. To control the ensiling process, improve quality and to inhibit unwanted microorganisms, LAB can be used as additives in silage making. When using LAB as feed additives or starter culture in silage, antibacterial properties in addition to antifungal properties are desirable. Four strains of LAB were tested against one strain of *Clostridium butyricum* and three strains of *C. turybutyricum* with the agar well method.

Food and feed have been preserved by fermentation for a very long time, at first humans had no idea that microorganisms were responsible for what happened. Today we know that Lactic Acid Bacteria (LAB) and yeasts are part of the fermentation of many different foods and feed products (Adams & Moss, 2000). LAB are the microorganisms responsible for fermentation of silage by consuming the WSC that was added in the silage in the form of molasses. Ruminants have got such a capacity that they can efficiently use the NPN to synthesis microbial protein. For that reason feed grade urea is added in the grass to increase the amount of NPN.LAB is used in fermentation processes because of its ability to inhibit other microorganisms and because the lactic acid and other metabolites give the products a pleasant flavour and aroma.

Rapid and cost-effective evaluation of the improvement of the forage preservation, as well as determining the efficacy of additives (molasses, feed grade urea and bacterial inoculants) requires studies of the ensiling process at the laboratory scale. It has been assumed that small-scale silos provide a reliable prediction of the farm-scale silage fermentation process. Model silages have been used since the beginning of the 20th century, comprising different types of fixed-volume vessels like porcelain containers, test tubes and glass jars with different capacities ranging from 50 g to several kilograms.

CHAPTER 2

THEORITICAL BACKGROUND

2.1 SILAGE AS ANIMAL FEED

Forage is the most important source of nutrient for grazing animals and it is important that the animals should have access to good quality feed. During the winter, animals are fed preserved forage and high quality is essential to maintain animal health and to obtain good quality meat and milk. The most common way to preserve forage used to be haymaking, which is a method dependent on good weather. Rain makes the grass wet, nutrients are drawn out and the risk of rotting and mould growth increases. While ensiling is not as sensitive to bad weather as haymaking and has been known since ancient times and is today the most common way to preserve forage (McDonald et al., 1991).

Ensiling has been used in the world since the eighteenth century but during the last 50 years it has been fully developed (McDonald *et al*., 1991). It is a preservation method where LAB ferment Water Soluble Carbohydrates (WSC) to organic acids, mainly lactic acid, under anaerobic conditions. The production of organic acids leads to decrease in pH and the grass is preserved (Weinberg & Muck, 1996). The low pH in combination with anaerobic condition and undissociated acids prevents growth of unwanted bacteria, moulds and yeasts (Scudamore & Livesey, 1998). LAB exist naturally on grass (epiphytic flora) and ensiling starts when air is excluded, for example when the grass is filled in silos or packed in plastic film (McDonald *et al*., 2002).

2.2 MILK PRODUCTION AND MILK QUALITY

Milk production of cows was increased with the feeding of pretreated silage as compare to control. Milk production of cows fed with inoculated silage was 17.7 kg per day per cow compared to 16.7 kg per day per cow for cows fed with the control silage. Milk samples from the morning and afternoon milking were taken to analyse the composition and quality. The crude protein, milk urea nitrogen (MUN) and fat were determined with the 605 Milko Scan Analyser. The intake of inoculated silage and control was 12.3 and 11.7 kg DM per cow per day respectively. Milk urea nitrogen (MUN) was found lower in the milk of cows fed with inoculated silage as compare to control group. (Meeske et al., 2002). Hence, pre-treatment of silage increases the milk production and DM intake of cows.

2.3 RUMINANTS GASTROINTESTINAL SYSTEM

Ruminants have quiet different gastrointestinal tract than the non-ruminants, they have large, complex and large number of microflora. Ruminants have got the ability to regurgitate the feed after eating. Therefore rumen is the main storage part of the GIT of ruminants.

2.3.1 Rumen As Anaerobic Chamber

Rumen is a combination of four compartmental anaerobic chamber of ruminant animals. Almost seventy percent of the total biomass in the world is not suitable for human consumption. Fortunately, ruminant animals such as cattle, buffaloes, sheep and goats have got such a natural modification in their digestive system that they can easily digest fibrous forage components like cellulose, hemicelluloses and starches, and utilize the resulting nutrients for growth, production and reproduction. The symbiotic relationship of host ruminant animals and microorganisms favours the conversion of organic matter of plant to edible products in the rumen. In cattle rumen has the capacity of 50-70 liters of liquid, organic matter, microbes, gases and end products of fermentations. Rumen is large chambered vat filled with organic matter and gases and it constitutes almost 54 % of total digestive tract (Yokoyama and Johnson, 1988).

A thick muscular wall separates the four compartments of the rumen. Digesta in the rumen is mixed and baffles with the help of pillars present in the rumen, these pillars also facilitate the maximum exposure of plant material with the microorganisms for the degradation process. Bacteria fungi and protozoa are the main population of microorganisms found in the rumen. In the presence of bacterial enzymes polysaccharides are converted in to mono saccharides ie glucose which can be easily fermented to gases like carbon dioxide and methane, ammonia, heat, lactate and volatile fatty acids. Major organic acids, acetate, propionate and butyrate are produced as a result of fermentation of monomers like glucose, fructose, maltose, sucrose, cellobiose and cellodextrine. These volatile fatty acids are the source of energy for the both host animal and microbes. As a result of symbiotic relationship microbes produce protein for the host animal and that protein is absorbed in the lower gastrointestinal tract. In this relationship microbes get ideal environment, anaerobic, supply of masticated feed, removal of gases and acids, constant temperature and pH and flushing out the microbial products and indigestible feed particles (Owens and Goetsch, 1988).

2.3.2 Environment of the Rumen

No mammalian enzyme is secreted in the fore stomach compartments (rumen, reticulum and omasum). The abomasum, final compartment that is similar with human stomach, secretes mammalian enzymes. Ingested feed stuffs stay in the first three compartments for longer period to reduce the particle size for effective enzymatic action (Yokoyama and Johnson, 1988).

In rumen, pH ranges between 5.5-7 and temperature remains constant between 38-41 °C (Yokoyama and Johnson, 1988). pH of rumen increases by production of sufficient amount of saliva, and acids produced by the process of fermentation are absorbed by rumen epithelium. Rumen is considered to be the anaerobic chamber but it is not completely deprived with oxygen, Oxygen enters with the ingestion of feed particles and that is utilized by aerobes or fecultative anaerobes or directly removed by eructation.

2.3.3 Microbial Community in the Rumen

Strict anaerobes from rumen were first isolated by Robert Hungate, by using growth stimulating media and anaerobic culturing techniques. Furthermore in the rumen, many other types were found like Gram positive, Gram negative, cocci, rodes and crescent shaped organisms were found in clumps or singly (Stewart and Bryant, 1988). Rumen bacteria are classified as amylolytic, methanogenic, cellulolytic, hemicellulolytic, lactilytic, and proteolytic. This classification is based on morphology, substrate utilization and end product formation.

2.4 SILAGE AS PRE-FERMENTED ANIMAL FEED

To aid the silage fermentation process grass is commonly harvested and ensiled with the use of an appropriate silage additive. The additive may stimulate lactic acid bacterial proliferation within the silo, restrict the growth of putrefactive microorganisms or provide a source of nutrients for the bacteria. In the rumen of animals process of fermentation occurs but silage pre-fermented animal feed facilitates that process.

The three main groups of microorganisms significant in silage fermentation are:

a) Lactic acid bacteria

b) Endospore forming bacteria (Clostridia and Bacilli)

c) Coliform bacteria

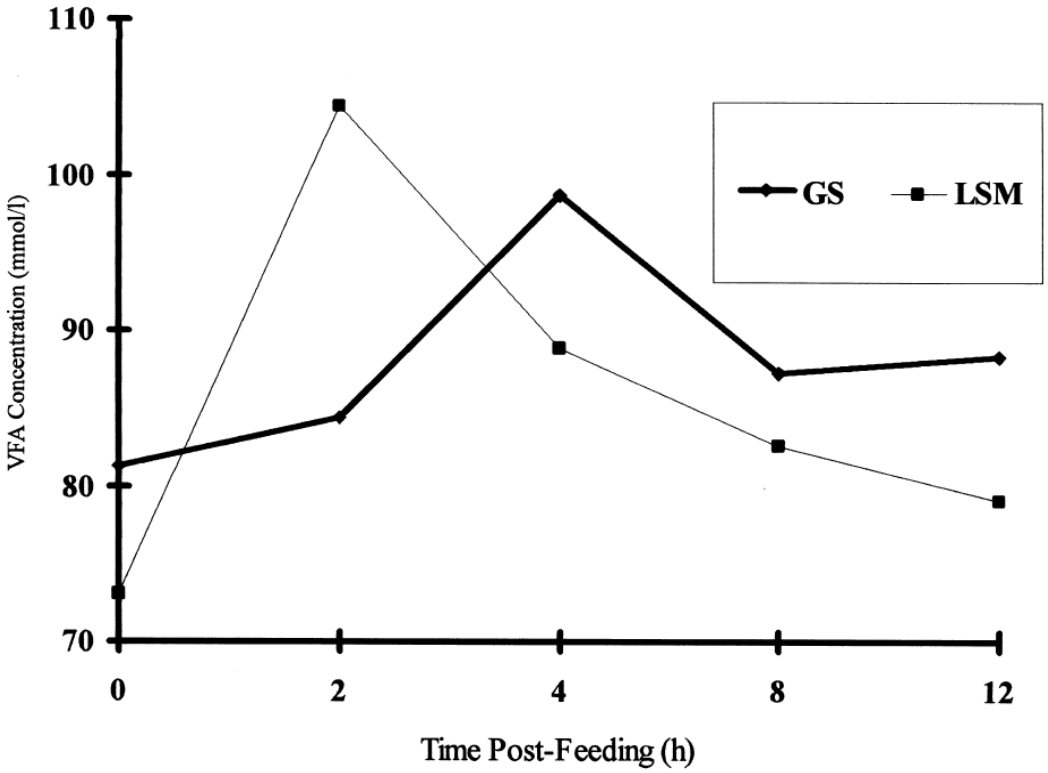

Figure 2.1 Total VFA concentrations in rumen liquor of cows on GS (—◆—) or LSM (—■—) forages at 0, 2, 4, 8 or 12 h post-feeding (Fitzgerald and Murphy, 1999).

2.5 FUNDAMENTAL BASIS OF SOLID STATE FERMENTATION

Solid-state fermentation (SSF) involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water. Although droplets of water may be present between the particles, and there may be thin films of water at the particle surface, the inter-particle water phase is discontinuous and most of the inter- particle space is filled by the gas phase. The majority of the water in the system is absorbed within the moist solid-particles.

The substrates used in solid state fermentation processes are often products or byproducts of agriculture, forestry or food processing. Typically the source of nutrients comes from within the particle, although there are some cases in which nutrients are supplied from an external source. Usually a polymers give the solid structure to the

particle and this polymer may or may not be degraded by the microorganism during the fermentation process.

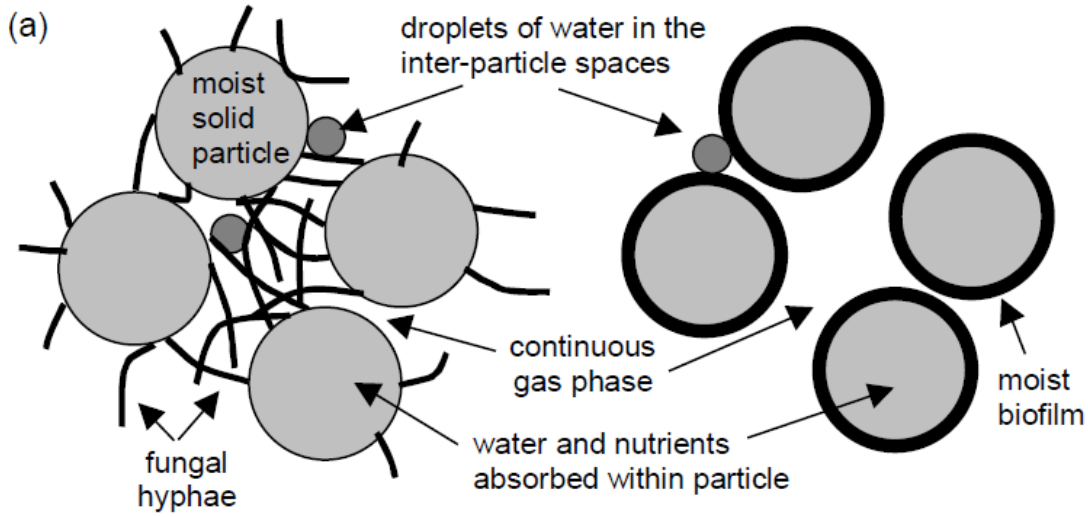

Figure 2.2 Features of solid-state fermentation (SSF) systems (following the terminology of Moo-Young et al. 1983).

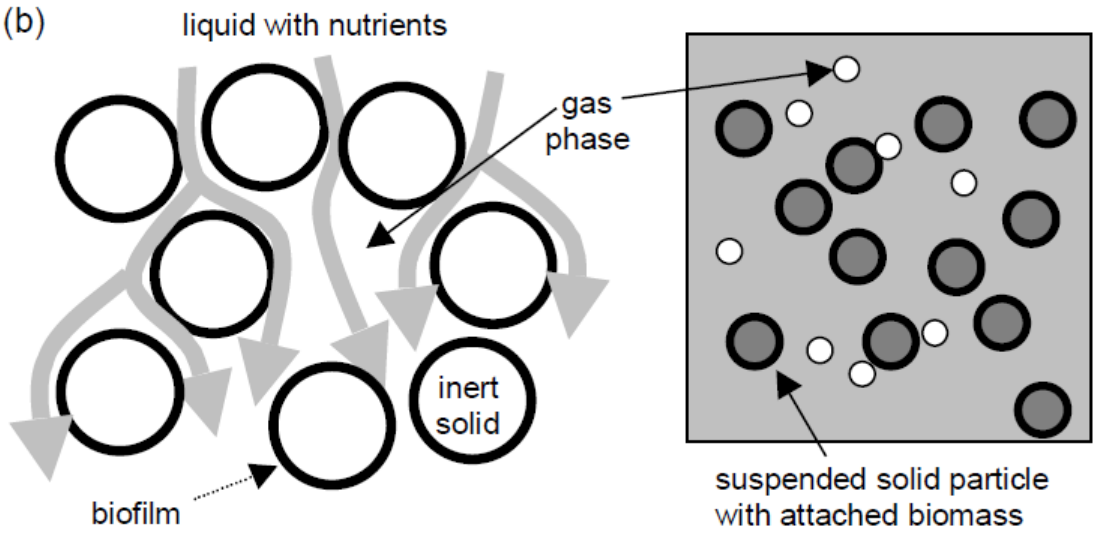

Figure 2.3 Features of solid-state fermentation (SSF) systems (following the terminology of Moo-Young et al. 1983).

2.6 PROCESS OF ENSILING

Ensiling is defined as a forage preservation method which is based on a spontaneous lactic acid fermentation under anaerobic conditions. The epiphytic lactic acid bacteria (LAB) ferment the water-soluble carbohydrates (WSC) in the plants to lactic acid, and

to a lesser extent to acetic acid. Due to the production of these acids, the pH of the ensiled material decreases and spoilage micro-organisms are inhibited. Once the fresh material has been stacked and covered to exclude air, the ensiling process can be divided into 4 stages (Weinberg and Muck, 1996; Merry *et al.*, 1997).

The silage process has four phases (Driehuis & Oude Elfink, 2000; Weinberg & Muck, 1996):

• Aerobic phase – In this phase pH ranges between 6.0-6.5. Oxygen is still present in the plant material but it is consumed by aerobic microorganisms and respiration of the plant material.

• Fermentation phase – During this phase oxygen is consumed and anaerobic microorganisms like lactic acid bacteria, but also clostridia that not are wanted in the silage, start to grow, pH decreases to 3.8-5.0.

• Storage phase – In this phase few changes occur in the silage if no oxygen enters the silo.

• Feeding out phase - The silage is exposed to oxygen when animals are fed. When oxygen enters in the silage, aerobic microorganisms start to grow again and the silage will be destroyed due to an increase in pH and growth of yeasts and moulds.

2.6.1 Silage Microflora

The silage microflora plays a major contributing in the successful outcome of the conservation process. The flora can basically be divided into two groups, namely the desirable and the undesirable micro-organisms. The desirable micro-organisms are LAB. The undesirable ones are the organisms that can cause anaerobic spoilage (e.g. clostridia and enterobacteria) or aerobic spoilage (e.g. yeasts, bacilli, Listeria and moulds). Many of these spoilage organisms not only decrease the feed value of the silage, but also have a harmful effect on animal health or milk quality, or both (e.g. Listeria, clostridia, moulds and bacilli).

2.6.2 Lactic acid fermentation

Lactic acid producing bacteria involved in fermentation include; *Lactobacillus, Pediococcus,* and *Streptococcus.* In good quality silage, streptococci initiate the fermentation process. Pediococci and leuconostocs compete with, steptococci for viability and finally lactobacilli complete fermentation. Under aerobic conditions, lactic acid bacteria can produce both lactic acid and acetic acid and are classified as heterofermentors. Some lactic acid bacteria are capable of fermenting citric and malic acids found in some forages (McDonald, 1981). Approximately 10^3 to 10^4 cfu/g (the population of bacteria normally present) of lactic acid bacteria on chopped alfalfa before loading into the silo. The number of lactic acid bacteria needed to decrease the pH is approximately 10^8 cfu/g (Pitt et al., 1985).

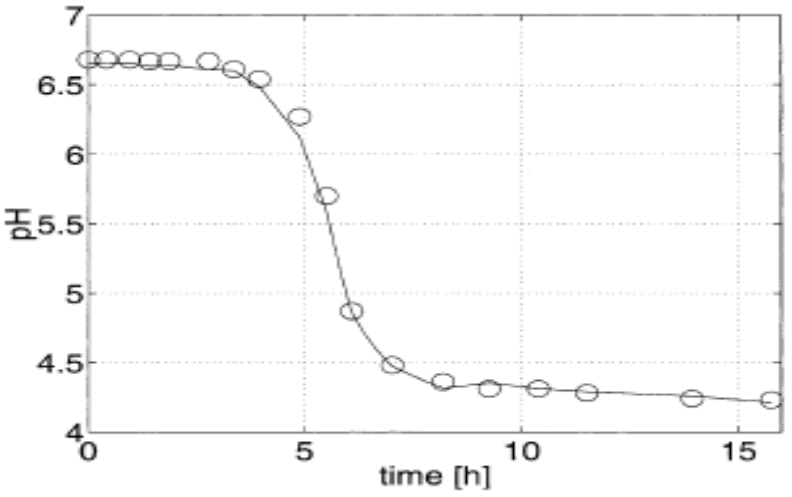

Figure 2.4 Variation of pH in function of time for the experimental case study; '6': experimental data; '—': simulation based on relations (14) and (15) and the experimental LaHtot values, linearly interpolated (Karen and Jan, 2002).

2.7 FACTORS INFLUENCING ON SILAGE FERMENTATION

The quality of lactic acid fermentation is largely depends on the following factors. These factors determine the extent of fermentation and end products like CP %, DM % and organic matter etc.

2.7.1 Effect of Temperature

The success of fermentation of silage is closely related to the conditions during the process of silage making. Temperature highly affects the performance of microbiology of additive used such as Lactic Acid Bacteria (LAB) in the ensiling process. Without any additive, well-fermented silage was obtained at 40°C because higher temperature depressed the activities of microorganisms as it was unfavorable for silage fermentation but with LAB additive, the quality of silage was reduced even if fermented at 40°C because LAB would be killed under high temperature. The temperature of ensiling process will affect the quality of silage due to the additive used in the silage (Setapar *et al.*, 2012).

2.7.2 Effect of Chopped length / Particle size

Similar to the human digestive system, chopped or chewed food will help the digestive system to absorb nutrient from that food more efficiently in the intestinal track. The chopped length also has a significant relationship with maturity of the forage harvested. Chopped length does not induce large difference in starch particle size distribution at the early maturity state. Late harvest of unchopped forage will give lower starch ruminal digestion. The size of forage chopped will give high impact on the effectiveness of the silage formulated. Besides that, it also depends on the type of forage taken as a raw material. By comparing grass and straw, the texture of the plant is different and thus gives different digestibility. Meaning to say that if the chopped length of grass is larger than straw, it will possibly give the same effectiveness in the digestive system of a cow (Setapar et al., 2012).

2.7.3 Fermentation Stimulants

These include bacterial cultures to establish lactic acid bacterial dominance. Being successful in improving the fermentation in small scale laboratory silos, the

effectiveness of inoculants in farm silos remain unproven, intake of inoculants treated silages improved compared with control silage although no apparent difference in fermentation quality was observed (Steen *et al.*, 1989).

Carbohydrate sources such as molasses which provide a fermentable substrate for microorganisms present in the silage and cell wall enzymes, such as cellulases and hemicellulases to break the polysacharrides into monosacharrides (Gordon, 1989).

Some forage crops may be low in WSC or may have lack of LAB which is responsible for the fermentation of the plants (McDonald, 1981), and silage additives / inoculants could be beneficial in this regard. To stimulate the fermentation process for the production of silage, a source of soluble carbohydrate such as molasses has been applied extensively as a silage additive (Thomas, 1978, Khattab *et al.,* 2000, Nkosi, 2003, Zobell *et al.*, 2004). Good source of carbohydrate, sugarcane molasses, which is a by-product of the sugar cane industry that contains 650 g/kg DM soluble carbohydrates (Ashbell *et al.,* 1995, Meissner, 1999), has been used to improve the fermentation process (Bolsen *et al*., 1996, Yunus *et al*., 2000, Van Niekerk *et al*., 2007, Nkosi *et al*., 2009).

Due to the viscosity of molasses, it is difficult to apply and should therefore be diluted preferably with a small volume of warm water to minimize effluent losses (Ashbell *et al*., 1995). With grass of low DM, a considerable proportion of molasses may be lost in the effluent during the first days of ensiling (Henderson, 1993). Furthermore, cell wall enzymes such as cellulases and hemicellulases, can be used as silage fermentation stimulants. Cellulase, hemicellulase and amylase enzymes have been widely tested as silage additives. These compounds have the potential to convert structural carbohydrates to soluble sugars which can be fermented by silage microflora. Many experiments have shown that their use increases the level of fermentable carbohydrates in silage, thus promoting extensive fermentation (Jakkolaa *et al*., 1991, Stokes, 1999). Fibrolytic enzymes and bacterial inoculants, has been proposed as a means of directly improving fibre digestibility as well as increasing the availability of water soluble carbohydrates to serve as a substrate for LAB (McDonald *et al*., 1991). In a study by Zahiroddini *et al*. (2006) the inclusion of enzymes with inoculants did not seem to be effective either in decreasing the NDF content or increasing the WSC content of barley silage. Other researchers (Ranjit & Kung, 2000, Kung & Ranjit, 2001) have applied

enzyme-containing inoculants onto barley silages with no effects on NDF and ADF concentrations. Zahiroddini *et al.* (2004) have found higher concentrations of plant fibre in silages treated with enzyme-containing inoculants ensiled in mini-silos or lab scale silo, but lower concentration of ADF in the same silages ensiled in large bag silos. They attributed this effect to the nature of ensiling environment.

2.7.4 Silage potential Inhibitors

Formic acid has been reported to reduce silage fermentation, and lowers the amount of acetic and butyric acids and the degree of proteolysis in the silo (Waldo, 1978 cited by Cole, 1992). Furthermore, increased DM intakes of growing cattle and dairy cows, coupled with improvements in animal performance have been associated with formic acid treated silages (Thomas & Thomas, 1985, Parker & Crawshaw, 1982), the improvements being greatest when the control silage is poorly preserved.

Chemical additives such as formic acid, sodium chloride, sodium acetate etc were used to improve the process of fermentation of ensiled high moisture by-products (Megias *et al*., 1998, Kholif *et al*., 2007). Application of formic acid resulted in a rapid acidification of fodder and partial inhibition of microbial growth (Woolford, 1984). Furthermore, experiments in laboratory and farm scale silos indicated that the addition of formic acid based preservatives at ensiling improved the fermentation pattern and aerobic stability of silage (Salawu *et al*., 2001, Filya & Sucu, 2007). Application of 4 ml/kg formic acid on wheat silages was found to be effective in improving silage quality and aerobic stability, but did not affect too much on organic matter digestibility (Filya & Sucu, 2007). Sterilants such as formaldehyde that inhibit the growth of microflora in general also restrict proteolysis in the silo. Problems of handling corrosive acids and poor intakes of the resultant silages have limited the use of chemical additives (Gwayumba, 1997). As a result, they have been replaced with biological additives such as microbial inoculants (Weinberg & Muck, 1996).

Formic acid was first applied in silage production in 1926, but did not become widely used until the introduction of crop harvesters, it reduces fermentation in the silo, and lowers the amount of acetic and butyric acids. Sterilants such as formaldehyde that inhibit the growth of microbes in general also restrict the amount of plant protein breakdown both in the silo and the rumen as well (Woolford, 1984).

2.7.5 Absorbents

Absorbents can be added to silages, particularly those that are not wilted or where acid additives are added to reduce the problems of pollution from effluent associated with these silages. They include barley, straw and sugar beet pulp. One of the major setbacks in ensiling agro-industry by-products is their high moisture contents, which requires that the by-product be dehydrated or mixed with a dry source (absorbent) to improve compaction and ensiling for high moisture crops (Khorvash *et al*., 2006).

Absorbents can be added to silages, particularly those that are high in moisture content (e.g. by-products) or where acid additives are added to reduce the problems of pollution from effluent associated with these silages. They include materials such as straw, bran, hay, barley straws, sugar beet pulp and poultry litter / manure. Nicholson *et al*. (1977) confirmed potato by-products to ensile satisfactory when mixed with dry roughage and observed positive results when fed to steers.

2.7.6 Aerobic Deterioration Inhibitors

Antibiotics and other antimicrobial agents such as ammonia can be added to silages to prevent the growth of spoilage microorganisms.

2.8 SILAGE ADDITIVES

Silage additives include feedstuffs, urea, ammonia, inoculants and acids. Their main functions are to either increase nutritional value of silage or improve fermentation so that storage losses are reduced / minimized. Response to additives depend on what kind of forage is being treated. Corn silage does not require any additive that improve fermentation since it ferments quite readily. Nutritional additives such as urea, ammonia and molasses however, have beneficial effects. Hay crop silages generally are more difficult to ferment and may respond to many silage additives, but animal response when fed treated silages is usually not different from animals fed untreated silage. Silage additives are not magic bullets and will not replace good silage management practices.

2.8.1 Effect of Molasses

In case of molasses addition, pH was found significantly low as compared to control after 30 days of anaerobic fermentation. However minimum level of lactic acid was found in control and maximum was observed in the silage having molasses application. It was also observed that flavour and colour of silage was desirable due to addition of molasses as compared to control. A minor increase in ash contents was observed due to molasses as compared to control (Qamar, 2009). The possible reason of increase in CP during ensiling may be the fact that proteolytic activity during fermentation process produces NH_3 but due to efficient fermentation and early stability of silage, this proteolysis activity is inhibited and the produced NH3 that helps in getting the aerobic stability because of its fungicidal properties. (Kung *et al*., 2000). The other possible reason of increase in CP contents is due to different types of bacteria present in the medium have no chance to perform their activity and they become the part of silage. These bacteria are protein in nature and contain more than 75% true protein (Yang *et al*., 2004).

2.8.2 Effect of Urea

Nutrient additives such as feed grade urea and molasses contribute to the nutritional needs of the animals consuming the silage. They can be either energy or protein yielding nutrients and include starches, cereals and nitrogen containing minerals such as urea. Many of the additives classed as absorbents (barley) or fermentation stimulants such (molasses) could also be described as nutrients. To sustain nutritional quality and enhance the fermentation process during ensiling, various additives (absorbents, feedstuffs, nutrients and absorbents etc.) have been used (Oude Elferink *et al*., 1999, Charmley, 2001). Urea is a common additive that provides both non-protein nitrogen (NPN) and the ammonia needed for optimal ruminal fermentation (Erfle *et al*., 1986, Leupp *et al*., 2006). Non-protein nitrogen sources (e.g. urea, anhydrous ammonia) not only increase the nutritive value, but also improve the aerobic stability of silage (Keller *et al*., 1994). The finding of Leupp *et al*. (2006) concluded that the addition of urea to wet beet pulp at ensiling increased the DM content, enhanced fermentation process, and also increased nutrient quality. However, the use of NPN in high moisture (> 70 %) silages is often discouraged due to inability to achieve a low enough pH (4.0) to minimize the microbial activity that causes nutrient losses (Valadares *et al*., 1999).

Nutrients such as ammonia, and minerals have also been used as additives during ensiling (McDonald, *et al*., 1991).

2.8.3 Inoculation of lactic acid bacteria / Mixed bacterial cultures

Successful silage requires epiphytic lactic acid bacteria (LAB) and water soluble carbohydrate (WSC) to produce sufficient lactic acid for rapid pH reduction and silage quality (Bureenok et al., 2011). LAB can be used as feed additives/starter cultures in silage production and the most important property is to compete with the epiphytic flora and dominate the fermentation process. In the ensiling study two strains of LAB that might be used as additives in the future were followed during the whole ensiling process to document their growth and ability to compete with the epiphytic flora. In the end, it is also important that the application of bacterial culture are able to inhibit growth of spoilage organisms such as yeasts, moulds and undesired bacteria. However, the addition of LAB inoculants to forage with low content of WSC (below 30 g/kg) has been shown to limit the effect upon silage fermentation process (Seale, 1986). In contrast, Rooke (1990) demonstrated that an inoculant of LAB could improve silage fermentation even at a very low concentration of water soluble carbohydrates (12.8 g/kg fresh grass). Haigh and Parker (1985) concluded that WSC content as low as 30 g/kg may be sufficient for a stable fermentation where an effective additive is added during ensiling process. In many cases, a source of readily fermentable substrate for LAB is included with commercial bacterial inoculants. This combination has proved to be effective in securing more stable silage fermentation process (Henderson, 1987). According to Whittenbury (1967, cited by Fish, 1991) the requirements of a quality silage micro-organism are as follows:

i) It must be fastly growing and able to compete with and dominate from other micro-organisms in silage.

ii) It must be homofermentative.

iii) It must be acid tolerant upto a silage pH of 4.0.

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

v) It should have no action on organic acids (in the silage). In addition, McCullough (1975) listed the following requirements for a cost effective quality inoculants.

i) The cost of the additive must be less than the silage lost without the additive.
ii) Addition of the additive must result in a more efficient fermentation than occurs natural process.
iii) The additive should produce a silage with a greater digestibility of energy and protein than untreated silage.

Weinberg *et al*. (2007) hypothesized that certain LAB strains interact with rumen micro-organisms to enhance rumen functions and animal performance. LAB are a genetically distinct group of bacteria that share the same properties, Gram positive, non-sporulating rods or cocci, catalase negative, acid tolerant, fermentative (lactic acid is the main metabolite) and prefer growing under anaerobic conditions but are aero tolerant (Wessels *et al.*, 2004). *Lactococcus, Leuconostoc, Pediococcus, Lactobacillus* and *Streptococcus* are example of bacterial genera that are members of the LAB group (Adams & Moss, 2000).

LAB can be found in different nutrient rich habitats like on mucosal membranes of animals and humans, on plants and in many feed and food systems (Holzapfel *et al.*, 2001).

The fermentation pattern of LAB can be attributed as homo- or heterofermentative. Homofermentative LAB use the glycolysis pathway with lactic acid as the main product. Heterofermentative LAB use the 6P-gluconate pathway or phosphoketolase pathway and the main end products are equal amount of lactic acid, carbon dioxide and ethanol (Adams & Moss, 2000).

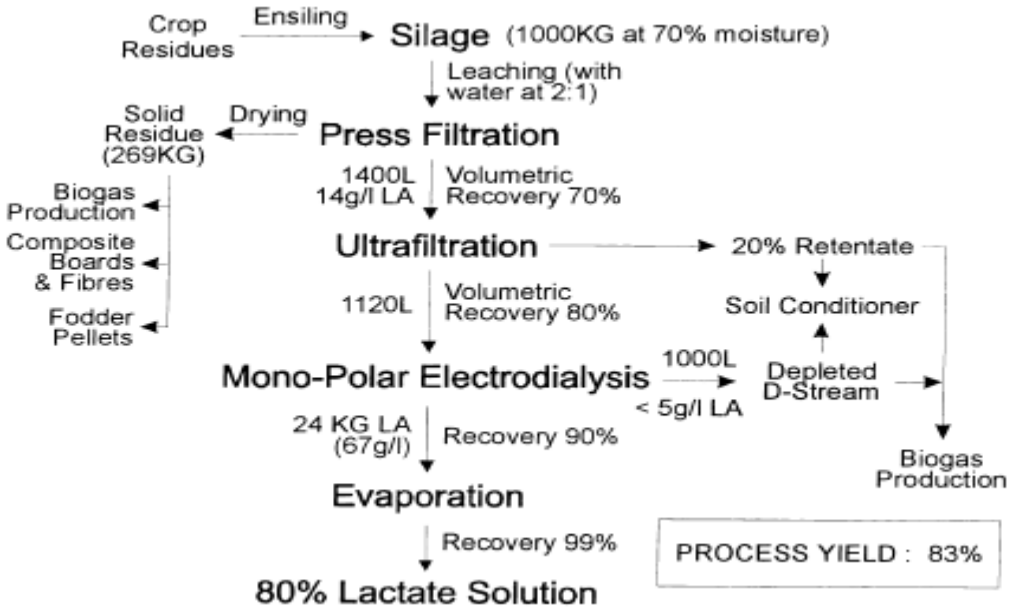

Figure 2.5 Process model for lactic acid extraction from silage. The basis of this process model is 1 tonne of silage at 70% moisture and at a lactic acid yield of 96 g LA/kg DM. Note: 2:1 is water to silage ratio (Danner et al., 2000).

2.8.3.1 Antimicrobial properties of Lactic Acid Bacteria

Chemicals have been used for a long time to preserve food and feed items. Today consumers are more concerned of how chemicals affect the body and the environment and they want food that is minimally processed with as little added chemicals as possible (Schnürer & Magnusson, 2005). At the same time the shelf life shall be the same as with chemical preservatives, as shall the taste, flavour and look. LAB can be used as bio preservatives because they may have got both antifungal and antibacterial properties (Schnürer & Magnusson, 2005). When LAB ferments food or feed they produce lactic acid that is the main substance that inhibit microbiological growth due to the lowering of pH (Adams & Moss, 2000). LAB produce a lot of other substances for example several bacteriocins, propionic acid, acetic acid and phenyl lactic acid (Stiles, 1996; Magnusson & Schnürer, 2005; Valerio *et al* 2004).

Bacteriocins are peptides or proteins that are bactericidal and they are often active only against bacteria closely related to the bacterium that produced them. Nisin is the only bacteriocin that is approved of as a food and feed additive but other bacteriocins

produced by lactic acid bacteria has the potential to be used as feed and food preservatives (Stiles, 1996). Acetic and propionic acid produced by heterofermentative LAB reduces growth of fungi and bacteria in combination with lactic acid by acidifying the cytoplasm of the microorganism (Schnürer & Magnusson, 2005). Phenyl lactic acid was discovered as one of the main factors for pro-longed shelf life in bread and also for anti fungal effects. It is also one of the metabolites forming cheese flavour (Valerio *et al*., 2004). Ström *et al* (2002) isolated a strain of *L. plantarum* that produced antifungal cyclic dipeptides; furthermore they have described the production of an antifungal acid in a *L. plantarum* strain.

2.8.3.2 Effect of inoculation of LAB on Aerobic Stability of silage

Aerobic stability is a term that animal nutritionists have used to define the length of time that silage remains cool and does not spoil after it is exposed to air, when opened for feeding (McDonald, 1981). Aerobic stability of silage is especially important in intensive animal production worldwide because large operations often contract for and take delivery of silage sufficient for 2 to 4 days of feeding and store it unprotected and, hot weather can lead to rapid aerobic deterioration of such silage (Pitt *et al*., 1991).

The inability to remove sufficient quantities of silage from silos between feedings can result in prolonged exposure to air. An air ingress as small as 100 to 150 mg O2/kg DM is enough to make silage highly susceptible to aerobic spoilage (Woolford, 1990). Upon exposure to oxygen, conditions become favourable for growth of aerobic bacteria, fungi and yeasts (Moon, 1981). 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 (Woolford, 1990). However, a high population of yeasts does not necessarily mean a silage will spoil (Nishino *et al*., 2003), instead, quantity of lactate-utilizing yeasts decides whether a silage will deteriorate or not upon aerobic exposure (Woolford, 1990). Thermophilic filamentous fungi are also found in deteriorating silage, however, their growth is generally lower and thus have a little effect on silage as feed (Fish, 1991). Regardless of the substrate utilized by these micro-organsisms, deterioration in forage crops is always accompanied by a loss of residual sugars and the evolution of ammonia and carbon dioxide (McDonald *et al*., 1991).

Aerobic deterioration of silage is indicated by an increase in temperature and pH caused by metabolism of organic acids and sugars by bacteria and yeasts that produce lactic

acid (McDonald *et al*., 1991). Furthermore, this deterioration of silage causes high DM losses and a risk of mycotoxin production in the feed, which are harmful to animal health (Filya, 2003). Ironically, silages that have undergone a clostridial fermentation are very stable when exposed to air because they have high concentrations of volatile fatty acids that have high antifungal properties (Woolford, 1990). Honig (1990) suggested that inoculation of silage with LAB might improve aerobic stability via competitive suppression of yeasts. However, in summary of studies conducted between 1990 and 1995, Muck and Kung (1997) reported that homolactic LAB inoculation of whole crop maize improved dry matter (DM) recovery and animal performance by 2 to 3 % and 3 to 5 % respectively. However, inoculants that contain mainly homofermentative LAB have often reduced the aerobic stability of silage because of insufficient production of volatile fatty acid (VFA) (Muck & Kung, 1997, Rust *et al*., 1989, Weinberg *et al*., 1993).

Inoculation with a homofermentative LAB inoculant probably reduced aerobic stability of silage. Weinberg *et al*. (1993) hypothesized that high levels of residual WSC, combined with high lactic acid concentrations and a lack of sufficient concentrations of protective VFA in the silage inoculated with a homofermentative LAB were associated with aerobic deterioration. In addition, inoculation with homofermentative LAB shifts the fermentation towards lactic acid rather than better inhibitors of yeasts. A relationship between acetic acid and stability was proposed by Danner *et al*. (2003) who claimed that increasing acetic acid concentrations inhibit spoilage organisms, thereby promoting exponential increases in stability.

Consequently the efforts for LAB inoculants that would inhibit the growth of yeasts and enhance aerobic stability was initiated. Inoculants containing the heterofermentative species, *L. buchneri,* have been marketed mainly on their ability to improve the aerobic stability (Weinberg & Muck, 1996, Ranjit & Kung, 2000). The explanation for aerobic stability enhancing effect of *L. buchneri* is that, in silages inoculated with this organism, the concentration of acetic acid is increased which reduce the activity of yeasts (Filya, 2003). According to previous research (Driehuis *et al*., 2001, Taylor *et al*., 2002, Nkosi *et al*., 2009) inoculation with *L. buchneri* typically results in acetic acid concentrations ranging from 36 to 50 g/kg DM, suitable to control yeast during aerobic exposure of silage. Furthermore the heterofermentative pathway of *L. buchneri* inoculants can cause greater silage pH and ammonia-N concentration (Neylon & Kung, 2003) and increased

losses of WSC and DM during fermentation (Adesogan & Salawu, 2004). Moreover, some heterofermentative LAB such as *L. reuteri*, *L. crispatus* and *L. Brevis* have been reported to produce ferulate esterases, which improve silage aerobic stability and increase digestibility and animal performance (Nsereko *et al*., 2008).
Although the fermentation efficiency of heterolactic bacteria is lower than homolactic bacteria (McDonald *et al*., 1991), any increase in dry matter losses during fermentation may be offset by improvements in the aerobic stability of the silage (Holzer *et al*., 2003).

Consequently, improved stability through elevated acetic acid levels may be possible without a reduction in the intake of silage. Inclusion of propionic acid bacteria in inoculants may also improve aerobic stability as propionate has also been shown to exhibit antifungal activity (Weinberg *et al*., 1995, Higginbotham *et al*., 1998). The beneficial effects of homofermentative LAB on fermentation and retention of nutrients in silages, along with the ability of heterofermentative LAB to improve the aerobic stability of silage, has led to the development of inoculants containing of mixtures of these bacteria (Ranjit & Kung, 2000). These inoculants are called dual-purpose inoculants and they improve the fermentation process as well as the aerobic stability of silage as treated with ryegrass (Ashbell *et al.*, 2002, Filya, 2003) and wet Bermuda grass silages (Adesogan *et al*., 2004). Combining *L. Buchneri* with other LAB to obtain positive characteristics when silages are exposed to air and active fermentation has been studied in cereal grain silages (Weinberg *et al*., 1999, Filya, 2003) and in grass silages (Adesogan *et al*., 2004).

2.9 ENGINEERING ASPECTS OF ENSILING

2.9.1 Biochemical Characteristics of Silage Feed

The reduced intake seen when silages are fed as opposed to other forms of forage have been attributed to their chemical composition. Low protein diets are associated with poor intakes by both monogastric and ruminant animals. Ruminants need for dietary protein are lower than those of monogastric animals since the microorganisms of the gut are able to utilise NPN in the diet and urea in the saliva for microbial protein synthesis.

A decrease in intake of mature sheep and cattle occurs when the dietary protein level falls below 8 - 10% (Blaxter and Wilson, 1963; Elliot and Topps, 1963). In the case of the lactating dairy cows where protein requirements are high, levels of dietary protein below 12% reduce intake (Bines, 1979). Protein deficiencies may be due to a reduction in bacterial and protozoal cellulytic efficiency in the rumen (Campling, Freer and Balch, 1962). Egan (1965) suggested that a protein deficiency would lead to depressed intake as key enzymes needed in the metabolic pathways to utilise digestion end-products would not be able to function without these limiting amino acids. A build up of digestion end-products would result, stimulating the chemo-receptors involved in intake regulation. Silage feeds are characterised by the rapid degradation of soluble protein and NPN in the rumen, resulting in a pronounced peak in rumen ammonia and a reduction in available amino acids and peptides. The fixation of ammonia by rumen bacteria requires energy. On many silage diets there is a lack of energy substrates in the rumen due to the poor ATP yield from silage fermentation end-products. This coupled with the decrease in the availability of amino acids and peptides results in a poor rate of microbial protein synthesis (Thomas and Thomas, 1985). A poor rate of microbial protein synthesis in the rumen could lead to a slowing of the rate of digestion and hence reduce voluntary silage intake (Chamberlain *et al.*, 1989).

2.9.2 Nutritive Problems of silage Protein and Carbohydrates

Three problems associated with ensiled forage feeds such as tropical corn have been found out. First, inherent low nutrient content of the forage is also evident in the ensiled crop, second, low protein available to the animal and third, these factors contribute to low intake by ruminants. Silages that are properly ensiled with minimal proteolysis may increase microbial protein synthesis by allowing the transport of the spared oligopeptides into the microbial cells (Stern and Hoover, 1979). Walker et al. (1975) found lower VFA concentrations in the ruminal fluid of cattle fed as silage diet. This implies that there is less carbohydrate available for synthesizing microbial protein. Although energy can be produced from the fermentation of amino acids, the yield is too low for enough protein synthesis. McDonald et al. (1981) found that the depletion of water-soluble carbohydrates during ensiling of forage may lead to low carbohydrate availability for protein synthesis.

2.9.3 Effect of end products of silage fermentation on intake

According to Charmley (2001) one of the major disadvantages associated with silage making is that the feeding value of silage is decreased relative to that of the original forage. However, silage research up to the present time has focussed on closing the gap between feeding value of the original forage and that of the resulting silage. Poorly preserved silages are consumed to a lesser extent than well preserved silages. Baker *et al*. (1991) produced two silages from the same sward grass, one was well ensiled using good silage techniques and an additive, while the second was made in a deliberate attempt to create a poor quality silage. When these were fed to dairy cattle, considerably more of the well produced silage was eaten, the main difference between the two being their amine content. According to Driehuis *et al*. (1999) reduced silage intake is found only with poorly preserved silage. Another fermentation end-product, butyric acid was first implicated as being responsible for reducing silage intake in 1963 (Charmley, 2001). Intake of silage by dairy cows declines as the concentrations of silage ammonia and butyric acid increase (Cushnahan *et al*., 1995). Low pH in silages is often associated with poor intake because low pH in rumen reduces cellulolytic activity and reduce intake (Charmley, 2001). However, silage pH alone could not account for a significant part of feed intake (Kawamoto *et al*., 2009), and its influence was indirect (Wilkins *et al*., 1971). According to Rooke (1995) there is no relationship between silage pH and rumen pH, because silage is neutralized by saliva upon consumption (Charmley, 2001). Some researches (e.g. Newbold *et al*., 1991) had reported that neutralization of silage with bicarbonate increased silage intake. Rooke (1995) also suggested that lactic acid may have a direct effect on palatability since a sour taste is associated with reduced palatability. Ammonia-N in silage is predominantly a product of clostridial fermentation of amino acids, and has been associated with reduced silage intake (Steen *et al*., 1998). It has been found that silage with a high CP content and high solubility can result in high rumen ammonia concentration leading to a reduced silage intake (Charmley & Veira, 1990). Under certain feeding situations, these conditions could lead to mild ammonia toxicosis which may depress feed intake (Charmley, 2001). Furthermore, various potential intake inhibitors with neuropharmacological effects, such as histamine and amines have been found in silage. These products are produced

by protein degradation during silage fermentation, and are typically found in butyrate silage (Ohshima *et al*., 1979).

2.9.4 Management of ensiling

2.9.4.1 Negative Biological Process in ensiling

These process occur by aerobic respiration of plant material due to improper sealing or improper removal of air during ensiling, due to these reasons the aerobic respiration continues longer than expected period of time. During the initial period of respiration small losses of DM and energy occurs. DM and fermentable carbohydrates are lost due to extended period of forage respiration that leads to scarcity of substrate availability for fermentation by lactic acid bacteria. Because of that pH remains high which leads to undesirable microbes such as clostridial fermentation and plant activity (Muck, 1988). Hemicellulose and starch are converted to monosaccharide by the actions of plant enzymes, they also change the protein into non protein nitrogen (NPN). Furthermore, protein content of silage will be lower when NPN will be converted into ammonia (Muck 1988). Apart from clostridial growth, silage quality will be decrease because of high pH and aerobic microbial activity. If the aerobic respiration stays longer than the expected period that can leads to production of maillard products, which effect the nitrogen content, making it unavailable. Mold is seen on the surface of aerated silage, which may contain mycotoxins that can cause digestive problems to ruminants (Muck, 1988).

2.9.4.2 Managing Negative Biological Process in ensiling

Plant respiration continues until the whole oxygen present in the grass will be utilized. Therefore, effectively compressing, packing and sealing the bunker or upright silo reduces energy and DM losses. In terms of plant proteolytic enzyme activity, for legumes, the optimum pH is around 6.0 and declines as the pH falls to 4.0 (McKersie, 1985). At day of ensiling proteolysis is greatest and decreases by day five, although activity never disappears. Drier silages have decreased proteolytic activity and do not require as a rapid decline in pH to reduce proteolysis (Muck, 1987). Lactic acid bacteria will produce acids and reduce the pH quickly, reducing proteolysis (Pitt, 1986).

2.9.4.3 Packing

Silage samples are packed in the anaerobic glass jars and entrapped air is removed with the help of vacuum pump as shown in the below Figure 2.6.

Figure 2.6 Packing of grass for ensiling

2.9.4.4 Storage and handling

Storage of experimental silages at room temperature in an open container resulted in significant changes in the chemical composition of the forage and it would seem reasonable to understand that this is a generic phenomenon relating to all silages. Storing the silage in a fridge was more convenient, and again had minimal effect on chemical composition. However, if such facilities are not available then evacuated bags were proved to be an effective way of storing silage at room temperature (Fraser *et al*.,

2003). Below Figure 2.7, elaborates the changes in pH and Oxygen concentrations in different steps of ensiling.

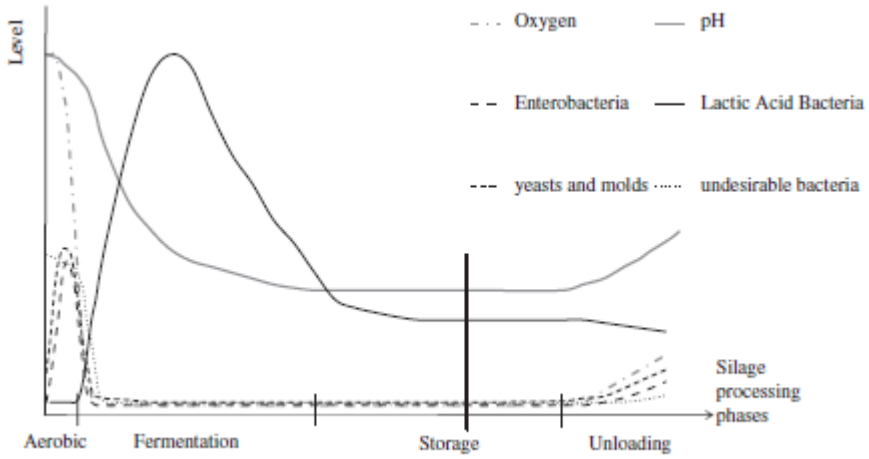

Figure 2.7 Theorical changes in oxygen content, pH values and different microbial populations at different steps of silage processing.Adapted from Pitt and Sniffen (1985).

2.9.4.5 Aerobic stability

Air has harmful effects on silage. Indirectly it reduces conservation efficiency, causes a loss of nutritive value and leads to a potential health hazard to livestock and personnel handling the feedstuff. Aerobic deterioration occurs to a varying degree in all silages with the possible exception of those which have sustained an extensive secondary fermentation. Air is important in a sense that it allows the growth of many aerobic micro-organisms which is otherwise reduced by ensilage (Woolfor, 1989).

2.10 SILAGE QUALITY PARAMETERS

To make good quality silage, environmental and microbial factors that influence the fermentation and ultimately, the nutrient value of the silage. These factors must be understood as an integrated package, as neglect of any one component can lead to a loss in this silage preservation process. Silage inoculants can facilitate the ensiling process, but they are not a replacement for paying attention to the fundamental factors (plant

maturity, oxygen exclusion, dry matter content) that are the key indicators for making good quality silage.

2.10.1 Taste and Smell of Feed

The sensation of taste would have been involved in the selection of these silages, it may have been assessed that the presence of butyric acid and possibly amines such as cadaverine, putrescine and in silage extracts would have reduced the sheep intake. The taste of a feed is probably the most important palatability factor when silages are fed to animals. Ruminant animals are known to be sensitive to sour, bitter, salty and sweet flavours (Jacobs et aI, 1978). Cattle are able to detect tastes with a greater sensitivity than sheep, with goats intermediate. Poorly preserved silages are eaten to a lesser extent than well preserved silages.

2.10.2 Physical Characteristics of Silage

The physical form of the feed can affect its voluntary intake by ruminant animals. Forages tend to be bulky fibrous feeds that has a physical limit on voluntary intake. Van Soest (1965) pointed out this limitation of forage intake to the cell wall constituents, the structural carbohydrates, as measured by the acid detergent fibre method. Increasing the crude fibre content of the feed of sheep not only decreased the daily intake of sheep but changed the feeding patterns, reducing both their size and length (Dulphy et aI, 1980).

2.10.3 DM contents

The voluntary intake of silage increases with increasing DM. The wetting of silage before feeding did not affect the DM intake of silage (Thomas et aI, 1961), whilst additions of water 12 hours before feeding to sheep reduced their intake (Dodsworth and Campbell, 1953). Extracellular water is not likely to limit voluntary potential of intake since it is rapidly absorbed across the rumen wall. Clancey et aI, (1977) concluded that water content percent is not involved in intake regulation. Wilting of grass has for many years been used as a means of improving fermentation quality. Wilting generally results in an increase in silage intake of between 5% (Rohr and Thomas, (1984) and 18% (Steen and Gordon, 1984), although generally a reduction in

live weight gain is seen when wilted and unwilted silage is fed. This improvement in intake is a consequence of improved fermentation (Wilkins, 1988) and the reduction in animal performance the consequence of a lowered digestibility of the feed (Gordon, 1989). Wilting of silage resulted in a 4% higher intake by lactating dairy cattle, but again animal performance in terms of milk yield was decreased by 2% (Rohr and Thomas, 1984). While wilted silages may enhance DM intake, Gordon (1989) calculated that the loss in animal product per hectare caused by wilting of grass prior to ensiling in dairy and beef industry could be as high as 13%.

2.11 CHARACTERISTICS OF BADLY PRESERVED SILAGE

Bad silage can be dangerous for the ruminant animals, it may contain pathogenic bacteria like *Listeria* and spore forming bacteria like *Clostridium botulinum, C. Butyricum, C. Tyrubutyricum*, mycotoxins, moulds and yeasts (Wilkinson, 1999). There is also a risk of finding spores from *Bacillus* and growth of enterobacteria might occur. *C. botulinum* produces a toxin; botulinum, that is the most powerful neurotoxin found in nature. Botulinum causes botulism, fatal muscular paralysis (Adams & Moss, 2000).

Spores from *Bacillus* spp. can be found in the grass silage and they can pass through the gastrointestinal tract of the animals unaffected and passed by faeces. The spores might then be transferred to the milk through faecal contamination of the udder and they also survive in the processing of the milk and cause spoilage and food borne diseases (Te Giffel *et al*., 2002). Almost all yeasts and moulds are strict aerobes and these can not cause any infection / problem if oxygen is removed. The problem with growth of moulds and yeasts occur when oxygen gains entry into the silage due to leakage / breakage in the plastic film or out take from the silo when feeding the animals (McDonald *et al*., 1991).

Enterobacteria are facultative anaerobes, which means that they can grow both in presence and absence of oxygen, they ferment sugars and the end product is acetic acid. They are also able to degrade amino acids (McDonald *et al.*, 1991). Badly preserved silage is silage where clostridial, enterobacteria, or both have dominated the fermentation process. The amount of LAB, lactic acid and WSC content are low while

acetic acid and butyric acid levels are high. This kind of silage is preserved with too low dry matter or too low levels of WSC (McDonald *et al*., 2002). A bad quality silage usually have pH values between 5.0 and 7.0, that is due to formation of butyric acid, that is weaker than lactic acid and not able to reduce the pH to the same extent. In this type of silage the levels of ammonia-N are high, the unwanted bacteria degrades amino acids and releases keto acids amines, ammonia-N, and fatty acids and the nutritional values are reuced (McDonald *et al*., 1991). High pH is not always equal to bad silage; it depends on the dry matter content of the grass / forage. Silage with pH 4.9 can be considered good if the dry matter is high.

Silage can also be deteriorated due to leakage of oxygen into the silo. Silages of this category should always be considered toxic because of growth of moulds, yeasts, and aerobic bacteria and should not be offered to animals (McDonald *et al*., 2002).

To improve the silage quality and the efficiency of the preservation and in terms of animal performance, LAB can be used as additives or starter cultures in the silage making process. The most common LAB used as starter cultures are *Enterococcus faecium*,*Lactobacillus plantarum, L. acidophilus, Pediococcus acidilactici* and *P. Pentosaceus* (Weinberg and Muck, 1996).

The inoculant should have several properties to be a suitable part of a starter culture / as additive (Weinberg & Muck, 1996):

- Produce large amounts of lactic acid in short time.
- Acid tolerant.
- Ability to grow at temperatures up to 50°C and in low water activity.

CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTION OF FRESH GRASS FOR ENSILING

Fatih University Istanbul, field area was selected for collection of grass. Grass was collected during different periods of spring season. Sunny day is considered to be the suitable one for cutting the grass sample for experiments. For the first set of experiment, 12 kg, in second and third 4 kg of fresh grass were used. If fresh grass has relatively high moisture content it needs to be wilted for some time. As soon as the crop is cut, the grass starts to lose nutrients, due to plant respiration and the breakdown of sugars and protein. Rapid wilting and ensiling minimises these losses by quickly creating acid levels that stop further respiration. These reactions require anaerobic (air-free) conditions, that is why quick consolidation in the clamp and sealing is crucial. Chopped length / particle size of grass sample was 2-3 cm, for the proper compaction and evacuation of air.

Figure 3.1 Bermuda Grass

3.2 EXPERIMENTAL SET UP

Three experiments were performed with fresh grass. Each set has three replicates and one control. Strict anaerobic conditions are required to start the ensiling process. In the 1st experimental set effect of molasses was studied. In this experiment three replicates with molasses application and one control were used, while each set contained 4 kg grass. In 2nd set of experiments the combined effect of molasses and urea was studied. Urea and molasses were applied together to 3 sets and one control with only molasses. Each set contained 1 kg of grass. The 3rd set of experiments were design to study the combined effect of urea, molasses and fermented juice of epiphytic lactic acid bacteria (mixed bacterial culture). 3 replicates with 1 kg of grass sample and the control has only urea and molasses were studied. All above sets of experiments were conducted in the different period of spring time.

Each set was wrapped in the triple layer of polythene bags, manually pressing to ensure the maximum removal of air entrapped among the particles. Each set was placed in the container to avoid breakage of polythene bags at room temperature (25-28 ˚C) for the period of 30 days.

3.3 PHYSICAL EXAMINATION OF GRASS / GRAB TEST

Grab test is a test used as field level examination to estimate the DM content of grasss. Grass sample is pressed in the hand. Chopped grass sample is taken in the hand and pressed with fingers. The texture of grass and extraction of juice by the fist after pressing gives the rough estimatiom of DM % and moisture %.

Table 3.1 Field test for DM % (Regan, 2003)

Amount of squeezing	**DM %**
Juice easily expressed by hand	<20
Juice expressed with some difficulty	20–25
Little or no juice expressed but hands moist	>25

This test is normally performed at farmer level but the accuracy of test depends on the experience of farmer or researcher. The test gives the rough estimation of DM and moisture content. This is easy, cost effective and less time consuming test.

Table 3.2 Field test for DM and Moisture % (Regan, 2003)

Amount of squeezing (Ball shape)	DM %
Ball retains its shape and some free juice expressed	<25
Ball retains its shape but no free juice is expressed	25–30
Ball slowly falls apart	30–40
Ball rapidly falls apart	>40

After pressing in the hand grass sample gets the shape of ball. Holding time of particles is observed, that gives the idea of moisture contents and DM of grass.

This test gives rough estimation about the forage for wilting or using as such (without welting). Wilting is process applied when the crops are harvested after the rain fall or silage making during rainy season when the moisture contents are too high.

3.4 ENSILING WITH DIFFERENT ADDITIVES

In this study 3 different types of additives have been used at laboratory scale silage production. Followings are the additives applied on the silage.

3.4.1 Molasses

Molasses dense solution was kindly supplied by Pakmaya, Pak Group. Molasses was diluted in warm distilled water in 1 ratio 2 and stirred on heating plate for 5 min for proper mixing. For each kg of fresh sample 30 ml of molasses solution was sprayed through a sprinkler.

3.4.2 Feed Grade Urea

15 grams of urea was dissolved in distilled water with final volume of 30 ml. Prepared solution was sprayed on grass. Sprinkling was done layer by layer on the fresh grass. For each kg of fresh grass 30 ml of urea solution was used.

3.4.3 Fermented Juice of epiphytic LAB

FJLB was prepared according to Bureenok et al., (2011) from 200 g fresh freshly harvested grass, which was macerated in 500 mL of sterilized distilled water with a blender. The juice was filtered through a double layer of cheesecloth; the filtrate was transferred to a glass bottle and 2% glucose was added, then it was incubated at 30 °C for 2 days. In this way stock solution was prepared and applied at the rate of 30 ml per kg fresh grass. The application was done by sprinkling on grass layer by layer for effective utilization of additive.

3.5 EXPERIMENTAL DESIGN

Three sets of experiments were design having 3 replicates each versus control.

Table 3.3 All experimental Sets

Parameters	Set 1	Set 2	Set 3	Control
Ensiling with Molasses				
Amount of grass (kg)	3	3	3	3
DM %	>40	>40	> 40	> 40
Moisture content %	< 60	< 60	< 60	< 60
Molasses ml / kg fresh grass sample	30	30	30	---
Ensiling With Feed Grade Urea and Molasses				
Amount of grass(kg)	1	1	1	.. 1
DM %	> 40	> 40	> 40	> 40
Moisture content %	< 60	< 60	< 60	< 60
Molasses ml / kg fresh grass sample	30	30	30	30
Urea soln ml / kg fresh grass sample	30	30	30	---
Ensiling with FJLB				
Amount of grass(kg)	1	1	1	1
DM %	> 40	> 40	> 40	> 40
Moisture content %	< 60	< 60	< 60	< 60
Molasses ml / kg fresh grass sample	30	30	30	30

Urea sol. ml/kg fresh grass sample	30	30	30	30
FJLB ml / kg fresh grass sample	30	30	30	---

3.6 SAMPLE PREPARATION

After 30 days of ensiling, 25 grams of sample is taken from 4 different places from each set. Sample was completely grinded. Macerated sample was put into the 100 ml of d mineralized water, and then centrifuged for 5-10 min for proper mixing (Sandra & Annette, 2010). Blend sample is filtered, and then used for different analysis, but fresh sample is used for the finding DM, moisture and ash contents.

3.7 ANALYTICAL PROCEDURE

pH was measured by digital pH meter. pH was measured pre and post ensiling. For the measurement of pH silage extract was used.

Moisture is evaporated from as such grass sample by oven drying. Total dry matter is determined gravimetrically as residue remaining after drying. Weighing may be made on hot sample or after cooling in desiccators. This procedure is used for determination of dry matter on forage samples or for dry weight determinations of fiber residues. The sample was put in oven for 3 hour at 105 C (AOAC, 1995).

It is used to find the moisture content of sample 5-10 grams of as such sample was taken.

The concentration of total nitrogen (N) was determined by the Total Kjeldahl Nitrogen procedure by Van-Soest *et al*. (1991).CP was calculated by multiplying N by the 6.25 conversion factor

The organic acids analysis was performed by HPLC (model of the instrument) according to Lı´vian et al (2011). The mobile phase consisted of H_2SO_4 0.005 mol/L solution. This solution was filtered through a 0.45 mm Millipore membrane and degassed by sonication for 10 min before use. Flow rate was 0.50 mL/min and injection volume was 20 μL. Organic acids (acetic and lactic acids) were analyzed by UV-Vis detection at 210 nm. The analytical column used was C18 at 55 ˚C under isocratic conditions.

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CHAPTER 4

EXPERIMENTAL RESULTS

Experimental studies for the investigation of potential usage of additives to increase the quality of silage were conducted in three groups of experiments. The first group of experiment was designed to examine molasses addition as water soluble carbohydrates to increase the production of fermentation end products such as acetate and lactate. The second group of experiment was conducted molasses together with urea to increase NPN (Non Protein Nitrogen), that will result in increased crude protein content of grass silage. The third group of experiment was performed molasses, urea together with fermented juice of lactic acid bacteria (FJLAB) to increase the formation of lactic acid. Experimental sets were examined pre-ensiling and at the end of 30 days of ensiling period for pH, moisture content, dry matter content, organic matter content as COD, crude protein content as TKN, acetic acid and lactic acid content of grass silage.

4.1 RESULTS OBTAINED FROM THE MOLASSES TREATMENT

30 ml of molasses solution having a COD value of 27 g/kg grass was applied in 3 replicates while 1 set kept as control. Below Table 4.1 shows the values of pH, DM %, moisture content %, organic matter as COD, crude protein as TKN and organic acids.

Table 4.1 Results with Molasses treatment on Grass Silage

Condition	pH	Moisture %	DM %	avg OM g/L silage extract By COD	CP % by TKN	acetic acid g / kg FM	Lactic acid g / kg FM	Effluent amount
Pre-ensiling	6.4	52	48	25.09	17.5	0.95	0.096	No
Control	4.52	56.6	43.4	38.2	14.3	11.05	4.57	No
Set 1	3.5	59.4	40.6	67.5	16.9	41.09	10.76	No
Set2	3.9	55.7	44.3	64.5	15.7	42.7	10.68	No
Set 3	4.1	54.7	45.3	58	13.1	30.59	7.74	No

A significant difference found among the parameters measured pre and post ensiling. From the Table 4.1, decline in ph, increment in the values of OM and organic acids was obseved, that is required for the preservation of grass and its quality.

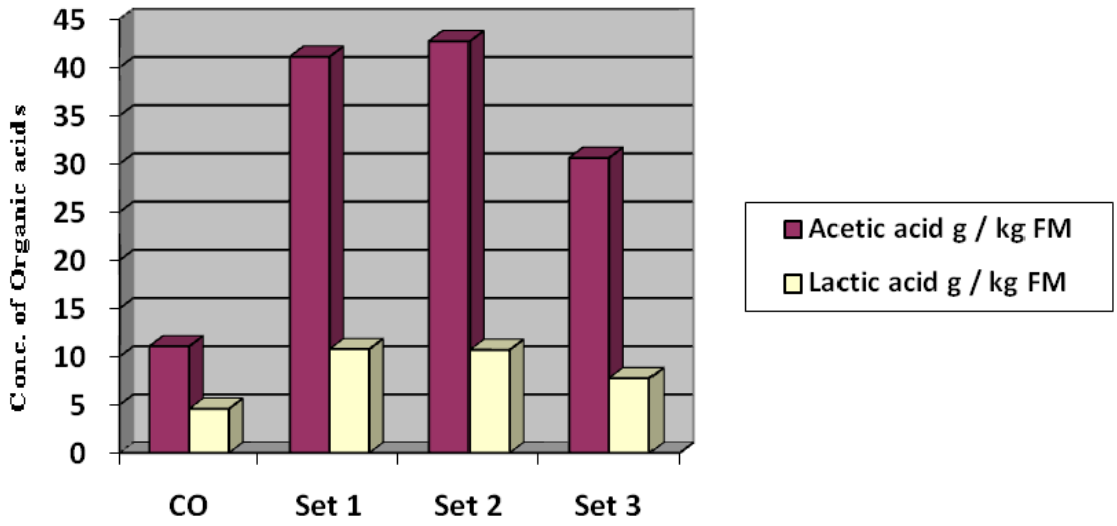

Figure 4.1 Organic acid contents of molasses treatment experiment

Organic acid measurements were presented in Figure 4.1. Apparently, control (without molasses) silage has very low amount of both acetic and lactic acid. In three replicates of molasses treatment resulted in an average acetic acid content of 38.1 g / kg FM silage and lactic acid content of 10 g / kg FM silage. Although a slight increase was observed in the lactic acid formation in post-ensiling process, molasses was mainly converted into acetic acid rather than lactic acid, which results in an increased concentration from 1.05 g / kg FM to 38.1 g / kg FM. This could be explained by the fact that proper anaerobic conditions could not be maintained in the plastic bags due to insufficient compression of the grass mass, resulting incomplete fermentation.

Acetic acid was found low concentration in control whereas high concentration with molasses tratment as shown in the Figure 4.1. For ideal conditions the lactic acid concenttration should be high. Therefore in other sets of experiments more additives were applied to have higher level of lactic Light yellow color with smell of fermentation was observed in the replicates with molasses treatment but control has the pungent smell.

Figure 4.2 Control Set, Grass Silage without molasses

Figure 4.3 Set 1, Molasses treated Grass Silage

Figure 4.4 Set 2 Molasses treated Grass Silage

Figure 4.5 Set 3 Molasses treated Grass Silage

Acetic and lactic acids were measured with HPLC, below figures show the standard curves for acetic and lactic acids.

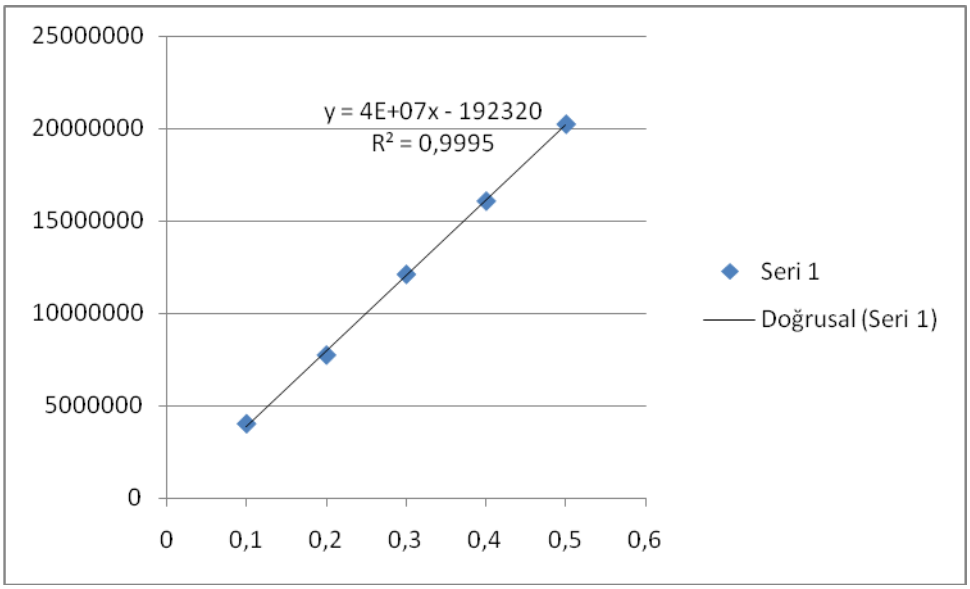

Figure.4.6 Standard curve for acetic acid by HPLC

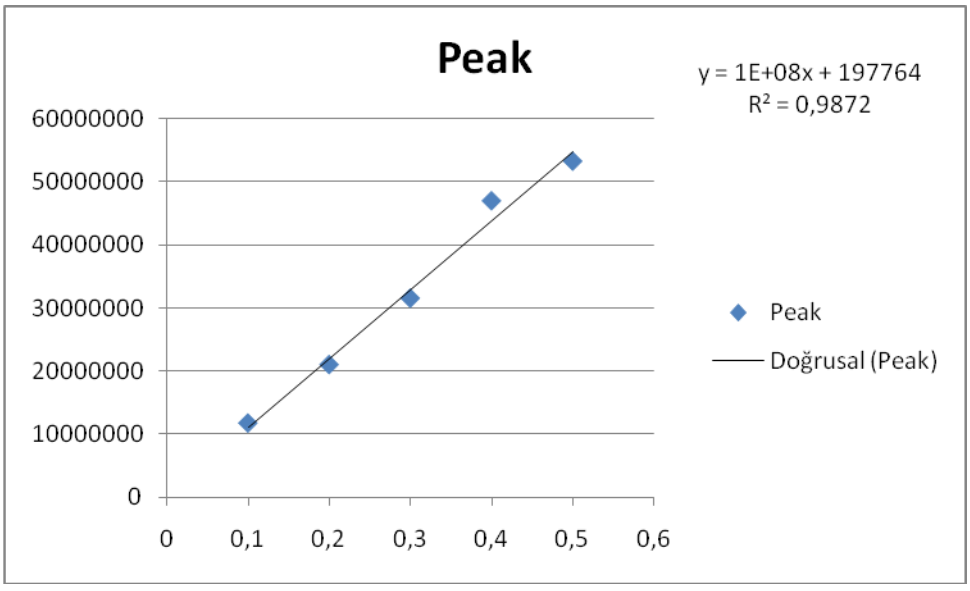

Figure 4.7 Standard curve of lactic acid by HPLC

The decline in pH values inhibit the spoilage microorganism proliferation, which allows the silage nutritive values to be preserved. Thus, the best silage forages are the ones with high concentration of soluble carbohydrates contents, which should be sufficient to promote the fermentation and produce enough organic acids to preserve the silage. According to Ferreira (2002), the minimum soluble carbohydrates contents recommended to ensure adequate fermentation for good silage, varies between 6% and 12% of the dry mass. Since the soluble sugar level is adequate, dry mass contents higher than 25% are sufficient to ensure a good silage production. In our samples pre and post ensiling the DM % remained more than 25 %, as it requires for good quality silage. There was no effluent found in both molasses treated silage and control.

4.2 RESULTS OBTAINED FROM MOLASSES AND UREA TREATMENT

During ensiling period urea decomposes to ammonia by urease therefore, decrease in pH level is delayed because of alkaline character of ammonia. From the below table 4.2 it is observed that addition of urea to silages causes increase in lactic, acetic and total organic acid as compared to control.

Ruminant animals have got such a capacity that they can effectively utilize the non-protein nitrogen in their rumen by ruminal microflora. They can convert that nitrogen into microbial protein, that is the one of the source of protein for these animals.

By adding feed grade urea in the silage that slows down the reduction in ph but it is effective in many ways like increase in CP and COD values.

By adding feed grade urea and molasses in the silage samples have resulted in the better production of organic acids like acetic and lactic acid as shown in the table 4.2 and Figure 4.6. The increase in the production of these acids is the key indicator for the better preservation quality and aerobic stability.

Table. 4.2 Results obtained from molasses and urea on grass silage with control

Condition	pH	Moisture %	DM %	avg OM g/L silage extract By COD	CP % by TKN	acetic acid g / kg FM	Lactic acid g / kg FM	Effluent amount
Pre-ensiling	6.4	52	48	25.09	17.5	0.95	0.096	No
Control	3.9	59.8	40.2	68.6	15.4	42.81	14.4	No
Set 1	3.6	63.5	36.5	84.2	12.1	50.73	16.59	No
Set2	4.1	66.1	33.9	79.9	16.4	45.12	15.86	No
Set 3	3.4	61.8	38.2	101.6	17	54.39	44.46	No

In the replicates feed grade urea and molasses were applied together, results have shown in the above table 4.2.As in the previous experiment CP value was decreased from 17 to 13, for now CP is not deceased but remained stable. CP value ranged between 16.3-17.5 % of the Bermuda grass (Deborah, 2008).

During process of fermentation crude protein of grass degrades, urea application makes it stable. Other parameters OM, moisture content were also improved. Light yellow color with smell of urea was observed in the replicate with urea and molasses treatment.

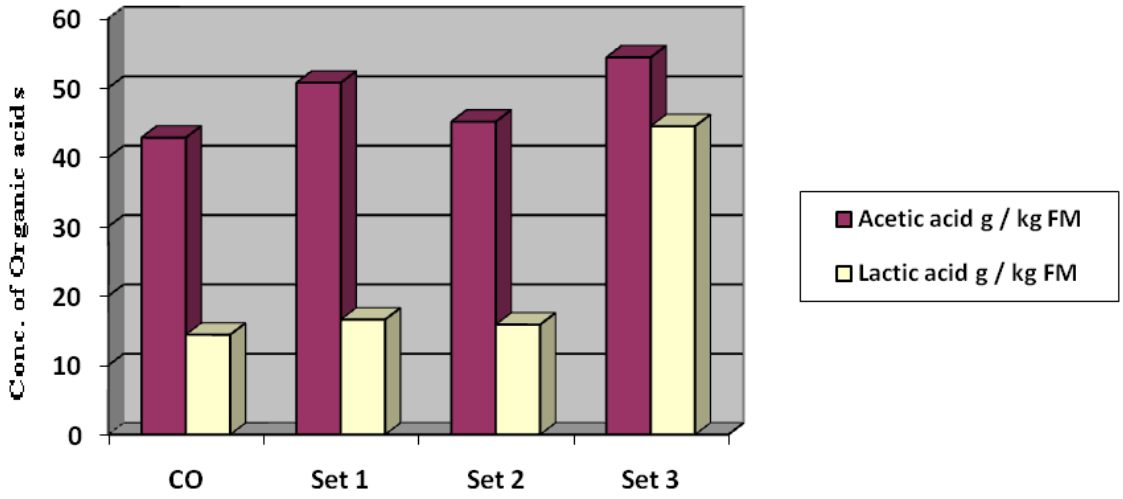

Figure 4.8 Organic acid contents of molasses and urea treatment experiment

Figure 4.9 Control, Grass Silage treated with only molasses

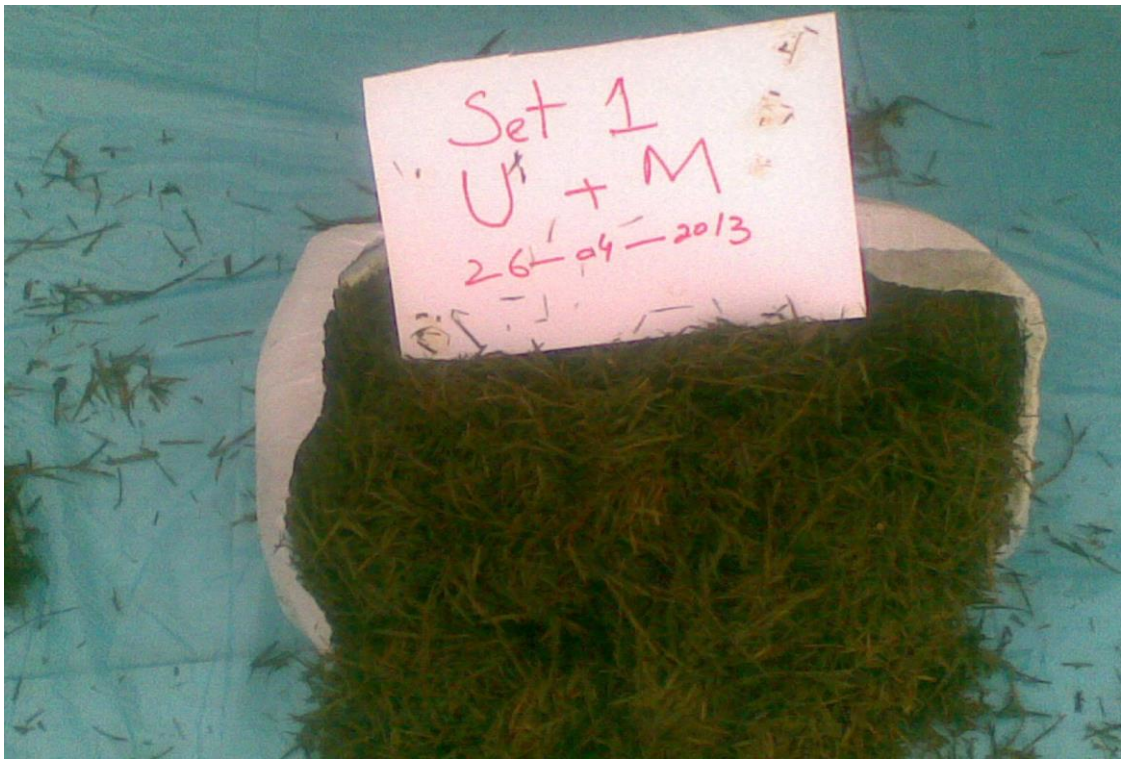

Figure 4.10 Set 1 Grass Silage treated with urea and molasses

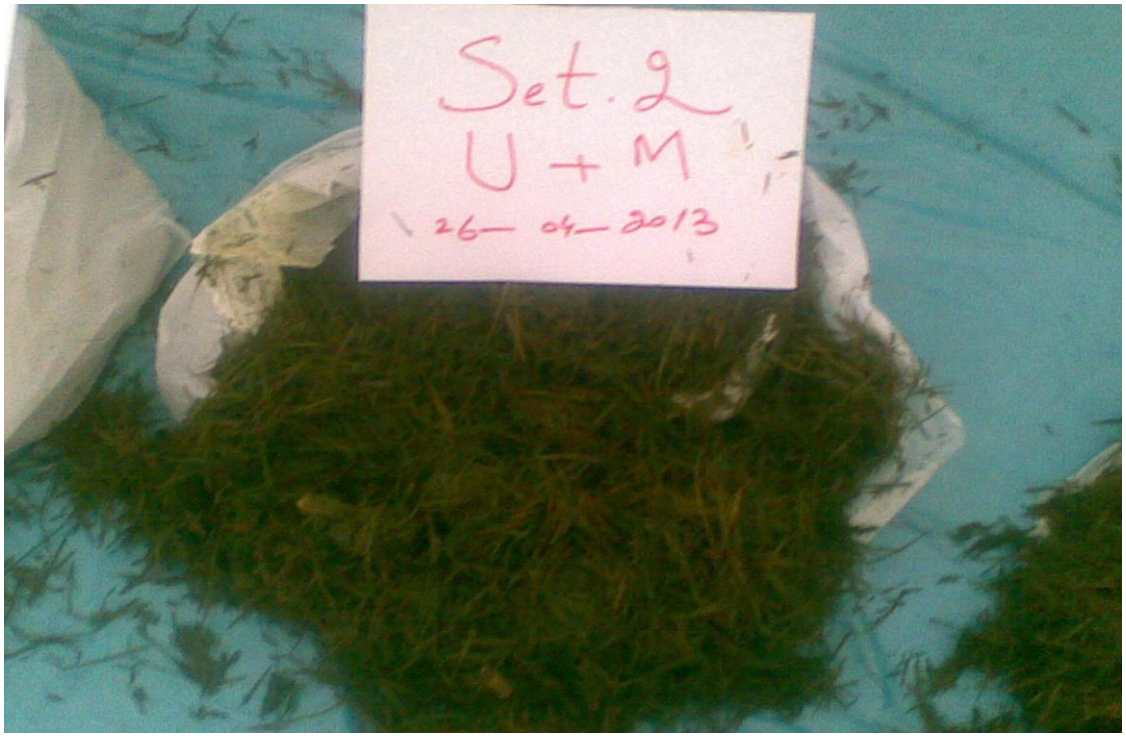

Figure 4.11 Set 2 Grass Silage treated with urea and molasses

Figure 4.12 Set 3 Grass Silage treated with urea and molasses

4.3 RESULTS OBTAINED FROM TREATMENT OF UREA, MOLASSES AND FJLB

Successful silage requires epiphytic lactic acid bacteria (LAB) and water soluble carbohydrate (WSC) to produce sufficient lactic acid for rapid pH reduction (Bureenok et al., 2011). The fermented juice of epiphytic lactic acid bacteria (FJLB) has been used as a silage additive for tropical grass silage. This solution / juice act as a mixed bacterial culture with dominating numbers of lactic acid bacteria. From below table 4.3 shows that, maximum amount of lactic acid was found in these sets of experiments .

Table 4.3 Results obtain from collective use of urea, molasses and FJLB

Condition	pH	Moisture %	DM %	avg OM g/L silage extract By COD	CP % by TKN	acetic acid g / kg FM	Lactic acid g / kg FM	Effluent amount ml
Pre-ensiling	6.5	55.5	44.5	27.86	15.3	0.95	0.096	NO
Control	4.2	64.2	57.5	38.2	15.7	30.6	10.7	NO
Set 1	4	67.5	32.5	89	16.5	49.9	29.6	NO
Set2	3.9	65.9	34.1	93.4	15.7	54.2	23.7	NO
Set 3	3.8	63	37	90.4	16.1	41.7	30.7	NO

The fermented juice of epiphytic lactic acid bacteria (FJLB) has been used as a silage additive for grass silage. This solution / juice act as a mixed bacterial culture with dominating numbers of lactic acid bacteria.

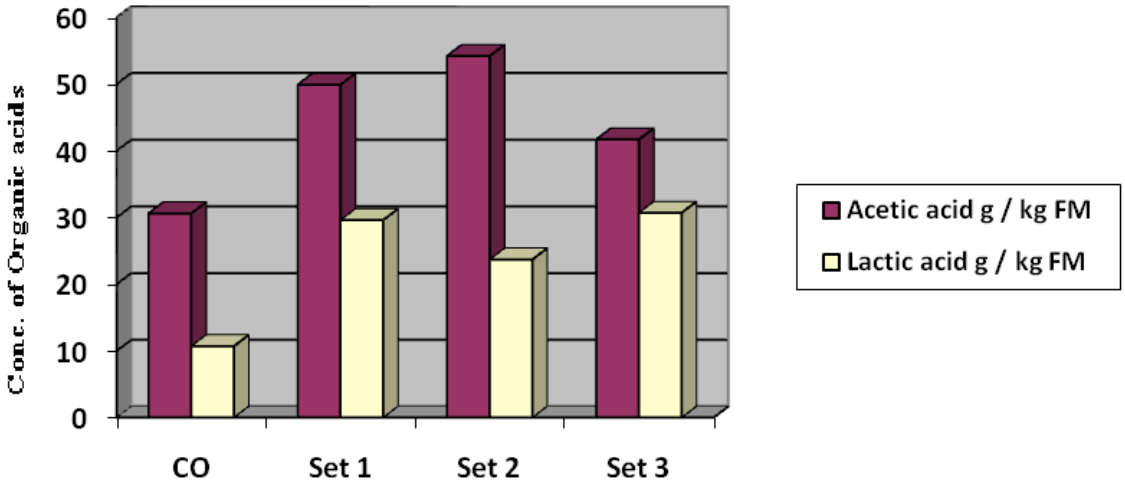

Figure 4.13 Organic acid contents of molasses, urea and FJLB treatment experiment

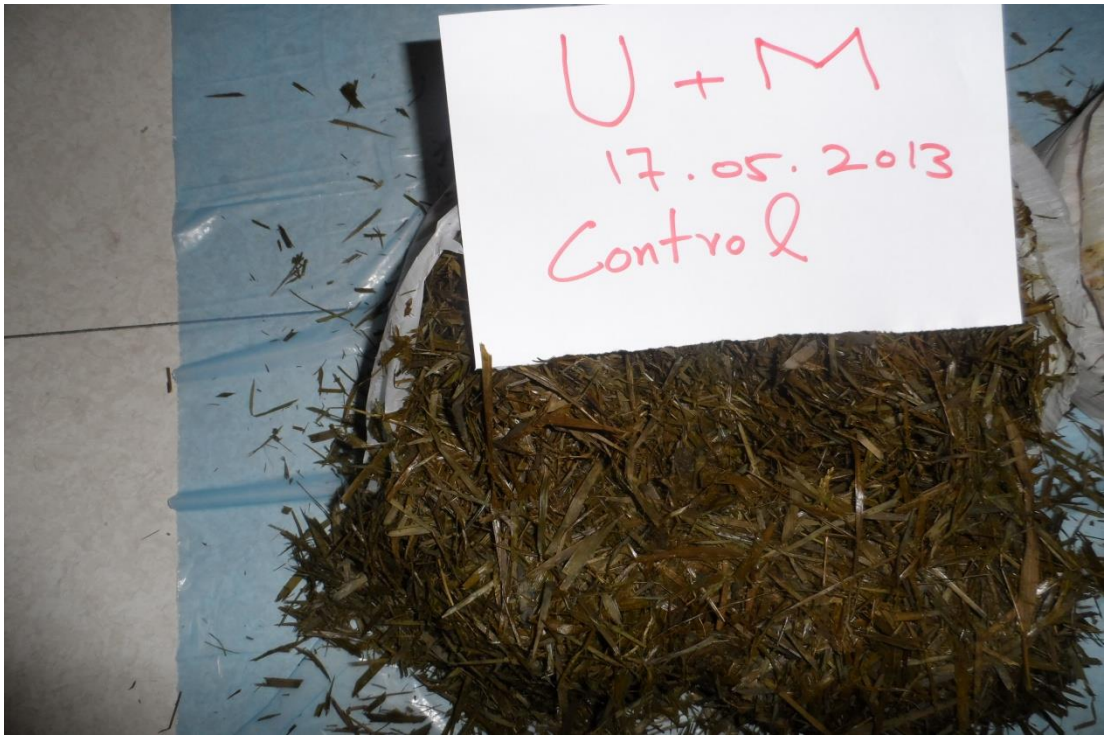

Figure 4.14 control set Grass Silage treated with urea and molasses

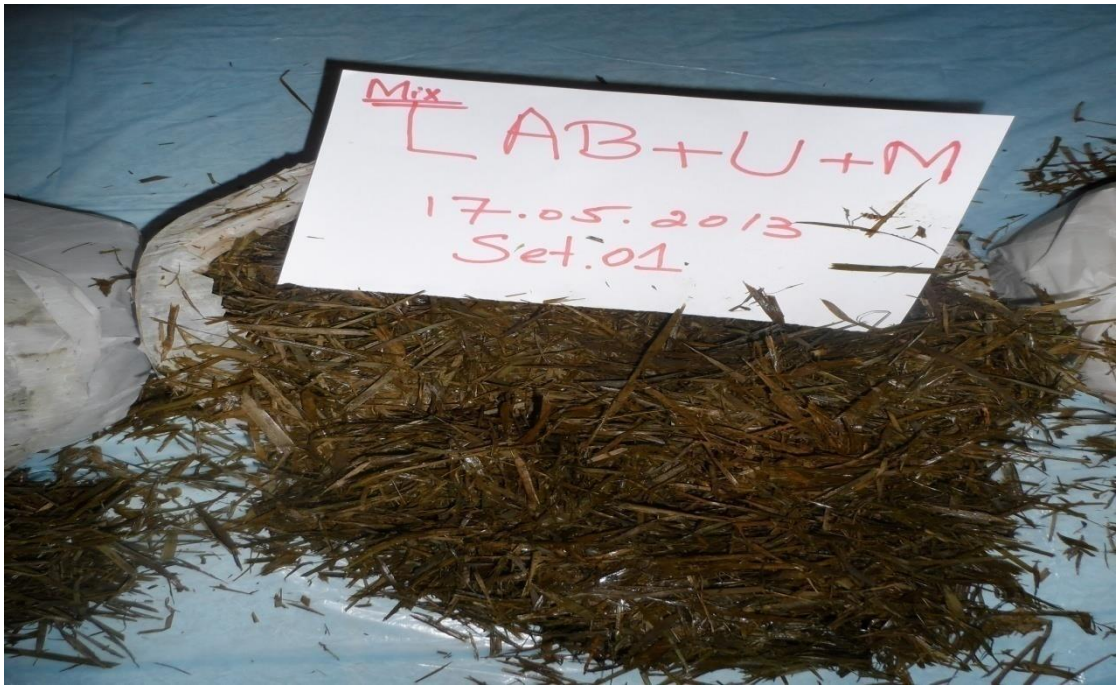

Figure 4.15 Set 1 Grass Silage treated of with urea, molasses and FJLB

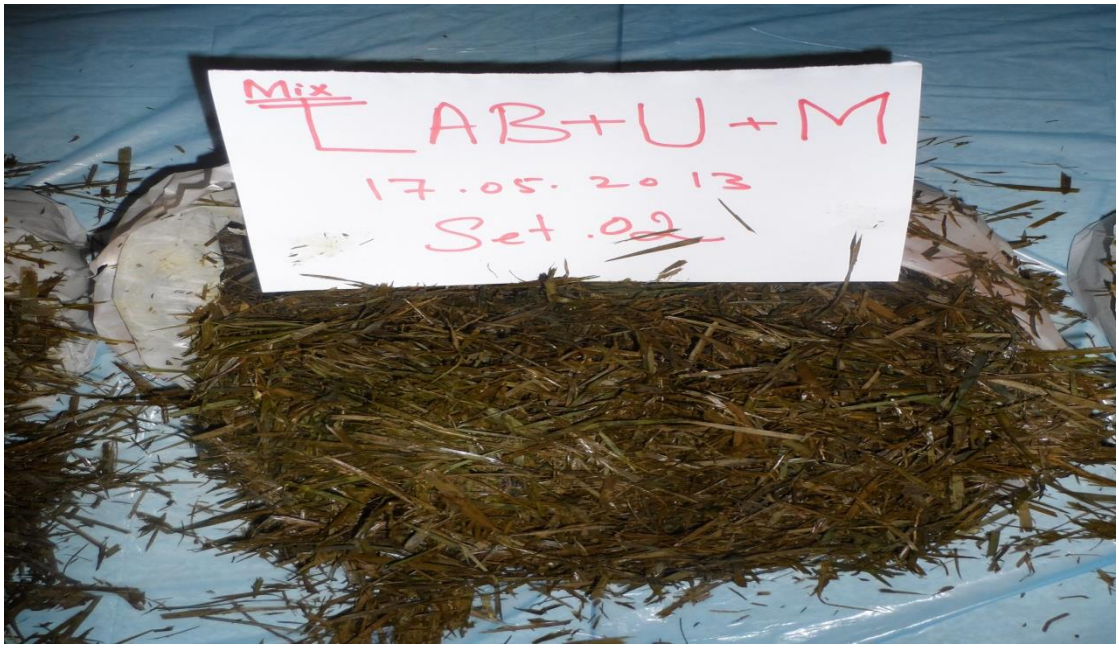

Figure 4.16 Set 2 Grass Silage treated with urea, molasses and FJLB

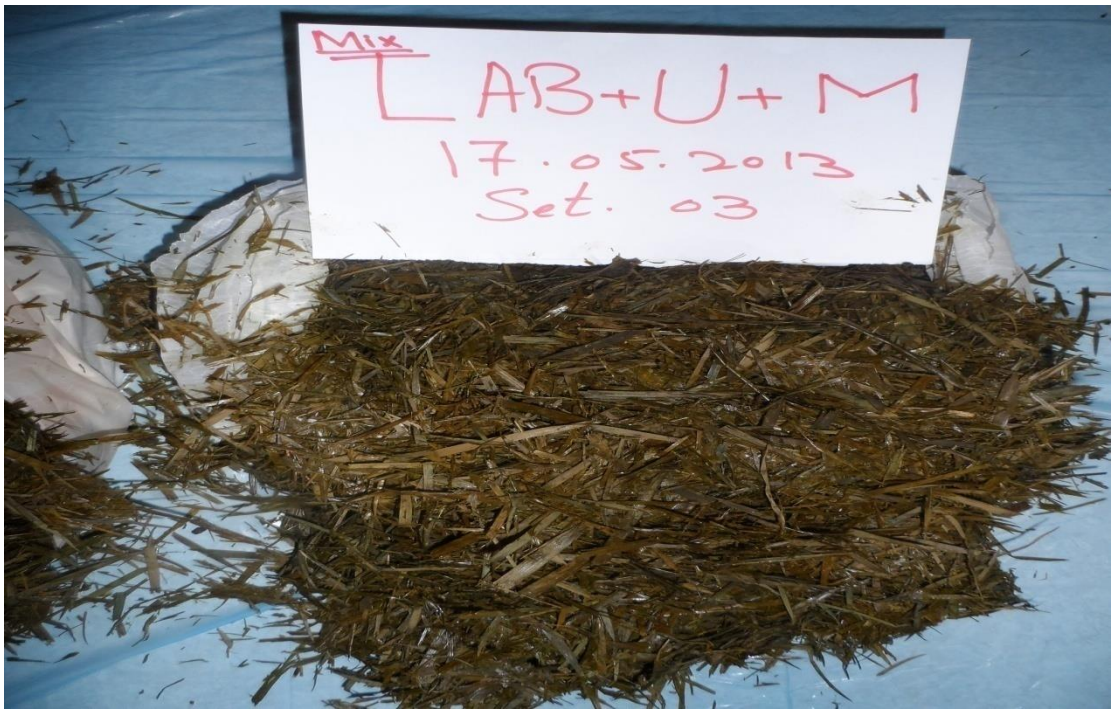

Figure 4.17 Set 3 Grass Silage treated with urea, molasses and FJLB

4.4 COMPARISON OF ABOVE 3 EXPERIMENTS

In the below Figure 4.18, the gradual increase in the average values of acetic and lactic acids have been observed. Therefore, simultaneouly application of these 3 additives showed the best results.

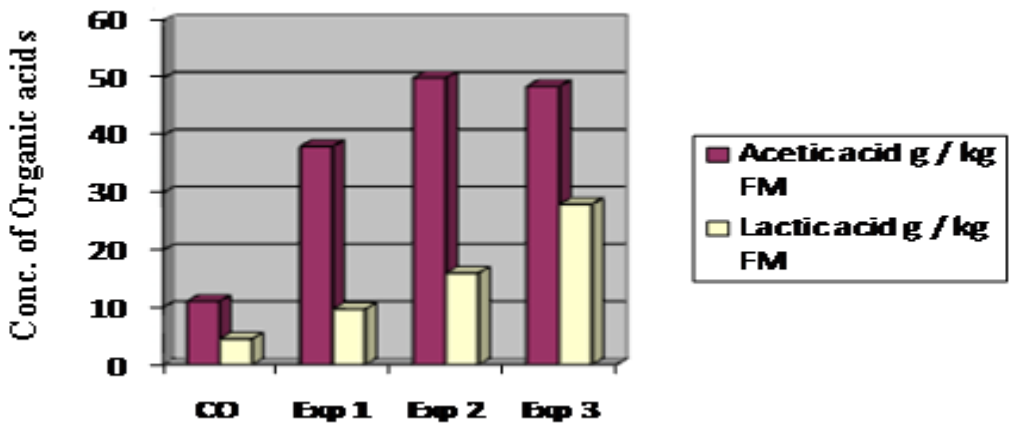

Figure 4.18 Organic acid contents of molasses, urea and FJLB treatment experiments (1,2 & 3).

Three groups of experiments were conduct in such a way that the effect of molasses, urea and FJLB were evaluated one by one while comparing with the control sets. The results showed that the control groups produced an unstable fermentation with low values of organic acids and TKN values. The gradual increase in the values of acetic and lactic acids have been observed in this study, however the values of acetic acid remained higher than the lactic acid. The possible reason for the higher value of acetic acid than lactic acid, could be because of improper evacuation of entrapped air while ensiling. The declined in ph values in more pronounced in the replicates as compare to control that is needed to stop the growth of clostridial species. The addition of FJLB has showed the maximum value of lactic acid that is needed for the better preservation and higher production yield (growth, maintenance and reproduction) of the ruminants.

CHAPTER 5

CONCLUSION

This study examined the effects of urea, molasses FJLB on fermentation dynamics and silage quality of Bermuda grass, which was ensiled in laboratory silos for 30 days at the room temperature (25-28 C˚) temperature. Three groups of experiments were conducted in such a way that the effect of molasses, urea and FJLB were evaluated one by one while comparing with the control sets. The results showed that the control groups produced an unstable fermentation with low values of organic acids and TKN values. The gradual increase in the values of acetic and lactic acids have been observed in this study, however the values of acetic acid remained higher than the lactic acid. The possible reason for the higher value of acetic acid than lactic acid, could be because of improper evacuation of entrapped air while ensiling. The reason could be the nutrient profile of land from where the grass has been collected. The declined in ph values in more pronounced in the replicates as compare to control, that is needed to stop the growth of clostridial species. The addition of FJLB has showed the maximum value of lactic acid that is needed for the better preservation and higher production yield (growth, maintenance and reproduction) of the ruminants.

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