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Bacteriological Studies of the Thermoduric Anaerobic and Non-thermoduric Anaerobic Population of Alfalfa Silage Prepared with the Addition of Preservatives

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BACTERIOLOGICAL STUDIES OF THE THERMODURIC ANAEROBIC AND NON-THERMODURIC ANAEROBIC POPULATION OF ALFALFA SILAGE PREPARED WITH THE ADDITION OF PRESERVATIVES

BY

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This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Grass silage is an important factor in the livestock industry because it furnishes a high quality succulent feed at a low cost. As it is known today, silage can be made from nearly any grass, legume or cereal crop. The legumes are particularly valuable because they produce a silage which has a high nutritive value.

The retention of plant nutrients, such as carotene and protein, is of utmost concern in the production of silage. The preservation of these forage nutrients is brought about by the fermentation reaction within the silage mass. The lactic acid bacteria, that are found on the green forage, convert the available plant carbohydrates into lactic acid. The major problem is to convert the available carbohydrates to lactic acid as soon as possible after ensiling the forage. The formation of other acids by microorganisms will result in the production of poor quality silage. Numerous investigations have been made in order to better understand the silage fermentation processes. The knowledge that is gained from these studies may be used to improve silage quality.

The purpose of this study is to evaluate some of the observations made when alfalfa is ensiled with various preservatives. The anaerobic microbiological population and biochemical properties of the silage are of particular interest.

REVIEW OF LITERATURE

The role of the microorganisms in the production of silage can be better understood by studying the reactions that occur within the silage mass. These reactions constitute the process known as silage fermentation.

Silage fermentation is the result of a series of microbiological and chemical processes. According to Morrison (32), the living plant cells that remain in the silage after ensiling continued to respire. The oxygen that was entrapped in the silage mass was rapidly used up and carbon dioxide was given off. Within five hours after ensiling, practically all the free oxygen had disappeared. The absence of oxygen prevented the development of molds. The acid-forming bacteria multiplied rapidly in the silage. These bacteria attack the sugars, some of the starches and pentosans in the green forage. This results in the production of organic acids, primarily lactic acid, some acetic acid and traces of other acids. Alcohol, usually ethyl alcohol, was also formed. The production of the acids was one of the most important processes in silage fermentation. The acidity that was produced prevented the growth of the undesirable bacteria that would have caused putrefaction of the silage. When enough acid was formed, the process was checked, and the fermentation finally ceased. If air does not gain entrance into the silage it will keep for a long period of time with little change.

Bacteriological Population of the Fresh Forage

The microorganisms that are found on the green forage prior to ensiling play an important role in the silage fermentation process. Thomas (40) stated that numerous different species of bacteria were found on the surface of the green forage. Studies by Allen *et al*. (3) also concluded that a large variety of organisms comprised the microflora of green plants. Thermophilic organism numbered from 1,660 to 2,070 per gram of forage. Aerobic sporeformers numbered from 250 to 2,400 per gram. The coliforms were found in numbers varying from 1,000 to 1,000,000 per gram. The lactobacilli varied from 1,000 to 10,000,000 per gram. Yeasts and molds on the green forage numbered up to 25,300 per gram of forage. The mixed aerobic microflora numbered from 1,630 to 42,800,000 per gram. *Lactobacillus plantarum* was the predominant species of lactobacilli isolated. The coliforms were identified as *Aerobacter aerogenes* var. *graminis*. Kroulik *et al*. (24) identified the coliforms they had isolated as belonging to the species *Aerobacter cloacae*.

Allen *et al*. (3) found small numbers of sporeforming obligate anaerobes on fresh forage. They numbered from 10 to 100 per gram. The predominant species present was identified as *Clostridium sporogenes*. Langston *et al*. (27) found less than 100 anaerobic spores per gram of fresh orchard grass. Studies by Martos (31) indicated that the original chopped fodder contained not more than 50 to 100 anaerobes per gram of forage.

Species of streptococci, micrococci and the Actinomyces were also isolated from the green forage. Kroulik et al. (24) reported that the predominating organisms on the green plants prior to ensiling were, aerobic, chromogenic, nonsporeforming, rod-shaped bacteria. The coliforms comprised a large portion of the microflora. From the washings of whole leaves of alfalfa, timothy and clover, Thomas (39) obtained eight different isolates. All were yellow pigmented species of bacteria.

Bacteriological Population During Fermentation

Following the ensiling of the green forage two microbiological processes take place within the silage mass. These two processes are known as the aerobic process and the anaerobic process.

The aerobic process, described by Bender et al. (6) started when the fodder was first placed in the silo. The plant cells continued to respire using the air that had been entrapped in the silage mass, forming carbon dioxide and water. Until the oxygen of the air had been used up, the aerobic organisms including the yeasts and molds continued to multiply. The aerobic period usually took place within one to eight days after ensiling. This period of time was reported by Allen et al. (3) as the lag phase. The lag phase is the time taken by the respiration of the plant cells and bacteria to use up the available oxygen. When the silage mass became anaerobic, the aerobic organisms, although unable to reproduce were still able to function as enzyme systems. These enzyme systems were able to form alcohol and various other metabolic end-products.

During the aerobic process a large variety of organisms are present in the silage mass. Studies by Burkey et al. (11) indicated that the bacterial flora of the forage when it was first placed in the silo consisted predominantly of chromogenic, pleomorphic and coliform bacteria. Most of the coliforms were of the Aerobacter species, although some were similar to Escherichia coli. Allen et al. (3) identified the coliforms as belonging to the Aerobacter aerogenes var. graminis group. The coliforms as well as the other aerobic organisms usually disappeared within two to five days after ensiling. These organisms were of little importance in the stored silage, even though they did attain numbers up to 1,000,000 per gram. Studies on the multiplication of bacteria in silage by Stirling (38) indicated that there was a rise in the total count of bacteria a few days after ensiling. The peak was reached in about two days. This period of rapid increase was followed by a gradual decline in numbers.

Unpigmented gram positive types such as the streptococci and lactobacilli, replaced the pigmented types that were predominant on the fresh forage (38). Streptococci, micrococci, and motile lactobacilli, were found by Cunningham and Smith (14) in recently ensiled forage prepared by the A.I.V.[1] method. Strains of Lactobacillus plantarum, Lactobacillus brevis, Streptococcus lactis, Leuconostoc

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[1]This is a method of silage preservation whereby a mixture of equal quantities of hydrochloric and sulfuric acid in a 2N solution is added to the fodder as it is ensiled. Bender, C.B., and Bosshardt, D.K., "Grass Silage: A Critical Review of Literature", J. Dairy Sci., 22: 637.

mesenteroides, and unidentified strains of micrococci and sarcinae were isolated from A.I.V. silage. Langston et al. (27) stated that the high acid producing lactobacilli usually do not become predominant until the cocci have reached high numbers and produce considerable amounts of acid. The cocci isolated were identified by Langston et al. (25) as belonging to the genera Streptococcus, Leuconostoc and Pediococcus. The streptococci were identified as Streptococcus faecalis, and Streptococcus liquefaciens. Two strains of Leuconostoc were identified as Leuconostoc mesenteriodes, and a variable strain designated as Leuconostoc type I. The strains of the Pedicoccus were not identified. Burkey et al. (11) also isolated pedicocci and streptococci from silage following the disappearance of the anaerobes. The pediococci were described as non-proteolytic diplococci that were similar in characteristics to the genus Pediococcus. He stated that the streptococci were identified as Streptococcus liquefaciens and several other unidentified species. Langston et al. (26) reported that after the cocci had reached high numbers and had produced considerable amounts of acid, the high acid producing lactobacilli became predominant. The lactobacilli that occurred most frequently were Lactobacillus brevis, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus arabinosus, and variable strains of Lactobacillus brevis and Lactobacillus casei. Allen et al. (1) and Burkey et al. (11) stated that the majority of the lactobacilli present were similar to or were strains of Lactobacillus plantarum. Burkey et al. (11) also isolated a number of un-named lancet (knife-shaped) or "diplo" short-rod forms.

tyrobutyricum. Burkey et al. (11) isolated a lactate-utilizing bacterium which he also identified as Clostridium tyrobutyricum, and an actively proteolytic bacterium similar to Clostridium sporogenes. Allen et al. (2) divided the anaerobic sporeforming bacteria that they isolated from 28 silage samples, into three groups: (a) Clostridium sporogenes, (b) Clostridium welchii, and (c) Clostridium butyricum. They also reported that the number of anaerobic spores remained small for the first few days after ensiling, and increased to approximately 1,000,000 per gram after 10 to 15 days. This was followed by a leveling off, but in some cases they increased to about 100,000,000 per gram for the next few weeks, and then slowly declined. The organisms that were most commonly isolated were classified as Clostridium sporogenes. Bryant et al. (10) reported that the spores of the anaerobic bacteria increased in numbers from 10,000 per gram at eight days after ensiling to 2,000,000 per gram at 33 days after ensiling. Langston et al. (27) reported that an increase in the number of spores of anaerobes did not begin until two days after ensiling in the poor quality silages and five and eight days in the intermediate quality silages. Following this a steady increase was obtained throughout the fermentation period.

Silage Quality

A standard criteria for the classification of silage on the basis of various biochemical and bacteriological properties was proposed as a method of evaluating the quality of silage. Langston et al. (27) classified silage into three groups on the basis of

quality. These groups were: Good, Intermediate, and Poor. This classification was based on pH, ammonia nitrogen, lactic acid, butyric acid and spore counts. Bacteriologically, the same general pattern of increase and decrease in numbers of bacteria was similar throughout the fermentation period in both the Good and Poor quality silages (31). Langston et al. (27) stated that, "The primary difference in quality of these silages can be correlated well with the increase of spore-forming anaerobes." Langston's (27) criteria for classifying silage is as follows:

Good Quality Silage:

(1) A pH from 3.9 to 4.8.

(2) Low ammonia nitrogen content, from 1.02 to 2.87 per cent.

(3) Traces of butyric acid.

(4) Spore count erratic, most samples none.

(5) Considerable amounts of lactic acid, from 3.03 to 13.16 per cent.

Poor Quality Silage:

(1) A pH from 5.2 to 5.7.

(2) Ammonia nitrogen content from 3.23 to 9.82 per cent.

(3) Spore counts high.

(4) Lactic acid content increased, then declined accompanied by a corresponding increase in the butyric acid content.

The Intermediate quality silage had characteristics that were between the Good and Poor quality silages.

Kempton et al. (22) stated that, butyric acid production in spoiled silages was associated with lactate-fermenting clostridia.

Colonies of bacteria isolated from spoiled silage resembled Clostridium tyrobutyricum. If the silage was going to spoil, the vegetative cells of the lactate-fermenting clostridia were detected within a few days after ensiling (22).

The pH of silage is an important factor in its production and preservation. The optimum pH for silage preservation described by Bender et al. (6) was between 3.5 and 4.0. A pH of 4.0 or below completely inhibited the harmful bacterial action which gave rise to butyric acid formation and protein breakdown. Studies by Marton (31) indicated that the presence of the anaerobic lactate-fermenting spore-forming bacteria in the silage caused a reduction in the acidity of the silage. This reduction in acidity permitted the growth of the spoilage organisms. The numbers of spoilage organisms increased until a pH of approximately 4.0 was reached. At a pH of 4.0 or below only the lactobacilli were capable of growing (4). At a pH of 4.5 to 5.2 the number of spores ranged from thousands to several hundred thousand per gram (4). Archibald (5) stated that, butyric acid does not develop to any extent unless the silage has a pH above 5.0.

Another factor related to silage quality is the content of fermentable carbohydrates in the silage. Bender et al. (6) stated that the low carbohydrate content of the silage can cause the production of poor quality silage. The production of good quality silage, therefore will depend upon the early conversion of available carbohydrates in the forage into lactic acid. Wilson et al. (41) stated that fermentable carbohydrates were necessary for the production of

the organic acids, which were responsible for the preservation of the silage.

Silage Preservation

The production of good silage will depend upon maintaining several biochemical and bacteriological properties that are characteristic of good quality silage.

Several factors, therefore will have to be considered: (a) The pH must be maintained at a low level, approximately 3.5 to 4.0 (6). (b) The spore counts will have to be decreased. This would involve decreasing the number of sporeforming anaerobes without inhibiting the development of the beneficial organisms, such as the lactic acid organisms. (c) The production of butyric acid must be retarded by lowering the pH (5,6) and by inhibiting the growth of the sporeforming anaerobes (22). (d) The supply of available carbohydrates may be increased so that the carbohydrates can be converted into lactic acid (6,41).

Using the previously mentioned factors as a guide for the production of good quality silage various methods of silage preservation have been used.

The addition of various acids to the forage is a popular method of silage preservation. The A.I.V. method will lower the pH of the silage to 3.6 to 4.0 (6). The use of phosphoric acid will lower the pH of the silage to 3.9 to 4.9 (21).

The addition of fermentable carbohydrates was another method used for the preservation of silage. Carbohydrates such as molasses

and whey have been used as silage preservatives (6).

Other methods of silage preservations have been used. Replacing the air in the silo with sulfur dioxide resulted in the pH of the silage being maintained at a level of 4.04 to 5.79 (23). Sulfur dioxide also promoted a rapid growth of lactobacilli and inhibited the growth of other bacteria (23). The use of chemical sterilizers such as formaldehyde and formic acid, and inoculating the silage with cultures of lactic acid forming bacteria as a method of silage preservation have been used. The results obtained did not warrant the future use of these methods (6).

In recent years the antibiotic principle has been used as a means of silage preservation. "Silotracin" (zinc bacitracin) is a silage preservative in a powdered form. It is designed to preserve silage by inhibiting the bacteria that are responsible for producing poor silage and encouraging the bacteria that are responsible for the desirable fermentation reactions (13). Studies by Rusoff et al. (36) using "Silotracin" as a preservative for grass silage indicated that silage treated with "Silotracin" was of good color, pleasing odor and had a pH below 4.5. The control (untreated silage) had a darker color, sharper odor and a pH of 5.4. "EN-SIL-AC", another commercial product in powdered form, has been used as a silage preservative. This product uses the principle of adding desirable cultures to the forage as it is ensiled. "EN-SIL-AC" is composed of a culture of Aspergillus oryzae and Torula dried yeast, as well as added nutrients.

EXPERIMENTAL PROCEDURE

By the addition of various preservatives further information was obtained concerning the effect of the preservatives on the anaerobic microbiological populations and biochemical properties of silage.

In this study 14 different samples were prepared. These samples were designated A through P omitting F and H. Samples A and G received no treatment and served as the controls. One preservative treatment was used in each of the remaining samples. Table I shows how each of the samples was prepared using the various preservatives.

Forage Preparation

First cutting alfalfa used for this study was cut just prior to the blossoming stage. It was wilted in the field for one and one-half days, chopped and brought into the laboratory where the preservatives were added.

Because this first group of samples with low moisture was so uniform in the physical and chemical characteristics, pH, color, and odor, a second group of silage samples was prepared. The forage used in the second series was third cutting alfalfa. The chopped forage was not wilted but was brought directly to the laboratory where the various preservatives were added.

Preparation of Silage Samples

Samples from the low moisture forage were prepared by packing the forage mixed with respective preservatives into separate pint and

TABLE I. RATES OF APPLICATION OF PRESERVATIVES PER POUND OF SILAGE

Sample	Preservative	Rate of Application per pound of Silage[a] (grams)
A	None	0
B	EN-SIL-AC[b]	2.0
C	Silotracin[c]	1.5
D	EN-SIL-AC-	2.0
	Silotracin	1.5
E	Silotracin	1.5
	Ground corn[d]	25.0
	Molasses[e]	12.5
G	None	0
I	Dry ice[f]	2.0
J	Lactose[g]	12.5
K	Silotracin	1.5
L	EN-SIL-AC	2.0
M	Ground corn	25.0
N	Molasses	12.5
O	Silotracin	1.5
	Ground corn	25.0
	Molasses	12.5

TABLE I. (Continued)

Sample	Preservative	Rate of Application per pound of Silage[a] (grams)
P	EN-SIL-AC	2.0
	Ground corn	25.0
	Molasses	12.5

a Rate of application according to the manufacturer's recommendation.

b EN-SIL-AC: A commercial preservative manufactured by Dr. Mac Donald's Vitamized Feed Co., Ft. Dodge, Iowa.

c Silotracin: A commercial preservative manufactured by the Commercial Solvents Corporation, New York, N.Y.

d Ground corn: R.J. Baker, and H.H. Voelker, "Preservatives for Alfalfa Silage," (Abstract) J. Dairy Sci., 41: 734, 1958.

e Molasses: A.J. Barnett, Silage Fermentation, Academic Press Inc., New York, 1954.

f Dry ice: H.H. Voelker, (Personal Communication).

g Lactose: A.J. Barnett, Silage Fermentation, Academic Press Inc., New York, 1954.

one-half gallon fruit jars. Samples representing each day of the first week of the fermentation period were packed into pint jars. Samples representing the last five weeks of the fermentation period were packed into the one-half gallon jars. Weekly samples from high moisture forage were prepared by packing the forage with the respective preservative into separate one-half gallon fruit jars.

The jars had been sterilized by steaming to prevent contamination of the forage. After packing, the jars were labeled and "escape valve"[2] lids were attached. The jars were stored at room temperature until time for sampling. The sampling period lasted 42 days. Samples prepared from low moisture forage were taken daily for the first week and then weekly for the remainder of the fermentation period. Samples prepared from high moisture forage were taken weekly throughout the fermentation period.

In the following experiment two groups of organisms were studied. The first group consisted of those silage organisms isolated from roll tubes incubated at 32° C. This group will be called the non-thermoduric anaerobes. The second group of organisms consisted of those silage organisms which were able to survive heating for 20 minutes at 80° C., and were isolated from roll tubes incubated at 32° C.

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[2]This was a device inserted into the lid of the sample jars that would allow any gas that would accumulate to escape from the jars. This device was made by drilling a hole into a metal fruit jar lid. A length of one fourth inch rubber tubing was inserted into the hole. Into the rubber tubing a length of glass tube was inserted. Another piece of rubber tubing with a small longitudinal slit in it was attached to the upper end of the glass tube. A short piece of solid glass rod was inserted into the open end of the rubber tube.

This group will be called the thermoduric anaerobes. The purpose of the heat treatment was to destroy the vegetative cells so that only the sporeforming anaerobes would be present. For the purpose of clarification these two groups will be called: (1) the non-thermoduric anaerobes, those organisms that did not receive the heat treatment, and (2) the thermoduric anaerobes, those organisms that survived the heat treatment.

Preliminary Analysis

A preliminary analysis was conducted to obtain information concerning the moisture content, pH, thermoduric anaerobic and non-thermoduric anaerobic population of the fresh forage prior to ensiling.

To determine the moisture content of the low moisture forage at the time it was packed into the jars, a 50 gram sample of fresh forage was dried to a constant weight in an oven at 85° C. The moisture content of the high moisture forage was determined by the Oil Distillation Process (12).

Preparation of Samples for Analysis

To demonstrate the effect of various preservatives on the thermoduric anaerobic and non-thermoduric anaerobic population of the silage, bacterial counts were made throughout the fermentation period.

The preparation of the silage samples was the same for both groups of organisms. An 11 gram sample was weighed out and placed into a Waring blendor jar containing 99 ml. of sterile phosphate buffered

distilled water. To control the speed of mixing the blendor was attached to a variable transformer with the voltage adjusted to 20 to 30 volts. The sample was mixed for a period of five minutes. After mixing, the sample was transferred to a sterile four ounce screw-cap jar. This sample constituted the initial 1:10 dilution, (11 grams of silage per 99 ml. of phosphate buffered distilled water). From this initial dilution successive dilutions were prepared in order to obtain thirty to three hundred colonies per tube (37).

Preparation of Dilutions

The following dilutions were used for each group of organisms:

(1) Non-thermoduric Anaerobes: Dilutions of 1:10,000, 1:100,000, and 1:1,000,000 were used for the first three days of the fermentation period. For the remainder of the fermentation period dilutions of 1:1,000,000 and 1:10,000,000 were used.

(2) Thermoduric Anaerobes: Dilutions of 1:100 and 1:1,000 were used throughout the fermentation period. These dilutions were heated in a water bath for 20 minutes at 80° C.

Preparation of Media

The media used for the thermoduric anaerobic and non-thermoduric anaerobic counts are described by Langston et al. (27). The media were prepared in the manner similar to that used by Hungate (17). In this study nitrogen gas was used to flush out oxygen that gained entrance into the media.

The non-thermoduric anaerobic medium was prepared by mixing

the ingredients and diluting the mixture to the desired amount with distilled water. The medium was boiled to dissolve the agar and a 0.0001 per cent resazurin solution was then added. The dissolved medium was dispensed into heavy-wall Pyrex glass Erlenmeyer flasks. The contents of each flask were heated to boiling to drive off the dissolved oxygen. A solution of cysteine-HCl was added as nitrogen was passed into each flask through a straight Pasteur pipette, connected to the nitrogen source by a rubber tube. The pH of the medium was adjusted to 7.2. As the Pasteur pipette was withdrawn from the flask a rubber stopper was inserted into the flask. The stoppers were wired in place to prevent them from blowing out during sterilization. The medium was sterilized in the autoclave for 20 minutes at 15 pounds pressure. After sterilization the medium was cooled in a water bath to a temperature of 45° to 50° C. The wire was cut and as the stoppers were removed nitrogen was passed into the flasks to prevent the entrance of oxygen. A sterile solution of sodium carbonate, that had previously been flushed with nitrogen to remove all traces of oxygen, was added to each flask. The medium was then ready for use in preparing the non-thermoduric anaerobic culture tubes. The only difference in preparing the thermoduric anaerobic medium was that it contained soluble starch in addition to the other ingredients.

Preparation of Culture Tubes

The procedure of Hungate (17) modified by Bryant et al. (8) was used to prepare the culture tubes. Culture tubes 20 X 175 mm., that had been previously sterilized without stoppers were used for

preparing roll tubes. The culture tubes were stoppered with sterile rubber stoppers immediately upon their removal from the autoclave.

The anaerobic procedure involved the use of gaseous nitrogen to displace any oxygen that had gained entrance into the media or roll tubes. While high purity nitrogen was used, the precaution of scrubbing out any oxygen by passing the nitrogen through heated copper shavings packed into a Pyrex glass column, was used. The effluent end of the column containing the copper shavings was connected to a glass manifold. The glass manifold contained six outlets and attached to each outlet was a Pasteur pipette. The gas would then pass out through the Pasteur pipettes into the culture tubes or flasks containing the media. The purpose of the glass manifold was to facilitate the preparation of several dilutions in a series.

Two methods of preparing culture tubes were used. One method involved adding the desired dilution of inoculum to empty, sterile culture tubes as nitrogen was passed into the tubes. After the addition of the desired dilution of inoculum, approximately seven ml. of culture media were added. The culture media were held at a temperature of 45° to 50° C. by keeping the flasks containing the media in a hot water bath. The flasks containing the culture media were continuously flushed with nitrogen to exclude the entrance of oxygen.

The second method of preparing culture tubes involved flushing empty, sterile culture tubes with nitrogen and then adding approximately seven ml. of culture media. The tubes were then stoppered with sterile rubber stoppers and stored in the 45° C. incubator. The

desired dilution of the inoculum was added later.

In both methods of culture tube preparation, after the addition of the desired dilution of inoculum or the culture media, a rubber stopper was inserted into the culture tube as the Pasteur pipette was withdrawn. A roll tube was prepared in a manner similar to the procedure described by Hungate (17), with the exception that as the tube was rolled, an even film of agar covered the entire inside surface of the tube.

Roll tubes of both the thermoduric anaerobes and non-thermoduric anaerobes were incubated at 32° C. for three days. After the incubation period the roll tubes were removed from the incubator, colony counts were made and the tubes were stored in the refrigerator for future studies.

Further studies were made only on colonies isolated from those roll tubes that were prepared from the low moisture forage. The analysis of the high moisture silage consisted only of colony counts of the thermoduric anaerobes and non-thermoduric anaerobes at weekly intervals throughout the fermentation period.

Procedures Used for the Classification of Isolated Strains

Colonies selected from roll tubes were transferred into agar deeps of Trypticase Soy Agar (9) and incubated for three days at 32° C. At the end of the incubation period the type of growth, with relation to oxygen, was noted. Those tubes showing aerobic growth were discarded. Gram stains were made from colonies showing anaerobic growth. All cultures that were not contaminated were selected for use

in further studies.

To insure the purity of each culture several passages were made through Trypticase Soy Agar (9) deeps. Gram stains were made of each successive passage. After the final passage a gram stain was made as a criterion in classifying each culture on the basis of its morphology and reaction to the gram stain.

Further studies were initiated, to group the isolated organisms on the basis of common characteristics. These studies included: the gram stain, morphology, carbohydrate utilization, reaction in litmus milk, gelatin liquefaction, hydrogen sulfide production, nitrate reduction, and indole production. The following additional tests were used for the non-thermoduric anaerobes: utilization of ammonium phosphate, hydrolysis of starch, hydrolysis of urea, and growth in 4.0 and 6.5 per cent sodium chloride. For the thermoduric anaerobes the following additional tests were used: utilization of arabinose, fermentation of lactate and digestion of Brain Heart Infusion Agar.

The following methods and materials were used for the culture studies:

(1) Gram stain: The procedure described in the Manual of Pure Culture Study (29) was used to ascertain the morphology of both groups of organisms and their reaction to the gram stain.

(2) Motility: The motility of the organisms that were isolated from the non-thermoduric anaerobic roll tubes, was determined from a deep of Cystine Trypticase Agar Base (33). The motility of the thermoduric anaerobes was determined from a deep of Trypticase

Agar Base (33).

(3) Carbohydrate utilization: The carbohydrate utilization reactions of the non-thermoduric anaerobes were determined from a deep of Cystine Trypticase Agar (33) medium plus the desired carbohydrate. Trypticase Agar Base (33) medium plus the desired carbohydrate was used for the thermoduric anaerobes.

(4) Reaction in Litmus Milk: The Litmus Milk was prepared by the method described in the Manual of Microbiological Methods (30). The procedure used for determining the reactions in Litmus Milk was the procedure described in Products for the Microbiological Laboratory (33). This procedure was used for both groups of organisms.

(5) Gelatin liquefaction: Gelatin liquefaction for both groups was determined from a Nutrient Gelatin (16) deep, using the procedure described in the Manual of Pure Culture Study (29).

(6) Hydrogen sulfide production: The production of hydrogen sulfide for both groups of organisms was determined from a deep of Trypticase Lactose Iron Agar (33).

(7) Indole production and nitrate reduction: The reduction of nitrate to nitrite and the production of indole was determined by inoculating separate tubes of Indole-Nitrate medium (33). The reagents described in the Manual of Pure Culture Study (29) were used to test for indole production and nitrate reduction.

(8) Hydrolysis of starch: The hydrolysis of starch was determined from a Starch Agar (16) deep and tested for the hydrolysis of starch with iodine (29).

(9) Growth in salt solutions: The growth in 4.0 and 6.5 per cent sodium chloride was determined by inoculating tubes of Nutrient Broth (16) containing 4.0 and 6.5 per cent sodium chloride and observing for growth.

(10) Fermentation of lactate: The ability of the thermoduric anaerobes to ferment lactate was determined by a rise in pH of the medium. The medium and procedure described by Bryant et al. (9) was used.

(11) Digestion of Brain Heart Infusion Agar: The digestion and blackening was determined from deeps of Brain Heart Infusion Agar (16).

(12) Hydrolysis of urea: The hydrolysis of urea was detected by a change in the color of inoculated Urea Broth (16) from yellow to red.

RESULTS AND DISCUSSION

Analysis of silage samples consisted of determining the effect of the various preservatives on the bacteriological populations and biochemical properties of the silage samples. This analysis consisted of pH determination, non-thermoduric anaerobic and thermoduric anaerobic counts of silage samples at the various stages of the fermentation. The organisms that were isolated from the different silage samples, prepared from the low moisture forage, were tentatively identified. Identification was based on cultural, morphological, and physiological characteristics.

Preliminary Analysis of the Forage

The preliminary analysis of the forage consisted of moisture determination, non-thermoduric anaerobic, thermoduric anaerobic counts, and pH determination of the forage prior to ensiling. The results of the preliminary analyses are given in Table II.

TABLE II. BACTERIOLOGICAL AND BIOCHEMICAL STUDIES OF FORAGE PRIOR TO ENSILING

Sample	Moisture %	pH	Anaerobes per gram of Forage	
			Non-Thermoduric (000)	Thermoduric (000)
Fresh Forage*	58.8	6.08	26	< 1
Fresh Forage	81.0	6.08	81	< 1

* Wilted

The moisture content of the wilted (low moisture) forage was lower and the moisture content of the non-wilted (high moisture) forage was higher, than the recommended levels. Forage that is to be ensiled should have a moisture content of approximately 70 per cent (11, 19, 20, 35, 42).

The low number of anaerobes per gram of fresh forage appeared to be normal, as a large number of thermoduric anaerobes or non-thermoduric anaerobes would not be present because of the lack of anaerobic conditions on the fresh forage. Allen et al (3), Langston et al. (27) and Martos (31) reported not more than 100 spores or spore-forming anaerobes per gram of fresh forage.

The pH of both the high and low moisture forage was the same. This would indicate that the pH of fresh forage is not affected by the time of cutting (first, or third crop) or moisture content.

Bacteriological Analysis of Low Moisture Silage

The data on the bacterial counts and pH for the various silage samples for the fermentation period are given in Table V, Appendix I.

When comparing the number of non-thermoduric anaerobes for each sample it was noted that there was a general pattern in the number of anaerobes throughout the fermentation period. This general pattern consisted of three phases of increases and decreases in the number of non-thermoduric anaerobes. First there was an initial increase the first few days after ensiling with the maximum number being attained during the first week of the fermentation period. Figure 1 shows the day during the fermentation period when the maximum number of

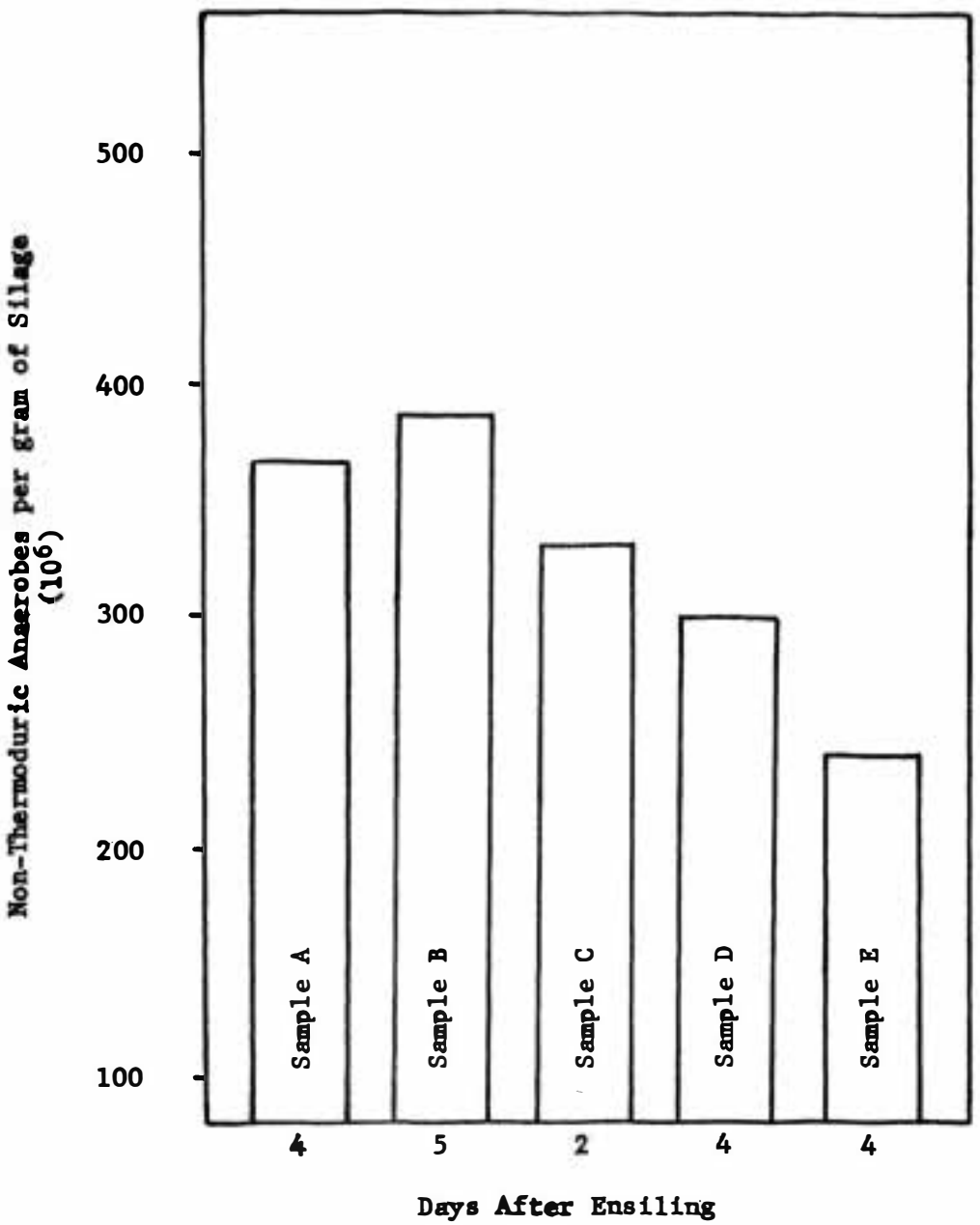

Figure 1. Maximum Number of Non-Thermoduric Anaerobes From Each Silage Sample Prepared From Low Moisture Forage

non-thermoduric anaerobes was attained.

The non-thermoduric anaerobes do not reach a peak in numbers until after the silage mass has become anaerobic, which usually takes from one to eight days after ensiling. This period is known as the lag phase, and has been described by Allen et al. (3), as that time taken by plant and bacterial respiration to utilize the available oxygen that remained in the silage mass.

This initial increase was followed by a decrease in numbers until the end of the first week in all samples except B, in which the decrease was reached at the beginning of the second week. A second increase in numbers began at 14 days, except for samples B and E, in which the second increase took place at the beginning of the third week. This was followed by a second decrease the fourth week after ensiling, in all samples except E where there was an increase in numbers. At the fifth week of the fermentation period, a third increase in numbers took place except for sample E, where there was a decrease in numbers.

After the initial increase in numbers of non-thermoduric anaerobes, the successive increases, for the majority of samples, were lower than the preceding increase. There was a general decline in numbers after the initial increase.

When comparing the number of thermoduric anaerobes throughout the fermentation period it was noted that there was no general pattern of increases and decreases in the number of organisms. In only sample A was there a rapid initial increase in numbers the first week after

ensiling. The maximum number of thermoduric anaerobes in samples A, B and E were reached during the first week after ensiling. The day of the fermentation period when the maximum number was attained can be seen in figure 2. The peaks in numbers for samples C and D were reached the fourth week of the fermentation period. This pattern in numbers appeared to follow the normal trend. Allen et al. (2) stated that the number of anaerobic spores remained small for the first few days after ensiling but increased for 10 or 15 days, in some cases there was an increase in numbers for the next few weeks.

Tentative Classification of Isolated Strains

Non-thermoduric anaerobic and thermoduric anaerobic organisms, for tentative classification, were isolated from roll tubes representing each sampling period. Bergey's Manual of Determinative Bacteriology (7) was used for the tentative classification.

The non-thermoduric anaerobes studied were gram positive cocci, facultative, anaerobic, and arranged in pairs and short chains. At the end of the study 14 cultures of non-thermoduric anaerobes were tentatively classified. Table III lists all the characteristics for each isolate classified. Eight strains had characteristics similar to Streptococcus lactis. Five strains were identified as having characteristics similar to Streptococcus cremoris. The strains that were tentatively classified as Streptococcus lactis were differentiated from Streptococcus cremoris by their ability to grow in 4.0 per cent sodium chloride. One strain was classified as Streptococcus faecalis by its ability to grow

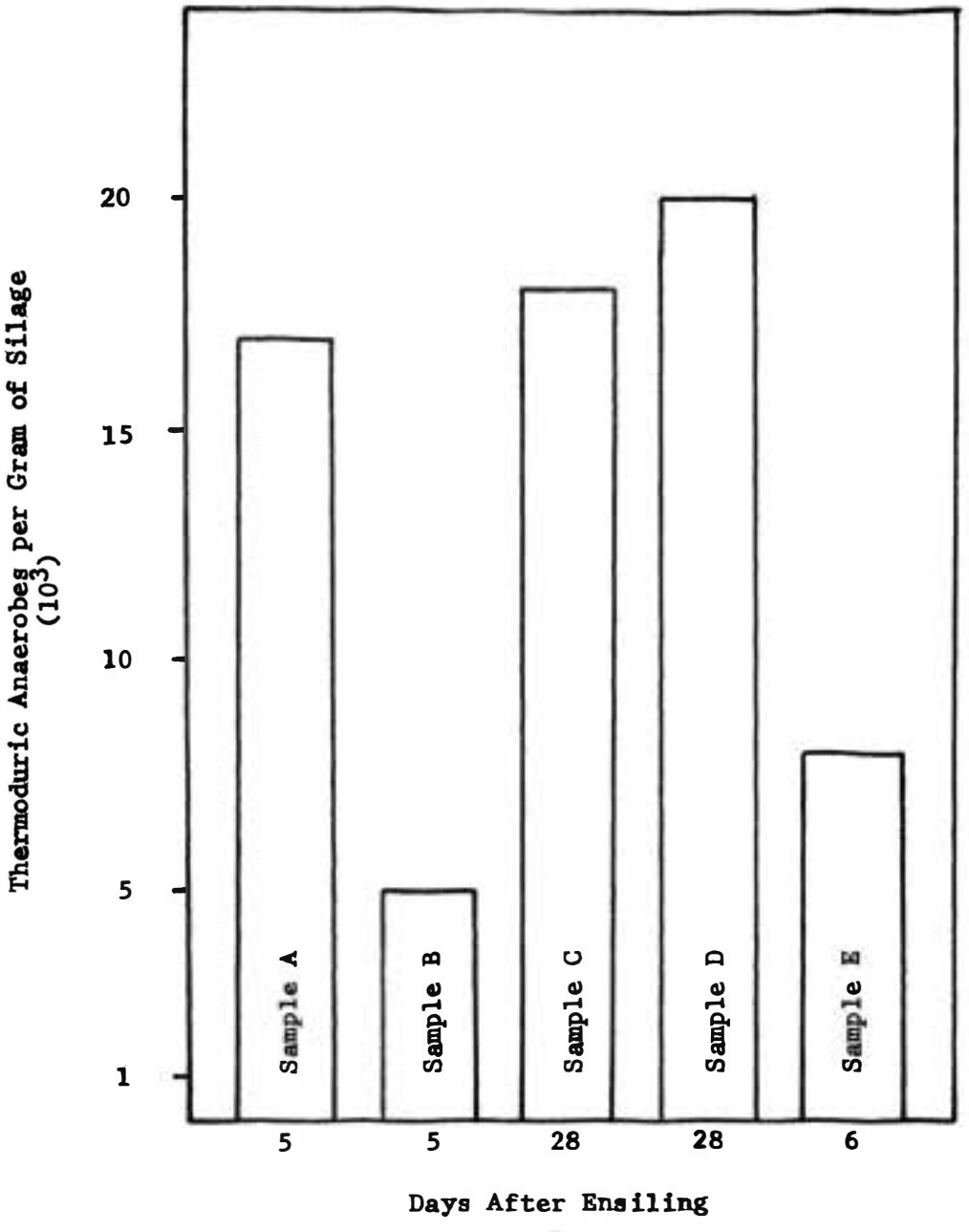

Figure 2. Maximum Number of Thermoduric Anaerobes From Each Silage Sample Prepared From Low Moisture Forage

TABLE III. SOME CULTURAL, MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF NON-THERMODURIC ANAEROBIC STRAINS ISOLATED FROM GRASS SILAGE PREPARED FROM LOW MOISTURE FORAGE*

Strain	Growth	Motility	Dextrose	Lactose	Sucrose	Maltose	Sorbitol	Xylose	Galactose	Raffinose	Mannitol	Gelatin	Lit. Milk	H_2S Prod.	NO_3 Red.	$NH_4H_2PO_4$	Indole	Urea	Starch	4.0% NaCl	6.5% NaCl	Classification
1F	F	-	A	A	A	A	-	A	A	-	A	-	R	-	+	-	-	-	-	+	-	S. lactis
2F	F	-	A	A	A	A	-	A	A	-	A	-	R	-	-	-	-	-	-	-	-	S. cremoris
D1	F	-	A	A	A	A	A	A	A	-	A	-	R	-	-	-	-	-	-	+	-	S. lactis
E2	F	-	A	A	A	A	-	A	A	-	A	-	R	-	-	-	-	-	-	-	-	S. cremoris
D3	F	-	A	A	A	A	A	A	A	-	A	-	R	-	+	-	-	-	-	-	-	S. cremoris
C4	F	-	A	A	A	A	A	A	A	A	A	-	R	-	+	-	-	-	-	+	-	S. lactis
D4	F	-	A	A	A	A	A	-	A	-	A	-	R	-	+	-	-	-	-	+	-	S. lactis
B5	F	-	A	A	A	A	-	A	A	A	A	-	R	-	+	-	-	-	-	+	-	S. lactis
C7	F	-	A	A	-	A	-	A	A	-	A	-	R**	-	+	+	-	-	-	+	+	S. faecalis
B14	F	-	A	A	A	A	A	A	A	-	A	-	R	-	+	-	-	-	-	-	-	S. cremoris

TABLE III. (Continued)

Strain	Growth	Motility	Dextrose	Lactose	Sucrose	Maltose	Sorbitol	Xylose	Galactose	Raffinose	Mannitol	Gelatin	Lit. Milk	H_2S Prod.	NO_3 Red.	$NH_4H_2PO_4$	Indole	Urea	Starch	4.0% NaCl	6.5% NaCl	Classification
A35	F	-	A	A	A	A	A	A	A	-	A	-	R	-	+	-	-	-	-	-	-	S. cremoris
C35	F	-	A	A	A	A	A	A	A	A	A	-	R	-	+	-	-	-	-	+	-	S. lactis
A42	F	-	A	A	A	A	A	A	A	-	A	-	R	-	+	-	-	-	-	+	-	S. lactis
C42	F	-	A	A	A	A	A	A	A	-	A	-	R	-	+	-	-	-	-	+	-	S. lactis

* All strains isolated were gram positive non-pigmented cocci, arranged singly, in pairs, and chains.

** Reduction with the production of acid and gas.

Key:

F Facultative
\- Negative
\+ Positive
A Acid
R Reduction

in 6.5 per cent sodium chloride, to utilize ammonium phosphate, and to produce acid and gas in litmus milk.

A total of 15 strains of thermoduric anaerobes were tentatively identified at the completion of the study. Three strains of spore-forming anaerobes, Clostridium sporogenes, Clostridium tyrobutyricum and an unknown Clostridium strain, that were obtained from Dr. Langston, were used for comparison with the unknown strains isolated from the roll tubes.

The results of the tentative classification of the thermoduric anaerobes are given in Table IV. All strains were gram positive rods, occurring most frequently singly or as pairs, some however were found in short chains.

One strain was tentatively identified as Clostridium sporogenes on the basis of its ability to liquefy gelatin. This strain was differentiated from Clostridium perfringens (welchii), because it lacked the ability to produce acid from lactose. The other isolated strains of thermoduric anaerobes had the ability to ferment calcium lactate. This ability was detected by a rise in the pH of the inoculated medium. A rise in pH from 6.68 to between 7.59 and 8.42 was detected.

Bergey's Manual of Determinative Bacteriology (7) differentiates Clostridium tyrobutyricum from the other Clostridium species by its ability to ferment calcium lactate. Bryant et al. (9) however, showed that strains of Clostridium butyricum also have the ability to ferment calcium lactate. Other characteristics of strains isolated by Bryant et al. (9) were similar to strains isolated in this study, but did not

TABLE IV. SOME CULTURAL, MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THERMODURIC ANAEROBIC STRAINS ISOLATED FROM GRASS SILAGE PREPARED FROM LOW MOISTURE FORAGE*

Strain	Growth	Spore	Dextrose	Lactose	Sucrose	Maltose	Sorbitol	Xylose	Galactose	Raffinose	Mannitol	Gelatin	Lit. Milk	H_2S Prod.	Arabinose	Lactate	Indole	NO_3 Red.	Brain Med.	Classification**
C1	F	-	A	A	A	A	S	-	A	-	A	-	R	-	A	+	-	+	-	
C2	F	C	A	-	A	A	-	-	A	-	A	-	R	-	A	+	-	+	-	
C3	F	C	A	-	A	A	-	-	A	-	A	-	R	-	A	+	-	+	-	
B5	O	C	S	S	A	A	-	S	S	-	A	-	-	-	A	+	-	+	-	
C5	M	-	A	-	A	A	-	-	S	-	A	-	-	-	A	+	-	+	-	
D6	M	-	A	-	A	A	-	A	-	-	A	-	-	-	A	+	-	+	-	
E6	M	C	A	-	A	A	-	S	-	-	A	-	-	-	A	+	-	+	-	
E7	M	C	A	-	A	A	-	A	-	-	A	-	-	-	A	+	-	+	-	
B14	M	C	A	-	A	A	-	S	A	-	A	-	-	-	A	+	-	+	-	
B21	F	C	A	-	A	A	-	S	S	-	A	-	-	-	A	+	-	+	-	
B28	F	C	A	-	A	A	-	S	S	-	A	+	S	-	A	+	-	+	-	Clostridium sporogenes
C28	M	C	A	-	A	-	-	-	-	-	-	-	-	-	A	+	-	+	-	
B35	M	C	A	-	A	A	-	-	S	-	A	-	-	-	A	+	-	+	-	

TABLE IV. (Continued)

Strain	Growth	Spore	Dextrose	Lactose	Sucrose	Maltose	Sorbitol	Xylose	Galactose	Raffinose	Mannitol	Gelatin	Lit. Milk	H_2S Prod.	Arabinose	Lactate	Indole	NO_3 Red.	Brain Med.	Classification**
A42	M	-	A	-	A	A	-	-	-	-	A	-	-	-	A	+	-	+	-	
C42	F	C	A	S	A	A	-	-	A	-	A	-	S	-	A	+	-	+	-	
AKn	F	Cl	A	A	-	-	-	A	A	-	-	-	-	-	A	-	-	+	-	
BKn	O	C	-	-	-	-	-	-	-	-	-	-	-	-	A	-	+	-	-	
CKn	F	C	A	-	-	A	-	A	A	-	-	-	-	-	A	-	-	+	-	

* All Strains isolated were non-motile, gram positive rods, arranged singly in pairs or chains.

** All strains were tentatively identified as either Clostridium butyricum or Clostridium tyrobutyricum unless stated otherwise.

Key:

F	Facultative	A	Acid
O	Obligate	R	Reduction
M	Microaerophilic	+	Positive reaction
C	Central	-	Negative reaction
Cl	Club shaped	S	Slight reaction

Known Cultures:

AKn	Clostridium sporogenes
BKn	Clostridium tyrobutyricum
CKn	Unknown Clostridium

agree with the characteristics described in Bergey's Manual of Determinative Bacteriology (7). This was also true for the cultures of Clostridium received from Dr. Langston. Richard (34) stated that many of the cultural, morphological and physiological characteristics of single stains of butyric acid-forming bacteria varied from time to time.

It was possible therefore, without further tests, to tentatively identify the remaining isolates only as strains of either Clostridium tyrobutyricum or Clostridium butyricum. The difficulty in differentiation between the two species was the variation in their action on some of the carbohydrates.

Biochemical Analysis of Low Moisture Silage

The effect of the various preservatives on the pH of the silage samples can be seen in figure 3. The pH of all samples except C and D, declined steadily throughout the fermentation period. It is interesting to note the rapid decrease in pH early in the fermentation period of samples B and E. This rapid decrease in pH in sample E could be attributed to the molasses in the preservative. The molasses provided a readily available source of carbohydrate. The carbohydrate in sample B consisting of corn sugar, dried whole whey, and feeding cane molasses, could be responsible for the rapid decrease in pH. This decrease in pH is the result of the ability of the acid-producing bacteria to convert this readily available supply of carbohydrate into acid. Langston et al. (27) stated that this rapid lowering of the pH is

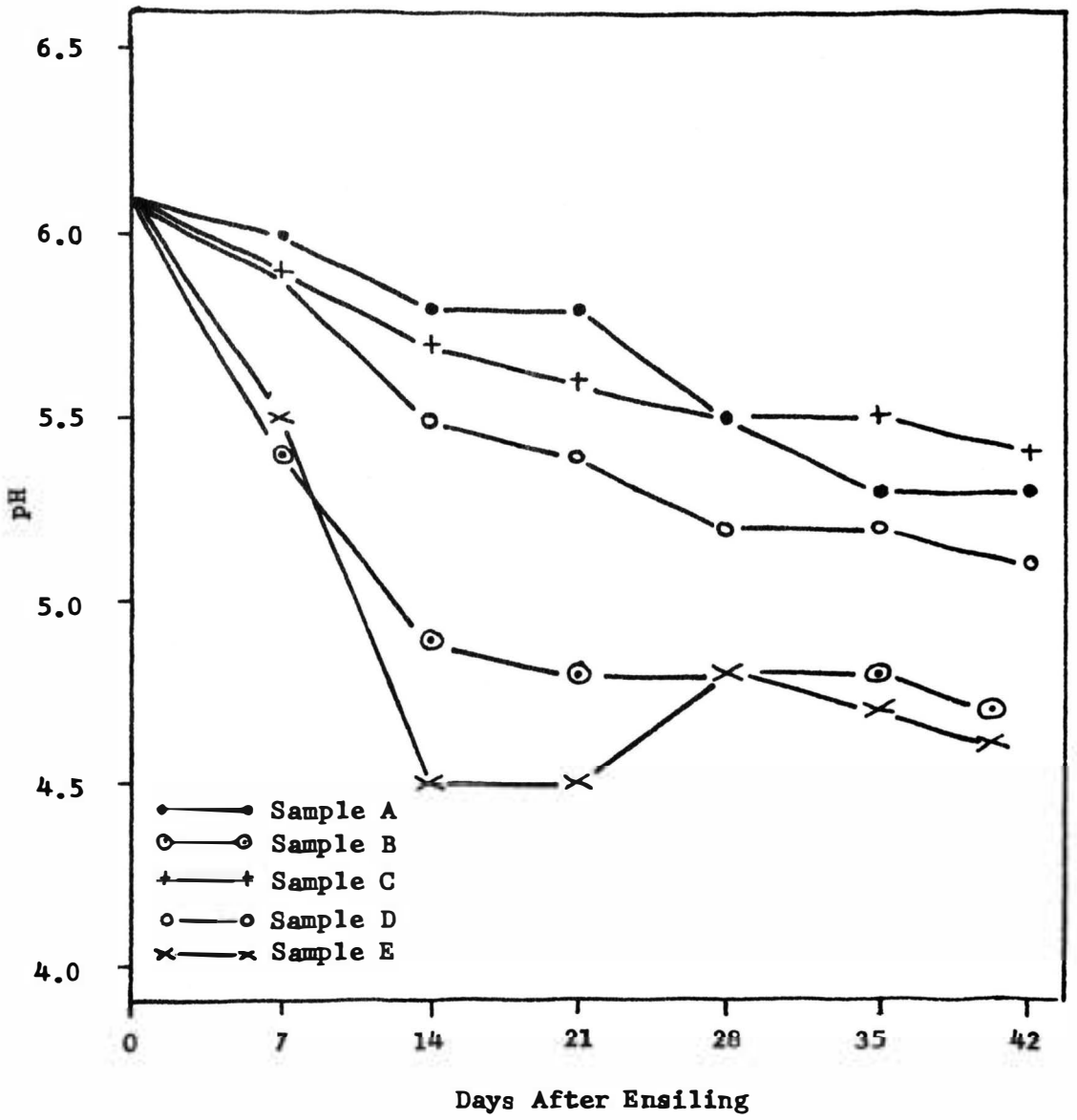

Figure 3. Comparison of pH Values for Each Silage Sample Prepared from Low Moisture Forage

essential for the production of good quality silage.

It appeared that the rapid decline in pH early in the fermentation period combined with a low pH at the end of the period had an inhibitory affect on the maximum number of thermoduric anaerobes. Allen et al. (4) stated that the growth of sporeforming anaerobes was affected by a low pH. At a low pH only the lactobacilli are capable of growing. There does not appear to be any direct effect of pH on the numbers of thermoduric anaerobes and non-thermoduric anaerobes at the different stages of the ensiling period.

As indicated previously the strains of non-thermoduric anaerobes isolated from the silage were acid-forming cocci. It appears therefore that the samples with the high number of non-thermoduric anaerobes would also be the samples with the rapid decrease and constant low pH levels. Sample B followed this pattern of high numbers in conjunction with low pH levels. Sample E, however, had the desirable pH characteristics, but the number of non-thermoduric anaerobes were low compared with the other samples. Langston et al. (26) stated that usually the cocci that are found in silage attain high numbers and produce considerable amounts of acid.

Bacteriological Analysis of High Moisture Silage

The results of the bacterial counts and pH determinations are given in Table VI, Appendix I. There appears to be a general pattern of steady decrease in the number of non-thermoduric anaerobes after the first week of the fermentation period. The first week showed the initial count for all samples except J and N, to be greater than

5,000,000 per gram of silage. After the first week there was a steady decrease until the numbers were less than 500,000 per gram. The initial counts for samples J and N were considerably lower than for the other samples.

The maximum number of non-thermoduric anaerobes for each silage sample was attained during the first week of the fermentation. Figure 4, shows the week of the fermentation period when the maximum number of non-thermoduric anaerobes was attained. In samples J, N, O and P it appeared that the maximum number was reached during the first week after ensiling. At the time of sample analysis the number of non-thermoduric anaerobes were beginning to decline. This would account for the low maximum number of non-thermoduric anaerobes.

It is possible that the preservative used in preparing the silage samples had a stimulatory effect on the number of non-thermoduric anaerobes. That is, a rapid increase in numbers was promoted during the first week of the fermentation. There is also the possibility that the preservatives in the silage samples had an inhibitory effect on their growth. The maximum number of non-thermoduric anaerobes was lower in all samples than in the control (sample G). This can be seen in figure 4.

The same general pattern of a steady decrease in the number of organisms that was characteristic of the non-thermoduric anaerobes, was not characteristic of the thermoduric anaerobes. Figure 5 shows the week of the fermentation period when the maximum number of thermoduric anaerobes was attained. There were several samples, however that

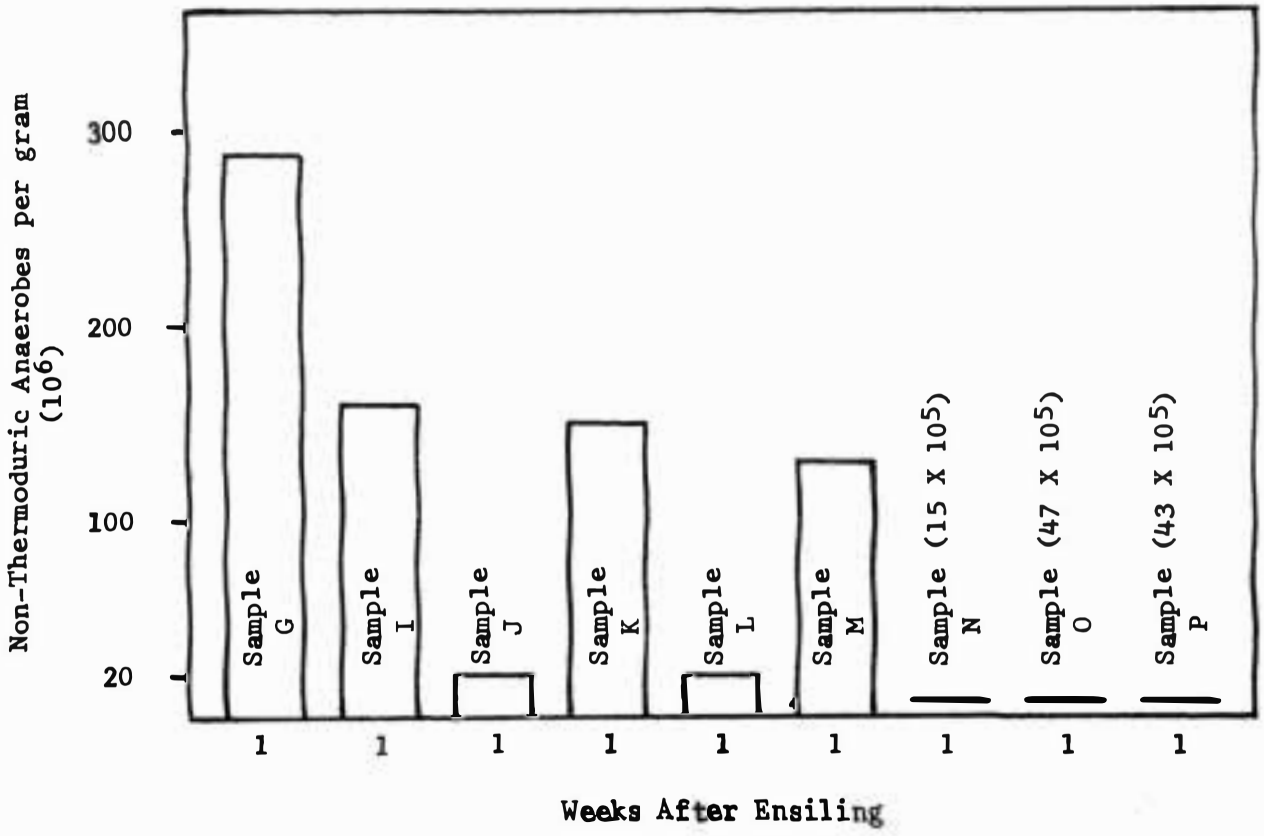

Figure 4. Maximum Number of Non-Thermoduric Anaerobes for Each Silage Sample Prepared from High Moisture Forage

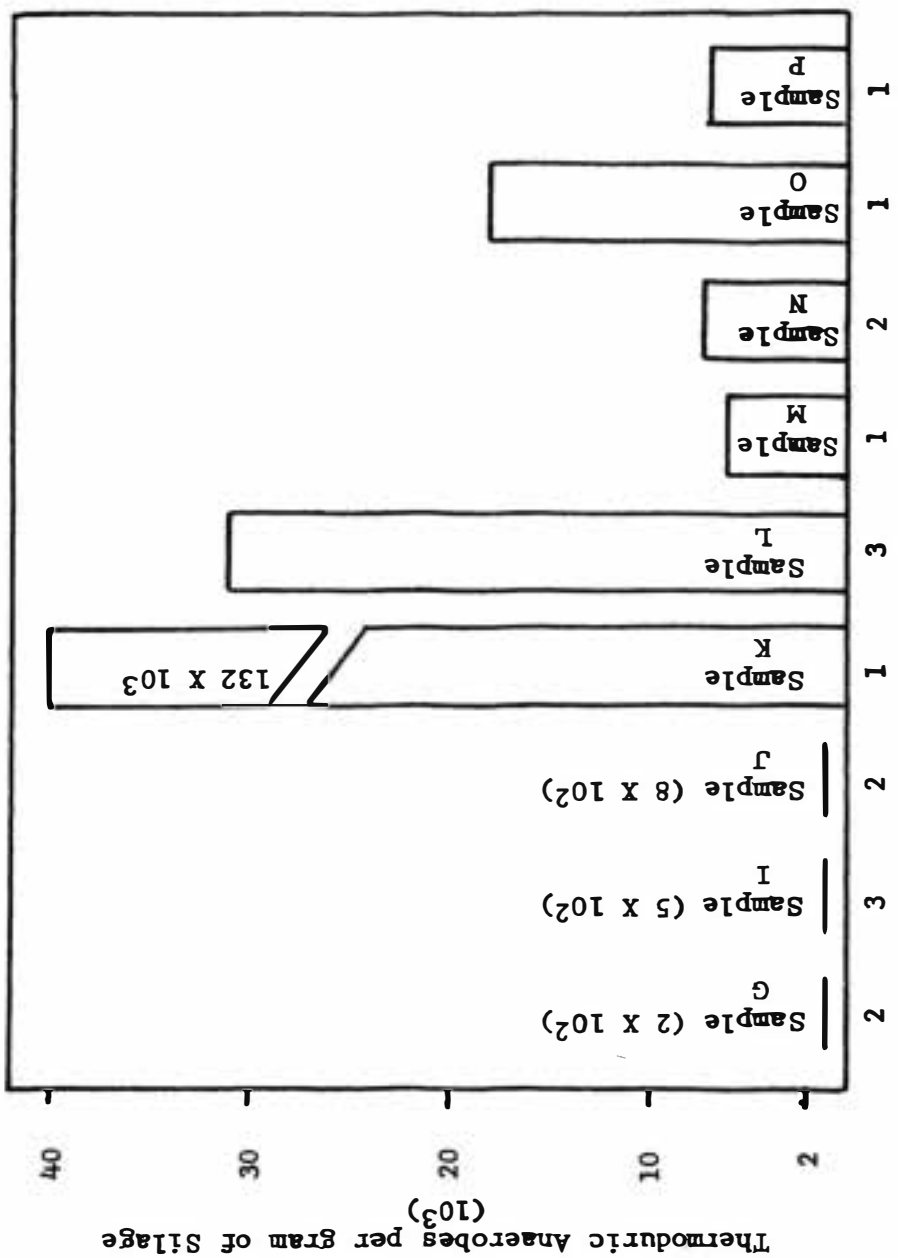

Figure 5. Maximum Number of Thermoduric Anaerobes for Each Silage Sample Prepared from High Moisture Forage

showed similar characteristics. In samples G, I, and J, the number of thermoduric anaerobes remained below 1,000 per gram throughout the fermentation. Samples M, N, O, and P had a similar pattern of a decrease in the number of thermoduric anaerobes. These samples were characterized by a small initial number the first week after ensiling. There was a steady decrease in numbers until less than 1,000 per gram were reached the third week. In all samples, except M, there was a slight increase in numbers the sixth week of the fermentation period. Sample K was characterized by a high initial number of thermoduric anaerobes. There was a decrease the second week followed by a slight increase the third week. Throughout the fourth and fifth weeks there was a steady decrease in numbers. The sixth and final week was characterized by a slight increase in the number of thermoduric anaerobes. The initial number for sample L was low and decreased the second week. After the second week the number of thermoduric anaerobes followed the same pattern as found in sample K.

In all samples except L and N the maximum number of thermoduric anaerobes was reached during the first week of the fermentation period. This is not characteristic of the sporeforming anaerobes in silage fermentation. Langston et al. (27) stated that the number of anaerobic spores increased steadily throughout the fermentation period. It is possible that the preservatives kept the number of thermoduric anaerobes at a low level by providing a substrate that was unfavorable for their growth.

Biochemical Analysis of High Moisture Silage

The results of the biochemical analysis is given in Table VI, Appendix I. There was a similar pattern in pH of each silage sample. This can be seen in figure 6. In all samples except K, L and N, there was a steady decrease the first and second weeks after ensiling. Samples K, L and N showed an increase the second week. This decrease in pH the first few weeks after ensiling was followed by a rise the third or fourth week in all samples. Following the rise there was a gradual decrease and leveling off in all samples except G and K. These two samples were characterized by continued rises and falls in pH throughout the fermentation period.

It should be mentioned that during the third and fourth weeks of the fermentation period there was a sudden increase in the atmospheric temperature. The silage samples were maintained at room temperature, therefore this sudden rise in temperature might be responsible for the fluctuation in pH during the fermentation period.

The pH of sample J was between 4.0 and 4.5. Because of this low pH it appears that the preservative in sample J would be a beneficial additive to the forage as a method of improving silage quality. Langston et al. (27) stated that good quality silage has a pH level of 4.0 to 4.5. The preservative in samples N, O, and P would also be a beneficial preservative for maintaining a low pH level. The reason for the rapid decrease could be attributed to the readily available supply of carbohydrate. In sample J, lactose was the carbohydrate and in sample N, O and P the carbohydrate was molasses.

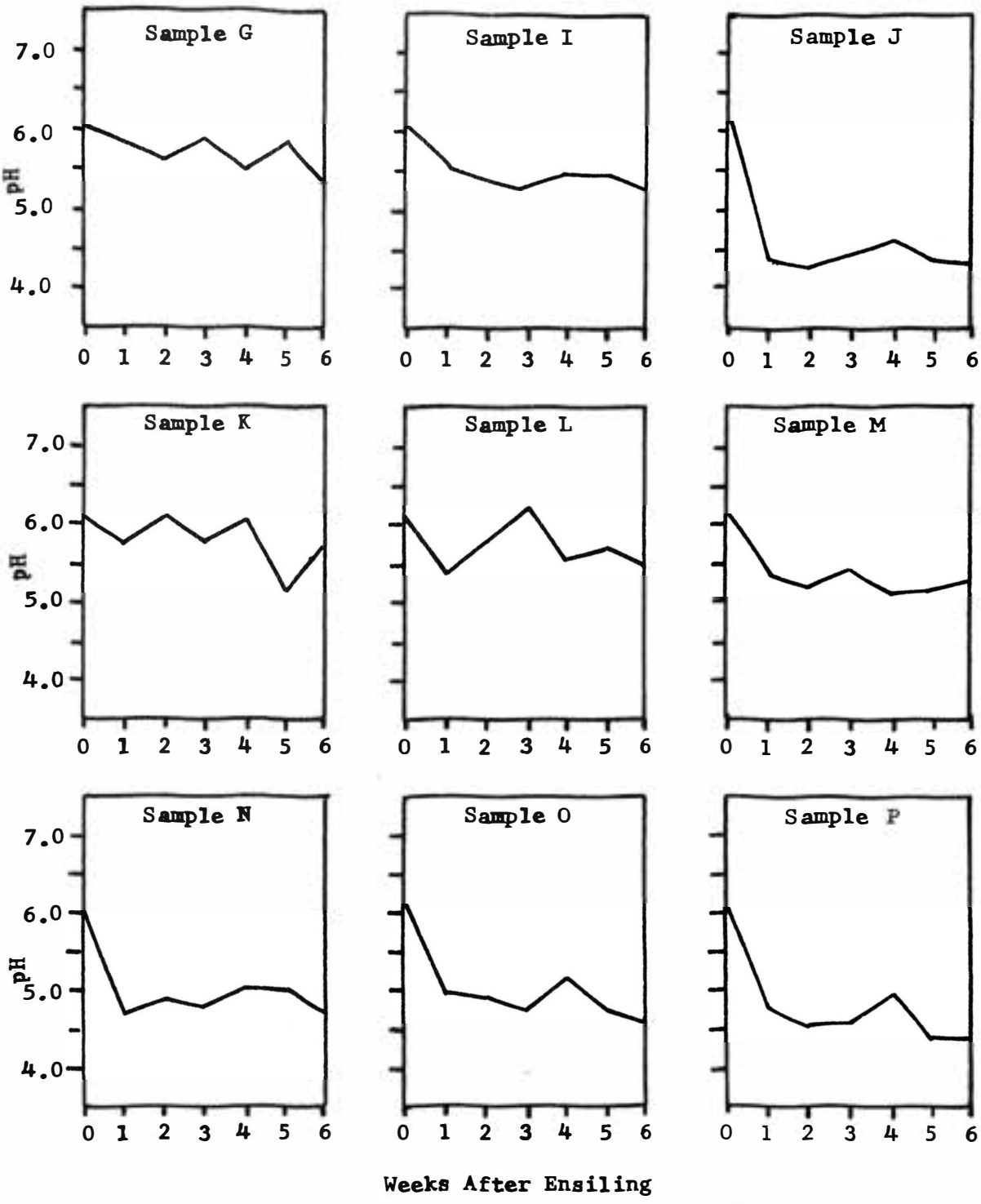

Figure 6. Comparison of pH for Each Sample Prepared from High Moisture Forage

It is interesting to note, see figure 4, that the maximum number of non-thermoduric anaerobes in samples J, N, O, and P were lower than the maximum number of non-thermoduric anaerobes for the other samples. It is possible that the low pH value of these samples was responsible for maintaining the low maximum number of non-thermoduric anaerobes. This is not the case with the thermoduric anaerobes, as seen in figure 5. Samples J, N, O and P that were characterized by a low pH, had high thermoduric anaerobic counts. It appears that there is no direct comparison between the pH of the silage samples, the number of non-thermoduric anaerobes, and the maximum number of thermoduric anaerobes.

Bacteriological and Biochemical Comparison of High and Low Moisture Silage

The effects of the preservatives, Silotracin, EN-SIL-AC, and the Silotracin, ground corn, molasses combination on high moisture and low moisture silage were discussed previously. A comparison was made between the high moisture and low moisture silage in regard to the number of non-thermoduric anaerobes, thermoduric anaerobes and pH, when using the previously mentioned preservatives. The results of this comparison are given in figures 7, 8 and 9.

It is interesting to note that in the low moisture silage the pH for all the samples showed a more rapid decrease with a lower final pH than the high moisture silage. It appears that these three preservatives were more effective in producing a desirable pH when they were used with a low moisture forage.

The numbers of non-thermoduric anaerobes in the low moisture

silage samples were erratic from the second week until the end of the fermentation period. The numbers of non-thermoduric anaerobes in the high moisture silage samples were characterized by a steady decline followed by a leveling off near the end of the fermentation.

The low moisture samples, except Silotracin, were characterized by a steady low level of thermoduric anaerobes after the first week of the fermentation period. After the first week the high moisture silages were characterized by erratic decreases and increases in the number of thermoduric anaerobes. A slight increase in numbers was observed at the end of the fermentation. Langston et al. (27) stated that good quality silage was characterized by low levels of spore-forming anaerobes. The EN-SIL-AC and Silotracin, ground corn, molasses combination appears to be more effective when used with low moisture forage. These preservatives are effective in keeping the number of thermoduric anaerobes at low levels.

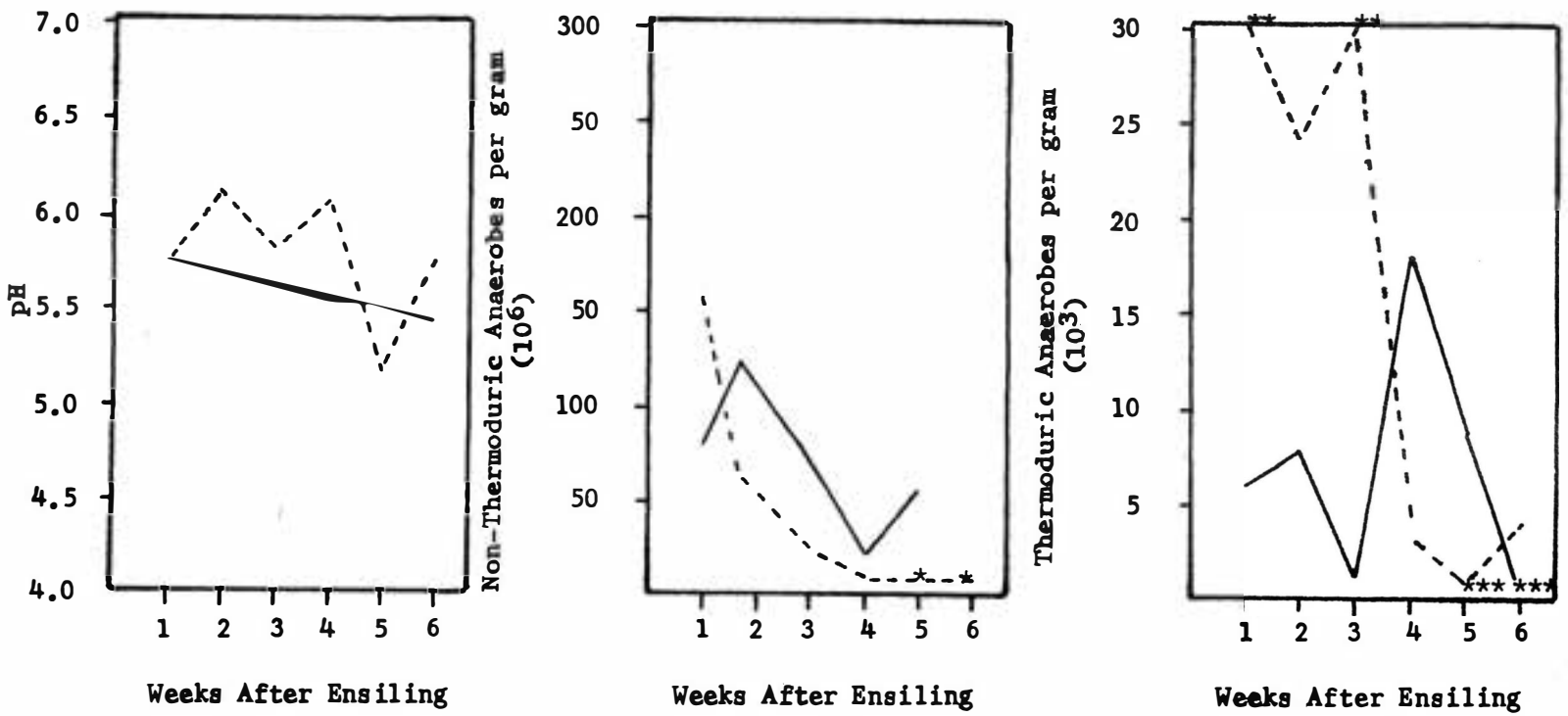

Figure 7. Comparison of Bacteriological and Biochemical Properties of Low Moisture and High Moisture Forage Using Silotracin as a Preservative

——— Low Moisture Silage Sample C
- - - - - - - High Moisture Silage Sample K

* Less than 1 X 10^7
** Greater than 30 X 10^3
*** Less than 1 X 10^3

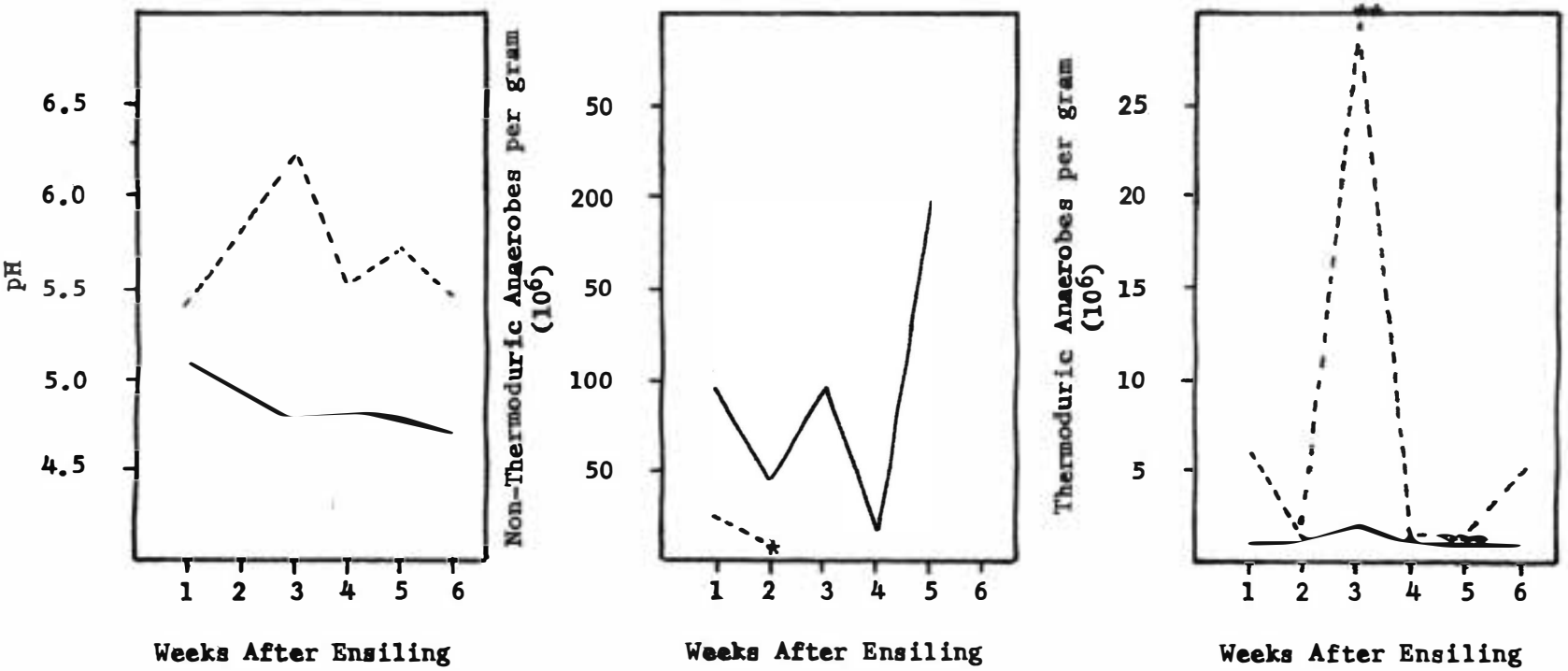

Figure 8. Comparison of Bacteriological and Biochemical Properties of Low Moisture and High Moisture Forage Using EN-SIL-AC as a Preservative

——— Low Moisture Silage Sample B

- - - - - High Moisture Silage Sample L

* Less than 1 X 10^7

** Greater than 30 X 10^3

*** Less than 1 X 10^3

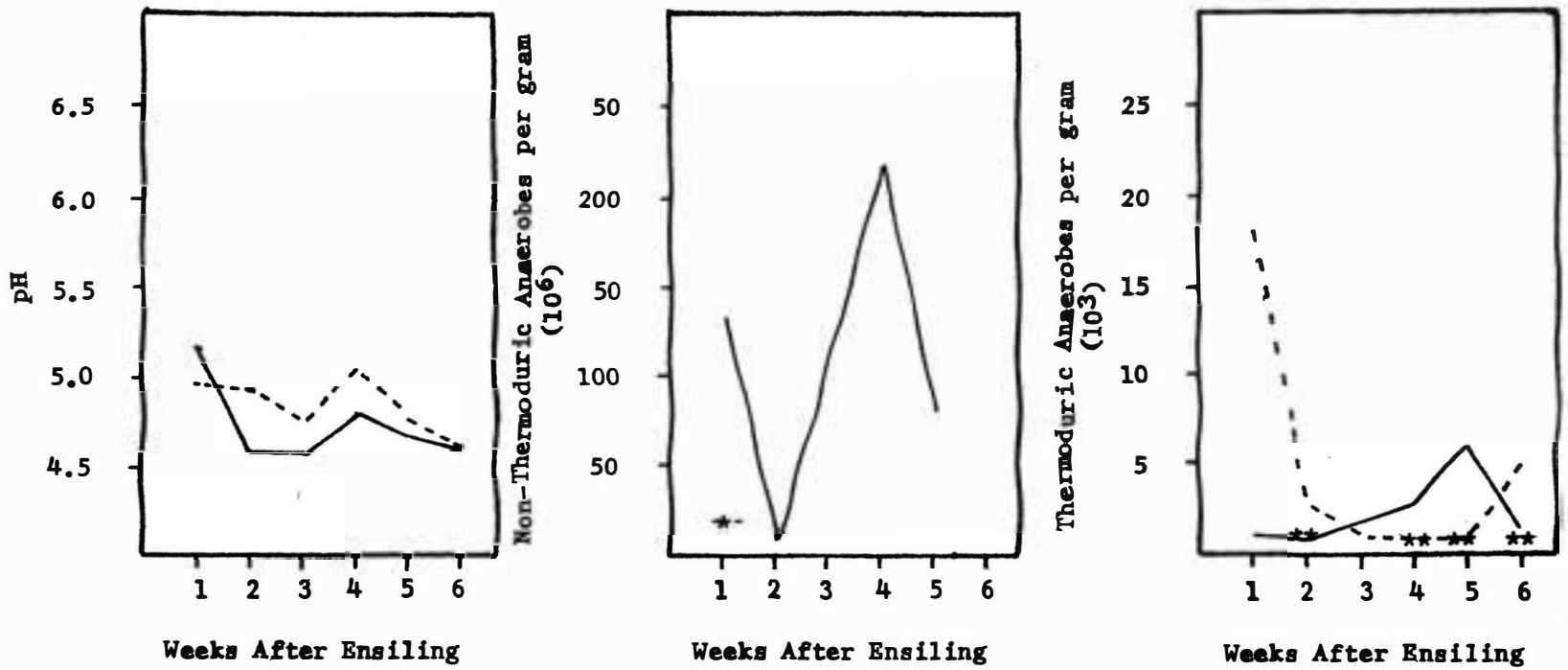

Figure 9. Comparison of Bacteriological and Biochemical Properties of Low Moisture and High Moisture Silage Using a Combination of Silotracin, Ground corn and Molasses as a Preservative

——— Low Moisture Silage Sample E

- - - - High Moisture Silage Sample O

* Less than 1 X 10^7

** Less than 1 X 10^3

SUMMARY AND CONCLUSIONS

First and third cuttings of alfalfa, having a moisture content of 58.8 and 80.0 per cent respectively were used for this study.

Silage samples were prepared by mixing various preservatives with the forage. The forage was then packed into fruit jars and maintained at room temperature for the fermentation period.

A preliminary analysis was conducted on both the low moisture and high moisture forage to determine the actual moisture content, the pH, thermoduric anaerobic, and non-thermoduric anaerobic population of the forage prior to ensiling.

An analysis of the silage samples was made to obtain some information on the effect of preservatives on the thermoduric anaerobic and non-thermoduric anaerobic population of the silage at the various stages of the fermentation period. An analysis was also made to determine the effect of the preservatives on the pH of the silage during the fermentation.

In the majority of samples the maximum number of thermoduric anaerobes and non-thermoduric anaerobes, in both the high moisture and low moisture silage, was attained the first week after ensiling.

The non-thermoduric anaerobes were tentatively identified as Streptococcus lactis, Streptococcus cremoris, and Streptococcus faecalis. The thermoduric anaerobes had characteristics similar to both Clostridium tyrobutyricum and Clostridium butyricum. It was possible to tentatively identify the isolates only as either Clostridium tyrobutyricum or Clostridium butyricum.

All of the cultures tentatively identified were isolated at various stages during the fermentation.

The preservatives containing a readily available supply of carbohydrate maintained a low pH level throughout the fermentation period.

There did not appear to be a direct comparison between the pH and the non-thermoduric and thermoduric populations.

The low moisture silage was characterized by erratic numbers of non-thermoduric anaerobes, and in general, low numbers of thermoduric anaerobes. The pH pattern of the low moisture silage was characterized by a lower pH level than the high moisture silage.

It appears that silage quality can be improved by ensiling high moisture forage with mild antibiotics or carbohydrates as preservatives.

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APPENDIX

APPENDIX I.

TABLE V. BACTERIAL COUNTS AND pH OF SILAGE SAMPLES PREPARED FROM LOW MOISTURE FORAGE

Sample	Age (Days)	pH	Anaerobes per gram of Forage	
			Non-Thermoduric (000)	Thermoduric (000)
	1	6.08	21,000	1
	2	6.15	16,000	<1
	3	6.21	264,000	1
	4	6.05	373,000	4
	5	6.01	280,000	17
A	6	5.79	122,000	1
	7	5.77	282,000	6
	14	5.81	272,000	6
	21	5.77	80,000	2
	28	5.49	190,000	<1
	35	5.33	130,000	<1
	42	5.30	204,000	<1
	1	6.10	4,000	1
	2	5.80	146,000	1
	3	5.40	170,000	2
	4	5.17	172,000	4
	5	5.05	389,000	4
B	6	5.12	200,000	5
	7	5.07	94,000	1
	14	4.91	42,000	1
	21	4.77	96,000	2
	28	4.81	15,000	1
	35	4.79	197,000	<1
	42	4.70	*	1
	1	6.19	22,000	6
	2	6.12	335,000	7
	3	6.13	139,000	5
	4	6.00	139,000	7
	5	5.64	155,000	8
C	6	5.68	92,000	3
	7	5.77	82,000	6
	14	5.70	127,000	8
	21	5.61	72,000	1

TABLE V. (Continued)

Sample	Age (Days)	pH	Anaerobes per gram of Forage	
			Non-Thermoduric (000)	Thermoduric (000)
	28	5.53	23,000	18
	35	5.52	56,000	9
	42	5.43	*	1
	1	6.18	10,000	3
	2	6.19	93,000	3
	3	6.21	103,000	3
	4	5.88	292,000	3
	5	5.81	184,000	6
D	6	5.65	116,000	5
	7	5.53	82,000	15
	14	5.53	126,000	6
	21	5.42	*	2
	28	5.23	71,000	20
	35	5.18	10,000	8
	42	5.09	192,000	20
	1	6.22	30,000	6
	2	5.98	48,000	5
	3	5.62	90,000	2
	4	5.23	243,000	3
	5	5.10	219,000	5
E	6	4.97	118,000	8
	7	5.17	132,000	1
	14	4.59	6,000	1
	21	4.57	*	*
	28	4.83	217,000	3
	35	4.67	80,000	6
	42	4.59	*	1

* Colonies were spreading and could not be counted.

TABLE VI. BACTERIAL COUNTS AND pH OF SILAGE SAMPLES PREPARED FROM HIGH MOISTURE FORAGE

Sample	Age (Days)	pH	Anaerobes per gram of Forage	
			Non-Thermoduric (000)	Thermoduric (000)
G	7	5.82	292,000	0
	14	5.62	145,000	<1
	21	5.86	30,000	0
	28	5.49	200	0
	35	5.84	1,400	0
	42	5.31	100	<1
I	7	5.53	160,000	<1
	14	5.40	135,000	<1
	21	5.32	1,700	<1
	28	5.47	100	0
	35	5.47	1,000	0
	42	5.32	100	0
J	7	4.42	200	<1
	14	4.33	100	<1
	21	4.47	100	<1
	28	4.63	100	<1
	35	4.36	100	<1
	42	4.34	100	<1
K	7	5.73	155,000	132
	14	6.12	71,000	24
	21	5.82	26,500	35
	28	6.08	11,300	4
	35	5.17	190	1
	42	5.79	100	4
L	7	5.39	22,500	6
	14	5.82	4,600	1
	21	6.20	2,800	31
	28	5.53	400	1
	35	5.72	330	1
	42	5.45	100	5

TABLE VI. (Continued)

Sample	Age (Days)	pH	Anaerobes per gram of Forage	
			Non-Thermoduric (000)	Thermoduric (000)
M	7	5.37	127,000	6
	14	5.17	5,900	1
	21	5.40	5,200	<1
	28	5.11	3,000	<1
	35	5.13	206	<1
	42	5.31	100	<1
N	7	4.67	1,500	4
	14	4.87	100	7
	21	4.77	100	<1
	28	5.02	*	<1
	35	5.03	100	<1
	42	4.72	100	2
O	7	4.97	4,700	18
	14	4.92	100	3
	21	4.79	100	1
	28	5.14	100	<1
	35	4.77	*	<1
	42	4.63	100	5
P	7	4.80	4,300	7
	14	4.55	2,300	7
	21	4.60	100	<1
	28	4.96	100	<1
	35	4.42	100	<1
	42	4.42	100	4

* Colonies were spreading and could not be counted.