1959

# Some Bacteriological and Biochemical Aspects of Alfalfa Silage Prepared At Different Moisture Levels

Gene W. Ronald

# SOME BACTERIOLOGICAL AND BIOCHEMICAL ASPECTS OF ALFALFA SILAGE PREPARED AT DIFFERENT MOISTURE LEVELS

BY

GENE W. RONALD

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Department of
Bacteriology, South Dakota State
College of Agriculture
and Mechanic Arts

August, 1959

**SOME BACTERIOLOGICAL AND BIOCHEMICAL ASPECTS OF ALFALFA SILAGE PREPARED AT DIFFERENT MOISTURE LEVELS**

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.

Thesis Adviser

Head of the Major Department

## ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. E.C. Berry, Dr. R.J. Baker, Dr. D.F. Breazeale and Dr. R.M. Pengra for their frequent help and suggestions in the preparation of this manuscript. Also, personal thanks to William Stoll for his able assistance in various phases of this study.

GWR

TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

# INTRODUCTION

The preservation and utilization of leguminous forage crops has become an important aspect of dairy farming. The economic role of the dairy industry is one of importance, therefore, the cost of feeding dairy cattle and the production of good quality milk on the farm are of prime concern to the industry as a whole.

In this respect leguminous plants, such as alfalfa, would seem ideal as a winter roughage feed for dairy cattle because of the high carotene and protein content of the plants. The problem, however, lies in trying to preserve these important qualities of the plants.

Preservation of the protein and carotene content of the ensiled alfalfa is effected through a fermentation process whereby the lactic acid producing organisms, present on the plants, convert the available carbohydrate of the plants to lactic acid and acetic acid.

The problems involved with the production of a good quality silage are evident when individual alfalfa samples, apparently alike in all respects, do not produce uniform products. The necessity of understanding the basic mechanisms of the silage fermentation process, in order that a better quality silage may be produced with some degree of consistency, is obvious.

The object of this study is to elucidate the facts observed in the experimental trials of ensiling alfalfa in terms of microbiological populations and some biochemical analyses.

# REVIEW OF LITERATURE

## Bacteriological

The role of microorganisms in silage fermentation is generally recognized as one of major importance. Work, concerning the microflora, has been done on green plants prior to ensiling and at various stages throughout the fermentation period.

Kroulik et al. (17) observed that the predominant organisms on green plants were aerobic, chromogenic, nonsporeforming, rod-shaped bacteria. Coliform bacteria, in large numbers, were shown to be present on green plants, and a few studied belonged to the genus Aerobacter. Studies of similar nature by Allen et al. (3) showed that lactic acid organisms are present in numbers ranging from 1,000,000 to 10,000,000 per gram. They reported the predominant organism in this group as Lactobacillus plantarum. In this study coliforms ranging from 1,000 to 1,000,000 per gram were reported to be present on green plants prior to ensiling. These consisted primarily of Aerobacter aerogenes var. greminis. They reported also, that the predominant species of anaerobes present was Clostridium sporogenes.

Stirling (27) worked with ten different types of fresh forage in an attempt to determine the number of lactic acid producers. The highest counts obtained ranged from 101 to 300 per gram. He stated "the counts of lactobacilli may not always be sufficiently high to ensure preservation when the fodder is made into silage." Stevens and Kuhlman (19) stated that the incorporation of commercial lactic acid starter cultures at the time of ensiling increases the acidity of the

silage. In contrast to both of these lines of reasoning, however, Langston et al. (18) maintained that forage high in lactic acid producing bacteria is no better than forage which contained small numbers of these organisms. They stated "this indicates that inoculation of the original material with lactic acid bacteria would be of little or no value." In support of this statement Stone et al. (29) observed that all green plant material suitable for ensiling contained enough lactic acid producing organisms and that the fermentation does not vary as to the type of organisms present but rather according to the conditions under which the forage is stored.

Many authors have approached the problem of defective silage from the view point of the anaerobic population in the silage. They have shown that spores of the anaerobic organisms do exist on fresh alfalfa forage. Allen and Harrison (1) worked with fresh grass and reported that the number of anaerobic spores appears always to be small but that this group of organisms increases in number after the first few days until approximately 1,000,000 per gram are present. They maintained that these high numbers are usually present for weeks. Clostridium sporogenes was the prominent species of this group. Bryant and Burkey (10) isolated several strains of proteolytic, sporeforming anaerobes. They stated that the predominant species was Clostridium tyrobutyricum, van Beynum and Pette. Rosenberger (23) contended that the dominant anaerobes isolated from silage were proteolytic and lactate attacking species. The organisms involved in their study did not multiply below pH levels of 4.0 to 4.2. Some of the butyric acid producing types of anaerobes could ferment lactate in pure cultures,

in the presence of acetate or pyruvate.

Hunter (15), in studies involving prepared silages made from alfalfa forage and supplemented with various additives, suggested another thought to the overall picture of protein breakdown in silage. He stated that protein decomposition may result from microbial or plant enzymes or their associated action. He felt that the role of the plant enzyme is of minor importance, and suggested that the possibility exists whereby the acid producing organisms may possess the ability to utilize protein in the absence of the available carbohydrate.

Working with A.I.V.[1] silage, Cunningham and Smith (11) reported the microflora consisted mainly of lactic acid bacteria such as lactobacilli, streptococci, micrococci, and sarcinae. They stated that both homofermentative and heterofermentative types are represented, the former by Lactobacillus plantarum and the latter by Lactobacillus brevis. They went on to say that the streptococci, micrococci and motile lactobacilli were found in fodder recently ensiled, and lactobacilli and sarcinae in the older samples.

Allen et al. (4) utilized silages to which various preservatives had been added and reported that in every case lactic acid organisms

---

1. This is a method of silage preparation whereby the addition of mineral acids, such as hydrochloric acid or phosphoric acid, are utilized for rapid lowering of the pH value of the silage. W.H. Peterson, G. Bahtedt, H.P. Bird, and W.H. Beeson, "The Preparation and Nutritive Value of A.I.V. Silage for Dairy Cows", J. Dairy Sci., Vol. 18, 63-78, The American Dairy Association, Ohio State University, Columbus, 1935.

People doing the work. Realizing the need for a more standardized procedure for judging silage quality, Langston et al. (18) suggested the following criteria for the evaluation of silage:

Good Quality Silages:

(1) pH values 3.9-4.8.

(2) Ammoniacal nitrogen 1.0-2.9 per cent

(3) Lactic acid 3-13 per cent.

(4) Spore counts low or none at all.

Poor Quality Silages:

(1) pH values 5 and above.

(2) Ammoniacal nitrogen 3.0-9.8 per cent.

(3) Butyric acid present.

(4) Spore counts high.

Silage quality may be influenced by various factors, i.e., temperature, moisture content, types of forage and atmospheric conditions within the container. Nilsson et al. (21) studied silage fermentation under various temperatures, ranging from 2°C. to 37°C. and found that lactic acid production was faster at higher temperatures. In all cases the silage was of poor quality at high temperatures. They reported that the silages were best at temperatures below 20°C. Silage quality was based on ammoniacal nitrogen and butyric acid content.

Controlled temperature conditions were utilized to compare the ability of crops containing small quantities of sugar and those of high sugar content to produce good quality silage. Toth et al. (30)

conducted these studies and reported that fermentation progressed more rapidly in all forages at the higher temperatures. It was shown that after a peak of lactic acid production was established the lactic acid content of the silage tended to decrease. In the silage held at lower temperatures the fermentation progressed more slowly; once the lactic acid production reached a peak it tended to stay there considerably longer. An interesting statement was made by these workers, "When a lactic acid content of 1.6 per cent to 2.6 per cent is reached, the lactic acid production ceases even if sugar is available for further fermentation." They concluded from this work that in order for a low carbohydrate plant to produce good quality silage, sugar in available form must be supplemented, since in practice it is hard to maintain temperatures below 20°C.

In other studies on fermentation processes in silage, Rydin et al. (25) studied the effects of various carbohydrates, as supplemented, for the production of good quality silage. The materials utilized were starch, cellulose, barley meal, straw powder, beet pulp, and malt. At low temperatures (16°C) the carbohydrates of the plants were sufficient for good quality silage. At temperatures of 24°C to 37°C carbohydrate additives were necessary. Of all the additives utilized only malt had a favorable effect. This they attributed to the amylase content of the malt. Temperature is not important if the carbohydrate content of the plant is high. Watson and Ferguson (31) stated that the addition of about one per cent (15-2 pounds/ton) molasses has resulted in silage of excellent quality. They placed a great deal of emphasis on the silo filling process. Hegsted et al. (14) compared silage

preserved by the A.I.V. process and molasses methods. They reported that protein and carotene are better preserved by the former process but that larger amounts of non-volatile fermentative products were formed in the latter method. Bender and Bosshardt (8) reported that there were losses of dry matter as a result of the production of such volatile materials as carbon dioxide, hydrogen, methane and ammonia by bacterial action and cell respiration. They also attributed the loss of some soluble materials to drainage from the silo.

Moisture content is another factor influencing the quality of silage. Archibald and Kurmeski (5) reported that high moisture content in silage is undesirable because of "its association with high fiber content, which lowers the feeding value, and with butyric acid which is responsible for the bad odor in poor quality silage." They stated that it is "significantly correlated with high carotene" but add that "the lower levels of carotene found in silage are high enough to supply a cow's requirements." The work of Autrey et al. (6) corroborates their conclusions. They stated that wilted silage regardless of treatment had lower pH values than the unwilted silage and that the wilted samples produced the most consistent increase in lactic acid. Barnett (7) is of the opinion that butyric acid is formed from either lactic acid or soluble carbohydrates. It occurred from lactic acid when insufficient soluble carbohydrates were present.

## EXPERIMENTAL PROCEDURE

Many of the underlying principles of the silage fermentation process are still very obscure. By varying the moisture content of the silage samples in this study, a better understanding of the affects of moisture on the silage fermentation process occurs. For the purpose of this study four silage samples were obtained, ranging in moisture content at intervals of ten per cent, from 70 per cent for sample A to 40 per cent for sample D. The respective silage samples will here after be referred to as samples A,B,C, or D.

### Forage Preparation

First cutting alfalfa at approximately the half-bloom stage of maturity was utilized for this study. Freshly cut alfalfa was brought into the laboratory for further wilting to the desired moisture content of samples B,C, and D. Sample A was prepared immediately as the moisture content of the forage was correct. To hasten the wilting process and also to facilitate better packing in the silage containers the forage was chopped into approximately quarter-inch lengths with a paper cutter. The chopped forage was spread upon laboratory tables in a thin layer for the wilting process. Electric fans were placed in position to blow across the forage and hasten the drying process. During wilting, the forage was stirred frequently to prevent heating of the lower layers.

Samples were taken at approximately hourly intervals for moisture analysis. This procedure involved an American Moisture Tester which utilized an infrared-bulb as a source of heat and incorp-

orated an exhaust fan to keep moisture from condensing on the sample. The pan, containing the sample to be analyzed, occupies a position between the infrared-bulb and the exhaust fan. The 10 gram sample is weighed directly into the pan on the moisture tester. At the end of the period of analysis, approximately 30 to 45 minutes, the per cent moisture is read directly from a scale on the tester.

When the moisture levels of the forage neared the desired point, it was immediately packed into pint and half gallon glass jars. The glass jars had been previously steamed as a precaution to reduce the possibility of contaminating microorganisms entering the study from the jars. The forage was packed tightly in the jars, excluding as much air as possible. The jars were then tightly sealed, labelled and stored at room temperature until their respective sampling times. This procedure was carried out on every jar in each of the four silage samples. Enough individual replicates were prepared from each of the four samples (A,B,C, and D) so that the jars had to be opened only at the time of sampling. Once a sample was taken, that particular replicate could be discarded.

## Bacteriological Analysis

Preliminary studies were conducted on fresh, green alfalfa prior to ensiling to obtain some information about the bacterial population on the green plants. Two methods of preparing the samples were compared so the method giving the most consistent results could be applied to this study. The bacteriological analysis consisted of counts of organisms belonging to the following four groups: coliforms, lactic

acid producers, total aerobes, and proteolytic organisms.

The preparation of the alfalfa sample for each of the groups of organisms counted was the same and will, in general, apply to the preparation of all the silage samples for bacteriological analysis. Two 11 gram samples were weighed out and one sample was placed in a sterile four ounce bottle to which a 99 ml. sterile, buffered solution was added. The second 11 gram sample was placed in a Waring-blender jar to which a similar 99 ml. solution was added. This sample was comminuted approximately one minute in the blender and then poured into a sterile four ounce bottle. Preparing the sample by using 11 grams of alfalfa per 99 ml. of solution, resulted in an initial dilution of 1:10 (one gram of alfalfa to 10 ml. of solution). From this initial dilution, successive dilutions could be made to cover any desired range. The range selected was such that a plate containing 30 to 300 colonies would be available for counting (26).

Preparing the plates for counting the individual groups of organisms was done as follows:

1. Coliform organisms: Dilutions of 1:10, 1:100, 1:1000 and 1:10,000 were utilized for this group of organisms. These dilutions were plated out and poured with Violet Red Bile Agar (12) which is specific for this group of organisms. The plates were incubated at 32°C (2) for 24 hours and counted.

2. Lactic acid producing organisms: For this group, dilutions of 1:100, 1:1000, 1:10,000 and 1:100,000 were used. The media utilized was V-8 juice Agar (13) which was formulated as recommended except the indicator was not added. The plates were poured and incubated at 32°C for 24 to 48 hours and counted.
3. Total aerobic organisms: Since this group would probably give higher counts, dilutions used were 1:1000, 1:10,000, 1:100,000 and 1:1,000,000. The media used for this group of organisms was Tryptone Glucose Yeast Extract Agar (26). The plates were poured and incubated at 32°C for 24 to 48 hours and counted.
4. Proteolytic organisms: Dilutions used for this group of organisms were the same as those used for the coliform group. The media was Tryptone Glucose Yeast Extract Agar plus five ml. of sterile, skim milk per 100 ml. of media (26). The plates were poured and incubated at 32°C for 24 to 48 hours and counted.

It should be stated here that slight variations in the range of dilutions, used for bacteriological analyses of the silage samples were necessary because of the various times the silage samples were analyzed. Bacteriological analysis was made every six hours for the first three days, daily for the remainder of the first week, and then at weekly intervals for a period of five additional weeks. The total

ensilage period was six weeks.

## Biochemical Analysis

Preliminary biochemical analyses were conducted on fresh alfalfa plants prior to ensiling. These studies consisted primarily of pH determinations and reducing sugar analysis. The methods described here for the green forage, prior to ensiling, were also the same for the silage samples.

1. pH determinations: Distilled water was added to a 10 gram alfalfa sample and was allowed to stand for 30 minutes. The pH was determined electrometrically using a Beckman Model G, battery operated pH meter.
2. Reducing sugar analysis: The method utilized was that of Wiseman et al (32). This method incorporates the use of an ion exchange mixture composed of Dowex 50 and Duolite $A_4$ ion-exchange resins. The purpose being to remove non-carbohydrate reducing substances present in the plants.

The original extraction was made by comminuting a 20 gram silage sample, in a Waring-blender for fifteen minutes, with approximately 400 ml. of 80 per cent ethanol. The extract was clarified with Darco G-60, an activated carbon compound, and filtered. The filtrate was made to a volume of 750 ml. An aliquot was de-ionized and filtered through a glass-wool plug in a powder funnel to remove the resin mixture. The filtrate was evaporated to near dryness on a

steam bath. After evaporation, 25 ml. of 1N hydrochloric acid was added and the sample allowed to stand overnight for inversion. After inversion the sample was neutralized with 20 per cent and two per cent solutions of sodium hydroxide. From this a five ml. aliquot was taken and prepared with a sugar reagent for titration with 0.005N sodium thiosulfite.

## RESULTS AND DISCUSSION

### Moisture Levels

Table I shows the actual results obtained by the laboratory wilting process in attempting to achieve the desired moisture content of the alfalfa plants prior to ensiling.

TABLE I. PROPOSED AND ACTUAL MOISTURE CONTENT OF ALFALFA FORAGE PRIOR TO ENSILING.

| Sample | Proposed Moisture % | Actual Moisture % |
|---|---|---|
| A | 70 | 70 |
| B | 60 | 61 |
| C | 50 | 53 |
| D | 40 | 44 |

Faster wilting would have been achieved by allowing the forage to lay in the field after being cut. For our purpose, however, this was not practical, because of the distance from the laboratory to the field and the necessity of chopping the cut alfalfa before ensiling. It was decided that the wilting process should be carried out under close supervision in order that the forage could be ensiled immediately when the moisture content of the forage neared the proposed level. The difference between the proposed moisture level and the actual moisture content of the forage was possibly caused by the difficulty of obtaining a representative sample. The difference was not great enough to have

an appreciable effect upon these studies.

## Preliminary Studies

The results of the method of sample preparation comparison studies on the green alfalfa are shown in Table II.

TABLE II. COMPARISON STUDIES ON GREEN ALFALFA PRIOR TO ENSILING.

| Date (1958) | Moisture % | Method of Sample Preparation | Groups of Organisms Counted: Coliform (000) | Lactics (000) | Total Aerobes (000) | Proteolytics (000) |
|---|---|---|---|---|---|---|
| June 7 | 70 | Rinse | --- | Spreader | 53 | --- |
| | | Blender | less than 1 | 20 | 59 | 40 |
| June 9 | 72 | Rinse | --- | 10 | 120 | 19 |
| | | Blender | 38 | 70 | 1,300 | 250 |
| June 10 | 80 | Rinse | --- | 14 | 1,600 | 60 |
| | | Blender | 100 | 590 | 7,900 | 3,000 |
| June 11 | 69 | Rinse | --- | 5 | 129 | 25 |
| | | Blender | less than 1 | 16 | 320 | 216 |

It was obvious from the results shown in Table II that comminuting the silage sample in the Waring-blender, as a method of preparing the samples for bacteriological analysis, consistently gave the higher counts. It was apparent, therefore, that the Waring-blender gave a better distribution of the organisms present on the forage. The microflora of green alfalfa was adequate prior to ensiling. It was not a matter of insufficient organisms that determined the quality, but rather the conditions under which the forage was ensiled.

Bacteriological and Biochemical Analysis of Silage Samples

It should be stated that only a presumptive identification of the organisms involved was made and that no attempt was made to identify the organisms as to their species. The presumptive identification was possible because the media, as used, is specific in one respect or another for those groups of organisms. Data on the bacterial counts and pH of the four silage samples, at their respective moisture levels, are shown in Table III.

It was interesting to note from these results that only in sample A did all the groups of organisms reach maximum growth before 72 hours of the ensilage period had elapsed, but in every case except one the organisms had established a peak growth by the end of the first week. The exception was the lactic group in sample D. Maximum growth was not established in this sample until the fourth week of the ensilage period. This can be seen in figure 1. The two groups of organisms in samples B and C were combined because they established peak growth at the same time. The only explanation offered for the lag of the lactic group in sample D was that this sample was of low moisture content (40 per cent). This may have been the primary reason for the lag of that group of organisms in this sample. If this was the case it is obvious then that wilting the forage prior to ensiling, does tend to have an inhibitory effect upon the development of the microflora present on the plants. This was further confirmed by the fact that the numbers obtained, of the various groups of organisms, are proportional to the moisture content of the silage. The moisture content of the silage plays an important part in the development and growth of the microbiological

TABLE III. BACTERIAL COUNTS AND pH VALUES OF SILAGE SAMPLES AT DIFFERENT LEVELS OF MOISTURE.

| Sample | Age (hrs.) | Moisture % | pH | Bacteria per gram of Silage | | | |
|---|---|---|---|---|---|---|---|
| | | | | Coliforms (000) | Lactics (000) | Total Aerobes (000) | Proteolytics (000) |
| | 0 | | 5.90 | 91 | 260 | 1,000 | 80 |
| | 6 | | 5.90 | 66 | 100 | 1,000 | 40 |
| | 12 | | 5.80 | 400 | 4,000 | 4,600 | 350 |
| | 18 | | 5.90 | 4,800 | 32,000 | 54,000 | 450 |
| | 24 | | 5.90 | 3,300 | 139,000 | 220,000 | 2,500 |
| | 30 | | 5.90 | 5,300 | 120,000 | 280,000 | 240 |
| | 36 | | 5.90 | 30,000 | 150,000 | 590,000 | 200 |
| | 42 | | 5.70 | 27,000 | 650,000 | 1,200,000 | 2,600 |
| A | 48 | 70 | 5.30 | 94,000 | 1,100,000 | 1,200,000 | 1,000 |
| | 54 | | 5.50 | 2,300 | 2,620,000 | 1,400,000 | 800 |
| | 60 | | 5.60 | 20,000 | 1,200,000 | 1,100,000 | 6,000 |
| | 66 | | 5.50 | 92,000 | 1,800,000 | 1,700,000 | 1,500 |
| | 72 | | 5.30 | 110,000 | 1,800,000 | 1,400,000 | 4,000 |
| | 96 | | 5.30 | 50,000 | 1,700,000 | 1,300,000 | 1,000 |
| | 120 | | 5.50 | 34,000 | 760,000 | 750,000 | 500 |
| | 144 | | 5.30 | 34,000 | 2,500,000 | 1,700,000 | 50 |
| | 165 | | 5.60 | 45,000 | 1,100,000 | 720,000 | 700 |
| | (weeks) | | | | | | |
| | 2 | | 5.50 | 8,100 | 270,000 | 200,000 | 3,000 |
| | 3 | | 5.20 | 280 | 600,000 | 10,000 | 50 |
| | 4 | | 5.10 | 24 | 120,000 | 130,000 | 11 |
| | 5 | | 5.00 | 19 | 110,000 | 130,000 | 140 |
| | 6 | | 5.00 | 12 | 24,000 | 20,000 | 23 |

TABLE III. (Continued)

| Sample | Age (hrs.) | Moisture % | pH | Bacteria per gram of silage | | | |
|---|---|---|---|---|---|---|---|
| | | | | Coliform (000) | Lactics (000) | Total Aerobes (000) | Proteolytics (000) |
| | 0 | | 5.80 | 450 | 780 | 2,600 | 250 |
| | 6 | | 6.0 | 450 | 1,000 | 1,700 | 25 |
| | 12 | | 6.0 | 190 | 1,000 | 1,800 | 70 |
| | 18 | | 6.10 | 430 | 12,000 | 11,000 | 140 |
| | 24 | | 6.0 | 7,400 | 56,000 | 510,000 | 160 |
| | 30 | | 6.0 | 8,200 | 57,000 | 110,000 | 220 |
| | 36 | | 5.90 | 8,100 | 220,000 | 180,000 | 1,300 |
| | 42 | | 5.90 | 13,000 | 570,000 | 490,000 | 1,100 |
| | 48 | | 6.00 | 29,000 | 460,000 | 410,000 | 400 |
| | 54 | | 5.9 | 12,000 | 430,000 | 320,000 | 1,200 |
| | 60 | | 5.8 | 34,000 | 650,000 | 740,000 | 800 |
| | 66 | | 5.9 | 42,000 | 560,000 | 430,000 | 700 |
| B | 72 | 61 | 5.9 | 136,000 | 840,000 | 570,000 | 6,000 |
| | 96 | | 5.8 | 110,000 | 530,000 | 460,000 | 1,000 |
| | 120 | | 5.8 | 52,000 | 770,000 | 560,000 | 2,000 |
| | 144 | | 5.6 | 18,000 | 1,100,000 | 620,000 | 800 |
| | 168 | | 5.50 | 11,000 | 1,100,000 | 510,000 | 1,200 |
| | (weeks) | | | | | | |
| | 2 | | 5.6 | 1,600 | 260,000 | 170,000 | 110 |
| | 3 | | 5.2 | 198 | 550,000 | 72,000 | 100 |
| | 4 | | 5.2 | 8 | 220,000 | 140,000 | 62 |
| | 5 | | 5.1 | 158 | 130,000 | 130,000 | 270 |
| | 6 | | 5.1 | 65 | 16,000 | 24,000 | 20 |

TABLE III. (Continued)

| Sample | Age | Moisture % | pH | Bacteria per gram of silage | | | |
|---|---|---|---|---|---|---|---|
| | | | | Coliforms (000) | Lactics (000) | Total Aerobes (000) | Proteolytics (000) |
| | 0 | | 5.9 | 190 | 240 | 4,800 | 60 |
| | 6 | | 6.0 | 370 | 1,500 | 1,300 | 70 |
| | 12 | | 6.0 | 870 | 1,800 | 4,600 | 100 |
| | 18 | | 6.0 | 2,800 | 5,100 | 4,100 | 490 |
| | 24 | | 5.9 | 130 | 2,900 | 4,500 | 500 |
| | 30 | | 5.9 | 550 | 9,700 | 29,000 | 500 |
| | 36 | | 5.9 | 660 | 11,900 | 28,000 | 400 |
| | 42 | | 6.0 | 2,100 | 13,000 | 16,000 | 400 |
| | 48 | | 6.0 | 2,600 | 42,000 | 54,000 | 700 |
| | 54 | | 5.9 | 1,500 | 670,000 | 48,000 | 1,000 |
| | 60 | | 6.0 | 1,500 | 56,000 | 55,000 | 500 |
| | 66 | | 5.9 | 6,900 | 91,000 | 110,000 | 500 |
| C | 72 | 53 | 5.9 | 6,100 | 58,000 | 57,000 | 500 |
| | 96 | | 5.8 | 3,500 | 110,000 | 62,000 | 500 |
| | 120 | | 6.0 | 1,400 | 200,000 | 140,000 | 500 |
| | 140 | | 6.0 | 1,300 | 190,000 | 150,000 | 200 |
| | 168 | | 5.8 | 1,400 | 140,000 | 99,000 | 800 |
| | (weeks) | | | | | | |
| | 2 | | 5.7 | 720 | 210,000 | 120,000 | 340 |
| | 3 | | 5.5 | 95 | 210,000 | 49,000 | 60 |
| | 4 | | 5.5 | 13 | 100,000 | 42,000 | 3 |
| | 5 | | 5.4 | 20 | 45,000 | 37,000 | 13 |
| | 6 | | 5.4 | 35 | 17,000 | 16,000 | 4 |

TABLE III. (Continued)

| Sample | Age (hrs.) | Moisture % | pH | Bacteria per gram of silage | | | |
|---|---|---|---|---|---|---|---|
| | | | | Coliforms (000) | Lactics (000) | Total Aerobes (000) | Proteolytics (000) |
| | 0 | | 6.0 | 2,200 | 3,200 | 4,900 | 630 |
| | 6 | | 5.9 | 500 | 1,900 | 4,500 | 170 |
| | 12 | | 5.8 | 3,100 | 4,300 | 4,300 | 120 |
| | 18 | | 5.9 | 740 | 1,300 | 4,200 | 100 |
| | 24 | | 5.9 | 150 | 1,300 | 2,500 | 500 |
| | 30 | | 5.9 | 520 | 3,400 | 4,200 | 200 |
| | 36 | | 6.0 | 1,500 | 11,000 | 9,600 | 700 |
| | 42 | | 6.0 | 1,500 | 8,400 | 6,000 | 100 |
| | 48 | | 5.9 | 4,100 | 14,000 | 12,000 | 500 |
| | 54 | | 5.9 | 2,500 | 10,000 | 15,000 | 1,000 |
| | 60 | | 5.8 | 5,700 | 33,000 | 40,000 | 500 |
| | 66 | | 5.9 | 1,100 | 20,000 | 32,000 | 2,000 |
| D | 72 | 44 | 5.9 | 3,500 | 27,000 | 17,000 | 1,000 |
| | 96 | | 5.9 | 700 | 38,000 | 42,000 | 500 |
| | 120 | | 5.9 | 2,600 | 72,000 | 27,100 | 500 |
| | 144 | | 5.9 | 640 | 33,000 | 21,000 | 400 |
| | 168 | | 5.9 | 1,600 | 47,000 | 63,000 | 200 |
| | (weeks) | | | | | | |
| | 2 | | 5.8 | 1,200 | 37,000 | 27,000 | 340 |
| | 3 | | 5.7 | 120 | 89,000 | 28,000 | 60 |
| | 4 | | 5.6 | 74 | 107,000 | 35,000 | 20 |
| | 5 | | 5.6 | 34 | 30,000 | 29,000 | 17 |
| | 6 | | 5.6 | 15 | 23,000 | 21,000 | 1 |

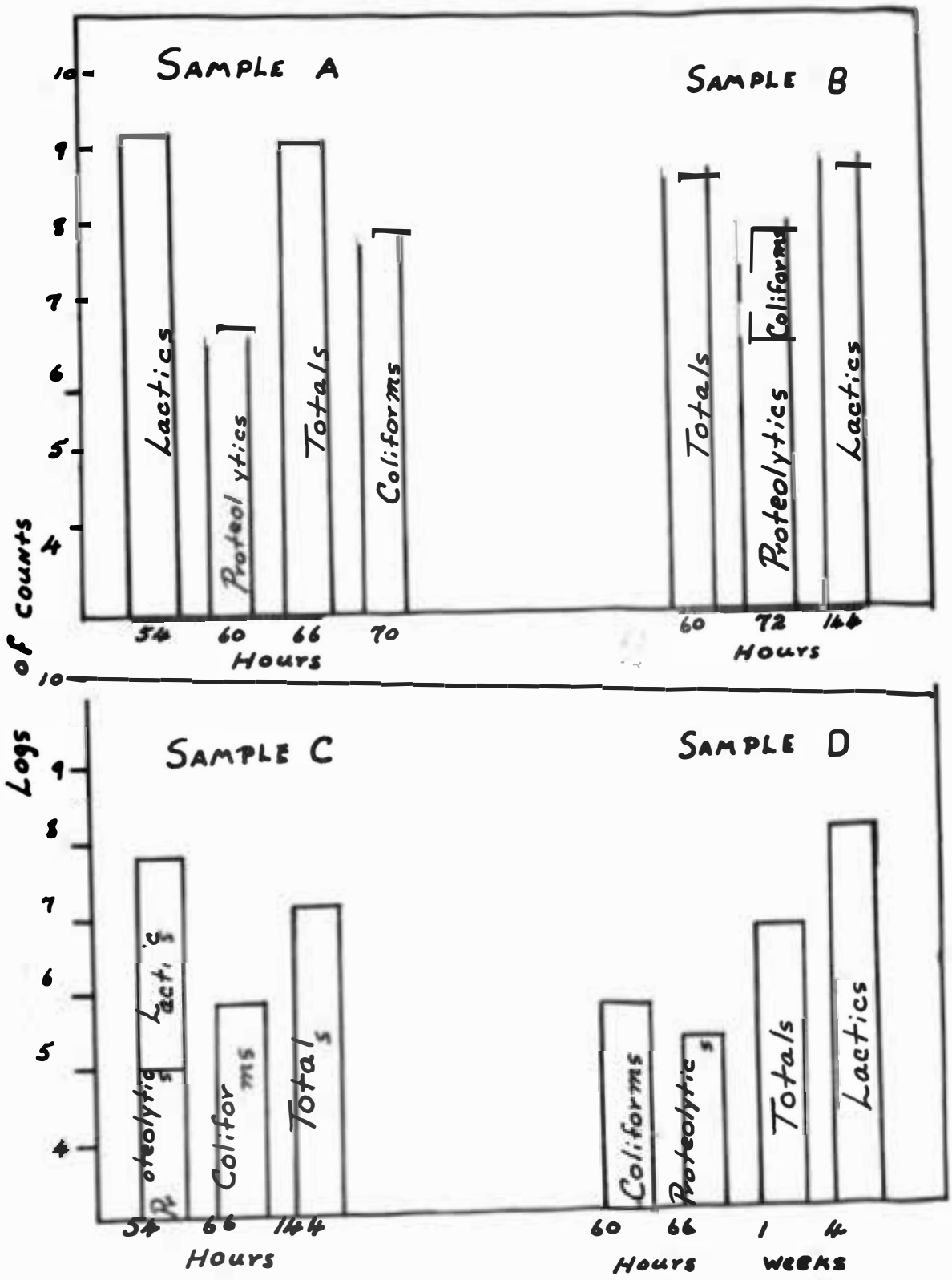


Figure 1. Maximum Numbers of Organisms in the four Silage Samples

flora of the plants (8,28). The relationship of the moisture content of the silage samples to the growth of the four groups of organisms can be seen in figure 2.

It is apparent from these results the growth of the organisms was very unstable during the first three days of the ensilage period. In almost every case the organisms in sample D (40 per cent moisture) showed an initial drop in numbers followed by a reasonably sharp rise. On the other hand the organisms of sample A (70 per cent moisture) all showed a sharp initial rise in growth usually followed by a leveling off or a decline in the numbers of organisms. It is possible to observe leveling off towards the end of the first week in all groups. The coliforms and proteolytics gradually declined throughout the ensilage period whereas the total aerobic and lactic acid producers tended to remain reasonably constant in number.

Also of interest in these studies was the fact that in none of the samples did the pH drop below 5.0. This presented a problem since many authors are of the opinion that the establishment of a low pH plays an important part in the development of a good quality silage (3,7,9,18,24). It was obvious that with a low pH a better preservative action would be imparted to the silage and it would tend to inhibit the growth of proteolytic organisms which are present on the plants. The reason for the failure of the pH to drop to approximately 4.0 was not clear. The pH in the lower moisture level samples remained higher than those in samples of a higher moisture content.

It was noticed, when sampling the jars of silage, after approximately 48 hours from the time of ensiling, that a strong positive

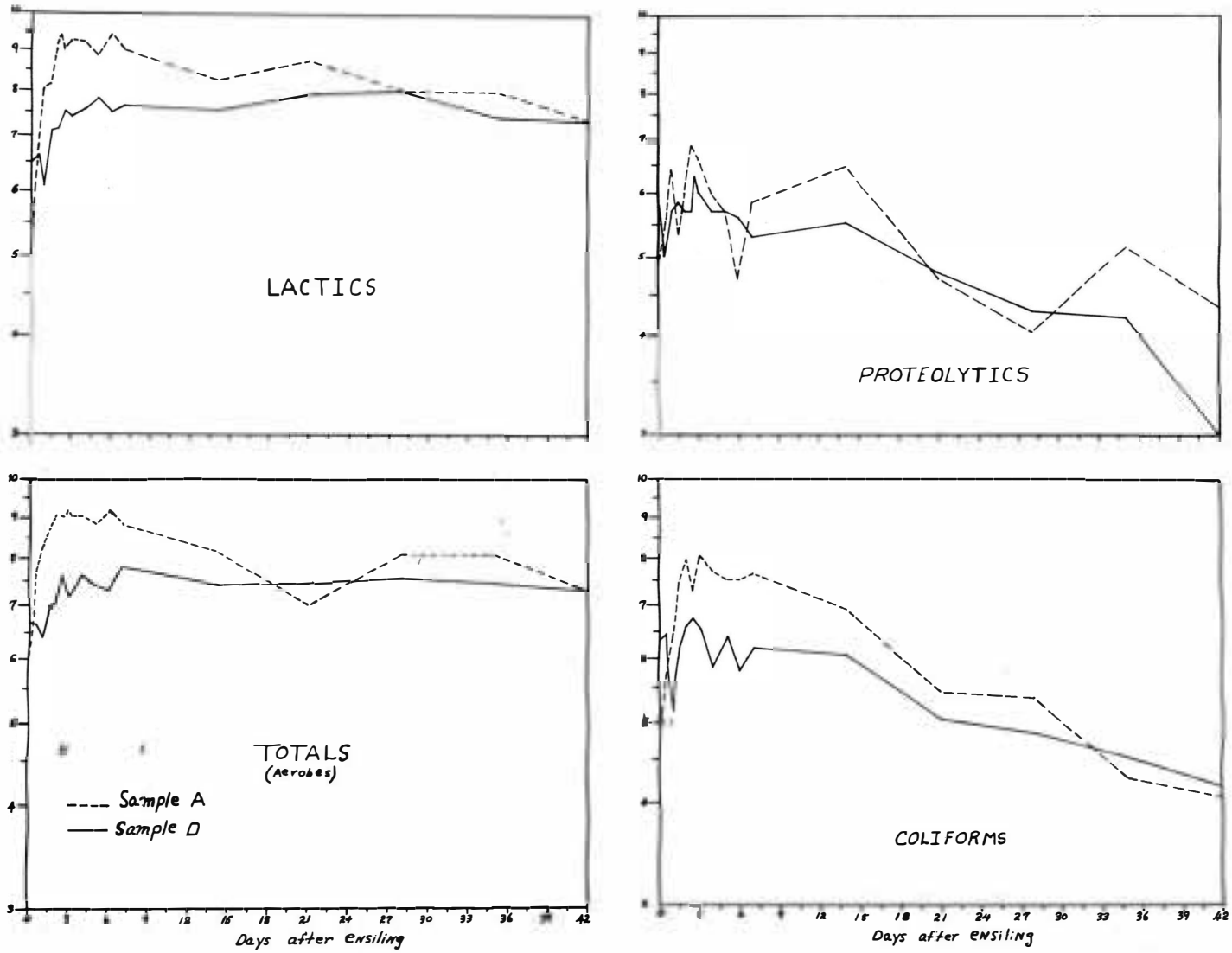


Figure 2. Comparison of the Numbers of Organisms in the High and Low Moisture Samples

pressure was established in the jars. It is very possible that this pressure if not relieved soon enough, could place too great a stress upon the microflora and tend to inhibit them (22). It is possible the type of gas present could also be the inhibiting factor. In future studies involving the use of glass jars as containers for the silage it might be worthwhile to equip the jar lids with some kind of an automatic pressure valve that would allow excess pressure in the jars to escape, thereby resulting in near atmospheric conditions within the containers.

Preliminary studies involving reducing sugar analysis on fresh, green alfalfa plants were run to obtain some idea as to the original reducing sugar content of these plants prior to ensiling. Results of these studies can be seen in Table IV.

TABLE IV. REDUCING SUGAR CONTENT OF ALFALFA PLANTS PRIOR TO ENSILING.

| Date | Sample | Sugar, % Dry matter |
|---|---|---|
| June 18, 1959 | 1 | 5.76 |
| June 22, 1959 | 2 | 3.18 |
| | 3 | 3.71 |
| | 4 | 3.33 |

It was apparent, from the results shown in Table IV, the carbohydrate content of alfalfa forage is not exceedingly high. The difference in the reducing sugar values of sample 1 and the others is

explained by the differences in age of the forage samples. Samples 2, 3, and 4 are four days older than sample 1 which, at the time of analysis, was approximately at the half-bloom stage of maturity. For purposes of comparison, Irvin et al. (16) reported reducing sugar values in alfalfa forage prior to ensiling, ranging from 5.33 per cent to 6.82 per cent. The results of sugar analysis, run on the weekly replicates of the four silage samples, can be seen in Table V.

The results of the fermentation process, in the four samples, were inconsistent. Theoretically, the reducing sugar content of the forage should decrease with the length of the ensiling period. The reasons for this not happening, however, may be caused by several factors. It is possible to experience variations, in individual replicates of a sample, because each replicate was prepared separately from the others. This may have been a result of inferior packing or inadequate sealing of the jar.

It was apparent that the average values of the reducing sugar content and pH values were inversely proportional to the moisture content of the silage sample. Wilting the forage prior to ensiling increased the reducing sugar content of the silage considerably above those samples of high moisture levels. It has been shown that wilting the forage prior to ensiling has an inhibitory affect upon the microflora of the plants. It seems logical, however, that a reduction in the number of beneficial organisms would be less important than inadequate supply of energy.

Some authors are of the opinion (21) that temperature is an

TABLE V. RESULTS OF REDUCING SUGAR ANALYSIS RUN ON THE WEEKLY REPLICATES OF THE FOUR SILAGE SAMPLES.

| Sample | Moisture % | Age (weeks) | pH | Sugar, % Dry matter |
|---|---|---|---|---|
| A | 70 | 1 | 5.5 | 1.73 |
| | | 2 | 5.5 | 0.53 |
| | | 3 | 5.2 | 0.58 |
| | | 4 | 5.1 | 0.64 |
| | | 5 | 5.0 | 0.24 |
| | | 6 | 5.0 | 0.76 |
| | | Average | 5.2 | 0.74 |
| B | 61 | 1 | 5.5 | 1.08 |
| | | 2 | 5.6 | 0.90 |
| | | 3 | 5.2 | 0.55 |
| | | 4 | 5.2 | 0.31 |
| | | 5 | 5.1 | 0.45 |
| | | 6 | 5.1 | 0.17 |
| | | Average | 5.3 | 0.57 |
| C | 53 | 1 | 5.8 | 3.10 |
| | | 2 | 5.7 | 3.30 |
| | | 3 | 5.5 | 2.48 |
| | | 4 | 5.5 | 2.61 |
| | | 5 | 5.4 | 2.37 |
| | | 6 | 5.4 | 2.29 |
| | | Average | 5.6 | 2.69 |
| D | 44 | 1 | 5.9 | 2.88 |
| | | 2 | 5.8 | 3.44 |
| | | 3 | 5.7 | 3.29 |
| | | 4 | 5.6 | 3.57 |
| | | 5 | 5.6 | 2.62 |
| | | 6 | 5.6 | 3.02 |
| | | Average | 5.7 | 2.83 |

important factor in the fermentation process. If the storage temperature is low enough the carbohydrate content of alfalfa forage is adequate to produce a good quality silage. It is conceivable that this may tend to explain the results obtained in this study. If this is the case then it appears as though carbohydrate supplementation is necessary for a consistently good product. Whether or not this is practical for large scale silage production, such as on farms, remains to be seen.

# SUMMARY AND CONCLUSIONS

First cutting alfalfa, approximately at the one-half bloom stage of maturity, was utilized for these studies.

The alfalfa forage was brought to the laboratory for further treatment. This involved chopping to approximately quarter-inch lengths and wilting. The wilting process resulted in four samples at different moisture levels.

The preliminary studies indicated the value of the Waring-blender for the preparation of samples for bacteriological analysis. In every case, bacterial counts were higher using this method of sample preparation.

Maximum numbers of organisms were established faster in the high moisture silage. The total number of organisms were, in every instance proportional to the moisture content of the silage.

The high moisture silage resulted in a sharp initial rise in the number of organisms. This was generally followed by a leveling off or decline in numbers. The initial number of organisms in the low moisture silage characteristically declined. This pattern was followed by an increase in numbers.

Proteolytic and coliform organisms declined slightly throughout the ensiling period.

Lactobacilli and total aerobic organisms tended to level off more and remain constant for the duration of the ensiling period.

Results of the sugar analysis on the silage samples showed inconsistencies in the fermentation process. Average reducing sugar

values for the silage samples were inversely proportional to the moisture content of the silage. This was also true for the average pH values.

The overall results of this study indicate the urgent need for a better understanding of the silage fermentation process. Basic knowledge of the fundamental mechanisms of this process is essential in order that some day good quality silage may be more easily produced.

## LITERATURE CITED


1. Allen, L.A., Harrison, J., "Anaerobic Sporeformers in Grass and Grass Silage", Annals of Appl. Biol., vol. 24, p. 148, Cambridge University Press, London, 1937.

2. _____ and Harrison, J., "The Characters of Some Coliform Bacteria Isolated from Grass and Grass Silage", Annals of Applied Biol., vol.23, p. 528, Cambridge University Press, London, 1937.

3. _____, Harrison, J., Watson, S.J. and Ferguson, O.J.S., "A Study of the Chemical and Bacteriological Changes Occuring in Grass Silage", J. Agr. Sci., vol. 27, p. 270, Cambridge University Press, London, 1937.

4. _____, Watson, S.J. and Ferguson, W.S., "The Effect of the Addition of Various Materials and Bacterial Cultures to Grass Silage at the time of Making of the Subsequent Bacterial and Chemical Changes", J. Agr. Sci., vol. 27, p. 294, Cambridge University Press, London, 1937.

5. Archibald, J.G. and Kuzmeski, J.W., "Further Observations on the Composition of Grass Silage", J. Dairy Sci., vol. 37, pp. 1283-1290, The American Dairy Association, Ohio State University, Columbus, 1954.

6. Autrey, K.M., Knodt, C.B. and Williams, P.S., "Grass and Legume Silage Studies Using 2-Quart Glass Jars as Miniature Silos", J. Dairy Sci., vol. 30, p. 775, The American Dairy Association, Ohio State University, Columbus, 1947.

7. Barnett, J.G., Silage Fermentation, Academic Press Inc., N.Y. 1954.

8. Bender, C.B. and Bosshardt, D.K., "Grass Silage: A Critical Review of the Literature", J. Dairy Sci., vol. 22, pp. 636-654, The American Dairy Association, Ohio State University, Columbus, 1939.

9. _____, Tucker, H.H., Krueger, W.C., Pfau, K.O. and Fox, A.S. "Molasses Hay Silage", J. Dairy Sci., vol. 19, pp.137-146, The American Dairy Association, Ohio State University, Columbus, 1954.

10. Bryant, M.P. and Burkey, L.A., "The Characteristics of the Lactate-Fermenting Sporeforming Anaerobes from Silage," J. Bact., vol. 71, pp.43-46, Williams and Wilkins Co., Baltimore, 1956.

11. Cunningham, A. and Smith, A.M., "The Microbiology of Silage Made by the Addition of Mineral Acids to Crops Rich in Protein: II. The Microflora", J. Dairy Res., vol. 11, pp. 243-265, Cambridge University Press, London, 1940.

12. Difco Manual, 9th. ed., Difco Laboratories Inc., Detroit, 1953.

13. Foster, E.M., Nelson, F.E., Speck, M.L., Doetsch, R.N. and Olson, J.C., Dairy Microbiology, pp. 141-142, Prentice-Hall, Inc. Englewood Cliffs, N. J., 1957.

14. Hegsted, D.M., Quackenbush, F.W. and Peterson, W.H., "Comparison of Alfalfa Silages", J. Dairy Sci., vol. 22, p. 489, The American Dairy Association, Ohio State University, Columbus, 1939.

15. Hunter, O.W., "Bacteriological Studies on Alfalfa Silage", J. Agr. Res., vol. 15, pp. 571-592, United States Government Printing Office, Washington, D.C., 1918.

16. Irvin, H.M., Langston, C.W. and Gordon, C.H., Development of Organic Acids in Silage, (Reprint), United States Department of Agriculture, Washington, D.C., 1956.

17. Kroulik, J.T., Burkey, L.A. and Wiseman, H.C., "The Microbial Populations of the Green Plant and of the Cut Forage Prior to Ensiling", J. Dairy Sci., vol. 38, pp.263-272, The American Dairy Association, Ohio State University, Columbus, 1955.

18. Langston, C.W., Irvin, H., Gordon, C.H., Bouma, C., Wiseman, H.C., Melvin, C.G., Moore, L.A., and McCalmont, J.R., "Microbiology and Chemistry of Grass Silage", Technical Bulletin No. 1187, United States Department of Agriculture, Washington, D.C., 1958.

19. Stevens, W.B. and Kuhlman, A.F., "Alfalfa Silage", J. Dairy Sci., vol. 19, pp. 611-617, The American Dairy Association, Ohio State University, Columbus, 1936.

20. Nilsson, P.H., "Some Characteristics of the Silage Microflora", Archiv. Für Mikrobiologie, Bd. 24, S. 396-411, Springer-Verlag, Berlin, 1956.

21. Nilsson, R., Tóth, L. and Rydin, C., "Studies on the Fermentation Processes in Silage: I. The Role of Temperature", Archiv. Für Mikrobiologie, Bd. 23, S. 366-375, Springer-Verlag, Berlin, 1956.

22. Porter, J.R., Bacterial Chemistry and Physiology, p. 203, John Wiley and Sons, Inc., New York, 1946.

23. Rosenberger, R.F., "The Development of Methods for the Study of Obligate Anaerobes in Silage", Proc. Soc. Appl. Bact., vol. 14, p. 161, The Society of Applied Bacteriologists, Crown Press, Reading, England, 1951.

24. Rydin, C., "Starch as a Source of Carbohydrates for the Lactic-Acid Fermentation", Archiv. Für Mikrobiologie, Bd. 27, S. 82-104, Springer-Verlag, Berlin, 1957.

25. _____, Nilsson, R. and Tóth, L., "Studies on Fermentations Processes in Silage: II. The Effect of Various Carbohydrates as Supplements", Archiv. Für Mikrobiologie, Bd. 23, S. 376-384, Springer-Verlag, Berlin, 1956.

26. Standard Methods for the Examination of Dairy Products, 10th. ed. American Public Health Association, Inc., New York City, 1953.

27. Stirling, A.C., "Bacterial Changes in Experimental Laboratory Silage", Proc. Soc. Appl. Bacteriol., vol. 14, p. 151, The Society of Applied Bacteriologists, Crown Press, Reading, England, 1951.

28. _____, "Lactobacilli and Silage Making", Proc. Soc. Appl. Bact., vol. 16, p. 27, The Society of Applied Bacteriologists, Crown Press, Reading, England, 1951.

29. Stone, R.W., BachDel, S.I. and McAuliffe, H.D., "The Fermentation of Alfalfa Silage", Pa. Agr. Expt. Sta. Bul., No. 444, Pennsylvania State College, College Station, 1943.

30. Tóth, L., Rydin, C. and Nilsson, R., "Comparisons of Different Types of Forage Crops", Archiv. Für Mikrobiologie, Bd. 25, S. 208-218, Springer-Verlag, Berlin, 1956.

31. Watson, S.J. and Ferguson, W.S., "The Chemical Composition of Grass Silage", J. Agr. Sci., vol. 27, p. 1, Cambridge University Press, London, 1937.

32. Wiseman, H.G., Mallack, J.C. and Jacobson, W.C., The Determination of Sugar in Silages and Forages, (Reprint), United States Department of Agriculture, Washington, D.C., 1958.