BACTERIAL, BIOCHEMICAL AND ENVIRONMENTAL INTERRELATIONS IN FRESH AND ENSILED FORAGES

By

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AN ABSTRACT

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This investigation was undertaken to make simultaneous bacterial, biochemical and environmental studies on a wide range of fresh and ensiled forages, using farm-sized silos as experimental vehicles.

The predominant bacteria on all fresh forages were classified as facultative-anaerobic species of Flavobacterium, on the basis of pigment production, and morphological and fermentative studies. Less than 0.1 per cent of the bacteria on the crop at the time of ensiling were capable of growing on lactobacillus selection medium.

In spite of differences in plant species, weather conditions, wilting and other harvesting procedures, the number of bacteria on the fresh crops could be predicted with reasonable error solely from the harvesting date. The predominant Flavobacterium type increased from 10^8 per gram of dry matter in late May, to 10^{10} in early August. The lactic acid bacteria showed a parallel increase from 10^4 to 10^6. However, it was found that the initial number of bacteria on the fresh crop bore no relationship to the final quality of the silage.

A significant correlation between the moisture content and pH of fresh crops was noted, in spite of differences in crop species. For crops of 60 per cent moisture, the regression line passed through a pH of 6.2 but falls to a pH of 5.7 when

the moisture content rises to 85 per cent. Since the low pH values of high moisture crops were not associated with higher numbers of acid-producing bacteria, it was concluded that plant enzymes may be more active in high moisture crops than in low moisture crops.

Silage quality was determined primarily by the amount of packing the silage received. Loosely packed silages overheated, but underwent an acetic acid fermentation. Well-preserved silages were optimumly packed, and contained as much as 300 micromoles of lactic acid per gram of fresh weight. When silage was packed too tightly, vegetative cells of Clostridium tyrobutyricum could be isolated immediately after ensiling. Although tightly-packed silages contained about 100 micromoles of lactic acid after 2-3 days in the silo, all the lactic acid subsequently disappeared, to be replaced by butyric acid. During spoilage, succinic acid also disappeared and some propionic acid was produced. Crops with a low dry matter content tended to undergo butyric spoilage only because they were more apt to be overpacked.

Excess production of volatile base could not be associated with any particular organism, but always occurred when the total hydrolysable carbohydrate dropped below 1 per cent of the fresh weight. High moisture crops tended to undergo a volatile base type of spoilage because the carbohydrate content was initially diluted.

Therefore, one silage was tightly packed and a butyric spoilage developed, but since the final carbohydrate content was 1.3 per cent, no excessive volatile base was produced.

Although Cl. tyrobutyricum developed in a bisulfite silage because it was too tightly packed, the bisulfite inhibited the formation of butyric acid. Bisulfite also inhibited the normal utilization of carbohydrate; hence, an appreciable amount of proteolysis and deamination occurred even though the carbohydrate content was above the critical 1 per cent.

The Flavobacterium group on the fresh forage did not multiply in the silage process, but did persist throughout the storage period in spite of the accumulation of acid.

The lactic acid bacteria increased tremendously soon after ensiling, frequently approaching 10^9 per gram of fresh material. Thereafter there was a relatively rapid decrease to an average of about 10^5 after three weeks in the silo. In most silages this was followed by a secondary fermentation which reached a peak of about 10^7 in four to five weeks, and subsequently declined. This pattern was found in both well-preserved and spoiled silages. Lactic acid bacteria did not develop at all in the overheated silages.

The lactic acid bacteria of fresh and ensiled forages were very variable morphologically. On a fermentative basis, they divided equally into two main types; one which fermented

all test carbohydrates and litmus milk, and one which fermented only simple sugars. There were no detectable differences in the lactic flora of fresh crops and of the primary and secondary fermentations.

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CHAPTER I

INTRODUCTION

Increasing emphasis on the practice of ensiling grasses and legumes has stimulated efforts to develop a method of ensiling which would consistently produce palatable silage of high nutritive value.

Silage preservation depends primarily on the attainment of a pH low enough to restrict the activities of spoilage bacteria. Providing there is sufficient fermentable carbohydrate present, the lactic acid produced by the metabolism of the indigenous microflora should lower the pH below the critical value of 4.0. Legumes are difficult to ensile because they have a low content of natural carbohydrate; hence several attempts to assure the production of good quality silage have been based on the addition of various carbohydrate sources such as molasses, beet pulp or whey.

The mechanical adjustment of pH by the addition of mineral acids has been practised extensively in Europe, and recently (Knodt et al. 1952) the control of spoilage bacteria by the use of selective bacteriostatic agents has been advocated.

It is also apparent that the efficacy of the fermentation is controlled by the moisture content of the crop. Within

limits, the moisture content can be controlled by wilting high-moisture crops before ensiling, or if necessary, water can be added during ensiling.

Unfortunately, no single method of making silage has been consistently satisfactory over a wide enough range of field conditions to be universally acceptable; hence spoiled silage still accounts for a substantial loss of farm income.

Successive failures have partially been due to a lack of fundamental knowledge of silage bacteriology. Although the predominant types of bacteria found at various stages of the fermentation have been briefly characterized, the precise role of each type has not been determined, nor have they been fully classified. Silage quality has often been correlated with environmental conditions or the chemical products of bacterial action, but the direct relationship between silage quality and the bacterial population requires further clarification. Furthermore, it has never been definitely proved that the fermentation occurring in large silos is identical with the fermentations in the miniature silos studied by most workers.

Consequently, this present investigation was undertaken in an attempt to interrelate the quality of forage crop silage with the bacterial and biochemical composition of the fresh and ensiled crops, and the environmental conditions at the time of ensiling. By sampling the fermentations which took place in a number of farm-sized silos located in Michigan's

Eaton and Ingham counties, a wide range of field conditions was included in this experiment.

The results of this study are presented and discussed in two main parts. The first part deals with the salient environmental conditions causing variation in the bacterial and chemical nature of fresh forages at the time of ensiling. The progressive changes occurring during the ensuing fermentations are presented in the second part.

CHAPTER II

LITERATURE REVIEW

The Bacterial Flora of Fresh Plants Prior to Ensiling.

The microorganisms responsible for silage fermentation are on the fresh plant material before ensiling. However, attempts to demonstrate the presence of typical silage lactic acid bacteria on fresh plants have produced conflicting conclusions.

Using dilution techniques, Allen and Harrison (1936b) identified the predominant organism on grass as Lactobacillus plantarum,* the same organism as found in silage. Stone et al. (1943) also concluded that fresh alfalfa contained large numbers of lactobacilli. Conversely, Stirling (1953) and Kroulik et al. (1955a) found that newly developed media which were selective for lactobacilli supported the growth of only a very small proportion of the organisms found on fresh plants. The few organisms Kroulik and co-workers did isolate did not belong to the groups of active acid-producing lactobacilli typical of the silage flora. Stirling was able to demonstrate

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*The term Lactobacillus plantarum will be used throughout to refer to the group of organisms Bergey's Manual (Breed et al. 1957) includes in this single species. Individual authors may have originally used a term now recognized as a synonym.

the presence of a small number of lactobacilli on a variety of forage crops regardless of the season or geographical location.

Kroulik et al. (1955a) characterized the predominant flora of fresh plants as chromogenic, aerobic, non-sporeforming rods which hydrolysed starch but did not generally produce acid from carbohydrates. Thomas (1950) believed that these chromogenic organisms were facultative anaerobes, and he further concluded that typical silage bacteria were derived from organisms found on fresh plants through the action of specific lysins found in plant juice. Similar yellow colonies from cotton plants have been identified as Xanthomonas malvacearum by Clark et al. (1947). The role played by these organisms in the silage process is unknown. Kroulik suggested that they may aid in the development of the anaerobic conditions necessary for acid production.

Since the type of fermentation may be a function of the number of bacteria on the fresh crop, Kroulik et al. (1955a) studied the effect of various environmental conditions on the total number of bacteria on alfalfa. They found that a substantial increase in the bacterial population accompanied an increase in the maturity of the plants, and that these aerobic, chromogenic bacteria increased in numbers if the crop was wilted. Variations in the bacterial population with the kind of plant, part of the plant, time of day and the season were less specific. As the season progressed, the

population became somewhat more varied and included a number of lighter yellow, buff, pink and brown colonies.

There are many coliform-like bacteria on fresh grasses. Allen et al. (1936a) classified this group as Bacillus aerogenes grammis because of their optimum temperature of 30°C, while Kroulik et al. (1955a) classified them as Aerobacter cloacae on the basis of their ability to liquefy gelatin.

Clostridium sporogenes has been implicated as a spoilage organism in silages which evidence a marked degree of proteolysis. Small numbers of this organism have been found on fresh grass by Allen and Harrison (1937).

The Chemical Composition of Fresh Forage Crops.

Related to the production of silage, carbohydrates are the most important constituents of fresh forages. Since green plants contain relatively small amounts of the simple sugars, the storage carbohydrates must participate in the formation of the large amounts of lactic acid produced in the conversion of fresh crops to good quality silages. Starch has long been considered the principal reserve carbohydrate of plants. However, Laidlaw and Reid (1952) found very little true starch in grasses and clovers, but considerable amounts of a 2-6 linked levan type of fructosan. Percival (1952) concluded that this fructosan was the reserve carbohydrate utilized in the production of silage. Laidlaw and Reid (1951)

also chromatographically identified glucose, fructose, sucrose, some oligo-saccharides, hemicellulose and cellulose in rye grass.

According to Watson (1949), the total carbohydrate expressed as "starch equivalent" increases as the plant matures. On the other hand, Barnett (1954) quoted several reports which indicated that the fructosan content apparently reaches a peak at the flowering stage and subsequently decreases as the plant matures. It may be significant that the peak fructosan content corresponds to the time that the crop is most ideal for silage making.

After photosynthesis has been retarded or stopped, plant enzymes continue the hydrolysis of reserve carbohydrate to simple sugars. Hence, if plants were cut and allowed to wilt in the field, especially overnight, there would be reason to expect an increase in the amount of simple sugars at the expense of the complex storage carbohydrates. The greater production of lactic acid observed in silages made from wilted crops has been ascribed to this higher level of simple sugars which are easily fermented. Brown (1893), Molisch (1921), Horn (1923), Ritschl (1929), Dickey et al. (1942), Stone (1943) and Speer (1950) all recorded sizable increases in reducing sugar content after wilting, with subsequent decreases in "starch" content. On the other hand, Wilson (1948) carried out exhaustive wilting experiments with several different crops and concluded that wilting did not

cause a measurable increase in fermentable sugar.

During the wilting process, considerable carbohydrate may be lost due to complete respiration to carbon dioxide and water. However, a decrease in pH during wilting would indicate the formation of organic acids by respiratory enzymes, in which case the carbohydrates would not have been wasted. Although Wilson (1948) did observe slight decreases in pH after wilting, this decrease could be accounted for by an increase in carbon dioxide as postulated by Small (1946). Small has also listed the pH values of the juices of plants and of various parts of plants.

Truog and Meacham (1919) studied the pH of plant juices which had been grown on limed and unlimed soil and harvested under various environmental conditions. The pH varied only within 0.2 of 1 pH unit regardless of the soil reaction and the amount of cloud cover when harvested. The juice was even allowed to stand for several hours without a change in pH being recorded.

Watson (1952) recorded increases in the per cent fibre and dry matter as grasses mature, with a resultant decrease in the per cent protein.

Fresh plant material does contain some acids. Annett and Russell (1908) have demonstrated the presence of small amounts of malic and succinic acids. Turner and Hartman (1925) added citric and malonic to this list, while Davies and Hughes (1954) were able to show the presence of acetic, lactic,

succinic, malic, citric and malonic acids by the use of chromatography. The malic acid content increased during the growth of the plant while citric acid decreased, but these two acids together accounted for 50 per cent of the plant acidity at every stage of growth (Davies and Hughes 1954). In addition to the above acids, Hulme and Richardson (1954) found traces of quinic and chlorogenic acids in meadow foxtail and meadow fescue.

Carbohydrate Metabolism in Silage.

During the course of silage fermentation, the carbohydrates of the fresh plants are metabolised to carbon dioxide, alcohol, volatile and non-volatile organic acids.

Previous to 1921, there was considerable disagreement over the relative role of plant and bacterial enzymes in carbohydrate metabolism. Babcock and Russell (1900) (1901) believed that plant enzymes were the main agents of fermentation because acids were formed even after the addition of antiseptics. On the other hand, Esten and Mason (1912) believed that the rapid multiplication of bacteria and yeasts which occurred during the silage fermentation must have been responsible for the parallel increase in acidity, and they also recognized the fact that these acids were important preservative agents. Samarani (1913) and Lamb (1917) agreed that respiration of plant cells could account for the alcohol produced early in the fermentation, but that

lactic acid was produced from carbohydrates by microorganisms. Samarani also postulated that acetic acid arose from the respiratory oxidation of alcohol. Still other workers (Sherman and Bechdel, 1918) admitted that their results were inconclusive.

In 1921, Hunter published conclusive proof that lactobacilli produced most of the acidity in a typical fermentation. He showed that the increase in acid production was always accompanied by an increase in acid-producing bacteria. No acids were produced in forage treated with chloroform, but when heat-sterilized forage was inoculated with fresh silage juice containing large numbers of microorganisms, a normal fermentation was resumed. Peterson et al. (1925) believed that alcohol in corn silage was also an end product of bacterial metabolism. As late as 1943, Pratolongo believed that "autolytic" plant enzymes produced lactic acid in silage, but the generally accepted view was recently summarized by Watson (1949), who stated that the respiration process results chiefly in the formation of large volumes of carbon dioxide.

Watson (1949) quoted Virtanen as having said that respiratory losses as carbon dioxide are inhibited by the addition of mineral acids at the time of ensiling, but Watson's own experience did not sustain this claim.

Alcohol is usually present in silage between the levels of 0.2 per cent and 0.8 per cent. Determinations on corn

silage showed that 72 per cent of the alcohol was present as ethanol, 21 per cent as methanol and 7 per cent as propanol (Watson 1949).

Of the organic acids present in silage, acetic, lactic, propionic, butyric and pyruvic acids are formed by the bacterial degradation of carbohydrates. Butyric acid may be formed directly from sugars or by the secondary fermentation of lactic acid. Valeric and other longer chain fatty acids are probably derived from amino acids by deamination (Barnett, 1954).

Silage quality has often been directly correlated with the amount of lactic acid present, and inversely correlated with the amount of butyric and higher acids. In a summary of many years experience, Archibald (1954) has stated that good silage has a lactic acid content of 3 to 5 per cent or more and a butyric acid content of 2 per cent or less, expressed on a dry weight basis. Barnett (1954) believed that as much as 8-9 per cent of the dry weight may be lactic acid, while Watson (1949) expressed the attainable limit of lactic acid as 2 per cent of the fresh weight, with 1 per cent of the fresh weight as the minimum for good quality silage. According to Barnett and Duncan (1953) even good quality silages may have a small amount of butyric acid. However, Barnett and Miller (1951) had shown that the presence of butyric acid in otherwise good silage did not affect its palatability, from which they concluded that although poor

silages have large amounts of butyric acid, their unpalatability stems from other side effects which accompany a butyric fermentation.

Lactic acid is a much stronger acid than acetic or butyric; hence its desirability in silage is a consequence of its ability to produce a low pH. Virtanen (Barnett, 1954) had found that proteolytic spoilage did not occur when the pH was lower than 4.0 while the production of undesirable volatile acids by clostridia and coliforms was inhibited below a pH of between 4 and 5. In general, good silage is characterized by a pH of 4.5 or less, the nearer to 4.0 the better (Archibald 1954). Barnett (1954) arrived at pH 4.2 as the critical value for silage preservation.

Nevertheless, the pH is not always an indication of quality. According to Lind (1953), lactic acid may be produced slowly in some silages, allowing a degree of butyric fermentation and proteolysis to occur before a suitably low pH has been attained. Spoilage of this type can be overcome by the addition of mineral acid at the time of ensiling, which lowers the pH below 4.0 immediately. McLean (1941) cautioned against the use of pH as an indicator of quality in overheated silages, which have very little nutritive value even though they may contain considerable lactic acid and have low pH values. On the other hand, legume silages rarely attain the critical pH, although the quality may be good. Watson (1949) believed that high pH values of

legume silages are due to the presence of buffering agents, notably calcium, but he admitted that this question requires further research.

The relationship between the pH and the various organic acids present can be illustrated by data presented by Watson (1949). The amount of lactic acid increased from 0.1 per cent at pH 5.0 to 2.2 per cent at pH values below 4.0. Conversely, the total volatile acids decreased from 1.5 per cent to 0.75 per cent over the same pH range. Between pH 4.0 and 4.5, the volatile acids were chiefly acetic, but the increase in volatile acid between pH 4.5 and 5.0 was largely due to butyric acid. However, molassed or acidified silage can exist at pH 5.0 with relatively less butyric acid than untreated silage. Common (1941) found that the buffering capacity of silage extracts was mainly determined by the ratio of volatile to non-volatile acids, and he has devised a formula relating the pH to the type of acids present. The relative amounts of volatile and non-volatile acids also accounted for the variations in the relationship between titratable acidity and pH observed by Pederson and Bagg (1944).

Stone (1943) has said that if the pH is not below 4.2 and there is no reserve sugar, the lactobacilli will attack lactic acid itself and the silage will not keep. This statement was made because it had been observed that poor quality silages made without preservatives or wilting had

little residual sugar. Archibald (1953) had also found more residual sugar in silages which had had preservatives added.

In particular, the residual sugar in SO_2 preserved silage was almost as high as the original sugar content. Archibald (1953) concluded that SO_2 prevented the breakdown of sugars, but did not prevent the utilization of cellulose and other complex carbohydrates. Knodt et al. (1952) claimed that the increased amount of residual sugar and the decreased production of lactic acid amounted to a saving in nutrient value in SO_2 treated silages. However, in 1930 Kirsch and Hildebrand (quoted by Watson 1939) had pointed out that lactic acid had almost as great a feeding value as glucose itself.

Barnett (1954) listed the total hydrolysable carbohydrate content of several silages. All values ranged between 5.30 and 9.65 per cent of the dry matter. Petersen et al. (1925) detected approximately 2 per cent reducing sugar even after 146 days in the silo. They concluded that the reducing sugar was probably glucose since a glucosazone was obtained with phenylhydrazine, but it may also have been fructose.

Nitrogen Metabolism in Silage.

There are three principal aspects to the study of nitrogen metabolism in silage, namely: (1) the loss of "crude" protein, (2) the conversion of "true" protein, and (3) the relationship between the end products of nitrogen metabolism and silage quality.

In a study of corn silage, Peterson et al. (1925) found that 14.7 per cent of the total nitrogen of the fresh plant was lost after 145 days in the silo, and their survey showed that four previous reports had listed losses of approximately the same order. Similar studies on hay crop silages were conducted by Taylor et al. (1940) who recorded losses of total nitrogen (expressed as crude protein) in the range of 12.8 to 17.3 per cent.

Some loss is unavoidable since the bacteria which produce the acid necessary for silage preservation require nitrogen for growth. In fact, nitrogen may have to be added to crops which have a low content of natural protein. For example, Cullison (1944) showed that a rapid fermentation of sweet sorghum could only be achieved by adding urea. However, Barnett (1954) has expressed doubt that the addition of urea to protein-rich crops would reduce nitrogen loss unless it could be proved that silage organisms preferred urea to plant protein. Urea added to grass actually produced an unpalatable, high pH silage (Archibald 1946).

Nitrogen losses were reduced when an earlier lactic acid fermentation was induced by crushing or macerating the plants (Barnett 1954).

Working with bottled grass sap, MacPherson (1952) found that 80 per cent of the nitrogen of fresh grass was contained in insoluble "true" proteins, whereas the nitrogen of the fermented product was only 60 per cent protein. The increase

in soluble nitrogen from 20 to 40 per cent was essentially all accounted for by increases in amino nitrogen and ammonia; hence, it was concluded that 25 per cent of the true protein was hydrolysed. Although Peterson et al. (1925) recorded a similar increase in soluble nitrogen in corn silage there was less proteolysis, since precipitation with tungstic acid indicated that approximately half of the soluble nitrogen was still protein in nature.

MacPherson (1952a) noticed that the bulk of protein breakdown occurred in the short time required for the pH to fall to a value between 4.5 and 5.0. He, therefore, concluded that proteolysis could be reduced by rapid acidification with mineral acid. Virtanen had already proved that this could be done on a large scale using HCl (Barnett 1954). In Virtanen's experience, no proteolysis occurred at pH 3.6. Watson and Ferguson (1937) concurred. The concentration of HCl used could not itself hydrolyse various vegetable proteins (Schmalfuss and Werner 1941).

Archibald (1946) found that losses of both true and crude protein were generally less when carbohydrates were used as preservatives. On the other hand, MacPherson (1952a) reported that proteolysis was unaffected by carbohydrate level.

Protein losses were greater when the crop was allowed to wilt (Swanson and Tague 1917) (MacPherson 1952b) (Autrey et al. 1947).

Even if much of the protein is hydrolysed to amino

acid , there is no great loss in feeding value. Kirsch and Jantzon (1933) showed that cattle responded equally well to diets of clover hay or clover silage which contained equivalent amounts of nitrogen, even though the silage contained less protein and more non-protein nitrogen. However, breakdown of amino acid is always indicative of spoilage. Deamination will produce ammonia and high molecular weight carboxylic acids such as isovaleric (Lind 1953). Decarboxylation produces other volatile basic amines. Archibald (1954) stated that the volatile base content expressed as ammonia should not exceed 0.5 per cent of the dry matter.

The relationship between pH and volatile base content has been summarized by Watson (1949). Where preservatives were used, the volatile bases accounted for less than 4 per cent of the total nitrogen at pH 4.0, increasing to 12 per cent at pH 5.0. In silages without preservatives, or in moldy silages, the volatile base content was generally higher, amounting to as much as 25 per cent of the total nitrogen at pH 5.0.

The relationship between silage quality and volatile base content has been measured in various ways. Watson (1939) estimated silage quality by the ratio of volatile base to amino acid. However, this ratio is misleading when applied to silages prepared by the addition of mineral acid, which have an unnaturally low amount of amino acid owing to the lower degree of initial proteolysis. Lind (1953)

proposed that the ratio of volatile base to total nitrogen be used to indicate the extent of protein loss. Barnett and Miller (1951) demonstrated the close relationship between the pepsin digestibility of a dried silage sample and the ratio of soluble to total nitrogen. In several cases, notably with overheated silages, this ratio gave a better indication of silage quality than the pH.

Bender et al. (1941) discovered that ammonia production was negligible in silages which had received added carbohydrate in the form of molasses. They concluded that fermentable carbohydrate suppressed ammonia formation. Barnett (1954) believed that the presence of fermentable carbohydrate directly inhibited the formation of deaminating enzymes irrespective of the pH. He therefore reasoned that a high residual carbohydrate level may explain the anomalous position of those silages which have a high pH yet are of good quality.

Hunter (1921) proved that carbohydrate breakdown was due to bacterial action. Using the same techniques, he showed that plant enzymes were responsible for most of the proteolysis in normal silages. MacPherson (1952a) expressed similar beliefs. By ensiling grass grown in a sterile chamber, Mabbitt (1951) was able to prove that both amino acids and volatile bases could be formed by plant enzymes. On the other hand, Peterson et al. (1925) believed that most changes in the nitrogenous constituents were due to bacterial

activity. Compromising, Barnett (1954) held that plant enzymes were responsible for the breakdown of protein to amino acids, but that putrefactive bacteria produced the excessive ammonia found in poor silages. Similar views had been expressed by Watson (1949).

Silage Microbiology.

Almost immediately after crops are ensiled, there is an enormous increase in bacterial numbers. Working with corn, Peterson et al. (1926) reported a 100 fold increase in the first 24 hours, and a further 1000 fold increase during the second day. A maximum count of 100 billion organisms per c.c. of silage juice was recorded on the seventh day. Thereafter, there was a gradual decline in numbers although there were still 10 million active bacteria per c.c. after 132 days. Similar changes in the bacterial population of grass and legume silages have been noted by Allen et al. (1937b), Stirling (1951) and Kroulik (1953).

Normal bacterial development is modified by wilting and the use of various additives. Stirling (1951) and Kroulik et al. (1955b) have shown that the bacterial population increases more slowly in silages made from wilted grass. However, since the wilted forage was well preserved, Kroulik concluded that the greater microbial activity in the high moisture silage was apparently not essential or even desirable. Stirling found that bacterial multiplication was stimulated

when the contents of the plant were made more accessible by maceration.

Allen et al. (1937a) studied the bacterial changes in silages which had received additions of molasses, mineral acids, whey and bacterial inocula. They found that the numbers of microorganisms were considerably higher than those observed previously in normal grass silage. Some increase in bacterial activity was expected due to a higher moisture content, but their data also indicated that silages could support higher numbers of bacteria when more carbohydrate was added. Kroulik et al. (1955a) were also able to show that lactic acid bacteria were favored by an abundance of carbohydrate.

It had been shown that the addition of mineral acids reduced, but did not eliminate bacterial activity (Cunningham and Smith 1939). When Knodt et al. (1950) introduced sulfur dioxide as a silage preservative, they believed that all fermentation would be inhibited by the formation of sulfurous acid. However, Alderman et al. (1955) concluded that the sulfite ion itself was bacteriostatic since some sulfur dioxide silages, although well preserved, had pH values above the critical limit of 4.0. Kroulik et al. (1955a) found that sulfur dioxide did not hinder the rapid development of lactobacilli in the first days of ensiling, but did inhibit subsequent bacterial activity. They concluded that sulfur dioxide selectively inhibited undesirable and unnecessary

bacteria.

Peterson et al. (1925), Thomas (1950) and Burkey et al. (1953) all agreed that the typical chromogenic organisms of the fresh plant disappeared with the increase in acidity.

Acetic acid was observed in silage before the main lactic fermentation had begun. Heineman and Hixson (1921) believed that this was the result of coliform activity. Allen et al. (1937b) identified the coliforms in grass silage as a type which would not grow at temperatures much above 30°C.; hence they believed that these organisms were destroyed by the normal temperature rise in the preliminary stage of the ensilage process. Burkey et al. (1953) claimed that it was the sharp decrease in pH which eliminated the coliforms as well as the chromogenic bacteria, since both groups persisted longer in silages made from wilted forages where acid development was slower. Hall et al. (1954) suggested that coliform bacteria may be susceptible to the lactic acid itself.

According to Allen and Harrison (1936b), most of the lactic acid is produced by strains of Lactobacillus plantarum. These workers placed the majority of 152 strains of silage lactobacilli in this species on the basis of their homofermentative degradation of glucose to inactive lactic acid and their comparative inactivity in litmus milk. L. plantarum has also been implicated in silages made with mineral acids (Cunningham and Smith, 1940), sugar beet pulp silage (Olsen, 1951) and sweet potato vine silage (Hall et al., 1954).

The strains of L. plantarum isolated by Allen and Harrison (1936b) showed considerable differences in ability to ferment carbohydrates. Cunningham and Smith (1940) found that the L. plantarum in mineral acid silage did not exhibit the preference for sucrose and maltose over lactose which Orla-Jensen had originally ascribed to this species. Working with mixed cultures of silage organisms, Salsbury et al. (1949) showed that simple sugars were generally fermented more readily than oligo- and polysaccharides.

Salsbury et al. (1949) believed that the comparatively low acid production from lactose by silage microorganisms might explain why dried whey was a less desirable preservative than molasses. However, Allen et al. (1937a) found that the lactobacilli isolated from silages made with whey differed from strains of L. plantarum previously isolated from untreated silages in that they could readily ferment lactose and rapidly produce an acid curd in litmus milk.

Fred et al. (1921) and Rogosa et al. (1953) believed that the L. plantarum species could be subdivided mainly on the basis of pentose fermentation. However, Bergey's Manual (Breed et al. 1957) has upheld Pederson's decision (1936) that the fermentation differences of various strains were not important enough, or constant enough, to warrant the creation of new species. Politi (1940) has proposed Lactobacillus sili as a new species for those silage lactobacilli which cannot be readily related to L. plantarum.

Peterson et al. (1925) found that most of the silage lactobacilli fermented xylose. These workers also reported that L. plantarum produced equimolar quantities of acetic and lactic acid from pentoses. The isotopic studies of Gest and Lampen (1952) have shown that this is accomplished by a splitting of the bond between carbons 2 and 3.

In a later publication, Peterson et al. (1928) showed that members of the L. plantarum group could use protein as a source of carbon with the liberation of ammonia. Gorini (1940) believed silage was rendered more digestible by the formation of amino acids from protein by certain strains of L. plantarum which possessed "acidoproteolytic" enzymes.

Peterson et al. (1925) believed that there was a second organism active in the production of lactic acid, but it was not clear whether this organism could produce a higher percentage of acid from a given quantity of sugar or whether there was a symbiotic relationship between this organism and the predominating type of lactobacilli. The possibility of a second organism being present in small numbers arose from the observation that a higher percentage of glucose was fermented by a large inoculum of silage juice than by a small inoculum which presumably contained only the preponderant type of lactobacilli routinely isolated in plate counts. Olsen (1951) found Lactobacillus casei in sugar beet silage.

Butyric and proteolytic spoilage has been associated with the presence of species of Clostridium. Beynum and

Pette (1935) (1936) isolated two types of butyric bacteria. Clostridium tyrobutyricum fermented lactic acid and was inhibited at a pH of 3.5, whereas Clostridium saccharobutyricum fermented sugars but not lactic acid, and could exist below pH 3.5. However, Sjoström (1942) demonstrated the presence of spores of C. tyrobutyricum in silages made with mineral acids which had pH values as low as 2.7. Lactate-fermenting sporeforming anaerobes have subsequently been isolated from silage by Martos (1949) and Rosenberger (1951). Bryant and Burkey (1955) concluded that Clostridium tyrobutyricum should be considered distinct from Clostridium butyricum on the basis of carbohydrates fermented. These authors also found that ATCC samples of Clostridium butyricum did ferment lactate in contradiction to the description of the species given in Bergey's manual.

Most reports of clostridia have been based on spore counts. Rosenberger (1951) has emphasized the fact that clostridia form spores in an actively growing state only when under duress. He has, therefore, devised media for enumerating proteolytic and lactate-fermenting anaerobes in the vegetative state, and he has cautioned against accepting data based on spore counts. Lind (1953) was able to show good correlation between the amount of butyric acid in silage and the number of obligate anaerobes in the vegetative state.

Allen et al. (1937b) and Bryant et al. (1952) have isolated proteolytic anaerobes from grass silages which were

similar to Clostridium sporogenes. Politi (1943) reported that some strains of proteolytic anaerobes in silage appeared to be closely related to Clostridium bifermentans.

Thermophilic bacteria in overheated grass silage were identified as strains of B. subtilis, although some of them were asporogenous (Allen et al., 1937a). Etchells and Jones (1949) classified the predominant organisms in steamed potato silage as thermophilic, facultative anaerobes belonging to group X of the genus Bacillus.

Several other bacterial species have been found in silage but their relative importance has not been determined. Allen et al. (1937a) made several silages with added whey which contained millions of Streptococcus lactis. Since very few of these organisms were isolated during the main fermentation, they were judged to be unimportant. However, Cunningham and Smith (1940) isolated S. lactis from silages made with mineral acids and in 1943 these authors recommended replenishing the microflora of steamed potatoes which were to be ensiled with a mixed culture of S. lactis and L. plantarum.

Beynum and Pette (1939) found that acid-producing diplococci preceded the typical lactic fermentation, while Burkey et al. (1953) demonstrated the presence of pediococci, streptococci and lancet or diplo-short-rod forms in the early periods of storage. These workers agreed that these organisms died out because they could not tolerate the high acidity produced by the lactobacilli.

After the main fermentation, heterofermentative rods predominate since they are even more acid resistant than L. plantarum (Peterson et al., 1925) (Cunningham and Smith, 1940) (Olsen, 1951). Burkey et al. (1953) also found Corynebacterium species in the later periods of storage.

The presence of these different groups of organisms in predominant numbers depended on such factors as the kind of forage, its stage of growth, and other conditions of ensiling and storage. In general, the bacterial flora of silages made from wilted forage were more complex than high-moisture silages (Burkey et al. 1953). Dobrogosz and Stone (1958) believed that pediococci were found in good silages and not in poor silages.

Leuconostoc mesenteroides (Betacoccus arabinosaceas) has been isolated from sugar beet silages (Olsen 1951) and silages made with the addition of mineral acids (Cunningham and Smith 1940).

Allen et al. (1937b) concluded that the miscellaneous flora of fresh grass, such as micrococci, yeasts and aerobic sporeformers exerted little influence on the resulting fermentation.

CHAPTER III

METHODS

Sampling Procedure.

Samples of the fresh plant material were taken at the instant the forage was being fed into the silo, when the silo was about three feet from being full. In upright silos, subsequent samples of the fermenting silage were taken at a mean depth of three feet so that the silage samples would correspond to the samples of fresh forage. At this depth, the samples appeared to be sufficiently isolated from surface effects. Furthermore, samples from this depth could not have been unduly influenced by the percolation of juices from higher layers. In the more tightly packed bunker silos, a sampling depth of one foot proved satisfactory. Silage samples were taken at irregular intervals, but as often as possible within 50 days after ensiling.

All samples were packed in one quart polyethylene freezer bags, sealed, and immediately placed in a portable dry-ice cold chest for transport to the laboratory.

At the time the samples of fresh material were obtained, the elapsed time between mowing and ensiling was estimated. The approximate composition of the crop, the quality of the stand and further details of the harvesting procedure were also

recorded. The temperature and relative humidity at the hour of ensiling were obtained later from the East Lansing weather bureau, but the number of days since the last appreciable rainfall and the amount of cloud cover had to be estimated locally.

When silage samples were taken, the general appearance was recorded and the quality was categorized as good, fair, poor or overheated.

Enumeration of Bacterial Populations

A 10g. portion of finely cut material was placed in a Waring blendor with 90 ml. of physiological saline and agitated for 10 minutes at a slow speed obtained by setting the Powerstat variable transformer to deliver 40 v. on a 115 v. line. Suitable dilutions were made, usually through one to one-hundred million, for the preparation of culture plates. All plates were counted after 5 days incubation at room temperature. Since the samples were not collected aseptically, counts of less than 1000 per gram were never reported. This parallels the procedure developed by Kroulik et al. (1955a).

The interval between sampling and the completion of plating rarely exceeded six hours. The remainder of the sample was resealed, quick frozen, and stored in deep freeze for future chemical determinations.

"Total" aerobic count

The total number of bacteria on fresh plants capable of

growing aerobically was determined with the tryptone-glucose-yeast (TGY) medium which Kroulik et al. (1955a) had found to be superior for this purpose.

TGY medium was also used to enumerate the aerobic bacteria in silage, since preliminary studies indicated that this medium gave consistently higher counts than the peptone-tomato agar employed by Stirling (1951). The ammonium lactate medium used by Stirling to count the gram-negative organisms on grass and in silage appeared to be less selective than claimed.

The formula for TGY medium is given in the Appendix.

"Total" anaerobic count

A total count of organisms capable of growing anaerobically on both the fresh and ensiled samples was made with anaerobic agar containing dextrose and Eh indicator (Baltimore Biological Laboratories) using Brewer anaerobic petri dish covers.

The count for the whole plate was obtained by multiplying the number of colonies on 10 or 15 sq. cm. in the center of the plate by 6.35 or 4.23 respectively.

Lactic acid bacteria count

The population of lactic acid bacteria on the fresh plant material was determined with the lactobacillus selection (LBS) medium developed by Rogosa and co-workers (1951), but at the author's suggestion the 2 per cent glucose in the original formula was replaced by 1 per cent glucose, 0.5 per cent

arabinose and 0.5 per cent sucrose. The complete formula is given in the Appendix.

For enumerating the lactic acid bacteria in the silage samples, both LBS medium and the V8 agar developed by Fabian et al. (1952) were used, since Rosen et al. (1956) demonstrated that LBS medium restricted the growth of a number of strains of lactobacilli. Since the V8 medium was relatively non-selective, its value as a counting medium was restricted to those samples where acid-forming bacteria predominated. The formula for V8 agar is also listed in the Appendix.

Characterization of Bacterial Populations

For every sample of fresh and ensiled forage, eight isolations were made from each counting medium. To make the isolates as representative as possible, a rectangular area containing about eight colonies was marked off on a plate from a suitable dilution showing discrete colonies, and transfers were taken from all the colonies in this area. Each isolate from TGY, LBS and V8 media was replated once in the isolation medium, whereas the isolates from anaerobic agar were similarly purified by dispersion in tubes of the same medium which had been melted and cooled.

Representative colonies were subsequently transferred to tubes of storage medium. Isolates from TGY and anaerobic media were stored in the same media at 4°C while isolates from LBS and V8 media were stored in micro assay culture agar

(Difco) at room temperature.

All incubation times given in the following paragraphs refer to room temperature.

Characterization of isolates from TGY medium

Fermentation tests were conducted in purple base broth (Difco). The following substrates were used at levels of 1%: D-glucose, D-fructose, D-lactose, D-sucrose, D-maltose, D-mannitol, D-sorbitol, glycerol, D-xylose and inulin. The media were dispensed in 4 ml. quantities into 12 by 100 mm. test tubes containing inserts. Tubes of litmus milk (BBL), nitrate broth (Difco) and nutrient gelatin (Difco) were similarly prepared, but without inserts.

All tubes were inoculated by needle from a 24 hour streak culture on TGY agar. This 24 hour growth was also used to prepare gram strains, employing the Hucker modification, and for the catalase test.

The sulfanilic acid - alpha-napthylamine test for the reduction of nitrate to nitrite was performed after 3 days incubation. After 4 days incubation, observations were made for the production of acid and gas in the carbohydrate media, for changes in the indicator or any change in the physical characteristics of litmus milk, and for the liquefaction of gelatin.

Pigment production on TGY agar was noted.

Characterization of anaerobes

The anaerobes were classified as obligate or facultative by their zone of growth in tubes of anaerobic agar. Gas production and pigmentation were also noted.

Gram stains were prepared from 24 hour growth in anaerobic agar.

The obligate anaerobes were tested for proteolytic and lactate-fermenting ability by Rosenberger's (1951) methods. The modified media employed are described in the Appendix.

Characterization of lactic acid bacteria

Fermentation tests were conducted in the modified microinoculum broth employed by Rosen et al. (1956). The following substrates were used: L-arabinose 1.0 per cent, D-glucose 1.0 per cent, dextrin 1.0 per cent, D-lactose 1.0 per cent, D-sucrose 1.0 per cent, D-mannitol 1.0 per cent, and salicin 0.5 per cent. The media were dispensed in 4 ml. quantities into 12 by 100 mm. test tubes. Arabinose was sterilized at 115°C for 12 minutes.

Tubes of litmus milk and indole-nitrite media (BBL) were similarly prepared.

Inocula were prepared by transferring from the stock culture into 4 ml. of modified microinoculum broth. When abundant growth was observed (usually 24 hours) one drop of this suspension was added to each tube. This inoculum was also used for the gram stain and catalase test.

The test for nitrite was made after 3 days incubation at room temperature. Observations for acid production from carbohydrates and changes in litmus milk were made daily for 2 weeks.

Chemical Determinations

The frozen samples were thawed in a refrigerator for 2-4 hours, cut into 1/4"-1/2" lengths with hand scissors while still in a semi-frozen state, divided into 2 portions and immediately refrozen.

One portion was thawed for the pH, Kjeldahl nitrogen and moisture determinations. Carbohydrate analysis was made on the dry residues from the moisture determination.

The second portion was used to prepare the extract for the organic acid measurements and the extract for the total acidity, amino acid and volatile base determinations. The extractions were usually begun while the samples were still frozen.

Kjeldahl nitrogen

Total nitrogen was determined on 5 g. moist samples. Digestion with sulfuric acid in the presence of K_2SO_4 and $CuSO_4$ was continued for 90 minutes after the boiling solution had cleared. An excess of NaOH and a small measure of granulated zinc were added to the flasks before distillation. The distillate was trapped in standard acid, and the excess acid was titrated with standard base.

Results were expressed as crude protein, calculated as 6.25 times the nitrogen content.

pH

Three to 5 g. of material were added to 20 ml. of distilled water in a 50 ml. beaker. Preliminary trials indicated that the relative amounts of material and water were not critical. The mixture was briefly stirred and the pH was measured with a Beckman model G pH meter.

Moisture

The moisture content was calculated from the weight loss of 5 g. samples which had been heated in a dry oven at 105°C for 24 hours. The weighing dishes used had previously been tared after being dried to constant weight at 105°C and cooled in a dessicator.

Carbohydrate

The total hydrolysable carbohydrate was determined by the phenol-sulfuric acid method developed by Koch et al. (1951). The experimental details outlined by Barnett (1954) were followed from the preparation of extracts to the development of the colored solutions.

The color densities were determined with a Bausch and Lomb "Spectronic 20". For the samples of fresh forages, the per cent carbohydrate was calculated by the formula given by Barnett from the optical densities of the standard and sample at 425 mμ. The per cent carbohydrate in the silage

samples was obtained from the optical density at 490 mμ. with reference to a standard curve which had a range of 10 to 60 micrograms per aliquot.

Organic acids

The micromoles of butyric, propionic, acetic, formic, lactic and succinic acids per gram of dry matter were determined by the Wiseman and Irvin (1957) chromatographic method.

Total titratable acidity, volatile bases, and amino acids

The Woodman (1925) modification of the Foreman method was used to measure the total acidity, volatile bases and amino acids. The experimental details have been outlined by Barnett (1954). Extracts were made in 2 litre screw-cap bottles on a shaker which reciprocated through 5 inches 75 times a minute. Results were expressed as micromoles of acid or base per 100 gm. of dry matter by an adaptation of the formula given by Watson and Ferguson (1937).

Statistical Calculations

The formula used for computing regression lines was:

(1) $$y - My = a(x - Mx)$$

in which My and Mx represent the true means of the independent and dependent groups of values respectively.

The slope "a" of regression lines was calculated from the formula:

(2) $$\frac{\Sigma xy - \frac{\Sigma x \Sigma y}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

Correlation coefficients were calculated from the formula:

(3) $$r = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{N}}{\sqrt{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{N}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{N}\right]}}$$

Standard error was calculated from

(4) $$\sigma e = \sqrt{\frac{\left[\Sigma y^2 - \frac{(\Sigma y)^2}{N}\right] - a\left[\Sigma xy - \frac{\Sigma x \Sigma y}{N}\right]}{N - 2}}$$

In formulae (2) (3) and (4), x represents the variation from an assumed mean of a single item in the dependent variable group of values, and y the same variation in the independent variable group. N represents the total number of samples.

Significance was determined by reference to the table of values for this purpose given by Snedecor (1938). The term "highly significant" was applied to "r" values which were well beyond the point of 1 per cent significance.

CHAPTER IV

THE BACTERIAL FLORA OF FRESH FORAGES

Introduction

It has been shown that the number of bacteria on fresh forage fluctuates with the plant species, the part of the plant, the maturity of the crop, the time of day, the season, the amount of wilting and other details of the harvesting procedure (Kroulik et al. 1955a). It is also logical to expect that the bacterial population of fresh crops at the moment of ensiling may vary with the pH and carbohydrate content of the plant juice, the temperature, rainfall and other environmental factors.

This present investigation was an attempt to determine the net effect of all these environmental factors on the number of bacteria on fresh forages at the time of ensiling. Ideally, there would be one or two dominant factors from which the number of bacteria could be predicted without appreciable error due to the sum of all other factors.

It was hoped that the morphological and fermentative characterization of the predominant bacteria of fresh plants would lead both to their classification and to an understanding of the role these bacteria play in the ensuing fermentation. Similar studies of the lactic acid bacteria found on fresh

plants were made to determine whether or not they were typical silage types.

In addition, the data obtained in this section will be used to investigate the relationship between silage quality and the number and type of bacteria on the fresh crop.

Results

A total of 33 samples of different forages were collected and analysed as outlined in Chapter III. All environmental statistics, plate counts on TGY, AA and LBS media, and other analytical data are listed in the Appendix. Bacterial counts are given on a dry matter basis to eliminate the partial effect of wilting, rainfall and humidity, which is due only to a change in dry matter content.

It was found that the harvesting date was the most important factor affecting the number of bacteria on fresh plants. This relationship is illustrated in Figure I. The regression line and correlation coefficient for TGY medium was found to be:

$$y = .033x + 7.83; \qquad r = .79$$

Similarly for AA medium:

$$y = .030x + 6.63; \qquad r = .75$$

and for LBS medium:

$$y = .028x + 4.39; \qquad r = .61$$

With 31 degrees of freedom, all r values are very significant at 1 per cent.

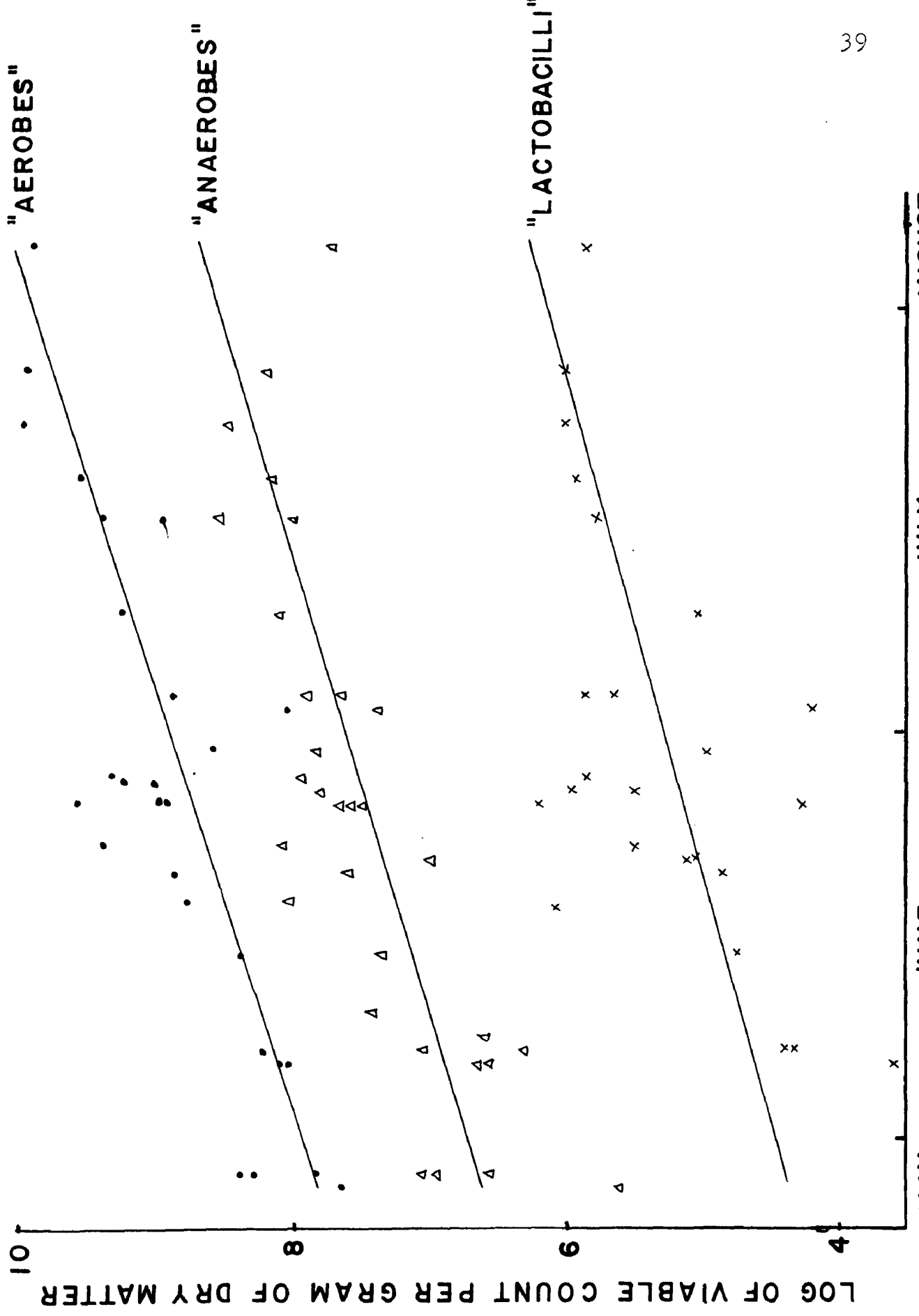

Figure 1. The relationship between the number of bacteria on fresh forages at the time of ensiling and the harvesting date.

Most of the 225 organisms isolated from TGY medium were chromogenic, the number of each color being given in Table I. All cultures were short, gram negative rods which often exhibited polar staining.

Almost 60 per cent (132) of all strains did not produce acid in any of the carbohydrates tested. The remaining 40 per cent produced acid in various carbohydrate broths, but essentially none of the strains produced gas, even from glucose. A graphic presentation of carbohydrate preference of the 40 per cent which did produce acid indicated that the simple sugars were fermented most often (Figure 2). Both fermentative and non-fermentative strains were found in each of the color categories of Table I.

All cultures were variable with respect to nitrate reduction and gelatin liquefaction.

The organisms capable of growing anaerobically were all facultative with respect to oxygen requirement. Although all cultures in AA medium were colorless, most exhibited yellow colonies when transferred to TGY medium, indicating that the bacteria isolated from both media were similar. When grown on the surface of AA medium, those isolates from both AA and TGY media classified as "dark yellow" in Table I retained their coloration. However, "light yellow" isolates from both sources were colorless even on the surface of AA medium.

The bacteria isolated from LBS medium are compared to

TABLE I

PIGMENTS PRODUCED BY THE BACTERIA OF FRESH FORAGES ISOLATED FROM TGY MEDIUM

Color	Number of isolates	Per cent of isolates
Dark Yellow	80	35.6
Light Yellow	77	34.2
Yellow-Orange	20	8.9
Orange	10	4.5
Pink	8	3.6
Brown	5	2.2
No Pigment	25	11.1

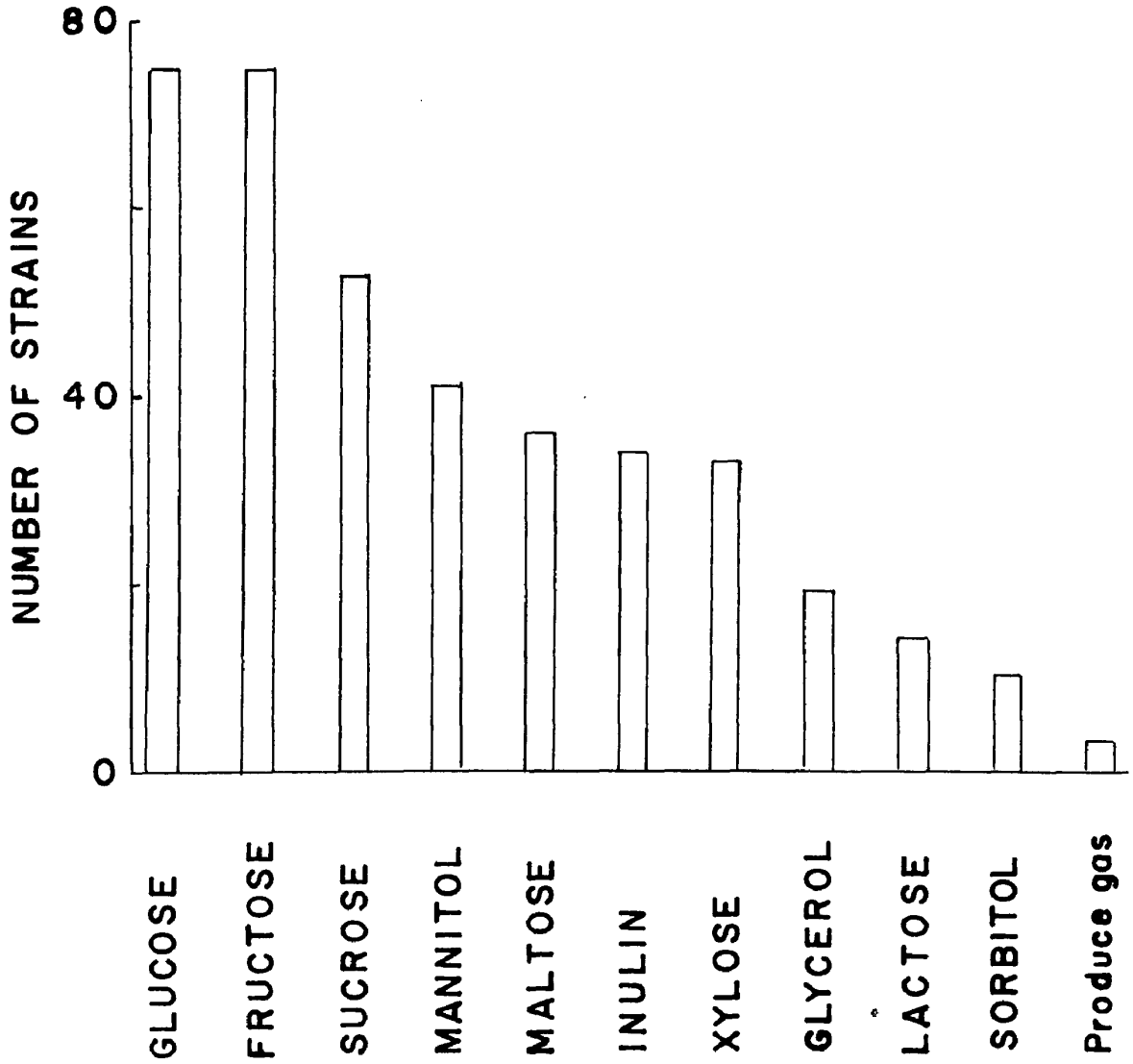

Figure 2. Carbohydrate preference of the acid-producing strains of the chromogenic bacteria of fresh forages.

the typical silage bacteria in Chapter IX.

Discussion

Good correlation has been found between the bacterial numbers on fresh forages and the harvesting date, in spite of marked differences in other environmental conditions, plant species and crop composition. This indicates that the harvesting date exerts the controlling influence on the bacterial composition of the raw material from which silage is made.

Since the regression lines for lactic acid bacteria and the predominating plant flora have similar slopes, the harvesting date apparently does not affect one group more than the other. Probably only a factor which selectively favored the lactic acid bacteria would be worth further investigation.

Kroulik et al. (1955a) found that bacterial numbers increased with the maturity of the plant more than with the season. The data presented here neither deny nor confirm this, since "harvesting date" is the sum of maturity and season, in which the contributions of the individual factors could not be separated.

If a second controlling factor existed, it could be detected by a study of the deviations from the main regression line of bacterial numbers vs. harvesting date. For example, since Kroulik et al. (1955a) found that wilting greatly

increased the number of bacteria on fresh alfalfa, it might be expected that wilted crops would have higher counts than the bacterial numbers vs. harvesting date relation would predict. Although this could not be demonstrated routinely, it was noted that all crops which were field chopped (essentially no wilting) had less than the minimum numbers of lactobacilli even though one such sample was obtained as late as July 16.

Exhaustive attempts to correlate these deviations with other factors were unsuccessful.

The quantitative results showed that fresh plants contained almost as many bacteria capable of growing anaerobically as aerobically. Subsequent qualititative investigations indicated that the bacteria growing anaerobically were the same as those growing aerobically; the higher count on aerobic media may mean that some strains were strictly aerobic. These data are in accordance with the results of Thomas (1950) who found that the predominating chromogenic bacteria of fresh crops were facultative anaerobes, and at variance with the results of Kroulik et al. (1955a), who believed that they were strict aerobes.

The predominating bacteria on fresh forage crops can, therefore, be described as gram-negative, rod shaped bacteria; which may show polar staining; which characteristically produce yellow, orange, red, or brown insoluble pigments, the hue often depending upon the nutrient medium; which

generally do not ferment carbohydrates and which are aerobic to facultatively anaerobic. This description contains all the essential characteristics of the genus Flavobacterium (Breed et al. 1957).

Since the preponderant number of bacteria on fresh crops do not produce acid, they are of little help in the preservation of silage. However, most liquefied gelatin. Where they predominate for long periods of time in silage made from wilted crops (Burkey et al. 1953), they may be responsible for some of the excessive proteolysis which occurs in this type of silage (MacPherson 1952b). Since they are facultative anaerobes, it can not be the attainment of anaerobic conditions which causes their demise in silage.

CHAPTER V

THE CHEMISTRY OF FRESH FORAGES

Introduction

This study of the chemical composition of fresh forages was slanted to detect evidence of any fermentation which might have occurred up to the time the crop was placed into the silo. As in the previous chapter, an attempt was made to show what environmental factors controlled the amount of fermentation in this pre-ensiling period. Since plant enzymes are active in this period, this preliminary fermentation need not be correlated with changes in the bacterial flora to be significant in the silage process.

Results

It was observed that high-moisture crops have lower pH values than low-moisture crops at the moment of ensiling. This relationship is depicted in Figure 3, where the regression line was:

$$y = -.22x + 7.46$$

The correlation coefficient was a highly significant -.69.

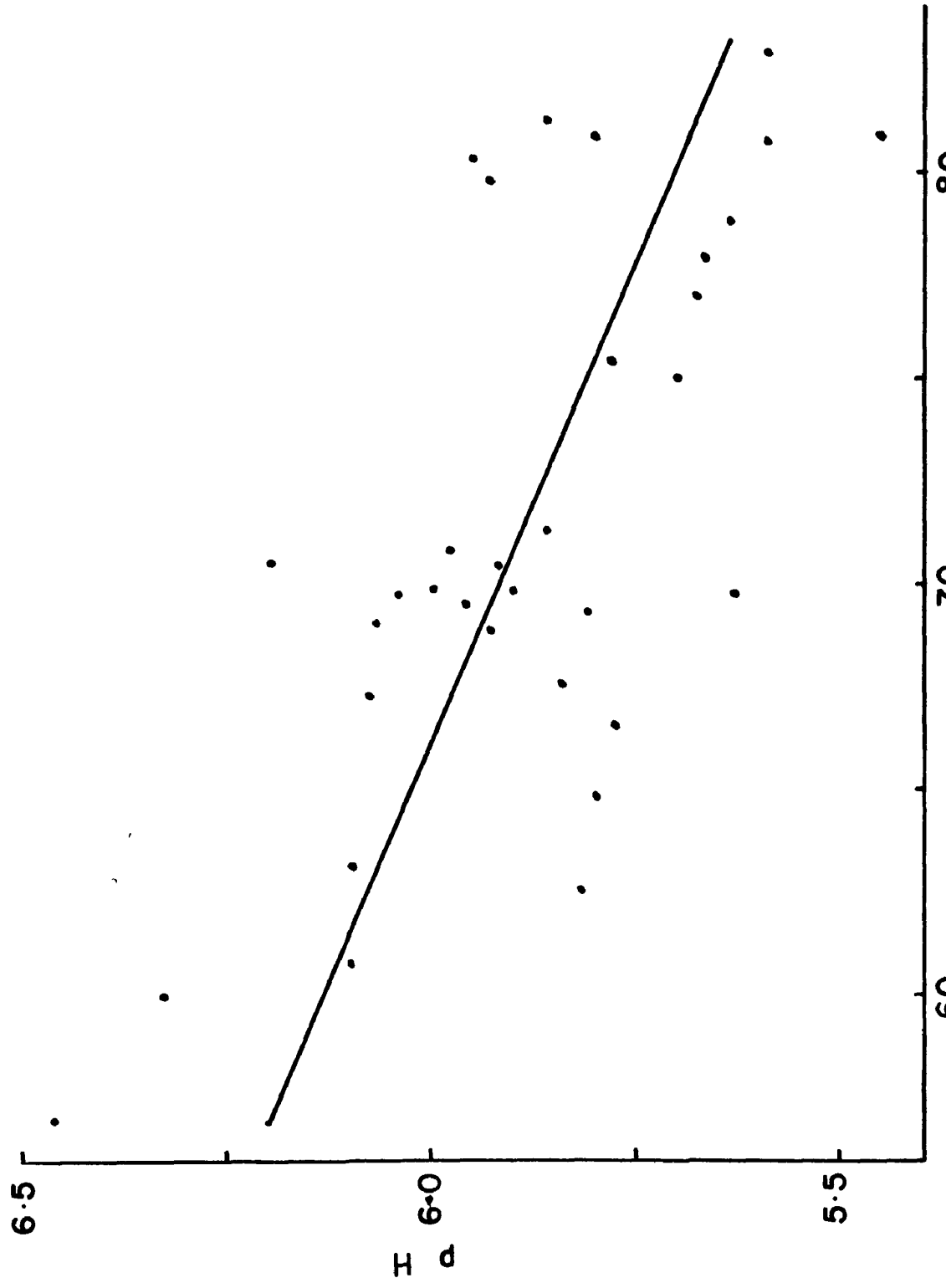

Figure 3. The relationship between the pH and moisture content of fresh forages at the time of ensiling.

Discussion

Kroulik et al. (1955b) found that high-moisture silages undergo a more rapid fermentation than corresponding wilted silages, and that the higher rates of fermentation were associated with higher numbers of acid-forming bacteria.

Results obtained from a study of different crops at the time of ensiling have shown that high-moisture crops also undergo a more rapid fermentation in the interval between cutting and ensiling. However, samples with low pH values did not have correspondingly greater numbers of lactic acid bacteria; therefore, it is proposed that this pre-ensiling fermentation may be the result of plant enzyme activity.

Since plant enzymes are generally held responsible for the breakdown of protein to amino acids (Mabbitt 1952) those samples showing greater enzymatic activity (ie., lower pH values) might have been expected to have higher amounts of amino acid. This could not be proved. With one exception, all samples of fresh plant material contained about the same small amount of volatile base and amino acid.

CHAPTER VI

GENERAL FERMENTATION INDICES

Introduction

The pH is generally considered a sufficiently good index of silage quality (Watson 1949).

From a nutritional standpoint, the most important consideration in the evaluation of silage is the dry matter content. In poor quality silages, much dry matter can be lost due to the formation of volatile acids and bases. Since many spoiled silages have a high moisture content, dry matter loss is further aggravated by run off (Barnett 1954).

This chapter is inserted to prove that farm-sized silos are suitable experimental vehicles even though the sampling error was expected to be large. It will be shown that changes in the pH and dry matter content in large silos follow the accepted patterns in relation to silage quality.

Results

The Appendix contains all the analytical data for the 14 silos on which the most complete observations were made. A general description of these silos and silages is given in Table II. The numbers assigned to the various silos in Table II will be used throughout this chapter and all

TABLE II

GENERAL DESCRIPTION OF SILOS AND SILAGES

Silo Number	Silo Type	Date Filled	Crop	Quality of Crop	Quality of Silage
*1	upright	May 29	alfalfa	medium	fair
2	upright	June 26	June clover	excellent	excellent
3	upright	June 21	alfalfa brome	excellent	excellent
**4	upright	July 16	oats	excellent	good
5	upright	July 3	alfalfa alsike	excellent	good
6	upright	June 29	alfalfa	medium	good
**7	upright	July 3	mammoth clover	excellent	good
8	bunker	June 8	alfalfa	excellent	spoiled
9	bunker	June 9	alfalfa	excellent	spoiled
10	bunker	June 14	alfalfa brome	excellent	spoiled
11	bunker	July 2	alfalfa	excellent	spoiled
12	bunker	June 20	alfalfa alsike	medium	spoiled
**13	upright	June 21	alfalfa	poor	overheated
14	upright	June 18	alfalfa	medium	overheated

* Sodium bisulfite added, 7 lbs. ton

** Water added at the time of ensiling

succeeding ones.

Figure 4 depicts the comparative changes in pH of a well-preserved silage and a silage which spoiled. All spoiled silages (silos 8, 9, 10, 11, 12) exhibited an initial drop in pH in the early days of the fermentation, but the pH subsequently rose to final values above 5.0. Conversely, all well-preserved silages (silos 2, 3, 4, 5, 6, 7) quickly attained and maintained a pH between 4.0 and 4.5. The overheated silages (silos 13, 14) and the bisulfite silage (silo 1) were intermediate in both pH and quality.

Figure 5 compares the dry matter content of the acceptable silages to the spoiled silages. Silos 4, 7 and 13 are not included in this chart because their dry matter content was artificially reduced by the addition of water at, or immediately after, ensiling. Silo 1 is not included because it rests on the borderline between the acceptable and spoiled groups.

All 5 spoiled silages reveal a marked loss of dry matter, while 4 of the 5 acceptable silages show some increase in dry matter content.

Discussion

Although the spoiled silages did not always have low dry matter content originally, the final dry matter content of the five spoiled silages ranged from 14 to 21 per cent, while the dry matter content of the acceptable silages ranged

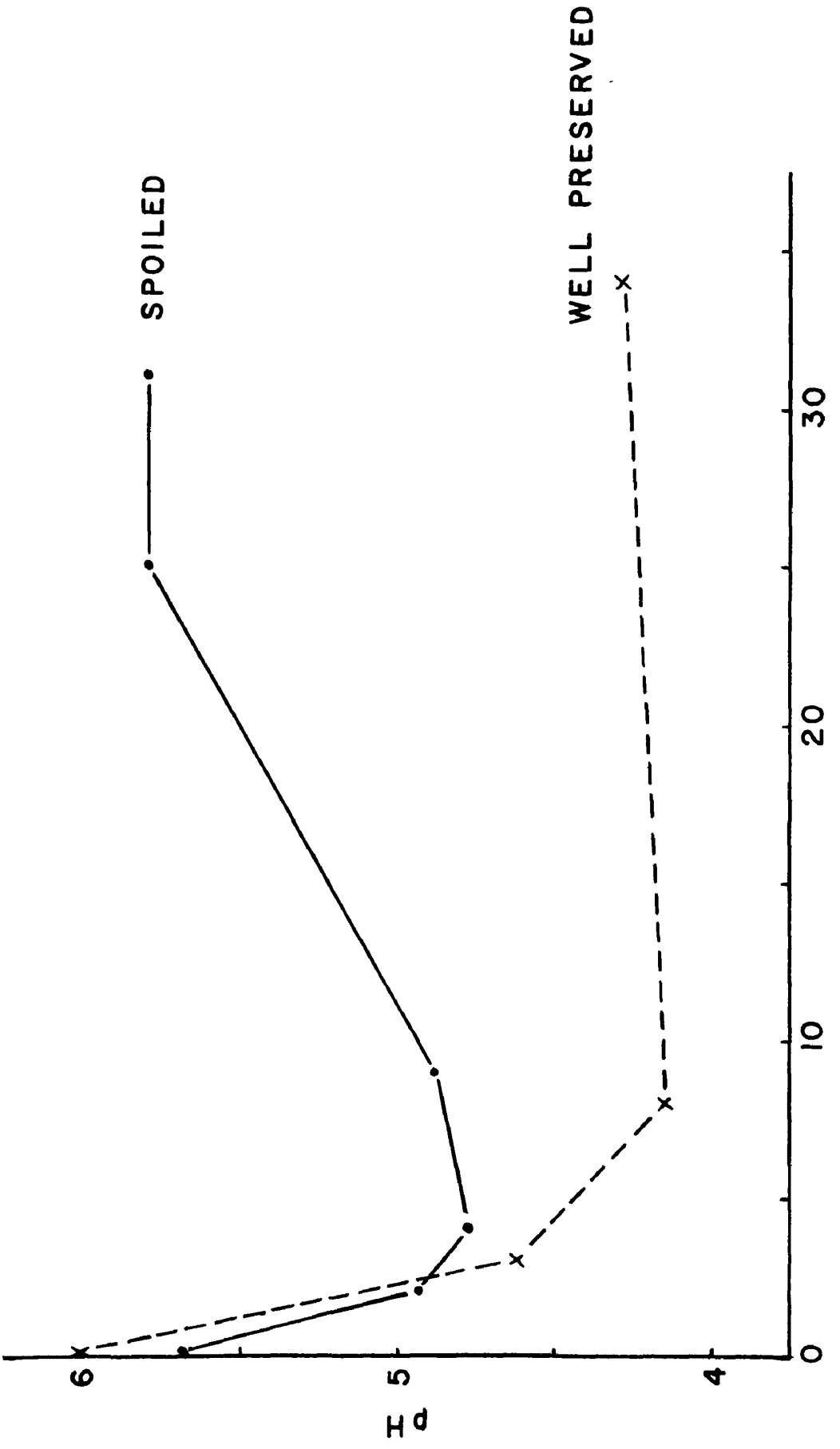

Figure 4. Comparison of the pH changes in a well-preserved silage (silo 7) and a spoiled silage (silo 9).

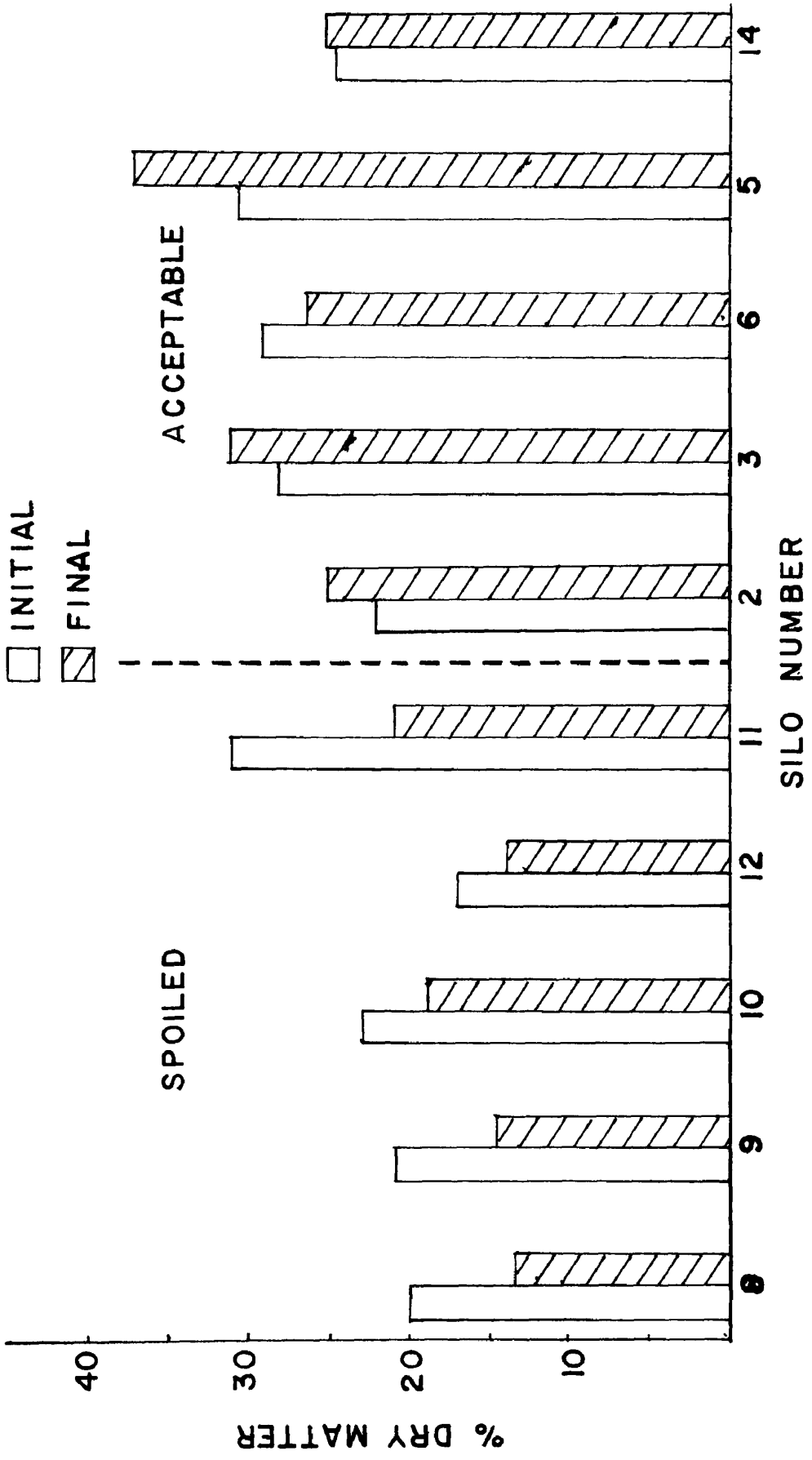

Figure 5. Changes in the dry matter content of silages during storage.

from 25 to 37 per cent.

Four of the 5 acceptable silages exhibited increases in dry matter content. Since all samples were taken near the top of the fermenting mass, the apparent gain in dry matter is actually a loss of moisture through downward percolation or upward evaporation. The amount of moisture lost from acceptable silages through drainage is nil. Since the only acceptable silage showing a loss of dry matter was sealed against evaporation, it is concluded that acceptable silages lose some moisture from the upper layers through evaporation.

On the other hand, all of the spoiled silages drained excessively; hence the actual loss of dry matter is probably even greater than recorded, due to the simultaneous loss of moisture by both percolation and evaporation.

Regardless of the actual changes in dry matter content, the apparent changes are lucid enough to conclude that spoiled silages are characterized by a measurable loss of dry matter.

Silage quality is usually estimated by the final pH value. Curves of the change in pH during fermentation indicate that spoiled silages initially undergo a typical increase in acidity, but that the pH subsequently rises as the silage begins to spoil. It has been noted that silages of doubtful quality have final pH values between the excellent and spoiled ranges.

All the spoiled silos happened to be of the bunker or pit type, while all the acceptable silow were uprights

(Table II). Paradoxically, the spoiled silages belonged to the more progressive farmers who had invested in the self-feed bunker silos in order to increase production. The spoilage they experienced was admittedly due to lack of experience rather than to any inherent fault in bunker silos.

All the bunker silos were filled with field chopped legumes and packed with tractors. In all cases the packing was excessive, and in one silo the resultant silage had to be chopped out with an ax. Although the fresh crops were not unreasonably high in moisture content, packing by tractors brought about maceration of the plant cells. The plant juices so freed from the cells were able to run off; hence all the bunker silos drained profusely.

These results have shown that the fermentations in these farm-sized silos were comparable to the fermentations which have previously been studied in experimental and miniature silos.

CHAPTER VII

CARBOHYDRATE METABOLISM IN SILAGE

Introduction

The study of carbohydrate metabolism in silage involves the conversion of the simple sugars and reserve polysaccharides of the green plant into the fermentation acids of silage.

Results

Typical acid production in a high quality silage is illustrated in Figure 6. All other well-preserved silages show similar, steady increases in lactic acid and a small constant level of succinic acid. Formic acid was present in trace amounts in all samples.

In all spoiled silages (Figure 7), there was an initial lactic fermentation. The lactic acid was later replaced by butyric and propionic acids. There was approximately half as much propionic acid as butyric acid in each case. In the spoilage cycle, the succinic acid is also eliminated.

Figure 8 shows that the overheated silages underwent an acetic acid fermentation for the most part, although a small amount of lactic acid did accumulate. Succinic acid again remained at a low level.

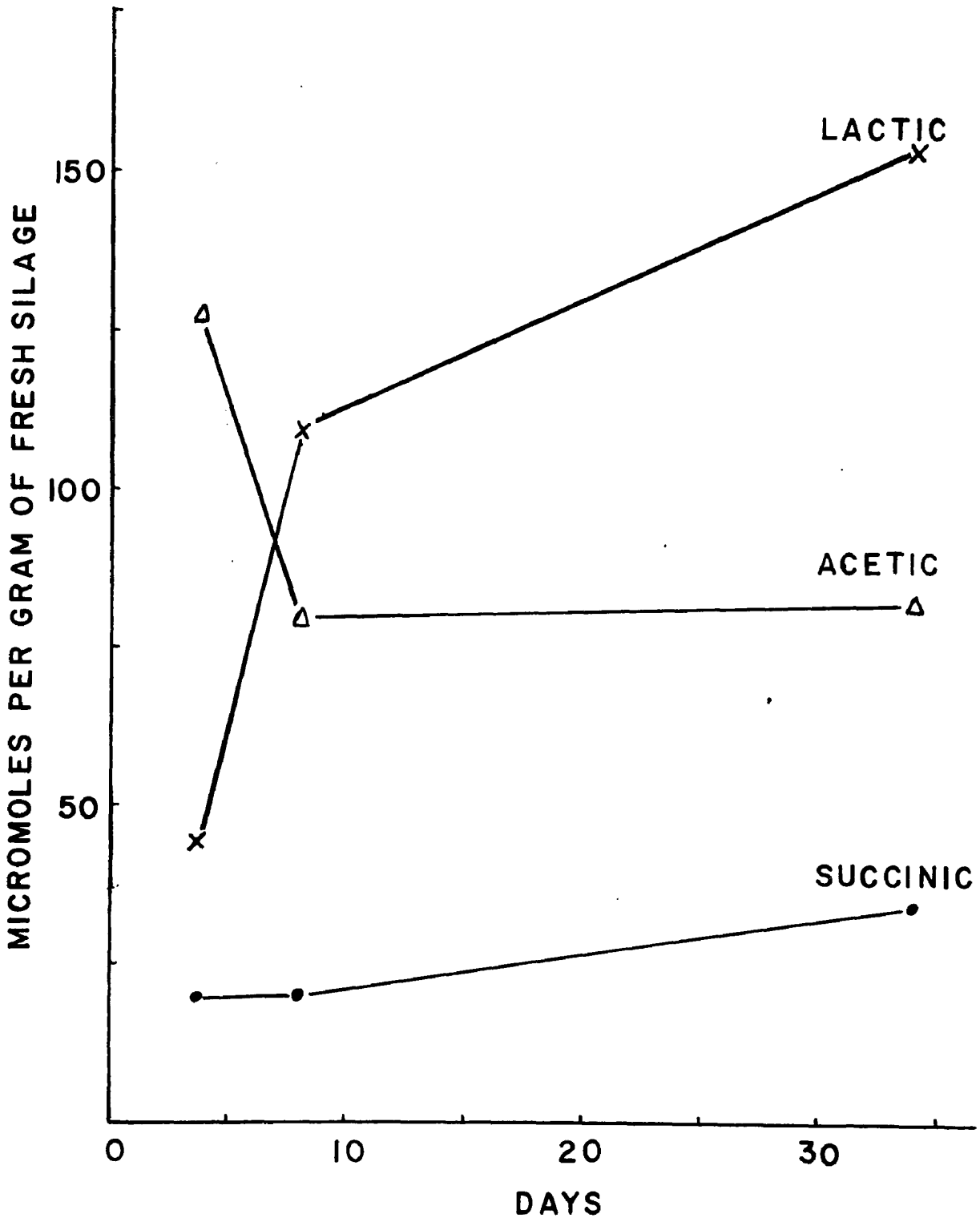

Figure 6. The production of organic acids in a well-preserved silage (silo 7).

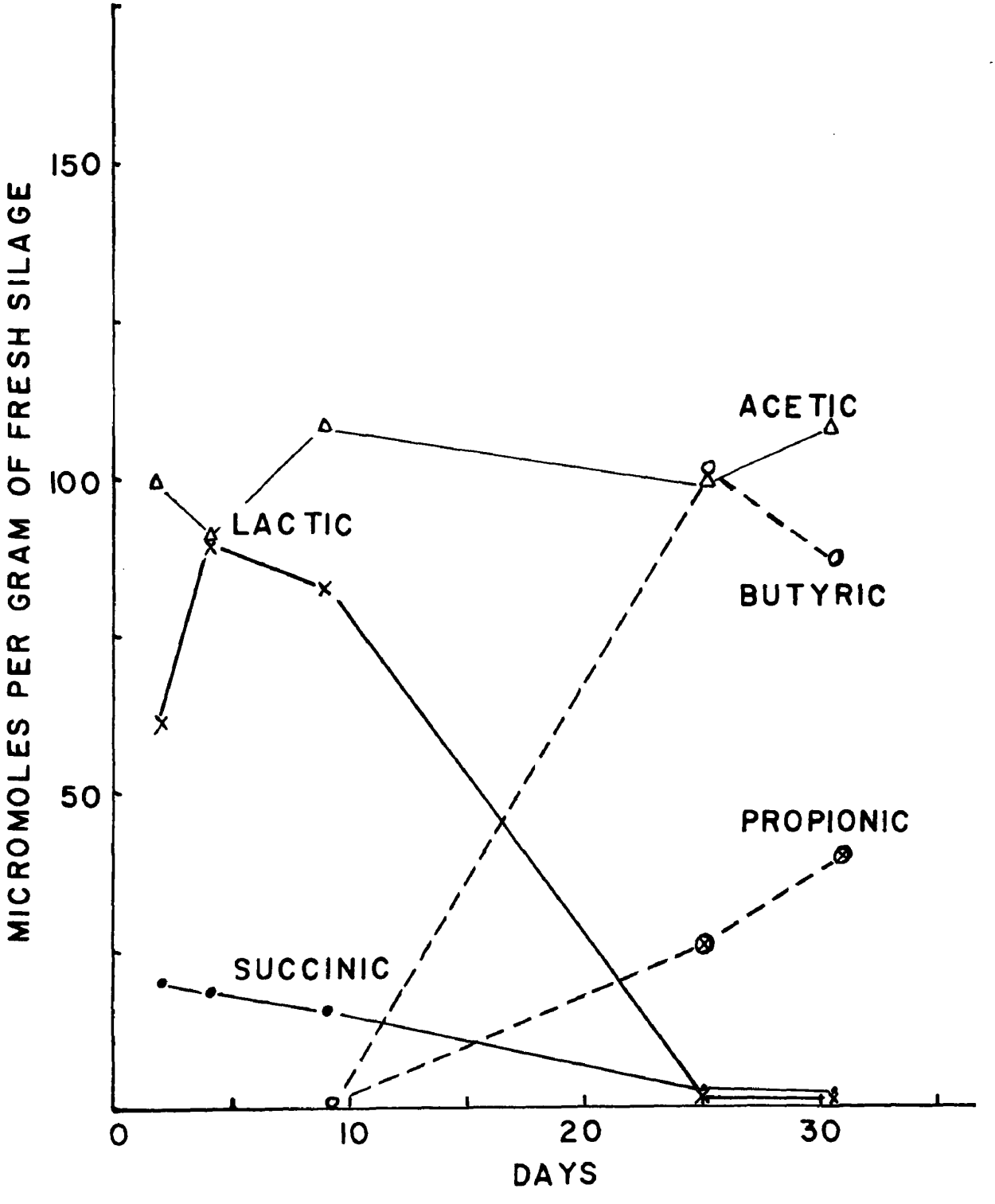

Figure 7. The production of organic acids in a spoiled silage (silo 9).

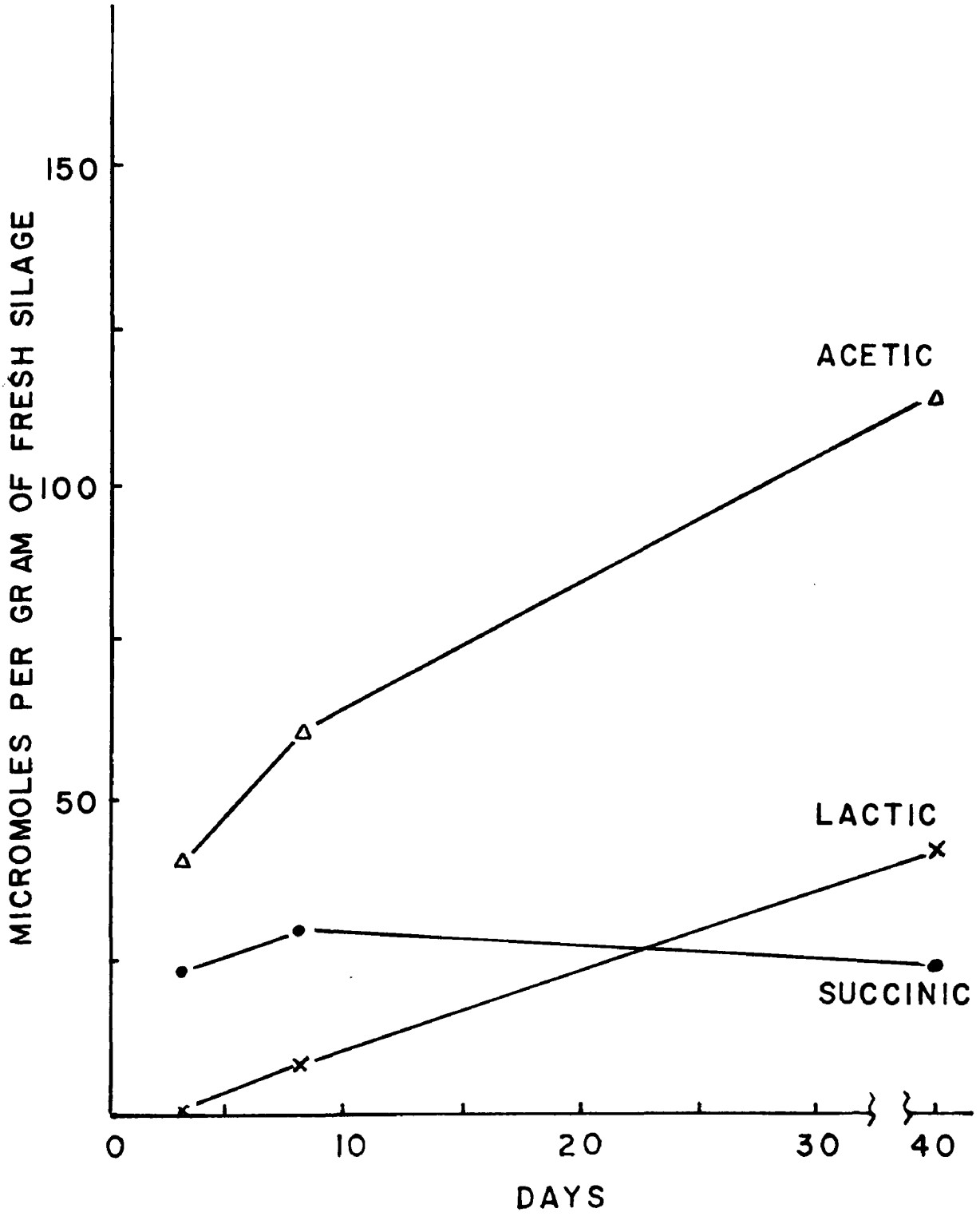

Figure 8. The production of organic acids in an over-heated silage (silo 13).

The amount of acetic acid in silages was not related to quality. Good, spoiled and overheated silages all formed a maximum acetic acid concentration in the range of 88 - 213 micromoles per gram of silage. Peak acetic acid concentration was observed early, intermediately or late in individual silages in no apparent pattern.

Accid production in the bisulfite silage was similar to the well-preserved silages, except for a trace of butyric.

The relationships between the pH and the relative amount of volatile and lactic acids is pictured in Figure 9. The regression line for this relationship was found to be:

$$y = 33.15x - 98.7.$$

The coefficient of correlation of .62 was highly significant and the standard error of estimate was 17.7.

Since the total hydrolysable carbohydrate content did not appear to affect the type of acids produced, but did play a governing role in nitrogen metabolism, the presentation and discussion of the amount of residual carbohydrate in the various silages will be found in Chapter VIII.

Discussion

Both well-preserved and spoiled silages underwent an initial lactic fermentation. In the spoiled silages, the butyric and propionic acid formation took place at the expense of succinic and especially of lactic acid. This suggests the lactate-fermenting activity of Clostridium

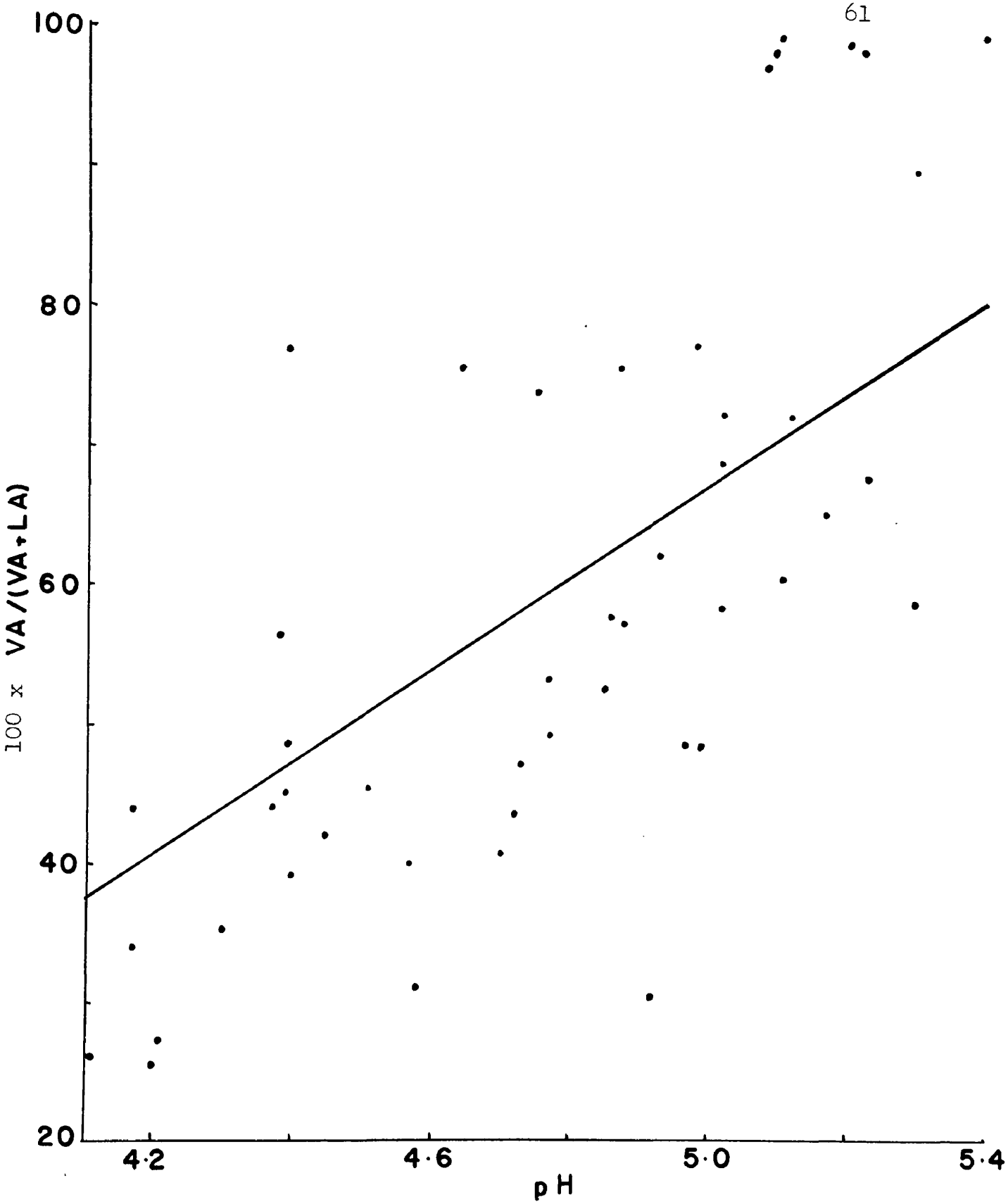

Figure 9. The relationship between the volatile and lactic acids in silage and the pH.

tyrobutyricum. There was no evidence of butyric formation early in the fermentation, which would have suggested the sugar-fermenting activity of Clostridium saccharobutyricum.

The amount of acetic acid formed apparently bears no relation to silage quality. Overheating did not prevent the formation of acetic acid even though very little lactic acid accumulated.

Although the quality of the bisulfite silage was only fair, there was no appreciable butyric fermentation.

Common (1941) related the pH of silage to the relative amounts of volatile and non volatile acid through the equation:

$$\frac{VA \times 100}{VA + RA} = 38.30\ pH - 119.93.$$

He found the standard error of estimate to be 6.07. Common believed that this relatively large standard error was due to the presence of succinic acid in the RA (residual acid) and to inaccuracies in the Woodman (1925) process, such as the error caused by the partial volatility of lactic acid.

The results obtained here offered an opportunity to test Common's hypothesis relating silage acids and pH, using a true figure for lactic acid (LA) in place of the RA obtained by the Woodman procedure. In this case the equation was found to be:

$$\frac{VA \times 100}{VA + LA} = 33.15\ pH - 98.7$$

The standard error of estimate was calculated as 17.7;

considerably larger than the 6.07 reported by Common.

The larger standard error is due to two main factors. First, these results have shown that some of the volatile acid in spoiled silages has come from the succinic acid of the plant. Thus, although succinic acid is not included in LA in normal silages, it is included in VA in spoiled silages; hence in spoiled silages the ratio $\frac{VA \times 100}{VA + LA}$ is relatively greater than would be predicted.

Secondly, this relationship was not valid in very immature silages which had only undergone the initial acetic acid fermentation at the time of sampling. However, these samples should probably not be classified as "silages" and could logically be excluded. These two factors account for the group of samples bunched in the upper right corner of Figure 9.

In conclusion, the relationship between the pH of silage and the various acids, which was derived by Common (1941), has been firmly supported. However, the inclusion of succinic acid in the calculations is probably not an error as Common believed since it has been shown that succinic acid is inseparably included in the butyric and propionic acids of spoiled silages.

CHAPTER VIII

NITROGEN METABOLISM IN SILAGE

Introduction

This chapter deals with the relationship between silage quality and the end products of nitrogen metabolism. From the work of others it was expected that amino acids would comprise the main product of proteolytic activity in well-preserved silages, whereas spoiled silages would be characterized by large amounts of volatile base (chiefly ammonia) derived from amino acids by deamination.

It was also expected that deamination would occur only when the supply of fermentable carbohydrate had been depleted (Bender 1941).

Results

The accumulation of amino acid and ammonia in a typical, well-preserved silage is depicted in Figure 10. All well-preserved silages (silos 2, 3, 4, 5, 6, 7), both overheated silages (silos 13, 14) and one spoiled silage (silo 11) showed a similar pattern. A substantial accumulation of amino acid accompanied by a slight accumulation of volatile base was characteristic of all these silages.

The other spoiled silages (silos 8, 9, 10, 12) exhibited

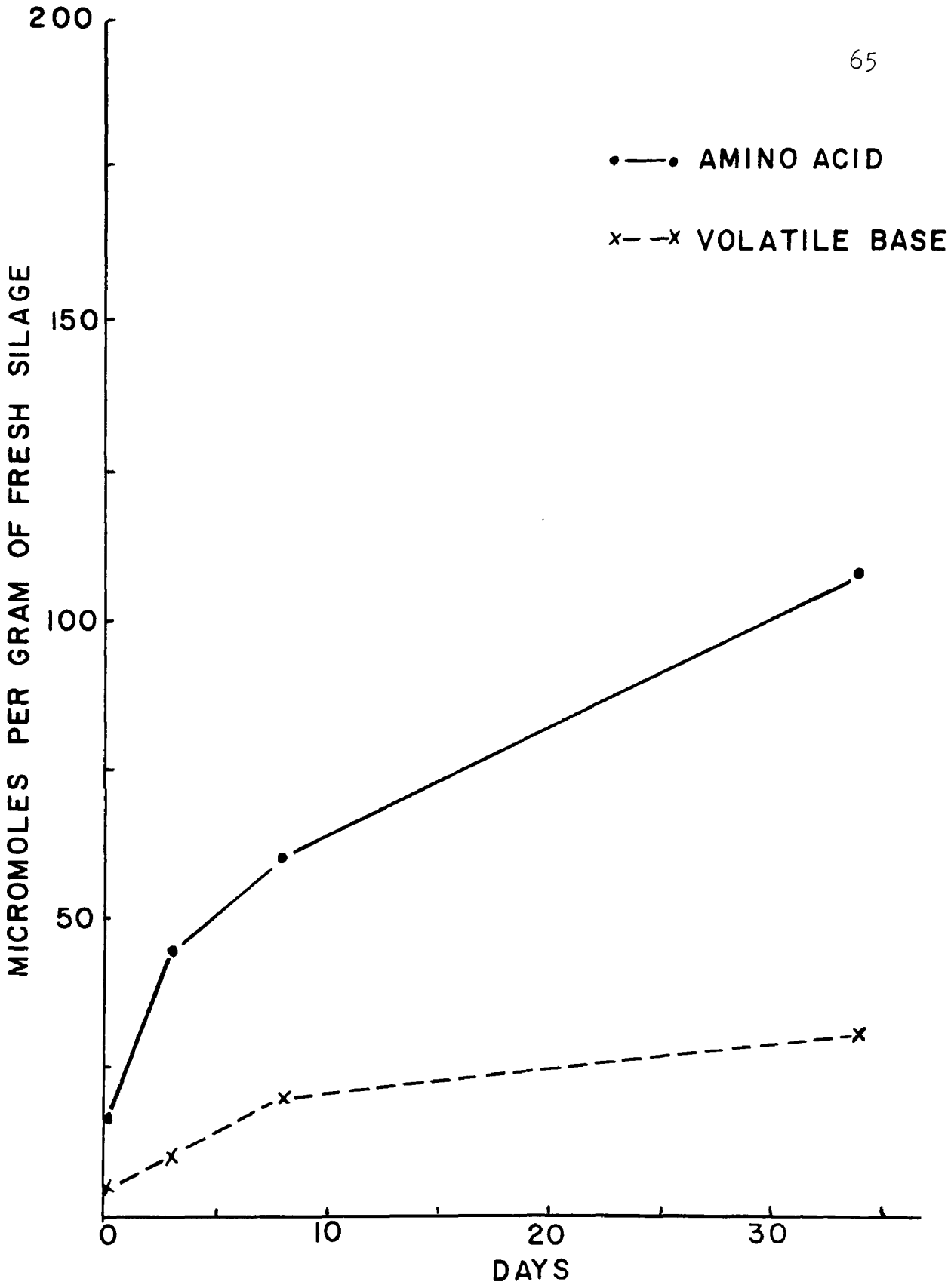

Figure 10. The production of amino acid and volatile base in a well-preserved silage (silo 7).

considerably more breakdown to amino acid in the early stages, followed by the loss of most amino acid with a concurrent rise in the volatile base content (Figure 11).

The bisulfite silage (silo 1) contained elements of each type as illustrated by Figure 12. There was a greater accumulation of amino acid throughout the storage period than in any other silage. There was less volatile base than was found in the typical spoiled silages, but considerably more than in any of the well-preserved silages.

Silages exhibiting the pattern depicted in Figure 11 (silos 8, 9, 10, 12) had residual carbohydrate levels ranging from 0.6 to 1.1 per cent. All other silages had between 1.2 and 3.7 per cent residual carbohydrate.

Discussion

MacPherson (1952a) believed that plant enzymes were responsible for the breakdown of protein to the amino acids and ammonia found in well-preserved silages, but that excess deamination may be brought about by bacteria in the absence of sufficient fermentable carbohydrate. He also reported that proteolysis stopped when the pH was lowered to between 4.5 and 5.0. Consequently, there was considerably more proteolysis in wilted silage where sufficient acidity was produced more slowly (MacPherson 1952b).

In the analysis of good quality silages reported here, there was a gradual increase in both amino acid and volatile

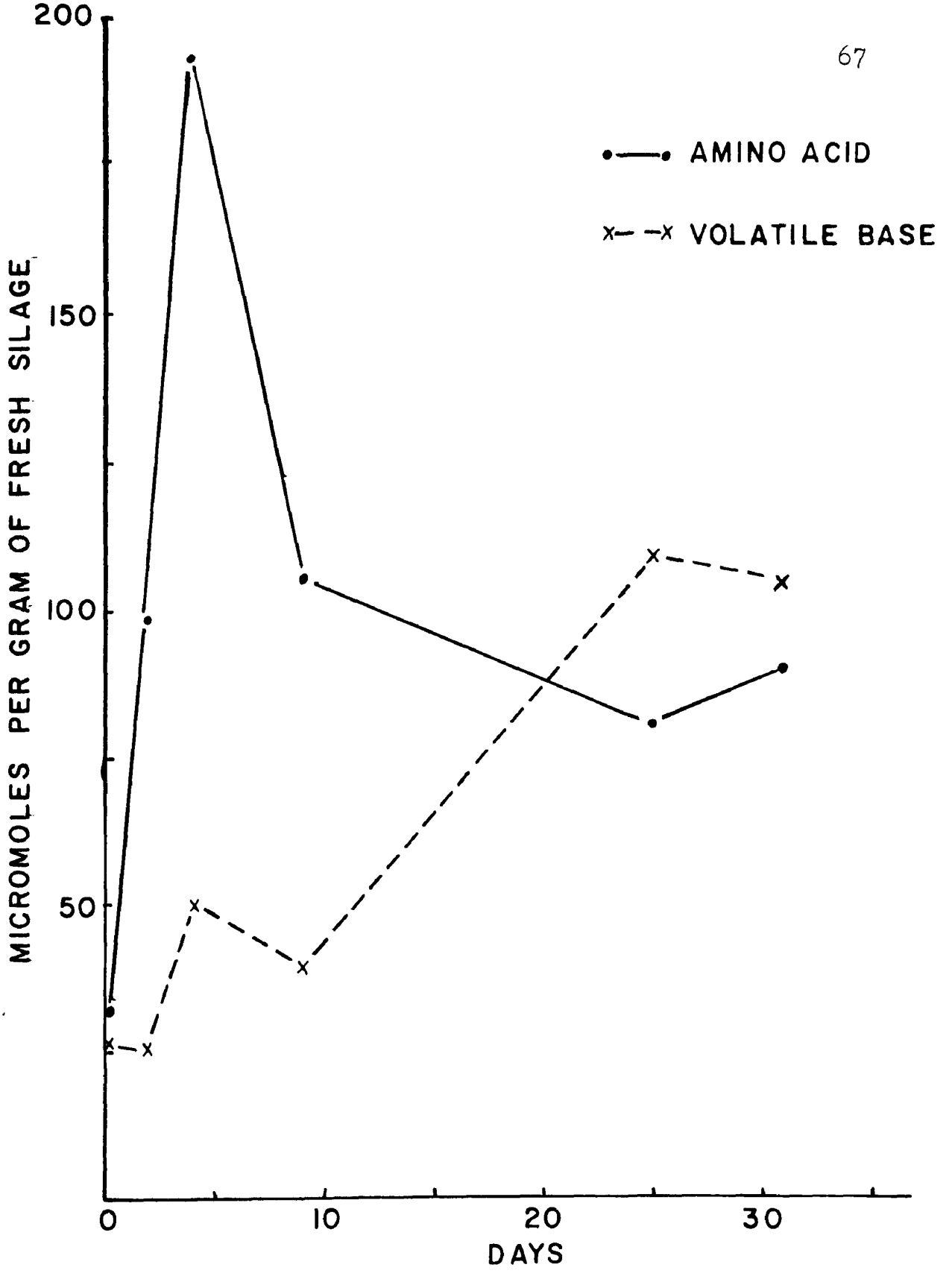

Figure 11. The production of amino acid and volatile base in a spoiled silage (silo 9).

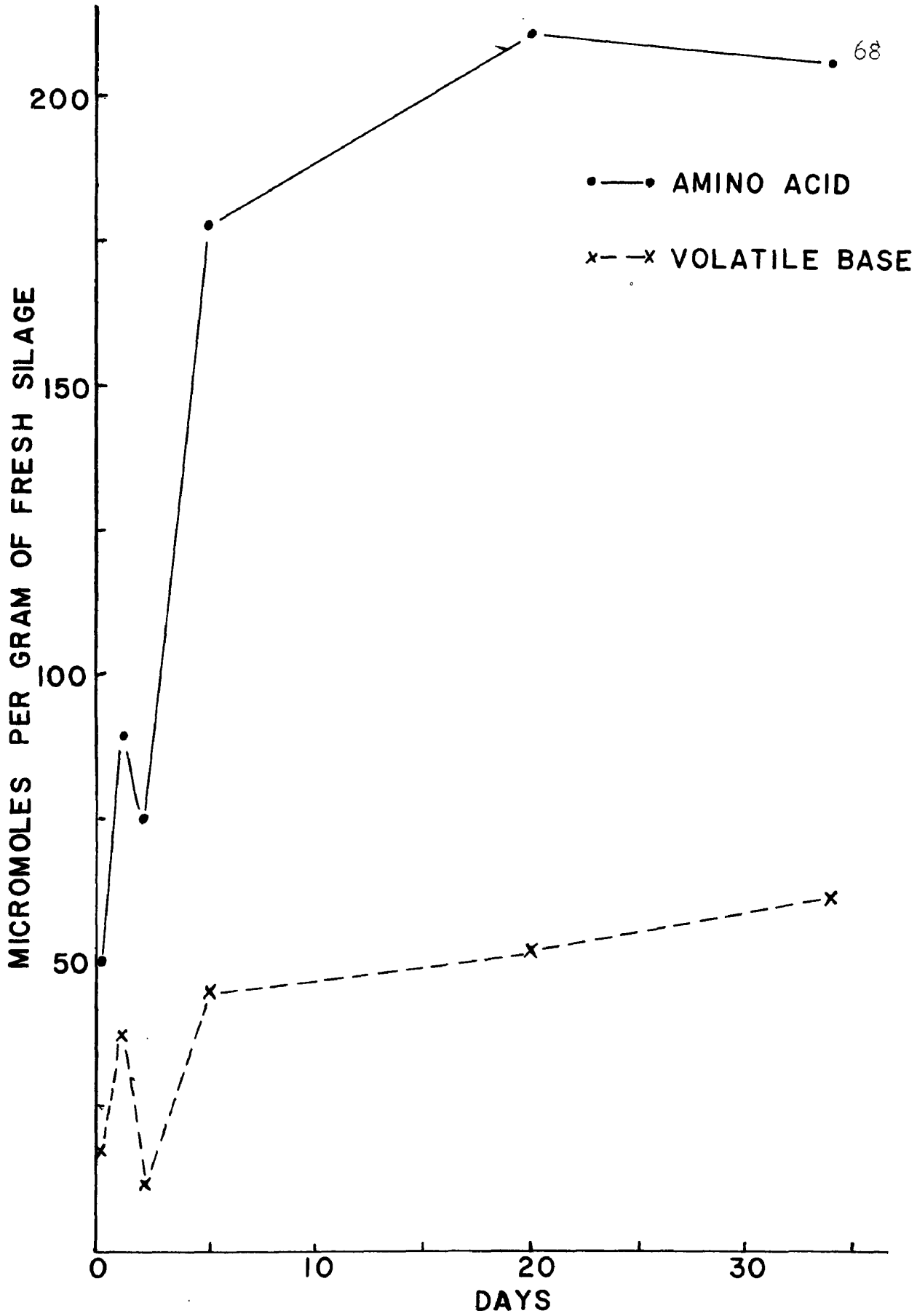

Figure 12. The production of amino acid and volatile base in a bisulfite-treated silage (silo 1).

base content throughout the storage period, even when the pH was as low as 4.0 to 4.5. Volatile base production was never excessive in the good quality silages.

In poor quality silages, where considerable volatile base was formed, there was also a larger amount of amino acid produced initially. The rate of deamination exceeded the rate of amino acid formation as the fermentation progressed, leaving a net decrease in amino acid.

However, in the bisulfite silage, the rate of amino acid formation exceeded the deamination rate throughout the storage period, even though the deamination rate was much greater than in the well-preserved silages.

In normal silages without additives, volatile base formation occurred when the supply of fermentable carbohydrate became too reduced. Normal silages with high amounts of volatile base had less than 1.1 per cent carbohydrate on a wet weight basis.*

Although silo 11 underwent a butyric fermentation, it retained a carbohydrate content of 1.3 per cent; hence there was no excessive amount of deamination. It is concluded that a butyric acid fermentation need not imply protein spoilage.

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*From the basis of a livestock feed, the absolute quantity of carbohydrate is important; but considering silage as a bacteriological medium, carbohydrate content must be described in terms of concentration.

In the bisulfite silage, there was considerable volatile base formed without a butyric fermentation. Although there was 1.8 per cent residual carbohydrate, its utilization may have been blocked by the presence of bisulfite as suggested by Archibald (1953).

Perhaps, when the supply of carbohydrate has been exhausted or blocked, silage bacteria utilize amino acids as a source of carbon and liberate the excess nitrogen as ammonia.

When the pH attains exceedingly high values, such as in the 12th. day and 19th. day samples of silo 12 (see Appendix), all the volatile base is lost. These samples were in advanced stages of decomposition, and were included only for comparison.

CHAPTER IX

SILAGE BACTERIOLOGY

Introduction

Biochemical studies have shown that spoiled silages and well-preserved silages underwent basically different fermentations. Although the silage fermentation has been proved to be essentially a bacterial process, previous workers have found that differences in the bacterial flora of silages of different quality have not been as great, or as basic as expected.

This investigation was primarily concerned with three aspects of silage microbiology. First, since all silages underwent an initial lactic fermentation, the numbers of lactic acid bacteria in different silages were compared. Studies based on the morphology of the predominant silage bacteria have been inconclusive; hence the bacteria isolated from the media selective for lactic acid bacteria have been compared on the basis of their fermentative capacities.

Secondly, the lactic acid bacteria isolated from samples of the fresh plant were compared to the predominant silage types; and the relationship between the number of bacteria on the fresh crop and the ensuing fermentation was investigated.

Thirdly, the number of obligate anaerobes was determined by vegetative methods. Although lactate-fermenting, sporeforming anaerobes have often been detected by spore counts, high spore counts are not necessarily indicative of high lactate-fermenting activity.

Results

The number of silage bacteria capable of growing on LBS medium is shown in Figure 13. Well-preserved and spoiled silages were indistinguishable, but the overheated silages were characterised by the absence of any increase in lactic acid bacteria over the number present on the fresh crop. With the exception of the overheated silages, the population of lactic acid bacteria decreased markedly after the initial rapid development of the first few days. After 3 to 5 weeks there was a further increase in lactic acid bacteria in most silages (silos 1, 2, 3, 5, 6, 8, 11, 12). There was no difference between the counts on LBS and VS media.

A comparative study of the fermentative capacities of 542 isolates from VS and LBS media is given in Table III. The largest single group fermented all sugars very rapidly and are very active in litmus milk. In general, Group 1 and Group 2 of Table III form one division of types which can be called "highly fermentative". In contrast, the second division composed of Groups 3, 4, 5, and 6, are "weakly fermentative". Most of the miscellaneous group differ from one of the other

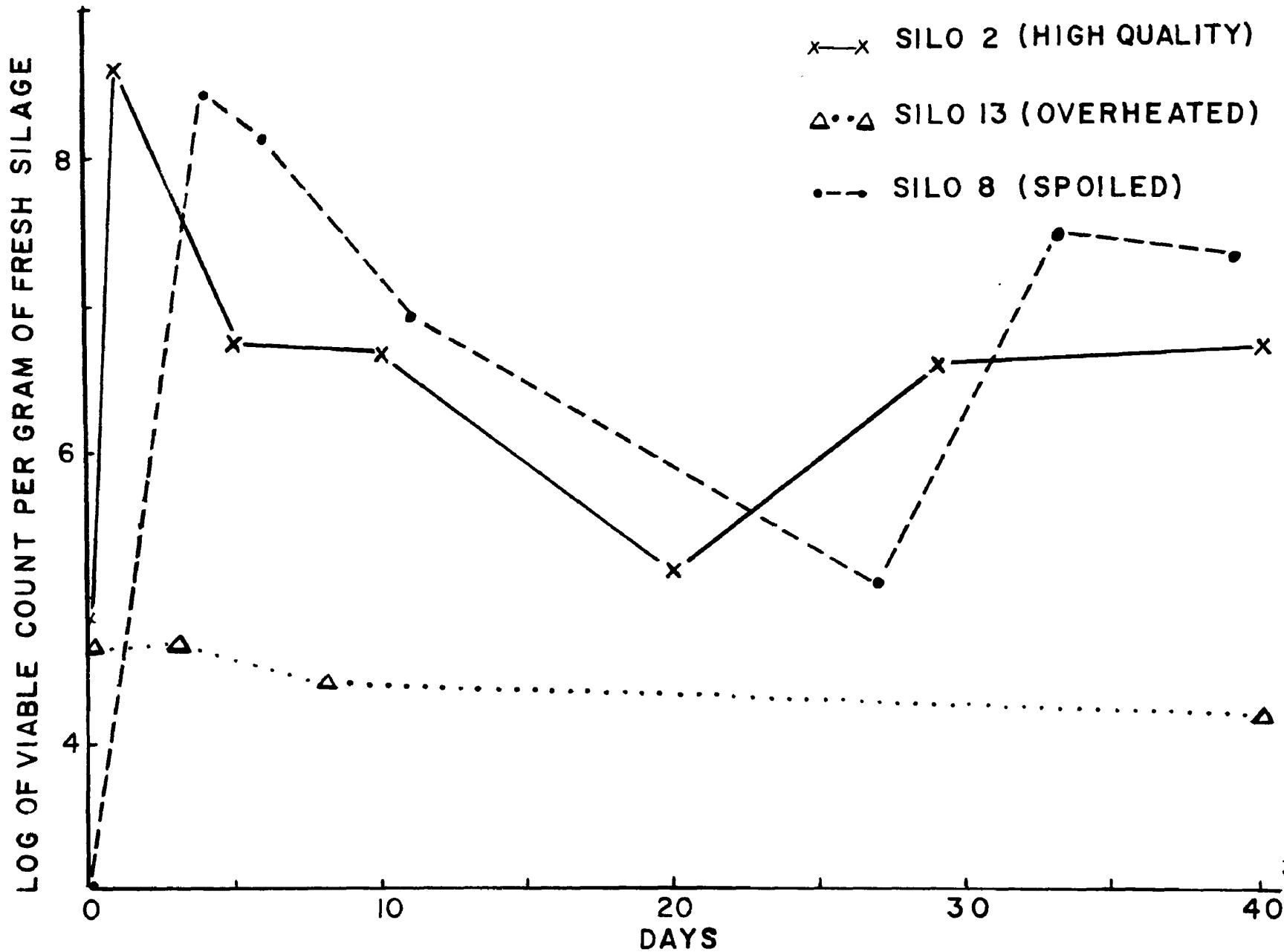

Figure 13. The population of lactic-acid bacteria in well-preserved, spoiled, and over-heated silages.

TABLE III

FERMENTATIVE ABILITY OF SILAGE MICROORGANISMS ISOLATED FROM VS AND LACTOBACILLUS-SELECTION MEDIA

Group	1	2	3	4	5	6	yeasts	Misc.	Totals
Strains, Number (Per cent)	91 (17)	71 (13)	20 (4)	70 (13)	34 (6)	72 (13)	9 (2)	175 (32)	542 (101)
Glucose	+++	+++	+++	+++	+++	+++	-	+++	
Arabinose	+++	+++	+++	+++	+++	+++	-	+++	
Lactose	+++	+++	++	+	+	-	-	*	
Sucrose	+++	+++	+++	-	-	-	-	*	
Dextrin	+++	+++	-	-	-	-	-	*	
Salicin	+++	+++	+++	+++	-	-	-	*	
Mannitol	+++	+++	-	-	-	-	-	*	
Litmus milk, acid	+++	-	-	-	-	-	-	*	
" curd	+++	-	-	-	-	-	-	*	
" reduction	+++	-	-	-	-	-	-	*	

+++ = Positive reaction in 1 - 3 days
++ = Positive reaction in 4 - 6 days
+ = Positive reaction in 7 -14 days
- = Negative reaction after 14 days
* = Variable

groups in the fermentation of only 1 carbohydrate. When these were included with the group they most nearly resembled, the relative percentage in each group did not change significantly.

Morphological studies of the lactic acid bacteria were inconclusive. They included large diplococci, pediococci, lancet-rods and diplo-rods. Each morphological type was found in each fermentation group of Table III. All were catalase negative, and less than 1 per cent reduced nitrate.

Table IV indicates that the fresh plants contained about the same number of highly fermentative types as the silages.

All attempts to show that the number or type of lactic acid bacteria varied with quality met with failure. Furthermore, the percentage of highly fermentative bacteria was the same throughout the storage period.

A total of 171 obligate anaerobes was isolated. All fermented lactate and none hydrolysed gelatin. Essentially all the bacteria growing in AA media were of this type in silages with a high content of butyric acid. These organisms were also found in preponderant numbers in the spoiled silages before the butyric fermentation had begun, and in the bisulfite silage where butyric acid never occurred. A few were also isolated sporadically from the well-preserved and overheated silages.

The chromogenic organisms found on the fresh plants

TABLE LV

COMPARISON OF THE LACTOBACILLI OF FRESH AND ENSILED FORAGES

Division	Group	Fresh Crops		Silages	
		Strains		Strains	
		Number	Per cent	Number	Per cent
Highly Fermentative	1	5	7	91	17
	2	14	19	71	13
			26		30
Weakly Fermentative	3	10	14	20	4
	4	13	18	70	13
	5	1	1	34	6
	6	4	6	72	13
			38		36
Miscellaneous		25	35	175	33

persisted throughout the storage period, but they were only detected when they were present in predominant numbers. For example, in silo 8, there was a rapid multiplication of lactic acid bacteria and no chromogenic types were found on TGY medium after 24 hours. However, after 55 days in the silo, the count on LBS medium dropped below the count on TGY medium and chromogenic types were isolated again. In overheated silos, the number of lactic acid bacteria never exceeded the count on TGY medium and chromogenic organisms were isolated at every stage.

Discussion

Most workers noted a tremendous increase in the number of lactic acid bacteria immediately after ensiling, followed by a gradual decrease during the remainder of the storage period. Kroulik et al. (1955b) found that the numbers decreased more rapidly than expected up to about the 50th day. Thereafter, a secondary increase in bacterial numbers occurred in most silages. Kroulik's work with experimental silos has been substantiated by the results obtained from these full-sized silos.

Burkey et al. (1955) and Dobrogosz and Stone (1958), attempted to follow the changes in silage bacteria on a morphological basis. In some samples, Burkey classified as many as 87 per cent of the bacteria as "variable rods". Results of this present investigation were similarly

inconclusive, although all of the various descriptions of bacteria given by Burkey were noted.

However, when arranged in order of their ability to produce acid from various carbohydrates and in litmus milk, they divided readily into two main divisions; those which fermented all the test carbohydrates readily, and those which fermented only the simple sugars. All the lactic acid bacteria produced acid in pentose (arabinose) as well as in glucose. This concept has been unchanged since the work of Peterson et al. (1925).

The most common lactic acid bacteria were those of Group I, which were active in all carbohydrates and litmus milk. This group is unlike the strains of L. plantarum in normal silages described by Allen et al. (1937b). These same workers (1937a) found organisms with these characteristics only in silages which had received various additives such as whey. Salsbury et al. (1949) also believed that normal silage bacteria preferred the simple sugars, which would mean the organisms in Division 2 would be predominant.

Kroulik et al. (1955b) found that the lactic acid organisms on fresh plants were not "acid producers" like those in the initial days of the ensiling process, and that the bacteria in the later stages of storage were generally "weak acid producers". In this report, it was found that the fermentative capacity of the lactic acid bacteria of fresh plants was as great as the silage bacteria, and that the types

of bacteria in silage after 50 days of storage were not consistently different from those isolated earlier.

There were no detectable differences in the number or type of lactic acid bacteria between well-preserved silages and silages which eventually spoiled. Lactic acid bacteria developed normally in bisulfite silage, whereas overheated silages did not undergo a lactic fermentation, and correspondingly did not possess many lactic acid bacteria. It is concluded that silage quality is unrelated to the number or type of lactic acid bacteria present, except when overheating occurs.

The predominant anaerobe in butyric acid silage resembles Clostridium tyrobutyricum. Since it predominates, it can be isolated in the vegetative stage in an anaerobic agar. However, counts of anaerobes are not significant, since the count on anaerobic agar was often higher than on LBS and V6 media without signifying the presence of obligate anaerobes.

It is significant that Clostridium tyrobutyricum was the predominant organism in spoiled silages before butyric acid was produced. Evidently, the ascendancy of this organism is favored by the method of ensiling, rather than by failure to attain a low pH.

The presence of Clostridium tyrobutyricum in the bisulfite silage may mean that bisulfite inhibits its lactate-fermenting ability but not its growth.

Although the initial bacterial flora fluctuated with the harvesting date, silage quality was dependent only on what occurred after ensiling.

It also appeared that some of the chromogenic bacteria of fresh plants could persist throughout the ensiling period in spite of the accumulation of acid. Whenever the count on LBS and V8 media dropped below the count on TGY medium, the chromogens reappeared. Since lactic acid bacteria also grow on TGY medium, they merely occluded the Flavobacterium species of the fresh plant. A medium which would support the growth of chromogens while restricting lactic acid bacteria might be of value in determining the exact fate of these organisms which comprised the predominant bacterial type on the fresh plant.

Since quantitative and qualitative studies failed to uncover any specific organism which is exclusively present in high-ammonia silages, it is suggested that deamination is brought about by the normal silage flora in the absence of fermentable carbohydrate.

CHAPTER X

GENERAL CONCLUSIONS

Fresh forage crops contained large numbers of chromogenic bacteria which were classified as species of Flavobacterium, and a smaller number of lactic acid bacteria which had fermentative capacities similar to the typical lactic acid bacteria of silage.

As the harvesting date advanced from May to August there was an appreciable increase in the number of each bacterial type on the crops at the time of ensiling. However, examination of the resultant silages failed to show any relationship between silage quality and the number of lactic acid bacteria originally present. This is further evidence that the inoculation of silage with lactic acid bacteria would be useless if used in conjunction with unfavorable harvesting conditions, and unnecessary under optimum conditions.

The predominant Flavobacterium of the fresh crop could apparently persist throughout the fermentation regardless of acid production. It might appear that they were eliminated quickly in those silages where the lactic acid bacteria multiplied rapidly, but they were actually only overwhelmed. They reappeared later in every fermentation whenever the count on LBS medium dropped below the count on TGY medium.

By the time they were ensiled, high moisture crops had

lower pH values than low moisture crops. Since the apparent increase in acidity was not associated with higher numbers of acid-producing bacteria, it was assumed that acid-producing plant enzymes were very active in the high-moisture crops.

Spoilage entailed the loss of a considerable amount of dry matter, while well-preserved silages appeared to gain in dry matter content because of moisture loss. Therefore, single dry matter determinations after the storage period would have shown that all spoiled silages had less than 20 per cent dry matter. From similar data it has often been concluded that a high moisture content causes spoilage. Since there was no correlation between the moisture content of the fresh crops and silage quality, it was concluded that high moisture silage is only the result of spoilage. However, high moisture crops are predisposed to spoilage because they can be over-packed more easily. Over-packing always led to spoilage.

It was found that over-packing favored, or even caused, the development of lactate-fermenting Clostridium tyrobutyricum in the vegetative state immediately after ensiling and before butyric acid appeared. The addition of bisulfite inhibited the formation of butyric acid, but since the bisulfite silage was over-packed, Clostridium tyrobutyricum still appeared.

A summation of the biochemical changes which occurred

in a spoiled silage is given in Figure 14. In Phase I (0 - 10 days), the initial drop in pH corresponded to the formation of lactic acid.

During Phase II (10 - 25 days), the lactic and succinic acids were utilized by Clostridium tyrobutyricum, and butyric and propionic acids were produced. Since butyric acid is a weaker acid than lactic, the pH rises. Amino acids were deaminated in this interval, but this reaction does not contribute to the rise in pH because volatile base and high molecular weight acids are produced in equivalent amounts. Therefore, the pH remained low in silo 1 because no butyric acid was formed, even though there was considerable volatile base produced. Conversely, there was no appreciable deamination in silo 11, but the pH rose because butyric acid was produced.

In Phase III (25 - 55 days), the pH gradually decreased. Because the pH was relatively high during this period, volatile bases were lost, leaving an acidic residue. In some cases, the level of volatile base did not drop in absolute quantity, but a loss of base could be calculated from the excess of amino acid lost to volatile base gained. When advanced spoilage occurred, the pH rose above 8, in which case essentially no acids were present, and most of the volatile bases had also been lost.

As mentioned above, vegetative cells of Clostridium tyrobutyricum were found in over-packed silos from the

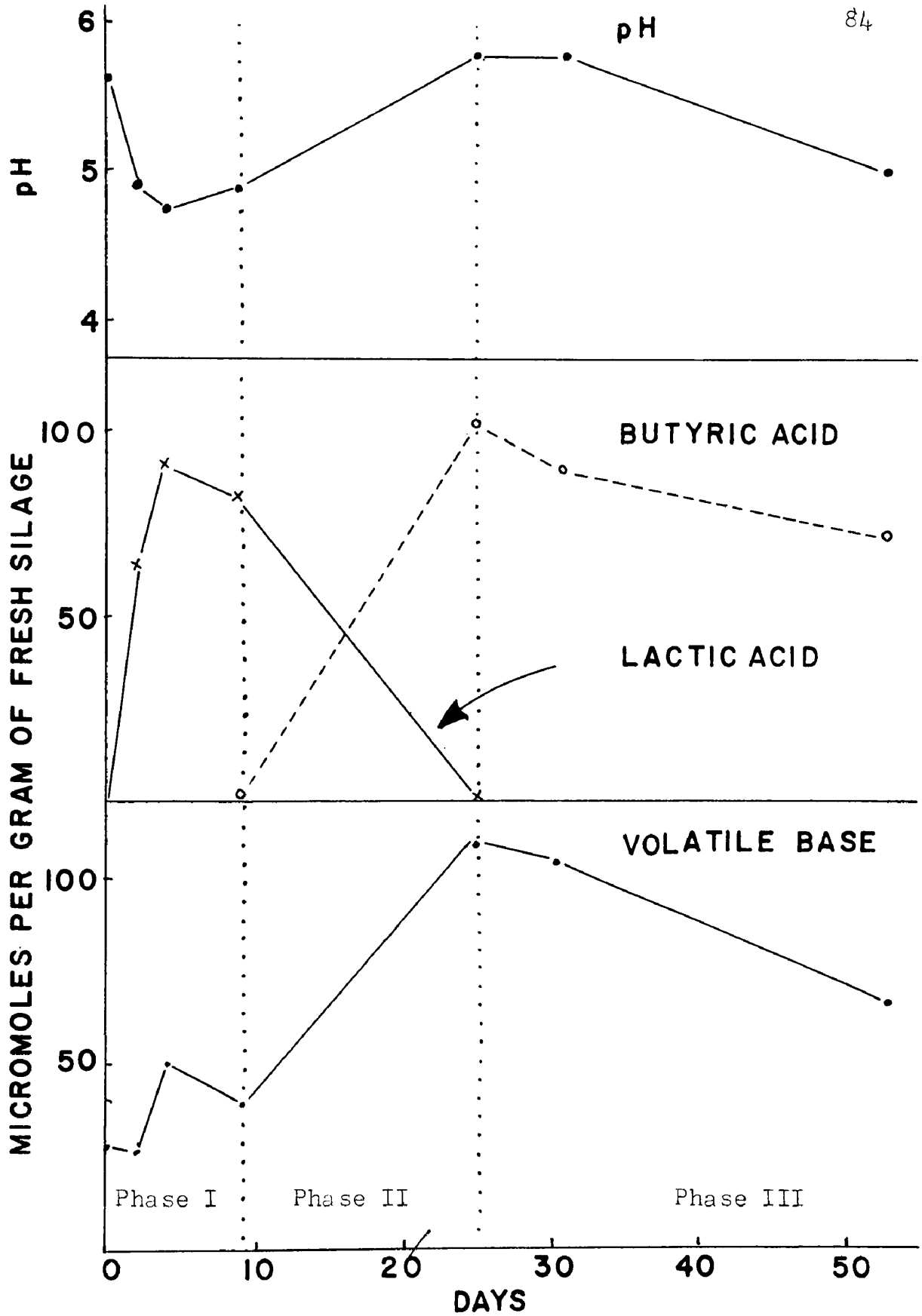

Figure 14. Summation of the biochemical changes in a spoiled silage (silo 9).

beginning of the fermentation, but there was no apparent factor which precipitated the formation of butyric acid. Conversely, deamination was precipitated when the carbohydrate level dropped below 1 per cent, but there was no noticeable alteration of the bacterial flora. Therefore, deamination and butyric acid formation could occur separetely; not necessarily together as in Figure 14. Butyric acid was produced in silo 11 because the silage had been too tightly packed, but deamination did not take place because the carbohydrate level remained above 1 per cent.

The population of lactic acid bacteria exhibited two peaks during the storage period of both well-preserved and spoilage silages. Although this indicated a secondary fermentation, and other workers had noticed this same phenomenon, there was no corresponding changes in the biochemical composition of either type of silage. Furthermore, the lactic acid bacteria in the main and secondary fermentations were indistinguishable.

CHAPTER XI

SUMMARY

Fresh forages have been found to contain a large number of chromogenic bacteria which were classified as species of Flavobacterium, and a small number of typical silage bacteria.

The number of both types of bacteria on fresh forage at the time of ensiling can be predicted from the harvesting date. Bacterial numbers increased 100 times between late May and early August. However, the initial number of bacteria on the fresh crop bore no relationship to the final quality of the silage.

The Flavobacterium group did not multiply during the silage process, but they evidently persisted in low numbers throughout the storage period in spite of the accumulation of acid.

By the time they were ensiled, forages with a high moisture content had developed low pH values. Since there was no corresponding increase in acid-forming bacteria, it was concluded that plant enzymes may be very active in high-moisture crops prior to ensiling.

When the silage was too tightly packed, Clostridium tyrobutyricum developed immediately after ensiling, eventually leading to the conversion of all lactic and succinic acids

to butyric and propionic acid. Forages with a high moisture content tended to undergo butyric spoilage because they were more apt to be packed too tightly.

Excessive production of volatile base occurred when the hydrolysable carbohydrate content fell below 1 per cent of the total mass. Due to a high content of water and a relatively smaller concentration of carbohydrate, high-moisture forages were also more likely to undergo volatile base spoilage.

The one example of bisulfite silage was also tightly packed; hence Clostridium tyrobutyricum developed. However, the bisulfite evidently inhibited the fermentation of lactic acid to butyric acid. Bisulfite also apparently interfered with the normal utilization of carbohydrate, since volatile base was produced even though the carbohydrate level remained above the critical level of 1 per cent of the wet weight.

Quantitatively, the lactic acid bacteria in both well-preserved and spoiled silages revealed two peaks; one in the first few days of ensiling and the second after 3 to 5 weeks. The significance of this secondary fermentation was not determined.

Qualitatively, the lactic acid bacteria divided into a group which rapidly fermented all carbohydrates and was active in litmus milk, and a second main group which preferred simple sugars. Bacteria of each type were isolated at every stage of the fermentation from every quality of silage.

Overheated silages were intermediate in quality and pH. Acetic acid was the only acid produced in appreciable quantity; hence they contained only a few lactic acid bacteria.

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APPENDIX

LACTOBACILLUS SELECTION MEDIUM

Rogosa et al., (1951)

Trypticase (BBL)	10 g.
Yeast extract (Difco)	5 g.
KH_2PO_4	6 g.
Ammonium Citrate	2 g.
Glucose	10 g.
Sucrose	5 g.
Arabinose	5 g.
Tween 80	1 g.
Sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)	25 g.
Glacial Acetic Acid (99.5%)	1.32 ml.
Agar	15 g.
*Salt Solution	5 ml.
Distilled Water	to 1 liter
pH	adjusted to 5.40

Do Not Autoclave

*Salt Solution

$MgSO_4 \cdot 7H_2O$	11.5 g.
$MnSO_4 \cdot 2H_2O$	2.4 g.
$FeSO_4 \cdot 7H_2O$	0.68 g.
Distilled Water	to 100 ml.

V8 MEDIUM

Fabian *et al.* (1953)

Tryptose	10 g.
Glucose	5 g.
Agar	18 g.
Filtered V8 Juice	125 ml.
Distilled Water	to 1 litre
Brom Cresol Green	0.1 g.
pH	adjusted to 5.6

TGY MEDIUM

Kroulik *et al.* (1955)

Tryptone	5 g.
Glucose	1 g.
Yeast Extract	3 g.
Agar	15 g.
Distilled Water	to 1 litre
pH	adjusted to 7.0

MEDIUM FOR THE ANAEROBIC FERMENTATION OF LACTATE

Rosenberger, (1951)

Sodium lactate syrup (70%)	14.3 ml.
Sodium acetate	8.0 g.
Yeast extract (Difco)	20.0 g.
$(NH_4)_2SO_4$	0.5 g.
p-amino-benzoic acid	100 ug
Cysteine hydochloride	0.5 g.
Thioglycollic acid	0.5 ml.
Resazurin	5.0 mg.
$MnCl_2.4H_2O$	0.15 g.
NaCl	2.5 g.
K_2HPO_4	1.0 g.
$(NH_4)_2MoO_4$	0.005 g.
$FeSO_4.7H_2O$	0.04 g.
$MgSO_4.7H_2O$	0.80 g.
Agar	2.0 g.
Tap water	to 1 liter
pH	adjusted to 6.0

MEDIUM FOR THE STUDY OF ANAEROBIC PROTEOLYSIS

Rosenberger, (1951)

Trypticase (BBL)	10 g.
Phytone (BBL)	5 g.
Gelatin	120 g.
Yeast extract (Difco)	10 g.
Cysteine hydrochloride	0.5 g.
Resazurin	5.0 mg.
Tap water	to 1 liter
pH	adjusted to 7.0

WATER AGAR

Rosenberger, (1951)

Thioglycollic acid	0.5 ml.
Cysteine hydrochloride	0.5 g.
Agar	10 g.
Resazurin	5.0 mg.
Tap water	to 1 liter
pH	adjusted to 7.0

ANALYSES OF THE FRESH CROPS AT THE TIME OF ENSILING

Sample	1	2	3	4	5	6	7	8	9	10
Date	May 28	29	29	29	June 6	6	7	7	8	10
Time of Day	1600	1330	1400	1630	0900	1630	1400	1600	1400	1500
Air Temp. (°F)	67	73	73	73	70	79	58	62	70	86
Humidity (%)	50	36	36	36	75	48	72	72	33	53
Cloud Cover (10ths.)	1	0	0	1	2	0	10	10	0	0
Days since Rain	2	3	3	3	5	5	0	0	1	4
Composition*	LW	LG	LG	L	LG	LG	LG	LG	LG	LG
Method of Harvest**	DC	DC	DC	DC	DC	S	DC	S	DC	DC
Wilted (Hours)	0	2	3	0	0	0	0	0	0	0
Bacterial Count (LBS)	<3***	<3	<3	<3	3.6	<3	4.4	4.3	<3	
Bacterial Count (TGY)	7.67	8.29	8.43	7.85	8.09	8.07	7.43	8.24	7.09	
Bacterial Count (AA)	5.63	7.07	6.97	6.58	6.57	6.66	6.33	7.05	6.60	7.46
pH	5.86	5.92	6.00	5.93	5.78	5.93	5.45	5.59	5.95	5.64
Moisture (%)	81.19	70.43	69.79	79.72	75.41	68.84	80.90	80.62	80.34	78.93
Protein (%)	4.56	4.92	6.03	5.30	4.76	5.38	3.32	3.28	4.60	4.36
Carbohydrate (%)	4.93	7.22	5.17	2.35	3.20	2.90	2.22	3.12	3.83	4.28

ANALYSES OF THE FRESH CROPS AT THE TIME OF ENSILING

Sample	11	12	13	14	15	16	17	18	19	20
Date	June 14	18	20	21	21	22	25	25	25	26
Time of Day	1700	1400	1615	1500	1500	1400	1200	1240	1330	1500
Air Temp. (°F)	78	86	82	87	87	91	67	67	67	78
Humidity (%)	59	63	40	38	38	49	62	62	62	62
Cloud Cover (10ths.)	10	10	0	0	0	0	10	10	10	0
Days since Rain	3	1/4	2	3	3	4	1	1	1	1
Composition*	LG	L	L	LW	LW	GL	L	LW	GL	GL
Method of Harvest**	S	S	DC	S	S	S	S	S	S	S
Wilted (Hours)	0	2	0	2	3	4	0	2	3	0
Bacterial Count (LBS)	4.75	6.06	4.88	5.12	5.09	5.51	6.22	<3	4.29	5.98
Bacterial Count (TGY)	8.41	8.80	8.89	7.97	7.87	9.43	9.60	8.95	8.98	9.03
Bacterial Count (AA)	7.37	8.04	7.61	6.06	7.00	8.12	7.66	7.61	7.52	7.67
pH	5.68	5.70	5.59	5.80	5.82	6.10	5.80	5.84	5.96	5.63
Moisture (%)	76.95	75.46	82.96	64.81	62.45	63.14	80.80	67.55	69.46	69.71
Protein (%)	4.21	3.46	3.96	5.55	5.08	5.49	3.29	4.91	4.63	4.06
Carbohydrate (%)	2.93	4.27	2.57	5.38	5.67	4.79	1.56	5.71	5.06	4.30

ANALYSES OF THE FRESH CROPS AT THE TIME OF ENSILING

Sample	21	22	23	24	25	26	27	28	29	30
Date	June 26	27	29	July 2	3	3	9	16	16	19
Time of Day	1630	1400	1500	1030	1000	1010	1300	1435	1320	1100
Air Temp. (°F)	77	77	73	76	84	84	78	79	70	85
Humidity (%)	66	64	41	73	72	72	45	46	46	83
Cloud Cover (10ths.)	10	5	0	0	0	0	0	0	0	0
Days since Rain	1	1/4	1	4	5	5	1		2	2
Composition*	C	C	LGW	L	C	L	LW	0	LW	LW
Method of Harvest**	DC	S	S	S	S	S	S	DC	S	S
Wilted (Hours)	0	0	0	0	0	0	0	0	0	0
Bacterial Count (LBS)	5.50	5.86	4.99	4.21	5.65	5.86	5.05	<3	5.79	5.94
Bacterial Count (TGY)	9.26	9.34	8.62	8.06	8.90	9.03	9.26	8.96	9.42	9.58
Bacterial Count (AA)	7.84	7.96	7.85	7.39	7.94	7.67	8.23	8.03	8.56	8.18
pH	5.67	6.04	5.86	6.07	5.98	5.81	5.90	6.46	6.08	5.78
Moisture (%)	77.85	69.70	71.27	68.97	70.78	69.32	69.79	57.03	67.24	66.54
Protein (%)	3.60	4.54	4.39	5.03	3.74	4.78	4.34	5.03	4.72	5.13
Carbohydrate (%)	3.94	5.24	4.37	4.72	5.49	4.20	4.92	8.50	3.80	4.08

ANALYSES OF THE FRESH CROP AT THE TIME OF ENSILING

Sample	31	32	33
Date	July 23	27	Aug. 5
Time of Day	1030	1050	1000
Air Temp. (°F)	72	80	66
Humidity (%)	83	82	77
Cloud Cover (10ths.)	0	5	0
Days since Rain	1	5	
Composition*	LW	LW	LW
Method of Harvest**	S	S	S
Wilted (Hours)	0	0	0
Bacterial Count (LBS)	6.00	6.00	5.87
Bacterial Count (TGY)	9.99	9.93	9.91
Bacterial Count (AA)	8.50	8.18	7.73
pH	6.20	6.10	6.33
Moisture (%)	70.44	60.70	60.65
Protein (%)	4.52	5.47	5.38
Carbohydrate (%)	4.40	4.79	5.37

*Composition, L = Legume (alfalfa)
G = Grass (orchard or brome)
W = Weeds
C = Clover (mammoth or June)
O = Oats
predominant component listed first

**Method of Harvest
DC = Direct Chopped
S = Swathed (one load at a time)

***Bacterial numbers are expressed as logarithms

ANALYSES FOR SILO 1

Days ensiled	0	1	2	5	20	34	44
Bacterial count (LBS)	<3***	-	7.26	8.52	6.85	5.00	5.26
Bacterial count (V8)	-	-	6.95	8.43	7.11	5.48	5.36
Bacterial count (TGY)	7.16	8.76	8.26	8.36	8.04	6.11	5.32
Bacterial count (AA)	5.88	-	7.60	8.22	8.35	>6	6.25
pH	5.93	5.08	5.43	4.70	4.62	4.72	4.72
Moisture (%)	79.72	81.75	80.83	81.28	80.77	81.26	80.82
Protein (%)	5.30	4.43	4.44	4.45	4.64	4.77	4.81
Carbohydrate (%)	2.35	2.3	0.9	2.0	2.4	1.5	1.8
Total acid (µM)	165	239	253	418	468	495	495
Volatile base (µM)	17	37	12	46	53	61	72
Amino acid (µM)	50	89	75	179	212	207	215
Butyric acid (µM)	0	0	0	0	0	1	1
Propionic acid (µM)	-	0	0	0	0	2	0
Acetic acid (µM)	58	26	91	59	111	119	
Formic acid (µM)	-	6	1	0	1	6	5
Lactic acid (µM)	-	81	39	189	182	191	189
Succinic acid (µM)	-	36	31	33	22	41	27

***Bacterial numbers expressed as logarithms in all succeeding tables.

ANALYSES FOR SILO 2

Days ensiled	0	1	5	10	20	29	40
Bacterial count (LBS)	4.85	8.60	6.74	6.71	5.20	6.57	6.73
Bacterial count (V8)	-	8.74	6.67	5.78	4.70	6.43	6.57
Bacterial count (TGY)	8.60	8.79	-	6.96	6.30	6.45	6.54
Bacterial count (AA)	7.19	8.75	6.76	7.46	4.98	6.49	6.54
pH	5.67	5.08	4.75	4.39	4.21	4.20	4.39
Moisture (%)	77.85	70.80	80.68	78.68	74.34	69.21	75.42
Protein (%)	3.60	4.80	2.75	3.34	4.24	5.26	4.03
Carbohydrate (%)	3.94	3.2	1.7	1.5	2.1	2.2	1.2
Total acid (µM)	104	171	187	314	400	406	448
Volatile base (µM)	8	16	20	25	32	27	25
Amino acid (µM)	-	51	47	86	124	95	189
Butyric acid (µM)	-	0	0	0	0	0	0
Propionic acid (µM)	-	0	0	0	0	0	2
Acetic acid (µM)	-	73	108	152	109	106	137
Formic acid (µM)	-	5	0	4	0	6	9
Lactic acid (µM)	-	2	38	120	300	329	180
Succinic acid (µM)	-	26	9	21	35	43	32

ANALYSES FOR SILO 3

Days ensiled	3	10	32	45
Bacterial count (LBS)	5.88	5.65	3.78	3
Bacterial count (V8)	4.78	6.04	<3	<3
Bacterial count (TGY)	7.28	7.30	4.60	5.90
Bacterial count (AA)	6.88	6.91	<3	<3
pH	4.73	4.45	4.17	4.11
Moisture (%)	72.14	72.50	70.49	68.93
Protein (%)	4.21	3.91	3.99	4.16
Carbohydrate (%)	3.2	2.6	3.3	2.6
Total acid (µM)	235	281	380	424
Volatile base (µM)	19	30	35	35
Amino acid (µM)	73	92	96	109
Butyric acid (µM)	0	0	0	0
Propionic acid (µM)	0	0	0	0
Acetic acid (µM)	63	79	88	80
Formic acid (µM)	0	1	4	6
Lactic acid (µM)	69	105	198	245
Succinic acid (µM)	25	34	29	32

ANALYSES FOR SILO 4

0	1	14	22
<3	8.26	4.87	6.78
-	8.49	4.48	6.61
8.59	10.05	6.96	6.85
7.67	8.94	6.71	6.25
6.46	6.23	4.92	4.37
57.03	72.87	67.28	72.01
5.03	3.53	4.29	3.61
8.50	5.4	3.6	1.9
71	157	339	388
26	30	64	43
26	69	144	137
-	0	0	0
-	0	0	0
-	44	51	109
-	7	7	9
-	0	133	149
-	33	28	19

ANALYSES FOR SILO 5

Days ensiled	0	10	20	25	33
Bacterial count (LBS)	5.34	7.88	7.68	7.52	8.71
Bacterial count (V8)	-	-	7.95	7.71	<3
Bacterial count (TGY)	8.52	8.92	8.61	6.79	9.01
Bacterial count (AA)	7.16	5.	8.01	7.40	8.68
pH	5.81	4.70	4.97	4.40	5.28
Moisture (%)	69.32	72.78	76.31	73.18	63.00
Protein (%)	4.78	4.18	4.23	4.65	5.79
Carbohydrate (%)	4.2	1.9	1.4	3.5	2.9
Total acid (μM)	69	243	229	335	286
Volatile base (μM)	27	31	33	38	42
Amino base (μM)	37	86	104	70	123
Butyric acid (μM)	-	0	0	0	0
Propionic acid (μM)	-	0	0	0	0
Acetic acid (μM)	-	63	92	108	80
Formic acid (μM)	-	2	1	2	1
Lactic acid (μM)	-	94	99	172	113
Succinic acid (μM)	-	17	18	23	24

	ANALYSES FOR SILO 6					ANALYSES FOR SILO 7			
Days ensiled	0	2	19	33	39	0	3	8	34
Bacterial count (LBS)	4.40	7.30	<3	3.90	<3	5.11	9.43	<3	<3
Bacterial count (V8)	-	7.30	<3	3.30	<3	-	9.18	<3	<3
Bacterial count (TGY)	8.08	7.36	7.04	6.59	5.11	8.36	9.39	7.11	6.00
Bacterial count (AA)	7.31	7.69	>4	5.87	4.40	7.40	8.42	<3	4.87
pH	5.86	5.23	4.85	4.77	4.39	5.98	4.64	4.16	4.30
Moisture (%)	71.27	72.16	71.10	71.81	73.43	70.78	77.80	77.82	73.82
Protein (%)	4.39	4.60	5.15	4.72	4.43	3.74	2.87	3.08	3.53
Carbohydrate (%)	4.37	1.7	2.0	1.4	1.2	5.49	2.0	1.8	1.8
Total acid (µM)	182	223	305	310	367	70	164	213	346
Volatile base (µM)	25	39	54	59	57	5	11	20	30
Amino acid (µM)	59	83	105	107	111	16	44	60	108
Butyric acid (µM)	-	0	0	0	0	-	0	0	0
Propionic acid (µM)	-	0	0	0	0	-	0	0	0
Acetic acid (µM)	-	67	86	91	127	-	128	79	83
Formic acid (µM)	-	9	5	2	2	-	7	6	2
Lactic acid (µM)	-	36	82	81	138	-	44	109	156
Succinic acid (µM)	-	9	25	36	27	-	20	21	34

ANALYSES FOR SILO 8

Days ensiled	0	2	4	6	11	27	33	39	49	55
Bacterial count (LBS)	<3	-	8.43	8.15	6.95	5.10	7.51	7.34	7.65	6.71
Bacterial count (V8)	-	-	8.43	7.60	6.66	5.17	7.50	6.90	7.36	<3
Bacterial count (TGY)	6.38	-	8.11	-	6.71	5.85	-	7.53	6.99	6.82
Bacterial count (AA)	5.89	9.03	8.60	8.32	6.60	6.65	7.61	7.44	7.74	>5
pH	5.95	5.30	4.58	4.51	5.11	4.98	5.22	5.93	5.20	5.10
Moisture (%)	80.34	79.25	79.25	81.92	82.75	79.46	78.68	84.34	84.96	86.42
Protein (%)	4.60	4.37	4.29	3.22	3.28	4.24	4.46	3.63	2.97	2.52
Carbohydrate (%)	3.83	2.1	2.0	2.2	0.9	1.9	2.1	0.5	0.7	0.8
Total acid (μM)	165	270	336	233	167	407	363	316	235	224
Volatile base (μM)	28	50	25	22	22	55	94	137	143	71
Amino acid (μM)	39	88	119	78	60	179	119	120	54	37
Butyric acid (μM)	-	0	0	0	33	0	91	99	100	90
Propionic acid (μM)	-	0	0	0	7	7	58	67	39	34
Acetic acid (μM)	-	55	54	70	94	213	151	131	142	117
Formic acid (μM)	-	4	1	3	1	3	4	3	10	7
Lactic acid (μM)	-	41	122	87	82	65	4	3	3	2
Succinic acid (μM)	-	26	35	31	25	43	30	0	0	1

ANALYSES FOR SILO 9

Days ensiled	0	2	4	9	25	31	53
Bacterial count (LBS)	-	9.04	8.20	6.83	4.97	4.78	4.43
Bacterial count (V8)	-	9.15	7.90	7.00	<3	<3	<3
Bacterial count (TGY)	-	8.34	7.60	6.48	7.62	6.38	6.89
Bacterial count (AA)	6.79	8.77	8.40	6.40	6.68	5.90	6.10
pH	5.64	4.93	4.77	4.88	5.80	5.80	5.04
Moisture (%)	78.93	83.62	83.99	85.18	54.37	84.38	85.43
Protein (%)	4.36	3.64	3.30	2.88	3.70	4.16	3.68
Carbohydrate (%)	4.28	1.4	1.1	1.1	1.3	0.8	0.7
Total acid (µM)	143	239	444	263	250	260	207
Volatile base (µM)	28	26	50	39	109	104	65
Amino acid (µM)	33	98	194	106	81	91	43
Butyric acid (µM)	0	0	0	0	102	89	72
Propionic acid (µM)	-	0	0	2	26	40	25
Acetic acid (µM)	-	99	81	108	100	109	125
Formic acid (µM)	-	6	9	4	6	9	4
Lactic acid (µM)	-	63	91	83	0	2	2
Succinic acid (µM)	-	21	19	16	3	5	0

ANALYSES FOR SILO 10

Days ensiled	0	4	7	22	41
Bacterial count (LBS)	4.11	7.30	7.03	4.81	<3
Bacterial count (V8)	-	7.18	7.04	4.86	<3
Bacterial count (TGY)	7.77	7.40	5.78	6.43	7.14
Bacterial count (AA)	6.73	8.20	6.82	6.94	<3
pH	5.68	4.99	4.86	6.40	5.43
Moisture (%)	76.95	79.26	77.96	81.22	81.21
Protein (%)	4.21	3.98	4.38	3.84	3.71
Carbohydrate (%)	2.93	1.2	0.8	1.3	1.1
Total acid (μM)	98	336	292	256	270
Volatile base (μM)	71	31	52	99	110
Amino acid (μM)	33	141	104	86	61
Butyric acid (μM)	-	0	0	99	108
Propionic acid (μM)	-	0	0	36	23
Acetic acid (μM)	-	111	115	68	132
Formic acid (μM)	-	3	16	2	2
Lactic acid (μM)	-	120	96	2	0
Succinic acid (μM)	-	36	21	8	0

ANALYSES FOR SILO 11

Days ensiled	0	1	9	14	31
Bacterial count (LBS)	3.70	8.92	<3	5.61	3.60
Bacterial count (V8)	-	8.89	<3	<3	<3
Bacterial count (TGY)	7.56	8.43	6.00	6.40	6.28
Bacterial count (AA)	6.88	9.26	6.53	6.22	5.56
pH	6.07	5.17	5.02	4.72	5.08
Moisture (%)	68.97	70.46	76.67	75.61	78.70
Protein (%)	5.03	4.54	3.65	4.02	3.27
Carbohydrate (%)	4.72	3.5	1.6	1.7	1.3
Total acid (μM)	42	225	256	262	187
Volatile base (μM)	19	30	49	52	42
Amino acid (μM)	32	78	93	93	58
Butyric acid (μM)	-	0	0	11	42
Propionic acid (μM)	-	0	7	6	19
Acetic acid (μM)	-	63	100	59	109
Formic acid (μM)	-	5	5	5	2
Lactic acid (μM)	-	36	74	83	3
Succinic acid (μM)	-	43	26	30	21

ANALYSES FOR SILO 12

Days ensiled	0	2	12	26	43	12*	19*
Bacterial count (LBS)	4.11	8.77	6.14	4.48	5.56	8.10	7.78
Bacterial count (V8)	-	8.52	6.04	<3	5.32	-	7.30
Bacterial count (TGY)	8.12	6.26	6.32	7.08	7.49	9.	9.82
Bacterial count (AA)	6.84	8.19	-	6.34	7.25	8.11	9.19
pH	5.59	4.57	6.83	5.87	5.78	8.50	8.30
Moisture (%)	82.96	80.59	81.52	77.88	86.22	83.52	86.17
Protein (%)	3.96	3.98	4.01	4.99	3.83	3.95	3.06
Carbohydrate (%)	2.57	1.2	0.9	1.1	0.6	1.0	1.1
Total acid (µM)	167	328	286	349	350	89	67
Volatile base (µM)	20	35	111	174	177	58	15
Amino acid (µM)	11	132	111	96	76	72	39
Butyric acid (µM)	-	0	126	119	128	6	5
Propionic acid (µM)	-	0	21	44	65	5	4
Acetic acid (µM)	-	94	96	175	160	56	23
Formic acid (µM)	-	5	2	5	0	0	4
Lactic acid (µM)	-	149	2	6	1	0	3
Succinic acid (µM)	-	18	1	10	4	0	1

*Taken from a badly decomposed, black, slimy layer

ANALYSES FOR SILO 13

Days ensiled	0	3	8	40
Bacterial count (LBS)	4.66	4.72	4.43	4.20
Bacterial count (V8)	-	<3	<3	3.79
Bacterial count (TGY)	7.45	7.26	8.09	4.70
Bacterial count (AA)	6.58	5.44	6.10	4.98
pH	5.82	6.13	5.30	4.39
Moisture (%)	62.45	72.97	70.28	63.13
Protein (%)	5.08	3.94	4.50	4.96
Carbohydrate (%)	5.67	2.9	3.7	3.1
Total acid (µM)	57	157	235	374
Volatile base (µM)	10	30	54	57
Amino acid (µM)	26	63	102	104
Butyric acid (µM)	-	0	0	1
Propionic acid (µM)	-	0	0	0
Acetic acid (µM)	-	40	60	115
Formic acid (µM)	-	4	2	29
Lactic acid (µM)	-	1	7	40
Succinic acid (µM)	-	23	29	24

ANALYSES FOR SILO 14

0	9	37	43
5.45	<3	5.77	<3
-	<3	5.58	<3
8.19	6.48	7.56	6.19
7.43	5.95	6.32	6.08
5.70	5.02	4.87	5.02
75.46	75.56	76.80	75.29
3.46	3.62	3.53	3.73
4.27	1.1	2.1	1.9
52	202	196	173
11	27	36	35
33	60	58	57
0	0	0	0
-	0	3	0
-	89	74	68
-	8	8	8
-	44	27	29
-	25	24	10