

THE INFLUENCE OF THE GRAM-NEGATIVE BACTERIA
ON THE SAUERKRAUT FERMENTATION

By

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A THESIS

Submitted to the School of Graduate Studies of Michigan
State College of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

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AN ABSTRACT

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J. W. Fabian

A new V-8 medium has been developed for the enumeration and isolation of lactic acid bacteria. The characteristic appearance of the subsurface colonies of Lactobacillus plantarum and other lactobacilli suggests that this medium may be employed satisfactorily for routine enumeration of the lactic acid group of bacteria.

Aerobacter cloacae and Flavobacterium rhenanus were identified as members of the Gram-negative group that were found to be increasing in numbers during the beginning stages of the normal sauerkraut fermentation. The increase of these bacteria was determined by observing an increase in the number of lenticular-shaped colonies on the V-8 medium while the total count was decreasing. A. cloacae seems to be more prevalent since it was found during the beginning of five out of the six fermentations studied whereas F. rhenanus was isolated from only one of the fermentations.

The measurement of the oxidation-reduction potentials during the normal fermentation showed that the Eh potential decreases with the growth of the Gram-negative bacteria. The low potentials obtained for A. cloacae in pure culture, however, were not reproduced during the normal sauerkraut fermentation. The reason for this might be due to the presence of inhibiting substances in the cabbage which caused the accumulation of hydrogen peroxide.

When a large inoculum of A. cloacae was placed in sterile cabbage juice with an inoculum of Leuconostoc mesenteroides or

other acid-forming bacteria, the formation of acid was retarded. With a smaller inoculum, A. cloacae apparently produces favorable conditions for the growth of L. mesenteroides as indicated by a greater acid formation in mixed culture.

The introduction of large numbers of A. cloacae into the natural sauerkraut fermentation showed that this organism was capable of causing dark sauerkraut and at the end of two weeks produced a sharp radish-like flavor which was undesirable. The exposure of such sauerkraut to the air also intensified darkening.

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INTRODUCTION

In the manufacture of sauerkraut, it is necessary to allow the shredded cabbage to undergo a bacterial fermentation. When proper conditions are maintained, the fermentation proceeds spontaneously after salting, and within a short time the product is ready for the market. In the preservation of most vegetables, the emphasis is upon the destruction or suppression of microbial growth rather than its propagation. In this respect, the sauerkraut fermentation is different, since the bacterial growth enhances its palatability as contrasted to the rotting and putrefactive changes that spoil other vegetable products salted under the same conditions.

If one follows the titratable acidity throughout the course of the sauerkraut fermentation, there is a very consistent increase of acid after the salting period. The organisms concerned with the production of acidity have previously been identified (47) as Leuconostoc mesenteroides, Lactobacillus plantarum, and Lactobacillus brevis. These organisms have been shown to appear subsequent to a group of organisms that have been classified as the Gram-negative group. The importance of the acid-forming bacteria has been established definitely. Very little work has been done, however, on the importance of the Gram-negative group in the sauerkraut fermentation. It has always been assumed that these Gram-negative bacteria were a heterogeneous group present on the exterior of the cabbage and were not important, since they disappeared shortly after the salting period.

Since little is known concerning the significance of these Gram-negative bacteria, it was the purpose of this study to determine the numbers and types of Gram-negative bacteria growing at the beginning of

the fermentation and to determine the influence of their growth.

The interest concerning the Gram-negative bacteria evolved as the result of preliminary investigations beginning in 1949 using a new V-8 medium (16). In studying the sauerkraut fermentation, it was found that the V-8 medium satisfactorily indicated the growth of the Gram-negative bacteria as well as the growth of the acid-forming bacteria. Further use of the V-8 medium was made inasmuch as these Gram-negative organisms could be enumerated and isolated easily for identification by its use.

REVIEW OF LITERATURE

The study of the sauerkraut fermentation dates back to Conrad (12) who in 1897 isolated a Gram-negative, motile, non-spore-forming rod from the center of a head of cabbage. He called this organism Bacterium brassicae acidae and believed that it was important in the natural sauerkraut fermentation. The presence of Gram-negative bacteria at the beginning of the sauerkraut fermentation was also determined by Butjagen (9), Pederson (47, 56), Perekalin (59), Priem, Peterson, and Fred (62), Keipper and Fred (30) and others.

"Duggeli (14) studied the microflora of healthy green plants, seeds, and fruits and determined that certain bacteria exist on the surface of the plant in the form of a zoogloea. "Duggeli found Bacterium herbicola aureum most frequently. "Hüttig (29) later reported that variation in the medium, the temperature, etc., may bring about complete conversion of Bacterium herbicola to Streptococcus lactis or vice versa.

Round (65) showed that lactose-bile fermenting organisms were found in small numbers at the start of the fermentation. These increased rapidly for the first few days and quickly disappeared after the sauerkraut showed an increase in acidity. "Gruber (22) isolated an organism he called Pseudomonas brassicae acidae, which resembled the organism isolated by Conrad (12). Both of these organisms had characteristics of the coliform bacteria.

Members of the coliform group were isolated from fresh vegetables by Burri (8). He believed that these organisms were a part of the natural microflora. Similarly, Keipper, Fred and Peterson (32) isolated the coliform group from the outside leaves of fresh summer cabbage.

They indicated that this group amounted to 40 percent of the initial microflora. These workers (32) found also that the bacterial numbers on the inside of the cabbage were usually low in total count and that the majority belonged to the lactic acid-forming bacteria. They isolated an organism that possessed characteristics similar to those of Bacterium herbicola aureum (Duggeli) and suggested that these organisms be placed in the genus Flavobacterium according to Bergey's classification (5).

Holtman (28) showed that Serratia marcescens was the cause of pink sauerkraut, thus indicating the growth of another Gram-negative organism.

Pederson (47) reported that the Gram-negative bacteria were undoubtedly of little significance in an ordinary fermentation since they fail to grow after a small amount of acid is produced. In later work (56) he noted bactericidal substance in cabbage juice that caused a marked reduction in the number of Gram-negative bacteria within 6 to 24 hours. He stated that the substances were different from those found in onion juice by Fuller (20)

Gram-positive lactic acid bacteria also were isolated throughout this period of bacteriological investigation. Wehmer (70, 71) isolated a non-motile, non-spore-forming, non-gas-producing rod which he named Bacterium brassicae.¹ Even though he isolated yeasts and Gram-negative bacteria from his fermentation studies, he ascribed the fermentation of sauerkraut to Bacterium brassicae.

Butjagen (9) agreed with Wehmer in regard to the value of the Gram-positive rods, in that the Gram-negative bacteria disappeared early during

¹now Lactobacillus plantarum, according to Bergey (5)

the fermentation.

Henneberg (23), Round (65, 66), Murray (44), and Holtman (27), indicated that the normal fermentation was carried out by Gram-positive, non-motile, acid-forming bacteria.

Orla-Jensen (45) identified Streptobacterium plantarum² as one of the organisms that was isolated from the sauerkraut fermentation. LeFevre (35) studied the lactic acid bacteria and believed that the organism isolated by Wehmer (71) was related to the organism isolated by Henneberg (13, 24). Brunkow, Peterson and Fred (7) also believed that the normal fermentation was carried out by Gram-positive, non-motile, acid-forming bacteria. These workers inoculated sauerkraut with selected cultures of lactic acid bacteria and reported that this improved the final product. They observed that the inoculation reduced the number of foreign organisms and the duration of their existence in the fermentation. Pederson (48, 52, 53), Fred, Peterson, and Viljoen (19), and LeFevre (34) and Henneberg (23) also studied the effect of inoculating cabbage shreds with lactic acid bacteria. Some of these workers claimed good results, but others indicated that it was questionable whether the quality had been improved.

The most comprehensive and complete study of the acid-forming bacteria was made by Pederson (47). He showed that the first acid-forming bacteria to appear were the gas-producing cocci, the predominant species of which was Leuconostoc mesenteroides. He attributed the pleasant flavors of sauerkraut to this organism. However, he indicated that this group was unable to complete the fermentation. He characterized these bacteria by stating that they grow at lower temperatures than the lactobacilli and ferment the sugar to an acidity of 0.7 to 0.9 percent.

²now Lactobacillus plantarum, according to Bergey (5)

Pederson further indicated that this group of bacteria was followed by two species of non-gas-producing rods, L. cucumeris and L. plantarum, and three species of gas-producing types, L. pentoaceticus, L. fermentatae and L. buchneri, which may be discussed simply as non-gas-producing rods and gas-producing rods, respectively. The effect of the gas-producing rods was similar to that of Leuconostoc species, but they were capable of producing a higher acidity. The non-gas-producing rods, on the other hand, were capable of completing the fermentation.

As a consensus of previous work, the following is a classification of the organisms generally found on the cabbage as presented by Keipper, Fred and Peterson (32). The organisms are grouped in four divisions apart from the small number of molds and yeasts which are occasionally found.

1) The aerobic spore-formers, usually soil contaminants; 2) The colon-aerogenes types; 3) The chromogenic forms which make up a large part of the native flora. (These may be considered as strains of the yellow or red Bacterium herbicola and Bacterium fluorescens;) 4) The lactic group, which is represented by two great sub-groups, the low acid-tolerant organisms, of which the coccus-like forms belonging to Leuconostoc mesenteroides are typical, and the high acid-tolerant organisms, L. pentoaceticus, L. cucumeris, L. plantarum, and other members of the Lactobacillus group.

A great deal of work on the sauerkraut fermentation has been devoted to the study of factors influencing the quality of the market product. Peterson, Parmele and Fred (60) indicated that sauerkraut made from late cabbage was better than from early cabbage. These views are in accordance

with those of Keipper, Peterson (31) and Fred who indicated that the percentage of lactic-type organisms was higher on the outside of fresh, fall cabbage than on fresh summer cabbage.

Pederson (51, 54, 55, 57, 58) studied various factors influencing the quality of sauerkraut. He found that the rate of fermentation increased at a higher temperature and that abnormal sauerkraut may be caused by improper distribution of salt. Cleanliness and proper covering of the sauerkraut were also important considerations in good quality sauerkraut. Hof (26), however, found that sauerkraut made with or without salt showed no important differences in bacterial flora or acidity.

Martin, Peterson and Fred (41), Parmele, Fred and Peterson (46), and Holtman (27), indicated that the quality of sauerkraut was largely dependent upon the temperature at which the fermentation was carried out. A temperature of 65° F produced the best quality.

LeFevre (36, 37) discussed the production of sauerkraut and gave statistics stating the economic importance of sauerkraut in the United States.

PRELIMINARY EXPERIMENTS

Development of the V-8 Medium

The need for a medium which would be suitable for determining acid-forming bacteria was evident in making preliminary bacteriological studies of the sauerkraut fermentation. Many media were suggested for the isolation and enumeration of lactic acid bacteria. It was felt that the media in use were not specific enough for the detection of these organisms since they allowed other microorganisms to grow abundantly. Some media supported growth of the lactic acid bacteria, but the colonies were often small and difficult to count.

In preliminary work by the author, using tryptone glucose extract agar (Difco), the overgrowth of surface bacteria prevented an accurate enumeration of the acid-forming bacteria. This was observed even though a suitable indicator was incorporated to show the formation of acid. To eliminate this opprobrium, experiments were started in 1949 to find a new medium which would: (a) permit the growth of the acid-producing bacteria quickly and to a larger size than any of the previously used media, (b) permit the growth of acid-producing bacteria to the exclusion of other bacteria which made plate counts and isolation difficult or impossible.

Six different media were prepared, using V-8 vegetable juices³ as a base. The composition of each medium is listed in Table 1.

³V-8 juice is a trade name used for a blend of eight vegetable juices first manufactured by Standard Brands at Terre Haute, Indiana. It is now manufactured by the Campbell Soup Company, Philadelphia, Pennsylvania.

Table 1
The composition of six V-8 media

Medium	Tryptose (gm)	Glucose (gm)	Beef extract (gm)	Agar (gm)	V-8 juice (ml)	pH
A	10	5	3	18	500 unfiltered	4.6
B	10	5	3	18	250 filtered	5.1
C	10	5	-	18	250 filtered	6.1 adjusted
D	10	5	-	18	125 filtered	5.6
E	10	5	3	18	500 filtered	4.5
F	10	5	3	18	500 filtered	5.8 adjusted

In testing these six media with L. plantarum, media D and F gave the best results when compared with tomato juice agar. Since these media were to be used primarily as plating media for pickle and sauerkraut fermentations, it was desirable to have an indicator present to distinguish the acid-producing bacteria from other bacteria. Accordingly, brom cresol green was chosen because the color range was from 3.8 to 5.4 and the pH of media D and F were near this range. Test organisms used were two cultures of L. plantarum, one from a pickle fermentation and the other from a sauerkraut fermentation. Also, the following species of the genus Bacillus were used: cereus, subtilis, mesentericus fuscus, vulgatus, megatherium, and pumilus. Experiments using these eight organisms and 0.005, 0.0075, 0.01, 0.02, 0.04, and 0.06 gm, respectively, of brom cresol green per 100 ml of media D and F, showed that 0.01 gm of the indicator gave the best results. Quantities less than 0.01 gm were not satisfactory due to

a lack of contrasting color. Larger amounts had too great an inhibiting effect on L. plantarum. These experiments also indicated that 0.1 percent brom cresol green inhibited the various species of Bacillus which are commonly found in the soil.

Another series of tests was made with L. plantarum using media D and F with and without the 0.1 percent brom cresol green indicator and another series in which lactose was substituted for dextrose. Thus, there was medium D with and without 0.1 percent brom cresol green in which dextrose was present and a duplicate set in which lactose replaced dextrose. A duplicate of this series was made also with V-8 medium F using tomato juice agar for comparison.

The results from these experiments showed that the presence of the indicator did not reduce materially the number or size of the colonies. They showed also that medium D with dextrose had the most colonies per plate and that medium F with lactose had slightly fewer than D, but much larger and more distinct colonies. The colonies on medium F were three to four times larger than those in tomato juice agar.

In order to check the range of optimum pH, a series of tubes was used containing V-8 broth at various pH values. Also, the amount of turbidity was measured by means of a photelometer. The range of pH used was 4.5 to 6.5, measuring at pH intervals of 0.4 pH. The following species of the genus Lactobacillus were used: plantarum from four different sources, casei, arabinosis 17-5, dorner, and leichmanni.

Table 2 lists the optimum pH ranges of the various lactobacilli.

Table 2

Optimum pH range of various lactobacilli

Organism	Optimum pH range
<u>L. plantarum</u>	5.3-5.6
<u>L. casei</u>	4.9-6.1
<u>L. dörner</u>	5.4-6.1
<u>L. arabinosis</u>	5.3-6.5
<u>L. leichmanni</u>	5.4-5.6

From these data it was concluded that a pH of around 5.5 to 5.7 was suitable to cultivate the above lactobacilli at an optimum pH. The pH of medium F, which had been tested at 5.8, was changed to 5.5 to 5.7. This range was used in all subsequent work. It should be noted that the V-8 medium gave identical results at pH 5.5 and at pH 5.7. However, a pH 5.6 to 5.7 was chosen because it was found in practice that the media with pH values below 5.5 produced smaller colonies than those with slightly higher pH values.

Discussion

Medium F was selected as the preferred medium even though the total count was slightly lower than that obtained with medium D. Medium F contained 10.0 gm tryptose, 5.0 gm lactose, 3.0 gm beef extract, 15 gm agar, 500 ml filtered V-8 juice, 500 ml distilled water, and 0.1 gm brom cresol green. Approximately 4.8 ml of 3N NaOH was added also to

adjust the pH properly. The slightly higher count on medium D seemed to be outweighed by the superior colony size of medium F, since the total counts obtained on the latter compared favorably with those obtained on tomato juice agar.

Figures 1 and 2 show a 48 hour culture of L. plantarum with maximum halo formation on medium F.

Figure 1

Forty-eight hour culture of L. plantarum on V-8 medium F

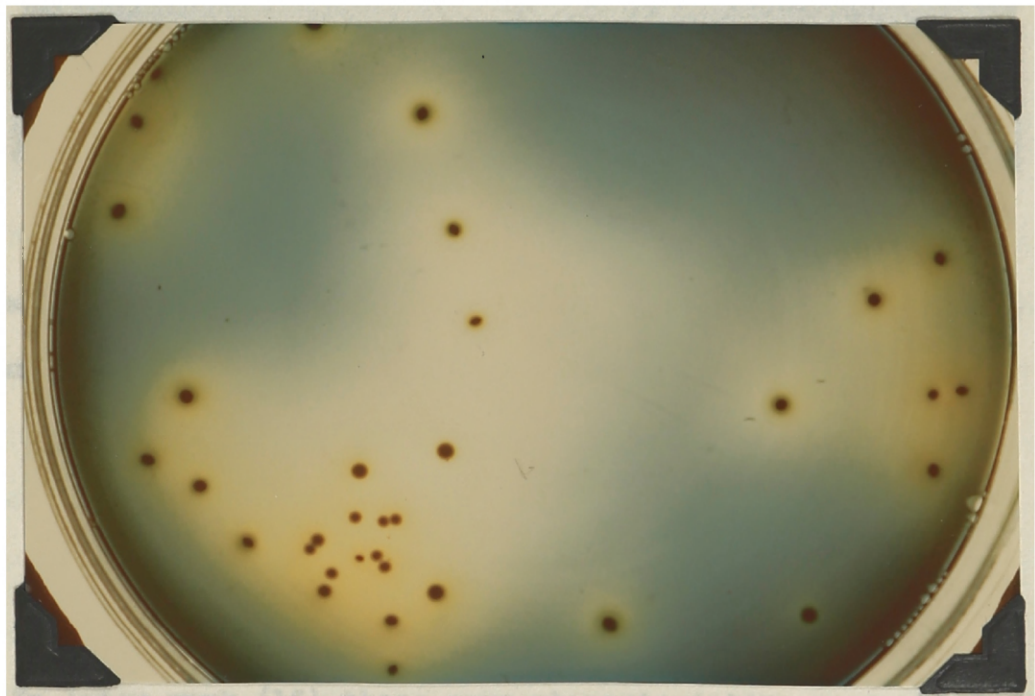
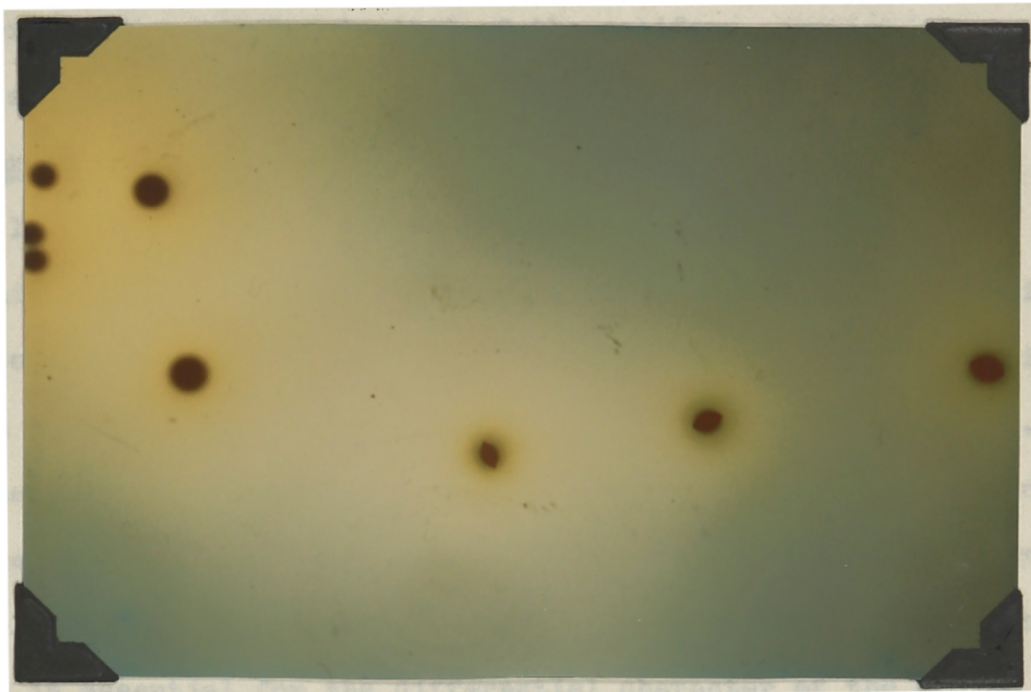


Figure 2

Forty-eight hour culture of L. plantarum on V-8 medium F



As shown in the two colonies toward the center of Figure 2, the following cultural details appear to be characteristic of L. plantarum:

1. Oval jet black subsurface colony from 2 to 3 mm in diameter.
2. Dark, fuzzy area bordering the colony.
3. Bright yellow halo

In later work (16) these same characteristics were obtained for seven other lactobacilli cultures. When the growth of these seven lactobacilli cultures and three other lactic acid organisms on V-8 medium F were compared with their growth on ten other media proposed for cultivation of acid-forming bacteria, the V-8 medium was superior for

enumerating and isolating the lactobacilli. These results were based on colony characteristics, total counts, and colony size. These characteristics of the lactobacilli on the V-8 medium indicated a possible means of differentiation similar to that of the coliform organisms on eosin-methylene blue agar (Difco).

Summary

1. The use of a new V-8 medium for the enumeration of the lactic organisms (Lactobacillus, Leuconostoc, Streptococcus, etc.) indicated that the total counts obtained with this medium compared favorably with other media commonly used for determination of this group of bacteria. Preliminary tests showed that the media without brom cresol green resulted in greater total counts than those containing the indicator. However, brom cresol green should be used in the recommended quantity since the indicator is essential for the differential character of the medium.

2. It was shown that most of the lactic acid fermenting organisms produced a peculiar characteristic colony on the V-8 medium. The appearance of this colony was a means of differentiation between the lactic acid organisms and other organisms. In comparing different media containing indicators, only the V-8 medium could differentiate between miscellaneous acid-forming bacteria and the true lactic types as determined by the typical colony produced on the V-8 medium.

The appearance of the lactic organisms on the V-8 medium was somewhat variable depending on the particular organism. In general, however, the lactic organisms appeared dark green to jet black. However, in some instances the typical yellow halo was weak or only slightly green. With

the stronger acid producers the halo was bright yellow. Most of the lactobacilli were easily characterized by observing a bright yellow halo surrounding a jet black colony. A weaker acid producer, such as Leuconostoc, appeared similar; however, the colony was generally green with weaker acid production. The above description of the lactic organisms applied only to plates with less than 120 colonies. A greater number of colonies obscured these characters and differentiation was not possible.

3. Attempts to cultivate many species of the genus Bacillus showed that the V-8 medium inhibited surface and subsurface growth. This in itself was a great advantage of the V-8 medium, since incubation of the plates for two to five days was desirable for the determination of the lactobacilli. Other media permitted "spreaders" which grew abundantly after 24 to 48 hours and obscured the growth of the lactic acid organisms.

Development of Sampling Technique

The procedure outlined by Pederson (49) for making sauerkraut consisted of covering and properly weighting the exposed surface to prevent the growth of undesirable aerobic organisms. Pederson stated that the inclusion of air within the fermenting shreds permitted the growth of undesirable organisms. Thus, the problem of obtaining representative samples from a properly prepared fermentation became evident. The sampling procedure required a device that would permit the removal of a representative portion of the fermenting liquor without introducing air. The requirements for a suitable sampling device became more involved when considering possible oxidation-reduction measurements, since the latter required a relatively large sample.

After studying the problem thoroughly and trying many different methods, a technique was devised that permitted a suitable sample to be withdrawn periodically and then returned after the Eh had been taken. The device was constructed so that it insured a representative sample by withdrawing and returning 170 to 210 ml of the fermenting liquor at the desired interval. Anaerobic conditions were obtained by maintaining an atmosphere of oxygen-free nitrogen within a closed container adapted for withdrawing the sample. Thus, the general operation of the sampling device involved flushing the system out with oxygen-free nitrogen gas, sampling by vacuum, and returning the sample by nitrogen pressure. Stier and Scalf (68) devised a similar technique whereby yeast samples were obtained under anaerobic conditions using purified dry nitrogen to maintain anaerobic conditions.

Figure 3 shows two sample containers from a group of five used in the experiment. The two sampling devices in Figure 3 were identical except that the sample container, E, the flask at the left, did not contain platinum electrodes as the sample container pictured at the right of the same figure. Figure 4 is a schematic drawing of the sampling device with the parts labeled. All the glassware and rubber connections used for this apparatus were sterilized in the autoclave prior to each sampling. When making oxidation-reduction measurements, however, the apparatus was only sterilized prior to the first sample.

Much of the success in sampling depended on the tip of the sampling tube shown in Figure 5, which was fastened to the end of the sampling tube S. After the cabbage had been fully salted and packed into gallon jars, a 5 ml pipette was forced into the jar and withdrawn in order to make an opening. The sampling tube fitted with the sampling tip then was introduced into this opening. A wooden cover, provided with a hole for the sampling tube, was placed on top of the cabbage. Sufficient weights were applied until the cabbage juice came near the top of the wooden cover.

Before sampling the system was flushed out with oxygen-free nitrogen gas. The removal of the oxygen was accomplished by passing the gas from the nitrogen tank through a series of two gas-washing bottles. The first bottle contained pyrogallol and the second served as a trap to catch any pyrogallol that might have passed accidentally from the first bottle. Glass beads were added to the flask containing the pyrogallol solution to disperse the gas from the nitrogen tank.

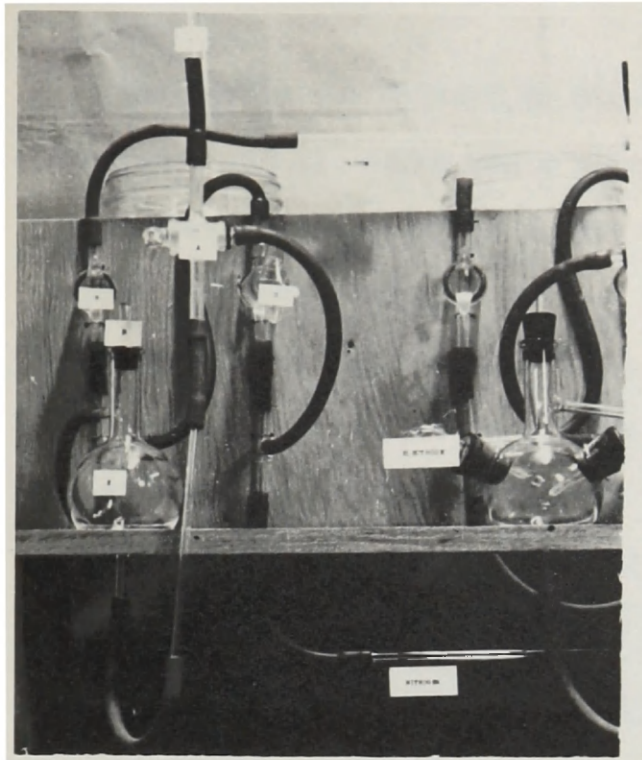


Fig. 3. Device used for obtaining samples during the sauerkraut fermentation

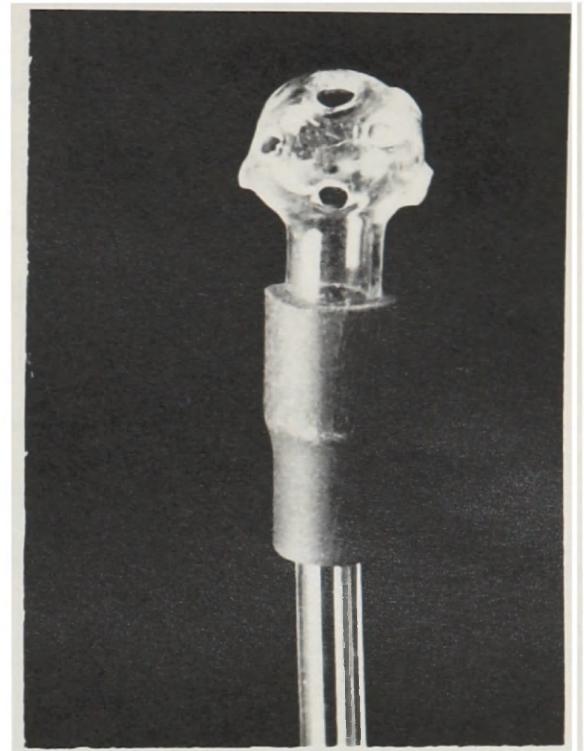


Fig. 5. Tip of sampling tube

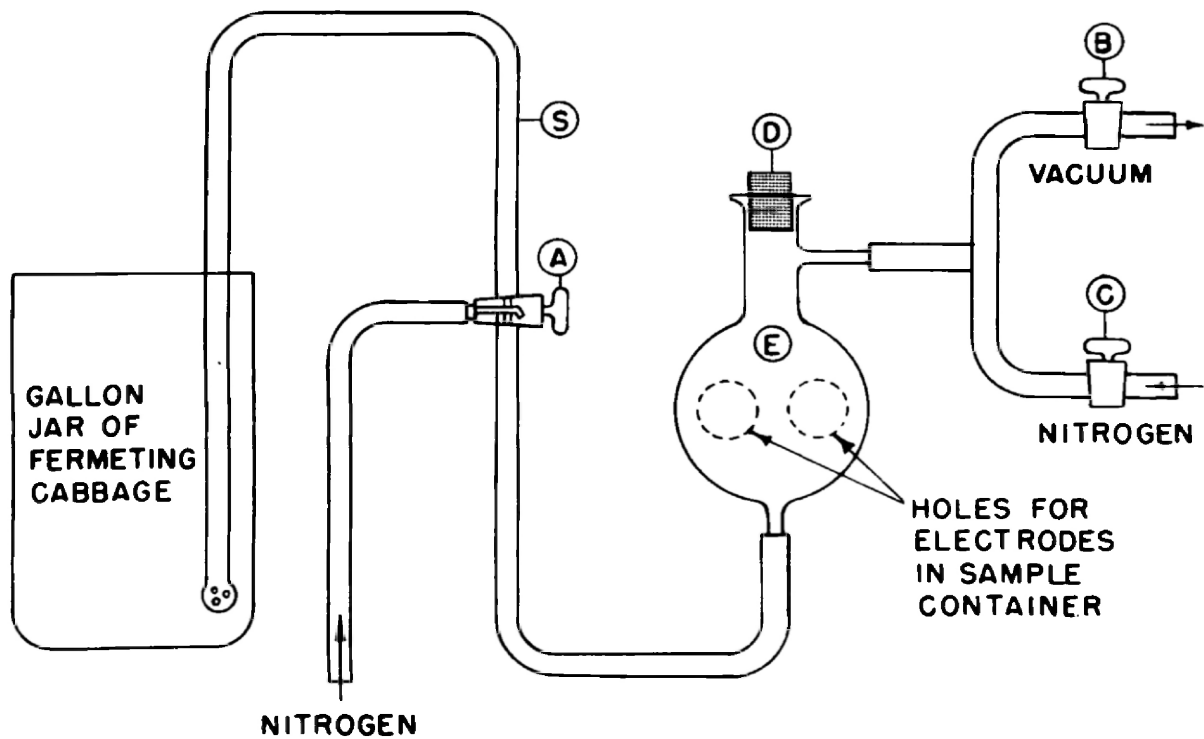


Fig. 4. Schematic drawing of sampling device

The system was flushed by directing the gas through valve A (Figures 3 and 4), which was a three-way through glass stopcock. The gas passed directly through the sample container E and out at D where the stopper had been removed. Valve C was opened to flush out the tubing which connected sample container E.

To discontinue flushing, valve C was closed first. Then, valve A was opened from sample container E to sample tube S which concurrently stopped the flow of nitrogen from A to E. The stopper at D was replaced immediately.

The sample was withdrawn by opening valve B to the vacuum supply. A water aspirator was suitable for this purpose. As soon as the sample container E was filled, the vacuum was turned off and valve A slowly turned to admit nitrogen into the sample container E, the same procedure as used in flushing. A slight trickle of gas through valve A into the sample container E was maintained which resulted in a positive nitrogen flow. The rubber stopper D could be removed without the possibility of contamination by oxygen from the air. Samples for bacteriological analysis and acid determination were removed at D.

The sample was returned to the gallon jar by turning valve A to a vertical position, allowing the passage from E to S. The nitrogen was turned on at valve C, forcing the sample back into the jar by nitrogen pressure. As soon as the sample was returned, valve C was turned off. When another sample was desired, the vacuum was turned on immediately at B.

Bacteriological Studies

There was considerable confusion concerning the bacteriological investigation during preliminary analysis. Since the work on the V-8 medium still had not progressed to the point of application, all the preliminary investigations were made with tryptone glucose extract agar (Difco) with 0.04 gm brom cresol purple added per liter of medium. The results with this medium appeared confusing. It was a general observation that whenever surface colonies appeared, as in the first stages of the fermentation, there were never any acid-forming bacteria indicated. The acid-forming bacteria were indicated only after the fermentation had progressed and when few non-acid-forming bacteria were present. Later, when the V-8 medium was employed, the acid-formers generally could be detected earlier when comparing the two media. The relationship between the Gram-negative bacteria and the acid-forming bacteria in plate culture on tryptone glucose extract agar will be discussed later.

With further use of the V-8 medium it was noted that a peculiar type of colony predominated before the acid formers appeared. The colonies were lenticular in shape and they were much different from those of other bacteria which presented a variety of characters on the medium. The most noticeable characteristic of these lenticular shaped colonies was the white color that was evident against the dark blue background of the V-8 medium. Figure 6 shows the characteristics of the lenticular-shaped colonies as they appeared during the beginning stages of the sauerkraut fermentation.

Figure 6

The lenticular-shaped colonies appearing in the beginning stages of the sauerkraut fermentation



The predominance of these lenticular colonies was determined by comparing their numbers with the total count. In this way it became evident that these lenticular colonies increased as the total count decreased. However, in subsequent determinations it was noted that the total count did not always decrease. If there was no decrease, the total count generally remained the same or increased slightly as compared with the greatly increasing number of lenticular shaped colonies.

The appearance of these lenticular colonies in plate culture varied only slightly from one fermentation to another. Most of the colonies were about 2 mm in diameter; however, some appeared disc-like with a diameter of almost 7 mm. When the subsurface growth of these organisms

extended to the surface of the agar plate, they formed a mucoid mass that spread from 10 to 15 mm. Sometimes these subsurface colonies were quite small. However, after 4 days incubation at room temperature, they generally were found to be from 2 to 4 mm in diameter as noted in Figure 6.

Six sauerkraut fermentations were carefully examined to confirm the appearance of these lenticular colonies. In all six of the fermentations the lenticular colonies increased and predominated during the early stages of the fermentation. The increase and predominance of the lenticular-shaped colonies was noted also when salting shredded cabbage in 5 and 10 gallon containers. No apparent difference was noted between the relative number of lenticular shaped colonies and the total count in the different containers.

From this work it was found that these organisms predominated for periods ranging from 12 to 54 hours. With further experimentation, however, the range seemed to be from 35 to 45 hours. In all cases their growth terminated shortly after the appearance of the acid-forming bacteria. Later, in one fermentation, it was not possible to follow the lenticular-shaped colonies because the acid-forming bacteria appeared so quickly. The acid bacteria normally do not appear so quickly, therefore, this was an exception.

In Figure 7, the relationship between the lenticular-shaped colonies and the total count is presented in graphic form. It was possible to determine the total number of bacteria concurrently with the number of lenticular colonies on the V-8 medium; however, the total number could be determined better on tryptone glucose extract agar (Difco).

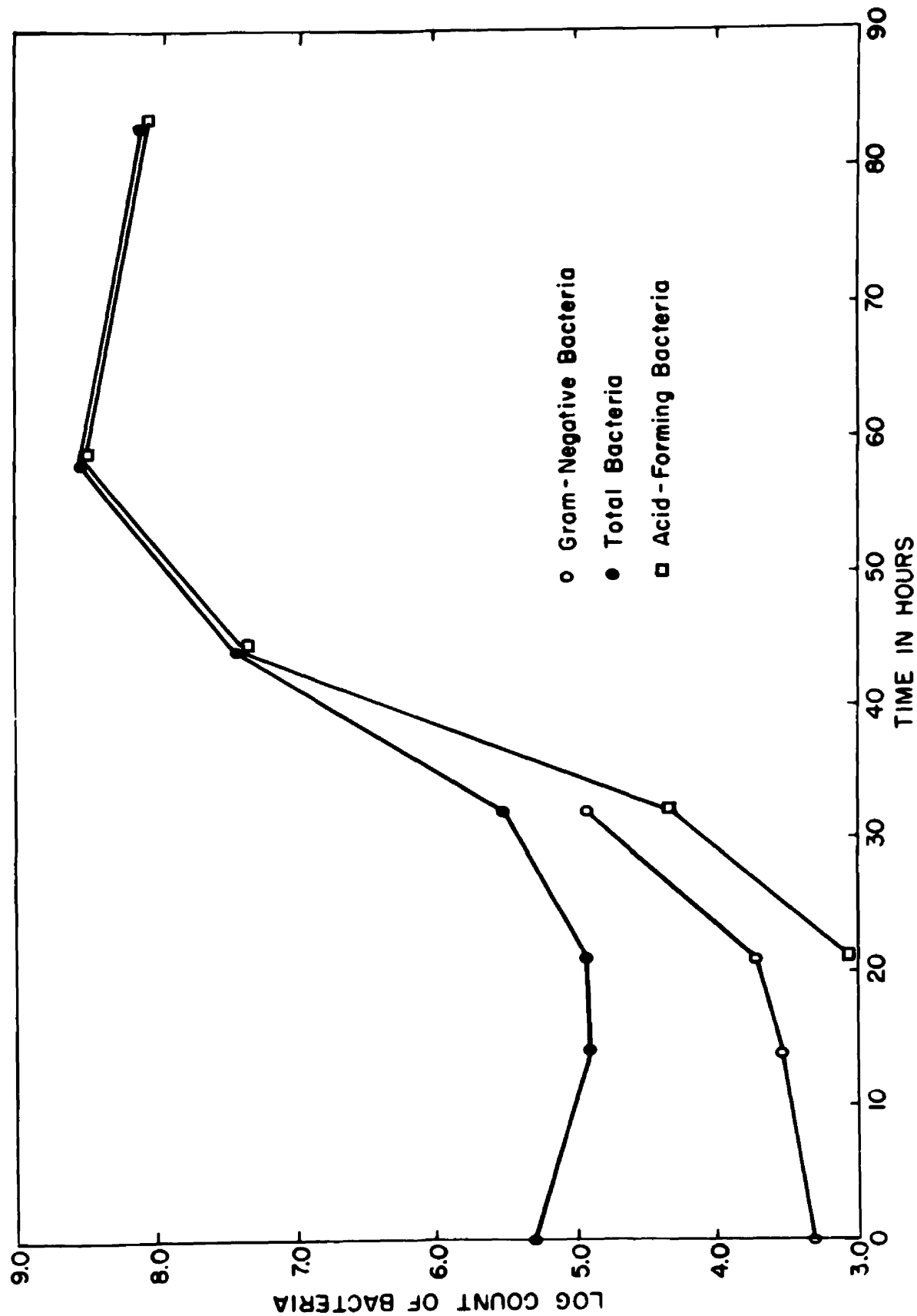


Fig. 7. Bacterial growth during the beginning stages of the sauerkraut fermentation as determined with the V-8 medium

Isolation of Bacteria

In subsequent work these lenticular-shaped colonies were isolated in order to characterize these bacteria. In every case the isolations were made from the second series of plates where a definite increase in these bacteria was evident. Typical acid-forming bacteria were isolated also during the fermentation from the first plates that gave typical colonies of acid-forming bacteria.

Isolation was carried out by picking the colonies from the V-8 medium and transferring them to a dilution blank. Aliquots were plated out using the V-8 medium. Well isolated colonies were chosen 24 to 48 hours later and the plating procedure was repeated. After repeating the plating procedure for the third time, well isolated colonies were selected and transferred to lactose motility medium (Difco) for initial observations. The isolates were given numbers which are used throughout the paper when referring to these organisms.

Transfers were made from the motility medium to nutrient broth. Gram stains were made of all the organisms at the end of 20 hours incubation. The acid-formers were cultivated on a medium composed of peptonized milk, glucose, and yeast extract due to poor growth encountered in nutrient broth. The incubation temperature for all these studies was 25° to 27°C. This was the range of the room temperature and was chosen for convenience. All subsequent work including fermentation studies were also carried out at this incubation temperature.

Table 3 shows the characteristics of the various isolates as they appeared on the V-8 medium and the results of the Gram stain. All the lenticular-shaped colonies were Gram-negative, whereas, the acid-forming bacteria were Gram-positive.

Table 3
Bacterial cultures isolated

Fermentation	Characteristics of colonies on V-8 medium	
	White-lenticular	Acid-forming
I	1-23	24-25
II	46-72, 82, 83	73-81, 84, 85
III	100-106	107, 108
IV	200-201	202, 203
V	501-506	507a, 508a
VI	601-605	0
Gram character	Gram-negative	Gram-positive
Total number of isolations	73	38

The Growth of the Gram-negative Bacteria on Tryptone Glucose Extract Agar

Since the lenticular-shaped colonies were members of the Gram-negative group, experiments were conducted to determine whether Gram-negative bacteria affected the growth of acid-forming bacteria when associated in plate culture. These tests were conducted in an attempt to explain the failure of the tryptone glucose extract agar to indicate the growth of the acid bacteria when brom cresol purple was added as an indicator of acid formation. The Gram-negative and the acid-forming bacteria were apparently in association during the beginning stages of the fermentation as shown by the results with the V-8 medium.

The procedure consisted of streaking the Gram-negative bacteria over the surface of a 24 hour growth of the acid-forming bacteria. The latter was prepared by inoculating a 24 hour broth culture into melted tryptone glucose extract agar containing 0.04 gm brom cresol purple per liter. After solidification, the plates were incubated at room temperature. The growth of the acid-formers produced sufficient acid to change the brom cresol purple indicator to a bright yellow within 24 hours. At the end of this time the Gram-negative organisms from an agar slant were spread on the surface of these same plates. After 4 to 6 hours incubation at room temperature the surface that had been streaked showed a definite purple coloration and after 8 hours the purple color was as dark as that of the control. It was interesting to note that the purple area immediately below the surface of the streak spread farther from the streak as time progressed. After 48 hours the colonies of the acid-formers directly underneath the purple portion of the medium were larger than those in the yellow portion.

The results of cross-streaking indicated that the acid-forming bacteria could not be detected on the tryptone glucose extract agar, containing brom cresol purple, during the initial stages of the fermentation due to the growth of these Gram-negative organisms. The latter prevented the acid-forming bacteria from producing an acid halo. It seemed probable that after the Gram-negative organisms died the acid-formers could be detected by their characteristic acid halo.

Summary

These preliminary investigations showed by using the V-8 medium that a peculiar lenticular-shaped colony predominated and multiplied prior to the appearance of the typical acid-forming bacteria. The increase of these lenticular-shaped colonies was noted on the V-8 medium even though the total number of bacteria decreased or remained constant during the first stages of the sauerkraut fermentation. The bacteria which formed these lenticular-shaped colonies were identified as members of the Gram-negative group. These organisms were enumerated on the V-8 medium in association with the acid-forming bacteria. However, enumeration of the acid-forming bacteria was not possible by employing the tryptone glucose extract agar (Difco) with 0.04 gm brom cresol purple per liter. The growth of Gram-negative organisms on this medium prevented the acid-formers from producing an acid halo by which they could be distinguished.

PROCEDURE

1

Identification Studies

Experimental

The similar morphology of the lenticular-shaped colonies appearing on the V-8 medium suggested that the organisms which produced them might be identical species or possibly members of the same genus. In order to prove this, and to serve as a basis for comparing their physiological activity, a pure culture study was made of 14 representative Gram-negative cultures, chosen from the six fermentations listed in Table 3. The biochemical tests used in this study were those suggested by the manual of pure culture study (67).

The cell morphology of all the Gram-negative bacteria studied in pure culture was determined from 12 to 48 hour cultures. In all cases the cell size varied from 1.0 to 1.3 microns in width by 1.3 to 1.5 microns in length. In most cases the cells were in pairs and appeared much like a diplobacillus or even diplococcus. A great number of cells that were found singly appeared almost spherical. Since these organisms appeared to be so similar to some acid-formers, such as Leuconostoc mesenteroides, the Gram stain was the only definite way of differentiating the acid-formers from the Gram-negative bacteria.

The lactose motility medium was used as a stock culture medium and in general separated the Gram-negative from the Gram-positive organisms since most of the former grew with diffuse growth and the latter grew only along the streak of inoculation. The growth characteristics of the Gram-negative organisms on the lactose motility medium were observed. There was diffuse growth with gas bubbles after 24 hours. Acid was

formed in most cases in 24 hours but a few organisms produced acid slowly. After several weeks the acid generally dissipated and the surface of the medium became white and viscid. A few of the organisms giving identical biochemical tests did not become viscous. Acid was formed in every case except for cultures 501 and 506 which also failed to conform to the above description that typifies motility.

The optimum temperature for growth of the Gram-negative bacteria was between 25° and 30° C. Growth was also good at 20° C and generally poorer at 37° C. The optimum temperature for growth of the acid-forming bacteria was also between 25° and 30° C. These temperatures for optimum growth were determined by incubating the organisms in broth culture at 20°, 25°, 30° and 37° C and by observing the amount of growth produced after 24 hours.

The catalase test was made by employing catalase meters. Table 5 gives the volume of oxygen produced by a given amount of hydrogen peroxide added to a 24 hour culture of each of the 14 organisms. The amount of oxygen evolved was measured over a 4 hour period.

Smith tubes containing dextrose broth were employed to determine the ratio of CO₂ and H₂ produced over a period of 72 hours. Carbon dioxide was analyzed at the end of the incubation period by adding KOH to absorb CO₂. The remaining gas indicated the percentage produced. The percentage of each gas was calculated as a percentage of the total gas production measured in mm. Table 4 gives the ratios of gas obtained for the Gram-negative bacteria.

Table 4

Ratios of carbon dioxide and hydrogen produced by the
Gram-negative organisms

<u>Culture</u>	<u>Mm of gas in tube</u>	<u>Mm absorbed by KOH</u>	<u>Percent CO₂</u>	<u>Percent H₂</u>
15	38	23	60.5	39.5
18	no gas	--	----	----
69	19	12	63.2	36.8
102	25	16	64.0	36.0
105	50	33	66.0	34.0
106	42	27	64.3	37.5
200	no gas	--	----	----
201	69	42	60.8	39.2
501	no gas	--	----	----
506	no gas	--	----	----
601	26	18	69.2	30.8
603	50	32	64.0	36.0
604	25	16	64.0	36.0
605	18	11	61.1	38.9

Results and Discussion

The biochemical reactions listed in Table 5 show that cultures 15, 69, 102, 105, 106, 201, 601, 603, 604, and 605 conformed satisfactorily to the physiological characteristics of Aerobacter cloacae. In every case dextrin was fermented with only a very weak formation of acid in contradiction to Bergey's manual (5) that describes growth with acid and gas. The only other discrepancy was observed in litmus milk. According to Bergey, A. cloacae produces acid, coagulation, gas and slow peptonization in litmus milk. The above organisms produced slight acid, gas, fine curd formation but no typical peptonization. Instead of the typical peptonization in litmus milk the reaction remained slightly acid with a clear ring at the surface extending to a depth of 2 mm. This ring appeared clear and somewhat viscous.

These results indicated that A. cloacae predominated in five of the six fermentations studied. In two of the fermentations there was an indication that other Gram-negative organisms might be involved since organisms 18 and 200 had different characters than 15 and 201 which were isolated at the same time and were shown to be A. cloacae.

In the one fermentation where A. cloacae failed to appear, another Gram-negative organism was isolated. The pure culture study of 501 and 506 indicated that these organisms were Flavobacterium rhenanus. Since this organism did not grow well below the surface of the lactose motility medium, it was necessary to determine the motility by means of a hanging drop. All the characteristics of F. rhenanus were closely allied to the description given by Bergey (5) except that peptonization in milk occurred over an extended period while Bergey describes only an alkaline reaction.

Table 5

Biochemical reactions of the Gram-negative bacteria

Biochemical Test	Organism													
	15	18	69	102	105	106	200	201	501	506	601	603	604	605
Fermentation of:														
Arabinose	(/)*	/	(/)	(/)	(/)	(/)	-	(/)	/	/	(/)	(/)	(/)	(/)
Xylose	(/)	/	(/)	(/)	(/)	(/)	-	(/)	/	/	(/)	(/)	(/)	(/)
Glucose	(/)	/	(/)	(/)	(/)	(/)	/	(/)	/	/	(/)	(/)	(/)	(/)
Fructose	(/)	/	(/)	(/)	(/)	(/)	/	(/)	/	/	(/)	(/)	(/)	(/)
Galactose	(/)	/	(/)	(/)	(/)	(/)	/	(/)	/	/	(/)	(/)	(/)	(/)
Mannose	(/)	/	(/)	(/)	(/)	(/)	/	(/)	-	/	(/)	(/)	(/)	(/)
Lactose	(/)	/	(/)	(/)	(/)	(/)	-	(/)	-	-	(/) ^{slow}	(/) ^{slow}	(/) ^{slow}	(/) ^{slow}
Sucrose	(/)	-	(/)	(/)	(/)	(/)	-	(/)	/	/	(/)	(/)	(/)	(/)
Maltose	(/)	/	(/)	(/)	(/)	(/)	/	(/)	/	/	(/)	(/)	(/)	(/)
Raffinose	(/)	/	(/)	(/)	(/)	(/)	-	(/)	-	-	(/)	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dextrin	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	-	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}	(/) ^{weak}
Glycerol	/ ^{weak}	(/)	/ ^{weak}	/ ^{weak}	/ ^{weak}	/ ^{weak}	/ ^{weak}	/ ^{weak}	-	-	/ ^{weak}	/ ^{weak}	/ ^{weak}	/ ^{weak}
Mannitol	(/)	/	(/)	(/)	(/)	(/)	/	(/)	/	/	(/)	(/)	(/)	(/)
Salicin	(/)	/	(/)	(/)	(/)	(/)	/	(/)	-	-	-	(/)	(/)	(/)
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Gelatin hydrolysis	14 days	-	14 days	16 days	16 days	16 days	16 days	16 days	4 days	20 days	20 days	20 days	20 days	20 days
Indole formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-red reaction	-	/	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer reaction	/	-	/	/	/	/	/	/	/	/	/	/	/	/
Koser citrate	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Catalase (ml. of gas)	7.5 ml	12.5 ml	3.4 ml	2.3 ml	2.2 ml	2.0 ml	11.4 ml	1.2 ml	0.4 ml	0.7 ml	2.2 ml	1.9 ml	1.4 ml	1.2 ml
Litmus milk	gas sl.acid	sl.acid	gas sl.curd	gas sl.curd	gas sl.curd	gas sl.curd	sl.acid	gas sl.curd	pept** d.blue	pept** d.blue	gas sl.curd	gas sl.curd	gas sl.curd	gas sl.curd
Nutrient broth	viscous	viscous	turbid pellicle	very viscous	very viscous	very viscous	floccu- lation	viscous	floccu- lation	floccu- lation	viscous	viscous	viscous	viscous
Lactose motility medium	/	/	/	/	/	/	?	/	?	?	/	/	/	/

* (/) acid and gas, / acid
** peptonization

Mixed Culture Study of Gram-Negative and Acid-Forming Bacteria

The predominance of the Gram-negative bacteria before the appearance of the acid-forming bacteria during the beginning stages of the sauerkraut fermentation suggested that the Gram-negative bacteria might either effectively inhibit or stimulate the acid-forming organisms. Thus, in effect, the growth of the Gram-negative bacteria would either hinder or favor the ultimate formation of acid throughout the fermentation.

If the Gram-negative organisms produced antibiotic substances capable of inhibiting or destroying the acid formers, the total acidity would consequently depend upon: (a) resistance of the acid-forming bacteria to the antibiotic, (b) initial number of Gram-negative bacteria or (c) growth period of the Gram-negative bacteria producing the antibiotic. However, the association of the bacteria, might be beneficial whereby the acid-forming bacteria were dependent upon the transient appearance of these Gram-negative forms which possibly synthesize growth factors or change the physical properties of the fermentation to the advantage of the acid-forming bacteria.

In order to obtain evidence indicating the ultimate effect of the Gram-negative bacteria, representative cultures were chosen from the Gram-negative group and grown in a mixed culture with members of the acid-forming group. Sterilized salt-expressed cabbage juice was used as the test medium. The effective growth of the acid-forming bacteria was determined directly by titration of acid. The incubation temperature for the mixed culture study and all subsequent work was carried out at 25-27° C.

In addition to the mixed culture, the two groups of organisms were grown in pure culture. A comparison of the acid formation in pure and in mixed culture was made over a period of 7 to 14 days, sampling at intervals of 24 hours. When the quantity of acid produced in mixed culture deviated from the quantity in pure culture, the results were interpreted as evidence of factors which influenced the growth of the acid-forming bacteria. The amount of acid produced was used as a criterion in this manner since the Gram-negative organisms only slightly affected the titratable acidity as compared with the total effect of acid-forming bacteria.

Experimental

The organisms chosen for this work were the same organisms isolated from the six fermentations listed in Table 3. In addition to the isolated acid-forming bacteria, known cultures of Leuconostoc mesenteroides, L. brevis and L. plantarum were included.

Salt-expressed cabbage juice was prepared by adding salt to shredded cabbage at the rate of 2 percent by weight.⁴ One-half to 2½ hours after salting, the shreds were placed in a large piece of cheesecloth and the juice was expressed by hand. The juice was pipetted in 10 ml amounts into 20 mm diameter test tubes and sterilized at 15 pounds pressure for 15 minutes. After cooling, a series of tubes was inoculated with a drop of the respective 24 hour culture. The medium for inoculation of both organisms consisted of 2 percent peptonized milk, 3 percent glucose, and

⁴The actual percentage of salt, as determined by titration, using standard silver nitrate, was in the range of 2.863 percent.

0.2 percent yeast extract. This medium gave excellent growth after 24 hours incubation.

The total acid-formed per 10 ml sample during intervals of 24 hours by each of the organisms in pure and mixed culture was determined by titration using 0.1566 N NaOH and phenolphthalein as the indicator. In this way representatives of each series indicated the total acid formed as if removed from a single fermentation. This technique was chosen to eliminate the introduction of contaminants since each tube containing 10 ml would thus be exposed only briefly during inoculation.

Results and Discussion

The results of the entire mixed culture study are tabulated in Table 6 which shows the effect of the Gram-negative organisms in culture with the acid-forming bacteria. These results indicated that the Gram-negative organisms in all 42 cross inoculations either inhibited, retarded, or had no effect on the formation of titratable acidity. Figures 8a and 8d show representative examples of complete inhibition and of no inhibition in graphic form.

In the nine cases where the acid formation was inhibited completely, A. cloacae was the cause of only three. In effect, A. cloacae caused complete inhibition of acid in only 7 percent of the total mixed culture studies. However, in the case of this 7 percent the inhibited acid former was L. brevis. Neither the acid formers isolated in this study nor known cultures of L. mesenteroides were inhibited completely by A. cloacae.

Of the 24 cases where acid formation was retarded by the Gram-negative organisms, 63 percent of these cases resulted in lower amounts of acid formed during the incubation period. This indicated the un-

Table 6

Effect of Gram-negative organisms in culture
with acid-forming bacteria

<u>Complete inhibition of acid formation</u>	<u>Retarded formation of acid</u>	<u>No inhibition</u>
501 - L. b.	x102 - 108	501 - 108
501 - L. p. 100	x201 - 203	501 - 202
501 - 108	x102 - 203	18 - L. p. 100
501 - 508a	x201 - 508a	200 - L. p. 100
200 - L. b.	501 - 208a	200 - 108
x102 - L. b.	x102 - 108	x15 0 L. p. 100
x601 - L. b.	x102 - 202	18 - L. m.
x201 - L. b.	x102 - 508a	200 - L. m.
501 - L. m.	x601 - 108	x15 - L. m.
	x601 - 202	
Total 9	x601 - 508a	Total 9
	x201 - 108	
	x202 - 202	
	18 - 108	
	18 - L. b.	
	18 - 508a	
	200 - 508a	
	x102 - L. m.	
	x601 - L. m.	
	x201 - L. m.	
	x15 - L. b.	
	x102 - L. m. 100	
	x601 - L. p. 100	
	x201 - L. p. 100	
	Total 24	

Note:

L.b. - L. brevis

L.p. - L. plantarum

L.m. - L. mesenteroides

x - A. cloacae

501 - F. rhenanus

desirable nature of the Gram-negative bacteria since their growth prevented the normal amount of acid from being formed. In the other 37 percent of the cases, the acid tended to rise near that value obtained by the acid bacteria in pure culture only after an extended period. Figures 8b and 8c are examples of each of these effects in graphic form.

In 5 percent of the total cross inoculation studies, A. cloacae had no effect on the formation of acid. In this case the formation of acid proceeded as if A. cloacae were not present.

The cause for the slow formation of acid or even complete inhibition in mixed culture remained as a point of conjecture. The large inoculum used in all this work was intentionally employed to show the effects of a large number of organisms as well as the products of their growth. Therefore, the inhibiting effects were possibly due to the by-products of the Gram-negative bacteria as well as to the immediate effects of a large number of Gram-negative bacteria.

A. cloacae produced a large quantity of gas in pure culture. This organism might be the cause of the gas production during the beginning stages of the sauerkraut fermentation. Preuss, Peterson and Fred (61) studied the gas from normal fermenting sauerkraut and concluded that this consisted of almost 100 percent CO₂. It is believed that carbon dioxide is not an inhibiting by-product since Longsworth and MacInnes (40) reported that CO₂ is essential for the growth of L. acidophilus under anaerobic conditions.

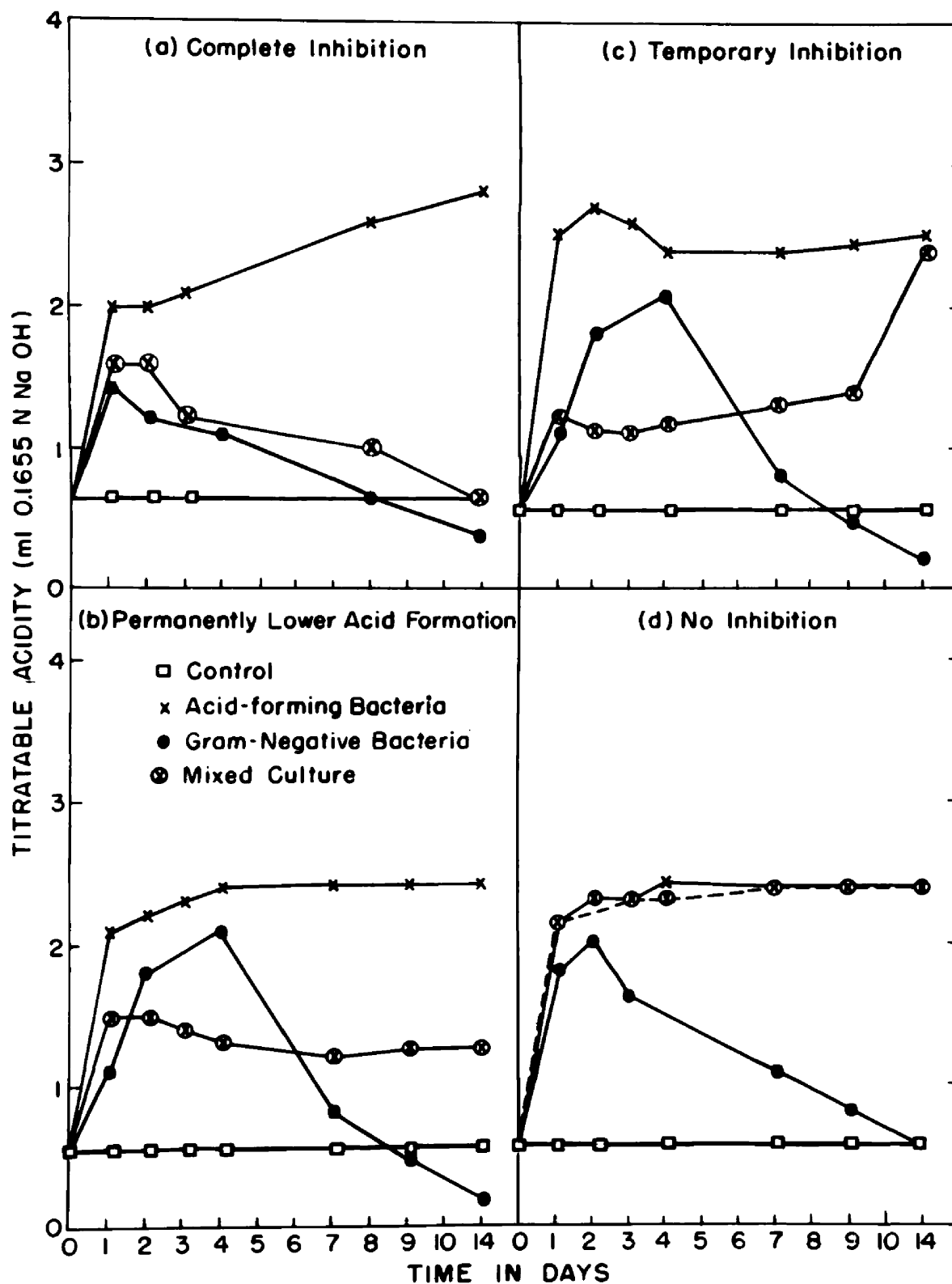


Fig. 8. Influence of Gram-negative bacteria on the titratable acidity of acid-forming bacteria in sterile cabbage juice

Oxidation-Reduction Potentials

Literature Review

Dubos (13) noted that a broth culture became increasingly toxic for Pneumococcus, Streptococcus, and Staphylococcus aureus. He reported that this broth could be restored by autoclaving, boiling, or reducing with hydrogen as well as by adding small amounts of reduced cysteine. He suggested that these findings could be accounted for by assuming that the bacterial species could multiply only in media when the oxidation-reduction potential was below a critical value. The favorable growth conditions obtained by the above procedure may be attributed to the establishment of a proper reduction potential in the medium. He further indicated that the same result was obtained by using a large inoculum owing to the reducing properties of bacterial cells.

Quastel and Stephenson (63) reported that when 0.1 percent cysteine was added to the broth only a very small inoculum was sufficient to initiate "aerobic growth". Webster (69) observed that an inoculum of at least 100,000 cells was necessary for growth to develop under aerobic conditions. On the other hand, growth occurred with an inoculum of only a few cells when the culture was incubated under anaerobic conditions or in the presence of sterile blood.

Allyn and Baldwin (1,2) indicated that the oxidation-reduction character of bacteriological media exerted an important influence on the growth of certain aerobic bacteria (Rhizobia).

Brown and Baldwin (6) studied the oxidation-reduction character of several culture media and showed that the addition of 0.005 to 0.02

percent thioglycollic acid permitted good growth of bacteria that were facultative with respect to oxygen. No growth was obtained unless thioglycollic acid was added to the mannitol-nitrate medium.

Gillespie (21), however, observed that it required smaller numbers of pneumococci to start growth on agar than to initiate growth in broth.

Knaysi and Dutky (33) showed that the limiting factor in the growth of Bacillus megatherium in vacuum was the oxygen content and not the oxidation-reduction potential of the culture medium.

Reed and Orr (64) found that some 15 species of pathogenic clostridia grew luxuriantly from small inocula in a simple, slightly alkaline peptone solution, provided it was poised at a favorable oxidation-reduction potential.

Clifton, Cleary and Beard (10), and Clifton and Cleary (11) presented evidence that the oxidation-reduction potentials are a resultant of the metabolic activities of the bacterial cells.

Faville and Fabian (17) indicated that low oxidation-reduction potentials were consistently obtained during the beginning stages of the cucumber fermentation. They showed that the growth of A. aerogenes exhibited a great reducing ability in pure culture. Other organisms were shown to exhibit reducing conditions, also, but none had reducing abilities which equalled that of A. aerogenes. These workers also studied the oxidation-reduction potentials of L. plantarum. They showed that this organism grew at a high potential and possessed little or no reducing activity. No attempt was made, however, to determine whether the low oxidation-reduction potential in the beginning stages of the cucumber

fermentation was favorable or detrimental to the growth of the acid-forming bacteria.

The immediate interest in oxidation-reduction resulted from the inhibition of acid formation observed in the mixed culture study. Since A. aerogenes created such intensely reducing conditions (17), it was believed that A. cloacae might similarly reduce the oxidation-reduction potential.

In the present study when A. cloacae was grown in mixed culture with the acid-forming bacteria, the inference was made that an adverse state of oxidation might have been created which inhibited the formation of acid. Interest in such a relationship was extended to the normal sauerkraut fermentation in an attempt to further indicate the effects of the Gram-negative group.

Experimental

The platinum electrodes used in the work on oxidation-reduction potentials were prepared by sealing a 4 cm length of platinum wire in the end of 4 mm glass tubing, according to the method outlined by Allyn and Baldwin (2). Two platinum electrodes were used in each container. One electrode was placed near the surface and the other at the bottom in order to note any characteristic differences between the reducing activities in the upper and lower strata. The upper electrode was placed 1 cm from the surface of the medium, and the lower electrode 1 cm from the bottom. Mercury was used as a suitable electrical contact between the wires and the platinum electrodes.

The test container consisted of freezing tubes 12 cm long and 3.5 cm in diameter. The container was closed by means of a four-holed rubber stopper that contained the two platinum electrodes, salt bridge, and a short piece of glass tubing provided with a cotton plug which was used for inoculating the container with the desired organism.

The two test media used for studying the oxidation-reduction potentials were a broth medium and salt expressed cabbage juice. The former was prepared by adding the following to 1 liter of distilled water: 20 gm peptonized milk, 30 gm glucose, 2 gm yeast extract. The salt-expressed cabbage juice was prepared by adding salt to the cabbage shreds at the rate of two percent by weight. The juice was expressed by hand after the cabbage had been in contact with the salt $\frac{1}{2}$ to $2\frac{1}{2}$ hours. These media were dispensed in 50 ml quantities in the containers and sterilized at 15 pounds pressure for 15 minutes.

After sterilization the media were held at 26° C. for 36 hours in order to obtain satisfactory agreement of the initial oxidation-reduction measurements. Measurements were made at the end of this time. If satisfactory readings were obtained, the 24 hour inoculum was introduced. One ml of a 1/100 dilution of a 24 hour culture served as an inoculum for all the oxidation-reduction studies.

The salt bridge used in this work was the sintered glass-tipped bridge as described by Longworth and MacInnes (38). This bridge was made by fusing a mixture of 30 percent powdered alundum and 70 percent powdered pyrex into the end of a piece of 4 mm glass tubing by means of an oxygen torch. In early oxidation-reduction work by various experimentors, however, an agar salt (KCl) bridge was employed (2, 10, 17, 25).

A comparison of these two types of salt bridges proved that the sintered glass-tipped salt bridge described by Longworth and MacInnes (38) was superior. Evidence for this was shown by comparing the amount of salt that diffused from each type of salt bridge over a period of 24 hours. This test was conducted by determining the amount of salt that diffused into 10 ml of distilled water by titration with 0.1711 N AgNO_3 and dichlorofluorescein as the indicator.

Table 7

A comparison of salt diffusion from two types
of salt bridges

Salt bridge	Amount of 0.1711 N AgNO_3 used
I. Sintered glass tipped salt bridge	
A	0.4 ml
B	4.5 ml
C*	1 drop
D*	2 drops
E*	1 drop
F	0.4 ml
G*	2 drops
H*	2 drops
I*	2 drops
II. Agar (KCl) bridge	
A	2.8 ml
B	2.8 ml
C	2.5 ml

*Chosen for subsequent work

From Table 7 the comparison of the amount of salt diffusion from these two salt bridges may be noted. These results showed that the sintered glass-tipped salt bridge permitted only slight salt diffusion as compared with the diffusion of salt noted with the agar salt bridge. Sintered glass-tipped salt bridges C, D, E, G, H, and I were chosen for subsequent work, since these tips permitted the smallest amount of salt to diffuse into the medium during the 24 hour period.

Another advantage of the sintered glass-tipped salt bridge was the convenience of preparation. The agar salt bridge had to be prepared by aseptically filling the glass tubing with sterile agar and then inserting it into the test medium through a hole in the top. The sintered glass-tipped salt bridge, on the other hand, was sterilized when already in the rubber stopper. The electrical circuit was completed by filling the reservoir on the sintered glass-tipped salt bridge with a saturated solution of KCl and introducing an agar salt bridge as a contact between the sintered glass-tipped salt bridge and the standard calomel cell. The agar salt bridges were used repeatedly by keeping their agar contact immersed in saturated KCl when not in use.

Figure 9 shows the six test containers as they were used for making oxidation-reduction measurements. The potentials of the two electrodes in each container were determined by means of six corresponding double throw knife switches as outlined by Hewitt (25). Figure 10 shows the circuit diagram for one of the test containers.

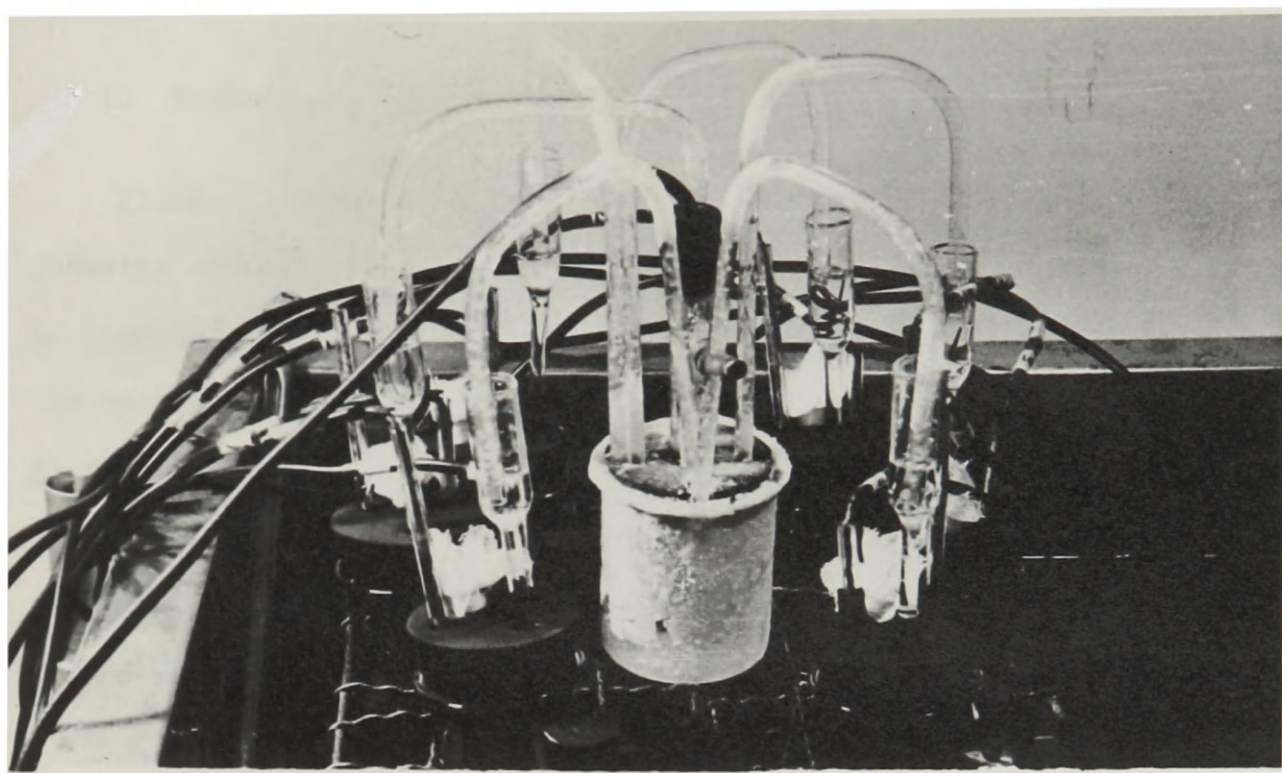


Fig. 9. Six culture containers used for determining oxidation-reduction potentials in pure culture

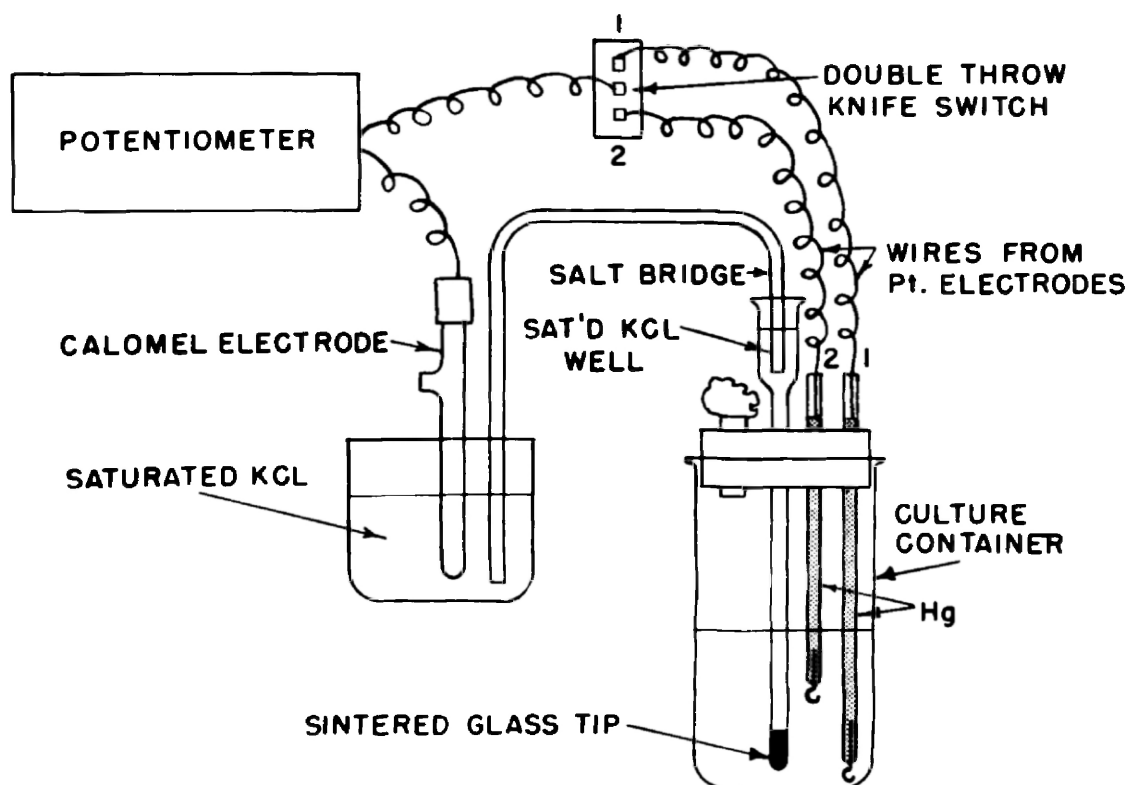


Fig. 10. Schematic drawing of one culture container

1. Determination of Oxidation-Reduction in Pure Culture

Figure 11 shows that a definite difference was obtained between the reducing ability of A. cloacae as compared with those of L. mesenteroides, L. plantarum, L. brevis, and other acid-forming bacteria isolated from the sauerkraut fermentation. In the oxidation-reduction (Eh) test medium, A. cloacae was capable of lowering the potential only 5 hours after inoculation. By 8 hours, the medium attained a maximum low of -0.200 volt as also noticed in the case of A. aerogenes by Faville and Fabian (17). The oxidation-reduction curve for A. cloacae was much different from those of L. mesenteroides, L. plantarum, and L. brevis, where only weak reducing activity was noticed.

The technique employed using two platinum electrodes at different depths in the medium indicated that the acid-forming bacteria differed in their reducing ability in the upper and lower strata. Even though identical oxidation-reduction measurements were obtained in the upper and lower strata in some cases, this physiological characteristic was still evident. When upper and lower potential readings were the same, only one graphic value was plotted. When these values differed, the lower electrode was plotted as usual and the upper potential was plotted by using a dotted line with the same symbol used to designate the organism.

Figure 11 shows that the actual potentials obtained for the lower electrodes were dependent on whether there was a difference between the upper and lower values. When low values were obtained in the lower stratum, the upper stratum was invariably much higher. When high values were obtained at the lower stratum, the upper potential was the same value.

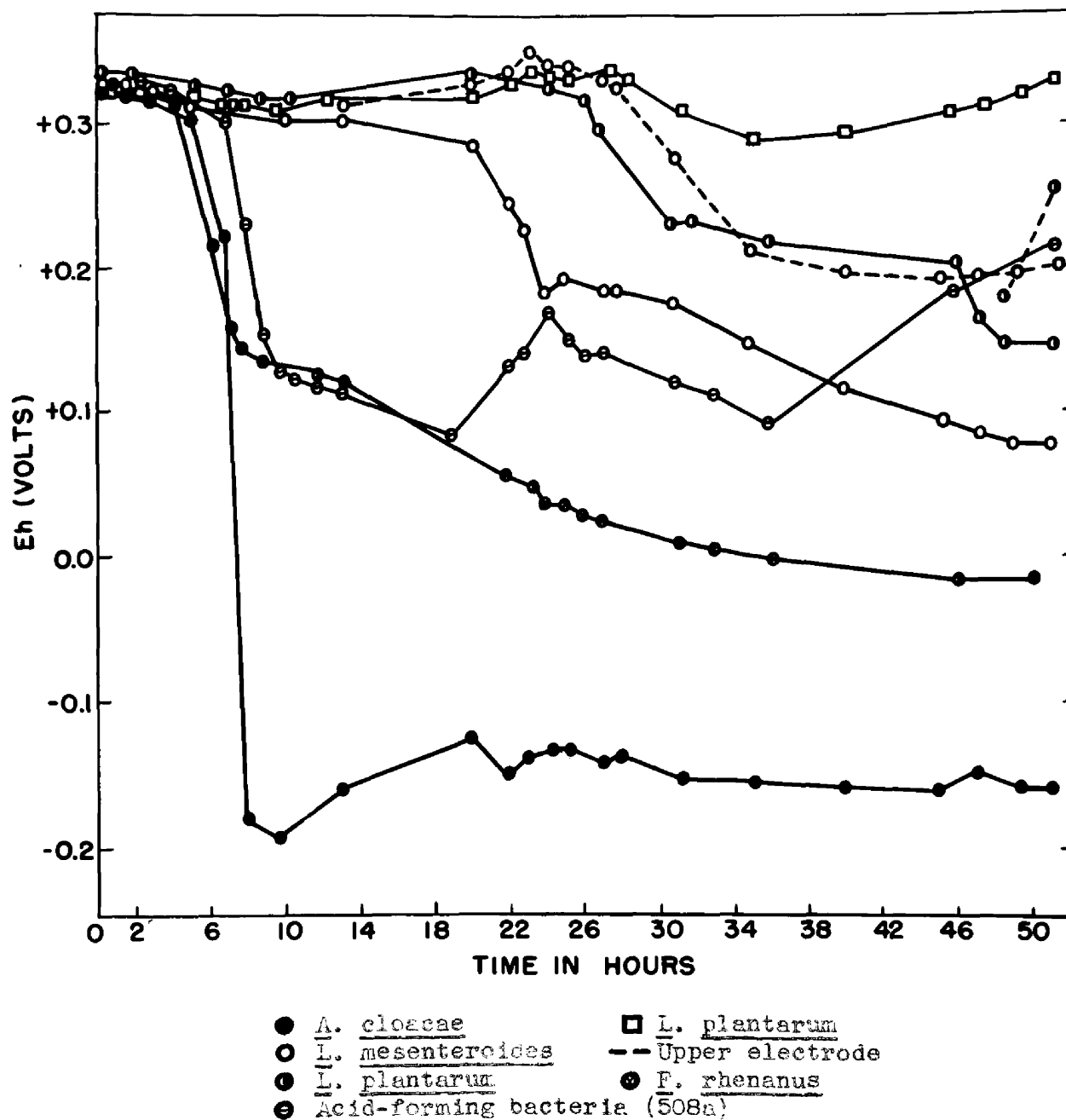


Fig. 11. Oxidation-reduction potentials in pure culture using an Eh test medium

It is believed that this condition is caused by stratification in the first case and mixing of the strata in the second. Stratification would yield lower potentials at the lower stratum due to the exclusion of air.

Longsworth and MacInnes (39, 40) studied the oxidation-reduction potential of L. acidophilus under anaerobic conditions. They reported that there was a slight drop in the curve before appreciable amounts of acid were produced and an abrupt drop during the time that the rate of acid production was increasing. They reported, also, that a minimum was obtained in the Eh curve corresponding to a maximum rate of acid production. Under anaerobic conditions there was a minimum of -0.175 volt after 15 hours. At the end of 50 hours the potential increased only 0.010 volt.

These differences observed between the upper and lower strata presented the question of which time potential curve represented the reducing ability of the organism. It was believed that the values obtained where mixing did not take place indicated the proper values for that organism. The increase of potential was believed to be due to the formation of peroxide (25) at the surface of the medium. Therefore, such determinations should be carried out under anaerobic conditions as in the work of Longsworth and MacInnes (39, 40). Avery and Morgan (3) found that conditions favoring the formation and accumulation of peroxide in broth cultures of Streptococcus pyogenes and Diplococcus mucosus were dependent upon access of air.

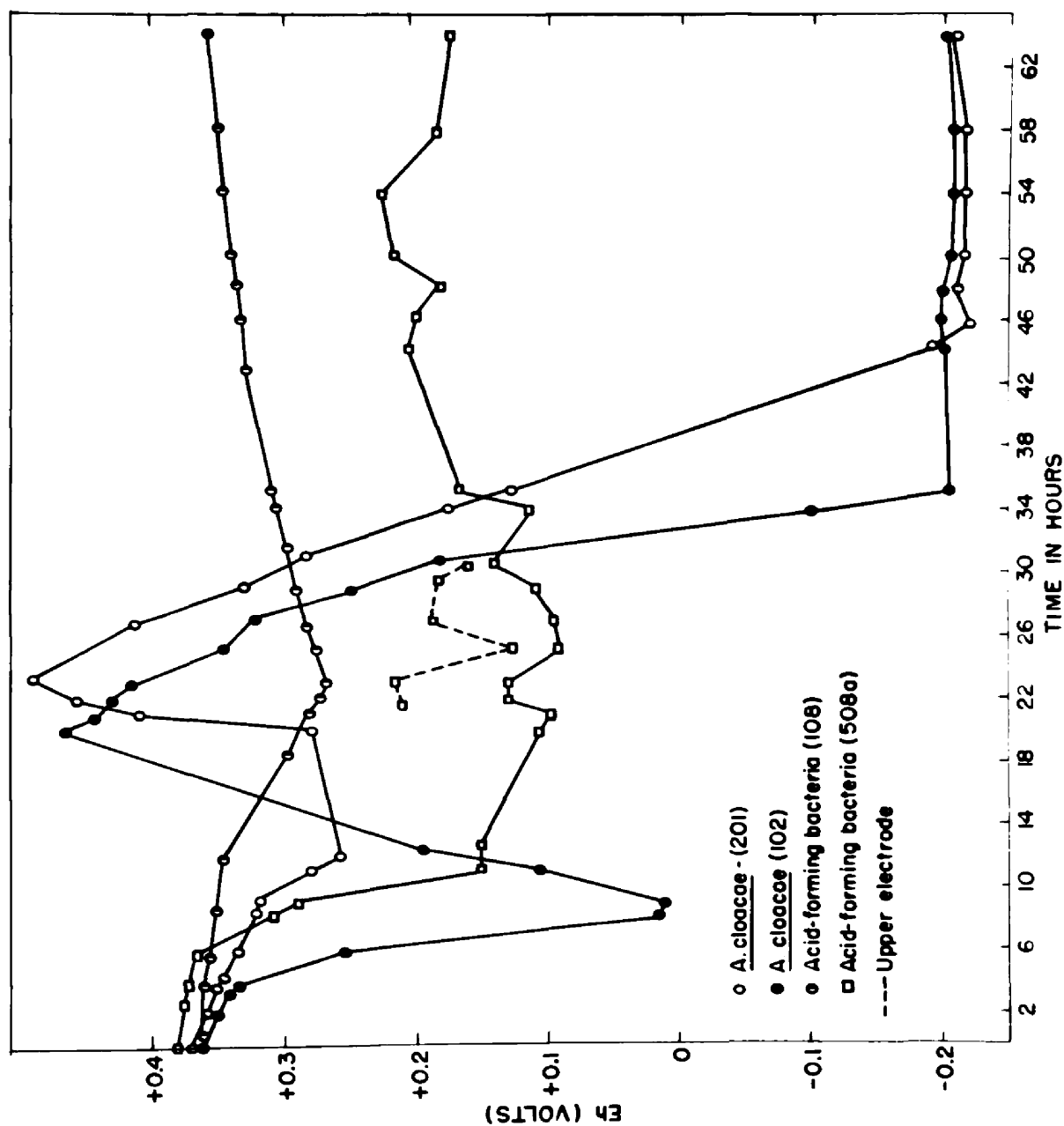
In cases where mixing does not take place, it is believed that the potential then exhibits the normal reducing capacity as if removed from contact with the air. Thus, measurements of the different strata

properly indicated the physiological activity of the organism when actually in contact with the air. According to McLeod and Gordon (42, 43), anaerobes form peroxide in the presence of oxygen and are thereby inhibited. Microaerophilic organisms form peroxide, too, but they are not as sensitive to the compound. The latter organisms, therefore, may be expected to continue growth even though they form peroxide. This phenomenon is suggested by the increase of the oxidation-reduction potential for the acid-forming bacteria shown in Figures 11 and 12. The time potential curves obtained for the acid bacteria using salt-expressed cabbage juice were nearly identical to that employing the Eh test medium. The results in Figure 12 show that A. cloacae began to lower the potential as in the Eh test medium. However, the potential began to rise shortly after adding the inoculum and soon exceeded the values of the control by 0.100 volt. This latter increase was only temporary for the potential descended again and then followed the usual course as in the Eh test medium.

This rise in potential with A. cloacae was very different from that obtained in the Eh test medium with the same organism. Searching for an explanation of this phenomenon it is believed that the cabbage juice may possibly contain a thermostable inhibitor for the catalase system whereby the accumulation of peroxide takes place. Avery and Morgan (3) found that conditions favoring the formation and accumulation of peroxide in broth cultures included also the absence of catalase, peroxidase, and other catalysts capable of decomposing hydrogen peroxide.

It is believed that the substance inhibiting the catalase system may act by blocking or tying up the catalase enzyme in a manner similar to

Fig. 12. Oxidation-reduction potentials in pure culture using sterile cabbage juice



that which Baldwin (4) classified as competitive inhibition. Depending on the initial amount of the competitive substrate, inhibition of the catalase system continues until the amount of catalase exceeds the amount of the substance capable of blocking or combining with catalase. The latter is suggested in Figure 12 by the secondary drop in potential that indicates the removal of peroxide.

2. Determination of Oxidation-Reduction in Mixed Culture

The apparent differences between the reducing ability of A. cloacae and the acid-forming bacteria suggests that the former may inhibit L. mesenteroides or other members of the acid-forming group by virtue of its greater reducing ability. In order to obtain evidence of this phenomenon, L. mesenteroides and A. cloacae were grown in mixed culture in the usual containers provided for measuring oxidation-reduction potentials. Salt-expressed cabbage juice was used as the test medium. The effective microbial growth was measured by removing a 1.0 ml sample and titrating the total acid with 0.0156 N NaOH using phenolphthalein as an indicator. The inoculum was 1 ml of a 1/100 dilution of a 24 hour culture. In cases of the mixed culture 1 ml of each organism was introduced into the same container.

The results given in Figure 13 indicated that the initial lowering of the oxidation-reduction potential by A. cloacae favored the development of titratable acidity. These results were exactly opposite those given in Table 6 in which case the organisms were grown in mixed culture and the titratable acidity was retarded. It is important to note that these differences may be ascribed to a difference in technique. In the work with

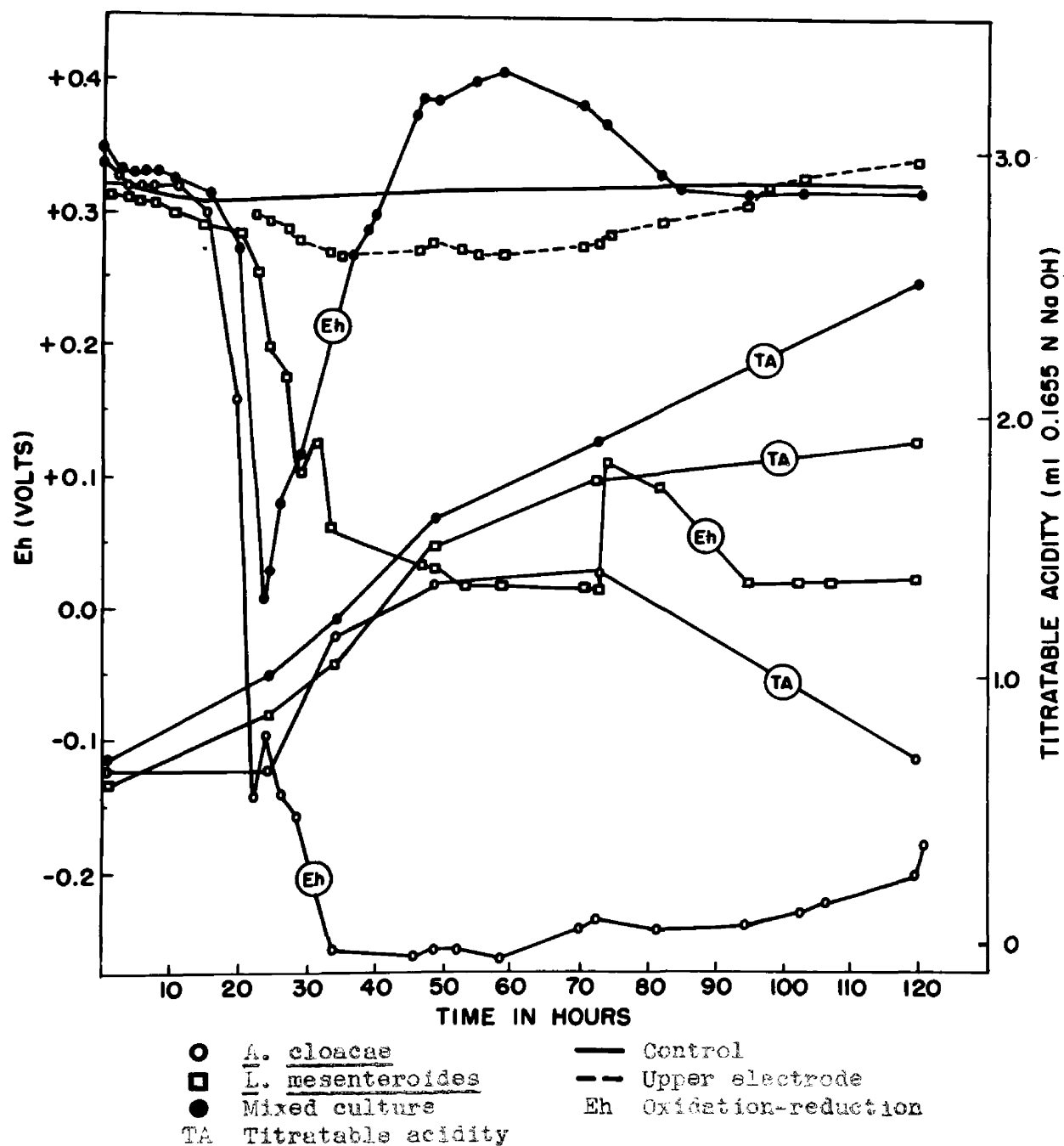


Fig. 13. Relationship between titratable acidity and oxidation-reduction potentials of *A. cloacae* and *L. mesenteroides* in pure and in mixed culture using sterile cabbage juice

mixed cultures, given in Table 6, the inoculum was a single drop of a 24 hour culture in 10 ml sterile salt-expressed cabbage juice. In this work on oxidation-reduction potentials the inoculum was 1 ml of a 1/100 dilution which reduced the initial number of organisms per ml by approximately 25 times.

Even though the initial number of organisms was much lower, it was believed that the dilution also might have removed inhibiting substances produced by A. cloacae. Due to the possible removal of inhibiting substances, it is believed that this experiment properly indicated the physiological relationship between the oxidation-reduction potentials and the growth of the L. mesenteroides, which is representative of the acid-forming group. It is believed that the initial lowering of the potential by A. cloacae favored the development of L. mesenteroides as was evidenced by the increased acid formation.

Figure 13 shows an increase of potential after 40 hours in the case of the mixed culture that is difficult to explain when considering the potentials of each organisms in pure culture. It is believed, however, that the actual increase in potential was caused by the growth of L. mesenteroides. The formation of gas by A. cloacae created a turbulence that increased the surface contact of the medium thereby increasing the peroxide formation of L. mesenteroides in mixed culture.

3. Comparison of Oxidation-Reduction Potentials During Fermentation of Normal and Inoculated Sauerkraut

In view of the increased rate of acid formation when A. cloacae was grown in mixed culture with L. mesenteroides, it was of interest to determine whether the Gram-negative group that grew so abundantly on the V-8 medium could similarly cause a low oxidation-reduction potential and favor the growth of the acid-forming bacteria during the normal sauerkraut fermentation. Along with correlating the oxidation-reduction potential with the growth of the Gram-negative bacteria (lenticular-shaped organisms on V-8 medium), A. cloacae was inoculated in another batch of cabbage using the normal fermentation as a control. This was done in order to emphasize directly the effects of the growth of the Gram-negative group, since A. cloacae had been identified as a member of the Gram-negative group growing during the beginning stages of 5 out of 6 fermentations studied.

The sampling technique, which had been used for preliminary bacteriological studies, was modified so that two platinum electrodes could be introduced into the sample container and the oxidation-reduction potential, acid titration, and bacterial analysis could be made simultaneously. It was believed that by employing the V-8 medium, the growth of the Gram-negative organisms might be followed, thereby, noting how they affect the oxidation-reduction potential in the normal and inoculated sauerkraut. The appearance of the acid-formers also might be followed similarly, in every case referring the number of acid-forming bacteria to the total acid produced.

The sampling device was modified by making two holes in the side of the sample container with an oxygen torch. The platinum electrodes were introduced by securing them within a one-holed rubber stopper. The system was flushed out with purified nitrogen and the sample withdrawn by the method previously described.

As soon as the sample was withdrawn, the rubber stopper D (see Figure 4) was removed and the sintered glass-tipped salt bridge introduced and held firm by another one-holed rubber stopper. An agar salt bridge completed the circuit from the calomel cell to the sintered glass-tipped salt bridge, as shown in Figure 14. Oxidation-reduction measurements were made immediately. The sample was returned to the gallon jar by nitrogen pressure every few hours and immediately re-sampled to insure representative fermenting conditions within the sample container. Bacteriological analysis and acid titration were made three times daily. The titration of acid was made by withdrawing a 1 ml sample at D and titrating the acid with 0.0156 N NaOH using phenolphthalein as the indicator.

The inoculum of A. cloacae was prepared by salting cabbage shreds at 2 percent by weight and dispensing 50 ml of the expressed juice in a suitable flask. After sterilization the cooled cabbage medium was inoculated with A. cloacae from an agar slant. After being incubated for 24 hours at 27° C the total amount of inoculum was then placed in a 5 gallon crock and mixed well with the salt and cabbage shreds. This mixture was transferred to a gallon jar and the sample tube S inserted and connected to the sample container as shown in Figures 3 and 4.

Figures 15 and 16 show that the oxidation-reduction potential is greatly affected by the addition of the 24 hour inoculum of A. cloacae.

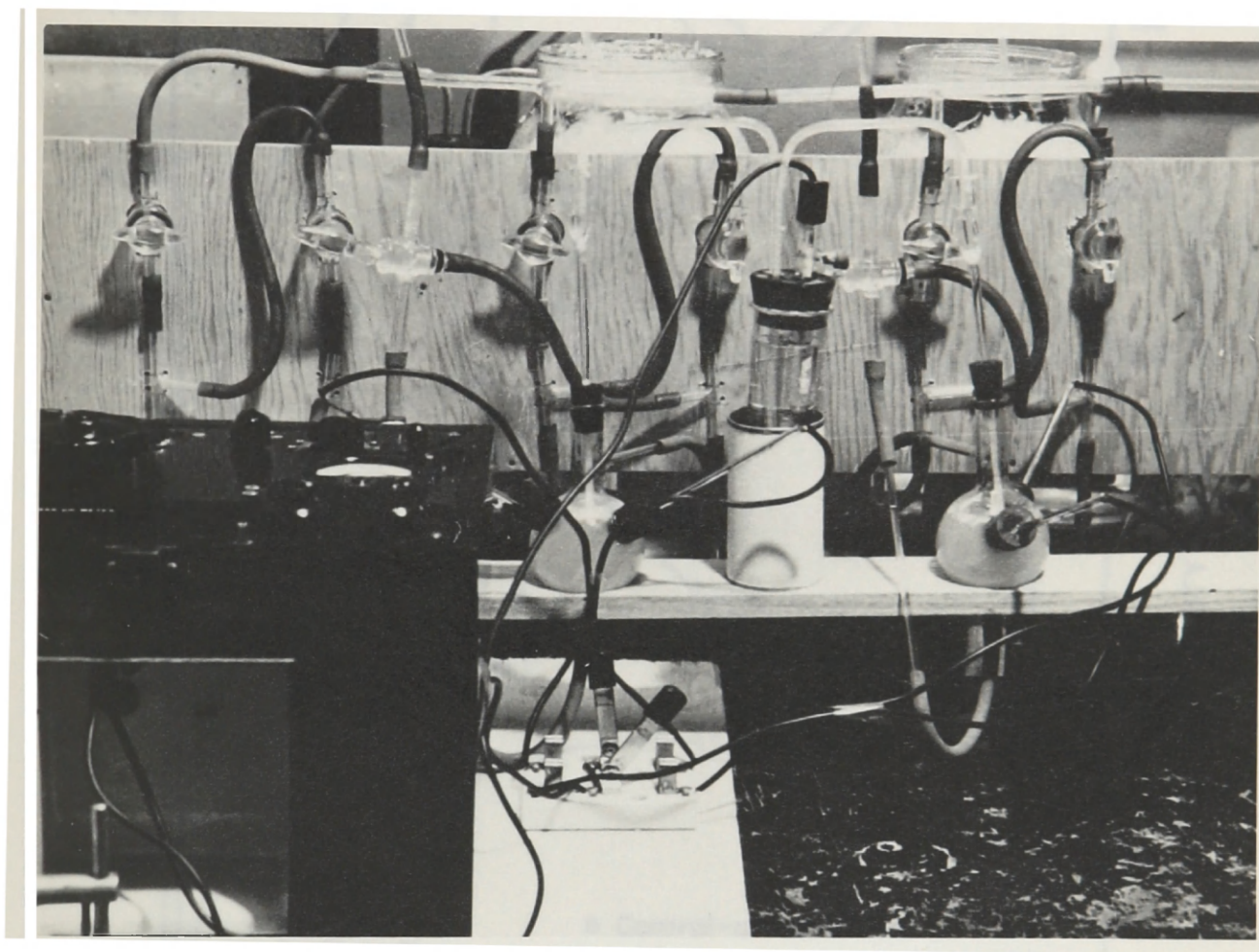


Fig. 14. Apparatus used for determining oxidation-reduction potentials during the sauerkraut fermentation

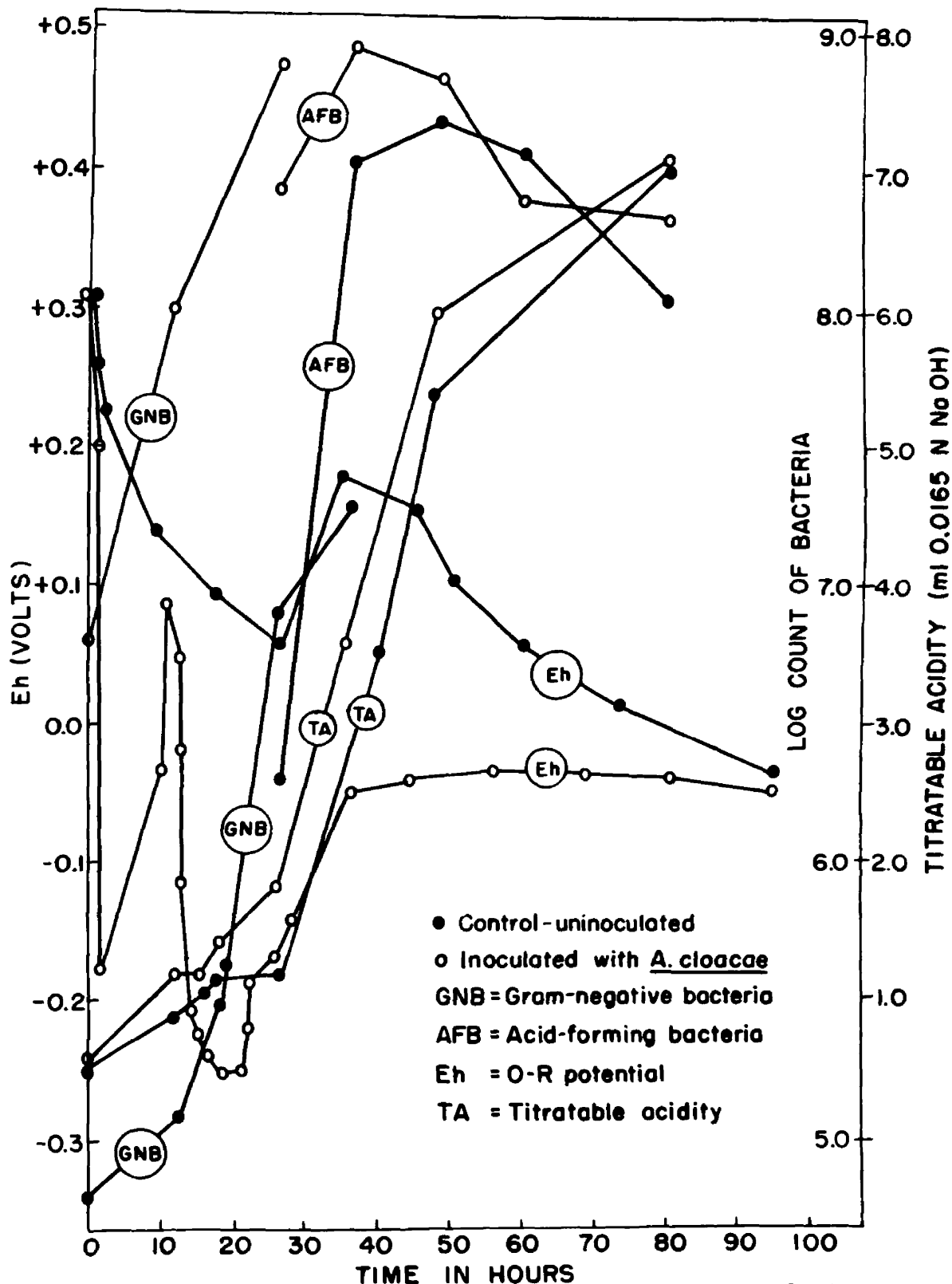


Fig. 15. Relationship between acid formation, oxidation-reduction potentials and bacterial microflora in normal and in inoculated sauerkraut

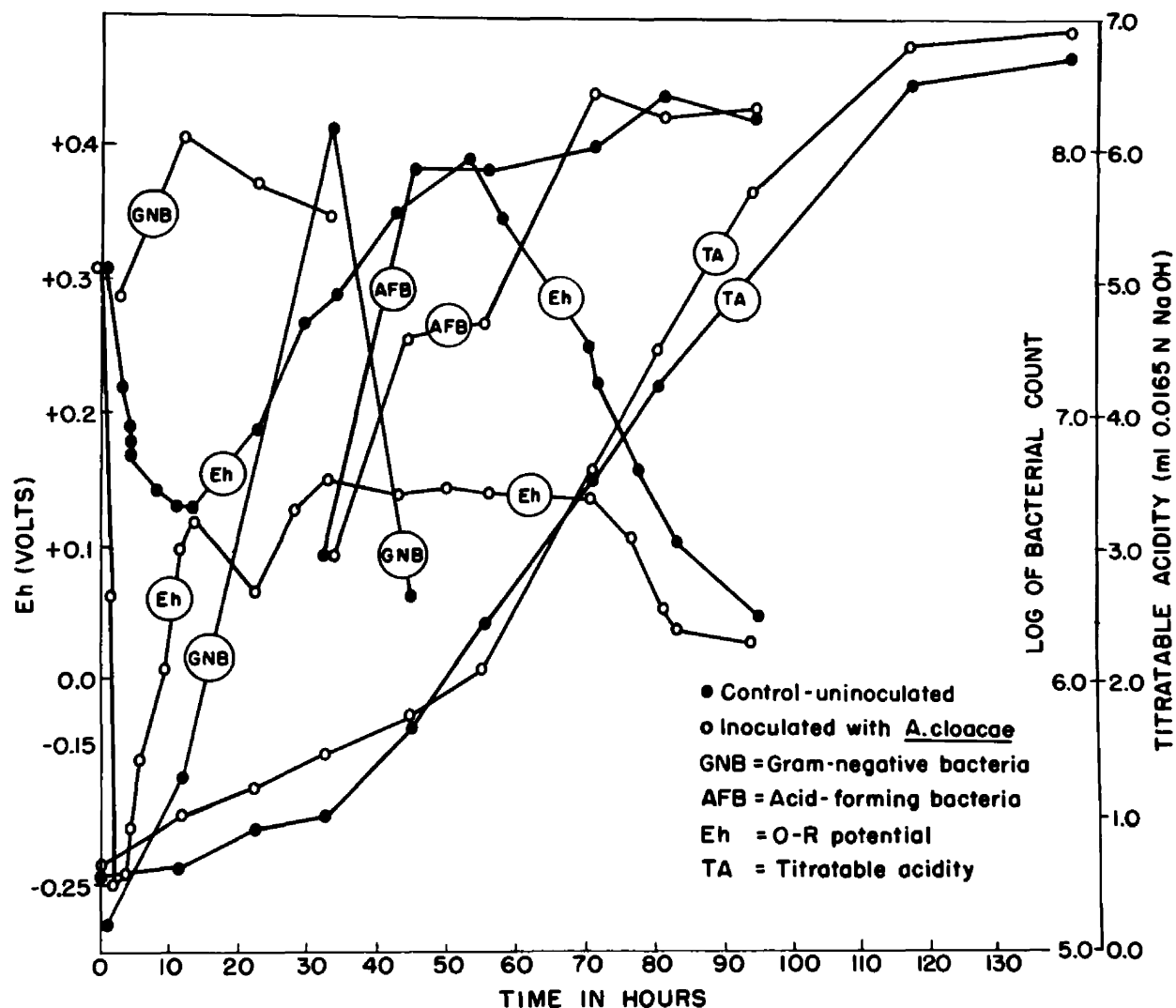


Fig. 16. Relationship between acid formation, oxidation-reduction potentials and bacterial microflora in normal and in inoculated sauerkraut

Only two hours after the addition of the inoculum, the oxidation-reduction potential decreased 0.400 to 0.450 volts; the control decreased only about 0.050 volts. This drop in potential, corresponding to the addition of the inoculum, seemed to be due to the addition of reducing substances. Another possible explanation is that the inoculum contained an enzyme (catalase) which might have been capable of affecting the oxidation-reduction reaction of a substrate which acts competitively (see page 49).

The growth of A. cloacae was poorer in the inoculated sauerkraut shown in Figure 16 than in the inoculated sauerkraut shown in Figure 15. The greater growth of A. cloacae in the latter sauerkraut may be correlated with the secondary drop in oxidation-reduction potential that was observed after 13 hours. The acid-forming bacteria also appeared sooner where the greater growth of A. cloacae took place. It appeared that the lower potential obtained in this case might have favored the growth of the lactic acid bacteria. Figure 15 shows that the titratable acidity also increased faster than in the normal sauerkraut. The titratable acidity also increased slightly during the growth of A. cloacae; however, this increase might have been due to the formation of CO₂.

A. cloacae did not grow well in the inoculated sauerkraut shown in Figure 16 and the acid-forming bacteria in this instance did not appear as soon as they appeared in the inoculated sauerkraut shown in Figure 15. Actually, the acid-formers appeared sooner in the normal sauerkraut, shown in Figure 16, than in the inoculated sauerkraut in this instance. The appearance of the acid-forming bacteria during a period of high oxidation-reduction potential was different from the inoculated sauerkraut shown in Figure 15 and from the study of oxidation-reduction potentials in mixed

culture shown in Figure 13 especially since the Gram-negative bacteria grew with increasing potential.

The oxidation-reduction potential of the normal sauerkraut fermentation also decreased during the initial stages of the fermentation; however, the potential never decreased enough to equal that which was characteristic of the reducing conditions found with A. cloacae in pure culture. Actually the identity of the Gram-negative bacteria growing during this period was not known.

It is interesting to note that at the end of 80 hours the oxidation-reduction potentials of both the normal and inoculated sauerkraut tended to become equal.

The work on oxidation-reduction potentials with sauerkraut inoculated with A. cloacae also brought forth the possibility that this organism might be the cause of the darkening of sauerkraut and abnormal flavor under certain conditions. This observation, though not directly concerned with oxidation-reduction, is mentioned here since the literature indicated only limited knowledge of the organisms causing these defects. As a result of the inoculation studies with A. cloacae, it is believed that this organism is the cause for much of the dark sauerkraut and abnormal flavors described by Pederson (49, 50).

Two days after the inoculation with the 50 ml sample a slight brown coloration was noted throughout the shredded cabbage. As time progressed the darkening became more evident. These results were obtained in four studies where A. cloacae was introduced into the fermentation. In one experiment the wooden top was removed from the gallon jar two weeks after the start of the fermentation. In this case the browning increased at

the surface and extended down into almost half of the contents. The normal fermentation showed no discoloration during the two-week observation period. At the end of 2 weeks the cabbage to which A. cloacae had been added had a raw cabbage flavor with the sharpness of a radish. The texture of this cabbage was examined also, but no changes were noted. The normally fermented cabbage made at the same time and kept under the same conditions, but which was not inoculated with A. cloacae and which served as a control, had normal flavor and texture.

GENERAL DISCUSSION OF RESULTS

Isolation and identification of lenticular-shaped colonies on a V-8 medium indicated that A. cloacae and F. rhenanus (Gram-negative organisms) grew rapidly during the beginning stages of the sauerkraut fermentation. A. cloacae was believed to be the more prevalent organism since it was found during the beginning of five out of the six fermentations studied whereas F. rhenanus was isolated from only one of the fermentations.

Aerobacter has been isolated in connection with other fruit and vegetable products, also. Faville and Hill (18) identified Aerobacter sp as one of a group of bacteria causing spoilage in unpasteurized orange juice. Etchells, Fabian and Jones (15) showed that Aerobacter was the cause of gassy fermentation of cucumbers.

Factors influencing the growth of either Aerobacter or Flavo-bacterium in this work are not known. However, it was generally observed, that as soon as the lactic acid bacteria appeared, the Gram-negative bacteria quickly disappeared.

The identification of Aerobacter in the present study is in accordance with the work done by Round (65) who showed that lactose-bile fermenting organisms increased rapidly for the first few days of the sauerkraut fermentation and then quickly disappeared shortly after an increase in acidity. Gruber (22) and Conrad (12) also isolated colon-type organisms from the sauerkraut fermentation. Keipper, Fred and Peterson (32) indicated that the coliform group of bacteria may amount to 40 percent of the initial microflora on the outer leaves of summer cabbage. These workers also isolated numerous bacteria that conformed to the characteristics of Bacterium herbicola aureum (Duggeli) and suggested that their organisms and Bacterium herbicola aureum (Duggeli) might be placed in the genus

Flavobacterium according to Bergey's classification (5). Their work substantiates the present study in which A. cloacae and F. rhenanus were isolated and identified.

The results of growing the Gram-negative bacteria with the acid-forming bacteria in mixed culture indicated that a large number of Gram-negative bacteria or their by-products inhibited the formation of acid to some degree. In some cases the acid formation was retarded only temporarily whereas in others the amount of acid produced was lowered permanently.

The study of oxidation-reduction potentials in a mixed culture of A. cloacae and L. mesenteroides showed that acid was produced more quickly by the latter when the potential was concomitantly lowered by A. cloacae. These results were not in accordance with the results of the mixed culture study mentioned above. The reason for this might be the size of the inoculum. In the first mixed culture study, each organism was inoculated by means of 1 drop of a 24 hour culture in 10 ml sterile cabbage juice. In the oxidation-reduction culture study the amount of inoculum was decreased by approximately 1/25. In comparing these two studies it appeared that the inoculum in the first study contained substances that prevented the formation of acid. These substances were produced possibly as a result of bacterial growth of the Gram-negative organisms and were possibly removed by dilution in the second study.

The inhibition by-products of bacterial growth in this case would be similar to that described by Dubos (13) when he reported that broth cultures became increasingly toxic for Pneumococcus, Streptococcus, and Staphylococcus aureus. He further found that this broth could be restored by autoclaving, boiling or reducing with hydrogen.

The acid-forming bacteria also appeared sooner during the sauerkraut fermentation when an inoculum of A. cloacae was added to the salted cabbage shreds. A drop in the oxidation-reduction potential corresponding to the addition of A. cloacae again indicated that the growth of the acid-forming bacteria was favored by a low potential. The appearance of A. cloacae during the beginning stages of the sauerkraut fermentation may actually be beneficial; however, any extended growth of this organism would definitely be detrimental.

Although the addition of A. cloacae to the sauerkraut fermentation caused a faster growth of the normal acid-forming microflora, the sauerkraut began to darken within a day after salting. At the end of two weeks the sauerkraut was quite dark when compared with the control. When the surface of the inoculated sauerkraut was exposed to the air, the darkening was intensified. The flavor of the sauerkraut also was abnormal and possessed a sharp radish-like taste.

Pederson (49, 50) suggested that the darkening of sauerkraut was caused by the growth of many undesirable bacteria or yeasts. He also noted off-flavors in the sauerkraut. He reported that gas-producing lactobacilli or rod-shaped bacteria, if allowed to develop too early in the fermentation, produced a rather sharp or biting sauerkraut. Although Pederson did not attribute these defects to any particular organism, the darkening and the off-flavor might have been due to the growth of A. cloacae.

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SUMMARY

Under the conditions of these experiments the results may be summarized as follows:

A. cloacae and F. rhenanus were identified as members of the Gram-negative group that were found to be increasing in number during the beginning stages of the sauerkraut fermentation. The increase of these bacteria was determined by observing an increase in the number of the lenticular-shaped colonies on the V-8 medium with a corresponding decrease of total count.

A. cloacae was isolated in five of the six fermentations studied and is considered of greater prevalence than F. rhenanus since the latter was isolated in only one of the six fermentations.

The study of A. cloacae and F. rhenanus in mixed culture with the acid-forming bacteria indicated that the presence of a large number of Gram-negative bacteria, or their by-products, prevented the normal formation of acid by the acid-forming bacteria. When the amount of inoculum of A. cloacae was further reduced, acid was produced at a faster rate in the cabbage juice with the mixed culture of A. cloacae and L. mesenteroides than when either organism was used separately. The measurement of the oxidation-reduction potentials in mixed culture also indicated that the greater formation of acid may be attributed to the lowering of the oxidation-reduction potential by A. cloacae which may favor the growth of L. mesenteroides.

The introduction of A. cloacae into the sauerkraut fermentation indicated that the growth of this organism lowered the oxidation-reduction potential. The lowering of the potential, and/or other factors, appeared to favor the growth of the acid-forming bacteria since the acid-forming

bacteria appeared sooner than in a similar fermentation where A. cloacae was not added. In one of the fermentations, A. cloacae did not grow well and in this instance the growth of the acid-forming bacteria was delayed rather than favored.

The oxidation-reduction potential decreased during the beginning stages of the normal sauerkraut fermentation with the growth of the Gram-negative organisms, however, the low potentials obtained for A. cloacae in pure culture were not reproduced in the normal sauerkraut fermentation. The reason for this might be due to the presence of inhibiting substances in the cabbage which caused the accumulation of hydrogen peroxide.

The introduction of A. cloacae into the natural sauerkraut fermentation showed that this organism was capable of causing dark sauerkraut and at the end of two weeks produced a sharp radish-like flavor which was undesirable. The exposure of such sauerkraut to the air also intensified darkening.

CONCLUSIONS

These experiments indicated that the Gram-negative bacteria, such as A. cloacae and F. rhenanus, multiply during the beginning stages of the normal sauerkraut fermentation. A. cloacae was isolated in five of the six fermentations studied and is considered of greater prevalence than F. rhenanus since the latter was isolated in only one of the six fermentations.

In small numbers, A. cloacae apparently produces favorable conditions for the growth of L. mesenteroides. However, if A. cloacae is present in large numbers or if conditions are favorable for an abnormal amount of growth, this organism can cause darkening of the sauerkraut, a sharp radish-like flavor, or even a permanently lower titratable acidity.

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