A Study of the Microorganisms from Grass Silage

I. The Cocci

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In a natural silage fermentation, the mass is acidified by lactic and acetic acid forming bacteria that ferment sugars in the plant material. When forage is ensiled the plant cells continue to respire for a time, using up the oxygen and giving off CO₂ and heat. As conditions become favorable, acid producing bacteria increase rapidly and, at the end of 3 or 4 days, each gram of silage will contain several hundred million bacteria. These organisms produce acid until the sugar is exhausted or until the pH becomes unfavorable for further growth.

A recent study (Langston et al., 1958) on the microorganisms in orchard grass and alfalfa silages showed that the total numbers of acid producing bacteria had little bearing on the final quality. The increase in numbers of these organisms showed similar trends in all silages studied. The counts reached about the same maxima in both good and poor quality silages, and the few silages that had relatively high initial counts of acid producing bacteria were no better in quality than those with few or none. The difference in quality of the silages was correlated directly with the appearance and increase of sporeforming anaerobes. The poor quality silages had high numbers of these organisms.

It is not clearly understood why some silages show a rapid drop in pH and others yield only a limited amount of acids. The carbohydrate source, however, is not always the limiting factor. Various reasons have been put forth to account for the variability in acid production. They are: (1) variability in sequence changes of microorganisms, (2) antagonism among certain groups of bacteria early in the fermentation process, (3) deficient nutrients in the plant material for bacterial growth, and (4) occurrence of weakened strains of bacteria.

Although the preservation of forage by natural fermentation is an old and useful method of preserving fodder, little work has been done to characterize the types of organisms responsible for the acid production. Many of the early workers investigating the microorganisms in silage spoke only generally of "lactic ferment" or "acidifying bacteria."

The object of this study was to learn more about the types and occurrence of microorganisms in silage and their taxonomical relationship. The data presented are the results of detailed colonial, morphological, and physiological studies on the cocci important in the silage fermentation.

**Materials and Methods**

Preparation of silages and techniques used in isolating and grouping the lactic acid bacteria from the silages have been outlined in an earlier publication (Langston et al., 1958).

This phase of work includes detailed studies on representative strains of lactic acid bacteria from 30 silages. The strains were picked from highest dilution roll tubes (Trypticase) and plates (Rogosa et al., 1951).

Preliminary grouping was based on the following tests: Gram stains, catalase production, growth at 45 C, growth in 6.5 per cent of sodium chloride, hydrolysis of arginine, reduction of nitrate, production of gas from glucose, and reaction in litmus milk.

About 440 strains from the 3142 isolates grouped as indicated above were chosen for further study. The strains were replated at least twice to insure purity and all tests were standardized as far as possible. Unless otherwise stated, the media were inoculated with one drop of culture (18 to 20 hr incubation) that had been transferred 2 or 3 times in tomato juice glucose broth. Except for temperature studies all tests were incubated at 30 C.

All of the strains studied were gram-positive and nonsporeforming. During the preliminary study, several strains were observed to produce catalase and reduce nitrate. Some of these strains were chosen for detailed study. This proved to be a judicious choice because, as will be shown later, most of the strains that had these variable characteristics were similar in most respects to the true lactic acid bacteria. Characteristics studied:

1. Colony formation. Colony formation (arrangements, size, and color) was observed with the aid of a wide-field microscope.

2. Gram stain (Burke modification), form, size, and arrangement of cells. Smears were made from 18 to 24-hr cultures grown in tomato juice glucose broth and semi-

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solid deeps. Tomato juice glucose broth had the following composition and will be designated as medium A: Tryptase, 10 g; phytone, 5 g; yeast extract, 5 g; glucose, 5 g; sodium chloride, 5 g; potassium phosphate (dibasic), 1.5 g; “Tween 80” (sorbitan monoooleate),\(^2\) 0.5 ml; tomato juice, 200 ml; bromeresol purple, 0.016 g; and distilled water to make 1 L. The medium was adjusted to pH 7 and dispensed into test tubes in 9-ml quantities. It was autoclaved at 15 lb pressure for 15 min. The semisolid medium was similar to medium A with the addition of 5 g agar and deletion of the bromeresol purple.

3. Motility. Hanging drop slides and in some cases semisolid medium were used to detect motility. Flagella stains were prepared in the following manner: Cells were washed from the surface of slants with saline solution (0.85 per cent) centrifuged twice, and made to a slightly cloudy suspension with distilled water. The slides were prepared and stained by the method described by Leifson (1951). Best results were obtained with cells 8 to 12 hr old. The slant medium was similar to medium A with the deletion of bromeresol purple and the addition of 15 g of agar.

4. Catalase production. Catalase production was determined on broth cultures and on agar streak plates containing low carbohydrate (0.05 per cent). The broth and plating media were similar to medium A. To determine catalase from cultures growing in broth, a few drops of the broth were transferred to a spot plate and 3 per cent hydrogen peroxide was added. The evolution of gas constituted a positive test. Precautions should be taken in reading tests, especially when the cultures are weakly catalase positive. Flaming of the pipette before transferring the culture to the spot plate should be avoided and the test should be observed for at least 5 min before discarding. Streak plates were flooded with 3 per cent hydrogen peroxide and observed for gas evolution. Strains that produced catalase always showed greater activity on plates than in broth.

5. Production of gas. To detect gas, medium A was used with the following modifications: Bromeresol purple was deleted, glucose increased to 20 g, and 20 g of agar added. The medium was dispensed into test tubes in 7-ml quantities. An agar layer (2 per cent) and oil seal were used to prevent loss of gas. Heavy inoculations were made and the tubes were incubated for 2 weeks. It was not uncommon for some cultures to push the agar and oil layers to the top of the tubes.

6. Growth at 15 C and 45 C. Medium A was used with the following modifications: Five grams of agar were added; the tomato juice was filtered, and bromeresol purple deleted. The medium was dispensed in 9-ml quantities into test tubes. After sterilization and inoculation (inoculating loop), the tubes were sealed with rubber stoppers to avoid evaporation. A water bath was used to determine growth at 45 C (1 week of incubation) and growth at 15 C (2 weeks’ of incubation) was determined in a Cenco \(^3\) refrigerating incubator.

7. Growth in 0.5 per cent of sodium chloride. The organisms were tested for their ability to grow in sodium chloride in medium A. The salt concentration was increased from 5 g to 65 g per L. The medium was dispensed in 9-ml quantities into test tubes and incubated for 2 weeks.

8. Growth at pH 9.6. After the medium was autoclaved and dispensed in 9-ml quantities into test tubes, sterile sodium hydroxide was added aseptically to produce the desired pH. The tubes were incubated for 2 weeks.

9. Reduction of 0.1 per cent methylene blue. Skimmed milk\(^4\) containing 0.1 per cent methylene blue was inoculated with test cultures and observed for changes from light blue to colorless. The incubation period was for 1 week.

10. Reaction in litmus milk. Litmus milk\(^5\) was inoculated with test cultures and incubated 1 month. Tubes were examined and medium changes recorded at 24 hr, 48 hr, 1 week, and 1 month.

11. Final acidity in skimmed milk. Dehydrated skimmed milk\(^6\) was reconstituted, dispensed in 9-ml quantities into test tubes, sterilized, and inoculated with test cultures. After 1 month of incubation, the contents of the tubes were washed into small beakers. Clots when formed were broken up by magnets kept in motion by a Mag-mix.\(^7\) The pH was recorded and titration values obtained by titrating electrometrically to the phenolphthalein end point with \(\frac{1}{10}\) NaOH. Acid values from the controls were subtracted from the cultures to obtain correct acidity values (acidity values are expressed as per cent lactic acid). A Beckman\(^8\) pH meter was used.

12. Final acidity in glucose broth. Procedures for obtaining final acidity values in glucose broth were essentially the same as those described for skimmed milk. The medium had the following composition: Tryptase, 10 g; yeast extract, 5 g; NaCl, 5 g; glucose, 20 g; and distilled water to make 1 L. The medium was adjusted to pH 7 and dispensed into test tubes (18 by 150 mm) in 15-ml quantities. The tubes were inoculated with test cultures and incubated for 2 weeks.

13. Production of ammonia from arginine. The method and medium described by Niven et al. (1944) was used to determine ammonia production from arginine. After 3 days of incubation, ammonia was detected by placing 3 or 4 drops of culture into a spot plate and adding 1 drop of Nessler’s reagent.

14. Production of acetyl-methylcarbinol. Test cultures were inoculated into the following medium: polyptone,\(^9\) 7 g; glucose, 5 g; potassium phosphate (dibasic),

\(^{2}\) Atlas Powder Company, Wilmington, Delaware.
\(^{3}\) Central Scientific Company, Chicago, Illinois.
\(^{4}\) Precision Scientific Company, Chicago, Illinois.
\(^{5}\) Beckman Instruments, Inc., Fullerton, California.
5 g; and distilled water to make 1 L. The pH of the medium was adjusted to 7.0. After inoculation the tubes were incubated 48 hr. To detect acetylmehtylearbinol, 1 ml of culture was placed in a test tube and 0.6 ml of a 5 per cent α-naphthol solution in 100 ml of alcohol and 0.2 ml of 40 per cent potassium hydroxide were added. The development of a red color in the mixture from 2 to 4 hr constituted a positive test. No tubes were read later than 4 hr after the addition of the reagents.

15. Gelatin liquefaction. Cultures were inoculated into tubes of gelatin medium that had the following composition: Beef extract, 3 g; peptone, 5 g; gelatin, 120 g; and distilled water, 1 L. The pH of the medium was adjusted to 7.0. After 48 hr incubation the tubes were chilled to determine if the gelatin had been liquefied.

16. Production of mucoid colonies on 10 per cent sucrose plates. The medium described by McCleskey et al. (1947) was used to determine dextran production.

17. Nitrate reduction. Nitrate reduction was determined after incubation for 3 days in indole-nitrate medium. Tests were made for nitrite and residual nitrate.

18. Indole test. The test for indole was carried out in indole-nitrate medium after 24 and 48 hr incubation. Kovacs reagent was used to determine indole production.

19. Carbohydrate fermentations. Fermentation reactions were carried out in the following basal medium: Trypticase, 10 g; yeast extract, 5 g; NaCl, 5 g; and distilled water to make 1 L. Substrates were added at the 1 per cent level. Rhamnose, glucose, lactose, sucrose, trehalose, raffinose, melezitose, starch, dextrin, inulin, salicin, esculin, sodium hippurate, glycerol, mannitol, inositol, sorbitol, and sodium lactate were sterilized with the base. Arabinose, xylose, ribose, fructose, galactose, mannose, sorbose, maltose, cellobiose, and melibiose were sterilized separately (Seitz filtration) and added to the autoclaved base. The material was then tubed aseptically into test tubes in 7-ml quantities. The tubes were incubated for 2 weeks. Fermentation reactions were detected through the use of a Beckman pH meter.

Hydrolysis of sodium hippurate was detected by adding 0.5 ml of 50 per cent sulfuric acid to 2 ml of culture. Appearance of benzoic acid crystals constituted a positive test. Hydrolysis of esculin was determined by adding 0.5 ml of ferric citrate to 7 ml of culture. The typical blackening reaction of the medium constituted a positive test.

20. Fermentation products. Strains of lactic acid bacteria were grown in medium similar to that described by Harrison and Hansen (1950b). However, the glucose was sterilized separately and added to the autoclaved base and tomato juice was deleted except in cases where acid production was very low. Initial and residual glucose was determined by the anthrone method described by Morris (1948). Aliquots of the medium were acidified to pH 1.0 with concentrated sulfuric acid and extracted with anhydrous ether for 36 hr in a water bath at 60°C (Langston, 1955). Aliquots of the extract were titrated to determine total acids produced and a fraction was removed for chromatographic analysis of the individual acids. Butyric, propionic, acetic, formic, succinic, and lactic acids were determined by a method similar to the one described by Neish (1950).

The remaining acid solution was brought to about half volume on the steam bath, boiled for a few minutes, and neutralized with zinc carbonate (Brin et al., 1952). The zinc lactate crystals were usually collected in three fractions and characterized by the water of crystallization and optical rotation.

Results

The genera Streptococcus and Leuconostoc are of prime importance in many phases of agriculture and industry from a standpoint of desirable or undesirable fermentations. The genus Streptococcus includes the homofermentative cocci which produce primarily dextro-lactic acid as a by-product of glucose fermentation. Sherman (1937) separated the streptococci into four groups on the basis of certain physiological characteristics. In contrast, the genus Leuconostoc includes the heterofermentative cocci which usually produce a limited amount of levo-lactic acid, CO₂, acetic acid, and alcohol from glucose. Three species have been recognized in the genus Leuconostoc (Hucker and Pederson, 1930).

The lactic acid producing cocci from the forages were identified and divided into five more or less distinct groups. Included were strains of (a) Streptococcus faecalis, (b) Streptococcus liquefaciens (Streptococcus faecalis var. liquefaciens), (c) Leuconostoc mesenteroides, (d) variable Leuconostoc (Leuconostoc, type I), and (e) Pediococcus. All cultures had the following characteristics in common: colonies were usually small and subsurface and the commonly observed forms were lenticular or circular. The margin of the colonies were usually entire although some were slightly wavy or undulate. Surface colonies were uniform, smooth, and round. Most of the colonies observed had a whitish to grayish appearance. All strains were facultative anaerobes, gram-positive, and nonsporeforming. Growth on the surface of agar slants was thin to moderate. None of the strains reduced nitrate to nitrite or produced indole. (a) S. faecalis and (b) S. liquefaciens were usually oval shaped and occurred singly, in pairs, and sometimes short chains (4 to 6 cells). They averaged about 0.7 μ by 0.8 to 1.1 μ. None of the strains produced gas from glucose or dextran on 10 per cent sucrose agar and no catalase was observed. All of the cultures produced ammonia from arginine, grew in 6.5 per cent of sodium chloride and at pH 9.6, and reduced 0.1 per cent methylene blue in milk.

(a) Streptococcus faecalis. The strains of S. faecalis
were further characterized by their ability to completely reduce litmus milk prior to acidification and curdling, and most of the strains produced acetylactic acid. They grew at 15°C and eight of the 13 strains showed growth or slight growth at 45°C. None liquefied gelatin. The final pH in glucose broth ranged from 3.8 to 4.3 (avg 4.2), and in skimmed milk, 4.4 to 5.8 (avg 4.8). Titratable acidity values in glucose broth and skimmed milk ranged from 0.40 to 0.86 (avg 0.55) and 0.13 to 0.66 (avg 0.46), respectively.

All of the strains (table 1) fermented ribose, glucose, fructose, mannose, galactose, maltose, lactose, trehalose, cellulose, dextrin, salicin, esculin, glycerol, and mannitol; most fermented arabinose, xylose, rhamnose, sucrose, starch, and sorbitol. They varied with respect to melibiose and raffinose and none fermented sorbose, melezitose, inulin, sodium hippurate, inositol, or sodium lactate.

Dextro lactic acid (table 2) was the main by-product of glucose fermentation produced by three strains of *S. faecalis*. Recoveries showed that 91.2 to 96.0 per cent of the glucose fermented was converted to lactic acid. Traces of other products were also present. These included propionic, acetic, formic, and succinic acids.

(b) *Streptococcus liquefaciens*. *S. liquefaciens* acidified, curdled, and peptonized litmus milk. The litmus was completely reduced prior to curdling. All strains liquefied gelatin, produced acetylactic acid, and grew at 15°C and 45°C.

Final pH values in glucose broth ranged from 4.2 to 4.3 and titratable acidity values from 0.47 to 0.61 (avg 0.55). Carbohydrate fermentations (table 1) were similar to those of *S. faecalis* with the exception that *S. liquefaciens* failed to ferment arabinose but did ferment melezitose and sodium hippurate.

*Streptococcus liquefaciens* gave a carbon distribution (table 2) similar to that of *S. faecalis*, however, two of the strains studied produced inactive lactic acid. Although organisms in this group usually produce dextro-lactic acid, it is not unusual for strains to produce a mixture of the dextro- and levo- types (Long and Hammer, 1936). The two strains that gave inactive lactic acid were replated and picked to insure purity and the by-products redetermined. This procedure was repeated twice and in each trial inactive lactic acid was obtained.

The strains of *S. faecalis* and *S. liquefaciens* described here are generally in close agreement with earlier descriptions presented by Sherman et al. (1937), Smith and Sherman (1942), Harrison and Hansen (1950a) and as

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### TABLE 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>Streptococcus faecalis</em></th>
<th><em>Streptococcus liquefaciens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture reaction</td>
<td>pH Range</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1-; 12+</td>
<td>4.3-5.8</td>
</tr>
<tr>
<td>Xylose</td>
<td>3-; 10+</td>
<td>4.3-5.1</td>
</tr>
<tr>
<td>Ribose</td>
<td>13+</td>
<td>4.3-4.5</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>2-; 11+</td>
<td>4.6-5.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>13+</td>
<td>4.0-4.3</td>
</tr>
<tr>
<td>Fructose</td>
<td>13+</td>
<td>4.0-4.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>13+</td>
<td>3.9-4.5</td>
</tr>
<tr>
<td>Galactose</td>
<td>13+</td>
<td>3.8-4.6</td>
</tr>
<tr>
<td>Lactose</td>
<td>13+</td>
<td>4.1-4.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3-; 11+</td>
<td>4.1-4.5</td>
</tr>
<tr>
<td>Trehalose</td>
<td>13+</td>
<td>3.9-5.0</td>
</tr>
<tr>
<td>Cellulobiose</td>
<td>13+</td>
<td>4.1-4.5</td>
</tr>
<tr>
<td>Melibiose</td>
<td>5-; 8+</td>
<td>4.4-5.2</td>
</tr>
<tr>
<td>Raffinose</td>
<td>6-; 7+</td>
<td>4.3-5.5</td>
</tr>
<tr>
<td>Melezitose</td>
<td>13-</td>
<td>4.6-5.6</td>
</tr>
<tr>
<td>Starch</td>
<td>1-; 12+</td>
<td>4.3-5.6</td>
</tr>
<tr>
<td>Dextrin</td>
<td>13+</td>
<td>4.3-5.6</td>
</tr>
<tr>
<td>Salicin</td>
<td>13+</td>
<td>4.2-4.6</td>
</tr>
<tr>
<td>Esculin</td>
<td>13+</td>
<td>4.2-4.6</td>
</tr>
<tr>
<td>Sodium hippurate</td>
<td>13-</td>
<td>4.5-5.2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>13+</td>
<td>5.4-5.8</td>
</tr>
<tr>
<td>Mannitol</td>
<td>13+</td>
<td>4.4-5.0</td>
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<td>Inositol</td>
<td>13+</td>
<td>4.4-5.0</td>
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<tr>
<td>Sorbitol</td>
<td>1-; 9+</td>
<td>5.0-5.2</td>
</tr>
</tbody>
</table>

None of the strains fermented sorbose, inulin, or sodium lactate.

The heterofermentative cocci studied were divided into 2 groups mainly on the basis of dextran production. Strains of *L. mesenteroides* produced this material readily. A closely related group (*Leuconostoc, type I*) produced dextran only after serial transfers in enriched media.

(c) *Leuconostoc mesenteroides*. Cells of *L. mesenteroides* were usually oval to slightly elongated and occurred singly, in pairs, and short chains. They averaged about 0.7 to 0.8 μ by 1.2 μ. It was not uncommon to find elongated cells up to 3 or 4 μ in length. The strains produced gas from glucose, grew at 15 C and 45 C and in 6.5 per cent of sodium chloride. Only slight acid reactions were observed in litmus milk. Final pH and titratable acidity values in glucose broth ranged from 4.1 to 4.4 (avg 0.43) and 0.41 to 0.63 (avg 0.53), respectively. None of the cultures produced ammonia from arginine, acetyl methylcarbinol, liquefied gelatin, or grew at 45 C. The unusual feature of this group was the ability of some strains to produce catalase. Five of the 10 strains studied exhibited this property. Weak catalase reactions were first observed in tomato juice glucose broth. When the strains were streaked on low carbohydrate medium (0.05 per cent glucose) they gave a relatively strong reaction. Two strains that failed to produce catalase in the tomato juice glucose broth gave a positive reaction on the low carbohydrate medium. Some of the cultures exhibited a “delayed reaction” in production of O2 following the addition of hydrogen peroxide. The test material should be observed for several minutes before recording results. Visual observations indicated that the amount of gas evolution by the more active strains of *Leuconostoc* was similar to that produced by cultures of *Pediococcus*. Catalase production by organisms in this genus comes as no surprise since various workers have shown that other facultative lactic acid producing bacteria (pediococci, lactobacilli) exhibit this property.

Two colonial types were observed when strains of *L. mesenteroides* were streaked on 10 per cent sucrose agar. The colonies resembled type B and D described by McCleskey *et al.* (1947). Although, they noted varia-

### TABLE 2

*Per cent acids and optical type of lactic acid produced by Streptococcus*

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Per Cent Glucose Fermented</th>
<th>Per Cent Fermented Glucose Converted to</th>
<th>Optical Type of Lactic Acid Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butyric acid</td>
<td>Propionic acid</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>T-10</td>
<td>51.8</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>T-16</td>
<td>47.8</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>T-843</td>
<td>45.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Streptococcus faecalis

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Per Cent Glucose Fermented</th>
<th>Per Cent Fermented Glucose Converted to</th>
<th>Optical Type of Lactic Acid Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butyric acid</td>
<td>Propionic acid</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>T-848</td>
<td>49.3</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>T-865</td>
<td>51.2</td>
<td>0</td>
<td>0.02</td>
</tr>
<tr>
<td>T-873</td>
<td>41.6</td>
<td>0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Streptococcus liquefaciens

### TABLE 3

*Final pH of broth cultures after 14 days incubation at 30 C*

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>Leuconostoc mesenteroides</em></th>
<th><em>Leuconostoc Type I</em></th>
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<tbody>
<tr>
<td></td>
<td>Culture reaction</td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>Avg</td>
</tr>
<tr>
<td>Arabinose</td>
<td>3- ; 7+</td>
<td>4.3-4.9</td>
</tr>
<tr>
<td>Xylose</td>
<td>10+</td>
<td>4.4-5.1</td>
</tr>
<tr>
<td>Ribose</td>
<td>5- ; 5+</td>
<td>4.2-5.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>10+</td>
<td>4.0-4.4</td>
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<tr>
<td>Fructose</td>
<td>10+</td>
<td>4.2-4.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>10+</td>
<td>3.7-4.5</td>
</tr>
<tr>
<td>Galactose</td>
<td>10+</td>
<td>4.2-4.7</td>
</tr>
<tr>
<td>Maltose</td>
<td>10+</td>
<td>3.6-4.4</td>
</tr>
<tr>
<td>Lactose</td>
<td>2- ; 8+</td>
<td>4.3-5.1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10+</td>
<td>4.2-4.5</td>
</tr>
<tr>
<td>Trehalose</td>
<td>10+</td>
<td>4.0-4.4</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>2- ; 8+</td>
<td>4.4-4.9</td>
</tr>
<tr>
<td>Melibiose</td>
<td>3- ; 7+</td>
<td>4.3-4.7</td>
</tr>
<tr>
<td>Raffinose</td>
<td>3- ; 7+</td>
<td>4.3-4.5</td>
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<tr>
<td>Starch</td>
<td>10-</td>
<td>37- ; 6+</td>
</tr>
<tr>
<td>Dextrin</td>
<td>10-</td>
<td>32- ; 11+</td>
</tr>
<tr>
<td>Salicin</td>
<td>10+</td>
<td>4.3-4.7</td>
</tr>
<tr>
<td>Esculin</td>
<td>10+</td>
<td>19- ; 24+</td>
</tr>
</tbody>
</table>

None of the strains fermented rhamnose, sorbose, melizitose, inulin, sodium hippurate, glycerol, inositol, sorbitol, or sodium lactate.
tions in fermentation reactions that we did not observe. Carbohydrate fermentations (table 3) were almost identical with those described in Bergey’s Manual (Breed et al., 1957) for L. mesenteroides. All strains fermented xylose, glucose, fructose, mannose, galactose, maltose, sucrose, trehalose, salicin, and esculin. They varied but usually fermented arabinose, ribose, lactose, cellobiose, melibiose, raffinose, and mannnitol. None of the strains fermented rhamnose, sorbose, melezitose, starch, dextrin, esculin, sodium hippurate, glycerol, inositol, sorbitol, or sodium lactate.

Strains of L. mesenteroides converted less than 43 per cent of the glucose fermented to lactic acid (table 4). Acetic and traces of other acids were also found. All of the cultures tested produced the levo- form of lactic acid.

(d) Leuconostoc, type I. Leuconostoc, type I, showed greater morphological variation than was found with L. mesenteroides. The cells varied from about 0.4 to 0.7 μ by 0.8 to 4 μ. Under some conditions of growth it was difficult to determine if the organisms were actually cocci or rods. Cultures grown in stabs were usually more coccoïd in shape than those grown in broth. This relationship did not always hold true, however, and it was not unusual to find within a smear small oval and elongated rod forms from solid or liquid media. This morphological variation presented some problems in the early phase of work. It was difficult to determine if they were more closely related to the heterofermentative cocci or rods. The problem was further complicated in that although they usually fermented sucrose they failed to produce dextran on plates containing 10 per cent sucrose. Later results showed that they were closely related to the genus Leuconostoc and, as will be shown later, the cultures produced dextran after serial transfers in enriched media. The cells occurred singly, in pairs, and occasionally short chains and clumps. Some cells tapered and longer ones had a tendency to bend.

Physiological reactions were similar to those of L. mesenteroides with the following exceptions: 12 of the 43 strains showed slight growth at 45 C; some gave a reduced, acid, and curdled reaction in litmus milk; and a slightly wider range was noted for titratable acidity values in glucose broth (0.23 to 0.77).

Carbohydrate fermentation reactions (table 3) were usually similar to those produced by L. mesenteroides although some of the strains failed to ferment xylose, maltose, sucrose, and esculin and a few showed slight growth in starch and dextrin.

The cultures fermented less glucose than L. me-

TABLE 3
Per cent acids and optical type of lactic acid produced by Leuconostoc

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Per Cent Fermented Glucose Converted to</th>
<th>Optical Type of Lactic Acid Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Butyric acid</td>
<td>Propionic acid</td>
</tr>
<tr>
<td>T-13</td>
<td>95.6</td>
<td>0 2.6</td>
</tr>
<tr>
<td>T-60</td>
<td>77.0</td>
<td>0 3.0</td>
</tr>
<tr>
<td>T-79</td>
<td>78.0</td>
<td>0 3.9</td>
</tr>
<tr>
<td>T-328</td>
<td>86.0</td>
<td>0 4.0</td>
</tr>
</tbody>
</table>

Leuconostoc mesenteroides

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Per Cent Fermented Glucose Converted to</th>
<th>Optical Type of Lactic Acid Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-17</td>
<td>44.5</td>
<td>0 0.08</td>
</tr>
<tr>
<td>T-40</td>
<td>68.0</td>
<td>0 0.01</td>
</tr>
<tr>
<td>T-57</td>
<td>43.0</td>
<td>0 5.2</td>
</tr>
<tr>
<td>T-312</td>
<td>76.0</td>
<td>0 0.1</td>
</tr>
<tr>
<td>T-323</td>
<td>51.0</td>
<td>0 0.4</td>
</tr>
<tr>
<td>T-357</td>
<td>62.0</td>
<td>0 0.07</td>
</tr>
<tr>
<td>T-1600</td>
<td>60.0</td>
<td>0 2.0</td>
</tr>
<tr>
<td>R-610</td>
<td>58.0</td>
<td>0 0.06</td>
</tr>
<tr>
<td>R-343</td>
<td>59.0</td>
<td>0 1.0</td>
</tr>
<tr>
<td>R-392</td>
<td>75.0</td>
<td>0 0.2</td>
</tr>
</tbody>
</table>

Leuconostoc type I

Later, the cultures produced dextran after serial transfers in enriched media. The cells occurred singly, in pairs, and occasionally short chains and clumps. Some cells tapered and longer ones had a tendency to bend.

Physiological reactions were similar to those of L. mesenteroides with the following exceptions: 12 of the 43 strains showed slight growth at 45 C; some gave a reduced, acid, and curdled reaction in litmus milk; and a slightly wider range was noted for titratable acidity values in glucose broth (0.23 to 0.77).

Carbohydrate fermentation reactions (table 3) were usually similar to those produced by L. mesenteroides although some of the strains failed to ferment xylose, maltose, sucrose, and esculin and a few showed slight growth in starch and dextrin.

The cultures fermented less glucose than L. me-

(e) Pediococcus. The high lactic acid producing cocci that form tetrads were first described as spoilage agents in beer and through the years the relationship of these organisms to other types of homofermentative lactic acid bacteria has been confused. In the 6th edition of Bergey’s Manual of Determinative Bacteriology (Breed, Murray, and Hitchens, 1948), the genus Pediococcus was included in the appendix in the family Micrococaceae.
A critical review and detailed study of the group by Pederson (1949) suggested that they should be placed in the family of lactic acid producing cocci and rods. The 7th edition of Bergey's Manual (Breed et al., 1957) now includes the genus *Pediococcus* in the tribe *Streptococcae* of the family *Lactobacillaceae* along with the genera *Diplococcus*, *Streptococcus*, and *Leuconostoc*.

The pediococci examined in this study were rather uniform in their morphology. The cells occurred singly, in pairs, as tetraads (especially in acid medium), and occasionally short chains (4 to 6 cells). They averaged 0.6 to 0.7 μ by 0.8 to 1.1 μ. All of the cultures grew in 6.5 per cent of sodium chloride and at 15 C. None liquefied gelatin, gave gas from glucose, or produced acetyl-methylcarbinol. All of the cultures examined produced inactive lactic acid (table 5) and most of the glucose fermented was converted to lactic acid along with acetic and traces of other acids. Pederson's (1949) study on 121 strains of the genus *Pediococcus* from fermenting vegetables showed some differences among strains, but on the basis of carbohydrate fermentations no significant trends were demonstrated. He concluded that all of his strains should be considered as belonging to one species. His strains produced inactive lactic acid, a trace of volatile acid, and a small amount of CO₂. They fermented glucose, fructose, mannose, galactose, maltose, and usually arabinose, sucrose, lactose, raffinose, salicin, and amygdalin. Most strains failed to ferment mannitol, α-methylglucoside, inulin, dextrin, and starch. Pederson brought out that slight differences in ability to utilize certain sugars and related compounds should not be considered sufficient grounds for excluding a strain from the species or establishing a new species.

The silage isolates had many characteristics similar to those outlined above. Some additional characteristics not noted by Pederson were observed in the present work. For comparative purposes and to show certain variable characteristics, the 79 strains in this study were divided into five groups.

In table 6 it may be noted that the majority of the strains produced ammonia from arginine. However, all strains in group 4 failed to hydrolyze this compound. Some of the strains in each group produced catalase with the exception of those in group 5. Organisms in group 5 also grew at 48 C and the other groups failed to grow at this temperature. Strains varied in litmus milk, some produced only a slight acid and others caused an acid curdled reaction. Many of the cultures reduced the litmus in the bottom of the tubes and a few were capable of almost complete reduction of the litmus. The curdling reaction in litmus milk always occurred after 1 week of incubation. Final pH and titratable acidity values ranged from 3.6 to 4.1 and 0.78 to 1.4 per cent, respectively. Strains in group 4 gave the lowest pH and the highest titratable acidity values.

All of the strains (table 7) fermented glucose, fruc-
TABLE 7
Final pH of broth cultures after 14 days of incubation at 30 C (Pediococcus)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Group 1 pH</th>
<th>Group 2 pH</th>
<th>Group 3 pH</th>
<th>Group 4 pH</th>
<th>Group 5 pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture reaction</td>
<td></td>
<td>Culture reaction</td>
<td></td>
<td>Culture reaction</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>Avg</td>
<td>Range</td>
<td>Avg</td>
<td>Range</td>
</tr>
<tr>
<td>Arabinose</td>
<td>11+</td>
<td>3.6–4.5</td>
<td>1–2</td>
<td>20+</td>
<td>3.9–4.6</td>
</tr>
<tr>
<td>Xylose</td>
<td>11–</td>
<td>4.0–4.3</td>
<td>4.2</td>
<td>21+</td>
<td>4.2–4.7</td>
</tr>
<tr>
<td>Ribose</td>
<td>11+</td>
<td>4.0–5.0</td>
<td>4.6</td>
<td>19–</td>
<td>2+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>11+</td>
<td>3.8–4.1</td>
<td>4.0</td>
<td>21+</td>
<td>3.9–4.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>11+</td>
<td>4.0–4.3</td>
<td>4.1</td>
<td>21+</td>
<td>3.9–4.5</td>
</tr>
<tr>
<td>Fructose</td>
<td>11+</td>
<td>3.5–4.1</td>
<td>4.0</td>
<td>21+</td>
<td>3.9–4.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>11+</td>
<td>3.7–4.2</td>
<td>4.1</td>
<td>21+</td>
<td>3.8–4.2</td>
</tr>
<tr>
<td>Galactose</td>
<td>11+</td>
<td>3.6–4.1</td>
<td>4.0</td>
<td>21+</td>
<td>3.9–4.2</td>
</tr>
<tr>
<td>Maltose</td>
<td>11+</td>
<td>3.8–5.5</td>
<td>4.4</td>
<td>21+</td>
<td>4.2–4.7</td>
</tr>
<tr>
<td>Lactose</td>
<td>10+</td>
<td>3.8–4.4</td>
<td>4.0</td>
<td>14–7+</td>
<td>4.1–4.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10+</td>
<td>3.8–4.2</td>
<td>4.1</td>
<td>1–20+</td>
<td>4.1–4.3</td>
</tr>
<tr>
<td>Trehalose</td>
<td>11+</td>
<td>3.9–4.3</td>
<td>4.1</td>
<td>21+</td>
<td>3.9–4.2</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>10+</td>
<td>4.0–4.4</td>
<td>4.2</td>
<td>9–12+</td>
<td>4.0–4.5</td>
</tr>
<tr>
<td>Melibiose</td>
<td>10+</td>
<td>4.2–4.9</td>
<td>4.4</td>
<td>8–13+</td>
<td>4.1–4.5</td>
</tr>
<tr>
<td>Raffinose</td>
<td>11+</td>
<td>4.2–4.6</td>
<td>4.1</td>
<td>21+</td>
<td>4.3–4.7</td>
</tr>
<tr>
<td>Salicin</td>
<td>11+</td>
<td>4.1–4.6</td>
<td>4.1</td>
<td>21+</td>
<td>4.3–4.7</td>
</tr>
<tr>
<td>Eucalin</td>
<td>11+</td>
<td>20+</td>
<td>21+</td>
<td>5.2–5.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>

None of the strains fermented sorbose, melezitose, starch, dextrin (all strains in group 4 gave a slight positive reaction), inulin (exception of one strain in group 1), sodium hippurate, mannitol (exception of one strain in group 5), inositol, sorbitol, or sodium lactate.
tose, mannose, galactose, cellubiose, salicin, and esculin. They usually failed to ferment sorbose, melezitose, starch, dextrin, inulin, sodium hippurate, mannitol, inositol, sorbitol, or sodium lactate. Variations were observed in the fermentation of arabinose, xylose, ribose, rhamnose, maltose, lactose, sucrose, trehalose, melibiose, and raffinose. The greatest variation occurred in groups 3, 4, and 5. Pederson (1949) reported that the majority of his strains fermented sucrose, lactose, and raffinose. Many of the strains in the above mentioned groups failed to ferment these compounds. Furthermore, Pederson showed that all of his strains fermented maltose. None of the silage strains in group 5 fermented this sugar. Some of the strains in group 2 and most of the strains in groups 3, 4, and 5 were not able to ferment melibiose. The greatest variation in the ability to ferment carbohydrates occurred in group 4. None of these strains fermented arabinose, xylose, ribose, sucrose, melibiose, raffinose, or glycerol and it is interesting to note that they also failed to hydrolyze arginine. This group deserves further attention with regard to final pH values in carbohydrates. It may be seen in table 6 that organisms in this group produced lower pH and higher titratable acidity values than were found in the other groups. However, in table 7 it may be seen that the same organisms gave the highest final pH in glucose and other carbohydrates. This seems to illustrate variability among strains in response to conditions for growth.

Whether the group differences indicated are important enough to warrant their division into new species or subspecies remains to be seen. Certainly, too few strains have been studied to draw any definite conclusions. At this time, however, the authors agree with the suggestion of Pederson that the strains studied should be considered as belonging to one species (*Pediococcus cerveisiae* Balecke). When a greater number of strains are studied from different environments, it is possible that the group differences indicated above will become important.

**DISCUSSION**

The results showed that the cocci isolated from forages fell into two primary groups. Those which produce mostly lactic acid from glucose and those which produce limited amounts of lactic acid along with CO₂, acetic, and traces of other acids.

The 13 strains of *S. faecalis* examined compared favorably with the published data of other workers. The only variation of significance as compared to the work of Sherman et al. (1937) and described in *Bergey's Manual* (Breed et al., 1957) is that 12 of the 13 silage strains hydrolyzed starch and none of their strains were able to hydrolyze this carbohydrate. The hydrolysis of starch by this group would suggest some similarity to *Streptococcus bovis* but other basic characteristics described earlier discount this relationship.

Recent work has been submitted which indicates that *Streptococcus faecium* (Orla-Jensen, 1919) should be given species rank along with *S. faecalis*. For many years these organisms were regarded as being synonymous. The following characteristics have been used to differentiate between the organisms: *S. faecalis* grows in the presence of 1/2500 potassium tellurite, usually shows a vigorous fermentation of mannitol and sorbitol, fails to ferment arabinose, and gives a strong reduction of litmus milk prior to acid and curdling. *Streptococcus faecium* is inhibited by 1/2500 potassium tellurite, always ferments arabinose, varies on mannitol, usually does not ferment sorbitol, and may show only acidity in litmus milk with little or no reduction. Although four of the silage strains resembled *S. faecium* in that they fermented arabinose and failed to ferment sorbitol, they must be regarded as *S. faecalis* because all of the strains studied were able to grow in the presence of 1/2500 potassium tellurite and gave a strong reduction of litmus milk prior to acid and curdling. The cultures also exhibited a-hemolysis on blood agar. The diversity of fermentative reactions within this species was pointed out by Harrison and Hansen (1950a). In fact, they showed greater variation in carbohydrate fermentation among strains of *S. faecalis* isolated from the intestinal flora of healthy turkeys than was reported here or by Sherman et al. (1937).

The 6th edition of *Bergey's Manual* (Breed et al., 1948) regards *S. faecalis* and *S. liquefaciens* as separate species. The 7th edition of *Bergey's Manual* lists *S. liquefaciens* as a variety of *S. faecalis*. Apparently the editors felt that differences were not great enough to warrant species distinction. Although it is indicated that the primary differences between the two organisms are that of gelatin liquefaction and peptonization of milk, it is interesting to note that strains of *S. liquefaciens* isolated from forages varied from *S. faecalis* in that they consistently fermented melezitose and sodium hippurate. Harrison and Hansen (1950a) also showed this distinction with regard to the fermentation of melezitose. They did not, however, test the ability of their strains to hydrolyze sodium hippurate.

A comparison of the data obtained in this study with the detailed work of Hacker and Pederson (1930) on the genus *Leuconostoc* showed that strains of *L. mesenteroides* from forage are identical to theirs in most respects. The forage strains differed mainly in their ability to produce catalase. Members of the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* have been shown to possess the enzyme catalase (Harrison and Hansen, 1950a; Felton et al., 1953; Dacre and Sharpe, 1956; Vankova, 1957). Since this property has become so common among the facultatively anaerobic lactic acid producing bacteria, it is doubtful that it should be used as one of the main lines of identification and classification. Harrison and Hansen (1954) in reference to the genus *Lacto-
bacillus stated that the presence of catalase excludes any member from this group. By the same token this should also apply to the genus Leuconostoc. If taxonomists agree with this opinion then it will be necessary to remove the catalase producing strains to new and different genera. If, however, the decision is made to maintain the organisms in their present taxonomic positions, it will be necessary to change the genera definitions to include strains which are able to produce catalase.

The evidence is clear that Leuconostoc, type I, should be regarded as a variable of Leuconostoc mesenteroides. This is obvious not only from the standpoint of glucose fermentation and by-product distribution but also from reactions in different carbohydrates. The sucrose fermenting, nondextran producing strains of Leuconostoc and Lactobacillus studied by Pederson and Albury (1955) gave submaximal amounts of acid in glucose broth (average of about 0.24 per cent). Some of the forage strains also produced low acid in glucose but the range was from 0.23 to 0.77 per cent. It was interesting to note that when their strains recovered the ability to produce dextran that they were also able to utilize certain sugars that they had not previously fermented. They suggested that the changes observed in variants may involve dormant or latent enzyme formation, spontaneous mutation or a combination of the two systems.

Other investigators have also observed variations in dextran production among the Leuconostoc and Lactobacillus. Niven et al. (1949) studied the lactic acid bacteria which caused surface discoloration on sausages and found that 14 of 20 cultures of the genus Lactobacillus studied produced large mucoid colonies on 5 per cent sucrose gelatin medium. Ten strains of Leuconostoc, on the other hand, failed to produce mucoid colonies on this same medium. Carr (1957) studied strains of lactic acid producing cocci and rods from apple juice, cider, and berries and reported that they fell into two groups which he considered belonging to the species L. mesenteroides. The groups showed variation in fermentation patterns and none of the strains produced dextran in the presence of sucrose. One strain, a rod that he designated as Lactobacillus pastorianus, was able to produce slime from sucrose.

Other workers (Ward, 1892; Perquin, 1940; Kobayasha, 1944; Deibel and Niven, 1959) have reported incidence of lactobacilli that produced mucoid colonies from sucrose. Continued reports of lactobacilli that synthesize dextran from sucrose have narrowed the taxonomic differences between the genera Lactobacillus and Leuconostoc and more work is needed to clearly define the relationships and boundaries of these groups.

Following the review and description of the genus Pediooccus by Pederson (1949), various investigators have examined his strains and have isolated and studied strains from a variety of sources. They have been found in fermenting forages, fermenting vegetables (Pederson, 1949), the rumen (Bauman and Foster, 1956), summer sausages (Deibel and Niven, 1957), the cecal feces of turkeys (Harrison and Hansen, 1950a), and in cheese (Dacre, 1958). Pederson indicated that these organisms may be important in vegetative fermentations when more is learned about their true role in nature. In view of the part played by this group in the silage fermentation, his prediction is true. They occur in fermenting forage in high numbers and because of their ability to produce and withstand high acid conditions they are undoubtedly important in the preservation process.

In Pederson's (1949) work on the genus Pediooccus, he stated they were catalase negative. Later work by Felton et al. (1953) showed that catalase was produced by these organisms when they were streaked and grown on low carbohydrate medium. Weak catalase activity in the group had been shown earlier, however, by Harrison and Hansen (1950a). Work by Jensen and Seeley (1954) further defined the group. They determined nutritional requirements of 34 strains and several physiological characteristics not determined by Pederson. Later work was presented by Dacre (1958), on a strain of Pediooccus isolated from cheddar cheese. It was concluded that the strain studied was a variant of the type species, Pediooccus cerevisiae Bakele. It was catalase negative, failed to grow in 6.5 per cent of sodium chloride, and showed variation on certain carbohydrates. This strain showed some characteristics that resembled the forage strains described in groups 4 and 5 (table 7).

With the exception of the pediococci, the cocci described in this paper occurred early in the forage fermentations, then were lost when the higher acid producing lactobacilli appeared. It was not unusual to find pediococci both early and late in the fermentation process. The effect the cocci have upon the fermentation of forage will be discussed in a subsequent paper.

Acknowledgments

The authors wish to express their appreciation to Dr. P. Arne Hansen of the University of Maryland for his helpful suggestions and critical comments on the manuscript, and to Barbara Stafford and Patricia Augustine whose technical assistance aided materially in this work.

Summary

Cultural and physiological data have been presented on the homo- and heterofermentative cocci isolated from forages.

Results obtained with cultures of Streptococcus faecalis and Streptococcus liquefaciens compared favorably with the published reports of other workers.

Leuconostoc mesenteroides and variant strains of this species were described. The strains of L. mesenteroides
examined showed only minor variations from their usual description. The most striking feature exhibited was the ability of some strains to produce catalase. Data were presented to show that atypical, sucrose fermenting, nondextran producing cocci isolated from forage were similar to *L. mesenteroides*. The nondextran producing cocci recovered their ability to produce dextran after serial transfers in enriched media.

Strains of the genus *Pediococcus* studied were divided into five groups on the basis of physiological characteristics. Although group variations were observed, it was concluded that a greater number of these organisms should be studied before specific names are applied to them.

REFERENCES


