A Study of the Microorganisms from Grass Silage

II. The Lactobacilli

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Few workers have attempted to perform a systematic study of the lactic acid bacteria in silage. As a result, the sequence changes, comparative growth rates, and the effect individual species have upon the fermentation process remain relatively obscure. Many investigators have reported that lactic acid in silage is produced mainly by *Lactobacillus plantarum*. The importance of the high acid producing lactobacilli in the fermentation process cannot be overlooked, in spite of the fact that few of these organisms are found on the fresh plant material. It should be noted, however, they usually do not become predominant until after the cocci have reached high numbers and produced considerable amounts of acids (Langston *et al.*, 1958; Langston and Bouma, 1960). This sequence change from lower acid producing cocci to higher acid producing lactobacilli is recognized in many types of fermentations.

The object of this work was to gain a better understanding of the types of lactobacilli that proliferate in silages and their contribution at different stages of the fermentation process.

Materials and Methods

Procedures and methods used in this study, unless otherwise specified, are reported in an accompanying paper (Langston and Bouma, 1960).

Results

The cultures of lactobacilli from the forage studied have been identified as (a) *Lactobacillus brevis*, (b) *Lactobacillus brevis* (variable), (c) *Lactobacillus casei* (d) *Lactobacillus casei* (variable), (e) *Lactobacillus plantarum*, (f) *Lactobacillus arabinosus* (*Lactobacillus plantarum*), and (g) branching lactobacilli.

The organisms were facultative anaerobes, gram-positive and nonsporeforming. Colonies were usually small, lenticular, and subsurface. Growth on the surface of slants was thin to moderate. None of the cultures liquefied gelatin, gave a positive Voges-Proskauer test, or produced indole. Some of the organisms (although presently excluded from the genus *Lactobacillus*) were able to reduce nitrate to nitrite and a few produced catalase.

(a) *Lactobacillus brevis*. Orla-Jensen (1919) included the lactic acid, gas producing rods in the genus *Betabacterium*. Three species were described which produced considerable fermentation products other then lactic acid. With the exception of one species, the group preferred the pentoses and usually failed to ferment salicin and the alcohols. Pederson (1938) in a later paper reviewed and studied over 300 strains of heterofermentative lactobacilli and proposed a classification for them. From this study he concluded that the gas producing lactobacilli, including intermediate strains, were closely related and proposed a key to the species based upon a combination of characters. The most important differential tests that he used for separating strains into four species were growth temperatures and action on arabinose, lactose, sucrose, and raffinose. With only minor variations, the strains to be discussed below are in close agreement with the species *L. brevis* described in *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957).

Eighty-two cultures of *L. brevis* were examined. They varied in their morphology but were usually short to medium rods with rounded ends. They occurred singly and in pairs. The usual size of the cells was about 0.7 to 0.9 μ by 1.3 to 4 μ. It was not unusual, however, to see cells 10 to 15 μ in length, and occasionally long filamentous ones were observed. All of the cultures produced CO_2 from glucose and grew at 15 C. The majority of the strains were unable to grow at 45 C and none grew at 48 C. The cultures showed little activity in litmus milk. None grew rapidly; a few curdled milk after a weeks incubation, but others failed to produce more than a slight acidity after 1 month. Nineteen of the 82 strains failed to hydrolyze arginine and 22 were not able to grow in 6.5 per cent of sodium chloride. None were motile and all cultures failed to produce catalase or reduce nitrate. The cultures gave a final pH in glucose broth ranging from 3.7 to 4.2 (avg 4.0), and titratable acidity values from 0.76 to 1.2 (avg 0.92, as per cent lactic acid).

The cultures usually fermented arabinose, xylose, ribose, glucose, fructose, galactose, maltose, melibiose, esculin, and mannitol; the majority failed to ferment mannose, lactose, sucrose, raffinose, melezitose, and salicin; none fermented rhamnose, sorbose, trehalose,

TABLE 1

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

Substrate	*Lactobacillus brevis*			*Lactobacillus brevis* (Variable)			Basal Medium pH
	Culture reaction	pH		Culture reaction	pH		
		Range	Avg		Range	Avg	
Arabinose	3−; 79+	3.6–5.9	4.2	11−; 4+	4.1–4.6	4.4	6.4
Xylose	82+	3.9–5.8	4.3	15+	4.2–4.7	4.5	6.4
Ribose	2−; 80+	3.9–4.8	4.3	6−; 9+	4.1–4.9	4.7	5.4
Glucose	82+	3.9–5.2	4.3	15+	4.0–4.4	4.3	6.7
Fructose	1−; 81+	4.3–5.3	4.5	15+	4.3–4.5	4.4	6.6
Mannose	75−; 7+	4.4–5.7	5.0	15+	4.1–4.6	4.4	6.8
Galactose	1−; 81+	3.9–5.5	4.4	15+	4.1–4.8	4.5	6.7
Maltose	1−; 81+	3.9–5.9	4.3	15+	3.9–5.2	4.4	6.8
Lactose	63−; 19+	4.0–5.8	5.1	6−; 9+	4.3–5.6	5.0	6.9
Sucrose	68−; 14+	4.0–5.3	4.4	15+	4.1–4.5	4.4	7.0
Trehalose	82−			9−; 6+	4.2–4.5	4.3	7.0
Cellobiose	82−			15+	4.1–4.9	4.5	6.9
Melibiose	4−; 78+	4.1–5.5	4.6	14−; 1+		4.3	6.9
Raffinose	74−; 8+	4.3–4.8	4.6	14−; 1+		4.2	7.0
Melezitose	73−; 9+	4.1–5.4	4.4	15−			7.0
Salicin	78−; 4+	4.4–5.5	5.2	15+	4.4–5.1	4.6	7.0
Esculin	12−; 70+			1−; 14+			
Mannitol	32−; 50+	4.6–5.9	5.4	14−; 1+		4.9	7.0

None of the strains fermented rhamnose, sorbose, starch, dextrin, inulin, sodium hippurate, glycerol, inositol, sorbitol, or sodium lactate.

cellobiose, starch, dextrin, inulin, sodium hippurate, glycerol, inositol, sorbitol, or sodium lactate (table 1). The by-products of glucose fermentation were similar (table 2). The organisms produced inactive lactic acid ranging from 40.0 to 45.8 per cent, and acetic acid ranging from 4.4 to 7.6 per cent of the glucose fermented. Traces of propionic, formic, and succinic acids were also observed.

(b) *Lactobacillus brevis* (variable). The atypical strains of gas producing lactobacilli showed considerable variation in morphology. They usually occurred singly and in pairs with round to tapered ends. Cultures grown in stabs were shorter and more coccoid than those grown in liquid broth. Size of the individual cells varied from about 0.6 to 0.8 μ by 0.9 to 3 or 4 μ. Some of the cells had a tendency to bend and occasionally cells were observed that had swollen ends. All of the cultures produced CO_2 from glucose, grew at 15 C, and in 6.5 per cent of sodium chloride. None were motile, produced catalase, or reduced nitrate. Three of the cultures grew well and eight gave slight growth at 45 C. The majority of the cultures showed no change in litmus milk. Two of the 15 strains failed to hydrolyze arginine.

Final pH values in glucose broth ranged from 4.1 to 4.3 (avg 4.2) and per cent titratable acidity values from 0.46 to 0.66 (avg 0.54). The majority of the cultures formed acid in xylose, ribose, glucose, fructose, mannose, galactose, maltose, lactose, sucrose, cellobiose, salicin, and esculin; most failed to ferment arabinose, trehalose, melibiose, raffinose, or mannitol; and none fermented rhamnose, sorbose, melezitose, starch, dextrin, inulin, sodium hippurate, glycerol, inositol, or sodium lactate (table 1).

TABLE 2

Per cent acids and optical type of lactic acid produced by Lactobacillus brevis and Lactobacillus brevis (variable)

Culture No.	Per Cent Glucose Fermented	Per Cent Fermented Glucose Converted to						Optical Type of Lactic Acid Produced
		Butyric acid	Propionic acid	Acetic acid	Formic acid	Succinic acid	Lactic acid	
		Lactobacillus brevis						
T-64	56.2	0	0	5.2	0.1	0.6	40.0	Inactive
T-516	57.8	0	0.07	6.5	0.3	1.0	43.0	Inactive
T-548	56.7	0	0	5.9	0.06	0.5	42.0	Inactive
T-1041	48.5	0	0.07	4.4	0.2	1.0	44.3	Inactive
T-1589	61.4	0	0.1	4.9	0.05	0.3	42.6	Inactive
T-1948	51.2	0	0.07	7.6	0.09	0.6	41.4	Inactive
R-379	74.4	0	0	6.1	0.08	0.5	45.8	Inactive
R-589	63.3	0	0.06	4.4	0.08	0.3	43.8	Inactive
		Lactobacillus brevis (variable)						
T-322	51.2	0	0.04	3.7	0.1	0.4	30.1	Inactive
T-1563	66.2	0	0.1	2.5	0	0.3	45.3	Inactive
T-1812	45.4	0	0	3.3	0	0.1	44.6	Inactive
T-1849	55.8	0	0.2	5.6	0.2	1.0	47.0	Inactive
R-915	51.2	0	0.08	3.3	0.2	0.4	41.7	Inactive

Limited amounts of inactive lactic acid were formed (30.1 to 47.0 per cent) along with acetic and traces of other acids (table 2).

When these organisms were first streaked on 10 per cent sucrose agar, two strains gave isolated colonies that

were slightly raised and shiny. Because of the reactions observed with atypical *Leuconostoc* (Langston and Bouma, 1960) and the fact that they were induced to produce dextran following serial transfers in enriched media, it was decided to subject the 15 organisms in this group to similar treatments. After 8 transfers in tomato juice glucose broth, 8 of the 15 strains produced dextran. The other 7 strains produced dextran after 14 further transfers in orange juice glucose broth.

(c) *Lactobacillus casei.* Tittsler *et al.* (1947) made an extensive study on about 200 strains of homofermentative lactobacilli which comprised most of the species. They proposed a key to differentiate the species in the genus based on certain cultural and physiological characteristics. Two types of *L. casei* were recognized based on growth temperature and certain carbohydrate fermentations. Their results correlated well with Orla-Jensen's (1919) earlier work who also described two types. His separation was based on the fermentation of rhamnose and growth at 45 C.

The strains of *L. casei* isolated from silage did not always meet the exact requirements of the species described by the above authors.

The cultures occurred as short or long rods, singly, in pairs, and sometimes in chains. The usual cell size varied from 0.6 to 0.8 μ by about 1.2 to 7 or 8 μ. Many cultures exhibited cells up to 40 or 50 μ in length and occasionally strains were observed that had long interwinding cells. In older cells the staining was uneven and showed granulation. Gram-negative elements were also in evidence.

All of the cultures grew at 15 C and 45 C, in 6.5 per cent of sodium chloride, and gave a reduced, acid curdled reaction in litmus milk. The coagulation of the milk usually occurred after an incubation period of 1 week or longer. Final pH and per cent titratable acidity values in skimmed milk ranged from 4.0 to 4.6 (avg 4.3) and 0.52 to 0.86 (avg 0.70), respectively. Final pH values in glucose broth ranged from 3.5 to 3.7 (avg 3.6). Per cent titratable acidity values in glucose broth ranged from 1.3 to 1.8 (avg 1.6). Eighteen of the 22 cultures produced ammonia from arginine and two of the cultures were able to reduce nitrate. It should be mentioned, however, that one of the two cultures lost its ability to reduce nitrate when tested after being held in stock medium for a period of about 3 years. None of the strains exhibited motility, produced CO_2, or grew at 48 C.

The majority of the strains fermented glucose, fructose, mannose, galactose, maltose, lactose, sucrose, cellobiose, salicin, esculin, and sodium hippurate. Variations were observed but most of the strains failed to

TABLE 3

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

Substrate	*Lactobacillus casei*			*Lactobacillus casei* (Variable)			Basal Medium pH
	Culture reaction	pH		Culture reaction	pH		
		Range	Avg		Range	Avg	
Arabinose	22−			39−; 2+	4.2–4.3	4.3	6.4
Xylose	21−; 1+		4.7	38−; 3+	4.0–4.5	4.2	6.4
Ribose	20−; 2+	4.2–4.9	4.6	12−; 29+	4.0–4.8	4.3	5.4
Rhamnose	20−; 2+	5.6–5.7	5.7	29−; 12+	3.9–5.7	4.9	6.7
Glucose	22+	3.8–4.8	4.3	41+	3.8–4.8	4.3	6.7
Fructose	22+	3.6–4.7	4.2	41+	3.8–5.0	4.3	6.6
Mannose	22+	3.7–4.9	4.1	41+	3.5–5.6	4.1	6.8
Galactose	22+	3.7–5.6	4.4	41+	3.7–5.6	4.4	6.7
Sorbose	21−; 1+		4.1	41−			6.5
Maltose	1−; 21+	3.7–5.1	4.4	4−; 37+	3.7–5.6	4.5	6.8
Lactose	22+	3.8–5.9	5.0	29−; 12+	4.1–4.9	4.6	6.9
Sucrose	22+	3.9–5.7	5.1	16−; 25+	3.9–5.7	5.0	7.0
Trehalose	16−; 6+	3.8–5.8	4.9	4−; 37+	3.8–5.8	4.5	7.0
Cellobiose	1−; 21+	3.8–5.0	4.4	1−; 40+	3.8–5.8	4.4	6.9
Melezitose	19−; 3+	4.0 4.4	4.2	35−; 6+	4.2–5.0	4.7	7.0
Starch	21−; 1+		5.1	10−; 31+	4.7–5.6	4.8	7.1
Dextrin	21−; 1+		4.8	12−; 29+	4.6–5.3	4.9	7.0
Inulin	21−; 1+		3.8	40−; 1+		5.8	7.0
Salicin	22+	4.1–5.2	4.6	3−; 38+	4.1–5.7	4.7	7.0
Esculin	22+			41+			
Sodium hippurate	5−; 17+			41−			
Glycerol	21−; 1+		5.6	22−; 19+	4.1–5.4	4.7	7.0
Mannitol	21−; 1+		4.3	35−; 6+	4.4–5.4	4.7	7.0
Sorbitol	21−; 1+		4.6	40−; 1+		4.3	7.0

None of the strains fermented melibiose, raffinose, or inositol.

TABLE 4

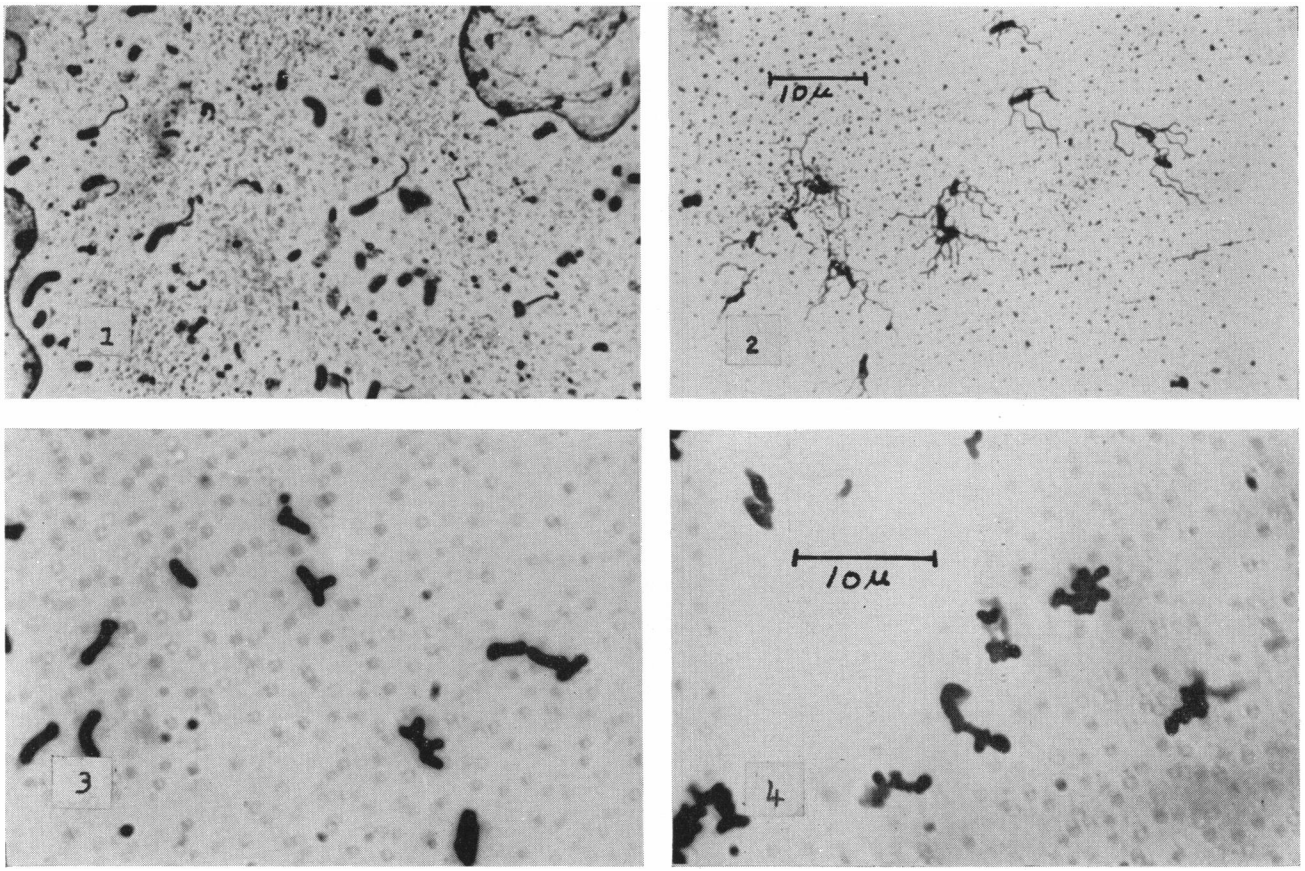
Per cent acids and optical type of lactic acid produced by Lactobacillus casei and Lactobacillus casei (variable)

Culture No.	Per Cent Glucose Fermented	Per Cent Fermented Glucose Converted to						Optical Type of Lactic Acid Produced
		Butyric acid	Propionic acid	Acetic acid	Formic acid	Succinic acid	Lactic acid	
		Lactobacillus casei						
T-2126	96.7	0	0.4	2.3	0.3	2.0	86.3	Inactive, dextro
R-443	87.1	0	0.1	1.3	0.2	2.1	87.0	Inactive, dextro
R-841	84.4	0	1.0	1.2	0.3	0.5	90.2	Dextro
T-1194	96.3	0	0.1	2.2	0.04	0.3	88.3	Dextro
R-903	88.5	0	0.8	1.3	0.4	1.3	88.9	Dextro
		Lactobacillus casei (variable)						
T-2119	50.0	0	0	0.9	0	0	76.3	Dextro
T-741	97.5	0	0	1.3	0	0.1	77.2	Dextro
R-198	97.6	0	0.07	2.4	0.07	2.0	65.7	Dextro
R-906	70.0	0	0.1	1.4	0.08	0.5	54.8	Dextro
R-1002	79.5	0	0.3	2.6	0.06	0.4	71.7	Inactive, dextro
T-125	52.8	0	0.3	2.7	0.3	2.8	75.4	Inactive, dextro
T-1963	64.0	0	0.4	2.7	0.3	0.9	88.8	Dextro

ferment arabinose, xylose, ribose, rhamnose, sorbose, trehalose, melezitose, starch, dextrin, inulin, glycerol, mannitol, and sorbitol. None fermented melibiose, raffinose, or inositol (table 3). The cultures produced mostly lactic acid from the glucose fermented (table 4). Small amounts of acetic and other acids were also found. The optical type of lactic acid produced was usually of the dextro- form. Two cultures (Strain T-2126 and Strain R-443) produced a mixture of D- and L-forms on the first crystallization of the zinc lactate, however, the second and third crystalizations gave principally the dextro-form. The main variation of the silage strains as compared to the work of other investigators was in the fermentation of certain substrates, namely trehalose, mannitol, and esculin.

(d) *Lactobacillus casei* (variable). A relatively large number of cultures were isolated from forages that were closely enough related to *L. casei* to include them in this group as variables. They did have some characteristics, however, that resemble *L. plantarum*.

The morphological characteristics of the variable *L. casei* strains were similar to the ones described for *L. casei*.



Figures 1 to 4. Figures 1 and 2, Leifson's stain, 8- to 12-hr cultures grown in tomato juice glucose broth. Figures 3 and 4, Gram stain, cultures grown in tomato juice glucose broth. *Figure 1. Lactobacillus casei* (variable), monotrichous flagella. *Figure 2. Lactobacillus casei* (variable), peritrichous flagella. *Figure 3.* Branching lactobacilli (44 hr old). *Figure 4.* Branching lactobacilli (1 month old).

All but 1 of the 41 cultures grew at 15 C, 25 grew at 45 C, and 1 at 48 C. They showed little change in litmus milk, the majority produced a slight acid reaction while only nine were able to curdle the milk. Most of the cultures were able to grow in 6.5 per cent of sodium chloride. Two of the strains reduced nitrate and none were able to hydrolyze arginine. In glucose broth the final pH and per cent titratable acidity values ranged from 3.6 to 4.2 (avg 3.9) and 0.71 to 1.4 (avg 0.98), respectively. None produced CO_2 or catalase. Twelve of the cultures were motile (figures 1 and 2). Some of the cells contained a single flagellum, others two or three lophotrichous flagella but the majority possessed peritrichous flagella.

Table 3 shows the carbohydrate fermentation reac tions of the variable *L. casei* group. Many of the carbohydrates fermented were similar to the ones fermented by *L. casei* although differences were observed in some substrates. These included ribose, rhamnose, lactose, sucrose, trehalose, starch, dextrin, sodium hippurate, and glycerol. Minor variations were observed among some of the other substrates.

The variable strains produced primarily lactic acid with some acetic and traces of other acids (table 4). Two strains (R-198 and R-906) produced abnormally low amounts of lactic acid from the glucose fermented. The values were much lower than would normally be expected from homofermentative lactobacilli. No attempt was made to determine products other than the ones listed. The strains usually produced the dextroform of lactic acid. Some produced inactive lactic acid with an excess of dextro- and a few produced principally inactive lactic acid.

Orla-Jensen (1919) indicated that there was close resemblance between his *Streptobacterium casei* (*L. casei*) and *Streptobacterium plantarum* (*L. plantarum*). In a later publication, Orla-Jensen (1943) described the species more clearly and showed that *L. plantarum* always fermented melibiose and *L. casei* failed to ferment this compound.

The fact that our variable strains failed to ferment melibiose, usually did not ferment the pentoses, and produced mostly dextro-lactic acid would place them with the *L. casei* group. However, because they produced submaximal amounts of acid from glucose (pH 3.6 to 4.2, per cent titratable acidity 0.71 to 1.4), fermented starch and dextrin, failed to hydrolyze arginine, and occasionally produced inactive lactic acid suggests that they would be related to *L. plantarum*.

(e) *Lactobacillus plantarum*. The largest percentage

TABLE 5

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

Substrate	*Lactobacillus plantarum*			*Lactobacillus arabinosus*			Basal Medium pH
	Culture reaction	pH		Culture reaction	pH		
		Range	Avg		Range	Avg	
Arabinose	40−; 62+	3.8–5.7	4.2	12+	3.9–4.5	4.2	6.4
Xylose	90−; 12+	4.1–5.1	4.4	12−			6.4
Ribose	3−; 99+	4.0–4.7	4.2	12+	4.0–4.2	4.1	5.4
Rhamnose	32−; 70+	3.6–5.6	4.9	12+	4.6–5.9	5.0	6.7
Glucose	102+	3.5–4.4	3.9	12+	3.6–4.0	3.8	6.7
Fructose	1−; 101+	3.7–4.8	3.9	12+	3.7–4.1	3.9	6.6
Mannose	1−; 101+	3.5–4.3	3.8	12+	3.7–4.0	3.8	6.8
Galactose	102+	3.5–4.4	3.9	12+	3.8–4.1	3.9	6.7
Sorbose	102−			11−; 1+		3.9	6.5
Maltose	5−; 97+	3.5–4.4	3.8	12+	3.6–3.8	3.8	6.8
Lactose	4−; 98+	3.6–4.8	4.0	12+	3.7–4.0	3.9	6.9
Sucrose	1−; 101+	3.6–4.6	4.0	12+	3.9–4.0	3.9	7.0
Trehalose	2−; 100+	3.6–5.2	3.9	12+	3.74–.0	3.9	7.0
Cellobiose	1−; 101+	3.6–5.8	3.9	12+	3.6–3.8	3.8	6.9
Melibiose	102+	3.7–4.4	4.0	12+	3.8–4.2	4.0	6.9
Raffinose	7−; 95+	3.7–4.4	4.0	12+	3.7–4.1	4.0	7.0
Melezitose	16−; 86+	3.8–4.9	4.0	12+	3.9–4.2	4.0	7.0
Starch	9−; 93+	4.8–5.8	5.3	12+	4.9–5.7	5.2	7.1
Dextrin	9−; 93+	4.8–5.7	5.1	12+	4.8–5.2	5.0	7.0
Inulin	97−; 5+	4.1–4.9	4.5	12−			7.0
Salicin	1−; 101+	3.5–5.0	4.1	12+	3.6–4.1	4.0	7.0
Esculin	102+			12+			
Sodium hippurate	94−; 8+			12+			
Glycerol	10−; 92+	4.3–5.8	5.2	12+	5.0–5.7	5.2	7.0
Mannitol	7−; 95+	3.8–4.3	4.1	12+	4.0–5.1	4.1	7.0
Inositol	101−; 1+		5.7	12−			7.0
Sorbitol	17−; 85+	3.6–4.5	4.0	2−; 10+	3.8–4.1	4.0	7.0

of organisms isolated from the forages belonged to the species *L. plantarum*. Generally, they were uniform in size ranging from about 0.7 to 0.9 μ by 1.3 to 8 μ. It was not unusual to see longer rods up to 15 to 20 μ in length and, occasionally, filamentous ones were observed. They usually occurred with rounded ends, singly, and sometimes in pairs and chains. Some cultures showed cells that tended to bend or even form ringlets.

None of the cultures produced CO_2, exhibited motility, hydrolyzed arginine, or grew at 48 C. Twenty-five of the strains reduced nitrate, three produced catalase, the majority grew at 15 C and in 6.5 per cent of sodium chloride, and about 50 per cent grew at 45 C. Most of the cultures gave a reduced, acid, curdled reaction in litmus milk. Reduction of litmus occurred in many cultures after 24 hr incubation. This varied, but sometimes up to one-half of the tube would be reduced. Only slight amounts of acid were observed in litmus milk at 24 and 48 hr. Some of the cultures were able to curdle the milk at 1 week and practically all at 1 month.

Final pH and per cent titratable acidity values in glucose broth ranged from 3.5 to 4.2 (avg 3.7) and 0.77 to 1.4 (avg 1.2), respectively. Similar values in skim milk ranged from 3.9 to 4.8 (avg 4.3) and 0.44 to 0.93 (avg 0.80).

Results from carbohydrate fermentations conformed closely to the reports of other investigators. The majority of the strains fermented ribose, glucose, fructose, mannose, galactose, maltose, lactose, sucrose, trehalose, cellobiose, melibiose, raffinose, starch, dextrin, salicin, esculin, glycerol, mannitol, and to a lesser extent, arabinose, rhamnose, melezitose, and sorbitol. Variations were observed but most of the strains failed to ferment xylose, sorbose, inulin, sodium hippurate, or inositol (table 5). The cultures produced inactive lactic acid and in some cases an excess of dextro-lactic acid (table 6). Some acetic and traces of other acids were also produced by these cultures. It is interesting to note the effect additions of tomato juice to medium had on the utilization of glucose. As mentioned in the experimental methods, tomato juice was not added except when cultures produced submaximal amounts of acids. The effect tomato juice had in stimulating cultures is shown in table 6. *Lactobacillus plantarum* (T-126) and *L. arabinosus* (T-253) were grown in medium containing tomato juice. The other cultures listed were grown in medium with no tomato juice added.

(f) *Lactobacillus arabinosus*. Fred *et al.* (1921) studied the pentose destroying bacteria from corn silage and sauerkraut. They identified and designated two new species: *L. arabinosus* n.sp. which fermented arabinose, lactose, and melezitose, but did not ferment xylose or dulcitol; and *Lactobacillus pentosus* n. sp. which fermented arabinose, xylose, and lactose, but failed to ferment melezitose, and varied on dulcitol. According to Pederson (1936) these are synonyms of *L. plantarum*.

Later work by Tittsler *et al.* (1947) showed some rather distinctive differences between *L. arabinosus* and *L. plantarum*, and Rogosa *et al.* (1953) concluded from their studies that *L. arabinosus* should be considered as a distinct species.

The strains we have designated as *L. arabinosus* are closely related to *L. plantarum*. Although characteristics were usually similar, some variations were observed which as a matter of clarity should be pointed out. *Lactobacillus arabinosus* always fermented arabinose, melezitose, esculin, and sodium hippurate and failed to ferment xylose. The majority of the *L. plantarum* strains failed to ferment arabinose and sodium hippurate, and some were not able to ferment melezitose. Rogosa *et al.* (1953) separated these groups nutritionally on the basis of esculin and hippurate hydrolysis and in their ability to ferment α-methyl-D-mannoside, arabinose, and melezitose. However, their strains of *L. plantarum*, like ours, also showed some variations in the fermentation of arabinose and melezitose, and hydrolysis of hippurate. Fermentation products of *L. arabinosus* were similar to those of *L. plantarum* (table 6).

(g) Branching lactobacilli. The last group of organisms to be discussed and probably the most unique ones isolated from silages exhibited the ability to branch. These organisms were first observed when checking the purity of fermentation flasks that had incubated for 2 weeks. The strains that exhibited branching properties were initially described as pleomorphic rods, occurring singly, in pairs, and occasionally short chains. Many cells had pointed ends, were curved, and club shapes were observed. The organisms were usually fatter than the other homofermentative lactobacilli studied. They varied from about 0.8 to 1.0 μ by 1.2 to 10 or 12 μ. When the cultures were transferred daily in tomato juice glucose broth, they showed little, if any, true

TABLE 6

Per cent acids and optical type of lactic acid produced by Lactobacillus plantarum and Lactobacillus arabinosus

Culture No.	Per Cent Glucose Fermented	Per Cent Fermented Glucose Converted to						Optical Type of Lactic Acid Produced
		Butyric acid	Propionic acid	Acetic acid	Formic acid	Succinic acid	Lactic acid	
		Lactobacillus plantarum						
T-126	87.4	0	0.2	0.6	0.01	0.7	87.7	Inactive
T-666	39.0	0	0.5	1.2	0.1	1.0	79.0	Inactive
T-645	37.0	0	0.4	0.5	0.06	0.8	85.1	Inactive
T-250	23.0	0	0	1.8	0.5	0	87.2	Inactive
T-1819	32.0	0	0	0.2	0.1	1.0	88.9	Inactive
T-1833	48.0	0	0	3.1	0	0.2	83.6	Inactive
T-2341	36.0	0	0.4	1.2	0.1	1.0	84.9	Inactive
		Lactobacillus arabinosus						
T-253	96.4	0	0.7	1.4	0.9	0.9	82.8	Inactive
R-614	30.0	0	0.2	0.8	0.08	0	72.0	Inactive

lateral branching, although some Y and T shapes occurred and an oaccasional cell had knobby protrusions which indicated the early phase of branching. At 44 hr (figure 3) the cells were swollen and exhibited Y, T, and bone shapes along with some that gave lateral branches. When the cultures were examined after 1 month incubation, the majority of the cells showed some lateral branching and many bizzare shapes occurred (figure 4). In many branched forms no septae were demonstrated by cell wall stains indicating that true branching was occurring.

Morphologically the silage branching cultures are similar to *Lactobacillus bifidus*. However, their physiological characteristics vary considerably. On primary isolation, the strains were facultative anaerobes in respect to their oxygen requirements. They were nonmotile, did not produce catalase, reduce nitrate or hydrolyze arginine. All of the cultures grew at 15 C, usually at 45 C, and in 6.5 per cent of sodium chloride. The majority of the cultures produced little if any acid in litmus milk.

Final pH in glucose broth and per cent titratable acidity values ranged from 3.8 to 4.0 (avg 3.9) and 0.92 to 1.0 (avg 0.98), respectively.

Most of the cultures (table 7) fermented rhamnose, glucose, fructose, mannose, galactose, maltose, mannitol, and sorbitol; they varied on sucrose, melibiose, raffinose, salicin, and esculin; the majority failed to ferment arabinose, xylose, ribose, lactose, trehalose, or cellobiose; and none fermented sorbose, melizitose, starch, dextrin, inulin, sodium hippurate, glycerol, or inositol.

All strains tested (table 8) produced primarily inactive lactic acid, some acetic, and traces of other acids. Taxonomically these organisms fit into the group of homofermentative, high acid producing lactobacilli.

TABLE 7

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

Substrate	Branching Lactobacilli			Basal Medium pH
	Culture reaction	pH		
		Range	Avg	
Arabinose	8−; 1+		5.4	6.4
Xylose	8−; 1+		4.9	6.4
Ribose	7−; 2+	4.4–4.6	4.5	5.4
Rhamnose	9+	4.1–5.0	4.7	6.7
Glucose	9+	3.9–4.5	4.1	6.7
Fructose	9+	4.1–4.2	4.2	6.6
Mannose	9+	3.7–4.1	4.0	6.8
Galactose	9+	4.1–4.6	4.3	6.7
Maltose	9+	4.0–4.4	4.2	6.8
Lactose	7−; 2+	4.2–4.4	4.3	6.9
Sucrose	3−; 6+	4.1–4.5	4.3	7.0
Trehalose	8−; 1+		5.1	7.0
Cellobiose	7−; 2+	3.9–4.2	4.0	6.9
Melibiose	4−; 5+	4.2–5.5	4.5	6.9
Raffinose	5−; 4+	4.2–4.3	4.3	7.0
Salicin	4−; 5+	4.2–4.4	4.3	7.0
Esculin	5−; 4+			
Mannitol	9+	4.2–4.6	4.4	7.0
Sorbitol	1−; 8+	4.0–4.8	4.4	7.0

None of the strains fermented sorbose, melezitose, starch, dextrin, inulin, sodium hippurate, glycerol, or inositol.

TABLE 8

Per cent acids and optical type of lactic acid produced by branching lactobacilli

Culture No.	Per Cent Glucose Fermented	Per Cent Fermented Glucose Converted to						Optical Type of Lactic Acid Produced
		Butyric acid	Propionic acid	Acetic acid	Formic acid	Succinic acid	Lactic acid	
T-1162	77.6	0	0.2	1.8	0.5	0	87.2	Inactive
T-1509	31.0	0	0.3	2.6	0.2	1.3	76.8	Inactive
T-388	88.4	0	0.2	1.0	0.1	0.4	88.5	Inactive

Discussion

The detailed study reported here on the microorganisms in forage has revealed that a variety of lactobacilli may be important in the silage fermentation process. Many of the species conformed closely to the usual descriptions reported for them, but a small group showed rather wide variations. In describing and attempting to place the organisms in a species, emphasis was placed on over-all characteristics. Failure to ferment a particular carbohydrate or minor variability in other physiological reactions did not necessarily delete an organism from a recognized or established group. Finer divisions within groups were possible in some cases, especially with regard to carbohydrate fermentations. However, the authors preferred to consider many of these differences as normal variations and did not propose subgroups unless it was justified on the basis of over-all reactions.

The principal heterofermentative lactobacilli isolated from forages were species of *L. brevis*.

Although some strain variations occurred, the results compared favorably with those obtained by Orla-Jensen (1919), Pederson (1938), Rogosa *et al.* (1953), and Mann and Oxford (1954). Most investigators have found that these organisms produce a vigorous fermentation of arabinose. Our results are consistent with this finding. The lowest average pH in the carbohydrates studied was in arabinose. Our cultures, like the ones studied by Mann and Oxford (1954), differed from those of Rogosa *et al.* (1953) in that they failed to hydrolyze sodium hippurate. Some of the silage strains also failed to hydrolyze arginine. Briggs (1953) and Niven *et al.* (1949) have also studied heterofermentative lactobacilli that failed to hydrolyze this compound. Although strain variations occurred, the fact that the organisms adhered to the usual carbohydrate pattern, produced CO_2, limited amounts of inactive

lactic acid, and gave a vigorous fermentation of arabinose justifies their inclusion in the species *L. brevis*.

Besides producing dextran, the variable strains of heterofermentative lactobacilli studied differed from *L. brevis* in that they always fermented sucrose, mannose, cellobiose, and salicin and usually did not ferment arabinose, melibiose, or mannitol. The failure of these organisms to ferment arabinose and their ability to ferment cellobiose and salicin is not suggestive of the heterofermentative lactobacilli. In spite of these differences, other characteristics indicate a close relationship to *L. brevis*. Niven *et al.* (1949) have studied dextran producing lactobacilli from sausages. They felt that their strains were more closely related to *L. brevis* than any of the other described heterofermentative species. The cultures exhibited an unusual fermentation pattern in that only glucose, fructose, mannose, and maltose were fermented by all of the strains. In a later paper Niven and Evans (1957) applied the name *Lactobacillus viridescens* n. sp. to organisms in this group. They differed from the silage strains in that they failed to ferment xylose or galactose and did not hydrolyze esculin or arginine. They also gave a higher final pH value in glucose broth.

With the exception of dextran production and variation on certain carbohydrates, the silage strains resemble the heterofermentative cultures studied by Rogosa *et al.* (1953) and designated as a new species, *Lactobacillus cellobiosus* n. sp. Like their strains, our strains fermented cellobiose and sometimes trehalose, they differed in that our strains usually did not ferment arabinose, melibiose, raffinose, or esculin.

Pederson and Albury (1955) showed that by serial transfers in tomato and orange juice media, nondextran producing strains of *Leuconostoc* could be adapted to produce dextran. They reasoned that, since some heterfermentative lactobacilli had been recorded as dextran producers, all might also be potential producers of this material. They examined 14 strains of lactobacilli obtained from different sources and found that, after 16 serial transfers in orange juice, followed by 8 transfers in tomato juice, all were capable of dextran production.

The production of dextran by lactobacilli has also been recognized by other workers: Olsen (1948), Kobayasha (1944), and recently by Deibel and Niven (1959), who studied homofermentative lactobacilli from commercial ham brines which synthesized a polysaccharide from sucrose.

The literature reveals that much confusion has existed in the identification of *L. casei* and *L. plantarum*. Certain overlapping characteristics have presented difficulties in properly separating these species. This has been true especially in the growth temperature limits and optical type of lactic acid produced from glucose.

Our cultures of *L. casei* showed some variation in temperature growth limits from those considered to be characteristic of the group. All strains studied grew at 15 C and 45 C but not at 48 C. The strains of *L. casei* studied by Orla-Jensen (1919), Tittsler *et al.* (1947), and Rogosa *et al.* (1953) that grew at 45 C also fermented rhamnose, in fact, Rogosa *et al.* (1953) recognized a new variety (*L. casei* var. *rhamnosus*) based on these characteristics. Although our cultures grew at 45 C, only two were able to ferment rhamnose. It should also be pointed out that the final acidity values in skim milk produced by our strains were lower than those reported by other workers. Orla-Jensen (1919), Tittsler *et al.* (1947), and Rogosa *et al.* (1953) all report acidity values in skim milk of about 1.5 per cent (as lactic acid) for *L. casei*. The value of the test as a differential characteristic has been questioned by Harrison and Hansen (1950 b). Our cultures of *L. casei*, like those studied by Wheater (1955) gave about the same amount of acidity in milk as was observed for *L. plantarum*. The cultures also varied in that most of the strains did not ferment sorbose, trehalose, mannitol, and sorbitol but did hydrolyze esculin. Failure to ferment mannitol and the ability to ferment esculin throws serious doubt on the validity of the group. In fact, Rogosa *et al.* (1953) stated that in their experience with about 1000 strains of *L. casei*, none had failed to ferment mannitol. This finding is in agreement with those of other workers. Nevertheless, our findings are convincing and the evidence at hand strongly suggests their inclusion along with the *L. casei* group. The distinguishing characteristics that identified our organism as species of *L. casei* were low final pH values in glucose broth, hydrolysis of arginine and sodium hippurate, and accumulation of dextro-lactic acid. The cultures also failed to ferment the pentoses, melibiose, raffinose, rhamnose, and glycerol.

Deibel and Niven (1958) have reported on variable motile organisms isolated from ham curing brines and the surface of cured unprocessed hams. The strains possessed characteristics similar to both *L. casei* and *L. plantarum* but were distinct from either group. The variable *L. casei* organisms isolated from silages have many characteristics in common with the ones described by the above authors. Twelve of the 41 strains studied were motile and, like the cultures of Deibel and Niven, the majority of our strains gave a slight acid reaction in litmus milk, failed to hydrolyze arginine and sodium hippurate, grew in high salt concentration, and produced dextro-lactic acid. Our strains differed from theirs in that many were able to grow at 45 C and lower final pH values were obtained in glucose broth. Although strain variations occurred, the fermentation pattern exhibited by our cultures were similar to theirs in many respects. The main differences were that our strains fermented rhamnose, sometimes dextrin and starch but usually did not ferment arabinose, xylose, inulin or mannitol. Many of the characteristics de-

scribed relate our group to *L. casei*. This is especially true because they failed to ferment melibiose and frequently produced dextro-lactic acid. Their failure to attack hippurate, starch, and dextrin and occasionally producing inactive lactic acid would identify them with *L. plantarum*. It should be mentioned that motile lactobacilli have been isolated from a variety of places and within different species. The prevalence of these organisms suggests the need for a comparative study of the groups to determine if species recognition should be given them. The strains studied by Mann and Oxford (1954), Cunningham and Smith (1940), and Hays and Reister (1952) seem to be more closely related to the motile culture of *L. plantarum* isolated from turkey cecal feces by Harrison and Hansen (1950a) and designated as a new variety *L. plantarum* var. *mobilis*. The silage strains and those studied by Deibel and Niven (1958) appear to be more closely related to *L. casei*. Motility has not been restricted to the above species because recently, Vankova (1957) described a motile, catalase positive strain of *Lactobacillus delbruckii*.

Orla-Jensen (1919) showed that the plant bacteria, *Streptobacterium plantarum*, had wide fermentative abilities. His strains generally preferred maltose and sucrose to lactose and they often fermented raffinose and sometimes inulin. The fermentation of sorbitol and rhamnose was more frequent with *S. plantarum* than with *S. casei*. A number of strains of *S. plantarum* were also active in the fermentation of arabinose and occasionally xylose. The silage strains of *L. plantarum* usually met these requirements. Although little difference was noticed in the fermentation of sucrose and lactose, the organism did prefer maltose to lactose. The fact that all of our strains fermented melibiose agrees with the previous work of Orla-Jensen (1919, 1943), Tittsler *et al.* (1947) and Harrison and Hansen (1950b). The main points of disagreement lie in the growth temperature limits and hydrolysis of esculin. All of the silage strains grew at 15 C, about 50 per cent at 45 C, and all hydrolyzed esculin. This is a wider temperature range than has been reported previously and is not surprising when one considers the wide temperature ranges found in fermenting forages. The strains studied by Harrison and Hansen (1950b) grew at 45.5 C but were unable to grow at 20 C. Other workers have reported lower temperature growth limits for these organisms (around 10 C) but usually have not found the range observed with the silage strains. The fact that our strains fermented esculin would place them close to *L. arabinosus* if one uses the scheme of Rogosa *et al.* (1953). Most of our strains, however, failed to hydrolyze sodium hippurate. From the results found in tables 5 and 6 it may be seen that strains designated *L. plantarum* and *L. arabinosus* are closely related, and on the basis of the results obtained it is difficult to justify them as separate species. Rogosa *et al.* (1953) used additional tests we did not employ and which they feel are reliable enough to separate them into distinct species.

Three of the silage strains of *L. plantarum* were catalase positive and a large number of them were able to reduce nitrate to nitrite. Other characteristics were similar to those described for the genus. As mentioned earlier, these strains are now excluded from the genus *Lactobacillus*. Other workers have previously reported on organisms in this genus which reduce nitrate and produce catalase (Biocca and Seppilli, 1947; Costilow and Humphreys, 1955; Dacre and Sharpe, 1956; and Vankova, 1957). Since these organisms appear to be rather common it seems logical that the genus description should be broadened to include them.

Confusion exists concerning the proper taxonomical position of *Lactobacillus bifidus*. Most of these organisms studied have fallen into two groups designated as *L. bifidus*, type I and II, by Weiss and Rettger (1938). *Lactobacillus bifidus*, type I, on continued subculture loses its obligate anaerobic requirements and tendency to branch, whereas *L. bifidus*, type II, retains its anaerobic requirements and branching characteristics. Further division was based on the proportions and amounts of volatile and nonvolatile acids produced, the optical type of lactic acid, and the fermentation of certain carbohydrates. The bifid type (type II) produced considerably more volatile acid than the nonbifid type (type I) and the dextro-form of lactic acid was found (Orla-Jensen, 1919; Pederson, 1945; Norris *et al.*, 1950; and Harrison and Hansen, 1954). The nonbifid strains (type I) produced mostly inactive lactic acid (Weiss and Rettger, 1934; King and Rettger, 1942; and Norris *et al.*, 1950).

Carbohydrate fermentations revealed that type II, the more anaerobic cultures, fermented arabinose, xylose, and melezitose but failed to ferment mannose. Type I failed to ferment the first three carbohydrates named, but did ferment mannose.

A recent publication by Clarke (1959) describes still another group from the rumen that shows characteristics similar to both type I and II. The rumen cultures resembled type II of Weiss and Rettger (1938) in morphology, and on subculture retained their bifid forms and obligate anaerobic requirements. They differed from type II, however, in that they produced optically inactive lactic acid. The yield of volatile acids was about 40 to 50 per cent of the glucose fermented. These strains also reduced nitrate to nitrite.

The silage branching lactobacilli resemble none of the groups thus far described, and, to the knowledge of the authors, this is the first report revealing their presence in silage. Most previous investigators agree that *L. bifidus* is an obligate anaerobe upon primary isolation. The silage branching strains were facultative

anaerobes. They differed further from *L. bifidus* in that they grew at 15 C and 45 C and produced a lower final pH in glucose broth (silage branchers 3.9; *L. bifidus*, 4.5 to 5.0). It was usually true that increased branching occurred as the cells aged and, although strain variation was observed, about the same amount of branching occurred on streaked plates as in broth, semisolid deeps, or anaerobic roll tubes. When growth of the silage strains was studied, turbidimetric measurements revealed that they grew as well at 30 C as at 37 C, although a few strains preferred 30 C.

In addition to the characteristics just pointed out, it should be noted that the silage strain behaved differently in carbohydrate fermentation reactions. Both type I and II strains of *L. bifidus* fermented sucrose, trehalose, lactose, raffinose, salicin, dextrin, and starch. The silage strains either failed to ferment these compounds or showed considerable variation on them. The *L. bifidus* cultures usually do not ferment mannitol or rhamnose, whereas these substrates and sorbitol were fermented by the silage strains. Further division of *L. bifidus* type I and II has been based on the fermentation of mannose, xylose, arabinose, and melezitose. Type II usually fermented the last three compounds but did not ferment mannose. Since the silage strains usually failed to ferment xylose, arabinose, and melezitose, but did ferment mannose, they are in this respect more closely related to type I.

Weiss and Rettger (1934) made a comparative study of *L. bifidus* and *L. acidophilus* and concluded that only slight differences existed between them. They suggested that *L. bifidus* should be regarded as a variant of *L. acidophilus*. When the silage strains were compared with *L. acidophilus* it was clear that we were not working with the latter organism. This was obvious not only from temperature growth limits but also in the fermentation of carbohydrates. *Lactobacillus acidophilus* (Tittsler *et al.*, 1947; Rogosa *et al.*, 1953) usually fermented trehalose, salicin, cellobiose, lactose, and sucrose. The silage strains either failed to ferment these compounds or showed considerable variability on them. *Lactobacillus acidophilus* also failed to ferment certain compounds which were in many instances fermented by the silage organisms. These included mannitol, melibiose, raffinose, rhamnose, sorbitol, and esculin.

The authors find it difficult to properly place these organisms among the already established species of lactobacilli. The fact that these strains have the ability to branch does not necessarily place them in the same group as *L. bifidus* because other workers have shown wide variation in morphology among the lactobacilli. Harrison (1952) noted pleomorphism among strains of *Lactobacillus fermenti*. Some cells showed extreme variances in length and tiny "buds" were attached to shorter rods. In occasional instances cells did show branching. Weiss and Rettger (1934) observed branching forms of *L. acidophilus*. They found Y and T forms, as well as clubbed, nobbed, and vesicular forms. The data strongly suggest that our branching strains should be included with the high acid producing homofermentative lactobacilli. Although they do not always behave in the accepted manner or show the normal fermentation pattern, they are more closely related to *L. plantarum* and *L. casei* than any of the other groups. Because of the variations in this group and the confusion that already exists among species in the genus *Lactobacillus*, the authors hesitate to apply a specific name until more detailed comparisons are made. It should be mentioned that the branching strains are extremely stable. The carbohydrates listed in table 7 were retested after the cultures had been held in the refrigerator for about 3 years. Only one strain varied and it lost the ability to ferment salicin. Repeated tests were also made on representative strains of the other lactobacilli studied. Few showed variations when retested. Occasionally an organism lost the ability to ferment a certain compound but in most cases it had originally given a slow or weak fermentation. None of the organisms tested gained the ability to ferment a compound.

The present investigation on silage lactobacilli has served to further point out the variability among organisms of this group. Because of these results and those of other investigators on organisms in the genus *Lactobacillus* which are motile, catalase positive, nitrate positive, and dextran producers, it seems reasonable that our taxonomical viewpoints should be emended and broadened to include these strains. Generally, workers in this field are in agreement that much is lacking in our present system of classifying the lactic acid bacteria. In fact, the majority of the species described in *Bergey's Manual* are insufficient for proper classification purposes. One needs only to spend a few minutes reviewing the genus *Lactobacillus* to realize the problems involved. In the majority of cases, too few characteristics are given and much work has been based upon sugar fermentation. As pointed out earlier, the lactobacilli are relatively stable in the fermentation of carbohydrates but these reactions should be coupled with cultural and other physiological characteristics to obtain a broad spectrum of reactions. Also, too few investigators have failed to determine by-products and optical activity of the lactic acid produced.

Although about 50 different characteristics were made on each strain in this study, it was difficult in some instances to place strains in a species with complete confidence. Some strains fit none of the known groups.

No claim is made here that all of the different species of lactic acid bacteria in silage have been isolated. The ones studied were peculiar to the grass ensiled, and the types which would grow under the isolation techniques and temperatures used. It is obvious, however, from

this study that a diversity of lactic acid bacteria is responsible for production of acids in forage. A subsequent paper will deal with the sequence changes of these organisms in forage and their importance in the fermentation process.

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SUMMARY

The hetero- and homofermentative lactobacilli from forage and silage have been described. The most frequently occurring lactobacilli in this study were (a) *Lactobacillus brevis*, (b) *Lactobacillus brevis* (variable), (c) *Lactobacillus casei*, (d) *Lactobacillus casei* (variable), (e) *Lactobacillus plantarum*, (f) *Lactobacillus arabinosus* (*Lactobacillus plantarum*) and (g) branching lactobacilli.

All of the cultures were typical of lactobacilli in that they were facultative anaerobes, gram-positive, nonsporeforming, nonpigmented, and produced mainly lactic acid from glucose.

Variable strains were described which were able to synthesize dextran, produce catalase, and reduce nitrate. The authors have suggested that the present taxonomical concept of the lactobacilli be broadened to include these variable strains.

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Thermal Death Time Curve of *Mycobacterium tuberculosis* var. *bovis* in Artificially Infected Milk[1]

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An accurate estimate of the slope of the thermal death time curve of *Mycobacterium tuberculosis* var. *bovis* in milk has not been reported, and only a few attempts to construct such curves for this organism have been made (North and Park, 1927; Oldenbush *et al.*, 1930). Ball (1943) concluded that none of the thermal resistance data reported in the literature was applicable to the construction of thermal death time curves for *M. tuberculosis* in milk. A possible nonlinearity in the curve at temperatures above 67 C was indicated by Faxholm (1949).

The project reported here was undertaken to study the characteristics of the thermal death time curve of *M. tuberculosis* var. *bovis* in milk.

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EXPERIMENTAL METHODS

Three strains of *M. tuberculosis* var. *bovis* were employed, strain USDA 854, strain ATCC 11756, and strain ATCC 12621. Cultures were carried on slants of Dubos medium (Dubos and Middlebrook, 1947), and cells for the test suspensions were prepared by obtaining a submerged, dispersed growth in Dubos Tween-albumin medium (Dubos and Middlebrook, 1947). Cells were harvested by centrifugation after 8 to 10 days of incubation on a shaker bath at 37 C, washed three times in physiological saline containing 0.05 per cent Tween 80,[3] and resuspended in the same saline-Tween diluent. The extent of cell clumping was controlled by shaking with glass beads and filtration through two sheets of Schleicher[4] 597 filter paper. The degree of cell clumping was determined microscopically. Viable cell counts were performed on Dubos oleic-acid

[3] Polyoxyethylene sorbitan monooleate, General Biochemicals, Inc., Chagrin Falls, Ohio.

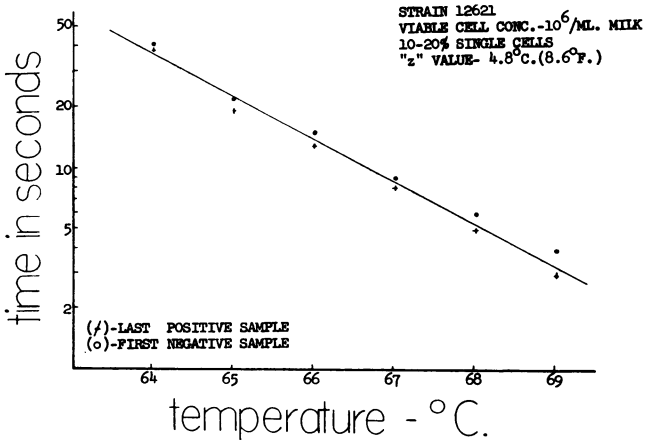

Figure 1. Thermal death time curve of *Mycobacterium tuberculosis* var. *bovis* 12621 in milk.

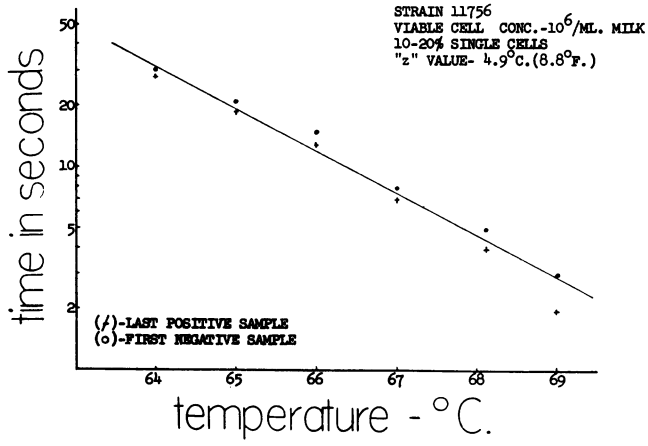

Figure 2. Thermal death time curve of *Mycobacterium tuberculosis* var. *bovis* 11756 in milk.