A COMPARATIVE STUDY OF MEMBERS OF THE LACTOBACILLUS GENUS, WITH SPECIAL EMPHASIS ON LACTOBACILLI OF SOIL AND GRAIN[1]

GEORGE A. HUNT AND LEO F. RETTGER

From the Laboratory of General Bacteriology, Yale University, New Haven, Connecticut

Received for publication March 4, 1930

Although lactic acid organisms were among the first to be studied, and though their isolation and their action on carbohydrates represent some of the first chapters in the history of bacteriology, they are still a much discussed and much disputed group. They have been described and classified in many ways by different workers. No group of lactic acid organisms shares in this controversy more than the lactobacilli.

Workers have been divided in their views. Some maintained that organisms of this genus, Lactobacillus, were not sufficiently distinct to warrant division into several species. Others have designated new species, on the basis of only very slight variations in morphology and cultural reactions.

Of the investigators who believed that the differences found are insufficient to divide the group into species, Rodella (1901 and 1908) and Heinemann and Hefferan (1909) may be cited. Each emphasized the pleomorphic nature of the Lactobacillus group and called attention to the presence of long rods and coccoid forms in the same culture. Heinemann and Hefferan concluded that these organisms had not been investigated thoroughly under uniform conditions, and that when so studied the differences were slight. They gave a composite description based upon a study of several organisms. They contended that

[1] This paper covers in part the work submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

the Bacillus of Massol, *B. acidophilus*, the Boas-Oppler bacillus, *B. panis-fermentati*, *Streptobacillus lebenis*, and *Leptothrix buccalis* were synonyms for one and the same organism, *B. bulgaricus*.

Henneberg (1904) and Rahe (1914 and 1918) held other views. Henneberg, working with twenty-two strains, made an extensive morphological and cultural study of this group. The information. contained in his orginal paper (*Zeitschrift für Spiritusindustrie*) formed the basis for many subsequent systems of classification. He divided *L. leichmanni* into 3 sub-groups according to the fermentation reactions on 14 carbohydrates.

Rahe divided his strains of *Lactobacillus acidophilus* into four sub-types, *L. acidophil-aerogenes* into eight, and *L. bulgaricus* into four sub-types. *L. bifidus* he did not subdivide, but classified as an undivided species. The differentiation was established entirely on the basis of fermentation reactions of five carbohydrates and of mannitol.

The difficulty encountered in establishing significant distinguishing characteristics is reflected in the literature in many instances. When a lactic acid organism was encountered as a contaminant in butyl alcohol and acetone fermentation, Thaysen (1921) believed it to be a new species and called it *B. volutans*. Later, work by Fred, Peterson and Stiles (1925) showed it to be a member of the Lactobacillus group and probably *L. leichmanni*.

Howe and Hatch (1917) isolated organisms of the Lactobacillus group from carious teeth and referred to them as *L. acidophilus* and *L. bifidus*. Rodriguez (1922), in a study of aciduric organisms isolated from deep foci of active dental caries, suggested that they be called *B. odontolyticus* (types I, II and III). McIntosh, James and Lazarus-Barlow (1922) found two types of organisms which they named *B. acidophilus-odontolyticus* (types I and II). Bunting, Nickerson and Hall (1926) called the organism isolated by them from caries, *B. acidophilus*. Morishita (1928, 1929) has noted that the organisms isolated by himself and others from carious teeth are, with few exceptions, not *L. acidophilus* as this species or type has been described by Kulp and Rettger (1924), and by others.

These scattered examples are but a few of many such instances.

Inasmuch as several investigations[2] made in this laboratory have dealt with organisms of the Lactobacillus genus found in the intestinal tract and in dental caries, it was thought advisable to make a study of some of the Lactobacillus organisms found in soil and grain, with particular emphasis on those which produce relatively large amounts of lactic acid from carbohydrates, and which bear such an important relation to lactic acid production as a commercial biological process.

The present paper covers one phase of an investigation conducted by the writers on *Biological Production of Lactic Acid by High Acid-producing Organisms of the Lactobacillus Genus.*[3] The 36 strains of Lactobacillus described here were obtained from the following sources: 18 isolated by the writers from soil, grain and fecal material; 9 from the American Type Culture Collection; 3 from the University of Wisconsin; 3 from Morishita's collection of dental organisms; and 3 from the laboratory stock of *L. acidophilus* and *L. bulgaricus*. A fuller account of the sources follows.

Sources of Lactobacillus strains

Numbers	*Authors' isolations*
11, 12, 13, 15	Lactobacilli isolated from drains of Yale University Armory.
23, 24, 28, 29	Lactobacilli isolated from stalls of Yale University Armory.
32, 34	Lactobacilli isolated from soil.
71, 72, 73	Lactobacilli isolated from bran.
85, 87	Lactobacilli isolated from cess pool.

[2] Rettger and Horton, (1914); Hull and Rettger, (1917); Rettger and Cheplin, (1920); Kulp and Rettger, (1924); Morishita, (1928, 1929).

[3] Work still in progress.

A7—Lactobacillus resembling A2 (see below) at time of isolation from a sample of barley. This culture was carried as a stock culture for two years before fermentation reactions described in this paper were determined.

A8—Lactobacillus resembling A4 (see below) at time of isolation from sample of corn meal. Strain carried as a stock culture for two years before fermentation reactions described in this paper were determined.

Alf—Lactobacillus isolated from alfalfa meal.

From the American Type Culture Collection:

A1—*Lactobacillus leichmanni* (368).
A2—*Lactobacillus delbrücki* (899).
A3—*Lactobacillus lycopersici* (4005).
A4—*Lactobacillus fermentatae* (4006).
A5—*Lactobacillus acidophil-aerogenes* (4007).
A6—*Lactobacillus acidophil-aerogenes* (4009).
A9—*Lactobacillus delbrücki* (4172).
A10—*Lactobacillus pentoaceticus* (367).
A11—*Lactobacillus plantari* (4377).

From Dr. Fred, University of Wisconsin:

F1—*Lactobacillus leichmanni* (isolated from corn meal).
F2—*Lactobacillus leichmanni* (isolated from corn mash).
F3—*Lactobacillus delbrücki* (Henneberg).

From Morishita, Yale University:

T1—*Lactobacillus odontolyticus* (33).
T2—*Lactobacillus odontolyticus* (19).
T3—*Lactobacillus odontolyticus* (BN).

From Yale University:

Laboratory of General Bacteriology:

RH—*Lactobacillus acidophilus*
K1—*Lactobacillus acidophilus* (Scavano).
K2—*Lactobacillus bulgaricus* (B-12).

All strains were Gram-positive rods occurring singly, in pairs or in chains. They varied greatly in size, at times even in the same culture (0.3 to 1.0 by 1.0 to 20μ). They were microaerophilic and fermented carbohydrates and alcohols with the production of lactic acid and varying amounts of acetic acid. Some of them formed two types of colonies in agar, described by Rettger and Horton (1914) as *x* and *y* types. The descriptions correspond with that given by Fred, Peterson and Stiles (1925) for lactic acid organisms isolated from soil (table 2).

ISOLATION OF LACTOBACILLUS STRAINS

The method of isolation was a modification of that used by other workers, namely the use of acid enrichment broth containing

a fermentable carbohydrate. However, in working with soil and grain as a source of organisms, much difficulty was encountered with fungi overgrowing the lactobacilli in the enrichment medium and in the plates which were subsequently poured. The following procedure was adopted with very satisfactory results.

Medium used:

Corn flour	50 grams
Peptone	10 grams
Water	1000 cc.

The mixture was boiled for fifteen minutes and adjusted to a reaction of pH 3.5 to 4.0 with lactic acid. The medium was dispensed in 10 cc. portions in test tubes, and sterilized for twenty minutes at 15 pounds extra pressure.

The tubes were inoculated with samples of grain or soil weighing about 1 gram, or with other test material in liberal amounts; a layer of sterile mineral oil 10 to 15 mm. thick was poured over the mash and the tubes incubated at 37 or 40°C. for forty-eight or seventy-two hours. Plates poured from these tubes often contained only lactobacilli in the higher dilutions. Molds were seldom seen on any of the plates.

The colonies selected for isolation were examined under the low power of the microscope and cut out of the agar by a sterile platinum spatula prepared by flattening a platinum wire slightly at one end. The agar block containing the colony was crushed in a tube of broth and immediately replated. Colonies selected from the second plating usually gave pure cultures. In some instances single cells were isolated by the improved Barber micropipette method for single cell culture study. For a full description of the technique employed here see Gee and Hunt (1928).

Several media were used for cultivation and preservation of stock strains. Yeast-water broth and agar containing glucose and peptone were found very favorable; they were prepared as follows: One pound of compressed yeast was added to 4 liters of water and suspended by shaking. Twenty-five cubic centimeters of chloroform were added to prevent bacterial growth. The

flask was incubated for forty-eight hours at 37°, the mixture being thoroughly shaken at intervals. It was then steamed in the autoclave until the chloroform had volatilized, and finally heated at 15 pounds pressure for an hour. After the yeast cells were allowed to settle, the supernatant fluid was removed, and clarified with egg albumin when this was found to be necessary. The resultant yeast water should have a clear amber or light brown color. To attain this end quickly the soluble starch which slowly precipitates and settles upon standing may be changed by the addition of a small amount of Takadiastase or filtered saliva when the yeast water is still warm.

The yeast water was made into the following medium:

Glucose	10 grams
Peptone	10 grams
Yeast water	300 cc.
Water	to 1000 cc.

This medium was adjusted to pH 6.8, tubed and sterilized in the autoclave.

Tomato juice broth as devised by Kulp (1927) was found to be almost as good as yeast water for some strains and better for others. It is prepared more easily than the other, and was used in many instances in this work to supplement the yeast water medium.

For the preservation of stock strains the following medium has many advantages:

Skimmed milk	1000 cc.
Peptone	10 grams
Glucose	10 grams
Litmus (enough to give a deep blue color).	

This was dispensed in test tubes containing about 0.5 gram of calcium carbonate per tube. The tubes were plugged and sterilized at 15 pounds for twenty minutes in the autoclave. When the seeded tubes were protected from excessive drying the organisms remained viable for at least seven months without apparent change of fermentation reactions. However, stock cultures were transferred at least once a month as a routine procedure.

With the use of the isolation technic described and with the yeast water and tomato juice media it was possible to isolate aciduric organisms of the Lactobacillus type from cracked wheat, rye meal, cracked corn, oats, corn flour, alfalfa meal, barley, bone meal, meat scraps, chicken feed and rolled oats. These samples represented grains purchased in a variety of wholesale and retail food stores and cereal mills located in the Eastern and Mid-Western sections of the United States.

Fifty samples of recently collected soil representing all but two types of surface soil found in Linn County, Iowa, were examined.[4] Organisms of the Lactobacillus group were present in all of these samples.

Samples of soil or grain kept in the laboratory in test tubes or bottles rapidly lost their viable lactobacilli. In fact, none of the fifty samples of soil contained viable lactic acid organisms of this type two months after the date of collection. However, lactobacilli were readily demonstrated in samples of grain which had been held in a feed store bin for several weeks or in a paper bag in the laboratory store room.. In such instances mouse feces were found to be present in the samples, and there was ample evidence that mice had been feeding on the grain. In one instance a bag of alfalfa meal remained on a shelf in a small store room or closet for several months during which time it was a source of food for mice, as was clearly indicated by the scattered meal on the shelf. A sample of this exposed meal revealed aciduric organisms, while a similar sample protected from mice by a wire screen showed none after weeks of storage.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

Morphological and cultural characteristics are shown in table 1. Tubes of glucose yeast-water broth were inoculated with the suspension of the washed organisms and incubated at one of four temperatures, 42, 37, 16 and 12°C., for forty-eight hours. The cultures were also plated out in glucose yeast-water agar; tubes of glucose yeast-water gelatine were inoculated and in-

[4] These were made available through the kindness of Prof. Anderson, Coe College.

TABLE 1

Giving certain morphological and cultural characteristics

CULTURE	MORPHOLOGY	COLONY FORMATION ON GLUCOSE YEAST WATER AGAR	GROWTH IN GLUCOSE YEAST WATER BROTH	GROWTH IN GLUCOSE YEAST WATER GELATIN AT 22°C.	pH AT 42° INCUBATION (GLUCOSE YEAST WATER BROTH)	pH AT 37° INCUBATION	pH AT 16° INCUBATION	pH AT 12° INCUBATION
A1	Short thick rod	x	MT.S	+	3.3	3.3	5.5	7.0
A2	Short thick rod in chains	x and y	C.S	3+	3.3	3.5	4.5	6.0
A3	Long rod single or pairs	x	MT.	+	5.5	6.0	6.0	7.0
A4	Short to long rods	y	ST.	3+	4.0	4.5	6.0	6.8
A5	Short rod in pairs	x and y	MT.S	4+	3.6	3.4	5.0	5.5
A6	Short rod in pairs	x and y	MT.S	4+	3.3	3.3	4.8	6:6
A7	Short rod streptococci-like chains	y	C.S	4+	4.4	4.6	6.0	7.0
A8	Short to long rod, single or in pairs	y	ST.S	3+	4.6	4.6	5.5	6.5
A9	Long rods, single or in pairs	x	C.S	0	4.0	4.4	7.0	7.0
A10	Very small rod, single or in pairs	y	ST	0	4.4	4.4	6.6	7.0
A11	Short rod in short chains	x	C.S	0	4.0	4.4	7.0	7.0
F1	Short to long rod	x and y	MT.S	0	4.0	4.0	7.0	7.0
F2	Short to long rod	x and y	MT.S	0	3.4	3.8	7.0	7.0
F3	Short rod in short chains	x and y	C.S	3+	3.4	3.4	4.5	5.5
Alf	Medium to short rod	y	ST	−	4.4	4.6	7.0	7.0
11	Medium rod, palisade-like growth	y	C.S	0	4.0	4.0	7.0	7.0
12	Short rod in pairs	x and y	C.S	0	3.4	3.8	7.0	7.0
13	Medium rod, palisade-like growth	y	C.S	0	3.6	4.0	7.0	7.0
15	Short rods in pairs	y	C.S	0	3.8	3.8	7.0	7.0
23	Short medium rod, occurring singly	y	C.S	0	5.0	4.6	7.0	7.0
24	Short thick rod in chains	x and y	C.S	0	4.6	4.6	7.0	7.0
28	Medium rod, palisade-like growth	x and y	C.S	0	4.6	4.4	7.0	7.0
29	Medium rod	y	C.S	0	4.0	4.0	7.0	7.0
32	Short medium rod, single or chains	y	ST	0	5.0	5.0	7.0	7.0
34	Medium rod	y	C.S	0	4.6	4.6	7.0	7.0
72	Medium rod palisade-like growth	x and y	C.S	0	3.4	3.8	7.0	7.0
85	Medium delicate rod	y	MT	0	3.4	4.2	7.0	7.0
87	Short to medium rod	y	ST.S	0	4.0	4.6	7.0	7.0

Key to table: ST = slight turbidity; MT = medium turbidity; C = clear; S = sediment.

cubated at 22°C. The acid production for the four temperatures of incubation, the type of colony in the agar, the growth in the gelatine, the appearance of growth in the broth, and the morphology of the organism found in an eighteen- to twenty-four-hour glucose yeast-water broth culture (prepared from stock cultures of the litmus milk containing glucose and calcium carbonate) are indicated in the table (1).

There were 4 *x* and 14 *y* types which remained reasonably constant, while 10 of the total number of 28 strains produced colonies which varied more or less between the *x* and *y* forms.

Nine members of the series produced visible growth in glucose yeast water gelatin at 22°C., whereas 18 refused to do so. All caused pronounced acid production in glucose yeast water broth at 37 and 42°, and 9 appreciable quantities at 16°C.; 19 failed to change the H-ion concentration of the medium at this temperature. Only three depressed the pH of the glucose broth at 12°.

Various writers have described members of the Lactobacillus genus as being extremely pleomorphic, the morphology of the individual strains varying from a distinct coccus form to long and short bacilli of extremely variable thickness. Such claims have recently been made by Rosebury, Linton and Buchbinder (1929). In some earlier publications of Rettger and his colleagues attention was called to very marked pleomorphism in the Bulgaricus-acidophilus group. More recent and intensive studies in our laboratory have shown, however, that under controlled uniform conditions of temperature and cultural environment the different strains are as a rule far less pleomorphic than has been so often claimed.

A certain amount of pleomorphism and cultural variation has been observed in the present investigation, and in some instances these seemed to vary with slight variation in environmental conditions, such as temperature, and with the intervals elapsing between transfers.

FERMENTATION STUDIES

Casein-digest broth (although not as favorable a medium for many strains of Lactobacilli as yeast water or tomato broth) was

used. Prepared from C. P. casein by the tryptic digestion method described by Kulp and Rettger (1924), it is free from carbohydrates and promotes growth of all strains in varying degress when a fermentable carbohydrate is added.

Two media were made from this for the determination of fermentation reactions. One was casein digest broth which was sterilized before the adition of the carbohydrate. The carbohydrate was added in the form of a sterile aqueous solution. The second medium was a casein-digest soft agar containing Andradè's indicator:

Casein-digest	equivalent to 10 grams casein
Peptone	10 grams
Agar	5 grams
Andrade's indicator	10 cc.
Water	to 1000 cc.

Here again the carbohydrates were added as sterile aqueous solutions after the medium had been tubed, plugged and sterilized in the autoclave. Xylose and arabinose solutions were sterilized by Berkefeld filtration; all of the others by autoclaving. The concentration of carbohydrate used was 0.5 per cent in both the broth and the soft agar media.

The inoculum was prepared from a twenty-four hours yeast water or tomato juice broth culture by centrifuging the culture, removing the supernatant fluid and washing by resuspending and centrifuging the residual cells three times in sterile saline solution (0.85 per cent). A final saline suspension was prepared having a turbidity of 8 or 10 on the McFarland nephelometer scale. Plates were then poured to determine the purity of the suspension and 0.1 cc. of the suspension inoculated into casein digest sugar broth (3 to 5 cc. volume); a stab inoculation was likewise made in Andrade soft agar medium, using a 4 mm. loopful of the suspension.

The Andrade soft agar was used in making simple fermentation tests with organisms which were supposedly of the Lactobacillus type, immediately after their isolation from the corn mash medium. The inoculum was washed in sterile saline, as described above. The carbohydrates employed in these tests were glucose, lactose,

maltose, sucrose and xylose. A carbohydrate-free control was also employed.

The casein-digest broth containing 0.5 per cent of a given carbohydrate and inoculated with the washed culture, was incubated at 37° for three days and the pH determined by the colorimetric method. A decrease in pH above any change observed in the sugar-free control was taken as an indication of carbohydrate utilization. In some instances growth took place without any decrease in the pH being observed. When possible in these cases, the reducing sugar was determined by the Somogyi (1926) modification of the Shaffer-Hartmann reagent. In no such instance did a quantitative determination of the reducing sugar reveal a loss in carbohydrate.

The following system is used in table 2 to indicate ranges of pH.

− = no change, pH 7.0
±? = doubtful (pH 6.6 to 6.7)
± = pH 6.5 to 6.1
+ = pH 6.0 to 5.6
++ = pH 5.5 to 5.1
+++ = pH 5.0 to 4.6
++++ = pH 4.5 or less.

The fermentation reactions were obtained in casein digest broth containing 0.5 per cent of the known carbohydrates and inoculated with washed cultures of the various strains.

An examination of the reactions in table 2 reveals several points of interest. A2, A5, A6, A7 and F3 resemble each other, although they are not identical. For instance, A2 and F3 slightly affect sucrose, while the others do not. F3 is the only one of these five strains which attacks dextrin, though this action is again slight. A2 and F3 act on mannitol more readily than do A5, A6 and A7. A5 and A6 are the only strains of these five which produce a visible change in milk.

A8 and A10 have some resemblences. A10 ferments raffinose, which is not acted upon by A8, but the latter ferments mannitol, salicin and milk, while A10 does not.

Although A1, F1 and F2 are all *L. leichmanni* strains, A1 differs from F1 and F2 in that it acts on lactose and salicin, as

well as on milk, although slowly. Except for the amount of acid produced, F1 and F2 are alike in their action.

A2, A9 and F3 are all *L. delbrücki* cultures, and although A2 and F3 resemble each other quite closely, A9 is different. A9 acts on lactose slightly, but does not ferment sucrose at all. It does not ferment melezitose or mannitol, and attacks raffinose feebly, while the other two are very active. A9 affects starch and milk,

Shown

CARBOHYDRATES, ETC.	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	F1	F2	F3	?
Glucose	+	++++	+++	+	++++	++++	++++	+	+	+	++++	++++	++++	++++	++
Levulose	+	++++	±	++++	++++	++++	+	+	+	++++	++++	++++	++++	++++	++
Maltose	+	++++	±	+	++	++	+	++	+	++++	++++	++++	++++	++++	++
Lactose	±	−	−	−	−	−	−	−	±?	−	−	−	−	−	++
Sucrose	+	±	±?	−	−	−	−	−	−	−	++++	++++	±	±	-
Mannose	+	++++	−	−	++++	++++	++++	±	±	±	++++	+	+	+++	++
Melezitose	−	++++	−	−	+++	+++	±	−	−	−	−	−	−	++++	++
Raffinose	±?	−	−	−	−	−	−	−	±?	±	++++	±	++++	−	=
Dextrin	+	−	−	−	−	−	−	−	±	−	++++	±	+	±	=
Arabinose	−	−	++	++++	−	−	−	++++	−	++++	−	−	−	−	-
Xylose	−	−	±?	−	−	−	−	++++	−	++++	−	−	−	−	:
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	+	-
Mannitol	±?	+++	−	−	±?	±?	±	±?	−	−	−	±	±	++++	++
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	-
Inosite	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Starch	−	−	−	−	−	−	−	−	+	−	−	−	−	−	-
Glycerol	−	±?	−	−	−	−	−	−	−	−	−	−	−	−	-
Salicin	±	++	−	−	++	++	+	++	±?	−	++++	−	−	++	++
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	-
Milk	+	−	−	−	+	+	−	+	+	−	−	−	−	−	-

while neither A2 nor F3 does. On the other hand, F3 ferments dulcitol, while A2 and A9 do not.

A4 and A8 resembled each other at the time of the isolation of A8. Similarly A2 resembled A7 at the time A7 was isolated. While A2 and A7 still resemble each other after being carried as stock cultures for two years, A4 and A8 have lost much of their similarity. A8 ferments four carbohydrates which are not acted upon by A4. The former also acts upon milk, while the latter does not.

Strain 11 resembles A11 in every way save degree of action.

Strain 13 resembles A1 except that A1 ferments mannitol and milk. Strains 87, 85, 29 and 24 are the same in their reactions as 13. Strains 34 and 71 were lost.

There is a general similarity in the fermentation reactions of the newly isolated strains 11, 12, 13, 15, 23, 24, 28, 29, 32, 72, 73, 85 and 87. The carbohydrates which are fermented are limited to glucose, levulose, maltose, lactose, sucrose, mannose, raffinose,

n reactions

RAINS

T3	K1	K2	RH	Alf	11	12	13	15	23	24	28	29	32	34	71	72	73	85	87
+++	++	+++	++	++++	++++	++++	++++	++++	++++	++	+	++++	+	−	−	+	++++	+++	++++
+++	+	−	+	+	++	±	++	++	±	+	+	+	+	−	−	+	+	+	+
+++	++	−	++	++++	++++	++++	++++	+++	+	+	+	++++	+	−	−	++++	++++	++++	++++
+++	++	+++	++	±	−	−	±	−	±	±	−	+	−	−	−	−	−	+	±
+++	+	−	±?	−	+	−	+	±	±	±	±	+	±	−	−	±?	+	+	±
+++	++	+++	+++	±	++++	±	++	+	±	+	+	+	±	−	−	±?	++++	+	+
+++	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
±?	+	−	++	−	++++	−	++++	±	−	+	±	+	±	−	−	−	+	+	+++
+++	−	−	±?	−	++++	−	++++	±	−	+	+	++++	+	−	−	−	+++	++++	++++
++	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
++	−	−	±?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
±?	−	−	−	−	−	−	−	−	−	−	−	−	±?	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+++	−	−	±?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
±?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+++	−	−	+	−	+++	−	+	++	−	±	−	+	−	−	−	−	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

dextrin and salicin. Arabinose, xylose, mannitol and milk were acted upon by many of the cultures carried in the stock medium, but they were not utilized, on the other hand, by the recently isolated strains.

The newly isolated strains described here fall into three groups: Type I includes 11, 15, 28 and 73. This group resembles A11 (*L. plantari*) of the type cultures. Type II includes 12, 23 and 72, which resemble culture Alf, but none of the type cultures. Type III includes 13, 24, 29, 85 and 87 and resembles A1 (*L. leichmanni*) of the type cultures.

Strains T1, T2 and T3 (teeth strains), K1 (*L. acidophilus*), K2 (*L. bulgaricus*), and RH (*L. acidophilus*) have one reaction in common which is not shared by most of the organisms isolated from grain and soil. The organisms of dental origin, those isolated from milk, and those from feces of animals on a high carbohydrate diet ferment lactose very readily. On the other hand, the soil organisms either do not ferment lactose at all or exert only a slight action.

SEROLOGICAL RELATIONSHIPS

Representative strains of type cultures and newly isolated organisms were selected for serological study. The antigens were prepared as follows: Twenty-four-hour old cultures of the organisms in yeast-water broth containing peptone and glucose were centrifuged, the supernatant fluid removed and the bacterial cells washed and centrifuged 3 times in sterile physiological saline solution. The residue was finally made up to a turbidity of 8 to 10 on the McFarland nephelometer scale with sterile saline, and a loopful of the final suspension plated out as a check on its purity. Rabbits were immunized with the unheated, washed antigens by repeated injections in the marginal ear vein until the agglutination titer of the serum against the homologous organism was at least 1:800.

The following eight cultures were used to prepare the immune sera which had the indicated agglutination titers:

A1	1:800
A5	1:1600
A9	1:1600
A10	1:800
Alf	1:1600
11	1:1600
72	1:1600
RH	1:800

AGGLUTINATION REACTIONS

The agglutination reactions of these sera against the strains of lactobacilli studied are shown in table 3. They were determined with washed antigens having a turbidity of 1:1.5. Four dilutions

TABLE 3

Showing agglutination reactions

SERA	ANTIGENIC STRAINS																																		
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	F1	F2	F3	T1	T2	T3	K1	K2	RH	Alf	11	12	13	15	23	24	28	29	32	34	71	72	73	85	87
A1	++	−	−	−	−	−	?		?	?	−	−	−	?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−
A5	−	−	−	−	++	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
A9	−	−	−	−	−	−	?	−	++	?	−	−	−	?	−	−	−	−	−	−	−	++	++	++	++	−	−	−	−	−	−	−	−	−	−	−
A10	−	++	−	−	−	−	++	−	−	++	−	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Alf	−	−	−	−	−	−	?	−	−	?	−	−	−	?	−	−	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	++	++	−	−	−	−	−	−	−	−	−	−	−
72	+	−	−	−	−	−	−	−	−	−	−	+	?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	++	−	−
RH	−	−	−	−	−	−	?	−	−	?	−	−	−	?	−	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 4

Giving complement fixation reactions

SERA	ANTIGENIC STRAINS																																		
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	F1	F2	F3	T1	T2	T3	K1	K2	RH	Alf	11	12	13	15	23	24	28	29	32	34	71	72	73	85	87
A5	−	−	−	−	++++	++++	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Alf	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
72	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	++++	−	−

of serum were used, namely 1:100, 1:200 1:400 and 1:800, and a control containing no serum. The readings were made after two hours incubation at 56°C. and again after remaining over-night at 3 to 4°C. The key to the symbols used in the table of agglutination reactions is as follows:

− = no agglutination
? = some agglutination in control tube
+ = agglutination in 1:100 or 1:200
++ = agglutination in 1:400 or 1:800

Most of the sera showed a marked proagglutination phase in the dilutions lower than 1:100, but all sera agglutinated the homologous organism at dilutions of 1:100 or above.

Several points should be noted in the table of agglutination reactions. Although A1, F1 and F2 are all type cultures of *L. leichmanni*, neither F1 nor F2 is agglutinated by A1 antiserum. This agrees as far as it goes with the interpretation of the results of the fermentation reactions. Strain A1 is unlike F1 and F2. Unfortunately no antiserum was prepared for either F1 or F2, and their serological similarity remains undetermined. It may also be significant that an agglutination reaction was obtained in the lower dilutions with strains 29 and 85. The last two organisms were found to be similar in their fermentation reactions to A1.

A5 and A6 are serologically the same. Although an agglutination was obtained in the lower dilutions with A5 antiserum and antigens of cultures of Alf and 24, neither of these cultures resembles A5 or A6 in its fermentation reactions. Strain 24 resembles A1 by the fermentation tests, while Alf falls into another group. There is no agglutination of either 24 or A1 by the antiserum of Alf.

A9 antiserum does not agglutinate A2 or F3 antigens. This agrees with the findings of the fermentation reactions. Although no two of these three strains show identical fermentation reactions, A2 more closely resembles F3 than A9 does either A2 or F3. Neither F3 nor A2 antiserum is available to determine whether A2 and F3 antigens and antisera cross agglutinate.

A9 antiserum also agglutinated 11, 12, 13 and 15 antigens even

in the higher dilutions. Inasmuch as No. 11 antiserum also agglutinates 11, 12, 13 and 15 antigens in the same high dilutions, it might be concluded that these cultures are serologically the same. Notwithstanding this fact, antiserum 11 does not agglutinate A9 antigen, and the fermentation reactions of these strains are quite different.

There is no serological similarity between cultures 11, 28, 73 and A11 as a group. Neither is there between Alf, 23, and 72, or between 13, 24, 29, 85, and 87. These groups have similar fermentation reactions within themselves. Antiserum RH does not agglutinate antigen K1, though both are *L. acidophilus*.

Strains 71, 72 and 73 are serologically similar, although 72 and 73 do not have similar fermentation reactions. While antiserum 72 agglutinates antigens A1 and F1 in the lower dilutions, and causes possibly a slight reaction with F2 antigen, there is no marked similarity in their fermentative properties. F1 more closely resembles 72 than does A1. However, A1, F1 and F2 are all *L. leichmanni* type cultures.

It is perhaps significant that antiserum A10 agglutinates antigens A2, A7 and F3 in dilutions as high as it agglutinates its specific antigen. Strain A7 was, as has been previously mentioned, similar in its fermentation reactions to A2 when first isolated. A2 and F3 are both *L. delbrücki*. While A9 is likewise an *L. delbrücki* type culture, it is not agglutinated. It has already been noted that A2 and F3 more nearly resemble each other in their fermentation reactions than either one resembles A9. However, A10 ferments the pentoses easily, while A2, A7 and F3 do not.

COMPLEMENT FIXATION REACTIONS

Kulp and Rettger (1924) failed to obtain satisfactory agglutination reactions with their strains of *L. acidophilus* and *L.bulgaricus* because of spontaneous agglutination. They finally resorted to the complement fixation test. Morishita (1928), in working with organisms isolated from dental caries, found the agglutination method satisfactory after *certain preliminary treatment* of the suspended antigen. In the present work only three strains

agglutinated spontaneously, and then not sufficiently to mask true agglutination. However, for the sake of comparison, complement fixation tests were made with four immune sera against the 36 antigens employed in the agglutination experiments.

The sera used were four of those which were employed in the agglutination work, namely A5, Alf, 11 and 72. They were run in two dilutions and well beyond the anticomplementary range. The antigens were prepared from the stock suspensions of washed viable organisms in sterile saline solution. The degrees of fixation are indicated in table 4 on the −, +, ++, +++, and ++++ basis.

The complement fixation reactions obtained here agree only in part with the agglutination reactions. Whenever a 4+ complement fixation was obtained, a 2+ agglutination reaction was observed. However, this is as far as the correlation between the two systems went. The 1+ complement fixations do not in any case agree with agglutinations reactions obtained in these instances.

While the complement fixation technique is a very delicate one, and to a certain extent confirms the agglutination reaction, it is also very cumbersome. The agglutination method may be used successfully with the strains of organisms used here. Strains that are naturally subject to self-agglutination may by certain treatment[5] be made to serve as satisfactory agglutination antigens.

DISCUSSION

Several points may be emphasized here. It is often very difficult to establish the identity of any one isolated strain of Lactobacillus definitely. At times there are greater differences between the fermentation reactions of two given strains of the same organism than are seen between two individuals of different species. Henneberg (1904) divided his *L. leichmanni* strains into three groups. *L. leichmanni* I did not ferment lactose, raffinose, dextrin or inulin, but attacked alpha-methyl glucoside.

[5] Rapid and repeated transfer of cultures (three to four hours) and centrifugation of the washed cells to remove the coarser clumps.

L. leichmanni II fermented all of these, while *L. leichmanni* III acted upon all but raffinose and the glucoside. Except for the amount of acid produced, *L. listeri* corresponded exactly with *L. leichmanni* II, save possibly in the instance of rhamnose; no data are given on the action of *L. leichmanni* II upon this sugar.

Of the fifteen organisms listed by Henneberg there are only four which ferment lactose strongly, eight which do not attack it at all, and two which act upon this sugar only to a limited extent. The lactose fermentation of one organism is not listed.

The evidence offered in this paper by the fermentation and agglutination reactions of known strains of lactobacilli is only in partial agreement with our knowledge of the sources of these strains. In some instances the fermentation reactions are confirmed by the agglutination reactions, while in others they are not. The agreements almost balance the disagreements. The complement fixation reactions only in part support the findings of the agglutination tests. The morphology of the organisms and colony formation merely serve as a preliminary means of identification and cannot be used by themselves as a means of distinguishing the individual members of the genus employed in this investigation.

Work with known members of this group is a discouraging preliminary to work with unknown members. If one cannot find agreement with known strains (sometimes subcultures of the same original strains from two different laboratories do not agree) then it is useless to place too much emphasis upon the fermentation or agglutination reactions. While there can be no question regarding the delicacy of the serological and fermentation methods, the significance of these sensitive reactions may perhaps be overemphasized in the instance of the lactobacilli when one considers the variability which some of these organisms at times show, at least in artificial culture.

The tendency of some writers, in describing a given organism of the Lactobacillus group, to conclude that it is in certain respects like *L. delbrücki*, or that it "resembles" *L. acidophilus*, is perhaps fortunate, and indeed judicious, particularly when their observations are based on incomplete data. To label an or-

ganism definitely as *L. acidophilus* or *L. delbrücki* simply because it bears certain general resemblances cannot be too strongly condemned.

There is one characteristic which most of the organisms isolated from soil, grains and fruits lack, but which is possessed by *L. acidophilus* and *L. bulgaricus* as described by Kulp and Rettger (1924), and by *L. odontolyticus* as described by Morishita (1929). The soil organisms employed here do not as a rule ferment lactose, while *L. bulgaricus*, *L. acidophilus* and *L. odontolyticus* utilize this carbohydrate readily. One is not justified, however, in arbitrarily taking one or two properties of an organism to the exclusion of all others, and concluding that all organisms possessing this property are the same. Rather, the writers wish to point out the dissimilarities between this large group of lactobacilli isolated from soil, and the more specific types of *L. acidophilus*, *L. bulgaricus* and *L. odontolyticus*.

Until further and more substantial data are available it is perhaps unwise to attempt further distinctions. However, it has been noted that the organisms isolated by the writers fall into several groups on the basis of their fermentation characteristics. Type I includes strains 11, 15, 28 and F3 which might be called *L. plantari*. Type II includes strains 12, 23, F2 and Alf, which act upon relatively few of the carbohydrates employed in these tests. Type III is the largest group including 13, 24, 29, 85 and 87 and might be designated as *L. leichmanni*. Type IV was not often met with, but like *L. pentoaceticus*, *L. fermentatae* and *L. lycopercici*, these organisms fermented one or more pentoses readily. A pentose fermenter was encountered and isolated in four instances, but A8 was the only strain available for these studies.

The ability of some organims to form gas from carbohydrates has been mentioned by some writers. None of the strains described in this paper possess this characteristic now. Even *L. lycopercici*, which produced large amounts of gas from tomato juice when first received in this laboratory, has lost this property. Hence it could not be used as a means of differentiation.

The question of source of the soil lactobacilli is an interesting

one. It was observed in this work that when a sample of grain was protected from animal contamination it ceased after a while to yield lactobacilli. This was first noted by the writers when they originally attempted to isolate what was then known as *B. volutans*. All efforts to isolate aciduric organisms from samples of corn and corn flour which had been in sealed jars and bottles in the laboratory for several months met with failure. When fresh samples were obtained from a feed store isolation was effected without difficulty.

Although it was surmised that samples from feed-stores and cereal mills were continually being infected by rodents, several attempts to isolate the organism from the intestinal contents of mice trapped in the store-room were not successful. It is possible, nevertheless, that the fecal material of certain animals is the ultimate source of these organisms. This speculation is strengthened when one considers the prevalence of the lactobacilli in surface soil of many varieties all of which were subject to fecal contamination.

SUMMARY

Thirty-six strains of Lactobacilli representing strains isolated from soil, grain, fecal material, milk products and dental caries are described. The media used and the methods of isolation are detailed.

The fermentation reactions of the organisms upon 20 carbohydrates and related substances, their action in milk, broth and gelatin, and their acid production at four incubation temperatures, are listed.

Agglutination reactions and complement fixation tests of the 36 strains with antisera of representative strains are also listed and results compared with fermentation reactions.

Lactobacilli isolated from soil and grain did not as a rule ferment lactose. This marks them as a group distinct from *L. acidophilus*, *L. bulgaricus* and *L. odontolyticus*.

Of the lactose non-fermenters, four types are more or less definitely indicated on the basis of their fermentation reactions.

REFERENCES

BUNTING, R. S., NICKERSON, AND HALL (1926) Further studies of the relation of *Bacillus acidophilus* to dental caries. Dental Cosmos, **68**, 931–942.

FRED, E. B., PETERSON, W. H., AND STILES, H. R. (1925) The Biochemistry of the granulated lactic acid bacteria from cereals. Jour. Bacteriol., **10**, 63–78.

GEE, A. H., AND HUNT, G. A. (1928) Single cell technic. Jour. Bacteriol., **16**, 327–351.

HEINEMANN, P. G., AND HEFFERAN, M. (1909) Study of *B. bulgaricus*. Jour. Inf. Dis., **6**, 304–318.

HENNEBERG, W. (1904) Zur Kenntniss der Milchsäurebakterien Brennereimasche, der Milch, des Bieres, der Presshefe, der Melasse, des Sauerkolhs, der sauren Gurken and des Sauerteigs sowie einige Bemerkungen ueber die Milchsäurebakterien des menschlichen Magens. Centralbl. f. Bakt., Abt. II, **11**, 154–170.

HOWE, P. R., AND HATCH, R. E. (1917) A study of microörganisms of dental caries. Jour. Med. Research, **36**, 481–491.

HULL, T. G., AND RETTGER, L. F. (1917) The influence of milk and carbohydrate feeding on the character of the intestinal flora. Jour. Bacteriol., **2**, 47–71.

KULP, W. L., AND RETTGER, L. F. (1924) Comparative study of *L. acidophilus* and *L. bulgaricus*. Jour. Bacteriol., **9**, 357–394.

KULP, W. L. (1927) An agar medium for plating *L. acidophilus* and *L. bulgaricus*. Science, **66**, 512–513.

MCINTOSH, J., JAMES, W. W., AND LAZARUS-BARLOW (1922) An investigation into the etiology of dental caries. I. The nature of the destructive agent and production of artificial caries. Brit. Jour. Exp. Path., **3**, 138–145.

MORISHITA, T. (1928) Comparative study of certain aciduric organisms of intestinal and dental origin. Proc. Soc. Exp. Biol. and Med., **25**, 654–656.

MORISHITA, T. (1929) Studies on dental caries, with special reference to aciduric organisms associated with the process. I. Isolation and description of organisms. Jour. Bacteriol., **18**, 181–198.

RAHE, A. H. (1914) An investigation into the fermentative activities of the aciduric bacteria. Jour. Inf. Dis., **15**, 141–150.

RAHE, A. H. (1918) The classification of aciduric bacteria. Jour. Bacteriol., **3**, 407–421.

RETTGER, L. F., AND HORTON, G. D. (1914) A comparative study of the Intestinal flora of white rats kept on experimental and on ordinary mixed diets. Centralbl. f. Bakt., I, Orig., **73**, 362–372.

RETTGER, L. F., AND CHEPLIN, H. A. (1920) The intestinal flora with special reference to the implantation of *Bacillus acidophilus*. Yale University Press, New Haven.

RODELLA, A. (1901) Ueber die sogenanten säureliebenden Bacillen im Säuglingsstuhle. Centralbl. f. Bakt., Abt. I, Orig., **29**, 717–724.

RODELLA, A. (1908) Magen Carcinoma und Milchsäure Bacillen. Centralbl. f. Bakt., Abt. I, **47**, 445–466.

RODRIGUEZ, F. E. (1922) Studies in the specific bacteriology of dental caries. Mil. Dent. Jour., **5**, 199.

ROSEBURY, T., LINTON, R. W., AND BUCHBINDER, L. (1929) A comparative study of dental aciduric organisms and *Lactobacillus acidophilus*. Jour. Bacteriol., **18**, 395–412.

SOMOGYI, M. (1926) Notes on sugar determination. Jour. Biol. Chem., **70**, 599–612.

THAYSEN, A. C. (1921) The bacteriology of the process for acetone and *n*-butyl alcohol manufacture. Jour. Inst. Brewing., **27**, 529.