COMPARATIVE STUDY OF LACTOBACILLUS ACIDOPHILUS AND LACTOBACILLUS BULGARICUS

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INTRODUCTION

For the past thirty years or more investigators here and abroad have given considerable time and thought to the study of the Gram-positive bacteria which make up the genus Lactobacillus (Committee on Classification, 1920).

Members of this genus comprise species which have been isolated from, and considered the most important fermentative agents in fermented milks from different parts of the world, as for example, mazun of Armenia, yogurt and kefir of Russia and the Balkans, leben raib of Egypt and gioddu of Sardinia. Members of this genus have received much attention in the study of the intestinal bacteriology of man and of many of the lower animals, the fermentation of ensilage and sauerkraut and the biology of salt-rising bread. They have been shown also to be a very important factor in the ripening of various kinds of cheese. At times their presence in soil has been noted; in fact they can as a genus be called almost "ubiquitous" bacteria.

This research deals with those members of the genus Lactobacillus which for the present may be considered as embodying three main types or species. The first of these is the organism found in fermented milks and called *B. bulgaricus* by Grigoroff (1905). The second is an intestinal bacterium, first isolated and studied by Moro (1900) and designated by him as *B. acidophilus*. The third species is another intestinal organism which is found particularly in the intestines of breast-fed infants and which was first studied by Tissier (1900) and by him given the name of *B. bifidus*.

The actual experimental work reported in this paper concerns the first two groups, *L. bulgaricus* and *L. acidophilus*. Such a study appeared important because of the recent work of Rettger and Cheplin (1921) on the implantation of *L. acidophilus* in the human intestine as a means of combating constipation, diarrhea and other intestinal disturbances. It is claimed by many that so-called auto-intoxication and general debility are caused by the absorption of accumulated products of bacterial putrefaction in the colon. Ever since Metchnikoff (1908) advocated the drinking of Bulgarian milk as an aid in securing longevity because of its apparent beneficial action upon the colon, there has been considerable interest in sour milk therapy, especially the use of *L. bulgaricus* milk.

Metchnikoff and his contemporaries thought the good effects of feeding Bulgarian milk were due to implantation and proliferation of *L. bulgaricus* in the intestine. More recently Rettger and his associates have shown that the beneficial influence results from increased growth of *L. acidophilus*, an organism native to the intestine and the growth of which is fostered in milk drinkers by the lactose contained in milk.

Without going further into a discussion of sour milk therapy, it will suffice here to note that the two opposing claims stimulated renewed interest in comparative studies of these two aciduric organisms. The generic term "Lactobacillus" has generally been used throughout this paper in accordance with the new classification adopted by the Society of American Bacteriologists.

In the present work the authors have attempted to acquire more complete first-hand knowledge regarding the characteristics of *L. bulgaricus* and *L. acidophilus*, particularly those which may be of importance in their differentiation.

GENERAL HISTORICAL REVIEW

For a complete historical review and bibliography, the reader is referred to the doctorate thesis of the Senior author in the Yale University Library.

A review of the literature covering the investigations of members of the Lactobacillus genus found in fermented milks, in the

intestines of man and animals, and of those obtained from other sources, reveals much confusion, especially in regard to nomenclature and identification. It appears that identical species isolated by different investigators from closely related sources have in many instances been given different names.

Grigoroff (1905), working in Massol's laboratory, reported two species which he had isolated from "podkvassa," the starter for the Bulgarian "kiselo mleko." His species "A" has been considered the type species of the Lactobacillus group and has been called *L. bulgaricus* (Massol).

Weigman, Gruber and Huss (1907) studied an organism isolated from Armenian sour milk which they named *Bact. mazun.* Heineman and Hefferan (1909) isolated a number of strains from various sources, which they considered identical with *L. bulgaricus.* Kindraczuk (1913) gave the name *B. carpathicus* to an organism similar to *L. bulgaricus,* which he isolated from "huslanka," a fermented milk of Bukowina and the East Carpathians.

Moro (1900) isolated an organism from the feces of breast-fed infants, the "Blaubacillus," to which he gave the name of *B. acidophilus.* Moro's organism was, as he supposed, derived from the mother's breast and was found in the mouths and stomachs of infants as well as in the intestinal contents. He stated that it was not a single species but a group of closely related forms which preferred an acid medium for growth.

Cahn (1901) noticed *L. acidophilus* in the feces of breast-fed and bottle-fed babies. Rodella (1901) isolated a similar organism from the same source and called attention to its pleomorphism. The organism varied in form from coccoid to very long rods in the same culture, some of the rods showing branches. Weiss (1904) noted that when large quantities of milk were ingested a marked increase of organisms of the lactobacillus type, corresponding to the *L. acidophilus* of Moro, was observed.

Tissier (1908) in a study of the intestinal flora of infants observed that at the age of about three days, breast-fed babies developed an intestinal flora in which one organism was

predominant. This had in general the staining reactions and morphological appearance of the lactobacilli. Tissier noted the presence of many bifurcated forms and, because of this characteristic gave his organism the name *B. bifidus*. Having isolated it, he discovered that it was anaerobic, in distinction from *L. acidophilus* which was facultative. This same worker also mentioned an organism, *B. exilis*, which he supposed occupied a half-way position between *L. bifidus* and *L. acidophilus*.

There was considerable controversy between Tissier and Moro as to the predominant organism in the feces of breast-fed babies, which was finally settled by Moro's admission that *L. bifidus* occurred in far greater numbers. However, both investigators concluded that *L. acidophilus* became predominant in the feces of babies whose chief food was cow's milk.

Rettger and his associates (1912, 1914 and 1921) have shown that the feeding of milk in large amounts, or of lactose, to white rats and human subjects brings about a transformation of the intestinal flora to one in which the lactobacilli of the acidophilus and bifidus type predominate.

Howe and Hatch (1917) in a study of dental caries noted the presence of large numbers of organisms similar to *L. acidophilus* and *L. bifidus*. Boas and Oppler (1895) isolated a species from cases of carcinoma of the stomach which was similar to the lactobacilli in general characteristics. Galt and Iles (1914) observed this organism in three cases of gastric carcinoma and concluded that it was identical with *L. bulgaricus*.

Döderlein (1892) discovered an "acid bacillus" in normal vaginal secretions. Jötten (1922) in an exhaustive comparative study of this organism and *L. acidophilus* concluded that the two species are identical.

EXPERIMENTAL

The present study does not include all known species of the Lactobacillus genus. It deals chiefly with varying strains of what have been designated as *Lactobacillus bulgaricus* (Massol) and *Lactobacillus acidophilus* (Moro) and which have been used in the preparation of sour milk for therapeutic purposes.

A few supposedly closely related forms have been included for the sake of comparison.

The experimental work naturally falls into the five following divisions:

1. Suitable media for use in the studies contemplated.
2. Methods of isolation, and a brief history of the strains of each species exployed, in so far as this was possible.
3. A detailed study of the morphological and cultural characteristics.
4. Determination of fermentative action in milk and various carbohydrates, etc.; and observations of some of the physiological actions of both species upon gelatin and upon milk proteins.
5. An attempted study of the antigenic properties of a few members of both species and complement fixation and agglutination studies with the sera of rabbits immunized by repeated intravenous injections of cultures.

It was hoped that through these experiments new and definite correlations might be established which would enable us more readily to differentiate between *L. bulgaricus* and *L. acidophilus*.

CHOICE AND DEVELOPMENT OF MEDIA

In the isolation and in the study of the cultural and biological characteristics of the *L. acidophilus* and *L. bulgaricus* species, much stress has been placed by various investigators on the medium used. Neither of these types gives a profuse growth in ordinary laboratory media, and some members of the *L. bulgaricus* group will not grow at all in such media. In studies of their action on carbohydrates and other fermentable substances, this has proved to be a very serious obstacle and, no doubt, is responsible for some of the contradictory results reported.

Investigators have employed a diversity of media, generally of special composition. The majority of these special media gave excellent results in so far as growth of the organism was concerned. However, many of the media were such that the carbohydrate constituents could not be controled. In addition, the method of preparation in some instances was rather

laborious and complicated. An attempt has therefore been made to develop a medium with complete control of the carbohydrate content and one which can be simply and easily prepared and yet give consistently good growths of both groups of organisms.

It was shown in a previous report (1922) that nutrient agar made with Difco pepton plus galactose produced a medium which gave sufficient growth of *L. acidophilus* for isolation work and cultural study. However, in the present work it was found that the majority of the strains of *L. bulgaricus* studied would not grow satisfactorily in this medium and that the fault lay not in the carbohydrate but in the pepton.

Opsine, a biuret-free protein digestion product of French manufacture, employed successfully by Robinson and Rettger (1916) was substituted for Difco pepton. An Opsine-meat-extract-agar containing 1 per cent galactose gave consistently good growths of both species of bacteria It was, however, impossible to secure a new supply of opsine as its manufacture had been discontinued, so another medium had to be developed.

Both species grew well in milk, especially *L. bulgaricus;* hence it was thought that a medium containing the nitrogen fractions of milk should furnish the proper food. Gorini (1914) emphasized the need of using a medium made from milk or milk constituents in any study of lactic acid bacteria. Ayers and Mudge (1920) advocated the use of milk powder agar in the determination of bacteria in milk. As a permanently transparent, carbohydrate-free basic medium was desired, it was apparent that one made from the digested proteins of milk should prove more or less satisfactory.

Cole and Onslow (1916) had devised a method for the preparation of a casein digest which in their hands proved to be a very superior medium for bacterial growth. Their method calls for a digestion period of fifteen days, which for practical purposes makes the method rather slow and cumbersome. A modification of this method was adopted.

In the first trials a mixture of 9 parts of casein and 1 part of egg albumen dissolved in 1 per cent sodium carbonate was

digested with fresh trypsin powder for forty-eight hours at 37°C. The resultant digest, when incorporated in a medium with galactose and meat extract, gave excellent growth of *L. acidophilus* and *L. bulgaricus*.

Further trials showed that the egg albumen could be omitted. After a number of experiments involving comparisons with other media, the following method was adopted as "standard" for the preparation of casein digest which was to be employed as the nitrogenous base of all media used in the comparative study of *L. acidophilus* and *L. bulgaricus* in this research.

Two hundred grams of powdered or finely granulated casein are dissolved in 2 liters of 1 per cent sodium carbonate in the following manner. The sodium carbonate solution is heated to boiling and the casein added, a small quantity at a time, with stirring after each addition until completely dissolved. This solution is then cooled to about 40°C. and a water solution (10 to 12 cc.) containing 3 grams of *fresh* Fairchild's trypsin added. After mixing well and adding 25 cc. of chloroform as a preservative, the mixture is introduced into a flask and placed in the 37°C. incubator where it is kept for forty-eight hours. At the end of the forty-eight hours incubation period the mixture is made neutral to litmus with HCl, heated in a double boiler to drive off all chloroform, and then filtered. When chemically pure casein is used the resultant filtrate is clear and has a light amber color. Commercial casein, because of its small lactose content, makes a darker colored product. The insoluble residue does not exceed over 5 per cent of the original casein and is usually considerably less.

In this investigation, a quantity of digest sufficient for several months use was made up at one time. Quantities equivalent to 10 grams of the original casein were placed in cotton-stoppered bottles and sterilized by autoclaving. The contents of each bottle were made up to 1000 cc. with water for each liter of medium desired.

It was determined that for routine use, a tryptic digest of Klim (powered skim milk) could be substituted for casein di-

gest. This gave even slightly better growth of *L. acidophilus* and *L. bulgaricus* than did casein digest media.

In the preparation of Klim digest, it has been found expedient to dissolve the Klim in *cold* sodium carbonate. The quantities employed are the same. It is difficult to secure a clear filtrate after digestion; therefore, the Klim digest was put up in 100 cc. amounts without filtering. During the process of media making, the fine precipitate in the digest was eliminated and the completed medium was practically clear.

The formula for the preparation of media from casein or Klim digest is as follows:

Digest solution corresponding to 10 grams of casein or Klim
3 grams of commercial meat extract
Agar to make the desired consistency
1 per cent carbohydrate (this amount can be cut in half when Klim is used)
Water to make 1000 cc.

No exhaustive chemical analyses of casein or Klim digests as prepared by the methods given, have been made. Qualitative tests have shown the presence of considerable free tryptophane in forty-eight hour digests. Viale (1922) has shown the presence of free tryptophane and cystine in milk, and it may be that these two amino acids, secured by the digestion of the casein, furnish the proper nitrogenous substance for speeding up the growth of *L. acidophilus* and *L. bulgaricus*. Koser and Rettger (1919) showed that tryptophane was the best single amino acid in combination with a synthetic salt mixture for certain bacterial growths. Broth made with a twenty-four hour casein digest, when incubated with *Bact. coli* and incubated for twenty-four hours gave a very intense color reaction for indol with Ehrlich's aldehyde reagent. Commercial pepton broth when employed under the same conditions, gave only a slight color after the same period of incubation. Davis (1917) states that the extent of indol production is a very good practical test for the biologic availability of a pepton.

In the study of the fermentative action of *L. acidophilus* and *L. bulgaricus*, the dilute agar medium devised by Hitchens

(1922) was used in place of broth. At least twice as much growth was obtained in the agar as in broth.

The results of media study in the present investigation may be summarized as follows:

1. Casein and Klim digest media appear superior to ordinary media for the cultivation of *L. acidophilus* and *L. bulgaricus*.

2. Galactose is apparently the most favorable carbohydrate for the development of both species. This supports earlier observations (Kulp and Rettger, 1922).

3. Hitchen's dilute agar is more suitable for obtaining abundant growth of these two organisms than broth.

Tryptic digest of casein or of milk powder (Klim), prepared as herein described, was employed as the nitrogenous base for all media used in this investigation, unless specifically noted otherwise.

METHOD OF ISOLATION

Beijerinck (1889) secured isolations of *L. caucasicus* by employing whey gelatin, made slightly acid with lactic acid. Colonies were picked off and transferred to sterile whey. Johannesen (1897) was the first investigator to use acid broth for the separation of acid-forming bacteria. Heymanns (1898) developed his glucose acetic acid broth for this purpose.

Veillon and Zuber (1898) grew their cultures in deep agar tubes by a modified method of Liborius. The colonies were withdrawn from the lower part of the tube with a sterile pipette or a platinum loop. Tissier isolated *L. bifidus* with the aid of the Veillon tube, while Moro employed acid beer wort broth and agar.

Rettger and his associates have shown that by feeding liberal amounts of dextrin and of lactose to white rats and human subjects the aciduric type of intestinal organisms can be made to develop in the intestines to the almost total exclusion of all other cultivable forms. Advantage was taken of this method to procure isolated strains of *L. acidophilus*. In certain instances where there had been no preliminary carbohydrate feeding, Heymanns' glucose acetic acid broth was employed

as an enrichment medium before dilution plates were prepared from the feces.

After incubation for forty-eight hours at 37°C., typical acidophilus colonies were removed by cutting out a block of agar immediately surrounding the colonies with the aid of a sterile platinum spatula, and transferred to a tube of sterile litmus milk. The agar and the enclosed colony were thoroughly broken up by crushing against the inner wall of the tube. This culture was then incubated until the milk showed an acid curd. To insure purity, this milk culture was plated again and re-isolation made. Usually this procedure was repeated four or five times. Gram stains were made as an additional check upon the purity of the culture.

All strains of *L. acidophilus* employed in this investigation were isolated from typical acidophilus colonies. All *L. bulgaricus* strains were received as pure cultures. These, however, were subjected to re-isolation tests to insure freedom from other forms.

The following is a list of all strains of both species employed in this investigation, with a short history of each.

Sources of bacterial strains employed

L. acidophilus strains R-1-1, S-1, R-J, B-A-3, R-1-1 F and S-d were isolated from the feces of white rats and human subjects. They soon acquired the property of curdling milk.

R-1-5, R-4-8, R-11, 12 X, 13, P, D, Rb-A, Rb-B and Rb-C were strains of *L. acidophilus* isolated from various sources: rats, humans, rabbits and dog—which had all the characteristics of the first group except that they curdled milk slowly and therefore could not be used in the practical production of acidophilus milk. L-43 was an organism secured from the Army Medical School and had been isolated from dental caries.

L. bulgaricus strains B-1, B-3, B-4, B-12 and B-13 were secured from the Dairy Division of the Bureau of Animal Industry. B-12 had been originally isolated by Dr. Lind of Copenhagen from Bulgaricus yogurt. B-13 was the organism which Freudenreich called *B. casei* (epsilon).

Strains F and H were secured from commercial firms which stated

that they were being used exclusively for the production of Bulgaricus milk and tablets.

MORPHOLOGY AND CULTURAL CHARACTERISTICS

Beijerinck (1889) stated that *L. caucasicus* is a non-motile, non-sporing facultative rod-shaped bacterium. The colonies were slow to develop, and at best remained very small. The optimum temperature was 45°C. Freudenreich (1897) reported that *L. caucasicus* grew very slowly at 22°C., faster at 35°C., producing small colonies with hair-like projections around the border. It did not grow upon potato. He described it as a Gram-positive rod with rounded ends, 1μ by 5–6μ, non-motile and without spores. Old cultures showed granules when stained. The organism was killed by exposure to 55°C. for five minutes and when dried, died off within two days at ordinary temperature.

Grigoroff (1905) described his Bacillus A (*L. bulgaricus*) as a long rod, non-motile, growing singly or in chains, and producing no spores. This organism stained well with all basic aniline dyes and was Gram-positive. It was a facultative anaerobe with an optimum temperature of 37°C. In a twenty-four hour broth culture there was turbidity which cleared up within a few days, leaving a heavy sediment. No growth was observed upon potato or in gelatin. Streptobacillus A of this investigator was a Gram-positive rod, growing in chains consisting of from 4 to 20 members. It appeared to be more heat-resistant than Bacillus A, withstanding a temperature of 60°C. for five hours, whereas Bacillus A was destroyed in one hour.

White and Avery (1910) placed the organisms which they studied in two groups, A and B. Those of group A revealed uniformly stained protoplasm when treated with Loeffler's methylene blue or Neisser's stain. Group B showed intensely staining granules. There was more abundant growth of B than A in gelatin, with no liquefaction in either case. In other respects, the organisms of both groups had the same general characteristics of the *L. caucasicus* group.

Moro (1900) described the "Blaubacillus" (*L. acidophilus*) as a non-motile, non-spore-forming rod, 0.6 to 0.9μ by 1.5 to 2μ. It was a facultative anaerobe, growing best at 37°C. Young cultures were Gram-positive, the bacilli becoming negative in old cultures. Morphology also changed in old cultures to a longer and more slender type with the disappearance of threads and the occurrence of degenerating forms. There was scanty growth on slant agar and no growth in gelatin or upon potato. In broth, there was marked turbidity at the end of twenty-four hours, the liquid clearing up quickly after that time, leaving a fine flaky sediment. Two kinds of colonies were observed in agar plates, one like a grain of sand and another with fine threads extending out from a solid center.

Finkelstein's (1900) organisms were quite similar to Moro's except that the former investigator noted occasional branching. Cahn (1901) did not find any branching forms, but in old cultures he observed marked involution forms. He noted a third type of colony formation on agar, namely, a small disc-like colony. Mereshkowsky (1905) labeled Moro's sand-grain colony type 1 and the filamentous colony type 2. Rodella's (1901) description of "Säuereliebender Bacillus im Säuglingsstuhle" agreed with Finkelstein's report.

Tissier (1900) described *L. bifidus* as a strict anaerobe and as being slender, from 2 to 6μ in length, non-motile and non-sporing. This organism appeared very often as a bifurcated (y-shaped) rod. It grew best at 37°C. and was not as strongly Gram-positive as *L. acidophilus*. A temperature of 60°C. killed this species very quickly. Under the microscope agar colonies appeared disc-shaped, with smooth edges and 2 to 3 mm. in diameter. Tissier's *B. exilis* was a small straight uniformly staining rod. It was Gram-positive, grew at 20°C. though the optimum temperature was 37°C. Aerobic colonies were very small and delicate, showing smooth edges.

Jacobson (1908) stated that *L. bifidus* could grow under aerobic conditions in symbiosis. This author noted two types of *L. acidophilus* colonies and three types of organisms: the first type was a large bacillus with square ends growing usually in long

chains; the second was a thin bacillus with round ends and producing no chains; and the third was a very short bacillus. He reported the Gram-staining property as being very variable and stated that no branching forms were observed.

Howe and Hatch (1917) described *L. bifidus* isolated by them as very pleomorphic. On the other hand they noted no marked pleomorphism in *L. acidophilus*. They stated that the size and arrangement of the latter was governed by the medium used.

Rettger and Horton (1914) used the term "X" type for the filamentous colonies of *L. acidophilus*, and "Y" type for the sand grain-like colonies. These investigators suggested a close relationship between the "Y" type and *B. exilis* of Tissier. Rahe (1914) found both "X" and "Y" types in his isolations of aciduric bacteria from the intestines. Sandberg (1904) had reported "X" and "Y" types of colony formed by the Boas-Oppler bacillus.

Jötten (1922) found the morphological and cultural characteristics of Döderlein's "Vaginalbacillus" to be identical with those of *L. acidophilus*.

MORPHOLOGY

All strains of the species employed in this study were Gram-positive rods with rounded ends. No motility was observed in hanging drop preparations taken from twenty-four hour sugar broth cultures, and there was no evidence of spore formation. When stained with Neisser's stain or Loeffler's methylene blue, some organisms stained evenly, while others showed granules. However, these characteristics did not appear to be stable, nor could they be used to differentiate species. Granule formation appeared to a greater extent in the older than in young cultures. No branched forms were seen. The orientation generally showed considerable variation, even in the same culture. In young cultures the width of the rod was quite constant. In old cultures, almost all shapes and sizes could frequently be observed. Generally the majority of organisms in old cultures became Gram-negative.

TABLE 1

	TWENTY-FOUR HOUR SUGAR BROTH CULTURES	FORTY-EIGHT HOUR SUGAR AGAR COLONIES	TWENTY-FOUR HOUR AGAR SLANT	YOUNG MILK CULTURES	OLD MILK CULTURES
B-1	Pairs + 0.8 x 1.5–2	Pairs + 0.8 x 2–4	Sh. ch. + 0.6 x 2–8	Sh. ch. + 0.6 x 2–5	No. ch. – 0.8 x 2–4
B-3	Lo. ch; filam. + 0.5 x 2–6	No. ch. + 1.0 x 2–6	No. ch. + 0.8 x 3–6	Sh. ch. + 0.6 x 4	No. ch. – 0.5 x 2–4
B-4	Sh. ch. + 0.6–0.8 x 3–8	No ch. + 1.3 x 3–8	No ch. + 0.8 x 3–10	ch. + 0.7 x 4–8	No ch. – 0.6 x 3–6
B-13	Lo. ch. + 0.6 x 3–10	Sh. ch. + 0.5 x 2–4	Ch.; rds. + 0.6–8 x 2–6	Ch. + 0.6–1 x 4–6	No ch. + & – 0.6 x 4–10
F	Sh. ch. + 0.8–1 x 3–8	No ch. + 1.0 x 6–8	Few ch. + 0.8 x 4–8	V. lo. ch.; filam. + 0.6–0.8 wide	Lo. ch.; filam. – 0.4 wide
H	Few ch. + 0.8 x 4–8	Few ch. + 0.6 x 3–8	Few ch. + 0.8–1 x 3–6	Few ch. + 0.6 x 3–8	No ch. – 0.5 x 2–8
R-1-1	No ch. + 0.6 x 1–2	No ch. + 1 x 2–4	Sh. ch. + 0.7 x 1.5–3	Lo. ch.; filam. + 0.5 wide	Slender rods – Gran.
S-1-3	Lo. ch. + 0.6 x 1.5	Few ch: + 1.0 x 2–6	Chains; filam. + 0.6 x 2–8	Lo. ch.; filam. + 0.6 wide	No ch. + & – 0.6 x 3–4
S-d	No ch. + 0.7 x 3–4	Lo. ch.; filam. – 0.7–1 x 2–6	Sh. ch. + 0.8–1 x 2–6	F. sh. ch. + 0.8 x 3–6	Sh. ch.; granular – 0.4 x 2–3

TABLE 1—*Continued*

	TWENTY-FOUR HOUR SUGAR BROTH CULTURES	FORTY-EIGHT HOUR SUGAR AGAR COLONIES	TWENTY-FOUR HOUR AGAR SLANT	YOUNG MILK CULTURES	OLD MILK CULTURES
Dog	F. sh. ch. + 0.5 x 1.5–2	No ch. + 0.8 x 2–4	F. sh. ch. + 7 x 1.5–4	Chains + 0.5 x 4	No ch. + & − 0.3 wide
R B	Pairs + 0.6 x 1–3	Pairs + 0.6 x 1.5–3	Sh. ch. + 0.6 x 1.5–3	Sh. ch. + 0.6 x 4–8	Sh. ch. + & − 0.5 x 4–8
R-1-5	Sh. ch. + 0.6 x 1.5–4	Sh. ch. + 0.6 x 1.5–3	Sh. ch. + 0.8 x 3–4	Chains + 0.6 wide	Chains + & − 0.6 x 3–4

The figures refer to the size in microns of individual bacteria.

+ and − mean Gram-positive and Gram-negative.

Ch. = chains; sh. = short; lo. = long; filam. = filaments; f. = few; v. = very; rds. = rods.

Gram stains of several members of each species were made from twenty-four hour broth cultures, agar colonies, agar slant growths and young and old litmus milk cultures. The size, orientation, and Gram-staining properties are given in table 1.

It is very evident that the morphological differences between these species are very slight and cannot be used as a basis for separation. The orientation and size of the various strains studied appear to be influenced to a large extent by the medium in which they are cultivated.

CULTURAL CHARACTERISTICS

The cultural characteristics of all organisms employed in this study were determined in sugar agar plates, potato, litmus milk, whey and in carbohydrate broth and gelatin. All cultures were incubated at 37°C. except where otherwise noted.

Potato. None of the strains of the two species grew on this medium.

Broth. Growth in whey-pepton and in sugar broths had the same general characteristics. The medium became turbid within twenty-four to forty-eight hours. On longer stand-

ing the liquid became clear. Some growth stuck to the walls of the test tubes; the remainder formed a sediment, either viscous or granular.

Litmus milk. This medium was curdled at 37°C. by all strains studied. The period of incubation before curdling occurred varied from twenty-four hours to several days.

The curd produced by *L. bulgaricus* was firm, with no extrusion of whey. *L. acidophilus* produced a softer curd, with slight extrusion of whey. The action by both organisms upon litmus was very much like that of *Streptococcus lacticus;* that is, reduction occurred with subsequent oxidation.

At room temperature (20° to 25°) growth, when evident, was very slow. B-3 curdled the milk in twelve days, B-4 and B-13 in thirty days; no other strain produced a curd after thirty days, and only a few caused a reddening of the litmus.

Slant Agar Growth. The cultures of both species showed practically the same characteristics. The agar was inoculated by drawing a loopful of a twenty-four hour broth culture over the surface. Growth was recorded after twenty-four hours incubation at 37°C. The growth was not heavy in any instance. B-13 and F gave a thin granular growth; while those of B-3, B-4 and B-12 were barely visible and appeared spicule-like. The growth of the remaining strains employed can best be described as being delicate and streptococcus-like to the naked eye or when slightly magnified.

Gelatin Stab Cultures. These were made from twenty-four hour slant agar cultures. The gelatin medium was incubated at 20°C. B-4, B-12 and S-1-3 did not reveal any growth in fifteen days, B-3, B-13, F, R-11 and R-1-1 showed development along the stab, with hair-like lateral branches. The growths of R-1-5, R B, B-1, P and B-A-3 were filiform. There was little or no development upon the surface of the medium and no evidence of liquefaction.

Agar Colonies. Characteristic colonies were formed by all of the *L. bulgaricus* strains but B-1. All colonies showed comparatively long interlacing and hair-like filaments radiating outward from a common center, best described as an extreme

"X" type. B-1 at times produced a solid colony with an even edge.

The different strains of *L. acidophilus* formed two main types of colonies, the almost smooth sand grain or streptococcus-like "Y" type and the filamentous "X" type; all graduations between these two were present. At times there also appeared small disc-like colonies. Surface colonies between the agar and the bottom of the Petri dish are often very filamentous and spreading. The variation in colony formation is a source of annoyance to anyone who is working with *L. acidophilus*, as the appearance of the "Y" type or disc-like colonies causes suspicion as to the purity of cultures. However, the colony type is not necessarily stable and re-isolations from any one type will at times produce colonies of either of the other two types. All primary isolations of the species labled *L. acidophilus* in this investigation were made from extreme "X" type colonies; yet the other two forms have appeared quite often in subsequent platings.

What the exact conditions are that determine the type of colony have not been discovered in this investigation. The "X" type appears more often in crowded plates, suggesting that metabolic products may influence the colony formation. This point, however, has not been confirmed.

PHYSIOLOGICAL ACTION

Fermentation studies. In the comparative study of members of the Lactobacillus genus, considerable weight has been given by many investigators to the fermentative action of these organisms in milk and other fermentable substances. Some workers have emphasized the ability to ferment definite substances as a basis of differentiation.

Grigoroff (1905) observed the necessity of sugar media for cultivation of his Bacillus A (*L. bulgaricus*) and Streptobacillus C. Bacillus A produced large quantities of lactic acid in milk, curdling it at a temperature of 42°C. in about five hours. This organism also attacked maltose, levulose and sucrose. It did not ferment rhamnose, dulcitol and sorbitol. The streptobacillus C curdled milk and attacked sucrose without inversion;

also glycerol and levulose. It had no action upon mannitol, maltose, rhamnose, dulcitol and sorbitol. From whey fermented by this organism, he secured a substance which gave the iodoform test for alcohol. Grigoroff makes no mention of gas formation.

Bertrand and Weisweiller (1906) reported that *L. bulgaricus* produced from 2.5 to 3 per cent lactic acid in milk, most of which was dextrorotatory. The organism also produced small amounts of succinic and formic acids in the same medium. These investigators claimed that this species acted by means of an intracellular lactase.

Cohendy (1906) isolated an organism from Bulgaricus milk which developed an acidity of 3.2 per cent as lactic acid in milk cultures incubated at 36°C. It also fermented maltose, sucrose levulose and glucose, and required carbohydrate for growth in any medium.

Bertrand and Duchacêk (1909) made an exhaustive study of the fermentative action of *L. bulgaricus* upon various carbohydrates and higher alcohols. They found that this organism produced inactive lactic acid with small amounts of formic, acetic and succinic acids from lactose, galactose, glucose, levulose and mannose. Levulose and mannose were less easily fermented than the others. Sucrose, maltose, the pentoses, sorbitol and mannitol were not acted upon. In milk the action of this organism was about the same except that the greater amount of the lactic acid was the dextrorotatory form.

Rahe (1914) made a thorough study of a number of species of the lactobacillus genus which he divided into three groups according to their action in milk and in a medium containing maltose.

Variety A clotted milk and produced no acid from maltose. Variety B clotted milk and produced acid from maltose. Variety C did not clot milk in six days and produced acid from maltose.

All strains of variety A were secured from type cultures of *L. bulgaricus*, fermented milks and bulgaricus tablets. There were 12 strains of this variety and it is of interest to note that

7 of these were reported as producing no acid in a levulose medium; 9 did not attack sucrose.

Strains of Variety B, 21 in number, were isolated from intestinal sources, with 2 exceptions. One of these was isolated from saliva and 1 from fermented milk. All of these fermented levulose, and all but 1, sucrose.

Variety C, 20 strains, with the exception of 2 from saliva and 1 from a tablet, were secured from intestinal sources. All of these strains fermented maltose and levulose, and all but 1, sucrose. Raffinose and mannitol were irregularly fermented by varieties B and C, while variety A did not attack these two substances at all. Variety A did not grow in sugar-free media; varieties B and C showed slight growth after seventy-two hours.

Moro (1900) stated that his original *L. acidophilus* produced acid in milk, curdling it in from three days to three weeks when incubated at 37°C. It developed considerable amounts of acid in carbohydrate media, from glucose and sucrose more readily than from lactose. No gas was formed by the organism in any medium studied. Kendall (1910) in a study of lactobacilli from the intestines corroborated Moro's conclusions.

Howe and Hatch (1917) reported that members of the Moro-Tissier group found in dental caries fermented, sucrose, glucose, lactose, maltose and raffinose with production of acid, but no gas.

Jötten (1922) observed that *L. acidophilus* and Döderlein's "Vaginalbacillus" fermented glucose, levulose, lactose, sucrose, maltose and mannitol. The pentoses and higher alcohols were not attacked. Glucose was fermented by a few strains of each species and glycogen by all strains of both. In milk culture incubated at 37°C. for fifteen days, the acidity ranged from 0.34 to 1.4 per cent, calculated as lactic acid.

Heineman and Ecker (1916) found that 3 strains of lactobacilli from gastric ulcers, which they called Boas-Oppler bacilli did not ferment maltose and produced 1.57 per cent levorotatory lactic acid in milk in from four to six days at 37°C.

The basic medium adopted for the fermentation studies in this investigation was a casein digest broth plus 0.1 to 0.15

per cent agar. All fermentable test substances were sterilized by filtration, to avoid decomposition of the carbohydrates. Five or 10 grams of test substance were dissolved in 100 cc. of distilled water. This solution was filtered through a sterilized Berkefeld candle into a sterile filtering flask. One-half cubic centimeter of this filtrate was added to each 5 cc. of sterile basic medium with a sterile pipette, making approximately a 0.5 or 1 per cent concentration of test substance. The medium prepared in this manner was always tested for sterility by incubation at 37°C. and at room temperature for at least two days before use.

Fresh skimmed milk, sterilized by autoclaving, was employed in the study of the action of *L. acidophilus* and *L. bulgaricus* on milk. Heavy inoculations are necessary in a study of these organisms, in order to secure good growth in a reasonably short period of time. In fact any thing short of the pipette method is inadequate. This applies not only to milk, but to all other media as well. The following method was used for supplying concentrated viable inoculum in small amounts without accompanying carbohydrates, etc. Young, vigorously growing broth cultures were centrifuged. After the removal of the supernatant liquid, the well-packed organisms were suspended in a sufficient amount of physiological saline solution to give the suspension a turbidity of about 6 on the McFarland nephelometer scale (1907); 0.1 cc. of this suspension was employed as the inoculum in all cases.

In order to determine to what extent the various test substances were broken down by heat during sterilization, control tubes of autoclave-sterilized casein-digest dilute agar containing the test substances were carried along with the adopted test medium in which the test substances had been sterilized by filtration. This brought out a very interesting point which will be discussed later.

The action of the species studied upon the various test substances in dilute agar was measured by the drop in the pH as determined by the colorimetric method of Clark and Lubs (1917). In milk, the acidity was determined by titration.

Preliminary experiments showed that 37°C. was the optimum temperature for all species used in this investigation. Therefore, the various casein digest dilute agar media were incubated at this temperature for seventy-two hours and the extent of growth and drop in the pH noted.

All determinations were carried out in duplicate or triplicate. A tube of uninoculated test medium as well as an inoculated tube of the basic medium were run with each strain studied. The purity and viability of each culture used for inoculum was tested by plating 0.1 cc. in galactose casein digest agar.

The following test substances of the highest purity were employed:

Hexoses
Glucose
Levulose
Galactose
Mannose

Disaccharides
Lactose
Maltose
Sucrose
Trehalose

Trisaccharides
Raffinose
Melezitose

Pentoses
Arabinose
Xylose

Polysaccharides
Dextrin

Alcohols
Glycerol
Mannitol
Dulcitol
Sorbitol
Rhamnose

Glucosides
Inosite

The results of several experiments conducted over a period of a year are summed up as follows:

ACTION IN VARIOUS CARBOHYDRATE DILUTE AGAR MEDIA

1. Dextrin was at best but slightly affected by the various strains; the results were variable.

2. Mannitol, arabinose, glycerol, dulcitol, inosite, rhamnose, xylose, sorbitol and melezitose were apparently not attacked by any of the strains.

3. All strains studied fermented levulose media with acid production when such media had been sterilized by heating. There were striking differences, however, when Berkefeld filtered

levulose was used. None of the *L. bulgaricus* strains fermented this medium when the pure unheated sugar was employed. Two samples of levulose which were not of highest purity were fermented by B-1 and F, even when these particular samples had been sterilized by filtration.

The fermentative action of some of the *L. bulgaricus* strains upon heated levulose was not as vigorous as upon other fermentable sugars; nevertheless, in practically every experiment in which heated levulose was employed there was an appreciable growth with a corresponding drop in the pH of the test medium. All the strains of *L. acidophilus* but two fermented both heated and unheated levulose media. The two exceptions were very slow growers, and this may in part at least account for their indifference.

4. Acid production in maltose media, which has been suggested by Rahe and others as an important differential test for the separation of the two species was somewhat disappointing from this standpoint. B-1 and B-13 attacked it vigorously while all other strains of *L. bulgaricus* failed entirely to act upon it. This is in agreement with previous experiences in this laboratory. These may be borderline types, especially B-1 which attacked sucrose. All strains of *L. acidophilus* employed fermented the maltose.

5. B-1 was the only *L. bulgaricus* species to produce acid from sucrose, while all strains of *L. acidophilus* gave positive acid production.

6. Lactose, glucose and galatose were broken down with production of acid by all members of the two species.

7. Trehalose showed the same variation with *L. bulgaricus;* as did maltose; *L. acidophilus* also was variable in attacking this sugar.

8. *L. bulgaricus* gave negative results in the raffinose medium; while *L. acidophilus* appeared quite variable in its action upon this sugar.

9. When there was acid production in any medium, the hydrogen-ion concentration varied considerably. However, the lower limit of the drop appeared to lie at about pH 3.6 to 4.0 for

all strains. This is in agreement with the findings of Clark (1916).

10. Glucose, galactose and lactose media in fermentation tubes were employed in an attempt to demonstrate gas production by members of these two species. There was complete absence of gas in all cases after twenty-four, forty-eight and seventy-two hour periods of incubation at 37°C.

11. With the exception of levulose and possibly mannose, sterilization of media containing the various fermentable substances by autoclaving at 15 pounds extra pressure for fifteen minutes did not appear to break down the test substances sufficiently to produce appreciable change in hydrogen-ion concentration when inoculated with members of the species that do not attack these sugars normally.

The study of the fermentative activity of the various strains of *L. acidophilus* and *L. bulgaricus* when grown in ordinary litmus milk gave the following results:

1. The milk generally curdled when the reaction reached about 0.5 per cent acid calculated as lactic acid by the titration method.

2. Acidity increased faster at 42° than at 37°C. but after a period of thirty days incubation the greatest acidity was obtained at the latter temperature.

3. All *L. bulgaricus* strains curdled milk within twenty-four hours at either 37° or 42°C.

The time required for curdling by *L. acidophilus* strains varied from forty-eight hours to ten days.

4. The acidity produced by different strains of *L. acidophilus* at 37°C. varied from 0.86 to 2.39 per cent; at 42°C. this acidity varied from 0.72 to 1.98 per cent.

5. *L. bulgaricus* strains developed at 37°C. from 1.82 to 3.15 per cent acidity; at 42°C. from 1.82 to 2.52 per cent acidity.

6. There was no formation of gas in milk by any strains employed in this investigation.

7. A distillate from whey broth cultures which had been incubated at 37°C. for twelve days gave an iodoform test for alcohol.

ACTION UPON MILK PROTEINS AND GELATIN; INDOL FORMATION

Grigoroff's (1905) Bacillus "A" did not produce indol and a casease could not be demonstrated. Cohendy (1906) stated that the species which he isolated did not visibly attack the casein in milk and had no action upon fibrin, egg white or sugar gelatin. Bertrand and Weisweiller (1906) reported that *L. bulgaricus* digested approximately one-tenth of the casein in ordinary milk culture.

Supplee (1917) noted that *L. bulgaricus* in a milk culture brought about a decrease in the casein and albumin nitrogen; there was a corresponding increase in pepton, diamino and ammonia nitrogen.

Tissier (1905) stated that *L. bifidus* attacked only proteoses, with liberation of ammonia. No indol was formed.

Kendall (1910) reported that *L. acidophilus* did not induce proteolysis and did not form indol from trytophane. Howe and Hatch (1917) in their study of the acidophilus-like organisms of dental caries, reported that these forms did not produce ammonia or indol in pepton media.

Because of the fact that normal members of these two species (*L. acidophilus* and *L. bulgaricus*) will not grow to any appreciable extent in sugar-free media it was necessary in this investigation to study their action upon gelatin and milk proteins in the presence of available sugars.

Ten per cent gelatin medium was made up with casein digest and 0.1 per cent galactose added before sterilization. This small amount of sugar had been determined as being sufficient for considerable growth of these organisms.

Skimmed milk was employed as the medium for observation of the action upon milk proteins.

Tubes of the sugar gelatin were inoculated with 0.1 cc. of a vigorous broth culture of the organism. Sufficient sterile lactic acid to make 3 per cent acidity was added to two tubes to be used as controls. All tubes were sealed by saturating the cotton plug with melted paraffin and then incubated at 37°C. for over a month.

The cultures were tested for liquefaction by removing to a cold place every four days and noting whether the medium became solid. No softening of the gelatin was induced by any of the strains employed after thirty days incubation. All cultures showed very good growth.

For the purpose of studying the action of both species upon the proteins of milk, the following procedure was adopted. Exactly 10 cc. quantities of skimmed milk were sterilized in cotton-plugged test tubes. Some of these were inoculated with 0.1 cc. broth cultures of F, H, B-1, Dog, R-1-1 and R-1-5. Varying amounts of sterile lactic acid were added to other tubes of milk so as to make the acid concentration in these tubes equal to 1, 2, 3, and 4 per cent. These acidified tubes and uninoculated, non-acidulated milk were taken as controls. All tubes were sealed with melted paraffin and placed at 37°C. where they were kept for three weeks.

At the end of this period the following nitrogen determinations were made. The entire contents of each tube were used for each determination so that no correction had to be made for evaporation. All determinations were made in duplicate.

1. Total nitrogen of the milk in two control tubes was determined by the Kjeldahl method.

2. Total protein nitrogen determinations were made of control tubes of 1, 2, 3 and 4 per cent acid milk and of each milk culture.

Ritthausen's method (Leffman and Beam) was employed. The proteins were precipitated by copper sulphate solution and sodium hydroxide. After filtering by decantation and washing Kjeldahl nitrogen determination was made upon filter paper and precipitate together.

3. Pepton and diamino nitrogen was determined in the filtrate from (2).

The filtrate was made acid with 0.5 cc. of 50 per cent sulphuric acid and 6 cc. of a 15 per cent solution of phosphotungstic acid added. After stirring well, this mixture was allowed to stand over night, so that the precipitate could settle out.

The supernatant liquid was poured upon a filter-paper and finally the precipitate itself was washed upon this paper by means of a wash-bottle containing a 2.5 per cent solution of phosphotungstic acid. After washing with this same solution, paper and precipitate were transferred to a flask and nitrogen determined by the Kjeldahl method.

The results shown in table 2 indicate some breaking down of the milk proteins, due to digestive action, by both *L. acidophilus* and *L. bulgaricus*. The acidified controls showed an

TABLE 2

Showing action of L. acidophilus and L. bulgaricus on milk proteins

	TOTAL NITROGEN	TOTAL PROTEIN NITROGEN		PEPTON DIAMINO NITROGEN		AMINO, AMMONIA, ETC., NITROGEN	
	mgm.	*mgm.*	*per cent* T. N.	*mgm.*	*per cent* T. N.	*mgm.*	*per cent* T. N.
Control	527.1	489.3	92.8	17.5	3.32	20.3	3.85
H	527.1	447.3	84.9	35.0	6.64	44.8	8.50
B-1	527.1	443.1	84.1	37.1	7.04	46.9	8.90
F	527.1	445.7	84.6	28.0	5.31	53.4	10.1
Dog	527.1	455.7	86.5	37.8	7.17	33.6	6.38
R-1-1	527.1	430.5	81.7	55.3	10.5	41.3	7.84
R-1-5	527.1	463.6	88.0	33.6	6.37	29.9	5.67
1 per cent lactic	527.1	480.2	91.1	25.9	4.91	21.0	3.99
2 per cent lactic	527.1	485.8	92.2	23.8	4.51	17.5	3.32
3 per cent lactic	527.1	472.5	89.6	38.5	7.30	16.1	3.06
4 per cent lactic	527.1	470.4	89.2	41.6	7.89	15.1	2.87

T. N. = total nitrogen.

Results are given in milligrams of nitrogen per 100 cc. of milk and in percentage of total nitrogen.

increase in the pepton and diamino nitrogen fraction, the greatest being in the case of the 4 per cent acid milk. With the exception of R-1-1, there was no great difference in this respect between the artificially acidified milk and the inoculated milk. However, taking into consideration that the acidity of the acidophilus milk was not over 2 per cent and that of the bulgaricus milk not over 2.8 per cent, there is some evidence that these organisms have produced more pepton and diamino acids from the milk proteins than is due to acidity alone.

The greatest evidence of digestion by these organisms is in the amounts of residual nitrogen, after the total protein and pepton-diamino fractions have been determined. The acidified milk gave practically the same amount as the control, while there was a considerable increase in the inoculated milk.

A preliminary experiment had shown that there was very little ammonia nitrogen in the inoculated milk; therefore, the greater part of this residual nitrogen was probably non-amino nitrogen. The increase of the inoculated milk over the control milk can be stated as due to a digestion of the milk proteins (presumably casein) by these organisms.

It may be stated that approximately from 2 to 6 per cent of milk proteins is broken down by *L. acidophilus* and *L. bulgaricus*.

Indol production of these species was studied by growing the various strains for five days at 37°C. in 0.1 per cent galactose casein digest broth, and in the same medium plus a trace of added tryptophane. Negative results were secured in every instance.

VIABILITY AND THERMAL DEATH POINTS

The viability of different strains of each species was determined in milk, in Klim digest and in whey pepton broths.

Milk cultures of *L. acidophilus* and *L. bulgaricus* were kept at room temperature and tested for viability by withdrawing 1 cc. and transferring to sterile litmus milk. Growth was indicated by the production of a typical curd. *L. acidophilus* was found to be decidedly viable after three months, while *L. bulgaricus* died off in about six weeks, under the conditions under which the experiment was carried out.

Viability tests on milk cultures kept at 37° and at 42°C. also showed a longer duration of life of *L. acidophilus* than of *L. bulgaricus* under the given conditions. All of the strains of the latter organism failed to revive after fourteen days incubation at both 37° and 42°C., while all of the 15 acidophilus strains grown at 37°C. gave the milk curdling test after eighteen days, and all but 3 were positive at the end of eighteen days incubation at 42°C. Furthermore, none of the *L. bulgari-*

cus strains developed on agar plates when plated out after thirty days incubation at 37° and 42°C., while 9 of the 15 strains of *L. acidophilus* employed gave colonies on the agar plates.

In Klim digest broth cultures kept at refrigerator (8° to 10°C.) and at room temperature there was little difference in the viability of the two species. The *L. bulgaricus* cultures remained viable three, four and five days in the ice box and two, three and four days at room temperature, and *L. acidophilus* three, four and five days and two, four and six days, respectively.

In pepton whey broth the viability of both species was distinctly greater than in Klim digest broth, due in part at least, to the buffering action of the pepton. *L. bulgaricus* remained viable for six, seventeen and forty-two, and three, eight and thirty days, and *L. acidophilus* for six, seventeen, thirty and forty-two, and three, six, seventeen and thirty-five days, respectively, at the ice box and room temperatures.

In ordinary sugar broths viability persists for only a very short time. Milk is preëminently the best medium for keeping the organisms of both species alive. According to Jones (1916) this is due to the buffer action of the milk proteins, which prevent dissociation of H-ions and therefore enable bacteria to grow and continue using carbohydrates.

The resistance of several strains to drying was determined in the following manner. A comparison was made between centrifuged twenty-four hour broth cultures suspended in saline solution and twenty-four hour pepton whey broth cultures. Pieces of sterile filter paper were saturated with very heavy cultures, prepared as noted. These pieces of paper were kept at room temperature and allowed to air-dry. Platings were made by mixing several pieces of paper in agar and incubating at 37° to 40°C. This procedure was repeated at intervals of a day or two until platings finally revealed no growth.

No colonies appeared on any of the plates from paper plated out after two days drying. Desiccation therefore, quickly killed off both species.

The thermal death point of three representative strains of each species was ascertained. According to the results, *L.*

acidophilus has a somewhat higher thermal death point than *L. bulgaricus*, namely 63° to 65°C. as compared to 57° to 61°C. This difference may possibly be explained as being due to a difference in the hydrogen ion concentration of the twenty-four hour cultures preliminary to centrifuging.

SEROLOGICAL RELATIONSHIPS

An exhaustive review of the literature covering the investigations dealing with *L. acidophilus* and *L. bulgaricus* reveals practically nothing in regard to their serological relationships. Jötten (1922) reports that the complement binding properties of *L. acidophilus* and Döderlein's bacillus justify the classification of these forms as one and the same organism.

Jötten immunized rabbits by giving 9 intravenous injections of saline suspension of agar slant cultures. He attempted agglutination studies with the sera obtained, but failed because the antigens agglutinated spontaneously. He was also unable to secure any positive results from the precipitin reactions. In complement fixation studies he obtained complete cross-fixation with sera and antigens of both species. However, he does not give complete details as to the technic which he followed.

In the present investigation an attempt was made to study the serological reactions of the sera of two lots of rabbits, one group of which had been immunized against *L. acidophilus* and the other against *L. bulgaricus* by repeated intravenous injections of cultures of these organisms.

The first experiment on serological relationships was begun with the idea of making an agglutination study. The antigens were secured in the same manner as the antigens for immunization. The organisms were suspended in phenolized saline for preservation. For use in the test, very light suspensions (nephelometer 0.3) were employed. The agglutination tests had to be abandoned, however, owing to spontaneous agglutination of antigens.

Complement fixation tests were conducted with autolyzed antigen which was prepared by suspending centrifuged cells

from sugar broth cultures in saline solution, adding sodium carbonate to give a pH of 9.5, and keeping the alkaline suspension at 37°C. for four days. The extraction of soluble antigen was in all probability due to solution by the carbonate as well as to actual autolysis. The antigens were tested before incubation by Gram staining and found to be made up of even staining, strongly Gram-positive rods. After the four days incubation they were again examined. Practically all of the cells were Gram-negative and some showed complete disruption. Two

Complement fixation results

		F SERUM DILUTED 1:25 0.4 CC. USED	D SERUM DILUTED 1:10 0.4 CC. USED	R-1-1 SERUM DILUTED 1:20 0.3 CC. USED
Bulgaricus	B-1	0	3+	4+
	B-3	4+	3+	4+
	B-4	4+	3+	4+
	B-12	4+	3+	3+
	B-13	4+	2+	4+
	F	4+	3+	3+
	H	4+	3+	2+
Acidophilus	R-1-1	3+	4+	4+
	D	3+	4+	4+
	S-1	3+	3+	4+
	R-1-5	3+	4+	4+
	R B	4+	4+	4+
	B-A-3	0	3+	+
	L-43	0	0	2+

of the antigens (R-1-1 and Dog), were practically clear solutions after incubation: the third showed more or less turbidity.

Four rabbits were immunized by the same method as was used before, with R-1-1, Dog, F, and H strains. Intravenous injections were made at four day intervals until 9 injections had been given. Eight days after the last immunizing dose, the animals were bled and the sera secured as in the former experiment. No preservative was added to these sera.

Serum H did not prove to be strong enough for use. There-

fore three sera were employed in the complement fixation tests. F was bulgaricus immune serum, D and R-1-1 were acidophilus sera. These sera were not anticomplementary in any dilution employed.

The homologous antigen and thirteen heterologous antigens were tested for their complement fixing powers with each serum. Three tenths cc. of antigen was adopted as the antigenic unit. B-A-3 and L-43 were markedly anticomplementary in amounts over 0.6 cc., while none of the others showed anticomplementary action in 1 cc. amounts.

This table shows positive fixation between heterologous antigens and sera in nearly all tests. B-A-3, L-43 and possibly B-1 apparently do not belong to either of these two groups, in so far as serological tests show. If any weight is to be given to cross fixation, this set of experiments indicates a close relationship between the other 11 strains employed (6 of *L. bulgaricus* and 5 of *L. ancidophilus*).

DISCUSSION

The results of this investigation indicate a very close relationship between the members of the *L. acidophilus* and *L. bulgaricus* types studied.

The strains used are fairly representative of the organisms found in fermented milks and in the intestines of man and animals, characterized by the production, in part or altogether, of sea-urchin-like colonies in agar and in gelatin media. The *L. bulgaricus* type appears to be more constant than the *L. acidophilus* type in this respect.

The morphology of the two groups is quite similar.

There are differences in their action upon maltose, sucrose and unheated levulose. Other physiological reactions differ only in degree.

The results of complement fixation tests show cross-fixation between heterologous immune sera and antigens. There is a quantitative difference in some cases, but this is no greater than would be expected between different strains of the same species.

The one outstanding difference between the two types is their habitat. In no case in the course of this investigation has an organism been isolated from feces which had the characteristics of a typical *L. bulgaricus* strain. Herter and Kendall (1909), Rahe (1915) and Rettger and Cheplin (1921) have proved that *L. bulgaricus* cannot be implanted in the intestines of man or animals. The present authors in the course of an unfinished experiment were not able to recover a non-maltose fermenting *L. bulgaricus* from the feces of white rats after two weeks feeding of large milk cultures of *L. bulgaricus* plus lactose.

On the other hand, the ingestion of milk or broth cultures of *L. acidophilus*, or the use of a high lactose or dextrin diet alone, (Rettger and Cheplin) brings about a transformation of the intestinal flora to such an extent that *L. acidophilus* becomes the predominating organism. Therefore, it can be concluded that the normal habitat of *L. acidophilus* is the intestine of man and animals. Other Gram-positive bacteria present are not to be considered as the same species. These can be excluded by means of their colony formation. This will eliminate such types as L-43 and B-A-3 employed in this study.

From the manner in which the organism grows in milk this medium can be considered as giving optimum conditions for the growth of *L. bulgaricus*. However, this does not explain its original source.

The question of the origin of *L. bulgaricus* can only be a matter of conjecture. Was it always a so-called saprophyte, living in milk, possibly coming from soil? Or, is it a degenerate form of *L. acidophilus*, requiring milk as a food because of long acclimatization to this medium? The authors offer the latter as a suggestion for the following reasons.

1. In milk, *L. bulgaricus* has lactose and the hydrolysis products of this sugar, galactose and glucose, to supply its carbohydrate requirements. There is no levulose or maltose in milk. Therefore, because of years of sojourn without these sugars, *L. bulgaricus* may have lost its power to utilize them.

2. As has been stated, *L. bulgaricus* grows best in milk. On the other hand *L. acidophilus*, which in primary isolation grows

poorly in milk, can become acclimated so that it develops very rapidly.

3. *L. acidophilus* grows well in practically all ordinary sugar media. In the intestine it has a great variety of nitrogenous food to choose from and therefore can adapt itself to various nitrogenous constituents in media. *L. bulgaricus* on the other hand developed poorly or not at all in our experiments except where the nitrogenous constituents were derived from milk. Is this fastidiousness not due perhaps to the same cause as its inability to use sugar other than lactose or its immediate derivatives?

Heineman and Hefferan concluded that the whole group of lactobacilli should be placed in one species, *L. bulgaricus*.

From a study of the descriptions given of *B. lebenis*, *Streptobacillus* lebenis, *Bact. mazun*, *Bact. caucasicum*, *B. casei* (epsilon), etc., there can be little doubt that these organisms are identical with *L. bulgaricus* (Massol). The type isolated by various investigators from intestinal contents, which produces the "X" type colony, and probably the "Y" type as well, is without question *L. acidophilus* (Moro.) Furthermore, the Boas-Oppler bacillus and Döderlein's bacillus may be included in this division. It is quite probable that the aciduric silage bacteria, organisms isolated from dental caries, *B. panisfermentati*, etc., are not identical with either *L. bulgaricus* or *L. acidophilus*.

If *Bact. abortum* and *Bact. melitensis*, as well as *Bact. coli* and *Bact. aerogenes* and certain other closely related members of the colon-typhoid group are to be considered as separate species, then *L. acidophilus* and *L. bulgaricus* may also be considered as distinct and separate species.

However, the authors suggest that the Gram-positive rods designated *L. bulgaricus* and *L. acidophilus* be placed in the same species, with *L. acidophilus* as the central type. *L. bulgaricus* may be considered a variant of this type, variation being due to long culturing in milk. The chief differential characteristic of this variant is the fact that it has lost its ability to develop in the intestine. The central type, *L. acidophilus*, is a normal intestinal form which can continue to develop in the intestine, even when supplied as a milk culture.

SUMMARY AND CONCLUSIONS

In the comparative study of *L. acidophilus* and *L. bulgaricus* the following points received chief emphasis.

a. Development of suitable media
b. Source and habitat
c. Morphology and cultural characteristics
d. Physiological action
e. Viability and thermal death points
f. Immunological relationships

The results may be stated briefly as follows:

1. Casein digest media proved to be particularly valuable in the present study of *L. acidophilus* and *L. bulgaricus.*

2. These two species are quite similar in morphology and cultural characteristics. Differences, when observed, were quantitative rather than qualitative.

3. The action upon maltose, sucrose, and levulose appears to furnish a valuable means of separating strains of these two groups, which are now being employed in the production of fermented milks. In other physiological aspects they are very much alike.

4. The viability and thermal death points are somewhat variable for different strains of each species.

5. The immunological studies showed no greater difference between *L. bulgaricus* and *L. acidophilus* than would be expected to exist between members of one and the same species.

6. In addition to being unlike in their fermentative action upon the three sugars mentioned, they differ in another very important respect, namely in that *L. acidophilus* can and does live and develop in the intestine of man and animals, whereas *L. bulgaricus* is unable to do so.

7. The differences noted between these two types constitute evidence enough for classifying them as separate species. However, because of the various close relationships, it is suggested that these two types be placed in one and the same species of which *L. acidophilus* is the central type and *L. bulgaricus* a variant.

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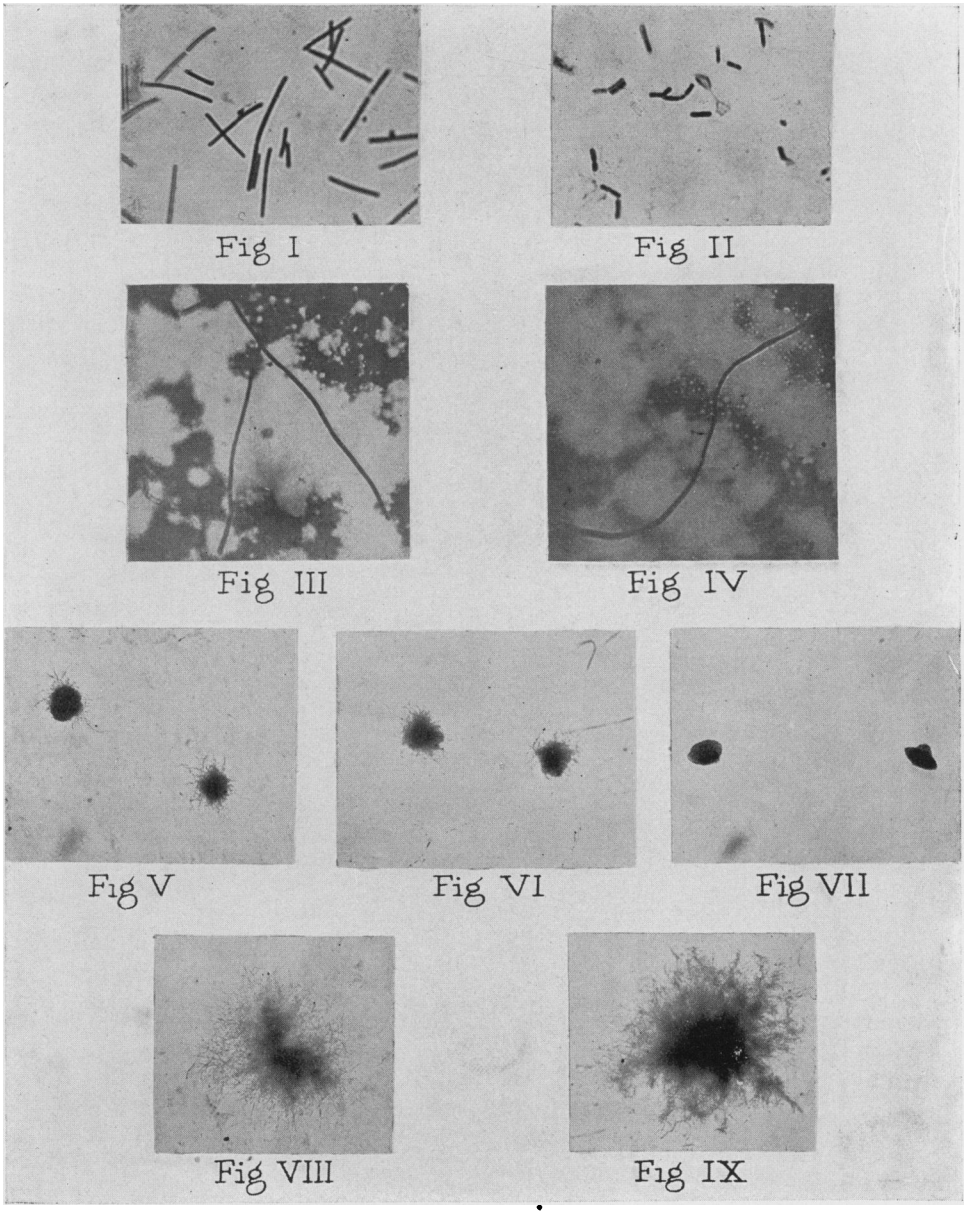
PLATE 1

FIG. 1. L. bulgaricus from a twenty-four hour broth culture (strain F).
FIG. 2. L. acidophilus from a twenty-four hour broth culture (strain R-1-1).
FIG. 3. L. bulgaricus from a twenty-four hour milk culture (strain F).
FIG. 4. L. acidophilus from a twenty-four hour milk culture (strain R-1-1).
FIG. 5. "X" type agar colonies of L. acidophilus (strain R-1-5).
FIG. 6. "X" type agar colonies of L. acidophilus (strain D).
FIG. 7. "Y" type and disc-like agar colonies of L. acidophilus (strain R-1-1).
FIG. 8. Agar colony of L. bulgaricus (strain B-12).
FIG. 9. Agar colony of L. acidophilus (strain S-d).



(Kulp and Rettger: Lactobacillus acidophilus and bulgaricus)