

## LACTOBACILLUS BIFIDUS<sup>1</sup>

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Received for publication June 2, 1934

The Lactobacillus genus constitutes one of the most widely distributed groups of known microorganisms. The group as a whole is primarily fermentative in character, and displays a marked degree of tolerance for acids, hence the term "aciduric," which is now generally applied to it. Members of this genus vary widely in their physiological activities and in their habitat. Some are characteristically milk and dairy products types; others are intimately associated with raw cereals and vegetables; others are of oral and dental origin; and still others are primarily intestinal in type. The intestinal types (*L. bifidus* and *L. acidophilus*) constitute the material for the present study.

An intensive review of the literature on aciduric bacteria of the intestine reveals almost hopeless confusion concerning the nature and biological position of *L. bifidus* of Tissier and, in spite of the large number of papers that have been published, its entity is still far from being established. For a more extensive review of the literature than space here permits the reader is referred to the senior author's doctorate thesis deposited in the Yale University Library.

Tissier (1899-1900) first isolated and described this organism and, because of its tendency to branch, called it *B. bifidus*. He classified it as a non-motile, non-spore-forming anaerobic pleomorphic rod which is Gram-positive in young cultures. In 1905 Tissier reported finding *L. bifidus* as the predominating organism in breast-fed, and *L. acidophilus* in bottle-fed, children.

<sup>1</sup> 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.

Moro (1900) reported the isolation of a Gram-positive rod from the stools of breast-fed infants which had many points of resemblance with *L. bifidus*. Because of its unusual acid tolerance, he called it *B. acidophilus*. Moro was of the opinion that his *B. acidophilus* was the predominant organism in the feces of breast-fed infants, and for a long time held to this view. Rodella (1908) could find no differences between the Boas-Oppler bacillus, *B. bifidus*, and *B. acidophilus*, in so far as their morphological and cultural characteristics were concerned. He found branching forms of *B. acidophilus*.

Many later investigators claimed to have isolated *L. bifidus*, but very few have given any detailed description of the organism. It has been described as an anaerobe, an aerobe, as branching, and as not branching; it has been placed by some in the *Lactobacillus* genus; by others among the bacteroides and again by others in the coli-typhi group.

Among the more recent contributions dealing with *L. bifidus* as a definite entity is that of Cruickshank (1925). This author reported finding *L. bifidus* present regularly in the stools of breast-fed infants, at times composing almost 99 per cent of the flora. He stated that *L. bifidus* is a strict anaerobe in primary culture, but that thereafter it grows readily in the presence of free oxygen. He described its varied morphology, but did not stress branching, thinking that this was unusual. He believed that the organism closely resembles *B. acidophilus*, but that it differs from the latter in morphology and certain cultural characteristics. According to him, *L. bifidus* is not a well-defined group serologically.

#### ISOLATION OF LACTOBACILLUS BIFIDUS

The following methods were employed: (1) that of Tissier (1899-1900) which involves the use of glucose-infusion agar in Veillon tubes; (2) the procedure of Torrey (1917) in which acid liver-infusion glucose agar to which 10 per cent of rabbit's blood is added is employed; (3) the Stransky and Maslowski (1927) modification of the Adam method (1921-22-23), which makes use of milk whey containing marble chips, followed, after twenty-four

hours' incubation, by inoculation into deep glucose agar tubes, and (4) the tomato-broth isolation method devised in this laboratory and based largely on the tomato-broth medium of Kulp (1927).

Since the tomato medium, both with and without agar, proved to be particularly useful and satisfactory in this investigation, the method of its preparation is given here briefly.

*Preparation of tomato-broth medium.* The contents of a large can of tomatoes are filtered through coarse filter paper. The clear filtrate is diluted with distilled water, 800 cc. of water being added to 200 cc. of the filtered tomato juice. To this are added 1 per cent peptonized milk (Difco), 0.5 per cent yeast extract (Difco), and 0.5 per cent peptone (Difco). The medium is adjusted to pH 8.0, tubed and autoclaved. For use as agar medium, 2 per cent Difco granular agar is dissolved in the liquid medium. The tubes are sterilized by autoclaving for fifteen minutes at 15 to 18 pounds extra pressure. The final pH of the broth and agar media is 6.8, the autoclaving having a definite pH depressant effect.

The tomato broth and agar offer a particular advantage in the isolation of intestinal lactobacilli in that they contain favorable food, especially a liberal amount of available carbohydrate which furnishes sufficient acid during the cultural period to depress the pH to 3.6 to 4.0, a level at which the lactobacilli are not destroyed, while other intestinal bacteria are held in check or actually killed.

*Isolation procedure.* A liberal amount of nursling's stool is placed in the tomato broth and the inoculated tubes held under anaerobic conditions at 37°C. for one week. Anaerobiosis is obtained by the use of anaerobic jars and a vacuum pump. At the end of the incubation period a loopful from each tube is streaked over the surface of tomato agar in Petri plates, which are then incubated anaerobically at 37°C. for seventy-two hours. Typical colonies are selected and grown in tomato broth. Alternate restreaking of agar and growth in the liquid medium are continued until pure cultures are obtained. This method, although relatively slow, has proven to be thoroughly dependable

for the isolation of *L. bifidus* from infants' feces. The Veillon tube method, while much shorter, does not lend itself well to isolation.

Most of the strains used in this investigation were obtained by the tomato-broth isolation method. Anaerobiosis was found to be necessary during isolation, and greatly facilitated growth during the next two or three transfers.

*Sources of strains of L. bifidus.* The fecal specimens from which all human strains were isolated were obtained from the Maternity Wards of the Philadelphia General Hospital. The human strains were numbered 1 to 31, in the order of isolation.

For comparison with the human strains, several strains of *L. bifidus* were obtained from white rats, according to the procedure of Rettger and Cheplin (1921). This method consisted in feeding the rats a diet high in lactose and making isolations by the Veillon tube and acid broth methods. The observations of Hull and Rettger (1914) and of Rettger and Cheplin that *L. bifidus* will develop in the intestine of rats receiving a high lactose diet, were confirmed; 13 strains were isolated from rat feces; these were numbered 1R to 13R, in the order of isolation. Attempts to isolate *L. bifidus* from the feces of normal monkeys, chimpanzees, baboons, horses, dogs, cats and cows were fruitless. Furthermore, no cultures of *L. bifidus* could be secured from other laboratories. Several strains of *L. acidophilus* were employed in this investigation. These were Scavano, R-1-1, R-1-5, TA, LWS and McGrath, all stock strains of this laboratory.

The stock strains of *L. bifidus* and *L. acidophilus* were kept in litmus milk, transfers being made every two weeks. *L. bifidus* grew well in this medium, the majority of strains producing curds within two to three days. In transferring the stock cultures, 1 cc. amounts were always used.

#### OXYGEN REQUIREMENTS

The literature appears to be about equally divided regarding the oxygen requirements of *L. bifidus*. Tissier (1899), Cahn (1901), Jacobson (1908), Distaso (1911), Logan (1913), Adam (1922), Stransky and Maslowski (1927) and others believed this

organism to be a strict anaerobe. On the other hand, Passini (1903), Mereshkowsky (1905 and 1906), Noguchi (1910), Basten (1914), Kuthe (1915), Torrey (1917) Howe and Hatch (1917), Brown and Bosworth (1922), Webster (1923), Cruickshank (1925), Gerstly, Howell and Nagel (1932) and others concluded that *L. bifidus* is either an aerobe or a facultative anaerobe.

Like *L. acidophilus*, *L. bifidus* requires an unusually favorable medium for maximum growth. The lack of such a medium has, without doubt, been the reason why many investigators have concluded that *L. bifidus* is an anaerobe. It grows aerobically on a rich and suitable medium, except in primary culture, when it requires anaerobic conditions. It is expedient to continue the anaerobiosis for several transfers after isolation. Triplicate cultures placed under strictly anaerobic, aerobic and partially anaerobic (air and 10 to 20 per cent CO<sub>2</sub>) conditions showed about equal growths, the partially anaerobic conditions proving slightly more favorable than the others. We may safely conclude that *L. bifidus* is a facultative anaerobe. In this respect it does not differ from *L. acidophilus*. Most of the present experimental work was carried out under partially anaerobic conditions.

#### MORPHOLOGY

According to Tissier and others, *L. bifidus* is decidedly pleomorphic, true branching, club and racquet forms being not at all uncommon. These claims, particularly those regarding true branching, have been disputed by various investigators; the morphology of *L. bifidus* received special attention, therefore, in this investigation.

Stained films made directly from the feces of breast-fed infants (fig. 1) reveal, as a rule, an almost pure picture (90 to 95 per cent) of slender, straight and comma-shaped, Gram-positive rods which vary from 2  $\mu$  to 8  $\mu$  in length (average 3  $\mu$  to 5  $\mu$ ); and from 0.5  $\mu$  to 0.7  $\mu$  in width. The rods possess rounded ends, with an occasional bulbous thickening at one extremity. The orientation of the individual cells is often such as to suggest branching in the form of a Y or T. Real branching may be seen at times; this is usually of the Y type. Chain formation is

absent, or at best very rare. Some of the cells stain unevenly and thus assume a granular or beaded appearance; others may be completely Gram-negative.

Films prepared from pure young cultures in broth, milk and agar reveal, on the whole, a morphology somewhat similar to that observed directly in nursling's stools. The rods are more variable as to length, some approaching coccoid forms and others attaining  $8 \mu$  to  $10 \mu$  in length. The staining is quite uniform in young cultures, granular or beaded forms being not as common as in fecal slides. While the cells are distinctly Gram-positive, it is relatively easy to decolorize them completely with alcohol. There is some tendency to chain formation, but not so marked as that displayed by *L. acidophilus*. No true branching has been observed by us in pure broth, agar and milk cultures of *L. bifidus*. Most of the rods are curved, comma and S forms being quite common. Older cultures show more variable cells, which tend toward the filamentous type.

The microscopic picture presented by young and old cultures of *L. acidophilus* does not differ markedly from that of *L. bifidus*. Both show a variable morphology. There is a greater tendency toward chain formation by *L. acidophilus* in the young than in the older cultures; branching is extremely rare. This organism resembles *L. bifidus* further in that older cultures lose their Gram-positive character and tend to become filamentous. Furthermore, granule formation is quite common. It would be very difficult to differentiate *L. bifidus* from *L. acidophilus* on the basis of morphology.

Repeated failures to demonstrate true branching of *L. bifidus* led the writers to revert to the method employed by Tissier for the isolation and study of this organism. Accordingly, nurslings' feces were inoculated into Veillon tubes containing glucose infusion agar, and the tubes incubated at  $37^{\circ}\text{C}$ . for seventy-two hours. Stained slides were prepared from the small, white oval and lens-shaped colonies (fig. 2). The microscopic picture observed in such films resembled that so clearly described by Tissier, except that the rods possessed rounded, instead of pointed, ends. Various kinds of branching forms were of common occurrence,

particularly the Y and T. The Y forms were the most numerous, and their ends were often distinctly thickened or club-shaped. Some cells exhibited branching and re-branching. While the branches were usually short, some attained considerable length.

Tissier's claims that *L. bifidus* reveals a decidedly varied morphology and true branching were further confirmed on examination of stained films prepared from primary colonies grown on Torrey's medium (fig. 3). The morphology of the cells in these colonies was even more extreme than that observed in the Veillon tubes, branching and re-branching being quite common. Subcultures prepared on the routine media from the Veillon tubes and the original platings with Torrey's medium showed little pleomorphism, and no branching.

It had been assumed by us that *L. bifidus* could be distinguished from *L. acidophilus* on the basis of extreme pleomorphism and branching. However, when human feces containing *L. acidophilus* were inoculated into Veillon tubes containing glucose infusion agar, and the tubes incubated for seventy-two hours, branching forms were readily obtained from the acidophilus colonies (fig. 4). Many types of cells were seen, including small and large Y and T forms, as well as clubbed, knobbed, vesicular and other forms. In fact the microscopic picture presented by *L. bifidus* was duplicated here. These observations were repeated many times with different samples of feces.

#### CULTURAL CHARACTERISTICS

The colonies of *L. bifidus* have been described as being oval or lenticular, and smooth. Most investigators have confined their studies on colonial form to growth in deep glucose infusion agar. It has been observed, however, that when a medium is used which contains a readily utilizable carbohydrate, and in which the organism grows abundantly, *L. bifidus* produces more or less rough colonies which may resemble those of *L. acidophilus*.

In the present investigation *L. bifidus* was observed to form small, white, round or oval colonies in deep glucose beef-infusion agar in Veillon tubes, which could be seen at times only by means of a magnifying lens. The colonies are fairly smooth, and almost

entirely solid; they usually possess a delicate plumose or filamentous border. Surface (streak) colonies on glucose beef-infusion agar are larger, quite granular, and have an irregular (not filamentous) edge. On tomato peptonized milk agar in poured plates the subsurface colonies (fig. 5) bear considerable resemblance to the xy type of *L. acidophilus*, being comprised of a solid center and filamentous border. The X or highly filamentous type of colony which is so common with *L. acidophilus* (fig. 6) has seldom been observed by us in *L. bifidus*. Many different factors influence the size of the colonies, such as crowding, length of time during which the strain has been artificially cultured, the medium used, temperature of incubation, and inherent peculiarities of the different strains. The fully developed colonies measure from 1 to 3 mm. in diameter.

Typical mature (seventy-two hours) surface colonies of *L. bifidus* on agar streak plates differ more or less, macroscopically, from those of *L. acidophilus*. The former appear quite solid, dark and granular, with a slightly irregular edge; they are disc-wheel-like and average from 2 to 3 mm. in diameter. *L. acidophilus* on the other hand, produces flat, thin, spreading colonies having a distinctly irregular or wavy edge, and are from 2 to 4 mm. in diameter. Under the microscope the colonies of the two types appear quite granular and possess a more or less plumose or filamentous border, the rough characteristic being much more pronounced in the acidophilus than in the bifidus colonies. (See figs. 7 and 8.)

*L. bifidus* produces a distinct growth in tomato broth in twenty-four hours, with general turbidity and a slight sediment formation. At the end of six days the broth is practically clear, the growth having settled out. The optimum temperature is 37°C. Very little or no growth occurs at 20° to 22°C. The 37° culture resembles that of *L. acidophilus* grown at the same temperature. No visible growth is produced by either organism in *sugar-free* meat extract, beef infusion or yeast-extract broth or agar during incubation at 37°C. for five days. In yeast-extract broth and agar containing galactose both organisms grow well.

Litmus milk was curdled at 37°C. by nearly all of the strains



of *L. bifidus*, and by all of the strains of *L. acidophilus*. The length of time required for curdling varied from twenty-four hours to several days. *L. acidophilus* regularly produced a solid curd and a pleasant sour odor. Only about half of the *L. bifidus* strains developed a solid curd, while the remainder formed a more or less lumpy curd. Torrey claimed to be able to differentiate *L. bifidus* from *L. acidophilus* by the growth produced on glucose blood liver-infusion agar. In our study the two organisms showed about the same type of colony, except that *L. bifidus* usually produced a greenish zone around the colony, while *L. acidophilus* did not. However, this difference may have been due to the more rapid development of *L. bifidus* on this medium.

#### FERMENTATION STUDIES

In these studies 31 strains of *L. bifidus* and 5 of *L. acidophilus* were used against twenty-one sugars and alcohols. The sugars were sterilized as 10 per cent water solutions, and added aseptically to yeast extract broth. Galactose, inulin, xylose, arabinose, levulose and rhamnose underwent a slight breakdown on steam sterilization; hence, solutions of these sugars were sterilized by filtration. The inoculums were prepared by centrifuging the growth obtained in tomato broth, washing with sterile physiological salt solution, recentrifuging and rewashing until the washings were neutral. The cells were then suspended in saline solution, each suspension being made up to a turbidity of 2.0 on the McFarland nephelometer scale, and 0.5 cc. of this suspension added to each medium. Brom-thymol-blue was employed as indicator. Uninoculated controls were run for each set of sugars, as well as an inoculated tomato-broth tube, to check the purity of each strain. Readings were made at twenty-four, forty-eight and seventy-two hours, and after one week. All of these tests were carried out twice, with a three months' interval between tests.

All of the strains of *L. bifidus* fermented galactose, inulin and levulose; nearly all attacked sucrose; a large majority formed acid in starch, dextrin, maltose, glucose, raffinose and trehalose. Some strains broke down lactose and salicin readily; an oc-

casional strain attacked mannitol, glycerol and rhamnose. None acted upon amygdalin, xylose, dulcitol, arabinose, inositol and melizitose. When the results of the fermentation tests with *L. acidophilus* are compared with those of *L. bifidus*, only one difference is observed; a greater proportion of the strains of *L. acidophilus* than of *L. bifidus* visibly attacked lactose. This may have been due, however, to the fact that the acidophilus strains have long been kept in milk and have therefore developed a pronounced lactose-splitting power. Some strains of *L. bifidus* differed markedly from some strains of *L. acidophilus*, in their carbohydrate properties, but at the same time different strains of *L. bifidus* differed materially from each other. With such differences between strains of the same species, one could hardly hope to differentiate these two organisms by the fermentation test. No gas formation occurred. The sugar-containing broths which were not fermented showed no growth; hence, growth in itself should be a good indication of the utilization of a carbohydrate by these two closely related organisms.

#### AGGLUTINATION TESTS

The immunological reactions of *L. bifidus* have been studied very little, and most of the investigators who made such studies used only a few strains, and generally only one antiserum. For the most part, the results seem to point to the fact that *L. bifidus* strains vary immunologically among themselves, and show a close general relationship to *L. acidophilus*. In the present investigation 12 antisera were prepared (9 of *L. bifidus* and 3 of *L. acidophilus*). These antisera were obtained in the usual way, rabbits receiving 3 injections weekly of large amounts of washed cells (dead and alive) in the marginal ear vein until a total of 16 injections had been given. Ten days after the last injection the rabbits were bled from the heart, and the serum separated and preserved with phenol. Antigens were prepared in distilled water, after some experimentation with physiological salt and various buffer solutions. The distilled water was found to be the most satisfactory for holding the lactobacilli in suspension. It may be objected to on the grounds that it is relatively free

TABLE 1  
Showing agglutination results

STRAINS	SERA											
	1	2	6	7	9	17	27	31	5R	Scav	LWS	TA
<i>L. bifidus</i>												
1	5+	3+	-	4+	2+	-	3+	3+	2+	2+	2+	5+
2	1+	5+	AC	AC	3+	AC	2+	3+	3+	2+	2+	4+
3	2+	4+	2+	3+	-	1+	2+	2+	1+	1+	2+	3+
4	2+	4+	3+	2+	3+	-	2+	3+	4+	3+	2+	4+
5	-	3+	5+	3+	-	4+	2+	4+	4+	4+	4+	4+
6	1+	AC	5+	1+	2+	AC	AC	AC	AC	AC	AC	AC
7	4+	-	-	5+	2+	-	-	2+	-	-	3+	4+
8	2+	4+	3+	3+	2+	2+	4+	2+	2+	2+	2+	1+
9	4+	-	2+	2+	5+	4+	2+	-	-	-	1+	1+
10	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
11	5+	5+	3+	-	2+	2+	2+	-	2+	-	2+	5+
12	5+	5+	-	1+	-	-	-	-	-	-	-	5+
13	5+	5+	-	3+	2+	-	-	1+	-	-	1+	4+
14	5+	-	-	-	-	-	-	2+	-	3+	3+	-
15	5+	5+	2+	3+	3+	1+	-	1+	-	-	-	-
16	5+	5+	2+	2+	-	4+	-	2+	2+	2+	-	4+
17	2+	-	3+	-	-	5+	-	2+	1+	-	-	-
18	5+	4+	5+	2+	-	2+	-	2+	1+	2+	-	-
19	3+	2+	-	2+	2+	1+	1+	-	-	-	-	-
20	1+	-	-	2+	-	-	2+	2+	4+	1+	-	-
21	1+	-	-	2+	-	3+	4+	2+	-	2+	2+	-
22	2+	-	2+	1+	-	-	-	3+	1+	2+	2+	-
23	2+	-	3+	4+	-	-	-	2+	1+	2+	3+	1+
24	5+	-	3+	2+	-	-	-	2+	2+	1+	2+	-
25	4+	5+	2+	2+	-	3+	-	-	-	-	-	4+
26	-	-	-	3+	1+	3+	-	-	-	-	2+	-
27	-	-	-	5+	-	-	5+	2+	2+	-	-	2+
28	AC	AC	AC	2+	AC	AC	AC	2+	AC	AC	-	AC
29	AC	AC	AC	5+	AC	4+	AC	4+	3+	2+	2+	1+
30	2+	-	AC	2+	AC	AC	AC	4+	2+	-	5+	3+
31	-	-	-	1+	1+	1+	3+	5+	2+	2+	2+	3+
1R	-	-	-	1+	3+	1+	1+	2+	4+	2+	2+	3+
2R	-	-	1+	2+	1+	1+	1+	4+	4+	2+	3+	3+
4R	-	-	1+	-	1+	1+	1+	4+	4+	2+	3+	1+
5R	-	-	5+	-	2+	-	-	5+	5+	2+	3+	3+
<i>L. acidophilus</i>												
SCav	-	-	-	-	-	1+	-	-	1+	5+	-	-
LWS	5+	5+	-	-	-	1+	-	-	2+	2+	5+	5+
TA	5+	5+	-	-	-	1+	-	-	1+	2+	4+	5+

Key: 1+, agglutination 1:20; 2+, agglutination 1:40; 3+, agglutination 1:80; 4+, agglutination 1:160; 5+, agglutination 1:320 or higher; AC, antigen clumpy (no reading possible); -, negative.

from electrolytes, but the water used here was only once-distilled and was far from being pure. Antisera for the following organisms were used: *L. bifidus*, No. 1, 1:1280; No. 3, 1:1280; No. 6, 1:640; No. 7, 1:640; No. 9, 1:2560; No. 17, 1:640; No. 27, 1:640; No. 31, 1:640; and strain 5R, 1:640; *L. acidophilus*, Scavano, 1:320; strain LWS, 1:320; and strain TA, 1:1280.

These antisera were set up against 36 strains of *L. bifidus* and 3 strains of *L. acidophilus*, starting with a dilution of 1:20 and doubling for each of 10 successive dilutions. Readings were made after twenty-four hours at 37°C. The results of the serological tests are given in table 1.

As has been emphasized by other investigators, *L. bifidus* does not comprise a single antigenic species. There is a very close general relationship between *L. bifidus* and *L. acidophilus*, antigenically. The results of these tests show, however, that there is so much variation serologically between different strains of each of these two organisms that agglutination tests cannot be used to distinction them.

#### INFLUENCE OF H-IONS ON GROWTH

Adam (1923) stated that *L. bifidus* will grow in a pH range of 4.2 to 8.6, while the growth limits of *L. acidophilus* are pH 4.1 and 7.2. In the present study of H-ion influence a series of tubes containing glucose (1 per cent), yeast extract (0.5 per cent) and peptone (1 per cent) and ranging from pH 3.6 to 9.0, were treated with 0.5 cc. of a suspension of washed bacterial cells. They were incubated at 37°C., and growth recorded after twenty-four, forty-eight and seventy-two hours, and one week.

The average limiting pH for *L. bifidus* was found to be 4.4, and for *L. acidophilus* pH 4.6. A few strains of *L. bifidus* grew as low as pH 3.8, and some of the *L. acidophilus* strains at pH 4.0. *L. bifidus* did not grow beyond pH 7.2, as a rule, although a few strains slightly exceeded this figure. *L. acidophilus* revealed an average upper pH limit of 6.8. Repeated observations showed the average optimum pH for growth of *L. bifidus* to be between 5.4 and 6.4, and for *L. acidophilus* between 5.8 and 6.6.

## BACTERIOSTATIC ACTION OF DYES

Cruickshank claimed that *L. bifidus* tolerates a concentration of 1:100,000 of crystal violet, while a solution of 1:1,000,000 of this same dye definitely inhibits *Staphylococcus albus*. Since the former organism is Gram-positive, one would assume that it is quite sensitive to crystal violet and closely related dyes. We attempted to test Cruickshank's conclusions, using glucose yeast-extract agar as the basic medium. Four Petri dishes were poured, containing different concentrations of crystal violet, namely 1:50,000, 1:100,000, 1:500,000 and 1:1,000,000 in one series, and basic fuchsin in another. The plates were streaked with liquid cultures of *L. bifidus*, *L. acidophilus*, *Staph. citreus* and *Bact. cloacae*. Control plates containing no dye were used in each case. There was complete or almost complete inhibition of growth of *L. bifidus* and *L. acidophilus* in all dilutions up to 1:1,000,000. *Staph. citreus* was inhibited definitely, showing a very slight growth in the 1:1,000,000 plates only, while *Bact. cloacae* was not inhibited in any of the concentrations of crystal violet employed. Basic fuchsin partially inhibited *L. bifidus*, *L. acidophilus* and *Staph. citreus* in dilutions of 1:50,000 and 1:100,000, and exerted little or no effect in the higher dilutions; *Bact. cloacae* was not inhibited at all. There was very good growth in all of the control plates. *L. bifidus* and *L. acidophilus* reacted as one would expect them to do towards crystal violet and related basic dyes, that is, like Gram-positive bacteria generally. Furthermore, the dye resistance tests revealed no difference in the tolerance of these two aciduric organisms for these dyes.

## INDOL AND PHENOL TOLERANCE

Kulp (1929) showed that *L. acidophilus* offers a much greater resistance to indol and phenol than *L. bulgaricus*. In comparing the resistance of *L. bifidus* and *L. acidophilus* to these two agents, the methods of conducting the tests and the concentrations of indol and phenol which we used were the same as those of Kulp, except that yeast-extract nutrient agar containing 1 per cent

glucose was employed by us in place of casein digest agar. Adequate control plates were included in the set up. The results obtained with *L. acidophilus* and *L. bifidus* were almost identical with those of Kulp for *L. acidophilus*. No appreciable difference could be observed between the resistance of the two organisms to the indol and phenol. Growth was inhibited by indol concentrations of 1:1100 and above, and complete tolerance was shown in concentrations of 1:2000 and less. Phenol in concentrations of 1:250 and above inhibited growth; normal growth occurred in concentrations of 1:400 and less. These results bear out the assumption that intestinal organisms are relatively resistant to the action of indol.

#### SEARCH FOR BACTERIOPHAGE

It was interesting to note that some strains of *L. bifidus* underwent complete lysis in broth during three days' incubation. This suggested bacteriophage action and led to a search for bacteriophage in the lactobacilli. Attempts to develop a phage from broth culture filtrates and from filtrates from suspensions of the feces of a breast-fed infant, for *L. bifidus*, *L. acidophilus*, *L. odontolyticus*, *E. coli*, *Proteus vulgaris* and *Staph. aureus*, resulted in failure. There was demonstrated, however, a definite inhibitory agent in broth filtrates of *L. bifidus*, which was sufficiently active to inhibit appreciably the growth of *L. bifidus*, and *L. acidophilus* when only one drop of filtrate was employed in 3 cc. of medium. This action could not have been due to acid. Many attempts to demonstrate the presence of filterable forms in the filtrates proved unsuccessful.

#### LYSOZYME STUDY

The lytic principle in the broth cultures could not be explained on the basis of bacteriophage action. It was observed that the lysis of cultures disappeared after the strains had gone through several transplants. This suggested the occurrence of a lysozyme, a substance first reported by Fleming and Allison (1922), and supposed to be present in all human tissues and in human secretions, except urine, sweat and cerebrospinal fluid. This sub-

stance or agent has the power to lyse certain bacteria and, according to Rosenthal and Lieberman (1931), is present in large quantities in the feces of breast-fed infants, having its origin in the human milk. These authors concluded that *L. bifidus* predominates in the feces of breast-fed infants because it is resistant to lysozyme action, while the other fecal forms are lysed by it.

Since egg-white is supposed to contain large amounts of lysozyme, fresh egg-white was added to liquid cultures of *L. bifidus* and *L. acidophilus*. No lysis occurred. Negative results were also obtained when egg-white was dropped on colonies in streaked plates. Egg-white, however, did inhibit the growth of these two organisms, which is in accord with the observations of Rettger and Sperry (1912). This appeared to support the claims of Rosenthal and Lieberman; but, when egg-white was tried against newly isolated strains of *E. coli* and the enterococcus no lysis occurred, either in broth, plates or washed suspensions. According to these observations, lysozyme plays no important, if indeed any, rôle in the predominance of *L. bifidus* in the feces of breast-fed infants. It also appears that lysozyme is not active against any of the ordinary intestinal organisms, and that it shows its lytic power only against special susceptible strains such as the "*Micrococcus lysodeikticus*" of Fleming.

#### LACTIC ACID PRODUCTION BY *L. BIFIDUS*

The investigation was almost completed up to this point when the paper of Curran, Rogers and Whittier (1933) on "The Distinguishing Characteristics of *Lactobacillus Acidophilus*" appeared in this JOURNAL. The properties ascribed by these authors to *L. acidophilus* agreed, on the whole, with those of *L. bifidus* as observed in the present study. Since considerable emphasis was placed by Rogers and his associates on the types and quantity of lactic acid formation by lactobacilli as a basis of classification, we undertook to make a roughly quantitative study of the acids produced by *L. bifidus*.

The methods described by Pederson, Peterson and Fred (1926) and by Pederson and Breed (1928) were employed. The following is a brief summary of the results obtained. The strains

of *L. bifidus* isolated by us produced a total acidity varying (with the different strains) from 0.05 to 0.4 per cent, in terms of lactic acid. The volatile acid fraction varied between 18 and 25 per cent of the total; it consisted largely of acetic acid, with small amounts of formic and butyric. Lactic acid constituted from 75 to 80 per cent of the total acid. The lactic acid proved to be of the inactive type.

These results agree in the main with those of Curran, Rogers and Whittier for *L. acidophilus*. The total acid production by *L. bifidus* was, on the whole, less than that of *L. acidophilus*, and the volatile acid fraction of *L. bifidus* was slightly higher than that of *L. acidophilus*. However, the differences cannot be regarded as significant, especially since some strains of both types yielded essentially the same results.

#### DISCUSSION AND CONCLUSIONS

*L. bifidus* may constitute from 90 to 95 per cent of the intestinal flora of breast-fed infants. It appears in the feces in from three to four days after birth and remains the predominating organism as long as breast milk is the sole diet. The results of this investigation indicate a very close relationship between this organism and *L. acidophilus* of Moro.

*L. bifidus* is isolated most readily by the acid broth preliminary enrichment method but, in order to observe its quantitative relationship to other intestinal bacteria, direct plating of the feces in special agar media, under anaerobic conditions is necessary. After primary isolation it grows under ordinary aerobic conditions, and in pure culture. *L. bifidus* should be classed, therefore, as a facultative anaerobe.

The morphological and cultural characteristics of the two organisms are essentially the same, the few differences observed being only of a quantitative nature.

While *L. bifidus* seemed to possess a slightly wider carbohydrate-decomposing range than *L. acidophilus*, there was no significant difference between the two types, in so far as fermentation properties are concerned.

There are apparently no greater serological differences between



*L. bifidus* and *L. acidophilus* than exist between members of one and the same species.

Both organisms reacted practically alike to acid, alkali, phenol, indol, crystal violet, basic fuchsin and lysozyme.

Qualitative and quantitative studies of the acids formed by *L. bifidus* agree in general with those reported by Rogers and his associates for *L. acidophilus*. The bulk of the acids produced by both types of organisms is inactive lactic acid.

While there are, on the whole, slight differences between *L. bifidus* and *L. acidophilus*, these can be accounted for by normal variation within the species or type. The authors are of the opinion that *L. bifidus* should be regarded as a variant of the species in which *L. acidophilus* is the central type.

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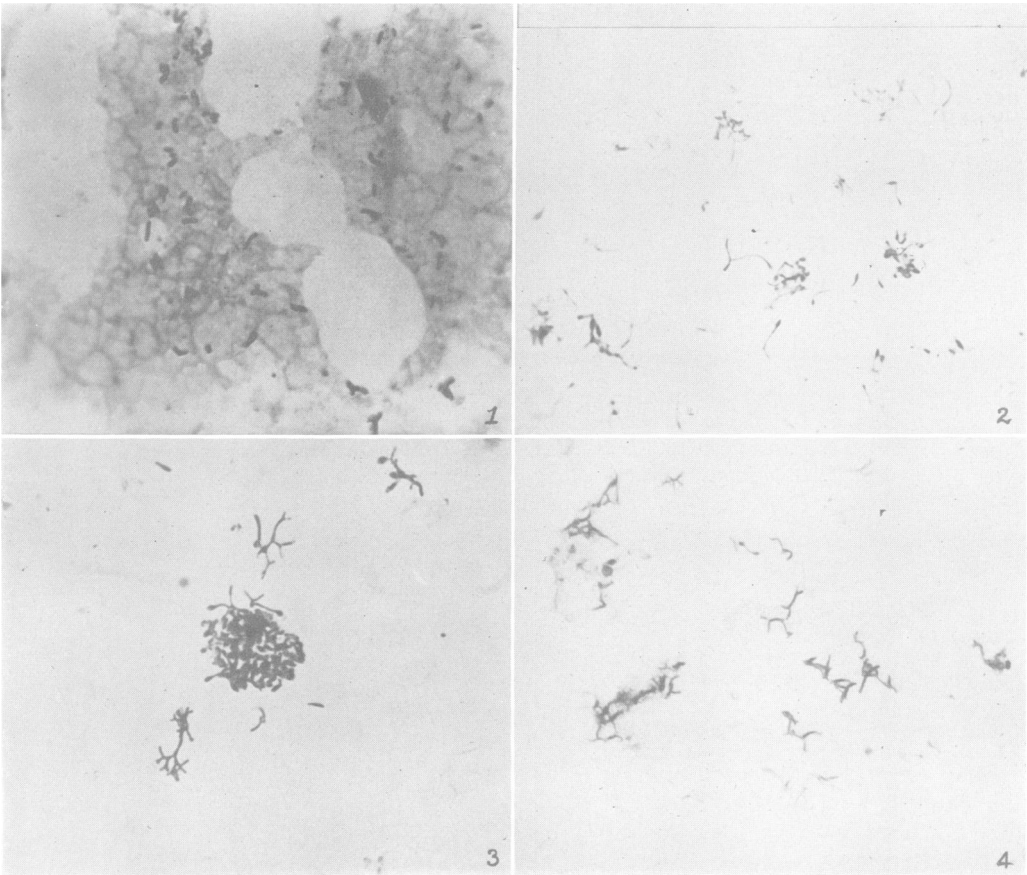
## PLATE 1

FIG. 1. *L. bifidus*. Gram-stained preparation made directly from nursling's stool.  $\times 1000$ .

FIG. 2. *L. bifidus*. Gram-stained preparation made from colonies in Veillon tube. Branching and club-shaped forms.  $\times 1000$ .

FIG. 3. *L. bifidus*. Gram-stained preparation made from colonies on Torrey's medium showing extreme branching and re-branching.  $\times 1000$ .

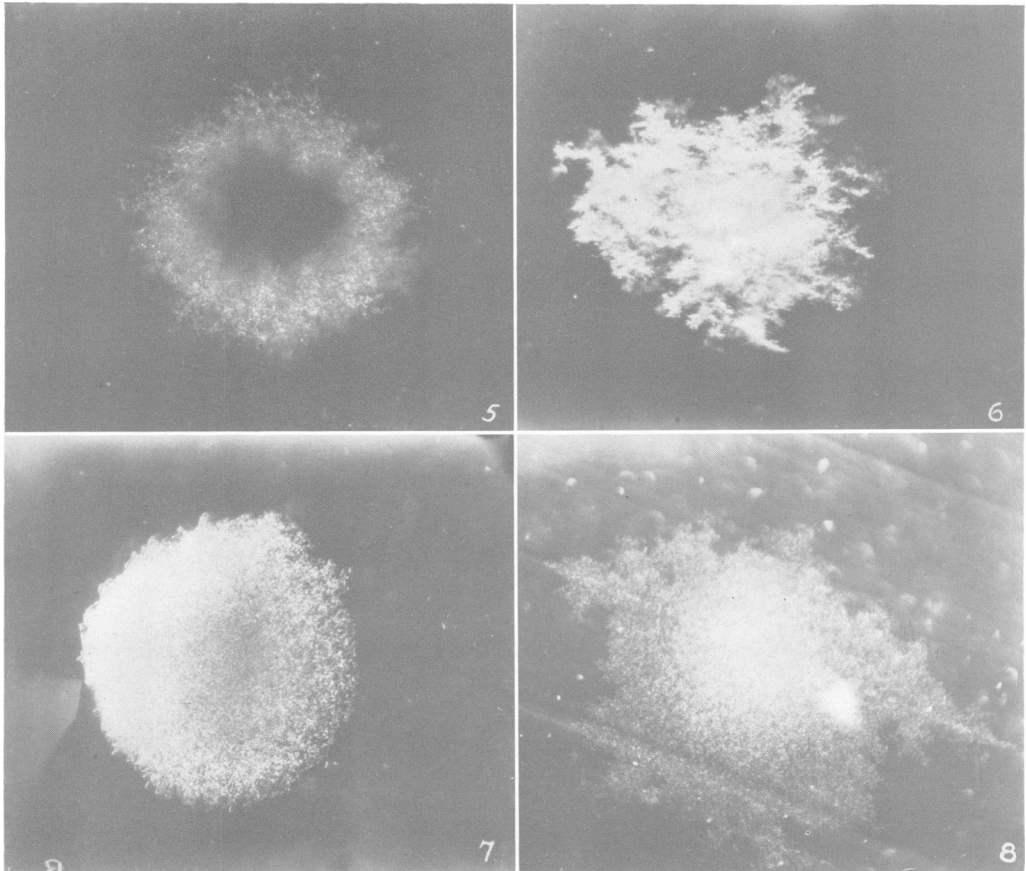
FIG. 4. *L. acidophilus*. Gram-stained preparation made from colonies in Veillon tube showing branching and re-branching.  $\times 1000$ .



(James E. Weiss and Leo F. Rettger: *Lactobacillus bifidus*)

## PLATE 2

- FIG. 5. *L. bifidus*. Subsurface colony in tomato agar.  $\times 125$ .  
FIG. 6. *L. acidophilus*. Subsurface colony in tomato agar.  $\times 48$ .  
FIG. 7. *L. bifidus*. Surface colony on tomato agar.  $\times 25$ .  
FIG. 8. *L. acidophilus*. Surface colony on tomato agar.  $\times 25$ .



(James E. Weiss and Leo F. Rettger: *Lactobacillus bifidus*)