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BACILLUS BIFIDUS: ITS CHARACTERS AND ISOLATION FROM THE INTESTINE OF INFANTS[1].

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(With Plate II and 1 Diagram.)

As a preliminary to the study of the intestinal flora in cases of diarrhoea in children, an investigation of the normal intestinal flora of both breast-fed and artificially fed infants under one year of age was undertaken. One of the early obstacles encountered was the fact that, although a Gram-stained film of the faeces of a breast-fed infant showed the presence in practically pure culture of a short Gram-positive bacillus, yet growths of this organism could not be obtained by the ordinary aerobic and anaerobic methods. First isolated by Tissier (36) in 1899, and named by him *B. bifidus communis* because of its supposed tendency to bifurcate, this organism has been the subject of much controversy, particularly by investigators in Germany and America. In this country, practically the only contribution on the subject is that of Logan (16) (1914), who, in an investigation on the intestinal flora of children, devoted much attention to the so-called aciduric group of organisms. Whereas he was able constantly to isolate *B. acidophilus* from the faeces of bottle-fed infants, he remarked that in breast-fed cases where the films showed very large numbers of Gram-positive bacilli morphologically resembling the beaded (so-called "punctate") form of *B. bifidus*, it was extremely difficult to isolate these organisms; thus successful isolation was effected in three only out of six breast-fed infants examined. Further, although Logan observed differences between the various strains of Gram-positive, acid-resistant bacilli, isolated from the faeces of breast-fed and artificially fed infants, with regard to certain of their cultural and fermentative characters, he did not consider these characters sufficiently constant to justify differentiation, and consequently he included *B. bifidus* and *B. acidophilus* in one group. There can be little doubt that *B. bifidus* and *B. acidophilus* both belong to a group—much larger than was at first thought—of organisms which are acid-tolerant, not acidophile, *i.e.*, which under cultural conditions survive a concentration of acid lethal for *B. coli*, but which do not find at such a degree of acidity the optimum reaction for growth. The facts, however, that *B. bifidus* is the predominant organism in breast-fed infants' faeces, while *B. acidophilus* predominates especially in the stools of artificially-fed infants, that *B. bifidus* is much more difficult to

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obtain in artificial culture than is *B. acidophilus*, and that definite differences are recorded in their cultural characters, have led to their being usually considered as separate organisms. The divergent opinions regarding *B. bifidus* are probably due to its close relationship to other members of the aciduric group, to its pleomorphism and to the difficulty of its cultivation. The following is a brief summary of knowledge regarding it. In 1899, Tissier (36), using anaerobic methods, first isolated the organism in pure culture and described its characters. In doing so he was able to disprove Escherich's (8) (1886) contention that *B. coli* is the predominant organism in the suckling's stool and that the Gram-positive appearance in the films of faeces was merely a "chromophilic" reaction of *B. coli*. This conclusion was come to, as Tissier pointed out, because Escherich examined only aerobic cultures and consequently was able to recover only coliform organisms. Tissier described *B. bifidus* as a strict anaerobe, pleomorphic, with a tendency to bifurcate at one or both extremities, forming small ovoid or lenticular colonies in sugar agar, producing acid but no clot in milk, and fermenting certain of the sugars without gas formation. In 1900 (37), he reported the isolation of another Gram-positive bacillus from the faeces of breast-fed infants; this organism, *B. exilis*, was a facultative aerobe, and although more slender than *B. bifidus*, resembled it closely in its cultural characters. In 1905, Tissier (38) recovered *B. bifidus* from the various parts of the alimentary tract, from stomach to rectum, of recently dead, bottle-fed infants; and in 1908 (40) he claimed that in children from one to five years of age *B. bifidus* was the predominant organism in the intestinal flora, varying from 50 to 90 per cent. of the total number present according as the diet was mainly protein or mainly carbohydrate. Moro in 1900 (23) had isolated from the stools of breast-fed infants a facultative anaerobic, Gram-positive organism with a tendency to grow in the form of long threads, which produced in deep beer-wort agar characteristic "feathery" colonies. From its supposed enrichment in acid medium, Moro called it *B. acidophilus*, but this was a misnomer, as Mereshkowsky (19) and later Distaso (6) showed that the organism was acid-tolerant or acid-resistant, not acidophile. Tissier (1900) denied that *B. acidophilus* could be isolated from purely breast-fed infants' faeces, but subsequent investigators (Cahn (3), Rodella (30) and Jacobson (10)) claim that they have recovered organisms of *B. acidophilus* type from the stools of such cases. Rodella believed that *B. bifidus*, *B. acidophilus* and *B. gastrophilus* of Boas-Oppler, were all the same organism, which varied in its morphology and cultural characters according to its position in the course of the intestine. Jacobson showed that, contrary to Tissier's statement, *B. bifidus* could grow on acid media, and that the deep colonies in solid media, although lenticular or ovoid at first, later developed buds or lateral offshoots. He agreed with Tissier that the bacillus was a strict anaerobe. He, however, isolated three times directly from the faeces and twice from cultures of *B. bifidus*, an organism which he called *B. intestinalis tuberculiformis*, a facultative aerobe with a tendency to grow into longer forms and of a greater vitality than *B. bifidus*. This organism,

both morphologically and in its cultural characters closely resembles Tissier's *B. exilis*, and it is probable that both were *B. bifidus* in its aerobic phase. Noguchi (25) commented on the close relationship existing between *B. bifidus*, *B. acidophilus*, *B. exilis*, *B. tuberculiformis* and the *B. infantilis* of Herter. He stated that *B. bifidus* could be changed from a strict anaerobe to (*a*) a strictly aerobic, spore-bearing, motile bacillus, producing dry, felted colonies on agar, and (*b*) a facultative anaerobe, a slender bacillus of *B. exilis* type, which after several subcultures could be converted to type (*a*). The cultural characters of type (*a*) resembled closely those of *B. mesentericus fuscus*. He further succeeded in bringing both types back to the strictly anaerobic state of the original *B. bifidus* by gradually diminishing the amount of oxygen with each successive sub-culture. There does not appear to be any confirmation of these results. In 1911 Distaso, in an extensive study of the acid-tolerant group of organisms, regarded *B. bifidus* as being distinct from *B. acidophilus* in that the former was a strict anaerobe which showed true bifurcation and formed only acetic acid from the fermentable sugars, whereas *B. acidophilus* produced in addition formic acid; also, an anti-serum to *B. bifidus* which agglutinated this organism was without effect upon *B. acidophilus*. No details are given of his agglutination experiments. Distaso and Jungano (7) do not appear to have added anything to the statements of Tissier. Orla-Jensen (26) found great difficulty in isolating *B. bifidus* and in his hands it tended to perish very quickly. He regarded it as a strict anaerobe and classified it as intermediate between the lactic acid and propionic acid bacteria. Logan remarked on the pleomorphism of the organism, but failed to find any bifid forms. Some of his strict anaerobes became after several sub-cultures facultative aerobes. The observations of several German workers (Blühdorn (2), Basten (1), Küthe (14)), who have studied the characters of *B. bifidus* are summarised by Lauter (15) (1921), who first used Tarozzi's medium as a means of isolating the organism, which he believed to be a strict anaerobe.

The question as to the source and mode of entry of *B. bifidus* into the intestine appears not to have been fully investigated. Noguchi suggested that it came from the lactating breast of the mother and in support of this theory quoted the facts that Scheurlen (32) isolated from breast cancers *B. mesentericus*, which, according to Noguchi, corresponds to the aerobic phase of *B. bifidus*, while later Rosenthal (31) found the same organism in the normal breast. Lauter examined (*1*) the vaginal secretion of the mother before the birth of the child, (2) the colostrum before the infant was put to the breast, and (3) the mouth and anus of the newly-born child. In two cases he isolated *B. bifidus* from the mouth of the child immediately after birth, but failed to recover it from any of the other sources. The organism appeared in the stools at the earliest 24 hours after birth and at the latest 89 hours. Tissier noted *B. bifidus* in the stools at the beginning of the third day and remarked that it became established as the predominant organism on the fourth or fifth day when the child was breast-fed.

The ubiquity of aciduric bacteria in the animal world has been proved in an extensive survey by Mereshkowsky and his co-workers (19-22). They isolated from the faeces of a wide variety of animals, ranging from molluscs to man, an organism which they called "*B. acidophilus No.* 1," which was of the bifidus type, but neither anaerobic nor bifid, while Webster (43) (1921) found both aerobic and anaerobic forms of the organism in the intestinal flora of mice. The author has likewise recovered from aerobic cultures of the faeces of mice and rabbits acid-tolerant organisms which in morphology and type of colony are indistinguishable from *B. bifidus*. It has been found in the present work that organisms resembling *B. bifidus* can be isolated in culture from the faeces of animals much more readily than from human faeces. A similar observation was made in the case of organisms of *acidophilus* type, confirming Smith's work (35).

In view of the confusion with regard to the cultural characters of *B. bifidus* and its relation to *B. acidophilus*, and the difficulty of recovering it in culture despite its preponderance in the suckling's intestine, a further investigation was thought desirable of methods of isolating this organism with a view to examining its characters.

The Isolation of *B. bifidus communis*.

The number and variety of methods devised for the isolation and cultivation of a particular organism are usually proportional to the difficulties encountered in obtaining it. This is well exemplified in the case of *B. bifidus*. Of the many methods recommended one may mention the following: Tissier, and following him, Cahn, Rodella, Passini (27) (1901) and Basten all used deep glucose-agar tubes inoculated with varying dilutions of faecal emulsion as the primary culture medium. Torrey (41), in an investigation on the effect of different diets on the intestinal flora of dogs, recommended acid liver-glucose-agar to which 10 per cent. of rabbit blood was added. According to this method tubes of liquefied medium are inoculated with varying quantities of the faeces, then plates are poured and incubated for three to four days under semi-anaerobic conditions which are secured by growing on the opposite half of the plate an organism of *B. subtilis* type. More recently Zeissler and Käckel (44) claimed good results with "Adam-bouillon" (acidified lactose-broth with marble chips *plus* human blood), using this as a primary culture medium for 24 hours and then inoculating into deep glucose-agar tubes. The objection to most of the methods mentioned—and all of them have been tried in the present investigation—is that a large amount of medium is used and much time expended in the attempt to isolate the organism. Further, it is not easy to isolate from the depths of a glucose-agar tube colonies of a particular organism where other organisms in any considerable number are present, such as *B. coli* and enterococci. Blühdorn, using the methods recommended by Burri, Lentz and Buchner, failed to isolate the organism. In 1921 Lauter advocated the use of Tarozzi's medium to which was added 0·5 per cent. "acetic acid" for the

initial cultivation. After incubation of this primary culture for three to four days at 37° C. shake-cultures were made from it into deep glucose-agar, in which isolated colonies were usually seen after four to six days' incubation. By means of a modification of Lauter's procedure the isolation of *B. bifidus* in pure culture from the faeces of ten young breast-fed infants (7 to 14 days old), and in culture associated with enterococci from the faeces of four other cases, has been effected with comparative ease.

Method. A deep tube containing 20 c.c. of 1 per cent. lactose or glucose broth, neutral to litmus paper, along with a small piece of fresh, sterile rabbit kidney and sealed with a half-inch layer of sterile vaseline, is inoculated by means of a piece of quill tube drawn out into a capillary pipette with 0·5 c.c. of a fairly opaque faecal emulsion; the vaseline is melted to seal the track of the pipette and the tube is then incubated for six to eight days at 37° C. Gas is evolved during the first few days and is conveniently expelled by remelting the vaseline seal. Plates of 1 per cent. glucose-agar or Loeffler's serum are then stroked from the primary culture and incubated (*a*) anaerobically, *e.g.* in a McIntosh and Fildes' jar, and (*b*) aerobically. After 48 hours at 37° C. or sometimes earlier small, glistening, greyish colonies of pin-head size and with regular contour are seen on the surface of the anaerobic plate and occasionally on the aerobic one. The only other organism which is likely to be present on the plate is the enterococcus, which forms larger, whitish colonies. These, however, are as a rule scanty and readily distinguishable from the abundant growth of the small diphtheroid-like colonies of *B. bifidus*. After three to four days' incubation the colonies have become somewhat larger and, from being 1–2 mm. in diameter after 48 hours, may increase to 3–4 mm. Sub-cultures are made from these on glucose-agar slopes, glucose broth, and milk and these are incubated under aerobic conditions. Rich inoculation is advisable, since sub-cultures from a single colony frequently fail to grow. Growth appears as a rule on the glucose-agar slope and is practically certain to occur in the glucose broth and milk tubes within two days at 37° C. After one or two further sub-cultures on glucose-agar at intervals of two days, the organism will grow readily on ordinary agar and has now become acclimatised to aerobic conditions, so that it can be grown on most of the ordinary culture media. Likewise its viability is considerably increased and cultures on agar remain alive for several weeks when kept at room temperature. As an alternative to the use of sugar-bouillon, serum-water and ascitic fluid were tried along with fresh kidney tissue (the original medium of Tarozzi), but were found to give less certain results. The organism was also isolated by inoculating a tube of acid blood-glucose-agar (0·2 c.c. $N/1$ acetic acid to 10 c.c. neutral glucose-agar, to which was added 1 c.c. of fresh rabbit blood, the mixture being immersed in boiling water for one minute and quickly cooled). This was inoculated, poured into a Petri's capsule, and incubated in the anaerobic jar for four days. The colonies of *B. bifidus* appeared as minute white dots with a greenish halo. This method, however, is not so effective for eliminating coliform organisms and enterococci as that

described above, so that pure cultures from the primary growth are much more difficult to obtain; also, the first sub-culture must be incubated under anaerobic conditions. Filtrates obtained by passing watery extracts of breast-fed infants' faeces through a Berkefeld filter, alone or added to various media, human milk, and many of the reputed anaerobic methods, were also tried but without success.

Morphology of *B. bifidus*.

A film made from the faeces of a breast-fed infant, seven to twelve days old, and stained by Gram's method, shows a practically pure culture of a rather slender, straight or slightly curved, Gram-positive bacillus of variable length—from 2–3 μ to 6–7 μ, although the majority are 4–5 μ in length, and about 0·7 μ in breadth—(Plate I, fig. 1). The extremities are round (rather than,

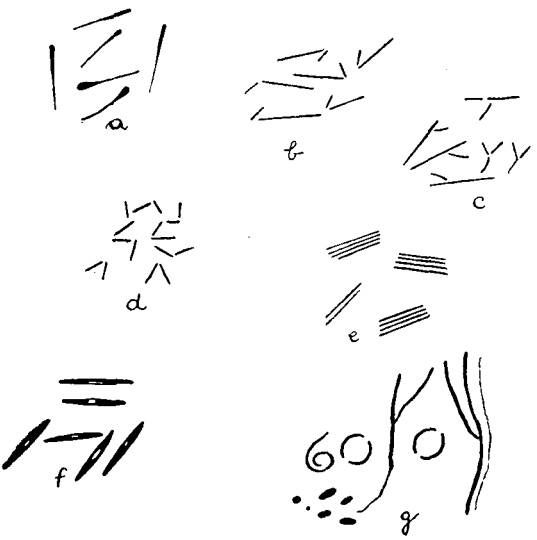

Diagram 1.

as Tissier says, "pointed") and often at one or both ends there is a bulbous or conical thickening (Diagram, *a*). The bifid character, that is, a bifurcation of one or other extremity, whence the organism derives its name, is rare in the direct film of faeces; but small bacillary or coccoid offshoots forming either an acute or obtuse angle with the main rod are frequently seen (Diagram, *b*); and perhaps more commonly a semblance of branching is given by the apposition of two or three bacilli forming a **Y** or a triad (Diagram, *c*). Diplo-bacillary forms are frequent in the direct film, the two members often lying at an obtuse angle, the "formes génicules" of Tissier (Diagram, *d*); but chain formation has not been noted. There is, however, a marked tendency for two, three or four bacilli to lie almost parallel to each other, similar to what is so characteristic of diphtheria bacilli (Diagram, *e*). Although *B. bifidus* is Gram-positive, many of the organisms in faecal films retain Gram's stain rather unevenly,

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so that a granular or beaded appearance is produced; these are probably degenerate forms. The organism is readily decolorised, as Tissier noted. Films made from the stools of older breast-fed infants (from one to nine months old) show, in addition, a small proportion (5 to 10 per cent.) of coliform organisms and numerous Gram-positive cocci.

Films from a young culture on agar of the organism isolated by the procedure described above, show the same rather pleomorphic bacillus, which both in morphology and in grouping most closely resembles the diphtheroid group of organisms (Plate I, fig. 2). The diplo-bacillary forms are again frequent and short chains of from three to five organisms are occasionally seen. Granular staining in Gram preparations is less evident and Gram-negative forms are very scanty in young cultures. When stained by methylene blue the granular appearance, and hence the resemblance to *B. diphtheriae*, is emphasised; but the organism does not show granules when stained by Neisser's method either in young or old cultures on serum. As in the direct film from faeces, bifurcation is simulated by the grouping of two or three bacilli, but true branching is rarely if ever present, although an irregular thickening of one extremity is occasionally seen. Noguchi's statement that bifurcation is most commonly found in deep glucose-agar cultures and not in fluid media, could not be corroborated with four strains tested by the author. The tendency to clubbing of one or both ends of the bacillus and to the formation of short bacillary offshoots from one pole is again evident. These forms of *B. bifidus* are more common when the medium is rather unfavourable for the growth of the organism, as in old fluid cultures, or in less nutritive media, *e.g.*, peptone water, or when the organism is grown at a lower temperature (20° C.). Tissier, who first noted this fact, also observed that the bifid and clubbed or candle-flame appearances are seen more frequently when *B. bifidus* is grown in symbiosis with other organisms.

In films made from older cultures in the depths of solid media (two to three weeks in deep agar or glucose-agar), many Gram-negative bacilli are present, often lying alongside Gram-positive forms, or a Gram-negative bacillus may be seen in a chain between two Gram-positive bacilli or, again, a long bacillus may be partly Gram-positive and partly Gram-negative. There is, too, an increased tendency to pleomorphism. Particularly in films made from deep colonies in glucose-agar, long curved threads are seen, which resemble the long forms of *B. acidophilus* and show the same irregularity of Gram-staining. Owing to the arrangement of these threads, the bacilli present a striking similarity to a streptothrix, but true branching has never been observed (Plate I, fig. 3). This tendency to grow into long forms was apparent also when the organism was grown on agar containing 1 : 100,000 crystal violet, which had only a slight inhibitory effect on *B. bifidus*, although *Staphylococcus aureus* was markedly inhibited by a concentration of 1 : 1,000,000 of the dye. It would seem, then, that these long, thread-like bacilli are involution forms of the organism. It was observed that in Gram-stained films from older cultures

where there were forms with clubbing of the ends or small terminal offshoots, these portions retained the Gram stain, while the rest of the bacillus was decolorised. Vesicular forms, *i.e.*, bacilli staining at both ends with the central part unstained and thus presenting a sausage-like appearance, were seen in old cultures and sometimes in direct films of faeces. Tissier regarded these as being dead or degenerate bacilli (Diagram, *f*). The point to be emphasised regarding the morphology of *B. bifidus* is its pleomorphism, in which it resembles *B. acidophilus*. It may vary in length from a coccoid organism to a long streptothrix-like thread, and it may assume many fantastic shapes—straight or comma forms, with bulbous extremities or lateral offshoots, curled-up little balls, or like two halves of a circle, and in old cultures whorls of long slender interlacing threads (Diagram, *g*). *B. bifidus* is a non-motile, non-sporulating, uncapsulated organism.

Cultural Characters. *B. bifidus* has been variously described as (1) a strict anaerobe (Tissier, Cahn, Lauter), (2) an organism requiring semi-anaerobic conditions for its cultivation (Torrey), (3) a facultative anaerobe (Passini, Basten, Küthe), and (4) a "pleobiotic" organism, which from being a strict anaerobe can be modified by selective cultivation to an aerobic spore-bearing phase and back to its original anaerobic state (Noguchi). From the results obtained by the method adopted in the present investigation for the isolation of *B. bifidus* it seems that for primary cultivation the organism requires fairly strict anaerobic conditions; but that after it has been isolated and has grown vigorously, it can be readily cultivated in the presence of air. In primary culture on the surface of glucose-agar it appears as a delicate, greyish, glistening growth; the colonies tend to remain discrete, but after many sub-cultures the growth is rather heavier and more confluent. Under the low power of the microscope, the surface colony is delicately granular, often with a brownish centre and a regular but finely serrated margin (Plate I, fig. 4). In deep agar or glucose-agar the organism forms dark brown lenticular or ovoid colonies about 1 to 2 mm. in diameter after 48 hours, but increasing in size after further incubation. At first the edge of the colony is well defined and regular, but after four or five days' growth there is frequently a lateral projection or bud giving the colony an irregular outline (Plate I, fig. 5). It bears no resemblance to the typical colonies of *B. acidophilus*, which are either of the delicate "feathery" type (Plate I, fig. 6), or of the heavier type with very irregular margin (the "crab" colony). In deep gelatine shake-culture incubated at 37° C. for 24 hours and then left for some days at room temperature, it grows as fine discrete colonies without causing liquefaction of the medium; in a gelatine plate kept throughout at room temperature the colonies are extremely small and scarcely visible to the naked eye. In fluid media (peptone water, bouillon and glucose broth) *B. bifidus* produces a granular turbidity after 24 hours. Growth is most rapid in glucose broth and after three to four days' incubation the organisms fall to the bottom of the tube, forming an abundant loosely granular deposit and leaving the supernatant fluid clear. In litmus milk acid is produced at first

slowly (after 72 hours or longer at 37° C.), but more quickly after repeated sub-culture. Although Tissier stated that there was never sufficient acid-production to cause coagulation of milk, two of the author's strains which had been repeatedly sub-cultured on agar for some six months, when tested again in milk, produced acid and clot in 48 hours. The addition of serum (5–10 per cent.) to solid media (agar or glucose-agar) enriches the growth of *B. bifidus*, so that the colonies become much larger and appear as small whitish balls. The presence of whole blood would seem also to favour the growth of *B. bifidus*, and the greenish halo surrounding the white colonies indicates the production of hydrogen peroxide and the consequent oxidation of the blood-pigment (M'Leod and Gordon(17)). The organism survives in cultures, on agar and glucose-agar or in fluid media at room temperature, for at least four weeks after it has been growing well and frequently sub-cultured. The optimum temperature for growth is about 37° C.; but it grows readily at 22° C. and more slowly at room temperature—16° to 18° C. A broth culture (24 hours at 37° C.) is killed by exposure to a temperature of 55° C. for half an hour, and does not survive heating at 70° C. for five minutes.

An attempt was made to isolate the aerobic spore-bearing variant of *B. bifidus*, which Noguchi claimed to have recovered from old cultures of the organism. Glucose-broth cultures, from two to four weeks old, were heated at 80° C. for five and ten minutes and a heavy inoculation then made into fresh glucose-broth tubes, which, however, invariably remained sterile, indicating that the organisms were all killed. Further, no spore forms were ever seen in films from old cultures. Although *B. bifidus* grows readily in the presence of oxygen, it would seem that it prefers at least semi-anaerobic conditions, since in deep glucose-agar tubes the colonies near the surface are always very small, but are of larger size in the depths of the tube. This fact is probably responsible for Cahn's belief that he was dealing with a mixed culture of *B. bifidus* and *B. acidophilus*. Further, it has been found by the writer that after several sub-cultures in deep glucose-agar, *B. bifidus* refuses to grow on the surface of agar or glucose-agar and has to be cultured in milk or glucose-broth before it can be grown again under strictly aerobic conditions.

Fermentation reactions. Eight strains were tested several times with lactose, glucose, mannitol, dulcitol, maltose and saccharose (neutral, 1 per cent. peptone water containing 1 per cent. of the substance and Andrade's indicator being used). Acid was invariably produced from glucose and maltose within 24 hours. Lactose was usually fermented later (second to fourth day): dulcitol was not fermented by any of the strains after 10 days at 37° C. Saccharose was fermented by seven of the strains, but the medium usually did not become acid until after 48–72 hours at 37° C. The strain which failed to ferment saccharose, as well as four others, did not form acid from mannitol; the rest rendered mannitol medium acid after 48–72 hours. Gas was not formed in any case. There was no formation of indol.

Agglutination. An antiserum to one of the strains of *B. bifidus* (Infant 1)

was prepared by injecting into a rabbit intravenously, unheated saline emulsions of 24 hours' cultures of *B. bifidus* on agar-slopes in increasing amounts, viz., 3, 4, 5 and 6 agar-slopes at 2-day intervals, and the blood was withdrawn three days after the fourth inoculation. By this method an antiserum was obtained which in a dilution of 1 : 2560 agglutinated the homologous organism.

In carrying out the agglutination test, saline emulsions of 24-hour cultures of the organism on agar-slopes were used unheated, 4 to 6 agar-slopes being required for each test to give an emulsion of sufficient opacity; 0·5 c.c. of the emulsion was added to an equal quantity of appropriate dilutions of the serum, the tubes were incubated in the water bath at 57° C. for three hours and the readings taken after half an hour further at room temperature. The flocculi of agglutinated organisms were as a rule rather small, but no test was considered positive if agglutination was not apparent to the naked eye. With some strains of *B. bifidus* it is difficult to get a homogeneous emulsion until the organisms have been repeatedly sub-cultured.

Eight strains of *B. bifidus* isolated by the anaerobic method from the faeces of babies and two strains isolated from mice were tested for their agglutinability by the antiserum. The results are tabulated below and show that not all the strains isolated by this method are agglutinated by an antiserum to one particular strain. The occurrence of a zone phenomenon with the mouse strains only was a striking feature. A large series of *B. acidophilus* strains isolated from children were also tested against the antiserum to *B. bifidus* and antisera to *acidophilus* strains, while the *bifidus* strains were tested against the *acidophilus* antisera. The results of this work will be published later, but it may be stated that there appears to be a close relationship serologically between *B. bifidus* and *B. acidophilus*.

Antiserum to B. bifidus (*strain Infant* 1). *Titre* 1 : 2560.

	Dilutions of antiserum						
Strain	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1250	1 : 2560
Infant 1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
„ 2	+ +	+ +	+ +	+ +	+	+	−
„ 3	+	−	−	−	−	−	−
„ 4	−	−	−	−	−	−	−
„ 5	+ +	+ +	+ +	+ +	+	−	−
„ 6	+ +	+ +	+ +	+	+	−	−
„ 7	+ +	+ +	+ +	+ +	+ +	+	+
„ 8	−	−	−	−	−	−	−
Mouse 1	−	+ +	+ +	+ +	+	−	−
„ 2	−	+ +	+ +	+ +	+	−	−

+ + = Complete sedimentation. + = Incomplete sedimentation. − = No agglutination.
The control tubes, in the absence of serum, showed no sedimentation in any case.

Physiological rôle of B. bifidus. It has been seen that in the faeces of young, breast-fed infants, *B. bifidus* is the predominant organism and the examination of stained preparations indicates that it may be present in practically pure culture. As regards the part which it plays in the intestine, little is definitely known. Tissier (1905) believed that its presence conduced to the normal state

of the bowel. He therefore fed infants suffering from chronic diarrhoea with cultures of a closely related organism (*B. acidi paralactici*) and recorded good results from the treatment. He believed that in this way putrefactive bacteria which were responsible for the diarrhoea were eliminated and *B. bifidus* again established as the predominant organism. The administration of cultures of lactic acid organisms (*B. acidophilus* and *B. bulgaricus*) has been extensively tried in recent years in cases of intestinal disorder in children and adults. While much improvement from the feeding of *B. acidophilus*—but not of *B. bulgaricus*—has been reported (Rettger and Cheplin (28), Kopeloff (12-13), Gompertz and Vorhaus (9)), it would appear that in adults these organisms predominate in the faeces only so long as considerable quantities of cultures are being administered. The relationship of an aciduric flora to the reaction of the faeces is obscure. Thus in experimental animals and in adults fed with cultures of aciduric organisms there is no appreciable increase of acidity of the faeces (Rettger and Cheplin). The faeces of breast-fed infants in which *B. bifidus* preponderates are distinctly more acid than those of artificially fed children; but it is not possible to say whether *B. bifidus* is the cause or an effect of the reaction. The particular constituents of human milk which favour the development of an acid reaction of the faeces and lead to predominance of *B. bifidus* are likewise not precisely known. Schlossmann (33) attributes the acidity of breast-fed infants' faeces to the low ratio in human milk of protein to fat (1 : 3 compared to cow's milk 1 : 1), while others regard the relatively high percentage of lactose as the determining factor. It is noteworthy in this connection that Rettger and Cheplin and also Torrey (42) found that lactose and dextrin, both of which are absorbed only to a relatively slight extent, were the only two carbohydrates which favoured the predominance of an aciduric flora in the intestines of rats, dogs and human subjects. Morris, Porter and Meyer (24) changed the intestinal flora in children from a proteolytic to an aciduric type by giving them a high carbohydrate diet. Although the reaction of the faeces was not affected by giving lactose or dextrin (Rettger and Cheplin), Cannon and McNease (4) showed that in rats fed with lactose the acidity of the intestinal contents in the caecum was considerably greater (pH 5·6) than in the lower part of the colon (pH 6·8), whereas with rats on a meat diet the reaction in caecum and colon showed only a slight difference. In view of the relatively high content of human milk in lactose and the fact that owing to its poverty in buffer salts its reaction is much more easily changed than is that of cow's milk (Clark (5), Marriott (18)), it is possible that the slowly absorbed lactose affords a suitable pabulum for the growth of *B. bifidus*, and that as a result of the acid so produced the large intestine is stimulated to hurry on its contents, thus leading to an acid stool. This explanation accords with the fact that diarrhoeal faeces tend to be more acid than those which have lain longer in the bowel (Robinson (29)). Whether it is the degree of acidity which inhibits the growth of other organisms (Tissier, Kendall (11)), or whether *B. bifidus* and similar acid-tolerant organisms are directly antagonistic to the proteolytic

bacteria (Rettger and Cheplin) has not been definitely proved. It is well known that inoculation of infants' faeces into neutral broth results in a heavy growth of coliform organisms and enterococci and very scanty Gram-positive bacilli; whereas, if sufficient acid be added to the same bouillon, the coliform organisms are entirely suppressed and the acid-tolerant organisms multiply rapidly. It may be that in the neutral broth the coliform bacteria inhibit the growth of the aciduric organisms in the same way as they inhibit the growth of *B. typhosus* (Smith (34)). Experiments by the author *in vitro* with a mixture of equal quantities of *B. bifidus* and *B. coli* in neutral broth failed to demonstrate any appreciable inhibitory effect of either organism on the other. On the other hand, when emulsions of similar opacity were made from 24 hour cultures on agar of *B. bifidus* and *B. coli* and equal quantities of each were added to glucose broth which was then incubated at 37° C., it was found that *B. coli* failed to grow when sub-cultures were made after 72 hours' incubation, whereas in the control tube with *B. coli* alone, this organism could still be sub-cultured after 1 weeks' incubation at 37° C. An accurate comparison of the hydrogen-ion concentration of the broth containing *B. bifidus* plus *B. coli* and *B. coli* alone, after incubation at 37° C. for 3 days, could not be effected owing to the high degree of acidity. Consequently flasks containing 50 c.c. of lactose and glucose broth were inoculated (*a*) with emulsions of *B. bifidus* plus *B. coli*, and (*b*) with *B. coli* alone and titrated with normal sodium hydrate after 4 days' incubation, by which time *B. coli* had disappeared in the sub-cultures made from the flasks containing both organisms. It was found that more than double the amount of alkali was required to neutralise the broth containing *B. bifidus* plus *B. coli* than was required for that containing *B. coli* alone. The increased acidity of the mixed culture is probably responsible for the disappearance of *B. coli*.

By the addition of acetic acid to glucose broth or peptone water, a degree of acidity was readily attained which inhibited the growth of *B. coli* while permitting *B. bifidus* to flourish. The amount of acid required to inhibit coliform organisms isolated from breast-fed infants' faeces varied slightly with different strains, but in every case, the addition of 0·4 c.c. of $N/1$ acetic acid to 10 c.c. neutral 1 per cent. peptone water or glucose broth (*i.e.*, "4 per cent. acid") inhibited the growth of these organisms while allowing *B. bifidus* to grow. This degree of acidity (pH 4·0–4·4 as tested by the usual indicators) was greater than the average acidity of the faeces of twenty young breast-fed infants, which had a pH value of 5·2; hence it would seem that in the intestine of the breast-fed infant there is a combination of factors which allow *B. bifidus* to grow exuberantly and which suppress most of the other organisms. It is conceivable, too, that the presence of an almost purely aciduric flora, together with the high degree of acidity in the breast-fed infant's intestine, acts as a protective agent, particularly against invasion by organisms of the coli group, and this may explain in part at least the relative immunity of the breast-fed child to gastro-enteritis.

SUMMARY.

1. *B. bifidus communis*, first isolated and described by Tissier, is the predominant organism in the intestinal flora of healthy breast-fed infants in the early weeks of life. In films of the faeces at this period Gram-positive bacilli may constitute almost 99 per cent. of the organisms present.

2. *B. bifidus* requires fairly strict anaerobic conditions for its isolation in primary culture; but thereafter it grows readily in sub-cultures in the presence of oxygen.

3. *B. bifidus* is a member of the acid-tolerant group of Gram-positive, faecal organisms. Although closely resembling *B. acidophilus*, it is distinct from the latter morphologically and also in certain of its cultural characters. The results of agglutination reactions indicate that different strains of *B. bifidus* are not serologically uniform.

4. Although *B. bifidus* is a very pleomorphic organism, no evidence was obtained in the present work that it could be changed into an aerobic, spore-bearing bacillus.

5. The predominance of *B. bifidus* in the intestinal flora of breast-fed infants appears to be closely related to the high degree of acidity of the faeces of these infants. It is probable that the predominance of *B. bifidus* over other organisms is an important factor in preserving a healthy condition of the intestinal tract in the breast-fed infant.

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DESCRIPTION OF PLATE II.

Fig. 1. Smear of faeces of breast-fed infant (2) aged 8 days. Gram stain. × 1000.
Fig. 2. *B. bifidus*, film of young culture on agar isolated from faeces of infant (2). Gram stain. × 1000.
Fig. 3. *B. bifidus*, film of old culture in deep glucose-agar. Gram stain. × 1000.
Fig. 4. *B. bifidus*, surface colonies on agar after 48 hours' incubation. × 25.
Fig. 5. *B. bifidus*, deep colony in glucose-agar after 3 days' incubation. × 60.
Fig. 6. *B. acidophilus*, deep colonies in glucose-agar after 2 days' incubation. × 100.

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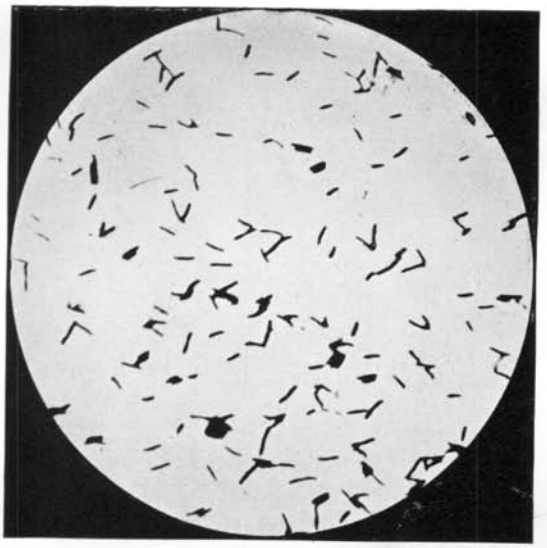


Fig. 1

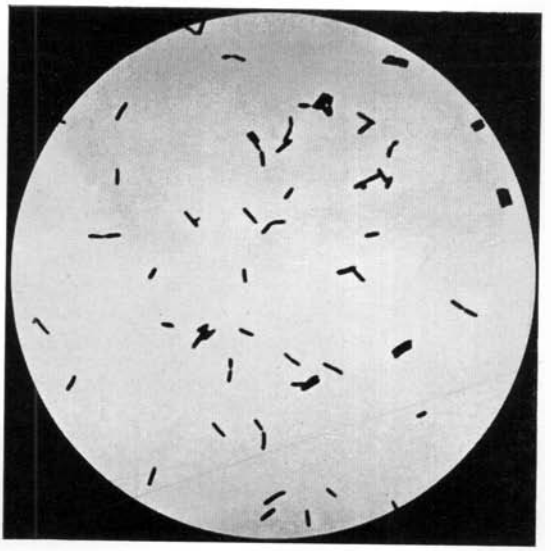
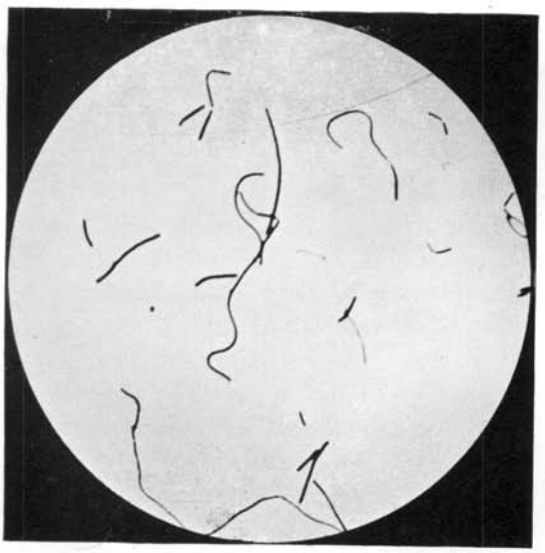


Fig. 2



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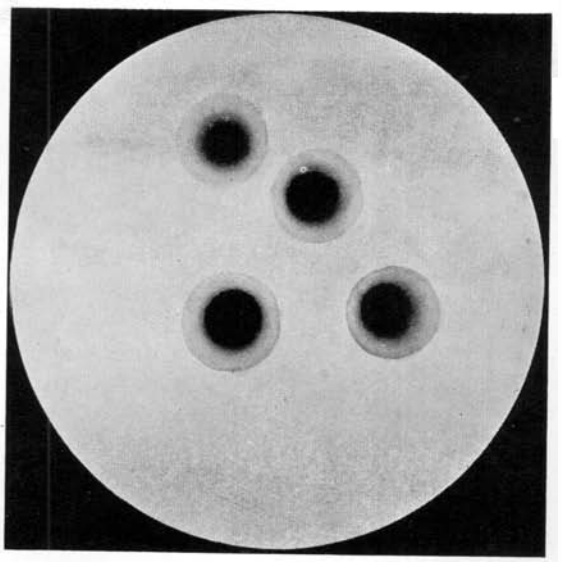

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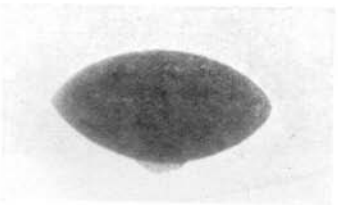

Fig. 5

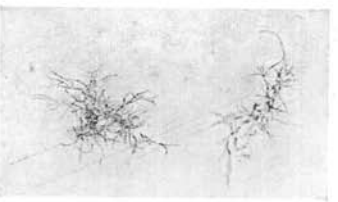

Fig. 6

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