

THE NORMAL BACTERIAL FLORA OF THE BEE.¹

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(PLATES III. AND IV.)

INTRODUCTORY.

THE object of the investigation, the main results of which are outlined below, was to determine as definitely as possible the nature of the normal flora of the alimentary tract of the honey-bee, *Apis mellifica*, in order to pave the way for a bacteriological study of Isle of Wight disease of bees.

This work was made necessary owing to the fact that no comprehensive account of the normal intestinal organisms of the bee could be discovered in the literature. Apart from the descriptions of the bacteria associated with certain diseases—more particularly brood diseases—and an analysis by Bahr (1919¹) of the “coli-like” organisms found in the bee, the only published work on the subject appears to be that of F. G. White (1906²), who gives an account of certain organisms isolated from pollen, comb, and the surface and intestine of the bee. It was found, however, that as regards the flora of the alimentary tract his description is inadequate, and that the majority of the organisms described by him are casual and inconstant components of the flora.

The work was carried out in the Pathology Department, Marischal College, Aberdeen, under the auspices of the Joint Committee on Animal Nutrition of the University of Aberdeen and the North of Scotland College of Agriculture.

Sufficient samples of bees have been examined from a variety of sources, the material being representative of some fifty to sixty stocks. The majority of bees were derived from Aberdeenshire and Kincardineshire, but a sufficient number of samples have been obtained from England and Wales to prove the general distribution of the organisms which have been studied.

In the majority of cases between six and a dozen worker-bees have been selected for each series of observations. In all, some 1200 bees have been examined by cultural methods and a larger number by direct

¹ Received July 1920.

observations on smears of the intestinal contents. Observations have been mainly directed to the chyle stomach and intestine. The contents of these organs were subjected to as thorough an examination as possible by—

1. Fresh preparations viewed as hanging drops ;
2. Direct smears stained by a variety of methods ;
3. Cultivation for periods varying up to fourteen days on a variety of solid and liquid media under aerobic and anaerobic conditions through a range of temperatures.

EXAMINATION OF STAINED FILMS.

A very considerable number of bees have been examined by stained films of various portions of the alimentary tract. The gut and chyle stomach were withdrawn as carefully as possible and their contents expressed upon a clean slide. Emulsions made with distilled water were prepared as films and stained.

Gram staining was found a very suitable method of surveying the flora of the gut. It was, however, found necessary to extract the dried films successively with xylol, spirit, and water before applying the stain. For examination of the flora of the chyle stomach where the "Gram-negative" elements are often obscured by débris, epithelial cells, and zymogen granules, which stain deeply with the usual counterstains, the "Gram" method is not so suitable. It was therefore used in duplicate with carbol-thionin preparations, or specially prepared and fixed films stained according to Giemsa. Both these methods gave excellent differentiation of the bacteria present.

Without overestimating the value of purely microscopical examination, it became evident that the normal flora of the gut and chyle stomach present certain very constant appearances suggesting the uniform occurrence of definite types mingled in very varying proportions. In individual films there may be present, sometimes in considerable number, a variety of microscopical types which depart from the above and represent the casually occurring element of the flora.

Almost always in examining Gram-stained films of the hind gut contents, the eye is struck by the occurrence of certain Gram-positive and Gram-negative bacilli, which conform to the four following types:—

Type I.—*Large form*, averaging $3.5 \mu \times 0.6 \mu$ in dimensions. It is regular in outline, possessed of a stiff appearance, and is usually bluntly rounded at the poles. The elements occur singly, or more frequently in slightly inflected pairs, and occasionally in longer systems or filaments. The Gram stain is often taken irregularly ; sometimes deeply Gram-stained granules at the poles alone remain. Such granules may also be shown by toluidine blue.

Type II.—*Small form*. Usually a slender and delicate bacillus of diphtheroid form averaging $2 \mu \times 0.3$ to 0.4μ in dimensions. The measurements are, however, very variable. There may be considerable irregularity of form, with swellings in the region of chromatic granules which are sometimes polar, sometimes central. Spindle, comma, and club forms are sometimes frequent. As the

bacillary pairs are sometimes united by their finer, sometimes by their clubbed extremities, a varied picture is produced. There is seldom any sign of chain or filament formation. In a large proportion of preparations the bacilli take a peculiar radial arrangement forming stellate groups about a central space or mass. This grouping is curious and characteristic.

While it is usually possible to distinguish the presence of these two elements, there is often difficulty in determining the nature of individual bacilli for the larger forms of Type II. greatly simulate those of Type I. In films stained with toluidine blue (Pugh's stain) there is a certain degree of differential staining, the bacilli of Type I. taking a hazy purple stain, those of Type II., apart from metachromatic granules, a clear blue.

Though both forms usually occur together, one usually predominates considerably over the other, not only in the individual bee but also in the stock from which it is derived.

Type III.—Among the multitude of Gram-negative organisms which is usually present, it is, as a rule, possible to distinguish dense clusters and scattered units of small or minute bacilli or cocco-bacilli varying in dimensions between $0.2\ \mu$ to $1.3\ \mu \times 0.1\ \mu$ to $0.25\ \mu$. They occur mainly as diplo-forms and frequently give indications of bipolar staining. There is a general outward similarity to the *B. influenzae* of Pfeiffer.

Type IV.—From the above may be usually distinguished another Gram-negative type. This is usually considerably longer and is very slender, many units not much exceeding $0.1\ \mu$ in width. Very frequently the poles are finely tapered. Here again diploforms abound. In some preparations the bacilli are gathered into loose sheaves in which delicate filamentous forms may be present.

These four bacillary types constitute the constantly occurring element of the intestinal flora sufficiently abundant to attract attention in a microscopical survey.

In individual films these are accompanied by other forms such as large Gram-positive and Gram-negative bacilli, occasionally diphtheroid forms and cocci. Organisms with the general morphology of the coli group are perhaps the most frequent and numerous constituents of the casual moiety of the flora. Various sporing forms and yeasts, and occasionally oidium stages of fungi may be seen. On the whole one is struck by the sparseness of such organisms.

Smears of the chyle stomach contents show little qualitative difference in their bacterial flora. The quantitative difference is, however, great. The total number of organisms is much lower than in the gut, and on the whole there is a greater preponderance of the Gram-negative over the Gram-positive types. The majority of the bacteria are located in the terminal region of the stomach, and in bees in the optimum conditions of activity the upper portion may approach sterility. On confinement there is a rapid increase of the bacterial flora ascending from below upwards. The same applies to the Malpighian tubules, which may show considerable numbers of bacilli within the lumina after confinement for a few days.

CULTURAL WORK.

Cultural work was opened with a large series of cultures on agar. Variations in the acidity of the medium, the temperature of incubation,

and the oxygen conditions of the culture were tentatively employed. These attempts showed that:—

1. Ordinary agar is inadequate for the culture of the essential organisms of the alimentary flora.

2. A considerable variety of bacteria may often be cultivated from the gut and chyle stomach upon this medium which are not related to the basal flora and show no constancy in bees derived from different stocks—though one or more may run through most of the bees of a particular hive. Some of these organisms can be identified and others placed in bacterial groups. The following may be mentioned: *B. vulgatus*, *B. mesentericus*, bacilli of the *proteus* type, *B. coli* (*communis* and *communior*), and many organisms of this group. A variety of sarcinæ, micrococci, and bacilli, including many chromogenic forms, were not considered deserving of special study.

While coliform colonies and those of a few other organisms may appear in large numbers in first cultures, the scantiness of the various casually occurring forms is rather remarkable. On the whole the chyle stomach is richer in such organisms than the intestine, and often a rich growth of coli-form bacilli is obtained from chyle stomach material when no such organisms appear in the hind-gut culture of the same bee. Seemingly the contents of the intestine form an unsuitable substrate for any but the hardiest organisms, or those specially adapted to its conditions. In all probability there is definite bactericidal action due, in part at least, to the amount of acid present.

Attention was next directed to the discovery of more suitable media.

A series of cultures on Löffler's blood serum medium resulted in the isolation of several diphtheroid forms, but again no satisfactory growth of the main flora resulted.

Use was next made of various enriched agar media such as tryptagar, nasagar, unheated egg-yolk agar, and Levinthal's medium. With these an advance was made. As Levinthal's medium¹ has proved satisfactory and has been mainly employed, the appearances on this will be outlined.

Levinthal plate cultures of the intestinal contents incubated for two to three days at 37° C. showed in addition to colonies of agar-culturable organisms a large number of minute colonies at first too small to be individually examined by the unaided eye. By the fourth day two forms of colony could be as a rule discriminated.

¹ This medium was prepared by the addition of 3 to 5 per cent of human or sheep's blood to the unclarified nutrient agar while liquid at a temperature of about 50° C. The blood and agar were well mixed, and the mixture was then gradually brought up to 100° C. in the Koch's steamer and kept at this temperature for twenty minutes, after which it was allowed to cool and set firmly. It was then reliquefied, and the clarified agar was separated from the coagulum by filtration through glass wool, to be finally tubed and sterilised in the Koch's steamer.

(a) Minute slightly raised transparent colonies. Under the low power of the microscope these were seen to have a regular circular outline (Plate III., Fig. 5). The young colonies were often almost hyaline, later developing a brownish granulation at the centre. The colonies increased but little in size upon further incubation, unless when stimulated by the beneficial changes wrought on the substrate by other organisms growing in their vicinity. In this case colonies 0.5 mm. in diameter sometimes resulted. These colonies were found to consist of minute Gram-negative bacilli morphologically identical with the Type III. bacilli of direct films of the gut contents.

(b) Slightly larger, much flattened colonies consisting of a convoluted sheaf of bacilli from which branched and filamentous processes were thrown out over the surface of the medium (Plate III., Fig. 3). These colonies, too, were transparent by transmitted light, dry and greyish by reflected light, and when densely sown gave the surface of the medium a frosted appearance. The bacilli of these colonies corresponded microscopically with the Type I. bacilli of the preceding section.

Growing within the colonies of small Gram-negative bacilli, or occasionally forming minute individual surface colonies, bacilli agreeing microscopically with the Type II. bacilli of intestinal smears were sometimes encountered. It was, however, by the use of deep glucose agar shake cultures that strong growth of what was apparently the Type II. organism was consistently obtained. The optimum effect was obtained when the liquefied medium was deoxygenated by ebullition under reduced pressure before use. Shake cultures of the intestinal contents in this medium showed after three to four days' incubation at 37° C. very numerous minute colonies in the deeper layers. On further incubation a number of large spherical or biconvex whitish colonies were produced, surrounded by a haze of much smaller colonies. Both the large and the small colonies were often found to consist almost entirely of the same organism—a small diphtheroid bacillus of characteristic grouping resembling the Type II. bacilli of smears of the gut contents. At other times colonies of larger bacilli (Type I.) formed a contamination.

Upon further incubation of the tubes there appeared in many cases a sudden production of elliptical colonies forming a disc 1 to 1.5 cm. beneath the surface of the medium. These colonies appeared about the seventh to ninth day and reached a maximum diameter of 1.5 to 2 mm. They were composed of delicate Gram-negative bacilli often showing finely tapered ends, and were identified with the Type IV. organism *infra*.

It was thus rendered probable that the four main types of bacteria noted in films of the alimentary materials had been obtained in culture, and this opinion was borne out by further work. In the succeeding section the cultural and other characteristics of these and certain other less uniformly distributed organisms will be considered in some detail.

DESCRIPTION OF THE FLORA OF THE ALIMENTARY TRACT.

For purposes of description the flora of the intestine and chyle stomach may be divided as follows:—

I. The adapted flora.

- (i) constantly occurring element;
- (ii) inconstant element.

II. The unadapted and casual flora.

To Group I. belong those organisms which, so far as present

knowledge goes, seem to be peculiar to the bee, and which seem to be specially adapted to the conditions pertaining in the alimentary tract of that insect. In Group II. are placed those organisms which are liable to be encountered at any time in bacteriological studies, and which do not seem to have any special predilection for the alimentary tract of the bee. As has been stated earlier, it is probable that this substrate is unfavourable to the majority of bacteria.

It has been considered right to introduce four new specific names, viz., *B. constellatus*, *B. rigidus apis*, *B. influenzoides apis*, and *B. tenuis apis*. Otherwise the formation of special nomenclature has been avoided.

I. (i.) *Adapted Flora (Constant).*

Bacillus constellatus (Type II. Bacillus).

Morphology.—A straight or slightly curved bacillus of variable dimensions. In films from strongly growing glucose agar cultures the rods average $1.5 \mu \times 0.4 \mu$, while in direct films from the gut they range between 0.5 to $4 \mu \times 0.3$ to 0.8μ . Both in the bee and in culture the organism shows considerable pleomorphism, and club, comma, spindle, and bizarre forms may abound even in young actively growing cultures. Under certain conditions—more especially in the presence of traces of oxygen—remarkable bifid and staghorn forms appear strongly reminiscent of those of *B. bifidus communis* (Plate III., Fig. 2 (b)). Such forms may be encountered in the chyle stomach.

The units occur singly, more frequently in linear pairs, sometimes united by clubbed sometimes by pointed ends. In liquid cultures short chains—often simulating streptococci—and filaments occur, but the tendency in this direction is not great.

Very characteristic is the radial grouping of the bacilli to be seen in many direct preparations from the hind-gut contents and in most glucose-agar cultures, the film being littered with fan or asterisk-like clusters (Plate III., Fig. 2 (a)). In some liquid cultures there also occurs a separation of small elements at right angles to the main axis of two or three larger bacilli (Plate III., Fig. 2 (b)). The organism shows no motility and no flagella have been observed. There is no evidence of spore formation though vacuolated and bloated forms appear in old cultures which have a slight superficial resemblance to spores.

Staining Reactions.—The organism stains readily with the ordinary bacterial dyes and retains the primary stain in Gram's method. In Gram-stained films which have been lightly treated with methyl violet, or in films stained with toluidine blue, the presence of polar or central granules may be clearly seen. This helps to impart to the more slender forms a markedly diphtheroid appearance.

Oxygen Requirements.—As a rule the bacillus is a very strict anaerobe, and growth is either completely inhibited or very slow and involuting in the presence of traces of oxygen. Thus in general only thoroughly deoxygenated media with added glucose are serviceable for cultures. There are, however, exceptions, and occasionally strains growing moderately well under full aerobic conditions are encountered. More frequently subaerobic strains are found which in shake cultures form a belt of growth at varying distances beneath the surface. All types may be met with in the same specimen, but as a rule bacilli derived from the chyle stomach are more tolerant to oxygen than those from the intestine.

Growth Characteristics.—As stated above only thoroughly deoxygenated media with glucose or other reducing agents are generally applicable to culture.

The surface colonies on glucose agar are raised, circular, white, and moist-looking, and are usually very irregular in size. Unless densely sown they remain discrete. The growth is very coherent, the entire colony often being removed by the needle, and does not readily form an even emulsion. Under the low power of the microscope the young colonies (Plate III., Fig. 1) are very highly refractile and have a curious bushy appearance, later becoming dense, brownish, and granular. The margin is fairly regular and sharply defined. Deep colonies show a very varied rate of growth which in first cultures is especially marked, a varying number of large spherical or lenticular colonies (1 to 1.5 mm. in diameter) lying in a nebula of minute colonies. The deep colonies often have a conglomerate structure.

In glucose gelatin at 22° C. there is a very slow non-liquefactive growth.

The organism does not take well in liquid media, and in carefully deoxygenated glucose broth growth may be invisible at the end of a week. It was, however, found that when sterile animal charcoal was added and the medium boiled under reduced pressure before use a much larger number of cultures were successful. Growth, once properly started, proceeds rapidly with the production of a heavy, often granular deposit of a whitish grey colour. Glucose and maltose are fermented with the production of acid. No gas is evolved. No other sugars have been found fermentable. Vigorous growth may be obtained at the bottom of open broth tubes in the presence of other organisms,—those which discharge the colour of litmus being most active in this respect.

No growth has been obtained on potato or in milk.

Isolation.—The organism is best isolated from glucose agar shake cultures of the hind-gut contents. The rate of growth is increased by boiling the medium under reduced pressure. In such a medium colonies are usually large enough for selection and subculture after two to three days at 37° C.

Surface first cultures on glucose agar by the usual anaerobic methods usually fail.

Vitality.—Cultures may remain alive up to the end of two months.

Bacillus rigidus apis (Type I. *Bacillus*).

Morphology.—A medium-sized, straight or slightly curved bacillus averaging $3.5 \mu \times 0.6 \mu$ in dimensions and possessed of a somewhat "raide" appearance (Plate III., Fig. 4 (a)).

There is a tendency both in the bee and in culture to form linear pairs, and filaments and chains of considerable length may be encountered. Coccoid and streptococcoid forms are not uncommon especially in liquid cultures. The rods are as a rule bluntly rounded at the ends. In anaerobic and subaerobic cultures the form and dimensions may become irregular, and slender bacilli and filaments appear frequently accompanied by bloated and spherical involution forms (Plate III., Fig. 4 (b)). No motility has been observed and spores do not appear to be produced.

Staining Reactions.—The usual bacterial stains are taken with avidity. To the Gram stain it reacts somewhat irregularly. Typically the primary stain is retained but usually a number of rods are more or less completely decolorised by the alcohol. In deep glucose agar and liquid glucose media the organism is usually Gram-negative. More deeply staining polar and central granules are seen by differential staining methods. The spherical involution forms stain very deeply with such stains as carbol-thionin and toluidine blue.

Oxygen Requirements.—The optimum oxygen conditions vary with different strains. Some are distinctly aerobic, but the majority probably thrive better in the presence of traces of oxygen than they do under atmospheric conditions. The organism is also a facultative anaerobe.

Growth Characteristics.—Ordinary agar is not a satisfactory medium, much better growth being obtained by the addition of glucose or the use of such media as Levinthal's agar or tryptagar. Surface colonies on these media are small, flattened, greyish, and dry looking, imparting a frosted appearance to the surface of the medium when thickly sown. Under the low power of the microscope the colony is usually found to consist of a contorted sheaf of bacilli (Plate III., Fig. 3) from which runners spread outwards. At other times the colony appears as an irregular mass of bacilli, or by the formation of sinuous wisps resembles a young colony of *B. anthracis*.

Buried colonies in glucose agar have usually the appearance of small flocculi, and when examined by the microscope show a small granular centre from which irregular arborescent processes arise.

No growth has been observed in gelatin or glucose gelatin at 22° C. In glucose-broth and glucose-gelatin incubated at 37° C.—which latter medium is very suitable—a highly flocculent whitish sediment is produced, the supernatant medium remaining clear; the sediment may be considerable. Glucose and maltose broth are rendered acid but no gas is evolved. No other sugars have been found fermentable and no change is visible in litmus milk.

A feeble, dry, and film-like growth had been noted on potato.

Isolation is most readily effected from surface cultures on glucose or Levinthal's agar, subcultures being made as glucose agar shakes. Surface subcultures often fail.

***Bacillus influenzoides apis* (Type III. Bacillus).**

Morphology.—A small bacillus ranging between 0.2 to 1.3 $\mu \times 0.1$ to 0.3 μ in dimensions. Frequently it is oval or coccoid and closely simulates the *B. influenzae* of Pfeiffer (Plate III., Fig. 6 (a)). In cultures and in the bee, microforms appearing as "pin-points" at a magnification of 2000 diameters are sometimes abundant. At other times it is slender. There is a great tendency to the formation of diploforms, but filaments or chains of more than three units are rare. In old cultures larger irregularly segmented forms may appear. In direct smears from the gut, the bacilli often appear in dense clusters, each cluster being composed of bacilli of uniform dimensions often differing markedly from those of other clusters in the same preparation. There is no evidence of spore formation. The organism is motile by means of a single polar flagellum (Plate III., Fig. 6 (b)).

Staining Reactions.—The stronger bacterial stains are taken with fair readiness, and distinct bipolar staining is sometimes to be seen. The primary stain is entirely lost in Gram's method.

Oxygen Requirements.—The organism belongs undoubtedly to the subaerobic organisms, thriving best at an oxygen pressure slightly below that of the atmosphere.

Cultural Characteristics.—Ordinary agar is unsuitable for culture, much better results being obtained with such enriched media as Levinthal's agar, egg-yolk agar, or with glucose agar. Levinthal's agar with 1 per cent. of added glucose has proved especially useful. On this medium the surface colonies are minute, circular, slightly raised, and almost transparent, resembling those of *B. influenzae*. Under the low power of the microscope the young colony (Plate III., Fig. 5) may appear almost homogeneous, later becoming mottled, and finally showing a brownish central granulation. Occasionally in the presence of other organisms, colonies 0.5 mm. in diameter may be produced. In first cultures of the intestinal contents the colonies often appear in vast numbers after three to four days' incubation at 37° C., but surface subcultures usually fail unless supported by a growth of other organisms.

Pure subcultures are best made on glucose-Levinthal plates and covered by a thin layer of the same medium poured over the inoculated surface, so that growth

occurs between two layers of the medium. The colonies are then usually visible on the third day, and have somewhat the appearance of crenated blood corpuscles when viewed with the microscope. After repeated subculture between layers of the medium a pure surface growth has been in several instances obtained.

Broth tubes inoculated with the pure culture show little or no growth; in glucose broth slow growth occurs without producing visible turbidity or sediment. Glucose is fermented without gas production. Growth also takes place in maltose broth, but the production of acid is doubtful. No other sugars seem to be affected.

No growth has been seen on gelatin or glucose-gelatin at 22° C. No growth has been obtained on potato and milk remains unchanged.

Isolation.—Subcultures from surface first cultures must be made almost as soon as the colonies are visible (third to fourth day) and covered by a thin layer of the medium as described above. The colonies are often too small for individual selection at the time of subculturing, and a number of strokes must be made and the subcultures examined later for contamination.

Vitality.—The organism is extremely delicate and susceptible to drying. On the most favourable media the organism rapidly dies out, and even though the colonies may continue to increase slightly in size up to the sixth day, the power to develop in subculture is often lost.

***Bacillus tenuis apis* (Type IV. *Bacillus*).**

Occurrence.—Present in the great majority of adult bees, but probably not in all.

Morphology.—In the bee and in buried glucose agar cultures it occurs as a very slender bacillus, often delicately pointed at the poles (Plate IV., Fig. 7 (a)). In such preparations the dimensions vary between 0.8 to 3 μ \times 0.1 to 0.3 μ . Diploforms are very frequent. In such cultures a few delicate filamentous forms may occur. In surface cultures on glucose agar a great tendency to the development of filaments and streptobacillary chains appears. The filaments are often much thicker than the bacillary forms; frequently they radiate from a dense central knob, while delicate bacilli and streptobacilli are interspersed among them (Plate IV., Fig. 7 (b)). In old cultures oval and spherical involution forms arise. The organism is non-motile and no spores are produced.

Staining Reactions.—The organism stains well with the usual basic stains. The filamentous forms often take the stain irregularly. Definite polar granules may occur. The primary stain of the Gram method is lost.

Oxygen Requirements.—The organism is a typical subaerobe, and early cultures are only possible at a definite reduced pressure of oxygen, the exact conditions varying with different strains. Growth under completely anaerobic conditions has not been obtained. The organism may be rapidly educated to atmospheric conditions and then flourishes more luxuriantly than during subaerobism.

Cultural Characteristics.—Upon ordinary agar little or no growth occurs. In 2 per cent. glucose agar shakes in the case of first cultures and early subcultures growth appears at the end of six to seven days. The colonies appear suddenly and grow fairly rapidly occupying a narrow zone 1 to 1½ cms. beneath the surface of the medium. In the case of later subcultures growth appears sooner and ascends to the surface. No growth occurs in the deeper layers of the medium. The buried colonies are oval, flattened, and biconvex and of a yellowish white colour. Under the low power of the microscope the colonies are dense and sharply defined. When once educated to surface conditions good growth is obtained in twenty-four to forty-eight hours. The surface colonies are large, circular, and often considerably raised. They are at first creamy white and moist looking, but later the colour deepens to orange and the surface becomes wrinkled and dry. On a moist medium the growth has a

creamy consistence and may spread out over the surface, but on a drier medium it is coherent and sticky, the colonies remaining discrete.

The glucose agar stab culture is characteristic, the growth ending abruptly at approximately 1.5 cm. from the surface. No growth was observed in gelatin at 22° C., but in glucose gelatin a slow, non-liquefying growth similar to that on glucose agar occurs. In glucose broth there gradually appears a uniform turbidity with a meagre deposit. There is no pellicle. Glucose appears to be the only sugar fermented; no gas is produced. No change is produced in milk. The organism thrives well on potato, giving rise to a thick slimy growth of a greenish brown colour.

Vitality.—Cultures may remain alive up to six weeks.

I. (ii.) *Adapted Flora (Inconstant)*.

Diphtheroid A.

Distribution frequently isolated from the intestine and chyle stomach of healthy bees. It does not appear to occur in great numbers.

Morphology.—A medium-sized bacillus exhibiting considerable pleomorphism. In rapidly growing cultures on Löffler's serum the units are short, oval, or lanceolate. Arranged in pairs they have the appearance of pneumococci. In more slowly growing cultures the bacillary form is much more marked. A considerable amount of clubbing and irregular segmentation may be present. In liquid serum cultures the units are mostly coccoid. The grouping is typically "diphtheroid." No spores are produced and the organism is non-motile.

Staining.—The usual bacterial dyes are taken with readiness and the primary Gram stain is retained. Such stains as toluidine blue and the Neisser stain demonstrate metachromatic granules which are best developed in Löffler serum cultures. The granules are large and usually closely beaded along the rod (Plate IV., Fig. 8 (a)), but typical dumb-bell forms may occur.

Oxygen Requirements.—All strains which have been studied were obligatory aerobes.

Cultural Characteristics.—The agar colony after two days' incubation at 37° C. is small, circular, and streptococcus-like, sometimes slightly raised, sometimes flattened and spreading at the margin. The growth is soft and readily emulsifies. On tryptic agar and Levinthal's agar the growth is similar but more luxuriant. On Löffler's serum medium growth is much more rapid, and raised creamy white confluent colonies are produced in twenty-four hours. On gelatin at 22° C. growth is very slow; no liquefaction results and the colony presents no special features.

In broth a slight diffuse turbidity is produced. Some strains ferment glucose and maltose without the evolution of gas, but in the majority of cases all sugars have remained unaffected at the end of a week. No change was observed in milk. Upon potato a white creamy growth resulted.

Diphtheroid B.

Occurrence.—Several times isolated from the intestine of healthy bees.

Morphology.—In general form this organism is very similar to *Diphtheroid A*. Upon Löffler's blood serum medium the rods are, however, considerably longer and more slender, more resembling the Klebs-Löffler type. In some cultures there is a considerable amount of true branching (Plate IV., Fig. 8 (b)).

Staining Reactions.—Very similar to those of *Diphtheroid A*, with the exception that the metachromatic granules are fewer and mostly confined to the poles of the rod.

Cultural Characteristics.—Upon agar and Levinthal's medium a moderate confluent growth of a pinkish colour is produced. At 22° C. on gelatin it grows

more slowly, again with the development of a pink pigment. Upon Löffler's serum a thick moist yellow or orange growth appears. In broth a yellowish deposit is produced. No fermentation of sugars has been observed.

***Streptococcus apis* (Var. ?).**

Occurrence.—Occurs with some frequency in the lower part of the chyle stomach and less frequently in the gut of the bee. In Isle of Wight disease the incidence of this organism appears to be increased.

Morphology.—The examination of a considerable number of strains has revealed a range of variation similar to that encountered in the pyogenes-conglomeratus group of human streptococci. Two main forms are to be found. In the first the cocci are small, round, regular in size, and the chains are usually of great length, forming tangled skeins and knots (Plate IV., Fig. 10 (a)). In the second the units are considerably larger and are less regular in size and shape many being oval or bilanceolate. The chains may be of considerable length but seldom reach that attained by the former (Plate IV., Fig. 10 (b)).

Capsules have never been demonstrated.

Oxygen Requirements.—When first isolated the organism is usually a definite subaerobe and grows best at an oxygen pressure below that of the atmosphere. Later all strains grow well under ordinary conditions. Some strains, but not all, are facultative anaerobes.

Cultural Characteristics.—With strains well established on artificial media fair growth may be obtained on ordinary agar, but glucose agar is much more satisfactory. Upon this medium the smaller types of cocci form minute, much raised colonies, which under the low power are highly refractile (Plate IV., Fig. 9); on a moist medium the colony may be festooned with chains. On the same medium the larger cocci form much larger, circular, and slightly raised colonies which have under the microscope a brownish colour and granular centre (Plate IV., Fig. 9). Every grade of intermediate colony is encountered. In suitable liquid media, more especially those containing fermentable sugar, growth is far more luxuriant than on the corresponding solid media. In glucose broth the small long-chained type produces in twenty-four hours abundant growth resembling that of *S. conglomeratus*. There is a heavy granular deposit but the supernatant fluid remains clear. The large cocci on the other hand produce a heavy turbidity and a flocculent or flocculo-granular deposit. The fermentative action of these cocci are summarised in the table below. No gas is evolved.

	Lactose and Saccharose.	Maltose and Glucose.	Mannite, Dulcitol, and Inulin.	Litmus Milk.
Small cocci . . .	A	A	O	A and C
Large cocci . . .	A or O	A	O	A or A and C

A = acid production and C = clot. O = no reaction.

No growth has been observed on gelatin at 22° C. Some strains are actively hæmolytic and may produce local abscesses in guinea-pigs.

Isolation.—The organism rarely appears in first cultures of the alimentary contents on agar or glucose agar and then the colonies are usually within those of coliform bacilli. Two methods of isolation have given satisfactory results.

(a) First cultures are made in glucose broth and this plated out on glucose agar after twenty-four hours' incubation at 37° C.

(b) First cultures are made in pure sterile unheated yolk of egg. After twenty-four to forty-eight hours' incubation the tubes which show streptococci

are plated out on glucose agar. This method gives the greatest number of positive findings.

The necessity for these manoeuvres appears to be that certain inhibitory substances in the bee must be diluted; that the organism is a subaerobe and oxygen must be partially excluded and that certain fermentable substances such as glucose must be present.

In addition to the foregoing, brief mention may be made of the following forms:—

1. A small bacillus morphologically identical with *B. influenzoides apis* which it also resembles in most cultural characteristics. The colony surface resembles that of *B. influenzoides* but under the microscope appears practically homogeneous and limpid. It is not often encountered.

2. A non-motile, non-sporing bacillus of irregular size and shape, averaging $1.5 \mu \times 1.1 \mu$ in dimensions. The majority of units are oval and may show pointed ends. The general appearance is yeast-like (Plate IV., Fig. 11 (a)). It is Gram-negative and, as a rule, takes a hazy and faint stain with the usual dyes. The staining is often polar or irregular. There may be a few meta-chromatic granules. The surface colony on Levinthal's (glucose) medium resembles that of *B. influenzoides* in minuteness and general form, but is very granular under the low power of the microscope. It has been encountered in 1 or 2 per cent. of the bees examined.

3. A plump, slightly or markedly curved bacillus or spirillum averaging $1.5 \mu \times 0.8 \mu$ in dimensions. In the bee and in cultures the organism frequently adopts a whorled grouping (Plate IV., Fig. 11 (b)). It is actively motile, but attempts to demonstrate flagella have not succeeded. It is Gram-negative, and no spores have been observed. The surface colony on Levinthal's medium is raised, circular, and has brownish transparence. The growth is firm and gelatinous. It has been found difficult to obtain pure subcultures of this organism. It is present in a considerable proportion of bees.

4. A Gram-negative, non-motile, spindle-shaped bacillus averaging $3.5 \mu \times 0.8 \mu$ (Plate IV., Fig. 12 (a)). It grows well on agar, upon which it produces largish circular colonies of the coliform type. Viewed by transmitted light these colonies have a curious appearance of spinning round. Spores are formed on the second day of incubation at 37°C . and are oval, single, and located towards the poles. In anaerobic culture and sometimes in the bee large involution forms appear (Plate IV., Fig. 12 (b)). In broth a heavy turbidity and sediment is produced. Glucose and maltose are rapidly fermented; lactose and saccharose more slowly. Gelatin is not liquefied. Milk is rendered acid and later digested. This organism has been fairly frequently encountered. It is doubtful whether it belongs to the specialised flora of the bee.

Several other organisms have been met with in a proportion of the bees examined, but it does not seem desirable to extend this list.

II. Casual Flora.

As has been stated, a considerable number of organisms—mostly agar-culturable—must be relegated to this group.

The following have been most frequently encountered: *B. vulgatus*, *B. mesentericus*, *B. subtilis*, *B. proteus*, *B. fluorescens liquefaciens*, *B. coli* and its congeners.

The last mentioned are by far the most frequent and abundant of the casual invaders of the alimentary tract. So far as an examination of these organisms has gone, the results have agreed with the

findings of Bahr⁽¹⁾ in his careful analysis of the coliform bacilli of the bee. Organisms of the true coli, the metacoli, and paratyphoid groups have all been frequently encountered. The diversity of forms, however, rendered any finer classification impossible without a protracted investigation.

Flora of Young Bees, Drones, and Queens.

The alimentary tract of a bee, when it first emerges from the comb, appears to be sterile. A period elapses before the adult flora is established. During this time, organisms are usually scanty but usually show a great diversity of type. Coliform bacilli are often among the first invaders. No important difference has been noted between the intestinal organisms of the queen and drone bees and the worker-bee.

CONCLUSIONS.

From what has been said it is seen that the intestine of the adult bee possesses a distinct fundamental flora of considerable constancy and relative simplicity. Further, the organisms are as a whole characterised by their predilection for glucose-containing media and the fermentation of this sugar without gas production. Further, the majority are either anaerobic or thrive best under reduced oxygen conditions. The organism to which the name *B. constellatus* has been given is probably closely related to *B. bifidus communis* of the breast-fed infant. It probably corresponds to the "Bacterium D" of White (1906²), though he states that there is no fermentative action on glucose. Whether the organism which has been referred to as *Streptococcus apis* (var.?) in this paper is really the variety of the *S. apis* (Maassen) isolated from foul-brood larvæ is doubtful. According to White (1920³) this organism is usually diplococcoid, but may occasionally form chains of two or more pairs. Growth occurs on gelatin at refrigerator temperature and liquefaction sets in. Mannite is fermented. The streptococcus of the adult bee differs in forming chains of great or considerable length and in not fermenting mannite. Attempts to obtain growth on gelatin at 22° C. have failed.

In concluding, I wish to tender my sincere thanks to those who have in one way or another assisted me in this work; to Dr Rennie, directing the Bee Disease Investigation, and to Miss Elsie Harvey, both of whom have kept me abundantly supplied with material and have helped me in many ways; to Prof. Shennan and the staff of the Pathology Department, who have offered me every facility for carrying out my work and have given much valuable advice and encouragement. Special thanks are also due to Mr A. H. E. Wood, Glassel, Aberdeenshire, to whose interest and support the investigation owes much.

While it is not pretended that the investigation has been by any means exhaustive, it is hoped that this survey of the intestinal flora will help to fill a gap in our knowledge of the bee, and may be helpful to other workers in apian bacteriology.

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DESCRIPTION OF PLATES III. and IV.

(N.B.—All Cultures figured were incubated at 37° C.)

PLATE III.

- FIG. 1.—*B. constellatus*. Young surface colonies on glucose agar after three days anaerobic incubation. (× 66.)
- FIG. 2(a).—*B. constellatus*. Bacilli from fourth day glucose agar shake culture. (× 1950.)
- (b).—*B. constellatus*. Bacilli from glucose broth culture after incubation for one week. (× 1950.)
- FIG. 3.—*B. rigidus apis*. Aerobic surface colonies from forty-eight hours culture on Levinthal's medium. (× 66.)
- FIG. 4(a).—*B. rigidus apis*. Bacilli from forty-eight hours surface culture on Levinthal's medium. (× 1950.)
- (b).—*B. rigidus apis*. Bacilli from forty-eight hours culture in glucose broth. Gram-staining was very irregular. Note spherical involution forms budded off from ends of rods. (× 1950.)
- FIG. 5.—*B. influenzae apis*. Surface colonies from first culture of hind-gut contents on glucose. Levinthal's medium after three days incubation. (× 66.)
- FIG. 6(a).—*B. influenzae apis*. Bacilli from forty-eight hours culture on glucose. Levinthal's medium. (× 1950.)
- (b).—*B. influenzae apis*. Same as Fig. 6(a), showing flagella. Film from which drawing was made stained by de Rossi's method.

PLATE IV.

- FIG. 7(a).—*B. tenuis apis*. Bacilli from 4-day-old subculture in glucose agar (deep colonies). (× 1950.)
- (b).—*B. tenuis apis*. Bacilli from second day surface colony on glucose agar. (× 1950.)
- FIG. 8(a).—Diphtheroid A. Bacilli from twenty-four hours culture on Löffler's serum. Drawing made from film stained with Pugh's toluidine blue. (× 1950.)
- (b).—Diphtheroid B. Bacilli from twenty-four hours culture on Löffler's serum. Drawing made from film stained with toluidine blue. Note branched forms. (× 1950.)
- FIG. 9.—*Streptococcus apis* (var.?). Colonies from mixed culture of large and small types of cocci on glucose agar after twenty-four hours incubation. One large, moderately raised colony of the large coccus type is seen among several small, much raised, highly refractile colonies of the small type of coccus. (× 66.)

- FIG. 10 (a).—*Streptococcus apis* (var. ?). Small coccus type. Chains from twenty-four hours culture in glucose broth. ($\times 1950$.)
- (b).—*Streptococcus apis* (var. ?). Large coccus type. Chains from twenty-four hours glucose broth culture. ($\times 1950$.)
- FIG. 11 (a).—Curious "yeast-like" bacillus referred to in text. Bacilli from third day culture on Levinthal's medium showing irregular shape and staining. ($\times 1950$.)
- (b).—*Spirillum* ?, referred to in text. Organisms from forty-eight hours culture on Levinthal's medium. Note whorled grouping. ($\times 1950$.)
- FIG. 12 (a).—Sporing bacillus, referred to in text. Bacilli from thirty-six hours culture on agar, three showing stained spores. ($\times 1950$.)
- (b).—Same organism as Fig. 12 (a). Involution forms from anaerobic agar culture. ($\times 1950$.)

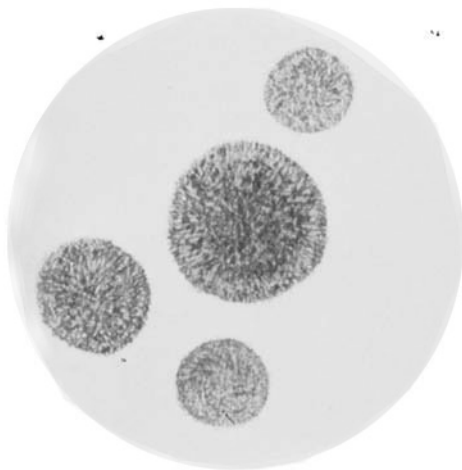


FIG. 1.

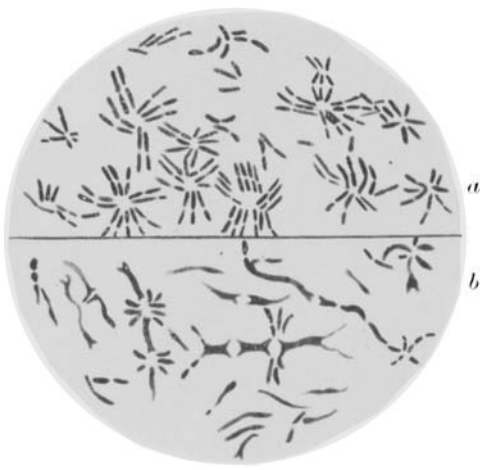


FIG. 2.



FIG. 3.

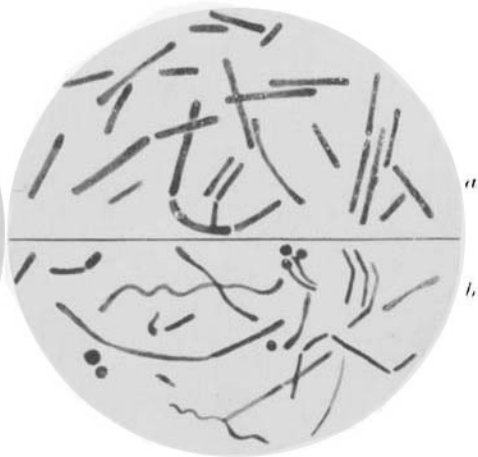


FIG. 4.

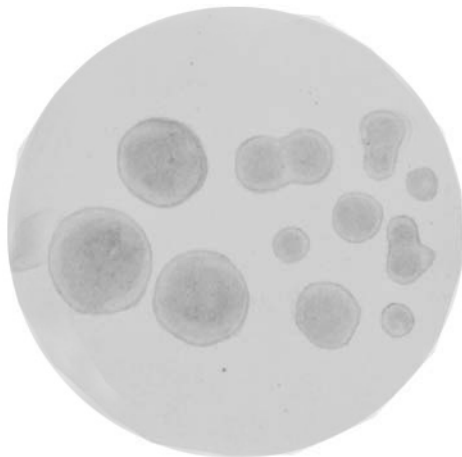


FIG. 5.

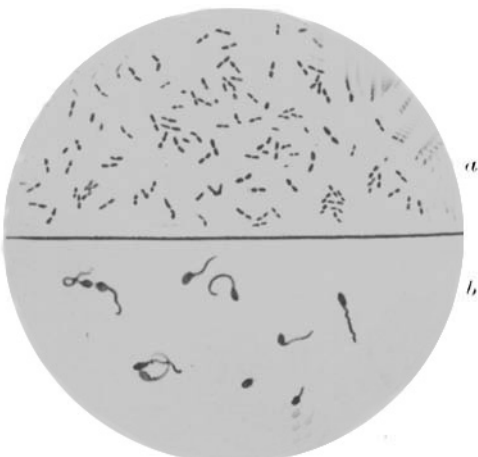


FIG. 6.

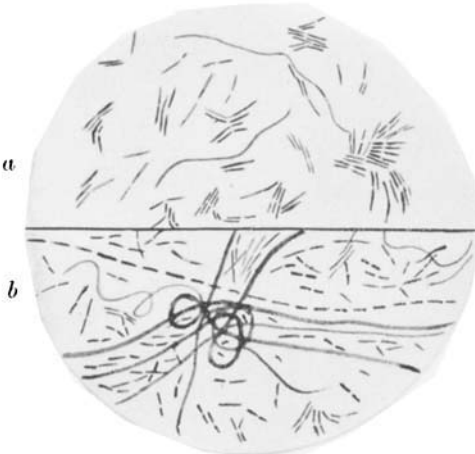


FIG. 7.

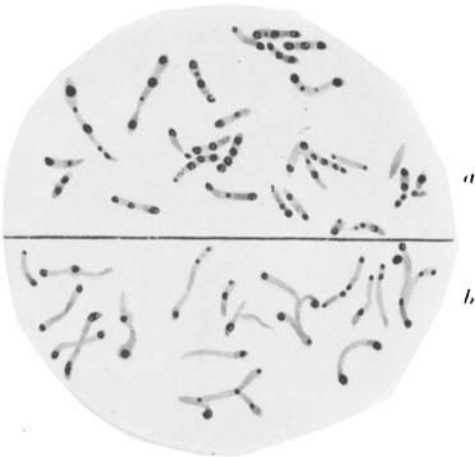


FIG. 8.

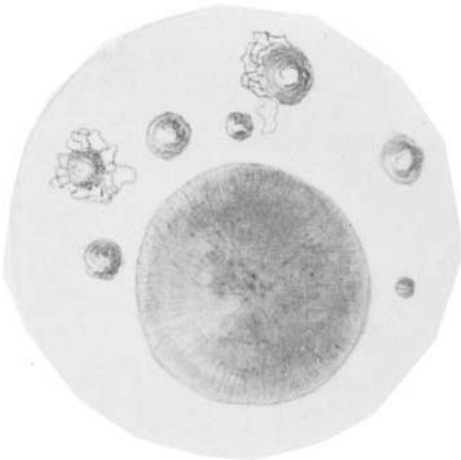


FIG. 9.

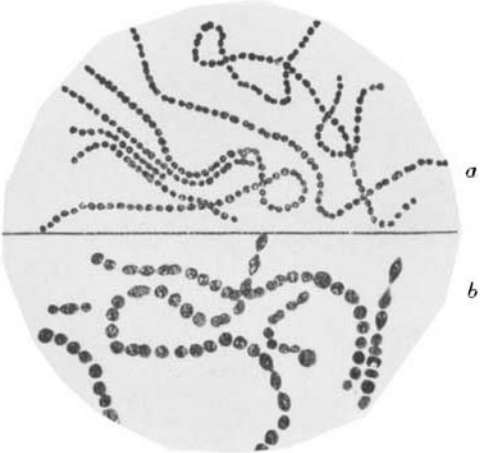


FIG. 10.

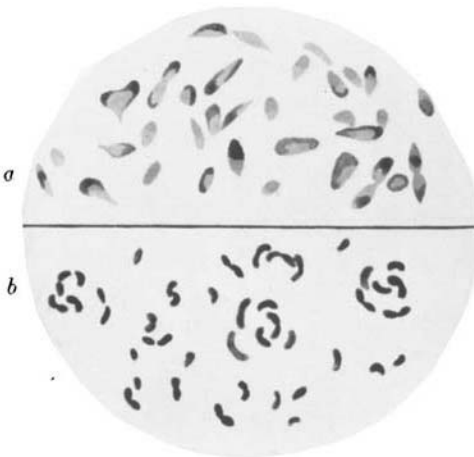


FIG. 11.

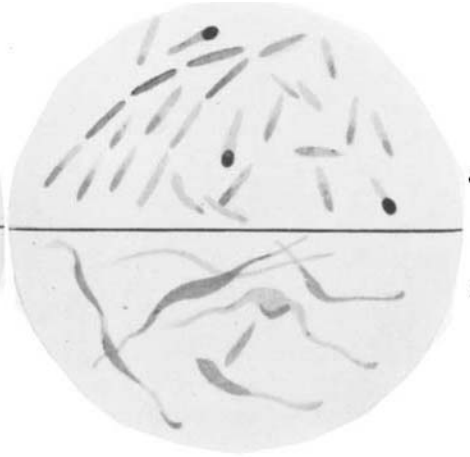


FIG. 12.