TURKEY LIVER
WITH CHARACTERISTIC BLACKHEAD LESIONS

BACTERIA AS SECONDARY INVADERS IN BLACKHEAD LIVERS OF TURKEYS

by

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REVIEW OF THE LITERATURE

A. Introduction

In 1895 Theobald Smith described a severe illness of turkeys which he named infectious enterohepatitis. However, the designation in more common usage by layman and specialist alike has come to be blackhead disease, or merely blackhead. This name, which persists in the literature and which is used repeatedly throughout this dissertation because of its brevity, is unfortunately not appropriate, since darkening of the head occurs only occasionally and is not the most characteristic symptom of the disease (Curtice, 1907a; Steiner, 1924; Tyzzer and Fabyan, 1927; and others). The usual symptoms are a ruffled appearance, lack of appetite, and especially sulfur-colored droppings. In addition, the sick turkeys appear to develop a leg weakness. They stand only in cases of extreme necessity; as the disease progresses they may be unable to stand at all, and eventually become prostrated and die.

Blackhead is similar in some respects to amoebiasis of man. The protozoan parasite, Histomonas meleagridis, gains admittance to the bird's body via the alimentary tract in contaminated food and drink and localizes in the cecum, a blind duct or pouch which opens into the gut at the juncture of the large and small intestine. The two ceca of the turkey

may be considered analogous to the appendix of man. The blackhead parasite produces ulceration of the cecal wall as does Endamoeba histolytica in the intestines of persons suffering from amoebic dysentery. It is believed that as a result of this ulceration the histomonad enters the circulatory system by which means it is carried to the liver where secondary foci are established (Smith, 1895), as occasionally happens with Endamoeba histolytica in untreated cases of amoebiasis of man (Strong, 1944). In blackhead disease the liver is almost always involved and becomes heavily infected with the parasite, and the pathognomonic stippled liver lesions are produced (frontispiece). If these blackhead lesions are cultured in suitable media, one may occasionally be able to isolate the causative organism, Histomonas meleagridis. Sometimes these lesions also yield bacteria and other protozoa. These organisms either accompany or follow the histomonad from the cecum to the liver. The part played by bacteria in this disease has never been completely elucidated.

The purpose of this thesis has been threefold: to determine the percentage of blackhead livers containing cultivatable bacteria, to identify these bacteria as to species where possible, and to determine if these bacteria are in themselves pathogenic. In addition, evidence will be presented which indicates that although bacteria may complicate matters, they are in reality opportunists and are not an initiating factor in blackhead disease.

B. The identity of the blackhead parasite

Shortly before the turn of the century, Samuel Cushman of the Rhode Island Experimental Station, in discussing some difficulties in turkey raising, referred to the "bowel troubles" of this bird. One cannot tell for certain whether the "bowel troubles" reported by Cushman (1893) were one or several entities. However, in at least one instance he is dealing with blackhead disease as we know it. He states: "When once started it is apt to be quickly fatal. There is diarrhea, weakness, no appetite, and the face and comb change from a bright scarlet to a dark purple." Later in this same paper he continues: "The bodies of the young turkeys that have died were examined, and in almost every instance the livers were found to be diseased." He did not know whether this ailment was caused by, as he put it, "contagious germs" or simply by overfeeding and other unfavorable circumstances.

Theobald Smith, who at this time was employed by the United States Department of Agriculture, received from Mr. Cushman in the autumn in 1893 the organs of an adult turkey which had suffered from "bowel trouble". The liver and ceca of this bird were extensively affected. As a consequence of this experience, Theobald Smith visited the Rhode Island Station in August of the following year and studied the disease at first hand. In the earliest scientific paper dealing with this disease Smith (1895) reported his findings with 18 cases, 16 of which showed liver involvement. He named the

disease infectious enterohepatitis, described its symptomology and pathology, and observed for the first time the etiological agent which because of its amoeboid appearance he named Amoeba meleagridis.

Curtice (1907a), also working at the Rhode Island Experimental Station, made an extremely important contribution: "As a result of the various observations, strong evidence was afforded that ordinary fowl carry and distribute the amoeba, yet nevertheless they rarely die of the blackhead disease, though a few cases, apparently identical with this disease in turkeys, have been encountered at the station and college poultry plants." In a subsequent publication Curtice (1907b) restated these findings and added: "Ordinary fields uncrossed by poultry were probably not infected" while "poultry yards are heavily infected." In short, he believed that the chicken is a host of the parasite and, like the infected turkey, broadcasts the parasite in droppings. He also found that no breed of turkey is immune and that older turkeys resist the disease more easily than the young. His work also indicated that the disease is not transmitted through the egg, since turkeys which were hatched from artificially incubated eggs and raised in confinement remained free of the disease. These facts have been verified by Smith (1915), Tyzzer and Fabyan (1922), Rettger, Kirkpatrick, and McAlpine (1929), and DeVolt and Davis (1936).

During the next decade the fine groundwork established

at the Rhode Island Experiment Station was eclipsed by somewhat vague and contradictory publications from this station by authors who unknowingly were troubled with mixed infections and who could not decide upon the nature of the blackhead parasite.

Cole, Hadley, and Kirkpatrick (1910), for example, stated that the organism observed by them to be the causative agent of blackhead was a phase in the life-cycle of a coccidium and not an amoeba as designated by Smith. They also believed that although this coccidium was the etiological agent in the majority of cases, other organisms frequently produce similar pathological conditions. Hadley and Amison (1911) considered that many cases of blackhead could be interpreted as infection with one or more species of flagellated organisms, and Hadley (1916a) concluded that many of the bodies which he had observed earlier and regarded as coccidia were actually flagellated protozoa. Finally, in a latter publication of the same year Hadley (1916b) summarized his beliefs when he stated: "aside from those cases in which it is identified with the schizont stage of Eimeria avium, Amoeba meleagridis Smith must be regarded as the late trophozoite (rounded) stage of the intestinal flagellate, Trichomonas." Jowett (1911a) reported his findings in South Africa. He stated: "The present writer's experience of the blackhead disease at the Cape is in entire agreement with the version last advanced by Hadley and Amison. - - - For our part, then, we regard the body termed by Smith the amoeba meleagridis, which is so constantly associated with

the lesions of the blackhead disease, as representing a stage in the life-cycle of a flagellate, in all probability a trichomonad, present in the intestinal contents of the bird." In a second paper Jowett (1911b) restated his previous conclusions and mentioned further that the trichomonad occurred in the feces of healthy as well as diseased turkeys.

It was not until the appearance of a series of papers by E. E. Tyzzer of the Harvard Medical School that the true nature of the blackhead parasite was elucidated. First Tyzzer (1919) noticed that although showing amoeboid movement, the blackhead parasite possessed an "extranuclear body" resembling a blepharoplast - - a structure characteristic of the flagellates, not amoeba. In an important publication, this author (Tyzzer, 1920) reported observing two forms of motility in the same organism. Using a warm stage at the body temperature of the turkey (41 to 42 C), he viewed not only only active amoeboid motility but in addition "rhythmic pulsating movements" which are associated with certain flagellates. Since the blackhead parasite, therefore, had characteristics in common both with amoeba (amoeboid movement and holozoic nutrition) and flagellates (pulsating movement due to flagella and the type of division), a new genus, Histomonas, was created by Tyzzer, but the species name, meleagridis, given by Smith retained, since this was certainly the organism first observed by Smith and recognized by him to be the causative agent of the disease. Subsequent statements continued to appear in the literature, however,

pointing to some existing uncertainties regarding the etiology of blackhead. In Germany, for example, Enigk (1935) considered blackhead to be caused by a fungus, whereas in this country Allen (1936) concluded that in addition to Histomonas meleagridis a species of pentatrichomonas, which she later identified as Trichomonas gallinarum (Allen, 1940), may produce similar lesions in the liver and ceca of poultry. She found that feeding cultures of this Trichomonas which she isolated from infected poults gave rise to the disease in healthy ones, and quite similar lesions resulted. On the other hand, Niimi (1936), in Japan, isolated Trichomonas and also the genus Chilomastix from blackhead livers of naturally affected chickens. These organisms of questionable etiological significance occurred in the liver lesions with H. meleagridis, the organism regarded by Niimi as the sole causative agent. In this country, DeVolt (1950) isolated Trichomonas and Chilomastix from blackhead livers of turkeys, and while collecting data for this thesis, I also encountered Trichomonas in this organ. This is not at all surprising since this genus comprises part of the normal intestinal fauna of poultry and like bacteria may therefore accompany or follow H. meleagridis from the intestine to the liver. (It is no wonder then that Jowett, 1911a, observed trichomonads in the feces of healthy as well as sick turkeys.)

Most parasitologists agree that Histomonas meleagridis is the sole organism causing blackhead disease. For example, in discussing the morphology of the blackhead parasite, Kenyon

(1926) states: "there is no trace of axostyle, undulating membrane, or basal fibre, so that its relation to Trichomonas cannot be upheld", and in their recent texts on parasitology and protozoology, Hegner (1938) and Kudo (1947) list H. meleagridis as the protozoon producing this disease.

C. The transmission of blackhead disease

Although Smith (1895) suggested that blackhead was picked up via the alimentary tract, it was not until the following year that Moore's (1896) experiments indicated this to be the case. Moore observed that healthy turkeys became sick when fed excrement and viscera from infected turkeys. As has already been mentioned, Curtice (1907b) observed that turkeys may pick up the disease from contaminated surroundings, that is, in yards that have harbored sick turkeys or even apparently healthy chickens. He stated that infected turkeys and chickens broadcast the parasite in their droppings.

Tyzzer and Fabyan (1920) demonstrated a uniform susceptibility of the normal turkey to blackhead throughout its period of growth. This is in disagreement with the findings of Curtice (1907b), who believed that older turkeys are more resistant to the disease than poults. Tyzzer and Fabyan produced a subcutaneous form of blackhead by inoculating infected liver tissue from acute cases subcutaneously into healthy turkeys. This form of the disease does not occur naturally, but the experiment demonstrated the infectivity of the organism

from diseased livers. Tyzzer, Fabyan, and Foot (1921) were able to induce blackhead artificially by inoculating infected liver tissue into the large wing vein of healthy turkeys. This method, however, resulted in only occasionally producing the disease; of ten turkeys inoculated, only three developed blackhead. In a later paper, Tyzzer and Fabyan (1922) produced blackhead by feeding infected livers to healthy turkeys and produced the ailment in a more natural manner when they contaminated food with soil taken from hen yards.

Drbohlav (1924) produced the disease by rectal implantation of a strain of H. meleagridis which he had succeeded in cultivating in test tubes.

Delaplane (1932) was able to produce blackhead disease by rectal inoculation of finely ground blackhead liver and also by inoculation of ground diseased ceca from turkeys or chickens harboring no protozoa other than H. meleagridis.

DeVolt and Davis (1936) also were able to produce blackhead in healthy turkeys by feeding them infected liver. These same workers were able to induce the disease by oral and rectal inoculation of test tube cultures of the histomonad. They succeeded in implantation of this parasite in the ceca of ten out of twelve poults so inoculated, and nine of these birds died from the disease.

Since cysts of Histomonas meleagridis never have been observed, the question of how yards could remain infective over a period of years was unsolved for some time. A new approach

to the study of blackhead began when Graybill and Smith (1920) discovered that incubator-bred poults fed embryonated Heterakis ova from turkeys sick with blackhead disease became infected also. (Heterakis is a nematode worm which is seldom troublesome in itself and which is prevalent in domestic poultry.) These authors did not seem to appreciate fully the importance of their observation however. They concluded that it was the lowering of the resistance of the poults by this nematode which allowed them to become more easily infected with the protozoa. They did not consider that the worm itself could be acting as an intermediate host for the blackhead parasite. It was not until later that the significance of the observation was recognized. Tyzzer and Fabyan (1922) showed that Heterakis ova which had been treated for three days with 1½ per cent nitric acid were able to bring about the disease when fed to healthy turkeys. Tyzzer (1929) demonstrated that H. meleagridis actually invades the tissues of this worm when the latter resides in the ceca of poultry infected with the protozoon. Up to the present time however, H. meleagridis has not been observed in Heterakis ova. Nevertheless, it is in the eggs of the nematode that H. meleagridis is thought to be able to survive for months in soil.

Niimi (1937) believes that the protozoon may survive longer than one year, perhaps three or four years in Heterakis ova. He does not think it likely that normally the disease is caused by direct injestion of the causative agent in its

free state, but only from the injestion of contaminated eggs of the helmith, and that "consequently Heterakis papillosa must be considered as a true vector of the blackhead-organism".

D. The cultivation of Histomonas meleagridis

Drbohlav (1924) was the first to cultivate the blackhead parasite. He propagated the flagellated stage of the organism which Tyzzer had found in the feces of chickens recovering from blackhead disease. The media employed were blood agar covered with Locke's solution and coagulated egg medium covered with the same solution. Ultimately coagulated white of egg covered with blood boullion with one per cent peptone proved to be of the best value. He found that the pH is important, the best range being between 7.2 and 7.8. "Parasites in culture tubes feed on bacteria; when blood agar is used, may take up red cells. Chickens inoculated per rectum with material from cultures, showed parasites in large numbers in three days. One died as early as the sixth day after inoculation."

DeVolt and Davis (1936) cultivated the histomonad in Locke's modified egg medium from the feces of infected poults and used these cultures for oral and rectal inoculation.

The first instance of isolation and subsequent cultivation from infected livers was reported by Bayon and Bishop (1937). They used tubes of inspissated whole egg slant covered with inactivated horse-serum diluted 1:8 in Ringer's solution to which was added a little rice starch. Only one

attempt was successful and these tubes contained bacteria as well as the histomonad.

DeVolt (1943) used a monophasic medium for culturing the parasite from liver lesions. This medium consists of turkey-serum diluted with Locke's solution to which rice starch is added at the time of inoculation. The amount of glucose employed in this medium has been reduced from 0.2 per cent, as tabulated in the original formula, to 0.02 per cent (DeVolt, 1950).

E. The role played by bacteria in blackhead disease

Smith (1895) wrote: "In the course of the investigations the possible bearing of bacteria upon the disease was not entirely overlooked and cultures mainly upon agar were made from the blood and the liver of a considerable percentage of the infected turkeys. Usually, bits of liver tissue were transferred to the culture tubes. Of the heart's blood, one or two loops were inoculated. The results obtained varied from case to case. As a general rule, tubes inoculated from birds that had just been killed remained free from growth, whereas the cultures of those which had died during the night and were examined next morning were quite regularly fertile." Most often encountered was B. coli communis. He evidently did not attempt to cultivate anaerobes, nor does it seem that he bothered with a thorough taxonomical study of the bacteria isolated. Theobald Smith expressed his feelings in regard to the part played by bacteria in this disease when he stated:

"The absence of any uniformity in the bacteriological results as well as the appearance of B. coli in the organs of the dead turkeys indicates that pathogenic bacteria were not associated with the protozoa in the cases examined."

Twenty years later Theobald Smith re-emphasized his earlier stand: "The presence of bacteria might be expected with such extensive destruction of tissue in ceca and liver. As a matter of fact they are rather uncommon in the liver necroses of turkeys chloroformed at the height of the disease. In turkeys which die during the night, smears and cultures may show next day several varieties of bacteria. The histological study brings strong evidence that bacteria have nothing to do with the lesions, and the frequent sterility of cultures of liver foci demonstrates it." However, in this same publication (Smith, 1915) the author recognized that bacterial complications may arise since he mentioned that in 1913 he had studied a dead turkey in which there were no fresh lesions of blackhead, and in reference to this case he stated: "The immediate cause of death was bacterial sepsis, for large colonies of bacilli were found both in lungs and liver."

This coincides more closely with the view held by Curtice (1907a) who writes: "An examination of many extensively diseased livers and ceca, and comparison with others less diseased, leads to the belief that death in the latter cases is not always produced directly as a result of blackhead disease, but is effected by secondary causes, such as climatic

changes, bacteria, and starvation, acting upon an already weakened body."

Tyzzer (1919) observed the disease in a baby chicken in which there was a complicating bacillary infection, but did not identify the bacterium involved.

Over a period of years Rettger and Kirkpatrick conducted an intensive search for a possible bacterial etiological agent. They employed both aerobic and anaerobic culture techniques, but found no bacterium as constantly occurring in blackhead livers. Since their results were negative, they reported their findings only briefly, stating that a coccus was observed in some cultures and that spirillum or spirochete forms were observed in several instances on India ink-stained slides (Rettger and Kirkpatrick, 1927).

Bayon and Bishop (1937) and Bishop (1938), while attempting to grow Histomonas meleagridis from blackhead liver lesions, observed that some livers contained cultivatable bacteria while others did not. In fact, H. meleagridis only appeared in tubes containing bacteria. (The presence of bacteria in a culture tube of course does not indicate that the tube is necessarily positive for protozoa.) Other investigators (DeVolt and Holst, 1950) also have observed that the media available at this time support growth of the histomonad only when bacteria are present. This is analogous with Endamoeba histolytica which will not grow unless in the presence of a compatable flora (Dobell and Laidlaw, 1926; Cleveland and Sanders, 1930a, 1930b; Chinn,

Jacobs, Reardon, and Rees, 1942; Rees, Boyicevich, Reardon, and Daft, 1944; Balamuth and Howard, 1946; and Shaffer and Frye, 1948) or other protozoa (Phillips, 1950; and Phillips and Rees, 1950).

F. Liver involvement in amoebiasis of man

Long ago Smith (1895) pointed out certain characteristics of the blackhead organism which parallel the behavior of Endamoeba histolytica in amoebic dysentery of man. Both organisms, H. meleagridis and E. histolytica, are intestinal parasites which the host picks up in contaminated food and drink, and both may pass through the ulcerated intestinal wall into the portal circulatory system and thence be carried to the liver where secondary foci are established. Liver involvement in amoebic dysentery is not as common as in blackhead however. In the latter disease liver involvement nearly always occurs while amoebiasis in the temperate zone produces liver involvement in only about five per cent of the cases. Even in the tropics where amoebic hepatitis may occur more often, liver involvement never reaches the extent as in blackhead disease. (For instance, Cort, 1928, reported hepatic involvement in 18 per cent of the cases studied by him in Siam, and Strong, 1944, found abscess of the liver in 23 per cent of the cases observed by him and Musgrave in the Philippines.)

Since bacteria have been encountered in blackhead liver lesions of turkeys, one would expect also to find bacteria

associated with Endamoeba histolytica in amoebic abscesses of man. This is actually the case. Rogers (1922) was one of the earlier workers to report quantitatively the number of amoebic livers containing bacteria. Out of 24 patients observed by him in India, nine, or 38 per cent, yielded pus at the time of operation which contained bacteria. Postmortem examinations told about the same story, since two out of five cases, or 40 per cent, of fatal amoebic hepatitis yielded liver abscesses which contained bacteria. Strong (1944) states: "In about 50 per cent of the abscesses, bacteria may be obtained by cultivation when sufficient material is inoculated."

While speaking of the high mortality occurring in untreated cases of amoebic hepatitis Manson-Bahr (1943) writes: "When death ensues it may be due to the pressure of the abscess itself, to secondary infection by streptococci or Bacillus coli, to gangrene of the abscess wall, to pneumothorax, to anemia, or to some intercurrent disease." He also reported a case where Bacillus enteritidis was involved, and Strong (1944) merely states: "Staphylococci, streptococci, and colon bacilli are not infrequently encountered."

It is surprising that no real systematic study dealing with the bacteria associated with Endamoeba histolytica in liver abscesses has been reported in the literature. Therefore, the work presented in this thesis, although dealing only with the bacteria found in blackhead liver lesions of turkeys, may nevertheless be of some value in suggesting what bacterial types might be expected to occur in liver lesions of man and other

animals affected with similar diseases.

II

MATERIALS AND METHODS

A. The technique employed in the isolation of bacteria from blackhead liver lesions

1. Source of material. From the beginning of April 1948 to May 1951, when the first and last samples were taken, there was available, except during the late winter and early spring months, an abundance of material with which to work. Over this period a total of 60 different infected turkeys representing 13 flocks were autopsied, and their livers sampled. This material came from three sources. Firstly, four flocks were provided by Dr. H. M. DeVolt for my exclusive use. Three flocks were raised in houses and yards on the grounds of the Livestock Sanitary Service Laboratory, College Park, Prince Georges County, Maryland. Since these houses and yards previously sheltered flocks in which the disease was present, these turkeys acquired the disease naturally --- from contaminated surroundings. The fourth flock was raised indoors in cages, also at the Livestock Sanitary Service Laboratory, and acquired the disease artificially -- through rectal and oral inoculation with test tube cultures of the histomonad. Secondly, concurrent with my studies, H. M. DeVolt was experimenting with certain new anti-histomonal drugs developed for blackhead prevention and therapy. The control birds from these and other

experiments were made available to the writer. Two of these flocks were raised in wire cages indoors and these poults also acquired the disease by inoculation. Several other groups of controls were from flocks raised in infected houses and yards, and these turkeys acquired the disease naturally. A third source of material provided turkeys from other sections of the state. The sick turkeys brought in by farmers and county agents which were diagnosed as blackhead victims by H. M. DeVolt were at the time of autopsy given to me for examination. In this manner eight liver samples representing five flocks from other parts of Maryland were obtained. These birds had of course picked up the disease in the usual way -- from association with sick turkeys or chickens, or from contact with surroundings contaminated by them.

In table 1 are listed in chronological order the 13 flocks represented in this survey. It may be observed that flocks two, three, and seven, as well as flocks eight and ten, are similar in regard to mode of infection and ownership.

2. The preparation and incubation of pour plates from blackhead liver samples. Turkeys which were sacrificed at the first appearance of symptoms by breaking the neck, and birds which died of the disease were carefully opened so as to keep contamination at a minimum. The appearance of the liver at autopsy, coupled with the observed symptoms up to the time of death was sufficient to identify the disease as blackhead. In order to prevent dust and feathers from falling upon the

TABLE 1

Information pertaining to the 13 turkey flocks covered in this survey

Flock number	Mode of infection	Location	Additional information
1	Artificial	College Park, Prince Georges County, Md.	Dr. DeVolt's flock, raised indoors
2	Natural	ditto	My flock, raised in yard
3	Natural	ditto	ditto
4	Natural	Garret County, Maryland	From a private farm
5	Artificial	College Park, Prince Georges County, Md.	My flock, raised indoors
6	Artificial	ditto	Dr. DeVolt's drug controls, indoors
7	Natural	ditto	My flock, raised in yard
8	Natural	ditto	Dr. DeVolt's drug controls, raised in yard
9	Natural	Baltimore County, Md.	From a private farm
10	Natural	College Park, Prince Georges County, Md.	Dr. DeVolt's drug controls, raised in yard
11	Natural	Frederick County, Maryland	From a private farm
12	Natural	Calvert County, Maryland	From a private farm
13	Natural	Montgomery County, Maryland	From a private farm

surface of the exposed liver, the breast-bone was not pulled far back from the liver at autopsy.

A representative portion of the diseased liver, usually from 3 to 8 grams in weight, was removed with a sterile scapel after first singeing the surface of the portion to be removed with the hot scapel blade. The sample was placed in a tared petri dish which was then reweighed in order to determine the exact weight of the liver specimen. The liver sample was then cut into a number of smaller pieces with the same scapel and removed from the petri dish with sterile forceps to a sterile dilution bottle containing glass beads. Sufficient 0.85 per cent sodium chloride was added to form an initial dilution of 10^{-1} of the sample. As a rule, little difficulty was experienced in macerating the specimen by shaking of this bottle. Occasionally however, older lesions required continued vigorous shaking before becoming macerated.

A loopful of the liver suspension was removed from this 10^{-1} dilution bottle to a microscope slide and the smear was stained with crystal violet. Usually a wet mount prepared from this dilution was observed for the presence of Histomonas meleagridis. In a few instances several mls from this same bottle were transferred to tubes of turkey serum - Locke's solution and rice starch medium (DeVolt, 1943) in an attempt to grow the histomonad. In every case 1 ml was transferred from the initial dilution to another bottle containing 99 ml of sterile isotonic saline.

From these two dilutions (10^{-1} and 10^{-3}) pour plates were prepared in the customary manner. Dilutions of the liver sample of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were made, unless the number of bacteria seen in the stained smear indicated a higher dilution range to be necessary. The plates were prepared in duplicate and incubated for two days at 41 to 42 C, the body (rectal) temperature of the turkey (Marsden and Martin, 1938). Two plating agars were used: eosine - methylene blue agar (Difco, dehydrated) incubated aerobically for the isolation of coliforms and other gram-negative rods, and Eugonagar (B.B.L., dehydrated), described by Vera (1947), incubated anaerobically for the isolation of more fastidious types. There were several reasons for employing this latter medium rather than a liver-infusion agar, which might seem more appropriate: Eugonagar is easier to prepare and is perfectly transparent, but more important, of the many agars tried for the isolation of fastidious facultative anaerobes and obligately anaerobic types in the cecal feces of turkeys, this medium consistently gave the highest or at least as high counts as the other media employed (Harrison, 1949). In addition, a single blood agar plate (Difco blood agar base to which was added 10 per cent cow blood) was streaked with a loopful of the 10^{-1} dilution and incubated aerobically. This medium was used in case there were present in the liver sample aerobes which could not grow on the E.M.B. plates. (In no instance were bacterial types isolated from the blood agar which were not encountered on the E.M.B. or Eugonagar plates.)

Anaerobiosis was produced according to the method of Weiss and Spaulding (1937), and about 10 per cent carbon dioxide was added to the anaerobic jar after the last evacuation, before adding the hydrogen.

After the incubation period, the counts on the E.M.B. and Eugonagar plates were recorded. Representative colony types were then picked and subcultured onto Eugonagar slants. Each colony so selected was numbered, and its appearance recorded.

The inoculated Eugonagar slants were labeled in a systematic manner: the number of the colony from which the respective tube was inoculated was attached by means of a hyphen to the number of the liver sample plated. Hence, the lineage of every culture may always be traced. For example, a tube labeled 6L-7 contains the progeny from colony number 7 of liver number 6L. Since the appearance of this colony, the medium from which it was isolated, etc. has been recorded, as well as information pertaining to liver 6L (table 2), a little of the background of culture 6L-7 is therefore at hand.

The cultures on Eugonagar were incubated aerobically or anaerobically depending upon the plates from which they were taken, and incubation was now at the customary 37 C.

It should be mentioned before proceeding further that in every instance the pour plates were in the incubator within 1½ to 3 hours after the arrival of the turkey at the laboratory. Turkeys which could not be handled immediately were discarded, no attempt being made to plate any livers except those from recently acquired birds.

TABLE 2

A listing of the blackhead liver samples obtained from the different turkey flocks and certain data pertaining to these liver samples

Flock number	Mode of infection of the flock	Sample number	Weight of sample in grams	Manner of death of the turkey from which sample was obtained
1	Artificial (Rectal inoculation with *Histomonas meleagridis*)	5L	---	Died of the disease
		6L	---	
		8L	4.0	
		9L	4.3	
		10L	3.2	
		11L	2.2	
		15L	2.5	
		16L	2.3	
		17L	2.0	
		18L	2.2	
		19L	1.8	
2	Natural (Contaminated surroundings)	31L	2.1	Sacrificed
		32L	5.2	Died
		33L	4.1	Sacrificed
3	Natural (Contaminated surroundings)	34L	4.5	Died of the disease
		35L	7.4	
		36L	5.2	
		38L	4.7	
		39L	4.9	
		40L	5.5	
4	Natural	37L	6.6	Sacrificed

TABLE 2 CONTINUED

Flock number	Mode of infection of the flock	Sample number	Weight of sample in grams	Manner of death of the turkey from which sample was obtained
5	Artificial (Rectal inoculation with *Histomonas meleagridis*)	41L	6.2	Died
		42L	4.7	Died
		43L	6.5	Sacrificed
		44L	6.1	Died
		45L	4.0	Sacrificed
		46L	4.7	Sacrificed
		47L	5.1	Sacrificed
		48L	5.2	Sacrificed
		49L	5.5	Sacrificed
		50L	4.5	Sacrificed
		51L	5.4	Sacrificed
		52L	---	Sacrificed
		53L	5.4	Sacrificed
6	Artificial (Rectal inoculation with *Histomonas meleagridis*)	54L	4.6	
		55L	4.6	
		56L	---	
		57L	5.0	Sacrificed
		58L	4.4	
		59L	6.0	
		60L	6.4	
		61L	4.9	
7	Natural (Contaminated surroundings)	62L	5.7	Sacrificed
		63L	6.9	Sacrificed
		64L	5.9	Sacrificed
		65L	5.5	Sacrificed
		66L	8.0	Died
		67L	6.0	Sacrificed
		70L	6.2	Sacrificed
		72L	7.0	Died
8	Natural	68L	4.8	Died
		71L	6.3	Sacrificed

TABLE 2 CONTINUED

Flock number	Mode of infection of the flock	Sample number	Weight of sample in grams	Manner of death of the turkey from which sample was obtained
9	Natural	69L 73L	5.2 8.2	Sacrificed
10	Natural	74L	3.7	Sacrificed
11	Natural	75L 76L 79L	5.0 6.5 4.7	Sacrificed
12	Natural	77L	4.9	Sacrificed
13	Natural	78L	5.6	Sacrificed

3. Preliminary studies with the bacteria isolated from blackhead liver lesions. Certain preliminary tests were undertaken so that the bacteria isolated could be grouped together for convenience sake. The extent of these preliminary investigations depended upon the number of infected livers available at the time for sampling. The preliminary tests usually included the following: oxygen tension preference, morphology, gram reaction, and some biochemical tests such as fermentation of the more common carbohydrates. With some bacteria, identification as to species was completed at this stage. The gram-negative rods with the characteristic appearance on E.M.B. agar, characteristic IMViC reactions, and which produced acid and gas in tubes of lactose broth were identified as Escherichia coli and no further tests applied. These cultures were then discarded except for one or two from each liver yielding this species. These were lyophilized for use in the pathogenicity tests to be described. The other bacteria were not as easy to identify, and representative strains were lyophilized so that they could be studied in more detail at a future date, as well as for use in the pathogenicity tests.

4. Limitations of the sampling technique employed. One could raise the question as to why the entire blackhead liver from each turkey studied was not mascerated, in this way avoiding the possible selection of a sample which perhaps was not truly representative of the liver as a whole. Although this might be conceded, the greater risk of contamination in

removing and handling an entire liver would more than offset such theoretical advantages.

Bacterial surveys of this type are of course always limited by the culture media used. The chance is ever present that bacteria may reside in the sample which are not capable of cultivation on the media employed, or for that matter on any artificial media. This was the case in this study, and later mention will be made concerning organisms which were observed microscopically but which were never isolated from the pour plates.

5. The plating of liver samples from healthy turkeys. Whether bacteria reside in the tissues of healthy animals is still controversial according to Tanner (1948) and it is not the purpose of this work to argue pro or con on the matter. However, the technique outlined on the previous pages was applied to 15 livers from apparently healthy turkeys. These samples served as controls in this survey. The majority of these liver samples did not give any colonies on the agar plates and can therefore be regarded as containing no cultivatable bacteria, at least not in numbers exceeding one organism per ten grams of liver sample. A few samples yielded an occasional plate which showed one or several surface colonies. These never appeared in a sequential dilution range, and when a film was prepared and viewed microscopically these were invariably seen to be cocci or aerobic spore-formers. These occasional colonies were therefore regarded as arising from chance contamination. It is felt, therefore, that any bacteria

from blackhead liver samples which occur in a definite dilution range represent an abnormal condition, and that these bacteria may be safely regarded as having accompanied, or followed, the histomonad from the intestine to the liver, unless they have perhaps lodged in the liver as a result of some chance independent concurrent infection, which is unlikely since the syptomology of all the turkeys tested was that of blackhead disease only.

B. The technique employed to determine whether the bacteria from blackhead liver lesions are in themselves pathogenic

Forty-nine bacterial cultures, representing the various bacterial types isolated from the blackhead liver samples, were injected into poults in order to determine if these bacteria were in themselves pathogenic.

The cultures were cultivated on either heart infusion agar (Difco, dehydrated) or Bugonagar for 18 to 24 hours at 37 C. Anaerobic strains were grown anaerobically according to the method of Weiss and Spaulding (1937), and as usual, about 10 per cent carbon dioxide added to the anaerobic jar after the last evacuation, before adding the hydrogen.

After the incubation period, a few ml of sterile 0.85 per cent sodium chloride were added to each slant and the growth rubbed free from the agar by means of a sterile inoculating loop. The resulting bacterial suspensions were transferred to sterile tubes and sufficient isotonic saline added to

produce a suspension that allowed 75 per cent light transmission as detected employing a Coleman Universal Spectrophotometer.

One-half ml of each bacterial suspension was injected into two 600 to 1200 gram poults via the large wing vein employing a 2 ml syringe with a 3/8 inch, #26 needle. The poults were then placed in cages and observed daily for about one month. Upon the appearance of illness the bird was sacrificed or allowed to die and then autopsied. Likely organs, especially the liver, were cultured in order to recover the injected bacterium. After the observation period, all remaining apparently healthy poults were also sacrificed and autopsied.

When a bacterium was recovered from the organs of a diseased poult it was compared to the strain originally injected. Such tests as gram reaction, morphology, and fermentation reactions were used for this comparison.

C. Identification of the bacteria isolated from blackhead livers

1. Gram-negative rods. The majority of the gram-negative rods isolated were strains of Escherichia coli, and as has been mentioned, they were identified as a result of the preliminary tests. These included the appearance of the colonies on E.M.B. agar (Difco, dehydrated), fermentation of lactose and sucrose (employing Difco phenol red broth base to which the carbohydrate was added in a concentration of 1 per cent), and the IMViC reactions. To test for the production of indole,

about 1 ml of Kovac's reagent (Manual of methods for the pure culture study of bacteria, 1945) was added to about 5 ml of a one day old and a two day old tryptone broth (Difco, dehydrated) culture. The methyl red and Voges-Proskauer tests were undertaken using MR-VP broth (Difco, dehydrated), and citrate utilization employing Simmon's citrate agar (Difco, dehydrated).

Three other species of gram-negative rods were encountered. They were studied using the tests just outlined, as well as some additional tests which will be mentioned when necessary in the results section of this dissertation.

2. Cocci. The 14 lyophilized cultures of cocci were opened into tubes of trypticase soy broth (B.B.L., dehydrated) and after incubation for 24 hours were streaked onto Eugonagar plates. Each culture (except 35L-8) gave rise to uniform colonies of all the same appearance. Cultures 6L-7, 10L-8, and 72L-1 produced colonies characteristic of the genus Micrococcus. They were round, smooth, convex, opaque, and greater than 1 mm in diameter, often 3 mm in diameter. Colonies of 72L-1 were pigmented yellow, the others white. Culture 35L-8 produced unusual colonies. They appeared bluish to the unaided eye, while under the hand lens appeared nearly translucent. They were about 0.5 mm in diameter and appeared rather flat. Their edge was not always entire, but somewhat lobate. An occasional colony was unusual in that within it there appeared to be a daughter colony which was smaller, more opaque,

whitish, and convex. The 10 other cultures produced uniform colonies. They were 1/3 to 1/2 mm in diameter, smooth, convex, opaque, and white. Occasionally a well isolated colony would have a diameter as great as 1 mm.

A single colony from each culture was transferred to a Bugonagar slant. From this medium stock cultures were prepared by inoculating yeast extract glucose slants of the following percentage composition: yeast extract, 2.2; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.01; glucose, 0.25; and agar, 1.5. After incubation for 24 hours these slants were stored in the refrigerator at 8 C. One culture, 16L-13, developed only feebly on this medium. The other cocci, however, grew well and remained viable for weeks in the refrigerator between transfers.

The cultures were prepared for inoculation into various test media by transferring a loopful of growth from the stock slants into trypticase broth. This general purpose medium had the following percentage composition: trypticase (B.B.L., dehydrated), 2; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.01; and glucose, 0.25. About 0.1 ml of an 18 to 24 hour culture of each coccus in this broth was inoculated by means of a pipette into fresh tubes of this same medium in triplicate. One set of tubes was then incubated at 10 C, another at 45 C, and the third set was heated to 61 C for 30 minutes and then cooled under the tap and incubated at 37 C for two weeks. These and other tests to be mentioned are employed for the identification of members of the genus Streptococcus (Sherman, 1937). Morphological

studies and gram stains were made from this medium (also from the yeast extract glucose agar), and in addition it was used to test for catalase production. About 2 ml of 3 per cent hydrogen peroxide were added to 10 ml, 24 hour cultures of the cocci in the trypticase glucose broth. The culture was considered catalase-positive if bubbles of gas appeared within a half hour at room temperature. The same medium to which was added 0.1 per cent KNO_3 was employed in testing for nitrate reduction. One and two week old cultures were tested for the presence of nitrite using the sulphanilic acid and dimethyl-a-naphthylamine reagents, and for nitrate employing diphenylamine and sulfuric acid (Manual of methods for pure culture study of bacteria, 1945).

To test for growth at a pH of 9.6 the trypticase glucose broth was adjusted to this value using the boric acid - potassium chloride - sodium hydroxide buffer described by Clark (1928). The broth was made up as follows: To 500 ml of trypticase glucose broth prepared double strength was added 60 ml of 0.5 M H_3BO_4, 60 ml of 0.5 M KCl, and sufficient 0.5 M NaOH (about 150 ml) to bring the pH to 10.0. Then water was added to bring the ingredients to the proper concentration, the broth dispensed in 10 ml amounts in test tubes, and sterilized at 12 pounds for 12 minutes.

In order to test the salt tolerance of the cocci, the trypticase basal containing 0, 2, 5½, 6½, 7, and 9 per cent added sodium chloride was employed. The trypticase glucose

broth and the salt solutions were prepared double strength, autoclaved separately, and then mixed together aseptically. Each of the broths was dispensed in 10 ml amounts into sterile test tubes which were incubated at 37 C several days to check sterility. Here again inoculation was by means of a pipette, and consisted of 0.1 ml of the respective 24 hour trypticase glucose broth culture.

In a like manner the following media were inoculated with each of the cocci: litmus milk; skim milk in duplicate, to test both for final pH after incubation for two weeks and the titratable acidity resulting at the end of this incubation period; and 0.1 per cent methylene blue milk. This later medium was prepared as follows: To 400 ml of fresh skim milk were added 10 grams of skim milk powder (Difco) and the resulting mixture shaken well and autoclaved at 12 pounds for 12 minutes. A second solution consisting of 0.5 gram of methylene blue (Difco, 84.4 per cent dye content) in 100 ml of water was prepared and also autoclaved. When cool the two solutions were mixed together aseptically and dispensed into sterile test tubes in 10 ml amounts. The inoculated tubes (0.1 ml of a 24 hour, 37 C, trypticase glucose broth culture) were incubated for two weeks, after a preliminary incubation period to check sterility.

To test for the liquefaction of gelatin two media were employed; nutrient gelatin (Difco, dehydrated) and a trypticase gelatin of the following percentage composition: trypticase, 0.5; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.01; glucose, 0.005; and

gelatin, 15. Esculin broth was prepared by adding to 500 ml of heart infusion broth (Difco, dehydrated) 1 gram of KH_2PO_4 and 0.5 gram of esculin and adjusting the pH to 7.4 with sodium hydroxide; this broth is similar to that employed by Plastridge et al. (1942). Sodium hippurate broth was prepared by adding to heart infusion broth sodium hippurate in a concentration of 1 per cent. These 4 media were inoculated with a loopful of culture taken from a 24 hour yeast extract glucose agar culture of the 14 strains of cocci. Esculin was considered split if a heavy black color resulted upon the addition of 1 drop of a 1 per cent solution of ferric citrate to 2½ ml of the 2 week old culture. The cultures were considered hippurate-positive if the addition of ferric chloride resulted in the formation of a heavy, buff precipitate.

The fermentation reactions of the 14 strains of cocci were determined by measuring the final pH in various substrates after incubation for two weeks. The glass electrode was used in taking the measurements. The medium employed had the following percentage composition: tryptone (Difco, dehydrated), 2; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.01; and sufficient substrate to form 1 per cent substrate. All substrates were autoclaved together with the base except xylose, arabinose, maltose, melibiose, and cellobiose which were sterilized separately by filtration and added aseptically to the autoclaved base. These tubes were also inoculated with a loopful of a 24 hour yeast extract glucose agar culture of each of the 14 strains

of cocci. Tryptone was used in lieu of trypticase since the fermentation reactions of cocci isolated from the cecal feces (Harrison and Hansen, 1950a) had been studied in this nitrogen source, and we desired to compare the fermentations of the liver cocci with these fecal cocci.

The hemolytic activity of the cocci was tested using the test tube method with sheep cells (Hansen et al., 1933) and using the plating technique employing horse blood (Brown, 1919).

Four ml of sterile defibrinated sheep blood were added to each of two, 15 ml graduated centrifuge tubes which were then centrifuged for 5 minutes in an International centrifuge. The serum was removed with a medicine dropper, 6 ml of 0.85 per cent sodium chloride added, the cells shaken, and recentrifuged. This process was repeated twice, the time of centrifugation being 10 minutes after the last addition of saline. The sheep cells were made up to a 5 per cent solution using isotonic saline and a hemolysin titration run as follows: One-half ml from an 18 hour, 37 C, heart infusion broth culture of each coccus was placed in a small test tube and ½ ml of the 5 per cent sheep cell suspension added. The tubes were then placed in a 37 C water bath and observed after 20 minutes, one hour, and three hours. Two controls were run simultaneously; one with the sterile broth and sheep cells, and the other with saline and sheep cells.

In order to detect hemolysis by the plate method, a loopful of culture was placed in a sterile petri dish and 5 per cent horse blood in heart infusion agar (Difco, dehydrated) was poured into the dish, mixed well, and allowed to solidify. The plates were incubated at 37 C and observed daily for three days, after which time they were read and then placed in the ice box for 24 hours and observed again. I incubated my plates one day longer than Brown (1919) recommended, because my streptococci produced such feeble reactions.

3. Gram-positive rods. The lyophilized cultures of the gram-positive, rod-shaped bacteria isolated from infected turkey livers were opened into tubes of trypticase soy broth (B.B.L., dehydrated) and incubated anaerobically at 37 C. At the end of two days' incubation the rods were transferred to Eugonagar (B.B.L., dehydrated) slants and incubated anaerobically for 24 hours. The cultures were then streaked from these slants onto plates of the same medium. After anaerobic incubation for two days a single colony was picked from each of these plates and subcultured into fresh tubes of Eugonagar. In some instances there appeared to be more than one colony type on the Eugonagar plates. When this occurred, one colony typical of each type was subcultured onto the agar slants, and each then carried and tested as an individual strain. It was felt that these different colony types were either due to an impure parent culture or resulted from variation of the

parent culture. In either case it was deemed advisable to handle each type separately rather than simplify matters, by keeping one and discarding the others in the perhaps erroneous assumption that the different colony types had little significance.

The problem of labeling these daughter strains was solved by merely adding lower case letters after the parent culture's number. For example, strains 39L-16a and 39L-16b are progeny arising from two different colony types derived from parent culture 39L-16.

The resulting 27 strains were transferred from the Eugon-agar slants to duplicate slants of yeast extract glucose agar (yeast extract, 2.2 per cent; K_2HPO_4, 0.2 per cent; $MgSO_4.7H_2O$, 0.01 per cent; glucose, 0.25 per cent; and agar, 1.5 per cent). One set was incubated aerobically and the other anaerobically. The growth on the surface of the slants and in the stabbed portion of the butts was compared in both sets of tubes in order to determine grossly the oxygen tension preferred by these bacteria.

Stock cultures of these rods were carried on yeast extract glucose agar slants. The tubes were incubated aerobically or anaerobically, depending upon their preference, at 37 C for 18 to 24 hours and then placed in a refrigerator kept at about 8 C. The anaerobic slants were kept within the anaerobic jars in which they had been incubated, the jar itself being placed in the refrigerator. These stock cultures were transferred

every one or two weeks. One culture, 18L-6, which grows only feebly on yeast extract glucose agar was kept on Eugonagar.

Morphology, the gram reaction, and catalase production were determined on the yeast extract glucose medium. A few ml of 3 per cent hydrogen peroxide were added to 24 hour slant cultures of the respective organisms, which were then observed over a half hour period at room temperature. A culture was considered catalase-positive if bubbles of gas appeared.

A broth of the following percentage composition was employed as the basal in the fermentation tests: yeast extract, 2.2; K_2HPO_4, 0.2; and $MgSO_4.7H_2O$, 0.01. Sufficient substrate was added to form 2 per cent substrate. All substrates were autoclaved together with the basal except xylose, arabinose, maltose, melibiose, and cellobiose which were sterilized separately by filtration and added aseptically to the autoclaved basal. The advantages which result when certain carbohydrates are autoclaved together with the nitrogen source have been pointed out in the researches of the Orla-Jensens (1932, 1933). Fermentation was detected by measuring the final pH developed in the various substrates after two weeks' incubation at 37 C. The pH was measured by means of the glass electrode. The tubes of the substrates were inoculated with growth taken from 18 to 24 hour Eugonagar slant cultures of the respective bacteria. This medium was used here rather than the yeast extract glucose agar because it yields a heavier cell crop.

The same basal as employed in the fermentation studies above was used in testing for nitrate reduction. To this basal were added 1 per cent glucose and 0.1 per cent KNO_3. One week old and two week old cultures were tested for the presence of nitrite and nitrate using the same reagents as employed in the case of the cocci.

In order to determine the temperature range permitting growth of the different strains, growth from 18 to 24 hour Eugonagar cultures was inoculated in septplicate into the same yeast extract basal containing 0.5 per cent glucose and 0.05 per cent sodium thioglycollate. Test tubes half full with this broth were cooled to about 40 C immediately after autoclaving, inoculated with the respective organism, and then sterile vaseline poured over the surface of the broth to form a layer about 3/4 inch thick. These tubes were incubated in water baths at 20, 30, 37, 41, 43½, 45½, and 49½ C for a week, except the 20 C tubes which were incubated two weeks.

To check for motility of the cultures they were observed in wet mounts microscopically. The yeast extract basal plus 0.5 per cent glucose was one of the media employed. The other was a broth of the following percentage composition: tomato juice (centrifuged), 20; yeast extract, 1; trypticase, 1; and glucose, 0.25. The morphology of the bacteria in this latter medium was also observed, after the coverslips were removed from the wet mounts and the film thus formed allowed to dry and stained with polychrome methylene blue (Lillie, 1942).

Tubes of fresh skim milk (autoclaved at 12 pounds for 12 minutes) were inoculated in duplicate in order to determine the final pH developed after two weeks' incubation at 37 C and also the titratable acidity formed after this same incubation period.

Gas production was detected using vaseline-sealed tubes of a broth of the following percentage composition: tomato juice (centrifuged), 20; yeast extract, 1; trypticase, 1; and glucose, 2. The obligate anaerobic strains were inoculated into this same medium containing 0.1 per cent sodium thioglycollate as an additional ingredient. Immediately after autoclaving, the broth was cooled to about 40 C, inoculated with the respective organism, and then the surface covered with sterile vaseline to form a layer about 3/4 inch thick.

Uninoculated tubes were of course prepared in a like manner to serve as controls. Gas, when produced, pushed the seal from the surface of the medium, and in some instances completely out of the tube. It was considered advisable that the tomato juice broth for this test be of the same composition as the broth used in the flask cultures for the determination of the products resulting from glucose fermentation by these gram-positive rods. The tubes were incubated at 37 C for two weeks.

Nineteen representative cultures of the gram-positive rods were grown in flasks in order to determine the products resulting from glucose fermentation by these bacteria. The

technique employed is outlined in the following paragraphs.

D. Determination of the products resulting from glucose fermentation by certain bacteria isolated from blackhead liver lesions

1. Preparing and dispensing the medium. A broth of the following percentage composition was employed for the cultivation of the facultative anaerobes: tomato juice (centrifuged), 20; trypticase, 1; yeast extract, 1; and glucose, 5. Obligate anaerobes and strains which grow considerably better under a reduced oxygen tension were cultivated in the above medium to which was added 0.1 per cent sodium thioglycollate.

Before making up to the final volume, the pH of the broth was adjusted to about 7.2 with sodium hydroxide. After thorough mixing, the broth was dispensed in 500 ml amounts into one-liter erlenmeyer flasks, each of which contained 16 grams of powdered calcium carbonate. The surface of the sodium thioglycollate broth was covered with about a 3/4 inch layer of melted vaseline. All the flasks were then stoppered with gauze-covered cotton plugs, and these plugs covered with heavy brown paper. Immediately the flasks were labeled with a wax pencil, weighed to the nearest gram, and sterilized by autoclaving at 15 pounds for 15 minutes. Immediately after autoclaving the flasks containing the vaseline-covered broth were placed in a trough of cold water so that the vaseline seal might quickly harden.

2. Inoculation and incubation. The flasks were inoculated with a heavy loopful of an actively-growing culture of the respective organism. The inocula were taken from 24 hour, 37 C, yeast extract glucose slants, except for strain 18L-6 which was taken from a Bugonagar slant. The anaerobes were inoculated exactly as the facultative anaerobes except it was, of course, necessary first to make a hole through the vaseline seal. This was accomplished by means of a hot inoculating loop. The anaerobe was then inoculated into the depths of the medium through this small hole.

The flasks were incubated at 37 C for 3 weeks. They were gently shaken periodically in order to agitate the layer of carbonate. The flasks containing the vaseline-covered broth were agitated by gently rotating the flasks about their vertical axes on a flat surface so that the vaseline seal would not sink or break up. The action of the generated acid upon the carbonate produced carbon dioxide which helps maintain anaerobiosis.

Flasks of uninoculated broth were incubated along with the cultures to serve as controls in these experiments. The control flasks were handled in a manner identical with the inoculated flasks, even to the extent of the periodic agitation.

3. Preparation of the cultures for analysis. After the incubation period, each flask, including the controls, was reweighed to the nearest gram and the loss of weight (resulting

primarily from evaporation) was corrected by adding water to the original weight. The flasks were then shaken, the carbonate and cells allowed to settle, and a 20 ml aliquot was pipetted off and placed in a clean 500 ml volumetric flask in order to determine the amount of glucose remaining in each flask. To this volumetric flask were added 400 ml of water, 20 ml of a saturated sodium fluoride (about 50 grams of NaF in 100 ml of water at room temperature), and 17½ ml of a solution of copper sulfate (40 grams of $CuSO_4.5H_2O$ in 1000 ml of water). The pH of the resulting solution was then adjusted to 6.5 with sodium hydroxide and sufficient water added to bring the level of the solution to the mark. The flask was then shaken and the flocculate allowed to settle; this usually took an hour or so. Duplicate 20 ml aliquots of the greenish, clear supernatent were used in the sugar analysis; Betrand's method described by Kertesz (1930) being employed. The quantity of glucose in the control flask differs from that originally added because the tomato juice and yeast extract contain some carbohydrate and because some glucose is lost by reacting with the peptones during autoclaving.

A second aliquot, in this instance 400 ml, was removed from each flask and acidified with concentrated C.P. sulfuric acid (5 to 10 ml) to a pH of 1.0. This aliquot was tested for volatile and nonvolatile acidity as outlined on the following pages.

4. Determination of volatile acidity. The acidified

400 ml aliquot from each flask was steam distilled (keeping the volume within the distillation flask constant) and about 1600 ml of distillate collected, which required about 3 hours. The control flasks were of course steam distilled since the uninoculated medium contains some volatile acid. The 1600 ml or so of distillate were shaken and 100 ml aliquots were titrated with about 0.05 normal sodium hydroxide using phenophthalein. Usually three titrations were made and the average employed in the calculations; the remainder was discarded. The per cent fermented glucose converted to volatile acid, calculated as acetic acid, is equal to the following relationship:

$$\frac{\left(\begin{array}{l}\text{Nr. of millieqvts.}\\ \text{of base nec. to}\\ \text{neutralize the}\\ \text{entire steam-}\\ \text{distillate from}\\ \text{the cult. flask.}\end{array} \times \frac{5}{4000}\right) - \left(\begin{array}{l}\text{Nr. of millieqvts.}\\ \text{of base nec. to}\\ \text{neutralize the}\\ \text{entire steam-}\\ \text{distillate from}\\ \text{the control flask.}\end{array} \times \frac{5}{4000}\right) 60}{\left(\begin{array}{l}\text{Nr. of grams of}\\ \text{glucose in the}\\ \text{control flask}\end{array}\right) - \left(\begin{array}{l}\text{Nr. of grams of}\\ \text{glucose in the}\\ \text{culture flask}\end{array}\right)} \times 100$$

5. Determination of nonvolatile acidity. The steam-distilled cultures were extracted with reagent grade diethyl ether in a liquid extraction apparatus in order to remove the nonvolatile acid present (figure 1).

The ether employed in the extractions was first tested for the presence of acidity by titrating with dilute base, and for the presence of peroxide by adding a few ml to an aqueous

solution of potassium iodide acidified with hydrochloric acid. The liberation of iodine (reddish-brown color) from the potassium iodide solution is indicative of peroxide.

An indication of the time required to extract all nonvolatile acid from the culture was determined experimentally as follows: To 1500 ml of distilled water in a 2 liter erlenmeyer flask were added 70 ml of lactic acid (C.P., 85.5 per cent). This results in about a 0.5 normal solution after autoclaving at 15 pounds for several hours. The solution of lactic acid was titrated again after standing several days to see if all lactide (Bancroft and Davis, 1931) had been converted to the free acid as a result of autoclaving. No increase in acidity was noted. Four-hundred mls of this aqueous lactic acid were then extracted with about 275 ml of ether. The rate of flow of ether through the acid solution was approximately 8 ml per minute. A titration of this acid was made at zero hours, before placing it in the extractor. Titrations after extraction for various time-intervals were made by removing 5 ml aliquots from the aqueous phase in order to plot a curve of the per cent lactic acid extracted vs. the time of extraction (figure 2). It was found that in 24 hours 92 per cent of the acid had been extracted; in 2 days, 99 per cent; and after three days' extraction almost 100 per cent of the lactic acid had been extracted from the aqueous phase. Therefore extraction of the cultures for 3 days should be sufficient to remove the nonvolatile acidity; I, however, extracted for 4 days to

allow a good margin of safety.

After completion of extraction, the extraction apparatus was removed from the boiling flask, about 60 ml of distilled water added to this flask, and the ether evaporated under the hood. The resulting amber-colored solution of nonvolatile acid was made up to 100 ml by adding more water. After mixing well, 5 ml aliquots of this acid were titrated with about 0.5 normal sodium hydroxide using phenophthalein. Three titrations were performed with each culture and the average value obtained used in the calculations. Multiplying the average number of ml base required to neutralize the 5 ml aliquot, the normality of the base employed, and the number of 5 ml aliquots present in the extract (20) gives the number of milliequivalents of nonvolatile acid in this extract. The per cent fermented glucose converted to nonvolatile acid, calculated as lactic acid, is equal to the following relationship:

$$\frac{\left[\left(\begin{array}{l}\text{Nr. of millieqvts.}\\ \text{of base nec. to}\\ \text{neutralize the}\\ \text{entire ether}\\ \text{extract from}\\ \text{the cult. flask.}\end{array} \times \frac{5}{4000}\right) - \left(\begin{array}{l}\text{Nr. of millieqvts.}\\ \text{of base nec. to}\\ \text{neutralize the}\\ \text{entire ether}\\ \text{extract from}\\ \text{the control flask.}\end{array} \times \frac{5}{4000}\right)\right] 90}{\left(\begin{array}{l}\text{Nr. of grams of}\\ \text{glucose in the}\\ \text{control flask.}\end{array}\right) - \left(\begin{array}{l}\text{Nr. of grams of}\\ \text{glucose in the}\\ \text{culture flask}\end{array}\right)} \times 100$$

6. Preparation of zinc lactate. The remainder of the aqueous solution of nonvolatile acid was heated toward boiling, sufficient C.P. zinc carbonate added to neutralize the acid, and norit for decolorization. The heating was continued for

5 minutes, and the mixture then filtered through a Büchner funnel using suction. The resulting pad of norit was washed once with a small amount of hot water. The clear solution of zinc lactate was decanted from the filter flask into a large evaporating dish and the crystals collected in two fractions. In some instances, where little nonvolatile acid was formed, the crystals were harvested in one batch. In some cases, where the zinc lactate was sufficiently concentrated, the first crystal fraction could be collected upon cooling the hot solution. However, usually crystallization was allowed to proceed after slow evaporation of the solution in the 37 C incubator. Each crystal fraction was filtered free of the mother liquor on a Büchner funnel using suction, washed with a few ml of cold water, and then placed in a clean, labeled petri dish. These petri dishes of crystals were kept at room temperature until dry, after which time each crystal fraction was pulverized with a mortar and pestle and then stored in tightly closed vials. The water of crystallization and the optical rotation of each fraction was determined as outlined on the following pages.

7. Determination of the water of crystallization. From about 0.3 to 0.4 gram of each fraction of dry, powdered zinc lactate was placed in a small tared crucible and weighed to the nearest milligram. The crucibles were then heated at 140 to 150 C for two hours after which time they were allowed to cool in a calcium chloride desiccator, and then quickly

reweighed to the nearest milligram. From the loss of weight, the per cent water of crystallization was calculated. The active salt (dextro or levo) contains two molecules of water of crystallization, while the inactive salt contains three molecules of water of crystallization (table 3).

8. Determination of the specific rotation. Sufficient distilled water was added with a medicine-dropper to cover the anhydrous lactate in the crucibles, and by means of a small stirring rod the lactate was broken up and suspended in the water. The contents of each crucible were then transferred with the medicine-dropper to 30-ml screw-top vials and each crucible was washed out thoroughly with distilled water and the washings added to the respective vials. Each vial then contained 15 to 20 ml of aqueous zinc lactate and each was labeled with the number of the culture, crystal fraction, and the anhydrous weight of the lactate therein. The vials were stored in the refrigerator at 8 C prior to rotation with a polarimeter.

The specific rotation of each batch of crystals was determined using a polarimeter with sodium light. An attempt was made to measure the angle of rotation of the solutions at, or about, 15 C. The vials were placed in a 15 C water bath, the contents poured into 25 ml volumetric flasks and the washings from the vials added to the flasks which were then filled with distilled water to the mark. These flasks were also kept

at 15 C. The contents of the flasks were shaken well and a portion was used to fill the decimeter tube. The angle of rotation was recorded. The polarimeter zero point was determined using distilled water and the instrument was checked using a sample of maltose of known concentration and known optical activity. (A 0.938 gram portion of this disaccharide was dissolved in 25 ml of 0.1 per cent ammonium hydroxide and the specific rotation determined to be +130.6, which compares well with the value of +130.4 as given on the label.)

From Lowry's (1930) data in the International Critical Tables a graph of specific rotation vs. concentration of the active zinc lactate has been prepared (figure 3). Purdie and Walker (1895) have also provided information of this nature, but their investigations were carried out at a somewhat lower temperature, namely 10 C, and therefore are not included in the figure.

9. Sources of error. The greatest source of error in the technique outlined occurs as a result of steam distillation. Four-hundred ml of a 0.5 normal solution of lactic acid and 400 ml of this same acid containing a known amount of acetic acid were steam distilled in the manner outlined. By observing the quantity of base required to neutralize the acid in the steam distillate and the acid remaining in the distillation flask it was discovered that the amount of fermented glucose converted to volatile acid (as acetic) will

TABLE 3

Some physical properties of active and inactive zinc lactate

Form of zinc lactate*	Molecular weight	Per cent water of crystallization*	Solubility in 100 ml water at 15 C**
Active zinc lactate, dextro or levo.			
$Zn(C_3H_5O_3)_2.2H_2O$	247.55	12.88	5.7 grams
Inactive zinc lactate.			
$Zn(C_3H_5O_3)_2.3H_2O$	297.57	18.18	1.67 grams

* From Bancroft and Davis (1931).

** From Hodgman (1950).

be approximately 3 per cent too high and the amount of fermented glucose converted to nonvolatile acid (as lactic) will be approximately 3 per cent too low. This is due to the fact that lactic acid is somewhat volatile, and therefore a small amount appears in the distillate.

A small additional error in the nonvolatile acidity occurs as a result of ether extraction. Although, as has been pointed out, almost 100 per cent of the lactic acid is extracted from the aqueous phase, it has been found that only about 98 per cent is actually recoverable from the ether extract in the boiling flask. This is probably due to formation of some lactide in the flask. Hence when the amount of glucose necessary to form the actual lactic acid present is compared with the amount of glucose necessary to form the recoverable lactic acid, it is found that this value will be approximately 1 per cent low.

To recapitulate then, although the values of volatile and nonvolatile acid tabulated in the results section of this dissertation are comparable and capable of duplication, the absolute values may be expected to be in error by a few per cent, about 3 per cent high in the case of the fermented glucose converted to volatile acid (as acetic) and approximately 4 per cent low in the case of the fermented glucose converted to nonvolatile acid (as lactic). Thus, if a culture is reported to convert 3.3 per cent of the fermented glucose to volatile acid (calculated as acetic acid), a figure of

3.2 per cent would be probably a closer value. This very small difference of course in no way affects the species identification or other conclusions recorded in this dissertation.

III

RESULTS AND DISCUSSION

A. The proportion of sampled blackhead livers yielding cultivatable bacteria, the types of bacteria isolated, their approximate numbers, and their pathogenicity

It may be seen in table 4 that of the blackhead livers studied, 29, or almost 50 per cent, yielded cultivatable bacteria. (That is, 29 liver samples produced colonies in a definite dilution range on the agar pour plates.) It is interesting in this connection that Strong (1944), referring to amoebiasis of man, mentions that in about 50 per cent of amoebic livers bacteria may be encountered. Evidently the chances of bacterial invasion of this organ are about the same in untreated cases of amoebic dysentery of man and blackhead of turkeys.

Some additional data may be gleaned from the table. For one thing, when turkeys are sacrificed at the appearance of symptoms and their livers immediately cultured there is less likelihood of encountering bacteria than when the livers are sampled after death of the bird. Of the 36 turkeys sacrificed at the appearance of symptoms, the livers of only 12, or about 33 per cent, yielded cultivatable bacteria. On the other hand, of the 24 turkeys sampled after death, 17, or about 71 per cent, yielded bacteria. Hence, the chances of finding bacteria in

TABLE 4

Comparisons of the number of blackhead livers free of cultivatable bacteria with those containing cultivatable bacteria

		Liver samples containing cultivatable bacteria	Liver samples free of cultivatable bacteria	Totals
Liver samples from turkeys acquiring the disease artificially (rectal inoculation with cultures of *H. meleagridis*)	From turkeys sacrificed at appearance of symptoms	5	13	18
	From turkeys found dead of the disease	10	4	14
Liver samples from turkeys acquiring the disease naturally (contaminated surroundings)	From turkeys sacrificed at appearance of symptoms	7	11	18
	From turkeys found dead of the disease	7	3	10
	Totals	29 (48%)	31 (52%)	60

blackhead livers is just about twice as great if the birds are allowed to die before culturing their livers. On the whole, this tends to verify the findings of Smith (1915) who stated that bacteria "are rather uncommon in the liver necroses of turkeys chloroformed at the height of the disease" while "turkeys which die during the night, smears and cultures may show next day several varieties of bacteria". If his statement, however, leans in either direction, it is in the direction of over-simplification; since, of the turkeys I cultured after death, about one-third were nevertheless sterile, and of those cultured after sacrificing at the appearance of symptoms, about one-third did contain bacteria.

The ratio of the number of livers containing bacteria to those lacking same is of a similar magnitude regardless of whether the birds were infected naturally or artificially, and hence these two modes of infection do not appear to affect the chances of bacterial invasion of the liver in this disease.

A varying number of colonies were picked from the pour plates and the resulting cultures were labeled in the manner outlined in the previous section; that is, the number of the colony selected was attached to the number of the liver sample. Thus, culture 17L-3 is descended from the third colony picked from the pour plates derived from liver sample 17L. Usually, from 10 to 20 representative colonies were selected from the pour plates, unless of course there were fewer than this number of colonies available.

In table 5 are listed the 29 liver samples which yielded bacteria, together with the bacterial types isolated, the plate count obtained, and the manner of death of the turkey from which each sample was acquired. (That is, whether the turkey was sacrificed at the first appearance of symptoms or was allowed to die of the disease.) For convenience, the samples are tabulated in three groups. The first group of 9 samples yielded gram-negative, lactose-fermenting rods only; the second group of 11 samples rendered gram-negative, lactose-fermenting rods together with one or more additional bacterial types as indicated; and the third group of 9 liver samples yielded bacterial types other than the gram-negative, lactose-fermenting rods.

It is apparent at a glance that these gram-negative rods were the bacterial type most frequently encountered, occurring in 20 out of 29 bacteria-infested livers. Although the identity of the isolated bacteria will be discussed in detail elsewhere, perhaps it will be apropos to mention here that these gram-negative, lactose-fermenting rods have been identified as strains of Escherichia coli. Thus, this species was the one most frequently encountered, being isolated from one out of every three blackhead livers sampled. As has been pointed out earlier in this dissertation ("Review of the literature", page 13), Theobald Smith isolated this bacterium from blackhead livers in 1895.

Cocci were next in prevalence, occurring in 13 livers. As has been pointed out, Rettger and Kirkpatrick (1927) isolated

TABLE 5

The types of bacteria isolated from 29 blackhead liver samples, the plate count obtained in each instance, and other pertinent information

Sample number	Manner of death	Plate count per gram sample		Bacterial types isolated
8L	Died	5,000		
9L	Died	120,000		
34L	Died	1,000,000		Gram-negative,
36L	Died	60		lactose-fermenting
66L	Died	16,000		rods
67L	Sacrificed	4,000		
68L	Died	2,000,000		
75L	Sacrificed	220		
79L	Sacrificed	15		
10L	Died	6,400		Micrococci
11L	Died	600,000		Streptococci
16L	Died	2,300	Gram-	Strep.&Pedio.
18L	Died	3,000	neg.,	G-pos.rods&cocci
19L	Died	1,000	lac-	Gram-neg. and
			tose-	Gram-pos. rods
37L	Sacrificed	230	fermen-	Gram-pos. rods
43L	Sacrificed	4,500	ting	Streptococci &
			rods	Gram-pos. rods
49L	Sacrificed	60	and:	Streptococci
60L	Sacrificed	300		Gram-neg. rods
62L	Sacrificed	10		Streptococci
69L	Sacrificed	85		Gram-pos. rods
5L	Died	-----		Streptococci
6L	Died	1,300		Strep. & Micro.
17L	Died	600		Gram-neg. rods
35L	Died	30		Streptococci
39L	Died	1,900		Cocci & Gram-pos. rods
55L	Sacrificed	35		Gram-pos. rods
57L	Sacrificed	10		Streptococci
71L	Sacrificed	10		Gram-pos. rods
72L	Died	140,000		Micrococci

cocci from blackhead liver lesions but did not identify them as to species. Gram-positive rods were encountered in 8 liver samples.

It should be noted that samples from turkeys which died of blackhead usually gave higher plate counts then samples from turkeys sacrificed at the appearance of symptoms. Of the former, 13 out of 17 gave plate counts of 1,000 or greater, while of the later only 2 out of 12 gave counts of this magnitude (table 5). Or to express the same thing in another way, the average plate count (geometric mean) of the samples from turkeys which died of blackhead was 10,000, while the average plate count (geometric mean) of the liver samples from turkeys which were sacrificed was only 100 per gram sample.

After preliminary tests, representative cultures were injected intravenously into young turkeys (pages 29 and 30) in order to determine their pathogenicity. A total of forty-nine cultures was so tested, and of these, six were found capable of producing illness or death.

In table 6, information pertaining to these six cultures is summarized. In no case did the affected poults void sulfur-colored droppings or at autopsy did their livers show blackhead lesions. They drooped, and exhibited anorexia and leg weakness. When reference is made to the heart as an organ from which the bacterium in question was recovered (the last column of table 6) it is heart's blood being referred to, not heart tissue.

TABLE 6

Information relating to the 6 bacterial cultures found to be pathogenic

Culture number	Date injected		Date poult became ill	Date poult died	Date poult was sacrificed	Organs from which the culture was recovered
17L-3	7/27/48	(a)	----	7/28	---	Liver
		(b)	7/30	----	8/3	Liver
72L-1	9/21/50	(a)	9/26	9/27	---	Liver
		(b)	----	9/26	---	Liver & heart
60L-4	9/21/50	(a)	10/11	----	10/11	None *
		(b)	----	----	10/19	None *
34L-1	5/10/51	(a)	----	5/11	----	Liver & heart
		(b)	5/11	----	5/11	Liver & heart
68L-1	5/10/51	(a)	5/12	----	5/12	Liver & heart
		(b)	5/11	5/12	----	Liver & heart
79L-1	5/10/51	(a)	----	5/13	----	Liver
		(b)	----	----	5/24	None

* Although not isolated from either poult, this bacterium has nevertheless been regarded pathogenic because poult (a) was observed at autopsy to have skin areas discolored green and areas of its liver discolored yellow, and the liver of poult (b) contained a number of small, hard necrotic lesions.

Only one culture, 60L-4, was not recovered from the poults at autopsy, but as is mentioned in the footnote to table 6, this culture was nevertheless regarded as pathogenic because of the pathological condition occurring in the two infected birds. Perhaps insufficient material was inoculated, or the organs were sterile at the time of autopsy. However, the other five cultures were recovered from the livers of the dead poults, and in several instances also from the heart's blood.

In order to fulfill Koch's last postulate it is necessary to identify the recovered bacteria as the same as were injected. This was accomplished by microscopical examination of gram-stains and some biochemical tests. For example, cultures 72L-1, 34L-1, 68L-1, and 79L-1 were tested in regard to their fermentative abilities before injection into the poults and after being recovered therefrom. The fermentation pattern (final pH) in each case was similar (table 7). In table 8 the reactions of 17L-3 in five different carbohydrates before injection and after recovery are tabulated, which show that here also the organism recovered was the same as was injected.

Because of its large, smooth, round, orange colonies, and other characteristics to be mentioned later, culture 72L-1 was identified as Micrococcus pyogenes var. aureus. The other three cultures listed in table 7 are gram-negative, lactose-fermenting rods which I have already indicated to be strains of Escherichia coli. These three strains were isolated from liver samples 34L, 68L, and 79L which fall in the first group in table 5, that is they yield this species only. Culture 60L-4 has been considered

TABLE 7

The final pH produced in various substrates by 4 pathogenic strains of bacteria before injection into poults and after recovery therefrom (Broth cultures, incubated at 37 C for 2 weeks; pH values representing significant fermentation have been underscored)

Strain number	Glycerol	Xylose	Arabinose	Rhamnose	Sorbitol	Mannitol	Inositol	Fructose	Glucose	Mannose	Galactose	Sucrose	Trehalose	Maltose	Melibiose	Lactose	Melezitose	Raffinose	Dextrin	Starch	Salicin	Control	Species
Control*	7.3	7.0	7.0	6.8	7.3	7.3	7.3	6.7	6.7	6.7	6.5	7.4	7.4	7.3	7.3	7.1	7.3	7.4	7.3	7.4	7.4	7.4	
72L-1 before injection*	5.5	7.7	7.8	7.7	7.9	5.1	7.8	4.7	4.7	5.2	5.2	5.2	4.9	5.2	7.9	5.1	7.0	7.9	7.8	7.8	7.2	7.9	*M. pyogenes* var. *aureus*
72L-1 from liver of poult (a)*	5.5	7.8	7.8	7.7	8.0	5.2	7.9	4.7	4.7	5.2	5.2	5.2	4.9	5.2	7.9	5.1	7.0	7.9	7.8	7.8	6.9	8.0	
72L-1 from heart of poult (b)*	5.5	7.8	7.8	7.7	8.0	5.2	7.9	4.7	4.8	5.2	5.2	5.5	4.8	5.2	7.9	5.1	7.1	8.0	7.8	7.8	7.0	7.9	
Control**	7.1	6.1	6.5	6.2	7.2	7.1	7.0	5.7	5.9	6.1	5.7	7.0	7.2	7.0	6.9	6.7	7.2	7.2	6.8	7.1	7.2	7.1	
34L-1 before injection**	8.3	5.3	5.1	5.3	8.0	5.4	8.4	4.9	4.9	4.9	5.0	8.5	5.2	5.3	6.6	5.3	8.6	8.5	8.0	7.0	6.6	8.5	
34L-1 from liver of poult (b)**	7.5	5.3	5.1	5.3	8.6	6.4	8.5	4.9	4.9	4.9	5.0	8.5	5.6	5.5	6.9	5.2	8.5	8.5	7.9	6.9	6.4	8.6	
68L-1 before injection**	7.4	5.3	5.1	5.3	8.5	5.4	8.5	4.9	4.9	4.9	5.1	8.6	5.7	5.4	7.0	5.2	8.6	8.5	8.2	7.8	6.6	8.6	*Escherichia coli*
68L-1 from liver of poult (a)**	8.4	5.4	5.1	5.6	8.6	5.4	8.5	4.9	4.9	4.9	5.1	8.6	5.0	5.4	6.8	5.2	8.5	8.5	7.3	6.9	6.5	8.5	
79L-1 before injection**	6.2	5.3	5.1	5.5	8.5	5.6	8.7	4.8	4.9	4.9	5.0	8.7	5.2	5.1	5.7	5.3	8.6	8.5	8.1	7.0	8.1	8.7	
79L-1 from liver of poult (a)**	6.7	5.3	5.0	5.7	8.2	5.5	8.6	4.9	4.9	4.9	5.0	8.6	5.1	5.1	5.7	5.2	8.6	8.7	7.6	6.7	8.0	8.8	

* Tryptone, 2%; K_2HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.01%; and substrate, 1%.

** Yeast extract, 2.2%; K_2HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.01%; and substrate, 2%.

TABLE 8

Fermentation reactions* of culture 17L-3 (Salmonella newport) before its injection into poults and after its recovery therefrom

	Glucose	Lactose	Sucrose	Maltose	Mannitol
17L-3 before injection	-/-	---	---	-/-	-/-
17L-3 from liver of poult (a)	-/-	---	---	-/-	-/-

* Phenol red broth base (Difco, dehydrated) to which sufficient substrate was added to form a concentration of 1 per cent.

-/- Acid and gas; --- No change.

Pseudomonas aeruginosa and 17L-3 was identified as Salmonella newport by means of serological typing and other tests. This species was also encountered in liver 19L. (These facts are mentioned at this time merely to facilitate discussion, as the taxonomy of the isolated bacteria will be considered in detail in subsequent sections.) The fermentation reactions of the cultures prior to injection and after recovery from the poults are similar, except perhaps in the case of Escherichia coli strain 34L-1, which manifests a somewhat weaker mannitol fermentation after recovery (table 7). The three pathogenic coli differ from one another in regard to melibiose fermentation; strain 79L-1 attacks this disaccharide while 34L-1 and 68L-1 do not (table 7).

It is apparent that the statement by Smith (1895); "the absence of any uniformity in the bacteriological results as well as the appearance of B. coli in the organs of dead turkeys indicates that pathogenic bacteria were not associated with the protozoa in the cases examined" will not always fit the situation, since, as I have shown, pathogenic bacteria may sometimes be associated with the blackhead parasite, and indeed even pathogenic strains of B. coli.

If it is correct to assume that the source of all bacteria recovered from blackhead livers is the cecum of the bird, then the "selectivity" of the liver is certainly well demonstrated in this study. For instance, three of the pathogenic bacteria isolated (Micrococcus aureus, Pseudomonas

aeruginosa, and Salmonella newport) were never encountered as part of the normal cecal flora (Harrison and Hansen, 1950a) and therefore must reside in the cecum in only relatively small numbers; and as another example, although E. coli, as I have indicated, is the bacterium most often found associated with the blackhead parasite in infected livers, it is one of the least dominant bacterial species in the cecum of the healthy turkey (Harrison and Hansen, 1950a). It must be kept in mind, however, that a blackhead cecum is not normal, and the cecal flora may change prior to hepatic involvement. A study of the flora of the blackhead cecum would certainly be of interest.

As we have seen, turkeys which are allowed to die of blackhead are more likely to contain bacteria in their diseased livers than are turkeys which have been killed earlier in the course of the disease, before the natural termination of this illness. Also, of the livers of both cases containing bacteria, the plate counts are usually greater in the instances where the turkeys died than where they were sacrificed. Two interpretations of cause and effect therefore present themselves: Are bacteria the cause and death the effect, or is death (or the prolonged illness) the cause and bacteria the effect?

The fact that of a total of 17 turkeys which died and yielded bacteria, 5, or about 30 per cent, contained bacteria which alone are able to bring about illness (and usually death) when injected into the circulatory system of young, vigorous

turkeys, leads one to speculate as to the effect of these bacteria, and those less pathogenic, on turkeys already seriously ill. In the cases of the turkeys donating liver samples 17L, 34L, 68L, 72L, and 79L death would have certainly ensued even if the effect of Histomonas meleagridis was eliminated. (These samples yielded five of the six pathogenic strains listed in table 6). In all probability, the presence of normally non-pathogenic bacteria in diseased livers hastens death. This evidence supports the view held by Curtice (1907a), who as a result of his casual observations of the degree of destruction in blackhead livers, theorized that death "is not always produced directly as a result of blackhead disease but is affected by secondary causes" such as bacteria. One fact that hasn't been mentioned should be brought to light here. In at least three instances (66L, 68L, and 72L) the turkeys died immediately before autopsy, actually while being carried from the turkey yard to the laboratory. In these cases the bacterial counts were considerable (table 5), yet were not likely to have occurred as post-mortem changes. It is interesting that in all three instances a single species of bacteria was isolated, and that in two of the three cases the bacterium isolated was pathogenic.

On the other hand, since the six pathogenic bacteria represent four distinct species, no single bacterial agent can be considered as being constantly associated with death in this disease; this is in agreement with the conclusions of

Kettger and Kirkpatrick (1927). In addition, since the livers of seven turkeys which died of the disease yielded neither cultivatable bacteria at autopsy nor were seen to contain bacteria as a result of microscopical examination, it is apparent that the protozoan alone can terminate the disease fatally, a finding which is in agreement with the conclusions expressed by Theobald Smith as early as 1895.

The reconciliation of the two opposing viewpoints of cause and effect is not at all difficult, since the evidence presented in this dissertation indicates that both interpretations represent what may actually happen in practice. My thesis therefore can be stated as follows: Although Histomonas meleagridis is the actual etiological agent, the initiating factor in this disease, and the organism which by itself can terminate the disease fatally; nevertheless, secondary or concurrent bacterial infection may play the decisive role, overriding the protozoan parasitism and punctuating the illness by a quick death.

This denouement only ammends Smith's (1895, 1915) work on this phase of blackhead disease and does in no way invalidate his fine pioneer study.

Since, as I have conclusively shown, when a sufficiently large number of blackhead livers are sampled about half may be expected to contain no bacteria, a means is at hand whereby one may readily obtain Histomonas meleagridis free of bacteria. (The method used to acquire bacteria-free liver suspensions

of H. meleagridis consists merely of sacrificing poults at the first appearance of blackhead and aseptically macerating their livers; a portion of the liver suspension is tested for bacterial sterility by plating on suitable media, see pages 19 to 23.)

The possibilities of the technique elaborated in this dissertation are being explored by direct injection of bacteria-free blackhead liver suspensions into the livers of healthy poults. DeVolt and Holst (1952), employing in part the technique outlined, already have produced liver lesions with bacteria-free blackhead liver suspensions in several experimental turkeys. Adequate controls were run parallel with the experiment. Although these findings will require further confirmation, the experiments performed so far have been in favor of the conclusions expressed in this dissertation.

B. The identity of the bacteria isolated from blackhead livers

1. Gram-negative rods. As has been mentioned previously, gram-negative, lactose-fermenting rods were the bacterial type most often isolated from the blackhead livers. They were encountered in 20 of the total of 60 livers sampled. In 9 cases they were the only cultivatable bacteria present, while in 11 cases they occurred together with one or more additional types. These gram-negative, lactose-fermenters showed the IMViC reactions characteristic of Escherichia coli and are

therefore considered to be strains of this species. The majority, those from liver samples 8L, 9L, 16L, 18L, 19L, 36L, 43L, 49L, 60L, 62L, 66L, and 69L, developed the characteristic coli-type colony on the E.M.B. agar plates; others, those from liver samples 10L, 34L, 67L, 75L, and 79L, although producing a metalic sheen were quite slimy; while colonies produced by E. coli from samples 10L, 11L, 37L, and 75L did not show much sheen and produced the pink, mucoid colony-type generally associated with Aerobacter aerogenes. Even when grown on heart infusion agar (Difco, dehydrated) cultures 67L-2, 75L-1, and 75L-8 are extremely stringy. The cultures of E. coli from 17 liver samples were tested for sucrose fermentation. It is interesting that the cultures from 11 livers were sucrose-negative (including the three yielding pathogenic strains 34L-1, 68L-1, and 79L-1), those from 5 were sucrose-positive, and one liver yielded both fermentative types. Hence, the majority of the coli strains encountered were non-fermenters of this disaccharide. This is in contradistinction to the case of E. coli isolated from the cecal feces of healthy turkeys. Here, fermenters and non-fermenters of sucrose occur in equal numbers (Harrison and Hansen, 1950a). The liver seems to be more "selective" toward the sucrose non-fermenters, although more work should be undertaken before the conclusions can be regarded as valid.

On the E.M.B. agar plates of the 10^{-2} dilution prepared from two blackhead livers (17L and 19L) there appeared small,

round, smooth, transparent colonies. Transplants from these two isolations were found to be non-fermenters of lactose, even after a week's incubation. The two cultures studied in some detail (17L-3 and 19L-6) were found to be gram-negative rods possessing peritrichous flagellation. They do not produce indole or urease, or liquefy gelatin. They are VP-negative, MR-positive, and ferment glucose with the formation of acid and gas. For these reasons the cultures were considered members of the genus Salmonella. Serological typing by the Army Medical School in Washington, D. C., revealed them to be cultures of Salmonella newport. They have the fermentation pattern considered characteristic of this species by Kauffmann (1941); fermenting arabinose, dulcitol, rhamnose, trehalose, and xylose, but not inositol. This bacterium was determined to be pathogenic by injecting culture 17L-3 into the wing vein of two healthy poults (table 6).

A third species of gram-negative rod, Pseudomonas aeruginosa, was isolated from one liver. This bacterium (culture 60L-4) produces a green fluorescent, extracellular pigment, liquefies gelatin, peptonizes milk, reduces nitrate, and does not form indole. It is motile, does not ferment maltose, and produces little or no acidity in glucose broth. It was identified employing the key in Bergey's manual (Breed et al., 1948). The pathogenicity of this strain has been discussed (table 6). This species was isolated from L.M.B. plates of a 10^{-1} dilution, and occurred in about equal numbers with Escherichia coli in this liver.

2. Cocci. Thirteen liver samples yielded cocci, four genera being represented. Whereas the cocci were occasionally the only bacteria isolated, in the majority of cases, actually eight out of the total thirteen, they occurred with one or more additional bacterial types, most often with Escherichia coli, as will be pointed out in the following paragraphs.

Liver sample 5L yielded Streptococcus liquefaciens in pure culture. Unfortunately none of the strains isolated from this liver were kept, and therefore this species is not represented in tables 9 and 10. However, the available information (resulting from the preliminary studies) leaves no doubt as to the identity of this coccus. The strains investigated grew at 10 C and 45 C, grew in the presence of 0.1 per cent methylene blue, grew at an initiating pH of 9.6, survived when heated at 61 C for 30 minutes, liquefied gelatin, peptonized milk, and fermented the following: sorbitol, mannitol, sucrose, trehalose, maltose, lactose, melezitose, starch, and salicin. Furthermore, they reacted with group D sera.

Sample 6L yielded Micrococcus and Streptococcus. The former was lyophilized and is characterized on subsequent pages. The streptococci, on the other hand, were not kept after the preliminary tests, but were definitely identified as S. faecalis employing most of the tests listed immediately above.

Micrococcus was isolated again from liver 10L, and this organism is characterized on following pages. It was isolated from a Mugonagar plate of the 10^{-2} dilution together with

Escherichia coli. The coliform was encountered on plates of the same dilution, but in greater numbers than the cocci.

Streptococci and Escherichia coli were encountered on Eugonagar plates of the 10^{-4} dilution in equal numbers from liver sample 11L. The cocci were identified as Streptococcus faecalis by means of the same tests as mentioned on the previous page.

Streptococci, some unidentified cocci resembling pediococci, and coliforms were isolated from 16L, the former two types from Eugonagar plates of the 10^{-3} dilution and E. coli from plates of the 10^{-2} dilution. A representative of each coccus type was lyophilized, revived at a later date and studied more thoroughly. The results of the detailed investigations are described on following pages.

Liver sample 18L yielded a gram-positive, anaerobic diplococcus together with members of the genus Lactobacillus and Escherichia coli. All three bacterial types were isolated from Eugonagar plates of the 10^{-3} dilution, but E. coli was outnumbered by the other two. The anaerobic diplococcus was assigned the name Diplococcus magnus, because the anaerobic diplococcus listed by Bergey (Breed et al., 1948) which it most closely resembles, is given this particular species designation. The organism measured about 1.3 micra in diameter and produced no acidity when grown in the presence of various sugars, including glucose. The lactobacilli are characterized in a subsequent section of this dissertation.

Sample 35L rendered two species of streptococci from the Eugonagar plates of the 10^{-1} dilution. Both streptococci are characterized on following pages.

Cocci and lactobacilli were encountered on Eugonagar plates of the 10^{-2} dilution from liver sample 39L. The lactobacilli occurred in slightly greater numbers than did the cocci. Two species of cocci have been recognized and are to be discussed in some detail.

Sample 43L yielded streptococci and E. coli in about equal numbers from the Eugonagar plates of the 10^{-2} dilution, and lactobacilli from this same medium of the next higher dilution, 10^{-3}.

From liver 49L were isolated streptococci and again E. coli. They occurred in about equal numbers on the Eugonagar plates of the 10^{-1} dilution.

Sample 57L yielded bacteria in very small numbers. Only one colony was found on each of the Eugonagar plates of the lowest dilution. Both turned out to be streptococci.

Bacteria were encountered from 62L also in very small numbers. From one of the 10^{-1} Eugonagar plates a single culture of Streptococcus was isolated, whereas a single E. coli colony occurred on one of the E.M.B. plates of the same dilution. The Streptococcus from this liver, as those from the preceeding three livers, have been identified as S. inulinaceus and are discussed on subsequent pages.

Liver specimen 72L yielded a pathogenic Micrococcus in

pure culture. Representatives of this organism were picked from Eugonagar plates of the 10^{-3} dilution, and one culture (72L-1) was studied in some detail (tables 9 and 10).

In October 1950 the lyophilized cultures of cocci were revived by shaking the entire contents of the respective ampoules into trypticase soy broth (B.B.L., dehydrated) and after incubation for 24 hours were purified by streaking out on Eugonagar plates. Unlike some of the lactobacilli to be described in a subsequent section, these cocci gave rise to one type of colony (see page 31). A single colony was selected and subcultured onto fresh Eugonagar slants. From these tubes the cocci were transferred to yeast extract glucose agar on which medium they were carried as stock cultures, being stored in the refrigerator at 8 C for several weeks between transfers.

In tables 9 and 10 are listed these hepatic cocci together with the outcome of various physiological and biochemical tests. Tabulated first are three cultures of micrococci which have been identified by means of the breakdown in Bergey's manual (Breed et al., 1948). They are unable to utilize $NH_4H_2PO_4$ as the sole nitrogen source and in one day produce turbidity in trypticase glucose broth containing 9 per cent salt. The first two have been identified as Micrococcus pyogenes, since they reduce nitrate, liquefy gelatin, and ferment mannitol. Culture 6L-7, produces pigment-less growth, is non-hemolytic, and non-pathogenic for poults (table 9), and ferments the widest variety of substrates (table 10).

TABLE 9

Characteristics of cocci" from blackhead livers

Culture number	Nitrate reduction	Gelatin liquefaction	Catalase production	Growth at 10 C	Growth at 45 C	Growth in pH 9.6	Growth in 0.1% M.B.	Action on lit. milk	Action in skim milk: Final pH	Action in skim milk: Acidity	Action in skim milk: Days to curdle	Growth in sodium chloride: 2 %	5.5 %	6 %	6.5 %	7 %	9 %	Esculin	Sodium hippurate	Species
6L-7*	-/-	-/-	-/-	-/-	---	-/-	---	slA R	5.5	0.29	12	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	M. albus
72L-1**	-/-	-/-	-/-	-/-	---	-/-	-/-	slA C P	5.1	0.43	1	-/-	-/-	-/-	-/-	-/-	-/-	---	-/-	M. aureus
10L-8*	---	-/-	-/-	-/-	---	-/-	-/-	slA R	6.0	0.21	---	-/-	-/-	-/-	-/-	-/-	-/-	---	-/-	M. flavus
16L-13*	---	---	-/-	-/-	---	---	---	slA	6.2	0.08	---	-/-	-/-	-/-	-/-	-/-	---	-/-	---	Unidentified cocci
38L-12*	---	---	---	---	-/-	---	---	A C R	4.6	0.60	1	-/-	---	---	---	---	---	-/-	---	
43L-8*	---	---	---	---	-/-	---	-/-	R A C	4.9	0.43	1	-/-	---	---	---	---	---	-/-	---	
49L-6*	---	---	---	---	-/-	---	-/-	R A	4.9	0.49	3	-/-	---	---	---	---	---	--/-	---	Streptococcus inulinaceus
57L-1*	---	---	---	---	-/-	---	-/-	R A C	4.9	0.44	1	-/-	---	---	---	---	---	-/-	---	
62L-2*	---	---	---	---	-/-	---	-/-	R A C	4.9	0.54	1	-/-	---	---	---	---	---	-/-	---	
30L-3	---	---	---	-/-	-/-	-/-	---	R A	5.6	0.21	---	-/-	-/-	-/-	-/-	---	---	-/-	---	
33L-6	---	---	---	-/-	-/-	-/-	---	R slA	5.8	0.16	---	-/-	-/-	-/-	---	---	---	-/-	---	
39L-2*	---	---	---	-/-	-/-	-/-	---	A C R	4.9	0.49	4	-/-	-/-	-/-	-/-	-/-	---	-/-	---	
35L-11	---	---	---	-/-	-/-	-/-	-/-	R A C	4.8	0.49	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	Streptococcus faecalis
35L-3*	---	---	---	-/-	-/-	-/-	-/-	R A C	4.8	0.54	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	
35L-12	---	---	---	-/-	-/-	-/-	-/-	R A C	4.9	0.49	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	
35L-4	---	---	---	-/-	-/-	-/-	-/-	R A C	5.0	0.49	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	
39L-4	---	---	---	-/-	-/-	-/-	-/-	R A C	4.9	0.49	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	
16L-1	---	---	---	-/-	-/-	-/-	-/-	R A	5.4	0.29	---	-/-	-/-	-/-	-/-	-/-	-/-	-/-	---	
35L-8*	---	---	---	-/-	---	---	---	slR	6.3	0.04	---	-/-	---	---	---	---	---	-/-	---	Streptococcus sp.

" All survive heating at 60 C for 30 minutes.

* Tested for pathogenicity and found to be not pathogenic for poults.

** Beta-hemolytic, and pathogenic for poults.

-/- Growth, or positive reaction;

--- No growth, or negative reaction;

-/- Questionable or feeble growth;

A Acid; slA Slight acid; C Curd; R Reduction;

slR Slight reduction; P Peptonization.

On the other hand, 72L-1 produces orange growth, is hemolytic for sheep and horse erythrocytes, and is extremely pathogenic for poults (table 6), and for these reasons has been given the variety name of aureus, whereas the former has been assigned the variety name of albus, although it is somewhat atypical in fermenting salicin. Gelatin liquefaction by 6L-7 has become less pronounced recently.

Some difficulty was experienced in the identification of 10L-8. An actively growing culture of this organism is not pigmented, but differs from the non-pigmented, nitrate non-reducing, ammonium phosphate non-utilizing M. candidus in liquefying gelatin and in not fermenting glycerol. However, it was observed that old cultures of 10L-8 sometimes may be considered to possess a very feeble yellow pigmentation; this is sometimes seen in a stock culture which has been kept in the refrigerator for several weeks. If one may consider this coccus to be pigmented yellow, then it fits well the description of M. flavus in Bergey's manual. At any rate, it has been assigned this epithet. (I feel that a property of this degree of elusiveness should not be assigned such a dominant position in the classification of members of the genus Micrococcus.) Whereas this culture originally liquefied gelatin, activity on this substance is now very weak, or absent.

Tabulated next are two cultures, neither Micrococcus nor Streptococcus, the first of which is a gram-positive, catalase-

TABLE 10

Final pH values of broth* cultures of cocci from blackhead livers after 14 days' incubation at 37 C
(pH values representing significant fermentation have been underscored)

Culture number	Glycerol	Xylose	Arabinose	Rhamnose	Sorbitol	Mannitol	Inositol	Fructose	Glucose	Mannose	Galactose	Sucrose	Trehalose	Maltose	Cellobiose	Melibiose	Lactose	Melezitose	Raffinose	Dextrin	Starch	Salicin	Inulin	Control	Species
Control	7.3	7.0	7.0	6.8	7.3	7.3	7.3	6.7	6.7	6.7	6.5	7.4	7.4	7.3	7.3	7.3	7.1	7.3	7.4	7.3	7.4	7.4	6.8	7.4	
6L-7	5.8	5.6	5.9	7.6	5.7	5.6	7.9	5.0	5.0	5.3	5.3	5.4	5.3	6.0	5.3	7.5	5.1	7.5	7.6	7.6	7.8	5.3	8.0	7.9	M. albus
72L-1	5.5	7.7	7.8	7.7	7.9	5.1	7.8	4.7	4.7	5.2	5.2	5.2	4.8	5.2	8.0	7.9	5.1	7.0	7.8	7.8	7.8	7.2	7.7	7.9	M. aureus
10L-8	6.9	7.3	5.8	7.3	6.2	6.2	7.6	6.0	5.2	6.4	5.4	5.3	6.1	5.2	7.7	7.5	5.5	6.4	7.6	6.9	7.7	7.7	7.5	7.7	M. flavus
16L-13	5.6	6.8	6.7	6.9	7.4	7.4	7.4	4.1	4.2	4.5	4.3	7.4	4.1	4.1	4.1	7.4	4.6	7.5	7.4	7.1	7.4	5.1	7.3	7.3	Unidentified cocci
39L-12	7.3	6.6	6.7	6.2	7.2	4.5	7.3	4.2	4.2	4.2	4.3	4.1	4.2	4.2	4.2	4.6	4.3	7.3	4.4	4.3	4.6	4.3	7.2	7.3	
43L-8	7.3	6.8	6.9	6.7	7.2	7.2	7.3	4.2	4.2	4.3	4.4	4.4	4.3	4.4	4.4	4.7	4.8	4.6	4.5	5.1	7.1	4.5	5.6	7.3	Streptococcus inulinaceus
49L-6	7.3	6.9	7.0	6.8	7.3	7.3	7.0	4.5	4.5	4.5	4.7	4.7	4.9	4.5	4.5	4.7	5.5	4.8	4.7	4.7	4.8	4.7	5.2	7.3	
57L-1	7.3	6.8	6.9	6.7	7.2	7.2	7.3	4.2	4.4	4.3	4.4	4.4	4.7	4.3	4.3	4.6	4.7	4.6	4.6	5.1	7.1	4.5	5.4	7.3	
62L-2	7.3	6.6	7.0	6.7	7.3	7.3	7.3	4.4	4.4	4.5	4.6	4.7	4.9	4.4	4.4	4.7	4.9	4.8	4.7	5.0	7.3	4.8	5.4	7.3	
39L-3	5.6	6.4	4.6	5.0	4.7	4.5	6.7	4.2	4.2	4.3	4.7	5.1	4.7	4.6	4.4	4.7	4.9	5.1	6.7	5.7	6.8	4.6	6.8	6.9	Streptococcus faecalis
39L-6	6.2	6.4	4.7	5.1	4.7	4.8	6.7	4.2	4.2	4.3	4.7	5.1	4.7	4.6	4.4	4.7	5.0	5.2	6.7	5.6	6.8	4.7	6.8	6.9	
39L-2	4.9	6.4	4.8	5.1	5.2	4.7	6.7	4.3	4.3	4.2	4.7	5.1	4.9	4.6	4.4	6.7	4.7	5.3	6.8	5.6	6.7	4.8	6.8	6.8	
35L-11	6.6	6.7	4.7	7.0	7.4	5.2	7.5	4.5	4.4	4.5	4.8	4.6	5.1	4.5	4.5	5.5	4.8	7.5	6.1	5.2	7.3	4.8	7.4	7.6	
35L-3	7.3	7.1	4.9	7.1	7.5	5.4	7.6	4.5	4.4	4.5	4.7	4.6	5.0	4.5	4.6	7.5	4.8	7.6	7.5	5.2	7.5	4.9	7.5	7.7	
35L-12	6.5	7.1	4.8	7.1	7.5	5.3	7.5	4.4	4.3	4.4	4.7	4.5	5.0	4.5	4.6	7.4	4.7	7.5	7.4	5.5	7.5	4.7	7.4	7.7	
35L-4	7.4	7.2	7.2	7.1	7.5	5.3	7.6	4.4	4.4	4.4	4.7	4.6	5.0	4.6	4.6	7.5	4.8	7.6	7.5	5.2	7.5	4.9	7.4	7.7	
39L-4	7.4	7.2	7.2	7.1	7.5	5.3	7.6	4.4	4.4	4.4	4.7	4.6	5.0	4.6	4.6	7.5	4.8	7.6	7.5	5.2	7.5	4.9	7.4	7.7	
16L-1	7.4	7.2	7.1	7.0	7.5	7.6	7.6	4.5	4.4	4.5	4.7	4.7	5.1	4.5	4.5	4.7	4.5	7.5	6.0	5.3	7.4	4.7	7.3	7.6	
35L-8	6.0	7.0	7.0	6.8	5.9	5.7	7.3	4.3	5.0	5.0	6.1	6.0	5.6	5.0	5.2	7.3	7.0	7.3	7.4	5.4	5.8	5.1	7.0	7.3	Streptococcus sp.

* Tryptone, 2%; K_2HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.01%; and substrate, 1%.

positive, facultative anaerobic coccus, producing the same type growth on agar as streptococci, growing along the entire stab in an agar butt, and resembling in some respects the Pediococcus strains isolated from the cecal feces of healthy turkeys (Harrison and Hansen, 1950a). Of interest is the fact that it is the only coccus isolated that fails to ferment sucrose, and in this respect is like the pediococci from feces. Like fecal strain 4-27, it can grow at an initial pH of 5.4. Whether growth occurs in salt depends upon the size of the inocula; a very small inoculum, a single loop of broth culture for example, fails to produce turbidity in trypticase broth containing 6½ per cent sodium chloride, whereas 0.1 ml allows growth to develop.

The second culture is also an unidentified coccus which in some respects resembles Pediococcus. It is a facultative anaerobe also, but is catalase-negative; it appears as small cocci mostly in pairs with adjacent sides somewhat flattened and is the most delicate strain of cocci encountered in this study, often losing its viability when stored for long in the refrigerator. It is felt that this organism is sensitive to some product or condition arising as a result of its growth, perhaps acidity, as is illustrated by the following simple experiment. After incubation at 37 C in tryptone glucose broth (0.5 per cent glucose) an attempt was made to subculture the coccus from this broth to yeast extract glucose agar

slants (0.25 per cent glucose). When one loopful from the 18 hour broth tube was inoculated onto the surface and stabbed into the butt of the yeast extract agar subsequent incubation revealed the development of only one colony on the slant and little growth in the stabbed portion of the tube. The transference of a number of loopfuls of the broth culture after 43 hours incubation, however, resulted in no growth at all on the agar. Normally, that is when transferred serially from slant to slant, 39L-12 grows well on the yeast extract glucose medium.

Streptococci were isolated from ten blackhead livers, Streptococcus faecalis from five, Streptococcus inulinaceus from four, Streptococcus liquefaciens from one, and an unidentified streptococcus from one liver sample. Representatives of S. faecalis from two of the livers were not lyophilized and therefore the strains listed in tables 9 and 10 are from only three of the five livers yielding this species (16L, 35L, and 39L). As has been already pointed out, the cultures of S. liquefaciens from liver sample 5L were not lyophilized and therefore also are not included in the tables; however, many of the reactions of one strain, 5L-1, have already been outlined (page 70). All four of the livers yielding S. inulinaceus are represented, 43L, 49L, 57L, and 62L, and a few words concerning this coccus may be of interest.

The "viridans" group of streptococci are characterized by Sherman (1937) as growing at 45 C but not 10 C, as being not beta-hemolytic, not tolerating 6.5 per cent sodium chloride,

0.1 per cent methylene blue, or a pH of 9.6, and being incapable of strong reduction, that is reduce litmus only after curdling milk. These are the criteria employed for the identification of this group of streptococci in Bergey's manual (Breed et al., 1948) as well. A member of this group is Streptococcus bovis to which Streptococcus inulinaceus is closely related. Orla-Jensen (1919) characterized and named both these organisms. He differentiated between the two on the basis of arabinose and starch fermentation, as well as on the basis of their behavior on casein. Streptococcus bovis attacks these substances, whereas the latter species does not. Orla-Jensen admitted that intermediates occur which are difficult to place with finality, and in this connection, Sherman (1937) implies, and Bergey (Breed et al., 1948) states that inulinaceus should be regarded as a variety of the former species. While not wishing to enter this discussion, we have labeled our strains Streptococcus inulinaceus since none ferment arabinose and only one attacks starch. The bovis-inulinaceus type differs from the other members of the "viridans" group, S. salivarus and S. mitis, by possessing a greater thermal resistance (surviving heating at 61 C for 30 minutes) and somewhat wider fermentative ability, and from S. equinus, a species we have encountered in the feces of healthy turkeys (Harrison and Hansen, 1950a), also by the wider fermentative range; this latter species, for example, does not ferment lactose.

Two properties of our S. inulinaceus cultures which are

unusual are the strong reduction of litmus in milk *prior* to the curdling of the casein; reduction usually taking place within 24 hours (the starch fermenter, 49L-6, is the weakest acid former in this substrate, requiring three days for curdling), and their tolerance of about 0.1 per cent methylene blue, reducing it in 24 hours or less. To my knowledge, these characteristics have not been reported in the literature, and are atypical of the "viridans" group as a whole. These aspects belie a kindredship with the "enterococci" which further complicate the systematics of the *bovis-inulinaceus* type. In this connection, I have recently become acquainted with an article by Shattock and Mattick (1943) in which these authors call attention to the reaction of three *S. bovis* strains with both *S. faecalis* and group D sera. These strains, which were isolated from human feces, "grew at 45 C, fermented raffinose but not mannitol, and did not grow at pH 9.6 or in 6.5 per cent salt". These workers, therefore, experienced no difficulty in distinguishing between *bovis* and *faecalis* on biochemical grounds, and concluded that the discussion of the place of the former species "should await the preparation of a suitable specific serum".

Three of the *inulinaceus* cultures were handled in a manner identical to the lactobacilli (see pages 42 to 50) in order to determine what end products were formed as a result of glucose fermentation. Culture 62L-2 fermented 38 per cent, 57L-1 fermented 50 per cent, and 43L-8 fermented 51 per cent of the

available glucose, a five per cent solution of this monosaccharide being employed. Culture 62L-2 produced dextro-lactic acid in theoretical yield, all the fermented glucose being converted to this end-product, while 43L-8 fermented 90 per cent to dextro-lactic acid with about 1 per cent of the fermented glucose converted to volatile acid. Culture 57L-1 produced a small amount of volatile acid, 7 per cent of the fermented glucose being converted to volatile acid calculated as acetic acid; 81 per cent of the glucose ended up as dextro-lactic acid in this instance.

On agar media S. inulinaceus may appear as a rod; therefore, unless noted in broth, may be mistaken for a Lactobacillus.

Streptococcus faecalis is represented by nine strains (tables 9 and 10), and six fermentative types may be distinguished. Cultures 39L-3 and 39L-6 are identical in regard to the substances they attack, and differ from 39L-2 only by fermenting melibiose. This latter culture is similar to the Streptococcus glycerinaceus of Orla-Jensen (1919, 1943) since it attacks glycerol, rhamnose, sorbitol, mannitol, and melezitose, but not melibiose. However, it has been included with the other strains of faecalis, as was done in the instance of culture 29-24 from feces (Harrison and Hansen, 1950a). This hepatic streptococcus differs from the others (table 9) also by the fact that it reduces the litmus in litmus milk only after curdling this substrate.

Cultures 35L-3 and 35L-12 ferment the same substances and differ from 35L-11 only in regard to the fermentation of melibiose (table 10). Also alike in fermentation reactions are cultures 35L-4 and 39L-4 which attack fewer substances than the aforesaid strains, and present an identical fermentation pattern as five cultures of faecalis isolated from the feces of healthy turkeys (Harrison and Hansen, 1950a). Culture 16L-1 differs from the others by not attacking mannitol (table 10).

The first three strains of S. faecalis listed in table 9 are somewhat atypical in that they do not grow in the presence of 0.1 per cent methylene blue; in fact, 39L-6 also will not develop in the presence of 6.5 per cent sodium chloride, and 39L-3 does so only feebly. In other respects, however, they behave as they should, presenting the proper fermentation pattern, growing well at 10 C, 45 C, and at a pH of 9.6, and surviving when heated to 61 C for 30 minutes. Two strains of faecalis were manipulated as outlined on pages 29 and 30, and were found to be non-pathogenic for poults.

Of particular interest is culture 35L-8, whose growth at 10 C and lack of same at 45 C might at first suggest a placement with the "lactic" group of streptococci, S. lactis and S. cremoris, but the fermentation pattern of this strain is far from typical of either species. For example, the fermentation of sorbitol, and of greater importance, the lack of lactose fermentation, set this gram-positive coccus apart from the "lactic" streptococci. Since

lactose is not attacked, no reduction of 0.1 per cent methylene blue in milk takes place; however, if glucose be added to this substrate then good growth and strong reduction of the dye will occur. Growth in 0.1 per cent methylene blue, as well as the temperature relationships mentioned above, are characteristics of the "lactic" group and again tempt one to place 35L-8 here, especially since Orla-Jensen and Hansen (1932) have described lactose-negative strains of S. cremoris and Yawger and Sherman (1937) have described lactose-negative strains of S. lactis. Two of the four cultures isolated by the latter investigators attacked mannitol, glucose, fructose, sucrose, maltose, and salicin, but did not attack glycerol, xylose, arabinose, lactose, raffinose, or inulin. No reference was made to sorbitol in their paper. It is apparent that the only discrepancy is the weak fermentation of glycerol by 35L-8; however, Orla-Jensen's (1919) strain #1 of this species produces a slight acidity in this substance, as well as starch, which is not attacked by this species according to Bergey's manual (Breed et al., 1948). It should be pointed out that 35L-8 forms relatively low acidity in the carbohydrates it ferments.

A very weak beta-hemolysis, or what is frequently termed an alpha-prime reaction, in horse blood agar pour plates is produced, but the coccus is not pathogenic for poults when injected intravenously. This facultative anaerobe has greater tendency toward chain formation than the "enterococci", but is not a long-chain streptococcus; in trypticase glucose broth,

for instance, it occurs as diplococci and in chains averaging six to ten units (figure 7D). The unusual colonial morphology has already been discussed (page 31). Although certain fundamental characters seem to indicate a possible kindredship with the "lactic" streptococci, the slight fermentation of sorbitol and lack of lactose fermentation will make it necessary to employ serological tests before this organism may be identified with certainty.

3. Gram-positive rods. Gram-positive, rod-shaped bacteria were isolated from the following eight livers: 18L, 19L, 37L, 39L, 43L, 55L, 69L, and 71L. These bacteria have been identified as Lactobacillus in all but one instance, 19L, in which case a Clostridium was involved.

The Clostridium from 19L occurred together with two gram-negative species, Escherichia coli and Salmonella newport. This obligate anaerobe was nonmotile, and the sugars it fermented were attacked with the production of acid and considerable gas. It measured about 0.8 μ X 3 to 5 μ, and formed no chains. The rectangular spores were located centrally and were of the same diameter as the cell; therefore, no swelling of the sporangium was noticeable. Employing the key in Bergey's manual (Breed et al., 1948) this bacterium most closely fits the description of Clostridium perfringens. Unfortunately, the culture was lost, and therefore is not available for further study or pathogenicity tests.

Although members of the genus Lactobacillus were one of the less dominant bacterial types encountered in blackhead livers, they were studied in considerable detail for several reasons. In the first place, since members of this genus are numerically the most important bacteria in the cecal feces of healthy turkeys (Harrison and Hansen, 1950a, 1950c), I was anxious to determine which of the fecal types were occurring in the livers. Secondly, reports in the literature dealing with the occurrence of lactobacilli in diseased tissues are extremely rare.

Aside from the possibility that certain Lactobacillus may be associated with dental caries, members of this genus have seldom been implicated as pathogenic agents. In his review concerning the parasitic lactobacilli, Rosebury (1944) mentions some cases where the injection of large doses of these bacteria produced sterile lesions in humans and rabbits, but these instances are not conclusive. However, Marschall (1938) found Döderlein's bacillus (Bacillus vaginalis, possibly a type of Lactobacillus acidophilus) in the blood and organs of a case of generalized sepsis with ulcerative endocarditis; he used cultural and serological tests for the identification of the organism.

More recently, Biocca and Seppilli (1947) isolated lactobacillus-like organisms from the blood of two fatal cases of generalized sepsis. Of particular interest is the fact that both patients had recent histories of dental infections.

Biocca and Seppilli concluded that their two strains were members of the genus Lactobacillus, although one strain was weakly catalase-positive and the other not unlike Streptococcus. These workers went to considerable pains to identify the acids formed by their strains, but the figures mean little to the reader, since unfortunately the most fundamental calculation, how much sugar was fermented, is omitted. These Brazilian workers erroneously labeled their organisms "Lactobacillus acidophilus-pathogenes"; there appears to be little similarity between their organisms and L. acidophilus however.

Johnson and Pollard (1936, 1940) isolated an organism which they named Lactobacillus meleagridis from the heart, liver, and yolk of a newly-hatched turkey. From their description there is nothing which indicates that the organism is not a Lactobacillus, but on the other hand, there is insufficient evidence presented to allow definite identification of their "diplo-bacillus", certainly not enough to warrant the establishment of a new species designation. It should be mentioned that although their strain was weakly motile, it may still be considered a Lactobacillus since motile strains of Lactobacillus brevis (Lactobacillus pentoaceticus) have been observed (Weinstein and Rettger, 1932) and also a motile strain of Lactobacillus plantarum (Harrison and Hansen, 1950b).

Before discussing the taxonomy of the lactobacilli cultures, it might be desirable first to describe a little of their history; that is, mention from what medium and dilution

they were picked, and what other bacteria occurred along with them in the livers. Accordingly, each liver sample yielding lactobacilli will be listed in chronological order.

The first instance of encountering lactobacilli from blackhead lesions occurred in June 1948 from liver sample 18L. Lactobacilli together with anaerobic cocci (page 71) were isolated from anaerobic Bugonagar plates of the 10^{-3} dilution. Escherichia coli occurred on the same plates, but in lesser numbers. After some preliminary investigations, representative cultures (18L-2 and 18L-6) were selected and set aside for further study by rubbing each from the surface of Bugonagar slants into fresh, sterile skim milk and lyophilizing the resulting bacterial suspensions. The lyophilization ampoules were sealed under a pressure of approximately 0.1 mm of mercury.

Not until September 1949 were lactobacilli again encountered. They were picked from the anaerobic Bugonagar plates of the 10^{-1} dilution (cultures 37L-10, 37L-11, 37L-13, 37L-14, and 37L-15) and from the 10^{-2} dilution (cultures 37L-3, 37L-4, 37L-6, 37L-7 and 37L-8) of blackhead liver sample 37L. Escherichia coli was encountered from the 10^{-1} dilution E.M.B. plates only, and therefore was outnumbered by the lactobacilli. In mid-October 1949 these ten representatives were also lyophilized.

Lactobacilli and cocci were encountered from a third liver in October 1949. The cocci were isolated from both

the E.M.B. and Eugonagar plates, but the lactobacilli were encountered on the latter medium only. The lactobacilli occurred in slightly greater numbers on the anaerobic Eugonagar plates of the 10^{-2} dilution, lactobacilli cultures 39L-14, 39L-15, and 39L-16 being isolated. This liver sample (39L) was one of the few from which were prepared aerobic as well as anaerobic Eugonagar pour plates. Three additional cultures, 39L-7, 39L-9, and 39L-10, were selected as representatives of the lactobacilli from the 10^{-1} dilution aerobic plates. These six strains were lyophilized at the same time as the ten cultures from the preceding liver.

Of the lactobacilli isolated from liver sample 43L toward the end of January 1950, one (43L-2) was kept for further study. This culture was picked from an anaerobic Eugonagar plate of the 10^{-3} dilution. Escherichia coli and streptococci were isolated in about equal numbers from the next lower dilution (10^{-2}) of this medium. Culture 43L-2 was lyophilized in the Spring of the same year.

Liver sample 55L yielded only lactobacilli, and these occurred in small numbers, since all anaerobic Eugonagar plates above 10^{-1} appeared sterile. In March 1950 one culture, 55L-5, was selected for additional study and lyophilized during the following month.

Liver sample 69L rendered Escherichia coli and lactobacilli in about equal numbers from the 10^{-2} dilution of the anaerobic Eugonagar plates. Culture 69L-3 was isolated from this sample

in June 1950, and after a few transfers lyophilized on the first of August.

The anaerobic lactobacilli isolated in the month of July 1950 from the Eugonagar plates of the 10^{-1} dilution from sample 71L were unfortunately lost prior to lyophilization and therefore are not included in this study. This was the last liver which yielded members of the genus Lactobacillus.

Three surface colonies appeared on the anaerobic Eugonagar plates prepared from liver sample 46L in February 1950. These, however, did not occur in sequential dilutions and were undoubtly contaminants; hence this liver sample has been included among those considered bacteria-free. Two of the cultures turned out to be aerobic micrococci which being of no interest were discarded; the other colony, however, gave rise to an anaerobic, rod-shaped bacterium which, although considered an air-borne contaminant, was kept and lyophilized (Spring, 1950). One usually does not consider anaerobic lactobacilli as air-borne contaminants, and therefore I was desirous to determine what type of lactobacillus could be picked up in this manner. Since the laboratory was used for diagnostic work on chickens and other poultry, the birds often being brought into the laboratory alive with the resulting stirring-up of feathers and dust, it seems possible that this bacterium may be of avian fecal origin. At any rate, the keeping of this culture (46L-6) proved to be worth-while, since as will

be seen, it is a representative of a rarer lactobacillus type; only two strains similar to it were isolated from the blackhead livers (tables 11 and 13). This culture is numbered 46L-6, indicating it to be the progeny arising from the sixth colony selected, when, as has been mentioned, only three colonies appeared on the plates from liver sample 46L. Three small particles of liver on the blood agar streak plate were mistaken for growth, transferred to Eugonagar slants, and labeled 46L-1 through 46L-3. The three actual colonies were the last transferred, and thus labeled in sequence.

Upon the completion of the liver sampling, the 22 lyophilized cultures, were, toward the end of January 1951, revived, and the next phase of study begun. The entire contents of each tube of lyophilized culture were shaken into a test tube of sterile trypticase soy glucose broth (B.B.L., dehydrated) and incubated at 37 C for two days under anaerobic conditions. From these broth tubes Eugonagar slants were inoculated and these incubated for one day under the same conditions. At the end of the incubation period, growth from each of the slants was streaked onto Eugonagar plates in order to "purify" each by picking off a single colony and transferring this to a fresh Eugonagar tube from which the stock culture on yeast extract glucose agar was prepared.

While the colonies of eighteen cultures on the streak plates appeared uniform, in three cases (37L-10, 37L-11, and 39L-16) two distinct colony types were observed, and in one

instance (37L-8) three types of colonies were distinguished. Unfortunately, no count was made of the different colony types and therefore an idea of their relative numbers is not at hand. However, it is felt that they did not arise from chance contamination for two reasons: firstly, being incubated anaerobically, the plates were unlikely to develop air-borne contaminants, and secondly, the different colony types occurred on the plates in considerable numbers. Admittedly, there was a great temptation to shrug off the entire matter as having little significance, thereby forbearing the addition of even more cultures to an already cumbersome collection of lactobacilli and other bacteria.

Culture 37L-10 gave rise to a large whitish colony, which has been labeled 37L-10a, and a smaller bluish colony, labeled 37L-10b. Culture 37L-11 produced a bluish colony, labeled 37L-11a, and a white, more opaque colony, labeled 37L-11b. In a like manner, 39L-16 yielded a white colony, labeled 39L-16a, and a bluish, more translucent colony, labeled 39L-16b. The three colony types developing from 37L-8 were a large bluish type, designated 37L-8a, a small, discrete, opaque type, designated 37L-8b, and a smaller bluish type, designated 37L-8c. (When reference is made to a large colony, it merely means the type in question was larger than the other types on the plate; all colonies were rather small and of a type characteristic of the lactobacilli.)

Transplanting and carrying as individual strains the

three colony types arising from one of the revived cultures (37L-8) and the two colony types arising from each of three other revived cultures (37L-10, 37L-11, and 39L-16) resulted in replacing the four parent cultures by nine offspring, thereby bringing to a total of twenty-seven, the collection of lactobacilli to be investigated.

As will become apparent, these 27 cultures vary from one another in morphology and certain physiological and biochemical characters, but they all have the general characteristics of the genus Lactobacillus; that is, they are gram-positive, non-motile, catalase-negative, nonsporeforming, nitrate non-reducing, rod-shaped bacteria which produce acid from a number of carbohydrates.

The fermentation reactions of the 27 strains of lactobacilli are listed in table 11. The basal medium employed was the yeast extract broth described in the "Materials and Methods" section of this dissertation, and the 22 substrates were added to form a concentration of 2 per cent. The final pH resulting in the different broths was measured after two weeks' incubation at 37 C employing the glass electrode. All cultures were incubated in the presence of the atmosphere except those marked with an asterisk which were incubated anaerobically.

The first 16 cultures tabulated (37L-3 through 46L-6) have been identified as strains of Lactobacillus fermenti. A hint of their heterofermentative nature is apparent from

the fact that they do not ferment salicin, cellobiose, trehalose, or the alcohols. A lack of the fermentation of these substances is characteristic of the heteroformentative lactobacilli (Orla-Jensen, 1919 and 1943; and Tittsler and Rogosa, 1946). The first 13 strains tabulated resemble each other closely; all ferment fructose, glucose, mannose, galactose, sucrose, and maltose; the vast majority ferment lactose and raffinose, whereas they vary in melibiose and only two attack dextrin. It should be noticed that mannose fermentation by some cultures is weak. Orla-Jensen (1919) called attention to the fact that freshly isolated mannose-fermenting strains of this species may lose this property later on. None of the fermenti cultures attack melezitose; this is in agreement with the findings of Orla-Jensen (1943).

Lactobacillus fermenti has a preference for anaerobic conditions, and although most of the first 13 strains listed in table 11 now are able to grow on aerobic agar slants, cultures 37L-8c and 37L-10a still require anaerobic incubation for surface development.

Cultures 37L-10b, 43L-2, and 46L-6 are tabulated together because they appear somewhat different from the aforesaid strains. Although 43L-2 behaves normally in regard to the sugars it ferments, the amount of acid formed is abnormally low, except with sucrose and maltose, in which it produces almost as low pH values as do the 13 strains tabulated above. Culture 37L-10b differs in that it does not ferment maltose,

TABLE 11

Final pH values of broth* cultures of lactobacilli from blackhead livers after 14 days' incubation at 37 C
(pH values representing significant fermentation have been underscored)

Culture number	Glycerol	Xylose	Arabinose	Rhamnose	Sorbitol	Mannitol	Inositol	Fructose	Glucose	Mannose	Galactose	Sucrose	Trehalose	Maltose	Cellobiose	Melibiose	Lactose	Melezitose	Raffinose	Dextrin	Starch	Salicin	Control	Species
Control	7.1	6.1	6.3	6.3	7.0	7.0	7.0	5.7	5.9	6.0	5.6	7.1	7.1	7.0	7.0	6.9	6.7	7.1	7.1	6.6	7.1	7.1	7.1	
Control**	6.7	6.5	6.6	6.5	6.7	6.7	6.7	6.1	6.2	6.2	6.1	6.6	6.6	6.6	6.6	6.6	6.4	6.7	6.7	6.6	6.7	6.6	6.7	
37L-3	7.1	6.0	6.2	6.2	7.1	7.0	7.0	4.2	4.1	4.4	4.6	4.2	7.1	4.1	7.0	6.4	4.2	7.1	4.2	6.5	7.1	7.1	7.1	Lacto-bacillus fermenti
37L-6	6.8	6.0	6.2	6.1	6.9	6.9	6.9	4.3	4.0	5.6	4.0	4.4	7.0	6.3	7.0	6.9	4.0	7.0	4.1	4.7	6.8	7.1	6.9	
37L-7	7.1	6.0	6.2	6.1	7.1	7.0	6.9	4.2	4.2	4.4	4.2	4.2	7.2	4.2	7.0	6.4	4.2	7.1	4.3	6.6	7.0	7.1	7.1	
37L-8c	7.1	6.0	6.2	6.2	7.0	7.0	7.0	4.2	4.2	4.5	4.4	4.2	7.2	4.1	7.0	4.2	6.0	7.1	7.0	6.5	7.1	7.2	7.1	
37L-10a	7.1	6.0	6.2	6.1	7.0	7.0	6.9	4.2	4.2	4.4	4.6	4.2	7.1	4.2	7.0	6.5	4.3	7.1	4.2	6.6	7.1	7.2	7.1	
37L-11a	6.9	5.9	6.1	6.1	6.9	6.9	6.8	4.2	4.0	5.6	4.0	4.2	7.1	4.0	7.0	6.3	4.0	7.0	4.2	6.4	7.0	7.1	7.0	
37L-11b	6.9	6.0	6.1	6.1	6.9	6.9	6.8	4.2	4.0	5.4	4.0	4.2	7.0	4.0	6.9	4.0	4.0	7.0	4.1	6.4	6.9	7.1	7.0	
37L-13	7.1	6.0	6.1	6.2	7.0	6.9	6.9	4.3	4.2	4.5	4.2	4.3	7.0	4.2	7.0	6.5	4.3	7.1	6.5	6.6	7.1	7.1	7.1	
37L-14	7.1	5.9	6.2	6.1	7.0	7.0	7.0	4.3	4.2	4.6	4.2	4.2	7.1	4.1	7.0	4.1	4.1	7.0	4.2	6.6	7.1	7.1	7.1	
37L-15	6.8	5.9	6.1	6.1	6.9	6.9	6.8	4.2	3.8	5.7	4.0	4.2	7.1	4.0	6.8	3.8	4.0	7.0	4.1	6.4	7.0	7.1	6.9	
38L-7	7.0	6.0	6.1	6.1	7.0	6.9	6.9	4.2	4.2	5.7	4.1	4.5	7.0	4.2	7.0	4.1	4.1	7.0	4.2	4.8	6.7	7.1	7.0	
55L-5	7.0	5.9	6.1	6.1	6.8	7.0	6.9	4.2	4.1	5.7	4.1	4.3	7.1	4.0	7.0	4.0	4.1	7.1	4.2	6.4	7.0	7.1	7.0	
63L-3	6.9	6.0	6.1	6.1	6.9	6.9	6.8	4.3	4.0	5.7	4.1	4.3	7.0	4.0	7.0	4.0	4.1	7.0	4.1	6.4	7.0	7.1	7.0	
37L-10b**	6.8	6.6	6.6	6.5	6.7	6.7	6.7	5.8	5.7	5.4	5.8	5.5	6.8	6.8	6.9	6.5	6.4	6.7	5.6	6.5	6.7	6.8	6.7	
43L-2**	6.8	6.5	6.8	6.5	6.7	6.7	6.7	5.0	5.3	5.4	5.4	4.3	6.8	4.4	6.8	6.5	5.4	6.5	5.4	6.5	6.7	6.8	6.7	
46L-6**	6.8	6.4	6.8	6.5	6.7	6.7	6.7	5.0	5.2	5.3	5.8	5.1	6.8	5.3	6.8	6.5	6.4	6.5	6.4	6.5	6.7	6.8	6.7	
37L-8a	7.0	6.0	6.1	6.1	6.9	6.9	7.0	4.2	4.1	4.6	4.2	4.1	7.1	4.0	6.1	4.0	4.1	7.0	4.1	4.3	7.0	5.4	7.1	Lacto-bacillus acido-philus
37L-8b**	6.6	6.4	6.5	6.3	6.6	6.6	6.6	4.4	4.3	4.5	4.3	4.4	6.7	4.3	4.6	4.5	4.3	6.5	4.3	4.3	4.3	5.1	6.6	
39L-9	6.9	5.9	5.6	6.0	6.8	6.9	6.9	4.7	4.6	4.7	4.7	5.2	6.9	4.9	5.8	6.4	4.8	6.9	4.7	4.9	4.8	5.2	6.9	
39L-10	7.0	5.8	5.9	6.0	6.9	6.9	6.9	4.8	4.8	4.8	4.9	5.1	7.0	5.1	6.0	6.4	5.1	6.9	5.0	4.9	4.8	5.0	7.0	
39L-16a	6.8	6.0	6.0	6.0	6.8	6.9	6.8	4.7	4.7	4.8	4.8	5.0	6.8	5.0	5.6	6.4	4.6	6.9	5.1	4.4	4.7	5.2	7.0	
18L-2	6.1	5.4	5.6	5.8	6.0	6.1	6.0	4.1	4.2	4.2	4.6	4.2	4.8	5.0	6.0	5.8	5.5	6.0	5.7	5.4	6.1	7.2	6.1	Lacto-bacillus bifidus
37L-4	6.1	5.4	5.6	5.8	6.1	6.1	6.0	4.1	4.2	4.2	4.5	4.2	4.8	4.8	6.1	5.8	5.0	6.1	5.2	5.4	6.2	7.2	6.2	
18L-6**	6.7	6.5	6.5	6.3	6.8	6.4	6.5	5.8	5.2	5.8	5.4	5.7	6.7	5.2	5.8	6.4	6.4	6.4	6.4	4.1	4.4	4.7	6.5	
39L-14**	6.7	4.7	4.7	6.3	6.7	6.7	6.7	4.4	4.8	4.6	4.7	4.2	4.6	4.5	4.9	4.6	4.8	5.2	4.5	4.7	6.7	4.7	6.4	
39L-15**	5.4	4.8	4.5	5.2	5.4	5.4	5.4	4.7	4.8	4.9	5.0	4.9	4.8	4.6	5.0	4.4	5.3	4.9	4.5	4.8	5.6	5.0	5.4	
39L-16b**	6.8	4.7	5.0	6.3	6.6	6.5	6.0	4.9	5.3	5.8	5.0	4.8	6.7	4.8	6.6	4.9	4.6	6.4	4.9	4.9	5.4	5.3	6.6	

* Yeast extract, 0.2%; K_2HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.01%; and substrate 2%.

** Incubated anaerobically.

and 46L-6 in that it does not attack sucrose. These latter cultures each lack fermentation of one of the disaccharides 43L-2 attacks to the greatest extent, even in preference to the monosaccharides.

Besides attacking fewer carbohydrates, 37L-10b, 43L-2, and 46L-6 are different in that they appear more fastidious than the other 13 strains. They are among the most anaerobic cultures of fermenti studied; up to the present time no surface growth on agar occurs when they are incubated in the presence of the atmosphere, and even under anaerobic conditions are the weaker acid formers (table 11). It is as if the nitrogen source were deficient in some necessary metabolite. This is borne out by the fact that upon the addition of tomato juice to a yeast extract glucose broth the pH after 24 hours' incubation at 37 C is about 4.8 as opposed to about 6.2 when tomato juice is absent (table 12). Hence, their low acidity in yeast extract without tomato juice cannot be explained on the basis of an abnormally high degree of sensitivity toward acid, but is probably due to a suboptimal diet. The response these cultures show toward tomato juice is indeed striking. When this enrichment is present in a yeast extract glucose basal, growth, as evidenced by turbidity is much faster (table 12).

Another manifestation of the fastidiousness of these three strains is the fact that they ferment less of the

TABLE 12

Growth and pH produced by four cultures of hepatic lactobacilli after 24 hours' aerobic incubation at 37 C in three different media

Culture number	Trypticase-glucose broth*	Yeast extract-glucose broth**	
		Without added tomato juice	With added tomato juice
Control	--- (6.6)	--- (6.4	--- (6.0)
37L-10b	-/- (6.5)	-/- (6.3)	-/- -/- (4.9)
43L-2	-/- (6.6)	-/- (6.1)	-/- -/- (4.7)
46L-6	-/- (6.6)	-/- (6.2)	-/- -/- (4.8)
18L-6	-/- (6.0)	--- (6.1)	--- (5.8)

--- No visible growth (turbidity);

-/- Fair growth (turbidity); -/- -/- Heavy growth (turbidity).

* Trypticase, 2%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.01% and glucose, 2%.

** Yeast extract, 2.2%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.01%; and glucose, 2%. (In the case where tomato juice was added, it existed in a concentration of 20%.)

available glucose than do the other 13 cultures of L. fermenti (table 13). Respectively, only 26, 19, and 17 per cent of the glucose present is attacked, and it must be remembered that the medium employed in these determinations contained tomato juice (see "Materials and Methods"). In short, as compared to the other fermenti cultures, these three strains manifest a weakness of fermentative power. Later on, when discussing another culture (18L-6), we will be confronted with this weakness to an even greater degree. Should the reader wonder why such a point is being made of this progressive loss of fermentative ability, it must be remembered that bacterial types are present in nature which taxonomists have assigned separate generic rank and which resemble the lactobacilli closely except for a dearth of fermentative power, for example, some of the Corynebacteriaceae (Breed et al., 1948).

The only absolute criteria of the heterofermentative nature of a lactobacillus is an evaluation of the quantity of lactic acid formed based upon the amount of carbohydrate fermented. Glucose or lactose are the carbohydrates usually employed. It may be seen in table 13 that these hepatic cultures of fermenti convert less than 45 per cent of the fermented glucose to nonvolatile acid, calculated as lactic acid. The levo and dextro forms of lactic acid are produced in almost equal amounts, there usually being a very slight excess of the dextro isomer formed however. Hence, the zinc lactate prepared from the acid was predominantly inactive.

TABLE 13

Characteristics of lactobacilli from blackhead livers

Culture number	Gas production (37 C)	Growth at - - 20 C	30 C	37 to 41C	43½ C	45½ C	49½ C	Skim milk: Final pH	Skim milk: Per cent acid calculated as lactic acid	Per cent glucose fermented	Per cent fermented glucose converted to - Vol. acid cal. as acetic acid	Nonvol. acid cal. as lactic acid	Optical type of lactic acid produced	Species
37L-3*	G	---	-/-	-/-	---	---	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	
37L-6*	G	---	-/-	-/-	-/-	-/-	---	6.2	0.15	100	2.3	39	I, D	
37L-7	G	---	---	-/-	---	---	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	
37L-8c*'	G	---	---	-/-	---	---	---	6.2	0.13	85	2.0	36	I, D	
37L-10a*'	G	---	-/-	-/-	-/-	---	---	6.4	0.05	58	2.0	38	I, D	
37L-11a*	G	---	-/-	-/-	-/-	-/-	---	5.8	0.13	100	3.6	42	I, D	
37L-11b	G	---	-/-	-/-	-/-	-/-	---	5.6	0.23	70	4.7	41	I, D	
37L-13	G	---	-/-	-/-	---	---	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	Lactobacillus fermenti
37L-14	G	---	-/-	-/-	-/-	---	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	
37L-15	G	---	-/-	-/-	-/-	-/-	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	
39L-7	G	---	-/-	-/-	-/-	-/-	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	
55L-5*	G	---	-/-	-/-	-/-	-/-	---	5.7	0.18	100	3.2	35	I, D	
69L-3*	G	---	-/-	-/-	-/-	-/-	---	5.7	0.18	100	2.8	43	I, D	
37L-10b*'	G	---	-/-	-/-	---	---	---	6.0	0.18	26	7.0	29	I	
43L-2*'	G	---	-/-	-/-	---	---	---	6.2	0.15	19	7.9	36	I, D	
46L-6*'	G	---	---	-/-	---	---	---	5.9	0.18	17	9.2	32	I, D	
37L-8a*	G	---	-/-	-/-	-/-	-/-	---	4.3	1.20	100	3.0	72	I	
37L-8b*'	n.g.	---	---	-/-	-/-	---	---	5.5	0.26	36	2.4	91	I	
39L-9	n.g.	---	-/-	-/-	-/-	-/-	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	Lactobacillus acidophilus
39L-10	n.g.	---	-/-	-/-	-/-	-/-	---	4.3	1.20	32	3.6	94	I	
39L-16a*'	n.g.	---	-/-	-/-	-/-	-/-	---	4.1	1.50	29	2.7	98	I	
18L-2*	n.g.	---	-/-	-/-	-/-	-/-	---	6.5	0.08	100	-0.1	78	I, L	
37L-4*	n.g.	---	-/-	-/-	-/-	-/-	---	6.4	0.08	78	0.4	86	I	
18L-6*'	n.g.	---	---	-/-	---	---	---	6.2	0.08	8.7	8.5	95	I, L	
39L-14	n.g.	---	-/-	-/-	---	---	---	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	Lactobacillus bifidus
39L-15'	n.g.	---	-/-	-/-	---	---	---	6.0	0.18	28	50	35	D	
39L-16b*'	n.g.	---	---	-/-	---	---	---	6.1	0.13	30	46	40	D	

* Tested for pathogenicity & found to be not pathogenic for poults;
G Gas produced; n.g. No gas produced; -/- Growth; --- No growth;

' Incubated anaerobically in flask culture;
n.t. Not tested; I Inactive lactic acid;
D Dextro-lactic acid; L Levo-lactic acid.

In fact, had not the zinc lactate been crystallized in several fractions, thereby utilizing the greater solubility of the active zinc lactate, the slight excess of the active isomer probably would not have been detected.

The heterofermentative nature of the cultures obviously could not have been elucidated by means of the per cent fermented glucose converted to volatile acidity, since in this respect they do not differ appreciably from the homofermentative species, little volatile acid being formed. The fastidious strains (37L-10b, 43L-2, and 46L-6), it will be noted, form somewhat greater amounts of volatile acid, respectively 7.0, 7.9, and 9.2 per cent (calculated as acetic acid). All the cultures produce considerable gas however. Oftentimes the vaseline overlay was pushed completely out of the test tube by the force of the gas generated. This is in contradistinction to the homofermentative cultures (except one) which produce little or no gas under the same conditions. Any remainder of fermented glucose must be converted to non-acidic compounds. (The L. fermenti studied by Jan Smit, 1915, from glucose formed considerable alcohol and some glycerol in addition to carbonic and lactic acids. Very little volatile acid other than carbonic was produced.)

A few observations regarding gas production by the hepatic fermenti strains may well be in order here. More gas was formed at 41 C than at 35 C, whereas gas never appeared at $43\frac{1}{2}$ C or higher, even in the cases of the strains which grew

well at 43½ C, producing as low a pH at this temperature as at 41 C, for example, cultures 37L-11b and 37L-15. (Perhaps the higher temperatures inhibit the enzyme system causing gas production.) It would be very interesting to know to what end product, or products, the portion of the fermented glucose normally going to gas, forms at the higher temperatures. May a heterofermentative species behave as a homofermentative species at the highest temperatures which still allow its development? In this connection, while studying the heterofermentative lactobacilli, Thiel (1940) observed that the ratio of lactic acid produced to the sugar disappearing from the medium is unaffected by temperature or oxygen tension.

The hepatic fermenti strains investigated also required varying times in order to form visible gas beneath the vaseline seal; cultures 37L-6, 37L-11a, 37L-11b, 43L-2, and 69L-3, for instance, pushed the vaseline overlay sometimes completely out of the tube in a day or less at 37 C. Cultures 37L-8a, 37L-10a, 46L-6, and 55L-5 required two days, and cultures 37L-8c three days to produce visible gas under the same conditions.

There is some variance in the temperatures permitting growth (turbidity and lowering of pH) by the fermenti strains. While seven cultures grew at 45½ C, an equal number would not grow much above 41 C. None grew at 20 C even after incubation at that temperature for two weeks. Three cultures (37L-7, 37L-8c, and 46L-6) show a very narrow temperature range permitting growth, not propagating at 30 C or 43½ C. The optimum

temperature for growth of this species from blackhead livers as evidenced by development of turbidity, seems to be near 41 C, which is not surprising, since this is the body (rectal) temperature of the turkey (Marsden and Martin, 1939). The fermenti studied by Smit (1915) and Pederson (1938) were able to grow a few degrees higher than our strains, whereas the Lactobacillus fermenti (Betabacterium longum) of Orla-Jensen (1919) agrees more closely with our strains in this aspect.

Little or no acid is formed in skim milk by the fermenti cultures described here; 37L-11b, the most active strain in this substrate, producing only 0.23 per cent acid (calculated as lactic acid) and a final pH of only 5.6 after incubation at 37 C for two weeks. Orla-Jensen (1919) has pointed out that the heterofermentative lactobacilli, as a rule, grow poorly in milk.

Since Lactobacillus fermenti has been encountered in blackhead livers, this species would be expected to occur normally in the turkey's cecum. This has been shown to be the case, and two representative strains from this organ (from cecal feces) have been described by Harrison and Hansen (1950c). Although the occasional xylose-fermenting strain of fermenti from the cecum has no counterpart in the blackhead livers studied, many hepatic cultures show a fermentation pattern identical to one of the fecal cultures described (29-35).

Lactobacillus fermenti may vary greatly in morphology

from strain to strain, as is apparent in the accompanying photographs. Unless stated otherwise, the photomicrographs were made of crystal violet-stained smears from 24 hour, 37 C, anaerobic, yeast extract glucose slant cultures. They were taken at an initial magnification of 700 and then enlarged to twice the size, resulting in an overall magnification of 1,400 diameters. Four strains of fermenti have been chosen as representatives of the morphological types of this species encountered in blackhead lesions.

Many strains are quite pleomorphic, containing cells of extreme variances in length -- from coccoid elements to filamentous forms, as typified by strain 37L-10a; short chains occur occasionally, and the longer cells are often somewhat curved (figure 4A). Most unusual are the tiny "buds" attached to the shorter rods. Other strains, typified by 37L-10b, although still manifesting quite a variability in cell length, are not as pleomorphic. Only in rare instances does a cell show branching, as may be observed in the case of the terminal cell of the centrally-located chain in figure 4B. However, tomato juice broth cultures of this same organism manifest a great pleomorphism, with rods of varying lengths, even coccoid elements, and especially, an extreme bending by some cells. At second glance it is apparent that many of the coccoid forms are merely tightly curled rods (figure 4D). (Other lactobacillus types, for instance some strains of plantarum, respond to tomato juice by also bending or curling to a great

degree.) It is worthy of note that the more bizarre forms from tomato juice broth are readily decolorized with ethyl alcohol when gram-stained, whereas the more uniform rods retain the crystal violet.

Other representatives of this species present a more conventional appearance. Strains 35L-5 and 69L-3 (figures 5C and 5D) are, for the most part, short, plump, uniform rods; culture 43L-2, on the other hand, consists of quite small discrete rods (figure 4C).

That the oxygen tension sometimes affects the cell shape is apparent when one compares figure 5A with figure 5C. Both photographs are of the same strain (55L-5) under identical conditions, except in the former figure the smear was prepared from an aerobic, rather than an anaerobic slant. There is a greater tendency toward the filamentous cellular form also with aerobic cultures of 69L-3 (figures 5B and 5D) and 37L-11b. Aerobic cultures of fermenti are more likely to develop somewhat swollen rods, as is the case with strains 37L-6 and 43L-2; swollen cells are seldom seen when the culture is grown anaerobically. In this connection, van Niel (1928) observed a great degree of pleomorphism by his Propionibacterium strains when cultivated on solid media aerobically, whereas the cells appeared more uniform under anaerobic conditions.

The hepatic fermenti were decidedly more anaerobic when first isolated. For example, of the lactobacillus cultures from liver sample 37L, strains 37L-7, 37L-15, and 37L-10 were

still obligately anaerobic after four or five transfers; the others from this liver had become capable of aerobic growth by this time however. Strains 55L-5 and 69L-3 were anaerobic when isolated, later adapting to aerobic conditions, whereas 39L-7 was capable of aerobic growth from the start, having been isolated from an aerobic Bugonagar plate. On the other hand, even at the time of this writing, 37L-8c, 37L-10a, 37L-10b, 43L-2, and 46L-6 produce no surface growth on agar unless cultivated under a reduced oxygen tension. It must be pointed out however, that anaerobic bacteria were stored on the yeast extract glucose agar slants in anaerobic jars in the ice box, being transferred to fresh anaerobic slants every two weeks. Had these lactobacilli been carried in deep butts rather than anaerobic jars, with the subsequent possibility of some air seeping to the organisms, perhaps a tolerance toward air would have occurred more quickly. As it was, the only time the cultures were exposed to the atmosphere was at the time of transfer.

Worthy of note is the fact that in the thin veil-like growth of strains 37L-11a and 37L-11b (observed in July 1951) more prolific daughter colonies developed; these daughter colonies did not occur on the parallel anaerobic slants, but only on the slants incubated in the presence of the atmosphere. I concluded that these outgrowths occurred as a result of a selective development of the more air-tolerant individuals in the two cultures. This observation indicates that even an

already somewhat air-tolerant strain may adjust itself in some way so that heavier aerobic growth results.

Listed next in table 11 are five cultures (37L-8a through 39L-16a) of Lactobacillus acidophilus isolated from two blackhead livers. I am using the definition of this species provided by Orla-Jensen et al. (1936) and recognized by Tittsler and Kogosa (1948). The cultures ferment the monosaccharides, and sucrose, maltose, lactose, raffinose, dextrin, and salicin. Two attack melibiose, four starch, and three cellobiose, one strongly, the others only weakly. Salicin fermentation reflects their homofermentative nature, and sets them apart from the cultures of fermenti tabulated directly above. The fermentation pattern presented by these hepatic acidophilus cultures, although agreeing in the main, does not fit exactly the fermentation pattern exhibited by fecal strains of this species from turkeys, but as has been already pointed out (Harrison and Hansen, 1950c) there is a great deal of variance among members of the acidophilus group in regard to their fermentation of the di-, tri-, and polysaccharides. The first five acidophilus strains tabulated are able to attack esculin; the activity of the other strains of this species has not been determined. The splitting of the glycoside was detected by means of the color test employing ferric citrate reagent rather than by the measurement of the final pH.

The homofermentative nature of the acidophilus cultures is apparent from the fact that more than 70 per cent of the

fermented glucose is converted to nonvolatile acid (calculated as lactic acid). It is interesting that the strain which produces the least amount of nonvolatile acid (37L-8a) forms some gas in addition (table 13). The other 3 cultures of acidophilus form no detectable gas, and convert almost every bit of the fermented glucose to nonvolatile acid, respectively 91, 94, and 98 per cent. The lactic acid formed is inactive. Very little of the fermented glucose is converted to volatile acidity (calculated as acetic acid), less than 4 per cent. Culture 37L-8b differs from the other four in being unable to produce sufficient acid in milk to cause curdling, and in showing a narrower temperature range permitting growth. It does not develop at 30 C or 45½ C, while the other four cultures do, although sometimes only poorly at the higher temperatures (table 13).

Cultures 18L-2, 37L-4, and 18L-6, also strains of L. acidophilus, which are unusual in some respects have been listed apart from the five strains tabulated directly above. Little or no growth occurs in skim milk (table 13); the first two bring about a very slight fermentation of xylose, arabinose, and rhamnose; whereas 18L-6 produces only little acid in the substrates it attacks, being particular weak in the di-, and trisaccharides (table 11). While a lack of raffinose fermentation is not unusual, only two strains investigated by Orla-Jensen et al., (1936) were unable to attack lactose.

Culture 18L-6 shows only displeasure toward the various

fare that have been offered it; even tomato juice broth does not fulfill its requirements, since only 8.7 per cent of the available glucose is attacked (table 13), and in this medium no appreciable turbidity or lowering of pH is discernable after 24 hours' incubation (table 12), although some turbidity does appear after incubation for several days. Since Bugonagar (a medium containing trypticase as a nitrogen source) allows good growth of this organism, 18L-6 was inoculated into a trypticase broth; with this nitrogen source growth is best, although admittedly still only weak (table 12). The fastidiousness of this strain is again apparent from the fact that it has the narrowest temperature range permitting growth, developing at neither 30 C nor 43½ C. This strain is related to the other acidophilus cultures in much the same manner as the three more fastidious fermenti strains (37L-10b, 43L-2, and 46L-6) are related to the others of this latter species.

The two acidophilus strains from liver 18L produce measureable amounts of levo-lactic acid in excess of the dextro form, and in this respect differ somewhat from the others, but are not atypical of the species in this regard since L. acidophilus may produce either pure inactive lactic acid or predominantly inactive acid with a slight excess of the levo isomer (Orla-Jonsen et al., 1936).

The flask containing culture 18L-2 yielded less volatile acid than the control flask, and since my analysis was at first

suspected to be at fault the experiment was repeated, but with the same results; volatile acid appears to be used up rather than generated, although admittedly only to a small degree (table 13). The other strain from this liver, 18L-6, also is somewhat unusual due to the fact that the acidity resulting from glucose fermentation totals to somewhat over 100 per cent. These phenomena may be expected if 18L-2 and 18L-6 are able to utilize carbon dioxide.

Of the hepatic acidophilus cultures investigated, 37L-8b most closely resembles the strains of this species we isolated from the cecal feces of healthy turkeys (Harrison and Hansen, 1950c).

Lactobacillus acidophilus, like the hepatic fermenti, also demonstrate morphological differences. For instance, when cultivated aerobically, cultures 18L-2 (figure 6A) and 37L-4 (figure 6B) appear as quite small, uniform rods varying somewhat in length, and occurring sometimes in short chains, whereas anaerobic incubation causes them to present a more coccoid appearance (figures 6C and 6D). On the other hand, strains 37L-8a, 37L-8b, and 39L-16a present the same morphological appearance whether cultivated aerobically or anaerobically. In addition, the latter three strains are larger and tend to form a greater number of filamentous cells, this latter trait being especially characteristic of 39L-16a (figure 8C).

For some time it has been known that the lactobacilli may vary morphologically on different substrates (Orla-Jensen, 1919).

The following observation, therefore, is not unique: culture 18L-6 consists of rather long rods when grown on anaerobic yeast extract-glucose agar (figure 7A), while on anaerobic Eugonagar, the preferred medium, the cells are tiny (figure 7C).

The acidophilus strains, like the fermenti, vary in regard to their sensitivity toward oxygen, especially when freshly isolated. Whereas 18L-2 grew on aerobic slants when first isolated, 18L-6 required anaerobic incubation for the first few transfers. In a like manner, strains 37L-4, 39L-10, and 39L-16a were aerobic from the beginning, while 37L-8a and 37L-8b remained anaerobic for a considerable period of time. All the hepatic acidophilus cultures now produce at least some aerobic growth when streaked onto the surface of agar slants.

Lactobacillus bifidus was encountered in one liver sample, and three strains of this heterofermentative organism (39L-14, 39L-15, and 39L-16b) are listed in table 11. The fermentation pattern of these cultures is not unlike that of L. acidophilus, except for one important difference: the strong fermentation of xylose and arabinose. This is one of the features pointed out by Orla-Jensen et al. (1936) and used by him to differentiate the two. Our cultures of this species, like acidophilus, and for that matter, fermenti, do not attack the alcohols; besides xylose and arabinose, they ferment the monosaccharides, although mannose-fermentation is lacking in one instance (39L-16b), and vary in trehalose, cellobiose, melezitose, and

starch. Orla-Jensen et al. (1936) observed the variable behavior in starch, and also called attention to the weak or lacking fermentation of mannose, which is so characteristic of this lactobacillus type, a similarity with fermenti and dissimilarity to acidophilus. None of the strains of bifidus studied by Orla-Jensen (1943) attacked trehalose, whereas two of my strains do so. Like my hepatic acidophilus, the bifidus cultures tested split esculin. Anna B. Orla-Jensen (1934) observed that Lactobacillus acidophilus (Thermobacterium intestinale) split this glucoside, but she did not test bifidus in this substance.

L. bifidus usually curdles milk, although my strains produce only slight acidity in this substrate. The strains here investigated show a narrower and somewhat lower temperature range permitting growth than do the acidophilus cultures, but too few strains have been studied to draw any definite conclusions as to whether this observation is merely coincidental or represents an actual difference between hepatic strains of acidophilus and bifidus.

The fundamental difference between acidophilus and bifidus, a difference so profound in nature as to have lead some investigators (Orla-Jensen et al., 1936) to assign these two organisms separate generic rank, concerns the manner in which fermentable carbohydrates are utilized. As has been pointed out, L. acidophilus forms mainly a single product, lactic acid, as a result of glucose breakdown; this organism is therefore

termed homofermentative. In juxtaposition, L. bifidus is called heterofermentative, since glucose breakdown by this organism leads to considerable amounts of more than one end product, namely, lactic and acetic acids, and sometimes small amounts of propionic acid. We have had no difficulty in differentiating acidophilus from bifidus on this basis, whether isolated from feces or blackhead livers. The bifidus cultures studied here, as well as those encountered from feces (Harrison and Hansen, 1950a), convert approximately half of the fermented glucose to volatile acid (calculated as acetic acid), convert the majority of the remaining fermented glucose to dextro-lactic acid (table 13), and are therefore phenotypic of the species as defined by Orla-Jensen et al. (1936). This is opposed to the situation as it concerns acidophilus, in which no appreciable amount of volatile acid is formed, and in which almost all the fermented glucose is converted to an equal mixture of dextro- and levo-lactic acids, or a mixture in which the levo isomer occurs in a slight excess.

Superimposed over the stable characters just mentioned (certain fermentation reactions and manner of glucose breakdown), there occur certain variable characteristics upon which some workers, unfortunately, have placed undue taxonomical importance. Heading the list of these variable traits are morphology and oxygen tension preference. L. bifidus is more anaerobic than acidophilus, and when freshly isolated from feces or blackhead livers remains an obligate anaerobe for

some time, before its continual cultivation on artificial media may render it finally capable of aerobic growth. At the same time, the characteristic pleomorphic, club-shaped and branching cell-forms usually observed in the freshly isolated state may give way to the monomorphic straight rods of the acidophilus-type after repeated transfers in the laboratory. These changes are very striking, and together with other observations, lead Rettger and co-workers (Weiss and Rettger, 1934; and Rettger et al., 1935) to conclude that bifidus and acidophilus were one and the same species. Prior to 1936, the techniques so successfully employed by Orla-Jensen for differentiating bacteria of this group were unknown to the American workers, in fact, even the monograph which appeared in the Proceedings of the Royal Academy of Science of Denmark (Orla-Jensen, 1919). Therefore in the 1930's much confusion existed in the literature regarding the taxonomy of these two lactobacilli types.

Lactobacillus bifidus strain 39L-15 has shown good bifid morphology on anaerobic yeast extract-glucose agar ever since being isolated two years ago, although it must be admitted, for about 14 months of that time the culture was in the lyophilized state. (Figure 7B was prepared from this culture at the time of this writing, October 1951.) Strain 39L-14 was isolated along with the above culture, and presented the same morphological appearance, but has not been viewed recently. Somewhat different is the case of 39L-16b; when observed in February 1951

this organism appeared very filamentous, like an actinomycete, no bifid forms being discernable (figure 8B), whereas now, eight months later, it appears similar to the two strains mentioned above, although it shows a greater tendency toward chain formation (figure 8D).

Up to the present time, all three strains grow on the surface of yeast extract glucose slants only when incubated under anaerobic conditions.

In the appendix to this thesis, a number of bifidus strains from the cecal feces of healthy turkeys are discussed, one of which may now be cultivated on agar slants in the presence of the atmosphere and which cannot be differentiated from acidophilus morphologically. The fecal strains are compared to the hepatic strains of bifidus.

Now that all the hepatic lactobacilli have been characterized and assigned an appropriate name, some interesting relationships existing between certain strains may be discussed with greater implicitness. The reader may recollect that earlier in the discussion of the lactobacilli (page 90), it was pointed out that a number of the cultures when revived from the lyophilized state, and eventually streaked out on agar plates, were observed to yield more than one type of colony.

For example, culture 39L-16, which when studied superficially before lyophilization was observed to be an anaerobe and appeared to be composed of many club-shaped and branching

cells, so that a likeness to diphtheroids and L. bifidus was noted, when reconstituted from the lyophilization ampoule, transferred several times anaerobically on Eugonagar, and eventually streaked out on this medium, was observed to form an abundance of two colony types: some were white -- one of these was selected and labeled 39L-16a, while others were bluish and more translucent -- one of which was picked from the plate and labeled 39L-16b. The former, strain 39L-16a, was capable of immediate aerobic surface growth on yeast extract glucose agar, and when viewed microscopically was observed to be composed of many branching, uneven-staining cells (figure 8A); whereas 39L-16b was an obligate anaerobe, and appeared entirely different morphologically (figure 8B). It was not long, however, before 39L-16a lost all semblance of bifid morphology and presented a more conventional appearance (figure 8C). Concurrent with this metamorphasis, 39L-16b lost much of its filamentous aspect and now shows striking bifid morphology (figure 8D); it seems to have acquired the very trait cast aside by 39L-16a!

These two offspring differ in ways other than morphology and oxygen tension preference. The homofermentative nature of 39L-16a and the heterofermentative nature of 39L-16b have already been contrasted, but shall be mentioned again briefly. Whereas both offspring ferment the same amount of glucose (table 13), one produces less than three per cent volatile acidity while the other forms almost 50 per cent volatile

acidity, and the lactic acid formed by the homofermentative strain is inactive while that produced by the other is predominantly the dextro isomer (table 13). Considerable acid is formed in milk by the homofermentative strain, whereas the heterofermentative organism does not grow appreciably in this substrate. In addition to the unlike behavior in xylose and arabinose, the two organisms differ further in regard to the fermentation of mannose, cellobiose, and melibiose (table 11). Also, as has been pointed out, the heterofermentative strain has a narrower temperature range permitting growth. All characters considered, it is obvious that the two organisms differ from each other greatly.

It is unfortunate that the parent culture's behavior in respect to fermentation and products arising from glucose breakdown has not been determined, so that it might be elucidated which of the two offspring most closely resembled the parent. In this connection, in regard to morphology and tolerance to the atmosphere 39L-16b, the heterofermentative bifidus type resembles the parent. It is interesting that from this same liver were isolated cultures which did not manifest this "activity", that is, did not give rise to more than one colony type. One of these, 39L-10, resembles closely 39L-16a and is phenotypic of L. acidophilus (as defined by Orla-Jensen), whereas another, 39L-15, is phenotypic of L. bifidus (as defined by this same worker) and resembles 39L-16b. The possibility that the parent culture was not pure is of

course not to be overlooked. However, I feel this not to be the case because the recognized changes in morphology and oxygen tension preference by these organisms does indicate some sort of "instability" in bifidus cultures, especially since Norris et al. (1950), by means of single cell isolation, were able to develop a monomorphic, acidophilus-like culture from a parent culture possessing the bifid morphology.

The differences between 37L-11a and 37L-11b, and 37L-10a and 37L-10b, while not as spectacular as in the instance cited directly above, and although not transcending established species types, are certainly measurable (tables 11 and 13). Strains 37L-10a and 37L-10b have already been discussed. Suffice to say here, the latter strain is a more fastidious counterpart of the former. It has a narrower temperature range permitting growth, utilizes less of the available glucose, converts less of the fermented sugar to nonvolatile acid, and forms less acidity in the substrates it attacks. Both strains are, at the time of this writing, anaerobic. The two colonies arising from 37L-11 gave rise to cultures which are practically identical, at least appear so with the tests employed in this study.

Three types of colonies were formed by 37L-8, and again these different colony types gave rise to strains possessing differences of such magnitude as to transcend species boundaries. The parent culture (37L-8) was capable of feeble aerobic growth when first isolated. It demonstrated considerable pleomorphism,

with filamentous and coccoid elements mixed together. After revival from the lyophilized state and eventual streaking on an Eugonagar plate (see page 91), three types of colonies were discernable: some were rather large and white -- one of which was subcultured and labeled 37L-8a, others were also white but smaller and discrete -- one of these was subcultured and labeled 37L-8b, while still others were small, bluish, and more translucent -- one of this type was subcultured and designated 37L-8c. At this time, all three strains preferred anaerobic conditions, and 37L-8a resembled its parentin that it manifested the same sort of pleomorphism, showing filamentous and coccoid cellular types. In juxtaposition, the other two differed from each other and also from 37L-8a in regard to morphology. Strain 37L-8b appeared as fairly large, long curved rods, whereas 37L-8c consisted of small uniform rods. No longer, however, does 37L-8a present the extreme pleomorphism, and now it resembles closely 37L-8b, although this latter organism's cells have an average diameter slightly greater than that of the former (compare figures 9A and 9B). Moreover, when stained with polychrome methylene blue the larger strain shows a striking metachromatic granulation (figure 9D); the other two offspring lack this feature. Strain 37L-8c is the smallest (figure 9C), the average cell length being only slightly greater then the average diameter of the cells of 37L-8b. At the time of this writing 37L-8c is unable to develop on the surface of agar unless incubation is under a

reduced oxygen tension, whereas its brothers are able to develop aerobically after prolonged incubation (several days). Strains 37L-8a and 37L-8b attack esculin, whereas 37L-8c does not.

In tables 11 and 13 one may compare certain characteristics of these three lactobacilli. It may be seen that 37L-8a is able to utilize all the available glucose (a five per cent solution of this monosaccharide in a tomato juice broth, see page 42) and this may be one of the reasons why it formed the larger colony type. In addition, this strain is the strongest fermenter of lactose in skim milk and can grow between the widest range of temperatures. As has been already pointed out, this homofermentative organism fits the description of L. acidophilus, and I have assigned it that epithet. It is interesting in this connection that this is the only acidophilus culture encountered from blackhead livers that produces visible gas, and in this manner belies its kinship to 37L-8c which is also a gas-former and a typical L. fermenti. However, a closer kinship exists between this organism (37L-8a) and 37L-8b, which has been also classified as L. acidophilus. This latter strain, it may be remembered, arose from the smaller white colony type, and paralleling this smaller colony size is its lack of lactose fermentation and poorer utilization of available glucose. It converts a greater portion of the fermented glucose to non-volatile acid (as lactic), which is not at all surprising since, unlike its brothers, it produces no visible gas. A few words concerning gas production by 37L-8a and 37L-8c may well be

apropos at this time. When tested for this characteristic as outlined on page 41, both strains were observed to generate gas in about equal amounts. In this respect the two behave as if they are one and the same organism. Now it must be remembered that 37L-8a utilized all the glucose presented it, whereas 37L-8c fermented only 85 per cent of the available glucose; therefore, the former strain is actually converting less of the fermented sugar to gas. Those of a speculative nature will no doubt attempt to explain this behavior in some manner. If strain 37L-8a actually should be impure, and consists of a mixture of cells of the 37L-8b and 37L-8c types, then one could conclude that the behavior we have associated with 37L-8a merely represents the additive properties of 37L-8b and 37L-8c, the latter strain fermenting glucose to the respective end products -- including gas, whereas 37L-8b ferments it without gas formation. However, if 37L-8a is merely a mixture of 37L-8b and 37L-8c other characters would be expected to be additive, which is not the case. Actually, 37L-8a possesses properties absent in both the other strains. For example, it forms a high degree of acidity in milk, whereas 37L-8b attacks this substrate only weakly and 37L-8c lacks lactose fermentation altogether. In addition, it is able to grow at 30 C and 45½ C, properties not shared by the other two. Finally, the morphological picture renders the hypothesis fallible; none of the cells in culture 37L-8a show metachromatic granulation, a property of cells of 37L-8b. However, the fact

that all characters are not additive may not necessarily mean that the explanation does not after all lie in the impurity of the culture, since perhaps 37L-8b and 37L-8c when grown together act upon each other as to modify somewhat each's characters. Additional work will be necessary in order to determine whether the behavior of 37L-8 is due to impurity or disassociation. Quantative studies with this, and the other "active" lactobacilli are contemplated for the future.

C. Microorganisms in blackhead livers which are not encountered in the agar pour plates

The degree of parasitism possible in a blackhead liver is amply demonstrated by the results obtained with liver sample 34L. This liver specimen was removed from a poult that had died of the disease. The plate count was approximately one million per gram of liver (table 5), a 4.5 gram sample being employed. Escherichia coli appeared to exist in pure culture, since every colony tested was able to ferment lactose with the production of acid and gas; the colonies tested further gave the typical reactions of E. coli, and the one strain tested for pathogenicity (34L-1) produced death after a single day (table 6). The plating technique, therefore, indicated that E. coli occurred in the liver with the protozoan parasite, Histomonas meleagridis. Microscopic examination of a stained film prepared from the initial 10^{-1} dilution allowed the same conclusion to be reached, since short, plump rods were easily

detected.

After plating the sample, the initial 10^{-1} dilution bottle and contents were placed in the 41 to 42 C incubator in order to see what organisms would grow under these conditions, and to attempt to cultivate the histomonad. After two weeks' incubation, a microscopic examination of several drops of the contents revealed the presence of many bacteria (short rods, probably E. coli) and Trichomonas. A few tenths of a ml of the suspension were transferred to DeVolt's (1943) monophasic medium and incubation continued at the same temperature. The mixed culture was carried through these tubes and observed periodically. After the second transfer, not only was E. coli and Trichomonas discernable, but also spirochetes (probably Borrelia) and histomonads.

It should be remembered that Rettger and Kirkpatrick (1927) observed spirochaetal forms in India ink-stained films prepared from blackhead livers. Their presence may be explained in the same manner as the other liver-residing microorganisms, by migration from the cecum. Jowett (1911b) reported seeing spirochetes in the cecal contents of healthy turkeys, and while making a survey of the flora of this organ I also observed them (Harrison and Hansen, 1950a).

It must be pointed out here that this degree of parasitism is rare, and seldom appears except in the livers of a few of the turkeys that have died of the disease. Incubation of the initial 10^{-1} dilution bottles from bacteria-free livers or

livers showing only a low plate count seldom reveal any other microorganisms.

In the manner outlined above, Trichomonas was observed also in liver samples 36L, 37L, and 40L; in 37L histomonads appeared as well.

Although it is perhaps a little far afield, I would like to record one more observation at this time. The trichomonads from liver 34L were observed to undergo a multiple division; large, extremely active protoplasmic masses were observed which often filled the entire oil-immersion field and which contained well over twenty individuals, that is, over twenty tufts of flagella and undulating membranes could be seen around the periphery of the mass. Although individual trichomonads appeared to continually try to pull away from the living mass, they were never actually observed to do so, and if the large body was accidentally crushed beneath the coverglass all movement would cease, no individuals remaining intact. After a number of transfers through the monophasic medium the trichomonads reverted to the orthodox manner of division and therefore the large bodies were never stained. Recently, Mr. Tromba in H. M. DeVolt's laboratory has observed the same phenomenon in freshly isolated cultures of this protozoan. As far as I am aware, Trichomonas of avian origin has never been reported to behave in this fashion.

IV

SUMMARY

If turkeys suffering from infectious enterohepatitis are killed at the first appearance of symptoms and their diseased livers examined, in the majority of cases only the protozoan parasite, Histomonas meleagridis, is discernable; in only about one-third of these cases are bacteria encountered. On the other hand, if turkeys are allowed to die of blackhead and then their livers sampled, the reverse is true. Now the livers not only contain Histomonas meleagridis, but the majority also yield bacteria; in over two-thirds of these cases bacteria are encountered.

These bacteria are opportunists and not an initiating factor in the disease. The chances of isolating them from blackhead livers increase in proportion to the length of the illness. In fact, the actual bacterial population, that is, the number of bacteria per gram of liver, increases as the disease progresses. However, some turkeys die of blackhead whose livers remain free of bacteria, only the protozoan parasite, Histomonas meleagridis, being discernable in the livers of these cases.

The discovery that early in the disease a high proportion of blackhead livers are bacteria-free provides a valuable tool

for acquiring Histomonas meleagridis inocula free of all contaminating bacteria. Hence, it becomes feasable to experiment with pure cultures of the histomonad, furnishing further evidence of the etiological role of this protozoon in blackhead.

Although most of the bacteria isolated from blackhead livers are in themselves not pathogenic, pathogenic strains are sometimes encountered. Therefore, secondary or concurrent bacterial infection may occasionally play the decisive role, overriding the protozoan parasitism and terminating the illness by an early death.

The following bacteria which are in themselves capable of producing illness, and usually death, when injected into the large wing vein of healthy poults have been isolated from blackhead livers: Micrococcus pyogenes var. aureus (once), Pseudomonas aeruginosa (once), Salmonella newport (twice), and strains of Escherichia coli (thrice).

Of the sixty livers sampled, twenty-nine yielded cultivatable bacteria, and, of these, twenty were found to contain Escherichia coli. This organism was the bacterial species most often encountered; in nine of the livers it was the only bacterium isolated, whereas in eleven cases it occurred together with one or more additional types. Other gram-negative rods were Pseudomonas aeruginosa and Salmonella newport.

Members of the genus Streptococcus were recovered from nine livers, and representatives of the following species, as well as an unidentified Streptococcus, were encountered:

S. faecalis, S. inulinaceus, and S. liquefaciens. Other cocci were Micrococcus pyogenes, varieties aureus and albus, and M. flavus, as well as one or two strains of unidentified cocci resembling pediococci.

Since members of the Lactobacillus are rarely encountered in pathological conditions, those of this genus isolated from blackhead livers were studied in considerable detail. The following species were identified: L. acidophilus, L. bifidus, and L. fermenti.

As a result of the investigations with the lactobacilli, it was observed that a number of cultures formed more than one recognizable type of colony when streaked out upon an agar plate. When these colony types were picked from a plate and carried as individual strains they were found to differ from one another not only in regard to colonial morphology but in other aspects as well; in several instances they differed to such an extent as to transcend established species boundaries. However, additional work will be necessary in order to determine whether this behavior is due to impurity of the parent culture or is due to disassociation of the culture.

In addition to the bacterial types mentioned in the preceding paragraphs which were picked from agar pour plates, other organisms were occasionally encountered when bits of the liver sample in the initial dilution bottle were incubated and transferred through a serum rice starch broth. These tubes occasionally rendered mixed cultures containing spirochetes

and other bacteria, trichomonads, and histomonads.

The results presented in this dissertation not only pertain to the immediate problem concerning the role of bacteria in blackhead of turkeys, but should be of value in suggesting the bacterial types which may be expected to occur in similar pathological conditions of higher animals, for example, the hepatic abscesses in amoebiasis of man.

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VI

APPENDIX

It has already been pointed out that the most numerous anaerobe in the cecal feces of healthy turkeys is Lactobacillus bifidus (Harrison and Hansen, 1950a and 1950c). In these earlier studies the lactobacilli showing decided anaerobic preferences and strongly fermenting the pentoses were considered as bifidus; actually, only several representatives of the bifidus fermentative type were grown in flasks and the products resulting from glucose fermentation determined. In the light of the variances in morphology and oxygen tension preference by this species it was deemed advisable to study in more detail representatives of the bifidus type from feces, especially since I was desirous to increase my collection of authentic bifidus strains. Accordingly, a number of the fecal lactobacilli possessing the bifidus fermentation pattern and considered members of this type were studied along with the hepatic lactobacilli.

The results are tabulated in tables 14 and 15; the four fecal strains are compared with the three hepatic strains which have been characterized on previous pages. Actually, the most important conclusion may be summarized in one sentence: In every case, the fecal lactobacilli which fermented xylose

TABLE 14

Final pH values in broth" cultures of Lactobacillus bifidus from cecal feces and blackhead livers of turkeys after 14 days' incubation at 37 C
(pH values representing significant fermentation are underscored)

Culture number	Glycerol	Xylose	Arabinose	Rhamnose	Sorbitol	Mannitol	Inositol	Fructose	Glucose	Mannose	Galactose
Control*	7.1	6.1	6.3	6.3	7.0	7.0	7.0	5.7	5.9	6.0	5.6
Control	6.7	6.5	6.6	6.5	6.7	6.7	6.7	6.1	6.2	6.3	6.1
20-32*	5.5	4.4	4.3	5.2	5.4	5.4	6.8	4.3	4.6	4.8	4.6
21-33*	5.6	4.5	4.4	5.2	5.4	5.4	5.4	4.3	4.6	4.6	4.6
29-4*	6.3	4.5	4.4	6.1	7.0	6.3	6.8	4.6	4.7	4.7	4.7
29-7*	7.0	4.5	4.4	6.1	6.3	6.3	6.8	4.6	4.6	4.7	4.5
39L-14	6.7	4.7	4.7	6.3	6.7	6.7	6.7	4.4	4.8	4.6	4.7
39L-15	5.4	4.6	4.5	5.2	5.4	5.4	5.4	4.7	4.8	4.8	5.0
39L-16b	6.6	4.7	5.0	6.3	6.6	6.5	6.0	4.8	5.3	5.8	5.0

TABLE 14 continued

Culture number	Sucrose	Trehalose	Maltose	Cellobiose	Melibiose	Lactose	Melezitose	Raffinose	Dextrin	Starch	Salicin	Control
Control*	7.1	7.1	7.0	7.0	6.9	6.7	7.1	7.1	6.6	7.1	7.1	7.1
Control	6.6	6.6	6.6	6.8	6.6	6.4	6.7	6.7	6.6	6.7	6.8	6.7
20-32*	5.2	4.9	4.6	4.8	4.5	4.5	5.0	4.5	5.2	5.3	4.6	5.4
21-33*	4.7	5.0	4.7	5.1	4.5	4.5	5.1	4.5	5.0	5.2	4.8	5.4
29-4*	4.8	5.3	4.8	6.3	4.7	5.5	7.0	4.7	5.5	6.3	4.7	7.0
29-7*	5.2	5.4	4.6	6.3	4.6	4.5	7.1	4.5	4.5	4.7	4.6	7.0
39L-14	4.5	4.6	4.5	4.8	4.2	4.6	5.3	4.5	4.7	6.7	4.7	6.4
39L-15	4.4	4.8	4.6	5.0	4.4	5.2	4.5	4.5	4.5	5.5	5.0	5.4
39L-16b	4.6	6.7	4.6	6.6	4.9	4.6	6.4	4.7	4.9	5.4	5.3	6.6

" Yeast extract, 2.5%; K_2HPO_4, 0.2%; $MgSO_4 \cdot 7H_2O$, 0.01%; and substrate, 2%.

* From cecal feces of healthy turkeys and incubated aerobically; Other cultures from blackhead livers and incubated anaerobically.

TABLE 15

Characteristics of Lactobacillus bifidus from cecal feces and blackhead livers of turkeys

Culture number	Skim milk		Per cent glucose fermented	Per cent fermented glucose converted to -		Optical type of lactic acid produced
	Final pH	Per cent acid calculated as lactic acid		Vol. acid cal. as acetic acid	Nonvol. acid cal. as lactic acid	
20-32*	5.3	0.23	46	43	40	D
21-39*	6.3	0.03	23	55	43	D
29-4*	6.1	0.13	46	43	39	D
39L-15	6.0	0.18	28	50	35	D
39L-16b	6.1	0.10	30	46	40	D

* From cecal feces of healthy turkeys.

All cultures incubated anaerobically at 37 C.

and arabinose and were considered L. bifidus are indeed typical examples of this species, since they are heterofermentative, converting approximately a half of the fermented glucose to volatile acid (calculated as acetic acid, table 15) and forming lactic acid which is predominantly the dextro isomer.

Since, as has already been pointed out in this dissertation, morphology and oxygen tension preference are not accurate criteria for the differentiation of these two lactobacilli types, the fermentation of the two pentoses may be of value as a rapid means of separating the two organisms. The fermentation of xylose and arabinose appears to be a stable trait, at least in the strains examined to date. It is independent of morphology and oxygen tension preference as is pointed out below.

Whereas the three hepatic bifidus cultures are still obligately anaerobic, the fecal strains can develop in tubes of broth in the presence of the atmosphere, and were, therefore, studied under aerobic conditions in regard to the fermentation reactions. However, all fecal strains except 20-32 which has recently become completely aerobic, produce little surface growth on agar or in broth having a large area exposed to the atmosphere, as is the case in flask cultures, and therefore require a reduced oxygen tension for good development under these latter conditions. Paralleling the aerobic nature of 20-32 is its complete lack of bifid morphology; it appears exactly like L. acidophilus in this

respect (figure 10B). On the other hand, the obligately anaerobic hepatic bifidus strains manifest a pronounced bifid morphology (figure 10D), whereas the intermediates in regard to oxygen tension preference (21-39, 29-4, and 29-7) are also intermediate in regard to morphology, showing only an occasional branching cell (figures 10A and 10C); but it should be emphasized that all seven cultures, nevertheless, bring about a strong fermentation of xylose and arabinose (table 14), and are no doubt still heterofermentative.

The seven strains investigated split esculin. None produce visible gas beneath the vaseline seal when cultivated in tomato juice glucose broth.

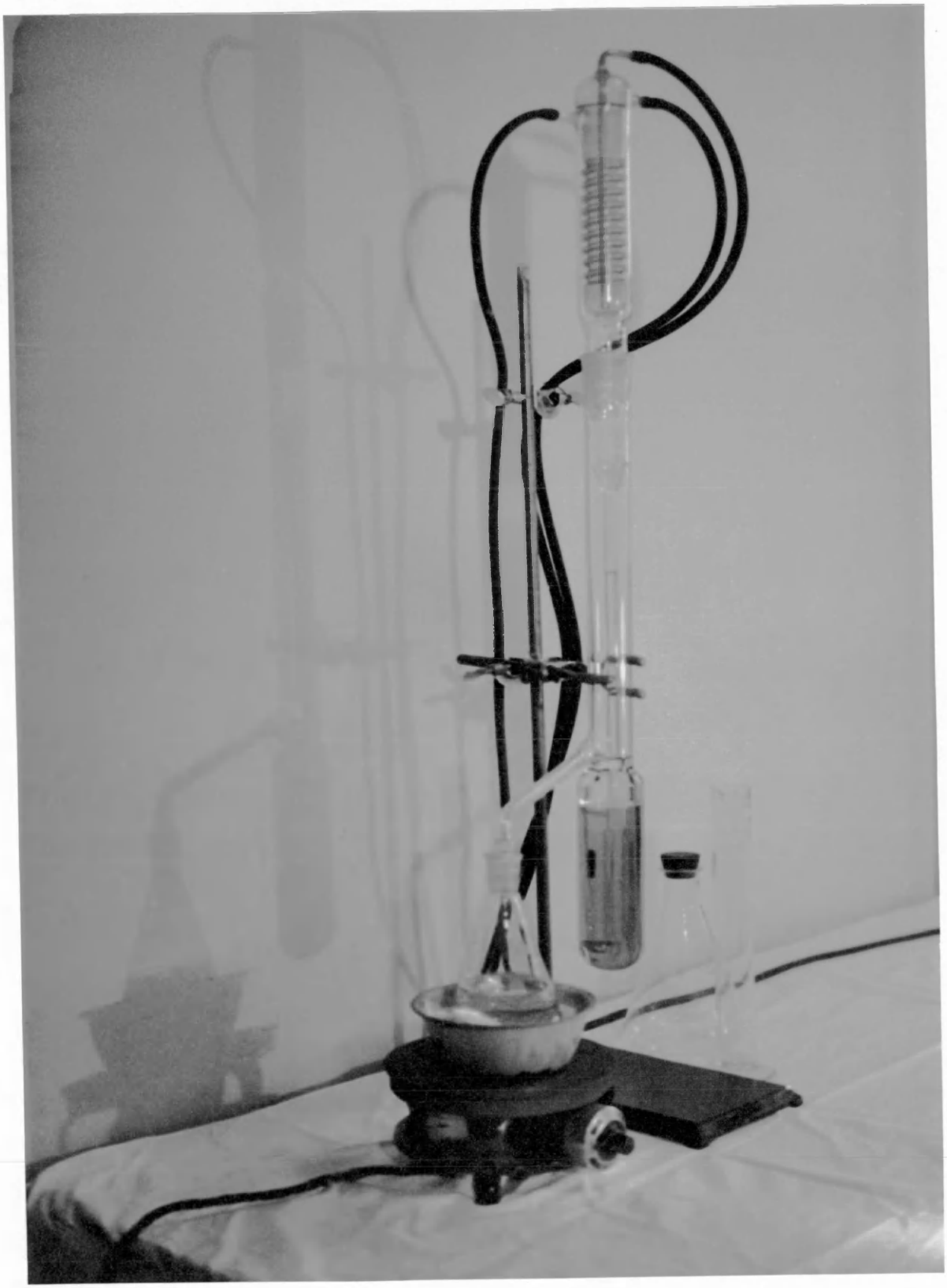
There appears to be little or no difference between the reactions of L. bifidus isolated directly from the cecal feces of healthy turkeys and those isolated from the diseased livers of turkeys affected with blackhead disease. The more aerobic nature of the fecal strains is probably only due to the fact that they have been maintained in the laboratory for a longer period of time.

VII

FIGURES

FIGURE 1

The liquid extraction apparatus employed to extract lactic acid from broth cultures of the Lactobacteriaceae.



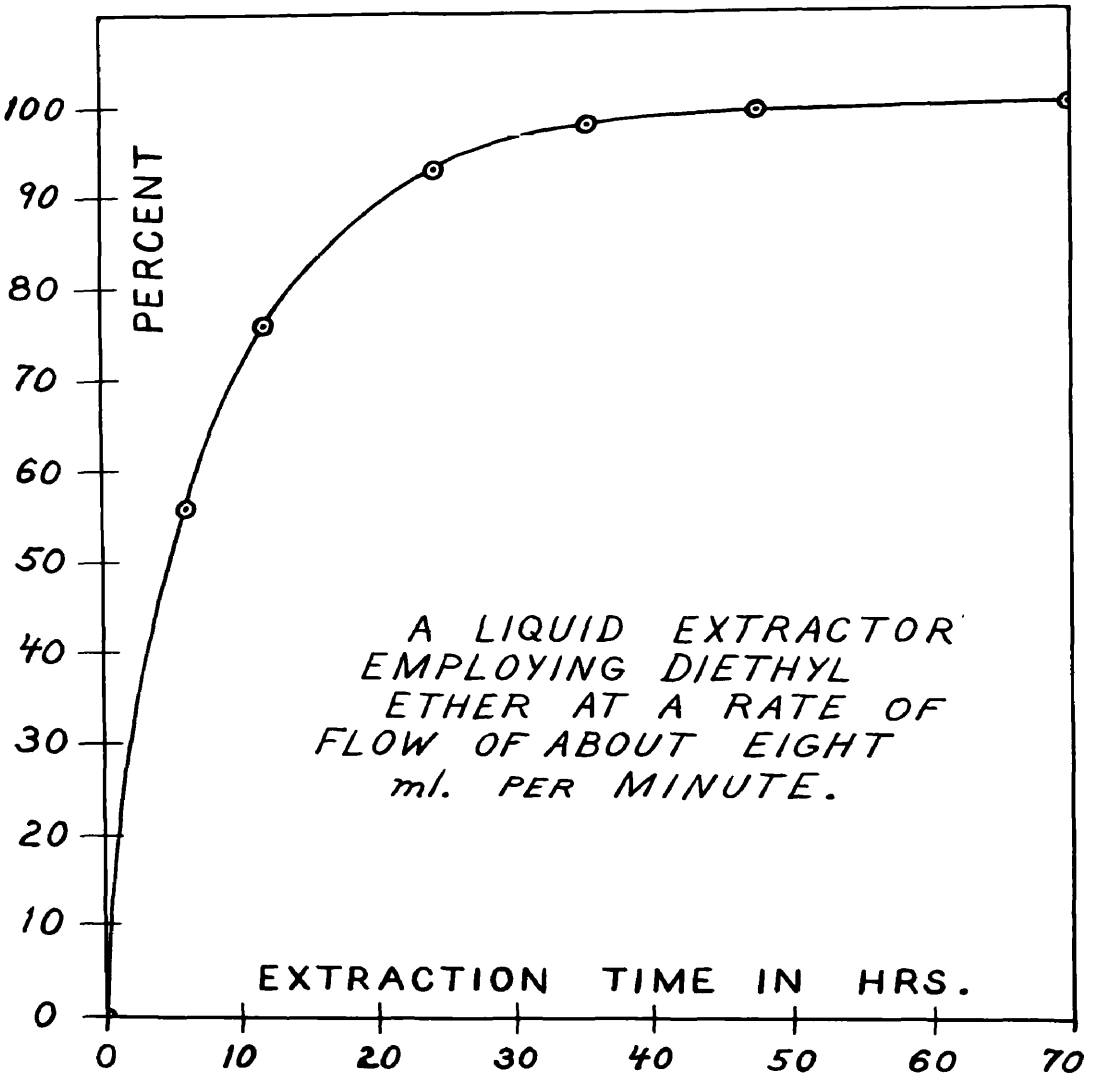


Figure 2. Per cent lactic acid extracted vs. extraction time in hours.

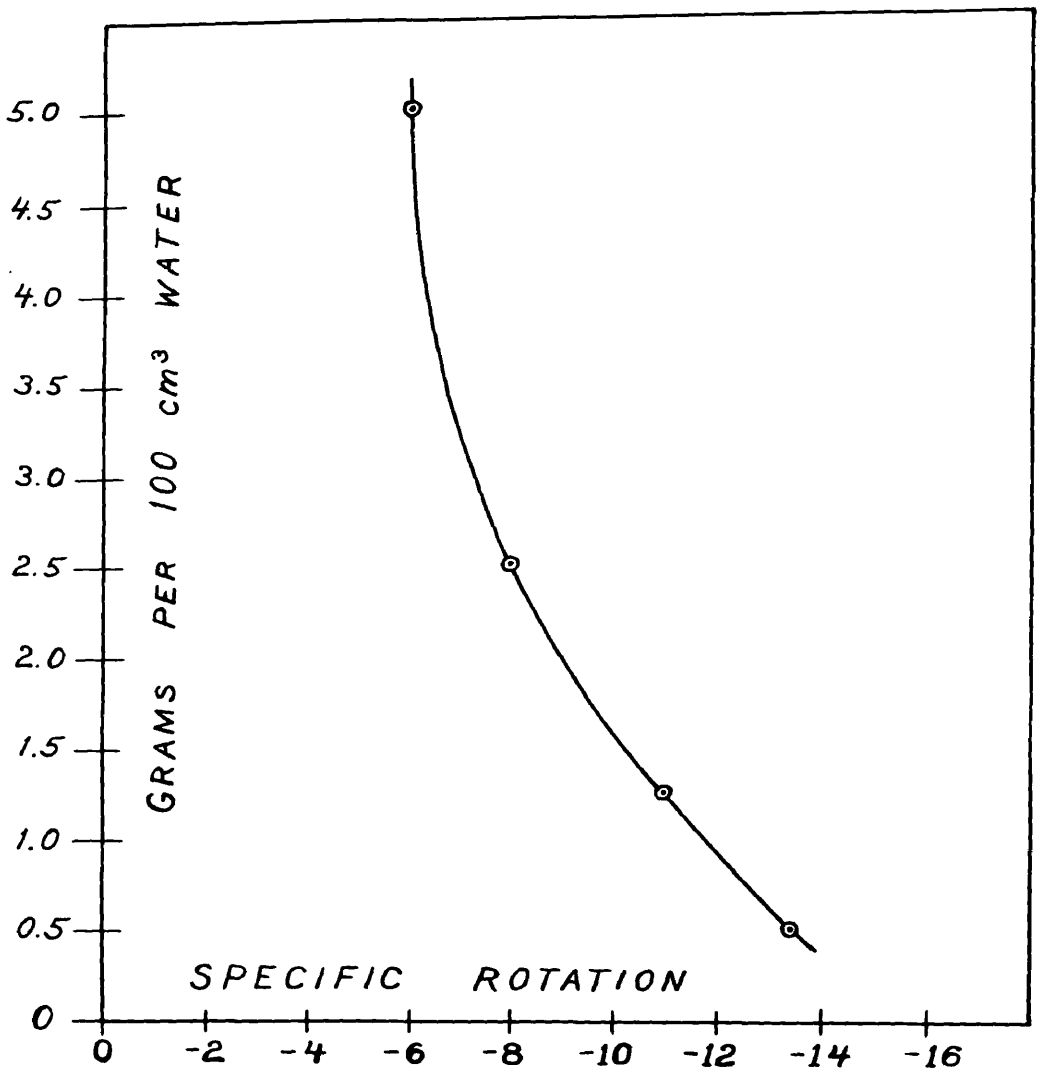


Figure 3. Concentration of $Zn(C_3H_5O_3)_2 \cdot 2H_2O$ from dextro-lactic acid vs. its specific rotation at 15C with sodium light.

FIGURE 4

The morphology of Lactobacillus fermenti from blackhead livers. All smears are stained with crystal violet (X 1,400).

Figure 4A.

Strain 37L-10a; anaerobic, 24 hour, 37 C, yeast extract glucose agar culture.

Figure 4B.

Strain 37L-10b; anaerobic, 24 hour, 37 C, yeast extract glucose agar culture.

Figure 4C.

Strain 43L-2; anaerobic, 24 hour, 37 C, yeast extract glucose agar culture.

Figure 4D.

Strain 37L-10b; anaerobic, 24 hour, 37 C, tomato juice broth culture.

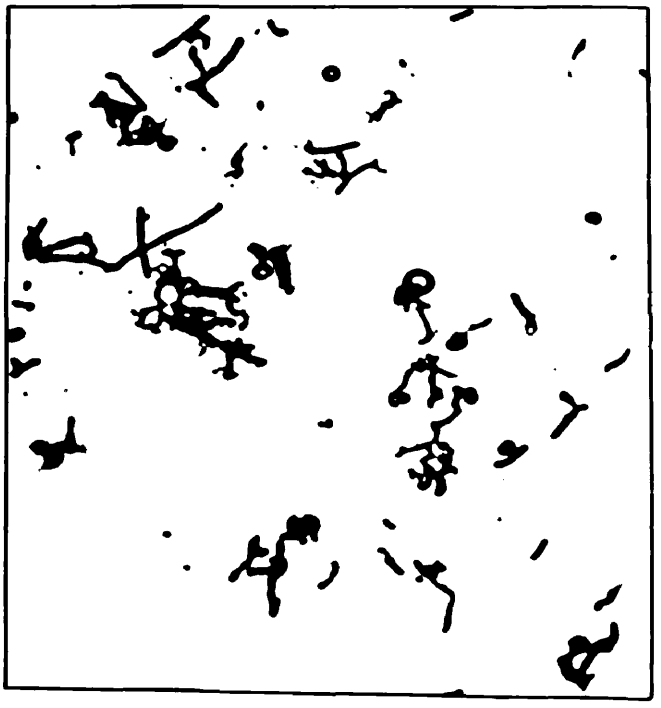


FIGURE 5

A comparison of aerobic and anaerobic morphology of two strains of Lactobacillus fermenti from blackhead livers. All smears are stained with crystal violet and are from 24 hour, 37 C, yeast extract glucose agar cultures (X 1,400).

Figure 5A.

Strain 55L-5; aerobic incubation.

Figure 5B.

Strain 69L-3; aerobic incubation.

Figure 5C.

Strain 55L-5; anaerobic incubation.

Figure 5D.

Strain 69L-3; anaerobic incubation.

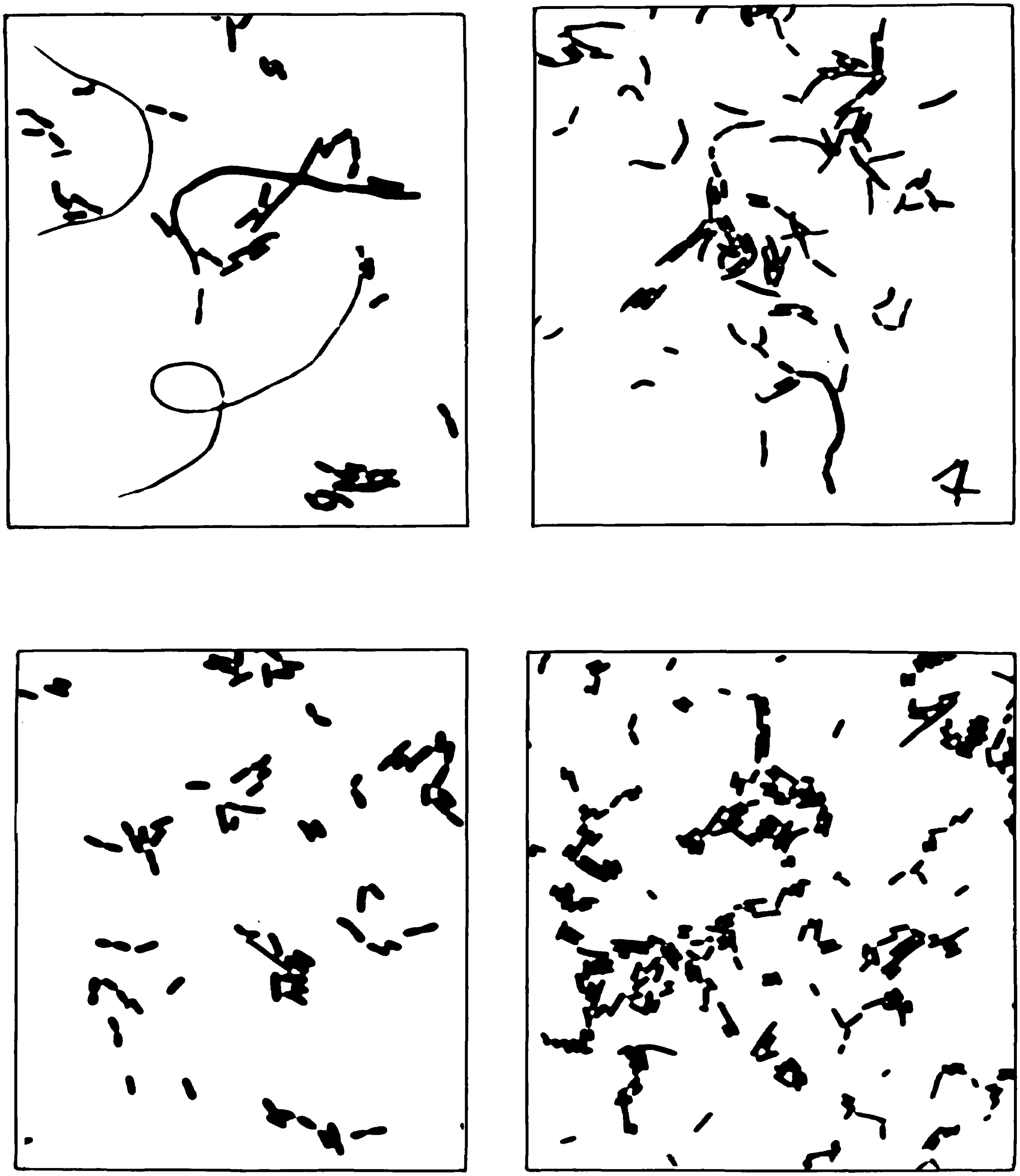


FIGURE 6

A comparison of aerobic and anaerobic morphology of two strains of Lactobacillus acidophilus from blackhead livers. All smears are stained with crystal violet and are from 24 hour, 37 C, yeast extract glucose agar cultures (XI,400).

Figure 6A.

Strain 18L-2;
aerobic incubation.

Figure 6B.

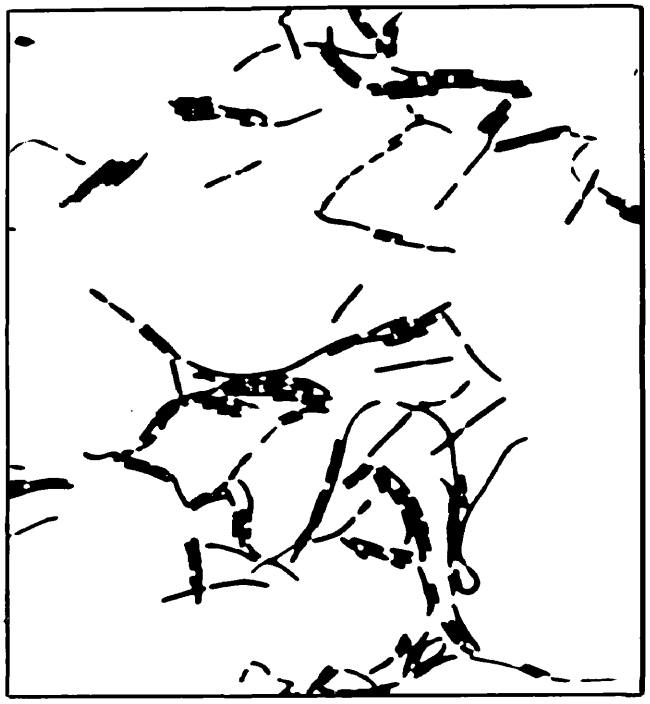
Strain 37L-4;
aerobic incubation.

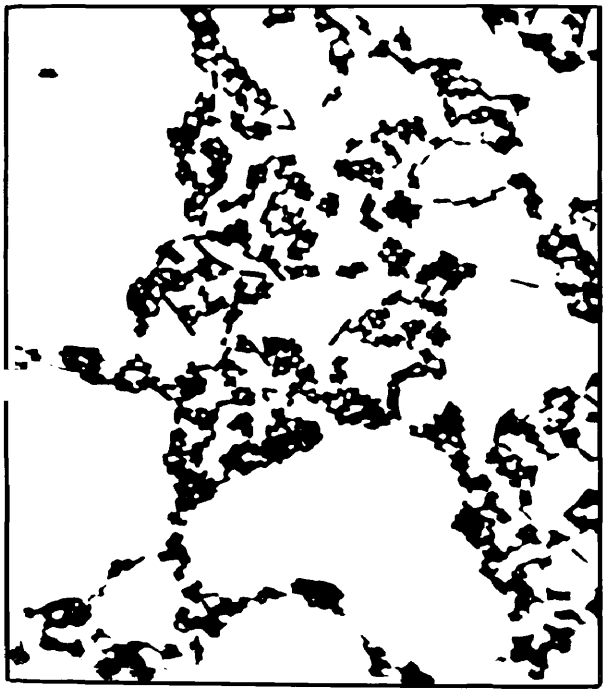
Figure 6C.

Strain 18L-2;
anaerobic incubation.

Figure 6D.

Strain 37L-4;
anaerobic incubation.





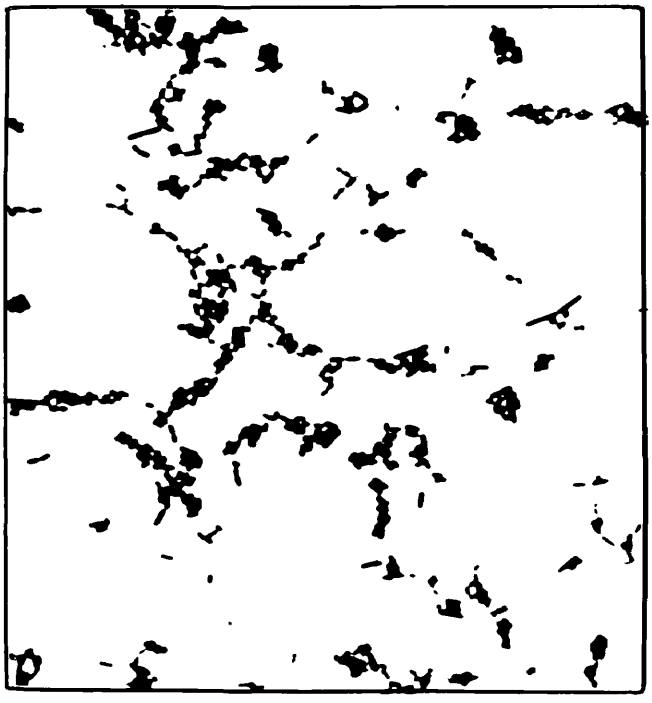


FIGURE 7

The morphology of several lactobacilli types and an unidentified Streptococcus from blackhead livers. All smears are stained with crystal violet and are from 24 hour, 37 C cultures (X 1,400).

Figure 7A.

Strain 18L-6 (Lactobacillus acidophilus); anaerobic, yeast extract glucose agar culture.

Figure 7B.

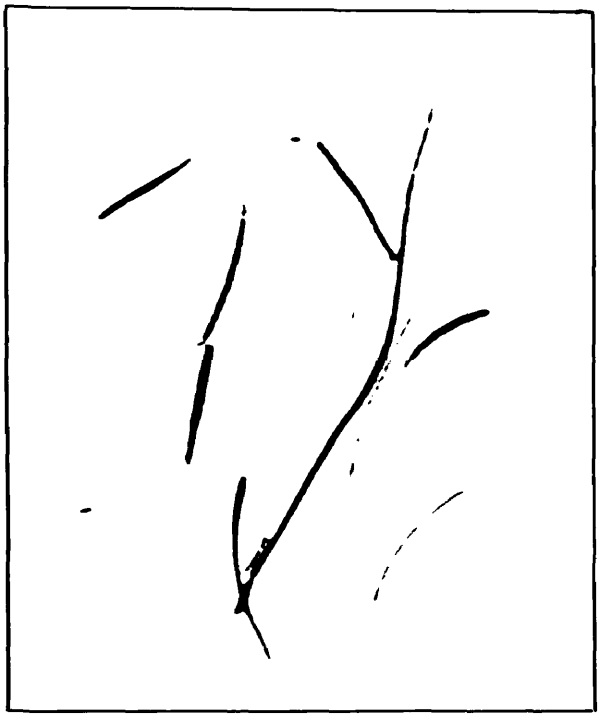
Strain 39L-15 (Lactobacillus bifidus); anaerobic, yeast extract glucose agar culture.

Figure 7C.

Strain 18L-6 (Lactobacillus acidophilus); anaerobic, Eugonagar culture

Figure 7D.

Strain 35L-8 (Streptococcus sp.); aerobic, trypticase glucose broth culture.



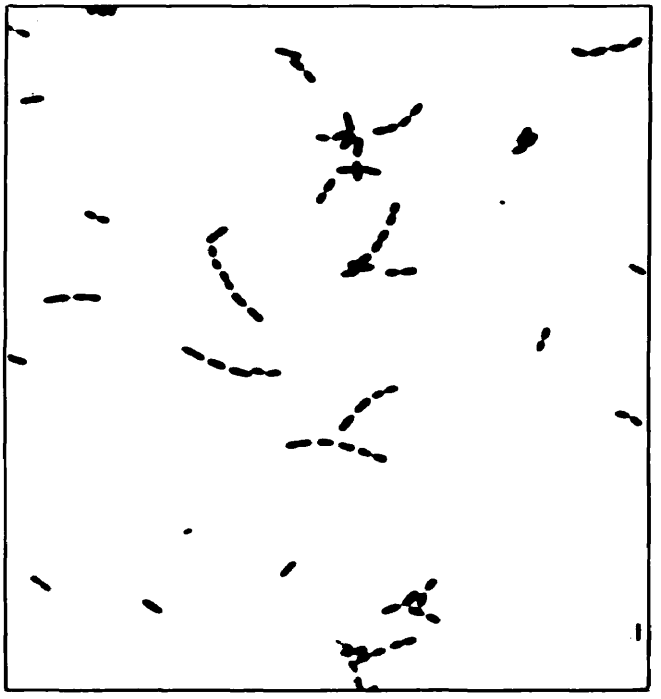


FIGURE 8

Morphology of the progeny arising from the two colony types which developed from Lactobacillus strain 39L-16. All smears are stained with crystal violet and are from anaerobic, 24 hour, 37 C, yeast extract glucose agar cultures (X 1,400).

Figure 8A.

Strain 39L-16a (Lactobacillus acidophilus); prepared February 1, 1951.

Figure 8B.

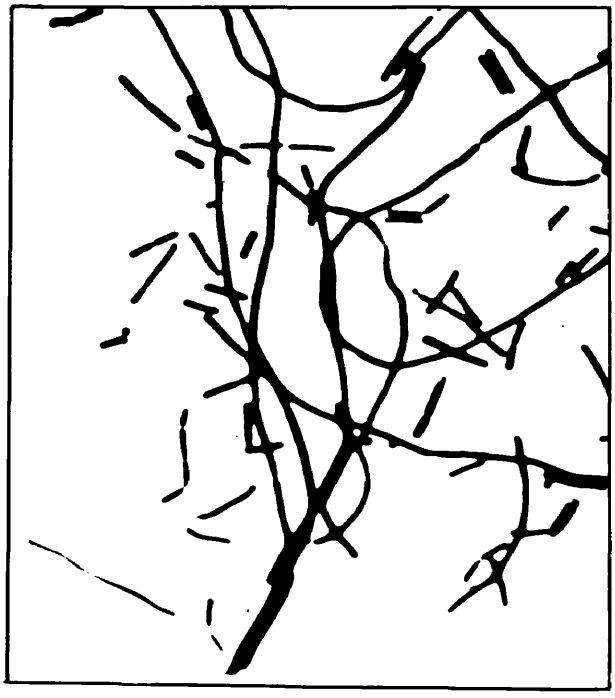
Strain 39L-16b bifidus);illus bifidus); prepared February 1, 1951.

Figure 8C.

Strain 39L-16a (Lactobacillus acidophilus); prepared October 14, 1951.

Figure 8D.

Strain 39L-16b (Lactobacillus bifidus); prepared October 14, 1951.



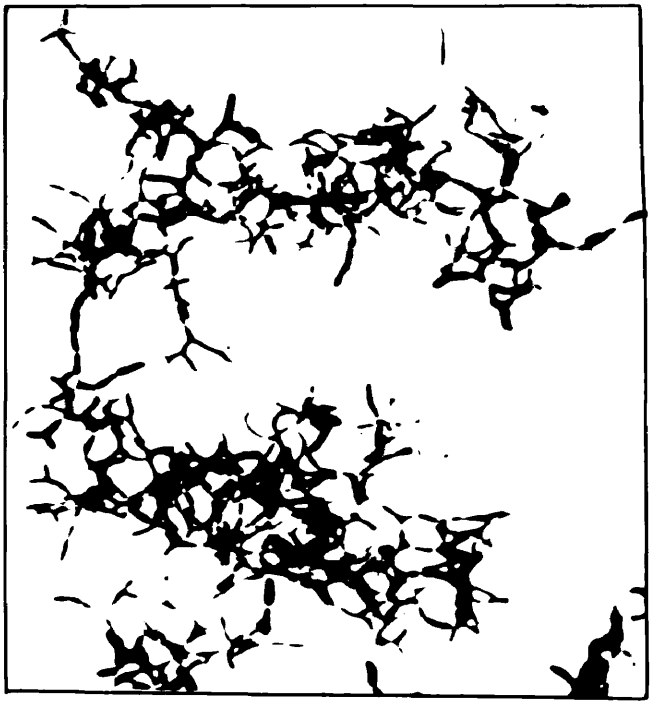


FIGURE 9

Morphology of the progeny arising from the three colony types which developed from Lactobacillus strain 37L-8. All smears are from 24 hour, 37 C, cultures (X 1,400).

Figure 9A.

Strain 37L-8a (Lactobacillus acidophilus); yeast extract glucose agar culture, stained with crystal violet.

Figure 9B.

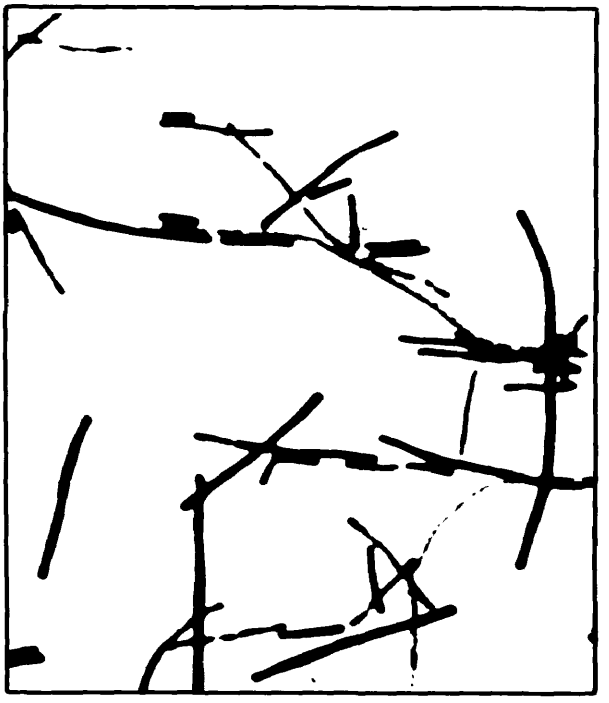
Strain 37L-8b (Lactobacillus acidophilus); yeast extract glucose agar culture, stained with crystal violet.

Figure 9C.

Strain 37L-8c (Lactobacillus fermenti); yeast extract glucose agar culture, stained with crystal violet.

Figure 9D.

Strain 37L-8b (Lactobacillus acidophilus); tomato juice broth culture, stained with polychrome methylene blue.



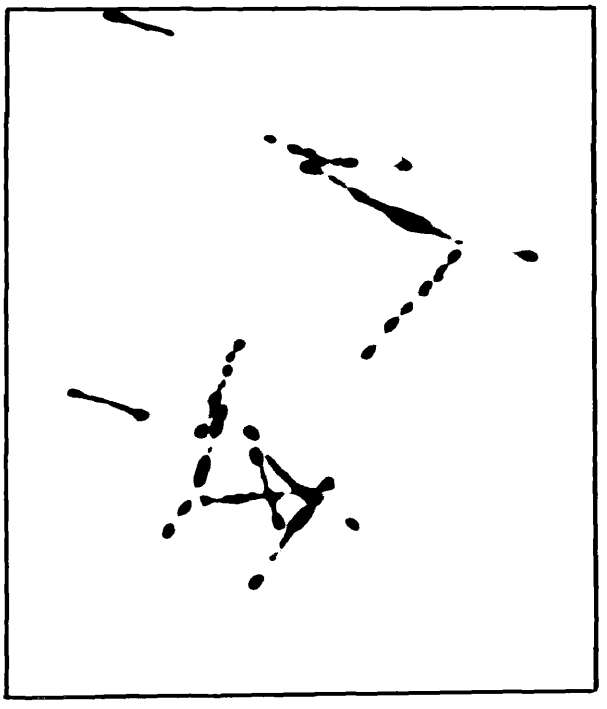


FIGURE 10

The morphology of four strains of Lactobacillus bifidus. All smears are stained with crystal violet and are from 24 hour, 37 C, yeast extract glucose agar cultures (X 1,400).

Figure 10A.

Strain 21-39; aerobic incubation.

Figure 10B.

Strain 20-32; anaerobic incubation.

Figure 10C.

Strain 29-4; anaerobic incubation.

Figure 10D.

Strain 39L-16b; anaerobic incubation.

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