1936

Studies on Lactobacillus cultures that actively coagulate milk

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NOTE TO USERS

STUDIES ON LACTOBACILLUS CULTURES THAT ACTIVELY COAGULATE MILK

By

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A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology

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Iowa State College
1936

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INTRODUCTION

It has long been known that certain species of the genus Lactobacillus are important in the dairy industry. Cultures of lactobacilli were early studied in connection with the ripening of Swiss, Cheddar and other types of cheese. Lactobacillus acidophilus (Moro) (Holland) is of particular importance in the production of fermented milk which has certain therapeutic uses. Lactobacillus bulgaricus (Grigoroff) (Holland) is also used in the production of certain types of fermented milk and in the manufacture of Swiss cheese.

The three types of lactobacilli are primarily important in the dairy industry because of their ability to ferment lactose actively. These microorganisms are definitely homofermentative, that is, lactic acid is the chief product formed in milk and there are only small amounts of additional compounds produced. Other lactobacilli are undoubtedly common in dairy products but the types that are active lactose fermenters have a much better opportunity to bring about conspicuous changes in milk and its derivatives than types that ferment lactose slowly or not at all.

The lactobacilli that ferment lactose actively are so closely related morphologically, culturally and biochemically that it is extremely difficult to pick out definite differentiating characters. In most of the previous work on this group of microorganisms, an adequate indentification has not been established on a definite species basis.

STATEMENT OF PROBLEM

The primary object of the work herein reported was to determine the general relationships of cultures of lactobacilli that actively coagulate litmus milk with reduction of the litmus and the formation of a smooth curd. Most of the cultures studied were isolated from dairy products and such other materials as ensilage, corn stover, and calf, dog, infant and rat feces. However, cultures of L. acidophilus and L. bulgaricus were obtained from various laboratories for comparative purposes. The cultures were studied particularly from the standpoint of their action in milk because of the importance of the organisms in dairy products.

HISTORICAL

von Freudenreich (77) was the earliest investigator of the Lactobacillus group of organisms. In his studies on Swiss cheese, in 1891, he isolated organisms which he thought were responsible for the ripening. Later, von Freudenreich (78) observed that bacterial enzymes might be important through their ability to increase the soluble nitrogen in milk. Smith (68), in 1897, also observed the proteolytic activity of certain lactic acid bacteria.

Escherich (17), in 1886, made the first study of the intestinal types of bacteria. However, he failed to isolate the organisms in pure cultures. Moro (53), in 1900, also studied the intestinal bacteria. He inoculated the feces of breast fed infants into beer wort bouillon for enrichment. By plating on acid beer wort agar, Moro obtained the "Blaubacillus" to which he gave the name Bacillus acidophilus. Tissier (72) worked with material from the same source and obtained an organism which he called Bacillus bifidus. He contended that this organism was the predominating one in the stools of breast fed babies and not Bacillus acidophilus as Moro claimed. Moro thought his organism was derived from the mothers' breasts and was found in the mouths and stomachs of infants as well as in the intestinal contents. He stated that it was not a single species but a group of closely related forms which preferred an acid medium for growth. Finkelstein (19) and Tissier confirmed the findings of Moro along this line.

In 1901, Cahn (10) observed Bacillus acidophilus in the feces of breast

and bottle fed babies. Rodella (62), also in 1901, isolated similar organisms from the same source and noted evidence of pleomorphism. These organisms varied in form from spheres to very long rods, while some of the rods showed branching. Later Weiss (81) observed that when large amounts of milk were ingested a marked increase in the organism of the Lactobacillus type was evident in the intestinal tract. He claimed that the organisms corresponded very closely to Bacillus acidophilus.

Thoni (71) was very much interested in the organisms that von Freudenreich (77) had isolated from Swiss cheese. He attempted to describe the organisms from the morphological standpoint, with the hope of classifying them on that basis. He also observed that the lactic acid producing rods made up 80 to 100 per cent of the flora in Swiss cheese after a few days of ripening.

The observations of Orla-Jensen (55) confirmed the work of von Freudenreich (77) on cheese ripening. The lactobacilli which Orla-Jensen isolated digested the casein in milk very rapidly when calcium carbonate had been added to the milk.

Henneberg (30) worked with 22 strains of lactobacilli isolated from various sources. He attempted to divide them into three groups, according to the fermentation reactions on fourteen carbohydrates. Of 14 organisms studied, only 4 actively attacked lactose; 8 did not attack it at all; and 2 acted upon the lactose only very slightly. The lactose fermentation of one organism was not given.

Grigoroff (21), in 1905, noted the necessity of sugar media for the cultivation of his Bacillus bulgaricus. He stated that his organism produced large quantities of lactic acid in milk and curdled the milk at 42°C. in

about 5 hours. The organism also attacked maltose, levulose, and sucrose but did not ferment rhamnose, dulcitol, and sorbitol.

Orla-Jensen (55) repeated the work of von Freudenreich (78) and confirmed the fact that Bacillus casei was the most important agency in the ripening of Swiss cheese. Cohendy (13) isolated an organism from Swiss cheese which he said was closely related to the lactobacilli but did not visibly attack the casein in milk, even after long incubation. On the other hand, Bertrand and Weisweiller (8) reported that Lactobacillus bulgaricus digested approximately one-tenth of the casein in an ordinary milk culture.

Samarani (64) reported that the Sardinian fermented milk, "Cieddu," contained only two forms of microorganisms. One form was a variety of Bacterium lactis acidi and the other a variety of Bacillus casei, which Samarani claimed was identical with the Lactobacillus studied by von Freudenreich.

Kuntze (48) studied two types of lactobacilli on the basis of their staining properties. One type, represented by Bacillus bulgaricus, stained solidly with Neisser's stain and alkaline methylene blue, while the other type, represented by Bacillus acidophilus, showed granules. Kuntze claimed these staining characteristics were important in the separation of Bacillus bulgaricus and Bacillus acidophilus.

Heineman and Hefferan (28), in 1909, found that microorganisms closely resembling Lactobacillus casei, Lactobacillus bulgaricus, and Lactobacillus acidophilus were very widely distributed. They were normally present in feces of various animals, in a variety of sour aromatic foods, in cattle

feed, in normal saliva and in various fermented milks. An organism closely related to Lactobacillus bulgaricus and to Lactobacillus casei was found widely distributed in milk, butter and cheese by Hastings and Hammer (26).

Except for carbohydrate fermentations, most of the early biochemical studies on the lactobacilli were limited to the principal non-volatile product of fermentation, namely, lactic acid. However, Heineman and Hefferan (28) reported that the volatile acid formed by Bacillus bulgaricus in milk was about 6 per cent of the total acid produced.

Bertrand and Duchaoêk (7) made an exhaustive study of the fermentative action of Lactobacillus bulgaricus on various carbohydrates. They found that this organism did not attack sucrose, maltose, the pentoses, sorbitol and mannitol; levulose and mannose were fermented to a very slight degree.

Kendall (39), in a study of lactobacilli from the intestinal tract, corroborated the work of Moro (53). He used n/20 acetic acid bouillon for isolation purposes. Kendall also proposed the term "aciduric" rather than "acidophilic" for the group. Lactobacillus acidophilus, which he isolated from the intestinal tract of man, failed to induce proteolysis in milk even after a long period of incubation. White and Avery (82) observed that organisms of the Lactobacillus bulgaricus type showed little or no action on casein or milk fat.

Stevenson (69) confirmed the findings of Heineman and Hefferan (28), and of Hastings and Hammer (26), as to the wide distribution of lactobacilli. He found them in market milk, Swiss cheese, human saliva, feces of cows and in the soil. For isolation purposes, he used yeast whey for enrichment and whey agar with chalk as a plating medium.

The action of the Lactobacillus group on milk proteins has been given considerable attention, since organisms included in this group or closely related to it are known to exist in various types of cheese. Hastings, Evans and Hart (25) observed that eight cultures of lactobacilli, which they had isolated, increased the soluble nitrogen after an incubation period of 3 months. The soluble nitrogen varied from 12.5 to 62.5 per cent over the control. Kendall, Day and Walker (40) used amino acid nitrogen determinations in measuring the extent of protein digestion by bacteria when grown in milk.

Evans, Hastings, and Hart (18), in their studies on the Bacterium casei group, concluded that these organisms were responsible for the pungent flavor that developed during the latter part of the ripening period of raw or pasteurized milk Cheddar cheese. Eldredge and Rogers (16) reported, from their studies on the Swiss type of cheese, that members of the Lactobacillus group were present during the ripening period. They noticed that cheese lacking in flavor and texture showed very few lactobacilli throughout the ripening. Studies on the cultural and physiological characters indicated that many of the organisms isolated belonged to the lactobacilli of the bulgaricus group. Eldredge and Rogers (16) observed that approximately 95 per cent of the lactose fermenting rods produced carbon dioxide, in varying amounts, in sugar whey broth.

Rahe (60) (61) attempted to make a thorough study of a number of lactobacilli, on the basis of their action in milk and in a medium containing maltose. However, his descriptions are so inadequate that it is extremely difficult to draw any conclusions in regard to his work.

The work of Evans, Hastings and Hart (18), Eldredge and Rogers (16),

Hart, Hastings, Flint and Evans (24), and Hucker (33) has confirmed the presence of Lactobacillus casei and Lactobacillus bulgaricus in Cheddar cheese.

Torrey (73), in 1915, investigated various methods of isolating aciduric organisms from fecal material. He concluded that Bacillus acidophilus was the only organism, aside from yeasts, capable of growing in acidulated dextrose broth with a pH around 5.5 to 6.0.

In 1916, the work of Hunter and Bushnell (35) again suggested the wide distribution of lactobacilli. They found that the Lactobacillus bulgaricus type was the predominating organism in the fermentation of normal ensilage. Their results were confirmed by the work of Sherman (65) and of Heineman and Hixson (29).

Clark (12), in discussing the acid production of Lactobacillus bulgaricus in artificial media, stated that he found the same pH in ensilage juice, in which certain lactobacilli may have been responsible for the acid production, as in artificial media.

Supplee (70), in his studies on nitrogen distribution in milk inoculated with Lactobacillus bulgaricus, noted a decrease in the casein and albumin nitrogen. There was also a corresponding increase in peptone, monoamino, diamino and ammonia nitrogen. Berman and Rettger (6), in 1918, concluded that an organism capable of inducing a change in the amino nitrogen in milk produced a proteolytic action. They used this method to measure the proteolytic action of an organism.

Lactobacillus bulgaricus is widely distributed in the soil where the temperature is normally lower than in the laboratory. In this connection,

Barthel (5) noted that with Bacillus bulgaricus better growth is obtained in acid soil (pH 5.0 to 6.0) at 22° to 24°C. than at 38°C., in spite of the thermophilic character of the organism in laboratory media.

Orla-Jensen (57) divided the lactic acid producing organisms into the following two groups: (a) Rod forms which belong to the genera Thermobacterium and Streptobacterium; in milk they form only traces of by-products in addition to lactic acid and are spoken of as the true lactic acid types. (b) Rod forms which belong to the genera Bifidobacterium and Betabacterium; in milk they form appreciable amounts of gas and other by-products in addition to lactic acid. Orla-Jensen characterized the Thermobacterium organisms as long rods, not grouped together, and with the appearance of very long thread-like cells. When stained with methylene blue, several species of Thermobacterium often showed dark granules. Organisms included in the genus Thermobacterium require a relatively high temperature for growth. They thrive best at 40° to 50°C., and do not grow under a temperature of 22°C. They form either laevo or inactive lactic acid, attack casein, and play an important part in the ripening of cheeses. Thermobacterium bulgaricum, isolated from yogurt, forms laevo lactic acid and very clearly displays dark granules in the cells. Organisms belonging to the genus Thermobacterium may also be found in human and animal feces.

Allen (2), in 1919, reported the isolation of several organisms, that were identical with Bacillus acidophilus, from the viscous starch and gluten liquors obtained in the wet process of manufacture of products from corn. He did not give a description of his organisms or of the method used in isolation.

Orla-Jensen (56) stated that he had never succeeded in finding Thermobacterium bulgaricum in the feces of adults, even after large doses of yogurt.

In some notes on the lactobacilli, Sherman (66) pointed out that Lactobacillus bulgaricus did not grow at 15°C. and very slowly or not at all below 20°C., while Lactobacillus casei and Lactobacillus acidophilus grew at 20°C. This was the first attempt to separate these organisms on the basis of growth temperatures.

Jötten (38) carried out fermentation and serological studies and observed that Lactobacillus acidophilus and Döderlein's "vaginal bacillus" fermented glucose, levulose, lactose, sucrose, maltose, and mannitol. He noted that the morphological and cultural characteristics of the two organisms were identical. His serological studies also indicated that the organisms were the same. In his complement fixation studies, Jötten obtained cross-fixation with the sera and antigens of both species. However, he did not give complete details as to the technic followed.

Weigmann (80), in 1924, investigated the breakdown of casein in milk by the lactobacilli. His results showed that the soluble nitrogenous products, such as amino acids, increased and had a marked influence on the characteristic flavor of cheese.

Kulp and Rettger (46) made a comprehensive study in the hope of differentiating between Lactobacillus acidophilus and Lactobacillus bulgaricus by the action of the organisms on maltose, sucrose and unheated levulose. They observed that Lactobacillus acidophilus fermented levulose, maltose and sucrose while Lactobacillus bulgaricus did not ferment levulose but did

ferment maltose and sucrose. Kulp and Rettger further reported that the organisms proteolyzed from 2.0 to 6.0 per cent of the milk proteins; this was calculated as the residual nitrogen after the peptone and diamino nitrogen had been determined. An attempt was also made to study the reactions of the sera of two lots of rabbits. One group had been immunized against Lactobacillus acidophilus and the other against Lactobacillus bulgaricus by intravenous injections of cultures of these organisms. The agglutination tests were unsuccessful, due to spontaneous agglutination of the antigens. Complement fixation studies were also attempted. The results showed a cross-fixation between heterologous immune sera and antigens. Quantitative differences were evident in some cases, but these seemed to be no greater than would be expected between different strains of the same species. No complete details were given as to the technic followed.

Pure cultures of Lactobacillus acidophilus were obtained by Kulp and Rettger (46) from rat feces by direct plating, after liberal feeding of dextrin or lactose, and by using Heymann's glucose acetic acid broth as an enrichment medium, without carbohydrate feeding. All the strains of Lactobacillus acidophilus used in the studies of Kulp and Rettger were isolated from typical colonies. These investigators reported that the growth of Lactobacillus acidophilus and Lactobacillus bulgaricus was very slow at 20° to 25°C. At such temperatures, one Lactobacillus bulgaricus culture curdled milk in 12 days while two cultures curdled milk in 30 days; none of the other strains produced a curd and only a few caused a reddening of the litmus.

Cannon (11) used the same principle as Kulp and Rettger in isolating

lactobacilli from fecal material. He isolated 64 strains from feces of adults, by using 0.25 per cent acetic acid dextrose infusion broth with a pH of 5.0. Transfers were made three times at 24 hour intervals before plating on whey or dextrose yeast agar.

In 1924, McIntosh, James and Lazarus-Barlow (50) compared the agglutination reactions of Lactobacillus acidophilus isolated from dental caries, and Lactobacillus acidophilus obtained from the intestinal tract. These investigators obtained cross agglutination of the two groups of organisms. In many instances, the results of the agglutination reactions indicated a close similiarity between the two groups.

Kendall and Haner (41), in their study of the nitrogen and carbohydrate metabolism of various strains of Bacillus acidophilus, indicated that these organisms, when grown for a long time on artificial media, showed an increased acid production from carbohydrates and also that these organisms were incapable of utilizing protein or the higher decomposition products of protein.

Waksman and Lomanitz (79) indicated that, because of the less vigorous formation of ammonia in the presence of carbohydrates, ammonia production serves as a good index of proteolysis only when no available carbohydrates are present. They suggested that in the presence of carbohydrates a study of ammonia production should be supplemented by a study of the formation of amino nitrogen.

In isolating lactobacilli from the feces of calves, Orcutt (54) suggested the use of standard agar plus horse blood, adjusted to a pH of 6.8 to 5.0. In this medium, he found that Bacillus acidophilus colonies formed small greenish zones.

Bunting, Nickerson and Hard (9) isolated and studied a number of organisms from dental caries. They called the organisms Bacillus acidophilus. Their identification studies were not complete and they only compared their organisms with the Lactobacillus acidophilus group from the standpoint of the outstanding characters.

Albus and Holm (1), in 1926, reported that sugar reactions were unreliable for the separation of the various species of lactobacilli. In this respect, they did not agree with Kulp and Rettger (46) and others who emphasized the importance of carbohydrate fermentations as a method of differentiating the various species of lactobacilli.

Kopeloff (42) stated that in the unpublished results of Zoller the non-volatile acid of Lactobacillus acidophilus was found to be entirely dextro lactic acid. Zoller claimed that steromorphism of the lactic acid constituted one point of differentiation between Lactobacillus acidophilus and Lactobacillus bulgaricus. Kopeloff further reported that, among the different strains of lactobacilli studied, the production of volatile acids was rather uniform, 5 to 10 per cent of the total acid being volatile. The volatile acids appeared to be about 50 per cent formic with nearly equal amounts of acetic and propionic.

Pederson, Peterson and Fred (58) stated that whenever a cell produced lactic acid it produced both the laevo and dextro forms ultimately. The ratios between the quantities of the two forms varied with the following factors: Kind of sugar fermented, temperature of incubation, the species of lactic acid organisms used and the presence of organisms which did not produce lactic acid. These investigators said that cultures under the same cultural conditions produced consistently the same ratio for the two forms of acid.

Sherman and Stark (67) studied the distribution of the Lactobacillus group of organisms, with particular reference to dairy products. They reported a predominance of Lactobacillus casei, as compared to Lactobacillus bulgaricus or Lactobacillus acidophilus, in both grade A and ordinary milk. Of the grade A samples, 71 per cent contained Lactobacillus casei in numbers as great as 1 per cc., while only 12 per cent contained Lactobacilus bulgaricus or Lactobacillus acidophilus in like numbers. In the ordinary milk, Lactobacillus casei was present in 94 per cent of the samples in numbers in excess of 10 per cc., while 2.3 per cent of the samples contained the other lactobacilli in like numbers. Sherman and Stark further observed that Lactobacillus bulgaricus and Lactobacillus acidophilus grew at 45°C.; Lactobacillus casei did not grow at this temperature but grew well at 15°C.; the other two types showed no growth at 15°C., even after prolonged incubation. The temperature relationships thus offer another means of differentiating certain of the lactobacilli.

Hyde (36), in 1927, concluded that the Lactobacillus types which are important in milk are able to break down the milk proteins with a corresponding increase in the soluble nitrogen; this occurred with and without the addition of calcium carbonate to the milk. No appreciable differences were noted, as far as the soluble nitrogen production was concerned, among the various types of lactobacilli studied.

Kopeloff and Bass (43), in 1927, reported that three cultures of Lactobacillus acidophilus gave dextro lactic acid in excess of laevo. Hyde and Hammer (37) studied 12 cultures of lactobacilli, 7 of which were isolated from sources that would be expected to yield Lactobacillus acidophilus,

while 5 were obtained from commercial or research laboratories. The lactic acid produced varied from practically pure active to practically pure inactive; the active acid was dextro rotatory in all cases.

Morishita (52) attempted to show differences between Lactobacillus acidophilus and the organisms of dental caries by agglutination reactions. He claimed that there was no cross agglutination between the two groups in dilutions of immune sera higher than 1:1000, while both groups were agglutinated by their homologous sera.

Peterson, Pruess, and Fred (59) studied the proteolytic action of 22 strains of lactobacilli in milk by measuring the production of non-protein nitrogen and amino nitrogen. They found that the non-protein nitrogen was the most abundant form of nitrogen produced, although in some cases there was a decrease rather than an increase of this constituent. The amino nitrogen increased to a certain point and then decreased.

Albus and Holm (1) were the first to study the growth of Lactobacillus acidophilus and Lactobacillus bulgaricus in media of lowered surface tension. Using the drop-weight method for measuring the surface tension, they found that Lactobacillus bulgaricus did not grow when the surface tension of the medium was depressed by sodium ricinoleate to less than 40 dynes. Hyde (36) studied 12 so-called Lactobacillus acidophilus cultures and obtained variable results with respect to growth in a medium at a surface tension of 37.4 dynes. Day and Gibbs (15) did not agree with Kulp and Rettger in regard to the fermentation reactions of Lactobacillus acidophilus and Lactobacillus bulgaricus on maltose, sucrose and levulose as a means of distinguishing between the two species. They also felt that surface tension methods were

inadequate for differentiating these organisms and that whatever differences were obtained were due to the varying toxic action of the surface tension depressant.

The work of Kulp (44) indicated that there are members of the genus Lactobacillus which appear to lie midway between typical Lactobacillus acidophilus and typical Lactobacillus bulgaricus. This investigator observed several of these borderline strains which morphologically and culturally resembled the above organisms, although their growth in culture media was very heavy as compared to either of the two species. Kulp indicated that there is a marked difference in growth between Lactobacillus acidophilus and Lactobacillus bulgaricus but that it should not be given much consideration as a differential characteristic. The borderline organisms failed to survive passage through the digestive tract. Surface tension studies classified them with Lactobacillus bulgaricus. No doubt they were closely related variants of this species.

Later, Kulp (45) attempted to separate Lactobacillus acidophilus from Lactobacillus bulgaricus by the determination of their tolerances for indol and phenol. He believed that the very slight tolerance of Lactobacillus bulgaricus for indol and phenol in vitro experiments explained why this organism was not able to survive passage through the digestive tract.

Rosebury, Linton and Buchbinder (63), in 1929, carried out some serological studies on 30 dental and intestinal strains of lactobacilli, with the idea of establishing a relationship according to their agglutination reactions. Organisms of each group showed marked cross-agglutination with the sera of the other group. On the basis of the results, the authors

felt that there was no need to differentiate between the aciduric organisms of dental caries and Lactobacillus acidophilus of the intestinal tract. Their studies confirmed the work of previous investigators who said that Bacillus acidophilus of Moro was found in the intestinal tract of all animals examined and also indicated that there are certain strains biologically distinct.

Hunt and Rettger (34), in 1930, studied some of the Lactobacillus organisms found in soil, grain and fecal material with reference to their ability to produce acid from lactose. They observed that the 18 strains isolated from soil and grain did not attack lactose very vigorously, while the fecal strains were much more active in this respect.

In 1933, Curran, Rogers and Whittier (14) studied the temperature relationship of various species of lactobacilli. This was done by heavily inoculating litmus milk tubes and holding them in incubators at 10°, 15°, 20° and 30°C., and in water baths at 37°, 40°, 43°, 46°, 48°, 50° and 52°C. Growth was determined by observing changes in the color of the litmus. The optimum growth temperature was between 37° and 40°C. and was uniform for the entire collection. None of the cultures grew at 52°C. and only a few at 50°C. The upper limit for a large number of the cultures was 46°C., and some grew at 43°C. but not at 46°C. In the lower temperature range there was a sharp differentiation, 79 per cent of the cultures failing to show growth in milk at 20°C. The remainder grew at 20°C. and many of these also grew at 15°C. or even 10°C. The results of the studies at low temperatures confirm the work of Sherman and Stark (67).

METHODS

Total Acidity

The total acidity of a skim milk culture was determined by titrating 9 gm. with n/10 sodium hydroxide, using phenolphthalein as the indicator, until a pink color appeared and remained for one minute. The result was calculated as the percentage of lactic acid.

Volatile Acidity

The volatile acidity was determined in a skim milk culture with the method outlined by Michaelian, Farmer and Hammer (51). A 250 gm. portion of the fermented milk, to which had been added 15 cc. of n/1 sulphuric acid and 250 cc. of distilled water, was steam distilled at constant volume until 1 liter of distillate was obtained; this required about 2 hours. The distillate was titrated with n/10 sodium hydroxide, using phenolphthalein as the indicator. The result was expressed as the cubic centimeters of n/10 sodium hydroxide required to neutralize the liter of distillate.

Acetylmethylcarbinol + Diacetyl

The acetylmethylcarbinol + diacetyl value was determined in a skim milk culture, using the procedure followed by Michaelian, Farmer and Hammer (51). A 200 gm. portion of the fermented milk was steam distilled, after

adding 40 cc. of a 40 per cent solution of ferric chloride to oxidize the acetylmethylcarbinol to diacetyl, and the distillate was collected in four 25 cc. fractions. A reagent to precipitate the diacetyl as nickel dimethylglyoximate was prepared by mixing 2 parts of a 20 per cent solution of hydroxylamine hydrochloride, 2 parts of a 20 per cent solution of sodium acetate, and 1 part of a 10 per cent solution of nickel chloride. Ten cc. of this mixture were added to the first 25 cc. fraction of distillate and if this showed a significant precipitate the reagent was added to the second fraction, etc. The distillate, with the added reagent, was allowed to stand 24 to 48 hours, in order to complete crystallization, and the nickel salt was then filtered into a weighed crucible. The salt was washed with distilled water, dried to constant weight at 110°C., and the results expressed as the milligrams of nickel dimethylglyoximate equivalent to acetylmethylcarbinol + diacetyl per 200 gm. of material.

Carbon Dioxide

The production of carbon dioxide was measured by means of Eldredge tubes. Ten cc. of skim milk or 10 cc. of skim milk to which 0.5 per cent peptone had been added were placed in one arm of each tube. The tubes were sterilized for 20 minutes at 15 pounds pressure, cooled to 37°C. and the milk inoculated with 0.5 cc. of a 48 hour milk culture. By means of a sterile pipette, 10 cc. of n/10 barium hydroxide were added to the empty arm of each tube. Uninoculated Eldredge tubes, prepared in the same manner, were used as controls. Absorption of atmospheric carbon dioxide was prevented by closing the upright tubes with sterile rubber stoppers after inoculation. At the end of the

incubation period, back titrations were made, using n/10 oxalic acid with phenolphthalein as the indicator. The carbon dioxide production was calculated from the difference between the inoculated and uninoculated tubes, and the results were expressed as the cubic centimeters of n/10 barium hydroxide neutralized by the carbon dioxide produced.

Fermentation Tests

In studying the fermentation reactions of the cultures, casein digest broth containing 1.0 per cent andrade's indicator was used as the basic medium. The broth was prepared from C.P. casein by the tryptic digestion method described by Kulp and Rettger (46). Five, 10 or 20 per cent solutions of the various sugars, depending upon their solubilities, were prepared in distilled water. All the sugar solutions were sterilized by filtration through Berkefeld filter candles. The sugar solutions were added aseptically to the sterile basic medium in test tubes, by means of a sterile distributing funnel, in sufficient quantities to yield a final concentration of 1.0 per cent.

Reference to the Preparation of Culture Media

The infusion agar was prepared according to the method of Zinsser and Bayne-Jones (83). A sterile lactose solution was added to the infusion agar by means of a sterile distributing funnel so that the final concentrations of the lactose in the medium was 1.0 per cent. The tomato juice agar medium was prepared as outlined by Kulp and White (47) and the casein digest agar medium was prepared according to the method given by Kulp and Rettger (46),

while the cabbage agar and whey agar used in the work were secured from the Difco Laboratories, Detroit, Michigan (3).

Fat Hydrolysis

In studying the action of various cultures of lactobacilli on fat, a nile-blue sulfate medium was used, as suggested by Hammer and Collins (23). The medium was prepared as follows: A 0.1 per cent aqueous solution of nile-blue sulfate was added to beef infusion agar in the proportion of 10 cc. to 100 cc. of the agar, and the medium put into bottles and sterilized. A fat emulsion was prepared by adding 0.2 per cent butter fat to 0.5 per cent agar solution and sterilizing; when the mixture was partially cooled it was thoroughly shaken to emulsify the fat. One cc. of the fat emulsion was placed in a sterile petri dish and 10 cc. of the agar, containing nile-blue sulfate, was poured into the dish and mixed with the emulsion. After the agar had solidified, it was streaked with the organisms.

Isomeric Form of Lactic Acid

The isomeric form of lactic acid was determined by the method outlined by Hammer (22). The fermented milk was heated in a water bath to facilitate the separation of the whey and then filtered through paper; in order to get an adequate separation of the whey and to liberate the lactic acid, 25 cc. of n/1 sulphuric acid were added to 800 cc. of fermented milk just before heating. The whey, to which n/1 sulphuric acid was added at the rate of 5.0 cc. per 100 cc. of whey, was evaporated to a comparatively small volume on a

steam bath. Plaster of paris was then mixed with the whey, usually at the rate of 15 gm. to 100 cc. of the original whey, to take up the remaining water. By breaking up the mass soon after setting had begun, a hard lump was prevented from forming and the material kept in a condition suitable for subsequent handling. The lactic acid was extracted by placing the whey and plaster of paris combination into a thimble and extracting with ether for approximately 25 hours in a Soxhlet fat extractor, with the solvent dripping on the material in the thimble. After transferring the ether and the dissolved material to a beaker, the ether was allowed to evaporate, water added, and then zinc carbonate added in excess.

The material was boiled with animal charcoal to decolorize, filtered, and the insoluble portion washed well with hot water. The filtrate was partially evaporated and allowed to crystallize; after the first crop of crystals had been removed, the filtrate was again partially evaporated and another crop obtained. The zinc lactate was crystallized as completely as possible, dried and finely ground, after which the salt was allowed to dry to constant weight in air.

The type of lactic acid was determined by drying a weighed portion of each salt to practically constant weight at 110°C. and determining the percentage of water of crystallization. The dextro-rotatory and laevo-rotatory forms of the active salts were distinguished by means of a polariscope.

Protein Digestion

An increase in amino nitrogen during the course of the fermentation of

milk was used to measure the proteolytic activity of the various lactobacilli. Three hundred cc. of fermented milk were removed to a large beaker and 200 cc. of 2.5 per cent trichloracetic acid were added to precipitate the proteins (31). The mixture was thoroughly stirred to break up all the lumps and heated to 40°C. for 30 minutes. Then the mixture was filtered through paper and the residue was thoroughly washed with distilled water and discarded. The filtrate, containing the soluble nitrogenous products and other soluble materials, was retained (31).

The filtrate was made barely alkaline to red litmus paper with 50 per cent sodium hydroxide solution, after which it was filtered through paper. The residue was discarded and the filtrate was placed in a 2 liter round bottom flask, with several glass beads, and subjected to vacuum distillation to remove excess water. With the temperature maintained between 40° and 50°C., the volume was reduced to 50 cc. The material was then made faintly acid to blue litmus paper with dilute acetic acid, and filtered through paper into a 100 cc. volumetric flask. The distilling flask was thoroughly rinsed and the volume brought to 100 cc. with distilled water. This volume contained the soluble nitrogenous materials from the 300 cc. of skim milk. Ten cc. of the filtrate were analyzed for amino nitrogen by the Van Slyke nitrous acid method (76), using the marco apparatus. The results were expressed as the number of grams of amino nitrogen in 300 cc. of the original skim milk culture. The difference, if any, between the control and the fermented milk gave the amount of amino nitrogen due to the activity of the organisms.

This modified method varied from the van Slyke nitrous acid method in the preparation of the sample of fermented milk.

EXPERIMENTAL

Selection of a Culture Medium for Isolation

Numerous types of culture media have been used by investigators for the isolation of Lactobacillus organisms. Obviously, a choice of a satisfactory isolation medium had to be made. Preliminary studies were carried out by selecting a number of strains of lactobacilli and preparing plates of an actively growing 48 hour milk culture of each strain with (a) beef infusion agar plus 1.0 per cent lactose, (b) cabbage agar, (c) casein digest agar, (d) whey agar and (e) tomato juice agar. All the plates were incubated at 37°C. for 48 hours, after which the colonies were counted with the aid of a colony counter. The results were expressed as the numbers of organisms per cubic centimeter of the cultures.

Eight cultures of lactobacilli were studied with respect to their ability to grow on different types of culture media; the data secured are presented in Table 1. Each of the cultures showed considerable variation in the numbers of colonies developing on the different media. When grown on beef infusion agar plus 1.0 per cent lactose, the cultures gave counts varying from 1,100,000 to 5,000,000 per cc., on cabbage agar the counts ranged from 16,500,000 to 350,000,000 per cc., on casein digest, from 180,000,000 to 350,000,000 per cc., on whey agar, from 40,000,000 to 380,000,000 per cc. and on tomato juice agar, from 200,000,000 to 450,000,000 per cc. It was evident that tomato juice agar, in most cases, gave higher

TABLE I

BACTERIAL COUNTS OBTAINED WITH DIFFERENT MEDIA ON 48 HOUR MILK CULTURES OF LACTOBACILLI

Plates incubated at 37°C. for 48 hours.

Culture No.	Beef infusion agar + 1% lactose	Cabbage agar	Casein digest agar	Whey agar	Tomato juice agar
a	5,000,000	300,000,000	200,000,000	270,000,000	350,000,000
b	4,000,000	250,000,000	250,000,000	360,000,000	410,000,000
c	1,500,000	20,000,000	180,000,000	220,000,000	370,000,000
d	3,000,000	45,000,000	240,000,000	290,000,000	250,000,000
e	1,200,000	350,000,000	310,000,000	50,000,000	330,000,000
f	4,300,000	150,000,000	350,000,000	40,000,000	200,000,000
g	3,500,000	22,200,000	280,000,000	300,000,000	370,000,000
h	1,100,000	16,500,000	200,000,000	380,000,000	450,000,000

counts per cubic centimeter than any of the other media employed. On the basis of these comparative studies, tomato juice agar was used throughout the work whenever a solid medium was desired.

General Methods of Isolation

The following four methods were used to isolate the various strains of lactobacilli:

a. When the original material was known to contain relatively large numbers of lactobacilli, it was plated directly, using tomato juice agar as the medium. After incubating the plates, colonies were picked into litmus milk.

b. When raw or pasteurized milk was used, a sterile 120 cc. bottle was filled with the milk and the stopper securely tied in with a cord. The bottle of milk was incubated at 37°C. until a stained preparation showed numerous Gram positive rods. Then the milk was plated on tomato juice agar, the plates were incubated and colonies picked into litmus milk.

c. When the material was likely to contain numerous organisms in addition to lactobacilli, a small amount of it was placed in a 120 cc. bottle that was almost full of sterile skim milk to which 0.5 per cent peptone had been added. After the milk was securely stoppered it was incubated at 37°C. until a stained preparation revealed numerous Gram positive rods. Plates were prepared with tomato juice agar, incubated and then colonies were picked into litmus milk.

d. When fecal specimens were used for the isolation of lactobacilli, a special enrichment procedure was followed. The fecal material was col-

lected in a sterile petri dish, to which a small amount of sterile physiological salt solution had been added to make a suspension. By means of a sterile pipette, 1.0 cc. of the fecal suspension was placed in a tube containing 10 cc. of 2.0 per cent dextrose broth, to which 0.5 cc. of n/1 acetic acid had been added. The dextrose acid broth containing the fecal material was incubated at 37°C. for 24 hours, after which 1.0 cc. of the broth was transferred to a second tube of broth and the broth incubated at 37°C. for 12 hours. Then 1.0 cc. of broth was transferred from the second tube to a third tube and this was incubated at 37°C. for 12 hours. The dextrose acid broth was finally plated on tomato juice agar and the plates incubated, after which colonies were picked into litmus milk.

Specific Methods of Isolation

Two samples of commercial acidophilus milk were plated on tomato juice agar. After the plates were incubated at 37°C. for 48 hours, numerous Lactobacillus colonies were present. Eighteen of these colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. Each culture gave a typical acid coagulation of the milk in less than 48 hours and the milk contained Gram positive rods. Of the 18 cultures isolated 3 were retained for study.

A small amount of ensilage was collected in a sterile, glass Mason jar and sterile water added in order to get a suspension of the organisms present. The suspension was separated from the solid material by pouring some of the liquid into a sterile petri dish. The liquid was plated on

tomato juice agar and the plates incubated at 37°C. After an incubation of 48 hours, many plates showed a mold growth which made it difficult to detect any Lactobacillus colonies present. One plate which contained very little mold growth showed a few Lactobacillus colonies. From this plate five colonies were picked into litmus milk and the milk incubated at 37°C. One milk tube showed marked proteolysis after a 24 hour incubation period. After 36 to 48 hours two other isolations failed to produce any changes in the milk. All of these milk tubes were discarded. Two of the isolations showed a typical Lactobacillus growth in the milk and Gram stained preparations of these revealed that they were characteristic Lactobacillus rod forms. The two cultures were retained for further study.

Two samples of corn stover were cut into very fine pieces, placed in two glass Mason jars and a small amount of water was added to each jar. The jars were incubated at room temperature (approximately 28°C.) until the stover juice contained numerous rods suggesting lactobacilli. The juice was plated on tomato juice agar and the plates incubated at 37°C. until characteristic colonies had developed. In many instances a heavy mold growth on the plates obliterated any Lactobacillus colonies that were present. Other plates, which showed only one or two molds, contained a few Lactobacillus colonies, and it was from these plates that five isolations were made into litmus milk. The milk was incubated at 37°C. for 36 to 48 hours. Two tubes of milk failed to show any change and one culture produced only a reddening of the litmus milk with no evidence of coagulation; these three tubes were discarded. The two remaining isolations coagulated the milk in

typical fashion and the milk contained organisms whose morphology suggested lactobacilli. One of these cultures was selected for study.

Strains of lactobacilli were isolated from seven samples of raw milk by filling sterile 120 cc. bottles with the milk and incubating at 37°C. for an indefinite period, usually for 7 to 8 days, until Gram positive rods made up a large percentage of the bacterial flora. The milk was then plated on tomato juice agar and the plates incubated at 37°C. for 48 hours. Mold colonies were present on several of the plates but the mold growth was not as extensive as that on the ensilage and corn stover plates. Several plates showed characteristic Lactobacillus colonies and were entirely free from mold growth. From these plates 12 colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. All of these cultures produced an acid coagulation in the milk with reduction of the litmus. After the cultures were checked for morphology, five of them were retained for study.

Strains of lactobacilli were obtained from two samples of pasteurized milk by following the same procedure as was used with raw milk. The plates poured with the fermented milk showed very few Lactobacillus colonies, although stained preparations of the milk from which the plates were prepared showed numerous Gram positive rods. There were very few mold colonies on the plates. Four characteristic Lactobacillus colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. Two of the cultures failed to produce sufficient acid to coagulate the milk even after they were incubated for 48 hours and were discarded. The re-

maining two cultures coagulated the milk with reduction of the litmus after an incubation period of 36 hours. The morphology of the cultures was checked and one of them was retained for study.

Lactobacilli were isolated from four samples of Cheddar cheese by the following procedure: A plug was taken from each cheese with a sterile trier, after scraping off a small area on the surface of the cheese with a sterile spatula. The lower end of each plug was cut into small pieces and placed in a sterile petri dish. Then with a heavy transfer needle, several small pieces of cheese were placed in a 120 cc. bottle that was nearly full of sterile skim milk to which 0.5 per cent peptone had been added. The four bottles of milk containing the four different samples of cheese were securely stoppered and incubated at 37°C., usually for 7 to 8 days, or until stained preparations of the milk revealed numerous Gram positive rods. Then the milk was plated on tomato juice agar and the plates incubated at 37°C. for 48 hours. Molds were present on some of the plates and on these it was difficult to observe any Lactobacillus colonies. However, many of the plates from the different bottles of milk showed a few Lactobacillus colonies. From these plates a total of 16 isolations were made into litmus milk. The tubes of milk were incubated at 37°C. for 36 to 48 hours. Four of the 16 cultures failed to produce acid in the milk after an incubation of 48 hours. The remaining cultures coagulated the milk with reduction of the litmus after 36 hours. Stained preparations from these cultures revealed characteristic Lactobacillus forms. Four of the 12 cultures were selected for study.

Eight cultures of lactobacilli were obtained from two samples of Swiss

cheese, using the same procedure as for Cheddar cheese. A few molds were noted on some of the plates that were prepared but they were not as numerous as on the plates from the Cheddar cheese. Some of the plates were free from molds and showed a few characteristic Lactobacillus colonies. Eight of these colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. Two cultures showed only a reddening of the litmus after 48 hours and were discarded. The remaining six cultures produced an acid coagulation in the milk after incubating 36 hours. A Gram stained preparation of each culture revealed that the organisms were all typical Lactobacillus forms. Four of the cultures were selected for study.

Two samples of feces were collected from two calves in the Ohio State University dairy herd. The calves were 6 and 12 weeks of age. The calf feces were treated as described under d. of "General Methods of Isolation." The broth from the third tube was plated on tomato juice agar and the plates incubated at 37°C. for 48 hours. The plates were examined and mold and yeast colonies were noted on some of them. Other plates failed to show any growth, while two showed numerous Lactobacillus colonies. Twelve colonies from these two plates were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. Four cultures did not coagulate the milk until after an incubation of 48 hours, while five produced only a reddening of the litmus; all these cultures were discarded. Three cultures produced a firm coagulation in the milk after 36 hours of incubation and stained preparations revealed characteristic Lactobacillus forms. One of these cultures was retained for study.

Two samples of dog feces were collected from the Ohio State Veterinary Clinic from two different dogs. These animals had been fed liberal amounts of acidophilus milk to correct intestinal disturbances. The same general procedure as for the calf feces was followed in passing the fecal specimens through a series of dextrose acid broth tubes. The third tube of broth was plated on tomato juice agar and the plates incubated at 37°C. for 48 hours. Four plates failed to show any growth except a few mold colonies. The other plates showed a few characteristic Lactobacillus colonies. Five colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. Three cultures failed to show any changes in the milk after 48 hours of incubation and were discarded. Two cultures coagulated the milk after an incubation period of 36 hours. These cultures contained characteristic Lactobacillus rod forms and one was retained for study.

Seven samples of infant feces were collected from a ward in Ohio State University Hospital. The ages of the infants ranged from 6 months to 2 years. Again following the general procedure suggested under d. of "General Methods of Isolation," the third tube of broth was plated on tomato juice agar and the plates incubated at 37°C. for 48 hours. Out of the 21 plates prepared from the different samples, 4 showed a few characteristic Lactobacillus colonies along with a few yeast colonies and the remaining 17 plates failed to show any lactobacilli. Five Lactobacillus colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. After 48 hours of incubation, one tube failed to show any change in the milk and another showed only a reddening of the litmus; these were discarded. The remaining three cultures from different samples of feces

coagulated the milk with reduction of the litmus within 48 hours. A stained preparation of each culture indicated that they were typical Lactobacillus forms. These three cultures were retained for further study.

Five samples of rat feces were collected from different rats used for nutritional studies in the Department of Agricultural Chemistry, Ohio State University. The animals were being fed liberal amounts of milk along with a modified diet. Using the procedure outlined under d. of "General Methods of Isolation," the third tube of acid broth was plated on tomato juice agar and the plates incubated at 37°C. for 48 hours. The plates were examined for typical Lactobacillus colonies but only a few were found. The presence of yeasts and other organisms made it difficult to isolate the organisms. However, from the various plates, three Lactobacillus colonies were picked into litmus milk and the milk incubated at 37°C. for 36 to 48 hours. One culture was contaminated and finally discarded. The other two cultures produced a typical acid coagulation in milk in less than 48 hours. These were checked for morphology by Gram stained preparations and one culture was retained for further study.

Sources of Organisms

A total of 36 cultures of lactobacilli were studied. Ten of these were obtained from various investigators. Six cultures of L. acidophilus were collected from the following sources: two from Kulp (rat and human strains) and one from Rettger, Yale University; one from Sarles, Iowa State College; one from Arnold, Colorado State College; and one from Myers, United

States Department of Agriculture. Two cultures of L. bulgaricus were also collected: one from Kulp, Yale University, and one from the Bureau of Dairy Industry, Washington, D. C. Two cultures of lactobacilli were obtained from Lane, Iowa State College: one was isolated from raw milk and one from Cheddar cheese. The remaining 26 cultures were isolated from various sources. Most of them were obtained from dairy products, since the main interest in the organisms was in their action on milk, but a number of cultures were isolated from feed and from human and animal feces. Five of the cultures were isolated from raw milk, one from pasteurized milk, three from commercial acidophilus milk, four from cheddar cheese, four from Swiss cheese, two from ensilage, one from corn stover, one from calf feces, one from dog feces, three from infant feces and one from rat feces. Table II gives the source of each of the organisms studied.

TABLE II

SOURCES OF THE ORGANISMS STUDIED

Culture No.	Source
1	L. acidophilus obtained from Kulp (rat strain)
2	L. acidophilus obtained from Kulp (human)
3	L. acidophilus obtained from Sarles (x-type)
4	L. acidophilus obtained from Arnold (milk)
5	L. acidophilus obtained from Myers (r-L-8A)
6	L. acidophilus obtained from Rettger (human strain)
7	L. bulgaricus obtained from Kulp (milk strain)
8	L. bulgaricus obtained from Rogers (cheese strain)
9	Lactobacillus obtained from Lane (milk strain)
10	Lactobacillus obtained from Lane (Cheddar cheese strain)
11	Lactobacillus isolated from raw milk
12	Lactobacillus isolated from raw milk
13	Lactobacillus isolated from raw milk
14	Lactobacillus isolated from raw milk
15	Lactobacillus isolated from raw milk
16	Lactobacillus isolated from pasteurized milk
17	Lactobacillus isolated from commercial acidophilus milk
18	Lactobacillus isolated from commercial acidophilus milk
19	Lactobacillus isolated from commercial acidophilus milk
20	Lactobacillus isolated from Cheddar cheese
21	Lactobacillus isolated from Cheddar cheese
22	Lactobacillus isolated from Cheddar cheese
23	Lactobacillus isolated from Cheddar cheese
24	Lactobacillus isolated from Swiss cheese
25	Lactobacillus isolated from Swiss cheese
26	Lactobacillus isolated from Swiss cheese
27	Lactobacillus isolated from Swiss cheese
28	Lactobacillus isolated from ensilage
29	Lactobacillus isolated from ensilage
30	Lactobacillus isolated from corn stover
31	Lactobacillus isolated from calf feces
32	Lactobacillus isolated from dog feces
33	Lactobacillus isolated from infant feces
34	Lactobacillus isolated from infant feces
35	Lactobacillus isolated from infant feces
36	Lactobacillus isolated from rat feces

STUDIES ON BIOCHEMICAL FEATURES

Because of the wide distribution of the lactobacilli in dairy products the action of these organisms on milk and its derivatives is of special importance. The results obtained in the studies on the chemical changes produced in milk are presented under various headings.

A. The Production of Total Acid, Volatile Acid and Acetylmethylcarbinol + Diacetyl in Milk

The 36 cultures of lactobacilli were investigated for their general action in milk by determining the production of total acid, volatile acid and acetylmethylcarbinol + diacetyl, after incubation at 37°C. for 7 days. The results obtained are given in Table III.

There was considerable variation in the amounts of total acid formed by the various cultures, the values ranging from 0.50 to 2.34 per cent calculated as lactic acid. No differences were noted in the appearance of litmus milk cultures of the organisms at the end of the incubation period and all the organisms produced a smooth curd with no evidence of gas or proteolysis. Cultures that produced relatively large amounts of total acid sometimes showed a tendency to "whey off." There was very little difference in the rate of coagulation of litmus milk with the various organisms. Culture 25 when first isolated coagulated milk rather slowly, but after the second transfer in litmus milk the rate of coagulation was about the same as for the other cultures.

TABLE III

THE PRODUCTION OF TOTAL ACID, VOLATILE ACID AND ACETYLMETHYLCARBINOL + DIACETYL IN MILK

Cultures incubated at 37°C. for 7 days.

Culture No.	Per cent total acid	Volatile acid*	mg. of Ni salt equiv. to amc + aa** per 200 gm.
1	2.02	39.0	trace
2	2.00	28.5	1.8
3	2.34	36.3	1.8
4	0.85	41.0	0.0
5	2.17	42.6	trace
6	2.10	35.7	1.2
7	2.31	31.8	0.0
8	1.50	34.5	trace
9	1.40	35.8	10.8
10	0.68	11.2	3.0
11	1.25	29.3	0.5
12	1.85	35.3	2.8
13	0.80	28.2	10.0
14	0.90	38.2	8.9
15	0.90	40.6	trace
16	1.11	28.6	1.3
17	0.96	27.3	0.0
18	1.21	16.9	trace
19	1.01	16.8	0.8
20	0.80	31.0	8.3
21	1.12	10.4	trace
22	1.34	30.9	1.5
23	0.90	22.4	0.0
24	0.80	21.5	2.1
25	0.50	10.0	trace
26	0.58	35.3	0.9
27	1.70	11.1	29.1
28	2.01	11.6	1.9
29	0.90	27.0	trace
30	1.19	27.5	0.0
31	2.05	36.0	11.8
32	1.80	29.4	17.4
33	1.50	27.8	0.0
34	1.90	29.1	0.0
35	2.10	28.9	5.5
36	0.80	21.4	1.6

*Volatile acid expressed as cc. n/10 NaOH required to neutralize the first liter of distillate obtained when a 250 gm. portion of culture was steam distilled after adding 15 cc. n/1 sulfuric acid.

**amc + aa = acetylmethylcarbinol + diacetyl.

All of the cultures produced an appreciable quantity of volatile acid. The value for the 36 cultures ranged from 10.0 to 42.6. A comparison of the production of total acid and of volatile acid is given in the following summary:

Cultures producing total acidities from	Produced volatile acidities from	Number of cultures
0.50 to 1.00	10.0 to 41.0	13
1.01 to 1.50	10.4 to 35.8	10
1.51 to 2.00	11.1 to 35.3	5
2.01 to 2.50	11.6 to 42.6	8

The summary shows that the production of volatile acid was not related to the total acid formed since the volatile acidities varied widely in each of the total acid groups and the minimum and maximum values for the different groups were much the same.

The yield of nickel salt equivalent to acetylmethylcarbinol + diacetyl with the various cultures ranged from 0.0 to 29.1 mg., with 7 of the 36 cultures giving values of 0.0. Only five of the cultures yielded 10.0 or more mg. of nickel salt and only one yielded more than 20.0 mg. The following summary compares the production of total acid with the values for nickel salt equivalent to acetylmethylcarbinol + diacetyl for the various cultures:

Cultures producing total acidities from	Yielded mg. of Ni salt from	Number of cultures
0.50 to 1.00	0.0 to 10.0	13
1.01 to 1.50	0.0 to 10.8	10
1.51 to 2.00	0.0 to 29.1	5
2.01 to 2.50	0.0 to 11.8	8

From the summary it is evident that the production of acetylmethylcarbinol + diacetyl was not correlated with the formation of total acid. Each of the total acid groups contained organisms that did not yield acetylmethylcarbinol + diacetyl and with three of the four groups, including those representing the highest and lowest acid producers, the maximum production by an organism in the group was essentially the same.

The results indicated that there was no direct relationship between the amounts of total acidity, volatile acidity, and acetylmethylcarbinol + diacetyl formed by the cultures, regardless of the sources from which the organisms were isolated.

B. The Production of Volatile Acid and Acetylmethylcarbinol + Diacetyl When 0.15 Per cent Citric Acid was Added to the Milk

The effect of the addition of citric acid on the production of volatile acid and acetylmethylcarbinol + diacetyl in milk was studied with the 36 cultures by adding 0.15 per cent citric acid to the milk at the time of inoculation, incubating at 37°C. for 7 days and then determining the volatile acid and acetylmethylcarbinol + diacetyl values. Table IV gives the data obtained.

TABLE IV

THE PRODUCTION OF VOLATILE ACID AND ACETYLMETHYLCARBINOL + DIACETYL WHEN 0.15 PER CENT CITRIC ACID WAS ADDED TO THE MILK

Cultures incubated at 37°C. for 7 days.

Culture No.	Milk alone*		Milk plus 0.15% citric acid	
	Volatile acid	mg. of Ni salt equiv. to amc + aa per 200 gm.	Volatile acid	mg. of Ni salt equiv. to amc + aa per 200 gm.
1	39.0	trace	35.0	11.1
2	28.5	1.8	19.3	trace
3	36.3	1.8	40.0	2.1
4	41.0	none	42.0	trace
5	42.6	trace	23.0	3.2
6	35.7	1.2	29.0	0.8
7	31.8	none	36.4	0.9
8	34.5	trace	37.1	1.9
9	35.8	10.8	12.2	2.0
10	11.2	none	15.1	1.0
11	29.3	0.5	23.5	0.9
12	35.3	2.8	30.4	trace
13	28.2	10.0	33.7	13.6
14	38.2	8.9	34.6	9.1
15	40.6	trace	44.5	2.1
16	28.6	1.3	27.3	8.0
17	27.3	none	30.2	1.5
18	16.9	trace	19.4	1.0
19	16.8	0.8	21.2	none
20	31.0	8.3	18.9	trace
21	10.4	trace	16.1	2.3
22	30.9	1.5	33.2	2.0
23	22.4	none	16.4	1.5
24	21.5	2.1	25.4	3.5
25	10.0	1.3	15.9	trace
26	35.3	0.9	31.4	1.1
27	11.1	29.1	21.2	1.8
28	11.6	1.9	13.5	2.1
29	27.0	trace	27.0	1.7
30	27.5	none	31.2	trace
31	36.0	11.8	39.0	9.3
32	29.4	17.4	37.2	21.2
33	27.8	none	19.8	trace
34	29.1	none	34.8	trace
35	28.9	5.5	29.5	3.5
36	21.4	1.6	28.4	2.2

*These data are taken from Table III.

The addition of citric acid to the milk apparently had little effect on the production of volatile acid by the organisms. The values obtained with the citric acid added ranged from 12.2 to 44.5 while those in the controls ranged from 10.0 to 42.6. Twenty-two of the cultures gave higher volatile acidities with citric acid while 13 gave lower values, and with 1 there was no difference but, in general, the differences were not significant. The addition of 0.15 per cent citric acid approximately doubles the citric acid content of the original milk so that if citric acid is a source of volatile acid the added citric acid should greatly increase the volatile acid formed.

The addition of citric acid had no significant effect on the production of acetylmethylcarbinol + diacetyl by the organisms in milk. The values for nickel salt obtained on the milk cultures with the citric acid added ranged from 0.0 to 29.1 mg. while those for the controls ranged from 0.0 to 21.1 mg. Twenty-six of the cultures gave higher values with citric acid added while 10 gave lower values. There was considerable variation in the amounts of acetylmethylcarbinol + diacetyl formed, both with and without citric acid added, and, in general, the differences between the values for the milk with citric acid and without the acid were not significant.

It was interesting to note that some of the cultures which produced relatively small amounts of acetylmethylcarbinol + diacetyl showed appreciable increases of these compounds when citric acid was added to the milk, while those cultures that produced comparatively large amounts of acetylmethylcarbinol + diacetyl did not show large increases of these compounds.

C. The Production of Acetylmethylcarbinol + Diacetyl When Various Concentrations of Acetaldehyde Were Added to the Milk

The effect of adding various concentrations of acetaldehyde on the production of acetylmethylcarbinol + diacetyl in milk was studied with 10 cultures of lactobacilli as follows: Five 100 cc. portions of sterile skim milk in bottles were inoculated with 0.5 cc. of an actively growing milk culture of an organism and incubated at 37°C. for 12 hours. Different concentrations of acetaldehyde were then added to four of the bottles and the remaining one was used as a control; the concentrations of acetaldehyde used were 0.05, 0.1, 0.3, and 0.4 per cent. After incubating at 37°C. for 7 days, acetylmethylcarbinol + diacetyl determinations were made on the various lots. The data obtained are given in Table V.

From the results, it appeared that the addition of various concentrations of acetaldehyde to the milk did not appreciably increase the production of acetylmethylcarbinol + diacetyl. The values for nickel salt, equivalent to acetylmethylcarbinol + diacetyl, varied widely both in the controls and in the milk to which the various concentrations of the acetaldehyde had been added; the values for the controls ranged from a trace to 23.2 mg. while those for the milk with acetaldehyde added ranged from 0.0 to 26.0 mg.

With 0.05 per cent acetaldehyde added the values for the nickel salt ranged from 1.8 to 25.1 mg. Eight of the cultures showed increases over the controls with the acetaldehyde added while two showed decreases but in no instance was the difference great.

TABLE (V)[3]

THE INFLUENCE OF VARIOUS CONCENTRATIONS OF ACETALDEHYDE ON THE PRODUCTION OF ACETYLMETHYLCARBINOL + DIACETYL IN SKIM MILK

Cultures incubated at 37°C. for 7 days.

Culture No.	mg. Ni salt equiv. to amc + aa per 200 gm.				
		Concentration of acetaldehyde			
	Control	0.05%	0.1%	0.3%	0.4%
9	8.4	9.1	8.0	trace	0.0
10	2.8	5.5	6.2	3.1	0.0
11	0.7	1.8	0.9	trace	0.0
13	9.1	10.4	10.9	6.3	0.0
20	5.1	2.0	0.0	0.0	0.0
21	trace	3.6	4.0	0.0	0.0
27	23.2	25.1	26.0	21.2	0.0
28	2.3	2.5	3.4	3.9	0.0
31	12.4	11.2	12.9	8.4	0.0
32	16.1	19.5	20.7	19.2	0.0

With 0.1 per cent acetaldehyde added to the milk the nickel salt values ranged from 0.0 to 26.0 mg. Eight of the organisms gave increases, as compared to the controls, in acetylmethylcarbinol + diacetyl with the aldehyde added while two gave decreases but in no instance was the difference great. One culture failed to produce any acetylmethylcarbinol + diacetyl with acetaldehyde while a considerable quantity was produced in the control.

With 0.3 per cent acetaldehyde added to the milk the acetylmethylcarbinol + diacetyl values ranged from 0.0 to 21.2 mg. nickel salt. In three instances there were increased amounts formed with the acetaldehyde while in seven there were decreases. Four of the organisms produced no more than a trace of acetylmethylcarbinol + diacetyl with the aldehyde added. In general, the results obtained suggest that the acetaldehyde was slightly toxic in the concentration used.

With 0.4 per cent acetaldehyde the milk failed to coagulate and none of the cultures produced any acetylmethylcarbinol + diacetyl. This indicates, that 0.4 per cent acetaldehyde in milk was definitely toxic to the organisms used.

From the results obtained it was evident that concentrations of 0.05 or 0.1 per cent acetaldehyde produced slight increases in acetylmethylcarbinol + diacetyl in milk in many instances, while in several instances small decreases were noted but in every case the difference was small. The addition of 0.3 or 0.4 per cent acetaldehyde to the milk appeared to be toxic for the organisms used.

D. The Production of Carbon Dioxide in Milk and in Milk Plus 0.5 Per cent Peptone

The production of carbon dioxide in milk and in milk plus 0.5 per cent peptone was studied with the 36 cultures, by means of Eldredge tubes using both 10 and 20 days of incubation at 37°C. The data are given in Table VI.

It appeared from the results that most of the organisms studied were capable of producing appreciable amounts of carbon dioxide in milk and in milk to which 0.5 per cent peptone had been added; only one organism (No. 16) failed to produce any detectable carbon dioxide in either type of milk. The values obtained for carbon dioxide varied widely both in the plain milk and in milk plus peptone. The values in the plain milk with an incubation period of 10 days ranged from 0.0 to 5.1, while in milk plus 0.5 per cent peptone they ranged from 0.0 to 5.5. When the incubation period was extended to 20 days the values for plain milk ranged from 0.0 to 8.1, while in milk plus 0.5 per cent peptone the values ranged from 0.0 to 9.7.

With an incubation period of 10 days, 3 of the cultures failed to produce any carbon dioxide in plain milk and 33 gave appreciable amounts, while in the peptone milk 8 cultures failed to produce any carbon dioxide and 28 of them formed appreciable amounts. There was considerable variation, both in the plain and in the peptone milk, in the amounts of carbon dioxide produced but, in general, the plain milk gave the higher values; in 28 instances the values for the plain milk cultures were the higher, in

TABLE VI

THE PRODUCTION OF CARBON DIOXIDE IN MILK AND IN MILK PLUS 0.5 PER CENT PEPTONE

Cultures incubated at 37°C. for 10 or 20 days.

Culture No.	cc. n/10 Barium hydroxide equiv. to CO_2 from 10 cc. of milk			
	Period of incubation			
	10 days		20 days	
	Plain milk	Milk plus 0.5% peptone	Plain milk	Milk plus 0.5% peptone
1	4.6	3.3	5.9	5.5
2	4.0	3.9	2.2	3.6
3	5.1	0.0	2.4	9.7
4	4.3	3.1	4.9	6.1
5	4.4	3.6	5.7	5.1
6	5.0	4.1	2.6	0.7
7	3.0	2.8	3.0	3.1
8	2.1	2.7	0.0	0.0
9	3.3	3.8	7.3	0.0
10	1.5	2.8	4.0	3.8
11	3.4	0.0	0.0	0.0
12	0.0	0.0	6.8	7.1
13	2.8	1.9	4.9	5.1
14	2.6	2.3	5.1	0.0
15	3.0	2.8	7.4	0.0
16	0.0	0.0	0.0	0.0
17	3.4	3.3	6.7	0.0
18	3.7	3.7	8.0	7.3
19	4.1	4.0	0.0	6.0
20	2.6	0.0	4.6	0.0
21	1.6	1.0	6.1	5.8
22	3.0	0.0	7.2	6.4
23	3.6	3.5	8.0	0.0
24	1.9	1.8	3.9	0.0
25	1.3	0.0	5.0	0.9
26	2.9	2.3	6.3	5.4
27	3.4	1.7	6.9	6.7
28	0.0	2.4	5.5	5.4
29	2.0	1.8	0.0	0.0
30	2.4	2.0	7.0	7.2
31	3.5	0.0	7.0	0.0
32	4.6	5.5	8.1	0.0
33	4.4	3.8	5.3	0.0
34	4.6	3.1	4.4	4.2
35	3.7	3.3	0.0	0.0
36	3.9	3.5	5.0	3.1

3 instances there were no differences and in 5 instances the peptone milk gave the higher values.

With an incubation period of 20 days, 6 of the cultures failed to produce any carbon dioxide in plain milk, and 30 cultures produced appreciable amounts, while in peptone milk 15 cultures failed to produce any carbon dioxide, and 21 formed varying amounts. There was considerable variation in the amounts of carbon dioxide produced both in the plain and in the peptone milk but, in general, the higher values were obtained in the plain milk; in 23 instances the values for the plain milk were the higher, in 4 there were no differences and in 9 the peptone milk cultures gave the higher values.

The results indicate that, in general, the lactobacilli produced greater amounts of carbon dioxide in plain milk than in milk to which 0.5 per cent peptone had been added and that the values secured after 20 days incubation were generally higher than those secured after 10 days.

E. The Production of Carbon Dioxide in Milk at Different Temperatures

The production of carbon dioxide in milk incubated at different temperatures was studied with 10 cultures of lactobacilli, by comparing the amounts of carbon dioxide formed at 21°C. with the amounts formed at 37°C., using a 15-day period of incubation at each temperature. The data obtained are given in Table VII.

All of the cultures studied were capable of producing appreciable

TABLE VII

THE PRODUCTION OF CARBON DIOXIDE IN SKIM MILK AT DIFFERENT TEMPERATURES

Cultures incubated at 21° or 37°C. for 15 days.

Culture No.	cc. n/10 Barium hydroxide equiv. to CO_2 from 10 cc. milk	
	21°C.	37°C.
10	5.0	6.8
13	4.5	5.1
14	1.8	4.6
20	0.7	8.0
21	2.5	7.4
26	1.9	4.0
27	2.2	4.9
28	4.1	5.3
29	1.6	5.0
30	3.0	6.4

amounts of carbon dioxide in milk incubated at either 21° or 37°C. The values for the cultures incubated at 21°C. ranged from 0.7 to 5.0 while the values for those incubated at 37°C. ranged from 4.0 to 8.0.

It was evident that all of the cultures produced less carbon dioxide 21°C. than they did at 37°C. and that there was more variation in the carbon dioxide values at 21°C. than at 37°C. It appeared that cultures of lactobacilli capable of growth in milk were able to produce appreciable amounts of carbon dioxide after a 15-day incubation at either 21° or 37°C., although greater amounts were formed at 37° than at 21°C.

F. The Fermentation Reactions with the Production of Acid

The 36 cultures of lactobacilli were studied from the standpoint of their general fermentation reactions, using casein digest broth plus 1.0 per cent andrade's indicator as the basic medium. This medium was prepared from C. P. casein by the typtic digestion method, described by Kulp and Rettger (46). Five, 10 or 20 per cent solutions of the various materials, depending upon their solubility, were prepared in distilled water. All the solutions were sterilized by filtration through Berkefeld filter candles and were added aseptically to the sterile basic medium in test tubes by means of a sterile distributing funnel, in sufficient quantity to yield a final concentration of 1.0 per cent. The cultures were prepared for inoculation as follows: Each culture was grown in 10 cc. of casein digest broth, to which 0.1 per cent glucose had been added, and incubated at 37°C. for 48 hours. The organisms were sedimented by centrifuging,

the supernatant fluid decanted off, and the organisms resuspended three times in 10 cc. amounts of sterile physiological salt solution and the last suspension was the inoculum used. One-tenth cc. of each culture was inoculated into 3 cc. of basic medium plus indicator plus 1.0 per cent of a sterile sugar solution. After the cultures had been incubated at 37°C. for 4 days, together with the uninoculated controls, they were observed for acid production. The results are given in Table VIII.

The data indicate that out of the 36 cultures studied, 2 of the organisms attacked arabinose, 35 dextrose, 27 dextrin, 3 dulcitol, 14 galactose, 6 inulin, 36 lactose, 23 levulose, 17 maltose, 2 mannitol, 10 raffinose, 11 salicin, 24 sucrose and 2 xylose. No gas production was noted in any of the cultures studied.

All of the six cultures of L. acidophilus obtained from the various research laboratories fermented dextrose, lactose, levulose and maltose, while none of them fermented arabinose, dulcitol, inulin and mannitol; five attacked dextrin, four galactose, two raffinose, two salicin, two sucrose and one xylose. The two cultures of L. bulgaricus obtained from research laboratories fermented dextrose, lactose, levulose and salicin but not arabinose, dulcitol, galactose, inulin, mannitol, raffinose and xylose; one fermented dextrin and sucrose but not maltose, while the other fermented maltose but not dextrin and sucrose.

Kulp and Rettger (46) claimed that L. acidophilus and L. bulgaricus could be separated on the basis of their ability to attack levulose. They stated that L. acidophilus fermented levulose while L. bulgaricus was unable to attack this sugar. However, when the sugar fermentation studies

TABLE VIII

FERMENTATION REACTIONS OF VARIOUS CULTURES OF LACTOBACILLI

Cultures incubated at 37°C. for 4 days.

Sugar	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-
Galactose	+	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	-	-	+	-	-	+	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levulose	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
Raffinose	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	-	+
Salicin	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+
Xylose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: Acid production indicated by +. No change in the sugar medium indicated by -.

were carried out with the pure cultures of L. acidophilus and L. bulgaricus obtained from various research laboratories, it was found that both these organisms attacked levulose.

The two cultures of lactobacilli obtained from Lane attacked dextrose, dextrin and lactose but not arabinose, dulcitol, galactose, inulin, mannitol, raffinose, salicin and xylose; one of the organisms fermented levulose, maltose and sucrose while the other did not.

The results revealed that there was a considerable variation in the fermentation reactions among the cultures of lactobacilli studied; this was true not only for the organisms isolated from several sources but also for the six cultures of L. acidophilus and the two cultures of L. bulgaricus secured from various research laboratories. Because of the variations, the fermentation reactions could not be used as a basis for separation of the lactobacilli into species.

G. The Lipolytic Activity of the Organisms upon Butterfat

The relative merits of various plating media for the detection of lipase producing bacteria were investigated by Turner (74). He concluded that nile-blue sulfate medium gave a sharper differentiation and a higher degree of sensitivity than other types of media used. In the nile-blue sulfate medium the dispersed fat appears as pink globules. When these globules are attacked by an organism capable of breaking down the fat the globules change from a pink color to a deep blue.

The lipolytic activity of 36 cultures were studied by streaking the

organisms upon nile-blue sulfate medium, which consisted of beef infusion agar containing nile-blue sulfate and dispersed butterfat. The general procedure employed was as follows: Approximately 12 cc. amounts of nile-blue sulfate medium were poured into 40 sterile petri dishes and allowed to harden. Each culture was then streaked on the surface of the medium, using one plate for each culture. Four uninoculated nile-blue sulfate plates were used as controls throughout this study. The plates were incubated at 37°C., examined after 4 days and then again after 6 days, using a small hand lens to study the color of the fat globules. From the results obtained, it appeared that very few of the organisms were able to grow on the medium and thus were unable to break down the dispersed fat in the medium.

The main interest in this study was to determine the ability of various strains of lactobacilli to attack fat. Since the organisms were unable to grow to any extent in the nile-blue sulfate medium as prepared by Hammer and Collins (23), an attempt was made to substitute tomato juice agar for the beef infusion agar in the medium. A series of plates was prepared with this medium and the organisms streaked on the surface of the agar in the manner previously described. The plates were incubated at 37°C. and examined in 4 days and again in 6 days. It was noted that 25 of the cultures, streaked on the nile-blue sulfate tomato juice agar medium, showed growth after 4 days and additional cultures showed growth after 6 days, but 4 failed to show any growth even after the 6-day period. The appearance of the fat globules in the medium were practically unchanged

by any of the organisms studied, therefore it was concluded that, with the possible exception of the four cultures which failed to grow in this medium, none of the lactobacilli studied were able to attack the fat.

H. The Isomeric Form of Lactic Acid Produced in Milk

The isomeric form of lactic acid produced in milk was studied with 30 of the cultures of lactobacilli. The organisms were inoculated into skim milk and the milk incubated at 37°C. for 5 days, after which the zinc salts were prepared. Determinations of the optical activity and percentage of water of crystallization in the zinc salts were made with all of the 30 cultures used and with 3 of them the percentages of zinc oxide in the zinc salts were also determined. The data obtained are given in Table IX.

The percentages of water of crystallization in the zinc salts varied from 12.87 to 16.95. These values indicate that the acids were largely active because the theoretical value for pure active acid is 12.89 and for pure inactive 18.18. Nineteen cultures gave zinc salts that were laevo-rotatory, indicating that the free acid was dextro-rotatory; the water of crystallization for this group ranged from 12.87 to 15.26 per cent. Nine cultures gave zinc salts that were optically inactive; the percentages of water of crystallization ranged from 15.30 to 16.78. Two cultures gave zinc salts that were slightly dextro-rotatory; the water of crystallization values were 16.95 and 15.91 per cent.

The three cultures of L. acidophilus produced dextro-rotatory acid;

TABLE IX

THE ISOMERIC FORM OF LACTIC ACID PRODUCED IN MILK BY CULTURES OF LACTOBACILLI

Cultures incubated at 37°C. for 5 days.

Culture No.	Water of crystallization in zinc salt		Average	ZnO in water free salt	Rotation of zinc salt
	Determination A %	Determination B %	%		
1	13.92	14.10	14.01		l
3	13.71	13.83	13.77		l
5	13.96	14.15	14.05		l
7	15.47	15.65	15.56		0
8	16.80	17.10	16.95	33.74	very slight d
9	15.71	16.11	15.91		very slight d
10	16.35	16.47	16.41		0
11	15.23	15.37	15.30		0
12	12.95	12.85	12.90		l
13	12.95	12.99	12.97		l
16	13.41	13.93	13.67		l
17	12.83	12.91	12.87		l
18	13.46	13.63	13.54		l
19	14.11	14.21	14.16		l
20	16.15	16.23	16.19		0
21	15.23	15.29	15.26	33.78	l
22	15.97	16.10	16.03		0
24	16.27	16.33	16.30		0
25	13.83	13.90	13.86		l
26	14.58	14.65	14.61		l
27	13.83	13.91	13.87		l
28	16.11	16.19	16.15		0
29	16.81	16.72	16.76		0
30	16.76	16.81	16.78		0
31	13.12	13.19	13.16		l
32	14.07	14.19	14.13		l
33	14.13	14.21	14.17		l
34	13.36	13.41	13.38	33.85	l
35	14.06	14.23	14.14		l
36	12.89	12.94	12.91		l

the percentages of water of crystallization in the zinc salts were 14.01, 13.77 and 14.05. These data indicate that the acid found was largely active.

One of the two cultures of L. bulgaricus obtained from research laboratories produced inactive acid and the other one slightly laevo-rotatory acid; the percentage of water of crystallization in the zinc salt from the inactive acid was 15.56 and from the laevo-rotatory acid 16.95. These data indicate that the acids formed by the L. bulgaricus cultures were largely inactive.

The sixteen strains of lactobacilli obtained from dairy products, such as raw and pasteurized milk, commercial acidophilus milk, Cheddar cheese and Swiss cheese, gave zinc salts in which the percentage of water of crystallization ranged from 12.87 to 16.41. Ten of these cultures formed dextro-rotatory acid, with the values for water of crystallization in the zinc salts ranging from 12.87 to 15.26 per cent; five formed inactive acid with the values for the zinc salt ranging from 16.03 to 16.41; and one formed laevo-rotatory acid with a zinc salt value of 15.91.

The three cultures isolated from ensilage and corn stover gave inactive acid; the water of crystallization values for the zinc salts ranged from 16.15 to 16.78 per cent.

The six cultures of lactobacilli isolated from fecal material formed dextro-rotatory acids; the water of crystallization values for the zinc salts ranged from 12.91 to 14.17 per cent.

The percentages of zinc oxide were determined in the water-free zinc salts prepared from the acids produced by three cultures (Nos. 8, 21 and 34). The values obtained were 33.74, 33.78 and 33.85 per cent, respectively; these suggest lactic acid, since the theoretical ZnO for anhydrous zinc lactate is 33.46 per cent.

From the results of the study with the various cultures of lactobacilli, it was evident that the type of lactic acid produced was not uniform but varied from pure active to practically pure inactive, with mixtures of these two acids present between the active and inactive acid. The active acid was a dextro form in nearly every instance.

I. The Increase of Amino Nitrogen in Skim Milk

The action of 26 cultures of lactobacilli in increasing the amino nitrogen of skim milk was studied as follows: A set of four bottles each containing 300 cc. of sterile skim milk was inoculated with 0.5 cc. of an actively growing milk culture; similarly, another set of four bottles containing 300 cc. of sterile skim milk, to which a small amount of calcium carbonate had been added, were inoculated with 0.5 cc. of the culture. All of the inoculated milk, together with uninoculated controls, was incubated at 37°C. for 2, 4, 6 and 8 weeks. At the end of each two week period one bottle of plain milk, one bottle of milk to which calcium carbonate had been added and an uninoculated control were removed from the incubator and the amino nitrogen determined. The results obtained are given in Table X.

TABLE X

INCREASE OF AMINO NITROGEN IN MILK BY VARIOUS CULTURES OF LACTOBACILLI

Cultures incubated at 37°C.

Culture No.	Period of incubation in weeks	Increase in amino nitrogen*	
		No calcium carbonate added	Calcium carbonate added
1	2	0.0281	0.0411
	4	0.0290	0.0422
	6	0.0299	0.0424
	8	0.0331	0.0454
2	2	0.0291	0.0313
	4	0.0316	0.0362
	6	0.0333	0.0384
	8	0.0391	0.0390
3	2	0.0250	0.0273
	4	0.0261	0.0296
	6	------	------
	8	0.0386	0.0415
4	2	0.0196	0.0271
	4	0.0232	0.0302
	6	0.0254	0.0376
	8	0.0293	0.0382
5	2	0.0313	0.0321
	4	0.0320	0.0325
	6	0.0386	0.02395
	8	0.0412	0.0445
6	2	0.0361	0.0379
	4	0.0372	0.0399
	6	0.0390	0.0435
	8	0.0473	0.0456
7	2	0.0162	0.0283
	4	0.0253	0.0356
	6	0.0294	0.0425
	8	0.0442	0.0518

TABLE X (CONT.)

INCREASE OF AMINO NITROGEN IN MILK BY VARIOUS CULTURES OF LACTOBACILLI

Cultures incubated at 37°C.

Culture No.	Period of incubation in weeks	Increase in amino nitrogen*	
		No calcium carbonate added	Calcium carbonate added
8	2	0.0332	0.0302
	4	0.0394	0.0364
	6	0.0443	0.0493
	8	0.0512	0.0500
9	2	0.0201	0.0234
	4	0.0194	0.0250
	6	0.0213	0.0271
	8	0.0172	0.0200
10	2	0.0163	0.0194
	4	0.0199	0.0199
	6	0.0216	0.0253
	8	0.0302	0.0296
11	2	0.0312	0.0332
	4	0.0284	0.0365
	6	0.0290	0.0416
	8	0.0291	0.0432
12	2	0.0231	0.0412
	4	------	------
	6	------	------
	8	------	------
13	2	0.0226	0.0232
	4	0.0284	0.0280
	6	0.0299	0.0316
	8	0.0316	0.0335
14	2	0.0308	0.0497
	4	0.0194	0.0236
	6	0.0370	0.0551
	8	------	0.0380
15	2	0.0191	0.0221
	4	0.0233	0.0280
	6	0.0112	0.0191
	8	------	0.0156

TABLE X (CONT.)

INCREASE OF AMINO NITROGEN IN MILK BY VARIOUS CULTURES OF LACTOBACILLI

Cultures incubated at 37°C.

Culture No.	Period of incubation in weeks	Increase in amino nitrogen*	
		No calcium carbonate added	Calcium carbonate added
19	2	0.0183	0.0171
	4	0.0201	0.0193
	6	0.0214	0.0162
	8	0.0110	0.0150
20	2	0.0316	0.0325
	4	0.0362	0.0383
	6	0.0370	0.0410
	8	0.0435	0.0455
21	2	0.0401	0.0452
	4	0.0421	0.0499
	6	0.0420	0.0000
	8	0.0000	0.0000
22	2	0.0325	0.0356
	4	0.0372	0.0382
	6	0.0370	0.0391
	8	0.0381	0.0390
23	2	0.0214	0.0216
	4	0.0169	0.0327
	6	0.0230	0.0444
	8	0.0281	0.0516
24	2	0.0213	0.0223
	4	0.0254	0.0327
	6	0.0171	0.0444
	8	0.0120	0.0531
25	2	0.0223	0.0196
	4	0.0234	0.0259
	6	0.0391	0.0243
	8	0.0376	------

TABLE X (CONT.)

INCREASE OF AMINO NITROGEN IN MILK BY VARIOUS CULTURES OF LACTOBACILLI

Cultures incubated at 37°C.

Culture No.	Period of incubation in weeks	Increase in amino nitrogen*	
		No calcium carbonate added	Calcium carbonate added
26	2	0.0256	0.0294
	4	0.0273	0.0334
	6	0.0280	0.0371
	8	0.0212	0.0360
27	2	0.0151	0.0234
	4	0.0215	0.0367
	6	0.0376	------
	8	------	------
30	2	0.0114	0.0153
	4	0.0256	0.0261
	6	0.0356	0.0332
	8	0.0410	0.0456
33	2	0.0351	0.0283
	4	0.0364	0.0315
	6	0.0482	0.0398
	8	0.0400	0.0451

*The results are expressed as the grams of amino nitrogen produced in 300 cc. of skim milk.

Most of the cultures were capable of increasing the amino nitrogen in the plain milk. The amino nitrogen in 300 cc. of milk incubated for two weeks ranged from 0.0114 to 0.0401 gm.; four weeks, from 0.0000 to 0.0421 gm.; six weeks, from 0.0000 to 0.0482 gm.; and eight weeks, from 0.0000 to 0.0512 gm. The values obtained varied considerably but in general, there was a gradual increase in amino nitrogen in plain milk during the eight weeks of incubation; in several instances there appeared to be a decrease toward the end of the period.

The amino nitrogen in the milk with calcium carbonate added incubated for two weeks ranged from 0.0153 to 0.0497 gm.; four weeks, from 0.0000 to 0.4999 gm.; six weeks, from 0.0000 to 0.0493 gm.; and eight weeks, from 0.0000 to 0.0518 gm. Again it was evident that the amino nitrogen content in the milk with calcium carbonate varied widely but in most cases there was a marked increase of amino nitrogen during the incubation period; in a few cases, however, a decrease of this compound was apparent at certain examinations.

In comparing the values of amino nitrogen in plain milk with those in milk to which calcium carbonate had been added, it appeared that the amino nitrogen values were higher when the milk was neutralized with calcium carbonate.

From the results obtained, it was noted that most of the cultures studied were capable of breaking down the milk proteins into amino nitrogen over a prolonged incubation period. Greater amounts of amino nitrogen were obtained when the acidity of the fermented milk was partially neutralized by the addition of calcium carbonate.

SPECIAL STUDIES UPON THE GROWTH AND MORPHOLOGY OF LACTOBACILLI IN MILK

A. The Influence of Deuterium Oxide upon the Growth and Morphology

The biological interest of deuterium oxide or heavy water, as it is sometimes called, cannot be overlooked in view of the fact that all living cells require water for existence. Experimental evidence indicates that high concentrations of heavy water administered to some animals are fatal. In the work of Lewis (49) certain plant seeds failed to sprout when placed in certain concentrations of heavy water. Likewise, the effect of this compound on bacteria showed marked variations. Some of the fluorescent organisms did not give out their fluorescent light in the presence of deuterium oxide. The work of Barnes (4) indicates that heavy water in low concentrations has a stimulating effect on the growth of microorganisms.

Through the courtesy and efforts of Dr. H. V. Moyer, Department of Chemistry, Ohio State University, who provided limited amounts of various concentrations of deuterium oxide, an attempt was made to study the effects of this compound upon the growth and morphology of six strains of lactobacilli.

The heavy water was mixed with Difco dehydrated whey broth so that the broth contained concentrations of deuterium oxide as follows: 0.13, 0.7, and 5.0 per cent. The control broth was prepared with distilled

water which was devoid of all deuterium oxide. The various lots of broth, in 10 cc. portions, were sterilized in sealed glass tubes at 15 pounds pressure for 20 minutes.

In studying the effect of deuterium oxide, four cultures of L. acidophilus (numbers 1, 3, 4, and 6), one culture of L. bulgaricus (number 7), and one Lactobacillus culture from acidophilus milk (number 19) were used. Each culture was inoculated into the various lots of whey broth, using 0.1 cc. of an actively growing broth culture for each tube, and the tubes were incubated at 37°C. At 12-hour intervals for a 60-hour period, 1 cc. was withdrawn from each tube and tomato juice agar plates were prepared, using dilutions of 1:1000, 1:10,000 and 1:100,000. All the plates were incubated at 37°C. for 48 hours, after which they were examined and the Lactobacillus colonies counted.

The results indicate that the various concentrations of deuterium oxide did not have any appreciable effect on the rate of multiplication of the six strains of lactobacilli in the whey broth. The colonies on the plates prepared from the cultures containing various concentrations of deuterium oxide were examined under the low power objective of the microscope and failed to show any differences in size or general appearance. The colonies on the lowest dilution plates, representing the various concentrations of deuterium oxide broth, were very small while those on the higher dilution plates were somewhat larger, but the differences in the sizes of the colonies were comparable to those in the control and, apparently, were largely determined by the numbers of colonies on the plates.

An attempt was made to study the effect of deuterium oxide upon the morphology of the six Lactobacillus cultures, using the same time inverval and the original concentrations of deuterium oxide broth. Gram stained preparations from the broth did not reveal any appreciable morphologic differences from the control cultures. However, at the end of the 48-hour interval, one of the cultures of L. acidophilus (number 1), showed marked granulation and was Gram negative in the 5.0 per cent concentration of deuterium oxide broth; at the end of the 60-hour incubation period, this granulation did not persist.

The results show that the various concentrations of deuterium oxide in whey broth gave no stimulating effect upon the growth or morphology of the organisms. These results are not in agreement with those of Barnes (4), since he found that deuterium oxide in low concentrations may have a marked stimulating effect upon living organisms. However, Barnes does not mention the procedure used in studying the effect upon organisms. Due to the cost and the difficulty of obtaining the higher concentrations of deuterium oxide, the experimental work could not be repeated. Thus it is noted that concentrations of 0.13, 0.7, and 5.0 per cent deuterium oxide in whey broth did not change the rate of multiplication of the Lactobacillus organisms when grown in this broth and then plated on tomato juice agar. Likewise, no marked morphologic changes were noted either in the size or the shape of the organisms.

B. The Influence of Temperature upon the Growth and Morphology

The growth and morphology of 36 cultures of lactobacilli were studied by inoculating 0.1 cc. of an actively growing milk culture into tubes of litmus milk. The two tubes of the litmus milk culture of each organism were incubated at 10°, 21°, 37° and 45°C. One uninoculated control tube of litmus milk was incubated with each set. All the tubes were incubated and observed daily, for an indefinite period, or until some visible changes were noted in the litmus milk, such as reddening of the litmus or acid coagulation. The data obtained are given in Table XIII.

The results indicated that at 10°C. 6 of the 36 cultures were capable of producing acid in sufficient quantities to change the color of the litmus in from 8 to 12 days. One culture produced a change in the litmus milk within 8 days, three cultures in 10 days, and two cultures in 12 days, but none of these cultures produced sufficient acid to coagulate the milk. The remaining 30 cultures failed to produce any change in the milk during 21 days incubation. Of the six cultures that grew at 10°C., three were from raw milk and three from Cheddar cheese.

The morphologies of the six cultures that grew in the litmus milk at 10°C. were studied. It was noted that all of the cultures showed spindle shaped rods, with considerable variation in the rods from different cultures.

Thirteen cultures produced acid at 21°C. within 4 to 10 days, and with 10 of these there was sufficient acid to coagulate the milk in from 10 to

TABLE XI

GROWTH OF LACTOBACILLI IN MILK INCUBATED AT DIFFERENT TEMPERATURES

Culture No.	Action at 10°C.		21°C.		37°C.		45°C.	
	Reddening of litmus	Coag. of milk	Reddening of litmus	Coag. of milk	Reddening of litmus	Coag. of milk	Reddening of litmus	Coag. of milk
	days	days	days	days	days	days	days	days
1	-	-	-	-	1	2	1	2
2	-	-	-	-	1	2	1	2
3	-	-	-	-	1	2	1	2
4	-	-	-	-	1	2	1	2
5	-	-	-	-	1	2	1	2
6	-	-	-	-	1	2	1	2
7	-	-	-	-	1	2	1	2
8	-	-	-	-	1	2	1	2
9	8	-	4	10	1	1.5	-	-
10	12	-	8	-	1.5	1.5	-	-
11	10	-	8	10	1	1.5	-	-
12	10	-	5	10	1	2	-	-
13	-	-	7	-	1	1.5	2	2.5
14	-	-	7	-	1	1.5	-	-
15	-	-	-	-	1	2	-	-
16	-	-	-	-	1.5	2.5	1	2
17	-	-	-	-	1	1.5	1	1.5
18	-	-	-	-	1	1.5	1	1
19	-	-	-	-	1	1.5	1	1.5
20	12	-	9	10	1	1.5	-	-
21	10	-	6	11	1	2	-	-
22	-	-	-	-	1.5	2	-	-
23	-	-	-	-	1.5	2	-	-
24	-	-	-	-	2	2.5	1.5	2
25	-	-	-	-	2	2.5	1.5	2
26	-	-	8	10	1	2	1.5	2
27	-	-	8	10	1	2	1.5	2
28	-	-	10	12	1.5	2	-	-
29	-	-	10	12	1.5	2	-	-
30	-	-	10	11	2	2.5	2	2.5
31	-	-	-	-	1	2	1.5	2
32	-	-	-	-	1	2	1.5	2
33	-	-	-	-	1	2	1	2
34	-	-	-	-	1	2	1	2
35	-	-	-	-	1	2	1	2
36	-	-	-	-	1	2	1	2

Note: - = no growth.

12 days. One culture produced a reddening of the litmus in 4 days and coagulation in 10 days. Another culture changed the litmus in 5 days and coagulated the milk in 10 days. One culture changed the litmus milk in 6 days and coagulated the milk in 11 days. Two cultures produced sufficient acid to change the litmus in 7 days but there was no coagulation even after 21 days; four changed the litmus in 8 days and three of these coagulated the milk in 10 days, while the other failed to produce coagulation in 21 days; one produced a reddening of the litmus in 9 days with coagulation in 10 days; three changed the litmus in 10 days and one of these coagulated the milk in 11 days and the other two in 12 days. Of the 13 organisms that grew at 21°C., 5 were from raw milk, 3 from Cheddar cheese, 2 from Swiss cheese, 2 from ensilage and 1 from corn stover.

In most instances, the morphology of the organisms incubated at 21°C. were comparable to the rod forms found in the cultures incubated at 10°C. Although there were many variations in the size of the rod forms, many of the cultures showed long spindle shaped rods.

At 37°C. all of the 36 cultures grew and produced a reddening of the litmus in from 1 to 2 days and coagulation of the milk in from 1.5 to 2.5 days. Twenty-seven cultures produced a reddening of the litmus in 1 day and of these, 8 coagulated the milk in 1.5 days, and 19 coagulated the milk in 2 days; 6 cultures changed the litmus in 1.5 days and of these, 1 coagulated the milk in 1.5 days, 4 coagulated it in 2 days, and the other culture coagulated the milk in 2.5 days; 3 cultures produced a reddening of the litmus in 2 days and coagulated the milk in 2.5 days.

The morphology of the cultures incubated at 37°C. were quite variable. There were many large thick rod forms as well as numerous slender rods. In many instances it appeared that both types of rods were present in the same culture.

At 45°C., 24 cultures produced a reddening of the litmus in from 1 to 2 days and produced sufficient acid to coagulate the milk in from 1 to 2.5 days, while 12 failed to show any change after 5 days. Sixteen cultures changed the litmus in 1 day, and 1 of them coagulated the milk in 1 day; 2 produced coagulation in 1.5 days; while 13 showed coagulation in 2 days. Six cultures changed the litmus in 1.5 days and coagulated the milk in 2 days. Two cultures showed a reddening of the litmus milk in 2 days and coagulation in 2.5 days. Of the 12 cultures that could not grow at 45°C., 5 were from raw milk, 5 from Cheddar cheese and 2 from ensilage. None of the organisms from Cheddar cheese grew at 45°C., while all the organisms from Swiss cheese grew at this temperature.

The morphology of the 24 cultures that grew at 45°C. were invariably large rod forms. These rods were much larger than those found in the cultures incubated at 37°C. Many pleomorphic forms were evident. Some of the cultures showed granules, while some stained very irregularly.

The results showed that all the lactobacilli grew well and coagulated the milk at 37°C., while at the other temperatures variable results were secured. None of the organisms that grew at 10°C. could grow at 45°C., and of the 13 that grew at 21°C. only 4 grew at 45°C.; 1 of these was from raw milk, 2 from Swiss cheese and 1 from corn stover. None of the

cultures of L. acidophilus or L. bulgaricus secured from various research laboratories grew at 21°C. while all of them grew at 45°C. The organisms from fecal material grew well at 37° and 45°C. but not at 10° or 21°C.

The morphology of the organisms varied widely among the organisms incubated at the different temperatures. However, it was noted that cultures incubated at 10° and 21°C. were rather uniform in size and shape of the rod forms. A similarity was also noted in the cultures incubated at 37° and 45°C., but most of these rods were much larger and more pleomorphic than the rod forms present in the cultures incubated at 10° and 21°C.

THE FLAVOR AND ODOR PRODUCTION IN MILK BY LACTOBACILLI

An attempt was made to compare the flavor and odor production of the 36 cultures of lactobacilli by the following procedure: A flask containing 200 cc. of sterile skim milk was inoculated with 0.1 cc. of an actively growing milk culture, the milk incubated at 37°C. for 48 hours and cooled to approximately 15°C. The cultures were then shaken thoroughly and judged by Prof. L. H. Burgwald, Department of Dairy Technology, Ohio State University. The following summary gives the flavor and odor of each culture examined:

Culture No.	Flavor	Odor
1	Sharp, clean, desirable acid	Acid, very slight, odor of a good butter culture
2	Clean, high acid	Mild acid
3	Clean acid	Slight acid
4	Clean acid	Slight acid
5	Clean acid	Medium acid
6	Sharp acid	Medium acid
7	Very sour	Strong acid
8	Very sour, some indications of an acetic acid flavor	Strong acid
9	Mild acid	Acid
10	Mild acid	Lacking in odor
11	Mild, slightly flat acid	Lacking in odor
12	Clean, mild acid	Considerable odor, resembling good butter culture

Culture No.	Flavor	Odor
13	Desirable, clean acid	Acid odor
14	Clean acid	Mild acid
15	Sharp acid	Strong acid odor
16	Clean, sharp acid	Strong acid odor
17	Clean, sharp acid	Strong acid odor
18	Clean, sharp acid	Pleasant acid odor
19	Clean, sharp acid	Pleasant acid odor
20	Mild acid	Pleasant acid odor
21	Mild acid	Pleasant acid odor
22	Mild acid	Pleasant acid odor
23	Mild acid	No odor
24	Mild acid	Slight acid
25	Mild acid	Pleasant acid odor
26	Mild, slightly flat acid	Unclean acid
27	Mild acid	Pleasant acid odor
28	Undesirable acid, chalky	Slight yeasty odor
29	Sharp acid	Acid
30	Sour acid	Strong acid
31	Clean, mild acid	Mild acid
32	Sharp acid	Pleasant acid odor
33	Sharp, desirable acid	Acid odor
34	Mild acid	No odor
35	Clean, mild acid	Considerable acid odor
36	Sharp acid	Acid, unclean odor

The summary shows that all the cultures gave acid flavors that varied from clean, mild and desirable to sharp, high acid undesirable. The six L. acidophilus cultures secured from research laboratories(Nos. 1 to 6) and the lactobacilli isolated from acidophilus milk (Nos. 17, 18 and 19), all gave desirable acid flavors that varied from milk to sharp acid odors that varied from mild to strong acid. One of the L. bulgaricus cultures secured from research laboratories (No. 7) gave a very sour flavor and strong acid odor while the other (No. 8) gave a very sour flavor with a suggestion of acetic acid and a strong acid odor.

The cultures isolated from raw milk (Nos. 9, 11, 12, 13, 14 and 15) gave clean, mild acid flavors, and odors that ranged from lacking to the odor of a good butter culture, except culture No. 15 which gave a sharp acid flavor and strong acid odor. The cultures isolated from Cheddar cheese (Nos. 10, 20, 21, 22 and 23) all gave mild acid flavors and the odors produced ranged from none to pleasant acid. The culture from pasteurized milk (No. 16) gave a clean, sharp acid flavor and a strong acid odor. Three of the cultures from Swiss cheese (Nos. 24, 25 and 27) gave mild acid flavors and slight acid or pleasant acid odors while the other cultures (No. 26) gave a mild, slightly flat acid flavor and an unclean acid odor.

The three cultures from feed gave undesirable flavors characterized as undesirable and chalky, sharp, and sour acid flavors and odors that were characterized as slight yeasty, acid, and strong acid respectively.

The cultures isolated from fecal material (Nos. 31 to 36) gave flavors that were generally clean and desirable with odors that ranged from none to an acid, unclean odor.

In general, it was noted that cultures which produced a clean, mild acid flavor usually gave a clean, desirable acid odor, while the cultures that produced an unclean, flat acid flavor gave an unclean odor, or in some instances were lacking in odor. It appeared from this study that cultures of lactobacilli which gave a desirable, pleasing flavor and odor in milk were similar to L. acidophilus. Cultures of lactobacilli that were lacking in a desirable flavor and aroma resembled the cultures of L. bulgaricus which produced a sharp acid and sometimes an unclean off-flavor. It was noted that all the cultures examined, regardless of the source from which they were isolated, gave an acid flavor which varied from a sharp to a mild acid. Likewise, an odor suggesting acid was evident in nearly every culture, although the odor ranged from none to a very strong acid odor.

THE ACTIVITY OF CLOSELY RELATED ORGANISMS IN MILK

In the isolation of the lactobacilli that actively fermented lactose it was noted that colonies which were typical Lactobacillus colonies often failed to give a rapid coagulation when inoculated into milk. This was especially true when isolations were attempted from certain materials, but in plating such a product as acidophilus milk all of the colonies gave a rapid coagulation as would be expected. Relatively few of the colonies, which did not produce a rapid coagulation, gave conspicuous changes in milk, such as proteolysis, or gas formation and most of them produced only a slight increase in the acidity or gave no change at all.

From a total of 64 characteristics Lactobacillus colonies picked into litmus milk from the various sources of material, there were some colonies that did not actively coagulate the milk within 36 to 48 hours. The following summary shows the action of 27 of such colonies that were picked into litmus milk:

Source of material from which the colonies were picked	No change in the color of the litmus	Reddening of the litmus	Partial coagulation of the milk
	Number of colonies picked into litmus milk		
Fresh ensilage	2		
Corn stover	2	1	
Pasteurized milk	2		
Cheddar cheese	4		
Swiss cheese		2	
Calf feces		5	4
Dog feces	3		
Infant feces	1	1	

The results indicated that 14 colonies failed to produce any changes in the litmus milk while 9 showed a reddening of the litmus but failed to produce sufficient acid to coagulate the milk, and 4 produced only a partial coagulation in the milk. All of the 27 colonies picked into litmus milk appeared to be characteristic Lactobacillus colonies, as indicated by their size and shape on the tomato juice agar medium.

It was interesting to note that 6 of the colonies which failed to show any changes in the milk were obtained from dairy products, 4 from feed and 4 from fecal material. However, 2 colonies that produced a slight amount of acid in the milk were obtained from dairy products, 1 from feed and 6 from fecal material, while the 4 colonies that produced only a partial coagulation in the milk were secured from fecal material.

These cultures were not studied further because the main interest was to select various strains of lactobacilli that would actively coagulate the milk.

DISCUSSION OF RESULTS

In the comparisons of the counts of lactobacilli cultures on different media the superiority of the tomato juice agar over the other media was shown not only in the higher counts obtained but also in the greater uniformity of the counts. Irregularities in counts were noted particularly with the cabbage and whey agars, while with the casein digest agar the counts were fairly uniform; the beef infusion agar plus 1 per cent lactose was distinctly inferior to the other media employed.

Lactobacillus cultures were obtained very readily by plating commercial acidophilus milk directly on tomato juice agar and also fairly easily by plating raw or pasteurized milk that had been incubated at 37°C. until the bacterial flora showed a large percentage of rod forms. However, the isolation of Lactobacillus from other materials was rather difficult because of the competition offered by the other types likely to be present. With Cheddar or Swiss cheese, the addition of small pieces of the cheese to sterile milk and incubating at 37°C. until a characteristic Lactobacillus flora predominated in the milk served as an enrichment procedure. Plating of such milk usually resulted in the isolation of typical Lactobacillus cultures. With material such as feed the presence of numerous other organisms made it necessary to allow the material to undergo a natural fermentation until the bacterial flora was made up largely of lactobacilli and then by plating on tomato juice agar characteristic Lactobacillus colonies were usually obtained. In the case of

fecal material, a medium was used in which the pH was much lower than the optimum conditions for the growth of organisms other than lactobacilli. Such a procedure inhibited most of the other organisms but allowed the lactobacilli to grow and become dominant after a few transfers and in most instances lactobacilli could be obtained by plating the material on tomato juice agar. The isolation of several cultures of lactobacilli from the feces of various animals indicates the ability of these organisms to tolerate the conditions that obtain in the intestines, but several samples of feces from different animals failed to yield any Lactobacillus types. A medium which would permit growth of the lactobacilli with the exclusion of the other types would be very useful in the isolation of lactobacilli from materials such as feces where the competition from other organisms makes direct plating on ordinary media impractical.

The wide variations that occurred among the cultures in the production of total acid, volatile acid and acetylmethylcarbinol + diacetyl indicate that these criteria are of little value in identifying the organisms; the variations were noted among the cultures from the same source and among those belonging to the same species as well as among those from different sources and different species. There was no direct relationship between the total acid, volatile acid and acetylmethylcarbinol + diacetyl formed by the various cultures of lactobacilli. Cultures that produced comparatively large amounts of total acid did not, in all cases, produce large amounts of volatile acid or acetylmethylcarbinol + diacetyl.

The addition of 0.15 per cent citric acid to milk increased the pro-

duction of volatile acid and acetylmethylcarbinol + diacetyl by a majority of the organisms studied. The increases, however, were insignificant and in a number of instances the addition of the acid resulted in a decrease in values for volatile acid and acetylmethylcarbinol + diacetyl. If the citric acid were the source of the flavor and aroma constituents, the addition of it to milk should result in a conspicuous increase in values for volatile acids and acetylmethylcarbinol + diacetyl, as is the case with the citric acid fermenting streptococci in butter cultures. The relatively high values for volatile acid and acetylmethylcarbinol + diacetyl obtained in plain milk with several of the cultures, suggest further studies to determine the sources of these materials. It would also be interesting to determine whether the lactobacilli that produce acetylmethylcarbinol + diacetyl are able to destroy it, as is the case with butter culture organisms.

The addition of 0.05 or 0.1 per cent acetaldehyde to milk resulted in an increase in the production of acetylmethylcarbinol + diacetyl with a majority of the organisms but the difference, in every case, was insignificant and in some instances the addition of the aldehyde resulted in decreases. The higher concentrations of acetaldehyde appeared to be toxic for the organisms. The failure to obtain significant increases in acetylmethylcarbinol + diacetyl by adding acetaldehyde suggests that the formation of these materials is not due to an acetaldehyde condensation.

Practically all the cultures of lactobacilli produced appreciable amounts of carbon dioxide in milk and in milk plus 0.5 per cent peptone. The results, however, varied widely with the various cultures isolated

from the same source and it was impossible to separate the cultures into the species L. acidophilus, L. bulgaricus and L. casei on the basis of their abilities to produce carbon dioxide, although Sherman (65), asserted that the L. bulgaricus types produced less carbon dioxide than the L. acidophilus types. In general, an incubation period of 20 days gave higher carbon dioxide values than an incubation period of 10 days in either the plain or peptone milk but in several instances the values after 20 days incubation appeared to be significantly lower than those after 10 days; this phenomenon is difficult to explain in view of the method by which the results were obtained. It is also difficult to explain why the values for the peptone milk were, in general, lower than those for the plain milk.

The 10 cultures of lactobacilli employed produced more carbon dioxide at 37°C. than at 21°C. and the values were more uniform at the higher than at the lower temperature. The values secured at 37°C. were all relatively high and with 5 of the 10 cultures the values at 37°C. were higher than those secured for the same cultures in the comparisons of the carbon dioxide production in plain and in peptone milk (Section D). With one culture, No. 29, the value after 15 days at 37°C. was 5.0 whereas the value after 10 days in plain milk was 2.0 and after 20 days was 0.0, in the comparisons of plain and peptone milk. These discrepancies suggest inaccuracies in the methods for determining the carbon dioxide value.

Many investigators have attempted to separate the various species of lactobacilli on the basis of their fermentation reactions, but the results obtained by the various workers are variable and only add to the con-

fusion prevailing in attempts to classify these organisms. The results of the fermentation studies in this work very clearly indicated that the various strains of lactobacilli that actively coagulated the milk could not be separated on the basis of their fermentation reactions. Lactose was attacked by all of the cultures studied and dextrose and dextrin were also frequently attacked. The other materials used in this study gave no indication as to a possible basis for separation. The failure of the cultures representing the same species (secured from various research laboratories), or of cultures isolated from a common source, to ferment the same carbohydrates further emphasizes the need of a better criterion for separating the cultures into species.

Since it is generally believed that the various species of lactobacilli play a very important part in the ripening of various dairy products, particularly cheese, the ability of the organisms to attack fat is important from the standpoint of flavor and aroma production. None of the organisms that grew on the nile-blue sulfate medium were able to attack butterfat but, on the other hand, the technic employed may not have been a fair test because of the inhibitory effect of the nile-blue sulfate. When tomato juice agar was substituted for beef infusion agar more of the organisms were able to grow on the medium but the growth was not extensive in any instance and 4 of the 36 cultures failed to grow during 6 days incubation. The results suggest that some other technic be employed in determining fat hydrolysis by organisms that are more or less fastidious.

The acid produced by the lactobacilli appears to be chiefly dextrorotatory lactic acid. Wide variations were noted in the determinations

on the zinc salts, both in the optical activity and in the percentage of water of crystallization. The values for the water of crystallization indicate that the acid formed ranged from pure active to practically pure inactive. In a number of instances the polariscopic reading on the zinc salts was 0 and, theoretically, the percentages of water of crystallization should have been 18.18, but the highest value obtained was 16.95. No explanation can be given for the failure of these values to check. The L. acidophilus cultures obtained from research laboratories seemed to produce practically pure dextro-rotatory acid while the L. bulgaricus cultures seemed to produce chiefly inactive acid. These results are in accordance with those of Kopeloff and Bass (43) in their study of pure cultures of L. acidophilus, in which they state that the cultures which they designated as L. acidophilus gave the dextro form of lactic acid. This did not agree with the work of Curran, Rogers and Whittier (14) who claim that the lactic acid produced by L. acidophilus is entirely of the inactive type, while cultures of uncertain identity, called L. bulgaricus or L. casei, produced a mixture of lactic acid of the inactive and active form. The active acid was a dextro form.

Since there was a variation in the lactic acid produced, which varied from an active to almost a pure inactive form by the various cultures used in the present study, it was interesting to note that cultures isolated from fecal material invariably gave the same form of acid as the cultures of L. acidophilus obtained from various research laboratories. Many of the cultures obtained from dairy products varied from an active to an inactive form of acid. In most instances, the dextro form of acid

was produced by cultures isolated from raw and pasteurized milk and commercial acidophilus milk. The d-form of acid was formed by most of the cultures from Swiss cheese, while the inactive acid was predominant with the organisms from Cheddar cheese. Cultures obtained from feed gave an inactive form. This work indicated that cultures designated as L. acidophilus as well as those obtained from fecal material produced an acid that was largely dextro form. Also, in most instances, cultures obtained from dairy products, with the exception of Cheddar cheese, gave an acid that was largely a dextro form. There were a few cultures that produced an acid that was largely inactive which might indicate that the cultures were either L. bulgaricus or L. casei.

Although there was no apparent proteolysis, the amino nitrogen determinations indicate that lactobacilli are able to partially break down the milk proteins. The ability of most of the lactobacilli to increase the amino nitrogen content in milk is significant from the standpoint of the ripening of cheese such as Cheddar and Swiss in which the protein breakdown is partially responsible for the development of a desirable texture and flavor. Although the results were variable there was, in general, a gradual increase in amino nitrogen during the incubation period of 8 weeks. With a few of the cultures there was an apparent decrease in amino nitrogen at certain examinations but these decreases were not pronounced and were not uniform and therefore are of no particular significance. In nearly every instance, greater amounts of amino nitrogen were formed in milk with calcium carbonate added than in plain milk. Undoubtedly, the acidity formed by the organisms had a tendency to

interfere with their maximum activity. No significant relationship seemed to exist between the amount of proteolysis and other biochemical features.

The failure of the deuterium oxide to exert any effect on the morphology or rate of growth of the lactobacilli should not be accepted as conclusive because the number of trials was limited and the concentrations used perhaps did not cover a wide enough range.

The growth of the lactobacilli in milk at various temperatures gives a suggestion as to a possible separation into species on the basis of growth temperatures. The L. acidophilus and L. bulgaricus cultures secured from various research laboratories, and the lactobacilli isolated from acidophilus milk and from fecal material, including 17 cultures, all grew well at 37° and 45°C., but failed to grow at 10° or 21°C. On the other hand, 13 of the remaining 19 cultures isolated from raw and pasteurized milk, Swiss cheese, Cheddar cheese and feed grew at 21°C. and only four of these grew at 45°C. The work of Sherman (66) indicated that lactobacilli, resembling L. bulgaricus, did not grow at 15°C. and grew very slowly below 20°C., while L. casei and L. acidophilus grew at 20°C. Later Sherman and Stark (67) asserted that L. bulgaricus and L. acidophilus grew at 45°C. but not at 15°C., while L. casei grew well at 15°C., but not at 45°C. This work was later confirmed by Curran, Rogers and Whittier (14). The findings reported in this work indicate that cultures that grew at 10°C. resembled the L. casei type, since they were obtained from raw milk and Cheddar cheese. Those that grew at 45°C. resembled the L. bulgaricus or L. acidophilus types. There appeared to be no means of

separating L. bulgaricus from L. acidophilus except on the basis of their sources. Therefore, cultures obtained from fecal material would be classed as L. acidophilus.

All the cultures produced an acid flavor in milk which varied from a mild to a very sour acid flavor. In most cases, an acid odor was evident, although it varied from flat or not detectable to a strong acid odor. In general, cultures that produced a clean, desirable acid flavor gave a clean acid odor, while those that produced a very sour or flat flavor usually gave an undesirable, unclean odor. There appeared to be little difference in the flavor and odor production among the various cultures isolated from different sources, and apparently there was no correlation between the flavor and odor and the production of total acid, volatile acid or acetylmethylcarbinol + diacetyl.

It was apparent that many of the Lactobacillus type colonies, isolated from various sources of material, did not actively coagulate milk, although the macroscopic appearance of the colonies appeared to be much the same. Since various investigators have reported the wide distribution of the Lactobacillus group of organisms, it was assumed that not all of them were capable of adapting themselves in milk. In fact, a few of the colonies isolated from dairy products did not produce any changes in the litmus milk, nor did some of the colonies obtained from other sources produce changes in the milk. On the basis of these observations, it would be safe to assume that not all characteristic Lactobacillus organisms are capable of coagulating milk rapidly. Therefore it is probable that there may be a group of organisms closely related to the Lactobacillus group which are not capable of adapting themselves in milk.

CONCLUSIONS

1. Tomato juice agar gives higher and more uniform counts with lactobacilli than does beef infusion agar plus 1 per cent lactose, cabbage agar, casein digest agar or whey agar; the results obtained with the beef infusion agar plus lactose are strikingly lower than the counts obtained on the other media.

2. Lactobacilli may be isolated from commercial acidophilus milk, raw milk, pasteurized milk, corn stover, ensilage, Cheddar cheese, Swiss cheese and calf, dog, infant and rat feces. When the material is known to contain numerous lactobacilli, the organisms may be isolated very easily by direct plating on tomato juice agar but when other organisms are dominant an enrichment procedure must be used before plating.

3. Appreciable quantities of total acid, volatile acid and acetylmethylcarbinol + diacetyl are formed in skim milk by most of the lactobacilli; the amounts of these materials produced by the different cultures varies greatly and there is no close correlation between the amount of total acid and the amounts of volatile acid or acetylmethylcarbinol + diacetyl produced by a culture.

4. The addition of citric acid to milk has no significant effect on the amounts of volatile acid and acetylmethylcarbinol + diacetyl formed by the organisms.

5. The addition of acetaldehyde does not significantly increase the production of acetylmethylcarbinol + diacetyl by the organisms in milk.

Low concentrations of the acetaldehyde result in slight increases in the amounts of acetylmethylcarbinol + diacetyl produced while the higher concentrations appear to be definitely toxic.

6. Most of the organisms produce carbon dioxide in plain milk and in milk to which peptone has been added. The values are generally higher for the plain than for the peptone milk and in either type of milk the values are generally higher after 20 days incubation than after 10 days.

7. The lactobacilli produce more carbon dioxide at 37°C. than at 21°C. during a 15-day incubation and the results secured at 37°C. are more uniform than those at 21°C.

8. The fermentation reactions secured with the lactobacilli are rather variable and cannot be used as a criterion for separation of the cultures into species. Dextrin, dextrose and lactose are the carbohydrates most frequently attacked.

9. None of the lactobacilli which grow on the nile-blue sulfate medium are able to hydrolyze butterfat.

10. The isomeric form of lactic acid produced by the various strains of lactobacilli is not uniform but varies from pure active to practically pure inactive with mixtures of these two acids present between the active and inactive acid. The active acid is dextro form in nearly every case. The percentages of zinc oxide in the zinc salts suggest that lactic acid is the acid formed by the cultures.

11. In general, the lactobacilli are able to gradually increase the amino nitrogen content in milk cultures during incubation at 37°C. for 8

weeks. The organisms produce greater amounts of amino nitrogen when the acid developed is partially neutralized with calcium carbonate.

12. Deuterium oxide has no effect on the morphology or rate of multiplication of lactobacilli.

13. The lactobacilli grow well and coagulate milk at 37°C. but the results obtained at other temperatures are variable. The L. acidophilus and L. bulgaricus cultures secured from various research laboratories and the lactobacilli from fecal material grow well at 37° and 45°C. but not at 10° and 21°C. In general, the lactobacilli from raw milk, Swiss cheese, Cheddar cheese and feed grow at 21°C. but not at 45°C.; a few of these organisms also grow at 10°C.

14. Lactobacilli give an acid flavor in milk which varies from a clean, desirable acid flavor to a sharp, very sour acid flavor. The odor varies from acid to flat; in some instances no odor is detectable.

15. In isolating lactobacilli, organisms may be encountered which resemble lactobacilli very closely but which do not coagulate the milk rapidly; some of these produce varying amounts of acid and others produce slight proteolysis.

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. B. W. Hammer for his assistance and helpful suggestions in the experimental studies and in the preparation of this manuscript. The author also acknowledges the help of Dr. H. C. Olson in the arrangement of the material in this thesis.