1943

A study of Bacterium linens

J. Oscar Albert
Iowa State College

NOTE TO USERS

A STUDY OF *Bacterium linens*

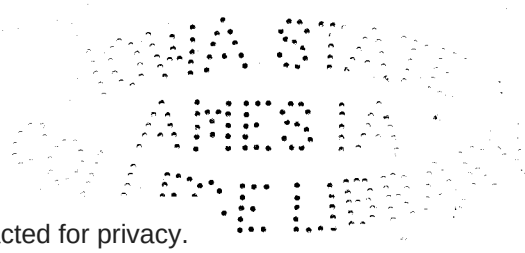
by

J. Oscar Albert

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology



Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1943

TABLE OF CONTENTS

INTRODUCTION

During the ripening of some cheeses, a reddish or reddish-brown slimy growth commonly develops on the surfaces. The material contains various microorganisms, among which Bacterium linens usually is present in relatively large numbers. This organism apparently plays a role in the ripening of certain cheeses, in which it aids in the protein breakdown and flavor development; it also aids in the production of the typical color at the cheese surfaces.

Bact. linens has received relatively little attention from the standpoint of its detailed study, particularly in view of its importance in the ripening of various cheeses and its possible importance in other connections.

STATEMENT OF PROBLEM

The work herein reported involved:

a. Development of an isolation procedure for *Bact. linens*

b. Studies on its distribution in dairy products and other materials

c. Studies on the general properties of the organism, and

d. Preparation of a description of the organism.

HISTORICAL

Duclaux (1), in 1893, investigated the complex problem of cheese ripening and noted the protective role played by certain color producing bacteria which appear on the surfaces of some soft cheeses. He believed that oxygen consumed by these organisms tends to prevent oxidation of the fat constituents.

The surface flora of tilsiter, romadour and rahmkase which had developed a reddish-yellow surface color during holding in the curing rooms was investigated by Wolff (15). When the colored material was cultured on cheese gelatin or cheese agar, he obtained various chromogenic organisms. These included a gram positive rod which produced a reddish-yellow to brown pigment; it was identified as organism IX. Later, Wolff (16) emphasized the importance of organism IX as a cause of color formation in the slime of cheeses. At the suggestion of Weigman, Wolff (17) named the organism Bact. linens.

Mazé (9) isolated 40 strains of red bacteria (bactérie du rouge) from camembert and brie cheeses and reported 8 of the strains were useful in cheese

ripening. He thought that the red bacteria represented a complex group of organisms. The color produced over the surfaces of cheeses was used as an index of the ripening progress. Mazé reported a general lack of resistance of the organisms to acid and classified the strains according to their acid resistance. The organisms were killed in 5 minutes at 65°C.

According to Weigmann (14) Bact. linens plays a major role in the color production and the protein breakdown of certain soft cheeses, such as limburger.

Steinfatt (13) investigated the proteolysis of milk by Bact. linens. After 2 months at 15°C. milk inoculated with the organism contained 50 per cent soluble nitrogen, 28 per cent amino nitrogen and 7 per cent ammonia. In symbiosis with various other organisms, proteolysis by Bact. linens was not increased. Steinfatt emphasized the importance of Bact. linens in cheese ripening and in color production.

That red bacteria (microbes du rouge) are essential for the ripening of soft cheeses was

emphasized by Kayser (4). These organisms retard growth of molds and produce ammonia through the decomposition of casein.

Grimmer and Schmid (2) investigated the products resulting from the decomposition of casein by Bact. linens. Leucine, isoleucine and tyrosine were found in appreciable amounts.

Rosell (10) indicated that agar made of 10 per cent cheese, 1 per cent potassium citrate and water gave good growth and increased color production by Bact. linens.

Changes in the flora on the surface of limburger cheese were studied by Kelly (5). Yeasts developed 2 or 3 days after the cheese were manufactured and in 4 to 5 days were present in large masses. After about 6 or 7 days Bact. linens appeared and by the eighth day reached large numbers. At this time a reddish color was present on the cheese and was due to the large numbers of Bact. linens. Kelly believed that this surface growth was largely responsible for protein breakdown in the cheese.

Later work by Kelly and Marquardt (6) indicated

that the high concentration of salt on the surface of limburger cheese suppresses many miscellaneous organisms found there and allows the development of yeasts which can tolerate the low pH produced by the lactic acid streptococci. The yeasts raise the pH to the point at which Bact. linens can grow. Acting on the protein, this organism raises the pH still higher and is believed to complete the ripening of the cheese. Maximum growth of Bact. linens was found to occur at pH 6.5; no growth occurred below pH 5.85 but growth did occur at pH 9.5.

Yale (18) reported that Bact. linens ordinarily is not the predominating bacterial species on the surface of limburger cheese, being less numerous than members of a group of alkali forming rods which fail to produce color or liquefy gelatin. Surface inoculation of limburger cheese made from raw milk with Bact. linens did not improve the quality, compared to cheese held under good factory conditions. The flavor of limburger cheese made from pasteurized milk inoculated with Bact. linens was usually slightly better than that of cheese made from the uninoculated milk.

EXPERIMENTAL

Development of an Isolation Technic for Bact. linens

The first isolations of Bact. linens were made on nutrient or cheese agar (15). Small portions of colored slime from the surfaces of cheeses were smeared on the media and the plates were incubated at 21°C. or 30°C. for a few days. With this procedure the organism commonly was obtained but because of the many other types of bacteria which developed well, including large numbers of micrococci, attempts were made to develop a special isolation technic which would aid in the isolation of the organism from materials in which it was present in relatively small numbers.

Trials with tryptone glucose extract agar.

In the early attempts to develop an isolation procedure, tryptone glucose extract agar (12) was used as the basic medium. It was modified in various ways in order to determine the effects of

various factors on growth of Bact. linens. Commonly, material from the surfaces of foreign type cheeses was smeared on the agar but when Bact. linens was believed to be present in large numbers in the material used, dilutions in sterile water were employed in making the inoculations.

Effect of sodium chloride. Various amounts of sodium chloride were added to the agar in an attempt to prevent growth of bacteria other than Bact. linens. Concentrations of 5, 7, 10 and 12 per cent were used and the plates were smeared and incubated at 8°, 10° and 21°C. In most of the trials micrococci developed rather well on the plates, while the growth of Bact. linens was retarded on plates containing 7, 10 and 12 per cent sodium chloride at 8° and 10°C. However, addition of 5 per cent sodium chloride was of some value in checking the development of certain non-salt resistant bacteria and apparently had no significant retarding effect on the growth of Bact. linens.

Effect of reaction of medium. A solution of 10 per cent lactic acid in water was added to the

agar in various amounts but it was soon evident that Bact. linens did not develop satisfactorily on media that were definitely acid, even when the plates were heavily inoculated with material from the surfaces of cheeses and incubated at 21°C. or room temperature.

Additions of alkali were more successful. To 100 ml. of tryptone glucose extract agar, 2.0, 3.0, 4.0 and 5.0 ml. of a 0.1 normal solution of sodium hydroxide were added at the time of pouring the plates. More than 3.0 ml. of the sodium hydroxide solution prevented or decreased growth of the desired organism, while less than 3.0 ml. apparently did not check development of the objectionable species. Addition of 3.0 ml. of the alkali solution gave a definitely alkaline reaction and was considered the maximum quantity without objectionable effects on growth of Bact. linens; moreover, organisms with little resistance to alkali were almost completely prevented from growing. Ammonium hydroxide also was used and yielded about the same results as sodium hydroxide but, because of various inconveniences

with it, sodium hydroxide was more extensively employed.

Effect of incubation temperatures. Temperatures of 8°, 10° and 21°C. were used for the incubation of plates prepared with tryptone glucose extract agar and smeared with materials from cheeses. No beneficial results were obtained from the use of the low temperatures; micrococci present on the plates grew more actively at the low temperatures than Bact. linens.

Combined effect of sodium chloride, high pH and temperatures. When more than 5 per cent sodium chloride was combined with more than 3.0 ml. 0.1 normal sodium hydroxide and the plates incubated at 8° or 10°C., the conditions were too severe and little or no growth of Bact. linens resulted. Here again the micrococci appeared to be the most resistant forms. Encouraging results were obtained when 5 per cent sodium chloride and 3.0 ml. 0.1 normal sodium hydroxide were added to 100 ml. of the agar and the plates incubated at 21°C. Under these conditions Bact. linens grew satisfactorily and the

growth of some undesirable species was much retarded.

Effect of sodium oxalate on color production. Seppilli (11) noted that the elimination of all soluble calcium salts from a culture medium helped certain pigment forming bacteria to produce a more intense color. He suggested that 0.2 per cent sodium oxalate be added to the media.

Tryptone glucose extract agar was prepared with the addition of 0.2 per cent sodium oxalate and inoculated with several strains of Bact. linens. All the strains showed a definite increase in color production on either plates or slopes of the oxalate medium incubated at 21°C.

Trials with cheese agar.

It was reported by Wolff (15) that Bact. linens showed a more luxuriant growth when cultivated on cheese agar than when grown on any other medium. He also noted that the color produced by the organism is intensified on such a medium.

Rosell (10) suggested the following formula for cheese agar:

Ripened cheese	10.0 per cent
Potassium citrate	1.0 per cent
Agar	1.5 per cent
Water to make	100.0 per cent

In preparing this agar, 100 grams of ripened cheese was ground in a mortar with 10 grams of potassium citrate and about 300 ml. of warm water. When the cheese was dispersed in the water, the mixture was placed in a cylinder at 50°C. for gravity separation of the fat. After about 30 minutes the fat layer was removed by suction and the aqueous phase was adjusted with water to 1 liter. Fifteen grams of agar was dissolved in the material and sterilization was carried out in the usual manner.

On the cheese agar Bact. linens developed readily and after a few days colonies were fairly large and showed a rather characteristic color. However, on the basis of colony appearance it still was impossible to distinguish with any great accuracy between colonies

of Bact. linens and colonies of certain micrococci.

Effect of oxalates. In order to increase the color production of Bact. linens, 0.2 per cent sodium oxalate, ammonium oxalate and calcium oxalate were used in the cheese agar. With sodium oxalate the intensity of the color produced by Bact. linens was significantly increased, but ammonium oxalate and calcium oxalate had no influence on the color production.

Effect of various gases. Incubating plates inoculated with Bact. linens in atmospheres of carbon dioxide, nitrogen or oxygen gave very different results. The inoculated plates were placed in a bell jar and the gas was allowed to run into the container for a few minutes. The inlet and the outlet tubes were then closed and the container was held at 21°C. or room temperature. With carbon dioxide or nitrogen there was no evidence of growth. With oxygen color production was more intense than when the plates were incubated in air. An increase in growth also was noted. With this technic of incubating the plates in an atmosphere of oxygen, colonies of Bact. linens

were readily picked out in an area seeded with other species, including micrococci.

Suggested isolation procedure.

On the basis of the tests carried out, a special isolation procedure for Bact. linens is suggested. It permits a selective growth of the desired organism and also increases its color production.

A special cheese agar having the following composition is employed:

Ripened cheese	10.0 per cent
Potassium citrate	1.0 per cent
Peptone	1.0 per cent
Sodium chloride	5.0 per cent
Sodium oxalate	0.2 per cent
Agar	1.5 per cent
Water to make	100.0 per cent

pH 7.4

To prepare the medium, 100 grams of cheese is suspended in 300 ml. of distilled water containing 10 grams of potassium citrate. When the cheese is well distributed the mixture is warmed to about 50°C. and placed in a cylinder for separation of the fat.

Thirty minutes are enough to give reasonably complete separation. The aqueous part only is used in preparing the agar so the fat is removed by suction. Ten grams of peptone, 50 grams of sodium chloride, 2 grams of sodium oxalate and 15 grams of agar are dissolved in 700 ml. of distilled water. The cheese suspension is added to this mixture and the reaction is adjusted to pH 7.4. The agar is dispensed in bottles and sterilized in the autoclave for 25 minutes at 15 pounds pressure.

Direct smears of the materials to be examined for Bact. linens are made on the surfaces of plates poured with the agar, or the materials are dispersed in sterile distilled water before making the smears. The plates are placed under a bell jar or otherwise enclosed, and oxygen is allowed to run slowly into the container for 15 minutes or more, depending on the number of plates to be incubated. The inlet and the outlet tubes are then closed; although 21°C. is a good temperature for incubation, since Bact. linens grows well there, room temperature has been extensively employed and ordinarily gives good results.

One week at either temperature usually is required for best growth and most characteristic color production. In general, any colony having a brownish-orange color and a shiny surface should be investigated. After microscopic observations reveal that the organism is rod shaped and gram positive, material from the same colony can be smeared on the surface of the cheese agar for purification. Only a short incubation period is necessary to produce colonies large enough to transfer to various media.

Distribution of Bact. linens

The distribution of Bact. linens was studied by examining dairy products and various material from dairy farms and other sources with the general isolation procedure suggested for the organism. Most of the studies were conducted at Ames, Iowa, but some were carried out at St-Hyacinthe, Québec.

Bact. linens in dairy products.

Foreign type cheeses. The surface growth on

various foreign type cheeses was examined for Bact. linens by smearing on plates, either directly or after dispersing in sterile distilled water. The cheese included blue cheese from Illinois, Iowa, Minnesota and Québec; brick cheese from Wisconsin; camembert cheese from Illinois, Ontario and Québec; limburger cheese from Illinois, New York and Wisconsin; and oka cheese from Québec. Bact. linens was readily obtained from most of the 51 samples investigated. Commonly, larger numbers of Bact. linens were obtained when the material used for smearing the plates had a reddish-brown color than when it did not.

Cheddar cheese. The interiors of cheddar cheese were examined for Bact. linens by obtaining a small amount of cheese with aseptic precautions, grinding it in a sterile mortar with a small volume of sterile sodium citrate solution and smearing the suspension on the plates. Of 35 samples of raw milk cheese made in Ontario and Québec, a rather large percentage yielded Bact.

linens in relatively small numbers. The cheese were from 2 to 18 months old and the organism was found as frequently in the old cheese as in the young cheese. Bact. linens also was found in some of a small number of cheese made in Iowa from pasteurized milk; the cheese were examined when they were from 15 hours to 2 months old. Since Bact. linens is easily destroyed by heat, its presence in pasteurized milk cheese undoubtedly is due to contamination of the milk subsequent to the heat treatment.

Milk and cream. Forty samples of raw milk from Iowa and Québec were examined for Bact. linens by smearing directly on plates, and the organism was present in somewhat more than half of them. It also was found in most of a small number of samples of raw sweet and sour cream which were examined in Iowa.

Bact. linens in materials other than dairy products.

Feeds. Bact. linens was recovered from most of 59 samples of various kinds of feeds, including

corn, oats, barley, wheat, etc., which were examined in Iowa and Québec by smearing directly on plates. From a considerable percentage of the samples, the organism was obtained in relatively large numbers.

Silage. Five samples of rather old, corn silage prepared in Iowa failed to yield Bact. linens when smeared directly on plates. However, 25 samples of rather old, corn and grass silage examined in Québec yielded the organism in a number of instances; when the silage was taken from the mangers, where it could have been contaminated with feeds, the numbers of organisms present were larger than when the silage was taken from the silo.

Green plants. Eight samples of green plants examined in Iowa yielded Bact. linens in about one-half of the instances.

Hay and straw. Nearly all of seven samples of hay and straw examined in Iowa yielded Bact. linens.

Water. From 17 samples of water used for

watering cows or standing in the stables or barn yards, Bact. linens was recovered rather regularly by smearing directly on plates; the samples were all from Québec. Water from a small Iowa lake did not yield the organism in cold weather but it often was obtained during the warmer season.

Mouths of cows. In the examination of mouths of cows for Bact. linens, a sterile, moist, cotton swab was smeared over the tongue or teeth of an animal, after which the cotton commonly was shaken in 10 ml. of sterile water and some of the water smeared on plates; in a few instances the swabs were smeared directly. With both methods the organism was recovered in relatively large numbers in 12 examinations made in Québec when the cows were in the stables. It was not found in 15 examinations made in Iowa when the cows were on pasture. In Québec the mouths of two calves receiving only milk yielded Bact. linens on culturing.

Manure. Samples of manure were examined for Bact. linens by smearing directly on plates. The organism was obtained from more than half of 50 samples collected in Iowa and Québec. Some of the samples were thoroughly dry when examined while others were fresh.

Air. The presence of Bact. linens in cheddar cheese made from pasteurized milk suggests that it may contaminate milk or cheese by falling from the air. Examinations of the air of dairy plants, cheese ripening rooms, wash rooms, stables, etc. were carried out by exposing plates for different periods in various locations. The organism was found in the air rather frequently in Iowa and Québec, but commonly it was present in relatively small numbers.

Soil. Thirty samples of soil were examined in Iowa and Québec. None of them yielded Bact. linens.

General statement.

The data indicate that Bact. linens is a widely distributed species. It commonly was found in milk and cream and on the surfaces of various foreign type cheeses, and also was encountered in the interiors of cheddar cheese, even in those made from pasteurized milk. Since the organism frequently is present in feeds, water and other materials around stables, there is abundant opportunity for contamination of milk and cream on the farms. The presence of the species in the air of stables, dairy plants, etc. provides an additional opportunity for the contamination of dairy products, not only during their production but also during their handling in dairy plants.

Special Studies on Bact. linens

General action in litmus milk.

In litmus milk in test tubes, Bact. linens slowly produced an alkaline reaction and then

later there was conspicuous proteolysis. On the basis of the color of the litmus, a change in pH usually was noted after 6 days, and a definite increase in pH generally was evident after 10 days, when a grayish or yellowish sediment was present and the first signs of proteolysis were evident. The proteolysis increased as the holding time was extended; generally, rather complete digestion required from several weeks to more than 1 month. The pH of milk showing the first evidence of digestion commonly was about 7.3, while after extended incubation at 21°C. the pH of milk cultures ranged from 8.0 to 8.3.

In plain skim milk in test tubes, no change was evident until after about 10 days had elapsed. Then the original white color of the milk often had changed to a slight orange-red. With some strains of Bact. linens a yellow-orange ring developed on the wall of the tube. The digestion proceeded as with litmus milk.

When either litmus milk or plain skim milk was in thin layers, for example, in a plate, Bact. linens produced changes in them much more rapidly.

Action on fat.

Since Bact. linens failed to produce a rancid odor when developing in milk containing appreciable amounts of fat, no lipolysis was expected with it. However, a number of representative cultures were examined for lipolysis with the methods suggested by Long and Hammer (8). Usually, the natural fat technic was first used and the plates were then flooded with a solution of nile blue sulfate but in some instances the indicator was added directly to the agar. None of the cultures investigated gave any evidence of being lipolytic.

Action on butter.

The action of Bact. linens on butter was investigated with five representative strains which were used to inoculate portions of highly

pasteurized cream. The cream was churned and worked with sterile equipment, and the unsalted butter was held in sterile containers at 21°C.

Each of the five strains produced a putrid condition in the unsalted butter. With four of them the defect was evident in 3 days while with one, 7 days were required for its development. However, under practical conditions, the organism probably is of no significance as a cause of the putrid defect in butter. Usually the condition is attributed to other species, and if Bact. linens were present in putrid butter in significant numbers it should have been noted in the many studies on butter of this type.

Protein breakdown in skim milk.

The protein breakdown in skim milk was investigated with five representative strains of Bact. linens. Each strain was inoculated into several 100-ml. portions of sterile skim milk in cotton stoppered bottles and the bottles were in-

cubated at 21°C. Analyses were made on the serum from each culture after periods ranging from 8 to 89 days. The serum was recovered from a culture by bringing the weight to the original with distilled water, adding 1.5 ml. of glacial acetic acid and heating in boiling water for 20 minutes with frequent agitation. The culture then was cooled and filtered through paper. The chemical analyses on the serum included determinations of total nitrogen, amino nitrogen and various fractions of proteins and protein decomposition products which were soluble or insoluble in trichloracetic acid, ethyl alcohol or phosphotungstic acid. The procedure developed by Lane and Hammer (7) was employed and is as follows:

Total nitrogen. Five ml. of serum was analyzed for total nitrogen by the Kjeldahl method.

Amino nitrogen. One ml. of serum was analyzed by the Van Slyke gasometric method.

Trichloracetic acid soluble and insoluble nitrogen fractions. Five ml. of serum was added to 40 ml. of water and 5 ml. of 20 per cent aqueous trichloracetic acid. After standing 10 to 12 hours the mixture was filtered through paper. A solution containing 5 ml. of 20 per cent aqueous trichloracetic acid and 45 ml. of water was used to wash the precipitate. The nitrogen was determined on the filtrate and on the precipitate.

Ethyl alcohol soluble and insoluble nitrogen fractions. Five ml. of water and 5 ml. of serum were added to 85 ml. of 95 per cent ethyl alcohol. After 10 to 12 hours the mixture was filtered and the precipitate was washed with a solution of 85 ml. of 95 per cent ethyl alcohol and 10 ml. of water. Both the filtrate and the precipitate were analyzed for nitrogen.

Phosphotungstic acid soluble and insoluble nitrogen fractions. Five ml. of serum was added to 45 ml. of water, 15 ml. of 25 per cent aqueous sulfuric acid and 10 ml. of 10 per cent aqueous phosphotungstic acid. After 10 to 12 hours the mixture was filtered and the precipitate was washed with a phosphotungstic acid solution made up with 50 ml. of water, 15 ml. of 25 per cent aqueous sulfuric acid and 10 ml. of 10 per cent aqueous phosphotungstic acid. The nitrogen was determined on the filtrate and on the precipitate.

The data, which are given in Tables 1 and 2, show that Bact. linens greatly increased the soluble nitrogen in milk in which it had grown. Commonly, the increase continued over a relatively long period. In Table 1 the increases from 30 days to 43 days often were relatively large and

Table 1. Protein breakdown in skim milk by Bact. linens - Trial 1

Culture No.	Incubation time (days)	ml. 0.1 normal acid equivalent to nitrogen of 5 ml. serum: Total nitrogen	Nitrogen fractionated into sol. and insol. portions with: Trichloracetic acid Sol.	Trichloracetic acid Insol.	Ethyl alcohol Sol.	Ethyl alcohol Insol.	Phosphotungstic acid Sol.	Phosphotungstic acid Insol.	mg. amino nitrogen in 5 ml. serum
1	8	3.8	3.0	0.7	2.5	1.3	1.0	2.6	0.67
	17	3.9	3.6	0.4	2.4	1.7	1.2	2.8	1.23
	30	6.4	6.1	0.5	4.7	1.9	3.3	3.3	3.25
	43	11.7	11.3	0.5	8.0	3.6	6.1	5.7	6.78
2	8	3.7	3.0	0.6	2.7	0.8	1.0	2.4	1.05
	17	5.2	4.7	0.6	2.8	2.2	1.4	4.0	1.63
	30	7.0	6.5	0.8	5.0	2.4	2.5	4.8	2.51
3	8	3.2	2.7	0.6	1.7	1.6	0.9	2.2	0.82
	17	5.4	4.8	0.8	3.2	2.2	1.7	3.6	1.58
	30	9.6	9.2	0.7	6.8	3.1	3.9	5.9	4.73
	43	10.9	10.3	0.7	8.5	2.5	6.1	4.8	6.44
4	17	5.4	4.6	0.8	3.1	2.1	1.1	4.1	1.18
	30	7.6	7.1	0.8	5.1	2.7	2.3	5.4	2.79
	43	10.0	9.2	0.7	6.5	3.5	3.9	6.3	4.28
5	17	2.3	2.0	0.5	1.2	1.1	1.1	1.4	0.83
	30	3.0	2.8	0.4	1.5	1.5	1.1	1.8	0.80
	43	5.4	5.2	0.4	3.5	3.5	2.1	4.0	2.54
Check	8	2.0	1.7	0.4	1.0	1.0	0.9	1.2	0.48
Check	43	2.2	2.0	0.4	1.2	1.0	1.1	1.2	0.48

Table 2. Protein breakdown in skim milk by Bact. linens - Trial 2

Culture No.	Incubation time (days)	ml. 0.1 normal acid equivalent to nitrogen of 5 ml. serum: Total nitrogen	Nitrogen fractionated into sol. and insol. portions with: Trichloracetic acid: Sol.	Trichloracetic acid: Insol.	Ethyl alcohol: Sol.	Ethyl alcohol: Insol.	Phosphotungstic acid: Sol.	Phosphotungstic acid: Insol.	mg. amino nitrogen in 5 ml. serum
1	51	12.5	12.0	0.6	9.0	3.5	5.5	6.7	6.87
	89	13.2	12.7	0.7	9.5	3.6	6.9	6.0	9.47
2	89	14.6	13.9	0.7	11.0	3.5	9.0	5.6	11.80
4	51	11.0	10.5	0.5	10.0	1.5	5.0	5.5	6.55
	89	14.0	13.5	0.7	10.4	3.8	8.0	6.3	11.27
5	51	5.0	4.0	0.8	3.0	2.0	2.5	3.0	1.52
	89	6.1	5.9	0.5	4.0	2.1	3.8	3.5	3.31
Check	51	2.0	1.5	0.5	1.0	1.0	0.6	1.5	0.43
Check	89	2.1	1.8	0.5	1.1	1.2	0.9	1.3	0.42

in Table 2 the increases from 51 days to 89 days sometimes were significant. There was some variation in the proteolyzing action of the different cultures; in both trials culture number 5 gave relatively low values for soluble nitrogen.

The distribution of the soluble nitrogen in the various cultures was essentially the same. Amino nitrogen was significantly increased in all instances, as were also the fractions soluble in trichloracetic acid and the fractions soluble and insoluble in ethyl alcohol and in phosphotungstic acid. As would be expected the largest fraction was the fraction soluble in trichloracetic acid while the fraction soluble in ethyl alcohol often was the second largest. The fraction insoluble in trichloracetic acid did not show any significant change during the incubation.

Effect of peptone and casein on color production.

Since the reddish-brown color produced on various cheeses by Bact. linens, either alone or in combination with other organisms, is different than the color commonly produced by Bact. linens on the usual media, an attempt was made to modify the color production of the organism on tryptone glucose extract agar. Hefferan (3) noted that Bacillus ruber indicus usually lacks pigment in ordinary meat bouillon but produces it in abundance in peptone solution. Accordingly, the effect of a large amount of peptone was investigated; a large amount of casein was included for comparison and a combination of peptone and casein also was employed.

One liter of tryptone glucose extract agar (12) was divided into 5 portions of 200 ml. each and the following additions were made:

(a) 10 per cent peptone

(b) 10 per cent casein

(c) 5 per cent peptone and 5 per cent casein

(d) 5 per cent peptone and 5 per cent casein; when the plates were poured, 0.2 ml. of rennet extract, which had been filtered through a bacteria-proof filter, was added

(e) none.

The portions were sterilized and plates poured. The casein remained in suspension. The various plates were inoculated lightly by smearing pure cultures over the surfaces. One-half the plates was incubated at 21°C. in oxygen while the other half was incubated at 21°C. in air. Ten strains of Bact. linens were investigated. The plates were examined 1 week after inoculation.

With incubation in either oxygen or air, cultures grown on the medium containing 10 per cent peptone showed a luxuriant growth and the color was very similar to that encountered on various cheeses. With the medium containing 10 per cent

casein the growth was rather poor but the color was somewhat like that found on the medium with 10 per cent peptone. Five per cent peptone and 5 per cent casein gave the same results as 10 per cent peptone. The rennet extract used in one portion of the medium did not influence the color production. On tryptone glucose extract agar alone, there was very little color produced.

The plates incubated in oxygen showed a more intense color than those incubated in air; however, the differences in the color production on the various media were evident with either type of incubation.

From the results it appears that addition of 10 per cent peptone or 5 per cent peptone and 5 per cent casein to tryptone glucose extract agar encourages the growth of Bact. linens and results in production of a color which has more similarity to the one appearing normally on various cheeses than has the color produced on the medium without the additions. They also suggest that the color pro-

duced by Bact. linens when it is growing on cheeses may be due in part to certain products resulting from the protein breakdown.

Action on alcohols.

The effect, on milk cultures of Bact. linens, of various materials that might be formed in or on cheeses during curing was investigated by adding the materials to the cultures, either at the time of inoculation or after growth was under way. Ethyl alcohol was included because it could be produced readily through the action of certain yeasts or other organisms on lactose. Litmus milk cultures of Bact. linens to which ethyl alcohol had been added in suitable concentrations developed a red color, rather than the blue color which is normal without the addition of alcohol. This general effect of added alcohol on the litmus milk cultures suggested an oxidation of ethyl alcohol to acetic acid.

A more detailed test was carried out using a

medium consisting of a 0.3 per cent solution of dehydrated yeast extract. The medium, in 250 ml. quantities, was sterilized in 6 liter Erlenmeyer flasks and the organism was permitted to grow for several days with frequent shaking of the flasks. Ethyl alcohol was then added and the frequent shaking was continued. Eventually, the medium was distilled after adding a small amount of sulfuric acid. The distillation involved a concentration and then distillation with steam. The distillate showed considerable acid and it was examined for acetic acid by the partition procedure.* The acid in the distillate proved to be practically 100 per cent acetic.

Detailed tests were then conducted with higher alcohols, using the procedure outlined. In general, the higher the alcohol the smaller the amount of acid found in the distillate, which suggests in-

---

* The actual partitions were carried out in the laboratories of the Bacteriology Section of the Iowa Agricultural Experiment Station.

creasing difficulty in the oxidation as the molecular weight increased. Propyl alcohol yielded largely propionic acid but there was some evidence of another acid, butyl alcohol yielded essentially only butyric acid and amyl alcohol yielded largely valeric acid with a trace of some other acid. In the tests with hexyl and heptyl alcohols only very small amounts of acid were found in the distillates and, accordingly, no attempt was made to carry out the partition procedure.

The results on the oxidation of different alcohols by Bact. linens suggest some interesting possibilities in connection with the production of various compounds in cheeses.

pH tolerance.

The ability of Bact. linens to grow at various pH levels was tested with 15 representative strains. A 2 per cent peptone solution was prepared and divided into ten lots. The reactions of eight lots were adjusted to pH values of approximately 3.0,

4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 9.8 with hydrochloric acid or sodium hydroxide and 0.1 and 0.5 per cent sodium hydroxide were added to the remaining two lots. The final pH values were obtained after sterilization, using a Beckman glass electrode pH meter. Each of the 15 strains was inoculated into tubes containing 10 ml. of the various lots of peptone solution and one tube of each lot was held as a control.

All 15 strains grew at a pH of approximately 6.0 but none of them grew at a pH of approximately 5.0. All of them grew at a pH of approximately 9.8 and with 0.1 per cent sodium hydroxide added to the medium but only eight of the strains grew with 0.5 per cent sodium hydroxide added. The most luxuriant growth was obtained when the peptone solution had a pH of approximately 6.0 or 7.0. The general results agree with those of Kelly and Marquardt (6) who found that Bact. linens did not grow below pH 5.85 but grew at pH 9.5 and grew best at pH 6.5

In order to study the survival of Bact. linens

in litmus milk in which Streptococcus lactis was growing, eight flasks, each containing 150 ml. of litmus milk, were prepared and sterilized. Each of four strains of Bact. linens was then used to inoculate two of the flasks. After incubating 3 days at 21°C., a bacterial count was made on the milk in one flask containing each strain; it was assumed that the milk in the other flask had essentially the same bacterial count. One flask containing each strain then was inoculated with S. lactis and counts of Bact. linens were made on each of the eight flasks after 7, 10, 13 and 17 days (4, 7, 10 and 14 days after adding S. lactis). The counts were made by smearing dilutions of the milk, or the milk direct in some of the later counts, on the surface of the special cheese agar and incubating the plates in oxygen at 21°C. Table 3 gives the data.

From the results it is evident that in the pure cultures in milk Bact. linens developed extensively; the maximum numbers noted with the various cultures

Table 3. Effect of S. lactis on survival of Bact. linens in litmus milk at 21°C.

Bact. linens Culture No.	S. lactis	Bact. linens per ml. after 3 days	7 days	10 days	13 days	17 days
6	not added	55,000,000	310,000,000	520,000,000	180,000,000	54,000,000
	added	55,000,000*	70,000	30	2	>1
7	not added	21,000,000	160,000,000	130,000,000	230,000,000	120,000,000
	added	21,000,000*	46,000	30	3	>1
8	not added	130,000,000	300,000,000	230,000,000	280,000,000	75,000,000
	added	130,000,000*	670,000	15	5	>1
9	not added	132,000,000	150,000,000	250,000,000	480,000,000	280,000,000
	added	132,000,000*	430,000	15	2	>1

* S. lactis was added after count of Bact. linens was made on a 3-day litmus milk culture.

were reached after 7, 10, or 13 days and there then was a decrease. With S. lactis added, the counts of Bact. linens decreased rather rapidly; after 10 days the counts were below 100 per ml., after 13 days they were below 10 per ml. and after 17 days they were less than 1 per ml.

Longevity in litmus milk.

The longevity of Bact. linens in litmus milk was studied by inoculating each of 15 representative strains into a test tube of the milk and holding at room temperature. From time to time transfers were made from each tube to fresh tubes of litmus milk; these were incubated at room temperature and later examined for growth. Each of the cultures was still alive after 4 months, which was as long as the tests were continued. During this period there was extensive concentration of the milk.

Effect of desiccation.

Eleven representative strains of Bact. linens

were investigated for their resistance to desiccation. Several strips of sterile filter paper were soaked for a few minutes in a 48-hour culture, in 1 per cent peptone solution, of each of the strains and then placed in sterile plates at room temperature to dry. Each strip was given ample room and drying appeared to be rather rapid. From time to time a strip of paper representing each culture was transferred to a tube of 2 per cent peptone solution. After incubating for 7 days at room temperature, each tube of peptone solution was cultured for the presence of Bact. linens, using the special cheese agar and incubating the plates in oxygen, to establish the identity of the species developing.

Transfers of the strips of paper to peptone solution were made after 1, 5, 11, 17, 22, 29, 36, 60 and 90 days. In each test with each of the 11 strains of Bact. linens, the organism was still active which indicates that in the dry state Bact. linens survives for extended periods.

Sodium chloride tolerance.

The sodium chloride tolerance of Bact. linens was investigated with both skim milk and 2 per cent peptone solution. In the case of the skim milk, the amounts of sodium chloride necessary to give the percentages desired to 10 ml. of the milk were weighed into cotton stoppered test tubes and sterilized. Then 10 ml. of sterile skim milk was added to each tube, with precautions against contamination. In the case of the 2 per cent peptone solution, the desired amounts of sodium chloride were added to relatively large volumes of the solution, after which 10 ml. portions were put into test tubes and the tubes sterilized. Fifteen representative strains of Bact. linens were inoculated into both skim milk and peptone solution containing 5, 8, 10, 12, 15, 20, 25, 30, 35 and 40 per cent sodium chloride; with 40 per cent sodium chloride, solution was not complete and with certain of the other concentrations crystallization was evident as drying occurred.

Growth of Bact. linens apparently was not delayed by concentrations of sodium chloride up to 15 per cent. In skim milk the organism eventually grew in all the concentrations employed, but with the higher concentrations growth was delayed. In peptone solution growth eventually occurred with all the strains in all the concentrations of 30 per cent or less, but with concentrations of 35 and 40 per cent 5 of the 15 strains failed to grow.

The results indicate the great resistance of Bact. linens to sodium chloride. They are in agreement with those of Kelly and Marquardt (6) who noted that Bact. linens continues to grow on the surface of limburger cheese even when the salt concentration reaches about the saturation point.

Heat resistance.

The heat resistance of Bact. linens was investigated with 12 representative strains. One-tenth ml. of a 2-day culture of each strain, grown in 1 per cent peptone solution at 21°C., was added to 10 ml. of a 1 per cent peptone solution and 2 ml. portions were

placed in sterile agglutination tubes. The tubes then were sealed and exposed in a water bath at 62.8°C. for 5, 10, 15 and 20 minutes. At the end of the exposures the sealed tubes were removed from the water bath and cooled rapidly in ice water. Then each tube was broken and the contents were transferred to a tube of 1 per cent peptone solution. After 3 days tubes showing growth were cultured for Bact. linens to establish the identity of the organism developing.

The results indicated that Bact. linens is very susceptible to destruction by heat. Only 2 of the 12 strains survived 62.8°C. for 5 minutes which was the shortest exposure used. The results were confirmed in additional trials. Apparently, the organism is readily destroyed by any adequate pasteurization procedure.

Catalase production.

Most of the strains of Bact. linens which were isolated were tested for catalase. The usual pro-

cedure was to add 3 drops of a 3 per cent solution of hydrogen peroxide to a depression in a black spot plate and then mix with it about 0.2 ml. of a 10-day litmus milk culture; the development of gas was then noted. Usually, gas was liberated rapidly in relatively large amounts.

All the strains of Bact. linens which were examined were catalase positive. Materials other than litmus milk in which Bact. linens had grown also were satisfactory for catalase tests. These included peptone solution and liquefied gelatin. Growth from agar slopes likewise rapidly liberated oxygen from hydrogen peroxide.

With litmus milk cultures of Bact. linens, the maximum gas liberation occurred after growth for about 10 days. Cultures grown 24 hours liberated only very little gas; as the culture aged the catalase activity increased until the culture was about 10 days old.

General Description of Bact. linens

The following description of Bact. linens is based on a study of more than 300 strains isolated from various materials in Iowa and Quebec. In its preparation the description of Wolff (15) was used for comparison.

Morphology (cultures grown at 21°C.)

Form and size: Rods; 0.5 to 0.75 by 0.5 to 4.0 microns (averaging about 0.62 by 2.5) when grown 1 to 2 days on T. G. E.* agar.

Arrangement: Singly, in pairs and short chains.

Staining reactions: Stains readily with common stains; gram positive.

Spores: None observed; the organism was easily destroyed by heat.

Motility: None observed.

Cultural characteristics (cultures grown at 21°C.)

Agar slant: On T. G. E. agar after 2 days, growth was abundant, glistening, filiform, non-viscid

---

* T. G. E. = Tryptone glucose extract (12).

and cream colored. With extended incubation, the color usually was brown. On special cheese agar in an atmosphere of oxygen, growth was bright orange to reddish-brown in 4 or 5 days.

Agar stab: Heavy surface growth on T. G. E. agar, with no growth along the line of inoculation.

Agar colony: On T. G. E. agar (surface inoculation) after 1 or 2 days, colonies were convex, glistening, smooth-edged and cream colored, becoming brown on extended incubation. Some cultures were viscid. Colony diameters were 2 to 5 mm. On special cheese agar with incubation in oxygen, growth was more vigorous than on T. G. E. agar in air and the color became bright orange to reddish-brown in 4 or 5 days (See Figures 1 to 4 inclusive).

Gelatin stab: Crateriform liquefaction, becoming infundibuliform on extended incubation. Rate of liquefaction varied considerably with different cultures, some completing it in 15 days, others not completing it even with long incubation.

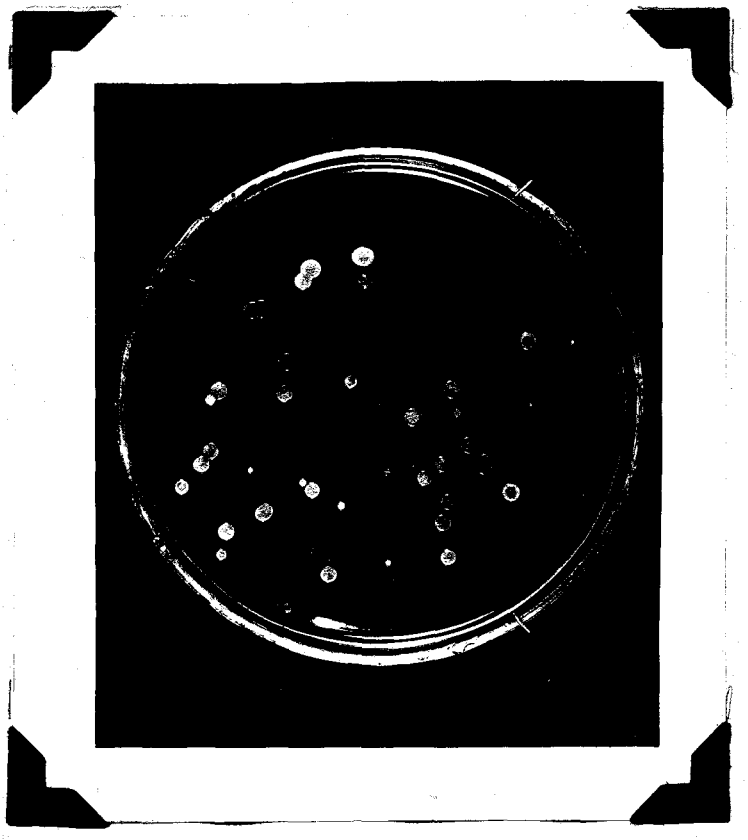


Fig. 1. *Bact. linens* on tryptone glucose extract agar; grown 6 days at 21°C. in air.

Fig. 2. Bact. linens on special cheese agar; grown 10 days at 21°C. in oxygen.

Fig. 3. Bact. linens on tryptone glucose extract agar plus 5 per cent peptone and 5 per cent casein; grown 10 days at 21°C. in oxygen. Plate heavily seeded to give mass of growth rather than well isolated colonies.

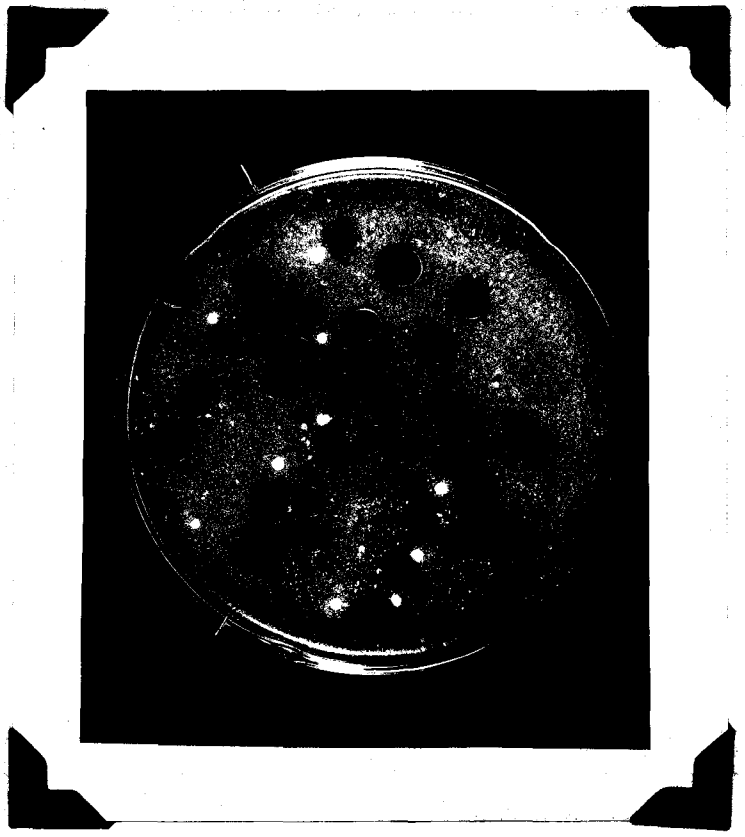


Fig. 4. Bact. linens on special cheese agar inoculated with material from bel paese cheese; grown 10 days at 21°C. in oxygen. Other species are evident on the plate.

Beef extract broth: Turbidity, sediment.

Potato: After 5 days growth was scanty, smooth, glistening and varying in color from grayish to brownish-orange. Medium became grayish. On extended incubation the growth sometimes became abundant.

Litmus milk: At the beginning the changes were very slow. After 6 or 7 days the reaction became alkaline and a yellow sediment appeared. After approximately 10 days some digestion was evident. Digestion generally required several weeks to more than 1 month for completion. Some cultures digested milk only slightly. A distinct ammoniacal odor, more or less objectionable, was produced in old cultures. Coagulation never occurred. Ropiness often was produced on extended incubation.

Biochemical features (cultures grown at 21°C.)

Indol: Not produced

Nitrates: Reduced to nitrites.

Methyl red reaction: Negative.

Voges Proskauer reaction: Negative.

Hydrogen sulfide: Excellent growth on peptone iron medium, both as broth and as agar; production of hydrogen sulfide was noted with some cultures but not with others.

Lipolysis: Natural fats not hydrolyzed.

Fermenting power: No acid or gas from arabinose, dextrin, dextrose, dulcitol, galactose, inulin, lactose, levulose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose or xylose.

Catalase: Rapidly produced in or on various media.

Growth conditions

Oxygen relationships: Aerobic.

Growth temperatures: Growth at 8° and 37°C. but not at 45°C., with the optimum at about 21°C.

Heat resistance: Heat resistance low, cultures being killed at 62.8°C. in a few minutes.

Identification procedure

Colonies that show the proper characteristics on the special cheese agar are inoculated into litmus milk. If the milk develops an alkaline reaction and later is proteolyzed and the cells are gram positive

rods, the litmus milk cultures are tested for catalase. If positive the cultures are inoculated into gelatin to determine their liquefying ability. Any additional tests that seem advisable are then carried out.

DISCUSSION OF RESULTS

The method developed for the isolation of Bact. linens, which includes the use of a special cheese agar and the incubation of the smeared plates in oxygen, offers some interesting possibilities from the standpoint of the study of the organism. It should aid in investigating the numbers and distribution of Bact. linens on and in various cheeses and in the milk from which the cheeses are made. The selective action of the medium is based on various factors, such as control of certain organisms with 5 per cent sodium chloride, a high pH or a combination of the two and the intensification of the color produced with an atmosphere of oxygen and with sodium oxalate.

The wide distribution of Bact. linens about the stables and in the air of dairy plants undoubtedly accounts for a more or less regular contamination of milk and cream with this species. Its high resistance to desiccation probably explains its presence in dried materials of different types,

including various feeds, hay and straw, and also in the air.

The protein is the principal constituent of milk attacked by Bact. linens. While fat and lactose are not attacked by the organism, the breakdown of protein is extensive. This proteolytic action accounts for the importance of the organism in the ripening of certain cheeses on which the organism is present in large numbers in the surface slime. The finding of Bact. linens in cheddar cheese suggests that its importance there needs investigation, although with the failure of the organism to resist high acidities any appreciable growth in cheddar cheese seems unlikely. However, there remains the possibility that in certain products the buffer capacity may permit the growth of the organism when it otherwise would not occur. Another possibility is that the production of an alkaline reaction by the organism may aid in its survival in acid media, and even the production of alkali by other species may tend to protect Bact. linens as well as the organism that was responsible for the production. Yale (18) reported an organism

on the surface of limburger cheese which might be of importance in this connection. Cultures which apparently were of this same general type were frequently encountered on the plates prepared with material from the surfaces of various cheeses. However, the oxygen requirements of Bact. linens suggest that even with no other limiting factor its growth in cheddar cheese would be greatly delayed if not prevented.

In all probability Bact. linens plays a conspicuous role in the color production on the surfaces of certain cheeses. Presumably, this color production adds to the general appearance of the cheese, at least in the eyes of certain consumers. From the studies carried out it seems that this color production is influenced by certain products of protein decomposition and, of course, these may be the result of the activity of either Bact. linens or some other ripening agent.

The action of Bact. linens on various alcohols suggests some interesting possibilities in connection

with dairy products which undergo extensive bacterial action. One species might produce an alcohol which later would be oxidized to the corresponding acid by Bact. linens or some similar species. Various micro-organisms growing on or in cheeses could be responsible for the alcohol production.

The salt resistance of Bact. linens undoubtedly is one of the characteristics which permits its growth on the surfaces of various cheeses where the salt content is comparatively high.

The active production of catalase by the organism may be of significance in clearing up the relationship of Bact. linens to other species, particularly in view of the fact that it is a gram positive organism.

SUMMARY

1. A satisfactory method was developed for the isolation of Bact. linens from various sources. A special cheese agar, in which are included potassium citrate, sodium oxalate and 5 per cent sodium chloride, is used and the smeared plates are incubated in oxygen. With this general procedure Bact. linens developed readily and the color production was much more intense than when tryptone glucose extract agar was used and plates incubated in air.

2. Bact. linens was found widely distributed in dairy products and materials about stables, especially in feeds, hay, straw, water and manure. It also was found in the mouths of cows and in the air of stables and dairy plants, but was not found in soil.

3. Bact. linens produced an alkaline reaction in litmus milk and then conspicuous proteolysis. On extended incubation it greatly increased the soluble nitrogen in milk, but different strains

varied considerably in the extent of the proteolysis. Amino nitrogen was significantly increased, as well as the fraction soluble in trichloracetic acid and the fractions soluble and insoluble in ethyl alcohol or phosphotungstic acid.

4. Bact. linens was not lipolytic. In unsalted butter at 21°C., it produced a putrid condition.

5. Color production on tryptone glucose extract agar by Bact. linens was increased by adding 10 per cent peptone or 5 per cent peptone and 5 per cent casein.

6. In a medium consisting of 0.3 per cent desiccated yeast extract in water, Bact. linens produced volatile acids from various alcohols. Ethyl alcohol yielded practically only acetic acid; propyl alcohol yielded largely propionic acid and there was evidence of some other acid; butyl alcohol yielded essentially only butyric acid; and amyl alcohol yielded largely valeric acid with a trace of some other acid. In the medium under the conditions used, hexyl and heptyl alcohols yielded very

little volatile acid.

7. In 2 per cent peptone solution Bact. linens grew at a pH of approximately 6.0 but not at a pH of approximately 5.0. It also grew at a pH of approximately 9.8. In litmus milk in the presence of S. lactis, Bact. linens decreased in numbers rather rapidly.

8. Bact. linens survived in litmus milk at room temperature for at least 4 months. When dried on filter paper it survived for at least 3 months.

9. Bact. linens grew in the presence of large amounts of sodium chloride; all strains grew in skim milk saturated with it and some grew in peptone solution saturated with it.

10. Bact. linens was rather easily destroyed by heat.

11. Bact. linens actively produced catalase when grown in or on various media.

12. A description of Bact. linens was prepared.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. B. W. Hammer for his counsel and suggestions in planning and directing the work herein reported and in the preparation of the manuscript; to Dr. H. F. Long for his help in the preparation of the manuscript.

LITERATURE CITED

1. Duclaux, E.
Sur le rôle protecteur des microbes dans la crème et les fromages. Ann. de l'Inst. Pasteur. 7:305-324. 1893.

2. Grimmer, W., and Schmid, Josef.
Beiträge zur Biochemie der Mikroorganismen. VII. Mitteilung. Zur Kenntnis von Bact. linens. Milchwirt. Forsch. 17:286-302. 1935.

3. Hefferan, Mary.
A comparative and experimental study of bacilli producing red pigment. Centbl. Bakt. Abt. 2, 11:456-475. 1904.

4. Kayser, Edmond.
Microbiologie appliquée à la transformation des produits agricoles. p. 423-448. Librairie J. B. Baillière et fils, Paris. 1931.

5. Kelly, C. D.
The microbiological flora on the surfaces of limburger cheese. Jour. Dairy Sci. 20:239-246. 1937.

6. Kelly, C. D. and Marquardt, J. E.
The influence of hydrogen ion concentration and salt on the surface flora of limburger cheese. Jour. Dairy Sci. 22:309-320. 1939.

7. Lane, C. B. and Hammer, B. W.
Bacteriology of cheese. I. Effect of pasteurizing the milk on the nitrogenous decomposition in cheddar cheese. Ia. Agr. Exp. Sta., Res. Bul. 183. 1935.

8. Long, H. F. and Hammer, B. W.
Methods for the detection of lipolysis by microorganisms. Ia. State College Jour. Sci., 11:343-351. 1937.

9. Mazé, P.
Technique fromagère. Ann. de l'Inst. Pasteur. 24:395-428. 1910.

10. Rosell, J. M., Urgel 71, Barcelone, Spain.
Unpublished information supplied in 1937.

11. Seppilli, A.
Influence du calcium sur la production des pigments bacteriens. Bollettino della sezione italiana, Società internazionale di microbiologia. 3:178-179. 1931.

12. Standard methods for the examination of dairy products. 8th ed. American Public Health Assn., N. Y. 1941.

13. Steinfatt, Fritz.
Uber Bacterium linens und seine Beziehungen zu einigen seiner Begleitorganismen in der Käserotschmiere beim Eiweissabbau in Milch. Milchwirt. Forsch. 9:1-50. 1930.

14. Weigmann, H.
Mykologie der Milch. p. 212-237. Verlag von M. Heinsius Nachfolger, Leipzig. 1911.

15. Wolff, A.
Welche Mikroorganismen beteiligen sich an der Bildung des rotgelben Farbstoffes auf der Oberfläche der Käse im Reifungskeller? Milchwirt. Centralbl. 5:145-154. 1909.

16. Wolff, A.
Nachtrag zu der Frage: Welche Mikroorganismen beteiligen sich an der Bildung des rotgelben Farbstoffes auf der Oberfläche der Käse im Reifungskeller? Milchwirt. Centralbl. 5:414-416. 1909.

17. Wolff, A.
Milchwirtschaftliche Bakteriologie. Centbl. Bakt. Abt. 2, 28:417-422. 1910.

18. Yale, M. W.
The surface flora and the use of pure cultures in the manufacture of limburger cheese. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 268. 1943.