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# The relationships of a lipolytic organism to rancidity of butter

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THE RELATIONSHIPS OF A LIPOLYTIC ORGANISM  
TO RANCIDITY OF BUTTER

BY

124  
87-35

RALPH VICTOR HUSSONG

A Thesis Submitted to the Graduate Faculty  
for the Degree

DOCTOR OF PHILOSOPHY

Major Subject: Dairy Bacteriology

Approved:

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## INTRODUCTION

The work presented in this paper is the outgrowth of finding the same species of bacterium in two samples of rancid butter examined in the Dairy Bacteriology Laboratory of the Iowa Agricultural Experiment Station. The organism was present in rather large numbers in each sample and this suggested a relationship to the rancid condition. A few preliminary churnings were made, using pasteurized cream inoculated with the organism isolated. The butter from these churnings became rancid within three days, when stored at 15.5 C., while the control butter was unchanged. This indicated more definitely that the organism isolated was responsible for the rancidity in the original butter. Preliminary inoculation of the organism on a medium designed to detect fat hydrolysis showed that it rapidly hydrolyzed fat.

The organism isolated from the rancid butter possessed two unusual characters similar to those of an organism which had frequently been found in previous years on plates poured from both normal and abnormal dairy products, but whose possible relationship to the production of rancidity in butter had not been suspected. The most prominent of these characteristics was the production of a ring of acid curd at the surface of a litmus milk culture. The ring seldom extended entirely

over the surface of the milk but adhered to the sides of the test tube. Only after long incubation periods did the entire tube of milk coagulate. The other character in which the organism isolated from the rancid butter was similar to the organism previously isolated, was the production of an odor resembling that of the flower of the common Mayapple or mandrake (*Podophyllum peltatum*).

Organisms identical with, or very closely related to, the two cultures secured from rancid butter were later isolated from a number of samples of normal and abnormal dairy products secured from widely separated regions of the United States. After 51 cultures had been isolated a systematic study of them was undertaken. Before this had proceeded far it was evident that the apparently pure cultures were producing different colony types when plated on agar. A preliminary survey of these variant cultures revealed that certain of them possessed biochemical characteristics which were unlike those of the parent culture. Some of the variant cultures were found to remain stable during a number of transfers in litmus milk.



STATEMENT OF PROBLEM

The purpose of the investigation was:

- (1) To identify a group of organisms which was isolated from various normal and abnormal dairy products and which was capable of hydrolyzing fat and producing rancidity in butter. This part of the problem was complicated by the knowledge that pure cultures of the organisms were capable of producing a number of stable colony types. The colony types varied not only in appearance but in biochemical activity. If the colony types had been isolated from different sources with no knowledge of the colony variation of pure cultures the differences were such that they would have been considered separate species.
- (2) To study the colony variations occurring in pure cultures of the organism.
- (3) To determine the biochemical activity of a number of stock cultures and the variant colonies secured from some of them.
- (4) To study the changes occurring in butter made from cream containing the organism.

### III. HISTORICAL

#### A. Fat-splitting organisms and rancidity of butter.

The bacteriology of rancid butter has been the subject of numerous investigations most of which were carried out during the last part of the nineteenth and the first part of the twentieth century.

Marpmann (24) described a spore-forming organism which he thought was the cause of the lactic acid fermentation; he named it Bacillus limbatum acidi lactis. The organism is of interest because von Klecki (19) considered it similar to a species which he isolated from rancid butter.

Conn (4) made butter from pasteurized cream which had been inoculated with a Micrococcus. This butter developed a rancid taste and a bad odor. The organism produced butyric acid in milk.

von Klecki (19) described five species of bacteria which he had isolated from rancid butter. Two of the species isolated were cocci and were named Diplococcus butyri and Tetrococcus butyri. One species was a spore-forming rod which von Klecki thought was similar to Bacillus limbatum acidi lactis Marpmann and which he called Bacillus limbatus butyri. The two remaining species were non-spore-forming rods and were designated

Bacillus butyri I and Bacillus butyri II.

Eijkman (6) studied a differential medium for the isolation of fat-splitting bacteria. From his data he concluded that Bacillus procyaneus, Staphylococcus pyrogenes albus, Bacillus fluorescens, and Bacillus prodigiosus could hydrolyze fat strongly while Bacillus indicus and Bacillus ruber possessed a weaker hydrolyzing action.

Orla-Jensen (25) reported that under ordinary conditions the bacteria which produced rancidity in butter were Bacillus fluorescens liquefaciens and Bacillus prodigiosus.

Schreiber (27) isolated 30 organisms from fat which had been in contact with the earth for a long time. Of these only two species of bacteria, Bacillus fluorescens and Bacillus ? were able to decompose fat. After the organisms had been grown on laboratory media for a long time the first lost the power of decomposing fat.

de Kruiff (20) described nine species of fat-splitting bacteria which he had isolated from soil, sewage, water, old butter, and animal feces. Eight of the species were meagerly described and were not given specific names. The remaining species was identified as Bacillus fluorescens liquefaciens. All of the species studied grew at 37° C.

Hass (17) isolated a fat-splitting organism from milk; he named it Bacteridium lipolyticum. The organism grew at 37° C.

Evans (7) reported that a variety of Bacillus abortus hydrolyzed fat; it was designated Bacillus abortus var. lipolyticus.

Haag (13) studied the splitting of fat by bacteria and concluded that of the organisms studied only Bacillus pyocyaneus was capable of bringing about this change.

Henneberg (16) studied a number of organisms which he isolated from various grades of milk. The organisms were divided into the following groups: micrococci (including Sarcina); streptococci; Bacterium alcaligenes; fluorescens; Proteus, cloacae, and Corynebacterium. Fat was decomposed by one species of Micrococcus; one species of Sarcina, four species of streptococci; six species of Alcaligenes; two species of fluorescens; two species of Proteus; and one species cloacae. The organisms were incompletely described and were not named.

#### B. Aroma producing bacteria.

Reinmann (26) in a study on rancidity of butter mentioned a coli-like bacterium which produced a strawberry aroma. When grown in butter the organism caused the butter to become rancid. The other characters of the organism were not given.

Eichholz (5) isolated an organism which produced a strawberry-like aroma in all of the media tested except potato. He considered it a new species and named it Bacterium fragi. Its

optimum growth temperature was 26-29° C. Although it could withstand 37° C. for several days it did not grow at this temperature.

Gruber (11) isolated an organism which produced a strawberry-like aroma. He named the organism Pseudomonas fragariae.

Later Gruber (12) isolated another species capable of producing strawberry-like aroma; it was secured from milk. He called this species Pseudomonas fragariae II.

Huss (18) also reported the isolation of an organism which produced a strawberry-like odor in various media. He designated the organism Pseudomonas fragaroidea.

#### G. Bacterial disassociation

Variations in the types of colonies produced by pure cultures of bacteria have received considerable attention during recent years. Hadley (14) reviewed the literature dealing with this subject up to 1927. In his review Hadley used the terms smooth and rough (designated S and R) to describe the colonies produced. These terms were first used by Arkwright (1). Another colony type which Hadley included was the intermediate or O type. This was first described by Firtsch (8) in 1888. The descriptions of the S and R type colonies are fairly definite but is difficult to interpret the exact nature of the O type colony.

Soule (28) in his study of Bacillus subtilis added two more colony types to those previously recognized. These were the mucoid or M type and the phantom or P type.

Hadley (15) later, in a study of the filterable forms of bacteria, added another colony type to those already in existence. This was the G type which he thought represented the gonidial phase in the life cycle of bacteria. It is probable that this is the same as the phantom colony type described by Soule (28).

Kuhn and Sternberg (22) reviewed the literature of another school of bacteriologists who base their differentiation of the variation in cultures of bacteria on the morphology of the cells present in the cultures rather than on the character of the colonies. Kuhn (21) has divided the cells occurring in a culture of bacteria into four groups as follows: The A forms are large globular elements that occur in cultures of many species and were considered by Kuhn to be protozoa or myxomycetes and are reported to form spores which invade normal bacteria causing them to swell and burst; The invasion of the normal cells by spores of the A forms, and their subsequent bursting Kuhn considered to represent bacteriophage action; The B forms are probably the same as the normal or S type of other investigators; The C forms were reported to be filterable through porcelain filters and to represent the metachromatic granules often seen in bacterial cells. They are probably identical with

the P colonies of Soule (26) and the G colonies of Hadley (12). The D forms represent the filamentous and branched cells often found in bacterial cultures.

In general, work on colony variation has been carried out by using various outside agents, such as dyes, inorganic salts, variation in pH, and toxic materials, to either accelerate the variation or to suppress one type and allow the others to develop. However, some of the investigations reported involved none of these agents and deal with the colony variations occurring either in rapidly growing or old cultures of bacteria.

#### IV. METHODS

##### A. Method of making experimental butter.

In investigating the action of various cultures in butter, the experimental butter was made from sweet cream which had been pasteurized at 62.2° C. for fifteen minutes, cooled and inoculated with a young litmus milk culture of the organism to be studied. The inoculated cream was incubated for about 18 hours at 5° C. before it was churned.

The churn used was a small motor-driven shaker to which was attached glass jars containing the cream to be churned. For small amounts of cream one quart fruit jars, fitted with glass tops, were used. Salt-mouth bottles having a capacity of four liters were employed for larger amounts of cream; the bottles were stoppered with corks covered with sterile parchment.

All of the material coming in contact with the butter was sterilized before use. These materials included wash water, paddles, working bowls, salt, and the final packages in which the butter was placed. The finished butter was stored either in Petri dishes or, where larger amounts were desired, wrapped in parchment paper. The butter was stored at various temperatures depending on the experiment being



conducted.

The exposure used in pasteurizing the cream was such that the resulting butter had a pronounced heated flavor and odor. The heated flavor and odor persisted for several days after the butter was made but the intensity decreased so that after four or five days it was very slight.

B. Determination of numbers of bacteria in butter.

The number of bacteria present in the butter was determined by means of the plate count. A small sample of the butter was carefully melted in a Petri dish and after a thorough mixing of the melted butter one milliliter was transferred to a water blank which had been warmed to 45° C. Dilutions were then made in the usual manner using previously warmed water blanks. The nutrient medium used was beef infusion agar adjusted to pH 6.8-7.0. The plates were incubated four days at 21° C. In the case of butter made from cream containing butter culture as well as the Mayapple organism only the Mayapple colonies were counted.

C. Determination of the total acid volatile acid in butter.

The total amount of acid present in the butter was determined by dissolving 10 grams of butter in 15 ml. of ethyl alcohol and 35 ml. of ethyl ether and titrating with tenth normal sodium hydroxide, using phenolphthalein as an indicator.

The amount of alkali required will be referred to as the acid value of the butter.

The amount of volatile acid present in butter was determined by steam distilling 500 grams of butter and titrating the first liter of distillate with tenth normal sodium hydroxide, using phenolphthalein as an indicator. The amount of alkali required to neutralize the acid in the liter of distillate will be referred to as the volatile acid value.

D. Detection of fat hydrolysis by bacteria in media other than butter.

In studying the relationship of bacteria to rancidity in butter various strains of the Mayapple organism were inoculated into cream and the cream churned. Such a procedure, however, is very time consuming and it is desirable to use some more convenient method for the detection of fat hydrolysis. A procedure for this has been described by Turner (30). The method is based on the fact that Nile-blue sulfate acts as a specific dye for staining unsaturated fatty acids. The bacteria to be tested are grown on the surface of agar containing Nile-blue sulfate and an emulsion of fat. After fat-splitting organisms have grown on this medium the unsaturated fatty acids liberated are stained a deep blue by the absorption of the dye from the surrounding medium. The absorption of the dye from the agar leaves a clear zone extending out

from the area of bacterial growth. The emulsion of fat used was made either by emulsifying cottonseed oil with india gum and, after sterilization, adding this to the agar or by adding cream to the agar.

In the studies herein carried out the usual method of preparing an emulsion of fat was as follows: Two to four per cent of olive oil or butter fat was added to 100 ml. of 0.5 per cent agar solution and sterilized. After sterilization the mixture was cooled below the solidifying point of the agar. To make the emulsion all that was now necessary was to shake the mixture vigorously. This gave an emulsion which was finely enough dispersed to give excellent results when mixed with the nutrient agar. When butter fat was used it was necessary to warm the mixture to a point slightly above the melting point of the fat before shaking because of the tendency to churn.

Nile-blue sulfate was added to beef infusion agar at the rate of 0.8 ml. of a 10 per cent aqueous solution of the dye to 10 ml. of agar. The Nile-blue sulfate was added either before or after the agar was sterilized. When added after sterilization of the agar a sterile solution of the dye was used.

To prepare plates for inoculation with the organisms one milliliter of the fat emulsion was placed in a Petri dish and 10 ml. of agar, to which the dye had already been added, was then poured into the Petri dish and the two mixed. The plates were inoculated, after the agar had solidified, by streaking

a loopful of a litmus milk culture of the organism over the surface of the agar. One plate could be used for a number of cultures by marking the bottom into a number of areas.

#### E. Measurement of protein hydrolysis.

The action of the organisms on the protein of milk was determined in the following way: Sterile skim milk was inoculated with the organism to be studied and incubated at 20° C. for one week. The fermented milk was acidified with glacial acetic acid, at the rate of one milliliter of acid to 250 ml. of milk, and heated to 60° C. in a water bath to firmly flocculate the insoluble material. Following heating the milk was cooled in a water bath, filtered through paper and the whey used for the determination of the soluble and the amino nitrogen. The Gunning-Kjeldahl-Arnold method was used for the soluble nitrogen and the Van Slyke procedure for the amino nitrogen. The results of the analyses are expressed as the increase or decrease in the milligrams of nitrogen per 10 ml. of whey, as compared with the uninoculated control.

## V. RESULTS OBTAINED

### A. Distribution of the organism in dairy products

Organisms resembling the cultures secured from rancid butter have been isolated from a considerable number of samples of various dairy products. Some of these samples, mainly ice cream, were apparently normal at the time the organisms were isolated but most of the cultures were secured from materials which were abnormal in some respect at the time the plates were poured.

#### 1. Presence of the organism in normal dairy products

In connection with a study of the variations in the bacterial content of ice cream samples were shipped to the laboratory from various ice cream plants, both in Iowa and in other states. Triplicate sets of plates were poured from the samples and one of the sets was incubated at 7.2° C. On the plates incubated at this temperature colonies developed which were similar to the colonies developing on the plates poured from the rancid butter. The colonies were picked into litmus milk and, after incubation, the characteristic acid ring and Mayapple odor were present. Cultures were secured from 12 samples of normal ice cream. Eleven of these samples came from

Iowa factories and the remaining one came from Tennessee.

2. Presence of the organisms in various abnormal dairy products.

The preliminary work indicated that the organism was responsible for a rancid condition in butter and as the isolation of cultures proceeded other samples of rancid butter were examined. A number of samples of abnormal milk, cream and evaporated milk was also examined.

a. Presence in abnormal butter. Abnormal butter from the following sources was examined.

1. Twelve samples of butter, most of which were considered to be rancid when received, were sent to the laboratory for analysis. Five of these samples were from centralizers, six were from cooperative creameries, while the source of the remaining sample was unknown.

Two of the samples of centralizer butter came from Illinois and the Mayapple organism was secured from one of them. The other sample yielded an organism which was also capable of producing rancidity in butter but it belonged to an entirely different species. Samples of centralizer butter were also secured from Kansas, Minnesota and Virginia, one sample from each state, and cultures of the Mayapple organism were secured from each of them.

The six samples of butter coming from cooperative creameries were from Iowa plants and were all considered to

be rancid when received. A typical Mayapple organism was isolated from each of them. Most of these samples were made from sweet cream and the butter had a low salt content.

The remaining sample of butter was more tallowy than rancid when received and repeated attempts to isolate a Mayapple organism from it were unsuccessful.

2. Samples of butter from a number of Iowa plants are sent to the Iowa Butter Control Laboratory each month for analysis. These samples are packed in pint glass jars and sent without refrigeration. Occasionally a sample of this butter is rancid when received. Nine of these rancid samples have been plated and typical Mayapple organisms isolated from all of them.

3. Ten samples of butter were secured from a study on keeping quality of this product. The butter came from cooperative creameries and was of good quality when received. The usual procedure was to hold the samples at room temperature to determine the length of time they would remain in a marketable condition; at stated intervals the butter was judged. Each of the seven samples which developed definite rancidity yielded the Mayapple organism. A sample which developed a yeasty condition and two others which became malty did not yield the Mayapple organism.

4. Four samples of butter made in the laboratory during a miscellany of studies on butter deterioration became rancid

during storage and each yielded the Mayapple organism. Three of the samples were made from pasteurized cream and the fourth was made from raw cream.

5. Three samples of butter made on farms from raw cream were studied. None of them yielded the Mayapple organism. All of the butter was heavily salted and was presumably made from rather sour cream. These factors alone or combined probably inhibited the growth of the Mayapple organism if it was originally present in any of the samples.

b. Presence in milk. Various samples of milk were examined for the presence of the Mayapple organism. The milk may be divided into (1) samples which were abnormal when received and (2) normal samples which were held in the refrigerator until spoilage had occurred.

1. Two samples of ropy milk were brought to the laboratory for examination. One of these samples was from a pasteurizing plant in another state which was having trouble with ropiness in their pasteurized product. An examination of this milk revealed that Bacterium viscosum was the organism causing the ropy condition. Cultures of the Mayapple organism were also secured from this milk.

The second sample of ropy milk was from an Iowa city. A large number of extremely ropy colonies developed on plates poured from the milk. When these colonies were picked into litmus milk and the changes occurring noted, it was evident,



from the ring of acid curd at the surface and the odor, that they were of the Mayapple type.

2. Two samples of skim milk were allowed to remain in the refrigerator until an abnormal odor had developed. When these samples were plated a large percentage of the colonies developing were of the Mayapple type.

A sample of high grade raw milk, originally brought to the laboratory for a bacterial count, developed the characteristic Mayapple odor after standing in the refrigerator for several days. The Mayapple organism was easily secured from this milk by plating and picking the resulting colonies.

c. Presence in abnormal cream. Three samples of cream, all showing abnormal odors which suggested either rancidity or the Mayapple odor, were examined. The samples were from pasteurized supplies and the abnormalities developed after the samples had been held in an ice box for several days. A typical Mayapple organism was secured from each of the three samples. The plate count on one of the samples, using beef infusion agar and an incubation of four days at room temperature, was 407 million bacteria per milliliter. The Mayapple made up 32 per cent of the total flora of this cream.

d. Presence in abnormal evaporated milk. Milk evaporated to different densities was prepared for use in an ice cream investigation. This milk was not sterilized after concentrating but was held in a room slightly above the freezing point.

After several days storage some of the milk developed a May-apple odor. The Mayapple organism was isolated from milk with both high and low concentrations of solids; the odor seemed to be most pronounced in the milk with the lowest solids content.

### B. Identity of the organism

After 51 cultures of the Mayapple organism had been isolated their systematic study was undertaken with the object of identifying them. The cultures were purified by repeated platings before being subjected to this study. The Mayapple organism was found to produce three distinct types of colonies, S, O and R, when grown in laboratory media. Each of the types differed somewhat in biochemical activity and, because of this, descriptions will be given separately. Only the characteristics in which differences were noted will be repeated in the descriptions. The description of the types follows:

#### S TYPE

##### MORPHOLOGY

- Form . . . . . The organism was rod-shaped with rounded ends.
- Size . . . . . The organisms varied in size from 0.5 to 0.8 by 1.0 to 3.0 microns.
- Arrangement . . . . . When grown in milk and bouillon the organism occurred singly, in pairs,

and in chains. Certain cultures were made up almost entirely of short chains.

Staining . . . . . The organism was gram negative. It stained readily with other stains.

Motility . . . . . The organism was motile by means of a polar flagellum.

Spore formation. . . . . The organism did not form spores.

#### CULTURAL CHARACTERISTICS

Agar slopes . . . . . The growth on agar slopes was abundant, greyish-white, smooth and glistening. The growth from some of the cultures studied was ropy but with others it was not.

Agar plate colonies . . . . . The smooth type colony was convex, glistening, smooth-edged, smooth-surfaced and opaque; in some cultures it was ropy. This type of colony was considered to be the normal type because it was encountered most often in isolations from various dairy products.

Gelatin . . . . . Gelatin was slowly liquefied, the type of liquefaction changing from crateriform to stratiform. Even after long periods of incubation the liquefaction never extended to the bottom of the tube.

- Bouillon . . . . . In bouillon a pellicle was usually formed and this was followed by a turbidity which gradually extended to the bottom of the tube. The pellicle was delicate and was easily broken, after which it settled to the bottom of the tube. Unless the tube was shaken the turbidity was ordinarily very slow in extending down the tube.
- Potato . . . . . The growth on potato was echinulate to arborescent, raised, and glistening, and became slightly brown on extended incubation.
- Litmus milk . . . . . The action of the organism in litmus milk was quite characteristic. The first change noted was the formation of an acid ring at the surface. After considerable time a coagulum was formed at the surface next to the wall of the tube. Only after extended incubation was all of the milk coagulated. Accompanying this change was the production of a

characteristic odor resembling that given off by the flower of the May-apple. Results of the soluble and amino nitrogen determinations indicated that there was a slight proteolytic action on the nitrogenous constituents of the milk.

BIOCHEMICAL FEATURES

- Gas formation . . . No gas was formed in milk or from any of the fermentable substances used.
- Indol . . . . . The organism did not produce indol.
- Voges-Proskauer . . The organism was V-P negative.
- Methyl red . . . . The organism was M-R negative.
- Nitrates . . . . . The organism did not reduce nitrates to nitrites.
- Sp.* Ammonia . . . . . The organism produced ammonia from peptone.
- Reaction change . . Acid was formed in bouillons containing arabinose, galactose, and glucose. Acid was not formed in bouillons containing glycerol, levulose, mannitol, sorbitol, lactose, maltose, sucrose, salicin, raffinose, inulin, dextrin, or starch. After long incubation periods the reaction of the cultures

in which acid had been formed became slightly alkaline. Tubes containing substances which were not fermented became alkaline rather quickly

Action on fat . . . The organism hydrolyzed butter fat and olive oil.

#### Temperature relationship

The organism was psychrophilic. No growth was observed at 37° C. but the organism grew well at 20° C. and at 10° C., and slowly at 3-5° C.

Oxygen relationship....The organism was aerobic.

Heat resistance . . The organism was very sensitive to heat.

Only one of the five cultures tested survived 68.2 C. for 10 minutes and most cultures were killed by very much shorter exposures than this. Incubation of slope cultures at 37° C. for several days killed the organism.

O TYPE. The O type of Mayapple organism is characterized as follows:

O type colony. The intermediate type of colony was thin, translucent, smooth-edged and had a smooth surface. The consistency of the colony was comparable to that of a drop of a weak starch gel which has been allowed to flow in a thin

layer. These colonies were never ropy and, in general, remained quite stable.

Litmus milk . . . . Litmus milk cultures of this type are rarely coagulated and exhibit only a slight reduction of the litmus in the bottom of the tube. This type is less proteolytic than either the S or R type.

Action on fat . . . Usually the O type does not hydrolyze fat although some strains have this power.

In other characteristics the O type colonies resemble the S and R types.

R TYPES. Three types of R colonies varying in degree of roughness and opacity were isolated during the investigation. The characters given below are only those which are different from the S and O types.

R1 type colony. This type was thin, translucent, spreading, flat and bluish, with irregular edges and a smooth surface. It was found to be relatively stable and possibly was an intermediate type between the S and R colonies. The R1 colonies usually were larger than the smooth colonies and never became as opaque. By following them through several platings a tendency to change into one of the other rough types was noted.

R2 type colony. This colony was thin, translucent, rough-edged and wrinkled and was larger than the smooth type. The

colony was very tough and usually the entire colony could be removed from the agar with a needle. With extended incubation some of the R2 colonies became tinged with brown and had the appearance of a thin, transparent brown membrane.

R3 type colony. This colony was encountered only a few times and was found to be unstable. It was opaque, white, raised, the surface was rough and the margin was irregular. It probably was a mixture of smooth and rough colonies.

Litmus milk . . . . The action of the R colonies on litmus milk differed only in degree from the action of the S colonies. They were less proteolytic than the S type and were more proteolytic than the O type colonies.

Action on fat . . . . There was some variation in the action of the R colonies on fat. Part of the cultures yielded R types which hydrolyzed fat while the R types from other cultures did not produce this change.

The systematic study of the Mayapple organism isolated from various normal and abnormal dairy products revealed that it was an aroma-producing, polar-flagellated, psychrophilic, fat-splitting bacterium. There have been a number of species described which show some of the characteristics of the Mayapple type.



Identification of the Mayapple organism with organisms already described is complicated by two factors: (1) The incomplete descriptions of the previously described species, including their action on fat and, (2) The fact that three stable variants could be secured from the Mayapple organism. The variant types of the Mayapple organism possessed characteristics which, had the variants been secured from separate sources, would have justified placing them in different species.

The following possibilities may be considered in the identification of the Mayapple organism: (1) That the organism is a previously undescribed species and that a new species name should be given it; and (2) that each of the colony types had been described as separate species and could be brought together in one species with the knowledge of colony variation now available.

There were two main characteristics by which the Mayapple organism could be traced to previously described species. The first of these is its action on fat and the second is the odor produced in various media and which is here called a Mayapple odor.

Some of the fat-splitting organisms which have been described and their possible relationships to the Mayapple species are given below.

Since the work of Orla-Jensen (25) there has been a tendency to consider Pg. fluorescens as the most important species con-

cerned in the production of rancidity in butter. Although the Mayapple organism qualifies for the genus *Pseudomonas* it cannot be considered identical with *Pg. fluorescens* as it did not grow at 37° C., did not reduce nitrates, and produced an acid reaction in litmus milk.

Haag (13) has reported that *Pseudomonas pyocyanens* (*Pseudomonas aeruginosa* (Schroter) Migula) was capable of splitting fat. The Mayapple organism differs from this species in the following respects: slow liquefaction of gelatin, inability to reduce nitrates, fermentation of arabinose, galactose, and glucose, and inability to grow at 37° C.

*Pseudomonas non-liquefaciens* Bergey et al (*Bacillus fluorescens non-liquefaciens* Eisenberg) has been isolated from rancid butter by various workers and considered to be able to hydrolyze fat. In some respects the description of this organism agrees with that of the rough type of the Mayapple organism. The colonies were described as being fern-like, gelatine was not liquefied and nitrates were not reduced. Litmus milk was reported unchanged which is unlike the action of the Mayapple type.

There have been a few aroma-producing organisms isolated whose descriptions fit rather closely the description of the Mayapple organism. It is unlikely that any group of individuals would all describe in the same way the odor produced by the Mayapple organism. For this reason too much importance should

not be placed on the kind of odor produced by the organism and more emphasis should be placed on its biochemical activity. The several species which have been reported to produce a strawberry-like odor seem to agree in several characters with the various colony types of the Mayapple organism encountered during this investigation.

Grüber (12) isolated an organism, Pseudomonas fragariae II, from pasteurized milk which had been held for a considerable period of time. A strawberry odor developed in this milk and Grüber attributed this odor to the growth of Ps. fragariae II. When the organism was grown in other media the strawberry odor also developed. When grown on gelatin plates the colonies were large, round, arched, dirty-white, and glistening. The organism was rod-shaped, was motile by means of a polar flagellum, peptonized gelatin, and coagulated milk by the production of acid. It grew best at 18-22° C. and did not grow at temperatures above 34° C. This organism agrees rather closely with the S type colony of the Mayapple organism.

Grüber (11) isolated another strawberry-aroma producing organism, Pseudomonas fragariae, from beets. This organism was a non-spore-forming, fluorescent, gelatin non-liquefying species with 1 to 9 polar flagella. The colonies were described as dew-drop like and bluish, round, raised, shining and translucent. Both salted and unsalted butter containing the

organism developed a strawberry odor after 14 days. This description agrees in many respects with the description of the O colony type of the Mayapple organism which, as will be shown later, did not produce a rancid condition in butter but did produce the typical Mayapple odor.

Richholz (5) isolated a strawberry-aroma producing organism, Bacterium fragi, from milk which had been held several days at  $3\frac{1}{2} - 7^{\circ}$  C. It was a non-spore-forming organism, which was killed by heating for 30 minutes at  $50^{\circ}$  C. and by 10 minutes at  $75^{\circ}$  C. Richholz described the colonies as forming rosette and daisy-like patterns on gelatin. On agar streaks the growth was easily removed but was neither sticky nor slimy. The organism was not fluorescent. Neither acid nor gas was formed in milk and the milk became alkaline. The description of this organism agrees in many respects with the description of the E colony type of the Mayapple organism.

From the comparison of the characteristics of the three Mayapple types with the three described species it is evident that they are identical or at least are closely related. Two of the species were isolated from milk and it is not unlikely that they were different colony types of the same organism. The other species was isolated from beets but its characteristics are so nearly identical with the other two species that it is also possible for it to be a variant colony type of one

of the other two species.

Obviously if the Mayapple organism is identical with the three species given above some of the names must be dropped. Nicholz (5) was the first to describe and name one of the strawberry-aroma producing organisms. He used the name Bacterium fragi. The characteristics of the organism are such that it probably belongs in the Genus Pseudomonas and the name Pseudomonas fragi is suggested for it.

C. Variation in the types of colonies produced by the organism.

1. Sources of the cultures used for variation studies.

The systematic study of the organism was carried out with 51 cultures isolated from the sources already mentioned. From these cultures four were selected to be used in the work dealing with colony variation. Each of the four cultures selected hydrolyzed fat and produced rancidity in butter made from cream containing them. The sources of the four cultures are as follows:

Culture A was isolated from a sample of rancid butter and was considered to be the cause of the rancid condition. Culture B and C were isolated directly from plates poured from two samples of normal ice cream.

Culture D was isolated from a sample ofropy milk. It was considered to be the cause of the outbreak of ropiness and a

large percentage of the organisms appearing on plates poured directly from theropy milk was of this type.

The studies on the types of colonies occurring in pure cultures of the Kayapple organism were divided into (1) those on cultures purified by plating and (2) those on cultures purified by single cell isolations.

## 2. Method of studying variations.

Variations in the types of colonies produced were studied by using beef infusion agar plates (pH 6.8) poured from two day old litmus milk cultures of the organism and incubated at 20° C. After a few platings had been made the amount of the inoculum could be so controlled that the colony distribution was satisfactory. After colonies had developed on the plates a system of selective picking was used whereby one of each of the colony types appearing on the plates was picked into litmus milk. This procedure will be referred to as "successive selective platings" in the following descriptions. These new litmus milk cultures formed the inocula for the next plates in the series. At no time during the dissociation work was any material such as dye, inorganic salts, or disinfectants used to bring about changes in colony form although litmus was used in the milk.

The value of purifying cultures of bacteria by some technic which utilizes single cell isolation has been repeatedly

emphasized during recent years. Some investigators think that this is the only procedure which will result in pure cultures. Other bacteriologists, who consider that all bacteria have an invisible or filterable stage in their life cycle, believe that this method of purification does not necessarily yield a pure culture, the objection being that some of the invisible forms may be carried along with the single viable cell and contaminate the culture. While this is a valid objection there is not enough evidence as to the existence of these forms to permit a definite conclusion.

In spite of the objections to the single cell procedure the method was used for the purification of some of the variant types of the *Neurospora* organisms. The method of Wright and Nakajima (33), in which the single cell is inoculated into a small droplet of medium and inoculated over a moist chamber, was employed. A Lathx micromanipulator was used for carrying out the mechanical part of the process.

The variant type s used for single cell isolations were secured from the studies dealing with colony variation in cultures purified by plating. From three of these cultures, (A, B, and D), S, O, and R variants had already been secured and attempts were made to secure single cells from each of them. Only S, and O type variants were secured from culture O and these were used for single cell isolations.

Some of the variant cultures produced a shiny material

which made single cell isolations impossible. With these cultures the cells stuck together and a droplet could not be secured which contained one organism.

3. Study of colony variations in cultures of the Mayapple organism purified by plating.

Culture A. After several preliminary platings culture A was plated on beef infusion agar and the plates incubated at room temperature. Following a two day incubation period a large percentage of the colonies appearing on the plates were of the S type, although a few O and a few H1 colonies were present. A representative of each of these types was picked into litmus milk and incubated at room temperature for two days. The cultures originating from the colonies picked served as the starting material for the platings which are recorded below.

S type. An S colony from the original plating was passed through eight successive selective platings. The colonies appearing on the plates of the entire series were largely of the same type as the parent colony. On five of the eight sets of plates only S type colonies were observed. Variant colonies appeared on platings two, three, and seven. The variant colonies appearing on platings two and three consisted of a few O colonies. These were picked into litmus milk and passed through several successive selective platings and the plates examined for variant colony types. None of the platings made



using the O colonies as inocula showed any variation in colony type. From these results it was concluded that the O culture had become stabilized.

The variant colonies appearing on plating seven consisted of a few O and a few R3 colonies. Neither of these types was tested for purity because at this point the entire series of platings was discontinued and the cultures existing were used as material from which to isolate single cell cultures.

O type. The O type colony coming from the original plating was passed through eight successive selective platings. There was no evidence of colony variation on any of the plates in the series. The colony from the last plating was used for the isolation of single cells.

R type. The R1 colony coming from the original plating was passed through eight successive selective platings and here again no variations were noted. The culture from the last plating was used for the isolation of single cells.

Culture B. Culture B was repeatedly plated to make certain that it was pure. After purification it was again plated on beef infusion agar and the plates incubated at room temperature for two days. Here, as with culture A, there were three types of colonies, S, O, and R1, on the plates. Each of these types was carried through eight successive platings and the plates examined for evidences of colony variation. No

variation was observed in any of the plates in the series. A colony from the eighth plating of each type was used for the isolation of single cells.

**Culture C.** The first plating of culture C, after purification, yielded two types of colonies S, and O. Both of these types were carried through a series of eight platings. There was no evidence of variation on any of the plates in the series. A colony from the eighth plating of each type was used as material for the isolation of single cells.

**Culture D.** Following purification, the first plating of culture D yielded three colony types, S, O, and R1. Each of these colony types was used for subsequent platings.

S type. The S colony was carried through eight successive platings. The first plating in this series was the only one which gave any evidence of variation. The variants here consisted of a few O colonies which when plated yielded only O colonies.

O type. No variation was observed on any of the plates in the series of eight platings to which the O type colony was subjected.

R1 type. The R1 type was passed through eight selective successive platings and the only variation noted occurred on the first

plating in the series. The variants consisted of a few S colonies which were found to be stable during a series of platings.

The data presented on the colony variation in cultures purified by plating show the variability in types of colonies produced by pure cultures of fat-splitting bacteria isolated from rancid butter, normal ice cream, and rony milk.

4. Study of colony variations in cultures of the Mavapple organism purified by single cell isolation.

Culture A. The first plating of the S type from culture A which had been purified by single cell isolation yielded three types of colonies, S, O, and R1.

S type. An S type colony from the first plating was plated and the plates yielded a large number of S colonies and a few R2 colonies. An S type colony from this source was passed through five platings without further evidence of variation on any of the plates. The next plating of this culture was made after a period of one month during which the culture was transferred occasionally in litmus milk. This plating yielded a large number of S colonies and a few O colonies. During the next two months there were no platings and the cultures were transferred occasionally in litmus milk. On the plates made after this period the S culture produced S and R1 colonies and the O culture produced mostly O colonies

with a few R1. The S and R2 type colonies which appeared on the second plating of the single cell culture will be considered next. When the R2 colony was plated four colony types were found on the plates, S, R1, R2 and O. The S and O type colonies from this source was passed through two platings each and no variations were found. The cultures were discarded. The R1 and R2 colonies from this source produced cultures which were very unstable and each of them yielded every colony type found in the entire investigation at some time during the subsequent platings. Diagram 1 shows the way transformations went. The colony types indicated on the left represent the starting type and the colony types on the right side represent the types secured from the original type.

O type. The single cell isolation from the O type colony of culture A gave no evidence of colony variation until the fourth plating which yielded R1 in addition to O colonies. The R1 colony used gave no evidence of variation during three platings, after which it was discarded. The O colony from the fourth plating yielded R1 and O colonies on the first plating. An O colony from the above source yielded no more variant colonies during the remainder of the study but there was a tendency for the colonies to increase in thickness and at the last plating it more nearly approached the appearance of the S type colony than the original O type.

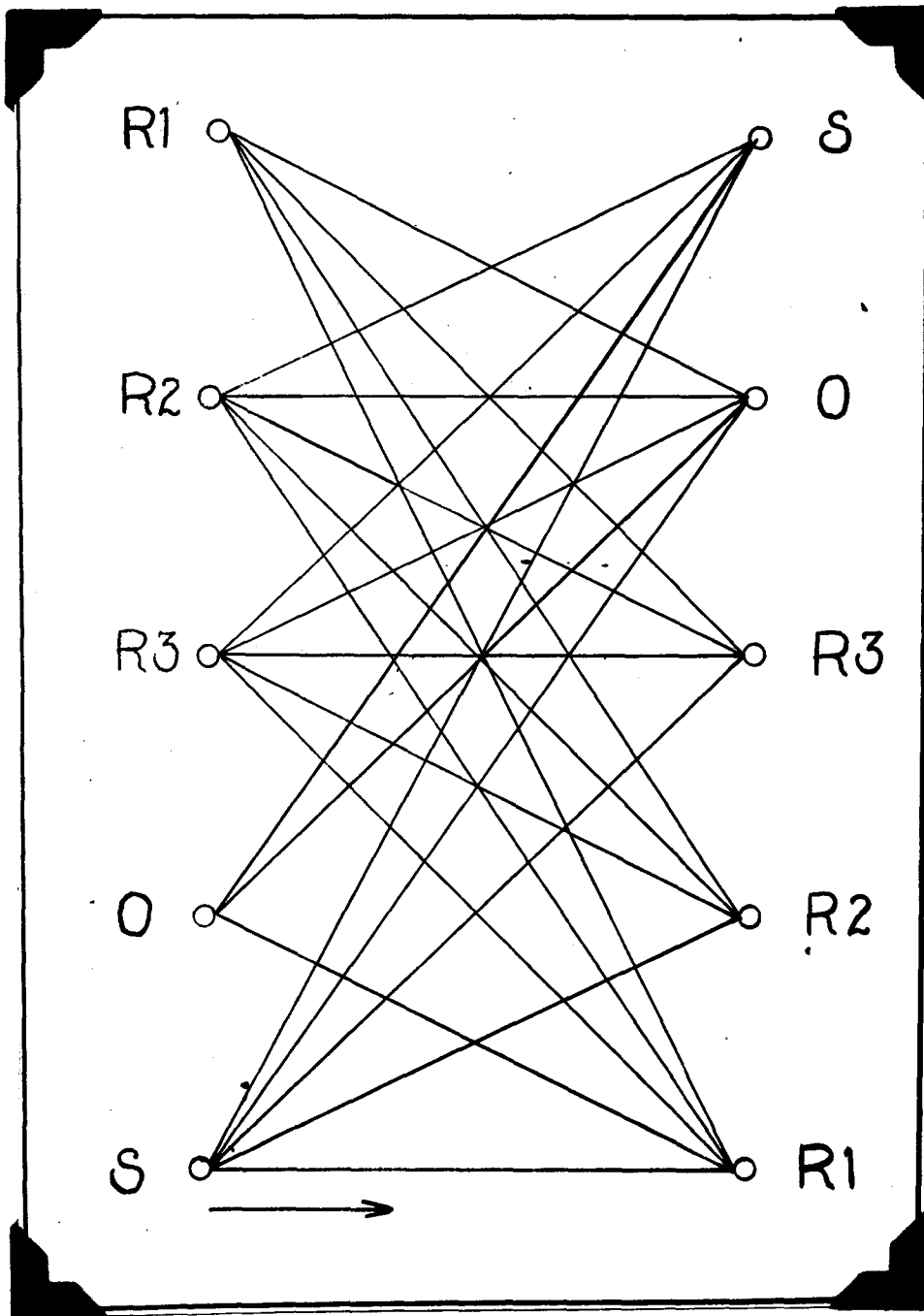


Diagram 1.

Diagram showing transformation of colony types. The changes go from left to right as indicated by the arrow at the bottom of the chart.

R type. The single cell culture from the R type was subjected to 12 consecutive platings and no evidence of colony variation was found on any of the plates in the series. At one time in the trials a period of one month elapsed during which no platings were made, the culture being transferred occasionally in litmus milk. After this period the culture was plated once and no variants were found on the plates. A colony from this plate was carried in litmus milk for two months and at the first plating following this R1 and R2 colonies were secured. Further platings of these types were not made.

Culture B. Only one single cell isolation was made from culture B. This was from an S culture and was passed through eleven successive platings without any evidence of colony variation occurring on any of the plates. This agrees with the results secured with the S variant from a culture purified by plating where there was no evidence of colony variation after the first plating.

Culture C. Single cell isolations were made from the S and O variants of culture C. A rough type variant was not secured from this culture previous to the time the single cell isolations were made although one was secured from cultures purified by the single cell technic.

S type. The S type variant was passed through six platings before any evidence of variation occurred. Colony variants were

not secured even after the culture had remained in litmus milk for a one month period or after an additional two month holding period in litmus milk. However, the second plating after the two month resting period resulted in the appearance of S and O type colonies. The S culture was carried without variation through two more platings while the O type remained pure for one plating and then reverted entirely to the S type.

The results secured with the single cell cultures agree, in general, with those secured with cultures purified by plating. The only difference being in that variation was more evident with the culture starting from single cells.

O type. The O type variant of culture C proved to be extremely variable. Although no variant colonies were observed on the first two platings the third plating yielded both O and R1 colonies. Each of these again produced both O and R1 colonies when plated. These variant cultures were passed through seven successive platings and on each of the platings from each type there appeared both O and R1 colonies. From the sixth plating of the O type variant there was secured O, R1, and R2 colonies. This marked the first appearance of R2 colonies from culture C as R2 colonies were never secured from the strains of culture C that had been purified by plating.

A survey of the data secured using cultures purified by single cell technic reveals that in these cultures colony

variation was very evident. The results agree with those secured by the use of cultures purified only by plating.

The most striking thing about the single cell isolations was the fact that even greater variation was secured with the cultures purified by single cell means than with cultures purified by plating.

D. Differences in biochemical activity of the variant type colonies.

Numerous reports have been made concerning the differences in the biochemical activities of the dissociated strains of an organism. Hadley (14) has shown that during dissociation Bacillus pyocyaneus loses its power to produce the blue pigment pyocyanin. He also reported that the R type of B. pyocyaneus was much more inactive biochemically than the S type.

Mitsch (5) has indicated that two variants of Bacillus proteus secured by him (O and R types) have reduced gelatin liquefying power.

Braun and Schaeffer (3) reported a diminution of proteolytic activity in O types of Proteus 19 secured through the influence of phenol and on a starvation medium.

Baltzman (2) found that a variant from Vibrio comma showed a slower digestive action on gelatin and a slower coagulating action on milk than the culture from which it came.

Soule (25) working with B. subtilis, reported that the R type liquefied gelatin much more slowly than the S type.



Wahlin (31) studied an organism resembling Bacillus vulgatus and found that it dissociated readily but that there was no difference in the biochemical activities of the dissociants.

Gilbert (9) studied dissociation in an encapsulated Staphylococcus but found no differences in the cultural characteristics of the various colony types.

1. Ability of the organism and the various colony types secured from it to hydrolyze fat.

Because of the relationship of the Mayapple organism to the production of rancidity in butter and the hydrolysis of fat the action of the variants on these materials was investigated. The data presented in table 1 show the results of a study of the action of the four initial cultures and the variants secured from them on Nile-blue sulfate agar containing fat. The results are as follows: In general, the S, O, and R1, R2, and R3 types isolated from culture A hydrolyzed fat. One strain of the O type was secured which did not have this ability. It was found on plates poured from an S culture which hydrolyzed fat. Morphologically this O colony did not differ from the O colonies which hydrolyzed fat. Table 1 also shows that the S and R type colonies from culture B hydrolyzed fat while the O type colony did not produce this change. Both S and R colonies from culture C hydrolyzed fat while the O type colony did not. Later an R2 colony was secured which did not hydrolyze fat. The S type colony of culture D hydrolyzed fat while the O and

R types did not.

Table 1. Ability of the original cultures and the variants secured from them to hydrolyze fat.

Culture	Type of Colony					
	Original	S	O	R1	R2	R3
A	+	+	+(-)	+	+	+
B	+	+	-	+	-	-
C	+	+	-	-	-	-
D	+	+	-	-	-	-

+ = fat hydrolysis

- = no hydrolysis

These data indicate that there was a difference in the lipolytic activity of the different variants secured. Not only this but there was a difference in the lipolytic activity in the same colony types secured from different cultures. For instance, the O colonies of three of the cultures studied were unable to hydrolyze fat while O colonies of the fourth culture were variable in this respect, that is, some of the O colonies secured hydrolyzed fat while others did not.

2. Ability of the organism and the various colony types secured from it to increase the soluble and amino nitrogen content of milk.

When litmus milk cultures of the Mayapple organism became

old a thin layer of yellowish whey that collected at the surface of the coagulated milk suggested proteolysis. This condition was noted with both S and R types. The action of all of the variants on the nitrogenous constituents of milk was studied by determining the soluble and amino nitrogen content of milk in which the organism had been grown. The four cultures used for studying colony variations, and the variants secured from them, were investigated. Soluble nitrogen determinations were made on the original cultures and the variants secured from platings one and two. Amino nitrogen determinations were usually made on cultures secured from platings one to seven inclusive.

Table 2. Action of the parent cultures on the nitrogenous constituents of milk.

Culture	Increase over the control per 10 ml. of whey	
	Amino N mgms.	Soluble N mgms.
A	1.88	16.16
B	0.76	21.10
C	0.17	1.76
D	1.49	12.63

Table 2 gives the soluble and amino nitrogen increases in milk secured with the four parent cultures. The results show considerable variation among the different cultures with respect

to their ability to hydrolyze milk protein. Cultures A, B, and D gave large increases over the controls in the amount of soluble nitrogen while culture C possessed a relatively weak action on milk protein as judged by the increase in soluble nitrogen. The values for the amino nitrogen show definite increases with cultures A, B, and D while culture C gave only a very small increase in amino nitrogen.

Table 3 presents the results of the soluble and amino nitrogen determinations on the variants from culture A. From the results secured it is evident that there was a marked difference in the proteolytic activity of the different colony types. The original culture caused an increase of 16.16 mgus. of nitrogen per 10 ml. of whey. This value was comparable to the increases caused by the S and R1 type colonies from this culture. The O type colony produced a much smaller amount of soluble nitrogen in milk than either of the other variants. The results of the determinations on the second plating show that the S type colony was the most proteolytic of any of the colony types examined. The R1 type colony possessed a strong proteolytic action but it was definitely less than that shown by the S type. The O type colony was only about half as active as the R1 type. The amino nitrogen increases shown by the variant cultures are of the same order as the soluble nitrogen increases. The original culture showed an increase of 1.88 mgms. of amino nitrogen per 10 ml. of whey. The S

Table 3. Action of the variants of culture A on the nitrogenous constituents of milk.

Culture	Increase over control per 10 ml. of whey	
	Amino N mgms.	Soluble N mgms.
Original	1.88	16.16
First plating		
S	3.80	
O	1.01	8.49
R1	2.85	16.74
Second plating		
S	3.33	20.41
O	0.84	8.48
R1	2.98	17.24
Third plating		
S	3.51	
O	0.80	
R1	2.51	
Fourth plating		
S	3.73	
O	1.01	
R1	3.02	
Sixth plating		
S	3.44	
O	0.95	
R1	2.36	
Seventh plating		
S	2.59	
O	0.66	
R1	2.23	

type colonies in all six of the determinations made caused greater increases than the original culture. Next to the S type colony the R1 type produced the greatest increase in amino nitrogen. In each of the six determinations the O type colony produced the smallest increase in amino nitrogen of any of the three types examined.

The data concerning the degradation of milk protein by the variants of culture B are presented in table 4. The data secured with the original culture do not agree with those for culture A since with culture B, a high soluble nitrogen content was found but with it there was a very low amino nitrogen content. When the action of the various colony types is considered the same general situation exists, however, that existed with culture A. The values secured for soluble nitrogen show large increases for the S type and increases which are about one half of these for the R type culture. The amount of soluble nitrogen formed by the O type culture was very low, being 0.36 mgms. per 10 ml. whey in one case, and 0.16 mgms. in the other. This does not compare with the O type from culture A where the values for the O type were high. It should be noted here that the O type of culture A was able to hydrolyze fat while the O type from culture B was unable to do this. When the amino nitrogen values are considered it is evident that the same relationship between the types exists as for the soluble nitrogen. Here again the S type produced the highest

Table 4. Action of the variants of culture B on the nitrogenous constituents of milk.

Culture	Increase over the control per 10 ml. of whey	
	Amino N mgms.	Soluble N mgms.
Original	0.76	21.10
First plating		
S	2.06	15.18
O	-0.11	0.36
R	0.61	6.55
Second plating		
S	2.33	15.82
O	-0.10	0.16
R	0.49	7.67
Third plating		
S	1.97	
O	0.01	
R	0.63	
Fourth plating		
S	2.38	
O	-0.10	
R	0.77	
Sixth plating		
S	2.41	
O	-0.06	
R	0.47	
Seventh plating		
S	1.79	
O	-0.16	
R	0.05	

amino nitrogen content while the R type had a comparatively low value. The O type did not increase the amino nitrogen content of the milk but decreased it in every case except one. In the one case where the increase was noted it was so small that no significance can be ascribed to it. Probably also the slight decreases are not of much significance although they were fairly consistent.

The action of the variants of culture C on the nitrogenous constituents of milk is given in table 5. A survey of these data shows that with the original culture there was a very slight increase in both the soluble and amino nitrogen. These values are unusually low and there is the possibility an O type culture was used instead of an S type. The values for the soluble nitrogen produced in skim milk by the S types are lower than the values for the corresponding type of cultures A and B. The O type again showed little proteolytic activity. A rough type colony was not available from culture C, when the determinations were made. The increases in amino nitrogen in milk in which the variants from culture C had been grown were very low. However, the value for the S type was higher than the value of the original culture and this may be explained in the same way as the low soluble nitrogen values for the original culture. The values secured for amino nitrogen for the S types were far below those secured for the S type of culture A and B. Also the values for the O type were lower than the O type values of culture A but they agree with the



Table 5. Action of the variants of culture C on the nitrogenous constituents of milk.

	Increase over the control per 10 ml. of whey	
	Amino N mgms.	Soluble N mgms.
Original	0.17	1.76
First plating		
S	1.03	11.05
O	0.03	0.05
Second plating		
S	1.43	13.60
O	-0.08	1.09
Third plating		
S	1.57	
O	0.17	
Fourth plating		
S	2.07	
O	-0.34	
Sixth plating		
S	0.96	
O	-0.11	

O type values for culture B where there was a slight decrease in amino nitrogen. With the O type from culture C there was a slight decrease in amino nitrogen in three of the trials and a slight increase in two. The increases and decreases were both so small that they are of little significance as far as the increase or decrease is concerned but they are of significance when compared with the action of the S type cultures.

The data concerning the protein degradation by variants of culture D are presented in table 6. The analysis of the original culture revealed that it was the least active of any of the four parent cultures. The values for the soluble nitrogen show that the S type colony was less active than the parent culture but there was still considerable soluble nitrogen formed in the milk. The determinations on the R1 type colonies shows that they were only slight proteolytic. As was usually the case the O type colonies produced very slight changes in the amount of soluble nitrogen present. In the two determinations made one showed an increase of 0.06 mgms. of soluble nitrogen per 10 ml. of whey while the other showed a decrease of 0.56 mgms. The values for the amino nitrogen determinations of the S type are all considerably lower than the value for the parent culture. In four of the six determinations made on the R1 type there was a decrease in amino nitrogen rather than an increase. With one of the two remaining determinations the increase was very slight while with the other the increase

Table 6. Action of the variants of culture D on the nitrogenous constituents of milk.

Culture	Increase over the control per 10 ml. of whey	
	Amino N mgms.	Soluble N mgms.
Original	1.49	12.63
First plating		
Smooth	0.17	6.09
Thin	-0.15	- 0.56
Rough	-0.12	0.13
Second plating		
Smooth	0.60	6.51
Thin	-0.09	0.06
Rough	0.05	0.18
Third plating		
Smooth	0.90	
Thin	-0.15	
Rough	-0.27	
Fourth plating		
Smooth	1.01	
Thin	-0.04	
Rough	0.04	
Sixth plating		
Smooth	0.65	
Thin	-0.13	
Rough	-0.16	
Seventh plating		
Smooth	2.72	
Thin	-0.30	
Rough	2.97	

exceeded that of the parent culture. In each of the six determinations made on the O type colony there was a slight decrease in the amount of amino nitrogen present in the milk after the organism had grown. The action of the variant colonies on the nitrogenous constituents of milk correlated with their ability to hydrolyze fat. With each of the cultures used the S type colony always possessed a strong hydrolytic action for fat and a strong proteolytic action for milk protein. The action of the O type colonies on fat proved to be variable, and their action on milk protein also was quite variable. The O type colonies from cultures B, C, and D did not hydrolyze fat while from culture A, O type colonies were secured which did hydrolyze fat and others which did not hydrolyze it. The O type colonies from culture B, C, and D either decreased the amount of soluble and amino nitrogen present or at best there was only a very slight increase over the control. The O type culture from culture A which hydrolyzed fat also increased the amount of soluble and amino nitrogen to a marked extent. No data was secured on the protein-splitting action of the O type colony of culture A which did not hydrolyze fat.

E. Action of the Mayapple organism in butter made from cream containing the organism.

Series of churnings were made using cream which had been inoculated with Mayapple organism. The butter made during these trials was studied from the standpoint of the number of

organisms developing; the amount of total acid present after various storage periods; the amount of volatile acid present after various storage periods; and the change in the flavor and aroma of the butter. In some of the series the effect of various percentages of salt on these changes was also studied. Some of the butter was made from cream containing butter culture. When bacterial counts were made on butter in which butter culture was employed only the colonies of the Mayapple organism were counted; they could be easily distinguished from colonies of the butter culture organisms. The following tabulation gives the history of the various series of churnings:

The lots of butter in series A were made from three lots of cream inoculated with cultures E, G, and H. The butter was not salted and was divided into two portions which were stored at 15.5° C. and 7.2° C.

Series B contained lots of butter which were made from lots of cream inoculated with cultures E, F, G, and H. No salt was added to the butter. It was stored at 15.5° C.

In series C a single culture, F, was used as inoculum. After churning, the butter was divided into three portions. The first of these was not salted; to the second portion 2.5 per cent salt was added; with the third portion 10 per cent of butter culture was worked into the butter as thoroughly as possible. An uninoculated control was included in the series and was not salted. All of the butter was stored at 7.2° C.

In series D all of the cream was inoculated with one culture, H, and divided into three parts. The first part was churned and the butter not salted; the second part was churned and divided into two parts one of which received 1 per cent salt and the other 2.5 per cent salt; the remaining portion of cream received 10 per cent of butter culture immediately before churning and the butter was not salted. An uninoculated control was included in the series. All of the butter was stored at 7.2° C.

Series E was composed of butter from cream inoculated with variant cultures secured from culture A. Variant types S, O, R1 and two R2 cultures were used. None of the butter was salted. It was stored at 7.2° C.

1. Changes in the number of organisms present in the butter.

Table 7 shows the bacterial counts made on the experimental butter after various holding times. No bacterial counts were made on series A.

The counts made on the butter in series B indicated that large increases in the total number of organisms occurred. The initial counts on all of the butter immediately after making was roughly one million bacteria per ml. After three days at 15.5° C. two of the counts had increased to 52 million per ml. and the other to 18 million per ml. A still further increase was evident after seven days holding. The two lots of butter which had the high counts after three days again had the high

counts after seven days. These were of the order of 100 million bacteria per ml. of butter. The sample having the lowest count after three days had increased only 6 million bacteria per ml. during the added four days holding period.

The results obtained in series D shows the effect of salt on the bacterial content of butter. This is illustrated by the fact that the bacterial count on the unsalted butter was 295,000 per ml., that on the butter containing 1 per cent salt was 65,000 while the butter containing 2.5 per cent salt had a count of 25,000 bacteria per ml. The decrease in the number of bacteria must again be attributed to the addition of salt to the butter. The butter made from cream containing 10 per cent butter culture was churned separately and had a higher initial count than the salted butter. The butter culture did not seem to have much effect in restraining the development of bacteria for the count after seven days had reached a maximum of 11.4 million bacteria per ml.

The results of the bacterial counts on series E are much the same as those secured on the unsalted butter in the preceding series. In every case a large number of organisms were present. A count of 136 million bacteria per ml. was found in the butter made with the O type culture. This was a non-fat-splitting type and in spite of the high count the flavor and odor of the butter were normal, while all of the other butter was very rancid. After continued incubation a Mayapple odor developed in the butter con-

taining the O type culture.

2. Changes in the total acidity of butter containing the organism.

Another way of judging the changes taking place in butter containing the Mayapple organism was to determine the total amount of acid present in the butter. After the butter in the series of experimental butter had been held for various lengths of time the acid values were determined and the results secured are given in table 8.

The data on series A and B gives the amount of acid present in lots of butter containing various cultures of the Mayapple organism. The results regularly show a marked increase in the total amount of acid present after the organism had developed in the butter. In all cases the uninoculated control had acid values which were very low as compared with the values secured on butter containing the Mayapple organism. The values on series C show the effect of added salt and butter culture in preventing the development of acid in the butter containing the Mayapple organism. There the unsalted butter had an acid value of 23.4 after 40 days storage the butter containing the salt or butter culture had values of 5.7 and 9.75 respectively. The uninoculated control had a value of 3.4 which was lower than the other values recorded. The data on series D also show the effect of salt and butter culture on the development of acidity



Table 7. Growth of the Mayapple organism in butter, including the effect of salt and of butter culture on the growth.

Series	Cultures	Salt or butter culture added	Fresh		Age of butter	
			Bacteria per cc.	7 days	Bacteria per cc.	7 days
B	D1		745,000	52,000,000	100,000,000	
	R2		940,000	52,000,000	107,500,000	
	43		1,080,000	18,000,000	24,000,000	
C	13	2.5% salt	340,000	18,650,000		
	13		22,500	140,000		
D	43	1.0% salt	295,000	32,500,000	12,750,000	
	43	2.5% salt	65,000	4,950,000	800,000	
	43	10.0% butter culture	25,000	385,000	210,000	
E	R2		475,000	42,000,000	11,400,000	
	R2		205,000	25,000,000	15,750,000	
	R2		550,000	51,000,000	23,000,000	
	O		385,000	51,000,000	136,000,000	
	R1		1,020,000	35,000,000	32,000,000	
			1,425,000	67,500,000	69,000,000	

x = 4 days instead of 3  
 xx = 9 days instead of 3

in butter containing the Mayapple organism. After 40 days storage the unsalted butter had an acid value of 17.5. The data indicate that as the salt content of the butter was increased the amount of acid present decreased. This correlates with the number of Mayapple organisms present in the same lots of butter. The influence of butter culture seemed to be about equal to 2.5 per cent salt. In this series the uninoculated control again had a low acid value. The data on series E shows approximately the same thing as the unsalted inoculated butter in the preceding trials, namely an increase in the amount of total acid both after 21 and 28 days storage, with all of the types except the O type. This O type was a non-fat-splitting one secured from culture A and it will be recalled that the number of organisms present in the butter had reached 136 million per ml. after nine days storage. The acid value of this butter never exceeded that of the control in spite of the high bacterial content.

3. Changes in the volatile acidity of butter containing the organism.

A rancid condition in butter is usually associated with the presence of the volatile members of the fatty-acid series. Rancidity may be present, however, when it is impossible to detect an increase in the amount of volatile acid contained. The increase in volatile acid present gives some idea of the

Table 8. Changes in the total acid in butter containing the Mayapple organism.

Series A

Culture	Age of butter		
	21 days	42 days	91 days
	Acid value	Acid value	Acid value
Control	1.75	2.1	2.4
D1	10.75	14.4	29.8
25	16.20	21.4	39.15
43	16.45	22.5	48.4

Series B

Culture	Age of butter		
	16 days	180 days	
	Unsalted	Unsalted	Salted
Acid value	Acid value	Acid value	
43	10.05	19.6	12.4
25	8.8	15.3	11.5
13	16.4	29.1	11.85
D1	7.8		
Control	(4 weeks) 2.55	9.5 4.1	6.0 1.6

Series C

Culture	Age of butter	
	40 days	
Acid value		
13	23.4	
13 + 2.5% salt	5.7	
13 + butter culture	9.75	
Control	3.4	

Table 6 (Continued)

Series B

Culture	Age of butter	
	40 days	Acid value
43	17.5	
43 + 1.0% salt:	11.75	
43 + 2.5% salt:	7.55	
43 + 10.0% butter culture	7.3	
Control	2.0	

Series E

Culture	Age of butter	
	21 days	Acid value
R2	9.0	12.2
R2'	8.6	12.9
O	1.2	1.5
R1	6.7	9.1
S	6.1	9.1
Control	1.2	1.6

changes that have taken place in the butter and is, accordingly, of considerable value. Determinations of the volatile acidity were made on some of the experimental butter after various holding periods and the results are presented in table 9; the total acidities are also included.

The values in series B involve a number of samples of butter, each containing a different culture. These data are not comparable because the age of the butter varied considerably but they do give some idea of the powerful fat hydrolyzing action of the cultures used. The volatile acid value of the uninoculated control is 1.0 and the inoculated butter ranges in value from 25.0 to 72.0. From the data it will be seen that the ratio of volatile to total acid is rather constant. The values on series C, show the effect of the addition of salt and butter culture on the development of volatile acid. These determinations were made after the butter had been held at 7.2° C. for 36 to 43 days. The inoculated butter without salt or butter culture had a volatile acid value of 129.0 as compared with a value of 4.0 for the uninoculated control. The addition of 2.5 per cent salt to the butter kept the volatile acid value down to 34.0 while 10 per cent butter culture kept it down to 51.0. The data on series D, represent another trial in which the same culture was present in all of the butter and the effect of different amounts of salt and of butter culture are seen. The volatile acidity of the inoculat-

ed butter without salt or butter culture is high compared with that of the uninoculated control and as the amount of salt increased the amount of volatile acid present decreased. In this experiment the effect of 10 per cent butter culture in restraining the development of volatile acidity is about the same as the effect of 2.5 per cent salt.

4. Changes in flavor and aroma of butter containing the organism.

All of the cream used for making experimental butter was pasteurized at 185° C. for 15 minutes. The high temperature and long exposure was responsible for the presence of a very marked heated flavor and odor in the butter. The heated flavor and odor persisted for several days after the butter was made but its intensity decreased with the passage of time. Usually after three or four days the intensity had reached a point where it was no longer objectionable.

When the *Mayapple* organism was inoculated into the cream used for butter making some very definite off-flavors and odors were soon evident. Usually at about the time the heated flavor reached a minimum another off-flavor had developed. After three days at 60° C. the flavor was commonly detectable but was not pronounced enough to be definitely associated with any abnormality. After an additional incubation period of one day the off-flavor could definitely be called rancid. Following the first

Table 9. Changes in the total and volatile acidity in butter containing the Mayapple organism.

Series	Culture	Age of butter days	Total Acid value	Volatile acid value
B	Uninoculated control	40	2.55	1.0
	D1	28	7.8	25.0
	13	14	16.4	81.0
	25	16	8.8	28.0
	43	16	10.05	72.0
C	Uninoculated control	43	3.4	4.0
	13	36	23.4	129.0
	13 - 2.5% salt	40	5.7	34.0
	13 - 10% butter culture	42	9.75	51.0
D	Uninoculated control	47	2.0	3.6
	43	39	17.6	117.0
	43 - 1.0% salt	40	11.75	79.0
	43 - 10.0% butter culture	42	7.3	39.0

detection of the rancid flavor and odor the intensity rapidly increased until the butter was so badly off-flavor that it was no longer marketable.

During the time the rancid condition was developing there was a large increase in the number of Mayapple organisms in the butter. The development of the rancid condition can probably be correlated with the number of organisms present before there is any marked change in the other characteristics of the butter such as an increase in either the total amount of acid or the amount of volatile acid present.



## VI. SUMMARY

The data herein reported deal with the study and identification of an organism which was found in considerable numbers in two samples of rancid butter. The presence of the same organism in two samples suggested its relationship to the rancid condition. Preliminary churning trials, using pasteurized cream to which the organism had been added, revealed that the organism produced a marked rancidity in butter.

The organism possessed two unusual characteristics. The first of these was the production of a small ring of acid curd at the surface of a tube of litmus milk which did not extend to the bottom of the test tube until after long incubation periods. The second characteristic was the production of an odor which resembled the odor produced by the flower of the Mayapple or mandrake (*Podophyllum peltatum*). Organisms producing these changes in litmus milk had frequently been encountered on plates poured from dairy products but their relationship to the production of rancidity in butter had not been recognized.

Following the isolation of the organism from two samples of rancid butter a number of dairy products were examined for its presence. During this search the organism was isolated from 12 samples of normal ice cream; 31 samples of rancid butter; 5 samples of milk; 3 samples of cream; and 2 samples

of evaporated milk.

After 51 cultures had been secured from the various dairy products they were subjected to a systematic study. The organism was found to be a psychrophilic, aroma-producing, gram negative, non spore-forming, polar-flagellated rod which was capable of hydrolyzing fat. A number of bacteria capable of hydrolyzing fat have been described but the descriptions did not fit the description of the organism isolated. Eichholz (5) described an organism which produced a strawberry-like aroma in various media and Grüber (11, 12) described two such organisms. The characteristics of the described species seemed to agree rather closely with those of the organism isolated from rancid butter and various other dairy products. If the different colony types which were secured from the organism isolated are considered, each type can be identified as one of the three organisms mentioned above. Thus the O type can be identified with Bacterium fragi Eichholz; the S type can be identified with Ps. fragariae II Grüber; and the R type can be identified with Ps. fragariae I Grüber. Bacterium fragi Eichholz was the first of these organisms to be described and this specific name should be retained. The characteristics of the organism are such that it seems to belong to the genus Pseudomonas. The name Pseudomonas fragi nov. comb. is therefore the one that should be used.

Another part of the study was concerned with the occurrence

of colony variations in four cultures of the organism isolated. Variations occurred in cultures purified by ordinary plating and in cultures purified by single cell technic. No outside agents were used to bring about colony variation although the litmus in the milk may have had some influence in doing this. Culture A yielded five different types of colonies; culture B yielded four types; culture C yielded three types; and culture D yielded three types. It was the occurrence of these various types which led to the identification with three previously described organisms.

Experiments designed to determine differences in the biochemical activity of the variant types revealed the following points. Variant types could be secured (from parent cultures with a marked fat-splitting action) which were unable to split fat. The S type colonies from each of the four cultures studied were all able to hydrolyze fat. O type colonies which were unable to hydrolyze fat were secured from all four of the cultures studied. However, an otherwise typical O type colony was secured from culture A which did hydrolyze fat. The fat-splitting ability of the R type colonies varied with the culture studied, the R. type colonies from cultures A and B were able to hydrolyze fat while those from cultures C and D were unable to bring about this change.

The data on protein decomposition by the variant cultures show that there was considerable difference in their ability

to hydrolyze this material. In general the original cultures and the S type variants caused considerable increase in the amount of soluble and amino nitrogen when they were grown in skim milk. With all of the O type variants studied there was a marked decrease in the amount of both soluble and amino nitrogen produced as compared with the amount produced by the S type variant from the corresponding culture. With two of the O type variants there was actually a slight decrease in the amount of amino nitrogen present. All of the R type variants studied possessed a proteolytic activity which was midway between the S and the R type variants from the corresponding culture. The R type more nearly approached the S type than the O type.

The section of the work dealing with butter made from pasteurized cream containing the organism showed that the organism could grow in butter held at low temperatures until large numbers were present. The effect of salt was very marked in restraining the development of the organism in butter. As the salt content of the butter increased the number of organisms decreased. The effect of salt on the organism was evident within a very short time after the salt was added to the butter. Butter culture did not seem to have a very marked action in restraining the growth of the organism in butter.

The total amount of acid in butter containing the organism increased rapidly until after a relatively long storage period, there was a large amount of acid present in the butter even at

the low temperatures used. The amount of acid present was greatly influenced by the addition of salt to the butter. In no instance when salt was present did the acidity ever reach the high level that it reached in unsalted butter. As the salt content of the butter increased the amount of acid developing decreased. When 10.0 per cent butter culture was added to the cream before churning the resulting butter never contained as much acid as the control without butter culture. The same inhibiting action was produced when the butter culture was worked into the butter after churning.

The volatile acid content of butter containing the organism was very much higher than the volatile acid content of the uninoculated controls. Determinations made on butter containing salt revealed that the amount of volatile acid present was greatly influenced by the salt. With increasing salt concentrations the amount of volatile acid present decreased. Butter culture also inhibited the development of volatile acid.

Butter made from cream containing the organism regularly exhibited a characteristic sequence of changes. The first of these was the disappearance of the heated flavor and odor resulting from the high pasteurization exposure. At about the time that the heated flavor had reached a minimum an abnormal flavor had appeared. When first noticed this was not definite but with continued storage the abnormal flavor was identified as a rancid condition. Following the first appearance of the

rancid condition it increased rapidly and made the butter unmarketable.

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