THE CAUSE OF EYES AND CHARACTERISTIC FLAVOR IN EMMENTAL OR SWISS CHEESE¹

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INTRODUCTION

Due to a lack of the proper natural inoculation in the milk, the Swiss or Emmental cheese industry in the United States has had only a limited success. The peculiar sweetish flavor which is characteristic of the best cheese of this type is very commonly lacking in our American-made cheese. It is also frequently deficient in eye development, and in fact in some cases the cheeses are entirely "blind." Swiss cheese is made only during certain seasons in America, because of the uncertainty of obtaining the proper development of eyes and flavor. It would seem that this industry could be put on a sounder as well as a more scientific basis by the use of cultures which would cause proper ripening in the cheese. With such cultures at hand it should be possible to make Swiss cheese of a uniform and high-grade quality throughout the year; such practice should result in raising very materially the quality, as well as the quantity, of our American-made Swiss cheese.

Von Freudenrich and Orla-Jensen (1906) in their work in Switzerland have isolated propionic acid-producing bacteria which they consider the cause of eyes in Emmental cheese. The essential organism, called by them *Bact. acidi-propionici* (a), was found to ferment lactates with the production of propionic acid, acetic acid, and carbon dioxide. Other varieties of propionic

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bacteria were found but they did not appear to have much influence on the ripening of cheese.

In the early experiments conducted by the Department of Agriculture on Swiss cheese, some cultures of propionic acid bacteria were obtained from Professor Burri of Berne in the hope that these could be introduced and used in the manufacture of Swiss cheese in this country. The experiments conducted with these cultures, however, were not encouraging; in fact it was not established experimentally that they were able to cause the development of eyes when used for starters in the manufacture of Swiss cheese. Following the methods of Von Freudenrich and Orla-Jensen, cultures were isolated which corresponded to their published descriptions of the propionic-acid bacteria. These were also used in the manufacture of experimental Swiss cheese with negative results. These findings do not discredit the work of Von Freudenrich and Orla-Jensen, since it is entirely possible that the cultures used belonged to varieties which do not play important rôles in the ripening of Emmental cheese. The experiments referred to were carried on a few years after the death of Professor von Freudenrich; Professor Orla-Jensen at that time was not able to furnish cultures of these organisms.

I. CONCERNING THE OCCURRENCE AND NUMBERS OF LACTATE-FERMENTING BACTERIA IN EMMENTAL CHEESE

That there exist in Emmental or Swiss cheese bacteria which ferment lactates with the production of volatile acids has been shown by Von Freudenrich and Orla-Jensen (1906), who succeeded in isolating such organisms in pure culture; and the theory was advanced that the production of eyes is due to the carbon dioxide liberated by these bacteria in the transformation of lactic acid to propionic and acetic acids, according to the formula:

They also determined the approximate number of lactate-fermenting organisms in Emmental cheese by means of dilution cultures in a calcium lactate broth. By such methods they were

able to demonstrate that these bacteria occur in numbers from 10,000 to 200,000 per gram of cheese.

Troili-Petersson (1909) using the same methods found approximately the same numbers of lactate-fermenting bacteria as did Von Freudenrich and Orla-Jensen. In a previous report from these laboratories, Eldredge and Rogers (1914), who worked with American cheese of the Emmental type, found this type of organism present in somewhat smaller numbers than was reported by the European workers, and in fact apparently entirely lacking in some cheese.

Modification of the lactate broth of Von Freudenrich and Orla-Jensen

For the growth and isolation of lactate-fermenting bacteria from Emmental cheese, Von Freudenrich and Orla-Jensen (1906) used a calcium lactate broth of the following composition:

	per cent
Pepton (Witte).....	2.0
Sodium chloride.....	0.5
Dipotassium phosphate.....	0.2
Calcium lactate.....	2.0

Although such a mixture is obviously faulty, due to the incompatibility of the calcium and phosphate ingredients, resulting in a heavy precipitate of an insoluble calcium phosphate upon sterilization, the broth as used by Von Freudenrich and Orla-Jensen, so far as we are aware, has not been modified by subsequent workers who have used it extensively for studies of the propionic and butyric acid-forming groups of bacteria. Only recently Boekhout and De Vries (1917) have employed it in an extensive study of the bacteria responsible for gas formation in cheese.

It need hardly be mentioned, assuming that the several components of the broth are in fact of value, that the ingredients added should not be rendered inert by precipitation. This may be obviated by the use of another salt of lactic acid, such as sodium lactate, in place of the calcium. The commercial sodium lactate syrup may be used if desired, but we have found it convenient to prepare the sodium lactate just before use by neutralizing the desired amount of lactic acid with sodium hydroxide. The sodium lactate broth has been found to be in all respects as good

as that made with the calcium salt, and in one very important respect to be superior.

In the preparation of the calcium lactate broth no attention, so far as the published papers indicate, has been paid to its reaction; and considering the reactions of the individual ingredients employed there would seem offhand to be little need for concern about this point. It was noted, however, when broth was tested for its hydrogen-ion concentration, by means of the colorimetric method of Clark and Lubs, (1917) that a value of about $\text{pH} = 5.2$ was always obtained. This result (from $\text{pH} = 5.1$ to $\text{pH} = 5.3$) was found with Witte pepton as well as with a variety of American brands.

An inquiry was therefore made into the reactions of the individual components and of combinations of the several components. Calcium lactate broth was made and at the same time solutions of the various ingredients were prepared separately in the same concentrations as they occur in the broth. These were all sterilized in the autoclave for twenty minutes at 15 pounds pressure. After cooling, the following results were obtained:

	<i>pH</i>
2.0 per cent pepton.....	6.8
0.5 per cent sodium chloride.....	7.3
0.2 per cent dipotassium phosphate.....	8.2
2.0 per cent calcium lactate.....	7.3
Von Freudenrich and Orla-Jensen broth.....	5.2

As is shown by these data, the reaction of the finished broth is much more acid than is any one of its several components. Another lot of broth made with the same ingredients, with the exception that sodium lactate was substituted for the calcium salt, gave a reaction of $\text{pH} = 7.2$.

It would appear then that the explanation is to be found in the reaction between the phosphate and the calcium lactate; and such it seems is the case, as is indicated by the result given below. These solutions were sterilized as were those reported above.

	<i>pH</i>
0.2 per cent dipotassium phosphate.....	8.2
2.0 per cent calcium lactate.....	7.3
0.2 per cent dipotassium phosphate + 2 per cent calcium lactate..	4.8

The marked acidity of the lactate-phosphate mixture is probably explained by the formation of acid phosphates and lactic acid along with the insoluble calcium phosphate.

Aside from the case of this particular broth, the principle here illustrated should be given more general consideration in the formulation of culture media. It would seem, *à priori*, that there is danger of such a shift in the hydrogen-ion concentration upon sterilization of any medium which contains calcium or magnesium and a phosphate, if the calcium-magnesium portion is in excess of the phosphate. This principle is violated in many of the synthetic media which are recorded in bacteriological literature. It is obvious also that the buffering effect of the phosphate is lost in such a combination.

In the recommendation that sodium lactate be used in the place of the calcium salt, it has been assumed that the dibasic phosphate employed in this medium serves some useful purpose. As a matter of fact, in pure culture, the lactate-fermenting bacteria of Swiss cheese grow very well in broth containing only pepton and either calcium or sodium lactate. This does not prove that the simplified medium would be just as good for quantitative estimations in which the seedings are very light.

In the work here reported quantitative determinations were made in a broth containing 1 per cent pepton, 1 per cent dried yeast and 1 per cent lactic acid (as sodium lactate). This broth supports a very active growth of the lactate-fermenters and is an excellent one for quantitative purposes.

Approximate numbers found

Quantitative dilutions of cheese were planted in broth composed of 1 per cent each of pepton, dried yeast, and lactic acid (in the form of sodium lactate). Dilutions of from 0.01 to 0.000,001 gram of cheese were tested. After incubation for four weeks at 30°C. the cultures were acidulated and subjected to steam distillation to determine the formation of volatile acids. Control flasks containing pepton-yeast broth without the lactate, inoculated with the same dilutions, were run in order to avoid any possible error through the measurement of the relatively small amounts of vola-

tile acids derived from the nitrogenous constituents of the medium.

Without going into details, it may be stated that of 16 samples of American-made Swiss cheese purchased on the open market all contained lactate-fermenting organisms in sufficient numbers to be revealed in 0.000,001 gram, the highest dilution used. These samples were representative of about the average run of domestic Swiss cheese; only samples which had sufficient eye development were taken, but the flavor varied from excellent to very poor.

Thus it will be seen that we have succeeded in demonstrating the presence of lactate-fermenting organisms in numbers considerably greater than has been reported by other investigators. Also, as will be shown later on, these bacteria have been isolated directly from cheese without previous enrichment in some selective broth.

Relation to previous work

Concerning the discrepancies between the results of various workers on this subject, we feel that these inconsistencies may well be reconciled through the observations made in connection with the work here reported. As has been noted, the reaction of the lactate broth, as employed by Von Freudenrich and Orla-Jensen and subsequent workers, is too acid for the best results. The error which may be introduced by this factor is well illustrated by the following test made on a pure culture of a lactate-fermenting organism from Swiss cheese: A broth culture one week old as tested by the dilution method, using the regular Von Freudenrich and Orla-Jensen broth (pH = 5.2), and another broth of the same composition except that sodium lactate was substituted for the calcium salt. This broth had a reaction of pH = 6.8. The result of this test showed that, whereas the sodium lactate broth gave a count of over 100,000,000 organisms per cubic centimeter the number as indicated by the calcium lactate broth was less than 1,000,000.

Aside from the error introduced through the unfavorable reaction of the calcium lactate broth, as it has been previously used, there are apparently other factors which make the dilution method

a rather uncertain one for the quantitative estimation of the lactate-fermenting bacteria of cheese. It has been noted on several occasions that the distillation for volatile acids gave negative results whereas further propagations from the culture used showed that lactate-fermenting bacteria were present. This phenomenon is probably to be explained by the presence in the culture of other organisms which consume the volatile acids. That this may sometimes be the case was indicated by the results obtained on certain samples of cheese in which volatile acids were produced from the high dilutions of cheese in lactate broth whereas the low dilutions, which contained a much heavier inoculation and a greater variety of organisms, gave negative results. In this work we have checked ourselves quite thoroughly against such errors by running all of our dilution cultures in triplicate, and also by making further examinations and propagations from dilution cultures which gave negative results. Thus we have on several occasions demonstrated the presence of the lactate-fermenting bacteria from cultures which gave negative results on the original test.

II. THE CAUSE OF EYES AND FLAVOR

In our work on Swiss cheese during the past few years the identity of the organism responsible for the development of the characteristic flavor, as well as the eyes, of Emmental cheese has been quite clearly established. The ability of this organism to play these rôles in the ripening of cheese has not only been established by carefully controlled laboratory experiments, but also under practical commercial conditions in factories located in widely separated areas of the country.

GENERAL CHARACTERISTICS

Morphologically the organism is a minute rod about twice as long as it is broad. It makes little or no growth on agar slope cultures; in agar stabs growth takes place throughout the length of the puncture but not on the surface. In agar shake cultures there is likewise no growth on the surface whereas good growth takes place throughout the medium; as incubation continues over

an extended period the growth is seen to become very heavy, barely below the surface of the agar. In a suitable nutrient broth a heavy slimy growth occurs at the bottom and the whole broth becomes turbid, with the usual exception of a narrow clear zone at the surface. Milk is rendered slowly acid and is usually curdled in from one to two weeks at 30°C. Growth in pepton milk is much better, curdling taking place in from four days to one week at 30°C. Small bubbles of gas may sometimes be seen in the curd. Gelatin is not liquefied. Glucose, lactose, maltose, sucrose, glycerol and salicin are fermented; raffinose, inulin, and mannitol are not.

One of the outstanding characteristics of this organism is the production of a large amount of catalase. Attention has previously been called to the relatively large amount of catalase which is found in Swiss cheese (Sherman 1919). The group of organisms herein described is the one which was shown to give this characteristic to cheese of the Emmental type.

Reference to the products produced by this bacterium indicates that it belongs to the group of propionic acid bacteria which was described by Von Freudenrich and Orla-Jensen. Lactates are fermented with the production of volatile acids, including propionic and acetic, and carbon dioxide. Also in the fermentation of lactose, volatile acids and carbon dioxide are produced.

Relation to previously described types

Whether this organism is identical with any of the types isolated by Von Freudenrich and Orla-Jensen cannot be definitely stated at this time. In general it appears to agree quite closely with their description of *Bact. acidi-propionici* (a) which they considered to be the true cause of the development of eyes in cheese. A few points, however, in their description do not agree with the characteristics which we have observed in our organism; they state that it causes no visible change in milk, whereas our organism in litmus milk develops an acid reaction after several days and causes coagulation on longer incubation. Further, from their results on the production of volatile acids it was noted that only a small amount of these substances was produced from glycerol,

while our organism causes an active fermentation of glycerol with the production of a considerable quantity of volatile acids. Finally, it may be stated that the organism with which we have been working causes the typical sweetish flavor in Emmental cheese, whereas the experiments of Von Freudenrich and Orla-Jensen did not give definite results on this point. Orla-Jensen (1912) has since stated more conclusively that the sweetish flavor is due to a factor other than the propionic acid bacteria.

It is of course recognized that an accurate comparison can not be made from published descriptions. It is hoped, therefore, that we may obtain from European workers cultures of the various types of propionic acid-forming bacteria so as to determine more definitely whether this organism is identical with any of the previously described types or whether it is a new variety. In this connection it may be noted that we have also isolated a variety of these lactate-fermenting bacteria, among which have been found quite distinct types. Although the characteristics of all of these varieties have not been studied in detail, they appear to agree in a general way with the types which have been isolated by European workers. It is hoped that further studies on these organisms, in comparison with types obtained from Europe, may be made in the future.

In keeping with the nomenclature used by the European workers for the group of propionic acid-producing bacteria, this organism will be tentatively designated as *Bact. acidi-propionici* (d).

The isolation of cultures

The direct isolation of this organism from cheese is difficult for various reasons, particularly because of its slow growth and its oxygen requirements. Though not a strict anaerobe it requires a considerably reduced oxygen tension. Although in pure culture this organism grows in all ordinary culture media, including even 1 per cent pepton solution, it is apparently not so easy to obtain growths from it when taken directly from cheese. On a few occasions colonies have been isolated

from agar plates made directly from cheese, but success by this method is rare.

On a number of occasions this organism has been isolated, directly from the cheese, by sealing agar dilutions in glass tubing of about 0.5 cm. diameter. With this method it is very easy to isolate the individual colonies by cutting the tube at the desired points. By sterilization of the outsides of these tubes by immersion in a strong disinfectant solution, and then rinsing with sterile water, there has been no difficulty in making isolations by this method without contamination. The medium which we have found very satisfactory for this purpose is one consisting of 2 per cent pepton, 1 per cent yeast, 1 per cent lactic acid (as sodium lactate) and 1.5 per cent agar. Although we have had fairly good success in making isolations by this method, it has by no means always proven successful.

By making enrichment cultures of the cheese in lactate pepton broth, as was done by Von Freudenrich and Orla-Jensen, the isolation of lactate-fermenting bacteria is much easier. We have isolated a variety of organisms belonging to this group from such enrichment cultures.

Our interest thus far has been more in the practical application of these bacteria in the cheese industry than in making a thorough study of their physiological characteristics. There is little doubt however, that by taking advantage of their known properties, a simple differential method could be developed which would be satisfactory for the direct isolation of this group of organisms from cheese.

Rôle in cheese

For studying the effect of this organism in cheese we have had at our disposal a supply of milk, obtained from the experimental herd of the Dairy Division, which was entirely lacking in the bacteria necessary for the development of the desired characteristics of Emmental or Swiss cheese. Cheese made from this milk by the Swiss method is always entirely lacking in the characteristic sweetish flavor, and is also frequently "blind." When the natural inoculation in this milk is such as

to cause a development of eyes in the cheese the resulting flavor is in no way similar to that characteristic of the typical Swiss cheese. This fact is important, since it shows that the formation of eyes may be due to bacteria other than the one herein described; it probably explains also the fact that American Swiss cheese is so frequently deficient in flavor even when abundant eye formation takes place.

In our laboratory work small cheeses of the Emmental type are made from about 200 pounds of milk. These cheeses are then handled in exactly the same way as are the large Swiss cheeses made under factory conditions, and they ripen in an entirely normal manner. From such experiments it has been demonstrated time and again that the organism described in this paper is responsible for the characteristic sweetish flavor of Swiss cheese and that it also causes the development of eyes. Its relation to the eye formation is shown in the photograph reproduced at the end of this paper; its relation to flavor production has been demonstrated in over 100 laboratory experiments in which one cheese in each experiment was inoculated while another cheese made from the same milk was left uninoculated.

That the use of this bacterium as a "starter" is practicable under commercial conditions has been demonstrated in a number of different factories. In all cases these factory experiments have shown a marked influence on the ripening of the cheese with respect to both eyes and flavor. The application of these results in cheese-factory practice will be treated more in detail in a future publication.

ACKNOWLEDGMENT

Should the work herein reported prove of value to the cheese industry, major credit therefor is due Mr. L. A. Rogers, in charge of the Research Laboratories of the Dairy Division, who recognized the possibilities of pure cultures in the manufacture of Swiss cheese and initiated work toward that end over ten years ago, and who has fostered and directed the work through

a period of many discouragements due to lack of facilities and frequent changes in the experimenting staff.

Acknowledgment is also due to Mr. K. J. Matheson, and his several collaborators, whose cordial coopération in conducting the cheese-manufacturing tests has made this work possible.

SUMMARY

1. Bacteria capable of fermenting lactates with the production of volatile acids have been found to be constantly present in normal cheese of the Swiss or Emmental type in numbers exceeding 1,000,000 per gram.

The discrepancies in the results of previous workers on this subject are probably explained by a faulty combination of salts contained in the medium used, resulting in the production of a reaction too acid for the optimum development of the organisms concerned.

2. The essential organism for the production of eyes and the characteristic sweetish flavor of Swiss cheese has been isolated and studied.

The organism concerned belongs to the group of propionic acid-producing bacteria, but appears to differ slightly in some of its characters from the several varieties of propionic bacteria which have been described in the literature.

Factory experiments have shown that pure cultures of the organism may be used successfully in practice to insure the proper ripening of Emmental cheese.

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PLATE 1

The lower row of cheeses were made from milk lacking in the bacteria essential for the proper ripening of Swiss cheese.

The cheeses in the upper row were made from the same milk as their respective "blind" controls, with the addition of a pure culture of the eye and flavor producing organism.