BACTERIAL FLORA OF ROQUEFORT CHEESE

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INTRODUCTION

It is well known that various kinds of hard cheese, such as Cheddar and Emmental, or Swiss, depend on a suitable bacterial flora for their normal development. The two types of cheese mentioned have been studied extensively by a number of investigators. It has been shown that in the manufacture of Cheddar cheese there must be a rapid development of the lactic-acid bacterium *Streptococcus lacticus*, and that during the ripening a development of other forms of cocci and lactic bacteria of the *bulgaricum*[1] type is necessary to produce the typical Cheddar flavor (*4*).[2] In Swiss-cheese manufacture the *Bacterium bulgaricum* is added, either in pure culture, as a starter, or unwittingly, with the rennet. What other microorganisms are responsible for the characteristic sweetish flavor, and for the development of the "eyes"; has not yet been established, but at any rate the ripening is due to the growth of bacteria, in distinction from the mold-ripened cheeses.

It is obvious that in cheeses which are mottled with molds the molds play an important part in the ripening. The mold which ripens Roquefort cheese, and also Gorgonzola and Stilton cheeses, has been named "*Penicillium roqueforti* (Thom)" (*6*). Currie (*1*) has shown that it hydrolyzes fat and the resulting acids have the peppery or burning effect on the tongue and palate which is characteristic of Roquefort cheese.

In the making of Roquefort cheese, as in the making of Cheddar cheese, a rapid development of lactic-acid bacteria is necessary to bring about the proper physical condition of the curd in the various stages of manufacture. This acidity results from the growth of *Streptococcus lacticus*. The importance of the *S. lacticus* and *Penicillium roqueforti* in the making and ripening of Roquefort cheese was recognized by Thom, who

[1] In earlier publications on the flora of Cheddar cheese these organisms were called "*Bacterium casei*," the name which Freudenreich applied to the lactic-acid-producing rod forms of Emmental cheese. He failed to recognize the organisms as similar to the one which he had isolated from kefir, and which he called "*Bacterium caucasicum*"; later it received the name "*bulgaricum*." There does not appear to be sufficient cultural, morphological, and chemical differences between the lactic-acid-producing rod forms from cheese and from other sources to justify the use of two species names, although the strains isolated from cheese are more hardy than those isolated from oriental milk drinks in respect to growth at low temperatures and in respect to growth on ordinary media. In this paper the species will be designated as "*bulgaricum*," the term accepted by common usage.

[2] Reference is made by number (italic) to "Literature cited," p. 233.

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made a study of mold-ripened cheeses. His observations (*6*) led him to believe that those two microorganisms were—

capable of ripening Roquefort cheese without the introduction of other enzyme producing or flavor producing organisms.

In the Dairy Division experimental work, when cheese of the Roquefort type was obtained, the question was raised as to whether the bacterial flora had anything to do with the difficulties which arose; whether cheese made from sheep's milk, according to the Roquefort way in France, differed significantly in its bacterial flora from cheese made in a similar manner from cows' milk in America. Accordingly, a study of the bacterial flora of Roquefort cheese, imported and experimental, was undertaken.

METHODS OF CHEESE EXAMINATION

The method of examination was similar to that used in the bacteriological analyses of Cheddar cheese (*4*). Plate cultures were made on infusion agar, and for comparison numerous colonies were "fished off" into litmus skim milk. Representative cultures of every type of organism appearing in considerable numbers were saved for detailed study.

Cultures for study were also obtained by the following method: Milk cultures were inoculated from dilutions of a cheese emulsion, the dilution increasing from tube to tube by a ratio of 10. The dilutions ranged from 1 to 10 to 1 to 1,000,000,000. The lower dilutions were inoculated into tubes which had been modified as follows to separate the mold growth from the bacterial growth: Ordinary test tubes were drawn out to form a constriction beginning about 4 cm. from the bottom of the tube and filled with skim milk to about 2 cm. above the top of the constriction. Since oxygen is required for mold growth, the part of the culture below the constriction was always free from molds. By breaking the tube at the constriction it was possible to obtain a subculture of bacteria free from molds. As soon as a milk culture in any dilution showed evidence of bacterial growth, it was plated out to obtain a pure culture of the predominating organism. Many of the cultures thus obtained duplicated the cultures obtained by the plate method, but sometimes an organism was obtained which would be missed if plate cultures alone were made.

The pure cultures were studied morphologically and biochemically. The detailed study of the different groups of organisms will be presented in separate papers, for the bacteria of Roquefort cheese are not of types peculiar to that kind of cheese, but are ripening agents common to many other kinds, each species differing in importance in different kinds of cheese, according to the various conditions to which it is subjected.

BACTERIAL FLORA OF IMPORTED ROQUEFORT CHEESE

Bacteriological analyses of a number of imported Roquefort cheese have been made and the data are presented in Table I. Most of the imported cheese obtained for study was well ripened when the analysis was made. Cheeses 13, 146, 148, and 150 were not well ripened, but they must have been several weeks old, for *Streptococcus lacticus*, which certainly must have been present in the cheesemaking, had disappeared entirely from every sample examined.

TABLE I.—*The bacterial flora of imported Roquefort cheese*

Cheese No.	Number of organisms per gram of cheese.		Cheese No.	Number of organisms per gram of cheese.	
	Cheese streptococci.	*Bacterium bulgaricum.*		Cheese streptococci.	*Bacterium bulgaricum.*
13	31,000,000	100,000	137	600,000	1,000,000
15	600,000	400,000	139	10,000	1,000
17	13,000,000	4,000,000	141	100,000,000	1,000,000
18	30,000,000	33,000,000	143	1,000,000	1,000,000
131	1,400,000	145,000	146	210,000	250,000
133	17,000,000	....	148	1,000,000	24,000,000
135	140,000,000	4,000,000	150	1,000	10,000

Two groups of bacteria were found to be the common flora of this type of ripened cheese: *Bact. bulgaricum*, and a group of organisms which here will be called "cheese streptococci." [1]

Table I shows that cheese streptococci in varying numbers, with 140,000,000 per gram as the highest number, were isolated from every one of the imported cheeses, and *Bact. bulgaricum* was isolated from all but one of them, with 33,000,000 per gram as the highest number. It most probably was present in the one cheese also, but failed to be isolated. Yeasts were isolated from a few of the cheeses, but in too small numbers to be considered of any significance.

BACTERIAL FLORA OF EXPERIMENTAL CHEESE MADE ACCORDING TO THE ROQUEFORT METHOD

For several years experimental cheese has been made in the Dairy Division according to the method by which Roquefort cheese is made, but with cows' milk instead of sheep's milk. The ripened cheese is very similar to the imported variety in appearance and flavor. The data obtained from a detailed study of the bacterial flora of the experimental cheese are given in Table II.

[1] The cheese streptococci are the subject of an accompanying paper (3). Culturally they are distinguished from *Streptococcus lacticus* by a failure to curdle litmus milk with the reduction of the litmus characteristic for that organism.

TABLE II.—*Bacterial flora of cheese made in the dairy division according to the Roquefort method. Number of organisms per gram of cheese*

Cheese No.	Age.	*Streptococcus lacticus.*	Cheese streptococci.	*Bacterium bulgaricum.*	Yeast.
1732...	These four samples were taken at progressive stages of the cheesemaking.	10, 000, 000	...	...	...
1732...		20, 000, 000	...	10	...
1732...		6, 500, 000	...	...	...
1732...		9, 000, 000	...	...	...
1732...	1 day...	100, 000, 000	...	...	...
1732...	2 days...	10, 000, 000	100, 000, 000	...	...
96. 115.	3 days...	10, 000	5, 000, 000	...	...
1732...	4 days...	1, 000, 000	100, 000, 000	...	...
1732...	6 days...	1, 000, 000	...	...	...
1732...	8 days...	100, 000	100, 000, 000	...	...
1732...	12 days...	100, 000	31, 000, 000	...	300
1732...	16 days...	...	...	10, 000	1, 000
1732...	23 days...	100, 000	10, 000, 000	...	...
1789...	25 days...	...	7, 500, 000	14, 000, 000	100, 000
1754...	35 days...	...	1, 000, 000	...	1, 000, 000
1727...	38 days...	...	120, 000	...	...
96. 121.	44 days...	...	1, 000, 000	...	400, 000
96. 17..	48 days...	...	40, 000	100	100, 000
96. 124.	2½ months...	...	10, 000	1, 000, 000	10, 000, 000
96. 125.	3 months...	...	100, 000	...	2, 500, 000
1621...	3⅓ months...	...	...	100, 000	40, 000
1601...	3¾ months...	...	...	...	1, 000
1590...	4 months...	...	1, 000, 000	470, 000	...
96. 12..	4½ months...	...	2, 150, 000	1, 000	...
96. 113.	6 months...	...	10, 000, 000	1, 000	...
96. 129.	6⅓ months...	...	170, 000	...	...

During the manufacturing of the cheese the *Streptococcus lacticus* which had been added as a starter was the only organism present in sufficient numbers to appear on the plates. These organisms gradually disappeared, and on the twenty-fifth day their numbers had so diminished that they no longer appeared on the plates.

A normal Roquefort cheese contains 4 per cent of sodium chlorid (NaCl) in 40 per cent of water, which can be regarded as a 10 per cent brine (*8*). An experiment was carried out to show the effect of a high concentration of salt on *Streptococcus lacticus.* Flasks of milk were inoculated with *S. lacticus,* incubated at 30° C. for about five hours, when sodium chlorid was added to make a concentration of 10 per cent. In five days all the organisms had been injured so that inoculations into tubes of litmus milk gave no growth. Later, a hardier strain of *S. lacticus* treated in the same manner was killed by the 10 per cent salt solution between the tenth and the seventeenth days. This experiment explains the complete disappearance of *S. lacticus* from Roquefort cheese during the first few weeks of ripening.

In the ripening cheese the cheese streptococci had multiplied to 100,000,000 per gram on the second day. A 6-months-old cheese still contained 10,000,000 of them, which demonstrates their hardiness in respect to a high concentration of the salt, quite unlike the sensitiveness of *S. lacticus.*

In the sample tested it was found that there were 10 cells of *Bact. bulgaricum* per gram of the curd just before cutting. By the twenty-fifth day the organism had multiplied to 14,000,000 per gram. Multiplication must have continued after the cheese was salted, on the sixth and eighth days. Later, this organism appeared in varying numbers. *Bacterium bulgaricum* is thus shown to be much more hardy than *Streptococcus lacticus* in respect to a high concentration of salt.

Yeasts were found more consistently in the cheese made in the Dairy Division than in the imported cheese, one cheese containing 10,000,000 per gram. The yeasts were either species of Saccharomyces which gave a gassy fermentation in dextrose, saccharose, and lactose, but not in maltose, or they were species which fermented dextrose alone.

In a general way it can be said that the bacterial flora of the cheese from the two sources was surprisingly alike. Fifty-three cultures of cheese streptococci isolated from domestic Roquefort cheese were subjected to many biochemical tests, and if slight differences in regard to fermentable substances were considered, they could be divided into seven strains. Forty-eight cultures isolated from the imported cheese, were distributed among the same seven strains, with one additional strain. The case was the same with the *Bacterium bulgaricum.* They could be divided into many strains on the basis of their fermentation reactions, but no strain was peculiar to one or the other source.

When compared with the bacterial flora of Cheddar cheese, the kinds of bacteria are the same, but the number of bacteria in Roquefort cheese is decidedly lower. Roughly it may be said that there are about one-fifth as many in Roquefort as there are in Cheddar cheese. The flora is also less constant in Roquefort cheese. Tables I and II show that occasionally from both the imported and the experimental cheese a sample which had only a few thousand bacteria per gram was found.

Unlike Cheddar cheese, the number of bacteria in Roquefort cheese appeared to have no influence upon the ripening. For example, in Table II cheese 1601 had so few bacteria that there was no growth in the cultures inoculated with the 1 to 1,000 dilution. Nevertheless it was a good cheese, with a fairly well-developed flavor. This suggests that the bacteria, with the exception of *Streptococcus lacticus*, play a very insignificant part in the ripening of Roquefort cheese, an idea that is supported by the fact that if any cheese, or any part of a cheese, fails to develop the mold, ripening takes place exceedingly slowly, but the curd remains hard and retains the acid flavor resulting from the decomposition of the lactose by *S. lacticus*, and the Roquefort flavor does not develop.

The only condition in the Roquefort-cheese curd detrimental to the activity of bacteria is the high concentration of the salt. The comparatively low numbers of bacteria are undoubtedly due to the salt, and quite probably the activity of those which are able to exist in the cheese is restrained by the salt. So far as the development of flavor is concerned, it appears to be a matter of indifference whether the bacteria are present or not, because the flavor produced by *Penicillium roqueforti* is so strong that it would mask any delicate flavors produced by the bacteria. Probably the flavor substances produced by bacteria, the acids, alcohols, and esters, are consumed by the mold. This study therefore confirms Thom's (*6*) opinion that the only bacteria essential to the making and ripening of Roquefort cheese is the *Streptococcus lacticus*.

INFLUENCE OF SLIME ON THE RIPENING PROCESS.

There is another problem in connection with the biology of Roquefort cheese ripening—viz, the influence which the organisms in the slime may have on the ripening process. Thom and Matheson (7) paraffined a large number of experimental Roquefort cheeses before slime had opportunity to develop, and they concluded that slime is not an essential factor in flavor production, but that it serves as an index of hygrometric conditions.

In the course of this study of the biology of Roquefort cheese ripening no evidence of any ripening changes proceeding from the exterior has been observed. When the cheese is cut through at any stage of the ripening, the appearance of the cut surface is uniform and no flavors have been observed in the outside layers more pronounced than in the interior. There is always a collection of moisture on the inside of the tin foil with which Roquefort cheese is covered—explained by the fact that the interior of the cheese has a higher temperature, owing to the fermentative processes, than the outside temperature of 7° to 10° C. at which the cheese is ripened. The condensation of moisture at the surface would be followed by a return movement toward the center to maintain the moisture equilibrium. This circulatory movement would tend to distribute through the cheese the enzyms which might be liberated by the organisms growing on the surface. This, it is argued by those who believe that the slime organisms are essential to Roquefort-cheese ripening, would bring about a uniform ripening throughout the cheese mass.

As a matter of general interest, the organisms making up the slime were studied. Immediately after the manufacture of the cheese *Oidium lactis* began to grow on the surface, and by the time the cheese was 2 days old it was well covered with oidium. On the sixth day the cheese was salted and the oidium was destroyed by the salt. Then came a growth of *Penicillium roqueforti* on the surface, which gave way to the growth of the typical reddish slime.

Smears were made of the slime from the surface of imported cheese and from the surface of experimental cheese in various stages of ripening. Microscopic examination showed that the slime was made up chiefly of bacteria, with scattered cells of yeasts, but in the smears from some of the cheeses yeast cells appeared in masses. Fragments of mycelium were occasionally seen. The bacteria in every smear were a mixture of rod and coccus forms. The organisms that made up the slime were isolated by plating on various kinds of agar, plain agar, Czapek's agar, cheese-infusion agar, and plain agar to which lactic acid was added to eliminate the growth of bacteria and to favor the growth of yeasts and molds. The isolated bacteria were submitted to various tests commonly used for the differentiation of bacteria. The most frequently isolated organisms found in the slime from the imported and the experimental cheeses was a micrococcus which gave an abundant yellowish growth on agar slope, liquefied gelatin very slightly, decomposed urea, and stopped the fermentation of carbohydrates at a hydrogen-ion concentration of about $P_H = 5.6$. Many other cocci differing only slightly from the description above were isolated from the slime. Micrococci identical with these cocci have been isolated commonly from aseptically drawn milk (*2*). It appears, then, that the udder is the source of the predominating flora of the cheese slime. There is apparently a selection of certain strains of udder cocci. The majority of them curdle milk, but no micrococcus isolated from cheese slime produced more than a slight acidity.

Another coccus fairly common in the slime from both imported and experimental cheese was exceedingly difficult to maintain on agar slopes. It formed a faint growth on agar and failed to attack the nitrogenous and carbohydrate test substances in broth cultures. It was surprising to find an organism that was capable of withstanding the rigorous conditions in the cheese slime and yet so delicate that it could scarcely be maintained under artificial cultivation.

The most frequently isolated rod form liquefied gelatin, decomposed asparagin, and gave an alkaline reaction in broths containing various carbohydrate test substances. Other rod forms differed slightly from this one.

Besides the types which have been briefly described as typical slime organisms, various kinds of cocci and rods were isolated only once or twice, which do not seem to be characteristic of the slime. For example, *Bacterium bulgaricum* was isolated from the slime once. Occasional colonies of mold, most frequently *Penicillium roqueforti*, appeared in the cultures.

None of the cheese-slime organisms were able to bring about any pronounced changes in milk in pure culture, but when pure culture of several types were inoculated together into milk their associative action digested the casein very slowly.

It would be a difficult matter to prove that these sluggish proteolytic enzyms either do or do not influence the ripening of the cheese. Gratz and Szanyi (*5*) made a careful chemical investigation of the action of the enzyms of the slime upon the ripening of the interior of hard cheeses of the Ovar and Trappist varieties on which the slime often becomes heavy. The authors concluded that in hard cheese of the type studied no ripening proceeds from the outside. The relative inactivity of the organisms of the slime, as compared with the vigorous activity of the *Penicillium roqueforti* suggest that in Roquefort cheese also the slime organisms are of minor importance, if indeed they have any influence in the cheese ripening.

It is said that if the *Penicillium roqueforti* fails to develop, there is, nevertheless, a softening of the curd after a long period of ripening. In such case the ripening is undoubtedly brought about by the bacteria of the interior of the cheese, probably aided to a considerable extent by the enzyms from the slime; but a ripe cheese of that kind is not a typical Roquefort cheese.

The Roquefort-cheese slime is normally of a reddish color, but no organisms producing red pigment were isolated from it, although apparently all forms seen in the smears grew in the plates. The only explanation that can be offered is that the cocci, rod forms, and yeast cells, all containing more or less yellow-and-orange pigment, may produce a reddish tinge when mixed in mass. It is quite possible that the pigment production of one or all of those species is altered by the intimate association with the other species.

SUMMARY

The microorganisms essential for the manufacture and ripening of Roquefort cheese are *Streptococcus lacticus* and *Penicillium roqueforti.*

Streptococcus lacticus decomposes the lactose during the manufacture of the cheese and thus produces the lactic acid necessary for the cheese making. These organisms disappear from the cheese after about two or three weeks, being killed by the high concentration of sodium chlorid.

The remaining flora of Roquefort cheese consists of cheese streptococci and *Bacterium bulgaricum*, organisms which are found in all kinds of ripening cheese. These organisms do not have any significant part to play in the ripening of Roquefort cheese.

The cheese slime consists of characteristic types of micrococci, rod forms, and yeast cells. The enzyms from the slime do not appear to be essential to the ripening of the cheese.

The flora of both the interior and the slime of the experimental cheese was identical with the flora of the interior and the slime of the imported cheese.

If the maker of Roquefort cheese will inoculate properly with *Streptococcus lacticus* and *Penicillium roqueforti*, and provide the proper condition of manufacture and ripening, he need have no other concern about biological ripening agents.

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