THE BUTTER AROMA BACTERIA

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It is not our present intention to consider the influence which the feeding of the cows or the various methods of ripening may have on the production of aroma in butter, and neither will we dwell on the various microorganisms, lactose fermenting yeasts and ester forming strains of B. coli which were supposed by former investigators to contribute towards aroma production but which we would now rather regard as harmful in butter. We will, however, at once pass on to discuss the remarkable capsule forming streptococci which were demonstrated almost simultaneously by Boekhaut (1917) in Holland, Storch (1919) in Denmark and Hammer (1919, 1920, 1921) in America, and to which all three investigators have ascribed an important influence in the formation of aroma in butter.

These bacteria form small amounts of acetic and carbonic acids and other volatile products, but hardly any appreciable quantities of lactic acid. According to Hammer, these substances would for the most part be formed at the expense of the citric acid in the milk, therefore he has called his bacteria Sc. citrovorus and Sc. paracitrovorus.

Practical trials with these aroma bacteria have, however, failed to give decisive results, so there is still a good deal to be said in favour of the current opinion that butter of good aroma can be produced only by the use of vigorous pure cultures of Storch’s cream ripening bacterium which one of us has investigated thoroughly and named Sc. cremoris (Orla-Jensen, 1919). On this account it may well be surmised that the butter aroma bacteria are really only weakened forms of Sc. cremoris. Clarity on this point would appear to be the first condition necessary for the solution of the aroma question.
Nothing is, however, more difficult than to assign a place in the system to a bacterium without any really characteristic properties.

The microscope affords but little help. The aroma bacteria resemble other streptococci in appearance. They have been stated to be specially slender, but are by no means so in pure cultures; their thickness varies very considerably with the conditions of nutriment. It is true that the aroma bacteria as a rule form well marked capsules but this is also the case with all the other lactic acid bacteria in the incubation stage proper, before they have produced appreciable amounts of lactic acid, while, as mentioned above, the aroma bacteria form very little acid; *Sc. cremoris* forms far larger capsules when appearing as the ropy milk bacterium. This is an example of the general rule in Bacteriology that systematic classification can only be based on the biological properties of bacteria.

As we have hitherto never met with any streptococcus which has not turned out to be a lactic acid bacterium, we started from the assumption that the aroma bacteria must be lactic acid bacteria. The fact that the small amount of acid formed by the aroma bacteria is mostly acetic acid does not invalidate this assumption, for every weakened lactic acid bacterium growing under unfavourable circumstances, forms relatively large amounts of acetic acid.

Our only firm basis for departure is the ability of the aroma bacteria to ferment citric acid. We have been able to confirm the accuracy of this observation. Freshly isolated cultures are able to cause a vigorous evolution of carbon dioxide in milk with added citrates but as happens with the Betabacteria, they completely lose the power to produce gas after propagation for some time as pure cultures. This property is thus not a constant one and therefore possesses only a minor significance for the purposes of classification.

In reality the only citric acid fermenting bacteria known are certain aerogenes bacteria with which, however, the aroma bacteria have no connection whatever. We have tried whether any of the pure lactic acid bacteria could ferment citric acid, and
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have found that certain Betacocci possess this property to a fairly marked degree, and as they, like the aroma bacteria, generally have a comparatively low optimum temperature and form a number of by-products, there is some likelihood that aroma bacteria are connected with the Betacocci.

We give here a rough summary of the new classification of the lactic acid bacteria (Orla-Jensen, 1919) for the benefit of those who may not be acquainted therewith.

<table>
<thead>
<tr>
<th>Sphere forms</th>
<th>Rod forms</th>
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<tbody>
<tr>
<td>a. Form only traces of by-products in addition to lactic acid</td>
<td>Streptococci (always dextro lactic acid)</td>
</tr>
<tr>
<td></td>
<td>Streptobacteria (dextro or inactive lactic acid)</td>
</tr>
<tr>
<td></td>
<td>Thermobacteria (laevo or inactive lactic acid)</td>
</tr>
<tr>
<td>b. A number of by-products in addition to lactic acid</td>
<td>Betacocci (always laevo lactic acid)</td>
</tr>
<tr>
<td></td>
<td>Betabacteria (inactive lactic acid)</td>
</tr>
<tr>
<td></td>
<td>(seldom inactive)</td>
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</table>

It will be seen that the Betacocci are primarily distinguished by the formation of laevo lactic acid, and if therefore we are to prove that the aroma bacteria belong to this group, we must first try to find a nutrient medium which suits them sufficiently well to allow of their developing their activities to the full and thus revealing their hidden characteristics.

As milk does not seem to be a favourable medium for aroma bacteria in pure cultures, we naturally tried yeast extract which has proved to answer admirably for many of the rod shaped lactic acid bacteria.

The yeast extract used was prepared by the autolysis of pressed yeast, and adjusted to the same hydrogen ion concentration and the same content of nitrogen, phosphoric acid and milk sugar as in the milk used. As the yeast extract also had approximately the same high content of buffer substances as milk, there was hardly any other physiological difference between the two nutrient media than that in the composition of the nitrogenous matter; the effect produced by mixing them in certain proportions is thus mainly to be sought in modifications in the nitrogenous nutrient. We must, however, remark that we did not succeed in preparing absolutely sugar free yeast extract; this invariably
TABLE 1

<table>
<thead>
<tr>
<th>KIND OF BACTERIA</th>
<th>NUMBER OF DAYS</th>
<th>% Yeast extract in milk</th>
<th>% Yeast extract</th>
<th>Per cent of yeast extract in milk</th>
<th>Pure milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>35%</td>
<td>10%</td>
<td>5%</td>
</tr>
</tbody>
</table>

*Sc. citrovorus* (Hammer)...
- 1: 3.2 7.8 6.2 4.3 3.4 3.8 2.5 1.5 0.9 0.7
- 7: 4.1 9.4 8.1 5.5 4.4 4.6 2.5 1.5 1.7 1.4

*Aroma bacterium* (2x)...
- 1: 0.7 3.4 2.7 1.8 1.8 1.7 1.4 1.0 0.8 0.5
- 7: 4.4 6.2 5.1 6.9 3.7 3.6 1.8 1.4 0.9 1.0

*Aroma bacterium* (6 Kirkedal 6)...
- 1: 1.8 5.0 5.0 3.4 3.2 2.7 2.7 2.0 0.7 0.5
- 7: 3.4 7.7 10.6 6.5 6.0 3.6 2.9 1.6 0.9

*Bo. arabinosaceus*...
- 1: 1.4 5.3 4.7 3.1 1.5 1.5 1.2
- 7: 6.5 7.2 7.3 7.2 6.4 5.6 4.1

*Bo. bovis*...
- 1: 0.7 1.9 2.0 2.4 2.6 1.9 0.7
- 7: 4.5 6.0 6.9 6.5 5.8 5.4 4.1

*Bb. caucasicum*...
- 1: 5.4 7.4 6.7 2.5 1.9 1.7 1.5 1.3 0.8
- 7: 7.9 10.4 11.0 9.5 5.0 5.0 3.5 3.2 2.0 1.7

*Bb. breve*...
- 1: 0.9 0 0 0 0 0 0 0 0 0
- 7: 15.6 17.3 11.7 6.5 5.0 4.7 4.2 3.7 0.6

*Bb. longum*...
- 1: 6.7 3.1 1.8 0.7 0.7 0.4 0 0 0 0
- 7: 6.7 9.2 10.1 10.1 4.3 4.0 4.0 2.2 2.2 0.5

*Sb. casei*...
- 1: 0.6 2.0 1.1 0.9 0.9 0.9 0.5 0.2 0.2

*Sb. plantarum* (1)...
- 1: 0 0 0 0 0 0 0 0 0 0
- 7: 11.8 12.1 14.2 11.0 9.2 7.6 5.2 4.4 1.6

*Tb. lactis*...
- 1: 7.0 11.8 12.2 11.9 11.9 11.5 11.7 10.6 10.4 9.7
- 7: 14.1 17.6 18.7 19.7 19.4 18.6 18.3 17.8 17.8 17.1

*Tb. bulgaricum*...
- 1: 4.5 10.0 12.2 12.2 12.2 12.2 11.9 12.2 11.5
- 7: 11.0 15.8 16.9 16.7 17.0 16.9 16.9 17.0 17.1 17.1

*Tb. Jugurt*...
- 1: 2.3 11.0 14.9 16.2 16.0 14.9 14.9 14.9 14.9 14.0
- 7: 7.6 23.6 24.5 27.3 25.2 24.8 26.1 25.5 25.5 26.1
but contained a trace of glucose in addition to yeast gum. The addition to milk of these carbohydrates, even in appreciably larger proportions than those in which they occurred in the yeast extract, did not however, improve in any way the nutrient value of the milk towards aroma bacteria or true lactic acid bacteria.

We now tried not only the cultivation of aroma bacteria in pure yeast extract but also in various mixtures of milk and yeast extract. For the sake of comparison we further tried representatives of the most important lactic acid bacteria in the same way. In table 1 the amounts of acid formed (the acidity of the inocu-

<table>
<thead>
<tr>
<th>KIND OF BACTERIA</th>
<th>NUMBER OF DAYS</th>
<th>Per cent of yeast extract in milk</th>
<th>% LACTIC ACID</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Pure yeast extract</td>
<td>50%</td>
</tr>
<tr>
<td><em>Tbm. helveticum</em></td>
<td>1</td>
<td>3.6</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8.8</td>
<td>16.4</td>
</tr>
<tr>
<td><em>Sc. faecium</em></td>
<td>1</td>
<td>2.0</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td><em>Sc. glycerinaceae</em></td>
<td>1</td>
<td>2.3</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.4</td>
<td>4.9</td>
</tr>
<tr>
<td><em>Sc. liquefaciens</em></td>
<td>1</td>
<td>1.9</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.0</td>
<td>4.7</td>
</tr>
<tr>
<td><em>Sc. thermophilus</em></td>
<td>1</td>
<td>2.0</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.6</td>
<td>6.4</td>
</tr>
<tr>
<td><em>Sc. mastitidis</em></td>
<td>1</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.2</td>
<td>3.8</td>
</tr>
<tr>
<td><em>Sc. cremoris</em></td>
<td>1</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5.0</td>
<td>7.4</td>
</tr>
<tr>
<td><em>Sc. lactis</em></td>
<td>1</td>
<td>2.5</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>
lated medium minus that of the not inoculated medium) are expressed as \( \% \) of lactic acid.

As is seen from table 1, we have just hit on exactly the right mixture, and it thus becomes possible to solve the problem in question. With 10 to 50 per cent of yeast extract in milk, the aroma bacteria form just as much acid as other streptococci, so that we are in a position to analyse the acid produced. It turns out to be mostly lactic acid, and laevo lactic acid at that, so there is no doubt at all that the aroma bacteria belong to the Beta-cocci, in which case they cannot be degenerated forms of \( \text{Sc. cremoris} \).

To determine whether the aroma bacteria ferment arabinose or not, i.e., whether they are more nearly related to \( \text{Bc. arabinosaceus} \) or to \( \text{Bc. bovis} \), we have tried out their behaviour towards various sugars admixed with yeast extract and milk from which we had fermented away the milk sugar with the help of Kefir yeast; we also found it to be very nearly as satisfactory to employ the more simply prepared mixture of yeast extract and the solution of peptonised casein containing various salts which we generally use in this laboratory.\(^1\) The results shown in table 2 were obtained with this solution containing 25 per cent of yeast extract.

\(^1\) Orla-Jensen: Dairy Bacteriology, p. 25. In different investigations we substituted for the extract of bakers’ yeast generally used in this laboratory, one which was manufactured some years back from brewers’ yeast (Cibus) and used for soup extracts; as a rule there was no difference in the amounts of acid produced.
It will be seen that the aroma bacteria display no marked propensity to ferment pentoses, for which reason they show closer affinity to *Bc. bovis* than to *Bc. arabinosaceus*.

The majority of the forms examined, however, differ, from *Bc. bovis* in having but little power to ferment laevulose, mannose and maltose, so possibly they constitute a distinct species. With the exception of 2 they ferment sucrose, and with the exception of 1 they ferment lactose. *Sc. paracitrovorus* has the power to ferment a larger selection of carbohydrates than the other aroma bacteria and also differs from them in growing very slowly at the ordinary temperature for which reason it can hardly have any practical significance.

Referring back to table 1, we see, as might be expected, that the development of the Betacocci is also greatly stimulated by the addition of yeast extract. This effect is even more pronounced in the case of the nearly related Betabacteria; as one of us has already mentioned, *Betabacterium caucasium* which constitutes the major portion of the Kefir grain, hardly grows at all in milk, and only forms appreciable amounts of lactic acid in the Kefir grains, i.e., in symbiosis with the yeast cells present therein which of course corresponds with the addition of yeast extract.

The rod shaped lactic acid bacteria called the Streptobacteria, among which *Sbm. casei* plays an important part in the ripening of cheese, also thrive much better in yeast extract than in milk, and best in milk containing about 25 per cent of yeast extract.

Even in the case of the lactic acid bacteria which grow better in milk than in yeast extract, the addition of a certain proportion of yeast extract has a favourable influence both on the rapidity of growth (amount of acid after twenty-four hours) and on the formation of acid (amount of acid after one week), but naturally the optimum proportion of yeast extract is somewhat lower for these than for those bacteria which prefer pure yeast extract to pure milk. Only *Sc. lactis* (the ordinary bacterium of sour milk) does not appear to improve on the addition of yeast extract.

It is particularly interesting to notice the stimulating effect

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3 Orla-Jensen, 1919, p. 177.
of even quite small amounts of yeast extract on the development of lactic acid bacteria. Even Sc. cremoris which prefers milk to yeast extract is noticeably stimulated both in growth and power to form acid by the addition of as little as 1 per cent of yeast extract.

If we are dealing with degenerated forms, the effect is much more marked. As regards the aroma bacteria we have even found cases of freshly isolated strains being able to coagulate milk on the addition of 1 per cent of yeast extract. We may further mention that a mixed culture of aroma bacterium and of a Sc. cremoris which ceased to grow at 6°C. in pure milk, formed notable amount of acid at 4°C., and even appreciable amounts at 2°C. (though only after a month's time) after the addition of 1 per cent of yeast extract.

As the yeast extract only contains 7 per cent of solids, and furthermore only a very small proportion of this can be assumed to be active matter, we must here be dealing with the effects of very minute amounts, which affords a striking parallel to the case of the vitamines. In this connection we may refer to Barthel's recent demonstration (1924) that extracts of moulds will also stimulate the development of lactic acid bacteria.

Now as the lactic acid bacteria and not least the butter aroma bacteria, are just as particular as to their nutritive requirements as the higher animals, while on the other hand, yeast extract is rich in vitamines B and D, it is highly probable that we are dealing with the effects of these very substances, and we reserve the right to investigate this matter further in the hope of finding an easy manner of estimating one of these vitamines.

In order to isolate the butter aroma bacteria Storch and others have proposed certain cumulative methods. These are, however, not applicable if the object is to form an opinion as to the proportion of aroma bacteria to other bacteria in a starter culture. To assume that aroma bacteria can be recognized by direct microscopic examination will lead to very serious mistakes. It might be thought that aroma bacteria would be easy to discover in litmus gelatin, but neither does this method lead to satisfactory results
as on the one hand the aroma bacteria are quite capable of turning litmus red when fresh from the starter culture, while on the other hand, cells of *Sc. cremoris* are often found in the starter culture which are very weak acid formers. In view of the above results we hoped a great deal from a casein pepton gelatin with yeast extract and cane sugar, as these substances promote the growth of aroma bacteria to a greater extent than that of the other bacteria of the starter. This medium, however, did not turn out to be very selective as most other lactic acid bacteria grew well on it simultaneously.

In order to judge of the number of aroma bacteria in proportion to other lactic acid bacteria, we have no better method than to plate the starter out on our ordinary glucose, casein, pepton gelatin, to isolate a large number of strains at random and to examine these in detail.

As these investigations will be further treated of in a separate paper we will not dwell on them here but only point out that the aroma bacteria seem as a rule to occur very scantily indeed in proportion to *Sc. cremoris*. From most starter cultures we did not succeed in isolating any aroma bacteria at all without previous enrichment. The fact that they were always present, however, was shown by the circumstance that when we inoculated the culture into milk with chalk, appreciable amounts of inactive (lævo + dextro) lactic acid were always formed besides the dextro acid.

The fact that the development of the aroma bacteria as well as the formation of acid can be furthered by the addition of a little yeast extract, seems to open up new possibilities in relation to the problem of cream ripening. One cannot of course add yeast extract to the cream, this would be too expensive and also cause a taint, but there would be no obstacle in the way of adding a little yeast extract to the milk used for the cultures or starters.

Unfortunately our laboratory experiments have not given any encouraging result in this direction. The vitality of the lactic acid bacteria does not seem to be enhanced by the use of yeast extract, but the bacteria would rather seem to become pampered so that
when afterwards cultivated in pure milk (or pure cream) they form a little less acid than previously to the cultivation with yeast extract.

Nevertheless we are of the opinion that by further investigations of the ripening problem, the addition of yeast extract as well as of citrates are matters which should also be tested out.

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