CCXLII. A SUBSTANCE INHIBITING BACTERIAL GROWTH, PRODUCED BY CERTAIN STRAINS OF LACTIC STREPTOCOCCI.

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AMONG the bacteria there are many instances where growth of one organism renders the medium unfavourable for growth of some other type of organism; but cases where an organism militates against growth of other strains of its own species, except by accumulation of the normal end-products of growth, are rare. The following account deals with an investigation into the biochemistry of two strains of lactic streptococci which produce during their growth some substance which very strongly inhibits the growth of other strains of lactic streptococci. The organisms were isolated from milk in the course of an investigation of trouble occurring during the manufacture of Cheddar cheese in a dairy factory [Whitehead and Riddet, 1933]. As is well known, development of lactic acid in the milk and curd is an essential part of the process of cheese manufacture, and the formation of acid can occur only if the lactic streptococci which are added as a "starter" culture grow readily in the milk and ferment the lactose. In the particular case under investigation most of the milk received at the dairy factory readily supported the growth of lactic streptococci and hence gave no trouble to the cheese-maker; but the milk from one particular source of supply proved entirely abnormal in that very little acid could be developed in it. The inhibitory action of the milk was so marked as to suggest the presence of some strong disinfectant, but none could be detected. Moreover, the bacteria present in the milk as contaminants were quite active, since added methylene blue was rapidly decolorised. The various types of bacteria present in the milk were isolated in pure culture, and it was found that there were present in large numbers some Gram positive cocci which, when allowed to grow in any sample of normal milk, rendered it unsuitable for the subsequent growth of lactic streptococci and hence prevented the development of acidity. Even when the cocci were destroyed by heat after they had grown for twelve or twenty-four hours the milk still retained its inhibitory power. Thus it became evident that the phenomenon was not due to an antibiosis between living organisms but to the production of some heat-stable substance which was inhibitory towards lactic streptococci.

Later, another Gram positive coccus which produced a similar effect was isolated from a sample of milk obtained from quite a different locality. There was a slight difference in this case in that the coccus itself produced appreciable quantities of acid. Thus milk in which this organism was growing appeared to allow acid production by lactic streptococci to proceed quite normally. When however the milk was pasteurised or sterilised, lactic streptococci were strongly inhibited just as was the case in the first example.

It was of interest therefore to classify these two unusual organisms and to attempt to determine the nature of the substance which they formed during their growth.

Nature and reactions of the two organisms.

Both organisms proved to be lactic streptococci. The only significant differences between them were in the rates of acid production in milk and the various sugars which they fermented. The first organism (9 S) produced acid very slowly, taking three days at 30° to clot milk; it had a tendency to grow in chains of six to ten units. The second organism (D1) produced acid much more rapidly; milk was clotted in one day at 30° or in two days at 20°. Both organisms produced *d*-lactic acid from lactose and hence are included in the group of lactic streptococci according to the classification of Orla-Jensen [1919]. The organisms, like all lactic streptococci, were rather strict in their growth requirements; they grew only very slowly in the absence of a fermentable sugar from the medium. The best growth (as small clear colonies) was obtained on an agar containing whey from milk and an extract of yeast. Neither organism liquefied gelatin or reduced nitrate. Both organisms rapidly produced a reducing potential in milk at 37° as evidenced by the rapidity with which their milk cultures reduced methylene blue to the leuco-base. The sugar reactions were determined by growth in a peptic digest of caseinogen (0.5 % total N) containing 2 % of the various sugars; the time of incubation was 14 days at 20°.

Table I.

Parts per thousand of lactic acid produced.										
	Gly- cerol	Xylose	Ara- binose	Rham- nose	Sor- bitol	Man- nitol	Fruc- tose	Glucose	Man- nose	Galac- tose
9 S	0.0	0.0	0.0	0.0	0.0	0.0	4.0	4.0	3.8	2.7
9 S	0.0	0.0	0.0	0.0	0.0	0.0	5.9	5.8	3.8	4.0*
D 1	0.0	0.4	0.4	0.4	0.0	0.0	5.8	5.2	5.2	3.4
	Sucrose	Maltose	Lactose	Raffinose	Inulin	Dextrin	Starch	Salicin		
9 S	0.0	0.4	4.5	0.0	0.0	0.0	0.0	0.0		
9 S	0.0	0.5	5.8	0.0	0.0	0.0	0.0	0.0		
D 1	6.7	3.6	5.9	0.0	0.0	2.9	1.3	3.8		

* Medium had 10 % yeast extract added.

According to Orla-Jensen's classification these reactions would indicate that 9S was a strain of *Strep. cremoris*. D1 resembled *Strep. mastiditis* in its reactions, except for its rather active habit of growth in milk. On the whole there was nothing to distinguish these two organisms from the majority of those present in the mixed cultures of lactic streptococci used as "starters" in cheese manufacture, except their ability to produce the inhibitory substance which had first drawn attention to them.

Method used for detection of the presence of the inhibitory substance.

The method used throughout the experiments for the demonstration of inhibition of acid production was as follows. 150 cc. of each milk to be tested were adjusted in temperature to 30°; 1 % of a twenty-four hour culture of lactic streptococci was added and the mixture was maintained at 30° for half an hour. 0.2 cc. of rennet solution was added, and after a further hour at 30° the clotted milk was cut into pieces about $\frac{1}{4}$ inch square. The mixture of curd and whey was incubated at 37° for two hours, and then the whey which had separated was drained off as completely as possible. The curd was kept at 30° for a further two hours, after which time the fresh lot of whey which had exuded was drained off and titrated with *N*/10 NaOH. A final titration was carried out

on the whey which drained from the curd during a further period of one hour at 30°. The two acidity values obtained were expressed as percentages of lactic acid. The figures obtained in this way give an indication of the total amount of acid formed by the streptococci during the period of the experiment; the difference between the figures gives a measure of the rate of acid formation.

The test described above is of course only of comparative value as between various samples of milk tested at the same time. Various factors (in particular, variations in the acid-producing power of the culture of streptococci used) make comparisons of tests carried out on different days impossible. Whitehead and Cox [1932] have indicated the technical applications of the above process as a means of making determinations of the activity or vitality of various cultures of lactic streptococci in several samples of the same milk. The process has been termed the "vitality test" and is of considerable use in the testing of "starters" used in the process of cheese manufacture. In the present instance the vitality test was used as a means of testing the inhibitory power of various milk samples, and of milk to which various additions had been made, on the production of acid by the same culture of lactic streptococci.

Influence of growth of Streptococcus 9 S on milk.

1. Fresh skimmed milk which had been steamed for half an hour was inoculated with a culture of 9 S in milk and allowed to stand at room temperature overnight. Next morning the milk still had an acidity within the range considered normal for fresh milk (actually 0.16 % as lactic acid) and contained approximately 50×10^6 cocci/cc. as determined by a direct count under the microscope. Methylene blue added to a sample of the milk was decolorised in half an hour at 37°. One portion of the milk was pasteurised at 63° for 30 minutes and a second portion was steamed for 30 minutes. Vitality tests were then carried out on the raw, pasteurised and steamed samples, and also on a portion of uninoculated milk, 1 % of a mixed culture of lactic streptococci being added to all the samples (Table II).

Table II.

Acidities as % lactic acid.

	Raw inoculated	Pasteurised inoculated	Steamed inoculated	Control
First acidity reading	0.16	0.13	0.15	0.33
Second acidity reading	0.18	0.11	0.15	0.54
Increase	0.02	-0.02	0.00	0.21

These results are typical of those obtained in many similar experiments. The apparent decrease in acidity in the pasteurised sample is not significant since the experimental error may on occasion amount to 0.05. The results indicate that the growth of 9 S had so influenced the milk as a medium for normal lactic streptococci that their power of producing acid was almost completely inhibited for a period of at least six hours. In some experiments the samples of curd were maintained at 30° for a further period of twelve hours. The acidity in the whey from the inoculated milk curds then reached a value of 0.8 to 0.9 % lactic acid as compared with a value of 1.1 to 1.2 % in the controls. Thus the action is inhibitory rather than bactericidal in nature. It is comparable with, though much more powerful than, the so-called bactericidal action of fresh milk and is evidently due to some heat-stable substance formed by 9 S during its growth, since the inoculated milk was still inhibitory even after it had been maintained at 100° for half an hour in order to destroy the bacteria.

2. In order to gain some idea of the concentration of inhibitory substance in a milk in which 9 S had grown, an experiment was performed in which various quantities of inoculated milk (containing on this occasion 26×10^6 of 9 S per cc.) were made up to 150 cc. with normal pasteurised milk (Table III).

Table III.

	Acidities as % lactic acid.								
	Percentage of steamed inoculated milk in normal past. milk				Percentage of past. inoculated milk in normal past. milk				Control normal past. milk
	10	20	30	40	10	20	30	40	
First acidity reading	0.32	0.29	0.23	0.20	0.26	0.20	0.16	0.15	0.33
Second acidity reading	0.60	0.47	0.33	0.26	0.47	0.30	0.21	0.18	0.54
Increase	0.28	0.18	0.10	0.06	0.21	0.10	0.05	0.03	0.21

These results indicate that relatively small quantities of the inhibitory substance are sufficient to produce a marked influence on the development of acidity. In some later experiments where more extensive growth of 9 S was permitted in the inoculated milk, quantities as small as 3 % of inoculated milk added to normal milk produced a mixture which was almost completely inhibitory to acid production. The results in Table III also indicate that the inhibitory substance is not completely heat-stable at 100°, for it is evident that there is a slight decrease in inhibitory power in inoculated milk which has been heated to that temperature.

Production of the inhibitory substance in media other than milk.

Several batches of medium were made consisting of a clear broth prepared by peptic digestion of sugar-free caseinogen to which were added 2 % of each of the five sugars which 9 S had proved capable of fermenting. After sterilisation, the media were inoculated with 9 S and incubated at 30° for four days. 25 cc. of each of the cultures were then made up to 150 cc. with normal pasteurised milk, and the mixtures were subjected to the vitality test. In every instance there was a complete inhibition of acid production, whereas in appropriate controls containing the uninoculated media, acid production was normal. 9 S gave practically no growth in the caseinogen broth alone and, as would be expected, produced no measurable amount of inhibitory substance. It was thus impossible to determine by an experiment such as this whether the substance was formed from any fermentable sugar or whether it was formed from protein material whenever growth of the organism was stimulated by the presence of a sugar.

Influence of the inhibitory substance on the increase in numbers of lactic streptococci in milk.

Although it seemed most likely that the results in the foregoing experiments were to be explained by inhibition of the growth of lactic streptococci, it was deemed desirable to determine whether this was actually the case or whether the streptococci were increasing in numbers without displaying their usual fermentative activity. Two identical samples of pasteurised milk were taken and to one was added 10 % of milk containing inhibiting substance; both were inoculated with 1 % of a twenty-four hour culture of normal lactic streptococci and incubated at 30°. At various intervals from the time of inoculation samples were taken, and the numbers of streptococci were estimated by direct count under the microscope according to the method of Breed and Brew [1916] (Table IV).

Table IV.

Time in hours	Numbers of streptococci in millions	
	Control	Experimental
$\frac{1}{2}$	22	17
$1\frac{1}{2}$	50	13
$3\frac{1}{2}$	285	20
$4\frac{1}{2}$	537	14
$6\frac{1}{2}$	804	14
10	—	200
12	1000	218
14	1000	436

The acidities of the control and experimental milks after fourteen hours were 0.84 and 0.52 % as lactic acid respectively. From these results it is evident that the inhibitory substance acts by delaying the multiplication of the lactic streptococci and not by interfering only with their fermenting power or otherwise modifying their habit of growth. The streptococci which finally grow in a medium containing the inhibiting substance have a normal appearance under the microscope.

*Partial isolation of the inhibitory substance and
determination of its properties.*

Two litres of sterilised skimmed milk were inoculated with 9S and allowed to stand overnight at room temperature. Next morning the milk contained approximately 170×10^6 cocci per cc. Vitality tests showed that 7 % of this milk culture added to normal milk was the minimum amount which would completely inhibit acid production by normal lactic streptococci for a period of six hours. Calcium chloride and rennet were added to the main bulk of the milk and the firm clot which formed was broken to allow the whey to separate. 1500 cc. of clear whey were obtained by filtration. Vitality tests showed that about 14 % of this whey added to normal milk gave a complete suppression of acid production by lactic streptococci. One litre of the whey was evaporated *in vacuo* at a temperature not exceeding 50° until its volume was reduced to 200 cc. This was poured into 800 cc. of absolute alcohol, and the bulky precipitate formed was filtered off. The filtrate was evaporated to dryness and extracted with absolute alcohol, and the insoluble material was again removed by filtration. The alcoholic solution was evaporated to dryness and the resulting glutinous residue (approximately 2 g.) was dissolved in 50 cc. of distilled water. Vitality tests showed that 2 % of this solution added to normal milk was the minimum amount necessary to inhibit acid production completely. Making allowance for the concentration, this corresponds to only 40 % of the original whey. Some of the inhibitory substance was shown to be present in the material soluble in 80 % alcohol but insoluble in absolute alcohol, so that it is possible that the substance (if it is a single entity) is not freely soluble in absolute alcohol. The concentrated solution of inhibitory substance, prepared as described above, was not inactivated when heated at 100° for 30 minutes.

In order to determine whether the substance were of protein nature some experiments were carried out to test the action of pepsin and trypsin upon it. The reaction of the concentrated extract was about p_H 4. A portion of it was adjusted to p_H 8 with NaOH to favour the action of trypsin. Portions of 5 cc. were treated according to the following scheme, then made up to 150 cc. with normal pasteurised milk and subjected to a vitality test (Table V).

Table V.

1.	5 cc. extract		
2.	"	+ 1/30 cc. 10 % pepsin solution	
3.	"	"	"
4.	5 cc. adjusted extract		
5.	"	"	
6.	"	"	+ 1/30 cc. trypsin solution
7.	"	"	" "

All the above tubes were incubated at 37° overnight, then tubes 3, 5 and 7 were pasteurised at 63° for 30 minutes.

Vitality tests on above samples made up to 150 cc. with normal pasteurised milk.

Acidities as % lactic acid.

	1	2	3	4	5	6	7	Control milk + pepsin	Control milk + trypsin	Control milk
First acidity reading	0.12	0.13	0.13	0.14	0.15	0.19	0.19	0.19	0.21	0.20
Second acidity reading	0.11	0.14	0.12	0.23	0.24	0.47	0.42	0.47	0.51	0.51
Increase	-0.01	0.01	-0.01	0.09	0.09	0.28	0.23	0.28	0.30	0.31

These results serve to define quite clearly the nature of the inhibitory substance. It is evidently a protein derivative (probably a polypeptide) which is readily destroyed by trypsin but is resistant to the action of pepsin. There is also an indication that incubation at 37° after adjustment to p_H 8 causes a weakening in power, probably due to a hydrolytic effect. In other experiments on similar lines, but in which the extract was heated to boiling-point after adjustment to p_H 8, the loss in power was greater, although with 9S the inhibitory action was never completely eliminated by this treatment.

Production of inhibitory substance by Streptococcus D1.

As has already been mentioned *Streptococcus* D1 differed from *Streptococcus* 9 S in that it formed rather more acid during its growth in milk. Milk in which the organism had grown did not appear inhibitory to the growth of normal acid-producing streptococci unless D1 had first been destroyed by pasteurisation. Thus in an experiment on milk in which D1 had grown until its numbers amounted to 50×10^6 per cc., the results of vitality tests were as follows.

Table VI.

Acidities as % lactic acid.

	Raw inoculated	Past. inoculated	Control
First acidity reading	0.28	0.11	0.38
Second acidity reading	0.59	0.10	0.66
Increase	0.31	-0.01	0.28

Thus in the case of this organism either its own acid-producing power counter-balanced the loss in acid production due to inhibition of the normal streptococci, or else the inhibitory substance was not finally formed in the milk until the latter was heated. In most other respects the substance proved to have properties very similar to those of the substance produced by 9 S. It differed mainly in its sensitivity to alkali. A concentrated extract of the substance in several experiments completely lost its power when it was adjusted to p_H 8 and incubated at 37° overnight.

Action of the inhibitory substances on pure cultures of bacteria.

The substances produced by both 9S and D1 were tested on various pure cultures. Both seemed to have a powerful action on the several cultures of lactic streptococci which were tested; neither restrained in any way the growth of a single strain of *Bact. coli* and of a strain of *B. subtilis*. The single instance up to the present where there has been a significant difference between them has been in their action on a strain of *Lactobacillus acidophilus*. The substance formed by D1 was markedly inhibitory towards growth of this organism whereas the substance formed by 9S was not. These observations need to be extended.

DISCUSSION.

It is evident from the foregoing results that the two strains of lactic streptococci described possess a property which has hitherto been unobserved with this species of bacterium. The significance of the formation of the inhibitory substances is evident in connection with any process in which lactic streptococci are used as biological agents for the development of acid. The formation of such substances in milk has already explained satisfactorily a difficulty which sometimes occurs during the manufacture of cheese. Moreover the reaction is of fundamental interest in the matter of the classification of the lactic streptococci. It remains to be seen whether the property of forming the inhibitory substance is a stable characteristic of these organisms. Up to the present time cultures in the laboratory, resown every two or three days in sterilised milk, have retained the property—in the case of 9S for seven months, and in the case of D1 for four months. The phenomenon would assume still greater significance if it were shown that normal lactic streptococci could ever acquire the property. Strains of streptococci vary so much in their properties from time to time that this is quite within the bounds of possibility.

If, however, these two strains of streptococci represent stable types the problem of their origin is of fundamental importance. Much attention has been devoted in the past to the question of abnormal milk which is inhibitory to the growth of lactic streptococci, since it influences the process of cheese manufacture so markedly. Leitch [1933] gave a comprehensive review of the matter and conducted experiments which indicated quite clearly that the inhibitory milk with which he was dealing was derived from cows suffering from udder disease, presumably streptococcal mastitis. Although he was of the opinion that the inhibitory factor was present in the milk as it came from the cow, it seems possible, in view of the fact that one of the streptococci described above is almost identical with *Strep. mastiditis*, that the inhibition observed by Leitch in milk might have been due to a formation of inhibitory substance by streptococci from the cow's udder, after the milk was drawn. Leitch also mentioned that he had obtained evidence that the inhibitory factor was associated with the milk-albumin. He did not give details of the evidence, but the suggestion that the inhibitory factor was of protein nature presents a further point of similarity with the present findings. The biochemistry of the streptococci which are the causative organisms in mastitis of the cow needs a more detailed examination before these points can be settled.

SUMMARY.

1. Two strains of lactic streptococci are described which produce during their growth substances having a marked inhibitory action on the growth of other lactic acid bacteria.

2. The nature of the inhibitory substances was investigated and evidence is submitted that they are of protein nature, possibly polypeptides, soluble in absolute alcohol and hydrolysable by trypsin and alkali but stable towards pepsin.

3. The significance of the properties of the organisms is discussed both from the point of view of the classification of the streptococci and from the technical standpoint, particularly with regard to processes like cheese manufacture in which lactic acid bacteria are used as a means for the production of acid.

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