CXXIII. OBSERVATIONS ON SOME FACTORS IN THE MILK OF INDIVIDUAL COWS WHICH MODIFY THE GROWTH OF LACTIC STREPTOCOCCI.

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It is well established that there are relatively wide variations in the chemical composition of milk taken from individual cows. Differences in the biological properties of individual milks have also been reported from time to time although these have never been correlated with chemical differences with certainty. From the point of view of those interested in the manufacture of cheese and butter, the most important way in which biological variations in milk are manifested is in their effect upon the growth and metabolic activity of the lactic acid bacteria and particularly upon the growth of the lactic streptococci which are commonly added to milk in an attempt to direct along certain lines the biological changes which take place in the manufactured products. Most of the papers that have been published on this question deal with the effect of particular samples of milk on the flavour of "starter" cultures (mixed cultures of lactic streptococci) produced when such milk was used as the bacteriological medium. Baker and Hammer [1926] found that the milk from certain cows produced "starter" cultures which were abnormal in flavour and aroma. They also noticed differences in the final acidity developed in the various milk samples by a given culture. They were unable to find any correlation between acid development and flavour, but they noticed that, in general, milk with a high content of total solids tended to permit the development of high final acidity. Knudsen and Sørensen [1929] also observed that milk containing a high percentage of total solids permitted the development of a high titratable acidity by lactic streptococci in 24 hours and suggested that the higher concentration of buffer substances in such milk explained the more active growth of the bacteria. They also found that certain samples of milk which had been drawn aseptically and sterilised immediately would not readily support the growth of certain strains of lactic streptococci until yeast extract or hydrolysed milk had been added. Gorini [1929] made a similar observation. Kelly [1930] noticed a very definite and constant difference in the flavour of a "starter" culture when it was propagated in the milk of two different herds; he took no account however of acid development. Farmer and Hammer [1931] found that starter cultures varied in their resistance to the germicidal action of raw milk and that different milks varied in their action on different cultures. The effect of the germicidal action which was still evident in milk kept at 68° for 30 minutes was no longer present when the milk was kept at 71° for 30 minutes. With

regard to germicidal or restraining power there seems to have been doubt for some considerable time as to whether the action was due to a germicidal substance or whether the various cells in milk were responsible. The work of Jones [1928] seems to show beyond doubt that a restraining substance quite distinct from blood complement exists in milk and incidentally that the substance varies in concentration from time to time in the milk of individual cows and even in milk from the different quarters of a single cow.

It is evident from these scattered observations that the growth of lactic streptococci in a medium composed partly or wholly of milk may be restrained through several causes.

(i) In raw milk or in milk heated only to a low temperature the germicidal action may lead to a delay in the growth of the organisms.

(ii) In some samples of milk there may be lacking certain substances which are necessary for the rapid growth of the bacteria. Even a low percentage of total solids may apparently lead to a check in acid development at some stage owing to insufficient buffering.

(iii) The cells which vary in number in the milk both of healthy and of diseased cows may restrain or destroy the bacteria by phagocytic action.

The influence of bacteria, present in milk as a contamination, on the subsequent growth of streptococci is not considered here, for it is intended to deal only with those factors which are present in the milk as it leaves the cow's udder.

The work to be described was originally undertaken with the object of determining whether any of these influences were of significance in the acid production which takes place in rennet curd during the process of cheese manufacture. There is sometimes great difficulty in promoting a sufficiently rapid rate of acid development in the curd in a cheese vat; and although this is often undoubtedly due to a lack of vitality in the lactic streptococci themselves, it seemed possible that the properties of the particular batch of milk used to form the curd might on occasions play a part. The results to be described show that this is indeed the case.

The test which was applied to different batches of milk consisted in the addition of a standard amount of a 24-hour "starter" culture, a treatment of the milk in somewhat the same manner as in the process of cheese manufacture, and a determination at definite times of the acidity of the whey which exuded from the curd. This procedure was followed in preference to the simpler method of determination of the acid production in the milk itself, because it was found that greater differences occurred in the acidities of different wheys than in the batches of whole milk, thus rendering the detection of variations more easy. In general the streptococci give lower acidities in a given time in whey from curd than in the corresponding whole milk. The reason for this will be discussed later. It also seems true that any inhibitory factor makes its influence more strongly felt in curd and thus leads to the greater differences which are observed. Moreover, the method used here demonstrates mainly the effect of restraining (or accelerating) factors in the milk on the rate of acid development in the initial and middle stages of the growth of the streptococci, in distinction to the methods of other workers, which without exception deal with the total acid development over a period of 18 to 24 hours. In the process of cheese manufacture it is the rate of acid development in the curd which is most important; hence the advisability of using a test which simulates the actual conditions and which enables one to determine the rate of acid development rather than the total capacity for acid development.

METHOD.

150 cc. of milk in a beaker were adjusted in temperature to 30°, and 1.5 cc. of a 24-hour culture of lactic streptococci in milk were added. The beaker was kept at 30° for 30 minutes, after which time 0.2 cc. of a rennet solution was added. The beaker was maintained at 30° for 1 hour to allow coagulation to take place, and then the curd was cut with a knife as evenly as possible into pieces about $\frac{1}{4}$ -inch square. The mixture of curd and whey was heated to 37°, and after 2 hours at that temperature the whey was drained from the curd as completely as possible. The beaker containing the curd was replaced in the 30° incubator for 2 hours. The concentration of acid in the whey which had exuded was then determined by titration, and all the whey was again drained off. After a further hour at 37° the whey which had exuded from the curd was again titrated. The acidities expressed as parts per thousand of lactic acid were taken as measures of the absolute quantities of acid produced and the difference between the two readings as the rate of acid development. It was found by experiment that differences of the order of 0.5 might occur between the results of duplicate tests, and that comparisons between tests on several batches of milk were justified only when all the tests were performed on the same day, mainly because of variations in the vitality of the "starter" culture from day to day.

RESULTS.

The individual raw milks of several cows were tested by this method. The milk was drawn from the cows direct into sterile vessels, an approximately equal quantity being taken from each quarter of the udder. The samples were placed immediately in a refrigerator and were used in the test within 2 hours of having been drawn. Microscopic examination showed that on every occasion the milks contained less than 10,000 organisms per cc. (the lower limit detectable by direct microscopic count). Since the cultures of lactic streptococci subsequently added contained on the average one billion cocci per cc. it was assumed that the number of contaminant acid-forming organisms present in the samples was in no case sufficient to influence the results of the acidity test. The following results were obtained in the first test (Table I). It will be observed that most

Table I.

Name of cow	Acidity as parts per 1000 lactic acid	
	1st reading	2nd reading
OD	2.7	5.7
R	2.5	4.5
G	2.2	4.2
WD	2.5	5.4
A.F.	3.2	6.6
K	2.4	5.1
I	2.6	5.7
T	1.1	1.2
L	2.7	6.3
F	2.6	5.7

of the milk samples gave substantially the same result, but that the milk from Cow T was outstanding in that the curd prepared from it gave practically no acid development whatever. On further test it was found that several cows in the herd gave milk which showed a similar inhibition of acid development. Upon investigation it was found that all the cows giving this abnormal milk had at some time suffered from mastitis, although at the time the test was made they were considered healthy. In some instances a considerable time had elapsed between recovery from the disease and the date of this test.

Microscopic examination of the milk used in the acidity tests showed however that whenever the phenomenon of inhibition occurred large numbers of leucocytes were present. Three cows which gave such abnormal milk were selected for testing purposes, and samples of milk from the individual quarters of their udders were taken on several occasions. The samples were subjected to the acidity test and to a reductase test (by the addition of methylene blue and incubation at 37°) and the numbers of cells present were estimated by the method of Breed [1911]. Table II gives typical results.

Table II.

Cow	Quarter	Cells per cc.	Reductase time (hrs.)	Acidities as parts per 1000 lactic acid	
				1st reading	2nd reading
L.C. Pure-bred Friesian	R. hind	640,000	>7	2.1	4.5
	L. hind	>60,000,000	$\frac{3}{4}$	1.2	1.4
	R. fore	370,000	>7	2.0	4.2
	L. fore	530,000	>7	2.3	4.2
T. Grade Jersey	R. hind	2,410,000	>7	1.9	3.9
	L. hind	>10,000,000	2	0.8	0.9
	R. fore	180,000	>7	2.3	3.8
	L. fore	1,970,000	>7	1.8	3.9
G. Grade Jersey	R. hind	5,550,000	4 $\frac{1}{2}$	1.4	2.3
	L. hind	140,000	>7	2.3	3.6
	R. fore	110,000	>7	2.5	3.9
	L. fore	70,000	>7	2.3	3.6

Many results such as these make it evident that there was an accurate correlation between a cell count above a certain value, a reduction of methylene blue within 7 hours and an inhibition of acid development in the rennet curd. The phenomenon was usually limited to milk from a single quarter of each abnormal cow, but on continued trial it was found that the condition was not constantly associated with any one quarter. Thus in the case of Cow T the following results were obtained on consecutive days:

Date	Abnormal quarter
7. viii. 32	L. hind
8. viii. 32	None abnormal
9. viii. 32	R. fore
10. viii. 32	R. hind
11. viii. 32	L. hind

On a subsequent day the milk from the L. fore quarter was also abnormal. The results of many tests on several cows showed that a cell count of five million per cc. was approximately the lower limit at which the phenomenon appeared. On odd occasions a milk containing fewer cells (of the order of two or three millions per cc.) gave a curd which inhibited the development of acid, but the inhibition was constantly found only when the numbers exceeded five millions. The milk of Cow T was examined daily for several months, and during that period it showed the phenomenon of inhibition at irregular intervals although at no time did the animal suffer from an acute attack of mastitis. The milk was normal also in appearance throughout the whole period, although the person who milked the cow occasionally observed small lumps during the process of milking. The cause of the inhibition became evident when a culture of lactic streptococci was added to a sample of milk which contained abnormal numbers of cells and the mixture incubated at 37° for a few hours. A stained smear of the milk examined under the microscope showed almost complete phagocytosis of the streptococci.

with consequent inhibition of their growth or fermentive activity. In some samples the organisms ultimately overcame the retarding effect of the cells and showed renewed growth. This was also evidenced in the acidity tests when the curd samples were allowed to remain in the incubator overnight. Some of the curds which had shown practically no acid development within the normal period of the test then gave a considerable development of acidity. Others which contained much larger numbers of cells still gave very little acid development. No attempt was made to differentiate the various types of cells in the milk samples, but certainly over 90 % of the cells in samples containing several millions per cc. were polymorphonuclear leucocytes. It seems to be quite definite from the results described that the leucocytes were sometimes able by their phagocytic action to prevent the development of streptococci for periods longer than 24 hours, while at other times they were capable of delaying activity for at least 6 hours.

Effect of heat upon abnormal milk.

It was of interest to determine what heat treatment of milk containing large numbers of cells sufficed to remove its inhibitory action on lactic streptococci. Milk which is pasteurised before consumption by human beings is usually heated at 63° for 30 minutes, while milk which is to be used for the manufacture of cheese is, in New Zealand, usually heated to 66–70° for 30 seconds. When abnormal milk such as has been described was heated for 30 minutes at 63° the inhibitory effect was entirely removed and normal results were obtained in the acidity test. Incubation of the heated milk with an addition of lactic streptococci showed also that phagocytic action was entirely absent.

Some experiments were then performed in which milk containing abnormally large numbers of cells was kept at various temperatures for a much shorter time, approximately 30 seconds, as is the practice in pasteurising milk by the flash process. The milk was run through the condensing space of a double surface condenser through the outer and inner jackets of which steam was passed. The rate of flow of the milk was controlled so that the desired temperature was recorded on a thermometer with its bulb immersed in the stream issuing from the lower end of the condenser. A second condenser through the outer jacket of which cold water flowed served to cool the milk immediately to a temperature of 30°. It was thus shown by means of acidity tests on abnormal raw milk and on the same milk pasteurised that the limiting temperature at which the inhibitory action was removed was with most samples 49–52°. After treatment at this temperature the majority of the samples tested still showed a slight inhibitory action, but at any higher temperature the milk after treatment gave a result in the acidity test which was within the normal range. Table III gives a set of typical results.

Table III.

Treatment		Acidity as parts per 1000 lactic acid	
		1st reading	2nd reading
Milk containing 24×10^6 cells per cc.	Raw	0.9	0.9
	Pasteurised 63° 30 mins.	2.5	5.4
	Pasteurised 49–52° 30 secs.	1.4	3.0
Normal milk	Pasteurised 63° 30 mins.	2.3	4.2

It is evident therefore that any of the heat treatments of milk normally used in practice would eliminate the inhibitory effect of leucocytes on the development of acidity in rennet curd. Even in raw milk it is unlikely that the

average number of cells in a mixed supply would be great enough to exert a marked effect on the growth of lactic streptococci, and as will be shown in a later section of this paper a number of cells less than that which gives phagocytosis is likely to lead to a stimulating rather than an inhibiting effect.

Differences between the milks of apparently normal cows.

During the course of the work on the milk of abnormal cows it was noticed that there were some cows whose milk, although normal in respect of its content of cells, yet gave consistently low results in the acidity test. The check in acid production was by no means so marked as with milk containing abnormal numbers of cells, but it was remarkably steady with any one animal over a period of many days. In the absence of any possibility of marked phagocytic action the possible explanations seemed limited to a deficiency in the milk or curd considered as a medium for the streptococci, or to an inhibitory factor in the milk. Six normal cows were selected as representative of those giving high and low results and samples of their milk were subjected daily to the acidity test. It was realised that the "starter" culture to be used would probably vary in activity from time to time, so two control milks were also used, one consisting of the mixed milk from the whole herd of about 50 cows and the other of a milk prepared from a dried skim milk. For the latter control one batch of powder was used throughout the whole period of the experiment. Each milk was tested both in the raw state and after pasteurisation at 63° for 30 minutes. Table IV gives particulars of the cows, and the graphs in Fig. 1 show the results obtained.

Table IV.

Cow	Breed	Average fat in milk %	Days between date of calving and beginning of experiment
1	Grade Jersey	4.8	77
2	Grade Jersey	5.1	54
3	Pure Friesian	3.4	227
4	Pure Friesian	3.2	76
5	Grade Friesian	3.2	111
6	Grade Jersey	4.5	60

Fig. 1.

----- Raw milk. ——— Pasteurised milk. Cells.

For simplicity only the increases in acidity between the first and second readings in the test are plotted, so that the results really indicate the rate of acid production in the curds at an arbitrarily chosen time after the start of the test. The numbers of cells in all the milk samples were estimated by the direct microscopic count as used for the counting of bacteria in milk and the logarithms of the number of cells are also plotted on the graphs. A consideration of the graphs leads to several interesting conclusions.

(a) The milk from any single cow tended to maintain a certain general characteristic level in rate of acid production in the curd in spite of slight fluctuation from day to day.

(b) With the milks of most of the cows pasteurisation tended to lead to a slightly higher rate of acid production. This is in agreement with the observations of Benson and Evans [1913]. It is probably due in part to a destruction of the bactericidal substance present in raw milk.

(c) The milks from individual Jersey cows gave curds in which the rate of acid development was in general lower than the rate in curds from Friesian milk. At first sight this seems opposed to the findings of Baker and Hammer [1926] who stated that milk with a high total solids content (*e.g.* Jersey milk) developed a higher acidity in 24 hours than milk with a low total solids content. There is however no real discrepancy, for it is found that although the acid in the moisture in Jersey milk curd develops at a slower rate during the cheese making process it ultimately reaches a higher value than the acid in the moisture of Friesian milk curd if sufficient time is allowed. The different rates of acid development in Jersey and Friesian curds are probably due to differences in moisture content. The greater the percentage of fluid in the medium, the more rapid will be the growth of the bacteria, and it is well known that curd prepared from milk of high solid content is less rich in moisture and even loses that moisture more readily than curd from milk of lower total solid content, all other conditions being equal. The same factor (*i.e.* difference in moisture content) probably explains the more rapid production of acid by bacteria in whole milk than in any type of curd.

(d) The curves showing the numbers of cells present in the milk samples show a tendency to follow the curves showing the rates of acid development. The tendency is evidenced both in general level and in many cases also in the fluctuations from day to day. The correlation is far from exact, especially as regards the daily variations in milks where the cells were low in number, and the estimation was necessarily subject to relatively large errors, but the general agreement in level is suggestive. The numbers of cells in practically every instance were within the range considered normal for milk from healthy animals, and whereas it has been shown that abnormal numbers of cells tend to inhibit the development of acid by lactic streptococci, the graphs under consideration indicate that, even when allowance is made for variations due to the changes in the vitality of the organisms and for the probable influence of total solid content, the presence of large numbers of cells within the normal range tends to render curd prepared from the milk a better medium for the organisms.

The foregoing tentative conclusion with respect to the influence of cells seemed so important that it was deemed advisable to carry out further experiments with the specific object of confirming or refuting it. Two experiments were therefore performed, the first with six Friesian cows and the second with six Jersey cows. Two of the animals had suffered from mastitis and were still yielding milk containing rather more than the normal numbers of cells, though in no case so many as five millions per cc. Table V gives the relevant particulars

Table V.

Cow	Breed	Average yield of milk per day lbs.	Average fat in milk %	Average total solids in milk %	Days between calving and beginning of experiment	Days between recovery from mastitis and beginning of experiment
F 1	Pure Friesian	45	3.0	11.1	93	—
F 2	Pure Friesian	47	3.5	12.2	59	—
F 3	Pure Friesian	49	3.2	11.2	93	—
F 4	Pure Friesian	48	3.3	11.9	91	—
F 5	Pure Friesian	42.5	3.4	11.6	68	—
F 6	Pure Friesian	53	3.6	12.4	95	63
J 1	Grade Jersey	37.5	4.3	13.9	120	—
J 2	Pure Jersey	32.5	6.5	15.9	108	—
J 3	Pure Jersey	33	4.9	14.3	41	—
J 4	Grade Jersey	25	5.5	14.6	201	—
J 5	Pure Jersey	18.5	7.7	17.7	116	—
J 6	Grade Jersey	25	4.5	13.9	94	8

Fig. 2.

Fig. 3.

of each animal and Figs. 2 and 3 give graphs of the results. On this occasion only the pasteurised milk was tested.

It will be observed that the results of the previous test were substantially confirmed. The Friesian milks again gave a higher general rate of acid production, and perhaps even more definitely in these experiments the rates of acid production showed a correlation with the numbers of cells present in the milk samples. There were one or two anomalies (*e.g.* with the milk from Cow J 4), but in general the correlation was remarkably good. It seemed to make no difference whether the cells were present normally in the milk or were due to recent infection of the udder (*e.g.* in the cases of Cows F 4 and F 6). It seems possible, therefore, that large numbers of cells in the milk of certain cows indicate some previous infection even though no clinical symptoms were ever observed.

The results in any case promise to provide a link, for the first time so far

as we are aware, between physiological processes in the cow and the properties of milk as a bacteriological medium. It seems hardly likely that the cells themselves furnish nutriment for the bacteria especially in raw milk where they are in the living state. Furthermore the fact that the correlation between acidity development and cell numbers holds even with milk which has been pasteurised indicates that the stimulating effect is probably due to some chemical entity stable to moderate degrees of heat. The alternative explanation that absence of cells coincides with presence of some inhibitory factor does not seem so satisfactory. Most probably the presence of the stimulating substance in the milk is conditioned by the same physiological influences which affect the numbers of cells. From this aspect the problem appears to be one for the physiologist.

SUMMARY.

1. Milk containing numbers of leucocytes in excess of five millions per cc. gave rise to a rennet curd in which lactic streptococci were not able to develop normal quantities of acid. The influence of the cells was shown to be most probably due to phagocytosis of the streptococci.

2. Cows which were not suffering at the time of the experiments from evident disease of the udder often secreted milk normal in appearance but containing more than five millions of cells per cc.

3. The inhibitory action of the cells was eliminated by a heat treatment of the milk at 49–52° for 30 seconds.

4. The rates of acid production in the rennet curds prepared from the milks of individual healthy cows varied to a significant extent from animal to animal and in one animal from day to day.

5. Some of the differences between milks from different animals were most probably connected with the relative amounts of protein and moisture in the curds.

6. After allowing for the differences due to the factors mentioned under 5 the different rates of acid production appeared to be connected to some extent with the number of cells present in the milks from which the curds were prepared. In most instances investigated the cells were present in numbers not greater than one million per cc. and the higher the number of cells within this range the more rapid was acid production in the curd.

7. The apparent stimulating effect of cells in small numbers in milk upon the growth of lactic streptococci was still manifest after the milk had been pasteurised at 63° for 30 minutes. Therefore, it seems evident that some stimulating substance, stable to moderate degrees of heat, must be secreted in the milk, and its presence must be governed by the same factors which govern the presence of the cells.

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