LII. ACID CHANGES IN MILK PRODUCED BY CERTAIN BACTERIA.

BY LESLIE HERBERT LAMPITT AND MARK BOGOD.

From the Laboratories of Messrs J. Lyons and Co. Ltd., Kensington, W. 14.

(Received February 17th, 1933.)

INTRODUCTION.

THE scientific literature concerned with the decomposition of milk by micro-organisms is very extensive, and reference to it demonstrates that the changes occurring have in most cases been correlated with variations in the acid content, although protein breakdown has, to a lesser extent, been studied. As a somewhat crude index of change, titration with alkali is very simple in application, and it is probably for this reason that it has been so widely adopted, and that, together with determinations of the hydrogen ion concentration, it has received more attention than any other available method.

In some cases a determination of a somewhat indefinite figure called volatile acidity has been used, but very rarely have even these two determinations—titratable acidity and volatile acidity—been carried out on one and the same series of samples. An unfortunate fact, too, is that this titratable acidity is often quoted as “lactic acid”; the serious error which this may entail has already been demonstrated [Lampitt and Bogod, 1930]. It is unnecessary to point out that it is impossible to define the course of any changes by determinations of the total “free” acids, and it would appear that only by determining a series of acids can a truer picture be obtained of the activity of specific organisms.

The present work serves to demonstrate the complexity of the acid changes which take place when organisms, chosen as representative of distinct groups, are inoculated into sterile milk, and is preliminary to a study of the mutual effect of the associative or symbiotic growth of certain bacteria, which has already received a certain amount of attention. The general methods adopted in this work have already been described by us [Lampitt and Bogod, 1932]. They consist of the determination of total acidity by normal and by electro-metric titration, of p_H , of lactic acid, of citric acid and of free fatty acid. Their application to the study of progressive bacterial action in milk and the souring of milk under different temperature conditions has been reported, and it would appear that the use of such a system of investigation gives a clearer idea of the complexity of the action than is given by the less comprehensive scheme of analysis adopted, *e.g.* by Marshall [1920] and by Porcher and Lambert [1930] in their respective works on association of *B. subtilis* and *S. lactis*, or by Vas [1924] in his study of *L. bulgaricus* and *S. lactis*.

Comparatively modern work of a fundamental nature in this field is not too extensive, but among the workers whose investigations about on that described herein may be mentioned Svanberg [1918; 1919] who studied the limiting values

for the p_H of milk under the influence of bacteria and also the velocity of growth of the bacteria under different p_H conditions. Thus, a p_H of 4.0 was found to be the limiting value and a range of p_H 5.5–6.4 the optimum for *S. lactis*. Baker and his co-workers [1920] showed the relation between bacterial growth and total acid production in milk. Sherman and Holm [1922], while studying the effect of sodium chloride on bacterial growth, found that *E. coli* failed to grow at p_H 4.8. Scheer [1922] used the p_H value in studying acid production by *E. coli* in buffered sugar solution and obtained a p_H of 5 in 20 hours, thereafter the acidity increased only slightly for 1 or 2 days, when acid production ceased. A study of the decomposition products of spore-bearing bacteria in heated milk by Lisk [1924] included the changes in p_H , titratable and volatile acidity; except in one case, an increase in titratable acidity was accompanied by a decrease in p_H . Cianci [1929] followed the progress of acid formation in 1 % lactose under the action of various bacteria including *S. lactis*, *E. coli* and *A. aerogenes* and also studied the action of *E. coli* in 1 % glucose broth. He found that p_H and acidity were constant after $7\frac{1}{2}$ hours. As will be shown later, with milk as the medium, these values only become constant after a much longer period.

The formation of acids volatile in steam from milk has been subjected to investigation, notably by Hammer and his associates. With Bailey [1919] the volatile acid production of starters and organisms isolated from these was studied; with Cordes [1921] the relation of volatile acidity to titratable acidity as produced by *S. lactis* was found to be constant and the relationship to vary for different starters. Hucker [1928] suggested the use of the volatile acidity-titratable acidity relationship as a means of differentiating streptococci.

Citric acid utilisation by bacteria has received the attention of various workers. Kicking [1922] reported that lactic acid-forming bacteria did not destroy citric acid, while peptonising bacteria did do so, and Koser [1924] found that typical coli of faecal origin were unable to affect sodium or potassium citrate, whereas the aerogenes-cloacae group all readily decomposed these salts. However, Hastings, Mansfield and Helz [1925] showed that citric acid fermentation depended upon the medium and found that the citrates in milk were not attacked by caseinogen-digesters, *S. lactis* or *A. aerogenes*, while *E. coli* had a marked positive effect.

EXPERIMENTAL.

The analytical methods used in this work have, but for one exception, been previously described [Lampitt and Bogod, 1932]. The exception, the volatile acidity, has been determined by a method described by Hammer and Sherwood [1923].

The bacteria (Bergey's nomenclature) used in the investigation were:

(1) *Streptococcus lactis*. This was obtained from the National Collection of type cultures and catalogued as *Streptococcus acidi lactici*, Thaysen, No. 662.

(2) *Alcaligenes faecalis*. From the N.C.T.C., catalogued as *Bacillus faecalis alcaligenes*, No. 415.

(3) *Aerobacter aerogenes*. From the N.C.T.C., catalogued as *Bacillus lactis aerogenes*, No. 418.

(4) *Escherichia coli*. From the N.C.T.C., catalogued as *Bacillus coli communis*, Houston 2, No. 692.

(5) *Bacillus subtilis*. From the N.C.T.C., catalogued as *Bacillus subtilis*, Hay, No. 85.

(6) A lipase-producing micrococcus of unknown species isolated from an air culture.

Certified milk was sterilised, and 700 cc. portions were measured into 1 litre Erlenmeyer flasks and inoculated with a culture of the bacteria in milk. Sufficient inoculum was added to give as nearly as possible 100,000 organisms per cc. The flasks were placed in a room thermostatically controlled at 30°. One sample was analysed immediately after inoculation and the remainder at intervals over a period of 2 weeks. For convenience in tabulation the determinations are recorded under letters as follows:

T. Titratable acidity, given as cc. 0.25 *N* NaOH required per 100 g. of milk.

E. Electrometric titration, given as cc. 0.5 *N* NaOH per 100 g. of milk.

H. p_H value.

L. Lactic acid as percentage content.

C. Citric acid as percentage of the original amount.¹

F. Free fatty acids given as cc. 0.1 *N* NaOH per 100 g. of milk.

V. Volatile acidity, given as cc. 0.25 *N* NaOH per 100 g. of milk.

The detailed results obtained are given in the following tables.

Table I. The total acidity determinations as given by T., E. and H. follow the normal course and show the attainment of a constant figure after approximately 6 days' incubation. The lactic acid content also increases to a reasonably constant figure in the same time. The volatile acidity, showing a marked increase,

Table I. *S. lactis*.

Period of incubation (hrs.)	T.	E.	H.	L.	C.	F.	V.
0	7.7	3.6	6.40	0.018	100	0.40	0.7
15	12.8	6.4	5.75	0.125	37.5	0.60	2.6
24	18.7	8.5	5.50	0.240	21.5	0.70	3.8
28	21.7	10.6	5.05	0.320	6.3	1.10	3.9
40	22.6	10.7	5.20	0.400	3.9	0.70	3.9
64	23.8	11.7	5.00	0.460	None	0.90	4.3
75	24.7	12.2	4.95	0.560	"	1.10	4.2
88	29.3	14.0	4.75	0.595	"	0.90	4.8
99	32.3	15.6	4.70	0.580	"	0.90	4.4
118	34.5	17.1	4.55	0.700	"	0.90	4.6
144	33.5	15.3	4.60	0.610	"	0.90	4.5
169	34.3	17.2	4.50	0.640	"	1.10	4.4
192	32.5	17.0	4.55	0.560	"	1.20	4.1
244	32.8	16.4	4.60	0.645	"	1.20	4.5
285	34.2	16.4	4.65	0.635	"	1.20	4.6
333	33.6	17.0	4.50	0.605	"	1.10	5.0

does not, however, represent a large proportion of the total acidity. The citric acid was found to disappear after 2 days; this does not agree with the work of Hastings *et al.* [1925] who stated that the citrates in milk were not attacked by *S. lactis*; Kicking [1922] also reported that lactic acid-formers did not destroy citric acid. Consequently this destruction of citric acid was confirmed by us on various occasions and further, in the communication previously referred to [1932] it was shown that *L. bulgaricus* which produced over 2 % lactic acid also destroyed citric acid in milk comparatively rapidly.

In order to determine any possible variation due to the initial bacterial concentration, lactic acid and citric acid were determined in two further series inoculated with *S. lactis* to give 10,000 and 1000 organisms per cc. respectively.

The progressive changes are shown in Table IA.

¹ The citric acid content varied for each supply of milk, results obtained being: 0.168, 0.155, 0.128, 0.181, 0.173, 0.154, 0.149, 0.131 and 0.190 %. In order, therefore, to compare results as between one organism and another, the results are expressed as stated.

Table IA. *Effect of variation of initial bacterial concentration.*

Period of incubation hrs.	Lactic acid %		Citric acid %	
	10,000 <i>S. lactis</i> per cc.	1000 <i>S. lactis</i> per cc.	10,000 <i>S. lactis</i> per cc.	1000 <i>S. lactis</i> per cc.
0	0.012	0.012	100	100
10	0.011	0.012	98.4	98.9
20	0.139	0.035	37.0	56.9
24½	0.241	0.198	22.1	23.8
30	0.274	0.269	9.9	11.6
34½	0.357	0.321	2.8	4.4
39	0.389	0.379	None	None
50	0.446	0.412	"	"
70½	0.455	0.483	"	"
94½	0.552	0.531	"	"
119	0.560	0.570	"	"

Table IA. If curves are drawn side by side with those obtained with an initial concentration of 100,000 per cc. (Fig. 1), it is apparent that the effect of decreasing the initial count is merely to delay the commencement of chemical change since once the action has commenced, the alterations follow a definite typical course.

Fig. 1. *Effect of variation of initial bacterial concentration.*

Table II. *B. faecalis alcaligenes.*

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V
0	6.5	3.4	6.60	0.017	100	0.35	0.6
15	6.6	3.6	6.35	0.022	100	0.28	0.6
24	6.8	3.6	6.35	0.035	98	0.40	0.6
28	6.8	3.4	6.50	0.017	98	0.42	0.7
40	6.7	3.1	6.60	0.022	97	0.48	1.0
48	6.6	3.3	6.45	0.015	96	0.52	0.6
64	6.4	3.3	6.45	0.022	95	0.54	0.7
76	6.3	3.5	6.40	0.030	93	0.50	0.6
100	6.7	3.1	6.60	0.015	93	0.60	0.8
120	6.2	3.3	6.50	0.012	93	0.38	0.7
169	5.9	2.6	6.75	0.022	89	0.60	0.7
192	5.8	2.8	6.70	0.012	88	0.52	0.6
285	5.4	2.8	6.70	0.022	86	0.40	0.7
333	5.5	2.4	6.80	0.019	85	0.46	0.8

Table II. The values for T., E. and H. show the development of a slight alkalinity which follows a decrease in the citric acid content. Ayers *et al.* [1919] also demonstrated this increase in alkalinity with a decrease in the citric acid content. The values for L., F. and V. show no appreciable change.

Table III. The formation of acid, although not as great as with *S. lactis*, is considerable. Half of the citric acid content has been destroyed during the 2 weeks' incubation. It will be noted that volatile acid production proceeds comparatively rapidly to a maximum value and then steadily decreases.

Table III. *Aerobacter aerogenes*.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
2	8.1	4.1	6.25	0.078	100	0.50	0.6
15	14.6	6.4	5.80	0.102	97	0.60	3.8
24	16.4	7.6	5.75	—	99	0.80	3.7
30	18.6	8.0	5.70	0.083	94	0.90	4.8
40	20.1	7.7	5.70	0.040	94	0.80	4.6
48	20.1	8.0	5.76	0.043	94	0.70	4.8
64	21.2	7.9	5.70	0.049	94	0.50	4.0
77	21.7	8.8	5.75	0.066	76	0.60	4.2
89	22.3	8.2	5.75	0.099	80	0.70	4.0
101	22.5	8.7	5.75	—	78	0.60	—
120	22.5	9.6	5.60	0.103	84	0.70	4.2
146	23.7	9.9	5.70	0.171	81	0.70	3.7
168	26.9	10.0	5.60	0.216	75	0.90	3.8
192	27.6	11.9	5.50	0.158	77	1.00	3.7
237	27.2	11.9	5.45	0.243	60	1.00	1.9
309	28.8	11.0	5.50	0.264	59	1.00	2.6
333	29.6	14.5	5.25	0.300	46	1.00	1.9

Table IV. Total acid formation here shows a rapid increase to a maximum value on the third day with little change thereafter. It will be noted that in this case the lactic acid content does not increase commensurately with the total acidity; indeed, the volatile acidity, of which the production is the highest of all those determined in this work, contributes more than the lactic acid to the total acidity. The destruction of citric acid is even more rapid than was the case with *S. lactis*.

Table IV. *E. coli*.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
2	8.4	4.2	6.25	0.028	100	0.60	0.7
15	15.2	7.5	5.70	0.050	4	0.60	6.1
24	19.7	9.1	5.50	0.065	None	0.70	5.1
30	21.7	10.0	5.35	0.068	2	0.50	8.0
40	22.3	10.3	5.35	0.060	None	0.50	8.8
48	22.7	10.6	5.30	0.068	"	0.50	8.3
64	26.0	10.9	5.30	0.065	"	0.50	8.2
77	24.8	11.5	5.30	0.060	"	0.80	8.3
88	25.7	11.3	5.30	0.055	"	0.80	8.6
101	24.5	11.5	5.30	0.060	"	0.80	8.6
120	24.1	11.8	5.30	0.070	"	0.80	8.5
166	25.3	11.1	5.25	0.088	"	1.00	9.2
192	23.7	11.8	5.30	0.060	"	0.60	9.2
285	25.1	11.8	5.30	0.083	"	0.70	8.5
333	25.7	12.5	5.20	0.073	"	0.80	10.2

Table V. In considering the results in Table V, it should be remembered that the organism was existing virtually under anaerobic conditions. In the first place, during sterilisation, dissolved oxygen was driven out and later, when the inoculated milk was being incubated, a layer of cream formed on the surface.

It is obvious that there has been little action taking place in so far as the observed chemical characteristics are concerned. A count, taken at the end of the incubation period, showed however that the bacteria had increased from 100,000 to 1,200,000 per cc.

Table V. *B. subtilis*.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
0	6.9	3.7	6.35	0.027	100	0.60	0.5
15	7.3	3.9	6.30	0.030	100	0.20	0.6
24	7.5	3.9	6.30	0.030	100	0.20	0.5
30	7.4	3.7	6.40	0.025	100	0.30	0.5
40	7.2	3.7	6.40	0.020	100	0.30	0.5
64	8.0	4.0	6.30	0.025	100	0.40	0.6
75	8.2	4.2	6.30	0.025	100	—	0.6
88	7.7	4.0	6.30	0.035	93	0.40	0.6
101	8.4	4.2	6.30	0.027	96	0.40	0.6
120	7.4	3.9	6.40	0.020	97	0.30	0.6
144	8.0	4.2	6.30	0.025	96	0.30	0.6
170	7.6	3.9	6.40	0.037	96	0.20	0.6
192	7.8	4.0	6.30	0.022	95	0.40	0.6
216	7.8	3.9	6.35	0.025	—	0.40	0.6
280	7.8	4.0	6.35	0.018	89	0.50	0.6
338	8.0	4.0	6.40	0.032	87	0.50	0.6

A further series inoculated with *B. subtilis* was investigated and precautions were taken thoroughly to shake the flask twice daily. In this manner it was ensured that the cream layer was broken up and dispersed through the milk, also that some air was dissolved. The results obtained are given in Table V A.

Table V A. It will be observed that in these circumstances the total acidity and lactic acid have been increased to some extent, but the effect on citric acid is comparable with the standard series: p_H and volatile acidity are again unaffected.

Table V A. *B. subtilis* (*shaking at intervals during incubation*).

Period of incubation hrs.	T.	H.	L.	C.	V.
0	6.6	6.30	0.018	100	0.6
98	7.2	6.30	0.028	92	0.8
147	8.2	6.50	0.060	89	0.9
194	9.6	6.45	0.086	88	0.9
288	10.8	6.40	0.081	84	0.9

Table VI. *Lipase-producing Micrococcus of unknown species*.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
0	7.0	3.5	6.35	0.018	100	0.90	0.3
15	7.0	3.7	6.30	0.030	100	1.00	0.3
24	6.6	3.4	6.40	0.028	100	1.10	0.2
29	6.8	3.7	6.25	0.030	100	1.10	0.2
40	8.1	3.8	6.40	0.028	100	1.20	1.0
50	6.6	3.4	6.50	0.020	100	1.00	0.6
64	6.8	3.8	6.25	0.020	100	1.00	0.6
76	7.1	3.8	6.30	0.030	100	1.00	0.9
88	6.9	4.1	6.15	0.020	100	0.90	0.8
119	7.6	4.0	6.15	0.040	100	1.10	1.0
144	8.4	4.3	6.10	0.033	100	1.10	1.3
170	8.3	4.1	6.15	0.040	100	1.20	1.1
192	7.6	4.2	6.15	—	97	1.30	1.4
216	7.4	4.2	6.15	0.028	96	1.30	1.4
278	8.2	4.4	6.10	0.045	96	1.30	1.6

Table VI. Here again a decided progressive action is not apparent; a more marked formation of fatty acids and volatile acids is, however, to be observed. The bacterial count at the end of the incubation period was 101,000,000 as compared with the original 100,000 per cc.

The graphs presented show the relationships existing among the various bacteria in regard to each specific determination. The electrometric titrations closely resemble the results for titratable acidity and have therefore not been graphically represented.

Fig. 2. The relative total acid-forming properties of the bacteria under test existing under the same temperature conditions are well contrasted. An apparent resemblance between *E. coli* and *A. aerogenes* is shown.

Fig. 2. Titratable acidity.

Fig. 3. *pH*. *B. subtilis*—no apparent change.

Fig. 3. The picture presented by these curves resembles that given by the previous one in regard to the relationships between organisms.

Fig. 4. A decided difference between the action of *E. coli* and *A. aerogenes* is here demonstrated and the chief characteristic of *S. lactis* is amply shown.

Fig. 5. A further distinction between *E. coli* and *A. aerogenes* is the destruction of citric acid, exceedingly rapid in the case of *coli* and with *aerogenes* only

Fig. 4. Lactic acid. *B. subtilis*, lipase-producing and *alcaligenes*—no appreciable change.

Fig. 5. Citric acid.

Fig. 6. Fatty acid. *B. subtilis* and *alcaligenes*—no appreciable change.

decreasing to about half the normal content. The citric acid loss with *B. subtilis* is very small, a fact which agrees with the work of Hastings *et al.* [1925] concerning the inactivity of caseinogen-digesters in connection with the citrates in milk, although Kicking [1922] reports to the contrary.

Fig. 6. The free fatty acid formation, which refers to the higher fatty acids, is greatest in the case of the lipase-producing *Micrococcus*.

Fig. 7. *E. coli* is shown to produce more of the volatile acids than any of the other micro-organisms tested. It will be noted that there is an apparent relation-

Fig. 7. Volatile acidity. *B. subtilis*—no appreciable change.

ship between the destruction of citric acid and volatile acid formation, since *coli* with the highest volatile acid production most rapidly destroyed the citrates; *S. lactis* is next and *A. aerogenes* is third in both respects.

We acknowledge with thanks the assistance of Messrs D. H. F. Clayson, D. G. Pirie and Miss D. A. Rudland in preparing cultures and performing inoculations and of Mr W. C. Wise for assistance with the analytical work.

Our thanks are also due to the Directors of Messrs J. Lyons and Co., Ltd., in whose laboratories this work was carried out, for permission to publish these results.

REFERENCES.

- Ayers, Rupp and Johnson (1919). *U.S. Dep. Agric. Bull.* No. 782.
 Baker, Brew and Conn (1920). *N.Y. Agric. Exp. Sta. Bull.* No. 74.
 Cianci (1929). *Boll. Soc. Ital. Biol. Sper.* 4, 1129.
 Cordes and Hammer (1921). *Iowa Agric. Exp. Sta. Res. Bull.* No. 66.
 Hammer and Bailey (1919). *Iowa Agric. Exp. Sta. Res. Bull.* No. 55.
 — and Sherwood (1923). *Iowa Agric. Exp. Sta. Res. Bull.* No. 80.
 Hastings, Mansfield and Helz (1925). *Proc. Soc. Amer. Bact.* (Abstr. from *J. Bact.* 11, 77.)
 Hucker (1928). *N.Y. Agric. Exp. Sta. Tech. Bull.* No. 143.
 Kicking (1922). *Biochem. Z.* 132, 210.
 Koser (1924). *J. Bact.* 9, 59.
 Lampitt and Bogod (1930). *Chem. Ind.* Special No. March, 510.
 — (1932). *Chem. et Ind.* 27, 3 bis, 777.
 Lisk (1924). *J. Bact.* 9, 1.
 Marshall (1920). *J. Dairy Sci.* 3, 406.
 Porcher and Lambert (1930). *Le Lait*, 10, 641.
 Scheer (1922). *Biochem. Z.* 130, 535.
 Sherman and Holm (1922). *J. Bact.* 7, 465.
 Svanberg (1918). Inaug. Diss. 4. Stockholm, *Abst. Bact.* 4, 284.
 — (1919). *Z. physiol. Chem.* 108, 120.
 Vas (1924). *Le Lait*, 4, 625.