Studies on Lactobacillus Cultures That Actively Coagulate Milk[1]

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IT HAS long been known that certain species of the genus Lactobacillus are important in the dairy industry. Cultures of *Lactobacillus casei* were early studied in connection with the ripening of Swiss, Cheddar and other types of cheese. *Lactobacillus acidophilus* is of particular importance in the production of fermented milk which has certain therapeutic uses. *Lactobacillus bulgaricus* is also used in the production of certain types of fermented milk and in the manufacture of Swiss cheese.

The species of lactobacilli mentioned above are primarily important in the dairy industry because of their ability to ferment lactose actively. These microorganisms are definitely homofermentative; that is, lactic acid is the chief product formed in milk and there are only relatively small amounts of additional compounds produced. Other lactobacilli are undoubtedly common in dairy products, but the types that are active lactose fermenters have a much better opportunity to bring about conspicuous changes in milk and its derivatives than types that ferment lactose slowly or not at all.

In view of the fact that little work has been done on the determination of the products other than lactic acid produced by lactobacilli, continued studies on the fermentation of milk by these organisms seem desirable.

The work herein reported involves a study of the amounts of total acid, volatile acid and acetylmethylcarbinol plus diacetyl produced in milk by representative strains of *L. casei* that actively coagulate milk.

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[1] Journal Paper No. J418 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 119.

METHODS

CULTURES USED

A total of 36 cultures of lactobacilli were studied. Most of the cultures were isolated from dairy products such as cheese and milk, while a small number came from feces and ensilage. Tomato juice agar was generally employed for the isolation of the cultures; the colonies were picked from the plates and propagated in litmus milk. Cultures identified as lactobacilli which did not produce relatively large amounts of acid in a comparatively short period were discarded.

CHEMICAL ANALYSIS

The test organisms were inoculated into flasks of sterile skim milk and held 7 days at 37° C. The following determinations were then made on the cultures:

Total acidity. Nine gms. of cultures were titrated with N/10 NaOH using phenolphthalein. The results are expressed as the percentage of lactic acid in 9 gms. of culture.

Volatile acidity. The method used was that outlined by Michaelian, Farmer and Hammer (1). The results are expressed as the cc. of N/10 NaOH required to neutralize the first liter of distillate when a 250-gm. portion of culture was distilled with steam.

Acetylmethylcarbinol plus diacetyl. The method employed was that suggested by Michaelian, Farmer and Hammer (1). The results are expressed as the milligrams of nickel dimethylglyoximate equivalent to acetylmethylcarbinol plus diacetyl per 200 gms. of material.

RESULTS

THE PRODUCTION OF TOTAL ACID, VOLATILE ACID AND ACETYLMETHYLCARBINOL PLUS DIACETYL IN MILK

The data on the amounts of total acid, volatile acid and acetylmethylcarbinol plus diacetyl produced in milk by the 36 cultures of lactobacilli are given in table 1.

There was considerable variation in the amounts of total acid formed by the various cultures, the values ranging from 0.50 to 2.34 percent calculated as lactic acid. No differences were noted in the appearance of litmus milk cultures of the organisms at the end of the incubation period and all the organisms produced a smooth curd with no evidence of gas or proteolysis. Cultures that produced relatively large amounts of total acid sometimes showed a tendency to "whey off." There was very little difference in the rate of coagulation of litmus milk with the various organisms. Culture 25 when first isolated coagulated milk rather slowly, but after the second transfer in litmus milk the rate of coagulation was about the same as for the other cultures.

TABLE 1. *The production of total acid, volatile acid and acetylmethylcarbinol plus diacetyl in milk*

Cultures incubated at 37° C. for 7 days

Culture no.	Percent total acid	Volatile acid[1]	Mg. of Ni salt equiv. to amc + aa[2] per 200 gm.
1	2.02	39.0	trace
2	2.00	28.5	1.8
3	2.34	36.3	1.8
4	0.85	41.0	0.0
5	2.17	42.6	trace
6	2.10	35.7	1.2
7	2.31	31.8	0.0
8	1.50	34.5	trace
9	1.40	35.8	10.8
10	0.68	11.2	3.0
11	1.23	29.3	0.5
12	1.85	35.3	2.8
13	0.80	28.2	10.0
14	0.90	38.2	8.9
15	0.90	40.6	trace
16	1.11	28.6	1.3
17	0.96	27.3	0.0
18	1.21	16.9	trace
19	1.01	16.8	0.8
20	0.80	31.0	8.3
21	1.12	10.4	trace
22	1.34	30.9	1.5
23	0.90	22.4	0.0
24	0.80	21.5	2.1
25	0.50	10.0	trace
26	0.58	35.3	0.9
27	1.70	11.1	29.1
28	2.01	11.6	1.9
29	0.90	27.0	trace
30	1.19	27.5	0.0
31	2.05	36.0	11.8
32	1.80	29.4	17.4
33	1.50	27.8	0.0
34	1.90	29.1	0.0
35	2.10	28.9	5.5
36	0.80	21.4	1.6

[1] Volatile acid expressed as cc. N/10 NaOH required to neutralize the first liter of distillate obtained when a 250 gm. portion of culture was steam distilled after adding 15 cc. N/1 sulfuric acid.

[2] amc + aa = acetylmethylcarbinol + diacetyl.

All of the cultures produced an appreciable quantity of volatile acid. The value for the 36 cultures ranged from 10.0 to 42.6. A comparison of the production of total acid and of volatile acid is given in the following summary:

Cultures producing total acidities from	Produced volatile acidities from	Number of cultures
0.50 to 1.00	10.0 to 41.0	13
1.01 to 1.50	10.4 to 35.8	10
1.51 to 2.00	11.1 to 35.3	5
2.01 to 2.50	11.6 to 42.6	8

The summary shows that the production of volatile acid was not related to the total acid formed since the volatile acidities varied widely in each of the total acid groups and the minimum and maximum values for the different groups were much the same.

The yield of nickel salt equivalent to acetylmethylcarbinol plus diacetyl with the various cultures ranged from 0.0 to 29.1 mg., with 7 of the 36 cultures giving values of 0.0. Only five of the cultures yielded 10.0 or more mg. of nickel salt and only one yielded more than 20.0 mg. The following summary compares the production of total acid with the values for nickel salt equivalent to acetylmethylcarbinol plus diacetyl for the various cultures:

Cultures producing total acidities from	Yielded mg. of Ni salt from	Number of cultures
0.50 to 1.00	0.0 to 10.0	13
1.01 to 1.50	0.0 to 10.8	10
1.51 to 2.00	0.0 to 29.1	5
2.01 to 2.50	0.0 to 11.8	8

From the summary it is evident that the production of acetylmethylcarbinol plus diacetyl was not correlated with the formation of total acid. Each of the total acid groups contained organisms that did not yield acetylmethylcarbinol plus diacetyl and with three of the four groups, including those representing the highest and lowest acid producers, the maximum production by an organism in the group was essentially the same.

The results indicated that there was no direct relationship between the amounts of total acidity, volatile acidity, and acetylmethylcarbinol plus diacetyl formed by the cultures, regardless of the sources from which the organisms were isolated.

THE PRODUCTION OF VOLATILE ACID AND ACETYLMETHYLCARBINOL PLUS DIACETYL WHEN 0.15 PERCENT CITRIC ACID WAS ADDED TO THE MILK

The effect of the addition of citric acid on the production of volatile acid and acetylmethylcarbinol plus diacetyl in milk was studied with the 36 cultures by adding 0.15 percent citric acid to the milk at the time of

inoculation, incubating at 37° C. for 7 days and then determining the volatile acid and acetylmethylcarbinol plus diacetyl values. Table 2 gives the data obtained.

The addition of citric acid to the milk apparently had little effect on the production of volatile acid by the organisms. The values obtained

TABLE 2. *The production of volatile acid and acetylmethylcarbinol plus diacetyl when 0.15 percent citric acid was added to the milk*

Cultures incubated at 37° C. for 7 days

Culture no.	Milk alone[1]		Milk plus 0.15% citric acid	
	Volatile acid	Mg. of Ni salt equiv. to amc + aa per 200 gm.	Volatile acid	Mg. of Ni salt equiv. to amc + aa per 200 gm.
1	39.0	trace	35.0	11.1
2	28.5	1.8	19.3	trace
3	36.3	1.8	40.0	2.1
4	41.0	0.0	42.0	trace
5	42.6	trace	23.0	3.2
6	35.7	1.2	29.0	0.8
7	31.8	0.0	36.4	0.9
8	34.5	trace	37.1	1.9
9	35.8	10.8	12.2	2.0
10	11.2	0.0	15.1	1.0
11	29.3	0.5	23.5	0.9
12	35.3	2.8	30.4	trace
13	28.2	10.0	33.7	13.6
14	38.2	8.9	34.6	9.1
15	40.6	trace	44.5	2.1
16	28.6	1.3	27.3	8.0
17	27.3	0.0	30.2	1.5
18	16.9	trace	19.4	1.0
19	16.8	0.8	21.2	0.0
20	31.0	8.3	18.9	trace
21	10.4	trace	16.1	2.3
22	30.9	1.5	33.2	2.0
23	22.4	0.0	16.4	1.5
24	21.5	2.1	25.4	3.5
25	10.0	1.3	15.9	trace
26	35.3	0.9	31.4	1.1
27	11.1	29.1	21.2	1.8
28	11.6	1.9	13.5	2.1
29	27.0	trace	27.0	1.7
30	27.5	0.0	31.2	trace
31	36.0	11.8	39.0	9.3
32	29.4	17.4	37.2	21.2
33	27.8	0.0	19.8	trace
34	29.1	0.0	34.8	trace
35	28.9	5.5	29.5	3.5
36	21.4	1.6	28.4	2.2

[1] These data are taken from table 1.

with the citric acid added ranged from 12.2 to 44.5 while those in the controls ranged from 10.0 to 42.6. Twenty-two of the cultures gave higher volatile acidities with citric acid while 13 gave lower values and with 1 there was no difference; in general, the differences were not significant. The addition of 0.15 percent citric acid approximately doubles the citric acid content of the original milk; therefore, if citric acid is a source of volatile acid, the added citric acid should greatly increase the volatile acid formed.

The addition of citric acid had no significant effect on the production of acetylmethylcarbinol plus diacetyl by the organisms in milk. The values for the nickel salts obtained on the milk cultures with the citric acid added ranged from 0.0 to 29.1 mg. while those for the controls ranged from 0.0 to 21.1 mg. Twenty-six of the cultures gave higher values with citric acid added, while 10 gave lower values. There was considerable variation in the amounts of acetylmethylcarbinol plus diacetyl formed, both with and without citric acid added and, in general, the differences between the values for the milk with citric acid and without the acid were not significant.

It was interesting to note that some of the cultures which produced relatively small amounts of acetylmethylcarbinol plus diacetyl showed appreciable increases of these compounds when citric acid was added to the milk, while those cultures that produced comparatively large amounts of acetylmethylcarbinol plus diacetyl did not show large increases of these compounds.

THE PRODUCTION OF ACETYLMETHYLCARBINOL PLUS DIACETYL WHEN VARIOUS CONCENTRATIONS OF ACETALDEHYDE WERE ADDED TO THE MILK

The effect of adding various concentrations of acetaldehyde on the production of acetylmethylcarbinol plus diacetyl in milk was studied with 10 cultures of lactobacilli as follows: Five 100-cc. portions of sterile skim milk in bottles were inoculated with 0.5 cc. of an actively growing milk culture of an organism and incubated at 37° C. for 12 hours. Different concentrations of acetaldehyde were then added to four of the bottles and the remaining one was used as a control; the concentrations of acetaldehyde used were 0.05, 0.1, 0.3, and 0.4 percent. After incubating at 37° C. for 7 days, acetylmethylcarbinol plus diacetyl determinations were made on the various lots. The data obtained are given in table 3.

From the results, it appeared that the addition of various concentrations of acetaldehyde to the milk did not appreciably increase the production of acetylmethylcarbinol plus diacetyl. The values for nickel salt, equivalent to acetylmethylcarbinol plus diacetyl, varied widely both in the controls and in the milk to which the various concentrations of the acetaldehyde had been added; the values for the controls ranged from a trace to 23.2 mg., while those for the milk with acetaldehyde added ranged from 0.0 to 26.0 mg.

TABLE 3. *The influence of various concentrations of acetaldehyde on the production of acetylmethylcarbinol plus diacetyl in skim milk*
Cultures incubated at 37° C. for 7 days

Culture no.	Mg. Ni salt equiv. to amc + aa per 200 gm.				
	Concentration of acetaldehyde				
	Control	0.05%	0.1%	0.3%	0.4%
9	8.4	9.1	8.0	trace	0.0
10	2.8	5.5	6.2	3.1	0.0
11	0.7	1.8	0.9	trace	0.0
13	9.1	10.4	10.9	6.3	0.0
20	5.1	2.0	0.0	0.0	0.0
21	trace	3.6	4.0	0.0	0.0
27	23.2	25.1	26.0	21.2	0.0
28	2.3	2.5	8.4	3.9	0.0
31	12.4	11.2	12.9	8.4	0.0
32	16.1	19.5	20.7	19.2	0.0

With 0.05 percent acetaldehyde added the values for the nickel salt ranged from 1.8 to 25.1 mg. Eight of the cultures showed increases over the controls with the acetaldehyde added while two showed decreases, but in no instance was the difference great.

With 0.1 percent acetaldehyde added to the milk the nickel salt values ranged from 0.0 to 26.0 mg. Eight of the organisms gave increases, as compared with the controls, in acetylmethylcarbinol plus diacetyl with the aldehyde added while two gave decreases, but in no instance was the difference great. One culture failed to produce any acetylmethylcarbinol plus diacetyl with acetaldehyde while a considerable quantity was produced in the control.

With 0.3 percent acetaldehyde added to the milk the acetylmethylcarbinol plus diacetyl values ranged from 0.0 to 21.2 mg. nickel salt. In three instances there were increased amounts formed with the acetaldehyde while in seven there were decreases. Four of the organisms produced no more than a trace of acetylmethylcarbinol plus diacetyl with the aldehyde added. In general, the results obtained suggest that the acetaldehyde was slightly toxic in the concentration used.

With 0.4 percent acetaldehyde the milk failed to coagulate and none of the cultures produced any acetylmethylcarbinol plus diacetyl. This indicates that 0.4 percent acetaldehyde in milk was definitely toxic to the organisms used.

From the results obtained it was evident that concentrations of 0.05 or 0.1 percent acetaldehyde produced slight increases in acetylmethylcarbinol plus diacetyl in milk in many instances, while in several instances small decreases were noted but in every case the difference was small. The addition of 0.3 or 0.4 percent acetaldehyde to the milk appeared to be toxic for the organisms used.

CONCLUSIONS

1. Appreciable quantities of total acid, volatile acid and acetylmethylcarbinol plus diacetyl are formed in skim milk by most of the lactobacilli; the amounts of these materials produced by the different cultures vary greatly and there is no close correlation between the amount of total acid and the amounts of volatile acid or acetylmethylcarbinol plus diacetyl produced by a culture.

2. The addition of citric acid to milk has no significant effect on the amounts of volatile acid and acetylmethylcarbinol plus diacetyl formed by the organisms.

3. The addition of acetaldehyde does not significantly increase the production of acetylmethylcarbinol plus diacetyl by the organisms in milk. Low concentrations of the acetaldehyde result in slight increases in the amounts of acetylmethylcarbinol plus diacetyl produced while the higher concentrations appear to be definitely toxic.

LITERATURE CITED

1. MICHAELIAN, M. B., R. S. FARMER AND B. W. HAMMER
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