OBSERVATIONS ON BACILLUS COAGULANS[1]

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Bacillus coagulans was described at the Iowa Agricultural Experiment Station (Hammer (1915)) as the cause of an outbreak of coagulation in evaporated milk packed by an Iowa condensery.

Cordes (1928) found *B. coagulans* responsible for an outbreak of "flat-sours" in evaporated milk. The cans from which the organism was secured had been subjected to 114.4°C. (238°F.) for twenty minutes in a batch sterilizer. However, milk cultures of the organism were killed when exposed in an autoclave to a temperature of 111.7° to 112.8°C. (233° to 235°F.) for fifteen minutes, the milk itself reaching 112.2°C. (234°F.), being between 100° and 112.2°C. (212° and 234°F.) for six minutes, and between 107.2° and 112.2°C. (225° and 234°F.) for four minutes.

B. coagulans was recently found to be the cause of an outbreak of coagulation in evaporated milk packed by a condensery in a neighboring state. A number of the cultures isolated were studied in order to (1) check and enlarge the description of the organism, and (2) secure additional information on the changes it produces in evaporated milk.

GENERAL CHARACTERS OF OUTBREAKS OF COAGULATION IN EVAPORATED MILK DUE TO B. COAGULANS

In 1915 an outbreak of coagulation in evaporated milk occurred in an Iowa condensery and extended over a period of several months, with the May and June milk showing the highest percentage of spoilage. *B. coagulans* was isolated from the spoiled milk. At the time coagulation was first encountered the heat

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treatments used varied with the milk, but were approximately 110° to 111.7°C. (230° to 233°F) for from thirty to thirty-six minutes. The exposures were increased, and although the percentage of spoilage decreased, the loss was still sufficient to be of considerable consequence. It was reasonably certain that some of the milk which spoiled was heated to 113.3°C. (236°F.) for thirty-six minutes. The milk which spoiled had been held at the condensery for at least ten days before it was sent out, and was then apparently normal, so coagulation did not occur rapidly. Most of the abnormal cans were firmly curdled, but a few showed a soft, flaky curd with considerable whey. The spoiled milk had a sweetish, cheesy odor, resembling to some extent the odor of Swiss cheese, and a sour, cheesy flavor. The odor and flavor were not disagreeable and there was no suggestion of putrefaction. Acid determinations on milk that coagulated during the outbreak, and on milk that coagulated following the inoculation of a pure culture of the organism, showed that the abnormal condition was accompanied by a considerable increase in acidity.

The outbreak supplying the spoiled evaporated milk from which *B. coagulans* was recently isolated occurred during the abnormally hot, dry weather of the summer of 1930. The outreak extended over a period of several weeks. The percentage of cans that spoiled varied greatly from one batch to another and, in one instance, was estimated by the plant managers to be as high as 60 per cent. The typical spoiled milk was firmly curdled, but in some cans the coagulation was less complete, due presumably to a less extensive growth of the organisms. The odor of the spoiled milk was distinctly cheesy, with a definite suggestion of acidity, and the flavor was sour and cheesy. At the condensery experiencing the difficulty it was noted that the acidity of the normal milk was 0.48 per cent while that of the spoiled milk was 1.05 per cent, calculated as lactic acid. Although the odor and flavor of the spoiled milk were decidedly abnormal, they were not suggestive of putrefaction; in a hard cheese they would have been quite acceptable.

The spoilage began with batches of milk subjected to a heat treatment which had been satisfactory over a long period. With

the appearance of the defect the temperature was increased somewhat, but coagulation of the milk continued. The plant reported considerable spoilage with one batch which was run through a standard heater for twelve minutes up to 97.8°C. (208°F.), a variable heater for twelve minutes at 97.8°C. (208°F.), and a cooker for fifteen minutes at 117.8°C. (244°F.).

EXPERIMENTAL

The cultures studied. As a rule the coagulated evaporated milk contained a sufficient number of viable organisms so that direct plating on beef-infusion agar gave a distribution of colonies satisfactory for isolation. With one rather old sample it was necessary to inoculate a beef-infusion agar slant with a small amount of the coagulated milk and then prepare plates from the growth developing on the surface of the slant. All of the cultures secured by these procedures were replated to insure their purity.

The observations made involved nine cultures isolated from seven lots of spoiled milk; cultures 1, 2, 3, 4, 5, and 9 were all from different lots while cultures 6, 7, and 8 were from the same lot. Eight of the cultures were isolated about one month after the milk had coagulated while one (culture 9) was isolated about five months after the milk had coagulated.

A detailed study of the morphology, cultural characteristics, and biochemical features of the nine cultures confirmed the original description of the organism. This description, enlarged by recent findings, is as follows.

DESCRIPTION OF BACILLUS COAGULANS

Morphology

Form and size. Rods; 0.5 to 0.7 by 1.6 to 7.1 microns when grown on beef-infusion agar (twenty-four hours at 37°C.); somewhat smaller when grown in milk (forty-eight hours at 37°C.).

Arrangement. Singly and in short chains.

Motility. Motile; flagellation peritrichous.

Staining reactions. Gram-positive in young cultures, often with distinct granulation; commonly Gram-negative in old cultures although a few Gram-positive cells sometimes persisted.

Spore formation. In old beef-infusion agar slant cultures and in coagulated evaporated milk some cells contained spores. Preparations made from agar or milk cultures grown under various incubation conditions regularly showed spores in less than half of the cells. The spores were small, round, did not bulge the cells and were sub-terminal.

Cultural characteristics

Agar slant. Beef-infusion and whey agars showed abundant, echinulate, white, non-viscid, shiny growth after two to three days at 37°C. Growth less abundant on standard agar.

Agar stab. Beef-infusion and whey agars showed heavy, white, non-viscid, surface growth with some growth along the line of inoculation after two to three days at 37°C.

Agar colony. After two to three days at 37°C. surface colonies on beef-infusion agar were shiny, white, non-viscid, round, about 1 to 2 mm. in diameter, with entire edge. Sub-surface colonies were round to oval, white, non-viscid, and smaller than the surface colonies.

Gelatin stab. On whey gelatin at 37°C. growth occurred; gelatin not liquefied.

Broth. Turbidity with sediment.

Potato. Dirty white, shiny, non-viscid, spreading growth.

Litmus milk. Litmus milk was reduced. Reduction was followed by coagulation and appearance of red band at top of milk. Red band increased in depth, curd contracted, expressing small amount of whey. No apparent proteolysis. Coagulation in four to eight days at 37°C. and in two to four days at 50°C.

Biochemical features

Indol. Not produced.

Nitrates. Not reduced.

Action on carbohydrates and alcohols. Glycerol, glucose, levulose, galactose, lactose, maltose, sucrose, salicin, raffinose, dextrin, and soluble starch fermented with the production of acid but no gas; arabinose, adonitol, dulcitol, inositol, mannitol, sorbitol, and inulin not fermented; starch hydrolyzed.

Oxygen relationship. Organism facultative; grew well aerobically.

Growth temperatures. Grew well between 37° and 55°C.; poorly, if at all, at 20°C.

Numbers of organisms in the spoiled evaporated milk. a. Milk which coagulated at the condensery. Several cans of evaporated milk, which had been processed during August, 1930, were sent to the laboratory for examination. Upon their arrival, October 15, 1930, they were placed in the cooler at about 7°C. and held there until plated on November 8.

After thorough shaking, 1 ml. of the contents of each can was removed, and dilutions plated on beef-infusion agar; the plates were incubated four days at 37°C. The results obtained are given in table 1.

The plate counts on the milk held for considerable periods after coagulation at the condensery varied from 7,000 to 77,000 per

TABLE 1

Plate counts on evaporated milk which coagulated at the condensery

DATE PROCESSED (AUGUST, 1930)	AGE WHEN PLATED	PLATE COUNT PER MILLILITER
	days	
7	93	23,000
7	93	22,000
7	93	77,000
7	93	31,000
20	80	58,000
22	78	7,000
25	75	11,000

milliliter and thus indicate that there should be little difficulty in securing *B. coagulans* from spoiled milk, even if the milk is not examined soon after spoilage occurs.

b. Milk which coagulated following inoculation with B. coagulans. Cans of normal evaporated milk were inoculated with *B. coagulans* and incubated at 37° or 50°C. for varying periods of time. Beef-infusion agar cultures grown at 37°C. for forty-eight hours were used for inoculation. This was carried out by covering a small area on an end of a can with concentrated HCl, flooding the area with solder, punching a hole in the center of the area with a nail which had been heated to redness and cooled, and then adding the organisms with a needle; after inoculation the can was imme-

diately resealed with solder and shaken thoroughly to distribute the organisms. The controls were punched and resealed, while unopened cans were also used in order to check the sterility of the original milk. After incubation under varying conditions plate counts were made on the coagulated milk, using beef infusion

TABLE 2

Plate counts on evaporated milk which coagulated following inoculation with B. coagulans

CULTURE USED TO INOCULATE	TEMPERATURE OF INCUBATION	TIME TO COAGULATE	PERIOD OF INCUBATION	PLATE COUNT PER MILLILITER
	°C.	days	days	
1	37	7	8	41,000,000
1	37	4	6	100,000,000
2	37	8	8	51,000,000
2	37	5	18	220,000
3	37	8	8	44,000,000
3	37	5	50	20,000
4	37	10	10	36,000,000
5	37	10	10	33,000,000
5	37	10	14	21,000,000
5	37	7	21	220,000*
5	50	4	50	300
6	37	10	10	33,000,000
6	37	8	14	29,000,000
6	37	7	39	110,000
6	50	4	39	21,000
7	37	6	18	12,000,000
7	50	3	50	400
8	37	7	21	200,000†
8	50	4	39	25,000

* Stored twenty-nine days at room temperature following period of incubation.

† Stored eighteen days at room temperature following period of incubation.

agar and an incubation of four days at 37°C. The data secured are given in table 2.

The results indicate that at 37°C. the number of organisms present soon after the milk coagulated was large (up to 100,000,000 per milliliter), and that continued incubation resulted in a decrease in the count. None of the cans incubated at 50°C. were plated soon after coagulation, but following the long incuba-

tion period the numbers were comparatively small. In general, the results indicate that there should be little difficulty in isolating *B. coagulans* from spoiled milk even if the examination is delayed. The time required for the inoculated milk to coagulate firmly varied from four to ten days at 37°C. and from three to four days at 50°C.

Total and volatile acidities of the spoiled evaporated milk. The total and volatile acidities were studied on a number of samples of

TABLE 3

Total and volatile acidities of evaporated milk which coagulated at the condensery

Date processed (August, 1930)	Treatment of cans: Period of holding, At condensery	At laboratory		Total acid as lactic	Volatile acid: 0.1 N NaOH required to neutralize 1,000 ml. steam distillate from 250 grams milk
	days	*days*	*°C.*	*per cent*	*ml.*
7	60	33	7	1.06	33
7	60	33	7	1.06	33
7	60	33	7	1.03	35
7	60	33	7	1.03	27
20	47	33	7	0.90	22
22	45	33	7	0.97	37
25	42	33	7	1.06	24
27	154	41	21	0.92	38
27	154	41	21	0.94	40
27	154	41	21	0.85	28
27	154	41	21	0.97	
27	154	41	21	0.48*	
27	154	41	21	0.49*	6
27	154	41	21	0.43*	
27	154	41	21	0.42*	
27	154	41	21	0.42*	

* Normal milk.

the spoiled evaporated milk. The total acidity was determined by titrating a 20-gram sample with 0.1 N NaOH, using phenolphthalein as an indicator, and calculating the acidity as lactic acid. The volatile acidity was determined by steam distilling a 250-gram sample of the milk after acidifying with 15 ml. N H_2SO_4; 1 liter of distillate was collected, a 100 ml. aliquot titrated with 0.1 N NaOH using phenolphthalein as an indicator, and the milli-

liters of 0.1 N NaOH that would have been required for the liter of distillate then calculated. The results obtained on milk which spoiled at the condensery are given in table 3, and those obtained on milk inoculated with *B. coagulans* in table 4.

TABLE 4

Total and volatile acidities of evaporated milk which coagulated following inoculation with B. coagulans

CULTURE USED TO INOCULATE	TEMPERATURE OF INCUBATION	TIME TO COAGULATE	PERIOD OF INCUBATION	TOTAL ACID AS LACTIC	VOLATILE ACID: 0.1 N NaOH REQUIRED TO NEUTRALIZE 1,000 ML. STEAM DISTILLATE FROM 250 GRAMS MILK
	°C.	*days*	*days*	*per cent*	*ml.*
1	37	4	6	1.06	
1	37	4	31	1.07	22
1	37	7	8	1.30	25
2	37	3	31	1.23	16
2	37	8	8	0.97	22
3	37	5	50	1.23	
3	37	4	31	1.28	18
3	37	8	8	1.05	28
4	37	4	31	1.15	19
4	37	10	10	0.95	23
5	37	10	14	0.86	
5	37	7	20	1.13	
5	37	10	10	0.87	20
5	50	4	50	0.95	
6	37	8	14	0.94	
6	37	10	10	0.93	21
7	50	3	50	0.98	
Unopened control.	37		10	0.49	
Unopened control.	37		31	0.43	
Unopened control.	37		50	0.48	
Opened and resealed control...	37		31	0.45	

The data in table 3 show that spoilage was accompanied by a large increase in total acidity, the abnormal milk showing acidities from 0.85 to 1.06 per cent while the normal product showed acidities from 0.42 to 0.49 per cent. The volatile acidities were also greatly increased, showing values from 22 to 40 while the

one determination on a normal product gave a value of 6. The results in table 4 are in general agreement with those in table 3, the total acidities of the spoiled milk ranging from 0.86 to 1.30 per cent and of the normal milk from 0.43 to 0.49 per cent while the volatile acidities of the spoiled milk ranged from 16 to 28. The inoculated cans held at 37°C. required from three to ten days to coagulate while the two cans held at 50°C. required three and four days, respectively.

The non-volatile acid in the spoiled evaporated milk. The results reported on total and volatile acidities in the spoiled evaporated

TABLE 5

Data on zinc salts prepared from the non-volatile acid in the spoiled evaporated milk

SAMPLE	WATER OF CRYSTALLIZATION IN ZINC SALT			ZnO IN WATER-FREE ZINC SALT	ROTATION OF ZINC SALT
	Determination A	Determination B	Average		
	per cent	*per cent*	*per cent*	*per cent*	
1	13.05	12.97	13.01	33.18	*l*
2	13.68	13.47	13.575	33.18	*l*
3	13.49	13.41	13.45	33.15	*l*
4	12.90	13.00	12.95	33.33	*l*
5	13.43	13.56	13.495	33.57	*l*
Theoretical for optically active zinc lactate.....			12.88	33.46	

milk indicate that a large percentage of the acid is non-volatile. Zinc salts were accordingly prepared from the residues remaining after the steam distillations, using the method outlined by Hammer (1920). The results obtained in the study of these salts are given in table 5; samples 1, 2 and 3 were from milk which coagulated at the condensery while 4 and 5 were from milk which coagulated following inoculation.

The percentages of ZnO in the zinc salts agree with the theoretical value for zinc lactate, and the percentages of water of crystallization indicate that the lactic acid was optically active; the rotation of the zinc salt was *l* showing that the rotation of the free

acid was *d*. The organism evidently produced lactic acid of the *d* type.

The volatile acid in the spoiled evaporated milk. The volatile acid present in the spoiled evaporated milk was investigated by the preparation and study of barium salts according to the methods used by Hammer and Sherwood (1923). The results obtained are given in table 6; samples 1 and 2 were from milk which coagulated at the condensery while sample 3 was from milk which coagulated following inoculation.

The percentages of barium in the barium salts suggest a mixture of acetic and propionic acids while the Duclaux values indicate largely acetic acid.

TABLE 6

Data on volatile acid in the spoiled evaporated milk

SAMPLE NUMBER	Ba IN BARIUM SALT			RESULT OF DUCLAUX DETERMINATION
	Determination A	Determination B	Average	
	per cent	*per cent*	*per cent*	
1	50.24	50.08	50.16	Largely acetic acid
2	50.01		50.01	Largely acetic acid
3	50.90		50.90	Largely acetic acid
Theoretical for Ba acetate.....			53.78	
Theoretical for Ba propionate..			48.46	

Proteolysis by B. coagulans. Although evaporated milk coagulated by *B. coagulans* does not show any evidence of being proteolyzed, members of the genus Bacillus often bring about protein decomposition in milk and, accordingly, a study was made of the soluble and amino-nitrogen contents of skim and evaporated milk coagulated by *B. coagulans*. A filtrate was secured from the milk by the method outlined by Hammer and Patil (1930); the soluble nitrogen was determined by the Kjeldahl method, and the amino nitrogen by the Van Slyke method. The results obtained are given in table 7.

The data show that in inoculated skim milk and in evaporated

milk which had coagulated at the condensery there was an increase in both the soluble nitrogen and the amino nitrogen. In the skim milk the soluble nitrogen after growth of the organism was roughly twice that in the uninoculated milk while the amino nitrogen was roughly three times that in the uninoculated milk. In the evaporated milk which spoiled at the condensery the soluble nitrogen was almost twice that in the normal milk and the amino nitrogen more than twice that in the normal milk. The odor and flavor did not suggest putrefaction with either the skim milk or the evaporated milk.

TABLE 7

Proteolysis by B. coagulans

MATERIAL EXAMINED	SOLUBLE NITROGEN		AMINO NITROGEN	
	Per 10 ml. of filtrate	Increase over control	Per 10 ml. of filtrate	Increase over control
	mgm.	*mgm.*	*mgm.*	*mgm.*
Skim milk inoculated with:				
Culture 1	13.457	6.205	1.206	0.768
Culture 2	13.345	6.093	1.178	0.740
Culture 3	14.660	7.408	1.233	0.795
Culture 4	13.120	5.868	1.260	0.822
Culture 5	13.429	6.177	1.096	0.658
Culture 6	13.820	6.568	1.123	0.685
Uninoculated control	7.252		0.438	
Can of evaporated milk coagulated at the condensery	24.330	10.090	2.014	1.103
Can of normal evaporated milk from same lot	14.240		0.911	

Composition of gas from cans of spoiled evaporated milk. The composition of the gas from cans of spoiled evaporated milk was studied by collecting the gas over water and subjecting it to the usual absorption materials. The results secured on milk which coagulated following inoculation with *B. coagulans* and on milk which coagulated at the condensery are shown in table 8.

Oxygen was absent from the cans (except for the small amount in the control that had been opened and resealed) whether the milk had spoiled or was normal. This indicates that in normal

milk the oxygen combines with the milk constituents. The coagulation of the milk was regularly accompanied by a large increase in the amount of carbon dioxide whether spoilage followed

TABLE 8

Gas content of cans of evaporated milk coagulated by B. coagulans

MATERIAL EXAMINED	TIME TO COAGULATE	PERIOD OF INCUBATION AT 37°C.	PERIOD OF HOLDING AT ROOM TEMPERATURE FOLLOWING INCUBATION	VOLUME OF GAS	OXYGEN		CARBON DIOXIDE		NITROGEN	
	days	*days*	*days*	*ml.*	*ml.*	*per cent of total gas*	*ml.*	*per cent of total gas*	*ml.*	*per cent of total gas*
Can of evaporated milk inoculated with:										
Culture 1	4	30	45	35.0			4.9	14.0	30.1	86.0
Culture 2	3	30	45	41.1			6.4	15.6	34.5	84.4
Culture 3	4	30	45	41.6			5.8	13.9	35.8	86.1
Culture 4	4	30	45	43.4			7.0	16.1	36.4	83.9
Unopened control		30	45	23.0			0.3	0.1	22.9	99.9
Opened and resealed control		30	45	34.1	0.4	0.1	0.5	0.1	33.2	99.8
Cans of evaporated milk which coagulated at the condensery:										
A			180	46.4			7.2	15.5	39.2	84.5
B			180	47.0			7.4	15.7	39.6	84.3
C			180	40.6			3.4	8.4	37.2	91.6
Cans of normal evaporated milk from same lot as cans A, B and C:										
D			180	37.6			0.6	0.2	37.0	99.8
E			180	41.4			0.6	0.2	40.8	99.8
F			180	41.0			0.6	0.2	40.4	99.8

inoculation or occurred naturally. In the spoiled cans carbon dioxide generally made up about 15 per cent of the gas. In one can which had spoiled at the condensery, approximately half the

usual amount of carbon dioxide was found; the total acidity of the milk from this can was also definitely lower than usual.

It should be emphasized that *B. coagulans* does not produce a bulging of cans of evaporated milk in which it grows.

Influence of temperature on the rate of coagulation of evaporated milk inoculated with B. coagulans. Tables 2, 4 and 8 present data on the time required at 37° and at 50°C. for the coagulation of cans of evaporated milk inoculated with *B. coagulans.* Thirty-four cans held at 37°C. coagulated in from three to ten days (average 6.7 days) while six cans held at 50°C. coagulated in from three to four days (average 3.7 days). Six inoculated cans of milk held at 21°C., and two cans held at about 7°C. failed to coagulate during a period of four months. These results show the great influence of temperature on the growth of *B. coagulans*; they suggest that wide variations are to be expected in the time required for spoilage of milk containing *B. coagulans,* and that milk containing this organism may fail to show spoilage if the holding temperature is comparatively low.

SUMMARY

B. coagulans was isolated from an outbreak of coagulated evaporated milk that was similar to the one which yielded the original culture of this organism. The description of the organism was checked and enlarged.

Rather large numbers of organisms were found in coagulated evaporated milk that had been stored for varying periods at different temperatures. The great viability of the organism makes its isolation from such milk comparatively easy.

The total and volatile acidities of coagulated evaporated milk were much higher than those of normal evaporated milk. The non-volatile acid was *d* lactic acid while the volatile acid was apparently made up of acetic and propionic acids.

Although evaporated milk coagulated by *B. coagulans* does not show any evidence of proteolysis, this organism greatly increased both the soluble and amino nitrogen in skim milk and in evaporated milk. The coagulated milk did not have an odor or flavor suggesting putrefaction.

No free oxygen was found in the gas from cans of normal or coagulated evaporated milk. One can, which was opened and then resealed without being inoculated, was found to contain a small amount of oxygen. The carbon dioxide content of the gas from cans of coagulated milk was found to be about 15 per cent while that of normal milk was much lower.

Temperature had a marked influence on the rate of coagulation of evaporated milk inoculated with *B. coagulans*.

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