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THE INHIBITORY ACTION OF VARIOUS
ORGANIC ACIDS ON SOME FOOD-
POISONING ORGANISMS WHEN TESTED
IN SOFT CUSTARD

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Joseph A. Stevens

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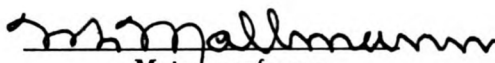
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THE INHIBITORY ACTION OF VARIOUS ORGANIC
ACIDS ON SOME FOOD-POISONING ORGANISMS
WHEN TESTED IN SOFT CUSTARD

By
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INTRODUCTION

Aside from the intoxication known as botulism, the majority of cases of food poisoning that come to the attention of the public is caused by the enterotoxic substance produced by micrococci, or is infection by certain members of the salmonella group. Of these cases, the foods which we have most frequently observed containing the causative agent are pies and pastry, especially those containing custard (16). The basic custard formula is used to produce such desserts as puddings and pies, and is used as a filling for cream puffs and eclairs.

The causative organisms can enter the custard from a number of sources: the milk (16, 23, 31), eggs (9), or sugar used to make the custard, the water supply (16) used to wash the utensils, the persons involved in its preparation (24), or the air.

Those strains of Micrococcus pyogenes, var. aureus and albus which produce the enterotoxin that causes food poisoning symptoms may be found in abundance in nature. In man they are carried in the throat (24), and are frequently found in the postnasal discharge following common colds, and can be isolated from pimples, carbuncles, boils, and other infections.

The percentage of the total number of strains of micrococci which are capable of producing enterotoxin is not known because

of testing difficulties. However, the number of strains of micrococci which produce enterotoxin is small as compared to the total number (12).

The symptoms of micrococcal food poisoning usually appear about three hours after the individual has partaken of the food containing the enterotoxin. The first symptom to appear is salivation, followed by nausea, vomiting, retching, abdominal cramping and diarrhea. All of these symptoms need not appear in order to confirm the presence of enterotoxin. Micrococcal food poisoning in otherwise normal individuals is usually not fatal, although a few fatal cases have occurred in which inadequate treatment was given.

Many different types of foods have been involved in micrococcal-type food poisoning. Kelly and Dack (23) mention outbreaks owing to contamination of cheese, cream-filled cake, chicken gravy, cream-filled éclairs (7) and tarts, custard-filled doughnuts, chocolate éclairs, milk (8), and tongue sandwiches. Ice cream (24), pineapple pudding (14), and bread pudding (28), also have been incriminated.

In upstate New York from 1935 to 1939, 17 outbreaks (that gave rise to 1246 cases) (7) of micrococcal food poisoning were reported that were due to contaminated cream-filled bakery goods.

The species of Salmonella most often responsible for producing food poisoning infections in man are S. paratyphi, S. schottmuelieri, S. typhimurium, S. choleraesuis, and S. enteritidis.

Although contaminated meat is most often incriminated (11), many Salmonella infection outbreaks have been due to foods other than meat (2, 28, 33, 34). Milk and milk products may become contaminated from a diseased cow or subsequently upon handling. The pasteurization process is effective in destroying salmonella organisms in such media.

Rodents (36), houseflies (26), and human carriers are the vectors by which salmonella organisms may gain entrance to foods such as custards.

The symptoms of Salmonella gastrointestinal infections are characterized by nausea, vomiting, abdominal pain and diarrhea. The symptoms usually appear 12 to 24 hours after the food is ingested.

At the present writing, the evidence points to the view that Salmonella food poisoning is actually an infection and that toxins or toxic substances play no role (10).

HISTORY

This writer's interest in the problem of diminishing the number of possibilities of obtaining food poisoning or food infections by ingesting contaminated soft custard pastries was stimulated by a suggestion from Dr. W. L. Mallmann. This suggestion was based on a bakery's claim in an advertisement in a Chicago newspaper that it had perfected a sugar cream filling for pies which inhibited the growth of all food poisoning or food infection type bacteria. The filling had the following formula:

10 lbs.	water
5 lbs.	granulated sugar
3 oz.	pure vanilla
1 oz.	salt
1 lb.	cereal starch
4 lbs. 4 oz.	whipped, fresh-frozen egg whites

It was decided to prepare this pie filling, sterilize it, and inoculate it with organisms of the type most frequently found (15, 21) in food poisoning or food infection cases. Therefore, one micrococcus and two salmonella species were used.

On hand in our laboratories was a known enterotoxin producing strain (Brail) of Micrococcus pyogenes, var. aureus which was used as the food poisoning type test organism. Cultures of Salmonella typhimurium and Salmonella schottmuelleri were used as the food infecting type test organisms.

None of the constituents of the cream filling formula was ever known to be of an inhibitory nature to microorganisms. Furthermore, the presence of the egg whites would supply the nitrogen requirements which Surgalla and Hite (35) found to be necessary for enterotoxin production by micrococci.

After 24 hours incubation at 37 C, 5 ml of this cream filling, inoculated with M. pyogenes, var. aureus, str. Brail, was swallowed rather hesitantly by this writer. Within 100 minutes, the bakery's advertisement was disproved. A headache, followed by a violent case of vomiting, and a severe case of diarrhea of six hours duration, firmly convinced this writer that the cream filling did support bacterial growth.

Since this type of pie filling did not inhibit microbiological growth, it was decided to investigate certain substances which might do so.

It followed, logically, to look into the use of various organic acids whose bacteriostatic properties (or those of their salts) were known; and whose previous use in foods was allowed by the Federal Food and Drug Act. It was also decided to use the sodium salt of dehydroacetic acid, which has only recently been developed (6). This acid and its salts have been used as mycostatic agents in bread and dairy products (3, 38).

Dehydroacetic acid* (3-acetyl-6-methyl-1,2-pyran 2,4 (3)-dione) and its sodium salt are white crystalline powders.

*Chemicals kindly supplied by the Dow Chemical Company, Midland, Michigan.

They have been designated as DHA and DHA-S, respectively, by the Dow Chemical Company. DHA, molecular weight 168.1, is only slightly soluble in water (0.1 g per 100 g water at 25 C), very soluble in organic solvents, and moderately soluble in oils and waxes. DHA-S, molecular weight 208.1, is very soluble in water, and only slightly soluble in organic solvents. These compounds were found to be stable when heated to 120-130 C for one hour (38, 40), and are odorless and tasteless in all concentrations suitable for use.

Extensive toxicological and pharmacological work has been done on DHA and DHA-S by Spencer (32), Seevers (29), Shideman (30) and Woods (39), et al. The majority of the work was done on animals, little on humans. The results of their work indicated that DHA and DHA-S would only be toxic when used in concentrations much higher than those necessary to produce stasis. Because of the solubility factor, only DHA-S was tested.

These chemicals were to be placed into soft custard in graded amounts, and their effectiveness noted.

It was decided to employ an adaption of the plate count method to observe the effect of the chemicals tested, as there is little or no agreement (17, 20) in the literature on the effectiveness of the kitten test as a means of determining the presence of enterotoxin in food. Further disagreement in the literature on the production of antibodies (19, 25) and the use of a serological test (1, 4, 5) induced this writer to

seek the more simplified plate count method to determine the presence or inhibition of microorganisms. Additional factors which prompted the use of the plate count method were the difficulty in feeding a definite amount of custard to kittens, and the amount of work in purifying the enterotoxin (13, 22, 37) and subsequent antibody production.

EXPERIMENTAL

The organic acid stock solutions were made by dissolving one equivalent weight of the acid in one liter of distilled water to produce a 1 N solution.

<u>Acid</u>	<u>Molecular weight</u>	<u>Equivalent weight</u>
Acetic	60.5	60.5
Citric	192.0	64.0
Lactic	90.08	90.08
Maleic	116.03	58.015
Malic	134.05	67.025
Propionic	74.08	74.08
Succinic	118.05	59.025
Tartaric	150.09	75.04

The DHA-S stock solution was a five percent solution, prepared by dissolving 0.60 gram DHA-S in 12.0 ml of distilled water. These solutions were autoclaved at 15 pounds pressure for 20 minutes.

The broth medium employed in the primary work was a one percent solution of dehydrated Bacto-peptone (commercially produced by the Difco Laboratories).

The soft custard employed in the second phase of the work was made up in multiples of the following formula:

Milk	244 grams
Eggs	48 grams
Sugar	25 grams

The soft custard medium was always freshly prepared.

Both the peptone medium and the soft custard were dispensed into a series of ten test tubes in portions of exactly 5 ml. In the cases where one of the organic acids was to be tested, the next step was to add exactly 5 ml of the 1 N acid solution into the first test tube in the series. The acid and the medium were then thoroughly mixed. (This procedure was standardized by aspirating the mixture into a pipette and then discharging it back into the test tube. This cycle was repeated five times to assure complete mixing.) The second of ten serial dilutions was then made by withdrawing 5 ml of the mixture from the first test tube and mixing it in the prescribed manner with the 5 ml of medium contained in the second test tube. Eight further serial dilutions were prepared in like manner.

In the case of DHA-S, the test tubes were prepared as follows:

<u>Medium</u>	<u>5% stock solution of DHA-S</u>	<u>Final concentration of DHA-S in medium</u>
3.9 ml	1.1 ml	1.1 %
4.1 ml	0.9 ml	0.9 %
4.3 ml	0.7 ml	0.7 %
4.5 ml	0.5 ml	0.5 %
4.7 ml	0.3 ml	0.3 %

Where the peptone medium was involved, sterilization was accomplished by autoclaving the test tubes containing the peptone medium and the organic acids or DHA-S at 15 pounds pressure for 15 minutes.

Where the soft custard medium was involved, sterilization was accomplished by the process known as inspissation, which

is usually employed in autoclaving media that contain eggs. This is done in the following manner: the autoclave is preheated, then loaded with the test tubes containing the soft custard medium and the organic acids or DHA-S. The door is closed, and all the outlet valves are closed tightly. The stem inlet valve is opened just a crack. The steam is allowed to enter the chamber slowly so that the pressure does not exceed 5 pounds in 15 minutes. At this rate, at the end of a 45-minute period, the pressure within the autoclave should be 15 pounds. This pressure is then maintained for a 20-minute period, at the end of which time the steam valve is closed, and the pressure within the autoclave is allowed to return to normal. When this procedure is followed carefully, the medium will appear as a firm custard, with no bubbles or frothiness. Because of the possibility of air pockets occurring in the chamber, sterility tests were made, employing test tubes which contained only 5 ml of the soft custard medium.

After the media were sterilized, they were allowed to cool to room temperature and inoculated. The inoculum used was the third of three successive 24-hour cultures of either Micrococcus pyogenes, var. aureus, Salmonella typhimurium, or Salmonella schottmuelleri, grown in 10 ml of peptone broth.

In the cases where the peptone medium was involved, the size of the inoculum was standardized by using one loopful of the organisms suspended in the peptone broth.

In the cases where the soft custard medium was involved, a straight wire was used as the inoculating needle. For standardization purposes a single stab into the soft custard medium was made after dipping the inoculating needle into the 24-hour old organisms suspended in peptone broth.

The inoculated test tubes were then incubated at 37 C for 24 hours.

Three groups of controls were set up, when using the soft custard medium, as follows:

Group I - the medium was inoculated, then immediately plated, i.e., an endeavor was made to determine the average number of organisms inoculated into each test tube.

Group II - the medium was inoculated, then incubated for 24 hours at 37 C, and plated. This would demonstrate whether the organism had grown in the medium.

Group III - controls were checks on the sterility of the medium. Number 1 consisted of 5 ml of soft custard plated immediately after inspissation. Number 2 consisted of 5 ml of soft custard that was incubated after it had been inspissated, at 37 C for 24 hours, then plated. Should either #1 or #2 show any bacterial growth, it would serve as an indication that the inspissation process had not served its purpose of sterilization.

At the end of the incubation period, the growth in the tubes was observed. In the cases of the test tubes containing peptone broth, this was done by holding the test tube up to a light source and classifying the growth according to the turbidity present, the range being - (negative indicating no apparent growth) to +4 (very turbid). Prior to reading the test tubes, they were all rotated in a manner that would re-suspend any cells that had settled to the bottom of the test tubes, or any that grew in the manner of a pellicle on the surface of the peptone broth.

In the cases of the test tubes containing soft custard, the plate count method was employed. This was accomplished in the following manner: 5 ml of sterilized peptone broth was added aseptically to each test tube. Then the soft custard medium was broken up into small pieces, using a sterile 5 ml pipette. The soft custard medium and the peptone broth were more or less homogenized by aspirating the mixture into the 5 ml pipette, and then discharging it back into the test tube. This cycle was repeated five times to assure uniformity of the suspension.

One ml of this suspension was then placed into a water dilution blank bottle containing 99 ml of sterile distilled water, and shaken 25 times. This produced a 1:100 dilution. Ten ml of this 1:100 dilution was added to a second water dilution blank bottle containing 90 ml of sterile distilled

water (to make a 1:1000 dilution of the original material) and shaken 25 times. This step was repeated once more, in order to establish a 1:10,000 dilution of the original material. One ml of each dilution was transferred aseptically into one of three sterile petri dishes; cooled liquid nutrient agar was then added, the plate swirled, and the agar allowed to solidify. The plates were then incubated at 37 C for 48 hours. At the end of the incubation period, the number of colonies per plate was read macroscopically.

TABLE 1

GROWTH OF ORGANISMS IN PEPTONE BROTH
CONTAINING VARIOUS CONCENTRATIONS OF ORGANIC ACIDS
(Read at 24 hours)

Conc. of acid in g/ml of peptone broth	<u>M. pyogenes,</u> <u>var. aureus</u>	<u>S. typhi-</u> <u>murium</u>	<u>S. schott-</u> <u>muelleri</u>	pH
<u>Acetic acid</u>				
1 Normal (.605 g/10 ml acid)				2.50
.03025	-	-	-	2.92
.01512	-	-	-	3.30
.00756	-	-	-	3.58
.00378	-	-	-	3.86
.00189	-	-	-	4.17
.00094	-	-	-	4.45
.00047	-	-	-	4.80
.00024	++	+	-	5.18
.00012	++++	+++	+	5.68
.00006	++++	++++	++	6.20
.0000	++++	++++	++++	7.0
<u>Citric acid</u>				
1 Normal (.640 g/10 ml acid)				1.89
.03200	-	-	-	2.15
.01600	-	-	-	2.49
.00800	-	-	-	2.84
.00400	-	-	-	3.28
.00200	-	-	-	3.78
.00100	-	-	-	4.30
.00050	-	+	-	4.73
.00025	++	+++	-	5.20
.00013	+++	++++	+	5.70
.00006	++++	++++	++	6.15
<u>Lactic acid</u>				
1 Normal (.9008 g/10 ml acid)				1.83
.04504	-	-	-	2.25
.00252	-	-	-	2.59
.01126	-	-	-	2.89
.00563	-	-	-	3.25
.00282	-	-	-	3.58
.00141	-	-	-	3.99
.00070	-	-	-	4.48
.00035	+++	++	-	5.00
.00018	++++	++++	+	5.60
.00009	++++	++++	+++	6.15

TABLE 1 (Cont.)

Conc. of acid in g/ml of peptone broth	<u>M. pyogenes,</u> <u>var. aureus</u>	<u>S. typhi-</u> <u>murium</u>	<u>S. schott-</u> <u>muelleri</u>	pH
<u>Maleic acid</u>				
1 Normal (.58015 g/10 ml acid)				0.82
.02901	-	-	-	1.10
.01450	-	-	-	1.40
.00725	-	-	-	1.75
.00363	-	-	-	2.31
.00181	-	-	-	3.18
.00091	-	-	-	4.04
.00045	-	++	+	4.80
.00023	++	++++	++	5.60
.00011	++	++++	++++	6.31
.00006	++++	++++	++++	6.75
<u>Malic acid</u>				
1 Normal (.67025 g/10 ml acid)				1.65
.03351	-	-	-	2.14
.01676	-	-	-	2.55
.00838	-	-	-	2.83
.00419	-	-	-	3.32
.00210	-	-	-	3.80
.00105	-	-	-	4.29
.00052	-	++	++	4.89
.00026	++++	++++	+++	5.95
.00013	++++	++++	++++	6.55
.00007	++++	++++	++++	6.83
<u>Propionic acid</u>				
1 Normal (.7408 g/10 ml acid)				2.49
.03704	-	-	-	3.04
.01852	-	-	-	3.40
.00926	-	-	-	3.70
.00463	-	-	-	3.97
.00232	-	-	-	4.22
.00116	-	-	-	4.50
.00058	-	-	-	4.80
.00029	+	+	-	5.15
.00015	+++	++++	-	5.60
.00007	++++	++++	+	6.10

TABLE 1 (Cont.)

Conc. of acid in g/ml of peptone broth	<u>M. pyogenes,</u> <u>var. aureus</u>	<u>S. typhi-</u> <u>murium</u>	<u>S. schott-</u> <u>muelleri</u>	pH
<u>Succinic acid</u>				
1 Normal (.59025 g/10 ml acid)				1.99
.02951	-	-	-	2.55
.01476	-	-	-	3.05
.00738	-	-	-	3.20
.00369	-	-	-	3.50
.00185	-	-	-	3.90
.00092	-	-	-	4.20
.00046	-	+	++	4.65
.00023	++++	+++	++++	5.25
.00012	++++	++++	++++	6.20
.00006	++++	++++	++++	6.60
<u>Tartaric acid</u>				
1 Normal (.7504 g/10 ml acid)				1.66
.03752	-	-	-	1.95
.01876	-	-	-	2.26
.00938	-	-	-	2.60
.00469	-	-	-	2.96
.00235	-	-	-	3.38
.00118	-	-	-	3.90
.00059	-	-	-	4.40
.00029	-	++	-	4.92
.00015	++	++++	+	5.52
.00007	++++	++++	++	6.03

TABLE 2
CONTROL TESTS ON BASIC SUBSTRATE
(Read at 48 hours)

Organism	Dilution		
	1:100	1:1,000	1:10,000
<u>Group I</u>			
<u>M. pyogenes</u> , var. <u>aureus</u>	TNTC (565)	62	7
<u>S. typhimurium</u>	TNTC (413)	48	4
<u>S. schottmuelleri</u>	242	28	4
<u>Group II</u>			
<u>M. pyogenes</u> , var. <u>aureus</u>	TNTC	TNTC	TNTC
<u>S. typhimurium</u>	TNTC	TNTC	TNTC
<u>S. schottmuelleri</u>	TNTC	TNTC	TNTC
<u>Group III</u>			
No. 1 (immediately plated out)	0	0	0
No. 2 (incubated 24 hrs., 37 C)	0	0	0

TNTC - Too numerous to count

- Group I - Inoculated, then immediately plated out (to determine the number of organisms inoculated into each tube)
- Group II - Inoculated, incubated for 24 hours at 37 C, then plated out (to determine if the organism grew in custard)
- Group III - Sterility controls (to determine that no air pockets were present in the autoclave)

TABLE 3

GROWTH OF MICROCOCCUS PYOGENES, VARIETY AUREUS IN CUSTARD
CONTAINING GRADED AMOUNTS OF ORGANIC ACIDS
(Read at 48 hours)

Conc. of acid in g/ml of custard	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Acetic acid</u>				
.03025	0	0	0	3.91
.01512	0	0	0	4.51
.00756	15	2	0	5.15
.00378	147	12	1	5.80
.00189	TNTC (315)	37	4	6.14
.00094	TNTC	TNTC	TNTC	6.40
<u>Citric acid</u>				
.03200	0	0	0	2.69
.01600	0	0	0	3.52
.00800	8	1	0	4.30
.00400	TNTC	TNTC	TNTC (3300)	5.15
.00200	TNTC	TNTC	TNTC	5.80
<u>Lactic acid</u>				
.04504	0	0	0	2.75
.02252	2	0	0	3.33
.01126	1	0	0	4.08
.00563	8	1	0	5.00
.00281	TNTC	TNTC	TNTC	5.70
<u>Maleic acid</u>				
.02900	0	0	0	1.23
.01450	0	0	0	1.87
.00725	24	4	0	3.43
.00362	TNTC	TNTC	TNTC	4.76

TABLE 3 (Cont.)

Conc. of acid in g/ml of custard	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Malic acid</u>				
.03351	0	0	0	2.40
.01675	0	0	0	2.88
.00837	8	1	0	3.62
.00418	TNTC	TNTC	TNTC	4.48
<u>Propionic acid</u>				
.03704	0	0	0	3.79
.01852	0	0	0	4.31
.00926	34	5	0	4.80
.00463	118	15	2	5.32
.00231	TNTC (398)	34	3	5.85
.00115	TNTC	TNTC	TNTC	6.20
<u>Succinic acid</u>				
.02951	0	0	0	3.15
.01475	19	2	0	3.59
.00737	24	2	0	4.09
.00368	TNTC	TNTC	TNTC	4.55
<u>Tartaric acid</u>				
.03752	0	0	0	2.30
.01876	0	0	0	3.01
.00938	5	1	0	3.92
.00469	9	1	0	5.00
.00234	TNTC	TNTC	TNTC	5.64

TNTC - Too numerous to count

TABLE 4
GROWTH OF SALMONELLA TYPHIMURIUM IN CUSTARD
CONTAINING GRADED AMOUNTS OF ORGANIC ACIDS
(Read at 48 hours)

Conc. of acid in g/ml of custard	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Acetic acid</u>				
.03025	0	0	0	3.91
.01512	2	0	0	4.51
.00756	0	0	0	5.15
.00378	56	7	2	5.80
.00189	TNTC (387)	36	2	6.15
.00094	TNTC	TNTC	TNTC	6.40
<u>Citric acid</u>				
.03200	0	0	0	2.69
.01600	1	1	0	3.52
.00800	8	0	0	4.30
.00400	TNTC	TNTC	TNTC (1740)	5.15
<u>Lactic acid</u>				
.04504	0	0	0	2.75
.02252	0	0	0	3.33
.01126	0	0	0	4.08
.00563	0	0	0	5.00
.00281	TNTC	TNTC	TNTC (1900)	5.70
<u>Maleic acid</u>				
.02900	0	0	0	1.23
.01450	0	0	0	1.87
.00725	34	1	0	3.43
.00362	TNTC	TNTC	TNTC	4.76

TABLE 4 (Cont.)

Conc. of acid in g/ml of custard	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Malic acid</u>				
.03351	0	0	0	2.40
.01675	0	0	0	2.88
.00837	4	0	0	3.62
.00418	TNTC	TNTC	TNTC	4.48
<u>Propionic acid</u>				
.03704	0	0	0	3.79
.01852	1	0	0	4.31
.00926	6	0	0	4.80
.00463	32	0	0	5.32
.00231	TNTC (648)	56	4	5.85
.00115	TNTC	TNTC	TNTC (780)	6.20
<u>Succinic acid</u>				
.02951	0	0	0	3.15
.01475	0	0	0	3.59
.00737	0	0	0	4.09
.00368	TNTC	TNTC	TNTC	4.55
<u>Tartaric acid</u>				
.03752	0	0	0	2.30
.01876	0	0	0	3.01
.00938	1	1	0	3.92
.00469	TNTC (989)	108	9	5.00
.00234	TNTC	TNTC	TNTC	5.64

TNTC- Too numerous to count

TABLE 5

GROWTH OF SALMONELLA SCHOTTMUELLERI IN CUSTARD
CONTAINING GRADED AMOUNTS OF ORGANIC ACIDS
(Read at 48 hours)

Conc. of acid in g/ml of custard	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Acetic acid</u>				
.03025	0	0	0	3.91
.01512	0	0	0	4.51
.00756	0	0	0	5.15
.00378	16	2	1	5.80
.00189	TNTC	168	14	6.14
<u>Citric acid</u>				
✓ .03200	0	0	0	2.69
.01600	0	0	0	3.52
.00800	12	2	1	4.30
.00400	TNTC	TNTC	TNTC (782)	5.15
<u>Lactic acid</u>				
.014504	0	0	0	2.75
.02252	0	0	0	3.33
.01126	0	0	0	4.08
.00563	3	0	0	5.00
.00281	TNTC	TNTC	TNTC	5.70
<u>Maleic acid</u>				
.02900	0	0	0	1.23
.01450	0	0	0	1.87
.00725	0	0	0	3.43
.00362	TNTC	TNTC	TNTC	4.76

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TABLE 5 (Cont.)

Conc. of acid in g/ml of custard	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Malic acid</u>				
.03351	0	0	0	2.40
.01675	0	0	0	2.88
.00837	18	3	1	3.62
.00418	TNTC	TNTC	TNTC	4.48
<u>Propionic acid</u>				
.03704	0	0	0	3.79
.01852	0	0	0	4.31
.00926	0	0	0	4.80
.00463	8	1	0	5.32
.00231	212	33	4	5.85
.00115	TNTC	TNTC	TNTC	6.20
<u>Succinic acid</u>				
.02951	0	0	0	3.15
.01475	0	0	0	3.59
.00737	0	0	0	4.09
.00368	TNTC	TNTC	TNTC	4.55
<u>Tartaric acid</u>				
.03752	0	0	0	2.30
.01876	0	0	0	3.01
.00938	0	0	0	3.92
.00469	52	7	1	5.00
.00234	TNTC	TNTC	60	5.64

TNTC- Too numerous to count

TABLE 6
GROWTH OF FOOD POISONING ORGANISMS IN
CUSTARD CONTAINING GRADED AMOUNTS OF DHA-S
(Read at 48 hours)

Conc. of DHA-S in custard (percent)	Dilutions			pH
	1:100	1:1,000	1:10,000	
<u>Micrococcus pyogenes,</u> var. <u>aureus</u> , Str. Brail				
0.3	TNTC	TNTC	TNTC	7.60
0.5	TNTC	TNTC	TNTC	7.55
0.7	TNTC	TNTC	TNTC	7.57
0.9	TNTC	TNTC	TNTC	7.52
1.1	TNTC	286	34	7.46
<u>Salmonella typhimurium</u>				
0.3	TNTC	TNTC	TNTC	7.60
0.5	TNTC	TNTC	TNTC	7.55
0.7	TNTC	TNTC	TNTC	7.57
0.9	TNTC	250	33	7.52
1.1	TNTC	180	21	7.46
<u>Salmonella schottmuelleri</u>				
0.3	TNTC	TNTC	TNTC	7.60
0.5	TNTC	TNTC	TNTC	7.55
0.7	TNTC	TNTC	222	7.57
0.9	TNTC	163	13	7.52
1.1	274	31	2	7.46

TABLE 7
PALATABILITY OF ACID-TREATED SOFT CUSTARD

Acid	Palatability	pH
<u>Acetic</u>		
.03025	-	3.91
.01512	-	4.51
.00756	-	5.15
.00378	-	5.80
.00189	+	6.14
.00094	+	6.40
<u>Citric</u>		
.03200	-	2.69
.01600	-	3.52
.00800	-	4.30
.00400	+	5.15
<u>Lactic</u>		
.04504	-	2.75
.02252	-	3.33
.01126	-	4.08
.00563	+	5.00
.00281	+	5.70
<u>Maleic</u>		
.02900	-	1.23
.01450	-	1.87
.00725	-	3.43
.00362	-	4.76
.00181	+	5.36

TABLE 7 (Cont.)

Acid	Palatability	pH
<u>Malic</u>		
.03351	-	2.40
.01675	-	2.88
.00837	-	3.62
.00418	-	4.48
.00209	+	5.18
<u>Propionic</u>		
.03704	-	3.79
.01852	-	4.31
.00926	-	4.80
.00463	-	5.32
.00231	-	5.85
.00115	-	6.20
.00057	+	6.40
<u>Succinic</u>		
.02951	-	3.15
.01475	-	3.59
.00737	-	4.09
.00368	+	4.55
.00184	+	5.13
<u>Tartaric</u>		
.03752	-	2.30
.01876	-	3.01
.00938	-	3.92
.00469	-	5.00
.00234	+	5.64

- Unpalatable

+ Presence of acid still detectable and slightly disagreeable

+ Palatable, even though the flavor of the acid was at times apparent

RESULTS AND DISCUSSION

The results of the primary work (Table 1) where the actions of various organic acids in peptone broth were tested on food poisoning and food infecting bacteria at first appear to be vague. However, closer over-all examination shows that S. schottmuelleri is on the whole much more susceptible to inhibition by the organic acids tested than M. pyogenes, var. aureus and S. typhimurium. This means that a much smaller concentration of the acid is required to keep down the growth. On the other hand, the other salmonella tested, S. typhimurium, proved to be the most resistant to the inhibitory actions of the organic acids. At times this resistance was rather great, as in the cases of citric and propionic acids, where it grew in concentrations four times as great as those which almost completely inhibited the growth of S. schottmuelleri.

The general effect appears to indicate that acetic, propionic and tartaric acids give the best results, closely followed by citric and lactic acids.

It is difficult to come to any sound conclusions on the basis of the presence of carboxyl groups (since three of these acids are monocarboxylic, one is di-carboxylic and the remaining is tri-carboxylic) except to point out that the monocarboxylic acids gave a much better result than the di-carboxylic acids

tested. It would be grossly unrealistic to make any assumptions on the part of tri-carboxylic acids from the results of this work as citric acid was the only one tested. A similar attempt to draw any definite conclusions at this point, on the basis of pH, is rather difficult since the range that induced stasis was tremendous. For example, in the maleic acid results, a pH of 4.04 is apparently necessary to produce inhibition of all three organisms tested, whereas in the cases of acetic and propionic acids, an acidity of only pH 4.80 was required to achieve the same effect. In addition, the results on the basis of pH varied even more greatly when the organisms were considered individually, e.g., M. pyogenes, var. aureus, str. Brail was inhibited at a pH of 4.48 using lactic acid, whereas the same effect was achieved at a pH of 4.92 using tartaric acid. Similarly, a pH of 4.04 using maleic acid inhibited S. typhimurium, while on certain occasions, acetic acid at a pH of 5.18 produced the same results. The pH range of inhibition regarding S. schottmuelleri is even more pronounced: inhibition occurred at a pH of 4.04 using maleic acid, and occasionally acetic acid inhibited the organism at a pH of 5.68. The controls for this portion of the work were tubes of peptone broth inoculated with the respective organisms. The growth that was achieved within 24 hours was considered to be +++, and all subsequent readings of the acid dilutions tubes were based upon this control, (i.e., no mechanical reading of turbidity was employed). The dearth of

DHA-S necessitated its non-inclusion in this primary phase. However, the results of the work done by Brunner and Mallmann (3) provided information comparable to that obtained in this primary phase on the organic acids.

Table 2 shows the types and results of the controls used in the second phase. The Group I controls indicate that the average numbers of organisms inoculated into the test tubes of soft custard were as follows:

<u>M. pyogenes</u> , var. <u>aureus</u> , str. Brail	630,000
<u>S. typhimurium</u>	862,000
<u>S. schottmuelleri</u>	614,000

The Group II controls indicate that the organisms grew in the soft custard, i.e., there were no natural inhibitory agents present in the custard. The Group III controls prove that the inspissation process not only "set" the custard, medium, but also simultaneously sterilized it.

In Table 3, the effects of the organic acids tested on M. pyogenes, var. aureus, str. Brail, in soft custards, are recorded. Although it has been previously stated that ten serial dilutions were prepared, only those concentrations showing inhibition, and the first (and sometimes second) concentrations showing lack of inhibition, are listed. When mixed with the soft custard medium, a much greater concentration of the organic acids was necessary to produce stasis of the organism than when the acids were mixed with the peptone broth.

The pH values indicate that the constituents of the soft custard medium either buffered or neutralized some of the effects of the acids on the organism. Acetic and propionic acids produced the best results, i.e., lower concentrations were necessary to produce inhibition. Next in value are lactic and tartaric acids. The greatest concentrations of acids needed to inhibit the growth of M. pyogenes, var. aureus, str. Brail, were demonstrated in the cases of the remaining acids: citric, maleic, malic and succinic. One startling difference is that citric acid when mixed with the soft custard medium gave nowhere near the (proportional) results one was led to expect after examining Table 1. It is difficult to evaluate the exact role pH plays in the inhibition of the organism. This becomes apparent in comparing the results observed when using maleic and acetic acid. Inhibition occurred in the former at the concentration where the pH of the soft custard medium was 3.43, whereas in the latter the pH was 6.14. When comparing the results of the acids on the basis of the number of carboxyl groupings present, it is noted that the monocarboxylic acids yielded the best results.

In Table 4, the effects of the organic acids tested on S. typhimurium, in soft custard, are recorded.

In Table 5, the effects of the organic acids tested on S. schottmuelleri in soft custard, are recorded.

Against both S. typhimurium and S. schottmuelleri, as well as previously mentioned in the case of M. pyogenes, var.

aureus, str. Brail, acetic and propionic acids gave the best results, closely followed by lactic and tartaric acids.

In Table 6, the effect DHA-S had on all three organisms, tested in soft custard, is recorded. S. schottmuelleri and S. typhimurium proved to be the more susceptible to static action of DHA-S, as a concentration of only 0.9 percent was required. A concentration of 1.1 percent was necessary to achieve the same effect against M. pyogenes, var. aureus, str. Brail. The pH of these concentrations was above neutrality, and this would indicate that the action of DHA-S is not based upon the hydrogen ion concentration but rather upon the chemical structure of the compound. This has previously been mentioned by Coleman and Wolf (6). It has been observed by Geiger and Conn (18) and later by Roblin (27) that such antibiotics as clavacin and penicillic acid were alpha, beta unsaturated ketones. The structural grouping found in these antibiotics, i.e., the $-\text{CH}=\overset{\text{I}}{\underset{\text{I}}{\text{C}}}-\text{C}=\text{O}$ grouping, is also observed in DHA-S. Geiger and Conn (18) observed that it is this grouping that in all probability is responsible for the antibacterial activity of clavacin and penicillic acid. Before concluding, it is necessary to add that in the concentrations of acetic, lactic, propionic and tartaric acids and DHA-S that proved to be effective against bacterial reproduction, the appearance of the custard was quite normal. The matter of taste, however, was quite a different story. The DHA-S apparently did not alter the flavor or taste of the soft custard,

but the organic acids certainly did, especially acetic and propionic acids. The concentrations that induced bacteriostasis were all unpalatable. The least objectionable in taste was citric acid. These data are contained in Table 7.

SUMMARY

1. Eight organic acids and DHA-S were tested in peptone broth and a soft custard medium for their effectiveness against food poisoning and food infecting organisms.
2. Acetic and propionic acids, closely followed by lactic and tartaric acids proved to be the best inhibitors in soft custard.
3. A 0.9 percent concentration of DHA-S was required to inhibit the Salmonella tested, while a 1.1 percent concentration was required to inhibit the enterotoxin-producing strain tested.
4. No correlation of results could be made on the basis of pH.
5. Of the organic acids tested, the consistently best results were achieved with the monocarboxylic acids.
6. The concentrations of organic acids that induced bacteriostasis rendered the soft custard unpalatable.
7. Soft custard containing a bacteriostatic concentration of DHA-S was completely palatable.

BIBLIOGRAPHY

1. Blair, J. E. 1939. The pathogenic Staphylococci. Bact. Rev. 3:97-146.
2. Brown, E. G., G. R. Combs and E. Wright. 1940. Food-borne infection with Salmonella aertrycke. J.A.M.A. 114:642-644.
3. Brunner, J. R. and W. L. Mallmann. 1950. Effectiveness of dehydroacetic acid as a mold inhibitor on butter and cheese surfaces. Mich. Agr. Exp. Sta. Quar. Bull. 33: 127-142.
4. Burnet, F. M. 1929. The exotoxins of Staphylococcus pyogenes aureus. J. Path. Bact. 32:717-734.
5. Burnet, F. M. 1931. The interactions of Staphylococcus toxin anatoxin and antitoxin. J. Path. Bact. 34: 471-492.
6. Coleman, G. H. and P. A. Wolf. Making proteinaceous and fatty foods resistant to microorganisms. U. S. Patent # 2,474,228.
7. Coughlin, F. E. and B. Johnson, Jr. 1941. Gastroenteritis outbreaks from cream-filled pastry. Amer. J. Pub. Health 31:245-250.
8. Crabtree, J. A. and W. Litterer. 1934. Outbreak of milk poisoning due to a toxin-producing Staphylococcus found in the udders of two cows. Amer. J. Pub. Health 24: 1116-1122.
9. Crowe, M. 1946. A localized outbreak of Salmonella food poisoning apparently transmitted by a hen's egg. J. Hyg. 44:342-345.
10. Dack, G. M., W. E. Cary and P. H. Harmon. 1928. Unsuccessful attempt to produce Salmonella intoxication in man. J. Prevent. Med. 2:479-483.
11. Dack, G. M. 1947. Problems and errors in assigning causes of bacterial food poisoning. Amer. J. Pub. Health 37:360-364.

12. Dack, G. M. 1949. Food poisoning. Rev. ed. Chicago: The Univ. of Chicago Press. 184 pp.
13. Davison, E. and G. M. Dack. 1939. Some chemical and physical studies of Staphylococcus enterotoxin. J. Infect. Diseases 64:302-306.
14. Doyle, H. S. 1946. An outbreak of food poisoning in Prince Albert, Saskatchewan. Canad. J. Pub. Health 37:65-68.
15. Draper, F. 1945. Gastro-enteritis and dysentery in children. The bacteriology of cases caused by Salmonella and dysentery organisms. Med. J. Australia 32:417-425.
16. Fuchs, A. W. 1941. Disease outbreaks from water, milk and other foods in 1939. Pub. Health Rep. 56:2277-2284.
17. Fulton, F. 1943. Staphylococcal enterotoxin - with special reference to the kitten test. Brit. J. Expt. Path. 24:65-72.
18. Geiger, W. B. and J. E. Conn. 1945. The mechanism of the antibiotic action of clavacin and penicillic acid. J. Amer. Chem. Soc. 67:112-116.
19. Glenny, A. T. and M. F. Stevens. 1935. Staphylococcus toxins and antitoxins. J. Path. Bact. 40:201-210.
20. Hammon, W. McD. 1941. Staphylococcus enterotoxin: An improved cat test, chemical and immunological studies. Amer. J. Pub. Health 31:1191-1198.
21. Haynes, W. C. and G. J. Hucker. 1945. Studies on Coccaceae XVIII. The enterotoxin producing micrococci. New York (Geneva) Agr. Exp. Sta. Tech. Bull. 275:1-82.
22. Holt, L. B. 1936. The purification of Staphylococcus toxoid. Brit. J. Expt. Path. 17:318-323.
23. Kelly, F. C. and G. M. Dack. 1936. Experimental Staphylococcus food poisoning. Amer. J. Pub. Health 26:1077-1082.
24. McCastline, W. B., R. Thompson and M. L. Isaacs. 1937. A food poisoning outbreak due to Staphylococcus. J. Bact. 33:50-51.

25. Minett, F. C. 1938. Experiments on Staphylococcus food poisoning. J. Hyg. 38:623-637.
26. Ostrolenk, M. and H. Welch. 1942. The house fly as a vector of food poisoning organisms in food producing establishments. Amer. J. Pub. Health 32:487-494.
27. Roblin, R. O., Jr. 1946. Metabolite antagonists. Chem. Rev. 38:255-377.
28. Rosenau, M. J. and H. Weiss. 1921. Food infections. J.A.M.A. 77:1948-1950.
29. Seevers, M. H., F. E. Shideman, L. A. Woods, J. R. Weeks and W. T. Kruse. 1950. Dehydroacetic acid. II. General pharmacology and mechanism of action. J. Pharmacol. Expt. Therap. 99:69-83.
30. Shideman, F. E., L. A. Woods and M. H. Seevers. 1950. Dehydroacetic acid. IV. Detoxication and effects on renal function. J. Pharmacol. Expt. Therap. 99:98-111.
31. Slocum, G. G. and B. A. Linden. 1939. Food poisoning due to Staphylococcus. Amer. J. Pub. Health 29:1326-1330.
32. Spencer, H. C., V. K. Rowe and D. D. McCollister. 1950. Dehydroacetic acid (DHA) I. Acute and chronic toxicity. J. Pharmacol. Expt. Therap. 99:57-68.
33. Spray, R. S. 1926. An outbreak of food poisoning probably due to rat virus. J.A.M.A. 86:109-111.
34. Stewart, H. C. and W. Litterer. 1927. An outbreak of gastro-enteritis. J.A.M.A. 89:1584-1587.
35. Surgalla, M. J. and K. E. Hite. 1946. Production of staphylococcal enterotoxin in chemically defined media. Proc. Soc. Expt. Biol. Med. 61:244-245.
36. Welch, H., M. Ostrolenk and M. T. Bartram. 1941. Role of rats in the spread of food poisoning bacteria of the Salmonella group. Amer. J. Pub. Health 31:332-340.
37. Wittler, R. G. and L. Pillemer. 1948. The immunochemistry of toxins and toxoids. V. The solubility of Staphylococcal toxin in methanol-water mixtures under controlled conditions of pH, ionic strength and temperature. J. Biol. Chem. 174:23-29.

38. Wolf, P. A. 1950. Dehydroacetic acid a new microbiological inhibitor. Food Tech. 4:294-297.
39. Woods, L. A., F. E. Shideman, M. H. Seevers, J. R. Weeks and W. T. Kruse. 1950. Dehydroacetic acid (DHA). III. Estimation, absorption, and distribution. J. Pharmacol. Expt. Therap. 99:84-97.
40. Summary of Toxicological Studies on DHA. The Dow Chemical Company, Midland, Michigan.

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