A STUDY OF THE LACTOBACILLI IN TOMATO WHEY MEDIUM AND SOY BEAN MILK

THESIS FOR THE DEGREE OF M. S.

Donald A. Grover

1934

Lactobacilli

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ACKNOWLEDGMENT

This opportunity is taken to acknowledge my indebtedness to E. D. Devereux for his constant and effective guidance throughout this work.

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INTRODUCTION

Soy bean milk has been used for centuries in the Orient as an article of food. Especially is this true of China where it serves the purpose of a milk substitute. More recently, interest in this milk has been developed in this country and certain of our larger Sanitariums have begun the production of soy bean acidophilus milk for use as a health beverage.

The milk in the raw state is not a good medium for the culture of the lactobacilli but with the addition of lactose it becomes suited to this purpose.

Tomato whey medium was added to the experiment after the work had begun because it was highly recommended for its ability to grow the members of the group covered in this report.

I. Experimental

The behavior of certain of the lactobacilli in soy bean milk and tomato whey medium presents many interesting features.

Commercially, common pasteurization seems sufficient to insure an acidophilus milk of good quality if the type of organism used is of the rapid growing variety. In the laboratory, however, where absolute sterility is needed, several problems were presented.

Attempts to produce such a sterile soy bean milk met with failure and spoilage occurred in as high as 95% of the trials made, when the amount to be sterilized averaged over a liter.

Upon examination these flasks showed a varied flora. Many types were isolated, of which three predominated, namely: 1. A gram positive spore former, 2. A gram positive streptobacillus, 3. A gram positive bacillus (long). Various other types were noted, but did not occur in as many instances as those listed above. Among the others isolated, members of the staphylococcus group appeared often. Occasionally a streptococcus form appeared and in a few stains a gram negative diplococcus was found. A pink surface mold seemed to be the organism most often involved in those flasks which spoiled after 72 hours or more.

Since the writer was little interested in the types

of organisms involved, but was trying to produce a sterile medium for other work, no attempt was made to classify the organisms isolated other than by noting their morphological form.

When it became evident that simple autoclaving was not sufficient to produce a sterile milk a fractional method was tried. Starting with two heatings of varied lengths, and an intervening incubation period varied from three to twelve hours, another heating period was added. A typical example of the method is given in the chart on the following page.

A slight decrease in spoilage occurred but the results were not sufficiently successful to guarantee a sterile product. Even had the method proved successful the change in the milk due to the continued heating would have forced its discontinuance.

The only explanation that could be given for the difficulty encountered in sterilizing the milk was that the size of the particle was such that it did not allow the heat to penetrate to the inner parts of the suspension. Many attempts were made to decrease the size of the particle involved. At first various sized screens were used in straining the milk, but little or no difference could be detected. Finally the 80 mesh screen was adopted for use in the routine production of raw milk.

CHART NO. I

F.N.	AMOUNT	F.H	F.I.	2.H.	2.I.	3.H.	3.I.	4.H.	REMARKS
I	1500 c.c.	15 lbs. 20 MIN.	3 HRS.	15 lbs. 25 MIN.	3 HRS.	15 lbs. 25 MIN.	OVER NIGHT	15 lbs. 25 MIN	Flask spoiled 3rd. day
II	2000 CC.	15 lbs. 20 MIN.	3 HRS.	15 lbs. 25 MIN	3 HRS.	15 lbs. 25 MIN.	OVER NIGHT	15 lbs 25 MIN	Flask spoiled 2nd day
III	2500 CC.	15 lbs. 20 MIN.	3 HRS.	15 lbs. 25 MIN.	3 HRS.	15 lbs. 25 MIN.	OVER NIGHT	15 lbs. 25 MIN.	Flask spoiled 5 TH. day

Key to Chart

F.N. - Flask Number
F.H. - First Heat (time in minutes)
F.I. - First Incubation Period (time in hours)
2 H. - Second Heat
2 I. - Second Incubation Period
3 H. - Third Heat
3 I. - Third Incubation Period
4 H. - Fourth Heat

The next step in controling the size of particle was in the use of a silk screen, but since this seemed to make little difference it was not included as a routine procedure.

The viscolizer in combination with the screens was included in the experiment but did little or not good in the production of a milk which could be readily sterilized.

All possible combinations of the above factors were used, as well as the elimination of the ingredients in the milk other than the beans. Accompanying charts indicate the combinations used and the results obtained.

After numerous trials using quantities of milk of a liter or more the larger quantities were substituted by smaller amounts ranging from 10 to 300 c.c. and a very great difference was at once noted. With the decrease in amount, keeping quality was greatly improved and very little spoilage occurred. Evidently the heat was now able to spread throughout the medium and a sterile product was assured.

Later in the work larger amounts of milk could be sterilized with ease. This can probably be explained by the fact that greater skill in keeping out contamination during the making process was acquired as the work progressed, and also because the beans were boiled before use during the later stages of the experiment.

During this work several organisms of the lacto-

CHART NO. II

Flask	(TIME IN HOURS) 24	48	72	96	AMOUNT	P	T	APPEARANCE	TYPE OF GROWTH
I	OK	Ap	-	-	1000 c.c.	15	25	Milk coagulated	gram+ strepto bacillus " " spore former
II	OK	Ap	-	-	1000 c.c.	15	26	" "	gram+ bacillus " " strepto-bacillus " " spore former
III	OK	Ap	-	-	1000 c.c.	15	25.	" " gas formed	gram+ strepto-bacillus " " spore former
IV	OK	Ap	-	-	2450 c.c.	15	35	Milk coagulated	gram+ bacillus " " strepto bacillus " " spore former
V	OK	Ap	-	-	2450 c.c.	15	35	" " gas formed	gram+ bacillus " " strepto-bacillus " " spore former
VI	OK	Ap	-	-	2450 c.c.	15	35	Milk coagulated	gram+ bacillus " " strepto-bacillus " " spore former

Key to Chart

I. 80 mesh screen plus sugar and salt.
II. 80 mesh screen and silk plus sugar and salt.
III. 80 mesh screen and viscolizer plus sugar and salt.
IV. Same as I.
V. Same as II.
VI. Same as III.

P - Steam pressure.
T - Time in minutes.

CHART NO. III

Flask	(TIME IN HOURS) 24	48	72	96	AMOUNT	P	T	APPEARANCE	TYPE OF GROWTH
I	OK	ap	-	-	1000 c.c.	15	25	Milk coagulated	gram + bacillus " " spore former
II	OK	ap	-	-	1000 c.c.	15	25	" "	gram + bacillus " " spore former
III	OK	ap	-	-	1000 c.c.	15	25	" " gas formed	gram + strepto bacillus " " spore former
IV	OK	ap	-	-	1000 c.c.	15	25	Milk coagulated	gram + spore former
V	OK	OK	ap	-	1000 c.c.	15	25	" " gas formed	gram + spore former " " strepto bacillus
VI	OK	OK	ap	-	1000 c.c.	15	25	Milk coagulated	gram + bacillos " " spore former
VII	OK	OK	ap	-	1000 c.c.	15	25	" "	gram + bacillus (short) " " spore former
VIII	OK	OK	ap	-	1000 c.c.	15	25	" "	gram + bacillos " " spore former
IX	OK	OK	ap	-	1000 c.c.	15	25	" "	Stophylococcus

Key to Chart

I. 80 mesh screen, plain milk.
II. 80 mesh screen, plain milk.
III. 80 mesh screen plus viscolizer, plain milk.
IV. 80 mesh plus salt.
V. 80 mesh plus silk plus salt.
VI. 80 mesh plus viscolizer plus salt.
VII. 80 mesh plus salt and sugar.
VIII. 80 mesh plus silk plus salt and sugar.
IX. 80 mesh plus viscolizer plus salt and sugar.

P - Steam pressure.
T - Time in minutes.

CHART NO. IV

Flask	(TIME IN HOURS) 24	48	72	96	Amount	T	P	Appearance	Type of Growth
I	ok	ap	-	-	1000 c.c.	30	15	Milk coagulated	gram + spore former " " bacillus " " staphylococcus
II	ok	ap	-	-	1000 c.c.	30	15	" "	gram + spore former " " bacillus " " strepto bacillus
III	ok	ap	-	-	1000 c.c.	30	15	" "	gram + spore former " " bacillus
IV	ok	ap	-	-	1000 c.c.	30	15	" "	gram + spore former " " bacillus
V	ok	ap	-	-	1000 c.c.	30	15	" "	gram + bacillus ?
VI	ap	ap	-	-	1000 c.c.	45	9	Milk putrified gas formed	gram + spore former " " bacillus
VII	ap	-	-	-	1000 c.c.	45	9	" " " "	gram + spore former " " bacillus
VIII	ap	-	-	-	1000 c.c.	45	9	" " " "	gram + spore former " " bacillus
IX	ap	-	-	-	1000 c.c.	45	9	" " " "	gram + spore former " " bacillus
X	ap	-	-	-	1000 c.c.	45	9	" " " "	gram + spore former " " bacillus

Key to Chart

I. 80 mesh screen no salt or sugar.
II. 80 mesh screen plus silk, no salt or sugar.
III. 80 mesh screen plus viscolizer, no salt or sugar.
IV. 80 mesh screen plus salt.
V. 80 mesh screen plus silk, salt.
VI. 80 mesh screen plus viscolizer, salt.
VII. 80 mesh screen plus salt and sugar.
VIII. 80 mesh screen plus silk, salt and sugar.
IX. 80 mesh screen plus viscolizer, salt and sugar.
X. 80 mesh screen plus viscolizer, salt and sugar. Whole bean milk.

P - Pressure in pounds per square inch.
T - Time in minutes.

bacillus group were used: two typical acidophilus types, one atypical acidophilus and two strains of bulgaricus.

Lactobacillus	acidophilus	# 1	M. S. C.
"	acidophilus	# 4	M. S. C.
"	acidophilus	# 1482E	Kopeloff (Atypical)
"	bulgaricus	# 1	M. S. C.
"	bulgaricus	# B4US	Kopeloff

Flasks of soy bean milk and cow's milk were inoculated simultaneously to furnish a comparison of the action of these types in the two media. The amounts of media, temperature of incubation and inoculum were kept as nearly constant as possible. The first irregularity noted was the speed at which soy bean milk coagulated as compared to that of cow's milk. The soy milk coagulated in from 12 to 24 hours, depending on the strain of organism used. Cow's milk showed no tendency to coagulate until sometime in the second day, often requiring more than 48 hours.

This led to the mistaken belief that the strains used, as a group, grew more rapidly in soy bean milk than in cow's milk. Plate counts did not verify this but stains showed that there was a slight increase in the size of the cells and length of chains in the acidophilus strains grown in soy bean milk, and much longer chains were demonstrable. The colony on the plating medium also was slightly

larger than those produced from the same strain grown in cow's milk.

The problem of selecting plating media for routine counts soon presented itself and several types were used, all of which were more or less successful.

Tomato agar medium was included because of the good results reported by various workers especially concerned with the lactobacillus group. Both new and old formulas were available and after many trials the following one was selected for this work:

300 c.c. Tomato juice

700 c.c. Distilled water

5 gms. Peptone

15 gms. Agar

Adjusted to a pH of 6.8 to 7.0.

A whey agar was also used in many of the counts, prepared after the formula reported by Kopeloff (1). The method is briefly as follows: Heat skimmed milk to 80° or 90° C. Acidify and allow the casein to settle. Filter first through absorbent cotton and then paper; neutralize the filtrate to pH 6.8 to 7.0. Before further filtering, add 0.5% peptone and autoclave the medium for fifteen minutes at 15 pounds extra pressure. Filter off the albuminous material through paper. Convert the resulting whey into agar by adding 1.2% standard shredded or powdered agar.

Bond of the Battle Creek Food Company laboratories offered for use in the work his formula for Tomato Whey Peptone Agar which he had used for some time in all routine soy bean milk counts. This medium was so successful that it was included as part of the problem covered by this report.

The whey contained in this medium was prepared in the manner outlined before, and needs no further discussion.

The tomato juice available for use was of the pureed type, slightly boiled to concentrate, and thus had a higher specific gravity than the juice obtained from ordinary canned whole tomatoes. The formula contained in this report has been adjusted to meet this situation and can be used as reported.

300 c.c. Tomato juice (whole canned tomatoes)
700 c.c. Whey
5 gms. Peptone
18 gms. Agar

After medium is in solution adjust to pH of 6.8 to 7.0 and filter out any further precipitate through filter paper.

In an attempt to shorten the procedure of producing this medium, a whey was produced from the dried commercial product sold by many feed houses. 50 gms. of dry whey was placed in a flask containing 1000 c.c. of distilled water. To this solution 5 gms. peptone was added and the resulting mixture heated in the autoclave for 10 minutes at 15 pounds

extra pressure and filtered through absorbent cotton while hot. This whey solution can be added to the tomato juice as in the previous formula.

The resulting medium gives as good counts as that made with fresh whey but the size of colony is diminished slightly. However, it compares favorably with other media in use.

The various media were compared and when series of plates, with all types of media included in the work and planted from the same dilution flasks, were counted, similarity was noted. Little or no difference could be detected as long as low dilutions and a microscopic method of plate counting were employed.

This method was a slight modification of the one reported by Breidigen and Chang (2) in their work on this group of organisms. What seemed to be abnormally high counts resulted, usually running from 1,000,000,000 to in one instance over 3,000,000,000 per c.c. As check counts were desired, higher dilutions, 1 - 1,000,000 or more, were employed and the standard plate counting method was used.

A great difference in counts appeared. With this method series of plates still roughly coincided, but differences often occurred. Counts now ran from 9,000,000 to 44,000,000 per c.c. This is quite readily explained when it is remembered that many colonies counted under the

microscopic method are invisible to the eye, even when aided by a hand lens, and so are missed.

The slight advantage of Tomato Whey Peptone medium was demonstrated by the increased size of colony it was able to produce. However, even with this medium it was a laborious task to count plates by the standard plate counting method.

	Comparative Counts	
Media	Stan. Plate Count	Microscopic
Whey agar	31,000,000	2,089,000,000
Tom. Whey Pep.	42,000,000	2,600,000,000
Tom. agar	26,000,000	1,828,400,000

The use of 10% CO_2 in the incubation of plates was discontinued as the advantage it displayed was not sufficient to warrant its use in the routine procedure. In its place a four day incubation period at 37° C. was used and in all cases was successful. In many cases growth was sufficient in three days.

In an effort to improve the formula for the Tomato Whey Peptone medium the concentration of its principal ingredients was varied. With the amount of tomato juice constant (30%), various concentrations of whey were used. Concentrations of 80%, 50%, 30%, and 20% were prepared and series of plates were run with the various strains of lacto-

bacilli. In all cases the counts were similar but there was a slight variation in colony size, decreasing with the greater dilutions of whey.

Next the tomato content was varied, the whey being left constant. Media containing 30%, 20%, 10% and 5% tomato juice were made and check counts were run. A slight decrease in count occurred after the dilution of 20% tomato juice was reached and again the size of colony varied, becoming slightly smaller.

In conclusion to this part of the work it might be stated that the original concentrations of ingredients in Tomato Whey Peptone medium gave the best results, but small dilutions of either or both of the principal constituents did little or no harm to the effectiveness of this preparation.

While the counts on the various media were similar for both soy bean and cow's milk, a great difference appeared in them as far as the production of acid was concerned.

Hammer (3) gives the acidity of fresh normal cow's milk as from 0.12% to 0.24%. This of course was calculated as lactic acid which is the usual method of recording acidities in milk and cream under dairy plant conditions. Titrations of skimmed milk used in this work were not done while the milk was in the fresh state but were made on

regular samples after the sterilization process. This method was used to eliminate any change occurring in the milk while being heated. The titrable acidities of skimmed cow's milk under these conditions usually were about 0.22%. In a few samples a slightly higher acidity was recorded. The samples on which higher acidities were found were usually more than eight hours old, thus sufficient time had elapsed for some bacterial action.

In soy bean milk a much lower initial acidity was found, 0.03% free titrable acid being the average. These titrations as in cow's milk were run after sterilization and were taken from regular samples.

When regular titrations of all cultural samples were included as a routine procedure it became evident why soy bean milk coagulated more rapidly than cow's milk. While an acidity of 0.25% to 0.45% was all that was necessary to precipitate the soy bean milk, cow's milk did not coagulate until an acidity of 0.5% to 0.65% was reached.

The pH values of both milks were compared. Again these were run after the sterilization process. Skimmed milk in the many trials attempted had an average pH of 5.8 to 6.0, which was fairly constant when fresh milk was used. Soy bean milk under the same conditions showed a pH of 5.6 to 6.0 with an average of about 5.8, which showed little tendency to fluctuate over the maximum or minimum, as freshly prepared milk was always used.

After many trials the average acidity required for the coagulation of soy bean milk was established at 0.35%, while the average pH at this point was 4.4.

The end points at which acid production ceased in the two milks varied widely according to the strain used. These titrations were made after the incubated cultures had stood at room temperature for ten days.

Chart No. V included in the report on the following page compares these acidities in soy bean and cow's milk.

The behavior of L. acidophilus #1482E was slightly irregular in soy bean milk. Many very high counts were obtained and some irregularity in acid formation was noted when the reactions of this organism were compared with those of the other acidophilus strains used. To check this, comparative tests were run of this strain and L. acidophilus #1, whose actions were typical for the group. Cow's milk was also included in this work and accompanying charts show the results obtained.

CHART NO. V

Comparative Titrable Acidities (End Points)

Strain	Medium	Acidity
L. acidophilus #1	Cow's milk	2.41%
L. acidophilus #4	" "	1.27%
L. acidophilus #1482E	" "	1.55%
L. bulgaricus #1	" "	1.62%
L. bulgaricus #B4US	" "	2.16%
L. acidophilus #1	Soy bean milk	0.80%
L. acidophilus #4	" " "	1.15%
L. acidophilus #1482E	" " "	1.68%
L. bulgaricus #1	" " "	0.54%
L. bulgaricus #B4US	" " "	0.84%

CHART NO. VI

Hours	Acidity	pH
0	.22%	5.8
24	.80%	5.0
48	1.76%	4.5
72	2.15%	4.0
96	2.20%	4.0
120	2.20%	4.0

L. acidophilus #1 -- Cow's Milk

Hours	Acidity	pH
0	.03%	5.6
24	.19%	4.8
48	.60%	4.2
72	.64%	4.0
96	.79%	3.8
120	.80%	3.8

L. acidophilus #1 -- Soy Bean Milk

CHART NO. VII

Hours	Acidity	pH
0	.22%	5.8
24	.42%	5.0
48	1.08%	4.5
72	1.36%	4.4
96	1.52%	4.2
120	1.55%	4.2

L. acidophilus #1482E -- Cow's Milk

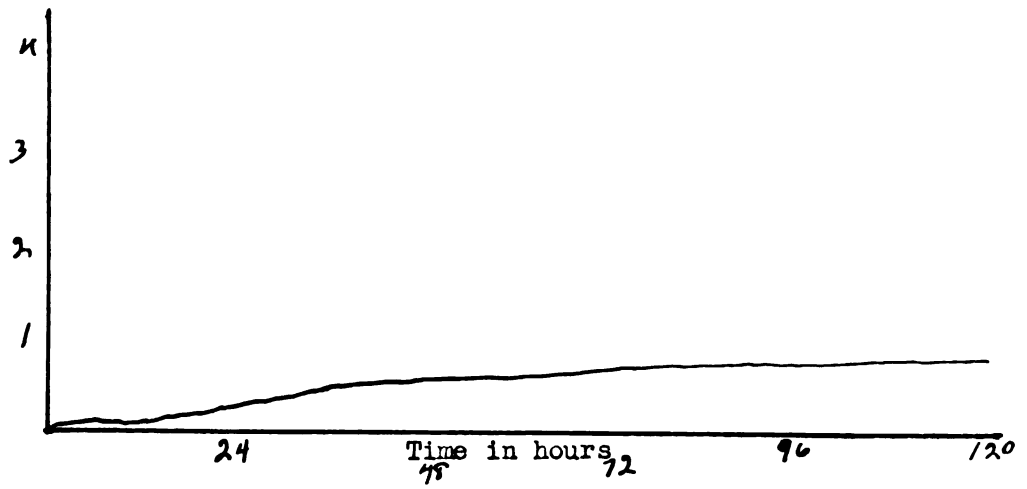
Hours	Acidity	pH
0	.03%	5.6
24	.82%	5.0
48	1.40%	4.6
72	1.65%	4.4
96	1.67%	4.4
120	1.68%	4.4

L. acidophilus #1482E -- Soy Bean Milk

CHART NO. VIII

L. acidophilus #1

% Acid — Titrable acidity in soy bean milk



L. acidophilus #1

% Acid — Titrable acidity in cow's milk

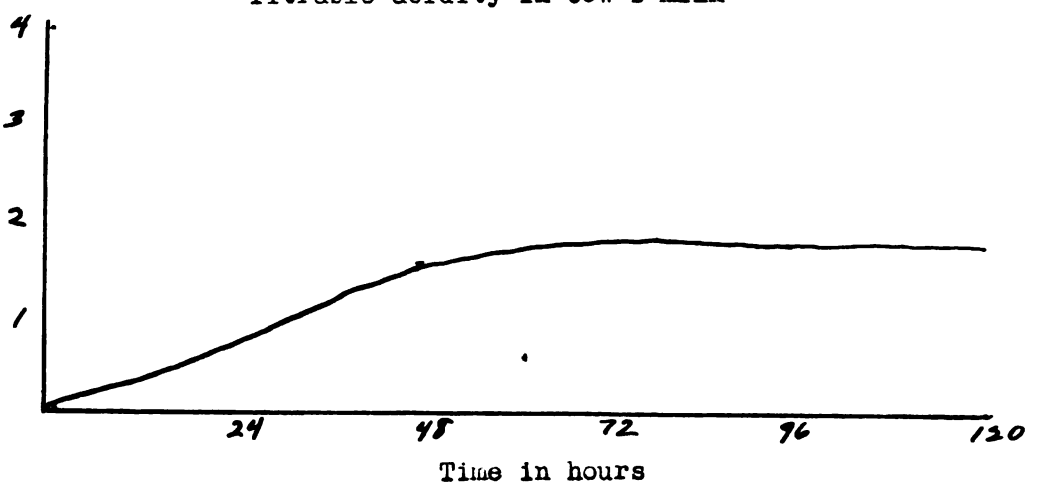
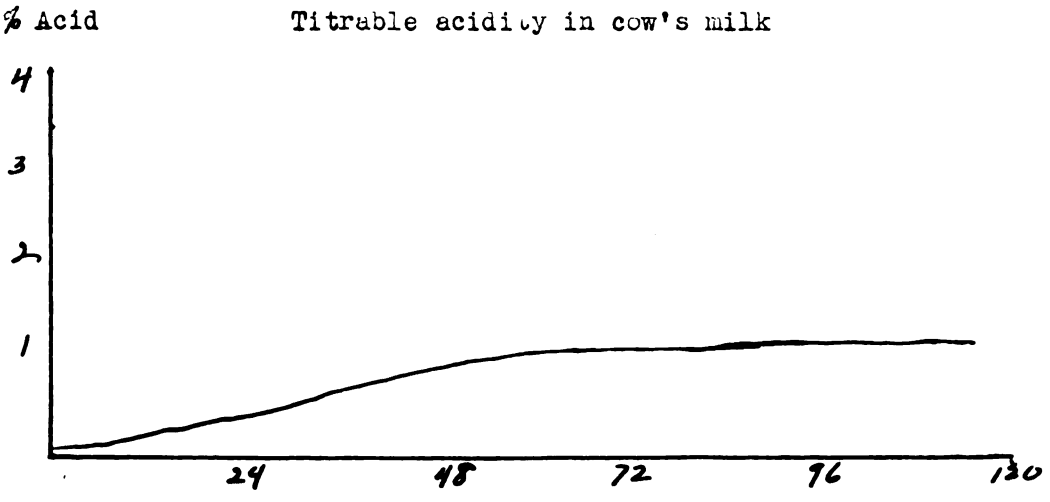
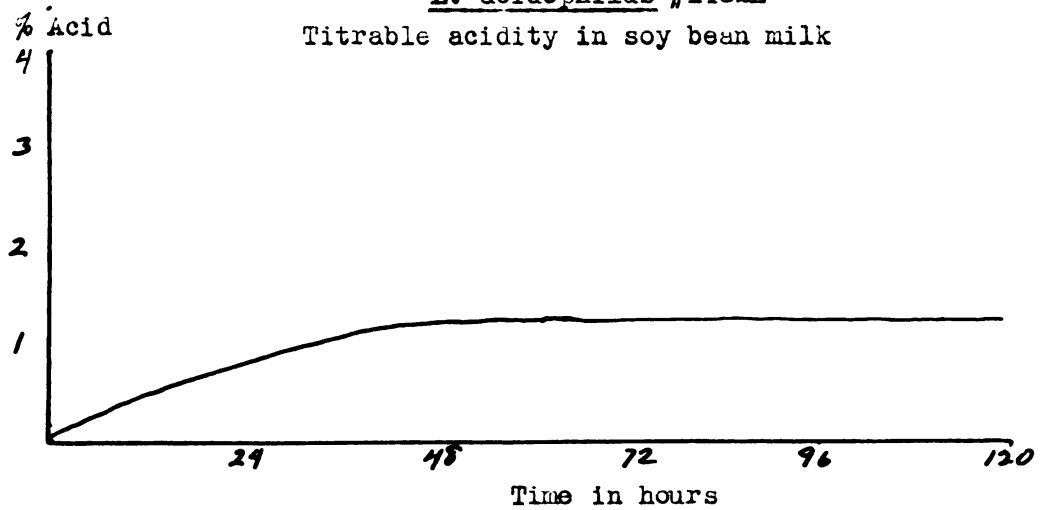


CHART NO. IX

L. acidophilus #1482E



L. acidophilus #1482E



An electrometrical method for determining pH was not available for use during this work, and as the color methods are at best only approximate, some error in readings was probable. However, the readings reported, being in most cases the result of three or more tests, are as accurate as the method employed.

The indicators used were Brom Phenol blue, with a pH range of 3.0 to 4.6, Merck's universal indicator (pH 4.4 to 9.0), and Methyl red indicator (pH 4.4 to 6.0). These overlap at either extreme and so were valuable in checking readings ranging in these regions.

The counts made from soy bean milk were in every way similar to corresponding counts made from cow's milk and were in some instances slightly higher. The titrable acidity of soy milk was in nearly all cases lower than those reported on cow's milk. The writer is not able to explain why this should be, as a complete chemical analysis of soy bean milk was lacking. Others working in the laboratory believe that the milk contains a buffer solution which keeps the free titrable acid at a low point. This explanation is tentative, and a better one in the author's opinion is that the lactose content in the soy milk (2% to 3%) was much lower than that of cow's milk, which ranges from 4% to 5%, but at the same time is in a much more readily usable state. This allows the lactobacilli to get a good growing start,

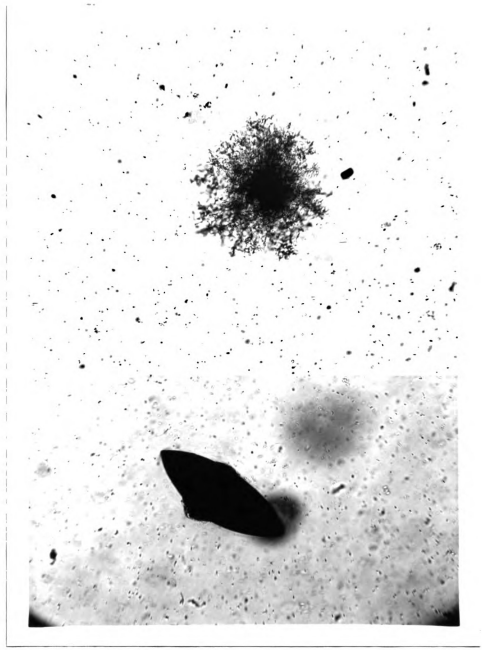
but the supply of lactose is soon exhausted and thus the acid content remains low. A peculiarity in taste is also noted here. While the total titrable acidity in the soy bean acidophilus milk is low the taste is much more sour than that of cow's milk of even higher acidity. There seems to be no explanation for this.

Many writers report that after six or more days in the icebox few acidophilus colonies appear on plates made from cow's milk cultures. This indicates that death of the lactobacilli is rapid in this medium. Soy bean milk under the same conditions demonstrates a much better keeping quality. Flasks containing L. acidophilus #1482E gave counts of more than a million after three weeks. In one case a flask of this culture gave a count of 800,000 per c.c. after six weeks in the refrigerator (ice box type).

A serviceable litmus soy bean milk can be prepared for use in the laboratory if so desired. The action of the lactobacilli in this medium is not the same as that typical for cow's milk, the whole medium becoming a brownish red instead of showing the pink and white areas. The reason for this is that the curd formed is very fine and does not acquire the rigidity that is produced in cow's milk. Precipitation is complete and the whey is not retained in the curd but is allowed to rise to the surface of the medium.

PLATES

Plate No. 1

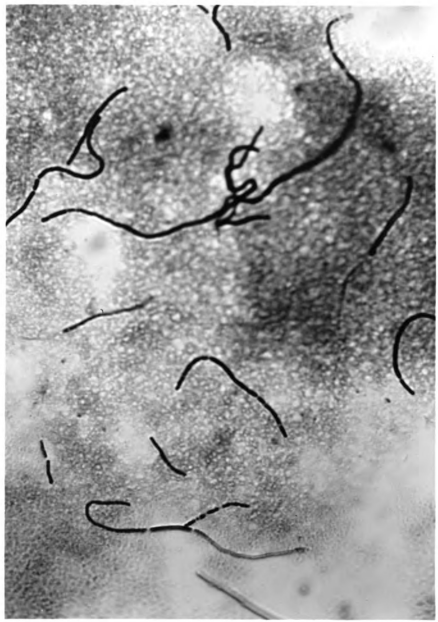


A typical acidophilus colony #1

An atypical colony #1482E

Magnification 75 x

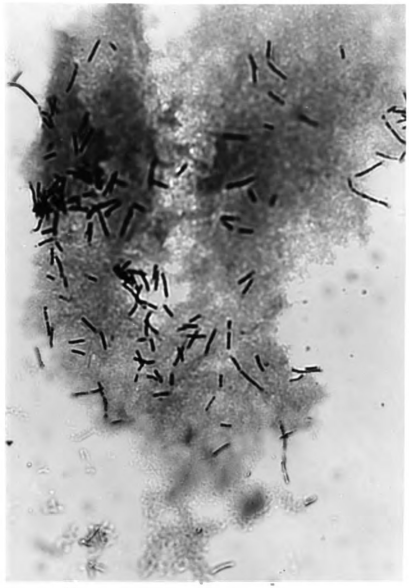
Plate No. 2



L. acidophilus #1 in soy bean milk

Magnification 1150 x.

Plate No. 3



L. acidophilus #1 in cow's milk

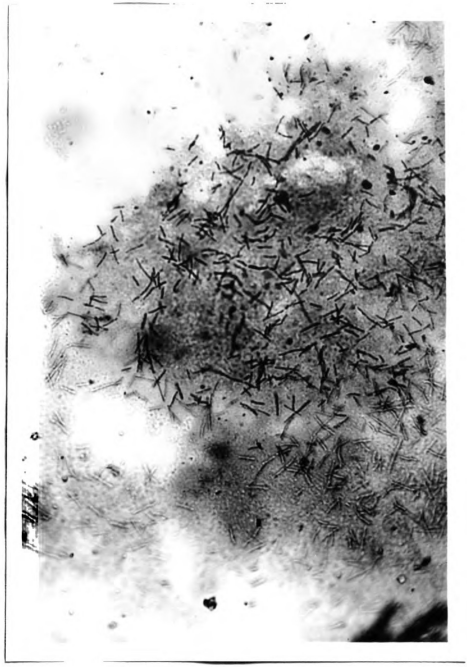
Magnification 1150 x.

Plate No. 4

L. acidophilus #1482E in cow's milk

Magnification 1150 x.

Plate No. 5



L. acidophilus #1482E in soy bean milk

Magnification 1150 x.

SUMMARY

1. Raw soy bean milk is not a good medium for the culture of the lactobacilli, but needs the addition of from 2% to 3% lactose.
2. Extremely high counts were found on plates counted by the microscopic method.
3. Counts made on cow's milk and soy bean milk were in most cases similar.
4. Soy bean milk coagulates more rapidly and at a lower titrable acidity than does cow's milk.
5. Certain strains of L. acidophilus show an increase in cell size and lengthening of the chain when grown in soy bean milk.
6. L. acidophilus #1482E behaves irregularly in soy bean milk both in growth and acid production.
7. Tomato whey is a good plating medium in which to culture the lactobacilli.

LITERATURE CITED

1. Kopeloff, N., "Lactobacillus Acidophilus", 1926, pg. 195 (Appendix).

2. Breidigen and Chang: A micromethod for the enumeration of micro-colonies. Jour. of Lab. and Clin. Med. 10: No. 11, p. 931.

3. Hammer, Bernard W., "Dairy Bacteriology", 1928, pgs. 22-24.