THERMO-TOLERANT ORGANISMS AS A CAUSE OF SO-CALLED PIN-POINT COLONIES[1]

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Received for publication August 5, 1926

INTRODUCTION

During the past decade the term "pin-point colony" has crept into bacteriological literature as descriptive of a certain type of small colony appearing on agar plates, especially in connection with milk analysis. Unfortunately the term is not well defined, but merely implies the existence of small colonies. Small colonies may be due to a wide variety of causes such as over crowding of plates, improper conditions of incubation, media requirements, etc., and not necessarily the result of the presence of a new and distinct type of bacteria in dairy products. If the term pin-point colony is to be used to designate an organism or group of organisms which characteristically produce very small colonies, it should be sufficiently well defined to prevent confusion with small colonies resulting from other causes. It is imperative, therefore, in order to discuss the subject, to come to some understanding as to just what constitutes the pin-point colony problem.

Bacteriologists for many years have observed very small colonies appearing in agar plates in the routine analysis of milk. If the colonies were large enough to count readily with the naked eye or with slight magnification, they were counted along with the other colonies and were given no further consideration. Within recent years, however, considerable complaint has been heard from laboratory workers, to the effect that pasteurized milk very

[1] Contribution No. 84 from the Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan, Kansas.

frequently showed excessively high bacterial counts by the agar plate method. The observation seemed to be unanimous that in such plates from pasteurized milk the majority of the colonies were very small. Some of the colonies were so small that they could be seen only with the aid of a hand lens and would escape observation with the naked eye. The term "pin-point" seemed to be fairly descriptive of these colonies and has come into general use among laboratory workers who examine milk and other dairy products. The important and distinctive feature about these organisms is their ability to survive pasteurization, and not the characteristically small colony which they produce on agar plates. Were it not for the fact that these organisms could survive the process of pasteurization as commonly practiced, they would never have attracted more than a passing interest. The failure to recognize this thermo-resistant characteristic has led to a great deal of confusion in that some workers have described as "pin-points" any organism which possessed the characteristic of producing a small colony.

The pin-point colony problem may be defined as a condition observed in the bacteriological examination of milk, resulting from the presence of microscopic colonies of organisms capable of resisting pasteurization at 145°F. for thirty minutes. They are characterized by the production of very small colonies on thinly seeded plain agar plates, and are difficult to detect with the naked eye. It will be noted that in this definition the identity of any particular organism is not implied. As will be pointed out later in this paper, it is believed that there are several organisms capable of producing this type of colony under the specified conditions.

The significance and importance of the pin-point colony problem can be grasped at once when it is realized that the presence of such colonies in the agar plates threatens to minimize the value of one of the most widely used procedures in public health laboratories—the plating of milk. The interpretative value of bacterial counts of milk is based on the assumption that the conditions which are conducive to low bacterial numbers are the conditions under which milk should be handled. The large

number of organisms which may be present in a sample of milk do not, in themselves, necessarily render the milk unsafe; they are significant only in that the conditions which favored their introduction and multiplication are not in accordance with commonly accepted ideas of milk sanitation.

The pasteurization of milk usually reduces the bacterial numbers, as determined by the plate method, from 90 to 99 per cent, a fact which is frequently used as concrete evidence of the value of the process. However, if the flora of the milk is constituted of organisms which produce the so-called pin-point colonies, no such reduction will be observed. In fact, cases have been reported in which there was an appreciable increase in bacterial numbers during the process of pasteurization, indicating growth of these organisms at the temperature of pasteurization.

As far as pasteurized milk and dairy products are concerned, the presence of pin-point colonies in the plates may, therefore, lead to an incorrect interpretation of the sanitary quality of the product. Pasteurization is almost universally regarded as a desirable process for improving the safety of market milk, yet in the presence of these organisms, its efficiency could not be judged by the bacterial count. Yet public health officials are accustomed to judge the thoroughness of pasteurization by the per cent of bacterial destruction. Many cities have standards which stipulate the maximum bacterial content of milk prior and subsequent to pasteurization. The market milk plant operator who is unfortunate enough to have these heat resistant types of bacteria well established in his plant, soon finds himself unable to meet the demands of the standards relative to bacterial numbers. In spite of the fact that the milk has been thoroughly pasteurized at 145°F. for thirty minutes, and its sanitary quality has been improved through the destruction of any disease producing bacteria which may have been present, the total bacterial count may still be nearly as high as that of the raw milk. When the milk is judged on a basis of its bacterial count, the improvement in its sanitary quality through pasteurization may not be recognized, if these organisms constitute a major part of the flora.

REVIEW OF LITERATURE

Many instances are reported of the isolation of thermo-resistant and thermophilic bacteria from milk. In fact it is possible to demonstrate the presence of such organisms in almost every sample of mixed market milk. The omnipresence of these types is usually considered to be of little significance, since they are usually present in small numbers and seldom find conditions favorable for their rapid multiplication. For a thermophilic organism to be of significance in milk, it must be able to grow rapidly under the conditions under which milk is usually handled. If an organism is capable of growing during pasteurization its presence in the milk supply or the equipment becomes a serious problem.

The literature on thermophilic and thermo-resistant types of bacteria is excellently reviewed by Morrison and Tanner (1922), and for this reason a detailed review of the literature on the thermophilic bacteria is not presented here.

Perhaps the first report of the pin-point colony problem was made by Jacobsen (1918), in connection with a study made in two Oregon dairies. His study of the reasons for wide variations in the bacterial content of milk coming from these two plants revealed the fact that in some cases the pasteurized milk from one of the plants had a higher bacterial count than the raw milk. Pasteurization of some of this milk at 144°F. for forty minutes under laboratory conditions showed bacterial counts of 197,000 before, and 246,000 after heating. The trouble reported by Jacobsen coincided with the experiences of many workers in milk laboratories, and although no definite reports were made, observations of this type were common. At the Kansas Experiment Station, this phenomenon appeared in 1921 in connection with some consecutive pasteurization experiments with ice cream mix.

In an article by Ayers and Mudge (1920) appears the statement: "There are certain types of lactic acid bacteria which grow slowly on extract agar, and their colonies may be either just visible or not show at all after forty-eight hours incubation at 37°C."

At the meeting of the Society of American Bacteriologists in 1922, two papers dealing with this subject were presented. Harding (1923) reported results of bacterial counts on samples taken from a continuous pasteurizer at frequent intervals during the day. A very marked rise was noted during the day's run. Yates (1923) described the high counts obtained from the pasteurized milk supply of Kansas City, Missouri as due largely to the presence of "pin-point colonies." He believed these were associated, and coincident with, the advent of the hydrogen-ion method of media standardization. His data also led him to believe that the outbreaks might have been associated with the use of chlorine solutions in sterilization of equipment. Ayers and Johnson (1923) encountered pin-point colonies in their study of the transportation of heated milk.

A study of the heat resistant organisms surviving pasteurization led Robertson (1923) to conclude that they had little to do with the keeping quality of the milk, since most of his cultures failed to produce sufficient acid to curdle the milk.

The general agitation among laboratory workers who had observed this phenomenon was brought to a focus at the meeting of the Society of American Bacteriologists in New Haven in 1923. Taylor (1924) submitted data showing the gradual increase in the count of pasteurized milk delivered from a continuous flow pasteurizer. Hungerford and Harding (1924) presented figures which also indicated the tendency of milk from a continuous flow pasteurizer to contain large numbers towards the end of the day. Repetition of this experiment for twenty days convinced these investigators that the most probable explanation was that growth of bacteria took place in the pasteurizer.

As a result of incubating 235 samples of milk at 145°F. for 3.5 to 24 hours, Adams and Harding (1924) demonstrated thermophiles in 28.2 per cent of raw, common milk; 43.7 per cent of class A milk, and 40.4 per cent of certified milk.

Harding and Ward (1924) incubated at high temperatures composite samples of pasteurized milk taken directly from pasteurizing tanks. They found that in each of 12 cases there was an increase in the number of bacteria over the count made immediately after pasteurization.

Tanner (1924a) concluded that thermophilic bacteria were not abundant in milk, but that they could be readily demonstrated after an enrichment period of twenty-four hours incubation at 55°C.

Tanner (1924b), in another article, reported the isolation from milk of the thermophiles which were capable of growing at pasteurizing temperatures. He called attention to the likelihood of the presence of these organisms causing misinterpretation of total bacterial counts on pasteurized milk.

Cooledge (1924) believed the appearance of pin-point colonies was associated with the reaction of the media. The same sample of milk plated on two media with reactions of pH 6.6 and 7.3, respectively, resulted in counts of 15,400 per cubic centimeter on the former, and 317,000 per cubic centimeter on the latter medium; the difference being due to the appearance of pin-point colonies. Cooledge further suggested that the appearance of these colonies in low dilutions was due to the ability of the large number of bacteria present to change the reaction of the media sufficiently to permit their development.

Yates and Glover (1924) in a comparison of pre-war media with Bacto-agar found that the latter gave higher counts with pasteurized milk. They believed that the pin-point colony phenomenon was not affected so much by the reaction of the medium as by the ingredients used in its preparation.

Van Horn (1924) found that pasteurized milk showed about three times as many colonies on Difco beef extract agar as when Liebig's beef extract was used. The raw milk, on the other hand, gave counts only slightly higher when the Difco extract was used.

Ayers and Johnson (1924) suggested the name *Lactobacillus thermophilus* for a Gram-positive, aerobic, non-spore-bearing rod which they found to be the cause of an outbreak of pin-point colonies in the analysis of milk from a commercial pasteurizing plant. The organism which they isolated in 37 out of 39 cultures had an optimum temperature between 50° and 62.8°C. and grew rapidly at pasteurizing temperature.

H. G. Harding (1925), in a thesis presented at the University of Illinois gave an excellent review of the literature bearing on the

subject of thermophilic bacteria in milk, as well as on the pin-point colony problem. He found the thermophilic flora of milk taken from the udder to be very small. Harding also found no relationship between the number of bacteria appearing on duplicate plates incubated at 37°, and at 50°C.

Swenarton (1925) expressed the opinion that pin-point colonies might be due to a variety of causes. Observations, based on data obtained in the bacteriological analysis of the Baltimore milk supply, showed that this phenomenon was most prevalent in the spring of the year. Of 52 cultures which this investigator isolated from typical plates, 96 per cent were streptococcus types. He suggested a possible relationship of these organisms to mastitis.

Johnson and Exworthy (1925) made isolations from pin-point colonies and found them to be thermo-resistant streptococci. The cultures isolated survived 62.5°C., and grew between 25° and 50°C. The authors concluded that the thermophilic streptococcus which they isolated was one of the causes of pin-point colonies.

Harding and Ward (1926) did not believe that the appearance of thermophilic bacteria in a milk supply could be attributed to any recognized factor. Their extensive observations, covering many city milk supplies, led them to conclude that the season of the year was not a factor in the pin-point colony problem.

Tanner and Harding (1926) were able to demonstrate thermophilic bacteria in every sample of milk examined. All the cultures which they isolated were motile, Gram-positive, spore-bearing rods which grew well at pasteurizing temperature (62.5°C.).

Fay (1926) observed pin-point colonies in the low dilution plates made in the bacteriological analysis of ice cream. These were shown to be thermo-resistant, but not thermophilic. Morphologically they were Gram-positive, short oval rods, resembling *S. lactis* in arrangement.

Thermo-tolerant organisms differ from true thermophilic types in that they are able to tolerate high temperatures but do not multiply at high temperatures. The so-called thermo-tolerant organisms are capable of withstanding for several hours a temperature which would kill most vegetative cells in a few minutes.

PRELIMINARY OBSERVATIONS

In previous experimental work at this station it has been frequently observed that successive pasteurization of ice cream mix and other dairy products occasionally failed to reduce the number of viable organisms. Approximately the same bacterial count would be obtained after the first pasteurization. The colonies appearing on the plates were very small, sometimes even escaping the observer's notice until examined with a hand lens. Another puzzling observation was the fact that such colonies appeared only on the low dilutions (1:10 and 1:100), none developing on the 1:1000 and higher dilutions. Swenarton (1925), in his study of the pin-point colony problem in the Baltimore milk supply, likewise observed that the highest counts were observed in the low dilutions.

The following is a specific instance representative of many subsequent observations. In examining the agar plates from an ice cream scoring contest, one sample had the rather low count of 2900 bacteria per gram. The 1:10 dilution plates averaged 285 colonies of regular size, the 1:100 dilution plates 30 colonies, and the 1:1000 dilution plates four colonies, the numbers indicating reasonably accurate dilutions. The plates were reëxamined more carefully with a hand lens to verify the low count, and on careful scrutiny it was observed that there were myriads of very small, clear colonies in the 1:10 dilution plates, which had entirely escaped previous notice. An attempt to estimate the number of small colonies indicated that there were approximately 20,000 of them in each plate. Examination of the 1:100 dilution plates showed that there were about 3000 of the small colonies present, which checked fairly well with the 1:10 dilution, in view of the fact that it was not feasible to make an actual count. From this it would be reasonable to expect approximately 200 to 300 of the small colonies to appear on the 1:1000 dilution, and 20 to 30 on the 1:10,000 dilution plates. On very careful examination of these plates, however, not a single pin-point colony could be found. Obviously, there must have been 200 to 300 organisms introduced into the 1:1000 plate, since the regular sized colonies

were present in expected numbers. For some reason, however, conditions were not favorable for the development of the organisms responsible for the pin-point colonies in the higher dilutions. This chance observation changed the count on the ice cream from 2900 to 300,000 per gram and, incidentally, made a difference of 20 points in the score of the product in the contest. This ice cream was made in one of the largest and best equipped ice cream plants in Kansas. Sanitary conditions were excellent and every effort was made to produce ice cream with a low bacterial content. It was the practice in this plant to pasteurize the mix at 165°F. for twenty minutes. In view of the special care given this contest sample it is reasonable to assume that the organisms survived pasteurization and did not gain access during subsequent handling. A sample of the same ice cream was submitted to another laboratory before the contest, and a bacterial count of 2400 per cubic centimeter was reported. Many such instances have been observed in connection with the work at this station and, no doubt, have been noted by many workers elsewhere.

Apparently there is something in the low dilution plates which supplies the requirements for the growth of pin-point colonies that is not present in high dilutions. Re-plating such samples on plain agar, to which had been added carbohydrate in the quantity which would be introduced with a 1:100 dilution of ice cream mix, made the conditions suitable for the development of the expected number of pin-point colonies in the high dilutions. When the amount of carbohydrate added was materially less than the quantity introduced with a 1:100 dilution of ice cream mix, the requirements for growth were not satisfied. This explains the development of 3000 small colonies on the 1:100 dilution in the example just cited, and the failure of the 200 to 300 expected colonies on the 1:100 dilution to appear. The addition of comparable quantities of gelatin, casein or butter fat did not induce growth of pin-point colonies in the high dilutions.

Attempts to grow these organisms at the temperature of pasteurization (63°) repeatedly failed with all the cultures isolated. They would survive the temperature of pasteurization,

however, for several hours. Milk inoculated with them could be repeatedly pasteurized without material reduction in bacterial numbers. In that they did not grow at pasteurizing temperature they do not correspond to the description which Ayers and Johnson (1924) give of pin-point colonies, yet their ability to survive pasteurization, and other typical characteristics which they possess render them a part of the pin-point colony problem.

Advantage was taken in isolating these organisms of their ability to survive several pasteurizations. Samples of milk and other dairy products were pasteurized in flasks by means of a water bath at 63°C. for thirty minutes, and cooled to room temperature. This was repeated several times until, in some cases, the samples had been pasteurized nine times. In all cases they were pasteurized at least three times. Agar plates were made before and after each pasteurization. From these the presence of heat resistant pin-point types could be detected, and if found they could be readily isolated.

Before the first pasteurization, a portion of the milk was set aside in an incubator at 63°C. for approximately eight hours, after which it was plated on carbohydrate media. By this method it was hoped to detect not only organisms which were sufficiently thermo-tolerant to survive several pasteurizations but also organisms which were sufficiently thermophilic to grow at pasteurizing temperature. The first attempts resulted in the isolation of several "pin-point" cultures from the pasteurized milk.

Duplicate plates were made from the incubated milk and one of these was incubated at 63°C. and the other at 37°C. The results on both plates showed the presence of thermophilic bacteria producing large colonies which developed on both plates; but none of the typical "pin-point" types could be found. Enrichment periods of twenty-four and forty-eight hours at 63°C. were then tried, but with the same results. After failure to isolate "pin-point" types from about 20 samples, the temperature of enrichment was reduced to 56°C., and later to 47°C., with much better results. After isolating from several samples, both by the consecutive pasteurization method, and also by enrichment

at 47°C., it was noted that by the latter method all the samples containing the desired types could be detected. Since it involved much less time and material, the enrichment method was adopted in all succeeding work. Small colonies which conformed to the general idea of "pin-points" were selected and inoculated into litmus milk or brom-cresol-purple milk.

EXPERIMENTAL WORK

Resistance of isolated cultures to pasteurization

The isolated cultures were inoculated into flasks of perfectly fresh whole milk which had been previously heated for thirty minutes at 100°C. From 3 to 5 cc. of a twenty-four-hour milk culture of "pin-points" were added to 100 cc. of heated fresh milk. After thorough agitation the inoculated milk was sampled for plating, then pasteurized in a carefully controlled water bath at 63°C. for thirty minutes. In order to approximate vat pasteurization more closely, the heating and cooling of the samples were conducted in such a manner that it required twenty minutes to reach 63°C. and twenty minutes to cool to room temperature. The samples were agitated during the heating period. As soon as one pasteurization was completed and the milk cooled, the flasks were sampled for plating, and the pasteurization immediately started again. One of the cultures was pasteurized in this manner eight times, five were pasteurized seven times, and the remainder were pasteurized three times. Referring to table 1, which shows the heat resistance of these cultures to pasteurization, it will be noted that an average of 99.84 per cent of the organisms survived the first pasteurization, 72.01 per cent of the second, and 46.33 per cent the third. When pasteurization was continued with six of the cultures, 37.3 per cent of the organisms survived the fourth pasteurization; 26.6 per cent the fifth; 15.2 per cent the sixth, and 4.5 per cent the seventh pasteurization. The sample which was pasteurized eight times was sterile.

It will be noted that in some cases the results show an apparent increase in bacterial numbers after pasteurization. With one possible exception (no. 17), it is believed that these are within the

TABLE 1

Effect of successive pasteurizations on pure cultures from pin-point colonies

EXPERIMENT NUMBER	PER CENT SURVIVING AFTER							
	First pasteurization	Second pasteurization	Third pasteurization	Fourth pasteurization	Fifth pasteurization	Sixth pasteurization	Seventh pasteurization	Eighth pasteurization
1	94.8	30.1						
2	46.6	21.3	20.0					
3	114.2	115.4	83.3					
4	54.5	31.8	0.6					
5	111.1	100.0	10.0					
6	104.6	100.0	115.3					
7	105.2	105.2	13.1					
8	88.8	96.2	103.7	74.0	70.3	70.3	11.1	
9	69.6	9.0	0.1					
10	100.0	63.6	36.3					
11	111.7	64.7	58.8					
12	81.2	68.7	68.8	31.2	9.3	1.8	3.1	
13	100.0	100.0	40.0					
14	90.9	63.6	54.6	13.6	3.6	0.1	0.0	
15	127.7	66.6	77.7	77.7	66.7	22.2	11.1	
16	84.6	46.1	5.3					
17	105.8	82.3	223.5					
18	60.0	50.0	40.0	13.0	4.0	0.8	1.6	
19	100.0	85.7	0.1					
20	100.0	85.7	35.7					
21	83.3	75.0	75.0					
22	85.7	71.4	11.4					
23	100.0	100.0	1.3					
24	84.2	68.4	9.4					
25	100.0	60.0	30.0					
26	68.0	72.3	78.7					
27	100.0	100.0	41.6					
28	106.0	69.6	9.0					
29	112.5	75.0	12.5					
30	133.3	53.3	60.0					
31	100.0	83.3	3.0					
32	100.0	100.0	100.0					
33	100.0	71.4	71.4					
34	88.2	29.4	22.0	14.7	4.4	0.7	0	0
Average..	99.84	72.01	46.33	37.36	26.68	15.20	4.52	0

limits of experimental error. Comparatively wide variations were frequently unavoidable due to the fact that the colonies were small and difficult to see. In culture no. 17 an increase of 223.5

per cent is reported after the third pasteurization. This at first was regarded as evidence of the ability of the culture to grow during pasteurization. However, later attempts to culture the organism at 63°C. repeatedly failed.

After the first pasteurization, there were 20 out of 34 cultures in which the number of organisms was not reduced at all, and eight of these were not affected by the second pasteurization. After the third pasteurization, however, many of the cultures began to decrease in number. Only eight of the cultures had more than 75 per cent of the original number after the third heating, and 19 of them had lost more than half their number. It is interesting to

TABLE 2

Frequency of isolation of pin-point colonies from dairy products

PRODUCT	NUMBER OF SAMPLES	ISOLATION OF PIN-POINTS		PER CENT SUCCESSFUL
		Successful	Unsuccessful	
Cream	35	20	15	57.1
Whole milk	56	21	35	37.5
Skim milk	4	3	1	75.0
Ice cream	2	2	0	100.0
Totals	97	46	51	47.4

note the continued resistance of two of the cultures (nos. 8 and 15) to the fourth, fifth and sixth heatings. The organisms included in this study unquestionably resist pasteurization, but with the possible exception of culture no. 17, there is no evidence in these results which indicates that they would grow at pasteurizing temperature. If thermo-resistant types, similar to the organisms isolated in this work, should dominate the flora, the bacterial count would not be appreciably reduced by pasteurization. The average efficiency of pasteurization as reported in table 1 was only sixteen-hundredths of one per cent for the first pasteurization. If organisms of this type should become established in a pasteurizing vat, even its continued use throughout the day would not successfully destroy all of them.

High bacterial counts, on dairy products which have been

carefully pasteurized, may be readily accounted for by the presence of such thermo-tolerant organisms. That these organisms are comparatively common in dairy products is indicated in table 2, which shows that they were isolated from 57 per cent of the cream samples, from 37 per cent of the whole milk samples and from 47 per cent of the total number of samples tested.

The per cent of the flora which is made up of these heat resistant types is shown in table 3 which gives the average total bacterial count on sucrose agar of 12 samples of milk before and after each of three successive pasteurizations. It will be noted that on the average, "pin-points," capable of resisting two successive pasteurizations, constituted about 12.2 per cent of the original total count.

TABLE 3

*Per cent of "pin-points" found in twelve samples of milk after successive pasteurizations**

BEFORE PASTEURIZING, TOTAL COUNT PER CUBIC CENTIMETER	AFTER FIRST PASTEURIZATION, "PIN-POINTS" PER CUBIC CENTIMETER	PER CENT OF ORIGINAL TOTAL	AFTER SECOND PASTEURIZATION, "PIN-POINTS" PER CUBIC CENTIMETER	PER CENT OF ORIGINAL TOTAL	AFTER THIRD PASTEURIZATION, "PIN-POINTS" PER CUBIC CENTIMETER	PER CENT OF ORIGINAL TOTAL
8,063,000	1,006,000	12.47	990,000	12.27	716,000	8.88

* Seven of these samples were pasteurized nine successive times. The reduction was marked after the fourth and fifth pasteurization, and only 0.37 per cent of the total count remained after the seventh pasteurization.

As previously pointed out, the pasteurizations in the foregoing experiments were conducted under laboratory conditions by means of flasks submerged in a water-bath. It was deemed advisable to determine whether the same results would be obtained in the pasteurization of larger volumes of milk in a vat under plant conditions. In coöperation with Prof. W. H. Martin of the Kansas Experiment Station, a 50-gallon rotary coil vat of skim milk was inoculated with several strains of pure cultures of "pin-points." After the inoculated milk was thoroughly agitated to insure equal distribution of the inoculum, about five gallons were drawn off into a sterile can for another part of the experiment. The remainder was sampled before and after each of

eight successive pasteurizations. The results are shown in table 4.

The 5-gallon portion of the inoculated milk previously mentioned was placed in a water bath and held at 63°C. for eight hours. Samples were taken each hour and plated immediately on sucrose agar. The results are shown in table 5.

An examination of the data in tables 4 and 5 shows that the cultures retained their thermo-resistant characteristics under vat conditions. More than 85 per cent of the organisms survived the first pasteurization, 28 per cent the second pasteurization, and 21

TABLE 4

Effect of eight successive vat pasteurizations of milk containing "pin-points"

Per cent of "pin points" surviving

FIRST PASTEURIZATION	SECOND PASTEURIZATION	THIRD PASTEURIZATION	FOURTH PASTEURIZATION	FIFTH PASTEURIZATION	SIXTH PASTEURIZATION	SEVENTH PASTEURIZATION	EIGHTH PASTEURIZATION
85.7	28.5	21.4	12.8	4.2	0.7	0	0

TABLE 5

The same milk used in table 4 held at pasteurizing temperature constantly for eight hours

Per cent surviving

FIRST HOUR	SECOND HOUR	THIRD HOUR	FOURTH HOUR	FIFTH HOUR	SIXTH HOUR	SEVENTH HOUR	EIGHTH HOUR
17.4	5.28	1.4	0.1	0.01	0.0004	0	0

per cent were still alive after three pasteurizations, but after seven pasteurizations all were dead. Table 5 shows that some of the organisms were sufficiently heat resistant to withstand continuous exposure to 63°C. even for six hours.

It was hoped by these two experiments to simulate (1) the thermal conditions existing in a pasteurizing vat which is in constant use throughout the day, and (2) the thermal conditions of a continuous pasteurizer in eight hours use. The results in tables 4 and 5 emphasize the fact that these organisms will not grow under these conditions, although, their resistance to heat enables them to persist in either type of pasteurizing equipment for several

hours. The use of pasteurizing equipment, pipelines, separators, etc., for dairy products heavily seeded with thermo-resistant organisms, would necessitate thorough cleaning after each operation in order to prevent contamination of succeeding batches.

In an effort to find sources of pin-point colonies for further study, milk samples were subjected to various temperature conditions in the hope of stimulating the growth of these organisms. The results reported in table 6 further substantiate the conclusion that these organisms do not develop at pasteurizing temperatures. None of the plates from the samples before heating gave any evidence of pin-point colonies, therefore, the counts before heating represent the total counts without reference to any special kind of colony. The results after heating, however, represent the number of pin-point colonies and do not include any of the other thermophilic types which appeared on the plates. Table 6 includes only the samples from which pin-point colonies were isolated and the figures are averages of the results from all the samples involved. Successful isolations were not obtained in the case of the 17 samples held at 63°C. for twenty-four and forty-eight hours, but they are included in this table merely to show the effect of pasteurizing temperature. There were, however, many large surface colonies of thermophilic organisms on the plates

TABLE 6

Effect of holding milk at high temperatures on the development of pin-point colonies

NUMBER OF SAMPLES	TREATMENT OF THE MILK	AVERAGE NUMBER OF BACTERIA BEFORE TREATMENT	AVERAGE NUMBER OF "PIN-POINTS" AFTER TREATMENT	PER CENT OF THE ORIGINAL NUMBER
5	Held at 47°C.— 8 hours	3,420,000	40,240,000	1,176.0
3	Held at 47°C.—16 hours	226,500	1,260,000,000	556,291.0
3	Pasteurized three times then held at 47°C.—16 hours	25,230,000	19,673,000	78.0
3	Held at 56°C.— 8 hours	99,800,000	1,855,000	18.9
2	Held at 56°C.—16 hours	6,600,000	242,000	3.6
17	Held at 63°C.—24 hours	168,000	0	0
17	Held at 63°C.—48 hours	168,000	0	0

made from milk incubated at 63°C. Isolations from these colonies resembled the spore bearing rods described by previous investigators, but when cultivated at 37°C. they either refused to grow at all or continued to produce large, spreading, surface colonies. Throughout the progress of this work continuous effort was made to isolate types of organisms which produce pin-point colonies and grow in milk at the temperature of pasteurization. From nearly all samples of milk large colonies of thermophilic types were isolated but only under conditions of overcrowding of plates, could they be induced to produce small colonies.

The effect of different media on the growth of organisms which produce pin-point colonies

It has been suggested that the cause of pin-point colonies could be traced to the kind of medium which has been used since the war. Previous to the German blockade, Witte's peptone was very largely used in this country for the preparation of media. At that time also it was the common practice to adjust the reaction of media according to the Fuller scale. During and since the war, however, American peptones have come into greater favor for use in media, and the reaction of media is now almost exclusively adjusted according to the hydrogen-ion concept. The concurrence of these changes in methods with the agitation over pin-point colonies, together with data which they had collected, led Yates and Glover (1924) to suggest a causal relationship between the new methods of media preparation and the presence of small colonies in the plates.

Observations made in the Kansas Experiment Station laboratory suggested that at least some of the so called "pin-points" were saccharophilic organisms, and that some were even obligate in this respect. As pointed out earlier in this paper, it was noted that small colonies appeared in large numbers on low dilutions, but failed to appear in the expected numbers in the higher dilutions. The fact that the other colonies of normal size were present in the higher dilution plates in the expected numbers eliminated the probability of failure on the part of the technician

to make the proper dilution. It was further shown in preliminary experiments that if one cubic centimeter of a 1:100 dilution of sterile ice cream or milk were added to the higher dilution plates, the pin-point colonies would appear in the expected numbers.

In order to determine the effect of various kinds of media on the growth of the organisms which had been isolated, 42 cultures, selected at random from about 200 cultures, were plated on 14 different media. Four different kinds of plain agar were prepared as follows: (1) made with Difco peptone and the reaction adjusted to pH. 7.0, (2) the same batch of media except that the reaction established at Fuller's scale, +1, (3) made with Witte's peptone and the reaction established at pH 7.0, and (4) the same medium as no. 3, except the reaction was adjusted to Fuller's scale, +1. These four media are represented in table 7 under columns A, B, C, and D respectively. The analyses reported in columns Am, Bm, Cm, and Dm were obtained from plates to which 0.1 cc. of a 1:10 dilution (0.01 cc. of milk) of sterile milk was added in addition to media A, B, C or D, respectively. The media in A, and Am; B, and Bm; C, and Cm; D, and Dm; were, therefore, identical except that the plates in one case contained the same amount of milk as is found in a 1:100 dilution plate. The Fuller scale media used in this work had a reaction of pH 5.8. In table 7 it will be noted that for nine of the cultures, the milk-media were not used.

In addition to these eight media, simultaneous analyses were made on six other media, viz., glucose, lactose, sucrose, casein, milk powder and whey agars. The carbohydrate media (glucose, lactose, and sucrose) were from the same batch as medium A, except that one per cent of the carbohydrate was added in each case. Casein agar was prepared according to Ayers (1911), except that the reaction was adjusted to pH 7.0. milk powder agar was prepared according to Zoller (1913), and the whey agar according to Bouska and Brown (1921). For economy of space the individual counts on these fourteen media are not given, but the averages for the 42 cultures are included in table 7.

In table 7 is given the per cent of cultures which failed to grow,

TABLE 7

Effect of various kinds of plain agar and other media on the growth of pin-point colonies

	MEDIUM													
	A	Am	B	Bm	C	Cm	D	Dm	E	F	G	K	M	W
Composition of medium	Plain agar Difco pepton pH 7.0	Same as A, plus 0.01 of sterile milk per plate	Plain agar Difco pepton Fuller scale + 1	Same as B, plus 0.01 cc. of sterile milk per plate	Plain agar Wittes pepton pH 7.0	Same as C, plus 0.01 cc. of sterile milk per plate	Plain agar Wittes pepton Fuller scale + 1	Same as D, plus 0.01 cc. of sterile milk per plate	Glucose agar	Lactose agar	Sucrose agar	Casein agar	Milk powder agar	Whey agar
Number of cultures	42	33	42	33	42	33	42	33	42	42	42	42	42	40
Per cent of cultures showing no growth	38.1	3.0	73.8	45.5	50.0	30.3	42.9	42.4	0	0	0	28.6	0	25
Per cent of cultures showing less than half normal growth	16.7	12.1	2.4	3.0	14.3	3.0	11.9	6.1	2.4	0	0	2.4	2.4	7.5
Per cent of cultures showing normal growth	45.2	84.9	23.8	51.5	35.7	66.7	45.2	51.5	97.6	100	100	69.0	97.6	67.5
Average count on all cultures plated	10,400*	6,300	8,800	4,500	10,100	6,400	10,000	7,800	12,500	12,800	12,100	9,500	12,500	11,400

* "000" is omitted from the average.

the per cent which showed less than half normal development, and the per cent which found the medium favorable for their development. Each culture was plated on each of the different media from the same set of dilutions. The plates were incubated forty-eight hours at 37°C. and the colonies counted. All plates showing no growth were examined after forty-eight hours further incubation at room temperature before they were discarded. It will be noted that nearly all the cultures found the carbohydrate media (glucose, lactose, sucrose, and milk powder agar) favorable for their development. The normal growth for each culture was judged by the average number of colonies developing on the carbohydrate media. On some of the less favorable media the organisms either failed to grow at all, or grew only to a slight extent. For example, in some cases where 300 to 400 colonies were expected on a plate, in the less favorable media only 20 or 30 colonies developed. Although this could not be regarded as failure to grow, the small number of colonies, being outside the expected limits or error of the method, indicated a very unfavorable medium. In table 7 such cultures are tabulated as showing less than half normal growth; however, in most of these cases the growth was less than one-fourth and in many cases less than one-tenth the expected development.

A study of the individual counts together with the summary presented in table 7 brings out the following interesting facts.

1. Plain agar in general was unfavorable for the growth of "pinpoints," 38 per cent of the cultures failed to grow on medium A, (Difco peptone, pH 7.0); 73 per cent refused to grow on medium B, (Difco peptone, Fuller scale +1); 50 per cent did not find medium C favorable (Witte's peptone, pH 7.0); and nearly 43 per cent of the cultures could not grow on medium D (Witte's peptone, Fuller scale +1).

2. The most unfavorable medium for the development of pinpoint colonies was medium B, (Difco peptone, Fuller scale +1); only 23.8 per cent of the cultures made normal growth on this medium.

3. The addition of 0.01 cc. of milk to the Petri dish made it possible for many of the cultures which otherwise could not grow

to develop in the plain agar. Media Am, Bm, Cm, and Dm, supported 85, 51, 66, and 51 per cent of the cultures respectively, as compared to 45, 24, 35, and 45 per cent for the same media without the milk. Sixteen cultures failed to grow at all on medium A, but the addition of milk enabled fifteen of these to grow. The amount of milk added to these plates (0.1 cc. of a 1:10 dilution of sterile milk), is the same quantity of milk carried over from the sample in a 1:100 dilution. In this connection it should be pointed out that the dilution of the milk sample under analysis in all these experiments was in no case less than 1:10,000, so that the amount of milk carried over from the sample was not sufficient materially to affect the carbohydrate content of the medium.

4. The carbohydrate in the milk apparently is the constituent which is of benefit to the culture. This was proven by adding the same quantity of lactose (0.01 cc. of a 5 per cent solution) to plates as would be introduced with 0.01 cc. of milk; the substitution of lactose enabled cultures to develop which otherwise could not grow on plain agar. Addition of amounts of butter fat or casein, equivalent to that introduced in 0.01 cc. of milk, failed to supply the requirements for growth on plain agar.

5. All of the carbohydrate media (glucose, lactose, sucrose, and milk powder agar) were favorable for the growth of pin-point colonies. It may be noted that none of the cultures failed to grow on any of these media, and only 2.4 per cent (one culture), made less than half normal growth on glucose and milk powder agars.

6. The colonies on the carbohydrate media were slightly larger than on plain agar. This was especially true of milk powder agar on which the colonies in some cases were nearly twice as large as on plain agar, and usually larger than on the other carbohydrate media.

7. Casein agar, and whey agar were somewhat less favorable media for these organisms, although in general they were better than plain agar. Even in those cases where normal growth was obtained in casein agar, the colonies were frequently even smaller than they were on the plain agar media. The colonies on casein

agar in some cases were too small to be seen with a Leitz binocular stereo-magnifier, using 30× magnification.

8. Whey agar was suitable for the growth of only 67 per cent of the cultures. If the culture would grow at all on whey agar, the colonies were usually as large as on the other kinds of media.

It is quite evident from this table that if it is desired to isolate this type of organism, a carbohydrate medium should be selected. Experience has proved that milk powder agar is the most desirable, since it enables the colonies to become considerably larger. It is also evident that if it is desired to prevent organisms of this type from showing on the plate, plain agar with high acidity should be used.

Even with a relatively unfavorable medium, the cultures are likely to grow in the low dilutions (1:100). The small amount of milk introduced in a 1:100 dilution contained sufficient carbohydrate to enable 51 per cent of the cultures to develop on medium Bm, where as only 24 per cent could develop on the same medium without the milk. It is believed that this fact may account, in part, at least, for the supposed appearance of these organisms after pasteurization. Plates made on the raw milk are likely to be counted on high dilutions, which do not permit the development of all the pin-point colonies, even though the organisms may be in the agar plate. After pasteurization, lower dilution plates are observed for counting and the "pin-points" are detected. This explanation, however, would apply only for the saccharophilic type of "pin-points" which are under discussion.

Destruction of the organisms

The resistance of these organisms to steam and to hypochlorite disinfectants was determined in the following manner. About 2 liters of water were inoculated with 2 to 5 cc. of fresh milk cultures, from each of ten of the most resistant strains in the collection. The water was sampled and found to contain approximately 3 million "pin-points" per cubic centimeter. This water was poured into a series of sterile beakers, and after a few seconds was poured out. The beakers thus contaminated with the organisms were treated with steam or with chemical disinfectant

(B-K) for varying lengths of time, from thirty seconds to five minutes. After treatment, 100 cc. of sterile water was poured into the beaker and subsequently used for plating. The detailed counts are not given because they are only significant in showing the length of time necessary to kill all the organisms. It was found that one minute exposure to steam or the disinfectant used was sufficient to destroy all the organisms left in the beaker.

Biochemical and morphological studies

Sixty-five of the cultures isolated were selected at random and studied morphologically and biochemically. Each culture was inoculated into fermentation tubes containing one of the following: arabinose, starch, dulcitol, glucose, sucrose, glycerol, inulin, lactose, rhamnose, xylose and salicin. Two tubes of Clark and Lubs media were inoculated, one used to determine the Voges-Proskauer reaction, and the other used for the methyl-red test for acid production.

The cultures were also plated on carbohydrate agar in sufficiently high dilutions to give plates containing relatively few colonies; the colonies on these plates were measured, using the low power lens of a microscope equipped with a standardized filar micrometer.

The cultures were studied morphologically for size, shape, arrangement and motility, and also for their reaction to Gram's stain.

Preparation of media for fermentation tests

A modification of Enlow's synthetic sugar-free medium was used for the fermentation tubes. The composition of the medium was the same as given by Enlow (1923) except that the agar was omitted, thereby giving a liquid instead of a semi-solid medium. Brom-thymol-blue was added to the medium before sterilization (1.2 per cent of a 0.2 per cent alcoholic solution) according to the method devised by Baker (1922). This medium was standardized to a reaction of pH 7.2 before sterilization. The medium was placed in Durham fermentation tubes and sterilized in the autoclav. One per cent of the various sugar and other test

solutions was subsequently added aseptically. All fermentation tubes were incubated forty-eight hours at 37°C. before inoculation, and thirty days after inoculation.

The tubes were inoculated from a twenty-four-hour milk culture by means of a very small (1 mm.) platinum loop. In order to be sure that the amount of lactose carried over with the milk was not sufficient to permit the production of acid in the medium, a control tube of sugar-free medium was inoculated using the same loop. In no case was there any acid production or even growth in the control tube. The organisms were so strictly saccharophilic that they would not develop at all in the sugar-free medium, and, therefore, grew only in those tubes in which the sugar added could be utilized as a source of energy.

TABLE 8

Per cent of 55 cultures giving positive reactions to various carbohydrate fermentations and other tests

ARABINOSE	STARCH	DULCITOL	GLUCOSE	SUCROSE	GLYCEROL	INULIN	LACTOSE	RHAMNOSE	SALICIN	XYLOSE	VOGES-PROSKAUER	METHYL-RED	GELATIN LIQUEFACTION	MOTILITY
20	40	0	100	80	0	0	100	0	36	0	0	29	0	0

Results of fermentation studies

None of the 55 cultures fermented dulcitol, glycerol, inulin, rhamnose or xylose. None of the cultures produced gas in any of the fermentation tubes. All the cultures fermented glucose and lactose with the formation of acid. The percents of the cultures fermenting the other substances are given in table 8.

Colony study

The cultures used in the fermentation studies were also plated on carbohydrate agar, in order to study the size and shape of the colonies, in which case it was found that the small size of the colony was a constant character. The sizes of the colonies were measured by means of a filar micrometer standardized for a low power lens of a microscope.

Two distinct groups of colonies were recognized, which, for sake of convenience, will be called type A, and type B. Type A was a very small spindle shaped colony, too small to be seen with the naked eye, and barely visible with a hand lens. The size of the average was 0.150 to 0.175 mm. in the longest dimension. In dealing with these colonies, it was not infrequent to regard plates as sterile, which, on more careful examination, were found to contain 200 to 300 colonies. In the original isolation of the cultures used in this work, it was frequently difficult to decide whether or not some of the colonies isolated were small enough to be classed as "pin-points." The standards used in isolation were (1) that the organism should be at least thermo-tolerant, if not thermophilic, and (2) that it should characteristically produce a very small colony on carbohydrate media in thinly seeded plates. When small colonies were found which were slightly larger than typical pin-point colonies, yet sufficiently small to be doubtful, isolations were made, but in these cases the fact was recorded that the colony was atypical either in size or some other respect. Subsequent plating of these cultures proved that the slightly larger size of the colony was a constant characteristic of the organism. They were accordingly grouped together and called type B colonies. The size of the average was 0.250 to 0.350 mm. in the longest dimension of the spindle shaped colonies. Although these colonies were nearly twice as large as the type A colonies they were barely visible with the naked eye, but easily counted with the aid of a hand lens.

When the results of the fermentation studies were compared with the two types of colonies, A, and B, the following observations were made.

1. Those cultures which produced a very small colony, not visible with the naked eye, and difficult to see even with a hand lens (type A, about 0.150 to 0.175 mm.), were characterized by their inability to ferment salicin or arabinose, by their ability to ferment sucrose, and by the fact that they usually gave a methyl-red negative test.

2. Those cultures which produced a slightly larger colony, barely visible with the naked eye, and easily counted with a hand

lens (type B, size 0.250–0.350 mm.) were characterized by inability to ferment sucrose, by ability to ferment salicin or arabinose, and by a methyl-red positive test.

Each culture was examined under the hanging drop for motility, and all were found to be non-motile.

All the cultures were non-spore-bearing, Gram-positive rods, but varied considerably in size and arrangement. None were distinctly round coccus forms, although many resembled *Streptococcus lactis*, being very short rods or elongated spheres in pairs and short chains.

The cultures were divided into three morphological groups, the descriptions of which follow:

Group no. 1. Short rods, with pointed ends, mostly in pairs, and short chains, rarely exceeding four cells in length. The size of the average was 1.06 by 0.71 micron. This type resembled in appearance *Streptococcus lactis*, the common milk souring organism.

Group no. 2. One of the most characteristic morphological groups was a chain of irregular shaped rods. The chains consisted of from four to ten irregular shaped cells. One cell in the chain might be a distinct rod, adjacent to it might be a perfectly round cell, while adjacent to the spherical cell, perhaps, would be found a club shaped rod with one bulging and one tapering end. In another part of the chain one would likely find a pair of very regular, rod shaped cells, having a distinct diplo-bacillus arrangement, not at all like its clubbed or spherical neighbors. It was a most peculiar arrangement, suggesting the probability of involution forms resulting from unfavorable media or growth conditions. The arrangement, however, was quite consistent on all media capable of supporting growth. On carbohydrate broth, the chains were longer and slightly less irregular, but conforming to the above description.

Group no. 3. Another characteristic shape and arrangement noted in these cultures was a long, rather narrow rod, appearing in pairs, but one of the cells much shorter than its mate. It had the appearance of a terminal daughter cell, but if it were a daughter cell, no cases were observed in which the younger cell had grown so as to approximate the size of the mother cell. The size of the average was 1.95 by 0.78 microns.

Upon analysis of the data, it was found that the morphology of the organisms from type A colonies was distributed as follows: 42 per cent belonged to group no. 1, 42 per cent to group no. 2, 10 per cent to group no. 3 while the remaining 6 per cent did not conform to any of the groups and were dissimilar to each other. Of the type B colonies, 70 per cent belonged to the morphological group no. 1, 20 per cent to group no. 2, and the remaining ten per cent did not conform to any of the groups and were dissimilar to each other.

Disregarding the few cultures which did not conform to the three morphological groups it would seem that there are three kinds of organisms chiefly responsible for the pin-point colonies encountered in this study, each of which may be divided into two sub-groups.

1. Organisms resembling *Streptococcus lactis* morphologically: (a) Methyl-red negative, ferments sucrose, but does not ferment salicin or arabinose, produces a very small colony which is too small to be seen with the naked eye, but visible with a hand lens; (b) methyl-red positive, does not ferment sucrose, but ferments either salicin or arabinose, and produces a colony which is comparatively very small, but may be seen with the naked eye.

2. Organisms having a very irreglar morphology, appearing in chains of four to ten cells: (a) and (b) as described above.

3. Slender rod shaped cells appearing in pairs, with one cell much shorter than its mate: (a) and (b) as described above.

The fact that each of the three morphological groups is about equally divided among the subgroups (a) and (b) which produce the large and the small colonies respectively and that each of these subgroups gives the same general biochemical reactions, would indicate that there are two forms of each of the three kinds of organisms.

DISCUSSION AND SUMMARY OF RESULTS

In this paper an effort has been made to outline the pin-point colony problem and to study the bacteriological aspects of the causative organisms in at least one phase of that problem. It is

not contended that the organisms studied in connection with this investigation are the sole cause of pin-point colonies. Other workers have described spore-bearing rods, capable of developing at the temperature of pasteurization which have nothing in common with the types herein reported, except the size of the colony produced. It is entirely reasonable to assume that there are many organisms responsible for pin-point colonies which, as yet, have not been studied.

It was through a search for the thermophilic spore-bearing rods described by other workers that this investigation was started. Every sample of milk and cream studied was incubated under conditions which made possible the development of such types. Many and varied forms of thermophilic spore-bearing rods were isolated, but when cultured at 37°C., would not produce pin-point colonies. It is believed, however, that the organisms herein described do account for the pin-point colonies encountered in previous work in this laboratory, and may perhaps account for the conditions observed elsewhere.

The fact that these organisms develop only on low (1:100) dilution plates when plain agar is used, together with the fact that they are very difficult to detect, renders it very easy to miss them entirely in thickly seeded plates of raw milk or raw cream. On the other hand, after pasteurization the 1:100 dilution plates will very likely contain relatively fewer colonies of normal size, and the counting of such plates might reveal several thousand pin-point colonies. Several samples of milk from which pin-point colonies were isolated after pasteurization, did not show the presence of these types in the raw milk. The 1:100 dilution plates from raw milk in some cases were so thickly seeded as to mask the presence of the extremely minute types, and in other cases, where the bacterial count of the raw milk was very high, the low dilution plates were so thickly seeded that the growth of "pin-points" was completely inhibited. After pasteurization the larger colonies, which previously dominated the low dilution plates, had been killed and the "pin-points" were more easily detected.

Many times during this investigation high counts, due to pin-

point colonies, were obtained on pasteurized milk, and the "pin-points" were not detected in the routine analysis of the raw milk. The inference was that the organisms had developed during pasteurization. However, attempts to culture the organisms in milk at 63°C. repeatedly failed. Usually such cultures would survive one or two hours exposure at 63°C., but quantitative determinations indicated gradual diminution of numbers instead of growth. The conclusion was, therefore, drawn that the organisms responsible for the pin-point colonies encountered in this work were thermo-tolerant, but not thermophilic. Tanner (1924) emphasized the necessity of differentiating between thermo-tolerant and thermophilic bacteria. It is believed that some, though indeed not all, of the so-called increases in count after pasteurization may be explained on this basis. Those cases in which the pin-point colonies have definitely been proved to be the result of spore-bearing, thermophilic rods, capable of growing at 63°C., no doubt represent another phase of the problem.

In this work it has been demonstrated that in 47.4 per cent of the samples studied, thermo-tolerant, saccharophilic organisms were present which characteristically produced pin-point colonies. The resistance of these cultures to pasteurization is shown by the fact that, on the average, 99.84 per cent of the organisms survived the first pasteurization, 72 per cent the second, and 46 per cent the third. There was no conclusive evidence that any of these could grow during pasteurization, even though some of the samples were pasteurized seven times successively. The isolated cultures failed to develop at temperatures above 47°C.

A study of the effect of various kinds of media on the growth of these organisms showed several interesting observations as follows: Media containing a very small amount of carbohydrate greatly increased the chance of "pin-points" appearing on the plate. The small amount of lactose carried over in a 1:100 dilution of milk adds sufficient sugar to the medium to permit many of the types to develop that could not grow on higher dilution plates. Thirty-seven per cent of the isolated cultures grew normally on plain agar, but the addition of 0.01 cc. of

sterile milk to the plate enabled 64 per cent of the cultures to grow. Casein agar and whey agar were also somewhat unfavorable for the growth of "pin-points," but more favorable than plain agar. The standardization of the reaction of media to Fuller scale +1, frequently, though not consistently, resulted in a sufficiently acid medium to discourage the development of "pin-points." There was no consistent marked preference for Difco or Witte's peptone.

The most resistant pin-point cultures isolated were killed by heating in the steamer one minute or by treating for an equal time with a commercial hypochlorite disinfectant.

Differentiation of these thermo-tolerant types was made at the time of isolation on a basis of the size of the colony produced. Biochemical studies showed that the smaller type which were visible only with a hand lens (type A) were characterized by fermentation of sucrose and failure to ferment arabinose, or salicin, and a methyl-red negative reaction. Those types which were also thermo-tolerant but produced a colony large enough to be detected with the naked eye (type B), were characterized by ability to ferment arabinose or salicin, inability to ferment sucrose, and by a methyl-red positive test.

Morphologically all of the cultures were found to be non-motile, Gram-positive, non-spore-bearing, short rods or oval cocci resembling short rods. Although a few cultures were found which did not conform, most of the cultures were classified into three morphological groups: (1) very short oval rods or elongated cocci, resembling *Streptoccoccus lactis;* (2) short chains, four to ten cells in length, having very irregular morphology, and (3) short rod shaped cells in pairs one of the cells being much shorter than its mate.

If the organisms are resistant to pasteurization, their subsequent destruction in milk is hopeless, and the solution of the "pin-point" problem must be through preventive measures. Some have suggested that a new medium be devised which would not permit the development of pin-point colonies; others have implied that their presence in the plate should simply be ignored. It should be borne in mind that the premises on which

bacterial counts are based are, that the conditions which make possible the entrance and development of large numbers of bacteria are not ideal conditions for the production and handling of such a delicate food. If milk contains large numbers of bacteria, regardless of whether they are "pin-points" or not, it indicates that the milk has not received proper attention. Deliberately to overlook the presence of certain types would be to ignore the undesirable conditions responsible for their presence.

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