A STUDY OF HEAT RESISTANT PSYCHROPHILIC BACTERIA ISOLATED FROM PASTEURIZED MILK

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The occurrence of thermoduric psychrophilic bacteria in pasteurized milk has been reported occasionally, but they have not been considered important in milk deterioration because spoilage is usually caused by the common non-thermoduric types. Improved machinery and processing methods, together with rigid sanitary control, have resulted in prolonging the shelf life of milk from some plants due to the elimination of the usual spoilage organisms. However, the current trend toward larger milk containers and larger volumes of sales through grocery stores and supermarkets tend to favor bacterial deterioration because of the relatively long exposure to favorable conditions.

The work reported herein was undertaken to determine what types of organisms in milk could survive pasteurization and grow at refrigeration temperatures and what defects these organisms could cause in milk.

REVIEW OF LITERATURE

There is considerable disagreement among various investigators as to the growth temperature requirements for psychrophilic bacteria. In many textbooks the requirement for the classification of an organism as a psychrophile includes an optimum temperature below 20°C(68°F) (8,16,20).

Ingraham (12) stated that "psychrophiles are usually defined as organisms that grow best below a certain temperature, usually 20°C or 12° to 18°C and sometimes as low as 5° to 10°C. Actually, however, there are very few reports of organisms with temperature optima below 20°C. Only three such organisms are listed in Bergey's Manual and these are questionable, since the temperature maxima were 30° to 40°C. One would not expect optimum and maximum temperature to differ so greatly. From a study of the literature one must conclude that there are very few or possibly no bacteria that fit the textbook definition of psychrophiles."

This statement supports the definition put forth by Foster _et al._ (11) and also that given in Standard Methods (1), both of which describe psychrophiles as bacteria which are capable of relatively rapid growth at low temperatures, generally within the range of 1.7° to 10°C (35°- 50°F).

There has also been some controversy as to whether the organisms which survive pasteurization are psychrophilic. Olson _et al._ (18) reported that psychrophilic bacteria are killed by proper pasteurization. Andrews and Kaufman (2) reported that 66 psychrophiles isolated from milk and water supplies did not survive pasteurization at 143°F for 30 minutes.

Thomas and Sekhar (23) showed that 206 representative strains of psychrophiles isolated from raw anf from pasteurized milk were all destroyed by laboratory pasteurization.

Rogick and Burgwald (21) reported that no psychrophiles were found in 4.1 ml of pasteurized milk taken from the LTLT vat or from the HTST pasteurization systems. They concluded that psychrophiles are not thermoduric.

Atherton et al. (3) reported that organisms surviving test tube pasteurization exhibited marked growth at 50°F but not at 45°F. Kennedy and Weiser (13) found that 2 of 15 pure cultures, isolated from pasteurized milk, survived laboratory pasteurization. These were called psychrophilic on the basis of their growth at 10°C. (50°F.). However, Olson et al. (17) observed that the "difference between 45°F-50°F is critical in plates incubated for psychrophilic counts. Counts using 50°F may include a significant portion of the thermodurics and not post-pasteurization contamination with psychrophiles." Davis (9) also stated that the use of growth at 50°F as a criterion for distinguishing the psychrophiles is responsible for the inclusion of several thermoduric types in this group.

Investigations b Boyd et al. (6) revealed that bacterial counts at 5°C (41°F) were consistently lower than those at 10°C (50°F) regardless of the incubation period. The incubation of plates at 10°C (50°F) resulted in the detection of a group of thermoduric organisms which was not found when agar plates were incubated at 5°C. (41°F).

Mikolajcik and Burgwald (14) reported that only 8 of 150 pure cultures of mesophilic-thermodurics isolated from milk grew at 45°F. Nineteen other cultures developed the ability to grow at lowered temperature although their growth was not vigorous. Boyd et al. (6) found that 17.9%

of cultures isolated from pasteurized milk and which were thermoduric grew at $5^{\circ}C$ ($41^{\circ}F$) during an incubation period of 20 days.

Nashif and Nelson (15) isolated a *Microbacterium* which resisted laboratory pasteurization and which grew at 3°-$5^{\circ}C$ (37.4°-$41^{\circ}F$) in less than 28 days. Stark and Scheib (22) isolated 486 bacteria cultures from butter, of which four survived LTLT pasteurization. All four were identified as *Flavobacterium synxanthum*. It's ability to grow at $5^{\circ}C$ was also demonstrated.

Boyd *et al*. (5), in conducting keeping quality tests on milk at $40^{\circ}F$ and at $33^{\circ}F$, reported that "the milk which had the best keeping quality at either storage temperature was spoiled eventually by the growth of a heat resistant sporeforming rod."

EXPERIMENTAL METHODS

Culture Media

Except for special agar media, the agar used throughout the experimental work was Plate Count Agar (1) (Difco number 0479-01). The litmus milk was prepared from skimmilk or from non-fat dried milk reconstituted by dispersing 10 parts in 100 parts of distilled water. The various media used for identifying the organisms were prepared according to the generally accepted laboratory procedures as presented by Pelczar and Reid (19).

Isolation of Cultures

The cultures used in the experimental work herein reported were isolated from samples of pasteurized milk which had been taken at various stages of the processing in the Oklahoma State University Dairy plant and which had been subjected to shelf life tests at 45°F. The pasteurization exposure used was 165°F for 16 seconds. Most of the isolates were from milk which had spoiled after 20 days or more of storage while a few were from milk which had been stored 50 days or more without spoilage.

In the initial trials, isolations were made by streaking spoiled samples on agar plates and incubating at room temperature (75°-85°F) for 3 to 5 days. Two colonies of each predominant type were inoculated into litmus milk. After incubation at room temperature for 5 to 7 days one tube of each type of reaction was saved for further study and what appeared to be duplicate tubes were discarded. The ability of each culture to survive HTST pasteurization was then determined.

Later the procedure was shortened by holding the laboratory pasteurized tubes at 45°F for 14-28 days instead of at room temperature. A loopful of milk from each tube showing no reaction was inoculated on agar and cultures which showed no growth were discarded. The isolation procedure was further simplified by inoculating a loopful of each sample of spoiled pasteurized milk into a tube of litmus milk and subjecting the inoculated milk to laboratory pasteurization at 161°F for 16 seconds. The tubes were held at 45°F for about 14 days and then streaked on agar plates and, after incubation at room temperature, predominant colony types were picked into litmus milk. To be present on the plates the organisms must have survived the pasteurization and they must have been able to grow at 45°F. To verify the heat resistance and psychrophilic character each culture was again laboratory pasteurized and incubated at 45°F.

This latter procedure was much preferred because it eliminated the non-thermoduric and non-psychrophilic organisms before the sample was streaked out. This simplified the isolation procedure and provided a better chance to isolate the thermoduric psychrophiles. Each culture was tested four to nine times for its ability to resist pasteurization and to grow at 45°F.

The isolated cultures were grouped according to their gram stain, cell morphology, colony characteristics and reaction in litmus milk. From each group two or three cultures were selected to conduct the experiments reported herein, except where a group consisted of only one culture.

Thermal Resistance

One loopful of fresh culture was used to inoculate 5 ml. of sterile litmus milk contained in 16 mm. screw capped test tubes. These tubes plus a control tube containing a dial thermometer were placed in a test tube support and heated in a water bath or in steam without pressure.

Long Time-Low Temperature Pasteurization (143°F for 30 minutes). The holding method of pasteurization (LTLT) was accomplished by heating in a one gallon capacity, thermostatically controlled pasteurizer designed to pasteurize milk in the home. This unit was filled with water at 120°F to a level which insured that the tubes would be immersed to a depth of approximately three and one-half inches. The test tube support containing the tubes and one control tube was placed in the pasteurizer and the lid fastened. The dial thermometer was inserted through a hole in the lid and into the control tube. The starting temperature of 120°F in the water allowed a come-up time to 142°F of about 12 minutes, which roughly simulates the come-up time for LTLT plant pasteurization. Although the temperature of the water bath remained constant at 143°F, the temperature inside the control tube remained constant at 142°F. Perhaps this difference in temperature was due to the fact that the control tube was open, which could cause a certain amount of cooling by evaporation. Following a timed exposure of 30 minutes at 143°F, the tubes were removed from the pasteurizer and partially submerged in ice water to drop the temperature to about 60°F.

High Temperature-Short Time Pasteurization. In trials involving HTST pasteurization, including 200°F for 5 minutes, heat treatments were accomplished by placing the test tube support and tubes in a container to which water had been added to a level of about one-half inch. This level was not of sufficient depth to touch the bottoms of the test tubes. The covered container with the thermometer inserted through the lid and into the control tube was heated to boiling so that the steam generated served as the heating medium.

When the temperature recorded by the thermometer reached a point 5 degrees below the desired temperature, the test tube support and tubes were transferred to a water bath adjusted to the desired temperature. Preliminary trials showed that the heat lag in the thermometer and the latent heat in the test tubes resulted in the attainment of the desired temperature if the tubes were removed at this stage. Also, the come-up time of about 30 seconds closely approximated that of the HTST pasteurizer used in the dairy plant. The exposure period was timed with a stop watch. At the expiration of the holding period, the tubes in the support were immediately cooled in an ice water bath.

Types of Spoilage

The types of milk spoilage caused by these thermoduric psychrophiles was determined by inoculation into homogenized milk, holding the milk samples at 45°F and observing organoleptically for defects.

Approximately 100 ml. quantities of fresh, pasteurized, homogenized milk from the Oklahoma State University dairy plant were dispensed into each of 38 sterile, 6 ounce prescription bottles with double parchment paper closures secured with rubber bands. A dial thermometer was inserted through the closure of one bottle. The bottles were then placed in an autoclave and heated in flowing steam for about 7 minutes or until the temperature of the control bottle reached 180°F. The bottles were then removed and placed at 45°F to cool. After cooling, thirty-five of the bottles were inoculated with selected cultures of thermoduric psychrophiles at the rate of about 0.5% of a litmus milk culture. Two bottles were not inoculated and served as controls.

These bottles were stored at 45°F and were tasted after 18 hours to detect any carry over flavors from the initial inoculation. Each bottle was then examined organoleptically at two-day intervals until a defect developed.

Growth on Agar at 45°F

To determine whether the cultures could form countable colonies under conditions used for psychrophilic counts, each culture was streaked on an agar plate and the plate incubated for 14 days at 45°F. About 10% of sterile litmus milk was added to the agar before pouring to compensate for the milk normally added in routine plating. The inoculated plates were held in metal petri plate containers during incubation to reduce dehydration and to lessen opportunity for air contaminations. The plates were examined at intervals and the rate and extent of colonial development observed.

Identification of Cultures

The media and procedures used were those which have received wide acceptance for the identification of unknown organisms (4, 10, 20). However, additional tests were used to detect spore formation.

After compiling the results of the tests, stains, and reactions Bergey's Manual (7) was used to identify the organisms.

RESULTS AND DISCUSSION

Isolation and Classification

From 227 different samples of pasteurized milk, which had been held at 45°F for varying periods of time, more than 700 bacterial cultures were isolated from agar plates inoculated with the milk. These samples were all from milk pasteurized at 165°F for 16 seconds in the Oklahoma State University dairy plant during a period of 18 months. From these isolates, 357 cultures were selected as representing the dominant types and the remaining cultures which appeared to be duplicates were discarded.

A number of these 357 isolates were eliminated because they failed to survive HTST laboratory pasteurization at 161°F for 16 seconds. Only those which were able to survive this exposure two or more times were selected as being thermoduric. Additional cultures were discarded because they failed to grow readily at 45°F. Finally, 135 cultures were obtained which survived the heat treatment and which grew at 45°F. These were considered to be thermoduric psychrophiles.

The gram staining reaction, cell morphology, colony characteristics, and reaction in litmus milk were used to classify the cultures into 15 types. From one to five cultures of each type, for a total of 35 cultures, were selected for more detailed study. These 35 cultures were then submitted to various tests commonly used for identification of bacteria. On the basis of the results obtained from these tests the cultures were divided into two groups: the spore producing and the non-spore producing types. The spore producers were further subdivided into seven types, designated as Types I, II, III, IV, V, VI, and VII. The non-spore producers were subdivided into eight types, designated as Types A, B, C, D. E, F, G, and H.

Spore Producing Types.

Eighty-two spore producing cultures were isolated from pasteurized milk which had been held at 45°F. These were classified into 7 types. The number of cultures in each type, the average shelf life of the samples from which they were isolated, the defects produced in milk by the cultures, and the growth on agar are shown in Table I.

The data show that Type I with 58 cultures was the one encountered most frequently, followed by Type II with eleven, Type VI with seven and Type III with three. Only one culture of each of the remaining three types was isolated. It should be noted that the average shelf life of the samples from which the cultures had been isolated was rather long, ranging from 22.7 to 68 days.

Action in Refrigerated Milk. All the spore producing cultures caused spoilage of milk at 45°F in from 2 to 12 days. Type I produced a distinct fruity flavor and odor followed by souring. Type II produced a yeasty, sour flavor defect, which was rather objectionable, accompanied by gas formation. Three types, III, IV and VI, caused sweet curdling followed by bitterness. Type IV required only 2 to 4 days to sweet curdle the milk. Type V resulted in souring in 6 days while Type VII appeared to be rather inert, requiring 12 days to produce an unclean, slight sour flavor defect.

TABLE I

GROWTH OF SPORE PRODUCING TYPES IN MILK AND ON AGAR AT 45°F

Type	Number of Isolates	Average Shelf Life of Source Samples Days	Development of Defects in Milk		Development of Visible Colonies on Agar Days			
			Days	Defects	5	7	10	13
I	58	33.3	4-10	Fruity, Sour	+	+	+++	++++
II	11	28.2	4-6	Sour, Yeasty, Gassy	+	+	++	+++
III	3	27.3	4-12	Sweet curdling, Unclean, Bitter	+++	++++	++++	++++
IV	1	68	2-4	Sweet curdling, Bitter	-	+	+	++
V	1	50	6	Sour	-	+	++	+++
VI	7	22.7	6	Sweet curdling, Bitter	-	-	+	+
VII	1	31	12	Unclean, Sour	+	+	+	+++

- = No visible growth
+ = Visible growth but no coutable colonies
++ = Barely countable colonies
+++ = Good growth; Distinct colonies
++++ = Abundant growth

It should be pointed out that, although the time required for occurrence of spoilage was only 2 to 12 days, a relatively heavy inoculation (0.5%) was used. Since the numbers of these organisms in plant pasteurized milk would ordinarily be expected to be rather small, spoilage of plant pasteurized milk by these types would likely occur only in milk which was heavily contaminated with these organisms or which had a long shelf life due to the absence of the common non-thermoduric spoilage types.

Growth Temperatures. The seven types of spore producers all grew at 45°F and at 98.6°F and Types IV, V, and VI also grew at 113°F.

Six of the seven types formed countable colonies on agar at 45°F in 13 days. Type VI showed growth, but the colonies were very small and would likely not be included in psychrophilic counts. Type III grew well at 45°F, producing visible colonies in 5 days. Types I, II, and V formed countable colonies within 10 days.

These results indicate that, with the exception of Type VI, the psychrophilic plate counts would include the spore producing thermoduric psychrophiles.

Thermal Resistance. The results obtained from heat resistance trials (Table II) showed that the seven types of spore producers were strongly thermoduric. All survived exposures of 20, 30, 40, and 50 seconds at 161°F and also exposures of 166°, 171°, and 176°F for 16 seconds. These exposures cover the range of HTST pasteurization exposures currently used in dairy plants. However, Type VII and one of four cultures of Type II were destroyed by exposure to 143°F for 30 minutes. Type VII and 3 of 4 cultures of Type II failed to survive an exposure to 200°F for 5 minutes.

The general results of the heat treatment trials indicate that the cultures were able to survive plant pasteurization and that they could pose

TABLE II

THERMAL RESISTANCE OF SPORE PRODUCING TYPES

Type	Heated at 161°F for				Heated for 16 seconds at			Heated at	
	20 sec.	30 sec.	40 sec.	50 sec.	166°F	171°F	176°F	143°F for 30 minutes	200°F for 5 minutes
I	+	+	+	+	+	+	+	+	+
II	+	+	+	+	+	+	+	±*	±**
III	+	+	+	+	+	+	+	+	+
IV	+	+	+	+	+	+	+	+	+
V	+	+	+	+	+	+	+	+	+
VI	+	+	+	+	+	+	+	+	+
VII	+	+	+	+	+	+	+	-	-

* - 3 of 4 cultures survived
** - 3 of 4 cultures were destroyed

a problem if present in large numbers in raw milk

Identification. The spore producing types were placed in the genus Bacillus because they were all aerobic, Gram positive or Gram variable, spore producing rod-shaped organisms which were catalase positive. On the basis of the results of various morphological, cultural and biochemical tests (Tables III and IV) an attempt was made to identify the cultures according to Bergey's Manual (7).

The following tentative identifications were made:

Type I - *Bacillus macerans*

Type II - *Bacillus polymyxa*

Type III - *Bacillus laterosporus*

Type IV - *Bacillus subtilis* var *aterrimus*

Type V - *Bacillus lentus*

Type VI - *Bacillus cereus*

Type VII - *Bacillus sphaericus*

Variations from the descriptions given in Bergey's Manual were noted as follows:

Type I - No gas in milk.

Type III - Brown, water soluble pigment formed.

Type IV - No acid from arabinose.

Type V - Did not produce urease. Very slow acid production in litmus milk.

Type VI - Acid in arabinose and xylose. Did not produce acetylmethylcarbinol.

Type VII - Produced acid slowly in litmus milk, hydrolyzed starch.

TABLE III

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF SPORE PRODUCING TYPES

Type	Gram Stain	Cell Morphology	Size in Microns	Motility	Colony Characteristics	Reaction in Litmus Milk
I	±	Rods	0.5-0.7 by 4-7	+	Thin, flat, translucent, greyish white, granular	Reduced, acid, fruity
II	±	Rods	0.5-0.7 by 2-6	+	Thin, flat, translucent, greyish white, granular	Reduced, coagulated, gassy, yeasty, Sl. proteolysis
III	+	Rods	0.7-1.0 by 2-6	+	Large, moist, translucent, grey, turning brown, thin dull grey margin; agar turns brown	Upper region turns brown followed by complete alkaline proteolysis
IV	+	Rods	0.6-0.8 by 2-5	+	Large, moist, translucent, grey, turning black; agar turns black	Reduced, complete proteolysis, Sl. alkaline, membraneous pellicle
V	+	Rods	0.6-0.7 by 2-4	+	Small, circular, entire, greyish white; more dense center	Reduced, acid, may or may not coagulate
VI	+	Rods	0.6-1.0 by 3-4	+	Small, circular, entire, dense, shiny white	Reduced, acid, coagulated, shrunken curd, proteolysis
VII	+	Rods	0.5-0.7 by 3-13	+	Small, circular, entire, white, more dense center	Partial reduction, slight acid

TABLE IV

BIOCHEMICAL CHARACTERISTICS
OF SPORE PRODUCING TYPES

	Types						
	I	II	III	IV	V	VI	VII
Catalase	+	+	+	+	+	+	+
Urease	−	−	−	−	−	−	−
Acetylmethyl-Carbinol	−	+	±	+	+	−	−
H_2S	+	+	+	+	+	+	−
Nitrates reduced	±	+	±	+	−	+	−
Idol	−	−	−	−	−	−	−
Starch	±	+	−	+	+	+	+
Gelatin	−	+	+	+	−	+	−
Dextrose	+	AG	+	+	+	+	−
Lactose	+	AG	−	−	+	−	−
Maltose	+	AG	±	−	+	+	−
Sucrose	+	AG	±	+	+	+	−
Arabinose	+	+	−	−	+	+	−
Glycerol	+	AG	−	+	−	+	−
Inulin	+	AG	−	−	+	+	−
Raffinose	+	AG	−	−	−	−	−
Salicin	+	AG	−	−	+	+	−
Xylose	+	AG	−	+	+	+	−

\+ = A positive reaction

− = A negative reaction

± = Some cultures positive; some negative

AG = Acid plus gas formation.

Non-Spore Producing Types

Fifty-three non-spore producing cultures were isolated from pasteurized milk which had been held at 45°F. These were classified into eight types. The number of cultures in each type, the average shelf life of the samples from which they were isolated, the defects produced in milk by the cultures, and the growth on agar are shown in Table V.

The data show that Type A with 17 cultures, Type D with 13 cultures, and Type B with 10 cultures were the most commonly encountered types, followed by Type H with 4, Type E with 2, and Types C, F, and G with one each. The average shelf life of the samples from which the cultures had been isolated was rather long, ranging from 22 to 44 days.

Action in Refrigerated Milk. The results of tests conducted in which milk was inoculated with each culture, stored at 45°F and the changes observed, are given in Table V. Types A, B, D, and E spoiled milk in 8-14 days with an unclean, sour defect. Type F caused ropiness plus a slight cheesy flavor in 14 days. Type H also caused a cheesy flavor, although no ropiness was present. Type G caused sweet curdling within 6 days. Type C was relatively inert, causing an unclean, sour, malty defect only after 14 to 24 days.

The fact that it requires from 6 to 24 days for spoilage to occur after a heavy inoculation with the organisms would seem to indicate that these types would not ordinarily be an important factor in milk spoilage unless relatively large numbers were present in the raw milk and competition from the non-thermoduric spoilage organisms were at a minimum.

Growth Temperatures. All eight types of non-spore producers grew at 45°F and at 98.6°F, but only Types G and H, which were cocci, grew at 113°F.

TABLE V

GROWTH OF NON-SPORE PRODUCING TYPES IN MILK AND ON AGAR AT 45°F

Type	Number of Isolates	Average Shelf Life of Source Samples Days	Development of Defects in Milk		Development of Visible Colonies on Agar			
			Days	Defects	Days 5	7	10	13
A	17	33.4	12-14	Unclean, Sour	-	+	+	+
B	10	27.9	8-12	Unclean, Sour, Feedy	+	+	+++	++++
C	3	31.0	14-24	Unclean, Sour, Malty	-	+	+	++
D	13	35.4	12-14	Unclean, Sour, Cheesy	+	++	+++	++++
E	2	35.5	14	Unclean, Sour	-	+	+	+++
F	3	44.0	14	Ropy, Sl. Cheesy	+	+	++	++++
G	1	22.0	6	Sweet curdling, Bitter	-	+	++	+++
H	4	33.3	12	Cheesy	+	+	+++	++++

- = No visible growth
\+ = Visible growth but no countable colonies
++ = Barely countable colonies
+++ = Good growth, Distinct colonies
++++ = Abundant growth

Seven of the eight types developed countable colonies on agar at 45°F in 13 days. Four, Types B, D, F, and H, showed visible growth after 5 days and large colonies at 13 days. Only one, Type D, had colonies large enough to be countable in 7 days, while five, Types B, D, F, G, and H were considered countable after 10 days. Type A showed visible growth at seven days, but only very small colonies after 13 days. These results indicate that most of the non-spore forming types would be included in routine psychrophilic counts.

Thermal Resistance. The effects of various heat treatments on the non-spore producing types is shown in Table VI. Types A, B, C, and F survived heat treatments covering the range commonly used for pasteurization of milk. They survived 161°F for as long as 50 seconds, 176°F for 16 seconds and 143°F for 30 minutes. It should be noted that one of the two cultures of Type F failed to survive 176°F for 16 seconds. Types E and H were rather resistant to pasteurization in that they survived 161°F for 50 seconds, 166°F for 16 seconds, and 143°F for 30 minutes. Types D and G were the least heat resistant. They survived 161°F for 20 seconds, but not for 30 seconds, they failed to survive 166°F for 16 seconds, and 3 of 4 cultures failed to survive 143°F for 30 minutes.

TABLE VI

THERMAL RESISTANCE OF NON-SPORE PRODUCING TYPES

Type	Heated at 161°F for				Heated for 16 seconds at			Heated at 143°F for 30 min
	20 sec.	30 sec.	40 sec.	50 sec.	166°F	171°F	176°F	
A	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+
D	+	–	–	–	–	–	–	±**
E	+	+	+	+	+	–	–	+
F	+	+	+	+	+	+	±*	+
G	+	–	–	–	–	–	–	+
H	+	+	+	+	+	–	–	+

* = one of two cultures did not survive
** = 3 of 4 cultures were destroyed

Identification. On the basis of the results of various morphological, cultural, and biochemical tests (Tables III and IV) an attempt was made to identify the non-spore producers according to Bergey's Manual (7).

The following tentative identifications were made:

Type A - *Microbacterium flavum*

Type B - *Arthobacter aurescens*

Type C - *Microbacterium lacticum*

Type D - *Corynebacterium equi*

Type E - *Arthrobacter* sp.

Type F - *Arthrobacter* sp.

Type G - *Streptococcus faecalis* var. *liquefaciens*

Type H - *Streptococcus faecalis*

Variations from descriptions given in Bergey's Manual were noted as follows:

Type A - Acid from maltose and lactose.

Type C - Starch not hydrolyzed.

Types E and F - The characteristics and reactions did not agree with the description given in Bergey's Manual for any organism. Due to the marked diversity of cellular forms observed with these types, the appearance of straight, curved, and swollen rods with some showing snapping division, and the presence of coccoid forms, they were considered to be members of either the genus *Corynebacterium* or the genus *Arthrobacter*.

However, because most of the *Corynebacterium* are pathogenic to either plants or animals and generally have high minimum growth temperatures, and because the genus *Arthrobacter* contains several heat resistant species and generally lower minimum growth temperatures, these two types were placed in the genus *Arthrobacter*.

TABLE VII

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF SPORE PRODUCING TYPES

Type	Gram Stain	Cell Morphology	Size in Microns	Motility	Colony Characteristics	Reaction in Litmus Milk
A	+	Rods	0.6-1.5 by 0.2-0.3	-	Circular, entire, convex, shiny yellow, translucent to slight dense	Reduction, slight acid
B	+	Rods	0.2-0.3 by 0.6-1.0	-	Smooth type; circular convex translucent, yellow, rough; opaque granular depressed into agar	Reduced, yellow ring and sediment, sl. proteolysis
C	+	Rods	0.2-0.3 by 0.6-2.0	-	Small circular, entire, convex, dull white	Reduced, acid, coagulation
D	+	Rods	0.3-0.8 by 2.0-5.0 (coccoid on old slant)	-	Large, moist, smooth, dense, pulvinate, tannish white, circular to irregular, entire, tends to run	Alkaline in 5-7 days, grey flocculent pellicle
E	+	Rods	0.6-0.8 by 2.0-3.0 (coccoid on old slant)	-	Small, dense, circular, white	Alkaline in 5-7 days, grey flocculent pellicle
F	+	Rods (capsules)	0.3-0.7 by 1.5-4.0	-	Circular, dense, moist, pulvinate, white, tends to run or drip	Thick, ropy, mucus-like, grey pellicle, reduced or alkaline
G	+	Cocci	0.6-1.0	-	Punctiform, translucent, grey	Reduced, coagulated, sl. acid slight proteolysis, shrunken curd
H	+	Cocci	0.6-0.7	-	Small, shiny white, convex, circular dense	Reduced, acid, coagulation

TABLE VIII

BIOCHEMICAL CHARACTERISTICS OF NON-SPORE PRODUCING TYPES

	Type							
	A	B	C	D	E	F	G	H
Catalase	+	+	+	+	+	+	-	-
Urease	-	-	-	+	-	+	-	-
Acetylmethyl-Carbinol	-	-	-	-	-	-	+	-
H_2S	±	+	±	+	+	+	+	- ±
Nitrates Reduced	-	+	-	±	-	+	-	-
Indol	-	-	-	-	-	-	-	-
Starch	-	±	-	-	-	±	-	-
Gelatin	-	+	-	-	-	-	+	-
Dextrose	+	+	+	-	-	-	+	+
Lactose	+	+	+	-	-	-	+	+
Maltose	+	+	+	-	-	-	+	+
Sucrose	+	+	+	-	-	-	-	+
Arabinose	+	±	-	-	+	-	+	+
Glycerol	±	+	±	-	-	±	+	+
Inulin	±	-	-	-	+	-	+	+
Raffinose	+	±	-	-	+	-	-	+
Salicin	+	±	±	-	+	±	+	+
Xylose	+	±	±	-	+	-	+	+

+ = A positive reaction
- = No reaction
± = Some cultures are positive; some are negative

SUMMARY AND CONCLUSIONS

Over 700 cultures were isolated from spoiled samples of pasteurized milk held at 45°F. What appeared to be duplicates were discarded. The remaining 357 cultures were tested for ability to survive laboratory HTST pasteurization (161°F for 16 seconds) and to grow at 45°F. Of the 135 cultures which proved to be thermoduric and psychrophilic, thirty-five cultures, representing 15 distinct types, were selected for detailed study in an attempt to identify them and to determine if they could survive pasteurization exposures commonly used and cause spoilage of milk at 45°F.

On the basis of the morphological and cultural characteristics, the biochemical reactions, and temperature relationships, the 35 cultures were divided into 7 spore producing and 8 non-spore producing types.

All the spore producing types caused defects in milk at 45°F within 2 to 12 days. The most common defects were fruity, sour and fermented, unclean and sour, and sweet curdling followed by bitterness.

All seven types grew at 45°F and 98.6°F while three grew at 113°F. Six of the seven types developed countable colonies on agar plates in 13 days at 45°F.

The spore producing types survived laboratory pasteurization exposures of 161°F for 50 seconds and 176°F for 16 seconds. Six types withstood 143°F for thirty minutes and five resisted 200°F for 5 minutes.

The spore producing types were tentatively identified as *Bacillus macerans*, *Bacillus polymyxa*, *Bacillus cereus*, *Bacillus lentus*, *Bacillus* subtilis var aterrimus, Bacillus sphaericus, and Bacillus laterosporus.

The eight types of non-sporeformers spoiled milk in 6 to 24 days following a heavy inoculation and storage at 45°F. The most common defects were unclean and sour, feedy and sour, ropy and slight cheesy, and sweet curdling followed by bitterness.

All eight non-sporeforming types grew at 45°F and 98°F, but only two types grew at 113°F. Seven types produced countable colonies in 13 days on agar held at 45°F. All eight types survived 161° for 20 seconds, while five survived this temperature for 50 seconds. Six types resisted 166°F for 16 seconds and three withstood 176°F for 16 seconds.

The eight non-spore producing types were tentatively identified as Microbacterium flavum, Microbacterium lacticum, Arthrobacter aurescens, Corynebacterium equi, Streptococcus faecalis, Streptococcus faecalis var. liquefaciens, and two Arthrobacter sp.

It appears that the thermoduric psychrophiles are of importance because they all caused spoilage of refrigerated milk and all survived the minimum exposure of pasteurization by the short time-high temperature method (161°F for 16 seconds). Several of the types also survived the maximum exposure currently used. While spoilage of pasteurized milk at temperatures below 50°F is commonly caused by non-thermoduric contaminants, deterioration due to the thermoduric psychrophiles can occur in milk free from post-pasteurization contamination.

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