

STUDY OF ENTEROCOCCI IN MILK

By

Ishwar Gopal Chavan

A THESIS

Submitted to the School of Graduate Studies of Michigan
State College of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

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THEMIS ABSTRACT

The Kitby, Mallmann and Fifield procedure for study of enterococci in river water, sewage and soil was used to determine the possible application of this test in measuring the sanitary quality of raw milk, the effectiveness of pasteurisation and the detection of post-pasteurisation contamination.

Dextrose aside broth is employed in the test as a presumptive medium and ethyl violet aside broth for confirmation. Because of the possibility that the presence of milk might upset the specificity of the aside-ethyl violet combination, all positive ethyl violet aside milk tubes were examined further to be sure that all turbidities were due to enterococci and not to streptococci or other bacteria that might be present.

Dextrose aside-ethyl violet aside broth test gave an accuracy of 96.2 percent for the samples and 97.2 percent for the cultures examined. The accuracy of the procedure compares favorably with the confirmation procedures used for the detection of coliform organisms in water.

Examination of raw milk for enterococci shows that if milk is drawn carefully, it contains very few enterococci. But further contact with the milking machine, milk cans, careless handling and inadequate cooling brings tremendous increase in numbers.

In regular low-temperature, long-time laboratory as well as plant pasteurisation more than 98 percent of the enterococci were killed and in regular high-temperature, short-time pasteurisation more than 99 percent of the enterococci were destroyed.

Low-temperature, long-time laboratory pasteurisation at 66.2 C for 30 minutes and high-temperature, short-time pasteurisation at 77.2 C for 16 seconds completely destroy enterococci in milk.

The thermal death time studies with pure cultures of Str. faecalis, Str. liquefaciens, Str. synagae and Str. durans reveal that the majority of the enterococci are susceptible to present pasteurisation temperatures but a small resistant minority survive long exposure periods.

The predominant subgroup of enterococci occurring in milk is Str. faecalis.

DEDICATED TO

Dr. Walter LeRoy Mallmann - An
inspiring teacher and sincere friend
with an ideal philosophy for
directing graduate studies.

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INTRODUCTION

There is a vast amount of confusing literature available on the subject of enterococci. Before Thiercelin (1899) isolated a coccus from human feces and used the term "enterococcus", Hirsh and Libman (1897) classified them as Streptococcus enteritis and later Andrewes and Horder (1906) placed them under the species name of Streptococcus faecalis.

The enterococci are markedly differentiated from the other known species of streptococci by their combination of low minimum and high maximum temperatures of growth and by their greater tolerance to salt, alkali, acid and bile. They also differ from most streptococci in having high thermal death points, in being resistant to relatively concentrated solutions of methylene blue and in having strong reducing action. They appear in diplococcal form or in short chains and are distinguished by their faculty of growing on ordinary media at room temperature.

The potential pathogenicity of the enterococci, combined with their high resistance and extreme ranges of temperature at which they grow is of particular interest to the dairy industry. Inasmuch as they are of fecal origin, their appearance in milk indicates fecal contamination. If allowed to grow extensively in milk, they give rise to taints and abnormal flavors. They ferment lactose, forming an acid and other objectionable by-products which make them very objectional to the fluid

milk industry. For example, Streptococcus liquefaciens rapidly coagulates milk, then actively proteolyzes it and is sometimes responsible for bitterness in pasteurized products.

The study of enterococci has been handicapped by the lack of suitable media. In 1950 Mallmann and Seligmann, Jr., demonstrated that azide dextrose broth (Difco) was an excellent enrichment medium for the growth of the enterococci and other streptococci. The concentration of 0.02 percent sodium azide used in azide dextrose broth (Difco) inhibits the growth of the coliform groups and allows the growth of streptococci. Hence the azide dextrose broth (Difco) is not only an enrichment medium for enterococci and other streptococci, but also a selective medium for the isolation of these organisms. Litsky, Mallmann and Fifield (1952), working on the development of a confirmatory medium, used 0.04 percent concentration of sodium azide which would allow Str. faecalis to grow and yet would inhibit the gram-negative organisms. Later on they found that 0.04 percent concentration inhibited the gram-negative bacteria but allowed the growth of some spore-formers, such as Bacillus subtilis. So they used another inhibitory agent, ethyl violet and thus developed ethyl violet azide broth as a confirmatory medium for the presence of enterococci when used with presumptive test in azide dextrose broth.

An attempt has been made to use these media for examining raw as well as pasteurized milk samples to determine the sources of enterococci in milk and possibly establishing an enterococci index for pasteurized milk within the limits using Sherman classification (1937) and Bergey's

manual (1948). The relative relationships of these organisms were studied as well as the thermal death time of pure cultures.

HISTORICAL BACKGROUND

Initial Isolations. The term enterococci was first used by Thiercelin (1899). His findings led him to believe that enterococci are casual agents in certain diarrhoeas, biliary infections and cases of appendicitis.

MacCullum and Hastings (1899) studied a case of acute endocarditis and isolated a new species of organisms. They named it Micrococcus zymogenes and described it as follows: "This micrococcus is very small, occurs mainly in pairs, sometimes in short chains, gram-positive, liquefies gelatin slowly and is especially characterized by its behaviour in milk, which it acidifies, coagulates and subsequently liquefies. It produces a milk curdling ferment and also a proteolytic ferment, each of which is separable from the bacterial cells. It remains viable for months in old cultures and is tolerably resistant to the action of heat and antiseptics. The micrococcus is pathogenic for mice and rabbits, causing either abscesses or general infections."

Hirsh and Libman (1897) described Str. enteritis as short-chained groups and English writers, Andrewes and Horder (1906) described Str. faecalis as a mannite fermenter and one of the most actively saccharolytic members of the streptococcus group.

Heineman (1920) demonstrated that Str. faecium occurs chiefly in human feces, but also in feces from other mammals. It ferments arabinose, mannite and saccharose but its fermenting power is quite variable. It occurs chiefly as a diplococcus, rarely in short chains.

Dible (1921), after studying the enterococcus and fecal streptococci, concluded that Str. enteritis comprises the large group of true chain-forming streptococci.

Knaysi (1941) describes the morphology of Str. faecalis as follows: "At the start, the cell resembles a flattened ellipsoid of revolution which gradually evolves to become an elongated ellipsoid of revolution. Strictly spherical cells are seldom encountered in growing culture of Str. faecalis.

Sherman and Wing (1935) described a new species of a hemolytic streptococcus which differed from the pathogenic species of hemolytic streptococci in its higher maximum temperature of growth, a higher thermal death point, and a more acid limiting pH of growth. It also differs from the human types in the hydrolysis of sodium hippurate and its failure to ferment sucrose. They suggested the name Streptococcus hemothermophilus but later (1937) changed the name to Streptococcus durans. The organism was thought to be closely related to the enterococcus group.

Sternberg (1896) isolated another streptococcus which he named Str. septicus liquefaciens and Orla-Jensen (1921) named a proteolytic organism Str. liquefaciens.

Common Habitat of Enterococci. Birge (1905) isolated from autopsied laboratory animals a micrococcus corresponding morphologically and culturally with that isolated by MacCullum and Hastings (1899) with the exception that this organism was non-pathogenic for laboratory animals. A rennin-like ferment was secreted by the organisms.

Bagger (1926) examined 92 strains of enterococci isolated from cases of peritonitis and 58 strains isolated from normal intestines. He described the enterococci as follows: "The organism is a gram-positive diplococcus, the individual elements being oblong or frequently rhomboid and characteristically inclined to one another. In fluid media, it forms short chains. The organism can grow at pH 5 to 10 and at temperatures between 10 and 48 C. It is exceptionally resistant to heat--two characteristics which differentiate it from ordinary streptococci and pneumococci." Gelatin was liquefied by 10 percent of the strains.

Porch (1941) demonstrated that the enterococci were the predominant group of streptococci recovered from the genito-urinary tract.

Ostrolenk and Hunter (1946) demonstrated that the fecal streptococci are common in the excreta of 10 animal species and found them occurring in significant numbers. They suggested that even though the streptococci are outnumbered by Escherichia coli, the resistance of enterococci to chemical agents and possibly to other environmental factors make them of sanitary significance as indexes of fecal contamination and pollution.

Smith (1939) studied the occurrence of Str. zymogenes in the intestines of animals and found that it occurred in the feces of the horse and the cow.

Alston (1928) studied 50 strains of streptococci isolated from the alimentary tract of man, dog and rat. Thirty percent of them were described as enterococci. The organisms he described were the small

cocci, oval in shape and occurring in pairs or short chains, non-hemolytic and capable of fermenting mannitol.

Gordon (1905) examined 20 normal stools and found at least 100,000 streptococci per gram of feces; in the majority of the stools they exceeded 1,000,000 per gram and in some they exceeded 10,000,000 per gram.

Smith and Sherman (1938) studied the hemolytic streptococci of the human feces and found that the commonest hemolytic streptococcus of the human intestine is Str. zymogenes and the second in frequency is the related "enterococcus", Str. durans.

Ayers and Johnson, Jr., (1924) examined 33 human fecal cultures and found that the predominant species was Str. faecalis or enterococcus. They concluded that Str. faecalis is similar to, if not identical with Str. lactis.

Donaldson (1917) stated that the French writers held the enterococcus to be a constant inhabitant of the normal intestine, and is more commonly found in the small intestine of infants three days after birth. He indicated that on the contrary, German workers who examined the stools of 3,530 persons, found no enterococci in the stools of the people having normal health. He found that the enterococcus was pathogenic for rabbits and probably was a variant of the Str. faecalis group.

Oppenheim (1920) studied the human fecal streptococci and observed that the mannite fermenting, non-hemolytic streptococci were the characteristic predominant types found in the feces of normal individuals. Hemolytic streptococci were found only occasionally in the stools of normal, healthy people.

Broadhurst (1915) used the fermentative tests in indicating the origin of a given streptococcus. She observed that a large number of mannite fermenters were from human feces and that strains from the throats of human beings failed to ferment mannite. Mannite fermenters were most often found in bovine mouths, milk and human feces. Strains found in milk were conspicuously not raffinose fermenters and rather commonly mannite fermenters. But in her conclusion, she stated that these fermentative tests did not seem to be definitely helpful in indicating the origin of a given streptococcus.

Classification of Enterococci. Prescott (1902) showed that the streptococci produce more acid than Bacillus coli in dextrose broth. He also observed that the colon bacilli appeared to be extremely sensitive to lactic acid of certain strengths and are therefore inhibited, if not actually killed, by the acid produced by the streptococci. Prescott and Baker (1904) were successful in cultivating streptococci in nearly pure cultures by using dextrose broth of slightly greater acidity than that produced by maximum numbers of B. coli. The incubation period for isolation was 24 hours. This rapid test helped to detect and separate streptococci from other microorganisms in polluted waters.

Welch (1929) classified streptococci of human feces on the basis of their fermentative ability and found that their fermentative characteristics were apparently constant. Morphology of the streptococci of the human feces did not help in their differentiation.

Avery (1929) (1929a) differentiated hemolytic streptococci of human and dairy origin by methylene blue tolerance and final acidity.

Methylene blue was bactericidal for the strains of hemolytic streptococci that fail to reduce it, but neither bacteriostatic nor bactericidal for the strains that caused its reduction. His work indicated that the saprophytic strains have a greater tolerance for the methylene blue dye than have the strains of parasitic origin.

Frobisher, Jr., and Denny (1928) working with a number of strains of M. zymogenes demonstrated that these organisms may not belong to a single species, since some produce alpha type, some beta type and some gamma type hemolysis on blood agar plates. There was so much resemblance between M. zymogenes and Str. liquefaciens that they considered them identical. They emphasized that M. zymogenes should be classed as a streptococcus and observed that there is no relation between hemolysin and proteolytic enzyme production by these organisms.

Elser and Thomas (1936) isolated Str. zymogenes from endocarditis. They found that all strains produce green discoloration on blood agar plates. Sherman, et al. (1937) differentiated Str. zymogenes from Str. liquefaciens, as being hemolytic. They observed two varieties of Str. zymogenes: (1) A hemolytic but apparently non-proteolytic form, not liquefying gelatin and causing no visible peptonization in milk. (2) A proteolytic but non-hemolytic type. Non-hemolytic strains of Str. zymogenes differed from Str. faecalis, in being hemolytic.

Gibson and Malek (1948) observed, in their studies of the bacteriology of milk, that many strains of Str. faecalis and its varieties are non-hemolytic but otherwise indistinguishable from Str. durans.

Pearl and Harriette (1931) examined 100 strains of fecal streptococci and 50 other strains with respect to their agglutinability by an immune serum specific for culture of fecal streptococci and found a definite parallelism between a serologic and bacteriophagic reactions of the fecal streptococci studied.

Torrey and Montu (1934) studied the cultural and agglutinative relationships of intestinal streptococci and demonstrated that there was not any specific serologic type of enterococcus or diplostreptococcus found associated with the lesions of nonspecific ulcerative colitis. They stated that M. zymogenes is a variant of the enterococcus.

Sherman and Stark (1931) stated that there were certain other streptococci besides Streptococcus thermophilus which grew at 45 C. and above. Sherman and Stark (1934) differentiated Str. lactis from Str. faecalis on the following basis: Str. lactis has a lower maximum growth temperature (41 to 43 C.), a lower thermal death time (65 C. for 30 minutes), a lower alkaline limit for growth (less than 9.6 pH), and a lower tolerance for sodium chloride.

Sherman, et al. (1937a) studied 434 strains of Str. faecalis and found that they all grew at 10 and 45 C. Some cultures were able to grow at 50 C. and all except five grew at 5 C. At least one of the strains was able to grow at 0 C. They included Str. faecalis, Str. zymogenes, and Str. liquefaciens in the enterococcus group. The organisms of the group have the faculty to cause a complete reduction of litmus in milk before curdling. All the cultures of Str. faecalis gave a prompt and complete reduction of litmus in milk, with the exception of the

narrow zone which is exposed to the air at the surface. The property of fermenting mannitol confirms in a broad sense but some strains failed to ferment mannitol.

Sherman and Gunsalus (1943) demonstrated that all members of Lancefield's groups B and D tested fermented glycerol and Gunsalus and Umbreit (1945) showed that the Str. faecalis ferment glycerol.

Chapman (1936), while studying the resistance of enterococci observed that the enterococci were more resistant to sodium carbonate, sodium bicarbonate and sodium chloride than Esch. coli, Aerobacter aerogenes, staphylococci and streptococci. Enterococci were still viable after contact for one hour with 3 percent sodium carbonate. He suggested that the interpretation of resistance to injurious agents will be simplified if enterococci are differentiated from other streptococci.

Evans and Chinn (1947) reviewed the literature on human infections with enterococci and found that they appear to be important casual agents in some cases of endocarditis, intestinal disorder, abdominal infections due to injury of the intestinal tract, infections of wounds inflicted during war and infections of the urinary tract. Distinguishing characteristics of the enterococci from other streptococci were as follows: reaction in serum of group D according to Lancefield's precipitin test, growth at 10 and 45 C, growth in media containing 6.5 percent sodium chloride, growth in media having an initial pH value of 9.6 and growth in media containing 40 percent bile. The types of hemolysis and liquefaction of gelatin were found not to be correlated with other significant characteristics.

Selective Media. Schuman and Farrell (1941) prepared a synthetic medium consisting of pantothenic acid, vitamin B₆, riboflavin, glucose, a salt mixture and 6 amino acids namely: arginine, glutamic acid, methionine, tryptophane, tyrosine and valine for the growth of Str. faecalis.

Niven, Jr., and Sherman (1944) studied the nutrition of the enterococci and found that there was no significant difference in nutritive requirements among strains of the four enterococcus species.

Houston and McCloy (1916) used Conradi-Drigalski's medium for isolating enterococcus from pus. This was a selective medium which inhibited many organisms including staphylococci. The medium turned red in the presence of the enterococci. The heat resisting properties of the enterococcus formed the basis of a method of further confirming organisms from pus, feces and sputum. They exposed a thick emulsion of the coccus in broth for one and a half hours to a temperature of 55 C and found that they survived.

Snyder and Lichstein (1940) found that 0.01 percent concentration of sodium azide in blood agar inhibited the growth of Bacterium proteus and other common gram-negative forms but allowed streptococci to grow.

Mallmann, et al. (1941) reported that slow oxidizing agents such as potassium dichromate and sodium azide, exert a bacteriostatic effect on gram-negative bacteria and allow the growth of gram-positive bacteria. The gram-positive cocci, particularly the streptococci, appear to be able to tolerate the slow oxidizing agents to a greater extent than do the gram-positive spore bearing bacteria.

Mallmann and Darby (1939) in their studies on media for coliform organisms established a broth medium containing 1 to 5,000 concentration of sodium azide to inhibit the coliform organisms and to allow the growth of the streptococci. Mallmann (1940) using this medium suggested a new yardstick for measuring sewage pollution.

Hajna and Perry (1943) developed a new medium known as "S F" medium (Str. faecalis) for streptococci index of pollution in swimming pools. They used sodium azide in concentration of 0.05 percent as an inhibiting agent for gram-negative bacteria and brom cresol purple as an indicator of an acid reaction. The medium was incubated at 45.5 C and an acid reaction was thought to be almost complete evidence of the presence of Str. faecalis.

Winter and Sandholzer (1946) used a modification of the White and Sherman sodium azide penicillin medium by doubling the penicillin content and adding 0.001 percent methylene blue. They isolated enterococci from raw sewage and fresh and salt water.

Mallmann and Seligmann, Jr., (1950) in a comparative study of media for the detection of streptococci in water and sewage using lactose broth, Mallmann's sodium azide broth (1940), Hajna and Perry's S F medium (1943), azide dextrose broth (Difco) and Ritter-Treece sodium azide broth (1948) demonstrated that the azide dextrose broth (Difco) was the best medium for quantitative determination of streptococci. S F broth was the poorest medium when incubated at 45 C. They suggested that the macroscopic determination of streptococci in azide dextrose broth, as indicated by turbidity due to growth, may be accurate in the absence of gram-positive

rods in the test material. In the presence of gram-negative rods the tubes should be checked microscopically.

Jolliffe (1948) demonstrated that the ethyl purple was an effective bacteriostat against gram-positive organisms and could be successfully used in media work.

Litsky, Mallmann and Fifield (1952) developed ethyl violet azide broth, a new medium for quantitative confirmation of the enterococci from azide dextrose broth. It was found that the enterococci were the only group that would grow in this medium. They demonstrated the specificity of this medium with samples from river water, sewage and soil. A comparison of methods for the detection of enterococci showed that the dextrose azide-ethyl violet azide broth test was the best and the easiest of those in use at present time.

Selective Isolation of Enterococci. Mallmann (1928) used glucose and lactose nutrient broths for quantitative measurement of B. coli and streptococcus indexes for swimming pool water. At the end of 48 hours incubation at 37 C. the tubes were removed and kept at room temperature for 3 days to allow the streptococci to settle at the bottom. The broth was decanted carefully and a loopful of thick creamy precipitate was stained with gentian violet. The smears were then examined for streptococci. He demonstrated that B. coli is not a universally reliable indicator of intestinal pollution in swimming pools. B. coli multiplies in the swimming pool and the presence of B. coli does not necessarily indicate pollution or danger, although the absence of B. coli is an

excellent index of safety. Streptococci are consistent indicators of intestinal pollution and their numbers are directly proportional to the number of bathers. Streptococci do not multiply in the swimming pool and hence their presence indicate an unsafe condition.

Mallmann and Sypien (1934) examined natural bathing places and demonstrated that the streptococci index fluctuated with the bathing load. Streptococci were not found at points where there was no bathing pollution. The colon indexes did not respond to changes in bathing load. The streptococci disappeared over night but the colon indexes and total count indexes sometimes showed an increase over night.

Ardrey (1936) found in a study of the source of streptococci in chlorinated swimming pools that the streptococci are not plentiful on the body surfaces except where contamination from the mouth, nose and fecal material has taken place. Streptococci, as found in swimming pools, were not indicators of the amount of intestinal pollution. He suggested that streptococci indicate the amount of pollution which has taken place from the noses and mouths of bathers and are therefore important in determining the safety of waters in regard to transmission of sinus and other respiratory diseases.

Ritter and Treece (1948) studied the sanitary significance of cocci in swimming pools and found that out of 79 strains of streptococci isolated from swimming pools 65.8 percent can be classified as Str. faecalis. They were resistant to 0.4 to 0.6 parts per million free available chlorine for 30 minutes to one hour. Tests for the temperature limits of

growth and for growth in alkaline medium were found to be most reliable in classifying the enterococci.

Seligman, Jr., (1951) used azide dextrose broth in the determination of the coccus index of swimming pool water. He showed the use of the coccus group as an indicator of pollution in swimming pool water. This group includes Str. salivarius, Str. mitis, Str. faecalis and M. epidermidis.

Mallmann and Litsky (1951) studied the survival of selected enteric organisms in various types of soil and found that the enterococci are the only organisms which could be used as the indicators of fecal contamination. They observed that the coliform organisms persist in soil for long periods and the enterococci were found to die out rapidly. They suggested that the enterococci seem to be good indicators of public health hazard from sewage in soils and on vegetables.

Driesens (1949) used sodium azide broth as a selective broth medium for studying the number of streptococci in raw and treated sewage, and longevity studies of streptococci in manured and unpolluted soils. He demonstrated that the streptococci may be found at high population levels in raw and treated sewage and in manured soils, and suggested for the purposes of evaluating irrigation water and garden soils, streptococci are shown to be of greater sanitary value than are the coliforms under certain conditions. He (1952) used the same medium to study the number of enterococci in the feces of the chicken fed with antibiotics.

Studies of Enterococci in Dairy Products. Kinyoun and Dieter (1912) believed that the presence of cocci which form chains in lactose bile

at 37 C is presumptive evidence that milk is contaminated with feces.

Rogers and Dahlberg (1914) studied 42 cultures from milk which formed chains in lactose bile at 37 C, 5 cultures from infected udders, 114 cultures from bovine feces and 39 cultures from the mouths of animals. They emphasized that the feces of the animal must be considered as a possible source of bacteria in milk and some of them would undoubtedly be found to be members of the lactic group.

Ayers and Johnson, Jr., (1914) while studying the ability of streptococci to survive pasteurization used 139 cultures of streptococci isolated from bovine feces, udders, mouths, and from milk and cream. These were heated in milk for 30 minutes under conditions similar to pasteurization. In the group tested 64.03 percent survived at 60 C, 33.07 percent at 62.6 C, 2.58 percent at 71.1 C and all of them were destroyed at 73.9 C. Using only 18 cultures from milk and cream, they found 100 percent survived at 60 C, 94.44 percent at 62.9 C, 50 percent at 68.3 C and all of them were destroyed at 73.9 C. They showed that streptococci isolated from milk and cream were slightly more resistant than those from the udder, mouth and feces. They suggested two classes of streptococci that survive pasteurization. (a) Streptococci which have a low thermal death time but because of certain resistant characteristics peculiar to a few cells are able to survive the pasteurizing temperatures. (b) Streptococci which have a high thermal death time above the temperature used in pasteurization. Heat resistance was found to be a permanent characteristic of certain strains of streptococci.

Evans (1916) studied 192 samples of milk freshly drawn from normal udders from 161 cows of 5 different dairies in 2 widely distant sections of the country and observed that 15.1 percent were long-chained streptococci and 58.8 percent micrococci.

Davis (1916) studied streptococci commonly occurring in both pasteurized and unpasteurized (certified) milk. He found that the strains varied among themselves, were more resistant to heat than human strains of hemolytic streptococci, non-pathogenic to rabbit, acidified and coagulated milk rapidly and formed short or long chains, but as seen in milk often appeared in pairs or a chain of few elements. He suggested that these organisms may be considered to have some sanitary significance.

Ayers and Rupp (1922) demonstrated that hippuric acid was hydrolysed by the 44 hemolytic streptococci isolated from the udders of cows, but not by 33 hemolytic streptococci of human origin.

Crowe (1923) used a special glucose-blood-agar medium for differentiating fecal streptococci. Blackening of the medium was found to be a confirmatory test for fecal streptococci. He described the organism as follows: "A characteristic organism is that small, heat-resisting, egg-shaped coccus, commonly known as the Str. faecalis and Str. zymogenes is a subsection of this group of organisms."

Brown, Frost and Shaw (1926) showed that many of the hemolytic streptococci were harmless to man and were frequently found in the best grades of raw and pasteurized milk.

Frost, Gumm and Thomas (1927) studied hemolytic streptococci in certified milk on five different farms. All strains were of the bovine

type with the exception of four cultures which proved to be Str. epidermicus Davis.

Long and Hammer (1936) worked with organisms important to dairy products and found an acid-proteolytic streptococcus which was identified as Str. liquefaciens Orla-Jensen. This organism coagulated milk by enzyme action rather than by the formation of acid. It remained viable at about 5 C over a long period of time but it was killed when heated for 40 minutes at 65.6 C.

Niven (1938) examined 68 raw and 245 pasteurized milk samples for the presence of hemolytic streptococci in milk. He observed that the prevailing types of hemolytic streptococci in raw milk were Str. mastitidis and the "animal pyogenes." The most common forms in pasteurized milk were Str. durans and Str. zymogenes.

Gibbons (1950) using Sherman's method for classification studied the species of enterococci in both egg products and chicken feces. The majority of the enterococci found in the egg products and chicken feces were non-hemolytic. Str. faecalis was found most frequently followed by Str. liquefaciens, Str. durans and Str. zymogenes. They concluded that the enterococci were a much better index of fecal contamination in egg products than the coliforms.

At present time, the coliform test of pasteurized milk is used as an index of improper sterilization of post-pasteurization equipment or contamination through defective handling or inadequate pasteurization.

Coliform organisms are widely distributed in the sense that there is much opportunity for their occurrence in raw milk, in dust, field

water, and on utensils, grain, mixed feed, bedding, plants and so in large numbers while their presence in raw milk may imply contamination of a fecal nature, this is not necessarily nor generally so.

Stark and Curtis (1936, 1936a) demonstrated that the addition of one ml. of sterile milk to 14 ml. of the brilliant green bile-lactose medium reduced the toxic action of brilliant green and allowed the growth of the sporulating, false-test organisms, and resulted in the growth of a larger number of the cultures which may be responsible for false test by means of "synergism".

It is repeatedly observed that besides coliform organisms Clostridium perfringes, Clostridium sporogenes and certain aerobic spore forming bacilli ferment lactose.

Sherman and Wing (1933) in their study of colon bacteria in milk concluded that the colon test is of no special value as an index to the sanitary conditions surrounding the production of raw milk of usual market grade.

As a matter of fact, the presence of E. coli in milk does not and can not mean anything until and unless E. coli when found, is traced back to its source and shown to be significant of something. There are so many chances that bacterial contamination will occur without the presence of E. coli that it would seem unwise and unsafe to rely alone upon its presence or absence as an "index to sanitation".

Taking into consideration the above facts, the coliform test does not fulfill the necessary requirements of an index of post-pasteurization contamination in milk.

EXPERIMENTAL PROCEDURE

Collection of Samples

Raw Milk

Samples were drawn aseptically with 10 ml sterile pipettes from milk cans at the Michigan State College Creamery as soon as they were received in the plant. Immediately after collecting they were transferred to sterile screw cap vials. Each vial was filled from two-thirds to three-fourths full. The sample containers and closures were handled aseptically. The temperature of the sample at the time of collection ranged from 10 - 12.8 C. All samples were immediately cooled and stored at about 3.3 - 4.4 C until tests were made.

Direct Microscopic Examination of Raw Milk for Total Bacterial Count

A welded loop calibrated to deliver 0.01 ml of milk was used to transfer a loopful of the well-mixed sample to a previously heated sterile slide. The milk was promptly spread evenly over a square centimeter area using a loop, inside diameter 4 mm., made of B and S gauge, no. 19 platinumiridium wire, fused on a 1 - 2 inch straight piece of similarly alloyed wire. The slides were dried at 40 - 45 C within an interval of 5 minutes without cracking and peeling the milk layer on the slide. The staining and counting of the bacteria were carried out according to the Standard Methods for the Examination of Dairy Products (1948).

Standard Plate Count for Raw and Pasteurized Milk

The procedure recommended in the Standard Methods for the Examination of Dairy Products (1948) was followed. Tryptone-glucose-extract-milk agar (TGE) was used for measuring the total count of the raw milk and the thermoduric count of the pasteurized milk.

Laboratory Pasteurization of Raw Milk

Exactly 5 ml of each raw milk sample was transferred into 5/8 by 6 inch uniform sterile tubes. The temperature of the sample was determined by placing a thermometer in a tube containing the same amount of milk. The tubes were placed into boiling water and as soon as the temperature reached 61.8 C in the control tube, the tubes were transferred to a water bath ($61.8\text{ C} \pm 0.1$) and were allowed to remain for intervals of 15, 25, and 30 minutes. At the completion of the pasteurization, the tubes were removed from the water bath, immediately cooled and stored in a refrigerator until they were checked for standard plate count, coliform index and enterococci index.

The phosphatase field test was performed according to the instructions supplied with the phosphatase field test kit. The tablets for the substrate and the BQC solution (2,4-di-bromo quinone chlorimide) were obtained from the Applied Research Institute, New York.

Coliform Index

The procedure recommended in the Standard Methods for the Examination of Dairy Products (1948) was followed, using brilliant green bile

lactose broth. The tubes were incubated at 35 C for 48 hours. The production of gas in the Durham's fermentation tubes was considered to be a positive test for the presence of coliform organisms. The coliform index was expressed as a most probable number of organisms per 100 ml of milk.

Enterococci Index

This index was determined by planting suitable dilutions of the milk in dextrose azide broth with confirmation in ethyl violet azide broth as recommended by Litsky, Mallmann and Fifield (1952). Three suitable dilutions were made and 1 ml of each dilution was transferred to dextrose azide broth (Difco). Five tubes of the broth were used for each dilution. Since the tubes contained milk, any attempt at measuring growth by turbidity was impossible. Each and every positive tube from dextrose azide broth was shaken thoroughly and 4 standard loopfuls (diameter 5 mm.) of the broth were transferred to ethyl violet azide broth. After thorough mixing, the tubes were incubated at 35 C for 48 hours. The ethyl violet azide broth used was made by the following formulation:

Ingredients	Grams per liter
tryptose	20
dextrose	15
sodium chloride	5
di-potassium hydrogen phosphate	2.7
potassium di hydrogen phosphate	2.7
ethyl violet	0.00120
sodium azide	0.4
PH	7.0

medium sterilized at 121 C for 15 minutes

In the later studies presented in this thesis on pasteurization, the development of turbidity in the ethyl violet azide broth was considered a positive test for the presence of enterococci. The enterococci index is expressed as a most probable number for 100 ml. of milk, following the table in Standard Methods for the Examination of Dairy Products (1948).

To determine if the turbidity in ethyl violet azide broth was sufficient evidence of the presence of the enterococci, pure cultures were isolated from the ethyl violet azide broth by smearing brain-heart infusion agar in such a manner that discrete colonies were obtained. After 24 hours incubation at 35 C, 4 single, well isolated colonies were transferred to brain-heart infusion broth, 6.5 percent sodium chloride tryptose phosphate broth and litmus milk. Brain-heart infusion broth was incubated at 45 C and the other media at 35 C for 48 hours. The litmus milk tubes were observed after 24 and 48 hours incubation. The observations were recorded as partial reduction, complete reduction, partial coagulation, complete coagulation, proteolysis and liquefaction of casein.

For further study, the enterococci isolated from the milk samples were separated into the following species Str. faecalis, Str. liquefaciens, Str. zymogenes and Str. durans. The identification followed methods proposed by Sherman (1937) and identification according to Bergey's Manual (1948), (Chart 1.).

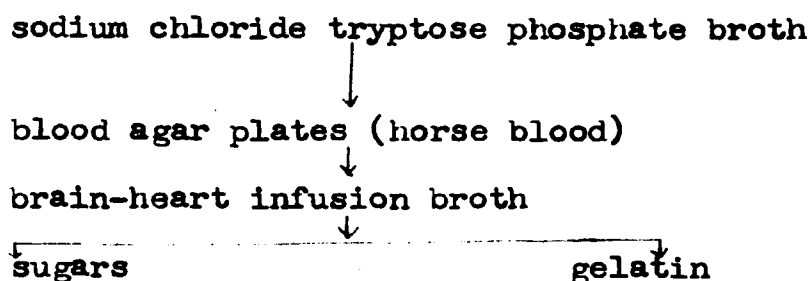
The resistance of enterococci to 6.5 percent sodium chloride was considered a significant test; hence, all cultures growing in sodium

CLASSIFICATION CHART 1

ENTEROCOCCI: DIFFERENTIAL CHARACTERISTICS

	<u>Str. faecalis</u>	<u>Str. zymogenes</u>	<u>Str. liquefaciens</u>	<u>Str. durans</u>
hemolysis	green ±	Beta ±	-	Beta +
gelatin liquefaction	-	+	+	-
litmus Milk strong reduction	+	+	+	-
maximum temperature or growth	45 C	45 C	45 C	50 C
survives 10 min. at temperature	62.8 C	62.8 C	62.8 C	65.6 C
mannitol Acid	+	+	+	rarely
arabinose Acid	±	±	±	-
lucrose Acid	±	+	+	rarely
raffinose Acid	±	±	±	-
Glycerol Acid	+	+	+	-
Sorbitol Acid	±	+	+	-

chloride tryptose phosphate broth were further identified. The following pattern was used:



One loopful of broth from a positive sodium chloride tryptose phosphate broth was smeared on blood agar plates in such a way that discrete colonies were produced. The blood agar plates were incubated at 35 C for 48 hours at which time they were examined for hemolysis. Four isolated selected colonies from each plate were transferred to mannitol, arabinose, sucrose, raffinose, glycerol and sorbitol tubes for the determination of acid production and to gelatin for the determination of liquefaction. All tubes were incubated at 35 C for 48 hours.

Study of Thermal Death Time

For the thermal death time determinations, 1 ml of a 24 hour culture was transferred to 250 ml sterilized skim milk and shaken thoroughly. Five ml of this dilution was placed in a sterile 5/8 by 6 inch test tube. Extreme care was exercised in the delivery of the milk to the tube in order to avoid deposition of milk on the sides of the tube. The tube was then preheated in boiling water to a desired pasteurization temperature, at which time the tube was transferred to the water bath--care being exercised not to spread the milk on the sides of the tube. The laboratory pasteurization was done as usual. The pasteurization was done in two ways;

namely, (1) the temperature was kept constant and the time varied, (2) the time was held constant and the temperature was varied. At the completion of each exposure, appropriate portions of the milk were transferred to tryptone-glucose-extract-milk agar plates, which were incubated at 35 C for 48 hours to determine the number of organisms that survive the pasteurization.

A representative culture of each of the 4 species of enterococci was tested.

Comparison of Coliform and Enterococci Indexes on the Basis of Practical Pasteurization as Well as Laboratory Pasteurization.

Raw milk samples from the Michigan State College Creamery were pasteurized in the laboratory at 61.8 C for 30 minutes. The coliforms and enterococci indexes were determined on the raw milk and the pasteurized milk.

To determine the effect of pasteurization under varied conditions, pasteurized milk samples were collected from dairies in the environs of Lansing and East Lansing. Nine dairies using the low-temperature, long-time pasteurization (61.8 - 62.8 C) for 30 minutes and three dairies using high-temperature, short-time pasteurization (71.6 - 72.2 C for 15 seconds) were checked. Coliform and enterococci indexes were made on all bottled milk samples obtained from these dairies.

Examination of Pasteurized Milk for Coliform and Enterococci Indexes in a Dairy Manufacturing Plant Using both High-Temperature, Short-Time Pasteurization and Low-Temperature, Long-Time Pasteurization for Different Times and Temperatures

This plant has 2 Majonniers cold-wall storage tanks with capacity of 2000 and 3000 gallons. The milk is usually held overnight at a

temperature varying from 3.9 - 7.2 C. The high-temperature, short-time pasteurizer was manufactured by Creamery Package Company. The pasteurizer has a capacity of 15,000 lbs. of milk per hour at regular pasteurization (72.2 C for 16 seconds). Two pasteurization times were used: (1) 72.2 C for 16 seconds for non-homogenized, (2) 77.2 C for 16 seconds for homogenized Vitamin D milk.

The holding pasteurizers were built by Creamery Package Company and are commonly called multiprocess tanks. The capacity of each tank is 500 gallons. The milk is generally held overnight and pasteurized on the following day. In these particular studies, the air space heater was not used. Samples of milk exposed to different times and temperatures were collected.

Sources of Enterococci in Raw Milk

Milk samples were collected aseptically with 10 ml sterile pipettes from the foremilk and strippings from 10 cows, from milking machines, small top milk pails, coolers and milk cans. Determinations were made on each sample for a total bacterial count by the direct microscopic technique and standard plate procedure. Coliform and enterococci indexes were also determined.

RESULTS

The results of the investigation are presented under the following headings:

A. Confirmation of dextrose azide-ethyl violet azide broths for the detection of enterococci in milk

B. Sources of enterococci in raw milk

1. Foremilk and strippings
2. Milking machine
3. Small top milk pail
4. Surface cooler
5. Milk cans

C. Comparison of coliform and enterococci indexes in pasteurized bottled milk

1. Low-temperature, long-time pasteurization at 61.8-62.8 C for 30 minutes
2. High-temperature, short-time pasteurization at 72.2 C for 16 seconds

D. Low-temperature, long-time laboratory pasteurization at

1. 61.8 C for 30 minutes
2. 62.8 " " " "
3. 63.4 " " " "
4. 63.9 " " " "
5. 64.5 " " " "
6. 65.0 " " " "
7. 65.6 " " " "
8. 66.2 " " " "
9. Summary of logarithmic averages for microscopic counts, standard plate counts, coliform and enterococci indexes

E. Low-temperature, long-time plant pasteurization at

1. 62.8 C for 30 minutes
2. Different times and temperatures above 62.8 C and above 30 minutes exposure

F. High-temperature, short-time plant pasteurization at

1. 72.2 C for 16 seconds
2. 76.6 " " " "
3. 77.2 " " " "
4. Summary of logarithmic averages for microscopic counts, standard plate counts, coliform and enterococci indexes

G. Thermal death time of enterococci when

1. Temperature of 61.8 C was constant and time of exposure was increased from 30 minutes upward.
2. Time of 30 minutes exposure was constant and temperature was increased from 61.8 C upward.

H. Percentage distribution of enterococci in raw milk

**A. CONFIRMATION OF DEXTROSE AZIDE-ETHYL VIOLET AZIDE BROTHS
FOR THE DETECTION OF ENTEROCOCCI IN MILK**

Litsky, Mallmann and Fifield (1952) reported a new test for the detection of enterococci in river water, sewage and soil. This test was used by Ellsworth (1952) for the study of fecal streptococci in the feces of calves fed antibiotics and by Radisson (1952) for studying enteric streptococci in the feces of the calves fed terramycin.

Up to now, this technique has not been used to detect the enterococci in milk. Because of the possibility that the presence of milk might upset the specificity of the azide-ethyl violet combination, all positive ethyl violet azide milk tubes were examined further to be sure that all turbidities were due to enterococci and not to streptococci or other bacteria that might be present. Further confirmation was made of all positive ethyl violet azide broth tubes from both raw and pasteurized milk samples.

TABLE 1

**CONFIRMATION OF DEXTROSE AZIDE-ETHYL VIOLET AZIDE BROTHS
FOR THE DETECTION OF ENTEROCOCCI IN MILK**

No. of Samples	No. of Cultures	Confirmed	Percentage Confirmation of Samples	Percentage Confirmation of Cultures
103	239	yes	96.2	97.2
2	4	doubt	1.9	1.6
2	3	no	1.9	1.2

B. SOURCES OF ENTEROCOCCI IN RAW MILK

Raw milk samples from the Michigan State College dairy herd, from foremilk and strippings, milking machines, small top milk pails, surface cooler, and milk cans were collected aseptically and were examined for microscopic count, standard plate count, coliform index, and enterococci index. The results are presented in Tables 2, 3, 4, 5 and 6.

The logarithmic averages of raw milk samples from the above studies are tabulated in Table 7. The results indicate that the milk obtained from the cow contained very few bacteria but as the milk came in contact with milk utensils, like milking machines, small top milk pails, etc. the number of bacteria increased materially. Milk cans were the greatest source of contamination.

TABLE 2

SOURCES OF ENTEROCOCCI IN RAW MILK, FOREMILK AND STRIPPINGS

Cow No.	Microscopic Count		Std. Plate Count		Coliform Index		Enterococci Index	
	Foremilk	Strippings	Foremilk	Strippings	Foremilk M.P.N.	Strippings M.P.N.	Foremilk M.P.N.	Strippings M.P.N.
523	6,000	6,000	4,000	700	0	0	0	0
	6,000	6,000	1,800	1,300	-	-	0	0
521	6,000	6,000	7,700	300	20	0	45	20
	6,000	6,000	2,600	800	-	-	0	0
490	6,000	6,000	5,000	1,100	540	45	20	0
	6,000	6,000	5,200	2,500	-	-	0	0
	6,000	6,000	5,000	200	-	-	0	0
517	6,000	6,000	3,600	700	0	0	0	0
	6,000	6,000	5,000	4,000	-	-	0	0
	6,000	6,000	1,500	0	-	-	0	0
525	6,000	6,000	8,000	500	69	79	0	0
	6,000	6,000	15,000	4,000	-	-	0	0
	6,000	6,000	6,000	1,000	-	-	0	0
Log. Average	6,000	6,000	5,389	570	2.83	1.87	1.7	1.26

TABLE 3
SOURCES OF ENTEROCOCCI IN RAW MILK, MILKING MACHINE

Cow No.	Microscopic Count	Std. Plate Count	Coliform Index M.P.N.	Enterococci Index M.P.N.
523	6,000	1,800	0	0
521	6,000	300	0	45
490	6,000	7,500	1600	40
517	6,000	1,100	41	20
525	6,000	4,300	1800 +	1700
524	6,000	400	0	480
Log. Average	6,000	1,404	22 +	55

TABLE 4
SOURCES OF ENTEROCOCCI IN RAW MILK, SMALL TOP MILK PAIL

No.	Microscopic Count	Std. Plate Count	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	6,000	6,000	920	45
2	6,000	4,500	170	18
3	6,000	3,500	79	93
4	6,000	600	45	130
5	6,000	800	130	170
6	6,000	27,000	79	330
7	6,000	3,000	79	110
8	6,000	6,500	45	45
9	6,000	300	0	110
10	6,000	3,300	1800+	0
Log. Average	6,000	2,737	90+	56

TABLE 5

SOURCES OF ENTEROCOCCI IN RAW MILK FROM SURFACE COOLER FOLLOWED
BY EACH COW

Cow No.	Microscopic Count	Std. Plate Count	Coliform Index M.P.N.	Enterococci Index M.P.N.
523	6,000	5,000	75	0
521	6,000	500	37	0
490	6,000	800	920	130
517	6,000	10,000	1600	78
525	6,000	2,900	920	20
524	6,000	1,100	120	78
Log. Average	6,000	1,999	277	16

TABLE 6

SOURCES OF ENTEROCOCCI IN RAW MILK, MILK CANS
(REFRIGERATED OVER NIGHT AT 5.5 C)

Can No.	Microscopic Count	Std. Plate Count	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	12,000	30,000	1800+	460
2	18,000	30,000	540	700
3	12,000	25,000	240	790
4	12,000	27,000	350	310
5	24,000	26,000	240	330
6	6,000	28,000	540	3,500
7	12,000	23,000	240	490
8	6,000	29,000	540	330
9	12,000	45,000	130	330
Log. Average	11,623	28,715	383+	553

TABLE 7
THE LOGARITHMIC AVERAGES OF RAW MILK SAMPLES
(TABLES 2-6 INCL.) FROM DAIRY HERD

	Logarithmic Average			
	Microscopic Count	Std. Plate Count	Coliform Index M.P.N.	Enterococci Index M.P.N.
Foremilk	6,000	5,389	2.83	1.87
Strippings	6,000	570	1.7	1.26
Milking Machine	6,000	1,404	22+	55
Small Top Milk Pail	6,000	2,737	90+	56
Surface Cooler	6,000	1,999	277	16
Milk Cans	11,623	28,715	383	553

C. THE COMPARISON OF COLIFORM AND ENTEROCOCCI INDEXES IN PASTEURIZED BOTTLED MILK

In this study, pasteurized bottled milk was picked from nine dairies using low-temperature, long-time pasteurization and three dairies using high-temperature, short-time pasteurization to compare the coliform and enterococci indexes on the basis of the most probable numbers per 100 ml milk. The results are given in Table 8. An examination of the results, shows that coliform organisms were present in milk from most of the dairies. This might be due to inadequate pasteurization, but contamination through defective handling or improper sterilization of post-pasteurization equipment is more likely. Enterococci were more susceptible to high-temperature, short-time pasteurization than to low-temperature, long-time pasteurization.

THE COMPARISON OF COLIFORM AND ENTEROCOCCI INDEXES IN PASTEURIZED BOTTLED MILK

[illegible]

D. LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION

The percentage-kill of enterococci by low-temperature, long-time pasteurization was determined by laboratory pasteurization of raw milk obtained from the Michigan State College Creamery. The tests were made in a Cenco-Dekhotinsky waterbath with a maximum variation in temperature of ± 0.1 C. (Figure 1.)

The five ml. test samples of milk were placed in sterile 5/8 x 6 in. test tubes using care not to deposit milk on the sides of the tubes. The tubes were then preheated in boiling water to the desired pasteurization temperature, at which time the tube was transferred to the above mentioned waterbath. The tests were made at temperatures of 61.8, 62.8, 63.4, 63.9, 64.5, 65, 65.6 and 66.2 C for 30 minutes. The number of samples tested was:

22	samples	61.8
8	"	62.8
9	"	63.4
4	"	63.9
5	"	64.5
6	"	65.0
4	"	65.6
2	"	66.2

The results of these tests are presented in Tables 9 to 16 inclusive.

In Tables 9, 10 and 11, maximum titres of high coliform and enterococci were not obtained due to the fact that dilutions used were too low. The logarithmic averages presented for these tables on raw milk, therefore, do not present maximum titres. They are presented, however, to show the effectiveness, in a relative manner, of 61.8 C. in the kill of the enterococci in comparison to the total count and the coliform index. In Tables

12 to 16 inclusive, maximum titres of enterococci were obtained, so that the logarithmic averages show comparative kills for each temperature.

In Table 17, the logarithmic averages of milk samples (Tables 9-16 inclusive) pasteurized at varying temperatures for a 30 minutes exposure period have been presented. The logarithmic averages of microscopic count and standard plate count do not show significant differences. Coliform organisms were completely destroyed. As far as enterococci are concerned, 98.72 percent of them were killed at a temperature of 61.8 C for a 30 minutes exposure period.



Figure 1. Cenco-Dekhotinsky constant temperature waterbath.

TABLE 9

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 61.8 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	40	50	+ -	1800+ 0	180,000 + 2300
2	Raw Past.	60	55	+ -	1800+ 0	180,000 + 1700
3	Raw Past.	35	40	+ -	1800+ 0	180,000 + 1300
4	Raw Past.	80	90	+ -	1800+ 0	180,000 + 450
5	Raw Past.	60	80	+ -	1800+ 0	180,000 + 780
6	Raw Past.	85	80	+ -	1600 0	1,300 200
7	Raw Past.	100	90	+ -	1800+ 0	180,000 + 1300
8	Raw Past.	60	50	+ -	920 0	35,000 450
9	Raw Past.	75	70	+ -	220 0	28,000 0
10	Raw Past.	80	60	+ -	1800+ 0	1400 450
11	Raw Past.	90	70	+ -	1600 0	92,000 200
12	Raw Past.	20	50	+ -	1600 0	18,000 + 2400

Continued next page

TABLE 9 - Continued

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
13	Raw Past.	24	30	+ -	1800 + 0	18,000 + 0
14	Raw Past.	36	30	+ -	1800 + 0	230 0
15	Raw Past.	300	250	+ -	1800 + 0	18,000 + 330
16	Raw Past.	260	200	+ -	1800 + 0	18,000 + 230
17	Raw Past.	96	100	+ -	1800 + 0	18,000 + 3500
18	Raw Past.	36	40	+ -	1800 + 0	18,000 + 5400
19	Raw Past.	114	110	+ -	1800 + 0	18,000 + 18,000 +
20	Raw Past.	84	82	+ -	1800 + 0	18,000 + 330
21	Raw Past.	24	36	+ -	920 0	4300 330
22	Raw Past.	21	15	+ -	69 0	9200 110
Log. Average Raw		61.770	62.867		1306 +	24,982 +
Log. Average Past.					0	321

TABLE 10

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 62.8 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	60	40	+ -	180 0	2,400 1300
2	Raw Past.	48	35	+ -	350 0	16,000 2400
3	Raw Past.	200	150	+ -	1800 + 0	3,500 0
4	Raw Past.	96	30	+ -	1800 + 0	2,400 130
5	Raw Past.	114	110	+ -	1800 + 0	18,000 + 2400
6	Raw Past.	84	82	+ -	1800 + 0	18,000 + 0
7	Raw Past.	96	100	+ -	1800 + 0	18,000 + 1700
8	Raw Past.	36	40	+ -	1800 + 0	18,000 + 270
	Log. Average Raw	80.768	62.31		1100 +	8,734 +
	Log. Average Past.				0	161

TABLE 11

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 63.4 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	324	1,770	+ -	1800 + 0	1,800,000+ 0
2	Raw Past.	96	100	+ -	1800 + 0	18,000+ 950
3	Raw Past.	36	40	+ -	1800 + 0	18,000+ 790
4	Raw Past.	114	110	+ -	1800 + 0	18,000+ 170
5	Raw Past.	84	82	+ -	1800 + 0	18,000+ 0
6	Raw Past.	24	36	+ -	920 0	4,300 170
7	Raw Past.	21	15	+ -	69 0	9,200 170
8	Raw Past.	60	40	+ -	180 0	2,400 490
9	Raw Past.	48	35	+ -	350 0	16,000 1,300
	Log. Average Raw	62.896	68.788		750 +	18,754+
	Log. Average Past.				0	110

TABLE 12

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 63.9 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	60	40	+ -	180 0	2400 340
2	Raw Past.	48	35	+ -	350 0	16,000 1300
3	Raw Past.	200	150	+ -	1800 + 0	3500 0
4	Raw Past.	96	30	+ -	1800 + 0	2400 0
	Log. Average Raw	86.230	50.100		672 +	4,238
	Log. Average Past.				0	26

TABLE 13

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 64.5 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	21	15	+ -	69 0	920 45
2	Raw Past.	24	36	+ -	920 0	4300 20
3	Raw Past.	60	40	+ -	180 0	2400 210
4	Raw Past.	48	35	+ -	350 0	16,000 1300
5	Raw Past.	1,440	2,310	+ -	1800 + 0	1,300,000 0
Log. Average Raw		73.120	70.540		373 +	11,460
Log. Average Past.					0	48

TABLE 14

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 65.0 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	30	24	+ -	1800 + 0	230 0
2	Raw Past.	24	30	+ -	1800 + 0	2400 0
3	Raw Past.	48	35	+ -	350 0	16,000 2400
4	Raw Past.	60	40	+ -	180 0	2400 1300
5	Raw Past.	200	150	+ -	1800 + 0	3500 0
6	Raw Past.	96	30	+ -	1800 + 0	2400 0
	Log. Aver- age Raw	58.434	40.685		933 +	2,372
	Log. Aver- age Past.				0	12

TABLE 15

**LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 65.6 C FOR 30 MINUTES**

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	24	36	+ -	920 0	4300 78
2	Raw Past.	21	15	+ -	69 0	9,200 78
3	Raw Past.	30	36	+ -	1800 + 0	230 0
4	Raw Past.	24	30	+ -	1800 + 0	2400 0
Log. Aver- age Raw		24.544	27.635		673 +	2,162
Log. Aver- age Past.					0	9

TABLE 16

LOW-TEMPERATURE, LONG-TIME LABORATORY PASTEURIZATION
AT 66.2 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	30	36	+ -	1800 + 0	230 0
2	Raw Past.	24	30	+ -	1800 + 0	2400 0
	Log. Aver- age Raw	26.833	32.863		1800 +	743
	Log. Average Past.				0	0

THE LOGARITHMIC AVERAGES OF MILK SAMPLES (TABLES 9-16 INCL.) PASTEURIZED
AT VARYING TEMPERATURES FOR A 30 MINUTES EXPOSURE PERIOD

Sample	No. of Samples	Temp. (Centi- grade)	Logarithmic Average				Percentage Reduction of Enterococci
			Micro Count (thousand)	Std. Plate Count (thousand)	Coliform Index M.P.N.	Enterococci Index M.P.N.	
Raw	22	61.8	61.770	62.867	1306 +	24,982 +	
Past.					0	321	98.72
Raw	8	62.8	80.768	62.310	1100 +	8,734 +	
Past.					0	161	98.16
Raw	9	63.4	62.896	68.788	750 +	18,754 +	
Past.					0	110	99.42
Raw	4	63.9	86.230	50.100	672 +	4,238	
Past.					0	26	99.39
Raw	5	64.5	73.120	70.540	373 +	11,460	
Past.					0	48	99.58
Raw	6	65.0	58.434	40.685	933 +	2,372	
Past.					0	12	99.49
Raw	4	65.6	24.544	27.635	673 +	2,162	
Past.					0	9	99.59
Raw	2	66.2	26.833	32.863	1800 +	743	
Past.					0	0	100.00

E. LOW-TEMPERATURE, LONG-TIME PLANT PASTEURIZATION

Raw milk was pasteurized in multiprocess tanks each having a capacity of 500 gallons. It was preheated up to 62.8 C and then was held for 30 minutes at this temperature. The samples were examined for microscopic count, standard plate count, phosphatase test, coliform index and enterococci index. Pasteurized milk samples were observed for thermoduric count, phosphatase test, coliform index and enterococci index.

The object of this study was to find out the behavior of coliform and enterococci under usual plant pasteurization conditions and to compare these results with the results obtained from the most accurate laboratory pasteurization of the milk samples. The results presented in Table 18 coincide with the results shown in Table 17.

The results tabulated in Table 19 indicate that the enterococci are not all killed at 62.3 C. for 38 minutes, 62.3 C. for 41 minutes, 62.8 C. for 35 minutes but at 66.7 C. for 30 minutes, 66.7 C. for 68 minutes, and when a temperature of 84.9 C was used and the milk after reaching the temperature, being immediately cooled, the enterococci were completely destroyed.

TABLE 18

LOW-TEMPERATURE, LONG-TIME PLANT PASTEURIZATION AT 62.8 C FOR 30 MINUTES

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.	Percentage Reduction of Enterococci
1	Raw Past.	1,100	1,150 12	+ -	1800 + 0	1,800,000 + 2,300	99.87
2	Raw Past.	168	150 25	+ -	1800 + 0	3,500,000 + 20	99.999 +
3	Raw Past.	156	1,430 6	+ -	1800 + 0	1,800,000 + 330	99.98 +
4	Raw Past.	250	300 5	+ -	1800 + 0	920,000 130	99.96
Log. Average Raw		291.365	521.585		1800 +	1,797,200	
Log. Average Past.			9.740		0	211	

LOW-TEMPERATURE, LONG-TIME PLANT PASTEURIZATION AT DIFFERENT TIMES AND TEMPERATURES

S. No.	Sample	Time (Minute)	Temperature (Centigrade)	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phospha- tase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.	Percentage Reduction of Enterococci
1	Raw Past.	38	62.3	180	650 22	+ -	1800 + 0	1,800,000 + 490	99.97 +
2	Raw Past.	41	62.3	444	910 3.4	+ -	1800 + 69	1,800,000 + 45	99.99 +
3	Raw Past.	35	62.8	500	450 4	+ -	1800 + 0	1,800,000 + 110	99.99 +
4	Raw Past.	38	62.8	1000	900 14	+ -	1800 + 0	1,800,000 + 220	99.99
5	Raw Past.	30	66.7	720	800 5	+ -	1800 + 0	790,000 0	100.00
6	Raw Past.	68	66.7	288	100 -	+ -	1800 + 0	3,500,000 0	100.00
7	Raw Past.	hitting	84.9	1,440	2,310 0.5	+ -	1800 + 0	1,300,000 0	100.00

F. HIGH-TEMPERATURE, SHORT-TIME PLANT PASTEURIZATION

A high-temperature, short-time pasteurizer, manufactured by Creamery Package Company, was used for this purpose. Three different temperatures were used, 72.2, 76.6 and 77.2 C. for 16 seconds. The whole process was automatic and there was no possibility of raw milk mixing with the pasteurized product. The number of samples tested was:

12 samples	72.2
7 " "	76.6
16 " "	77.2

The results are given in Tables 20, 21 and 22 respectively. Negative coliform indexes and phosphatase tests were obtained at all temperatures. The most probable number of enterococci obtained at 72.2 and 76.6 C. for 16 seconds is very low and at 77.2 C. the enterococci were completely destroyed, except in two samples.

In Table 23, the logarithmic averages of milk samples (Tables 20-22 inclusive) pasteurized at varying temperatures for a 16 seconds exposure period are presented. The percentage reduction of enterococci in high-temperature, short-time pasteurization is more efficient than in low-temperature, long-time pasteurization.

TABLE 20
HIGH-TEMPERATURE, SHORT-TIME PLANT PASTEURIZATION
AT 72.2 C FOR 16 SECONDS

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	350	1,085 34	+ -	1800 + 0	1,800,000 + 36
2	Raw Past.	1,263	2,160 20	+ -	1800 + 20	16,100,000 450
3	Raw Past.	1,788	2,460 13	+ -	1800 + 0	9,200,000 780
4	Raw Past.	2,580	5,000 18	+ -	1800 + 0	2,300,000 0
5	Raw Past.	828	1,400 10	+ -	1800 + 0	1,800,000 68
6	Raw Past.	850	2,000 20	+ -	1800 + 0	1,800,000 330
7	Raw Past.	696	1,490 31	+ -	1800 + 0	1,800,000 78
8	Raw Past.	492	1,530 40	+ -	1800 + 0	1,800,000 + 2400
9	Raw Past.	1,272	2,080 15	+ -	1800 + 0	1,800,000 + 330
10	Raw Past.	750	700 8	+ -	1800 + 0	1,800,000 + 110
11	Raw Past.	825	800 9	+ -	1800 + 0	1,600,000 230
12	Raw Past.	336	110 23	+ -	1800 + 0	1,800,000 + 130
	Log. Average Raw	843.662	1329.100		1800 +	2,501,600
	Log. Average Past.		17.753		2	140

TABLE 21

HIGH-TEMPERATURE, SHORT-TIME PLANT PASTEURIZATION
AT 76.6 C FOR 16 SECONDS

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phosphatase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	440	1,981 23	+ -	1800 + 0	1,800,000 + 0
2	Raw Past.	1,263	2,160 10	+ -	1800 + 0	16,100,000 680
3	Raw Past.	2,580	3,000 10	+ -	1800 + 0	16,100,000 0
4	Raw Past.	696	1,490 20	+ -	1800 + 0	1,800,000 + 78
5	Raw Past.	492	1,530 25	+ -	1800 + 0	1,800,000 + 230
6	Raw Past.	1,272	2,080 9	+ -	1800 + 0	1,800,000 + 230
7	Raw Past.	1,788	2,460 14	+ -	1800 + 0	9,200,000 0
Log. Aver- age Raw		1,016.000	2045.300		1800 +	4,249,700
Log. Aver- age Past.			14.651		0	22

TABLE 22

HIGH-TEMPERATURE, SHORT-TIME PLANT PASTEURIZATION
AT 77.2 C FOR 16 SECONDS

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phospha- tase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
1	Raw Past.	1000	1,250 2.7	+ -	1800 + 0	1,800,000 + 0
2	Raw Past.	200	169 5	+ -	1800 + 0	1,800,000 + 0
3	Raw Past.	300	297 9	+ -	1800 + 0	1,800,000 + 0
4	Raw Past.	325	311 5	+ -	1800 + 0	1,800,000 + 0
5	Raw Past.	440	1,981 23	+ -	1800 + 0	1,800,000 + 0
6	Raw Past.	1,263	2,160 10	+ -	1800 + 0	16,100,000 680
7	Raw Past.	2,580	3,000 10	+ -	1800 + 0	16,100,000 0
8	Raw Past.	1,788	2,460 14	+ -	1800 + 0	9,200,000 0
9	Raw Past.	720	800 15	+ -	1800 + 0	790,000 0
10	Raw Past.	552	1000 10	+ -	1800 + 0	9,200,000 0
11	Raw Past.	168	150 10	+ -	1800 + 0	3,500,000 0
12	Raw Past.	212	120 25	+ -	1800 + 0	790,000 0
13	Raw Past.	288	100 20	+ -	1800 + 0	3,500,000 0

Continued next page

TABLE 22 - Continued

S. No.	Sample	Micro. Count (Thousand)	Std. Plate Count (Thousand)	Phospha- tase Test	Coliform Index M.P.N.	Enterococci Index M.P.N.
14	Raw Past.	252	300 20	+ -	1800 + 0	1,800,000 0
15	Raw Past.	108	200 25	+ -	1800 + 0	1,600,000 0
16	Raw Past.	184	210 30	+ -	1800 + 0	1,800,000 45
17	Raw Past.	253	250 16	+ -	1800 + 0	1,800,000 0
18	Raw Past.	1,288	390 16	+ -	1800 + 0	1,600,000 0
	Log. Aver- age Raw	436.600	466.400		1800 +	2,669,400
	Log. Aver- age Past.		12.510		0	2

TABLE 23

THE LOGARITHMIC AVERAGES OF MILK SAMPLES (TABLES 20-22 INCL.) PASTEURIZED
AT VARYING TEMPERATURES FOR A 16 SECONDS EXPOSURE PERIOD

Sample	No. of Samples	Temp. (Centi- grade)	Logarithmic Average				Percentage Reduction of Enterococci
			Micro. Count (thousand)	Std. Plate Count (thousand)	Coliform Index M.P.N.	Enterococci Index M.P.N.	
Raw	12	72.2	843.622	1329.100	1800 +	2,501,600	
Past.				17.753	2	140	99.99 +
Raw	7	76.6	1,016,000	2045.300	1800 +	4,249,700	
Past.				14.651	0	22	99.9999 +
Raw	18	77.2	436,600	466.400	1800 +	2,669,400	
Past.				12.510	0	2	99.9999 +

G. THERMAL DEATH TIME OF ENTEROCOCCI

To study the thermal death time of four subgroups of enterococci, namely, Str. faecalis, Str. liquefaciens, Str. zymogenes, and Str. durans, pure cultures of these organisms were transferred to brain-heart infusion broth for 6 successive transplants and then a 24 hour culture was used for the thermal death time study. The results are shown in Tables 24 and 25. The thermal death point was determined, first, by holding the temperature constant (61.8 C) and determining the period of exposure necessary to obtain complete kill and second, by holding the time constant (30 min.) and increasing the temperature until complete kill was obtained. The Cenco-Dekhotinsky constant temperature waterbath, with a variation of ± 0.1 C, was used for this study.

TABLE 24

THERMAL DEATH TIME OF ENTEROCOCCI WHEN TEMPERATURE OF
61.8 C WAS CONSTANT AND TIME FACTOR WAS CHANGED

Name of Organism	Temperature (Centigrade)	Time (Minute)
<u>Str. faecalis</u>	61.8	64
<u>Str. liquefaciens</u>	61.8	64
<u>Str. zymogenes</u>	61.8	64
<u>Str. durans</u>	61.8	71

TABLE 25

THERMAL DEATH TIME OF ENTEROCOCCI WHEN TIME OF 30 MINUTES
WAS CONSTANT AND TEMPERATURE WAS CHANGED

Name of Organism	Time (Minute)	Temperature (Centigrade)
<u>Str. faecalis</u>	30	68.90
<u>Str. liquefaciens</u>	30	69.40
<u>Str. zymogenes</u>	30	68.90
<u>Str. durans</u>	30	69.40

H. PERCENTAGE DISTRIBUTION OF ENTEROCOCCI IN MILK

Fifty-two cultures of enterococci isolated from milk were classified by hemolysis on blood agar plates, liquefaction of gelatin, acid production in different sugars and reduction of litmus milk in 24 and 48 hours. The species percentage distribution of enterococci in milk is given in Table 26.

TABLE 26
PERCENTAGE DISTRIBUTION OF SUBGROUPS
OF ENTEROCOCCI IN MILK

Name of Organism	No. of Cultures	Percent
<u>Str. faecalis</u>	41	78.8
<u>Str. durans</u>	7	13.4
<u>Str. liquefaciens</u>	2	3.9
<u>Str. zymogenes</u>	2	3.9

DISCUSSION

Because enterococci are common in the intestinal tracts of man and animals, they should be good indicators of fecal contamination in foods and water. English bacteriologists use an enterococci test as a supplementary procedure for the sanitary testing of water.

Litsky, Mallmann and Fifield (1952) reported on the enterococci test for detecting pollution of river water, sewage and soil. Dextrose azide broth is employed in the test as a presumptive medium and ethyl violet azide broth for confirmation.

The Litsky, Mallmann and Fifield procedure was used for the examination of milk, to determine the possible application of the enterococci test in measuring the sanitary quality of raw milk, the effectiveness of pasteurization and the detection of post-pasteurization contamination.

The cultures isolated from the ethyl violet azide broth were considered to be enterococci if they grew at 45 C in brain-heart infusion broth and at 35C in 6.5 percent sodium chloride broth. The litmus milk reaction showed considerable variation as shown in Figure 2. This is due to the presence of different subgroups of enterococci in the same sample of milk. A gram stain of enterococci from dextrose azide broth and ethyl violet azide broth is shown in Figures 3 and 4.

A total of 246 cultures from 107 samples of milk were checked for identity. Four cultures from 2 samples were questionable and 3 cultures from 2 other samples were definitely not enterococci. These data show that in this study the ethyl violet azide broth confirmative test gave an

accuracy of 96.2 percent for the samples examined and 97.2 percent for the cultures examined.

The accuracy of the procedure compares favorably with the confirmation procedures used for the detection of coliform organisms in water. Thus for the studies presented later in this thesis, the dextrose azide broth-ethyl violet azide broth method was used as a quantitative procedure for determining the presence of enterococci in milk.

Examinations made of both foremilk and strippings showed that few enterococci were present. Only 1 out of thirteen samples of strippings showed enterococci and then only 20 per 100 ml. of milk. Coliform indexes were somewhat comparable.

The examination of milk from milking machines, small top milk pails, coolers and cans showed higher counts depending largely upon the cleanliness of the utensil and the care exercised in the operation. In nearly every instance the coliform count paralleled closely the enterococci index. The results indicate that the enterococci test is a measurement of the sanitary quality of milk but it is no better than the coliform test. Inasmuch as the coliform test is already established as a sanitary index there would be little reason even to suggest the enterococci test as a supplementary procedure.

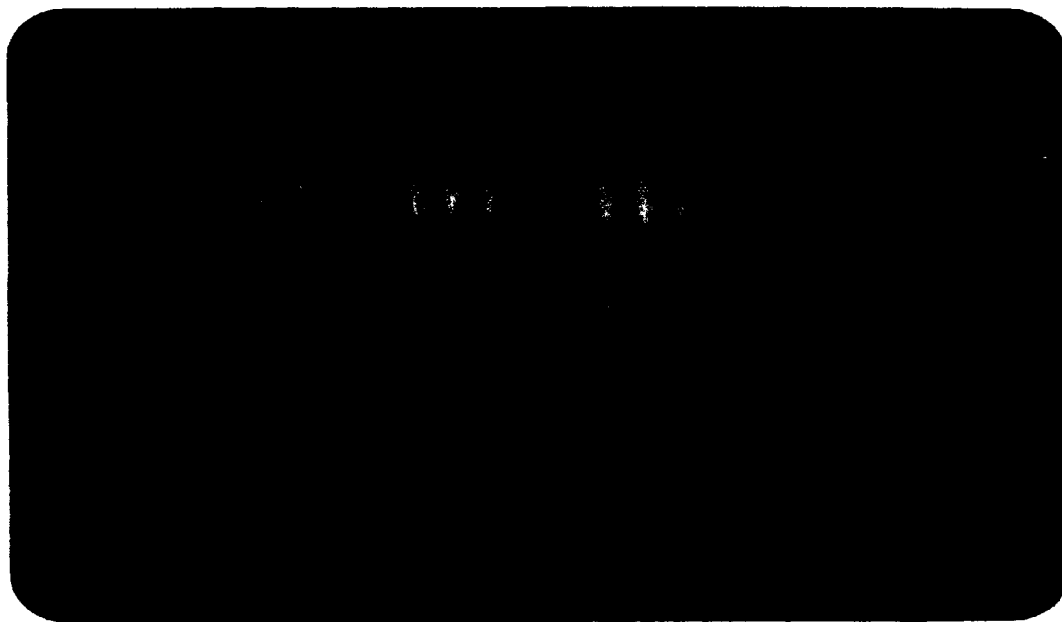


Figure 2. Variation shown by different subgroups of enterococci in litmus milk



Figure 3. Gram stain of enterococci from dextrose azide broth.

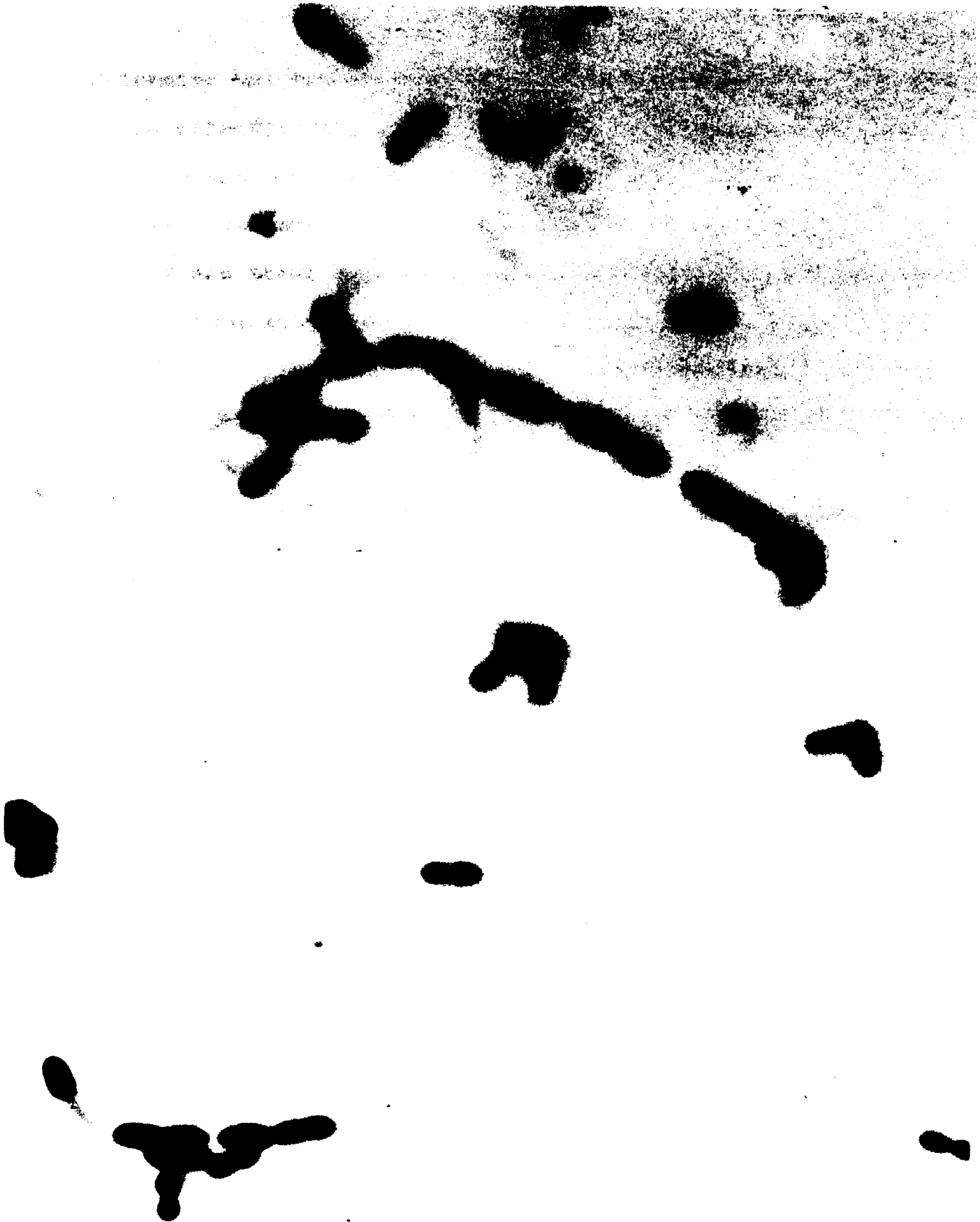


Figure 4. Gram stain of enterococci from ethyl violet azide broth.

To determine the possible application of an enterococci test for measuring the effectiveness of pasteurization, examinations of pasteurized market milks in the local area were made. In this study pasteurized market milk was obtained from nine dairies using low-temperature, long-time pasteurization and three dairies using high-temperature, short-time pasteurization. Examinations were made for both coliform and enterococci incidence. Both coliform and enterococci were found more frequently in milk pasteurized by low-temperature, long-time pasteurization. In general, the incidence of enterococci was higher than that for coliforms. The results indicate that in this particular survey post-pasteurization contamination was apparently quite common because it has been well established that coliform organisms do not survive pasteurization by either method. The presence of enterococci in this survey does not necessarily indicate post-pasteurization contamination. The results show however that enterococci are common to pasteurized milk and indicate the possible need of further study to determine the effect of pasteurization on the viability of the organisms.

Laboratory pasteurization tests were made on raw milk samples obtained from the Michigan State College Creamery. The laboratory pasteurization tests were made at temperatures of 61.8 C and above for an exposure period of 30 minutes. At a temperature of 61.8 C the percentage-kill of enterococci was 98.72. Tests made at 62.8 C gave a percentage-kill of 98.16. Successive tests made at temperature of 63.4, 63.9, 64.5, 65.0 and 65.6 C gave percentage-kill figures from 99.39 to 99.59. It is interesting to

note that the resistant minority at all temperatures irrespective of the initial population varied from 0 to 18,000+ organisms per 100 ml. of milk. An examination of these figures indicate that enterococci are apparently quite susceptible to pasteurization temperature but in most cases a few resistant cells existed. This resistant minority would be sufficient to exclude the test as a measurement of the effectiveness of pasteurization unless the number allowable was set at some figure above the average number observed.

In every instance, a complete kill of coliform organisms was obtained.

To study further the survival of enterococci in plant pasteurization, one plant was selected where various pasteurization temperatures are in current use for different milk or milks destined for further processing. To eliminate the possibility of recontamination by post-pasteurization which was quite evident in the first study of bottled pasteurized milk, samples were collected directly from the pasteurizer at the completion of pasteurization. When the milk was pasteurized at 62.8 C. for 30 minutes, the enterococci reduction varied from 99.87 to 99.99+ percent. The average enterococci index before pasteurization was 1,797,200 and after pasteurization of the milk was 211. The phosphatase test and the coliform index were both zero as would be expected. When the temperature of pasteurization was maintained at 66.7 C for 30 minutes or more, the enterococci were completely killed. These data are in agreement with the laboratory pasteurization tests.

Studies were also made at the same plant using high-temperature, short-time pasteurization. The results obtained at 72.2 C for 16 seconds are

comparable to those obtained with low-temperature, long-time pasteurization at 62.8 C. When the temperature was increased to 76.6 C for 16 seconds, 3 out of seven samples were negative for enterococci. When the temperature was increased to 77.2 C for 16 seconds only 2 out of 18 samples tested gave positive tests for enterococci. The temperature necessary for complete kill of enterococci in milk would appear to be a minimum temperature of 66.7 C for 30 minutes and 77.2 C for 16 seconds.

Lear and Foster (1951) demonstrated by use of the phosphatase test that the holding period may be a variable in different methods of pasteurization. For example, when milk is pasteurized at 63.5 C for 30 minutes, the percentage of total lethal heat contributed by one minute preheating for inactivation of phosphatase is 0.40 whereas when the milk is pasteurized at 73 C, the one minute preheating contributes 29.91 percent of the inactivation. This is in keeping with the report by Sanders and Sager (1948) U. S. Public Health Service Standards provide less safety margin at 71.1 C than at 61.7 C.

If milk were pasteurized at 77.2 C a considerably greater degree of safety would be provided and a negative enterococci test would definitely serve as an indicator of safety. Neither the phosphatase nor the coliform tests would serve as both tests are negative at a temperature of 72.2 C.

Under the laboratory and plant pasteurization conditions, the complete kill of enterococci as a group has been determined at a definite time and temperature both in low-temperature, long-time pasteurization and in the high-temperature, short-time process.

No attempt was made in these studies to determine the species of enterococci that survived the pasteurization process. It was felt essential to study thermal resistance of pure cultures of the four species of enterococci under identical pasteurization conditions.

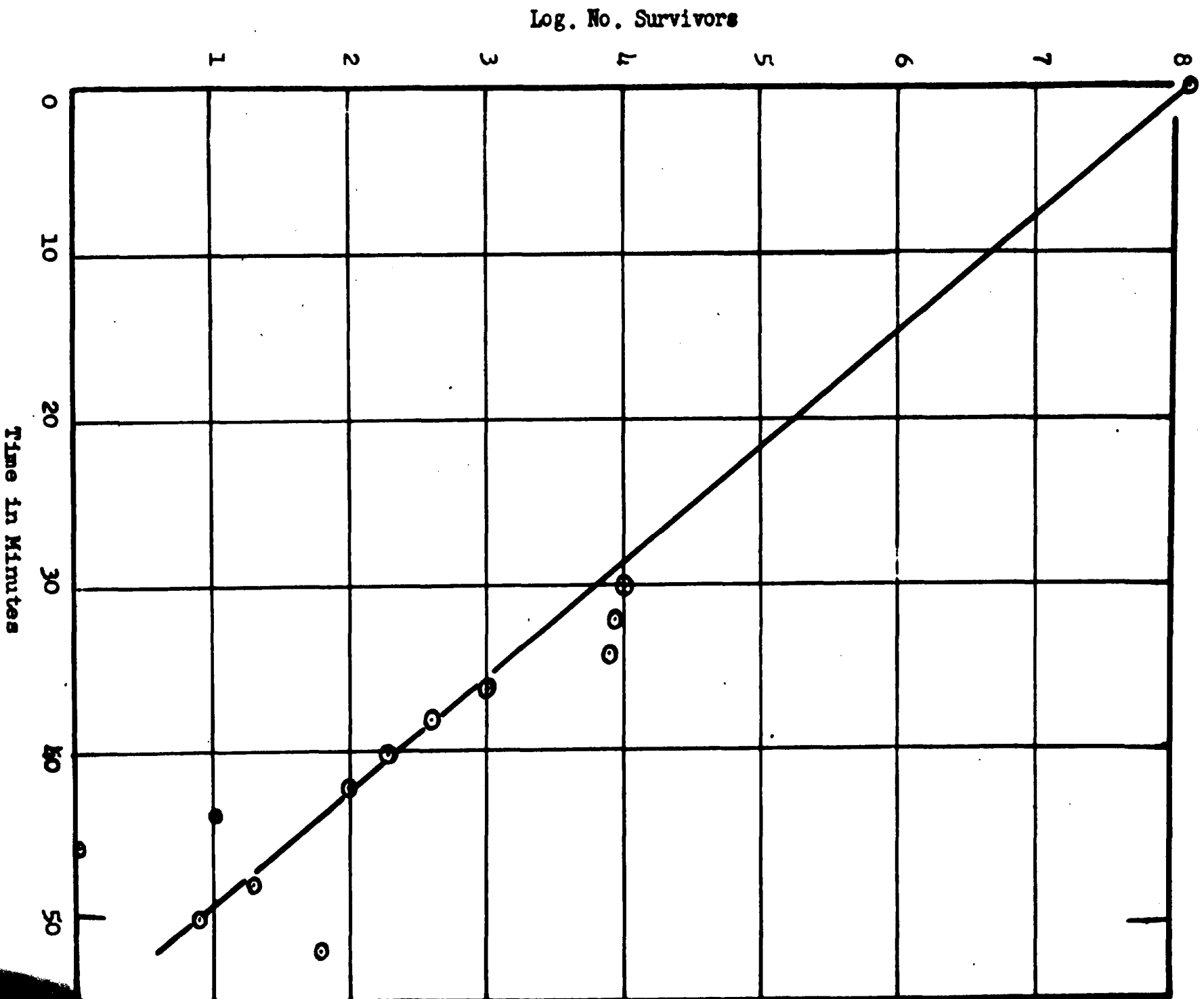
The thermal resistance of Str. faecalis and Str. liquefaciens is presented in Graphs I, II, III, IV. In a 30 minutes exposure period at 61.8 C, a kill of 99.99 + percent for both Str. faecalis and Str. liquefaciens was obtained. These results are comparable to those obtained in the low-temperature, long-time pasteurization of milk. When the period of exposure was increased, the reduction of the resistant minority was very gradual and a few organisms (less than 100 per ml. for Str. faecalis and less than 250 per ml. for Str. liquefaciens) survived up to 52 minutes, the maximum exposure period of the test.

When the temperature was increased to 64.5 C., the kill for both species was in excess of 99.98 + percent. The resistant minority decreased very gradually when the temperature for the 30 minutes test period was increased. Complete kill was not obtained until the temperature of 68.9 C for Str. faecalis and 69.4 C for Str. liquefaciens were used.

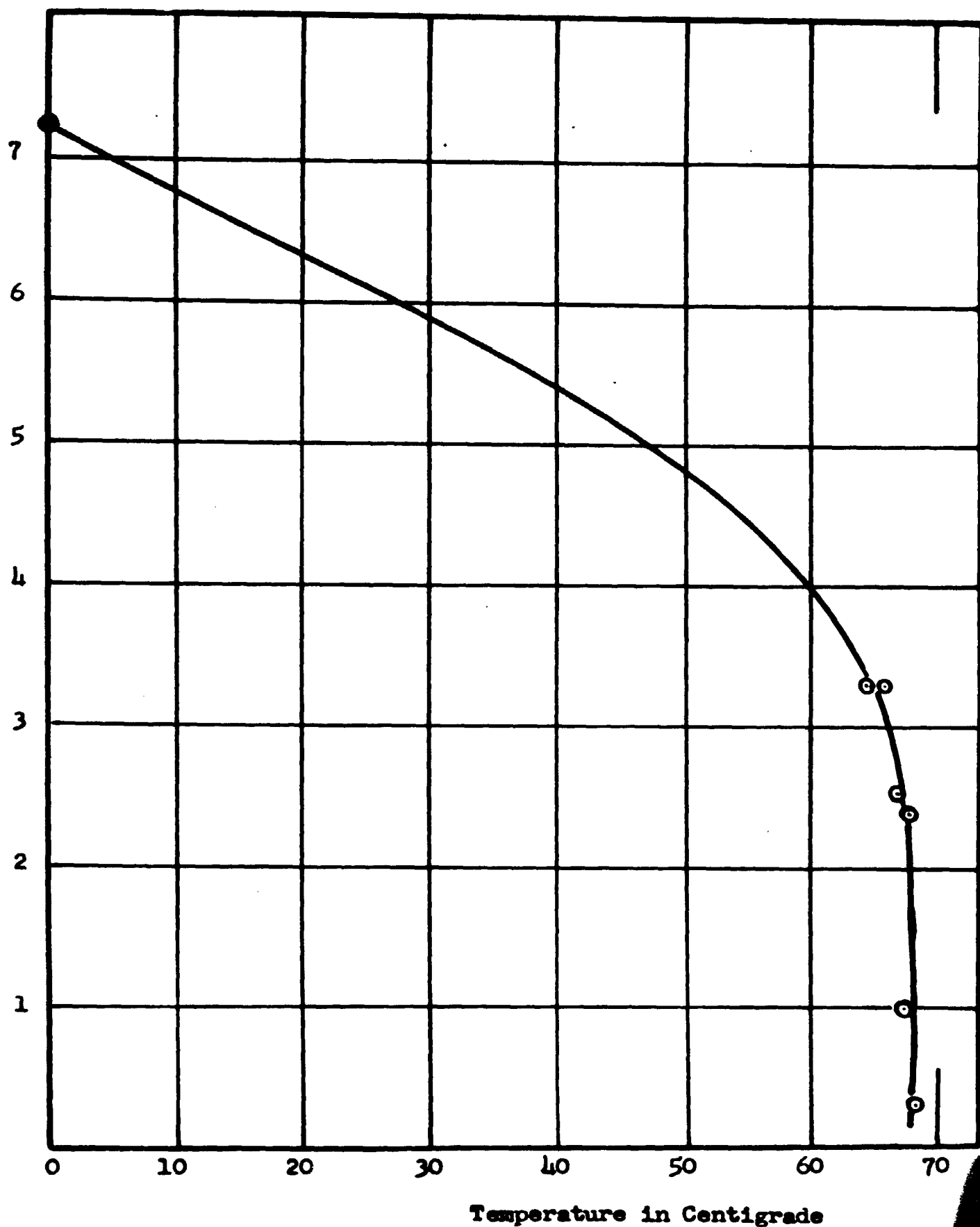
The pure culture study of thermal death time of enterococci shows that a resistant minority occurs in each of the subgroups of pure culture which ultimately increases the thermal death time and thermal death temperature.

At 61.8 C the pure cultures of Str. faecalis, Str. liquefaciens and Str. zymogenes were completely destroyed in a 64 minutes exposure period while Str. durans was killed in 71 minutes. In a 30 minutes exposure

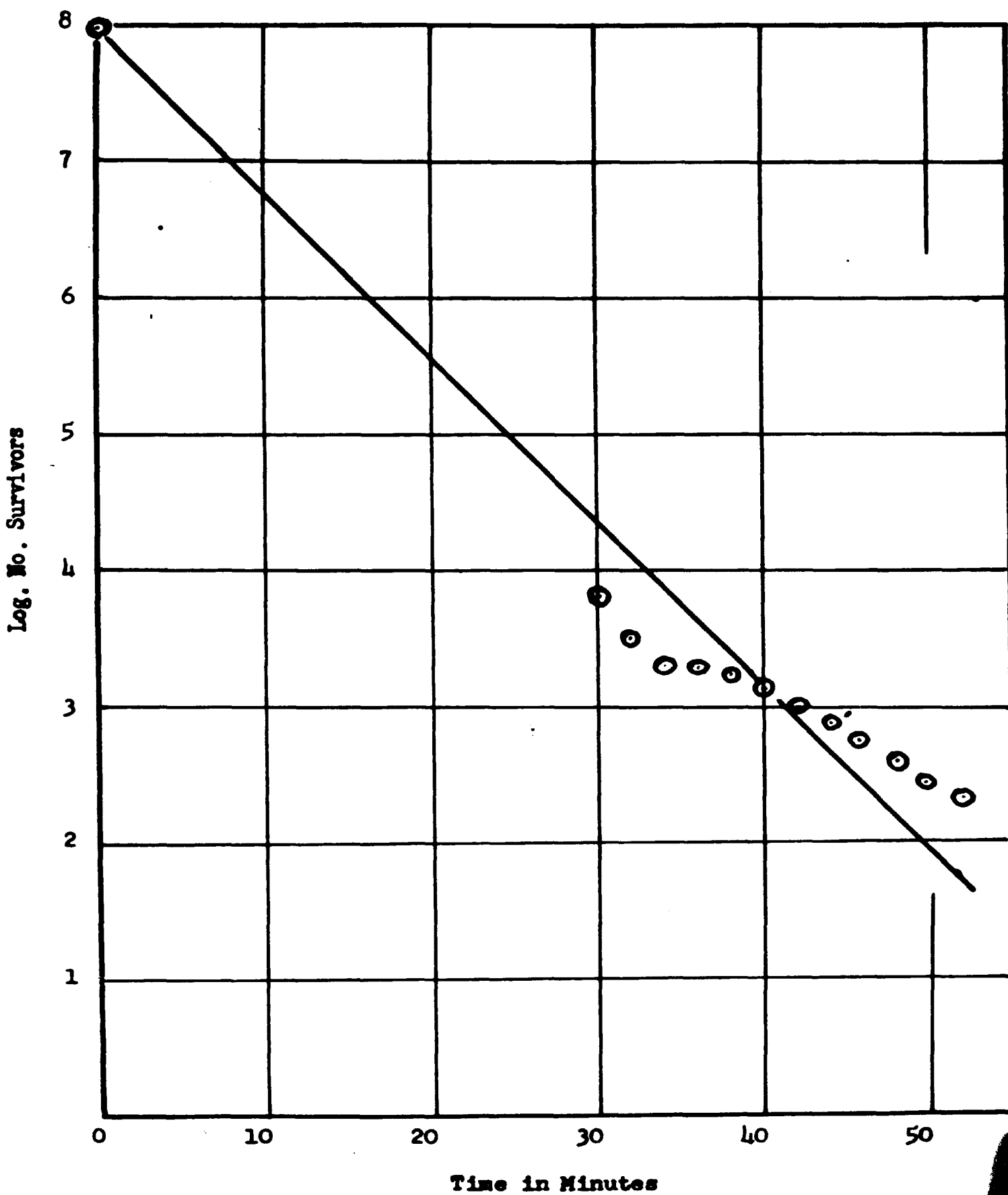
Graph I. The effect of time on Str. faecalis when exposed to a temperature of 61.8 C



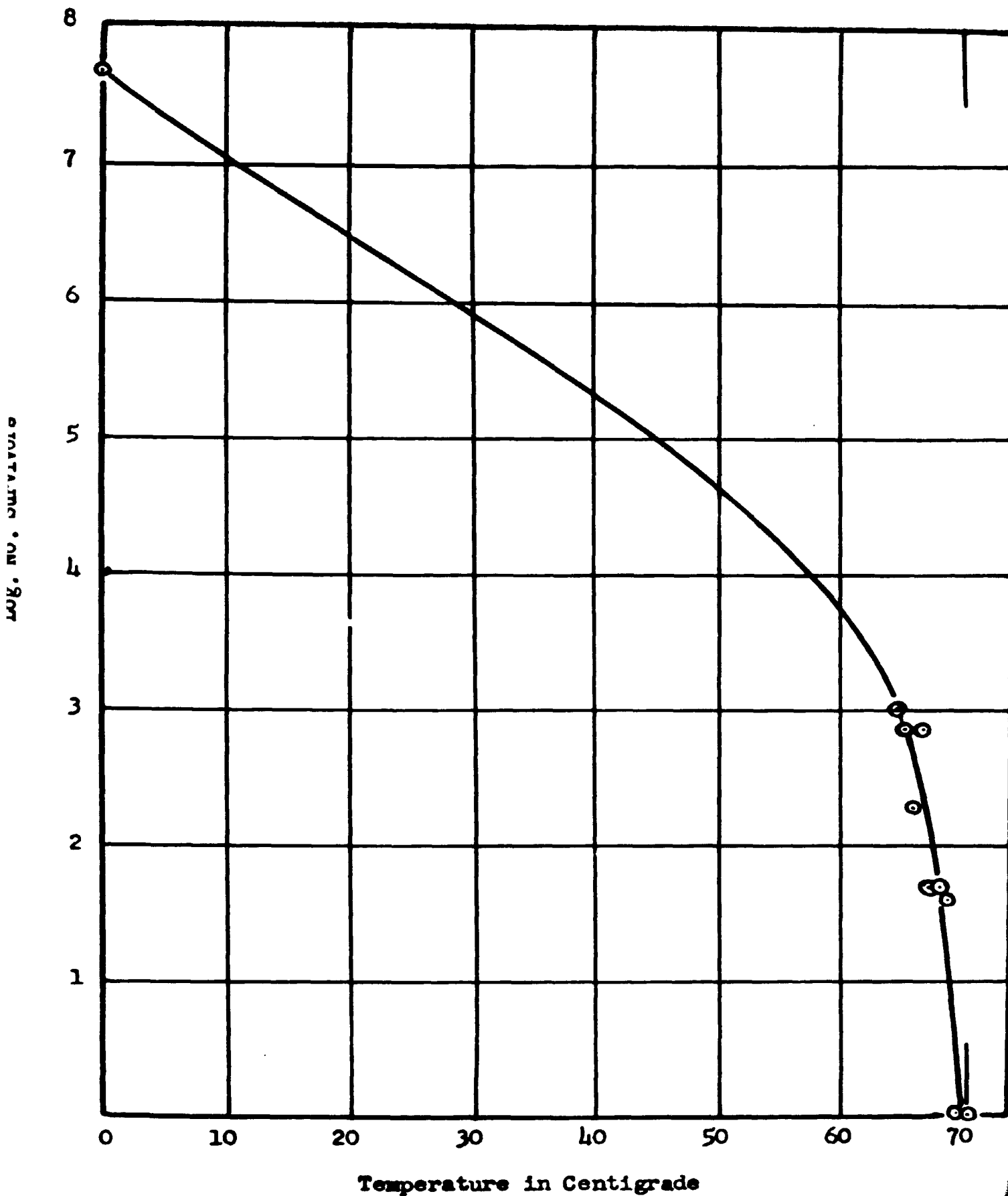
Graph II. The effect of successive increase in temperature on the viability of Str. faecalis for a 30 minutes exposure period.



Graph III. The effect of time on Str. liquefaciens when exposed to a temperature of 61.8 C



Graph IV. The effect of successive increase in temperature on the viability of Str. liquefaciens for a 30 minutes exposure period.



period, the pure cultures of Str. faecalis and Str. zymogenes were killed at 68.9 C while Str. liquefaciens and Str. durans were destroyed at 69.4 C.

These results prove that the enterococci on the whole at pasteurizing temperature are quite susceptible to heat but a resistant minority shows high resistance and survives at the present pasteurizing standards. This ability of a few bacteria to withstand the pasteurizing temperature may be due to certain resistant characteristics peculiar to a few cells. Hence, the maximum time and temperature required to bring about the complete destruction of enterococci is based on the resistant minorities and not the majority of the enterococci.

The percentage distribution of subgroups of enterococci in milk shows that the Str. faecalis is a predominating organism which constitutes 78.8 percent of the total cultures of enterococci studied. Next comes Str. durans and then Str. liquefaciens and Str. zymogenes. These results coincide with the statement made by Brown and Gibbons (1950) on the types of enterococci in eggs and egg products.

SUMMARY AND CONCLUSIONS

The dextrose azide-ethyl violet azide broth procedure for the detection of enterococci in milk was satisfactory.

Confirmation of enterococci by growth in ethyl violet azide broth gave an accuracy of 96-97 percent.

In regular low-temperature, long-time laboratory as well as plant pasteurization more than 98 percent of the enterococci were killed and in regular high-temperature, short-time pasteurization more than 99 percent of the enterococci were destroyed.

The high-temperature, short-time pasteurization was found to be more effective in destroying enterococci than was low-temperature, long-time pasteurization.

Low-temperature, long-time laboratory pasteurization at 66.2 C for 30 minutes and high-temperature, short-time pasteurization at 77.2 C for 16 seconds completely destroy enterococci in milk.

The thermal death time studies reveal that the majority of the enterococci are susceptible to present pasteurization temperatures but a small resistant minority survive long exposure periods.

The predominant subgroup of enterococci occurring in milk is Str. faecalis.

Examination of raw milk for enterococci shows that if milk is drawn carefully, it contains very few enterococci. But further contact with the milking machine, milk cans, careless handling and inadequate cooling bring tremendous increase in numbers.

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