STUDIES OF METHODS FOR CONTROLLING BACTERIOPHAGE IN LACTIC CULTURES

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INTRODUCTION

Before the advent of pasteurization of milk to be used in the manufacture of cheese and other cultured products, only raw milk was used. With the use of raw milk, even if the lactic culture used was attacked by bacteriophage, there normally would be enough wild strains of lactic streptococci naturally occurring in the milk to continue the production of a sufficent amount of acid to complete the cheese making process, but the finished product would be of very poor quality. Soon after the start of the practice of pasteurizing the milk to be used in cheese and other cultured dairy products, trouble was noted in the production of acid during the culturing process.

The manufacturers at that time thought the failure of acid development was due to the use of pasteurized milk, but on examination of various cheese factories it was revealed that outbreaks of bacteriophage attacks could not be correlated with the heat treatment of the milk.

Although it has been forty years since bacteriophage was first discovered and thirty years since the first bacteriophage for the lactic streptococci was isolated, the importance of bacteriophage in the dairy industry in this country was not fully realized until the past decade. Today, every section of the United States has been subjected to bacteriophage infection with sometimes very disastrous results and loss of money. Great amounts of time and money

have been used in this country, as well as others, on this problem, and as of yet, no suitable method has been devised to sufficently cope with it.

The present study was undertaken with one primary objective in mind, this was to find a specific substance or condition which would entirely destroy the bacteriophage present in a culture or milk to be used for the propagation of the culture without in any way damaging the acid and flavor producing ability of the host culture and that would also not be harmful for human consumption.

REVIEW OF LITERATURE

In 1915, an English investigator, Twort (63), reported an interesting phenomenon associated with the growth and development of the pus-producing cocci in which the cells would be destroyed during early growth. A year later in France, d'Herelle (18), began the first work on this phenomenon, which is today known as bacteriophage. It was not until 1926, that Hadley and Dabney (27) isolated the first bacteriophage active against *Streptococcus lactis*, the source of their material being sewage. Nelson, Harriman, and Hammer (43) were the first in this country to report bacteriophage active against the lactic acid organisms in butter cultures.

Parmelee, *et al*, (51) using an electron microscope, found that the bacteriophage of *Streptococcus lactis* are sperm shaped, about 220 millimicrons long, having a head diameter of 70 millimicrons and a tail that is 30 millimicrons wide and 150 millimicrons long.

Hook, *et al*, (28) analyzed *Escherichia coli* bacteriophage particles and found they had the following composition: 51 percent protein, five percent lipid, and 40 percent nucleic acid; one-sixth of the nucleic acid was ribonucleic acid and five-sixths was desoxyribonucleic acid. Polson and Wyckoff (53) found that the amino acid content of the virus protein of an *Escherichia coli* bacteriophage was almost identical with that of its host bacteria.

Krueger and Northrop(39) showed that in the presence of the homologus bacteriophage, the multiplication of

Staphylococcus aureus follows the normal growth curve much as though no bacteriophage were present, until approximately 100 bacteriophage units have accumulated for each bacterium present. When sufficent bacteriophage units have accumulated, lysis of the bacteria occurs very rapidly.

Ellis and Delbruck (21) found that the growth of bacteriophage is divided into three periods; absorption of the bacteriophage on the bacterium, growth upon or within the bacterium (latent period), and the release of the bacteriophage from the cell (burst).

Delbruck (14) found that bacteriophage can lyse bacteria in two ways, which he termed lysis from within and lysis from without. Lysis from within is caused by the infection of a bacterium by a single bacteriophage particle and the multiplication of this particle within the cell up to a maximum number. The bacteriophage particles are then liberated from the cell into the medium without deformation of the cell wall. Lysis from without is caused by absorption of bacteriophage above the maximum number. The cell contents are then liberated by a distension and destruction of the cell wall. The maximum yield of bacteriophage particles from a cell lysed from within is equal to the absorption capacity of the cell.

Delbruck reported later (17) that when two or more types of bacteriophage are present in a culture, the "depressor effect," or the competition for a common substrate between the bacteriophage type which penetrated into the cell walland the

excluded bacteriophage types, causes the penetration of the first type into the cell to make the cell membrane impermeable to any other bacteriophage type. Each bacteriophage type has a characteristic time of penetration and the change in permeability occurs uniformly at the end of this time interval for the entire cell membrane.

Delbruck (15) also found that with multiple bacteriophage infections, the cell reacts as if it were infected with only one bacteriophage particle. This is caused by the "Hypothesis of the Key-Enzyme," which is that each bacterial cell contains an enzyme which is necessary for bacteriophage growth and which is present in the cell in such limited amount that all of it is engaged by a single bacteriophage particle. The union between bacteriophage particle and enzyme is such that a bacteriophage type with greater affinity for the enzyme can displace from the cell a type with less affinity.

Ellis and Delbruck (21) found that the average latent period of a bacteriophage varies with the temperature in the same way as it varies with the host bacteria. The average latent period and the average burst size are neither increased nor decreased by a fourfold infection of the bacteria with their homologus bacteriophage. The average burst size is independent of the temperature.

Delbruck (16), using an Escherichia coli bacteriophage, found that the burst size, or the number of bacteriophage particles that comes from one bacterium upon lysis, ranges from 20 to 1,000 with an average of about 180 bacteriophage

particles. The burst size of a typical *Streptococcus lactis* bacteriophage averages about 90 bacteriophage particles per bacterium, and the average burst time is thirty minutes.

Whitehead and Cox (65), in an effort to cultivate a bacteriophage resistant cheese culture, isolated strains of *Streptococcus cremoris* from a culture after it had been attacked by bacteriophage and after secondary growth had appeared. One culture prepared in this manner was resistant to low concentrations of the bacteriophage strain with which it was treated during the course of its isolation, but high concentrations would lyse it. The process of immunization was not permanent as the supposedly immune strains gradually lost their resistance to the bacteriophage type with which they had been treated during the course of their isolation.

Babel (6), in an experiment using culture inoculations from 1.0 to 0.001 percent and a constant number of bacteriophage particles, found that secondary growth was most rapid in cultures receiving the greatest inoculation.

Mull (41) reported that secondary growth cultures are bacteria mutants which are resistant to the bacteriophage type which caused lysis of the culture. These mutants are present in relatively small numbers in many lactic cultures. Graham and Nelson (26) found that the acquisition of resistance is nearly always accompanied by an alteration in the physiology of the bacteria, and in some cases mutation to bacteriophage resistance appeared to be accompanied by loss of the ability to ferment lactose. Resistant strains generally grow and

produce acid slowly in milk, which explains the failure of the resistant mutants present to become dominant in cultures. Hunter and Whitehead (32) and Whitehead and Hunter (67) found that bacteriophage resistant cultures of *Streptococcus lactis*, developed by the action of bacteriophage on the sensitive bacteria, are susceptible to attack by a new race of bacteriophage.

Hunter and Whitehead (31) reported that secondary growth of *Streptococcus lactis* usually develops between 24 and 48 hours after bacteriophage has caused the lysis of the organism.

Mull and Nelson (42) found that the predominance in a multiple-strain lactic culture of an organism undergoing little or no mutation resulted in very slow recovery from bacteriophage attack. Some pure cultures showed no demonstrable recovery even after incubation for many days, apparently because of no mutation to resistant types.

Babel (5) prepared lactic cultures from the naturally occurring or wild strains of *Streptococcus lactis* isolated from raw sour cream or milk and found that such cultures were bacteriophage resistant upon isolation, but when used as cultures in commercial operations would become sensitive upon continued exposure to the bacteriophage.

Anderson and Meanwell (2) reported that cultures containing several strains of lactic acid streptococci fail to produce acidthe same as strains used as single strain cultures. Elliker (20) found this was caused by a nascent bacteriophage, which would normally attact but one of the bacterial strains.

Whitehead, et al, (66) reported that this nascent bacteriophage phenomenon was an apparent inhibition of a strain of bacteria by a heterologus bacteriophage, which can be brought about in the absence of the homologus bacteria. They concluded this phenomenon was actually an inhibition of the bacteria caused by a high concentration of the bacteriophage particles. Collins (11) reported that the nascent phenomenon could only be demonstrated in two of 14 strains of bacteriophage. He also found that the bacterial host is necessary only in supplying the necessary numbers of bacteriophage particles to bring about this phenomenon.

Potter and Nelson (55) found that calcium is necessary for the multiplication of lactic streptococcus bacteriophage. The calcium ion is not necessary for absorption or for the later stages of multiplication, but it's apparent function is in the bacteriophage penetration into the host cell or steps leading to penetration after absorption has occurred. The calcium may be replaced by barium, manganese, or strontium ions, but equivalent concentrations of the latter ions are less effective than the calcium. Magnesium and cobalt ions are not effective themselves, but are stimulatory when combined with low levels of calcium.

Shew (60), using a yeast lactose phosphate agar, was able to reverse the stimulatory effect of calcium by the addition of citrate. Stimulation of bacteriophage development was produced with a minimum of 0.001 M. calcium chloride, with a range of 0.02 to 0.007 M. being the optimum. He also

found that the optimum amount of calcium chloride varied with different media, according to the phosphate content.

Potter and Nelson (54) reported that maximum bacteriophage proliferation occurs in calcium concentrations in the medium of 0.004 to 0.3 percent, or when a two to five percent calcium chloride solution was used as a diluent for the bacteriophage to give a 0.004 to 0.3 percent calcium concentration in the medium after inoculation with the bacteriophage.

Collins, et al, (12) were able to maintain six lactic cultures out of eight carried in the presence of bacteriophage without risk of bacteriophage infection by using a defined medium containing very little calcium and supplemented with phosphate. They used Niven's medium (47) except they omitted sodium chloride, and substituted some amino acids of the L configuration for those of the DL configuration.

Nichols and Hoyle (44) isolated bacteriophage from whey after storage at one to five degrees C. for six years. They also reported the recovery of active bacteriophage particles from whey powder and from whey concentrates and the recovery of active bacteriophage from cheese after twelve months storage. Nichols and Wolf (45) found that active bacteriophage particles were present in quantities of cheese as small as 0.00005 gram after the cheese had ripened for three and one-half months. Prouty (59) dried bacteriophage filtrates on filter paper and found that the bacteriophage particles remained viable for 42 months at 0° C., 72 months at 37° C., and that the viability of the bacteriophage was extended to 78 months when stored at 12 and 25° C.

Ford and Babel (24) found that a culture of Streptococcus lactis inoculated once with bacteriophage required 132 transfers before the culture became free of bacteriophage when an incubation temperature of 21° C. was used. When the bacteriophage contaminated culture was incubated at 26°C. for nine transfers and then at 21°C. for subsequent transfers, the culture did not contain bacteriophage after 34 transfers. The same culture inoculated once with bacteriophage and propagated at 37°C. did not contain bacteriophage after ten transfers.

Nelson, et al, (43) found that lactic acid bacteriophage is not killed at normal pasteurization exposures (62.5°C. for 30 minutes) and is more resistant to heat than its homologus host. They also reported that some strains of lactic streptococci bacteriophage are resistant to 70°C. for ten minutes, but not for 15 minutes.

Overcast, et al, (50) found that bacteriophage can grow over the entire pH range of their homologus hosts. This range for most strains of Streptococcus lactis is from pH 4.8 to 9.4. These were considered the limits of bacteriophage growth as they are the limits of the optimum growth of their hosts. Hunter and Whitehead (30) and Prouty (58) report that a pH over 11.8 or under 2.5 is required to inactivate bacteriophage. The optimum pH is different for the bacteriophage than for its homologus host. Most lactic streptococci bacteriophage have an optimum pH of nearly 7.0 or slightly alkaline, while most Streptococcus lactis have an optimum pH of 6.0 or less. Krueger and Fong (38) found that increasing the hydrogen ion concentration prolongs

the log phase of the organism without a corresponding lengthening of the log phase of the bacteriophage, and that increasing the hydroxyl ion concentration causes no pronounced change in the curves of either organism growth or bacteriophage proliferation.

Hurd (33) found that bacteriophage of Streptomyces griseus can be inactivated with a 0.5 percent concentration of sodium chloride.

Perlman, et al, (52) were able to limit, but not inactivate, the growth of Streptomyces griseus bacteriophage by the addition of substances capable of sequestering calcium in the medium before infection of the host cell occurred. Among the sequestering agents found to be effective were citrate, oxalate, and phthlate.

Cherry and Watson (9) found that the absorption of bacteriophage by Streptococcus lactis is about three times as great in a medium consisting of one percent tryptone, 0.3 percent yeast extract, and 0.2 percent glucose as in the same medium when the tryptone is omitted.

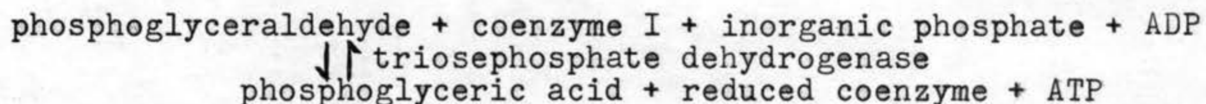
Hotchin (29), using a staphylococcus bacteriophage, found that neither phosphine, proflavine, 5-aminoacridine, nepacrine, rivonal, flavozole, rhodamine, fluorescein, propamidine isothionate, nor pentoamide isothionate inhibited a Staphylococcus bacteriophage at levels which permitted growth of the host cells

Dickinson (19) tried, about 500 compounds, which included the sulphonamides, amidines, pyrinidines, organometallic compounds, plant extracts, mould extracts, and antibiotics and found that they were all ineffective against a Pseudomonas bacteriophage at concentrations permitting the growth of the host cells. She found that proflavine exerted a viristatic effect while notatin and hydrogen peroxide were lethal in a defined medium, but not in a broth.

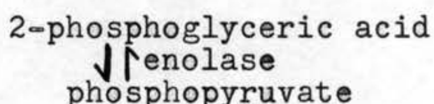
Olson (48) used various chemical substances in an effort to control Streptococcus lactis bacteriophage. Except for sodium chloride, which showed slight inhibition, all of his results were negative as the concentrations of the substances that would cause any degree of inhibition of the bacteriophage would greatly inhibit the growth of the homologus S. lactis. Likewise, any concentration of the substances which would permit normal growth of the Streptococcus lactis would not affect the bacteriophage proliferation. Among the substances used in his study were various surface tension depressants, alchols, aldehydes, ketones, osmotic pressure, mold inhibitors, germicides, metallic salts, salts of heavy metals, organic and inorganic acids, growth stimulators, anti-oxidants, enzymes and sodium compounds.

Nelson, et al, (43), found that Streptococcus lactis bacteriophage is inactivated by crystal violet, methylene blue, hydrogen peroxide, and potassium permanganate, but not at levels permitting growth of the S. lactis host.

Price (56) found that the use of either iodoacetate, fluoride, or azide against bacteriophage blocks the adenosine triphosphate formation, which is essential for bacteriophage multiplication, by inhibiting certain reactions in the Embden-Meyerhof glycolytic scheme. The iodoacetate prevents the reaction:



The fluoride inhibits the reaction:



The exact mechanism by which azide inhibits adenosine triphosphate synthesis is not known.

Collins (10) states that bacteriophage multiplication is closely related to bacterial multiplication, because when the omission of an individual component from a medium causes a decrease in bacteriophage multiplication, a parallel decrease in bacterial growth is also encountered.

Bennett and Nelson (7) atomized glycols into the air of a bacteriophage infected plant at the rate of 14.6 to 43.8 ml. per 1000 cubic feet of air space and could not detect any measurable bacteriophage inactivation. Aersols of calcium hypochlorite supplying 0.61 gram of available chlorine per 1000 cubic feet of air completely inactivated air borne bacteriophage within ten seconds. An aerosal supplying 0.7 gram of a quaternary ammonium compound, alkyldimethylbenzylammonium chloride, per 1000 cubic feet failed to inactivate

the bacteriophage and was intolerable to a person working in the room. When the bacteriophage was suspended in 1:2, 10^{-1}, and 10^{-2}, dilutions of whey filtrate at 25° C., 100 to 1000 ppm of the glycols were ineffective in inactivating the bacteriophage. Available chlorine from calcium hypochlorite in a concentration of 25 to 400 ppm inactivated the bacteriophage at pH 5.0 to 6.9 in 15 to 300 second exposures. Fifty to 1000 ppm of the quaternary ammonium compound inactivated the bacteriophage at a pH of 4.8 to 6.6 in 30 to 120 second exposures. The presence of organic matter decreased the effectiveness of both the hypochlorite and quaternary ammonium compounds. Prouty (57) recommends a concentration of 100 to 200 ppm of a quaternary ammonium compound and an exposure time of two minutes for a rinse solution to destroy bacteriophage.

Curry and Barber (13) found that the use of quaternary ammonium compounds in the culture medium to control bacteriophage is impractical. It requires a concentration of about 200 ppm of the quaternary compound to kill the bacteriophage, but the *Streptococcus lactis* host is killed in a 50 ppm concentration and it loses its acid producing qualities in concentrations ranging from 2.5 to 10 ppm.

Elford (23) reported that penicillin in concentrations up to 100 units per ml. in broth and synthetic media has no demonstrable effect, after 20 hours incubation at 37° C., on the activities of Staphylococcus K bacteriophage, coli bacteriophage, coli-dysentery bacteriophage, Streptococcal bacteriophage,

and a Bacillus bacteriophage. The simultaneus action of penicillin and bacteriophage on young cultures of Staphylococcus aureus produced, under certain conditions, a more rapid lysis than occurred in the presence of the penicillin or bacteriophage alone. This phenomenon occurred with other organisms, varying only in degree. Penicillin did not affect the absorption of bacteriophage by the organism. When the amount of antibiotic is sufficient to interfere adversely with the growth of the bacteriophage, then the multiplication of the cell decreases. He suggested that certain balanced intracellular reactions of metabolism are disturbed by the action of penicillin, and as a result, intermediates essential to growth, both of the cell and of the bacteriophage, cease to be available.

Smiles, et al, (61) using an electron microscope and ultraviolet light to observe the effect of bacteriophage and penicillin on Staphylococcus aureus, noted that the organisms would swell to almost twice their normal size, and immediately before lysis they became less opaque to electrons and their internal structure was evident.

Jones (34) found that streptothricin, streptomycin, and clavacin would cause inactivation of bacteriophage of Escherichia coli and Staphylococcus aureus, whereas penicillin and actinomycin were without effect. There appeared to be no correlation between the susceptibility of the host cells and that of the bacteriophage to an antibiotic agent.

Anderson (3) found that in the presence of 0.1 percent glucose, 0.1 mg. of penatin per ml. reduces the concentration of bacteriophage particles to one-thousandth of its original value in 18 hours at 21°C., while 0.001 mg. of penatin per ml reduced the concentration to one-tenth of its original value in the same period.

An Aspergillus mold was found by Asheshor, *et al*, (4) which produced two anti-bacteriophage substances, one an anti-Staphlophage substance, and the other an anti-Streptophage substance. Less than one part in ten million by weight would inhibit the Staphlophage, while the Staphlococcus required concentrations of one part in fifty thousand before being affected. The anti-Streptophage substance was less active, and was only inhibitory to three bacteriophage strains out of twelve tried.

Kassanis and Kleczkowski (35) isolated an inhibitor of plant viruses from the sap of *Phytolacca esculenta* by differential precipitation with ethanol followed by absorption on celite and elution with ten percent sodium chloride. The inhibitor was thought to be a glycoprotein.

Chantrill, *et al*, (8) examined the extracts of 288 plants, mostly of British origin, for suppressive action on the development of a bacteriophage strain of *Pseudomonas pyocyanea* and found that many of the plants possessed the property of inhibition and eight of them suppressed the growth of bacteriophage at concentrations less than one-tenth of that which affected the

host. Extracts of 142 of the plants were tested against Influenza A virus in embryonated eggs and twelve of them were found to suppress the virus multiplication. All extracts active against Influenza A virus were also active against the bacteriophage. Four extracts tested against the Influenza A virus in mice were ineffective. Eight extracts were investigated further; these were all inactivated by proteins and were only active when in direct contact with the virus in a protein-free medium. The inhibition activity was closely associated with the tannin content of the extracts and could not be separated from it. Commercial tannins were also highly active against the bacteriophage in protein-free medium. The eight extracts showing the greatest inhibition were obtained from: _Dryopteris filix-mos_, _Epilobium hirsutum_ L., _Lysimochia nummularia_ L., _Epilobium angustifolium_ L., _Sedum spurium_ M. Bieb., _Lysimochia vulgaris_ L., _Saxifrago ligulato_ Wall, and _Myrica Gale_ L.

Research being carried out at Michigan State College (25) using the extracts of 1,232 species of seed plants has shown that 405 species demonstrated varying degrees of inhibition toward _Mycobacterium tuberculosis_ and other organisms in vitro. A total of 562 of the extracts produced inhibition of one or more of the following microorganisms: _Mycobacterium tuberculosis_, _Micrococcus pyogenes_, var. _aureus_, _Salmonella typimurium_ and _Escherichia coli._

Takahashi (62) isolated an inactivator against the tobacco mosaic virus from bakers or brewers yeast.

Evans (22) and Krueger and Scribner (40) were able to completely inhibit bacteriophage by blood, pus, ascitic fluid, bile and saliva; they were able to partially inhibit the bacteriophage by urine.

Kleczkowski and Kleczkowski (36) prevented the multiplication of bacteriophage by adding 0.01 percent chymotrypsin to mixtures of Rhizobium bacteriophage and bacteria in liquid culture. Under these conditions the bacteriophage gradually became inactive. Chymotrypsin did not interfere with the combination of bacteriophage and bacteria. The bacteriophage particle combined with a bacterium and became inactive, the bacterium was thus protected and was able to multiply. Multiplication of the bacteriophage was unaffected by previous incubation of either the bacteriophage or host bacterium separately with 0.01 percent chymotrypsin and the chymotrypsin had no effect in an agar medium.

Kleczkowski and Kleczkowski (37) later found that pancreatic ribonuclease inhibits the multiplication of a Rhizobium bacteriophage in liquid cultures by preventing permanent combination between the bacteriophage and host. The addition of ribonuclease after the bacteriophage particle has combined with the bacterium does not prevent bacteriophage from multiplying, but it does decrease the rate of multiplication; the enzyme also interferes with the normal multiplication of the host bacterium. Experiments with ribonuclease in conjunction with chymotrypsin suggests

that bacteriophage and bacteria unites in the presence of ribonuclease, but that the union is only transitory and ends by the release of still active bacteriophage particles. In the presence of chymotrypsin, however, the bacteriophage particles become inactive during this transient union.

EXPERIMENTAL METHODS

A. Sources of culture and bacteriophage strains used

The butter cultures and cheese cultures used throughout this study were obtained from various commercial laboratories which supply cultures to dairy plants, and were among the cultures being carried by the Dairy Department at Oklahoma A&M College. Numerous pure *Streptococcus lactis* cultures were isolated and purified at the Oklahoma A&M College Dairy Department from various butter and cheese cultures, and from this group of pure cultures, one strain was selected for use in this experiment. This pure culture, Number 3, was very active, as one percent inoculation reduced litmus milk in three hours at 22° C., and coagulated the milk in eight hours under the same conditions. This strain of *S. lactis* was very sensitive to bacteriophage contamination, but of 16 strains of bacteriophage which would attack it, only one was selected for use in this study. This bacteriophage strain, Number 8, was very active, having a titer of 10^{-9}, and causing lysis of the host organism within three hours after inoculation and incubation at 22° C. Secondary growth was not produced until about four days incubation at 22° C. after this bacteriophage strain had lysed *S. lactis* 3.

Another pure strain of *Streptococcus lactis*, the W strain, was obtained from the Washington State College Dairy Department, as was its homologus strain of bacteriophage. This culture was apparently completely lysed by its homologus bacteriophage, but grew so slowly that it

was not as useful as S. lactis 3 for the experiments herein reported.

Eighteen other bacteriophage strains and their homologus cultures used during the course of this study were:

Culture:	Homologus bacteriophage strain:
Streptococcus lactis 3	1,7,10,11,13,15,17,21,26,30, 31,33,4D,6D,13D, and 8F
Streptococcus lactis W	26,30,6D,13D, and 8F
Butter culture 1	26
Butter culture 44	30 and 34
Butter culture 62	3
Butter culture 66	3 and 24
Butter culture 68	3
Butter culture 76	32
Butter culture 82	26

A number of these bacteriophage strains would lyse the same lactic culture, but the strains were considered to be different due to the different sources of their isolation.

The bacteriophage strains were obtained from slow lactic cultures from dairy plants experiencing trouble with slow acid production in cheese making. If the culture was not coagulated, it was acidified with a solution of 15 percent sterile citric or lactic acid. The cultures were then filtered through coarse filter paper into sterile 125 ml erlenmeyer flasks. This filtrate was then passed through a sterile Seitz filter into sterile four ounce screw capped prescription bottles and the resulting bacteria free filtrate diluted 50 percent with sterile litmus milk to help maintain the viability of the bacteriophage by the buffering action of the milk.

B. Testing for bacteriophage inhibition

The medium used in this experiment to test the action of bacteriophage filtrates on lactic cultures was prepared with non-fat dry milk solids and distilled water, using litmus as an indicator. The medium was prepared by mixing 50 grams of the NFDMS with 500 ml. of distilled water in a Waring blendor and adding aqueous litmus solution to give a light lavender color. This medium was then transferred in measured 10 ml quantities into screw capped test tubes 15 mm in diameter and 125 mm in length. These tubes were capped loosely and autoclaved for 15 minutes at 15 pounds pressure.

To test action of bacteriophage filtrates on lactic cultures, one drop (about 0.05ml or 0.5 percent) of the culture was inoculated into each of two screw capped test tubes containing the sterile litmus milk which had been tempered to 22° C. Then 0.5 percent (one drop or 0.05ml) of a bacteriophage filtrate was added to one of the tubes. Both tubes were then incubated at 22° C. Bacteriophage activity was denoted by comparing the rates of reduction and of coagulation of the milk. The coagulation was determined by inverting the tubes at intervals and noting the first signs of coagulated matter adhering to the sides of the tube. If the tube containing the bacteriophage filtrate reduced and coagulated the milk slower that the control, it was assumed that the action of bacteriophage was the cause of the difference in the rates of growth of the _S. lactis_ in the two tubes.

C. Bacteriophage titers

The enumeration of the bacteriophage, or the bacteriophage titers, was accomplished by placing serial dilutions of the bacteriophage into ten ml of sterile litmus milk in screw capped test tubes that had just previously been inoculated with the homologus host bacteria. The serial diluting fluid used was sterile buffered distilled water. The tubes were then incubated at 22° C. until the control tube had coagulated, and the highest dilution which prevented normal growth of the culture was considered to be the bacteriophage titer.

D. Activity tests

The activity test used was a modification of the seven-hour activity test used by Olson, et al (49). Exactly 17.6 ml of Grade A whole milk pasteurized at 62° C. for 30 minutes, and homogenized, was pipetted with a sterile Babcock milk pipette into sterile screw capped test tubes 15 mm in diameter and 125 mm in length. The tubes were then placed in ice water, and when the milk had cooled to the temperature of the water, the tubes were inoculated with about one percent of the culture to be tested, tempered to 31° C., and incubated at this temperature in a water bath for seven hours. At exactly seven hours from the time the tubes had been tempered at 31° C., they were taken from the incubator and placed immediately into ice water. When all of the cultures had cooled to the temperature of the water the titratable acidity was determined with 0.1 N sodium hydroxide, using phenolphthalein as the indicator.

The contents of each tube were dumped into 125 ml erlenmeyer flasks and the tubes rinsed with 10 ml of distilled water and this rinse also dumped into the erlenmeyer flask. The titratable acidity of each culture was divided by two to get the actual titratable acidity of each tube. The titratable acidity of the control tube, a tube of the same quantity of the same milk only not inoculated, was subtracted from that of each of the other tubes to determine the increase in acidity. The control tubes had been incubated along with the other tubes.

E. Standard plate counts.

The standard plate counts were run accordingly to the method given in the 1948 edition of Standard Methods (1), using tryptone glucose extract agar as the medium. The plates were incubated at 35° C. for 48 hours.

EXPERIMENTAL RESULTS AND DISCUSSION

A. The effect of grade of milk and pasteurization temperature of the milk on the activity and quality of lactic acid starters

An experiment was conducted to determine the influence of the grade of milk used and the heat treatment of the milk on the activity and quality of lactic acid cultures. It was thought that during the course of numerous propagations that those cultures carried in milk which had been pasteurized at a low enough temperature to possibly permit the survival of bacteriophage would develop a resistance to bacteriophage due to the continued exposure to them. It was likewise thought that a culture carried in milk pasteurized at 63° C. for 30 minutes would develop resistance to the "germicidal property" of such milk and would, therefore, subsequently grow better in milk during the cheese making process.

Grade A raw milk was obtained fresh daily from the Oklahoma A&M College milking herd. Samples of ungraded milk were obtained daily upon delivery at a local creamery. Both lots of milk were run through a centrifugal cream separator just prior to use each day and the skim milk used in this test.

Twenty ml portions of each grade of milk were transferred into each of 22 sterile screw capped test tubes (15 X 125mm). Eleven tubes of each grade of milk were pasteurized in flowing steam (99° C.) for 30 minutes and the remaining 11 tubes of each grade of milk were pasteurized at 63° C. for 30 minutes in an electric home

pasteurizer[1] with water as the heating medium. Immediately after pasteurization the tubes were placed in ice water. Standard plate counts were made on the raw and pasteurized milks.

The data in Table 1 show the quality of the milks used in this study and the bacterial efficiency of the heat treatments given to each grade of milk. Both the logarithmic and arithmetic averages are shown in the table. All averages described in this text are logarithmic. The logarithmic averages were calculated by the use of the log table on page 183 of U.S. Public Health Service Milk Ordinance and Code (64). The log of counts below 1000 were calulated by the use of a standard five place log table, using only the first two places. In order to get a true average, the counts which would normally have been recorded as less then 30 were recorded as the actual number of bacterial colonies appearing on the plates times the dilution used.

The standard plate counts per ml on the Grade A raw milk ranged from 1,100 to 360,000 and averaged 12,000. After pasteurization at 63° C. for 30 minutes, the standard plate counts ranged from 45 to 1,900 and averaged 224. After heating in flowing steam (99° C.) for 30 minutes, the counts on the Grade A milk ranged from 5 to 70 and averaged 25.

[1]Model PA-46D. Manufactured by the Waters Conley Company, Rochester, Minnesota.

The standard plate counts per ml on the ungraded raw milk ranged from 620,000 to 98,000,000 and averaged 6,600,000. After pasteurization at 63° C. for 30 minutes, the counts ranged from 10,000 to 7,700,000 and averaged 790,000. The counts on the ungraded milk pasteurized in flowing steam for 30 minutes ranged from 5 to 2,100 and averaged 39 per ml.

From the results in Table 1 it appears that it would be possible to have bacteriophage contamination in the cultures if any strains of bacteriophage should happen to be in the milk and if the low temperature of pasteurization, 63° C., would not be sufficent to kill them. Also, it is entirely possible that the cultures could become contaminated with bacteria which survived the pasteurization exposures.

The tubes were then tempered to 22° C. and one tube of each grade of milk at each pasteurization temperature was inoculated with one percent(.2ml or four drops) of a culture. One tube of each milk at each pasteurization temperature was not inoculated to serve as a control on the acid development of the other tubes. These control tubes were incubated the same as the inoculated tubes. All of the tubes were incubated at 22° C. for 16 hours. At the end of each incubation period activity tests were made on each culture. Ten butter and cheese cultures were carried for ten daily propagations, then nine weekly propagations, and finally nine daily propagations, making

TABLE I

Standard plate counts on Grade A and on ungraded milk before and after pasteurization

Propagation	Grade A Milk			Ungraded Milk		
	Raw Milk	Pasteurized at 63° C. 30 min.	Pasteurized in flowing steam 30 min.	Raw Milk	Pasteurized at 63° C. 30 min.	Pasteurized in flowing steam 30 min.
1	7,000	470	10	3,800,000	350,000	150
2	7,200	170	10	26,000,000	7,700,000	100
3	35,000	160	40	3,600,000	68,000	2,100
4	47,000	320	12	4,900,000	440,000	10
5	8,800	340	45	1,000,000	98,000	31
6	3,900	1,900	70	620,000	1,500,000	62
7	18,000	370	15	L.A.	7,400,000	12
8	35,000	1,100	65	1,600,000	210,000	30
9	72,000	250	20	1,300,000	2,100,000	15
10	11,000	90	25	11,000,000	240,000	5
21	4,300	80	25	98,000,000	14,000	15
22	6,800	450	15	90,000,000	73,000	10
23	1,100	110	40	1,600,000	24,000	12
24	8,400	450	50	1,600,000	23,000	720
25	360,000	70	20	11,000,000	160,000	70
26	2,400	70	45	8,500,000	10,000	110
27	6,800	45	70	42,000,000	160,000	30
28	28,000	L.A.	5	51,000,000	140,000	74
Log Average	12,000	224	25	6,600,000	790,000	39
Arith.Average	37,000	379	32	21,000,000	1,150,000	198

a total of 28 propagations. Between propagations the cultures were stored at 9° C.

The increase in titratable acidity of each culture during the course of the 28 propagations is recorded in Appendix Table I. A summary of this data is in Table 2. Activity tests were not run on propagations 11,12,13, and 18. The activity tests were not run on the 18th propagation due to the inconsistent coagulation of various tubes. In the Grade A milk pasteurized at 63° C. for 30 minutes, cultures 62,66,76, and 83 grew very slowly, 62 being the slowest. Cultures 76 and 84 carried in Grade A milk pasteurized at 99° C. for 30 minutes grew very slowly. In the ungraded milk pasteurized at 63° C. for 30 minutes, culture 62 grew very slowly. The irregular growth in propagation 18 was attributed to the very low activity of most of the cultures in propagation 17. This slight inhibition of most of the cultures in propagation 17 may have been caused by a number of things. The presence of antibiotics in the milk may have caused it, as the inhibition was present in both levels of pasteurization and certain antibiotics are heat stable. However, as the inhibition appeared in both grades of milk in just this one day of the study, this seems to be a very improbable answer. The slight inhibition may have been caused by bacteriophage

contamination, but the inhibition was present in the milks pasteurized at 99° C. for 30 minutes, which is believed to kill all bacteriophage. One logical reason for this inhibition would be that sometime during the incubation period of this propagation the temperature in the incubator fell below the normal 22° C., at which it was thermostatically controlled. This lower temperature would slow the growth of all the cultures, which would in turn favor the growth of any bacteriophage particles which are carried by some strains of lactic cultures. The variation in the bacteriophage resistance of the cultures would cause the variation in increases in titratable acidities in both the 17th and 18th propagations.

The significance between various cultures used in this study was not determined statistacially. If the difference in the titratable acidity between two cultures was 0.02 or more, this difference was termed significant. A difference of only 0.01 percent titratable acid could easily have been due to experimental error, as the direct titration method used to obtain the percent titratable acid is not an accurate quantitive measure of the actual acid present.

Culture Number one was rather slow at the start of the study, as indicated by the small increase in titratable acidity. This culture became more active during the course

TABLE 2

Average Increase in Titratable Acidity of 28 Propagations of Cultures Grown in two Grades of Milk Pasteurized at two Different Temperatures

Culture Number	Grade A Milk: Pasteurized at 63° C. 30 min.	Grade A Milk: Pasteurized in Flowing steam 30 min.	Ungraded Milk: Pasteurized at 63° C. 30 min.	Ungraded Milk: Pasteurized in flowing steam 30 min.
1	.46	.45	.46	.46
62	.42	.43	.44	.45
66	.45	.44	.45	.45
68	.47	.44	.49	.45
75	.54	.52	.54	.54
76	.54	.54	.53	.55
81	.51	.50	.52	.52
82	.42	.49	.52	.53
83	.52	.52	.53	.52
84	.49	.50	.52	.51
Average:	.48	.48	.50	.50

of the trial and as the average indicates, there was no significant difference at the end of the study between the cultures grown in the different grades of milk or between the same milk receiving the two different heat treatments. There appeared to be a greater difference among the cultures when propagated weekly than when propagated daily, the advantage was with the low temperature of pasteurization by 0.07 percent titratable acidity in each grade of milk. On the third propagation the culture carried in the ungraded milk pasteurized at 63° C. for 30 minutes was slowed considerably in activity, but it recovered to its original level of growth the following day. This decrease is indicative of bacteriophage contamination, but if this were the case, the culture demonstrated secondary growth at a remarkable rate.

The activity of culture number 62 also increased with continued propagation, but the cultures being carried in the low pasteurized milk of each grade were becoming progressively slower towards the end of the study. This slowness may have been caused by bacteriophage contamination as this culture was very sensitive to bacteriophage in the manufacture of cottage cheese. The growth of this culture in Grade A milk pasteurized in flowing steam was greatly inhibited on the 14th propagation, but recovered on the following propagation.

Culture number 66 did not increase significantly in activity during the trial, however, there was not enough difference between the average acidities to signify bacteriophage contamination.

The activity of culture number 68 showed a slight increase and finally a slight decrease during the course of the propagations. This latter decrease may have been caused by bacteriophage contamination. All four cultures of number 68 were greatly inhibited in propagation 17. The cultures carried in the low pasteurized milk of each grade recovered their original activity on the following propagation. However, it required seven transfers before the cultures carried in the milk pasteurized at high temperatures regained their original activity. The cultures carried in both grades of milk pasteurized at 63°C. for 30 minutes produced more acid than those carried in the milks pasteurized at 99°C. for 30 minutes, with the culture carried in the low grade milk demonstrating the greatest activity.

Cultures 75,76,81,82,83, and 84 all followed the same pattern during the course of the propagation in that the average increase in titratable acidity decreased from the first ten daily transfers, to the nine weekly transfers, and finally were the lowest in the last nine daily propagations. All of the cultures were relatively slow in the 11th propagation but recovered to their normal levels of growth following this inhibition, with the cultures propagated in both grades of milk pasteurized at 63°C. for 30 minutes recovering at a more rapid rate than the cultures

carried in both grades of milk receiving the higher heat treatment. In the 24th propagation, culture 82 carried in Grade A milk pasteurized at 62° C. demonstrated considerable inhibition, and this inhibition grew progressively worse during the final four propagations of this trial, until, on the 28th propagation, an increase of only 0.03 percent titratable acidity was produced by the culture. This culture was coagulated with 15 percent solution of sterile citric acid and the whey filtered through a sterile Seitz filter. The resulting filtrate contained a bacteriophage active against culture 82. On the 27th propagation, culture 82 carried in Grade A milk pasteurized in flowing steam for 30 minutes was greatly inhibited, but recovered on the succeeding propagation. Culture 84 carried in Grade A milk receiving the low heat treatment was inhibited slightly on the 26th propagation, but recovered on the succeeding transfers.

The data in Table 2, which is the average of all ten cultures, indicates that cultures carried in ungraded milk are more active than the same cultures carried in Grade A milk. This is true regardless of the heat treatment given the milk. However, as the final average shows, there is no difference between the two temperatures of pasteurization within each grade of milk.

Further analysis of the data in Appendix Table I are recorded in Table 3 and 4. The data in Table 3 were compiled by determining the number of times one culture developed significantly more acid(at least 0.02 percent

Comparison of Grades of Milk and Pasteurization Temperatures from the Standpoint of Culture Activity

Culture	Past. at 63°C. 30 min.		Past. at 99°C. 30 min.		Comparison of Grade of Milk		Comparison of Past. Temp.	
	Ungraded* Col.I	Grade A* Col.2	Ungraded* Col.3	Grade A * Col. 4	Ungraded* Col.1&3	Grade A* Col.2&4	63° C.* Col.1&2	99° C. * Col.3&4
1	9	9	11	6	20	15	18	17
62	13	8	7	9	20	17	21	16
66	8	2	13	6	21	8	10	19
68	9	2	12	8	21	10	11	20
75	8	6	13	2	21	8	14	15
76	6	7	12	2	18	9	13	14
81	13	7	13	2	26	9	20	15
82	11	3	13	4	24	7	14	17
83	10	3	8	4	18	7	13	12
84	9	5	14	3	23	8	14	17
Total:	96	52	116	46	212	98	148	162

* Number of instances in which the activity of the culture was significantly greater.

more) than another culture. This system was used to compare the two grades of milk and the two pasteurization temperatures.

The data in Table 3 indicate that cultures grown in ungraded milk were more active than the same cultures grown in Grade A milk at either pasteurization temperature. Cultures grown in ungraded milk produced significally more acid than cultures grown in Grade A milk 212 times, while cultures grown in Grade A milk produced significally more acid than cultures grown in ungraded milk only 98 times. The results also indicate that cultures grown in milk pasteurized at 99° C. for 30 minutes are more active than cultures grown in milk pasteurized at 60° C. for 30 minutes. This difference was very slight, and was only true with the ungraded milk.

Olson (48) has shown that when using the seven hour activity test, a culture must develop at least .50 percent acid to serve as a satisfactory lactic starter culture. Using this standard, the number of times each culture developed, at least .50 percent acid was determined and compiled in Table 4. The data indicate that, with a pasteurization temperature of 63° C. for 30 minutes, the cultures propagated in ungraded milk were satisfactory for use as cheese cultures 166 times, or 21 more times than the cultures grown in Grade A milk. With a pasteurization temperature of 99° C. for 30 minutes, the cultures propagated in ungraded milk were satisfactory 148 times, or 14 more times than the cultures grown in Grade A milk.

TABLE 4

Number of Cultures Developing .50% or more Acid *

Culture	Past. at 63° C. 30 min.		Past. at 99° C. 30 min.		Comparison of Grade of Milk		Comparison of Past. Tempt.	
	Ungraded Col. I	Grade A Col. 2	Ungraded Col. 3	Grade A Col. 4	Ungraded Col. 1&3	Grade A Col. 2&4	63° C. Col. 1&2	99° C. Col. 3&4
1	14	13	13	13	27	26	27	26
62	10	5	7	10	17	15	15	17
66	8	9	7	6	15	15	17	13
68	14	13	7	8	21	21	27	15
75	22	23	23	15	45	38	45	38
76	21	22	23	20	44	42	43	43
81	21	14	18	16	39	30	35	34
82	20	14	18	16	38	30	34	34
83	20	17	16	16	36	33	37	32
84	16	15	16	14	32	29	31	30
TOTAL:	166	145	148	134	314	279	311	282

* A culture producing 0.50% or more acid in the activity test used was considered to be satisfactory for use in cheesemaking

Upon comparison of the grades of milk using both temperatures, the ungraded milk produced 314 satisfactory cultures, or 35 more than the Grade A milk. Upon comparison of the temperatures of pasteurization using both grades of milk, 311 satisfactory cultures were produced in the milk pasteurized at 63°C. for 30 minutes, this was 28 more than the higher pasteurization temperature. It is also noted that very few satisfactory cultures were developed by cultures number 62, 66, and 68.

In summarizing the above data, it is quite evident that propagating cultures in ungraded milk or using a pasteurization temperature of 63°C. for 30 minutes is not detrimental to the culture. This is very remarkable due to the theoretical fact that there should be numerous bacteriophage particles in such culture medium.

Even though the cultures grown in ungraded milk seemed to produce more active cultures, this practice is not recommended for practical use due to the large possibility of contamination of non-lactic acid organisms as evidenced by the high plate counts on ungraded milk as shown in Table 1. One explanation for the greater activity of the cultures propagated in ungraded milk may be due to the possible protein degradation by the large number of organisms in the milk before pasteurization.

The fact that the cultures grown in this supposedly bacteriophage contaminated medium were very active, tends to indicate that the cultures had developed an acquired

y or resistance [illegible] phages as to the
dal properties of the milk. The following experiment
ertaken to substantiate this belief.

the 29th propagation, one percent of a
of [illegible] strain of 20 hour bacteriophage was added to one
each culture which had been prepared and inoculated
usual manner. The effect of the bacteriophage on
ture was determined after 12 hour incubation by
ng the period required for coagulation of the milk
s containing the culture and those contain-
culture plus the 20 hour bacteriophage. The
are shown in Table 5.

e results show that the culture grown in milk
ized at 62°C for 30 minutes, both Grade A
grade milk, were resistant to bacteriophage
e cultures carried in [illegible] grade milk pasteur-
[illegible] for 30 minutes [illegible] theorized
the Grade A milk pasteurized at 62°C for 30 minutes
the culture containing bacteriophage required
periods of time to coagulate the respective
s without bacteriophage. With two of these the
ence [illegible] it was five
and fourth [illegible] bacteriophage
[illegible] coagulate
[illegible] cultures
in Grade A milk pasteurized at 62°C for 30 minutes
apparently [illegible] all of the

TABLE 5

The Influence of the Grade and the Pasteurization Exposures of Milk used for Propagating Cultures on the Susceptibility to Bacteriophage

Hours Required for Coagulation by Cultures Grown In

Culture Number	Grade A Milk: Pasteurized at 63° C. 30 min.		Grade A Milk: Pasteurized in Flowing Steam 30 min.		Ungraded Milk: Pasteurized at 63°C. 30 min.		Ungraded Milk: Pasteurized in Flowing Steam 30 min.	
	C	C+P	C	C+P	C	C+P	C	C+P
1	13	13	12	12	14	15	12	12
62	16	17	12	12	14	14	12	12
66	14	14	12	12	14	14	12	12
68	13	13	12	12	12	12	12	12
75	12	12	12	12	12	12	12	12
76	12	12	12	12	12	12	12	12
81	12	12	12	12	12	12	12	12
82	24	29	12	12	12	12	12	12
83	12	13	12	12	12	12	12	12
84	18	29	12	12	12	12	12	12

C equals culture
C+P equals a mixture of 20 bacteriophage strains.

tubes containing the culture plus bacteriophage coagulated at the same time as the tubes containing only the respective culture.

With the ungraded milk pasteurized at 63° C. for 30 minutes, only one of the ten cultures containing bacteriophage required longer to coagulate than the respective culture without bacteriophage. The difference was only one hour. The remaining nine cultures were apparently not affected by the bacteriophage. All of the cultures grown in ungraded milk pasteurized in flowing steam for 30 minutes were apparently not affected by the bacteriophage as all of the tubes containing the cultures plus bacteriophage coagulated at the same time as the tubes containing only the respective culture.

It maybe noted further that all of the cultures grown in both grades of milk pasteurized in flowing steam for 30 minutes coagulated at the same time, 12 hours or less, while the cultures grown in both grades of milk pasteurized at 63° C. for 30 minutes required varying amounts of time to coagulate. The cultures grown in Grade A milk pasteurized at 63° C. for 30 minutes coagulated in from 12 to 24 hours. Three of the cultures grown in ungraded milk pasteurized at 63° C. for 30 minutes coagulated in 14 hours, the remaining seven cultures coagulated in 12 hours or less.

In conclusion, it has been demonstrated that cultures growing in the presence of bacteriophage or the "germicidal property," do not become resistant to them during numerous propagations. From the results of this experiment, it seems

apparent that cultures are less affected by bacteriophage when propagated in milk which has been pasteurized at 99° C. for 30 minutes than in milk pasteurized at 63° C. for 30 minutes.

After the last propagation, in order to determine if the cultures had become contaminated with other than the normal lactic acid organisms, each culture was streaked on tryptone glucose extract agar (1), incubated at room temperature (22° C.) for 48 hours and then examined for colonies which did not appear to be the normal lactic culture organisms. The results are shown in Table 6.

The data show that cultures grown in either grade of milk pasteurized at 63° C. for 30 minutes are very likely to become contaminated with other than the normal lactic acid organisms, while cultures grown in either grade of milk pasteurized in flowing steam for 30 minutes will be free of contamination if the standard procedures for propagating cultures are employed. Five of the ten cultures grown in Grade A milk pasteurized at 63° C. for 30 minutes were contaminated and all ten of the cultures were contaminated that had been grown in ungraded milk pasteurized at the low temperature. Also, none of the cultures that had been growing in either grade of milk pasteurized in flowing steam for 30 minutes was contaminated at the end of 28 propagations.

TABLE 6

Contamination of butter cultures after 28 preparations in two grades of milk receiving two pasteurization treatments

Culture Number	Grade A Milk: Pasteurized at 63°C. 30 min.	Grade A Milk: Pasteurized in flowing steam 30 min.	Ungraded Milk: Pasteurized at 63°C. 30 min.	Ungraded Milk: Pasteurized in flowing steam 30 min.
1	-	-	x	-
62	-	-	x	-
66	-	-	x	-
68	x	-	x	-
75	x	-	x	-
76	-	-	x	-
81	x	-	x	-
82	-	-	x	-
83	x	-	x	-
84	x	-	x	-

x= culture contaminated
-= culture not contaminated

The contamination in the cultures grown in either grade of milk pasteurized at 63°C. for 30 minutes is due to the large number of organisms, other than those which are normally present in lactic cultures, which survived this pasteurization exposure, as is noted in Table 1. This is especially true with the cultures grown in the ungraded milk pasteurized at 63°C. for 30 minutes.

Beginning with propagation 21 and continuing for the remaining 8 daily propagations, the cultures were judged for their desireability as butter cultures on the basis of body and texture and flavor. In addition to the normal propagation in the two grades of milk pasteurized at the different temperatures, the cultures were also propagated in 100 ml of Grade A skim milk heated in flowing steam for 30 minutes in standard one-half pint glass milk bottles. These cultures were then judged by competent judges. The cultures were given rates varying with their flavor intensities. A rating of 0 means that no flavor was produced; 1, very slight flavor; 2, slight flavor; 3, fair flavor; and 4, full flavor. The decimal fractions are the result of averaging the eight propagations and indicates the degree of variance between the two rates. The results are shown in Table 7, the data being the average of the last eight daily propagations.

TABLE 7

Average of Eight Flavor Ratings of Butter Cultures Grown in two Grades of Milk Pasteurized at two Different Temperatures

Culture	Pasteurized at 63°C. 30 min.	Pasteurized in flowing steam 30 min.	Pasteurized at 63°C. 30 min.	Pasteurized in flowing steam 30 min.
1	1.0	1.8	1.5	2.4
62	0.8	2.1	1.8	2.0
66	1.4	1.9	1.8	2.4
68	1.4	2.0	1.8	2.4
75	1.1	2.0	1.6	1.8
76	1.4	1.9	1.6	2.3
81	1.5	2.4	1.8	2.3
82	1.3	2.6	1.9	2.4
83	1.0	1.9	1.8	2.1
84	1.4	1.9	1.1	1.8
Total Average:	1.2	2.1	1.7	2.2

0 no flavor
1 very slight flavor
2 slight flavor
3 fair flavor
4 full flavor
The decimal fractions of some rates are the result of the averages and are indications of the degree of flavor intensity within that rate.

All of the cultures grown in Grade A milk pasteurized at 63°C. for 30 minutes produced very mild flavor with an average rate of 1.2. The cultures grown in the same milk only pasteurized at 99°C. for 30 minutes produced slightly better flavor with an average rate of 2.1. The average of the flavor rates of the cultures grown in ungraded milk pasteurized at 63°C. for 30 minutes shows that all of the cultures produced rather mild flavor, with an average rate of 1.7 while all of the cultures grown in the ungraded milk pasteurized at 99°C. for 30 minutes produced slightly better flavor, having an average of 2.2.

In summarizing Table 7, it is noted that the cultures grown in both grades of milk pasteurized at 99°C. for 30 minutes produced slightly better flavored butter cultures than the same cultures grown in each grade of milk pasteured at 63°C. for 30 minutes. There is also very little difference between the flavor quality of the cultures grown in either grade of milk pasteurized at 99°C. for 30 minutes, and between either grade of milk pasteurized at 63°C. for 30 minutes. It is noted that a higher average rate was produced by the cultures grown in ungraded milk than in Grade A milk receiving the corresponding heat treatments. From the results of this data it does not appear detrimental to propagate cultures in ungraded milk, but the higher temperature of pasteurization seems to produce the best flavored cultures.

Table 8 contains an itemized list of the flavor criticisms given the cultures and the number of times they occurred during the last eight daily propagations. The possible number of times each criticism could occur was 80 (10 cultures times 8 propagations). The cultures grown in Grade A milk pasteurized at 63°C. for 30 minutes were criticised 37 times for being flat, 12 times for being off flavor, 8 times for lacking flavor, 9 times for being green, and 6 times for having a coarse flavor, making a total of 72 criticisms. The cultures grown in Grade A milk pasteurized at 99°C. for 30 minutes were criticised 27 times for being flat, 8 times for being off flavor, 9 times for lacking flavor, 3 times for being green, and 7 times for having coarse flavor, making a total of 54 criticisms. The cultures grown in ungraded milk pasteurized at 63°C. for 30 minutes were criticised 29 times for being flat, 16 times for being off flavor, 4 times for lacking flavor, 11 times for being green, and 13 times for having coarse flavor; making a total of 73 criticisms. The cultures grown in ungraded milk pasteurized at 99°C. for 30 minutes were criticised 17 times for being flat, 16 times for being off flavor, 7 times for lacking flavor, and 14 times for being coarse; making a total of 54 critisims.

In summarizing the data in Table 8 it is noted that there is no significant difference between the total flavor criticisms given the cultures grown in each milk receiving the same pasteurization treatment. The cultures grown

TABLE 8

Flavor Criticisms, and the number of times they occurred, of Butter Cultures Grown in two Grades of Milk Pasteurized at two Different Temperatures

Grade A				Ungraded			
Past. at 63°C for 30 min.		Past. in flowing steam for 30 min.		Past. at 63°C for 30 min.		Past. in flowing steam for 30 min.	
Criticism	No. of times occurring	Criticism	No. of times occurring	Criticism	No. of times occurring	Criticism	No. of time occurring
flat	37	flat	27	flat	29	flat	17
off	12	off	8	off	16	off	16
lacking	8	lacking	9	lacking	4	lacking	7
green	9	green	3	green	11	green	0
coarse	6	coarse	7	coarse	13	coarse	14
TOTAL:	72		54		73		54

in the Grade A milk receiving both pasteurization treatments were criticised more often for being flat than the cultures grown in ungraded milk receiving the corresponding heat treatments. However, the cultures grown in the ungraded milk were criticised more often for being off flavor and coarse than the cultures grown in Grade A milk receiving the corresponding heat treatment. These two flavor defects are very undesirable and were probably caused by the growth of organisms which survived pasteurization.

The body and texture of the cultures grown in Grade A milk pasteurized at 63° C. for 30 minutes were criticised 3 times for being weak and 4 times for not being coagulated, the cultures grown in the same milk pasteurized at 99° C. for 30 minutes were criticised only once for being weak. The cultures grown in ungraded milk pasteurized at 63° C. for 30 minutes were criticised 3 times for having weak bodies; while the cultures grown in the same milk pasteurized at 99° C. for 30 minutes were criticised 5 times for having weak bodies. The weak bodies in the ungraded milk pasteurized at 99° C. for 30 minutes may have been caused by the high acidity of the milk and the high heat of pasteurization which would partially denature the milk proteins.

In conclusion it can be stated that cultures grown in milk pasteurized at 99° C. for 30 minutes produce better flavors than cultures grown in the same milk pasteurized at 63° C. for 30 minutes. Cultures grown in ungraded milk produce more flavor than the same cultures grown in Grade A milk receiving the corresponding heat treatments. When

determined chemically, however, when determined organoleptically, the cultures grown in Grade A milk had less serious criticisms than the cultures grown in ungraded milk.

B. <u>Heat resistance of bacteriophage</u>

In the manufacture of cheese it is often found that even if the culture employed is free of bacteriophage, there may be bacteriophage in the cheese milk. Also, if all the bacteriophage in the raw milk are not killed at normal pasteurization exposures (63°C. for 30 minutes), it was wondered what exposures would be required to insure bacteriophage free milk. Accordingly, it was decided to determine the temperature exposures required to destroy bacteriophage particles in milk.

Five bacteriophage strains and their homologus hosts were used in this study. All of the bacteriophage strains used were of high titer, 10^{-9}. One ml of each bacteriophage filtrate was added to 99 ml of sterile litmus milk and one and one-half ml portions of these bacteriophage dilutions were transferred into sterile 10 to 75 mm thin walled culture tubes. These tubes were then sealed in a flame and immersed in a water bath maintained at the desired temperatures. Temperatures of 63°, 66°, 68.5°, and 71°C. with exposure periods of 5, 10, 20, 30, and 40 minutes were employed. At the end of the prescribed heating period the tubes were removed from the hot water bath and placed immediately in water at about 4° C.

The survival of any bacteriophage particles was determined by breaking the culture tubes at the top, inoculating each tube with 0.1 ml of a 1.5 to 100 dilution of the homologus bacteria in sterile litmus milk. The tubes were then stoppered with sterile cotton plugs and incubated at 22° C. The presence of active bacteriophage particles was denoted by longer periods of incubation required to produce coagulation in these tubes than in a control tube which contained only the culture inoculated into the same milk. Likewise, the bacteriophage particles were considered to be destroyed if these tubes coagulated at the same time as the control tube inoculated with the culture only. A partial destruction of the bacteriophage particles was indicated when these tubes coagulated before a control containing the bacteriophage strain unheated with its homologus bacteria, but not coagulated before a control containing only the homologus bacteria. The results are shown in Table 9.

The results indicate that there is considerable variation in heat resistance of different bacteriophage strains. Normal pasteurization (63° C. for 30 minutes) did not kill or even partially destroy any of the bacteriophage strains used. At 66° C., one strain, 3-7, was partially destroyed on heating for 30 minutes and for 40 minutes, while the rest of the strains were apparently not affected. At 68.5° C. bacteriophage strain 3-7 was partially destroyed in 10 minutes, but was not completely inactivated until 40 minutes; bacteriophage strain 3-8 was partially destroyed in 20 minutes and was not completely inactivated even in 40 minutes;

TABLE 9

Heat Resistance of Bacteriophage

Temperature	Phage	5 min.	10 min.	20 min.	30 min.	40 min.
	3-7	-	-	-	-	-
	3-8	-	-	-	-	-
63° C.	3-15	-	-	-	-	-
	W-8D	-	-	-	-	-
	W-6D	-	-	-	-	-
	Phage					
	3-7	-	-	-	$\underline{x}$	$\underline{x}$
	3-8	-	-	-	-	-
66° C.	3-15	-	-	-	-	-
	W-8D	-	-	-	-	-
	W-6D	-	-	-	-	-
	Phage					
	3-7	-	$\underline{x}$	$\underline{x}$	$\underline{x}$	x
	3-8	-	-	$\underline{x}$	$\underline{x}$	$\underline{x}$
68.5°C.	3-15	-	-	-	$\underline{x}$	$\underline{x}$
	W-8D	-	-	-	-	-
	W-6D	-	-	-	-	-
	Phage					
	3-7	$\underline{x}$	x	x	x	x
	3-8	$\underline{x}$	$\underline{x}$	x	x	x
71° C.	3-15	-	-	-	x	x
	W-8D	-	x	x	x	x
	W-6D	-	-	x	x	x

x equals bacteriophage destroyed
$\underline{x}$ equals phage partially destroyed
$\underline{-}$ equals phage uneffected

bacteriophage strain 3-15 was partially destroyed in 30 and 40 minutes; while bacteriophage strains W-6D and W-8D were unaffected by this heat treatment in 40 minutes. At 71° C. bacteriophage strain 3-7 was partially destroyed in 5 minutes, and was completely destroyed in 10 minutes; strain 3-8 was partially destroyed in 5 and 10 minutes, and was completely destroyed in 20 minutes; strain 3-15 was completely destroyed in 30 minutes; strain W-6D was completely destroyed in 20 minutes; and strain W-8D was completely destroyed in 10 minutes.

The data show that even if the culture used in the manufacture of cheese is free of bacteriophage particles, some bacteriophage contamination can be expected in the cheese vat as normal pasteurization exposures will not kill any bacteriophage particles which may be in the raw milk. It is concluded that bacteriophage cannot be eliminated from the milk used in the manufacture of cheese by heat inactivation, as when the milk is heated over 63° C. the coagulation of the milk is disturbed producing a very low quality cheese (48). Milk used to grow lactic acid cultures should be heated to at least 71° C. for 30 minutes to completely destroy possible bacteriophage particles in the milk. However, pasteurization exposures above this are recommended in order to allow a margin of safety in insuring complete destruction of the bacteriophage particles.

C. The effect of incubation temperature on growth of bacteriophage

In some tests it was noted that the lytic actions of the bacteriophage strains used were different at different temperatures of incubation. In order to determine if this observation was of any practical importance, a study was made to determine the optimum growth temperature for five bacteriophage strains and for their homologus hosts. Another purpose of the study was to determine if lactic cultures could be incubated at a certain temperature that would allow normal growth of the culture organisms but would inhibit the growth of any bacteriophage particles which might be present in the culture.

The culture and bacteriophage strains used in this test were *Streptococcus lactis* 3 and its homologus bacteriophage strains 8, 30, and 31; and butter culture 76 and its homologus bacteriophage strain 32.

Two six-ounce screw capped prescription bottles, each containing 100 ml of sterile reconstituted skim milk, were tempered at each of the following temperatures: 22°, 27°, 32°, and 35° C. Each bottle was then inoculated with one percent culture and one bottle at each incubation temperature was inoculated with 0.5 percent of a high titer bacteria-free filtrate of the homologus bacteriophage strain. The bottles were then incubated at the above temperatures for 48 hours. Titratable acidities were determined for both the culture and the culture plus bacteriophage at each incubation temperature at time of inoculation, at 8 hour intervals during 24 hours and also at the end of 48 hours.

The increase in titratable acidity for each culture was determined, and this data recorded in Table 10. The average difference in titratable acidity between the cultures and cultures plus bacteriophage is recorded in the right hand column of Table 10.

The data show that increasing the incubation exposure is not a practical way to control bacteriophage multiplication. Two of the bacteriophage strains used seemed to be inhibited at incubation temperatures of 35° C., while the remaining temperatures were apparently not effective in inhibiting bacteriophage multiplication.

The average difference between the increase in titratable acidities of the culture and the culture plus bacteriophage incubated at 22° C. for 8 hours was 0.15 percent, 16 hours 0.37, 24 hours 0.41, and at 48 hours 0.42; incubated at 27° C. for 8 hours 0.30, 16 hours 0.39, 24 hours 0.40, and 48 hours 0.27; incubated at 32° C. for 8 hours 0.36, 16 hours 0.47, 24 hours 0.38, and 48 hours 0.09; and incubated at 35° C. for 8 hours 0.20, 16 hours 0.18, 24 hours 0.11, and 48 hours 0.01.

With increased incubation exposures the rate of secondary growth is also increased, which accounts for the smaller difference in titratable acidities at the higher temperatures and longer exposures. At 16 hours, which is the standard incubation time for lactic cultures (48), none of the incubation temperatures produced a small enough difference in acidity between the cultures and the cultures plus bacteriophage to be of any practical use in propagating lactic cultures.

Even though an incubation temperature of 35°C. indicates very much bacteriophage inhibition, this incubation temperature could not be used in commercial use because very poor quality cultures are produced when grown at this temperature (48).

After each culture and culture plus bacteriophage had incubated for 48 hours at each temperature, one percent of each of the bottles was inoculated into 10 ml of sterile litmus milk and incubated at 22°C. until the tubes containing the cultures had coagulated. The rate of growth in the tubes containing the culture plus bacteriophage was compared to that in the culture tube. These results are shown in Table 11.

The results show that an incubation temperature of 35°C. for 48 hours decreased the activity of the cultures as *Streptococcus lactis* 3 grew slowly on this transfer while butter culture 76 produced slight growth. This is comparable to the very slight growth produced by the cultures plus bacteriophages during this propagation. The two cultures incubated at 22°, 27°, and 32°C. for 48 hours demonstrated normal activity on this propagation, while the bacteriophage contaminated cultures incubated at 22°C. showed no growth and the bacteriophage contaminated cultures incubated at the remaining temperatures all showed very slight growth.

In conclusion, it has been shown that incubation exposures of lactic cultures 35° C. for 48 hours will almost completely inhibit the multiplication of the bacteriophage during that propagation, however, upon transfer and

TABLE 10

Influence of Incubation Temperatures on the Multiplication of Lactic Acid Bacteria and their Homologus Strains of Bacteriophage

Temperature of Incubation	Hours of Incubation	Streptococcus lactis 3	Bacteriophage 8	Bacteriophage 30	Bacteriophage 31	Butter Culture 76	Bacteriophage 32	Average Difference between Cultures and Phage Plus Culture
22° C.	8	.03	.03	.01	.02	.03	.00	.15
	16	.41	.04	.10	.08	.49	.02	.37
	24	.55	.10	.14	.12	.55	.11	.41
	48	.71	.28	.35	.27	.72	.29	.42
27° C.	8	.35	.03	.19	.07	.46	.02	.30
	16	.59	.06	.52	.11	.56	.09	.39
	24	.66	.08	.56	.17	.57	.14	.40
	48	.69	.50	.56	.35	.58	.20	.27
32° C.	8	.38	.00	.06	.08	.47	.04	.36
	16	.57	.04	.12	.13	.57	.12	.47
	24	.61	.25	.20	.17	.65	.33	.38
	48	.62	.55	.57	.44	.69	.62	.09
35° C.	8	.42	.02	.42	.42	.46	.07	.20
	16	.55	.07	.55	.55	.59	.37	.18
	24	.56	.20	.56	.56	.60	.54	.11
	48	.56	.52	.56	.56	.60	.60	.01

growth at normal incubation temperatures the bacteriophage particles again become active. It has also been shown that the activities of cultures are slowed when incubated at 35° C. for 48 hours. Incubation temperatures of 22°, 27°, and 32° C. for 48 hours had no serious abnormal effect on the activities of the cultures, but would not inhibit the multiplication of bacteriophage.

D. The effect of NFDMS content of reconstituted milk on bacteriophage activity

It has been shown (49) that with the increase in solids of the culture medium, there is an increase in activity of lactic cultures grown in that medium. It was thought that perhaps with the increased activity, the cultures could produce enough acid fast enough to get below the optimum pH of the bacteriophage before the bacteriophage could lyse the homologus culture.

Two hundred ml portions of reconstituted skim milk were prepared containing 6,9,12,15,18, and 21 percent solids. Each lot of reconstituted milk was prepared by mixing in a Waring blendor the required amount of non-fat dry milk solids to produce the desired percent solids when made up to 100 ml with distilled water. Each lot of reconstituted milk was divided evenly into two six-ounce screw capped prescription bottles, autoclaved at 15 pounds pressure for 15 minutes, cooled to 22° C., and inoculated with one ml of _Streptococcus lactis_ 3. One bottle of each lot of milk was also inoculated with 0.5 ml of the homologus bacteriophage strain, 3-8. The bottles were then incubated for 16 hours

TABLE 11

Second Propagation of Cultures and Cultures Plus Bacteriophage after incubation for 48 hours at four different temperatures

Tempt. of incubation		Bacteriophage 3-8	Bacteriophage 3-30	Bacteriophage 3-31		Bacteriophage 76-76
22° C.	4	0	0	0	4	0
27° C.	4	1	1	1	4	1
32° C.	4	1	1	1	4	1
35° C.	3	1	1	1	2	1

(Incubated at 22° C.)

0 no growth
1 very slight growth
2 slight growth
3 slow growth
4 normal growth, same as control

at 22°C. Titratable acidities were determined on each bottle before and after incubation, the difference between the two being the actual increase in titratable acidity as shown in Table 12.

The data indicate that increasing the solids in the milk does not prevent the growth of bacteriophage in lactic acid cultures.

The culture grown in 6 percent solids produced an increase in titratable acidity of 0.45 percent and the bacteriophage contaminated culture 0.17 percent, and the difference between the two was 0.28 percent; in 9 percent solids the culture increased 0.58, while the bacteriophage contaminated culture increased 0.21, and the difference was 0.37; in 12 percent solids the culture increased 0.71, while the bacteriophage contaminated culture increased 0.23, and the difference was 0.48; in 15 percent solids the culture increased 0.76, while the phage contaminated culture increased 0.25, and the difference was 0.51; in the 18 percent solids the culture increased 0.98; while the bacteriophage contaminated culture increased 0.35, and the difference was 0.63; and in the 21 percent solids the culture increased 1.12, while the bacteriophage contaminated culture increased 0.45, and the difference was 0.67.

It is probable that the increase in titratable acidity of the bacteriophage contaminated culture resulted from the growth of the culture organisms before becoming lysed by the bacteriophage. Even though this increase in titratable acidity became greater with the increased percent of solids in the milk, the difference between the titratable acidities

TABLE 12

Influence of Percent NFDMS in Reconstituted
Milk on Bacteriophage Activity

Increase in titratable acidity during incubation at 22° C. for 16 hours

	Percent solids in reconstituted milk					
	6	9	12	15	18	21
Culture alone	0.45	0.58	0.71	0.76	0.98	1.12
Culture plus bacteriophage	0.17	0.21	0.23	0.25	0.35	0.45
Difference	0.28	0.37	0.48	0.51	0.63	0.67

of the culture and the bacteriophage contaminated culture also became greater.

In summary, since it has been noted in Table 12 that the difference between the titratable acidities of the culture and the bacteriophage contaminated culture became greater with increased solids, it can be stated that increasing the solids content of the reconstituted milk does not prevent lactic cultures from becoming lysed by bacteriophage; in fact, the increased solids tends to favor the growth of bacteriophage.

E. Rate of bacteriophage growth

A study was conducted to determine the rate of growth and burst time of a bacteriophage strain. As this strain of bacteriophage was very active, the study was continued for a number of days to determine the time and rate of secondary growth of the host organism after being lysed by the bacteriophage.

A six ounce screw capped prescription bottle containing 100 ml of sterile reconstituted skim milk was tempered to 22° C., inoculated with one ml *Streptococcus lactis* 3, and 0.5 ml. of bacteriophage filtrate 3-8. The bottle was then incubated at 22° C. Titers were determined by the serial dilution method at time of adding the bacteriophage filtrate and at hourly intervals for 12 hours, and then at 12 hour intervals for another 84 hours. The results are shown in Table 13.

TABLE 13

Rate of Growth of Bacteriophage Strain 3-8

Hours from inoculation	Dilutions 4	5	6	7	8	9	10	11	12	13	14	15	Bacteriophage titer
0	-	-	x										10-5
1	-	-	-	-	x								10-7
2		-	-	-	-	x							10-8
3				-	-	-	x	x					10-9
*36				-	-	-	x	x					10-9
48				-	-	x	x	x					10-8
60				-	-	-	-	x					10-10
72				-	-	-	-	-	x	x			10-11
84				-	-	-	-	-	x	x	x	x	10-11
96						-	-	-	-	-	x	x	10-13

x equals normal culture growth, litmus milk coagulated and reduced

- equals not normal growth, litmus milk either not reduced or not coagulated, or both.

* The titers were determined hourly from 3 to 12 hours, and then at 24 and 36 hours, and the same titer produced.

The data show that the multiplication of bacteriophage strain 3-8 is rather rapid. The bacteriophage titer at the time of inoculation was 10^{-5}, at the end of one hour incubation it was 10^{-7}, two hours 10^{-8}, and three hours 10^{-9}, the latter being the titer of the bacteriophage strain used in the original inoculation. The titer was 10^{-9} for the remainder of 36 hours incubation, however, at 48 hours the titer of the bacteriophage had fallen to 10^{-8}. In 60 hours the titer was 10^{-10}, at 72 and 84 hours 10^{-11}, and in 96 hours incubation the titer had reached 10^{-13}.

The increase in titer during the first three hours of growth was expected, as the bacteriophage was very active. The growth of the bacteriophage, as indicated by the titer, from 36 hours on to the end of the trial is comparable to the results reported by Whitehead and Cox (64). This is shown by the fact that as the secondary growth organisms appeared at 48 hours the titer dropped because the secondary growth organisms were resistant to the bacteriophage. However, this resistance did not last very long, as the titers increased markedly from 60 hours on, which indicates that the secondary growth organisms were being attacked by the bacteriophage which had lysed the original culture.

The above results indicate that lactic acid cultures should not be incubated for longer periods than is required to develop normal growth, which is usually 16 to 24 hours at 22°C. (49). Longer incubation periods may increase the number of bacteriophage particles in the culture until there

is a sufficient number present to completely destroy the culture organisms.

F. The influence of products of biological growth upon the multiplication of bacteriophage

A vast amount of work has been done by numerous workers in isolating various anti-bacterial substances from biological sources. Numerous species of seed plants have been found to contain bacterial inhibitors (25), and some plants possess the property of bacteriophage inhibition at concentrations which will not affect the normal growth of the host bacteria (8). Anti-bacteriophage substances have also been isolated from mold growth (4). Yeast has also been found to contain an inactivator for plant viruses (62).

With the above results in mind, a study was undertaken using various biological growth products in an effort to find a way to inhibit or destroy bacteriophage particles without affecting the normal multiplication of the host bacteria in lactic cultures.

I. The effect of antibiotics

A total of 18 antibiotics were employed in a study to determine if there is a level at which the antibiotics would destroy the bacteriophage particles without slowing the homologus *S. lactis* culture appreciably.

The antibiotics were prepared in concentrations of 0.01 percent in 10 ml of sterile distilled water in a screw capped test tube. Preliminary tests were conducted to determine the levels at which the antibiotics would inhibit the

multiplication of Streptococcus lactis. This was done by placing sufficient quantities of the antibiotics to give a final concentration covering a wide range into screw capped test tubes containing 10 ml sterile litmus milk. These tubes were then inoculated with 0.05 ml of Streptococcus lactis 3, incubated at 22° C. for 16 hours, and the amount of growth in each tube compared with the growth in a control which contained the culture but no antibiotic. The concentrations of antibiotics to use were narrowed down in this manner until four concentrations were selected, the highest concentration being the maximum that the bacteria would tolerate, and the lowest of the four concentrations which would allow normal or near normal growth of the bacteria.

The desired concentrations were placed into each of two test tubes containing 10 ml of sterile litmus milk and their influence on bacteriophage determined. The amount of growth was rated as follows: 0 no growth (no reduction or coagulation); 1, very slight growth (slight reduction); 2, slight growth (complete reduction, no coagulation); 3, slow growth (coagulation, but not firm and requiring longer incubation to coagulate than control); and 4, full or normal growth (firm coagulation, same as control). The results are shown in Table 14.

The antibiotics were used in concentrations ranging from 0.25 to 60.00 parts per million, except sulfathiozole, which was used in four concentrations ranging from 180 ppm to

TABLE 14

Influence of Antibiotics on Multiplication of Bacteriophage

Antibiotic	Conc. (PPM)	C	C+P	Conc. (PPM)	C	C+P	Conc. (PPM)	C	C+P	Conc. (PPM)	C	C+P
Aureomycin	0.25	xxx	o	0.50	xxx	o	0.75	xx	x	1.0	x	x
Bacitracin	5.0	xxxx	o	10.0	xxx	x	15.0	xx	x	20.0	xx	x
Di penicillin G.	0.50	xxx	o	0.75	xxx	xx	1.0	xxx	xx	1.25	xx	x
K penicillin G.	0.25	xxxx	o	0.50	xxxx	o	0.75	xxxx	o	1.0	xx	o
Procain	0.50	xxx	o	0.75	xxx	o	1.0	xx	xx	1.25	x	x
Sulfathiozale	180.0	xxxx	o	200.0	xxx	o	220.0	x	o	240.0	o	o
Streptomycin	4.0	xxx	o	5.0	xx	x	6.0	xx	x	7.0	x	x
Tryathricin	10.0	xxxx	o	20.0	xxx	o	30.0	xx	o	40.0	x	o
Magnamycin	2.0	xxx	o	3.0	xx	o	4.0	xx	x	5.0	xx	x
Terramycin	1.0	xxx	o	2.0	xx	x	3.0	xx	x	4.0	x	x
Viacin	30.0	xxx	o	40.0	xxx	o	50.0	xxx	o	60.0	xx	x
Nystatin	5.0	xxxx	o	10	xxxx	o	20.0	xxx	o	40.0	xx	o
Nydrozid	5.0	xxxx	o	10	xxxx	o	20	xxx	o	40.0	xx	o
*Antibiotic A	5.0	xxx	o	10	xxx	x	20	xxx	x	40.0	xx	x
*Antibiotic B	5.0	xxxx	o	10	xxxx	o	20	xxx	o	40.0	xx	o
*Antibiotic C	5.0	xxx	o	10	xx	x	20	xx	x	40	x	x
*Antibiotic D	5.0	xxxx	o	10	xxxx	o	20	xxxx	o	40	xx	x
**Antibiotic E	5.0	xxxx	o	10	xxxx	o	20	xxx	o	40	xx	o

*Antibiotics A,B,C, and D above were numbers 9R3333, 52R6598, 53R2494, and 52R2493 supplied by Merck and Co., Inc., Rahway, New Jersey.

**Antibiotic E was special Antibiotic A6 supplied by Wyeth, Inc., West Chester, Pa.

o no growth
x very slight growth
xx slight growth
xxx slow growth
xxxx normal growth

C equals S lactis 3
C+P equals S lactis 3 plus phage 3-8

240 ppm. Seven of the antibiotics used in this study apparently did not affect bacteriophage multiplication at levels which destroyed or greatly inhibited the host cells. Five of the remaining eleven antibiotics apparently completely inhibited the bacteriophage at nearly the maximum levels tolerated by the host bacteria, but at these levels the growth of the host bacteria was too slow to be of any practical use as a lactic culture. These antibiotics and the levels at which they apparently completely inhibited the growth of bacteriophage were aureomycin, 1.00 ppm; procain penicillin G., 1.00 ppm; and 1.25 ppm; streptomycin, 7.00 ppm; terramycin, 4.00 ppm; and antibiotic C*, 40.00 ppm. Higher concentrations than these completely inhibited the bacteria, while lower concentrations did not produce any detectable inhibition of the bacteriophage.

The remaining six antibiotics apparently produced very slight inhibition of the bacteriophage, but not enough to be practical, and the concentrations of the antibiotics which would inhibit the bacteriophage would also slow up the *Streptococcus lactis*.

The data indicate that none of the antibiotics used in this study would inhibit bacteriophage at levels which would not affect the normal growth of the homologus bacteria, it is, therefore, concluded that the use of antibiotics is ineffective in controlling bacteriophage contamination in lactic cultures.

* Antibiotic Number 53R2494. Supplied by Merck and Co., Inc. Rahway, New Jersey

2. The effect of bacterial growth

Since certain bacteria produce anti-bacterial substances, there is a possibility that they also produce anti-bacteriophage substances. Accordingly, a study was undertaken whereby 195 bacterial colonies were isolated from plates poured for standard plate counts. These cultures were inoculated into sterile litmus milk and the tubes incubated at room temperature until the cultures demonstrated abundant growth. Then one ml of each culture was transferred into 100 ml quantities of sterile litmus milk in six ounce screw capped prescription bottles and incubated several days at room temperature. After the cultures had apparently produced maximum growth, those that had produced considerable acid were neutralized with sodium hydroxide to a pH of approximately 6.8, using bromo thymol blue as the indicator. Approximately 10 ml quantities of each culture were then placed into each of three screw capped test tubes and heated in flowing steam for 10 minutes. After cooling to 22 ^{o}C., two of the tubes were inoculated with Streptococcus lactis, and one of these two with a homologus bacteriophage filtrate. The influence of the bacterial cultures on bacteriophage was determined by the relative growth in these two tubes after incubation at 22 ^{o}C. for 10 hours. The third tube was incubated, but not inoculated, and was used to determine if the heated cultures contained any live bacteria.

Of the 195 bacterial cultures tried, none seemed to produce any bacteriophage inhibition; however, there were

four that demonstrated a rather marked inhibitory effect on the *Streptococcus lactis*, but the levels that would permit normal *Streptococcus lactis* growth apparently had no effect on the homologus bacteriophage.

3. The effect of products of mold growths

Numerous molds are known to produce antibiotics and a few have been known to produce anti-bacteriophage substances (4). From this it appeared logical that certain molds might produce materials which would inhibit bacteriophage without affecting the homologus lactic cultures. Accordingly, an experiment was conducted whereby 120 mold cultures were checked for possible anti-bacteriophage activity. These molds were isolated from plates poured for standard plate counts, and yeast and mold counts. The molds were first grown at room temperature in sterile litmus milk in screw capped test tubes until abundant growth had formed.

Then the tubes were shaken thoroughly and 1 ml of the mold growth was transferred to 100 ml sterile reconstituted skim milk contained in cotton stoppered one liter erlenmeyer flasks. The large flasks were used in order to provide a large surface area. The molds were incubated at room temperature for several days until apparently maximum growth had formed. The mold growths were then thoroughly mixed in a sterile Waring blender and the resultant mixture divided among three sterile six ounce screw capped prescription bottles. One bottle of each mold preparation was autoclaved for 15 minutes at 12 pounds pressure. Another bottle of it was passed

through a sterile Seitz filter into a sterile four ounce screw capped prescription bottle. The remaining bottle of it was stored raw or untreated at 9°C.

Preliminary tests were run with each mold preparation to determine if it would inhibit Streptococcus lactis in 10 percent concentrations and to determine if it was contaminated with bacteria. For this test, one ml of the sterile, the filtered, and the raw preparation was transferred into each of two test tubes containing 10 ml sterile litmus milk. One of these two tubes was inoculated with one drop (0.05 ml) of Streptococcus lactis 3. The tubes were inoculated in the order given above to prevent any possible transfer of contamination from the untreated or filtered mold mixtures to the sterile mixture, and from the tube containing the untreated mold preparation to the tube containing the filtered mold preparation. All the tubes were then incubated at 22°C. for 16 hours and checked for bacterial inhibition or bacterial contamination. Bacterial inhibition was evidenced by less or slower growth in the tubes containing the various mold preparations than in the control tube containing only the culture. Bacterial contamination was noted by any bacterial growth in the tubes containing only the raw or untreated mold preparations inoculated into sterile litmus milk. If it appeared that the mold growths were contaminated with bacteria, the mold was streaked on an agar plate and the isolated colonies picked into sterile litmus milk and the same procedure followed as before in preparing mold mixtures.

After the preliminary trials and after the mold had been repurified and cultured, one ml portions of the sterile, filtered and raw or untreated mold preparations were placed, in the order given, into each of two test tubes containing 10 ml sterile litmus milk and tested for their influence on bacteriophage.

Of the 120 mold cultures studied, only one appeared to completely inhibit bacteriophage multiplication. Three of the cultures were greatly inhibitory to Streptococcus lactis, but these cultures did not show any demonstrable inhibition of the bacteriophage in subsequent trials using low enough concentrations to permit growth of the S. lactis.

The mold culture which appeared to inhibit the bacteriophage had been isolated from a plate poured for the standard plate count which had incubated at room temperature for three days after it had been counted. This mold had the characteristics of the genus Asperigillus, but no attempt was made to positively identify it. This mold seemed to completely inhibit the bacteriophage in preliminary trials. However, later examinations of the mold by plating it out and by microscopic examination revealed that it was contaminated with bacteria. Preliminary trials, using this mold repurified, indicate some inhibition of the bacteriophage, but, there was not complete inhibition and it was not heat stable. Further work is being done with this mold.

4. The effect of products of yeast growth

It has been shown by Takahashi (62) that a virus inactivator is produced by yeast growth. Accordingly, an experiment was conducted using two forms of yeast in a study of their effect in inhibiting bacteriophage. One form was Difco's yeast extract and the other was ordinary powdered baking yeast obtained from a local grocery store.

The yeast extract was prepared in a 50 percent solution by mixing five grams of the yeast extract with five grams of distilled water. This mixture was then autoclaved at 15 pounds pressure for 15 minutes and cooled to room temperature. Concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0 percent were added to sterile litmus milk and the influence on bacteriophage determined, using bacteriophage 8.

The bakers yeast was prepared by placing the contents of one package of the yeast into 100 ml of sterile distilled water in six ounce screw capped prescription bottles. Three such bottles were prepared. One bottle was used raw or untreated, one was heated in flowing steam for 30 minutes, and the other was filtered through a sterile Seitz filter and the filtrate used. One ml (about 10 percent) of each of the various bakers yeast preparations was added to sterile litmus milk and the action on bacteriophage 8 determined. One tube was prepared by placing one ml of the untreated yeast preparation into 10 ml sterile litmus milk without added culture or bacteriophage and incubated with the other tubes to determine the action of the yeast only on the litmus milk.

The results indicated that the yeast extract contained no substance which was inhibitory to the multiplication of lactic acid bacteriophage at concentrations which would allow normal growth of the host bacteria. This was indicated in that five percent or less of the yeast extract demonstrated no inhibition to either the S. lactis or bacteriophage, while a ten percent concentration slowed the S. lactis growth without any demonstratable inhibition of the bacteriophage.

The results indicated that bakers yeast, in the concentrations used, had no effect on the bacteriophage, and the growth of S. lactis appeared to be normal.

5. The effect of plant extracts

A group of 29 domestic seed plants were gathered in the local area and tested for possible bacteriophage inhibition. These seed plants were gathered in the green state, using only the stems and the leaves in this experiment. The plants were mascerated in a food grinder and the plant juice separated from the pulp by filtration or by steeping with water for one hour and filtering out the pulp. These juices were then concentrated to 10 ml by boiling in a vacumn.

A preliminary experiment was conducted to determine the levels of tolerance by the S. lactis. Five tenths ml, one ml, and two ml, of the plant extracts were transferred into screw capped test tubes containing 10 ml sterile litmus milk.

The tubes were then autoclaved for 15 minutes at 12 pounds pressure, cooled to 22 °C, and inoculated with one percent *S. lactis*. After incubation for eight hours at 22 °C., the levels of tolerance of the *S. lactis* were noted by the comparative growth of these tubes with a control tube containing only the *S. lactis*. After the levels of tolerance of the *S. lactis* had been determined, the influence of the plant extracts on bacteriophage was determined.

The 29 seed plants used were ineffective in inhibiting bacteriophage in that the levels of the concentrates in which the *S. lactis* would grow would not inhibit the bacteriophage. There was an indication of some bacteria inhibition by four of the 29 concentrates used.

Among the plants used in this experiment were lambs quarter (*Chenopodium album*); prickley lettuce (*Lactuca scorialo*); bedstraw (*Galium rubioceae*); pepper grass (*Lepidium densiflorum*); spreading chervil (*Chaerophyllum procumbens*); yarrow (*Achillea millefolium*); buffalo pea (*Baptisia brocteata*); chickweed(*Stellaria media*); cornflower (*Centauria cyanus*); pigweed (*Amaranthus retroflexus*); little barley (*Hordeum pusillum*); squaw bush (*Rhus trilobata*); and little horseweed (*Ambrosia trifolia*). The remaining 16 plants were not identified.

A small quantity of various wild mushroom growths found growing on local lawns were gathered and tested for their influence of bacteriophage. These mushrooms were prepared by chopping them in a sterile Waring blendor with

the addition of a small amount of sterile distilled water. One portion of this finely chopped mass was placed in flowing steam in an autoclave for 30 minutes. Another portion was filtered through a sterile Seitz filter and the filtrate used. The remainder of the finely chopped mushrooms were used in the raw state.

Approximately 10 percent of these mushroom preparations were added to each of two 10 ml quantities of sterile litmus milk and tested for bacteriophage inhibition.

In concentrations of 10 percent the mushroom preparations demonstrated no apparent inhibition of the bacteriophage while allowing normal growth of the S. lactis. It is concluded, therefore, that in the concentrations used, mushrooms contained neither anti-bacterial nor anti-bacteriophage substance.

G. The influence of various chemical compounds on the multiplication of bacteriophage

It is a common practice in the field of bacteriology to inhibit the growth of certain organisms by chemical means while other organisms in the same medium continue to grow. Examples of this are the addition of sodium azide to blood agar to inhibit the growth of gram negative organisms and allow gram positive organisms to grow, and the addition of sodium desoxycholate to agar in the enumeration of coliform colonies (1). Also, sodium chloride has been used effectively in completely inhibiting the multiplication of bacteriophage of Streptomyces griseus without affecting the normal growth of the host (33). Since certain chemicals

apparently seem to have a selective action between bacteriophage and their homologus bacteria, as well as between different bacteria, a study was conducted using various sodium compounds and other chemical compounds to determine their influence on lactic Streptococcus bacteriophage.

The chemical substances used were usually prepared in 25 or 50 percent aqueous stock solutions, depending on their solubilities. All of the stock solutions were placed in six ounce screw capped prescription bottles and autoclaved for 15 minutes at 15 pounds pressure.

Preliminary trials were run to establish the approximate maximum concentration of each compound in milk at which the *Streptococcus lactis* could grow. These trials were conducted by placing various concentrations of each compound into sterile litmus milk, inoculating with *Streptococcus lactis* 3, incubating at 22° C., and determining the rate of reduction and coagulation by the organism. After the approximate maximum level of concentration of each compound tolerated by the *S. lactis* was established, four levels of concentrations were used to determine the effect of each chemical compound on the bacteriophage strain. These concentrations ranged from the maximum level tolerated by the *S. lactis* to the highest concentration which would permit normal growth of the culture.

1. <u>The effect of sodium compounds</u>

A total of 29 sodium compounds were tested for their influence on bacteriophage. These compounds were used in various ranges of concentrations, some being as low as 0.0001 percent. The results are shown in Table 15.

The data indicate that one of these compounds, sodium tetrapyrophosphate, apparently completely inhibited the bacteriophage without affecting the normal growth of the homologus bacteria. This compound, in a 1.5 percent concentration in milk, apparently completely inhibited the growth of bacteriophage without interferring with the normal growth of the <u>Streptococcus lactis</u>. At concentrations of 1.0 percent and 2.0 percent of this compound, the <u>S</u>. <u>lactis</u> culture showed normal growth, but the bacteriophage was only partially inhibited. A concentration of 2.5 percent of this compound apparently completely inhibited the bacteriophage and slightly inhibited the <u>S</u>. <u>lactis</u>. All concentrations used dissolved the casein in the milk; however, the casein was precipitated by the acid developed in the milk. The inability of the bacteriophage to multiply was apparently due to an inhibition by the substance rather than a destruction. This was determined by inoculating ten ml of sterile litmus milk in screw capped test tubes with one percent of these cultures and bacteriophage contaminated cultures that had previously been grown in various concentrations of sodium tetrapyrophosphate. These

were then incubated at 22°C. for 16 hours and the rate of growth noted. The tubes containing the culture alone on this second propagation demonstrated normal growth, indicating that the *S. lactis* was unaffected by the sodium compound. However, the tubes containing the bacteriophage contaminated culture upon this transfer showed no growth in any of the tubes at any concentration. The bacteriophage was, therefore, only inhibited while in the presence of sodium tetrapyrophosphate, and when the culture was transferred, as a cheese culture would be inoculated into cheese milk, the bacteriophage, which were still active, prevented multiplication of the *S. lactis*.

Two of the compounds, sodium citrate and sodium oxalate, slightly inhibited the bacteriophage in concentrations of 1.5 percent and .6 percent, respectively. However, these concentrations also slightly inhibited the growth of the homologus *S. lactis.* Higher concentrations greatly inhibited or prevented the growth of the *S. lactis*, while lower concentrations were ineffective in inhibiting the bacteriophage.

Seven of the remaining sodium compounds seemed to very slightly inhibit the bacteriophage, but concentrations which produced any useful degree of inhibition greatly or completely inhibited the homologus *S. lactis*. These seven compounds and the concentrations at which they seemed to very slightly inhibit the bacteriophage were sodium chloride, 2.0 percent and 3.0 percent; sodium diethyl dithio carbamate,

TABLE 15

Influence of Chemical Substances on Multiplication
of Bacteriophage 3-8 Effect of Various Sodium Compounds

Sodium Compound	Formula	Conc.(%)	C	C+P	Conc.(%)	C	C+P	Conc.(%)	C	C+P	Conc.(%)	C	C+P
Na. acetate	$NaC_2H_3O_2 \cdot 3H_2O$	0.25	xxxx	o	0.50	xxx	o	0.75	x	o	1.0	o	o
Na. azide	NaN_3	0.0001	xxxx	o	0.0005	xx	x	0.001	x	x	.002	o	o
Na. bicarbonate	NaHCO	0.20	xxxx	o	0.30	xxxx	o	0.4	xxx	o	0.5	xx	o
Na. tetraborate	$Na_2B_4O_7$	0.05	xxxx	o	0.10	xxxx	o	0.15	xx	o	0.2	o	o
Na. carbonate	Na_2CO_3	0.10	xxxx	o	0.20	o	o	0.3	o	o	0.4	o	o
Na. chloride	NaCl	1.00	xxxx	o	2.00	xxx	x	3.0	xx	x	4.0	o	o
Na. citrate	$2Na_3C_6H_5O_7 \cdot 11H_2O$	0.50	xxxx	o	1.00	xxxx	x	1.5	xxx	xx	2.0	xx	x
Na. desoxy-cholate		0.005	xxxx	o	0.01	xxxx	o	0.02	xxx	o	0.04	xx	o
Na. dichromate	$Na_2Cr_2O_7 \cdot 2H_2O$	0.0025	xxxx	o	0.005	xxx	o	0.01	x	o	0.02	o	o
Na. diethyl, dithio carbamate	$(C_2H_5)_2NC$ $5Na \cdot 3H_2O$	0.10	xxxx	o	0.20	xxx	x	0.3	o	o	0.4	o	o
Na. ferricyanide	$Na_4Fe(CN)_6$ $12H_2O$	0.0003	xxxx	o	0.0006	xxx	o	0.0012	xx	o	0.0024	x	o
Na. fluoride	NaF	0.025	xxxx	o	0.05	o	o	0.1	o	o	0.2	o	o
Na. Hydroxide	NaOH	0.0125	xxxx	o	0.025	xxxx	o	0.05	o	o	0.01	o	o
Na. lactate	$NaC_3H_5O_3$	1.0	xxxx	o	2.0	xxxx	o	3.0	xxx	o	4.0	xx	o
Na. molybdate	$Na_2MoO_4 \cdot 2H_2O$	.125	xxxx	o	.25	xxxx	o	.5	xxx	o	1.0	xx	o

TABLE 15 (CONT.)

Sodium Compound	Formula	Conc.(%)	C	C+P	Conc.(%)	C	C+P	Conc.(%)	C	C+P	Conc.(%)	C	C+P
Na. nitrite	$NaNo_2$	.05	xxxx	o	.1	xxx	o	.15	xx	o	.2	x	o
Na. oxalate	$Na_2C_2O_4$	.15	xxxx	o	.45	xxxx	x	.6	xxx	xx	.75	x	x
Na. phosphate, monobasic	$NaH_2Po_4 \cdot H_2O$	2.0	xxxx	o	4.0	xxx	o	6.0	xx	o	8.0	xx	o
Na. phosphate, dibasic	$Na_2HPo_4 \cdot 12H_2O$	1.0	xxxx	o	1.5	xxx	o	2.0	xx	x	2.5	xx	x
Na. phosphate, tribasic	$Na_3Po_4 \cdot 12H_2O$	.3	xxxx	o	.4	xxx	o	.4	o	o	.6	o	o
Na. tetra-pyrophosphate	$Na_4P_2O_7 \cdot 10H_2O$	1.0	xxxx	xxx	1.5	xxxx	xxxx	2.0	xxxx	xxx	2.5	xxx	xxx
Na. K-tartrate	$NaKC_4H_4O_6 \cdot 4H_2O$	2.0	xxxx	o	3.0	xxxx	o	4.0	xxxx	x	5.0	xxx	x
Na. salicylate	$NaC_7H_5O_3$	.05	xxxx	o	.1	xxx	o	.15	xx	o	.2	x	o
Na. sulfate	Na_2SO_4	2.0	xxxx	o	4.0	xxxx	o	6.0	xxx	x	8.0	xx	x
Na. tartrate	$Na_2C_4H_4O_6 \cdot 2H_2O$	2.0	xxxx	o	3.0	xxxx	x	4.0	xxxx	x	5.0	xxx	x
Na. thiosulfate	$Na_2S_2O_3 \cdot 5H_2O$	2.0	xxxx	o	2.5	xxxx	o	3.0	xxx	o	3.5	x	o
Na. tungstate	$Na_2WO_4 \cdot 2H_2O$	.2	xxxx	o	.3	xxx	o	.4	o	o	.5	o	o
Na.di(2-ethyl-hexyl) phosphate	(Tergitol P-28)	.1%	xxxx	o	.25%	xxxx	o	.5%	xxx	o	10%	x	o
Na.heptadecyl sulfate	(Tergitol 7)	.1%	xxxx	o	.25%	xxxx	o	.5%	xxx	o	10%	xx	o

C equals *S. lactis*
C+P equals *S. lactis* plus bacteriophage

o equals no growth
x equals very slight growth
xx equals slight growth
xxx equals slow growth
xxxx equals normal growth, same as control

0.2 percent; sodium potassium tartrate, 4.0 percent and 5.0 percent, sodium tartrate, 3.0 percent, 4.0 percent and 5.0 percent; diabasic sodium phosphate, 2.0 percent and 2.5 percent; sodium sulfate, 6.0 percent and 8.0 percent; and sodium azide, .0005 percent and .001 percent.

2. The effect of miscellaneous chemical compounds

The influence of 35 miscellaneous chemical compounds on the multiplication of lactic acid bacteriophage was determined in another trial. The results are shown in Table 16.

Only two of these miscellaneous compounds appeared to completely inhibit the bacteriophage, but the concentrations that inhibited the bacteriophage also greatly inhibited the homologus *Streptococcus lactis*. These two compounds were potassium iodide and diabasic potassium phosphate. They completely inhibited the growth of bacteriophage in concentrations of 0.12 and 2.0 percents respectively, and slightly inhibited the bacteriophage in concentrations of 0.06 and 1.0 percents respectively. Higher concentrations than those which completely inhibited the bacteriophage also completely inhibit the *S. lactis*. However, in each case, the concentration which completely inhibited the bacteriophage slowed the growth of the *S. lactis* to such a degree as to render it impractical as a lactic culture.

TABLE 16

Influence of Chemical Substances on Multiplication of Bacteriophage

Substance Used	Conc.	C	C+P	Conc.	C	C+P	Conc.	C	C+P	Conc.	C	C+P
2,3,5-Triphenyl, tetrazolium Chloride	.25%	xxxx	o	.5%	xxx	o	1%	xx	o	2%	o	o
Sucrose	21%	xxxx	x	24%	xxx	x	27%	xxx	x	33%	xx	o
Nitrofurzone	1 PPM	xxxx	o	2 PPM	xxxx	o	4 PPM	xxxx	o	8 PPM	xxx	o
Furamozone	1 PPM	xxxx	o	2 PPM	xxxx	o	4 PPM	xxxx	x	8 PPM	xxx	x
Furazolidane	1 PPM	xxxx	o	2 PPM	xxxx	o	4 PPM	xxx	x	8 PPM	xx	x
Sorbic Acid	100 PPM	xxxx	o	200 PPM	xxxx	o	400 PPM	xxx	o	800 PPM	xx	x
o-Phenylphenol	100 PPM	xxxx	o	200 PPM	xxxx	o	400 PPM	xxx	o	800 PPM	xx	o
Polyethoxy polypropoxy ethonal-iodine complex (Iosan)	9 PPM	xxxx	o	18 PPM	xxxx	o	27 PPM	xxx	o	36 PPM	xx	o
Tween 60	7%	xxxx	o	8%	xxxx	o	9%	xxx	x	10%	xx	x
Pyridine	10 PPM	xxxx	o	20 PPM	xxxx	o	40 PPM	xxx	o	80 PPM	xx	x
Hydrogen Peroxide	25 PPM	xxxx	o	50 PPM	xxxx	o	100 PPM	o	o	200 PPM	o	o
Hydrogen Peroxide plus catalase	1%	xxxx	xx	2%	xxxx	x	4%	xxxx	x	8%	xxx	o
Quinhydrone	.063%	xxxx	o	.125%	xxxx	o	.05%	xxx	o	.5%	xx	o
Lithium sulfate	.063%	xxxx	o	.125%	xxxx	o	.25%	xxx	o	.5%	xx	x
Mercuric oxide	.25 PPM	xxxx	o	.5 PPM	xxx	o	1.0 PPM	xx	o	2 PPM	o	o
Mercuric chloride	2.5 PPM	xxxx	o	5 PPM	xxx	o	10 PPM	xx	o	20 PPM	o	o
Iodine	25 PPM	xxxx	o	50 PPM	xxxx	o	100 PPM	xxx	o	200 PPM	xx	o

TABLE 16 (CONT.)

Substance Used	Conc.	C	C+P	Conc.	C	C+P	Conc.	C	C+P	Conc.	C	C+P
Potassium Iodide	.03%	xxxx	o	.06%	xxx	x	.12%	xx	xx	.24%	o	o
Potassium phosphate, monobasic	.03%	xxxx	x	.06%	xxx	o	.12%	xxx	o	.24%	xx	o
Potassium phosphate, dibasic	.5%	xxxx	o	1.0%	xxx	x	2.0%	xx	xx	4%	o	o
Methyl red	20 PPM	xxxx	o	40 PPM	xxx	o	80 PPM	o	o	160 PPM	o	o
Uranium Nitrate	.25%	xxxx	o	.5%	xxx	o	1.0%	xxx	o	2%	x	o
Methyl dodecyl benzyl trimethyl ammonium chloride (Spartec)	5 PPM	xxxx	o	10 PPM	xxxx	x	20 PPM	xxx	o	40 PPM	xx	o
Lauryl dimethyl benzyl ammonium chloride (Ster-bac)	5 PPM	xxxx	o	10 PPM	xx	o	20 PPM	x	o	40 PPM	o	o
Di-Isobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride monohydrate (Amerse)	5 PPM	xxxx	o	10 PPM	xx	o	20 PPM	o	o	40 PPM	o	o
Alkyl dimethyl benzyl ammonium chloride (Roccal)	.5 PPM	xxxx	o	1.0 PPM	xx	o	5 PPM	o	o	10 PPM	o	o
Methyl alkyl benzyl trimethyl ammonium chloride (Winroc)	5 PPM	xxxx	o	10 PPM	xx	o	20 PPM	x	o	40 PPM	o	o
Methyl dodecyl benzyl trimethyl ammonium chloride (Lo count)	10 PPM	xxxx	o	20 PPM	xxx	o	30 PPM	o	o	40 PPM	o	o
N(lauryl calamino formyl methyl) pyridinium chloride (Emolsept)	.5 PPM	xxxx	o	1.0 PPM	xx	o	5 PPM	o	o	10 PPM	o	o
Alkyl benzyl trimethyl ammonium chloride (Ortho ATM 50)	.1 PPM	xxxx	o	.5 PPM	xx	o	1 PPM	x	o	5 PPM	o	o

TABLE 16 (CONT.)

Substance Used	Conc.	C	C+P	Conc.	C	C+P	Conc.	C	C+P	Conc.	C	C+P
Trimethyl ammonium chlorides (Sterilix)	5 PPM	xxxx	o	10 PPM	xxxx	o	20 PPM	xxx	o	30 PPM	x	o
Water soluble alkyl aryl polyether alcohol (Triton X-100)	5 PPM	xxxx	o	10 PPM	xx	o	20 PPM	x	o	30 PPM	x	o

C equals <u>S. lactis</u> 3
C+P " <u>S. lactis</u> 3 plus phage 3-8

O equals no growth
x " very slight growth
xx " slight growth
xxx " slow growth
xxxx " normal growth, same as control

Nine of this group of compounds may have showed slight inhibition of the bacteriophage, but the action, if any, was so slight as to make the use of these compounds impractical. These nine substances and the concentrations at which they affected bacteriophage were: sucrose, 21, 24, and 27 percents; the nitrofurans, furamozone and furazolidone, each at 4 and 8 ppm; sorbic acid, 800 ppm; Tween 60, 9, and 10 percents; the quaternary ammonium compound, methyldodecylbenzyltrimethyl ammonium chloride (Spartec), 5 and 10 ppm; pyridine, 80 ppm; hydrogen peroxide plus the enzyme catalase, 1, 2, and 4 percents; and lithium sulfate, 0.5 percent.

The remaining 25 substances did not inhibit the multiplication of the bacteriophage to any degree at concentrations which permitted normal growth of the host Streptococcus lactis.

SUMMARY AND CONCLUSIONS

The objective of the present study was to attempt to discover an inhibitory substance or condition for the control of bacteriophage specific for _Streptococcus lactis_ which would not affect the normal growth of the host bacteria.

Cultures propagated in either grade A or ungraded milk pasteurized at a temperature which would not destroy bacteriophage (63° C. for 30 minutes), did not develop a resistance to bacteriophage. The sanitary quality of milk appeared to have no great effect on either the bacteriophage resistance or the activity of cultures. Cultures propagated in ungraded milk seemed to be slightly more active than cultures propagated in grade A milk. Cultures propagated in ungraded milk produced more flavor, but generally poorer flavor, than cultures propagated in grade A milk. Cultures propagated in milk pasteurized at 63° C. for 30 minutes, especially in ungraded milk, became contaminated with more non-lactic acid organisms than cultures propagated in milk pasteurized at 99° C. for 30 minutes.

There was considerable difference among the strains of bacteriophage in regard to their heat resistance. None of the five tested was destroyed by the normal temperature of pasteur-

ization (63°C.), even with 40 minute exposures. Only one strain was completely destroyed at 68.5^{o}C. and this one required an exposure of 40 minutes. All five strains were completely destroyed at 71°C., but the exposure period ranged from 10 to 30 minutes.

With increasing incubation temperatures from 22°C. to 35°C. there was a corresponding decrease in bacteriophage activity. However, there is still slight bacteriophage activity at 35°C. and this temperature is very unfavorable for the maintenance of satisfactory lactic cultures.

Increasing the total solids in the milk used for propagating cultures from 6 percent to 21 percent by the addition of non fat dry milk solids did not decrease bacteriophage activity and, the data show such a procedure may have favored the growth of bacteriophage.

The bacteriophage titer of a culture inoculated with 0.5 percent bacteriophage Number 8 was 10^{-9} upon incubation for 3 hours at 22°C. This was the titer of original inoculum, but the titer was increased to 10^{-13} upon incubation for 96 hours at 22°C.

None of the antibiotics used effectively inhibited bacteriophage at concentrations which permitted normal growth of the homologus _S. lactis_. However, five of the 18 antibiotics used apparently completely inhibited the growth of bacteriophage, but the concentrations inhibiting the bacteriophage also greatly inhibited the growth of the homologus _Streptococcus lactis_. These five antibiotics and the con-

centrations which apparently completely inhibited the growth of bacteriophage were aureomycin, 1.00 ppm; procain pencillin G., 1.00 ppm and 1.25 ppm; streptomycin, 7.00 ppm; terramycin, 4.00 ppm; and antibiotic C, 40.00 ppm.

Of 195 various bacterial cultures used, none produced any substances which inhibited bacteriophage in the concentrations used.

Of 120 mold cultures used, one seemed to contain some bacteriophage inhibiting properties. Further work is being carried on with this mold culture.

Neither yeasts, wild mushrooms, nor any of 29 seed plants produced any degree of bacteriophage inhibition at concentrations which would permit normal growth of the host bacteria.

Of 29 sodium compounds used, one sodium tetrapyrophosphage, apparently completely inhibited the growth of bacteriophage in concentrations of 1.5 percent while allowing normal growth of the host bacteria. However, this compound could not be used practically due to its harmful effect on the milk. This compound apparently completely inhibited but did not destroy the bacteriophage.

Two of the compounds, sodium citrate and sodium oxalate, slightly inhibited the bacteriophage in concentrations of 1.5 percent and .6 percent, respectively. However, these concentrations also slightly inhibited the growth of the homologous S. lactis. Higher concentrations greatly inhibited or prevented the growth of the S. lactis, while lower concentrations were ineffective in inhibiting the bacteriophage.

Seven of the remaining sodium compounds seemed to very slightly inhibit the bacteriophage, but concentrations which produced any useful degree of inhibition greatly or completely inhibited the homologous *S. lactis*. These seven compounds and the concentrations at which they seemed to very slightly inhibit the bacteriophage were sodium chloride, 2.0 percent and 3.0 percent; sodium diethyl dithio carbamate, 0.2 percent; sodium potassium tartrate, 4.0 percent and 5.0 percent; sodium tartrate, 3.0 percent, 4.0 percent, and 5.0 percent; diabasic sodium phosphate, 2.0 percent; sodium potassium tartrate, 4.0 percent and 5.0 percent; sodium tartrate, 3.0 percent, 4.0 percent, and 5.0 percent; diabasic sodium phosphate, 2.0 percent and 2.5 percent; sodium sulfate, 6.0 percent and 8.0 percent; and sodium azide,.0005 percent and .001 percent.

Of 30 miscellaneous chemical compounds used, two completely inhibited bacteriophage and eight slightly inhibited bacteriophage, however, concentrations which produced any degree of bacteriophage inhibition, also affected the normal growth of the homologus host. Bacteriophage was completely inhibited at concentrations in milk of 0.12 percent potassium iodide and 2.0 percent diabasic potassium phosphate. The eight compounds and the concentrations which slightly inhibited bacteriophage were: methyl dodecal benzyl trimethyl ammonium chloride (Spartec), 5.0 ppm and 10.0 ppm; lithium sulfate, 0.5 percent; pyridine, 80.0 ppm; tween 60, 9 percent and 10 percent; sorbic acid, 800 ppm; sucrose, 27 percent; furamazone, 4 ppm and 8 ppm; and furazolidone, 4.0 ppm and 8.0 ppm.

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APPENDIX

APPENDIX TABLE I

The effect of grades of milk
and pasteurization temperatures
of the milk on the activity of lactic acid starters.

Propagation number	Increase in Titratable Acidity Culture #1			
	High Grade Milk		Low Grade Milk	
	Past. 145°F 30 min.	Past. in flowing steam 30 min.	Past. 145°F 30 min.	Past. in flowing steam 30 min.
1	.26	.17	.29	.16
2	.34	.27	.40	.29
3	.27	.40	.16	.33
4	.34	.37	.42	.50
5	.26	.51	.40	.53
6	.35	.51	.43	.51
7	.42	.56	.37	.55
8	.47	.50	.33	.55
9	.46	.50	.39	.51
10	.48	.56	.50	.58
14	.39	.39	.57	.45
15	.50	.46	.54	.31
16	.50	.52	.57	.44
17	.51	.19	.51	.18
19	.55	.49	.55	.39
20	.55	.50	.54	.49
21	.52	.35	.52	.43
22	.51	.44	.51	.52
23	.55	.53	.55	.57
24	.50	.48	.48	.49
25	.55	.52	.52	.55
26	.56	.55	.51	.53
27	.55	.54	.51	.52
28	.55	.52	.52	.55
Average	.46	.45	.46	.46

TABLE I (CONT.)

Increase in Titratable Acidity
Culture #62

Propagation number	High Grade Milk: Past. 145°F 30 min.	High Grade Milk: Past. in flowing steam 30 min.	Low Grade Milk: Past. 145°F 30 min.	Low Grade Milk: Past. in flowing steam 30 min.
1	.24	.30	.22	.29
2	.49	.32	.55	.39
3	.41	.38	.52	.33
4	.25	.34	.49	.37
5	.28	.42	.23	.45
6	.39	.45	.48	.44
7	.39	.45	.52	.41
8	.40	.38	.51	.35
9	.33	.39	.36	.40
10	.52	.52	.56	.50
14	.44	.04	.41	.44
15	.50	.21	.47	.36
16	.47	.56	.53	.54
17	.32	.40	.32	.21
19	.47	.51	.46	.51
20	.51	.51	.56	.49
21	.30	.48	.37	.48
22	.46	.49	.52	.55
23	.55	.54	.55	.58
24	.40	.50	.33	.49
25	.53	.58	.50	.58
26	.47	.57	.37	.57
27	.46	.50	.32	.48
28	.44	.55	.46	.49
Average	.42	.43	.44	.45

TABLE I (CONT.)

Increase in Titratable Acidity
Culture #66

Propagation number	High Grade Milk Past. 145°F 30 min.	High Grade Milk Past. in flowing steam 30 min.	Low Grade Milk Past. 145°F 30 min.	Low Grade Milk Past. in flowing steam 30 min.
1	.37	.31	.29	.33
2	.48	.47	.42	.39
3	.37	.42	.37	.41
4	.40	.39	.42	.48
5	.23	.43	.45	.52
6	.43	.46	.50	.49
7	.53	.50	.44	.53
8	.51	.44	.45	.49
9	.37	.42	.38	.43
10	.56	.55	.57	.58
14	.57	.63	.64	.60
15	.50	.50	.52	.39
16	.52	.52	.57	.54
17	.23	.23	.17	.30
19	.49	.37	.45	.41
20	.50	.42	.48	.40
21	.37	.36	.51	.36
22	.48	.40	.49	.42
23	.53	.48	.50	.45
24	.47	.34	.27	.35
25	.55	.57	.49	.51
26	.49	.47	.52	.51
27	.48	.42	.33	.44
28	.48	.48	.46	.47
Average	.45	.44	.45	.45

TABLE I (CONT.)

Increase in Titratable Acidity
Culture # 68

Propa-gation number	High Grade Milk Past. 145°F 30 min.	High Grade Milk Past. in flowing steam 30 min.	Low Grade Milk Past. 145°F 30 min.	Low Grade Milk Past. in flowing steam 30 min.
1	.47	.36	.48	.35
2	.52	.52	.51	.46
3	.50	.48	.53	.45
4	.44	.46	.55	.49
5	.40	.48	.49	.50
6	.49	.52	.50	.52
7	.54	.53	.54	.57
8	.53	.50	.52	.42
9	.42	.46	.48	.48
10	.54	.55	.57	.58
14	.61	.60	.62	.61
15	.51	.41	.51	.42
16	.57	.45	.56	.54
17	.16	.16	.41	.32
19	.53	.38	.54	.43
20	.55	.46	.54	.41
21	.51	.31	.45	.39
22	.51	.36	.52	.30
23	.52	.47	.52	.40
24	.37	.36	.37	.38
25	.49	.56	.44	.52
26	.41	.54	.49	.48
27	.31	.25	.33	.40
28	.31	.33	.38	.40
Average	.47	.44	.49	.45

TABLE I (CONT.)

Increase in Titratable Acidity
Culture # 75

Propagation number	High Grade Milk: Past. 145° F 30 min.	High Grade Milk: Past. in flowing steam 30 min.	Low Grade Milk: Past. in 145°F 30 min.	Low Grade Milk: Past. in flowing steam 30 min.
1	.51	.48	.50	.33
2	.54	.54	.52	.54
3	.59	.49	.56	.55
4	.51	.51	.57	.55
5	.53	.54	.55	.55
6	.55	.56	.53	.56
7	.56	.49	.56	.58
8	.54	.56	.55	.56
9	.51	.50	.52	.53
10	.57	.56	.58	.56
14	.63	.65	.66	.64
15	.55	.56	.57	.56
16	.57	.57	.57	.58
17	.50	.49	.49	.54
19	.54	.48	.55	.54
20	.51	.49	.54	.54
21	.52	.46	.52	.56
22	.51	.45	.53	.52
23	.55	.50	.53	.56
24	.44	.46	.49	.51
25	.54	.53	.52	.54
26	.53	.55	.55	.53
27	.54	.53	.55	.56
28	.54	.52	.51	.56
Average	.54	.52	.54	.54

TABLE I (CONT.)

Increase in Titratable Acidity
Culture # 76

Propagation number	High Grade Milk Past. 145°F 30 min.	High Grade Milk Past. in flowing steam 30 min.	Low Grade Milk Past. in 145°F 30 min.	Low Grade Milk Past. in flowing steam 30 min.
1	.50	.48	.46	.49
2	.56	.48	.53	.55
3	.53	.52	.51	.55
4	.51	.54	.52	.56
5	.52	.52	.56	.56
6	.55	.56	.58	.57
7	.58	.57	.57	.58
8	.57	.57	.56	.57
9	.53	.50	.50	.52
10	.54	.58	.57	.56
14	.65	.65	.65	.67
15	.56	.56	.56	.56
16	.57	.58	.58	.57
17	.51	.44	.48	.52
19	.56	.52	.54	.55
20	.52	.57	.54	.54
21	.53	.54	.53	.53
22	.49	.51	.52	.52
23	.55	.54	.55	.55
24	.42	.47	.35	.50
25	.54	.54	.53	.56
26	.50	.52	.55	.54
27	.55	.54	.55	.56
28	.53	.54	.53	.54
Average:	.54	.54	.53	.55

TABLE I (CONT.)

Increase in Titratable Acidity
Culture #81

Propagation number	High Grade Milk Past. 145°F 30 min.	High Grade Milk Past. in flowing steam 30 min.	Low Grade Milk Past. 145°F 30 min.	Low Grade Milk Past. in flowing steam 30 min.
1	.49	.43	.38	.47
2	.57	.59	.51	.54
3	.48	.52	.50	.54
4	.49	.51	.52	.55
5	.54	.50	.52	.56
6	.56	.56	.52	.57
7	.56	.56	.58	.58
8	.53	.58	.54	.58
9	.50	.52	.52	.54
10	.54	.55	.57	.56
14	.61	.65	.65	.64
15	.60	.53	.51	.54
16	.56	.51	.55	.58
17	.43	.41	.46	.49
19	.43	.44	.53	.49
20	.47	.46	.52	.36
21	.49	.46	.51	.50
22	.49	.43	.52	.49
23	.55	.51	.55	.50
24	.45	.34	.43	.42
25	.53	.48	.51	.53
26	.39	.50	.53	.54
27	.53	.50	.55	.50
28	.50	.50	.51	.51
Average:	.51	.50	.52	.52

TABLE I (CONT.)

Increase in Titratable Acidity
Culture #82

Propagation number	High Grade Milk Past.145°F 30 min.	High Grade Milk Past.in flowing steam 30 min.	Low Grade Milk Past.145°F 30 min.	Low Grade Milk Past.in flowing steam 30 min.
1	.48	.50	.47	.46
2	.58	.53	.62	.50
3	.52	.52	.51	.55
4	.54	.50	.52	.53
5	.53	.53	.53	.56
6	.55	.56	.55	.58
7	.57	.58	.55	.58
8	.57	.56	.57	.59
9	.51	.52	.50	.54
10	.53	.59	.58	.55
14	.62	.64	.64	.64
15	.50	.57	.54	.56
16	.56	.56	.56	.54
17	.17	.17	.47	.43
19	.49	.43	.47	.48
20	.47	.45	.51	.46
21	.50	.49	.50	.54
22	.47	.46	.51	.50
23	.51	.48	.52	.48
24	.16	.36	.36	.44
25	.13	.52	.50	.53
26	.09	.54	.57	.54
27	.02	.13	.50	.53
28	.03	.51	.51	.53
Average:	.42	.48	.52	.57

TABLE I (CONT.)

Propagation number	Increase in Titratable Acidity, Culture 83: High Grade Milk, Past. 145°F 30 min.	High Grade Milk, Past. in flowing steam 30 min.	Low Grade Milk, Past. 145°F 30 min.	Low Grade Milk, Past. in flowing steam 30 min.
1	.48	.46	.48	.49
2	.55	.55	.54	.56
3	.49	.56	.51	.52
4	.54	.54	.56	.55
5	.53	.54	.54	.53
6	.55	.57	.57	.58
7	.58	.57	.56	.58
8	.56	.55	.56	.58
9	.49	.51	.51	.52
10	.55	.57	.58	.57
14	.64	.65	.64	.63
15	.51	.56	.55	.55
16	.56	.60	.56	.57
17	.39	.45	.49	.48
19	.52	.50	.50	.48
20	.45	.46	.50	.47
21	.48	.52	.52	.51
22	.50	.45	.51	.47
23	.53	.53	.57	.52
24	.44	.41	.44	.40
25	.53	.48	.52	.52
26	.54	.53	.55	.55
27	.54	.40	.49	.46
28	.52	.47	.52	.49
Average:	.52	.52	.53	.52

TABLE I (CONT.)

Increase in Titratable Acidity
Culture 84

Propagation number	High Grade Milk Past.145°F 30 min.	High Grade Milk Past.in flowing steam 30 min.	Low Grade Milk Past.145°F 30 min.	Low Grade Milk Past.in flowing steam 30 min.
1	.45	.48	.42	.48
2	.56	.51	.55	.56
3	.44	.56	.47	.48
4	.40	.49	.53	.51
5	.50	.50	.54	.54
6	.55	.56	.56	.56
7	.56	.54	.56	.58
8	.55	.55	.56	.64
9	.44	.51	.49	.49
10	.54	.54	.55	.54
14	.61	.64	.64	.67
15	.52	.52	.53	.52
16	.55	.57	.56	.58
17	.46	.43	.50	.45
19	.48	.45	.48	.48
20	.50	.45	.48	.44
21	.51	.38	.53	.51
22	.52	.46	.47	.50
23	.54	.50	.50	.52
24	.44	.38	.43	.45
25	.53	.46	.51	.52
26	.18	.47	.48	.54
27	.45	.50	.54	.50
28	.50	.52	.50	.18
Average:	.49	.50	.52	.51

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