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Development of cultural techniques for the study of *Streptococcus thermophilus* and *Lactobacillus* bacteriophages

by

Malireddy Srinivasulu Reddy

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY
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TABLE OF CONTENTS

	Page
DEDICATION	iii
INTRODUCTION	1
HISTORICAL REVIEW	3
EXPERIMENTAL MATERIALS AND METHODS	25
RESULTS AND DISCUSSION	71
SUMMARY AND CONCLUSION	260
LITERATURE CITED	263
ACKNOWLEDGMENTS	272

DEDICATION

TO MY WIFE
SYAMA

INTRODUCTION

The production of Swiss and Italian cheeses, as well as yogurt, is increasing yearly in the United States at dramatic rates. All of these popular dairy products require the use of *Streptococcus thermophilus* and several species of *Lactobacillus* such as *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus lactis*, as the prime starter cultures. There are only a few reports in the literature regarding bacteriophages of these commercially important lactic acid organisms. Most of the work on bacteriophages for these starters has been done in association with yogurt.

In the United States, Deane et al. (30), in 1953 surveyed Swiss cheese plants for the presence of *S. thermophilus* bacteriophage, but isolated phage from only one sample. Since then there have been no reports on *S. thermophilus* bacteriophage in the United States. Many people in the dairy industry believe that *S. thermophilus* bacteriophage is not prevalent and is not a problem during cheese making. Also, there are no reports in the literature concerning *L. bulgaricus* bacteriophage.

This investigation was to survey the incidence of *S. thermophilus* and *Lactobacillus* bacteriophage in Swiss and Italian cheese plants, and to study the associative growth relationships among these starter cultures in the presence and absence of their specific phage.

Previous workers used the cultural techniques developed for lactic *Streptococcus* bacteriophage to isolate *S. thermophilus* bacteriophage. It has been reported that plaque formation by *S. thermophilus* and *Lactobacillus* phage was difficult, so before conducting our survey on the incidence of these bacteriophages in the U.S. cheese plants, it was necessary to develop more reliable isolation and enumeration techniques.

Another part of this investigation was to study the conditions influencing proliferation, stability and enumeration of *S. thermophilus* and *Lactobacillus* phages. This information assisted in determining the appropriate time to sample whey during the manufacture of Swiss and Italian cheeses, and to ship the samples successfully to isolate these bacteriophages.

HISTORICAL REVIEW

History and Importance of Bacteriophage in the Dairy Industry

The first investigation in which the bacterial viruses were encountered was by Twort (92), in 1915. He demonstrated the existence of a transmissible lytic agent active against *Staphylococcus* organisms. Two years later, d'Herelle (41) isolated a dysentry bacteriophage, characterized it as an ultramicroscopic parasite of bacteria, and gave it the name "bacteriophage".

Bacteriophage active against lactic *Streptococcus* was first isolated by Whitehead and Cox (96), in 1935. The phage isolated by Whitehead and Cox (97) was active only against a particular strain of *Streptococcus cremoris* among several strains tested. Sutton (86), Whitehead and Hunter (100), and Mazé (54) isolated lactic *Streptococcus* bacteriophages from whey and demonstrated that these phages were specific for the cultures being used by the cheese factories. Failure of single strain starters as a result of bacteriophage infection was confirmed by Anderson and Meanwell (5), in 1942.

Johns (45) pointed out that the milk supply was a possible source of phage in cheese plants. Later Hunter (44) found multiplication during the cheese making process of phage present in the cheese milk and active against the single strain culture in use as starter. Several channels of infection of cheese milk with bacteriophage were listed by

Whitehead and Hunter (101), in 1945. In 1946, Babel (7) demonstrated that bacteriophage caused a complete cessation of acid production in the manufacture of Cheddar cheese. Until 1952 only phage active against _S. lactis_ and _S. cremoris_ used with Cheddar cheese and butter were known.

Pette and Kooy (65) in 1952, and Deane et al. (30) in 1953, isolated bacteriophage active against _S. thermophilus_ from Swiss cheese whey and yogurt. Kiuru and Tybeck (47), in 1955, isolated bacteriophage for _S. thermophilus_ from Swiss cheese starter cultures showing slow acid development. Bacteriophage active against _S. thermophilus_ also was isolated from yogurt by several other workers (23,24,37).

According to Deane et al. (30), during the manufacture of Swiss cheese, difficulty was encountered with 'dead milk' which exhibited slow acid development during the make procedure, and vats of cheese where the pH did not drop during the three hours after dipping. They also noted that acid production by _S. thermophilus_ was progressively lower on successive propagation. They attributed these abnormalities to the presence of _S. thermophilus_ bacteriophage. Pette and Kooy (65) announced that the presence of _S. thermophilus_ bacteriophage during the manufacture of yogurt caused a distinctly slow souring.

In 1968, Sozzi and Prella (83) isolated _S. thermophilus_ bacteriophage from 'soft-cheese' whey. _S. thermophilus_ bac-

teriophage also was isolated from Gorgonzola whey (84). These investigators concluded that slow acid development by lactic cultures used in Gorgonzola cheese was caused by bacteriophage infection. In 1955, Kiuru and Tybeck (47) isolated bacteriophage active against *Lactobacillus lactis* and *Lactobacillus helveticus* from starters showing a slow acid production. This was the first report of bacteriophage specific for *L. helveticus* and *L. lactis*. They found that the *Lactobacillus* phages had no effect upon each other's host bacteria. Tramer (90) believed yogurt does not suffer from phage attacks because of the rapid acid development. But he encountered a serious phage problem in a Swiss yogurt plant caused by phage build-up in a centrifugal pump used over an extended period for pumping inoculated milk. He did not specify the type of phage involved.

Conditions Influencing Proliferation and Enumeration of Bacteriophage

The calcium requirement for the proliferation of bacteriophage was first demonstrated by Stassano and de Beaufort (85) for *Shigella* bacteriophage. Later Rountree (75), and Smith (81) demonstrated that staphylococcal bacteriophages also require calcium for proliferation. In 1949, Shew (80) announced that eight strains of bacteriophage active against *S. lactis* need calcium for their maximum development. He judged the influence of calcium on bacteriophage on the basis

of plaque forming ability on solid media and clearing of turbidity in liquid broth. Reiter (72) demonstrated that the optimum plaque formation by lactic bacteriophages is obtained using 0.01 M $CaCl_2$ in an agar medium. According to Cherry and Watson (19) 0.005 to 0.05 M $CaCl_2$ was stimulatory to proliferation of one strain of bacteriophage active against S. lactis.

In 1949, Adams (2) observed that requirement of calcium varies among different strains of E. coli bacteriophage, and host organisms, i.e. E. coli, did not require this divalent cation for multiplication. Wahl (95) has reported that calcium is not necessary for adsorption of bacteriophage to sensitive bacterial cells, but it is necessary for the multiplication of bacteriophage. On the contrary, according to Delbrück (32), calcium is essential for the process of adsorption in coliphage T_4. Also, according to Cherry and Watson (19), the concentration of calcium that is necessary for maximum lysis facilitated greatest adsorption of bacteriophage by host cells. Collins et al. (24) concluded that calcium is not required for the growth of lactic streptococci in defined medium, but eight of ten homologous bacteriophages required the addition of calcium for proliferation. They also stressed the importance of this finding in developing a phage resistant medium low in calcium concentration for successfully propagating lactic streptococci with lessened dan-

ger of bacteriophage.

Potter and Nelson (69) determined the effect on plaque formation of different concentrations of $CaCl_2 \cdot 2H_2O$ as the cell diluent with eleven strains of lactic Streptococcus bacteriophages. They found that 2.5% $CaCl_2 \cdot 2H_2O$ solution as a diluent yielded a greater number of plaques and that the plaques were large in size. A decrease of plaque numbers and size was observed with high concentrations of $CaCl_2 \cdot 2H_2O$, i.e. 5.0%. Deane and Nelson (29) added $CaCl_2 \cdot 2H_2O$ to a final concentration of 0.33 per cent to the agar overlay to get a maximum number of discernible plaques.

In 1953, Potter and Nelson (70) announced that calcium ions are required for invasion, that is, penetration or steps leading to penetration of host cells after adsorption of lactic Streptococcus bacteriophage. They also said that strontium, barium, or manganous ions will partially replace calcium.

Das and Marshall (28), in 1967, studied the effects of supplemental calcium on staphylococcal bacteriophage proliferation in skim milk and found that the addition of 0.0005 M calcium borogluconate to trypticase soy broth produced an increase in phage titer about 1 millionfold, but it's addition to skim milk decreased bacteriophage titer about 1 hundredfold. Bacterial growth was stimulated by supplemented calcium in tryptic soy broth, but not in skim milk. In 1971,

Lowrie and Pearce (52) reported that the medium M16, when supplemented with 0.005 M calcium borogluconate yielded a maximum number of lactic Streptococcus bacteriophage plaques. They also reported that reproducible results will not be obtained if calcium is added as calcium chloride to the agar overlay, but calcium borogluconate, added to agar overlays, gives the highest plaque counts. Calcium chloride often causes precipitation in the agar medium, but addition of calcium borogluconate to the growth medium did not give visible precipitation.

Several reports in the literature have shown that the number of plaques obtained from a given bacteriophage suspension may vary considerably as the result of changes in the details of the enumeration technique (1,16,17,18,34,43,48, 105). Increasing the agar concentration or the thickness of the agar overlayer materially reduced the number of countable plaques (16,17,42,48). The amount of host inoculum used in the agar overlay had a pronounced effect on the enumeration of maximum number of bacteriophage particles (17,48). Turner and Nelson (91) conducted an extensive study on conditions influencing the maximum enumeration of lactic Streptococcus bacteriophages. The following facts were established from their research: 1. Maximum numbers of plaques were obtained when the total volume of the agar overlay was 3 ml; 2. The greatest number of plaques were obtained when the plate counts of bacteria in the agar overlayer were from 21 to 35

million; 3. When milk was used instead of water for preparing phage dilution, the total number of plaques was greatly improved; 4. No plaques were obtained when the medium was above pH 8 or below pH 5; 5. A pH of 5.8 to 6.0 gave the maximum number of plaques.

pH and Temperature Stability of Bacteriophages

Several workers have reported on the heat resistance of phages which attack lactic streptococci. In 1939, Whitehead and Hunter (99), working with nine phages, reported that the thermal death-points of lactic bacteriophages are in the region of 70-75 C. Nelson, Harriman and Hammer (58) demonstrated that filtrates of 'inhibitory principle' (later found to be bacteriophage) obtained from slow starter cultures, resisted heating at 70 C for 10 min at neutral pH. When the pH of the menstruum was lowered, the resistance of bacteriophage to heat was greatly reduced. These authors also reported that the thermal resistance of bacteriophage was much higher than that of their host bacteria at a given pH. According to Yakovlev (104), lactic bacteriophages could survive at 95 C for 2 h in the dried medium. In 1945, Nichols and Wolf (61) reported that lactic bacteriophages were not destroyed by heating for 50 to 60 min at 65 to 67 C in milk. However, these phages were inactivated in 7½ min at 75 C. These authors stressed the importance of this finding in the sterilization of milk for starter making.

According to Cherry and Watson (18), lactic bacteriophages were not affected by temperatures below 131 F, whereas rapid inactivation occurred between 131 and 149 F. The usual heat treatment given for sterilizing milk that is to be used for propagating starter cultures is sufficient to inactivate bacteriophage that may be present. The temperatures (145 F for 30 min or 161 F for 15 s) commonly employed to heat treat milk for the manufacture of several types of cheeses are not adequate to inactivate bacteriophage in milk. Since there is a direct relationship between the heat treatment and the firmness of coagulum produced by acid or rennet, it is not desirable to give a high heat treatment to the milk that is to be used for cheese making (9).

Deane et al. (30) studied the heat resistance of *S. thermophilus* bacteriophage. According to them, *S. thermophilus* bacteriophage showed no unusual heat resistance, being inactivated at much the same exposures found to inactivate moderately resistant lactic-group streptococci. From 99.5 to 99.96% of the phage particles were inactivated by holding at 61.7 C for 30 min. They announced that the usual pasteurization treatment of milk will not inactivate *S. thermophilus* bacteriophage, so milk must be free from bacteriophage to prevent starter failures. According to Kiuru and Tybeck (47), phages active against *L. lactis* and *S. thermophilus* were destroyed by a heat treatment at 72 C for 20 to 30 min or at

85 C for 3 to 5 min. Lactobacillus helveticus phage was inactivated at 63 C when held for 20 min or at 72 C for 30 min. They also indicated that the usual pasteurization methods are not sufficient to destroy these phages in milk.

According to Cherry and Watson (18), mass lysis of S. lactis by it's bacteriophage occurred at pH 6, 7, and 8, absolutely no lysis was observed at pH 4.0 and incomplete lysis was observed at pH 5.0. In 1951, Overcast et al. (63) pointed out that the optimum pH for lactic phage development, in litmus milk, is 6.5. The phages included in their study proliferated between pH 5.4 to 7.5. They also reported that one phage multiplied at a pH as low as 4.8 and at a pH as high as 9.4. Differences in rate of proliferation of some lactic bacteriophages also were observed at different pH's although the growth rates of some of the host bacterial cells were not significantly affected with a change in pH. Later, 1954, Zehren and Whitehead (106) by studying the action of different phages on one bacterial strain, concluded that the latent period and burst size were characteristic of the phage and not of the bacteria. According to them, the latent periods of phages of 6 lactic streptococci varied from 40 to 90 min and the burst size from 21 to 77 phage particles at 86 F. Kiuru and Tybeck (47) announced that strong acid (pH 3.0) and basic solutions (pH greater than 11.0) were effective in destruction of phage active against L. lactis and S. thermo-

philus. These phages were more stable at pH 7.0. These experiments were conducted using 0.5 per cent sodium lactate solution as the suspensory medium. Ciblis (21) determined the latent period and burst size of *S. thermophilus* bacteriophage. The latent period was about 80 min and the burst size was about 34 particles per infected bacterium.

Isolation of Bacteriophage

Several procedures are used in isolating bacteriophage from natural sources. The primary concern in isolating a new bacteriophage is to separate the phage from living bacteria. This can be achieved in three ways: filtration; chloroform treatment; and ultracentrifugation. All of these procedures have both advantages and disadvantages for the recovery of the maximum number of bacteriophage particles.

It is well documented that some bacteriophages stick to membrane filters and, thus, will not be fully recovered in the filtrate (53). Also, filtering any broth with a high density of bacterial cells is time consuming and inefficient.

Collins et al. (24) reported that when a *S. lactis* culture, grown along with it's specific phage, was filtered without prior neutralization through a sterile Selas microporous porcelain filter (porosity No. 03), the resulting filtrate usually contained few bacteriophage particles. But when the pH of the culture was adjusted to pH 6.7 for *S. lactis* strain W_2 and to approximately pH 7.0 for *S. lactis*

strain 505 prior to filtration, maximum numbers of bacteriophage particles were recovered in the filtrate. The retention of S. lactis bacteriophage by membrane filters was pronounced when the majority of the bacterial cells had not been lysed (24). If phage titers are low, all of the phage particles may be lost on the membrane filter (53). For some phages, the loss by filtration can be reduced by prewashing the membrane filter with a protein solution (53).

Membrane filtration of liquids such as milk and whey is tedious and time consuming. Deane et al. (30) prepared samples for S. thermophilus phage isolation by passing the whey through a filter bed of acid-coagulated milk. The clear filtrates, thus obtained, were passed through Selas microporous, porcelain filters (porosity No. 03) to obtain bacteria-free filtrates. To prepare cell-free filtrates of milk, it was first coagulated with lactic acid, and the resulting suspension then was passed through coarse filter paper prior to ultrafiltration (30). Several workers used this procedure to work with bacteriophages active against the starter cultures used in the dairy industry. This procedure of preparing samples for the isolation of bacteriophage is quite complex, and there is a good possibility that the majority of the phage particles will be lost during this multistep filtration procedure.

Samples may be prepared for the isolation of bacteriophage by treating the suspected material with chloroform (3).

Chloroform exerts it's bacteriostatic effect by being a lipid solvent. Since some phages are inactivated by chloroform, and because it's bacteriostatic effect is limited only to some bacteria, this method cannot be applied routinely for the isolation of unknown bacteriophages (53). It can only be used with chloroform resistant bacteriophages.

Even though preparation of cell-free filtrates of milk and whey are difficult and time consuming, so far none of the investigators working with bacteriophage active against dairy starter cultures have attempted to use the chloroform treatment procedure as a substitute to filtration.

Survey of Bacteriophage in the Dairy Industry

Although bacteriophages active against lactic streptococci were isolated from different cheese plants and from starter cultures by several workers, the only detailed survey was conducted by Moseley and Winslow (57), in 1959. They analyzed samples of milk, whey, and lactic cultures which were collected from 91 cheese factories in twenty states. Most of these cheese plants were experiencing a considerable degree of slow acid development. In addition, they examined twenty-three samples of whey obtained from twenty different cheese plants which were not experiencing any problem regarding acid production. Bacteriophage was detected in 93% of the samples obtained from plants experiencing slow acid

development and 74% of the samples obtained from cheese plants exhibiting normal acid development. In this investigation, sixteen different S. lactis and S. cremoris single strains were used to detect bacteriophage from different samples. They gave no information about how the samples were collected and shipped to the laboratory. It appeared that bacteriophages active against lactic streptococci were widely distributed and if the conditions were favorable they proliferated and caused slow acid development during cheese manufacture. Since lactic streptococci are extensively used in the manufacture of Cheddar, Cottage, Colby, and Edam cheeses, I would assume that this study mostly involved the plants which were making these products.

In the United States, in 1953, Deane et al. (30) surveyed Swiss cheese plants located in three states for the presence of Streptococcus thermophilus bacteriophage. Eighty-one samples of Swiss cheese whey were examined. Many of these Swiss cheese plants were experiencing some degree of slow acid development. The number of cheese plants surveyed was not given. The majority of the whey samples were collected, immediately after the curd had been dipped, in 6-oz prescription bottles, some of which contained 1 g of $CaCO_3$, and were mailed to the laboratory without refrigeration. Some of the samples they examined were just the cheese milk rather than whey. For the detection of bacteriophage, bac-

teria-free filtrates were prepared by extensive filtration and screened against 4 strains of *Streptococcus thermophilus*. Plaque assay was done by using the two-layer plate technique of Potter and Nelson (69). Several of the 81 samples inhibited slightly the growth of one or more of the *S. thermophilus* in litmus milk, but plaque formation was demonstrated with only one sample. According to the results obtained in the survey, *S. thermophilus* bacteriophage was not widely distributed and was not a serious problem in the dairy industry.

There is no other report in the literature since 1953 on *S. thermophilus* bacteriophage in the United States. However, a limited amount of work was done in Europe on this phage in connection with yogurt manufacture. Kiuru and Tybeck (47) announced that *S. thermophilus* bacteriophage was widespread, but only one *L. helveticus* phage was found from starter cultures which were used for Swiss cheese manufacture and showed slow acid production.

Influence of Bacteriophage on Associative Growth Relationship

Mixed lactic cultures, after several subcultures, occasionally lose their ability to produce normal acidity during cheese manufacture. The same culture may become normal after a few more transfers and produce enough acid. Cheese makers describe this as starter 'recovery'. Nichols and Ineson (60) interpreted starter 'recovery' in a more meaningful way. A

commercial starter originally contains a mixture of several strains of lactic streptococci. Many of these strains are active and capable of producing enough acid necessary for cheese making. Upon several subcultures, one strain in the mixed culture may become predominant owing to the differences in growth rates or response to temperature of incubation. If such a predominant strain is lysed by it's specific phage, the culture suddenly becomes slow in acid production. But upon successive transfers, the recessive or suppressed strains in the mixed cultures, which were contributing a limited amount of acid before, might grow and produce the desired amount of acidity. The starter then appears to be recovered. This theory was confirmed experimentally by Nichols and Ineson (60).

Based on this idea, several investigators used strain specific bacteriophages to study associative growth among mixed lactic cultures. Czulak and Hammond (27) suggested the use of different species of lactic streptococci in mixed lactic cultures to overcome the problem of strain dominance, and thus, the bacteriophage problem in the dairy industry. By using several strains of lactic streptococci and their homologous phages, Collins (23) determined the action of bacteriophage on mixed strain cultures. According to him, the strain dominance in a mixed lactic culture is due to the difference in acid tolerance among component strains. He also noted an

increase in titratable acidity after lysis of the dominant strain by it's specific phage in a mixed lactic culture. In mixed lactic cultures, after the dominant strain was lysed by phages, the mixture was immediately dominated by the other strain.

Lightbody and Meanwell (51), by using strain specific bacteriophages, also found that different strains of lactic streptococci may not grow in fixed proportion in a mixed culture after repeated transfers. They attributed this to the inhibitory substances produced by the dominant strain in a mixed culture. They also stated that there is no advantage of a mixed lactic culture over a single strain culture unless compatible strains are used in making the mixed culture.

In several cheeses and in a few cultured dairy products lactic cultures are not used. For instance, in Swiss, Italian cheeses and in yogurt, *S. thermophilus* and species of *Lactobacillus* are used as prime starters. The only exception being Iowa style Swiss cheese and a few Italian varieties of cheeses, where lactic cultures are also used as starters.

According to Pette and Lolkema (66), *L. bulgaricus* stimulated the growth of *S. thermophilus* by liberation of essential amino acids from milk proteins. Accolas et al. (1) announced that acid production by *S. thermophilus* was stimulated by the high temperature lactobacilli, *L. bulgaricus*, *L. helveticus*, and *L. jugurti*, as well as by mesophilic lac-

tic streptococci, in autoclaved milk and in milk heated at 80 C (176 F) for 30 min. Studies of Pette and Lolkema (66) indicated that *S. thermophilus* grown in association with *L. bulgaricus* produced more acid per cell than when grown alone.

Stimulation of *L. casei* by *S. lactis* was reported in the literature (38). Branen and Kenen (13) isolated and identified the stimulatory factor, a small peptide, from cell-free extracts of *S. lactis*. In 1971, Accolas et al. (1) observed that *L. lactis* and *L. bulgaricus* are stimulated by certain strains of *S. thermophilus*. They have also reported that *S. thermophilus* and *L. helveticus* stimulated acid production by some mesophilic lactic streptococci. A report in the literature indicated that a few strains of *S. lactis* (nonantibiotic producing) and *S. thermophilus* inhibited acid production by *L. helveticus* (68).

Control of Bacteriophage in the Dairy Industry

Babel (9) reviewed various methods of destroying, preventing, and limiting bacteriophage in the dairy industry. Nichols and Hoyle (59) typed a large number of strains of lactic streptococci and formulated a scheme for successful rotation of phage-unrelated single strain starters. Anderson and Meanwell (5) were the first to suggest, and Whitehead and Hunter (102) were the first to introduce rotation of phage-unrelated starters in the dairy industry. Now this is well established and practiced in several dairy plants (74).

Hunter (44) announced that renneted milk could support the growth of starter even in the presence of phage. It was after this discovery that the practice of adding rennet simultaneously with the starter, to eliminate the ripening period, was introduced in the dairy industry (26). Unfortunately this procedure cannot be applied to all types of cheeses (74).

In 1949, Reiter (72) suggested the use of lysogenic starter cultures in the dairy industry. Zehren and Whitehead (106), in 1954, mentioned the idea of selecting lactic starter cultures on the basis of latent period and burst size of their specific phages. According to these authors, a starter strain whose phage has a long latent period and small burst size is preferable in the dairy industry to a starter strain whose phage has short latent period and a large burst size. This indeed is a good criterion in the selection of starter strains. Another method by which the bacteriophage problem could be reduced is by employing multiple-strain starters. However, it has been shown that multiple-strain starters can be affected by phage (5).

There was a need for a method by which the starter cultures could be freed from phage. This was achieved by using media low in calcium and a medium containing calcium-binding agents for the propagation of starter cultures (9). Reiter (73), in 1956, developed a phage resistant medium by reducing the calcium concentration in milk. This medium supported the growth of lactic starter cultures but not their homologous

phages. The findings of Babel (8) and Crawford et al. (25) strengthened the usage of a phage resistant media for the propagation of starter cultures. Later, several media were developed, using calcium-binding agents, for the growth and propagation of bacteriophage-free cultures (39,40,36,62). Currently phage resistant media are extensively used in the dairy industry for the propagation of lactic *Streptococcus* cultures.

Finnish workers (46) have noticed interference with acid production by bacteriophages during the manufacture of Emmental cheese since 1953. Tybeck (93) determined the growth rates of strains of *S. thermophilus*, *L. lactis*, and *L. helveticus* and their homologous phages in the phage resistant medium designated "Cockade" P.R.M., developed by Reiter (74). She was especially interested in determining the value of "cockade" P.R.M. in controlling bacteriophage active against the starter cultures used in Emmental cheese. This investigation proved that the *S. thermophilus*, *L. lactis*, and *L. helveticus* strains tested did not produce acid in P.R.M., while one *S. lactis* strain grew almost normally even though phage proliferation was inhibited. Thus, "Cockade" P.R.M. was unsuitable for propagating starters made of *S. thermophilus*, *L. lactis*, and *L. helveticus* used in the manufacture of Emmental cheese. So, the phage resistant media employed for propagation of lactic *Streptococcus* cultures cannot be

applied directly to the control of phage active against starter cultures used in Swiss and Italian cheeses and yogurt.

Morphology of Bacteriophage Important in the Dairy Industry

Ruska (76) and Pfankuch and Kausche (67) were the first to publish electron micrographs of phages of Escherichia coli, in 1940. In 1941, Ruska (77) announced that the phages with which he worked were 250 to 400 nm in length and 60 to 100 nm head diameter. Ultrastructures of Streptococcus lactis bacteriophages were first studied by Parmelee et al. (64), in 1949. According to these authors, S. lactis bacteriophage particles are sperm shaped, 220 nm long, have a head diameter of 70 nm and a tail that is 30 nm wide and 150 nm long. Differentiation among several strains of bacteriophages was not possible on the basis of size and shape. Later, in 1959, Williamson and Bertaud (103) examined the ultrastructures of the bacteriophages of ten strains of Streptococcus cremoris and one strain of S. lactis and concluded that all these phages are identical in morphology. They could only find one phage, active against S. cremoris (type d_4), out of the eleven examined that was morphologically dissimilar in that it had a long flagellumlike tail with a width of 15 nm and a length of 560 to 610 nm. Sandine et al. (79), in 1960, published electron micrographs of bacteriophage active against a

strain of Streptococcus diacetilactis designated 18-16. This phage had a length of 180 nm with head and tail diameters of approximately 72 and 36 nm respectively. According to these authors, bacteriophages of S. diacetilactis were morphologically indistinguishable from those of previously described (64) S. lactis bacteriophages. Recently Bauer et al. (11) published a series of electron micrographs of phages active against S. lactis, S. cremoris, S. diacetilactis, and S. thermophilus. According to these authors, Streptococcus bacteriophages could be distinguished on the basis of their morphology. Electron micrographs of S. thermophilus bacteriophage were first published by Deane et al. (30), in 1953. The majority of the S. thermophilus bacteriophage particles measured had a head diameter of 90 nm and an overall length of 360 nm. The tails of this phage were slightly curled or tangled and overall the measurements are greater than those of the S. lactis bacteriophages reported by Parmelee et al. (64). The S. cremoris (type d_4) bacteriophage described by Williamson and Bertaud (103) is an exception. Bauer et al. (11) published electron micrographs of bacteriophages active against two strains of S. thermophilus. Streptococcus thermophilus phage had a flexible filament attached to the thin terminal tail plate. Distinct transverse striations were seen on the tail. The other phage of S. thermophilus designated 19S did not reveal any filament at the end of it's

tail. So, based on the morphological evidence, *S. thermophilus* bacteriophages can be classified into Bradley's Group B (12). According to Ciblis (21), *S. thermophilus* bacteriophage has a head diameter of about 70 nm and an undifferentiated tail about 237 nm long. Also, Gelin et al. (37) announced that *S. thermophilus* bacteriophage has an exceptionally long tail.

EXPERIMENTAL MATERIALS AND METHODS

Materials

Cultures

Twenty-nine *Streptococcus thermophilus* and seventeen *Lactobacillus bulgaricus*, two *Lactobacillus helveticus*, one *Lactobacillus lactis* and two *Streptococcus lactis* strains were included in this investigation. Most of these cultures were obtained from commercial culture manufacturers in the United States. All *S. thermophilus* cultures were purified by plating on tryptic soy agar (33) followed by inoculating individual isolated colonies into milk. *Lactobacillus* cultures were purified by picking individual colonies grown on LBS agar (BBL, Division of Bioquest, Cockeysville, Maryland). Culture identities were confirmed by use of tests outlined in Bergey's Manual of Determinative Bacteriology (14).

Culture propagation

The cultures were maintained by thrice a week transfers in reconstituted 11.0% nonfat dry milk (Matrix, Galloway West Co., Fond du Lac, Wis.) and incubated at 37 C for 12 h. Between transfers, they were stored at 5 C.

Phages

All the *S. thermophilus* bacteriophages included in this study were isolated in our laboratory from whey obtained from several Swiss and Italian cheese plants. Phages active

against *L. helveticus* and *L. lactis* were obtained from V. J. T. Kiuru, Helsinki, Finland.

Phage propagation

Streptococcus thermophilus bacteriophage was propagated along with active host (5 h old) in tryptic soy broth enriched with 0.5% yeast extract and 0.02% L-cystine, adjusted to pH 6.5±0.1. Hereafter this broth is referred to as enriched tryptic soy broth. Incubation was at 37 C for 10 h. The broth was then successively filtered through Whatman No. 42 filter paper and Millipore GS 0.22 µm filters (Millipore Corp., Bedford, Mass.). Phage stock thus obtained was stored at 5 C until further use. *Lactobacillus* bacteriophages were propagated essentially the same except that 0.5 ml of Tween-80 was added to every 100 ml of enriched tryptic soy broth.

Chemicals used

Reagent grade chemicals were used in all the experiments.

Preparation of maleate buffer

Maleate buffer was prepared by mixing 210 ml of 0.6 M solution of acid sodium maleate (24 g of NaOH + 58.8 g of maleic anhydride in 1000 ml of distilled water) and 170 ml of 0.6 M NaOH.

Methods

Phage enumeration technique

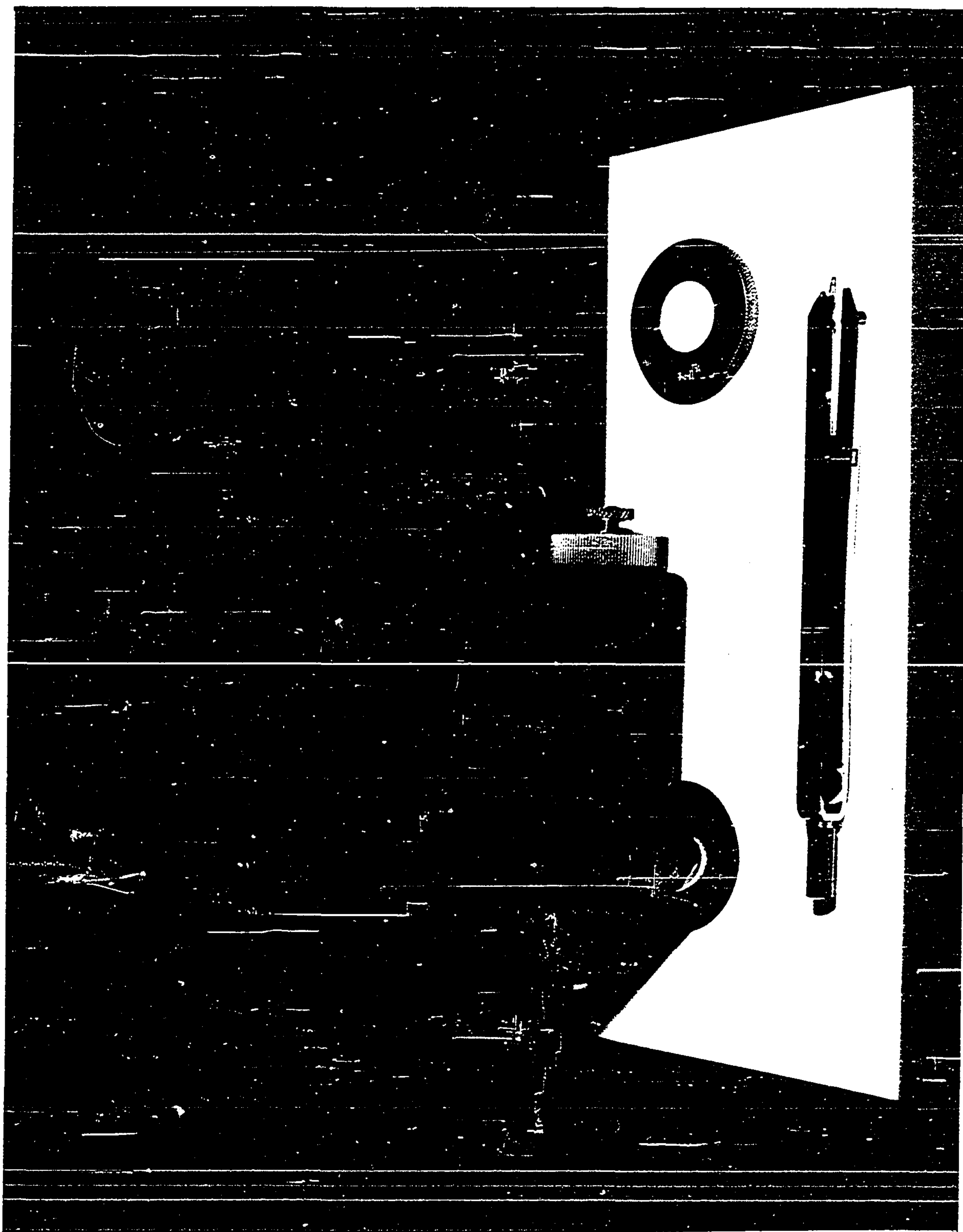
In the early stages of the experiments, the procedure described by Potter and Nelson (69) was used to isolate and enumerate *S. thermophilus* bacteriophage. Tryptic soy agar enriched with 0.02% L-cystine and 0.5% yeast extract was used as base medium. Hereafter this is referred to as enriched tryptic soy agar. Bacteriophage plates were incubated at 37 C for 12 h.

Plaque size determination

A simplified and dependable technique for the determination of phage plaque diameter was developed. Figure 1 shows the various appliances used. The upper left hand side of the figure shows the micrometer (Bausch and Lomb Inc., Rochester, New York). This micrometer is equipped with a removable battery illumination device. The upper right hand side of the figure depicts the calibration disc that is calibrated in millimeters. The calibration disc is used to check the micrometer division. Below the micrometer and calibration disc is the divisor. The whole apparatus is placed on white blotting paper.

The detailed procedure for determining the plaque diameter was as follows: 1. The divisor was very carefully adjusted to the size of the plaque; 2. Impressions were made on the blotting paper by putting gentle pressure on the divisor;

Figure 1. Apparatus used to measure the diameter of _S. thermophilus_ bacteriophage plaques. Micrometer equipped with battery illumination device, divisor, and blotting paper.



3. The micrometer was placed on the blotting paper in such a way that the impressions were in the middle of the open area of the micrometer; 4. The magnified centers of the impressions were matched with the calibrated scale of the micrometer and the diameter was read directly up to one-hundredth of a millimeter. To obtain the maximum precision and least error, diameters of ten contiguous plaques on each duplicate plate were determined. The average diameter of the twenty plaques represents the accurate diameter of the phage plaque. Using this procedure, the plaque diameters of *S. thermophilus* bacteriophage, under various growth conditions, were determined.

Determination of titratable acidity of milk

Titratable acidity of milk or milk culture was determined by titrating a 9 g sample with 0.1 *N* NaOH to the first persistent pink color using 10 drops of phenolphthalein indicator (1.0% in 95% ethyl alcohol).

Bacterial plate counts

The procedures outlined in Standard Methods for the Examination of Dairy Products (4) were followed. Enriched tryptic soy agar (fortified with 0.5% lactose and 0.5% glucose) was used for the determination of *S. thermophilus* bacterial counts. For the enumeration of species of *Lactobacillus*, LBS agar (10) was employed. Coliform and yeast and mold

counts were determined by using violet red bile agar (4), and potato dextrose agar (4). Sodium lactate agar (94) was used to enumerate propionibacteria.

Statistical analysis

The data was subjected to an analysis of variance (82). The bacteriophage counts were converted to logarithms for this analysis. Significant differences were established by a least significant difference test.

Effect of Various Growth Conditions on Plaque Formation of *S. thermophilus* Bacteriophage

Effect of preincubation of phage-host cells in distilled water and saline solution

The procedure described by Potter and Nelson (69) for the enumeration of bacteriophage was used except that distilled water is replaced by saline as diluent. Also, after the addition of host and specific bacteriophage dilution, the entire contents of the mixture were preincubated for 15 min at 37 C. All possible controls were included to study the effect of distilled water vs. saline and preincubation vs. no preincubation upon the number and size of *S. thermophilus* bacteriophage particles. Three *S. thermophilus* bacteriophages were included in this study.

Calcium chloride effect

Here the plating conditions included use of 0.85% saline solution as the diluent with preincubation for 15 min at 37 C.

To study the effect of different levels of calcium chloride, distilled water was substituted for $CaCl_2 \cdot 2H_2O$ as the negative control. Levels of $CaCl_2$ examined were: 0.5%, 1.0%, 2.0%, 2.5%, 3.0%, and 4.0%. Plates were incubated at 37 C for 12 h. The best level of calcium chloride that gave the maximum number and size of bacteriophage plaques was included for further experiments.

Host inoculum effect

To study the effect of amount of host inoculum on number and size of the plaques of *S. thermophilus* bacteriophage, host inoculum ranging from 0.1 ml to 1.0 ml in increments of 0.2 ml was included. The host was grown in milk at 37 C for 12 h, prior to inoculation. Other plating conditions included the use of 0.85% saline solution, preincubation, and 1.0% calcium chloride solution as the cell diluent.

pH effect

The influence of pH of the growth medium was studied by adjusting the pH of the enriched tryptic soy agar from 5.2 to 7.0 with successive increments of 0.2 units before sterilization. In this experiment, pH of both the basal agar, as well as the agar used for overlay, was maintained at the same pH. The rest of the plating conditions were according to the procedure of Potter and Nelson (69) except for the use of saline, preincubation, 1.0% calcium chloride solution, and 0.4

ml of the host inoculum.

Effect of different basal media

The influence of four different basal media on total number and size of *S. thermophilus* bacteriophage particles was determined. The media used were: Eugon agar (33), Elliker's Lactic Agar (35), Trypticase Soy Agar (10), and enriched tryptic soy agar. The pH of the media was adjusted to 6.6 ± 0.1. In this experiment, plating conditions included use of preincubation, 0.85% saline solution, 1.0% $CaCl_2 \cdot 2H_2O$, and 0.4 ml of host inoculum. Plates were incubated at 37 C for 12 h.

Effect of incubation temperature and environment

Three strains of *S. thermophilus* bacteriophages, after plating on semisolid agar, were incubated at 32 C, 37 C, 37 C in CO_2, and 45 C. The length of incubation was 12 h. All other plating conditions were similar to the previous experiment except for the variation of incubation temperatures.

Effect of different carbon sources

To study the effect of different carbon sources upon number and size of *S. thermophilus* bacteriophage plaques, 5 g/l of lactose, glucose, or sucrose were added to one liter of enriched tryptic soy agar. Plating conditions included use of 0.85% saline, preincubation, 1.0% $CaCl_2 \cdot 2H_2O$ as diluent, 0.4 ml host inoculum, and 12 h incubation at 37 C

in air. Enriched tryptic soy agar alone without the addition of any carbon sources served as control. The pH of the media was adjusted to 6.6 ± 0.1.

Another experiment was performed to study the use of two different carbon sources upon the number and size of bacteriophage particles. The effect was determined of addition of 5 g of lactose per liter of enriched tryptic soy agar vs. addition of 5 g each of lactose and glucose to enriched tryptic soy agar on the enumeration of *S. thermophilus* bacteriophage.

Effects of calcium chloride and calcium borogluconate

It has been reported in the literature (52) that the enumeration of lactic *Streptococcus* bacteriophage could be improved by the inclusion of 0.005 M calcium borogluconate in the agar. We wanted to compare the effect of calcium borogluconate vs. calcium chloride on total plaque numbers and diameters of *S. thermophilus* bacteriophage. From the previous experiments, 1% $CaCl_2$ improves the plaque numbers and size of *S. thermophilus* bacteriophage, so the effect of similar concentrations of calcium borogluconate on recovery of *S. thermophilus* bacteriophage was determined. Individually, both calcium chloride and calcium borogluconate were added to the agar overlay to give a final concentration of 0.005 M and 0.022 M. Enriched tryptic soy agar fortified with 5 g/l of each of glucose and lactose was used as growth medium. The

plates were incubated at 37 C for 12 h.

All the experiments conducted to study the effect of various growth conditions on plaque formation of _S. thermophilus_ bacteriophage were repeated three times using three strains of bacteriophage. The results were analyzed statistically.

Finalized "modified" two-layer plating procedure for the isolation and enumeration of S. thermophilus bacteriophage

A modified two-layer plating procedure was developed and was successfully used to isolate and enumerate _S. thermophilus_ phages. The procedure was as follows: Phage filtrates or chloroform treated materials were serially diluted in sterile 0.85% saline solution (A). To 2.6 ml of sterile 1% $CaCl_2 \cdot 2H_2O$ (B) in a screw-capped test tube, 0.4 ml of an active starter culture was added. The host organism was previously grown at 37 C for 12 h in 11% reconstituted nonfat milk solids. To this tube (B), 3 ml of the diluted phage filtrate (A) was added. After thorough mixing, this material (C) was preincubated (held for 15 min at 37 C) and then plated. For plating, 3 ml of enriched tryptic soy agar, fortified with 5 g/l each of lactose and glucose (tempered at 65 C) was added and mixed with the mixture (C) to form a soft lawn agar (D). Six milliliters of D were divided equally and transferred to two predried plates of enriched tryptic soy agar (pH 6.6$\pm$0.1). Plates were incubated at 37 C for 12-18 h.

If a 12-h period is inconvenient, plates could be incubated as long as 18 h. Plaques on both plates were counted, averaged, and multiplied by the appropriate dilution factor. Hereafter this method was used to enumerate S. thermophilus bacteriophage.

Spot test for the isolation and enumeration of Streptococcus thermophilus bacteriophage

A. Five and six-tenth's ml of sterile $CaCl_2$ saline mixture was pipetted into a wide mouth test tube. ($CaCl_2$-saline mixture was prepared by adding 100 ml of 1% $CaCl_2 \cdot 2H_2O$ solution to 100 ml of saline. This mixture was autoclaved at 121 C for 20 min).

B. Four-tenth's ml of an active, milk-grown Streptococcus thermophilus culture was inoculated into A.

C. Three ml of enriched tryptic soy agar fortified with 5 g/l each of glucose and lactose (tempered at 65 C) was pipetted into B, and mixed gently.

D. Three ml of mixture C was delivered onto predried tryptic soy agar plate.

E. A drop of suspected whey, milk culture, or broth culture, filtered or treated with chloroform, was spotted on the solidified overlay.

F. The plates were incubated, (right side up), at 37 C for 8 h.

G. A clear area or plaque(s) at the site of spot indi-

cated the presence of phage.

This procedure was used effectively to isolate phage from different sources, even if the concentration of phage was extremely low (10 or few hundred/milliliter).

Finalized "modified" two-layer plating procedure for the isolation and enumeration of *Lactobacillus* phage

Two phage strains active against *L. helveticus* and *L. lactis* were obtained from V. J. T. Kiuru, Helsinki, Finland. The technique used by previous workers to isolate and enumerate *Lactobacillus* phage was inadequate, so a suitable agar overlay technique for the isolation and enumeration of *Lactobacillus* phage was developed.

The modified procedure was as follows: Phage filtrates or chloroform treated materials were serially diluted in sterile 0.85% saline solution (A). To 3.0 ml of sterile 1% $CaCl_2 \cdot 2H_2O$ (B) in a screw-capped test tube, 0.1 ml *Lactobacillus* culture was added. With some strains only a loopful (internal diameter of loop was 4 mm) of *Lactobacillus* culture should be used. The host organisms (*Lactobacillus*) should have been previously grown for 12 h in 11% reconstituted nonfat milk solids. To this tube (B), a drop of sterile Tween-80 was added prior to the addition of 3 ml of the dilute phage filtrate (C). After thorough mixing, this material (C) was preincubated (held for 15 min at 37 C) then plated. For plating, 3 ml of enriched tryptic soy agar fortified with

5 g/l each of glucose and lactose (tempered at 65 C) was added and mixed with the mixture (C) to form a soft lawn agar (D). Six milliliters of (D) were equally divided and transferred to four prepoured plates of enriched tryptic soy agar (pH 6.6±0.1). In other words 1.5 ml D was delivered onto each plate. Extreme care was taken to distribute the 1.5 ml of soft agar on the prepoured plate by careful swirling of the plates. The prepoured plates should not be cold or dry at the time of overlaying. Solidified basal agar was tempered at 65 C and maintained strictly at that temperature. Addition of agar to mixture D, mixing, and overlaying was done as quickly as possible to prevent irregular distribution of 1.5 ml of agar on the prepoured plates. The plates were incubated at 37 C for 20-24 h in a carbon dioxide incubator. Plaques on four plates were counted, added, and the grand total was divided by 2 and multiplied by the appropriate dilution factor, to arrive at the *Lactobacillus* bacteriophage titer.

Spot test for the isolation and enumeration of *Lactobacillus* bacteriophage

A. Six ml of sterile $CaCl_2$ saline mixture was pipetted into a wide mouth test tube (A) ($CaCl_2$ saline mixture was prepared by adding 100 ml of 1% $CaCl_2$ solution to 100 ml of saline). This mixture was autoclaved at 121 C for 20 min.

B. One-tenth ml of active *Lactobacillus* culture was

added to (A). This mixture was designated (B).

C. Following the addition of culture, one drop of sterile Tween-80 was carefully added to (C).

D. The contents were mixed thoroughly, and 3 ml of enriched tryptic soy agar (tempered at 65 C) was pipetted into (C). The contents were mixed gently. This was designated D.

E. One and one-half ml of mixture D was delivered onto a prepoured tryptic soy agar plate and evenly distributed by swirling the plate quickly, so that the overlay would cover the entire surface of the prepoured plate.

F. A drop of whey, culture, or broth, filtered or treated with chloroform, was spotted on the solidified overlay.

G. The plates were incubated at 37 C in a carbon dioxide incubator for 16 h.

H. A clear area at the site of the spot indicated the presence of phage.

Effect of initial broth pH on proliferation of S. thermophilus bacteriophage

This experiment was devised to investigate the effect of the pH of the growth medium on the proliferation of *S. thermophilus* bacteriophage. This information should be of help in determining the appropriate time during the cheese making procedure to collect the whey sample for the isolation of phage. One hundred milliliter portions of enriched tryptic soy broth

were adjusted to pH 6.0, 6.5, and 7.0 and inoculated with 1.0% *S. thermophilus* host cells and a 2.0% filter sterilized phage preparation. The broths were incubated at 37 C for 12 h. After 4, 6, 8, 10, and 12 h of incubation, the entire contents of the flasks were quickly cooled and filtered through Whatman No. 42 filter paper and then through Millipore GS 0.22 µm filters. The number of phage particles in each sample were assayed by the modified two-layer plating procedure. The pH's of the broths also were determined at different stages of incubation. This experiment was conducted using two different strains of *S. thermophilus* bacteriophage.

Effect of pH, temperature, and time on storage stability of S. thermophilus bacteriophage

To study the effect of pH, temperature, and time on the viability of *S. thermophilus* bacteriophage, 0.5-ml portions of filter sterilized phage preparations were added to 9.5-ml volumes of sterile tryptic soy broth. The pH of the tryptic soy broth was adjusted before sterilization from 3.5 to 8.0 with successive increments of 0.5 units. The phage-inoculated tryptic soy broths of varying pH's, ranging from 3.5 to 8.0, were held at 5 C and 21 C for a period of 13 days. The bacteriophage counts were determined immediately after addition to tryptic soy broth and after 1, 4, 7, 10, and 13 days of storage. To determine the phage counts, only 1.0 ml was

removed from each test tube.

Effect of filtration on numbers of S. thermophilus bacteriophage

In all the previous investigations, phage levels seemed unreasonably low, so the effect of filtration on phage particle numbers was determined. The bacteriophage suspension (1.0%) in the presence of its homologous host culture (0.5%) was grown at 37 C for 12 h in enriched tryptic soy broth. The culture was filtered first through Whatman No. 42 filter paper and then through Millipore GS 0.22 μm filters. Bacteriophage counts were determined before filtration, after filtering through Whatman No. 42 filter paper, and after Millipore filtration. These experiments were repeated three times with 5 *S. thermophilus* bacteriophage strains.

Development of chloroform treatment procedure for the isolation of S. thermophilus bacteriophage

To avoid filtration, a simplified procedure using chloroform was developed to kill bacterial cells without materially affecting phage titer. It is performed as follows:

1. Add 0.5 ml chloroform to 15 ml of whey, broth, or milk. If viscous materials (coagulate milk or cheese slurries) are to be tested, 1.0 ml chloroform may be used.
2. Shake the mixture gently to assure uniform distribution of the chloroform. Allow the suspension to

stand undisturbed at room temperature for 5 min. Shake again for a few seconds and allow the mixture to settle and separate for 5 min more. A white, fairly firm precipitate may collect at the bottom of the tube. If an opaque medium is being tested, this precipitate will be difficult to see; hence, extra care must be taken while decanting.

3. Precoat inside of the centrifuge tube with antifoam (Foamkill, Nutritional Biochemicals). Antifoam should be sprayed until a large drop of it is collected at the narrower end of the centrifuge tube. Cover the top of the centrifuge tube with aluminum foil and autoclave it for 20 min. Then decant the supernatant from Step 2 into this sterile centrifuge tube. Aerate until most of the chloroform has been removed by bubbling sterile, filtered air through the mixture for approximately 20 to 25 min. An aquarium pump (Hush) can be used to achieve aeration. Sterilize Pasteur pipettes (plugged with cotton) separately at 121 C for 20 min and attach the wide end of the Pasteur pipette to the rubber tube of the aquarium pump. Start the pump after immersing the narrow end of the Pasteur pipette into the centrifuge tube (containing the experimental material). To determine if the remaining solution is

free from significant quantities of chloroform, this microtechnique can be used:

A. Add 1 drop of 5 N NaOH to 1 drop of aerated mixture of whey and chloroform in a small glass tube such as a Durham tube and mix thoroughly.

B. Add 2 drops of pyridine as an overlayer to the NaOH-whey mixture. Hold the mixture in boiling water bath for 1 min or heat it in a direct flame for a few seconds.

C. If chloroform is still present in significant quantities, a violet to red color will form in the pyridine layer. The intensity of the color is related to chloroform concentration.

D. With this method, it is possible to detect chloroform, in various liquids, of at least the following amounts:

Milk - 1 μg

Distilled water - 1 μg

Tryptic soy broth - 2 to 5 μg

Whey - 5 μg

E. Because of the relatively poor sensitivity of this detection procedure in whey and broth, aerate for an additional 1 or 2 min

after obtaining a 'negative' chloroform test.

Effect of chloroform on the quantitative recovery of S. thermophilus bacteriophage in different media

A S. thermophilus culture at 1.0% level and its specific phage at 2.0% level were inoculated into enriched tryptic soy broth and incubated at 37 C for 12 h. At the end of the incubation, 15-ml portions of the mixture were treated with 0.5 ml and 1.0 ml of chloroform respectively. The culture plus phage mixture without any chloroform treatment served as a control. Chloroform treated samples and the control sample were plated to determine the number of bacteriophage particles. To determine the effect of chloroform on S. thermophilus bacteriophage suspended in milk, a similar experiment to that outlined earlier was repeated using sterile skim milk as growth medium. Also, the effect of chloroform (0.5 ml) on bacteriophage active against L. helveticus, L. lactis, L. bulgaricus and two strains of S. lactis (C_2 and Ms) was determined.

Effects of two different concentrations of chloroform on starter cultures, associated flora, and on the enumeration of S. thermophilus bacteriophage from whey

Since it is reported in the literature that some microorganisms are resistant to chloroform treatment, this experi-

ment was devised to study the bactericidal effect of chloroform on dairy starter and associated flora. Five different batches of Swiss cheese whey were obtained from several commercial sources. The pH of the whey (100 ml) was adjusted to 7.0 and 1 ml of each of the following different active cultures were added: mixed lactic culture, *S. thermophilus*, *L. bulgaricus*, *Streptococcus durans*, *Streptococcus faecalis*, species of *Propionibacterium*, *Escherichia coli*, unidentified coliforms, unidentified yeasts and molds, *Brevibacterium linens*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Micrococcus varians*, and *S. thermophilus* bacteriophage. This experiment was adjusted so each batch of whey received a different species of the aforementioned microorganisms. Samples were thoroughly mixed, and from each batch of whey two 15-ml portions were treated with 0.5 and 1.0 ml of chloroform. The remaining portion of the whey to which no chloroform was added served as a control. Both the chloroform treated and nonchloroform treated samples were plated on violet red bile agar, potato dextrose agar, sodium lactate agar, and Elliker's lactic agar. Violet red bile agar plates were incubated at 32 C for 24 h for the determination of coliform counts. Yeast and mold counts were determined by incubating potato dextrose agar plates at 21 C for 5 days. For the enumeration of species of *Propionibacterium* sodium lactate agar plates were incubated in candle oats jar at 32 C for 7 days. To

enumerate organisms at different temperatures, plates poured with Elliker's agar were incubated at 21 C, 32 C, 37 C, and 45 C for a period of 5 days. *S. thermophilus* bacteriophage was enumerated by the modified double layer overlay technique.

Determination of S. thermophilus bacteriophage proliferation in skim milk

To every 100 ml of sterile skim milk (pH 6.3), *S. thermophilus* (early logarithmic growth phase cells at 1% level) and its specific phage (2% level) were inoculated. The mixture along with controls was incubated at 37 C for 12 h. *S. thermophilus* alone inoculated into skim milk served as a control. Bacteriophage counts, bacterial counts, pH and titratable acidities were determined both in phage inoculated cultured skim milk and in control at 2-h intervals from 0 to 12 h of incubation. Samples were prepared for phage assay using the chloroform treatment procedure outlined earlier. Bacterial counts were determined using enriched tryptic soy agar fortified with 0.5% glucose and 0.5% lactose. The pH of this agar medium was adjusted 6.6 ± 0.1.

Effect of the addition of lactose and sterile skim milk to enriched tryptic soy broth on the proliferation of S. thermophilus bacteriophage

Enriched tryptic soy broth, adjusted to pH 6.3, was dis-

pensed in 100-ml volumes into three prescription bottles. Five-tenths per cent of lactose was added to one bottle and to the second bottle 5.0% sterile reconstituted nonfat dry milk was added. The third bottle, without any additions, served as a control. To all three bottles, actively growing _S. thermophilus_ culture was inoculated at a 1% level along with it's specific phage at a 2% level. In addition to the tryptic soy broth, host-phage combinations of _S. thermophilus_ were also inoculated in sterile reconstituted nonfat dry milk. All these bottles were incubated at 37 C for 8 h. At the end of the incubation, bacteriophage counts, _S. thermophilus_ bacterial counts and pH were determined. Also, suitable controls were included to study the proliferation rates of _S. thermophilus_ in tryptic soy broth (with various additions) and in skim milk.

Effects of the addition of calcium chloride, and calcium borogluconate to enriched tryptic soy broth and sterile skim milk on the proliferation of S. thermophilus and S. thermophilus bacteriophage

To every 100 ml of sterile reconstituted skim milk (pH 6.3) and enriched tryptic soy broth, $CaCl_2$ and calcium borogluconate were added to a final concentration of 0.005 M and 0.022 M. Since the pH of the sterile skim milk was 6.3, in this experiment the pH of the tryptic soy broth also was adjusted to 6.3. _S. thermophilus_ (5 h old) at 1.0% and it's

specific phage at 2.0% level were inoculated into various batches of enriched milk and tryptic soy broth. The entire contents of the mixture, along with the controls were incubated at 37 C for 8 h. Addition of _S. thermophilus_ alone to various media, without it's specific bacteriophage, served as control. Eight hour length of incubation was preferred because it was shown in the previous experiment that the highest number of phage particles were obtained at 6 to 8 h of incubation in skim milk. At the end of the incubation period, phage counts, bacterial counts, and pH were determined in all the samples.

Effects of calcium carbonate, maleate buffer, and chloroform on bacteriophage, suspended and incubated at room temperature in whey

The effects of $CaCO_3$, maleate buffer (7.6 ml/100 ml whey) and chloroform (1.0 ml/100 ml whey) on the stability of bacteriophage were examined. The preparation of maleate buffer is presented under Materials section.

The maximum numbers of bacteriophage particles were present in whey samples obtained after 5 h of dipping during Swiss cheese manufacture. The pH of whey at this time was 6.0 ± 0.1. So, the amount and strength of buffer were adjusted so that the pH of whey was shifted to 6.6 ± 0.1, following the addition of specific amounts of maleate buffer. This pH should be favorable for the proliferation of _S. thermophilus_

and its phage. Wherever the effects of calcium carbonate and chloroform were to be examined, the pH of whey samples were adjusted to 6.6 with 1 N NaOH, and then specified amounts of $CaCO_3$ (1 g/100 ml) and chloroform (1.0 ml/100 ml) were added. After the addition of $CaCO_3$, chloroform, or buffer, 2 ml of phage preparation in milk was inoculated into 100 ml of whey. Immediately after the addition of bacteriophage to whey, a portion of the sample was removed, treated with chloroform and the initial bacteriophage counts determined. Phage counts also were determined after 3, 5, and 8 days of incubation at room temperature. The pH of whey samples also was determined from 0 to 8 days of incubation. The room temperature, during incubation period of 8 days, varied from 80 to 86 F.

<u>Effects of different concentrations of chloroform in the presence and absence of maleate buffer on the pH of whey incubated at room temperature</u>

To every 100-ml of whey, amounts of chloroform ranging from 0.2 ml to 1.0 ml with successive increments 0.2 ml were added. To one set of these bottles 7.6 ml of liquid maleate buffer was added. To prevent the escape of chloroform, the bottle caps were tightly fastened with adhesive tape. Whey without the addition of chloroform or buffer served as control. All these bottles along with controls were incubated at room temperature for 0 to 8 days. The pH was determined

at 2 day intervals.

Effects of different levels of calcium carbonate, buffer, and calcium carbonate on the pH of whey incubated at room temperature

The effects of buffer, various levels of $CaCO_3$, and buffer in the presence of various levels of $CaCO_3$ on the pH of whey incubated at room temperature were determined. To every 100-ml of whey (pH 6.6) $CaCO_3$ was added to a final concentration of 1, 2, 3, and 4 per cent. To one set of these bottles (with $CaCO_3$), 7.6 ml of maleate buffer was added. Wherever buffer was added, the initial pH of the whey (before adding buffer) was adjusted to 6.0 ± 0.1. With the addition of buffer, the pH of the whey was shifted to 6.6 ± 0.1. The volumes of buffer and whey were adjusted in such a way that the final volume of the mixture was always 100 ml. After the addition of these specific ingredients, the whey was incubated at room temperature for a period of 8 days. pH measurements were made at 2-day intervals from 0 to 8 days of incubation. The whey used in this experiment was inoculated with 1% of each of mixed lactic cultures and *S. thermophilus*, and 0.5% of *L. bulgaricus*.

Effects of different variables on the stability of S. thermophilus bacteriophage, suspended and incubated at room temperature in whey

Equal amounts of whey were dispensed into 5 prescription bottles. Calcium carbonate at 4% was added to one bottle. The second bottle received 4% $CaCO_3$ and 7.6% liquid maleate buffer. In addition to 4% $CaCO_3$ and 7.6% liquid maleate buffer, 2% of a *S. thermophilus* culture was inoculated into a third bottle. The fourth batch received 0.4% chloroform and 7.6% liquid maleate buffer. Whey alone, without any of these additions, served as the control. To each of the five bottles of whey (with different ingredients) specific *S. thermophilus* bacteriophage at the 2.0% level was added. Three different phages were investigated. Each of the 5 whey samples was incubated at room temperature from 0 to 8 days. The pH determinations were made at 24-h intervals. Viable phage counts were determined at 0, 3, 5, and 8 days of incubation.

Similar experiments were conducted using one phage strain of each of *L. helveticus*, *L. lactis*, and *L. bulgaricus*. Phage titers were not determined quantitatively in these experiments. Instead, specific dilutions of chloroform treated whey samples were spotted on lawns of host's cells, to determine the approximate concentration of bacteriophage. pH determinations were made at 0, 3, 5, and 8 days of incubation.

Whey sample shipping procedure

Four grams of $CaCO_3$ and 7.6 ml of maleate buffer were added to a sterile 6 oz prescription bottle. The cap was tightly closed and fastened with adhesive tape to prevent leakage. The amount of whey to be placed in the bottle was clearly marked on the bottle with an indelible pen. In addition to the bottle with liquid maleate buffer and $CaCO_3$, two other empty sterile 6 oz prescription bottles also were sent to each cheese plant. These additional two bottles were to be used for the collection of *S. thermophilus* and *L. bulgaricus* cultures. The marked bottles were packed and sent to each cheese plant by mail with the following instructions: 1. To the bottle containing the white liquid (liquid maleate buffer and calcium carbonate), add whey up to the indicated line, 100 ml). 2. Mix the ingredients gently to incorporate buffer. 3. Add 2 ml of active *S. thermophilus* ("coccus") culture. This must be the same coccus culture that was used in making the cheese. If a mixed rod and coccus culture is used in your plant add 2 ml of the mixed culture to the whey. 4. Close the cap tightly and fasten it with adhesive tape to prevent leakage. 5. If coccus and rod cultures are grown separately in your plant, add about 100 ml of each, separately, to the two additional bottles. If mixed bulk cultures are used, add about 100 ml to one of the bottles. 6. Please indicate the source and culture identification on the bottles, also note the type of cheese. 7. Order of prefer-

ence of selection of whey samples: A. whey from abnormally "slow" vat taken late in the make; B. Depending upon the variety of cheese being made, please take whey sample late in the make procedure or approximately 5 h after dip.

Survey of S. thermophilus bacteriophage from Swiss and Italian cheese plants

Whey samples from 32 different Italian and 13 Swiss cheese plants across the country were received along with their starter cultures. Samples were treated with chloroform. Each sample was checked for the presence of both *S. thermophilus* and *L. bulgaricus* phages against the cultures that were used in each individual plant. In addition, whey samples also were checked for the presence of phage against 24 different *S. thermophilus* (coccus) and 18 *L. bulgaricus* (rod) cultures which were received from three major culture manufacturers in the United States. Also, almost all of these cultures are currently being used extensively in the production of Swiss and Italian cheeses. Chloroform treated whey samples were first spotted on specific coccus and rod cultures that were used in the cheese plant. Later, the same sample of whey was spotted on lawns of 24 different coccus and 18 rod cultures. A clear spot at the site of inoculation was considered positive. The presence of phage was further confirmed by plaque assay.

S. thermophilus and *L. bulgaricus* strain specificity studies

Various phages isolated from different cheese plants were purified by repeated plating and picking the individual plaques (3 times). This was accomplished by gently touching the center of a clear plaque with a sterile needle and by dipping the needle in 3 ml of tryptic soy broth. From this broth serial dilutions of phage were made and plated by the modified two-layer plating procedure. After clear plaques were formed, the same procedure of purification was repeated three times. After obtaining a pure strain of phage, cross sensitivity studies were conducted. Each purified phage was spotted on the 24 different commercial coccus cultures to determine the specificity of *S. thermophilus* bacteriophage. Similarly, purified *Lactobacillus* bacteriophages were spotted on 18 different commercial rod cultures.

Electron Microscopy

Preparation of *S. thermophilus* bacteriophage samples for electron microscopy

Actively growing *S. thermophilus* at the rate of 1% and its specific phage at the 2% level were inoculated into 100 ml enriched tryptic soy broth and incubated at 37 C for 6 h. At the end of the incubation, the mixture was centrifuged at 5,000 rpm (RC2-B automatic refrigerated centrifuge, Ivan Sorvall Inc., Newton, Conn.) for 5 min and the supernatant

was carefully decanted. The residue left at the bottom of the centrifuge bottle was resuspended in phosphate buffer (pH 7.0), negatively stained with 2.0% phosphotungstic acid, and examined with an Hitachi HU-11C electron microscope.

Effects of storage and the pH of the suspensory medium on poly-tail formation in S. thermophilus bacteriophage

The procedure undertaken to prepare the sample for the examination of phage was the same as in the earlier experiment. After resuspending the residue left at the bottom of the centrifuge bottle in phosphate buffer (pH 7.0), the contents were divided into two equal portions. One portion was left at 4 C for 10 days. Another portion was divided into three equal amounts. The pH's were adjusted to 2.0, 7.0, and 11.0 with 2 N HCl and 2 N NaOH. These samples were negatively stained and examined with an electron microscope. The sample left at 4 C (pH 7.0) was examined after 10 days of storage.

Preparation of Lactobacillus bacteriophage sample for electron microscopy

Species of Lactobacillus (1%) along with their specific phages (2%) were grown in enriched tryptic soy broth plus 0.5% Tween-80 at 37 C for 12 h. At the end of the incubation, the mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was decanted into a sterile centrifuge tube. The clear supernatant thus collected was centrifuged at 30,000

rpm for 1 h. The sediment was suspended in a small portion of phosphate buffer (pH 7.0), negatively stained with 2% phosphotungstic acid and was examined under an electron microscope.

Associative Growth Studies

Effect of varying concentrations of S. thermophilus phage on acid production by S. thermophilus in skim milk

Nine screw-capped prescription bottles containing 100 ml sterile matrix milk were employed. One of these was inoculated with a 16-h old _S. thermophilus_ culture at 1% level. The second bottle was inoculated with chloroform-treated _S. thermophilus_ bacteriophage preparation in milk at the 2.0% level. These two bottles served as controls. The remaining bottles were inoculated with 1.0% active _S. thermophilus_ culture. The phage preparation was serially diluted in sterile skim milk from 10^{-1} to 10^{-7} dilution. Two per cent of each specific dilution was added to sterile skim milk, inoculated with _S. thermophilus_, and dispensed in prescription bottles. All the bottles, including controls, were incubated at 37 C for 12 h. Bacteriophage counts of the undiluted phage preparation were determined before inoculation. Titratable acidities were determined at 0, 2, 4, 5, 6, 8, and 12 h of incubation. A comparison was made between samples with and without phage, in relation to the amount of acid production.

This experiment was repeated using two different strains of *S. thermophilus* bacteriophage.

Effect of cellular lysis by specific bacteriophage on associative growth of S. thermophilus and S. lactis strains as measured by acid production

One hundred milliliters of sterile skim milk was inoculated with 0.5 ml each of *S. thermophilus* ST_A and *S. lactis* C_2. These organisms were transferred three times before inoculation. Specific bacteriophage preparations of *S. thermophilus* ST_A and *S. lactis* C_2 were inoculated at the rate of 1.0% each. Some samples received only *S. lactis* C_2 phage, whereas others received only *S. thermophilus* ST_A bacteriophage. Suitable controls were included in this experiment to study the detailed effect of lysis of *S. thermophilus* ST_A by specific phage and it's effect on acid production by *S. lactis* C_2, and vice versa. The different variables and controls included in this experiment are listed in Table 1. This experiment also was conducted by using two different phage strains unrelated to *S. thermophilus* ST_4 and *S. lactis* MS. After specific addition of host phage combinations, all the samples along with their controls were incubated at 37 C for 12 h. Titratable acidities were determined at 0, 2, 4, 5, 6, 8, and 12 h of incubation. Bacterial and phage concentrations of *S. thermophilus* and *S. lactis* were determined before inoculation.

Table 1. Variables and controls used to study the effect of cellular lysis by specific bacteriophage on associative growth of *S. thermophilus* and *S. lactis*.

Bottle[a]	*S. thermophilus*	*S. lactis*	*S. thermophilus* phage	*S. lactis* phage
1	ST_A	-	-	-
2	-	C_2	-	-
3	ST_A	C_2	-	-
4	ST_A	C_2	-	$C_2\emptyset$
5	ST_A	C_2	$ST_A\emptyset$	-
6	ST_A	-	$ST_A\emptyset$	-
7	-	C_2	-	$C_2\emptyset$
8	ST_A	-	-	$C_2\emptyset$
9	-	C_2	$ST_A\emptyset$	-
10	-	-	$ST_A\emptyset$	-
11	-	-	-	$C_2\emptyset$

[a]All strains inoculated into skim milk.

Effect of cellular lysis by specific bacteriophage on associative growth of L. helveticus and S. lactis

A similar methodical approach as in the previous experiment was followed. The different variables that were included in order to study the effect of cellular lysis by bacteriophage on associative growth relationships of *S. lactis* C_2 and *L. helveticus* are depicted in Table 2. A similar experiment was performed using strains of *L. lactis* and *S. lactis* MS.

Effect of cellular lysis by specific bacteriophage on associative growth of S. thermophilus ST_A *and L. helveticus*

Different variables and controls included in this experiment are presented in Table 3. The amount of host and phage inoculum, temperature and length of incubation, and frequency of titratable acidity determinations were similar to earlier experiments. Also, this experiment was repeated using strains of *S. thermophilus* ST_4 and *L. lactis*.

Effect of cellular lysis by bacteriophage on associative growth of S. thermophilus, L. helveticus, and S. lactis

One hundred milliliters of sterile skim milk was inoculated with 0.5 ml of each of *S. thermophilus* ST_A, *L. helveticus*, and *S. lactis* C_2. Specific bacteriophage preparations were added at the rate of 1.0% each. All the variables and controls included in this experiment are listed in Table 4.

Table 2. Variables and controls used to study the effect of cellular lysis by specific bacteriophage on associative growth of *L. helveticus* and *S. lactis*.

Bottle[a]	*L. helveticus*	*S. lactis*	*L. helveticus* phage	*S. lactis* phage
1	LH	-	-	-
2	-	C_2	-	-
3	LH	C_2	-	-
4	LH	C_2	LHØ	-
5	LH	C_2	-	C_2Ø
6	-	C_2	-	C_2Ø
7	LH	-	LHØ	-
8	-	C_2	LHØ	-
9	LH	-	-	C_2Ø
10	LH	C_2	LHØ	C_2Ø
11	-	-	LHØ	-
12	-	-	-	C_2Ø

[a]All strains inoculated into skim milk.

Table 3. Variables and controls used to study the effect of cellular lysis by specific bacteriophage on associative growth of *S. thermophilus* and *L. helveticus*.

Bottle[a]	*S. thermophilus*	*L. helveticus*	*S. thermophilus* phage	*L. helveticus* phage
1	ST_A	-	-	-
2	-	LH	-	-
3	ST_A	LH	-	-
4	ST_A	LH	ST_AØ	-
5	ST_A	LH	-	LHØ
6	ST_A	-	ST_AØ	-
7	-	LH	-	LHØ
8	ST_A	-	-	LHØ
9	-	LH	ST_AØ	-
10	ST_A	LH	ST_AØ	LHØ
11	-	-	-	LHØ
12	-	-	ST_AØ	-

[a]All strains inoculated into skim milk.

Table 4. Variables and controls used to study the effect of cellular lysis by specific bacteriophage on associative growth of *S. thermophilus*, *L. helveticus*, and *S. lactis*.

Bottle[a]	*S. thermophilus*	*L. helveticus*	*S. lactis*
1	ST_A	-	-
2	-	LH	-
3	-	-	C_2
4	ST_A	LH	C_2
5	ST_A	LH	C_2
6	ST_A	LH	C_2
7	ST_A	LH	C_2
8	ST_A	LH	C_2
9	ST_A	LH	C_2
10	ST_A	LH	C_2
11	ST_A	LH	C_2
12	ST_A	-	-
13	-	LH	-
14	-	-	C_2
15	-	-	-
16	-	-	-
17	-	-	-

[a]All strains inoculated into skim milk.

S. thermophilus phage	*L. helveticus* phage	*S. lactis* phage
-	-	-
-	-	-
-	-	-
-	-	-
$ST_A Ø$	-	-
-	LHØ	-
-	-	$C_2 Ø$
$ST_A Ø$	LHØ	-
$ST_A Ø$	-	$C_2 Ø$
-	LHØ	$C_2 Ø$
$ST_A Ø$	LHØ	$C_2 Ø$
$ST_A Ø$	-	-
-	LHØ	-
-	-	$C_2 Ø$
$ST_A Ø$	-	-
-	LHØ	-
-	-	$C_2 Ø$

Titratable acidities were determined at 0, 2, 4, 5, 6, 8, and 12 h of incubation. A similar experiment was also performed using strains of *S. thermophilus* ST_4, *L. lactis*, and *S. lactis* MS.

Effect of lysis of S. thermophilus on the growth and acid production of other S. thermophilus strains in associative growth

To every 100 ml of sterile skim milk, *S. thermophilus* and it's specific phage filtrate were inoculated at the rate of 0.5% and 1.0% respectively. Several controls were included in this experiment to prove the effect of lysis of one strain of *S. thermophilus* upon the growth and acid production by other strains of *S. thermophilus* when grown in association in sterile skim milk. Details on various controls included in the experiment are tabulated in Table 5. All the bottles, with specific additions of host-phage combinations, were incubated at 37 C for 12 h. Titratable acidities were determined at 0, 2, 4, 5, 6, 8, and 12 h of incubation.

A similar experiment was performed using strains of *L. helveticus* and *L. lactis*. In this experiment titratable acidities were determined at 0, 4, 6, 8, and 12 h of incubation.

Table 5. Variables and controls used to study the effect of lysis of *S. thermophilus* on acid production by other *S. thermophilus* strains.

Bottle[a]	*S. thermophilus* ST_A	*S. thermophilus* ST_4	*S. thermophilus* ST_A phage	*S. thermophilus* ST_4 phage
1	ST_A	-	-	-
2	-	ST_4	-	-
3	ST_A	ST_4	-	-
4	ST_A	ST_4	ST_AØ	-
5	ST_A	ST_4	-	ST_4Ø
6	-	ST_4	-	ST_4Ø
7	ST_A	-	ST_AØ	-
8	ST_A	-	-	ST_4Ø
9	-	ST_4	ST_AØ	-
10	-	-	-	ST_4Ø
11	-	-	ST_AØ	-

[a]All strains inoculated into skim milk.

Effect of cellular lysis by bacteriophage on associative growth of strains of S. thermophilus and species of Lactobacillus as measured by acid production

This experiment was devised to study the effect of using multiple strains of S. thermophilus and Lactobacillus, as opposed to single strains, in controlling bacteriophage problem. Two strains of S. thermophilus (ST_4 and ST_A), L. helveticus, L. lactis and their specific phages were used. The host inoculum was 0.5%, and phage was inoculated at the rate 1.0%. Titratable acidities were determined at 0, 4, 6, 8, 12, 16 and 20 h of incubation at 37 C. The details of the controls used in this experiment are presented in Table 6.

Effect of S. thermophilus and L. bulgaricus bacteriophage(s) on acid production and flavor development of yogurt

Homogenized whole milk fortified with 3% nonfat dry milk was heat treated at 82.2 C for 30 min and tempered at 45 C. Sixteen-hour cultures of S. thermophilus and L. bulgaricus were inoculated at the rate of 1.25% each. Before inoculation, bacterial cell concentrations were determined by plating on agar described by Lee et al. (50) for S. thermophilus and LBS agar for S. bulgaricus. Bacteriophage preparations were inoculated at the rate of 2.0%, wherever necessary. Bacteriophage suspensions were prepared by growing S. thermophilus (1%) or L. bulgaricus (1%) along with their specific phages (2.0%) at 37 C for 12 h in enriched tryptic

Table 6. Variables and controls used to study the effect of lysis by bacteriophage on associative growth of strains of *S. thermophilus* and species of *Lactobacillus*.

Bottle[a]	*S. thermophilus* strains	*L. helveticus*	*L. lactis*
1	ST_A	-	-
2	ST_4	-	-
3	ST_A, ST_4	-	-
4	ST_A	-	-
5	ST_4	-	-
6	-	LH	-
7	-	-	LL
8	-	LH	LL
9	-	LH	-
10	-	-	LL
11	ST_A, ST_4	LH	-
12	ST_A, ST_4	-	LL
13	ST_A	LH	LL
14	ST_4	LH	LL
15	ST_A, ST_4	LH	LL
16	ST_A, ST_4	LH	LL
17	ST_A, ST_4	LH	LL
18	ST_A, ST_4	LH	LL
19	ST_A, ST_4	LH	LL
20	ST_A, ST_4	LH	LL
21	ST_A, ST_4	LH	LL
22	ST_A, ST_4	LH	LL

[a]All strains inoculated into skim milk.

S. thermophilus phage strains	*L. helveticus* phage	*L. lactis* phage
-	-	-
-	-	-
-	-	-
$ST_A\phi$	-	-
$ST_4\phi$	-	-
-	-	-
-	-	-
-	-	-
-	LHØ	-
-	-	LLØ
-	-	-
-	-	-
-	-	-
-	-	-
-	-	-
$ST_A\phi$	-	-
$ST_4\phi$	-	-
-	LHØ	-
-	-	LLØ
$ST_A\phi$, $ST_4\phi$	-	-
-	LHØ	LLØ
$ST_A\phi$, $ST_4\phi$	LHØ	LLØ

soy broth. At the end of the incubation, the mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was decanted. The clear supernatant thus obtained was centrifuged at 30,000 rpm for 1 h. The sediment was suspended in sterile skim milk. This preparation was used as the phage inoculum for further experiments. Bacteriophage concentrations were determined before inoculation into milk.

After inoculating bacterial cultures and their specific phages into the fortified milk, the cultures were incubated at 45 C along with appropriate controls. Titratable acidities were determined at 0 h of incubation. Incubation was further continued until yogurt pH (without phage) was 4.7. In this experiment it took about 4.5 h. At the end of the incubation, titratable acidities, bacterial counts, and bacteriophage counts were determined. All the samples were stained with methylene blue and were examined under a microscope. In each sample, where the coccus and rod were used with and without their specific phages, the coccus to rod ratio was determined by counting and averaging ten different fields under the microscope. The samples were quickly cooled in an ice bath, and were stored at 5 C for 5 more days. A taste panel analysis was conducted on the 5th day. The pH of the samples also was determined.

In the same experiment, the effect(s) of *S. thermophilus* and *L. bulgaricus* phages inoculated at different stages

(after 1 and 2 h of incubation) during the yogurt manufacture also was determined. In a mixed culture, _S_. _thermophilus_ counts were obtained by plating dilutions on Lee's agar (50) and incubating it at 32 C for 24 h. _Lactobacillus_ _bulgaricus_ counts were determined by plating on LBS agar (10) with incubation at 37 C under CO_2 for 5 days.

For judging flavor and consistency of yogurt, four expert judges were included in the panel. As a control, a commercial yogurt sample also was supplied to the judges.

RESULTS AND DISCUSSION

Influence of Growth Conditions on Plaque Count and Diameter of *S. thermophilus* Bacteriophage

All the succeeding experiments were repeated three times using three *S. thermophilus* bacteriophages. In every trial total number and diameter of bacteriophage plaques were determined. The data were analyzed statistically to determine the significant differences among treatments, and treatment X bacteriophage interactions for both the plaque numbers and diameter.

Effect of saline vs. distilled water and preincubation vs. no preincubation

The results of these experiments are shown in Table 7. The values presented in Table 7 are the averages from three different trials. Statistically, treatment effects were significant for both bacteriophage numbers ($P<.005$) and plaque diameters ($P<.05$). A least significant difference test (LSD) revealed that the use of saline and preincubation of the host phage mixture yielded significantly more bacteriophage plaques than saline - no preincubation and distilled water - no preincubation. However, differences between saline - preincubation and distilled water - preincubation were insignificant. Since the highest treatment means with bacteriophage numbers were obtained with saline - preincubation treatment,

Table 7. Effect of preincubation of phage-host cells in distilled water and saline solution on plaque count and diameter with *S. thermophilus* bacteriophage.

Host		Distilled water		Saline solution[a]	
		Not Preincubated[b]	Preincubated	Not Preincubated	Preincubated
ST_2	Count/ml[c]	130×10^4	150×10^4	300×10^4	360×10^4
	Ave. dia.[c]	1.33	1.46	1.33	1.51
ST_4	Count/ml	110×10^5	120×10^5	200×10^5	200×10^5
	Ave. dia.	1.44	1.49	1.62	1.67
ST_A	Count/ml	40×10^5	78×10^5	69×10^5	130×10^5
	Ave. dia.	1.32	1.40	1.39	1.45

[a]0.85% NaCl in twice-distilled water.

[b]Preincubation wherein the phage was placed in contact with host cells for 15 min at 37 C before plating. No preincubation - phage placed in contact with host cells followed by immediate plating.

[c]Mean values obtained from three different trials.

this was preferred to other treatments in this experiment. Also, diameter means obtained with saline - preincubation treatment were greater and significantly different from other treatments. Interaction of bacteriophage and treatment with regard to diameters was insignificant ($P > .60$). Statistically significant results were obtained for bacteriophage X treatment interaction ($P < .025$) in regard to bacteriophage plaque counts. This was probably because S. thermophilus ST_A phage responded differently from other phages. Over-all the effect of using saline, and preincubating the phage-host mixture was considered superior to other treatments in yielding greater numbers and diameters of S. thermophilus bacteriophage plaques.

Effect of calcium chloride

According to previous investigators (69), use of 2.5% calcium chloride solution was optimum for obtaining the maximum number of lactic Streptococcus bacteriophage particles. This concentration of calcium chloride was employed by Deane et al. (30) to enumerate S. thermophilus bacteriophage. The results obtained from our study revealed that 1.0% calcium chloride was superior to other levels employed for the maximum enumeration of S. thermophilus bacteriophage. This is illustrated in Table 8. Phage counts and diameters presented in this table are the averages from three different trials.

Table 8. Effect of $CaCl_2$ concentration in agar overlayer on plaque count and diameter with S. thermophilus bacteriophage[a].

Host		Per cent $CaCl_2$ in diluent		
		0[b]	0.5	1.0
ST_2	Count/ml[c]	96×10^4	160×10^4	350×10^4
	Ave. dia.[c]	1.20	1.48	1.75
ST_4	Count/ml	130×10^5	160×10^5	220×10^5
	Ave. dia.	1.27	1.48	1.74
ST_A	Count/ml	29×10^6	41×10^6	46×10^6
	Ave. dia.	1.37	1.57	1.70

[a]Plating conditions include use of 0.85% saline solution as the diluent with preincubation for 15 min at 37 C.

[b]Distilled water substituted for $CaCl_2$ solution as the negative control.

[c]Mean values obtained from three different trials.

Per cent $CaCl_2$ in diluent			
2.0	2.5	3.0	4.0
260×10^4 1.60	260×10^4 1.42	200×10^4 1.12	140×10^4 1.03
180×10^5 1.69	160×10^5 1.64	160×10^5 1.58	97×10^5 1.29
26×10^6 1.37	17×10^6 1.33	11×10^6 1.27	8×10^6 1.06

Treatment effects were statistically significant for both bacteriophage plaque counts ($P<.005$) and diameters ($P<.01$). Also, a least significant difference test proved that treatment involving 1.0% calcium chloride was superior to other treatments in yielding both the maximum number and diameter of *S. thermophilus* bacteriophage plaques. Bacteriophage X treatment interaction was insignificant ($P>.90$) with plaque diameters, but highly significant ($P<.005$) with bacteriophage numbers. The use of 1.0% calcium chloride as cell diluent was superior to other concentrations used. Bacteriophage counts were obtained even when distilled water was used instead of calcium chloride as a cell diluent. This was not surprising because calcium ions might have been present in other ingredients of the medium used. The plaque diameters and numbers decreased gradually as the concentration of calcium chloride was increased beyond 1.0%. It appears that the higher concentration of calcium chloride may be inhibitory to *S. thermophilus* bacteriophage.

Amount of host inoculum

The results of this investigation are presented in Table 9. The bacteriophage counts and diameters presented in Table 9 are averages from three different trials. Statistically, treatment effects were significant for both bacteriophage numbers ($P<.005$) and plaque diameter ($P<.005$). Highest mean

Table 9. Effect of amount of host inoculum on plaque count and diameter with *S. thermophilus* bacteriophage[a].

Host		Amount of inoculum in ml		
		0.1	0.2	0.4
ST_2	Count/ml[b]	270×10^4	310×10^4	380×10^4
	Ave. dia.[b]	1.36	1.44	1.47
ST_4	Count/ml	220×10^5	240×10^5	270×10^5
	Ave. dia.	1.36	1.47	1.52
ST_A	Count/ml	42×10^6	48×10^6	66×10^6
	Ave. dia.	1.39	1.52	1.56

[a]Plating conditions include use of preincubation, 0.85% saline solution, and 1.0% $CaCl_2 \cdot 2H_2O$.

[b]Mean values obtained from three different trials.

Amount of inoculum in ml		
0.6	0.8	1.0
310×10^4 1.08	260×10^4 0.96	210×10^4 0.65
190×10^5 1.48	180×10^5 1.11	150×10^5 0.83
67×10^6 1.65	59×10^6 1.53	61×10^6 1.34

values for plaque counts were obtained with the 0.4 ml treatment. Also, a least significant difference test proved that the mean value obtained for 0.4 ml treatment was significantly different from other treatments except the 0.2 ml treatment. However, the mean diameter value obtained for 0.4 ml treatment was significantly larger and different from other treatments. Statistically insignificant ($P > .30$) interaction among bacteriophage and treatments was observed with plaque diameters. On the other hand interaction of bacteriophage X treatments was significant ($P < .005$). It was concluded that 0.4 ml of host inoculum is superior to other levels for the enumeration of maximum number of discernible plaques of _S. thermophilus_ bacteriophage. The decrease in diameter of _S. thermophilus_ bacteriophage plaques with an increase in host inoculum, was probably attributed to the disproportion of bacteriophage particle numbers to host cell numbers. Also, when a large amount of host inoculum was used in the agar overlay, the amount of acid production may be greater and thus the pH might be lowered at a faster rate. The low pH of the agar overlay may reduce the proliferation of bacteriophage.

Effect of the pH of the medium

The results of this experiment are presented in Table 10. Treatment effects were significant with both plaque counts

Table 10. Effect of medium pH on plaque count[a] and diameter with *S. thermophilus* bacteriophage[a].

Host		pH of medium		
		5.2	5.4	5.6
ST_2	Count/ml[b]	2.0×10^5	2.6×10^5	27.0×10^5
	Ave. dia.[b]	0.55	0.59	0.73
ST_4	Count/ml	-[c]	26.0×10^6	26.0×10^6
	Ave. dia.	-	0.75	0.82
ST_A	Count/ml	-	8.3×10^6	13.0×10^6
	Ave. dia.	-	0.56	0.58

[a]Plating conditions include use of preincubation, 0.85% saline solution, 1.0% $CaCl_2 \cdot 2 H_2O$, and 0.4 ml inoculum.

[b]Mean values obtained from three different trials.

[c]No visible plaques.

pH of medium			
5.8	6.0	6.2	6.4
34.0×10^5 0.86	41.0×10^5 1.19	46.0×10^5 1.30	53.0×10^5 1.39
25.0×10^6 1.17	30.0×10^6 1.31	32.0×10^6 1.34	34.0×10^6 1.44
36.0×10^6 0.91	58.0×10^6 1.14	74.0×10^6 1.33	93.0×10^6 1.56

Table 10. (Continued)

Host		pH of medium		
		6.6	6.8	7.0
ST_2	Count/ml	60.0×10^5	52.0×10^5	9.3×10^5
	Ave. dia.	1.59	1.65	1.76
ST_4	Count/ml	40.0×10^6	34.0×10^6	11.0×10^6
	Ave. dia.	1.53	1.59	1.67
ST_A	Count/ml	97.0×10^6	100.0×10^6	84.0×10^6
	Ave. dia.	1.63	1.72	1.76

($P < .005$) and diameters ($P < .005$). Plaque counts and diameters obtained at pH's below 5.8 were significantly lower than the ones obtained at other pH's. The highest treatment mean value was obtained with the plaque counts obtained at pH 6.6. On the other hand the largest mean diameter was obtained with pH 7.0 treatment. Bacteriophage X treatment interaction was insignificant ($P > .05$) for plaque diameters. However, the interaction of bacteriophage and treatment was significant ($P < .005$) with plaque numbers. Since the largest number of plaques were formed at pH 6.6, this was chosen superior to other pH's for the maximum enumeration of *S. thermophilus* bacteriophage.

Effect of different basal media

Table 11 presents the bacteriophage counts and plaque diameters obtained with different basal media. These values are averages from three different trials. Statistically, treatment effects were significant with both phage counts ($P < .01$) and plaque diameters ($P < .005$). Highest bacteriophage counts were obtained with enriched tryptic soy agar. Elliker's lactic agar (35) yielded plaques with significantly larger diameters. Bacteriophage X treatment interaction was insignificant both with diameters ($P > .05$) and with plaque counts ($P > .05$). Mean plaque counts obtained with enriched tryptic soy agar are large and significantly different from

Table 11. Effect of different basal media on plaque count and diameter with *S. thermophilus* bacteriophage[a].

Host		Basal agar			
		Eugon	Elliker's lactic	Trypticase soy	Tryptic soy[b]
ST_2	Count/ml[c]	14×10^5	38×10^5	13×10^5	41×10^5
	Ave. dia.[c]	0.91	2.23	1.24	1.54
ST_4	Count/ml	15×10^5	120×10^5	210×10^5	280×10^5
	Ave. dia.	0.87	1.63	0.75	1.59
ST_A	Count/ml	76×10^6	67×10^6	5.3×10^6	100×10^6
	Ave. dia.	1.42	1.79	0.56	1.60

[a]Plating conditions include use of preincubation, 0.85% saline solution, 1.0% $CaCl_2 \cdot 2H_2O$, 0.4 ml inoculum, and medium pH of 6.6 ± 0.1.

[b]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 1.5% Bacto agar.

[c]Mean values obtained from three different trials.

others. However, the largest mean value with diameters was obtained with plaques grown on Elliker's agar. Since there was an insignificant difference between the mean diameters obtained with Elliker's agar (35) and enriched tryptic soy agar, the latter was preferred as the best medium to enumerate maximum numbers of S. thermophilus bacteriophage particles. Eugon agar (33) was superior to trypticase soy agar (33) for the enumeration of bacteriophage strains ST_2 and ST_A. The improved capacity of enriched tryptic soy agar (33) over trypticase soy agar (33) was due to the addition of 0.5% yeast extract and .02% L-cystine to the former medium.

Effect of different incubation conditions

Effect of different temperatures of incubation and atmospheres (air and CO_2) on total number and diameter of S. thermophilus bacteriophage plaques revealed that 37 C (air) was optimum for the recovery of maximum number of plaques. These results are depicted in Table 12. Statistically, treatment effects for both plaque counts ($P<.005$) and plaque diameters ($P<.005$) were significant. Bacteriophage X treatment interaction was insignificant ($P>.3$) with diameters, but significant with plaque counts ($P<.05$). Both the means of plaques and diameters obtained with 37 C (air) treatment were large and significantly different from other treatment means, according to least significant difference test. Plaque

Table 12. Effect of plate incubation conditions on plaque count and diameter with *S. thermophilus* bacteriophage[a].

Host		Temperature and atmosphere			
		32 C - Air	37 C - Air	37 C - CO_2	45 C - Air
ST_2	Count/ml[b]	31×10^5	42×10^5	24×10^5	17×10^5
	Ave. dia.	1.12	1.48	0.87	0.84
ST_4	Count/ml	150×10^5	250×10^5	190×10^5	150×10^5
	Ave. dia.	0.79	1.42	1.00	0.90
ST_A	Count/ml	27×10^6	53×10^6	32×10^6	36×10^6
		0.71	1.27	0.81	0.95

[a]Plating conditions included use of preincubation, 0.85% saline solution, 1.0% $CaCl_2 \cdot 2H_2O$, 0.4 ml inoculum, medium pH 6.6 ± 0.1, and enriched Tryptic soy agar.

[b]Mean values obtained from three different trials.

counts obtained at 32 C and 45 C were significantly less than the counts obtained at 37 C in air. It was obvious that plaque counts at 32 C were lower because this temperature is not optimum for the growth of _S. thermophilus_. On the other hand rapid growth of _S. thermophilus_ at 45 C could be a possible reason for obtaining the decreased number and size of bacteriophage plaques. Also, it is possible that _S. thermophilus_ bacteriophage may proliferate rather slowly at 45 C. Over-all, 37 C (in air) was elected to be the optimum temperature of incubation for the recovery of maximum number of _S. thermophilus_ bacteriophage particles.

Effect of different carbon sources

Enriched tryptic soy agar fortified with 0.5% lactose, 0.5% sucrose or 0.5% glucose was used to study the effect of carbohydrates on number and size of plaques of _S. thermophilus_ bacteriophage. The results of this experiment are presented in Table 13. Statistically, treatment effects were insignificant with bacteriophage plaque counts ($P > .90$). However with plaque diameters treatment effects were significant ($P < .005$). The highest mean value was obtained with lactose treatment for plaque diameters. A least significant difference test proved that there was a statistically insignificant difference between the treatment effects of lactose and glucose with regard to diameters. Besides glucose, the treat-

Table 13. Effect on plaque count and diameter with *S. thermophilus* bacteriophage of addition of lactose, sucrose, or glucose to enriched Tryptic soy agar[a].

Host		Basal agar			
		Tryptic soy[b]	Tryptic soy + 0.5% lactose	Tryptic soy + 0.5% sucrose	Tryptic soy + 0.5% glucose
ST_2	Count/ml[c]	37×10^6	41×10^6	38×10^6	41×10^6
	Ave. dia.[c]	1.32	1.50	1.41	1.51
ST_4	Count/ml	140×10^6	140×10^6	120×10^6	140×10^6
	Ave. dia.	1.34	1.49	1.40	1.51
ST_A	Count/ml	160×10^6	200×10^6	180×10^6	180×10^6
	Ave. dia.	1.44	1.86	1.38	1.51

[a]Plating conditions included use of preincubation, 0.85% saline solution, 1.0% $CaCl_2 \cdot 2H_2O$, 0.4 ml inoculum, medium pH 6.6 ± 0.1, and incubation temperature 37 C in air.

[b]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 1.5% Bacto agar.

[c]Mean values obtained from three different trials.

ment mean obtained for lactose was significantly different from other sugars. Bacteriophage X treatment interaction was highly significant ($P < .005$) with diameters. This interaction was insignificant ($P > .10$) with plaque numbers. It appears that the addition of lactose at the 0.5% level to enriched tryptic soy agar will greatly improve the diameters of *S. thermophilus* bacteriophage plaques. Since lactose is a selective substrate for *S. thermophilus*, its growth may be enhanced and thus phage may proliferate at a higher rate. Also, glucose was as good as lactose in improving the enumeration of *S. thermophilus* bacteriophage.

In the next experiment, the effect on the enumeration of *S. thermophilus* bacteriophage of the addition of glucose and lactose to enriched tryptic soy agar was determined. The results of this experiment are illustrated in Table 14. Statistically, treatment effects were insignificant ($P > .05$) with plaque counts. The treatment effect on plaque diameters, however, were statistically significant ($P < .005$). The lactose and glucose treatment mean was significantly greater than the mean of lactose treatment alone with diameters. Interaction of bacteriophage and treatment was insignificant ($P > .60$) with diameters, whereas bacteriophage X treatment was significant ($P < .01$) with plaque numbers. So, it was concluded that the addition of 0.5% each of glucose and lactose to enriched tryptic soy agar improves the plaque diameters of

Table 14. Effect on plaque count and diameter with *S. thermophilus* bacteriophage of addition of lactose or glucose and lactose to enriched Tryptic soy soy agar[a].

Host		Basal agar	
		Tryptic soy agar[b] + 0.5% lactose	Tryptic soy agar + 0.5% lactose and 0.5% glucose
ST_2	Count/ml[c]	87×10^7	91×10^7
	Ave. dia.[c]	1.60	2.04
ST_4	Count/ml	35×10^7	41×10^7
	Ave. dia.	1.60	1.97
ST_A	Count/ml	37×10^7	35×10^7
	Ave. dia.	1.46	1.99

[a]Plating conditions included use of preincubation, 0.85% saline solution, 1.0% $CaCl_2 \cdot 2H_2O$, 0.4 ml inoculum, medium pH 6.6 ± 0.1, and incubation temperature 37 C in air.

[b]Enriched Tryptic soy agar - Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 1.5% Bacto agar.

[c]Mean values obtained from three different trials.

S. thermophilus bacteriophage.

Calcium chloride vs. calcium borogluconate

The importance of calcium ions for bacteriophage infection of lactic streptococci has been established (19). Potter and Nelson (69) and Deane and Nelson (29) used calcium chloride in the seed layer to increase the plaque counts of lactic *Streptococcus* bacteriophage. Lowrie and Pearce (52) noted that the addition of calcium borogluconate at 0.005 M to M16 plating medium increased the plating efficiency for lactic phages. These workers, however, did not compare the efficiency of calcium chloride vs. calcium borogluconate upon the enumeration of bacteriophage plaques. In our study two levels of calcium chloride and calcium borogluconate were included to study their effects on the enumeration of *S. thermophilus* bacteriophage. Since Lowrie and Pearce (52) announced that the addition of calcium borogluconate to the agar overlay at 0.005 M gives maximum number of plaques, in our study this level of calcium chloride and calcium borogluconate was employed. From our previous experiments it was known that the addition of 0.022 M $CaCl_2$ to the overlay yields the maximum number of *S. thermophilus* bacteriophage plaques. So, these concentrations of calcium chloride and calcium borogluconate also were used. The results of this experiment are presented in Table 15. The treatment

Table 15. Effect of different concentrations of calcium chloride and calcium borogluconate in agar overlayer on plaque count and diameter with *S. thermophilus* bacteriophage[a].

Host		Conc. of $CaCl_2$ or calcium borogluconate in agar[b] overlay			
		.005 M $CaCl_2$	.005 M calcium borogluconate	.022 M $CaCl_2$	.022 M calcium borogluconate
ST_2	Count/ml[c]	32×10^7	22×10^7	35×10^7	36×10^7
	Ave. dia.[c]	1.16	1.38	1.79	1.72
ST_4	Count/ml	123×10^6	280×10^6	190×10^6	130×10^6
	Ave. dia.	1.05	0.71	1.38	0.92
ST_A	Count/ml	56×10^7	41×10^7	51×10^7	40×10^7
	Ave. dia.	1.27	1.10	2.00	1.24

[a]Plating conditions included use of preincubation, 0.85% saline solution, 0.4 ml inoculum, medium pH 6.6$\pm$0.1, incubation temperature 37 C in air, and enriched Tryptic soy agar + 0.5% lactose.

[b]Enriched Tryptic soy agar - Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 1.5% Bacto agar.

[c]Mean values from three different trials.

effects were insignificant ($P > .50$) with bacteriophage numbers, whereas with diameters, the treatment effects were significant ($P < .001$). The highest significant treatment mean value for diameters was obtained with the 0.022 M $CaCl_2$ treatment. So, a 0.022 M $CaCl_2$ addition to the agar overlay was considered superior to calcium borogluconate in recovering plaques with large diameters. Bacteriophage X treatment interaction was highly significant ($P < .005$) with diameters, whereas insignificant ($P > .10$) with plaque counts. Calcium borogluconate was not found to be superior to calcium chloride in recovering maximum number or size of bacteriophage plaques. Calcium chloride when added at .022 M to the agar overlay significantly improved the size of *S. thermophilus* bacteriophage plaques.

Modified two-layer plating procedure

By taking into account the above findings, a modified two-layer plating procedure was developed for the isolation and enumeration of *S. thermophilus* bacteriophage. The details of this procedure are presented in the Methods section. This technique was far superior to the earlier procedure (30) that was used to isolate and enumerate *S. thermophilus* bacteriophage. Distinct and large discernible plaques were obtained at 12 h of incubation. Deane et al. (30) modified the plating procedure developed for the enumeration of lactic

phages by Potter and Nelson (69) for the isolation and enumeration of *S. thermophilus* bacteriophage by using trypticase soy agar with 0.02% L-cystine as a basal agar and incubating the plates at 36 C for 11 h. We found that the plating procedure developed by Potter and Nelson (69) for lactic phages was inadequate for the enumeration of *S. thermophilus* bacteriophage. This could be one reason Deane et al. (30) were unable to obtain plaque formation with several of the whey filtrates which were inhibitory to many *S. thermophilus* strains in fortified litmus milk.

With our modified plating procedure, plaques started appearing even at 6 h of incubation, but maximum number of plaques were obtained at 12 h of incubation. Bacteriophage plaque counts did not increase between 12 and 18 h of incubation. Hereafter in this investigation, this modified two-layer plating procedure was used to isolate and enumerate *S. thermophilus* bacteriophage.

Under normal circumstances in many laboratories and cheese plants it is not practical to use a detailed two-layer plating procedure for the isolation of phage, so a simplified spot test procedure for the enumeration of *S. thermophilus* bacteriophage was developed. The detailed description of this procedure was outlined under Materials and Methods section.

The modified double-layer plating procedure and spot test procedure developed for the *S. thermophilus* bacteriophage were also suitable for lactic *Streptococcus* bacteriophages.

Also, suitable qualitative and quantitative plating procedures were developed to enumerate bacteriophages active against species of *Lactobacillus*. Reducing the amount of agar overlay from 3.0 ml to 1.5 ml greatly enhanced the appearance and recovery of *Lactobacillus* phages. Tween-80 was added to the overlay to help the proliferation of species of *Lactobacillus*. The amount of host inoculum was a critical factor in isolating *Lactobacillus* phage. Inoculum levels as low as one loopful to 0.1 ml yielded discernible plaques. When the host inoculum exceeded 0.1 ml, plaques were not observed on the lawn of host cells. But the amount of inoculum should be determined for different strains of bacteriophage. The details of these procedures are presented under Methods section. Using these procedures several *Lactobacillus* bacteriophages were successfully isolated.

Effect of initial broth pH on proliferation of S. thermophilus bacteriophage

The highest titers of bacteriophage strains ST_2 and ST_4 in a relatively short time were obtained with initial broth pH 6.0, but at longer incubation times pH 6.5 seemed better

for ST_4. The counts increase up to 8 to 10 h. These data are presented in Table 16. Table 17 shows the pH's after incubation of these same two strains. The presence of the bacteriophage results in a slightly higher pH than those obtained without bacteriophage. The pH optimum for the maximum proliferation of S. thermophilus ST_2 and ST_4 bacteriophage was around or slightly below pH 6.0. These experiments were repeated a second time only up to 6 h of incubation. The pH values agreed with the experiment conducted the first time with a variation of $\pm$0.1 units. The trends of bacteriophage counts observed were in agreement with the first trial experiments.

Since the pH of the Swiss cheese whey should be around 6.0$\pm$0.1, at 5 h after dipping, it is presumed that the maximum number of bacteriophage particles may be present in Swiss cheese whey obtained at this stage of manufacturing (71). This assumption was tentatively confirmed by enumeration of phage particles in samples of whey obtained from a Swiss cheese plant experiencing a bacteriophage problem. Deane et al. (30) took samples of milk before setting, and whey samples after the curd had been dipped for the survey of S. thermophilus bacteriophage in Swiss cheese plants. They did not specify the exact time of sampling after the curd had been dipped. It is possible that the bacteriophage titers may be quite low at the time of dipping, especially when the initial concentration of bacteriophage is low in milk. This

Table 16. Effect of initial broth pH on proliferation of *S. thermophilus* bacteriophage[a,b].

Initial pH of the broth	Host-phage	Phage count X 10^5/ml after incubation (37C) at h				
		4	6	8	10	12
6.0	ST_2-ST_2Ø	31.00	100.00	110.00	110.00	100.00
	ST_4-ST_4Ø	18.00	19.00	20.00	26.00	27.00
6.5	ST_2-ST_2Ø	18.00	32.00	41.00	35.00	39.00
	ST_4-ST_4Ø	9.80	48.00	64.00	62.00	62.00
7.0	ST_2-ST_2Ø	4.80	7.60	19.00	20.00	18.00
	ST_4-ST_4Ø	0.23	6.40	18.00	21.00	28.00

[a]Initial count of *S. thermophilus* ST_2Ø at 0 time - 35 X 10^3/ml.

[b]Initial count of *S. thermophilus* ST_4Ø at 0 time - 27 X 10^3/ml.

Table 17. The pH of *S. thermophilus* cultures after incubation for specified lengths of time at 37 C with and without addition of *S. thermophilus* bacteriophage.

Initial pH of the broth	Host-phage	pH of the broth[a] after incubation (37C) at h				
		4	6	8	10	12
6.0	ST_2-ST_2ø	5.75	5.75	5.75	5.75	5.75
	ST_2	5.75	5.65	5.60	5.55	5.50
	ST_4-ST_4ø	5.70	5.65	5.65	5.65	5.65
	ST_4	5.70	5.55	5.45	5.30	5.25
6.5	ST_2-ST_2ø	6.20	6.15	6.15	6.15	6.15
	ST_2	6.15	6.10	6.05	6.00	6.00
	ST_4-ST_4ø	6.15	6.10	6.10	6.10	6.10
	ST_4	6.15	5.90	5.85	5.75	5.70
7.0	ST_2-ST_2ø	6.60	6.55	6.55	6.55	6.55
	ST_2	6.60	6.50	6.45	6.40	6.40
	ST_4-ST_4ø	6.50	6.40	6.30	6.30	6.30
	ST_4	6.50	6.40	6.30	6.25	6.20

[a]Enriched Tryptic soy broth - Tryptic soy broth plus 0.5% yeast extract and 0.02% L-cystine.

could be one reason Deane et al. (30) were unable to isolate *S. thermophilus* bacteriophage from several samples of Swiss cheese whey.

Effect of pH, temperature, and time on storage stability of S. thermophilus bacteriophage

Before receiving samples of whey from various cheese plants it was necessary to study the effect of pH of the suspensory medium, temperature of incubation, and length of incubation on storage stability of *S. thermophilus* bacteriophage. This study should be of great help in determining suitable conditions for shipment of whey samples to the laboratory. This experiment was conducted using tryptic soy broth as a suspensory medium. The results of these experiments are presented in Tables 18 and 19. *S. thermophilus* bacteriophage strain ST_2 exhibited greatest stability between pH 5.0 and 6.5. Considerable decreases in bacteriophage counts were obtained when the pH of the suspensory medium was above pH 7.0, and below 4.5. This decrease was pronounced when the temperature of incubation was at 21 C rather than 5 C. When the pH of the suspensory medium was between 5.5 and 6.5, the storage temperature did not have much effect on the stability of *S. thermophilus* ST_2 bacteriophage. The decreased stability of *S. thermophilus* ST_2 bacteriophage was more pronounced when the pH of the medium was acidic (4.5 to

Table 18. Effect of pH, temperature, and time on *S. thermophilus* (ST_2) bacteriophage.

Days	Temp. C	Viable phage counts at 10^3/ml at suspensory medium[a] pH			
		3.5	4.0	4.5	5.0
0	5	58.00	66.00	87.00	99.00
	21	62.00	69.00	85.00	100.00
1	5	28.00	48.00	68.00	99.00
	21	34.00	52.00	61.00	88.00
4	5	25.00	48.00	60.00	96.00
	21	3.70	41.00	55.00	91.00
7	5	20.00	50.00	63.00	97.00
	21	00.50	38.00	44.00	86.00
10	5	14.00	52.00	59.00	92.00
	21	00.23	30.00	41.00	86.00
13	5	10.00	43.00	45.00	85.00
	21	00.20	31.00	40.00	55.00

[a]Tryptic soy broth was used as suspensory medium, and 0.5 ml of phage preparation was inoculated into each 9.5 ml of broth.

Viable phage counts X 10^3/ml at suspensory medium[a] pH					
5.5	6.0	6.5	7.0	7.5	8.0
94.00	100.00	98.00	95.00	85.00	75.00
100.00	110.00	92.00	97.00	81.00	64.00
100.00	94.00	95.00	78.00	66.00	68.00
88.00	94.00	92.00	59.00	46.00	50.00
92.00	99.00	100.00	75.00	69.00	62.00
87.00	98.00	99.00	55.00	40.00	48.00
100.00	97.00	97.00	78.00	68.00	65.00
84.00	100.00	95.00	62.00	43.00	48.00
98.00	98.00	100.00	73.00	65.00	60.00
88.00	98.00	90.00	56.00	40.00	42.00
89.00	93.00	90.00	63.00	58.00	51.00
82.00	88.00	85.00	48.00	40.00	38.00

Table 19. Effect of pH, temperature, and time on *S. thermophilus* (ST_4) bacteriophage.

Days	Temp. C	Viable phage count X 10^4/ml at suspensory medium[a] pH			
		3.5	4.0	4.5	5.0
0	5	2.300	57.000	79.000	130.000
	21	2.500	54.000	76.000	120.000
1	5	0.550	49.000	68.000	120.000
	21	0.020	20.000	50.000	68.000
4	5	0.140	43.000	75.000	130.000
	21	NC	15.000	45.000	65.000
7	5	0.034	32.000	78.000	100.000
	21	NC	1.200	30.000	58.000
10	5	0.018	30.000	68.000	94.000
	21	NC	00.750	21.000	52.000
13	5	0.010	10.000	33.000	68.000
	21	NC	00.200	18.000	30.000

[a]Tryptic soy broth was used as suspensory medium, and 0.5 ml of phage preparation was inoculated into each 9.5 ml of broth.

Viable phage count X 10^4/ml at suspensory medium[a] pH					
5.5	6.0	6.5	7.0	7.5	8.0
140.000	130.000	130.000	180.000	160.000	160.000
140.000	130.000	140.000	170.000	170.000	160.000
130.000	130.000	130.000	170.000	160.000	140.000
120.000	130.000	120.000	110.000	100.000	86.000
140.000	140.000	140.000	150.000	150.000	110.000
100.000	95.000	88.000	81.000	83.000	53.000
110.000	120.000	130.000	130.000	130.000	98.000
78.000	76.000	84.000	42.000	47.000	38.000
110.000	110.000	120.000	120.000	130.000	88.000
70.000	70.000	82.000	40.000	38.000	32.000
73.000	80.000	77.000	78.000	86.000	52.000
60.000	61.000	64.000	41.000	36.000	32.000

3.5) rather than alkaline (7.0 to 8.0). Similar trends were obtained with ST_4 bacteriophage. However, this bacteriophage was more stable when stored at 5 C than at 21 C even when the pH of the suspensory medium was adjusted to 5.5 to 6.5. Also, the storage stability of ST_4 bacteriophage was much less than ST_2 phage at acidic pH's when the storage temperature was 21 C. Thus, the pH of the suspensory medium should be maintained above 5.5 to maintain maximum stability of *S. thermophilus* bacteriophage during shipment without refrigeration. These experiments were repeated using few salient variables. The similar trends in results were obtained.

Effect of filtration on S. thermophilus bacteriophage

In all of the previous experiments, bacteriophage counts were fairly low. So, the effect of filtration on numbers of *S. thermophilus* bacteriophage was determined. These data are presented in Table 20. The phage counts presented in this table are averages from three different trials. The per cent loss of bacteriophage particles due to filtration varied from 81 to 96%. Considerable losses in phage particle numbers were observed both while passing through Whatman No. 42 filter paper and Millipore GS .22 µm filters, but the maximum loss in phage numbers occurred during Millipore filtration. Electron microscopy revealed that *S. thermophilus* bacteriophage particles were large and had a peculiar property of

Table 20. Effect of filtration on phage particle number.

Phage	Number of particles[a,b,c]		
	No filtration	Whatman No. 42 filter paper	No. 42 and Millipore GS .22 u filters
ST_2	110.0 X 10^5	54.0 X 10^5	21.0 X 10^5
ST_4	82.0 X 10^5	35.0 X 10^5	5.3 X 10^5
ST_A	140.0 X 10^5	110.0 X 10^5	20.0 X 10^5
ST_C	120.0 X 10^5	61.0 X 10^5	5.8 X 10^5
ST_G	95.0 X 10^5	53.0 X 10^5	4.0 X 10^5

[a]Plaque count X 10^5/ml.

[b]Plaque counts are averages from three trials.

[c]Significant - $P < .005$.

adsorbing to cell debris and broken cell walls. These electron micrographs are shown in Figures 8 and 9. This could be part of the reason bacteriophage particles were lost during filtration. It was also possible that these phage particles might adsorb to the filters. Deane et al. (30) employed rigorous filtration procedures to prepare samples of milk and whey for the isolation of *S. thermophilus* bacteriophage. Part of the reason for their failure to isolate *S. thermophilus* bacteriophage can be attributed to the loss of bacteriophage particles due to filtration.

Development of a chloroform treatment for the isolation of S. thermophilus bacteriophage

Since chloroform is bactericidal to several genera and species of bacteria (53), it was used to kill the bacterial cells while preparing samples for the isolation of *S. thermophilus* bacteriophage. The chloroform resistance of several strains of *S. thermophilus* bacteriophage was determined in two suspensory media (tryptic soy broth and skim milk). The results of these experiments are presented in Tables 21 and 22. Statistically insignificant ($P > .10$) differences were observed among treatments indicating that chloroform did not exhibit any harmful effects on viability of *S. thermophilus* bacteriophage and the suspensory medium had no effect on the action of chloroform.

Chloroform (0.5 ml/15 ml) also did not inactivate phages

Table 21. Effect of different concentrations of chloroform ($CHCl_3$) on _S. thermophilus_ bacteriophage suspended in Tryptic soy broth.

Phage	Total number of plaques[a,b] obtained with		
	No $CHCl_3$ treatment	0.5 ml $CHCl_3$/15 ml broth	1.0 ml $CHCl_3$/15 ml broth
ST_2	30	31	34
ST_4	130	130	150
ST_A	74	68	83
ST_C	50	41	43
ST_G	62	64	70
X	37	39	38

[a]Plaque count X 10^5/ml.

[b]Plaque counts are averages from three trials.

Table 22. Effect of different concentrations of chloroform ($CHCl_3$) on *S. thermophilus* bacteriophage suspended in skim milk.

Phage	Total number of plaques[a,b] obtained with		
	No $CHCl_3$ treatment	0.5 ml $CHCl_3$/15 ml milk	1.0 ml $CHCl_3$/15 ml milk
ST_2	14	18	20
ST_4	35	55	52
ST_A	130	110	150
ST_C	25	18	28
ST_G	17	20	20
X	18	22	19

[a]Plaque count X 10^8/ml.

[b]Plaque counts are averages from three trials.

active against *L. lactis*, *L. helveticus*, *L. bulgaricus*, and *S. lactis* (C_2 and MS). These results are depicted in Table 23. The effect of chloroform on starter cultures and dairy associated organisms also was investigated. The results of these experiments are presented in Table 24. Chloroform was bactericidal to lactic streptococci, *L. bulgaricus*, *S. thermophilus*, yeasts and molds, *S. durans*, *S. faecalis*, coliforms, *S. aureus*, *S. epidermidis*, *M. varians*, *B. linens*, species of *Propionibacterium* and the other flora present in whey. Even 0.5 ml chloroform added to 15 ml of whey exhibited excellent bactericidal effect. *S. thermophilus* bacteriophage, however, was not affected with both the concentrations of chloroform. From these trials it was concluded that the chloroform procedure was ideal for preparation of samples of broth, milk, and whey for the isolation and enumeration of *S. thermophilus* bacteriophage. The chloroform procedure was more efficient than filtration for recovery of phage particles and could be easily and effectively used in most dairy laboratories. To appreciate the superiority of chloroform treatment procedure over filtration, comparative data showing the percentage loss of *S. thermophilus* bacteriophage by filtration and chloroform treatment were presented in Table 25. Also, chloroform was not detrimental to bacteriophages active against species of *Lactobacillus* and *S. lactis*. So, this chloroform treatment procedure will have wide application in the dairy industry,

Table 23. Effect of chloroform ($CHCl_3$) on bacteriophage active against species of *Lactobacillus* and *S. lactis*.

Phage	Total number of plaques[a] obtained with	
	No $CHCl_3$ treatment	0.5 ml $CHCl_3$/15 ml milk culture
L. helveticus	70×10^7	64×10^7
L. lactis	160×10^5	180×10^5
L. bulgaricus	120×10^4	130×10^4
S. lactis C_2	86×10^6	63×10^6
S. lactis MS	38×10^7	25×10^7

[a]Plaque counts are averages from two trials.

Table 24. Effect of two different concentrations of chloroform on starter cultures, associated flora, and on enumeration of *S. thermophilus* bacteriophage from whey.

Sample	Propionibacterium			Coliforms		
	Untr.	$CHCl_3$ tr.		Untr.	$CHCl_3$ tr.	
		0.5 ml	1.0 ml		0.5 ml	1.0 ml
A	34×10^6	<1/ml	<1/ml	200×10^5	<1/ml	<1/ml
B	79×10^5	<1/ml	<1/ml	35×10^5	<1/ml	<1/ml
C	47×10^6	<1/ml	<1/ml	80×10^4	<1/ml	<1/ml
D	130×10^6	2/ml	2/ml	200×10^5	<1/ml	<1/ml
E	120×10^5	<1/ml	<1/ml	110×10^5	<1/ml	<1/ml

[a]Not possible to count.

Yeast & mold			*S. thermophilus* bacteriophage		
Untr.	$CHCl_3$ tr.		Untr.	$CHCl_3$ tr.	
	0.5 ml	1.0 ml		0.5 ml	1.0 ml
120×10^4	<1/ml	<1/ml	NPC[a]	15×10^4	19×10^4
110×10^3	<1/ml	<1/ml	NPC	57×10^4	80×10^4
170×10^4	<1/ml	<1/ml	NPC	15×10^4	29×10^4
30×10^5	<1/ml	<1/ml	NPC	30×10^4	30×10^4
78×10^5	<1/ml	<1/ml	NPC	53×10^3	62×10^3

Table 24. (Continued)

Sample	Total count[b]					
	21 C			32 C		
	Untr.	$CHCl_3$ tr.		Untr.	$CHCl_3$ tr.	
		0.5 ml	1.0 ml		0.5 ml	1.0 ml
A	20×10^{7}	<1/ml	<1/ml	40×10^{7}	1/ml	3/ml
B	73×10^{6}	1/ml	<1/ml	98×10^{6}	<1/ml	<1/ml
C	20×10^{7}	1/ml	<1/ml	30×10^{7}	5/ml	5/ml
D	50×10^{7}	4/ml	1/ml	83×10^{7}	5/ml	3/ml
E	20×10^{7}	<1/ml	<1/ml	58×10^{7}	<1/ml	<1/ml

[b]Elliker's agar was used as growth medium.

Total count					
37 C			45 C		
Untr.	$CHCl_3$ tr.		Untr.	$CHCl_3$ tr.	
	0.5 ml	1.0 ml		0.5 ml	1.0 ml
60×10^8	2/ml	1/ml	170×10^6	<1/ml	<1/ml
45×10^8	3/ml	<1/ml	35×10^7	<1/ml	<1/ml
30×10^7	5/ml	2/ml	30×10^7	8/ml	<1/ml
20×10^8	2/ml	2/ml	73×10^6	4/ml	<1/ml
80×10^7	1/ml	3/ml	150×10^6	1/ml	1/ml

Table 25. Comparative percentage loss of *S. thermophilus* bacteriophage particles by filtration and chloroform treatment (0.5 ml $CHCl_3$/15 ml broth culture), calculated on the basis of three experimental trials.

Phage	% loss by filtration	% loss by chloroform treatment
ST_2Ø	81	0
ST_4Ø	94	0
ST_AØ	86	8
ST_CØ	95	18
ST_GØ	96	0

to isolate bacteriophage active against starter cultures.

Determination of *S. thermophilus* bacteriophage proliferation in skim milk

In this experiment the proliferation rate of *S. thermophilus* and its specific phage in skim milk was determined. In the previous experiments tryptic soy broth was used as basal medium to study phage proliferation rate. The highest bacteriophage counts were obtained after 6 h of incubation and after 6 h phage counts did not increase significantly. These results are illustrated in Table 26. There was an increase of 3 logs in bacteriophage counts even in the first 2 hours of incubation. Bacterial counts in the absence of phage increased gradually until 12 h of incubation. When the specific phage was added to the bacterial culture, there was a decrease of 4 logs in viable bacterial counts in the first two hours of incubation. After 4 h of incubation the bacterial counts of *S. thermophilus* were <10/ml, signifying the virulence of phage in skim milk. When phage was added, titratable acidities were low and did not increase beyond 2 h of incubation. Similar trends were observed with pH values. These observations have immense practical significance because *S. thermophilus* is essential for the development of acid and flavor in several cultured dairy products. Also, sterile skim milk (autoclaved) can be used as basal media to

Table 26. Growth of *S. thermophilus* (ST_A), *S. thermophilus* bacteriophage ($ST_A Ø$) and their effects on pH and titratable acidities produced in skim milk.

Time in hours	Bacteriophage counts/ml	Bacterial counts/ml in skim milk	
		Without Ø	With Ø
0	9×10^4	74×10^5	79×10^5
2	33×10^7	55×10^6	8×10^1
4	110×10^7	92×10^6	<10/ml
6	83×10^8	170×10^6	<10/ml
8	85×10^8	200×10^6	<10/ml
12	89×10^8	33×10^7	<10/ml

Titratable acidities produced in skim milk		pH of the skim milk	
Without Ø	With Ø	Without Ø	With Ø
0.17	0.18	6.35	6.35
0.23	0.20	6.00	6.25
0.34	0.20	5.65	6.25
0.39	0.20	5.55	6.25
0.46	0.20	5.20	6.25
0.53	0.20	5.00	6.25

enrich *S. thermophilus* bacteriophage in 6 h at 37 C.

Effect on proliferation of S. thermophilus bacteriophage of the addition of lactose and skim milk to enriched tryptic soy broth

Since the higher counts of *S. thermophilus* bacteriophage were obtained in skim milk than in tryptic soy broth in the shortest time, lactose or 5% sterile skim milk were added to enriched tryptic soy broth to see the effect they would have on the proliferation of bacteriophage. The results are presented in Tables 27 and 28. Addition of 0.5% lactose to enriched tryptic soy broth appreciably increased ST_2 bacteriophage counts. No such increase in phage titers were observed with ST_4 and ST_A bacteriophages. Significant increases in all bacteriophage titers were obtained when 5.0% sterile skim milk instead of 0.5% lactose was added to enriched tryptic soy broth. From these data it is evident that milk stimulated the proliferation of *S. thermophilus* bacteriophage. The phage counts obtained with skim milk alone were much higher than the counts obtained with enriched tryptic soy broth + 5.0% sterile skim milk, with bacteriophage strains ST_4 and ST_A; however, the reverse was true with ST_2 bacteriophage.

Addition of 0.5% lactose to broth improved *S. thermophilus* bacterial counts. This could be one possible reason

Table 27. Effect on proliferation of S. thermophilus bacteriophage of the addition of lactose or sterile skim milk to enriched Tryptic soy broth[a], as opposed to enriched Tryptic soy broth and skim milk.

Phage	Phage counts X 10^7/ml			
	Broth[b]	Broth + 0.5% lactose	Broth + 5.0% sterile skim milk	Skim milk
ST_2	5.9	76.0	160.0	100.0
ST_4	3.5	2.8	81.0	130.0
ST_A	7.0	7.0	29.0	390.0

[a]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine.

[b]Enriched Tryptic soy broth alone.

Table 28. Effect on pH and proliferation of S. thermophilus, in the absence and presence of its specific phage, of the addition of lactose and sterile skim milk to enriched Tryptic soy broth[a] and skim milk.

Bacterial strain		Broth[b]	
		Without ø	With ø
ST_2	Count/ml	50×10^6	130×10^3
	pH	5.95	6.15
ST_4	Count/ml	37×10^6	87×10^3
	pH	5.5	6.2
ST_A	Count/ml	8×10^7	180×10^1
	pH	5.95	6.30

[a]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine.

[b]Enriched Tryptic soy broth alone.

Broth + 0.5% lactose		Broth + 5.0% sterile skim milk		Skim milk	
Without Ø	With Ø	Without Ø	With Ø	Without Ø	With Ø
24×10^{7} 4.40	13×10^{1} 6.05	42×10^{7} 4.75	8×10^{1} 6.00	38×10^{7} 4.85	6×10^{1} 6.30
57×10^{7} 4.25	71×10^{4} 6.25	63×10^{7} 4.55	2×10^{1} 6.30	130×10^{7} 5.05	<10 6.25
52×10^{7} 4.35	130×10^{1} 6.25	65×10^{7} 4.65	20×10^{1} 6.30	23×10^{7} 5.05	12×10^{1} 6.25

for the increase of ST_2 bacteriophage counts. There was a reduction of three logs in bacterial counts after phage lysis in enriched tryptic soy broth. However, when lactose was supplemented at 0.5%, there was a reduction of 5 logs in bacterial counts. This further confirms that more bacteriophage particles were liberated and consequently more bacterial cells were lysed. Bacterial counts (in the absence of bacteriophage) were greatly increased when grown in enriched tryptic soy broth fortified with 5.0% sterile skim milk. Also, bacterial counts after phage lysis were low. Enriched tryptic soy broth fortified with 5.0% sterile skim milk was as good as skim milk in improving the bacterial counts; however, the bacteriophage counts were higher in skim milk than in enriched tryptic soy broth + 5.0% skim milk.

The pH data presented in Table 28 revealed that the addition of lactose or 5.0% skim milk greatly lowered pH of the broth in which _S. thermophilus_ was grown. When their homologous phage was present the pH's of the broths were high. So, from these data it appears that tryptic soy broth fortified with 5.0% sterile skim milk promoted the proliferation of both bacterial and phage counts of _S. thermophilus_. Addition of 0.5% lactose definitely had an effect on bacterial numbers, but in some instances increases in proliferation were not observed. So, hereafter lactose was added at 0.5% level to enrich tryptic soy broth.

Effect on S. thermophilus and its bacteriophage of the addition of calcium chloride and calcium borogluconate to enriched tryptic soy broth and sterile skim milk

Calcium salts are very important in the coagulation of milk. Calcium chloride is added to cheese milk during the manufacture of certain varieties of cheeses (71), so, the effect on proliferation of S. thermophilus and S. thermophilus bacteriophage of addition of calcium chloride and calcium borogluconate to enriched tryptic soy broth and sterile skim milk was investigated. The results of this experiment are presented in Tables 29, 30 and 31. The addition of calcium chloride to a final concentration of .022 M to enriched tryptic soy broth greatly improved proliferation of bacteriophage strains ST_4 and ST_A. Phage strain ST_2, on the other hand, showed no increase in phage titer following the addition of calcium chloride. Calcium borogluconate, however, when added to a final concentration of .022 M had a distinct stimulatory effect on ST_2 as well as ST_4 and ST_A bacteriophage proliferation, but the stimulation obtained with the addition of calcium chloride was far superior to calcium borogluconate in enriched tryptic soy broth.

Addition of calcium chloride (.022 M) to skim milk greatly enhanced bacteriophage titer of ST_2 bacteriophage, but with bacteriophage strain ST_4 stimulation was observed only when .005 M calcium chloride was added to milk.

Table 29. Effect on proliferation of *S. thermophilus* bacteriophage of the addition of two different concentrations of calcium chloride ($CaCl_2$) and calcium borogluconate (Ca bglu) to enriched Tryptic soy broth[a] and sterile skim milk.

Phage	Phage counts X 10^7/ml				
	Broth[b]	Broth + .005 M $CaCl_2$	Broth + .022 M $CaCl_2$	Broth + .005 M Ca bglu	Broth + .022 M Ca bglu
ST_2	76.0	70.0	61.0	19.0	180.0
ST_4	2.8	15.0	56.0	38.0	36.0
ST_A	7.0	230.0	280.0	170.0	140.0

[a]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 0.5% lactose.

[b]Enriched Tryptic soy broth only.

[c]Sterile skim milk only.

Phage counts X 10^7/ml				
Skim milk[c]	Skim milk + .005 M $CaCl_2$	Skim milk + .022 M $CaCl_2$	Skim milk + .005 M Ca bglu	Skim milk + .022 M Ca bglu
100.0	98.0	230.0	110.0	120.0
130.0	160.0	92.0	41.0	86.0
390.0	390.0	290.0	250.0	260.0

Table 30. Effect on the growth of *S. thermophilus*, in the absence or presence of its specific phage, of the addition of two different concentrations of calcium chloride ($CaCl_2$) and calcium borogluconate (Ca bglu) to enriched Tryptic soy broth[a] and sterile skim milk.

Bacterial strains	Bacterial counts/ml			
	Broth[b]		Broth + .005 M $CaCl_2$	
	Without ø	With ø	Without ø	With ø
ST_2	24×10^7	13×10^1	24×10^7	5×10^1
ST_4	57×10^7	71×10^4	57×10^7	2×10^1
ST_A	52×10^7	130×10^1	51×10^7	4×10^1

[a]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 0.5% lactose.

[b]Enriched Tryptic soy broth only.

Bacterial counts/ml					
Broth + .022 M $CaCl_2$		Broth + .005 M Ca bglu		Broth + .022 M Ca bglu	
Without ø	With ø	Without ø	With ø	Without ø	With ø
22 X 10^7	55 X 10^1	26 X 10^7	25 X 10^2	19 X 10^7	17 X 10^1
23 X 10^7	2 X 10^1	61 X 10^7	1 X 10^1	22 X 10^7	1 X 10^1
37 X 10^7	4 X 10^1	50 X 10^7	2 X 10^1	41 X 10^7	15 X 10^1

Table 30. (Continued)

Bacterial strains	Bacterial counts/ml			
	Skim milk		Skim milk + .005 M $CaCl_3$	
	Without ∅	With ∅	Without ∅	With ∅
ST_2	38×10^7	6×10^1	42×10^7	4×10^1
ST_4	130×10^7	<10	140×10^7	1×10^1
ST_A	23×10^7	12×10^1	19×10^7	2×10^1

Bacterial counts/ml					
Skim milk + .022 M $CaCl_2$		Skim milk + .005 M Ca bglu		Skim milk + .022 M Ca bglu	
Without ø	With ø	Without ø	With ø	Without ø	With ø
48×10^7	<10	46×10^7	10×10^1	44×10^7	4×10^1
89×10^7	5×10^1	140×10^7	<10	70×10^7	1×10^1
2×10^7	116×10^1	33×10^7	3×10^1	38×10^7	2×10^1

Table 31. Effect on pH of the addition of two different concentrations of calcium chloride ($CaCl_2$) and calcium borogluconate (Ca bglu) to enriched Tryptic soy broth[a] and skim milk when *S. thermophilus* was grown in the absence or presence of its specific phage.

Bacterial strains	Broth[b]		Broth + .005 M $CaCl_2$		Broth + .022 M $CaCl_2$	
	Without Ø	With Ø	Without Ø	With Ø	Without Ø	With Ø
ST_2	4.40	6.05	4.35	6.10	4.25	5.85
ST_4	4.25	6.25	4.25	6.20	4.25	5.80
ST_A	4.35	6.25	4.35	6.20	4.20	5.75

[a]Tryptic soy broth plus 0.5% Difco yeast extract, 0.02% L-cystine, and 0.5% lactose.

[b]Enriched Tryptic soy broth alone.

[c]Sterile skim milk only.

Broth + .005 M Ca bglu		Broth + .022 M Ca bglu		Skim milk[c]	
Without Ø	With Ø	Without Ø	With Ø	Without Ø	With Ø
4.40	6.10	4.45	5.50	4.85	6.30
4.35	6.20	4.60	5.50	5.05	6.25
4.40	6.15	4.45	5.50	5.05	6.25

Table 31 (Continued)

Bacterial strains	Skim milk + .005 M $CaCl_2$		Skim milk + .022 M $CaCl_2$	
	Without Ø	With Ø	Without Ø	With Ø
ST_2	4.85	6.15	4.70	5.70
ST_4	5.00	6.15	5.00	5.70
ST_A	5.05	6.10	4.85	5.70

Skim milk + .005 M Ca bglu		Skim milk + .022 M Ca bglu	
Without Ø	With Ø	Without Ø	With Ø
5.00	6.05	4.95	5.65
5.20	5.10	5.25	5.75
5.05	6.15	5.05	5.75

Addition of .005 M $CaCl_2$ did not improve proliferation of ST_A bacteriophage in milk, and .022 M $CaCl_2$ added to skim milk reduced the counts of both ST_4 and ST_A bacteriophage. The addition of calcium borogluconate to milk did not have any stimulatory effect on proliferation of any of the S. thermophilus bacteriophages. From these results it was concluded that the addition of calcium chloride to a final concentration of .022 M to enriched tryptic soy broth but not skim milk would have a distinct stimulatory effect upon proliferation of S. thermophilus bacteriophage. Higher bacteriophage counts were obtained in skim milk than in enriched tryptic soy broth with various additions of calcium salts. Enriched tryptic soy broth to which calcium chloride was added to a final concentration of .022 M could be used as the best enrichment broth for S. thermophilus bacteriophage.

From the results presented in Table 30, calcium chloride or calcium borogluconate added to enriched tryptic soy broth did not stimulate the growth of S. thermophilus. When the concentration of calcium chloride was raised to .022 M in sterile skim milk, the bacterial counts were reduced. This could be part of the reason for obtaining low phage counts when calcium chloride was added to skim milk; however, no such depressed effect of bacterial numbers was observed with increased additions of calcium borogluconate, except with strain ST_4. The pH data are presented in Table 31. It is re-

ported (28) that calcium borogluconate addition to trypticase soy broth enhanced the proliferation of Staphylococcus bacteriophage. Also, Lowrie and Pearce (52) announced that the addition of calcium borogluconate greatly improved plaque numbers of lactic Streptococcus bacteriophage. However, in our investigation no such pronounced effect of calcium borogluconate over calcium chloride in enhancing proliferation of S. thermophilus bacteriophage was observed. On the contrary, in some instances, addition of calcium chloride to tryptic soy broth was found to be superior to calcium borogluconate in promoting phage numbers.

Effects of calcium carbonate, maleate buffer, and chloroform on bacteriophage, suspended and incubated at room temperature

To develop a suitable procedure for shipping samples of whey from outlying cheese plants to a central laboratory without substantial reduction in phage numbers during their shipment, we tested the effect of calcium carbonate, maleate buffer, and chloroform on the storage stability of S. thermophilus bacteriophages in cheese whey. Calcium carbonate and maleate buffer were employed because of their buffering capacity. Deane et al. (30) received samples of whey in 6 oz prescription bottles with 1 g of calcium carbonate so we used the same amount (1%). Because chloroform was detrimental to bacterial viability but not to S. thermophilus bacteriophages, it was included in this study of additives. Chloroform was

used to arrest the growth of bacteria in whey during shipment. The results of this experiment are presented in Tables 32 and 33. The lowest bacteriophage counts were obtained with whey + 1% calcium carbonate after 8 days of incubation at room temperature. Whey without any addition of calcium carbonate was slightly superior, especially with bacteriophage strain ST_2. Samples to which buffer and chloroform were added maintained better stability of S. thermophilus bacteriophage. These data show that the stability of different strains of S. thermophilus bacteriophages differ widely with additives. According to the pH data presented in Table 33, it appears that pH of whey had distinct effect on viability of S. thermophilus bacteriophage. Also, the pH obtained with whey plus 1.0% calcium carbonate was much more acidic than the whey alone. Since calcium carbonate neutralizes developed acidity at the initial stages, the organisms tend to build up in high numbers and consequently more acid is produced. This effect may be enhanced by solubulization of $CaCO_3$ at low pH. This could be part of the reason for the failure of Deane et al. (30) to isolate S. thermophilus bacteriophage from several Swiss cheese whey samples. pH of the whey (to which maleate buffer was added) was $4.1\pm.05$ after 8 days of incubation.

Addition of 1.0% chloroform to whey maintained it's pH at $6.60\pm.05$ from 0 through 8 days of incubation, but with

Table 32. Effects of calcium carbonate, maleate buffer, and chloroform on bacteriophage incubated at room temperature in whey[a,b].

Days of incubation	Bacteriophage counts /ml obtained with					
	Whey			Whey + 1.0% $CaCO_3$		
	ST_2[c]	ST_4[c]	ST_A[c]	ST_2	ST_4	ST_A
0	52×10^5	200×10^5	360×10^5	52×10^5	200×10^5	360×10^5
3	26×10^4	63×10^2	10×10^1	52×10^4	70×10^4	20×10^2
5	110×10^3	2×10^1	4×10^1	21×10^3	15×10^2	<10
8	26×10^3	<10	6×10^1	130×10^2	<10	<10

[a]pH of the whey (92.4 ml) was adjusted to 6.0, 7.6 ml of maleate buffer added to arrive at pH 6.65 and then 2.0% phage preparation in milk was added.

[b]When buffer was not added, pH of the whey was adjusted to 6.65 with 1 N NaOH, $CaCO_3$ or $CHCl_3$ added, and then 2.0% phage preparation was inoculated.

[c]Strains of bacteriophage used.

Table 32. (Continued)

Days of incubation	Bacteriophage counts/ml obtained with					
	Whey + buffer			Whey + 1.0% chloroform		
	ST_2	ST_4	ST_A	ST_2	ST_4	ST_A
0	52×10^5	200×10^5	360×10^5	52×10^5	200×10^5	360×10^5
3	164×10^4	140×10^3	30×10^1	4×10^4	40×10^5	100×10^3
5	63×10^4	21×10^2	13×10^1	42×10^2	86×10^4	65×10^3
8	37×10^4	26×10^2	11×10^1	89×10^1	76×10^4	120×10^2

Table 33. Effects of calcium carbonate, maleate buffer, and chloroform on pH of whey[a,b] incubated at room temperature.

Days of incubation	pH of the samples					
	Whey			Whey + 1.0% $CaCO_3$		
	ST_2[c]	ST_4[c]	ST_A[c]	ST_2	ST_4	ST_A
0	6.65	6.65	6.65	6.65	6.65	6.65
3	4.20	4.00	4.05	4.35	4.25	4.25
5	4.00	4.00	4.05	3.90	3.75	3.85
8	4.00	4.00	4.05	3.65	3.75	3.75

[a]pH of the whey (92.4 ml) was adjusted to 6.0, 7.6 ml of maleate buffer added to arrive at pH 6.65, and then 2.0% phage preparation in milk was added.

[b]When buffer was not added, pH of the whey was adjusted to 6.65 with 1 N NaOH, $CaCO_3$ added, and then 2.0% phage preparation in skim milk was added.

[c]Strains of bacteriophage used.

pH of the samples					
Whey + buffer			Whey + 1.0% chloroform		
ST_2	ST_4	ST_A	ST_2	ST_4	ST_A
6.65	6.65	6.65	6.65	6.65	6.65
4.40	4.20	4.25	6.65	6.65	6.65
4.30	4.15	4.20	6.65	6.60	6.65
4.10	4.15	4.15	6.65	6.60	6.60

whey alone or whey plus maleate buffer, the pH dropped to 4.1 to 4.2. But these experiments show that the maleate buffer and chloroform were very successful in maintaining the viability of *S. thermophilus* bacteriophage.

Experiments were conducted to determine the effect of mixing maleate buffer and chloroform on stability of bacteriophage. Since 1.0% chloroform to some extent was detrimental to the ST_2 bacteriophage, we tried to determine the minimum level of chloroform, in the presence or absence of maleate buffer, that would maintain the whey pH. In this experiment only pH values were determined. The results of this experiment are shown in Table 34. From these data it was found that 0.4% chloroform, in the presence of maleate buffer, was sufficient to maintain a uniform whey pH up to 8 days. When maleate buffer was not added, the pH of the whey dropped from 6.65 to 6.30 after 8 days of incubation. The addition of maleate buffer was not necessary to maintain pH of the whey when higher levels of chloroform were used.

In a separate experiment, the optimum level of calcium carbonate, in the presence and absence of maleate buffer, that would maintain the whey pH was determined. Whey alone, without calcium carbonate, served as a control. The results of these experiments are tabulated in Table 35. With the addition of 4% calcium carbonate, the pH of whey was dropped to 4.65 after 4 days of incubation. Upon further incubation

Table 34. Effects of different levels of chloroform ($CHCl_3$) and chloroform and maleate buffer on the pH of whey incubated at room temperature.

Days of incubation	pH of the samples					
	Whey	Whey[a] + 0.2% $CHCl_3$	Whey[b] + 0.2% $CHCl_3$ + buffer	Whey + 0.4% $CHCl_3$	Whey + 0.4% $CHCl_3$ + buffer	Whey + 0.6% $CHCl_3$
0	6.65	6.65	6.65	6.65	6.65	6.65
2	4.25	4.90	6.10	6.45	6.50	6.60
4	4.10	4.00	5.95	6.40	6.50	6.60
6	3.95	3.95	5.30	6.35	6.50	6.55
8	3.90	3.95	5.15	6.30	6.50	6.55

[a]pH of the whey was adjusted to 6.65 with 1 N NaOH and then chloroform was added.

[b]pH of the whey was adjusted to 6.0±0.1, 7.6 ml of maleate buffer was added to arrive at pH 6.65±0.1, and then chloroform was added.

Table 34. (Continued)

Days of incubation	pH of the samples				
	Whey + 0.6% $CHCl_3$ + buffer	Whey + 0.8% $CHCl_3$	Whey + 0.8% $CHCl_3$ + buffer	Whey + 1.0% $CHCl_3$	Whey + 1.0% $CHCl_3$ + buffer
0	6.65	6.65	6.65	6.65	6.65
2	6.60	6.60	6.60	6.60	6.60
4	6.60	6.60	6.60	6.60	6.60
6	6.60	6.60	6.60	6.60	6.60
8	6.60	6.60	6.60	6.60	6.60

Table 35. Effects of different levels of calcium carbonate and calcium carbonate and maleate buffer on pH of whey incubated at room temperature.

Days of incubation	pH of the samples					
	Whey	Whey[a] + 1% $CaCO_3$	Whey[b] + 1% $CaCO_3$ + buffer	Whey + 2% $CaCO_3$	Whey + 2% $CaCO_3$ + buffer	Whey + 3% $CaCO_3$
0	6.65	6.65	6.65	6.65	6.65	6.65
2	4.20	4.60	4.70	4.65	4.75	4.75
4	4.10	4.15	4.10	4.40	4.50	4.65
6	4.00	3.65	3.75	4.25	4.40	4.45
8	3.90	3.55	3.70	4.20	4.35	4.40

[a]pH of the whey was adjusted to 6.65 with 1 N NaOH and then $CaCO_3$ was added.

[b]pH of the whey was adjusted to 6.0±0.1, 7.6 ml of maleate buffer was added to arrive at pH 6.65±0.1, and then calcium carbonate was added.

Table 35. (Continued)

Days of incubation	pH of the samples				
	Whey + 3% $CaCO_3$ + buffer	Whey + 4% $CaCO_3$	Whey + 4% $CaCO_3$ + buffer	Whey + 5% $CaCO_3$	Whey + 5% $CaCO_3$ + buffer
0	6.65	6.65	6.65	6.65	6.65
2	4.75	4.70	4.75	4.70	4.70
4	4.55	4.65	4.60	4.65	4.65
6	4.55	4.75	4.70	4.65	4.65
8	4.50	4.75	4.75	4.65	4.70

for 4 more days the pH of the whey came up to 4.75. This trend was observed even when maleate buffer was added along with 4% calcium carbonate. So, it was found that 4% calcium carbonate, added to whey, maintained the whey pH at 4.75 even after an extended length of incubation (8 days). From the previous experiments, it was known that S. thermophilus bacteriophage is relatively stable above pH 4.5.

The next experiment incorporated all of these findings in a study of the effects of certain variables on the stability of S. thermophilus bacteriophage, suspended and incubated at room temperature in whey. The results of these experiments are presented in Tables 36 and 37. Bacteriophage titers were <10/ml even at the third day of incubation in whey with phage strains ST_4 and ST_A. The stability of the bacteriophage was improved with the addition of 4% calcium carbonate. When the buffer was added to whey in addition to 4% calcium carbonate the stability of S. thermophilus bacteriophage was greatly improved. Also, in the same experiment the effect of addition of specific S. thermophilus culture to whey (to which 4.0% calcium carbonate and maleate buffer was added) upon phage titers was investigated. With bacteriophage strains ST_4 and ST_A an increase of 3 logs of phage counts was observed. Evidently these bacterial strains grew at the room temperature and thus allowed their phage to proliferate. ST_2 bacteriophage, however, did not proliferate,

Table 36. Effect of different variables on stability of S. thermophilus bacteriophage incubated at room temperature in whey.

Days of incubation	Bacteriophage counts/ml obtained with					
	Whey			Whey[a] + 4.0% $CaCO_3$		
	ST_2[c]	ST_4[c]	ST_A[c]	ST_2	ST_4	ST_A
0	96×10^3	100×10^3	150×10^3	96×10^3	100×10^3	150×10^3
3	10×10^1	<10	<10	42×10^3	88×10^2	53×10^3
5	<10	<10	<10	200×10^2	52×10^2	280×10^2
8	<10	<10	<10	160×10^2	170×10^1	220×10^2

[a]pH of the whey was adjusted to 6.65 with 1 N NaOH and then $CaCO_3$ was added.

[b]pH of the whey was adjusted to 6.0±0.1, 7.6 ml of maleate buffer was added to arrive at pH 6.65±0.1.

[c]Strains of bacteriophage used.

Table 36. (Continued)

Days of incubation	Bacteriophage counts/ml obtained with					
	Whey[b] + 4.0% $CaCO_3$ + buffer			Whey + 4.0% $CaCO_3$ + buffer + 2.0% specific *S. thermophilus* culture		
	ST_2	ST_4	ST_A	ST_2	ST_4	ST_A
0	96×10^3	100×10^3	150×10^3	96×10^3	100×10^3	150×10^3
3	38×10^3	92×10^2	51×10^3	55×10^3	88×10^6	170×10^6
5	220×10^2	65×10^2	44×10^3	170×10^2	61×10^6	130×10^6
8	220×10^2	69×10^2	290×10^2	100×10^2	58×10^6	120×10^6

Table 36. (Continued)

Days of incubation	Bacteriophage counts/ml obtained with		
	Whey + buffer + 0.4% $CHCl_3$		
	ST_2	ST_4	ST_A
0	96×10^3	100×10^3	150×10^3
3	44×10^1	150×10^1	220×10^1
5	2×10^1	10×10^1	18×10^1
8	<10	4×10^1	<10

Table 37. Effect of different variables on the pH of whey incubated at room temperature for specified lengths of time.

Days of incubation	pH of the samples					
	Whey			Whey[a] + 4.0% $CaCO_3$		
	ST_2[c]	ST_4[c]	ST_A[c]	ST_2	ST_4	ST_A
0	6.65	6.65	6.65	6.65	6.65	6.65
3	3.85	3.95	3.95	5.00	4.75	4.90
5	3.60	3.70	3.65	4.50	4.60	4.65
8	3.55	3.65	3.65	4.95	5.00	5.00

[a]pH of the whey was adjusted to 6.65 with 1 N NaOH and then $CaCO_3$ was added.

[b]pH of the whey was adjusted to 6.0 ± 0.1, 7.6 ml of maleate buffer was added to arrive at pH 6.65 ± 0.1, and then $CaCO_3$ was added.

[c]Strains of bacteriophage used.

pH of the samples								
Whey[b] + 4.0% $CaCO_3$ + buffer			Whey + 4.0% $CaCO_3$ + buffer + 2.0% specific *S. thermophilus* culture			Whey + buffer + 0.4% $CHCl_3$		
ST_2	ST_4	ST_A	ST_2	ST_4	ST_A	ST_2	ST_4	ST_A
6.65	6.65	6.65	6.65	6.65	6.65	6.65	6.65	6.65
5.20	5.00	5.05	5.15	5.05	5.05	6.60	6.65	6.65
4.90	4.80	4.75	4.90	4.85	4.80	6.60	6.60	6.65
5.05	5.15	5.00	5.10	5.10	5.00	6.60	6.60	6.60

still the viability of ST_2 bacteriophage under these conditions was satisfactory.

The combination of maleate buffer and chloroform exhibited marked detrimental effect on the stability of bacteriophage. The pH data presented in Table 37 shows that when buffer and 0.4% chloroform were added to whey, the pH was stabilized at 6.65 ± 0.05 from 0 to 8 days of incubation. So, the detrimental effect of buffer and chloroform in mixture on *S. thermophilus* bacteriophage was due to some adverse chemical reaction rather than pH. From these data it was obvious that the addition of 4.0% calcium carbonate, 7.6% maleate buffer, and 2.0% specific *S. thermophilus* cultures to whey will not only maintain the viability of *S. thermophilus* bacteriophage but also will permit them to proliferate during shipment.

Phages active against species of *Lactobacillus* also proliferated when 4.0% calcium carbonate, buffer, and 2.0% specific *Lactobacillus* culture was added to whey and incubated at room temperature. There was an increase of 4 logs in concentration of bacteriophages active against *L. helveticus* and *L. lactis* at the third and fifth day of incubation. *Lactobacillus bulgaricus* bacteriophage titer, however, increased two logs only. Also, at the eighth day of incubation there was a reduction of 1 log in titers of *L. helveticus* and *L. bulgaricus* bacteriophage. These results are presented in Tables 38 and 39.

Table 38. Effect of different additives on the stability of bacteriophage active against three species of *Lactobacillus* incubated at room temperature in whey.

Days of incubation	The highest dilution at which the phage plaques were obtained with					
	Whey			Whey[a] + 4.0% $CaCO_3$		
	L.L[c] Ø	L.H[d] Ø	L.B[e] Ø	L.L Ø	L.H Ø	L.B Ø
0	10^{-3}	10^{-2}	10^{-2}	10^{-3}	10^{-2}	10^{-2}
3	NP[f]	NP	NP	10^{-3}	10^{-2}	10^{-1}
5	NP	NP	NP	10^{-2}	10^{-2}	10^{-1}
8	NP	NP	NP	10^{-1}	10^{-1}	NP

[a]pH of the whey was adjusted to 6.65 with 1 N NaOH and then $CaCO_3$ was added.

[b]pH of the whey was adjusted to 6.0±0.1, 7.6 ml of maleate buffer was added to arrive at pH 6.65±0.1.

[c]L.L - *L. lactis*.

[d]L.H - *L. helveticus*.

[e]L.B - *L. bulgaricus*.

[f]NP - no plaques were obtained even at 10^{-1} dilution.

The highest dilution at which the phage plaques were obtained with					
Whey[b] + 4.0% $CaCO_3$ + buffer			Whey + 4.0% $CaCO_3$ + buffer + 2.0% specific *Lactobacillus* culture		
L.L ø	L.H ø	L.B ø	L.L ø	L.H ø	L.B ø
10^{-3}	10^{-2}	10^{-2}	10^{-3}	10^{-2}	10^{-2}
10^{-3}	10^{-2}	10^{-1}	10^{-7}	10^{-6}	10^{-4}
10^{-3}	10^{-2}	10^{-1}	10^{-7}	10^{-6}	10^{-4}
10^{-2}	10^{-1}	NP	10^{-7}	10^{-5}	10^{-3}

Table 39. Effect of different additives on the pH of whey inoculated with bacteriophage active against three species of Lactobacillus and incubated at room temperature.

Days of incubation	pH					
	Whey			Whey[a] + 4.0% $CaCO_3$		
	L.L[c] Ø	L.H[d] Ø	L.B[e] Ø	L.L Ø	L.H Ø	L.B Ø
0	6.65	6.65	6.65	6.65	6.65	6.65
3	3.45	3.45	3.45	4.30	4.40	4.15
5	3.10	3.20	3.15	4.25	4.25	4.45
8	3.10	3.10	3.15	4.60	4.50	5.40

[a]pH of the whey was adjusted to 6.65 with 1 N NaOH and then $CaCO_3$ was added.

[b]pH of the whey was adjusted to 6.0 ± 0.1, 7.6 ml of maleate buffer was added to arrive at pH 6.65 ± 0.1.

[c]L.L - L. lactis.

[d]L.H - L. helveticus.

[e]L.B - L. bulgaricus.

pH					
Whey[b] + 4.0% $CaCO_3$ + buffer			Whey + 4.0% $CaCO_3$ + buffer + 2.0% specific *Lactobacillus* culture		
L.L Ø	L.H Ø	L.B Ø	L.L Ø	L.H Ø	L.B Ø
6.65	6.65	6.65	6.65	6.65	6.65
4.75	4.65	4.55	4.65	4.70	4.50
4.55	4.55	4.40	4.50	4.60	4.45
4.50	4.50	4.50	4.60	4.60	4.60

Survey of S. thermophilus and Lactobacillus bacteriophage from Swiss and Italian cheese plants

Thirteen Swiss cheese plants and 32 Italian cheese plants were surveyed for the presence of phage active against *S. thermophilus* and species of *Lactobacillus*. Twenty-four commercial *S. thermophilus* cultures received from three different culture manufacturers were used as indicators for the detection of phage. In addition, the culture used in each cheese plant was also included as an indicator. The results of this survey are presented in Table 40. *S. thermophilus* cultures A to G are supplied by culture company 1, cultures designated H to T by culture company 2, and U to X by company 3. Ninety-two per cent of the Swiss cheese plants and 65% of the Italian cheese plants surveyed had *S. thermophilus* bacteriophage. Phages active against 14 out of 24 *S. thermophilus* commercial cultures used were isolated from whey samples. Twenty-eight out of 45 cheese plants had phage for the *S. thermophilus* culture that was being used in their cheese plant. Phages active against as many as six commercial starter cultures were obtained from several whey samples. There could be six different phages in a whey sample or one phage that is active against six different *S. thermophilus* cultures or the six cultures obtained from the three culture manufacturers with different strain designations may be the same culture. To clarify this, phage strains were purified

Table 40. Survey of incidence of *S. thermophilus* bacteriophage in Swiss and Italian cheese plants.

Plant No.	Type of cheese whey	*S. thermophilus* culture used in the plant	Bacteriophage active against commercial *S. thermophilus* cultures											
			1[a]							2[a]				
			A	B	C	D	E	F	G	H	I	J	K	L
1	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
2	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
3	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
4	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
5	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
6	Swiss	-	-	-	-	-	-	-	-	-	-	-	-	-
7	Swiss	-	-	-	-	-	-	-	-	-	-	-	-	-
8	Swiss	-	-	-	-	-	-	-	-	-	-	-	-	-
9	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
10	Swiss	+	+	+	-	-	-	-	-	+	+	-	-	-
11	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
12	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
13	Italian	+	-	-	-	-	+	-	-	-	-	-	-	-
14	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
15	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Italian	+	-	-	-	-	+	-	-	-	-	-	-	-
17	Italian	+	-	-	-	-	+	-	-	-	-	-	-	-
18	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
19	Italian	+	-	-	-	-	-	-	+	-	-	-	-	-
20	Italian	+	-	-	-	-	-	-	+	-	-	+	-	-
21	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-

22	Italian	+	−	−	−	−	−	−	−	−	−	−	−	−
23	Italian	−	−	−	−	−	−	−	+	−	−	−	−	−
24	Italian	+	−	−	−	−	−	−	−	−	+	−	−	−
25	Italian	+	−	−	−	−	−	−	−	−	−	−	−	−
26	Italian	+	−	−	−	−	−	−	−	−	−	+	−	−
27	Italian	−	−	−	−	−	−	−	−	−	−	−	−	−
28	Italian	+	−	−	−	−	+	−	−	−	−	−	−	−
29	Italian	+	−	−	−	−	+	−	−	−	−	−	−	−
30	Italian	−	−	−	−	−	−	−	−	−	−	−	−	−
31	Italian	+	−	−	−	−	−	−	−	−	−	−	+	−
32	Italian	−	−	−	−	−	−	−	−	−	−	−	−	−
33	Italian	+	−	−	−	−	−	−	−	−	−	+	−	−
34	Italian	+	−	−	−	−	−	−	−	−	−	+	−	−
35	Italian	+	−	−	−	−	+	−	+	−	−	−	−	−
36	Italian	+	−	−	−	−	−	−	−	−	−	+	−	−
37	Italian	−	−	−	−	−	−	−	−	−	−	−	−	−
38	Italian	−	−	−	−	−	−	−	−	−	−	−	−	−
39	Italian	+	−	−	−	−	+	−	−	−	−	−	−	−
40	Swiss	+	+	+	−	−	−	−	−	+	+	−	−	−
41	Swiss	+	+	+	−	−	−	−	−	+	+	−	−	−
42	Swiss	+	+	+	−	−	−	−	−	+	+	−	−	−
43	Swiss	−	−	−	−	−	−	−	−	−	−	−	−	−
44	Swiss	−	−	−	−	−	−	−	−	−	−	−	−	−
45	Swiss	+	+	+	−	−	−	−	−	+	+	−	−	−

[a]Cultures obtained from three different culture manufacturers.

Table 40. (Continued)

Plant No.	Type of cheese whey	*S. thermophilus* culture used in the plant	Bacteriophage active against commercial *S. thermophilus* cultures											
			2[a]								3[a]			
			M	N	O	P	Q	R	S	T	U	V	W	X
1	Swiss	+	–	–	–	–	–	+	–	–	–	–	–	+
2	Swiss	+	–	–	–	–	–	–	–	–	–	–	–	+
3	Swiss	+	–	–	–	–	–	+	–	–	–	–	–	+
4	Swiss	+	–	–	–	–	–	–	–	–	–	–	–	+
5	Swiss	+	–	–	–	–	–	–	–	–	–	–	–	+
6	Swiss	–	–	–	–	–	–	–	–	–	–	–	–	–
7	Swiss	–	–	–	–	–	–	–	–	–	–	–	–	+
8	Swiss	–	–	–	–	–	–	–	–	–	–	–	–	–
9	Swiss	+	–	–	–	–	–	–	–	–	–	–	–	+
10	Swiss	+	–	–	–	–	–	–	–	–	–	–	–	+
11	Italian	–	–	–	–	–	–	–	–	–	–	–	–	–
12	Italian	–	–	–	–	–	–	–	–	–	–	–	–	–
13	Italian	+	–	–	–	–	–	–	+	–	–	–	–	–
14	Italian	–	–	–	–	–	–	–	–	–	–	–	–	–
15	Italian	–	–	–	–	–	–	–	–	–	–	–	–	–
16	Italian	+	–	–	–	–	–	–	+	–	–	–	–	–
17	Italian	+	–	–	–	–	–	–	–	–	–	–	–	–
18	Italian	–	–	–	–	–	+	+	–	–	–	–	–	–
19	Italian	+	–	–	–	–	–	–	–	–	–	–	–	–
20	Italian	+	–	–	–	–	–	–	–	–	–	–	–	–
21	Italian	–	–	–	–	–	+	–	–	+	–	–	–	–

22	Italian	+	-	-	-	-	-	+	-	-	-	-	-	-
23	Italian	-	-	+	-	-	-	-	-	-	+	-	-	-
24	Italian	+	-	-	-	-	-	-	-	-	-	-	-	-
25	Italian	+	-	+	-	-	-	-	+	-	+	-	-	-
26	Italian	+	-	-	-	-	-	-	-	-	-	-	-	-
27	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
28	Italian	+	-	-	-	-	-	-	-	+	+	-	-	-
29	Italian	+	-	-	-	-	-	-	-	-	+	-	-	-
30	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
31	Italian	+	-	-	-	-	-	-	-	-	-	-	-	-
32	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
33	Italian	+	-	-	-	-	-	-	-	-	-	-	-	-
34	Italian	+	-	-	-	-	-	-	-	-	-	-	-	-
35	Italian	+	-	+	-	-	-	-	+	-	+	-	-	-
36	Italian	+	-	-	+	-	-	-	-	-	-	-	-	-
37	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
38	Italian	-	-	-	-	-	-	-	-	-	-	-	-	-
39	Italian	+	-	-	-	-	-	-	+	-	-	-	-	-
40	Swiss	+	-	-	-	-	-	-	-	-	-	-	-	+
41	Swiss	+	-	-	-	-	-	-	-	-	-	-	-	+
42	Swiss	+	-	-	-	-	-	-	-	-	-	-	-	+
43	Swiss	-	-	-	-	-	-	-	-	-	-	-	-	+
44	Swiss	-	-	-	+	-	-	-	-	-	-	-	-	-
45	Swiss	+	-	-	-	-	-	-	-	-	-	-	-	+

and cross sensitivity studies were conducted. Each purified phage was spotted on all 24 commercial indicator _S. thermophilus_ strains. The results of this experiment are presented in Table 41. _S. thermophilus_ bacteriophage A was active against cultures A and B (supplied by company 1), H and I (supplied by company 2), and X (supplied by company 3). Both strain specific and nonspecific _S. thermophilus_ bacteriophages were found. This information has great practical application. If there is a bacteriophage problem in the cheese plant, the cheese makers tend to change the cultures. Sometimes they change to a different culture supplied by the same culture manufacturer. Often they switch to cultures supplied by other culture manufacturers with a hope that the phage present in the cheese plant will not attack the new culture supplied by this second culture company. Sometimes this may solve the problem and other times it may not. A prior knowledge of the phage sensitivity patterns of various _S. thermophilus_ cultures is a prime requisite to control the bacteriophage problem in the dairy industry. It is better for a particular cheese maker to buy his cultures from one starter company than from several companies. It is also important for a starter culture manufacturer to know the phage sensitivity patterns of his starter cultures and to be in a position to recommend a scheme of culture rotation to cheese plants to whom he is supplying the cultures.

Table 41. Strain specificity of *S. thermophilus* bacteriophage.

Bacteriophage description	Commercial *S. thermophilus* cultures											
	1[a]							2[a]				
	A	B	C	D	E	F	G	H	I	J	K	L
AØ	+	+	−	−	−	−	−	+	+	−	−	−
BØ	+	+	−	−	−	−	−	+	+	−	−	−
EØ	−	−	−	−	+	−	−	−	−	−	−	−
GØ	−	−	−	−	−	−	+	−	−	−	−	−
HØ	+	+	−	−	−	−	−	+	+	−	−	−
IØ	+	+	−	−	−	−	−	+	+	−	−	−
JØ	−	−	−	−	−	−	−	−	−	+	−	−
NØ	−	−	−	−	−	−	+	−	−	−	−	−
OØ	−	−	−	−	−	−	−	−	−	−	−	−
RØ	−	−	−	−	−	−	−	−	−	−	−	−
SØ	−	−	−	−	+	−	−	−	−	−	−	−
UØ	−	−	−	−	−	−	+	−	−	−	−	−
XØ	+	+	−	−	−	−	−	+	+	−	−	−

[a]Cultures obtained from three different culture manufacturers.

Table 41. (Continued)

Bacteriophage description	Commercial *S. thermophilus* cultures											
	2								3[a]			
	M	N	O	P	Q	R	S	T	U	V	W	X
AØ	–	–	–	–	–	–	–	–	–	–	–	+
BØ	–	–	–	–	–	–	–	–	–	–	–	+
EØ	–	–	–	–	–	–	+	–	–	–	–	–
GØ	–	+	–	–	–	–	–	–	+	–	–	–
HØ	–	–	–	–	–	–	–	–	–	–	–	+
IØ	–	–	–	–	–	–	–	–	–	–	–	+
JØ	–	–	–	–	–	–	–	–	–	–	–	–
NØ	–	+	–	–	–	–	–	–	+	–	–	–
OØ	–	–	+	–	–	–	–	–	–	–	–	–
RØ	–	–	–	–	–	+	–	–	–	–	–	–
SØ	–	–	–	–	–	–	+	–	–	–	–	–
UØ	–	+	–	–	–	–	–	–	+	–	–	–
XØ	–	–	–	–	–	–	–	–	–	–	–	+

The results of this investigation indicate that *S. thermophilus* bacteriophage is widely distributed in Swiss and Italian cheese plants. This is not in agreement with the results of Deane et al. (30), who isolated phage from only one sample of Swiss cheese whey out of 81 examined. The results of Deane et al. (30) led some cheese industry personnel in the United States to believe that *S. thermophilus* bacteriophage is not prevalent and is not a serious problem in the dairy industry. The results obtained from our investigation proves the fallacy of this belief.

The samples of whey obtained from the 45 Swiss and Italian cheese plants also were checked for the presence of bacteriophage active against species of *Lactobacillus*. Here 17 commercial *L. bulgaricus* cultures and 1 *L. helveticus* culture was used as indicators to isolate the bacteriophage. These cultures were received from three different culture manufacturers. In addition, *Lactobacillus* cultures used in the cheese plants also were used as indicators. The results of this investigation are shown in Table 42. Phages active against *L. bulgaricus* were recovered from 45% of the Italian cheese plants and 15% of the Swiss cheese plants included in this study. Six out of 18 *Lactobacillus* cultures had phage.

Strain specificity studies are presented in Table 43. Both the strain-specific and nonspecific *L. bulgaricus* bacteriophages were observed.

Table 42. Survey of incidence of bacteriophage active against species of *Lactobacillus*.

Plant No.	Type of cheese whey	*Lactobacillus* culture used in the plant	Bacteriophage active against commercial *Lactobacillus* cultures								
			1[a]					2[a]			
			A	B	C	D[b]	E	F	G	H	I
1	Swiss	-	-	-	-	-	-	-	-	-	-
2	Swiss	-	-	-	-	-	-	-	-	-	-
3	Swiss	-	-	-	-	-	-	-	-	-	-
4	Swiss	-	-	-	-	-	-	-	-	-	-
5	Swiss	-	-	-	-	-	-	-	-	-	-
6	Swiss	-	-	-	-	-	-	-	-	-	-
7	Swiss	-	-	-	-	-	-	-	-	-	-
8	Swiss	-	-	-	-	-	-	-	-	-	-
9	Swiss	-	-	-	-	-	-	-	-	-	-
10	Swiss	-	-	-	-	-	-	-	-	-	+
11	Italian	-	-	-	-	-	-	-	-	-	-
12	Italian	+	-	+	-	-	-	-	-	-	-
13	Italian	+	-	+	-	-	-	-	-	-	-
14	Italian	-	-	-	-	-	-	-	-	-	-
15	Italian	-	-	-	-	-	-	-	-	-	-
16	Italian	-	-	-	-	-	-	-	-	-	-
17	Italian	-	-	+	-	-	-	-	-	-	-
18	Italian	+	-	+	-	-	-	-	-	-	-
19	Italian	-	-	-	-	-	-	-	-	-	-
20	Italian	-	-	+	-	-	-	-	-	-	+
21	Italian	-	-	-	-	-	-	-	-	-	-

22	Italian	-	-	-	-	-	-	-	-	-	-
23	Italian	-	-	-	-	-	-	-	-	-	-
24	Italian	+	-	+	-	-	-	-	-	-	-
25	Italian	+	-	+	-	-	-	-	-	-	-
26	Italian	-	-	-	-	-	-	-	-	-	-
27	Italian	-	-	-	-	-	-	-	-	-	-
28	Italian	-	-	-	-	-	-	-	-	-	-
29	Italian	-	-	+	-	-	-	-	-	-	-
30	Italian	-	-	-	-	-	-	-	-	-	-
31	Italian	-	-	-	-	-	-	-	-	-	-
32	Italian	-	-	-	-	-	-	-	-	-	-
33	Italian	-	-	-	-	-	-	-	-	-	-
34	Italian	-	-	-	-	-	-	-	-	-	-
35	Italian	-	-	-	-	-	-	-	-	-	-
36	Italian	+	-	+	-	-	-	-	-	-	-
37	Italian	+	-	+	-	-	-	-	-	-	-
38	Italian	-	-	-	-	-	-	-	-	-	-
39	Italian	+	-	+	-	-	-	-	-	-	-
40	Swiss	-	-	-	-	-	-	-	-	-	-
41	Swiss	+	-	-	-	-	-	-	-	+	-
42	Swiss	-	-	-	-	-	-	-	-	-	-
43	Swiss	-	-	-	-	-	-	-	-	-	-
44	Swiss	-	-	-	-	-	-	-	-	-	-
45	Swiss	-	-	-	-	-	-	-	-	-	-

[a]Cultures obtained from three different culture manufacturers.

[b]*L. helveticus*, all other cultures are *L. bulgaricus*.

Table 42. (Continued)

Plant No.	Type of cheese whey	*Lactobacillus* culture used in the plant	Bacteriophage active against commercial *Lactobacillus* cultures								
			2[a]						3[a]		
			J	K	L	M	N	O	P	Q	R
1	Swiss	-	-	-	-	-	-	-	-	-	-
2	Swiss	-	-	-	-	-	-	-	-	-	-
3	Swiss	-	-	-	-	-	-	-	-	-	-
4	Swiss	-	-	-	-	-	-	-	-	-	-
5	Swiss	-	-	-	-	-	-	-	-	-	-
6	Swiss	-	-	-	-	-	-	-	-	-	-
7	Swiss	-	-	-	-	-	-	-	-	-	-
8	Swiss	-	-	-	-	-	-	-	-	-	-
9	Swiss	-	-	-	-	-	-	-	-	-	-
10	Swiss	-	-	-	-	-	-	-	-	-	-
11	Italian	-	-	-	-	-	-	-	-	-	-
12	Italian	+	-	+	-	-	-	-	+	-	-
13	Italian	+	-	+	-	-	-	-	+	-	-
14	Italian	-	-	-	-	-	-	-	-	-	-
15	Italian	-	-	-	-	-	-	-	-	-	-
16	Italian	-	-	-	-	-	-	-	-	-	-
17	Italian	-	-	+	-	-	-	-	+	-	-
18	Italian	+	-	-	-	-	-	-	+	-	-
19	Italian	-	-	-	-	-	-	-	-	-	-
20	Italian	-	-	+	-	-	-	-	+	-	-
21	Italian	-	-	-	-	-	-	-	-	-	-

22	Italian	-	-	-	-	-	-	-	-	-	-
23	Italian	-	-	-	-	-	-	-	-	-	-
24	Italian	+	-	-	-	-	-	-	+	-	-
25	Italian	+	-	+	-	-	-	-	+	-	-
26	Italian	-	-	-	-	-	-	-	-	-	-
27	Italian	-	-	-	-	-	-	-	-	-	-
28	Italian	-	-	-	-	-	-	-	-	-	-
29	Italian	-	-	+	-	-	-	-	-	-	-
30	Italian	-	-	-	-	-	-	-	-	-	-
31	Italian	-	-	-	-	-	-	-	-	-	-
32	Italian	-	-	-	-	-	-	-	-	-	-
33	Italian	-	-	-	-	-	-	-	-	-	-
34	Italian	-	-	-	-	-	-	-	-	-	-
35	Italian	-	-	+	-	-	-	-	-	-	-
36	Italian	+	-	+	-	-	-	-	+	-	-
37	Italian	+	-	+	-	-	-	-	+	-	-
38	Italian	-	-	-	-	-	-	-	+	-	-
39	Italian	+	-	+	-	-	-	-	+	-	-
40	Swiss	-	-	-	-	-	-	-	-	-	-
41	Swiss	+	-	-	-	-	-	-	-	-	+
42	Swiss	-	-	-	-	-	-	-	-	-	-
43	Swiss	-	-	-	-	-	-	-	-	-	-
44	Swiss	-	-	-	-	-	-	-	-	-	-
45	Swiss	-	-	-	-	-	-	-	-	-	-

Table 43. Strain specificities of phages active against species of *Lactobacillus*.

Bacteriophage designation	Commercial *Lactobacillus* cultures								
	1[a]					2[a]			
	A	B	C	D[b]	E	F	G	H	I
BØ	-	+	-	-	-	-	-	-	-
HØ	-	-	-	-	-	-	-	+	-
KØ	-	+	-	-	-	-	-	-	-
PØ	-	+	-	-	-	-	-	-	-
IØ	-	-	-	-	-	-	-	-	+
RØ	-	-	-	-	-	-	-	+	-

[a]Cultures obtained from three different culture manufacturers.

[b]*L. helveticus*, all other cultures are *L. bulgaricus*.

Commercial *Lactobacillus* cultures								
2[a]						3[a]		
J	K	L	M	N	O	P	Q	R
-	+	-	-	-	-	+	-	-
-	-	-	-	-	-	-	-	+
-	+	-	-	-	-	+	-	-
-	+	-	-	-	-	+	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	+

The results of this survey should be of immense help both to the culture manufacturer and cheese maker.

Electron Microscopy

The technique used in this investigation to prepare bacteriophage samples for electron microscopy was quite effective and simple. Electron micrographs of bacteriophages active against *S. thermophilus* ST_4, ST_X, and ST_2 are presented in Figures 2, 3, and 4. These bacteriophages had the following measurements: ST_2 - head diameter - 60 nm, tail width - 10 nm, tail length - 236 nm; ST_4 - head diameter - 65 nm, tail width - 10 nm, tail length - 290 nm; ST_X - head diameter - 60 nm, tail width - 10.5 nm, tail length - 222 nm. These measurements were in agreement with the results of the previous workers (21). Also, ST_2 bacteriophage exhibited long and flexible tails. This is illustrated in Figure 5. In some preparations, lengths of ST_2 bacteriophage varied from 222 nm to 1025 nm. Bacteriophage active against ST_4 had a round ball-like structure at the distal extremity of it's tail. This could be an integral unit of ST_4 bacteriophage or receptor of *S. thermophilus* ST_4 that came off with the bacteriophage. No such structure was observed with other *S. thermophilus* bacteriophages.

Polytail formation

In several instances exceptionally long tails of ST_2

Figure 2. *S. thermophilus* ST_4 bacteriophage. Line scale equals 0.1 μm. PTA.

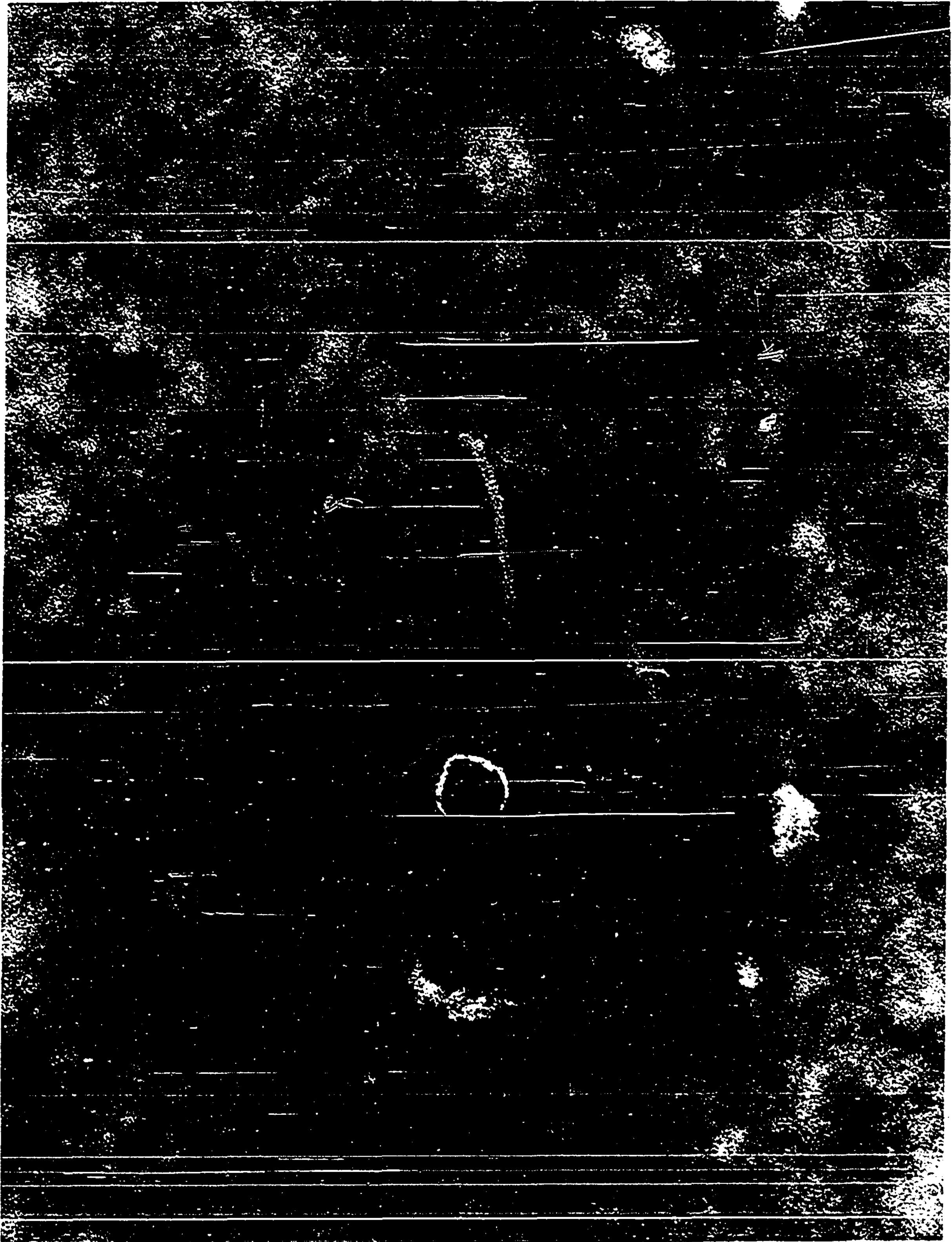


Figure 3. *S. thermophilus* ST_x bacteriophage showing distinct head and tail. Line scale equals 0.1 μm. PTA.

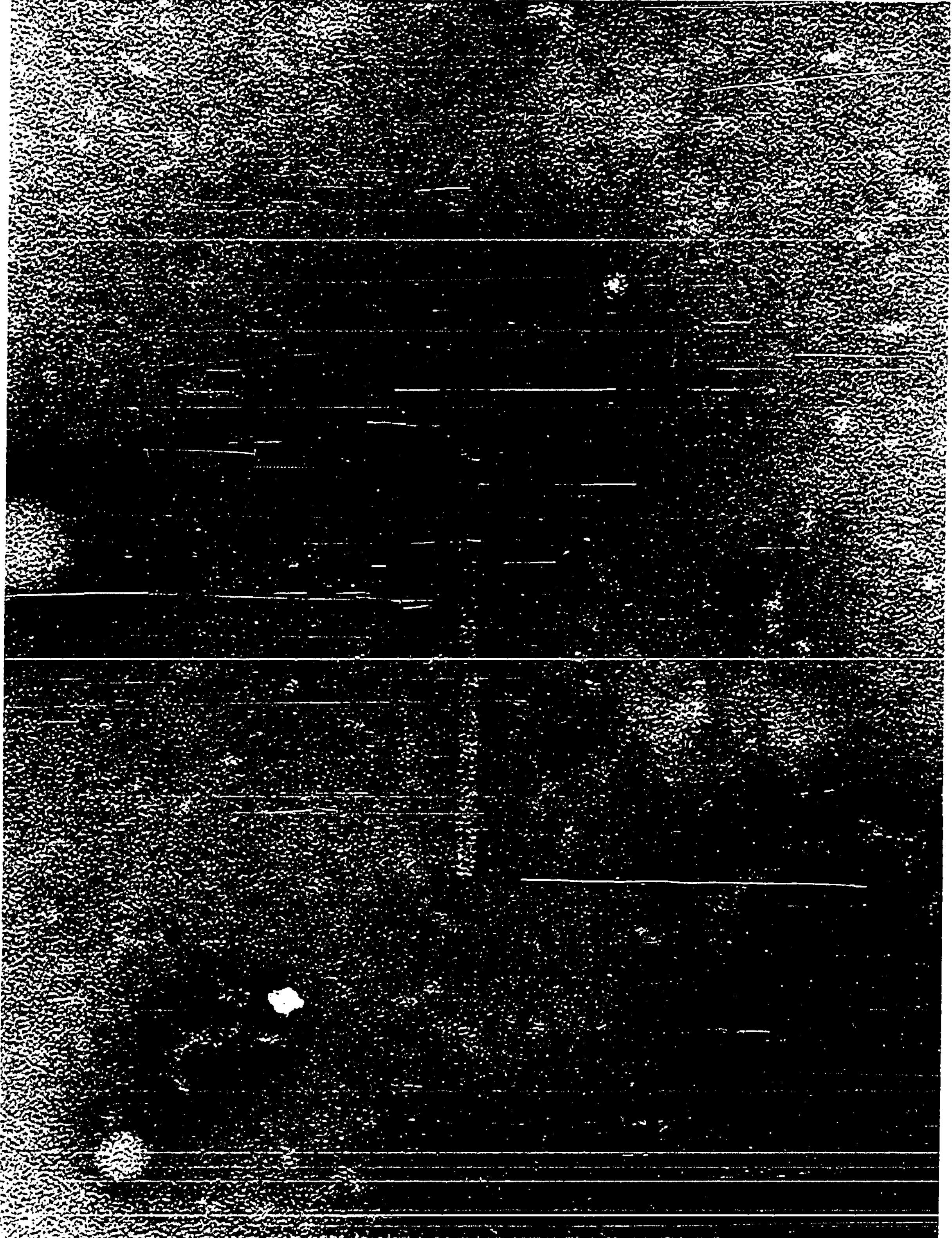


Figure 4. Polytail formation in S. thermophilus ST_2 bacteriophage upon extended incubation for 9 days in 2% ammonium acetate, pH 7.0. Line scale equals 0.1 µm. PTA.

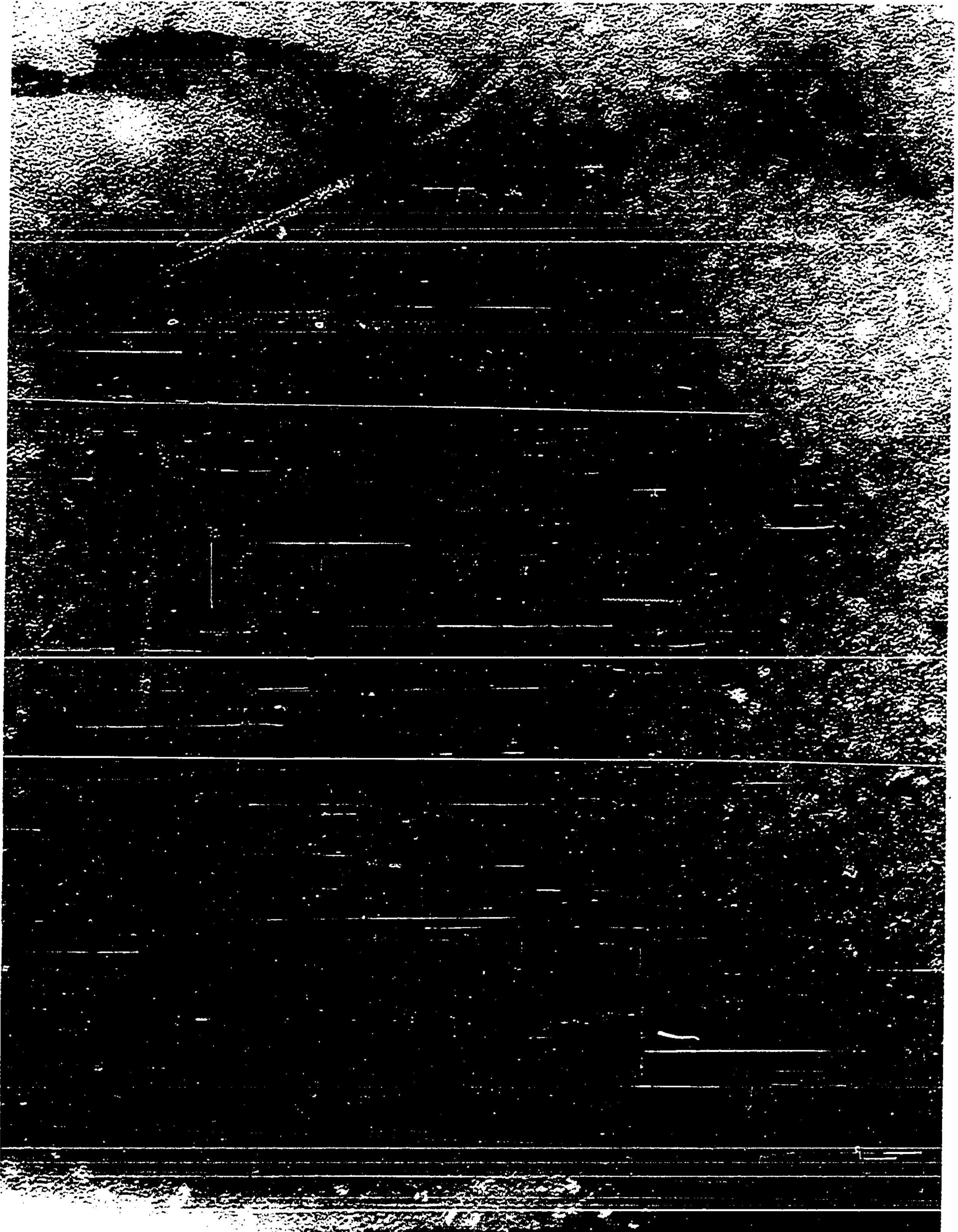
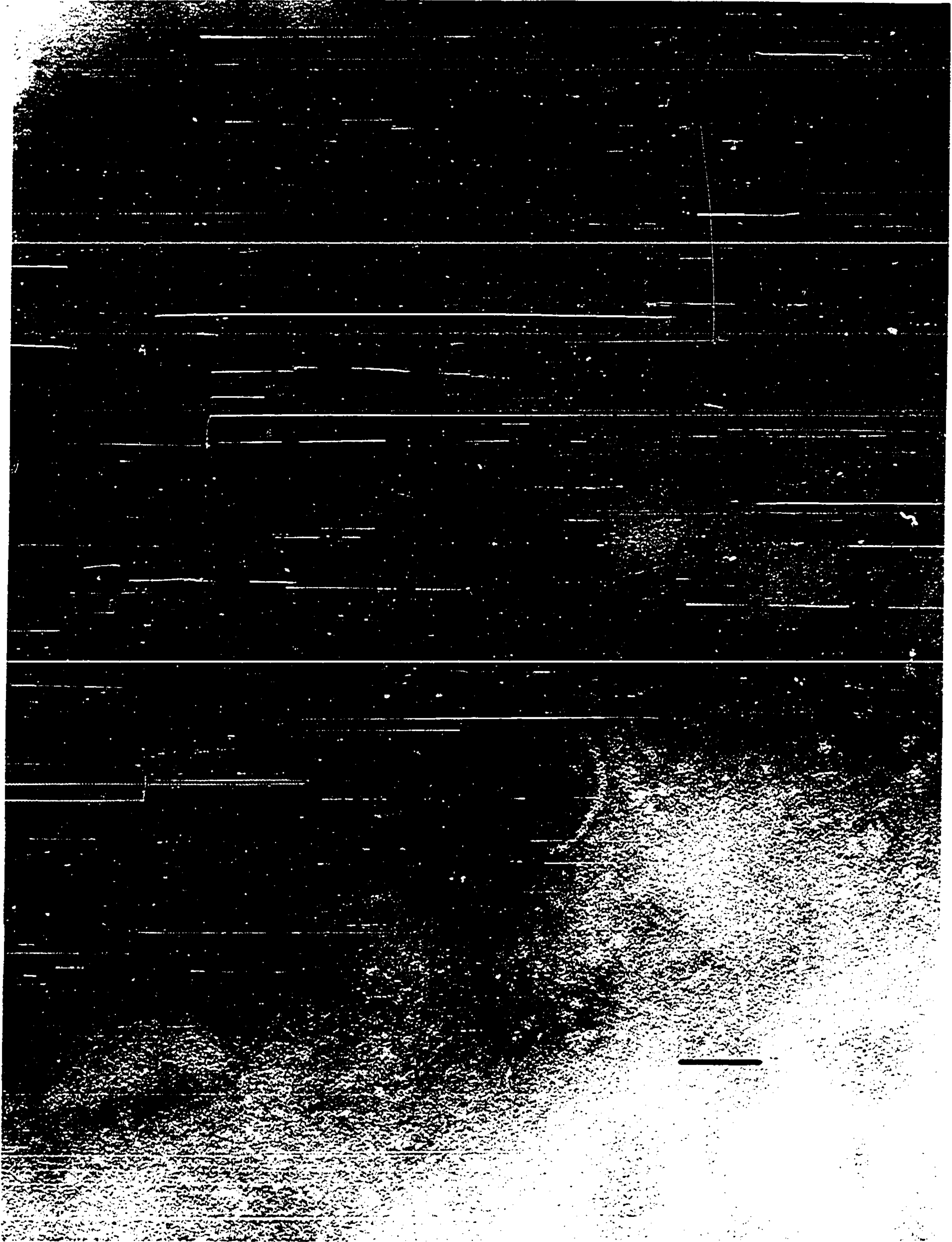


Figure 5. Cluster of *S. thermophilus* ST_2 bacteriophage particles showing long and flexible tails. Line scale equals 0.1 μm. PTA.



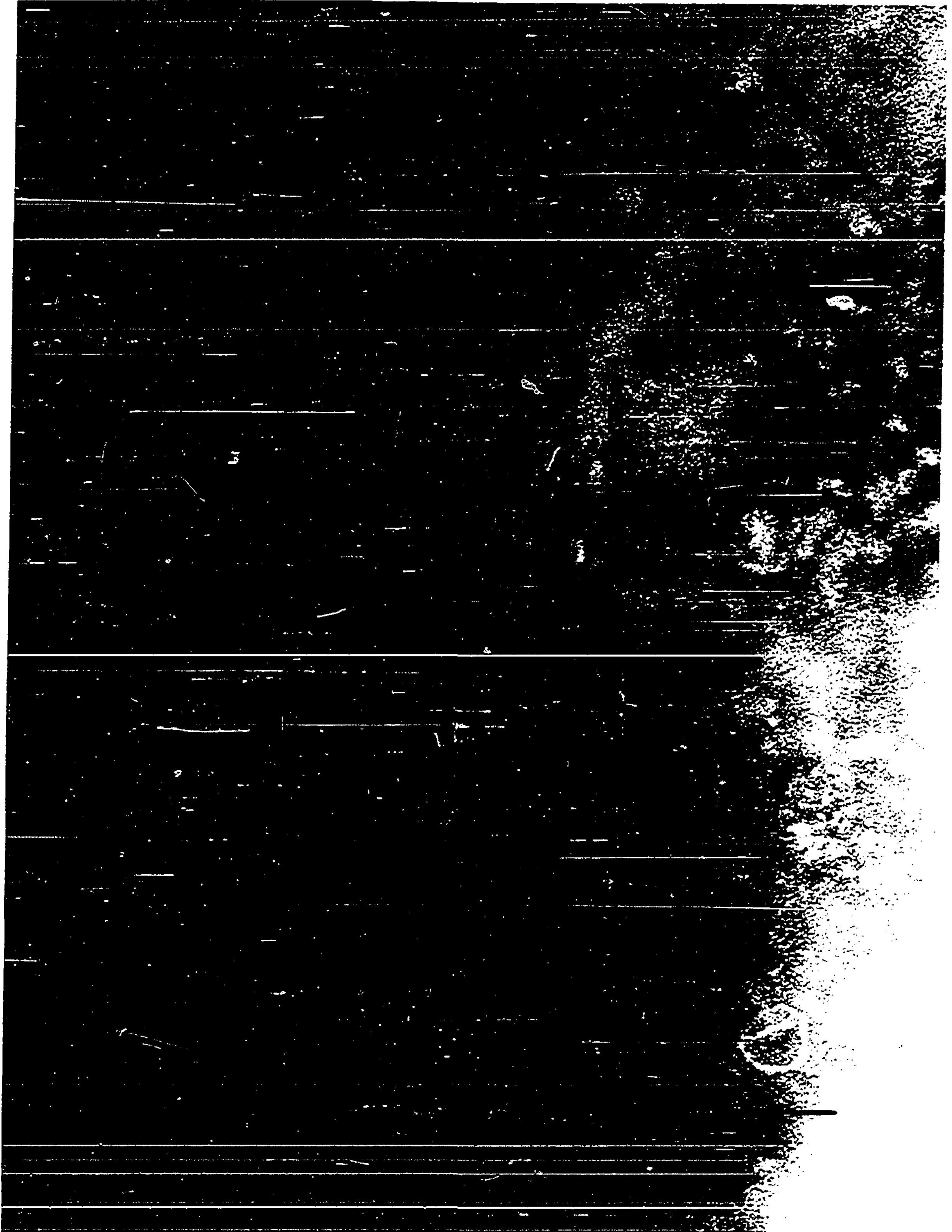
bacteriophage were observed. The width and structure of the abnormally long tail was similar to normal intact tails of ST_2 bacteriophage except for its length. For instance, ST_2 bacteriophage tail had a width of 10 nm and the total length of the tail was 236 nm. Whereas the polytails observed in the same preparation had a width of 10 nm (same as integral phage unit), but its tail length was 960 nm. In this instance the polytail was approximately four times longer than the normal phage tail. This is shown in Figure 4. However, the polytail presented in this micrograph did not have an intact phage head. Tikhonenko and Belyaeva (88) observed polyrod formation by phage No. 1 and No. 19 of _Bacillus mycoides_, when the phage preparation was heated to 90 to 100 C. However, no intact heads to these polyrods were observed. Polyrods were identical in structure and diameter to the intact phage rod, except that the polyrods were twice as long as normal rod. These authors have also noted that these polyrods showed a tendency to aggregate side by side, forming bundles. According to Tikhonenko (87) when suspension of phage No. 1 or phage No. 19 was heated to 90 to 100 C, the rod broke into separate units. When the suspension was cooled, the morphological units organized themselves spontaneously into hollow tubes of different lengths (polyrods), identical in structure and diameter to the tail rod. He also stated that the difference in lengths of polyrods was due to uncontrolled process

of self-organization, which was determined by electrostatic and thermodynamic properties of the identical subunits.

Polytail formation was observed in *S. thermophilus* bacteriophage even under the normal growth conditions. Also, often intact phage particles with polytails were observed. This is illustrated in Figure 6. The measurements of *S. thermophilus* ST_2 bacteriophage with a distinct head and a polytail were as follows: head diameter - 62 nm, tail width - 10 nm, and tail length - 1025 nm. The measurements of head and width of the tail were same as normal ST_2 phage particles. Whereas the length of the polytail was approximately 4½ times longer than the normal ST_2 phage particle. Effect of pH of the suspensory medium on polytail formation was investigated. The results of this study revealed that phage tails completely disappeared into the phage preparation at pH 2.0. At the alkaline pH (11.0) phage tails were intact but no polytails were seen. At neutral pH, a large number of phage particles were seen, and only a few of them exhibited polytail formation. So, polytail formation in *S. thermophilus* ST_2 bacteriophage was not dependent on pH of the suspensory medium.

The phage preparation, adjusted to pH 7.0, stored at 4 C for 10 days exhibited extensive polytail formation. This is illustrated in Figure 4. So, probably polytail formation in *S. thermophilus* phage was primarily a consequence of length

Figure 6. An intact *S. thermophilus* ST_2 bacteriophage particle showing distinct head and a long polytail. Line scale equals 0.1 μm. PTA.



of incubation. Polytail formation also was observed in *S. thermophilus* ST_G bacteriophage. This is depicted in Figure 7. Here the lengths of polytails ranged from 770 nm to 980 nm. The significance of polytail formation in *S. thermophilus* bacteriophage is not known.

While preparing the samples for the isolation of *S. thermophilus* bacteriophage by filtration, noticeable reductions in phage particle numbers were observed. Electron microscopy of unfiltered samples as opposed to filtered samples revealed that *S. thermophilus* bacteriophage had high adsorption rate to broken cell walls and cell debris. Approximately as many as 300 phage particles were adsorbed to a piece of cell debris. This is illustrated in Figures 8 and 9. Both the empty and full heads of phage particles were adsorbed to broken cell walls. This could be part of the reason for the reduction of *S. thermophilus* bacteriophage titer upon filtration.

Lactobacillus bacteriophage

Coetzee and Deklerk (22) published a report on temperate phages of *L. fermentii*. Later Sakurai et al. (78) isolated two temperate phages of *L. casei* and three of *L. salivarius*. Phage-like structures from *L. acidophilus* were isolated by Deklerk and Hugo (31). Tohyama (89) isolated twenty-three temperate phages of *L. salivarius* and studied their

Figure 7. Electron micrograph showing both the intact phage particles and polytails of *S. thermophilus* ST_G bacteriophage. Line scale equals 0.1 μm. PTA.

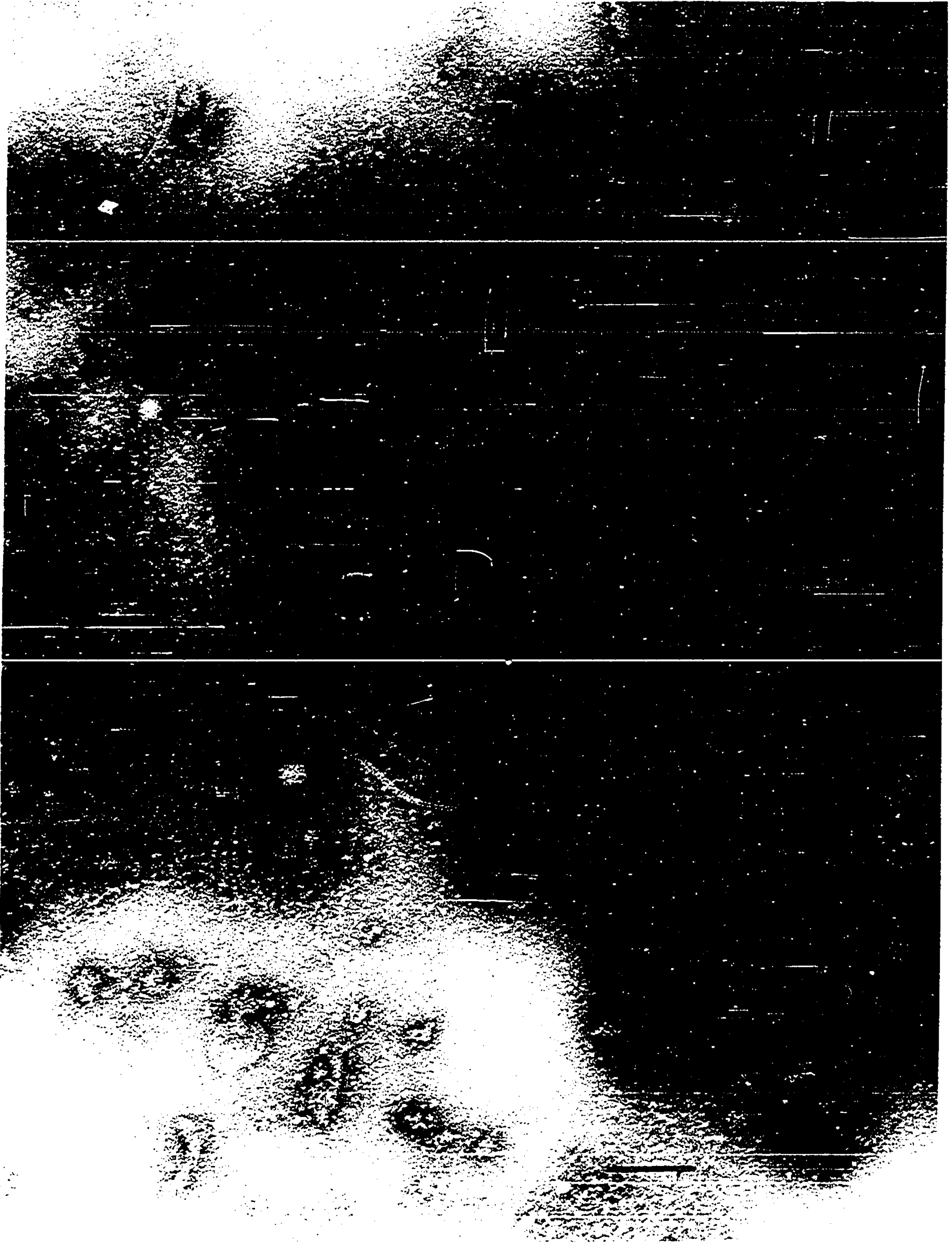


Figure 8. Electron micrograph showing numerous *S. thermophilus* ST_G bacteriophage particles, exhibiting both empty and full heads, adsorbed onto broken cell wall. Line scale equals 0.1 µm. PTA.

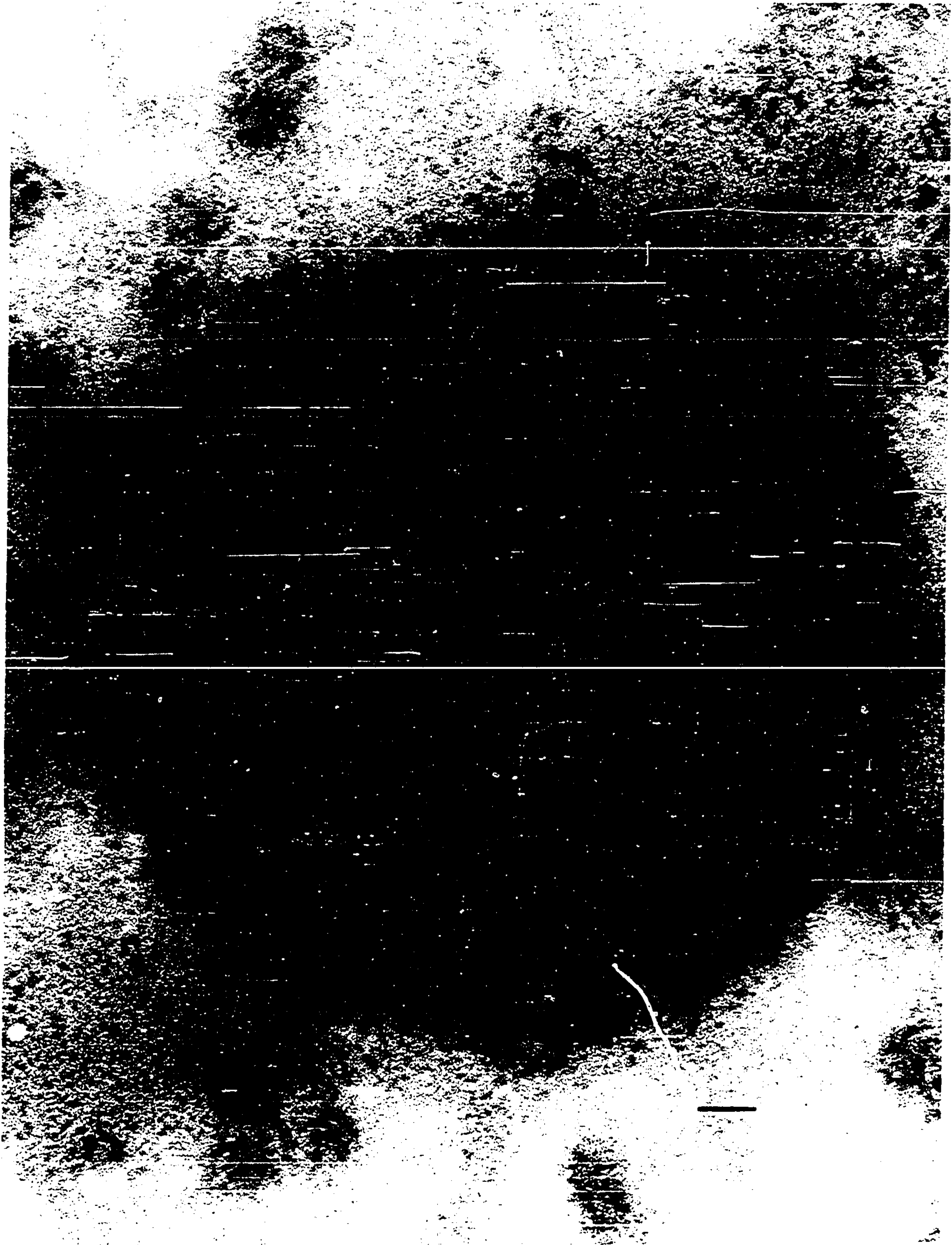


Figure 9. Electron micrograph showing high adsorption rate of *S. thermophilus* ST_A bacteriophage particles to cell debris. Line scale equals 0.1 µm. PTA.

morphological properties. In all these investigations ultrastructures of these phages were thoroughly studied. Phages active against *L. lactis* and *L. helveticus* were isolated first by Kiuru and Tybeck (47). However, they did not study the morphology of these bacteriophage strains. There are no reports in the literature concerning *L. bulgaricus* bacteriophage despite the commercial importance of it's host organism in the manufacture of yogurt, Swiss, and several Italian cheeses. So, in this investigation for the first time electron micrographs of *L. lactis*, *L. helveticus* and *L. bulgaricus* are presented. These are shown in Figures 10, 11, 12, and 13. These phages had the following measurements: *L. helveticus* - head diameter - 54 nm, tail width - 9 nm, tail length - 234 nm, width of the contracted tail sheath - 19.5nm, and length of the contracted tail sheath - 138 nm; *L. bulgaricus* LB_2 - head diameter - 50 nm, tail width - 5 nm, tail length - 175 nm, width of the contracted tail sheath - 17.5 nm, and length of the contracted tail sheath - 77 nm; *L. lactis* - head diameter - 50 nm, tail width - 7.5 nm, and tail length - 198 nm; *L. bulgaricus* LB_1 - head diameter - 59.4 nm, tail width - 6.6 nm, and tail length 198 nm.

Over-all, the phages active against species of *Lactobacillus* are relatively smaller and more slender than *S. thermophilus* bacteriophages. Phage active against *L. helveticus* and *L. bulgaricus* LB_2 exhibited contractile tail

Figure 10. *L. helveticus* bacteriophage with distinct contracted tail sheath and a rigid tail. Line scale equals 0.1 μm. Uranyl acetate.

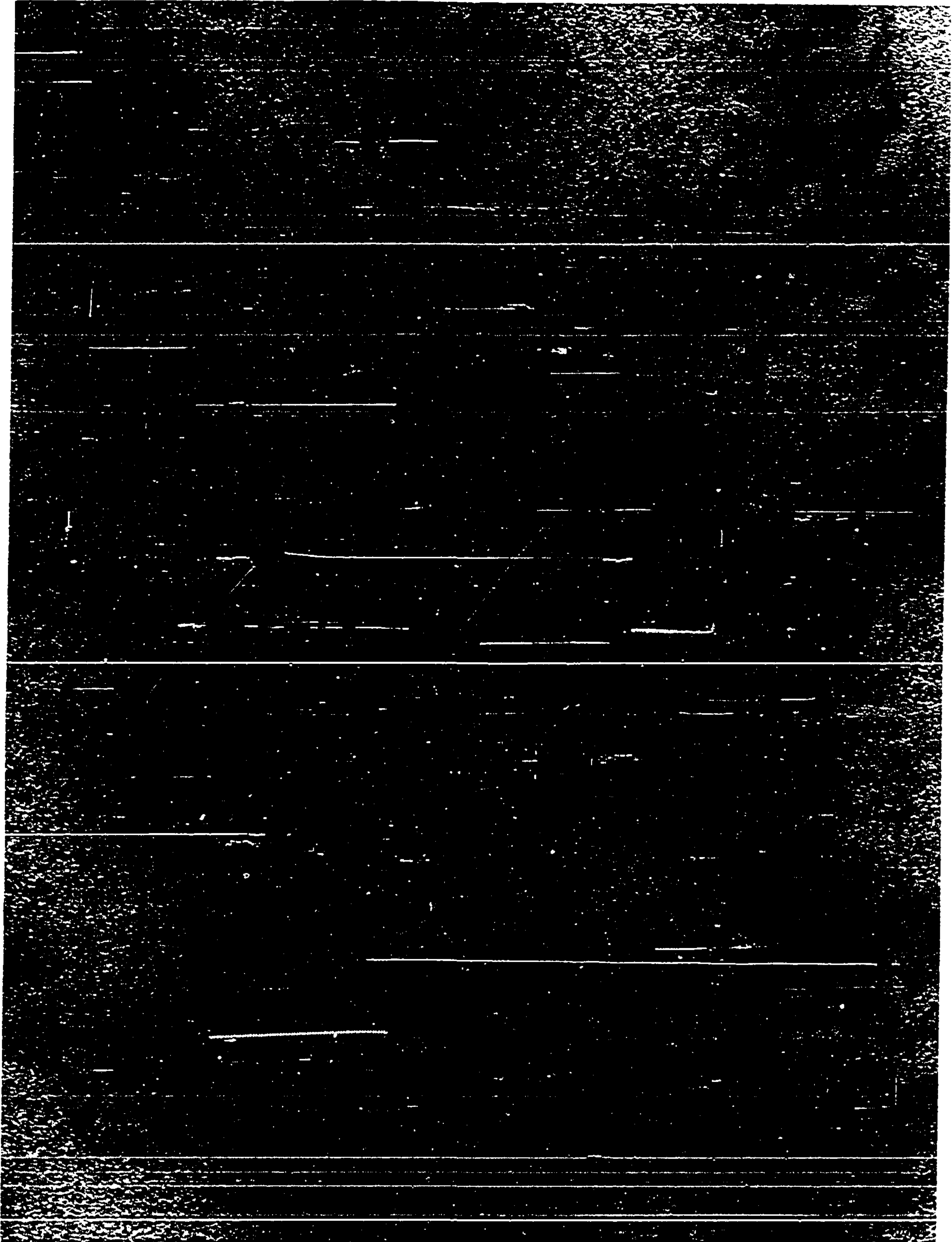


Figure 11. Particles of *L. bulgaricus* LB_2 bacteriophage with contracted tail sheaths. Line scale equals 0.1 µm. PTA.

Figure 12. Electron micrograph showing bacteriophage particles of *L. lactis*. Line scale equals 0.1 μm. PTA.

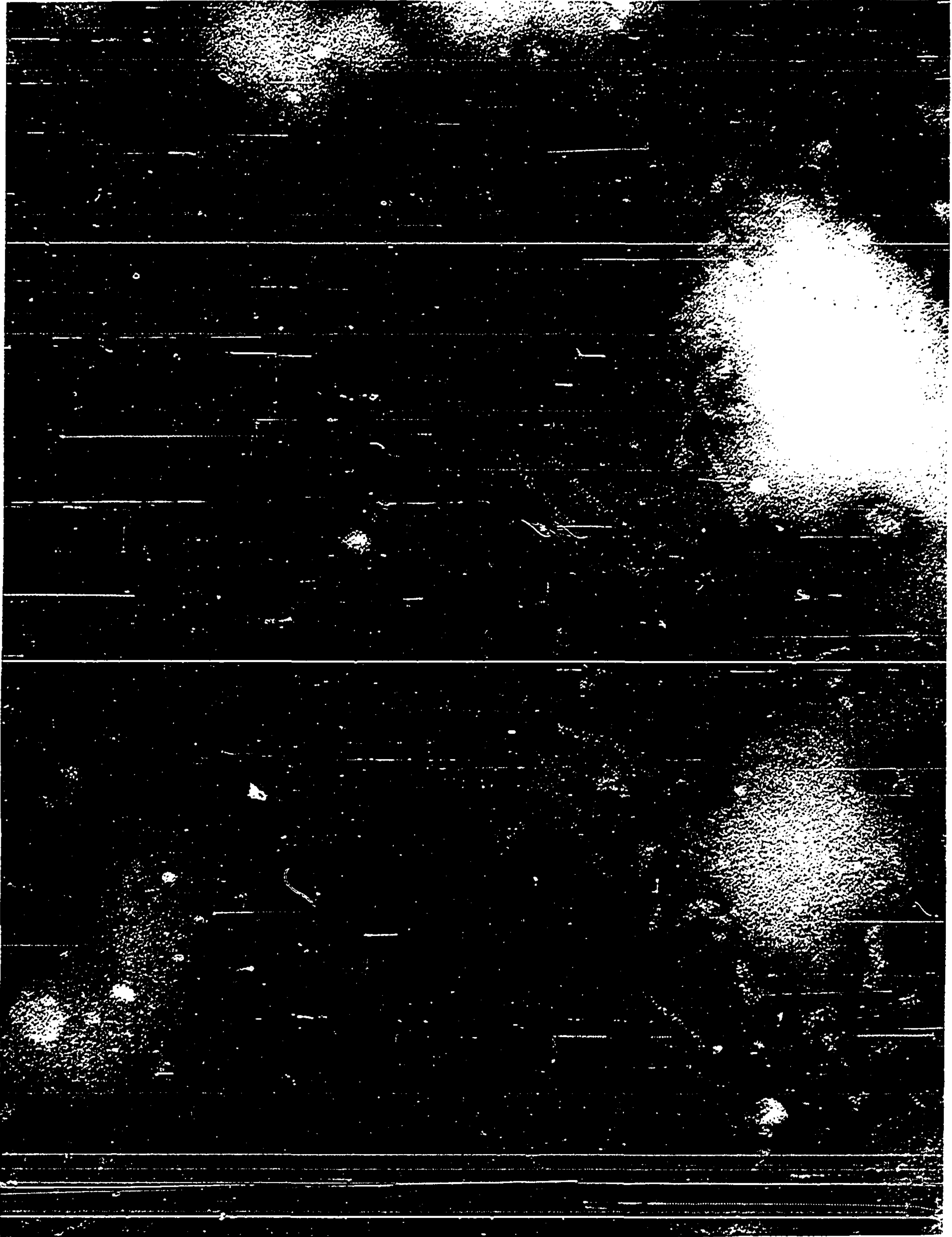
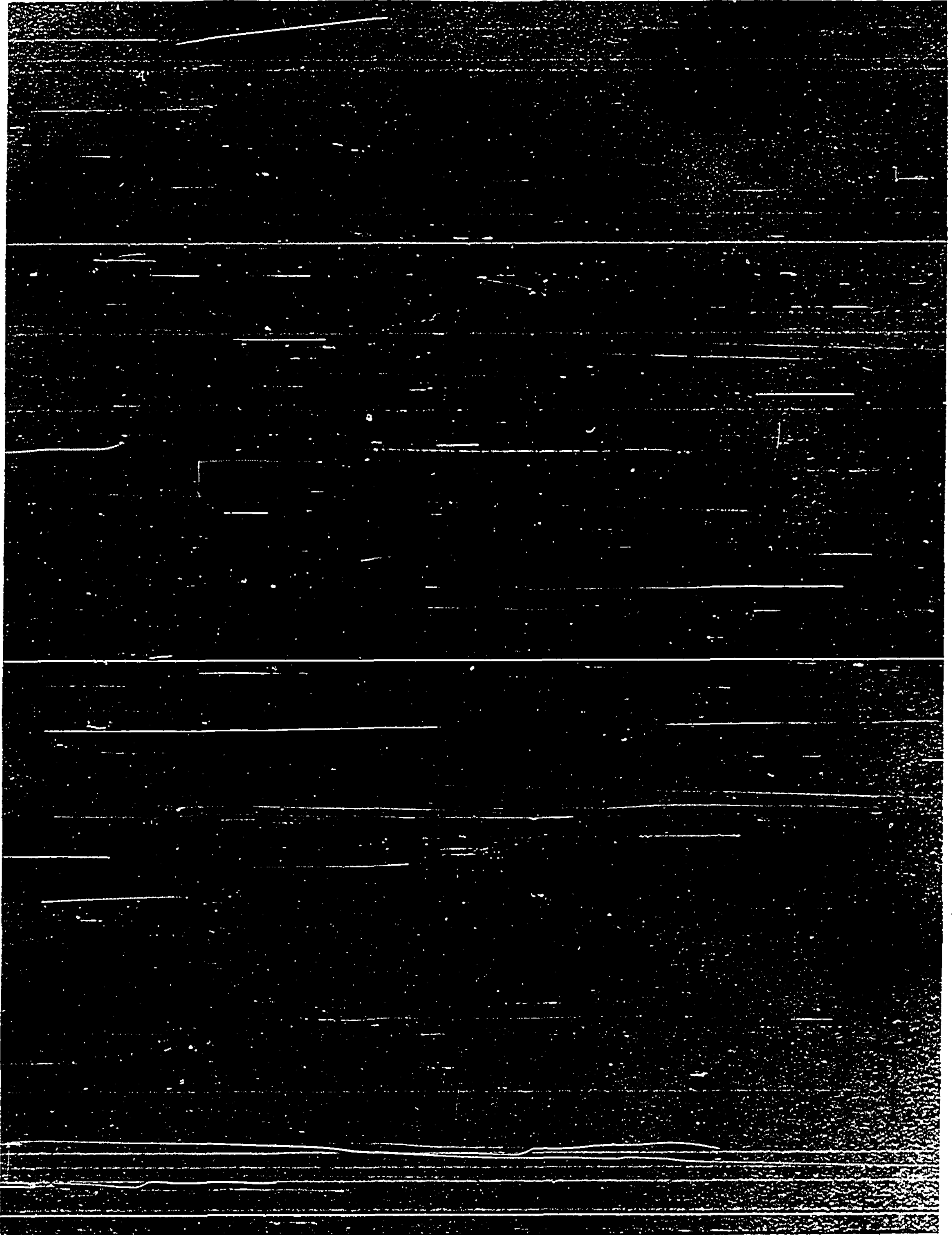


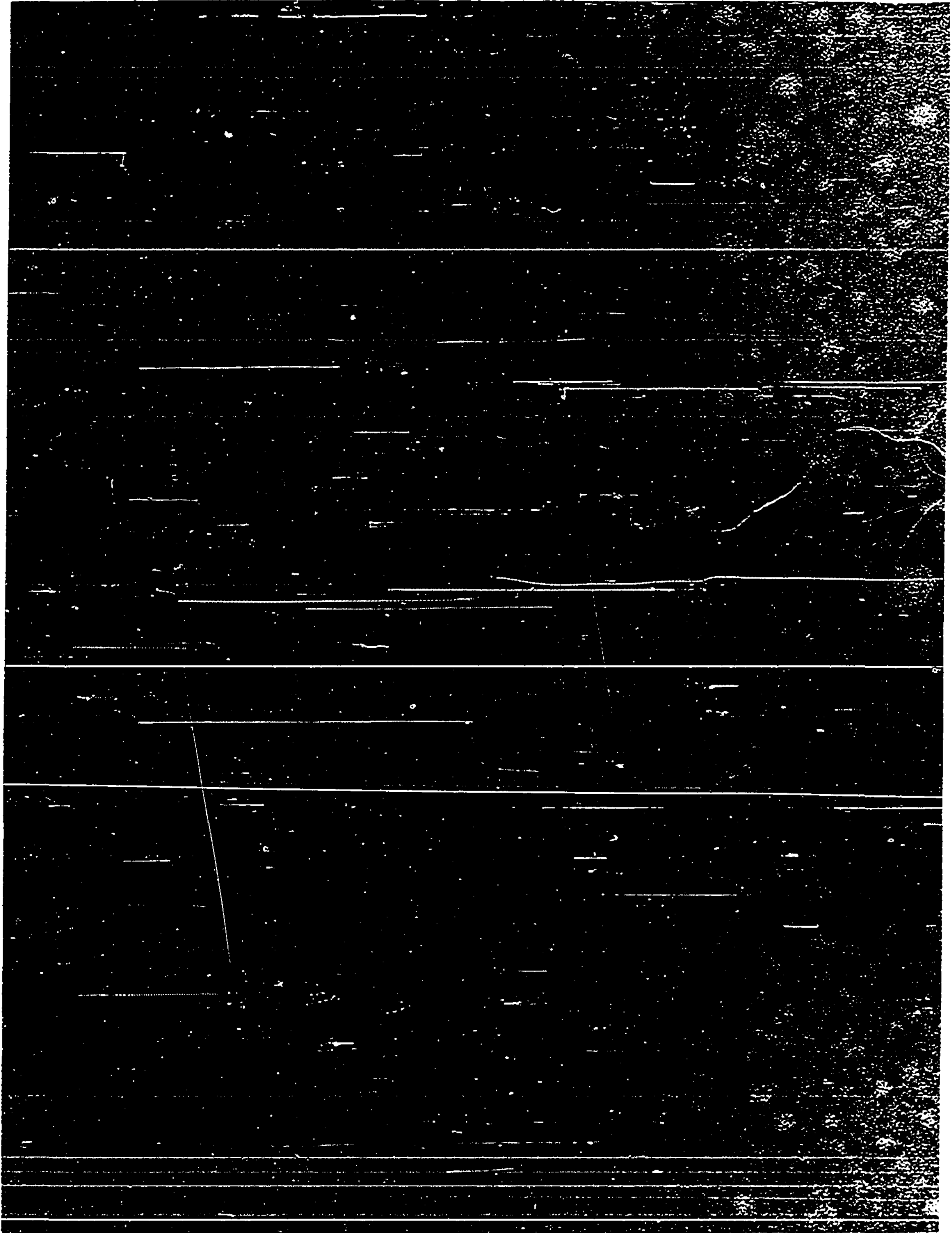
Figure 13. A group of *L. bulgaricus* LB_1 bacteriophage particles. Line scale equals 0.1 μm. PTA.



sheaths. A contractile tail sheath was first observed in T-even phage (87). It has been hypothesized that in T-even phages contraction of tail sheath assists the inner core of the tail to pierce the cell wall (49). The same role may be played by the contractile sheath of Lactobacillus phages. The specimens of L. helveticus and L. bulgaricus, negatively stained with 2% phosphotungstic acid, always revealed contracted tail sheaths. In none of the instances were tail sheath extending all along the length of tail observed. Also, the sheath was contracted both to the proximal and distal extremity of the tail. It has been reported that phage No. 1 of Bacillus mycoides was labile and its tail sheath becomes contracted even during preparation of electron microscopic specimens. This could be a possible explanation for the appearance of Lactobacillus phage sheath in contracted condition in most of our electron micrographs. However, L. bulgaricus phage when stained with uranyl acetate exhibited both the uncontracted long sheaths and contracted short sheaths. This is illustrated in Figure 14.

Lactobacillus bulgaricus LB_2 bacteriophage exhibited the following measurements: width of the uncontracted sheath - 17.5 nm, width of the contracted sheath - 20 nm, length of the uncontracted sheath - 175 nm, and length of the contracted sheath - 85 nm. The length of the tail sheath was reduced to approximately one-half of the normal uncontracted

Figure 14. Particles of *L. bulgaricus* LB_2 bacteriophage showing both contracted and uncontracted tail sheaths. Line scale equals 0.1 μm. Uranyl acetate.



sheath upon contraction. Also, the width of the tail sheath was increased from 17.5 nm to 20 nm upon contraction.

Associative Growth Studies

Streptococcus thermophilus, species of *Lactobacillus*, and occasionally lactic cultures are used in the manufacture of Swiss, Italian cheeses, and yogurt (71). Reports in the literature are limited concerning the effect of lysis of any of these species by their specific phages on the growth and acid production by other starters grown in association. The primary aim of this investigation was to study the associative growth effects, and if they existed, to relate these effects to the variations in acid production that frequently occur during the manufacture of fermented dairy products. To study the effect of cellular lysis by specific bacteriophages on the associative growth of *S. thermophilus*, *Lactobacillus*, and *S. lactis*, experiments were conducted using various double and triple combinations of these species.

Effect of varying concentrations of S. thermophilus phage on acid production by S. thermophilus in skim milk

Two different strains of *S. thermophilus* bacteriophage were included in this study. The concentration of *S. thermophilus* ST_A at the time of inoculation was 37 X 10^7/ml. Whereas the undiluted phage stock had a titer of 100 X 10^7/ml.

The per cent developed acidities produced by *S. thermophilus* ST_A in the absence and presence of varying concentrations of it's specific phage were presented in Figure 15. The results of this experiment showed that significant reductions in acid production occur even if 20 phage particles/ml of milk were present. When the concentration of *S. thermophilus* bacteriophage was raised to a level of a few millions/ml, *S. thermophilus* ceased to produce acid. The amount of acid production varied with the concentration of bacteriophage inoculated.

Figure 16 presents similar data obtained with *S. thermophilus* strain ST_4. Here the concentration of *S. thermophilus* ST_4 at the time of inoculation was 110 X 10^7/ml; it's phage was 53 X 10^7/ml. With this strain significant reduction in acid productions were obtained when 1100 phage particles/ml were present in milk.

Deane et al. (30) announced that the pasteurization of milk for cheese making cannot be considered adequate protection against *S. thermophilus* bacteriophage. The results obtained from the present investigation showed that even low titers of phage when present in milk might affect the production of acid by *S. thermophilus*, so the presence of *S. thermophilus* phage can be a potential hazard for the manufacture of several varieties of cheeses and yogurt.

Figure 15. Effect of varying concentrations of *S. thermophilus* phage (Ø) on acid production by *S. thermophilus* ST_A in milk.

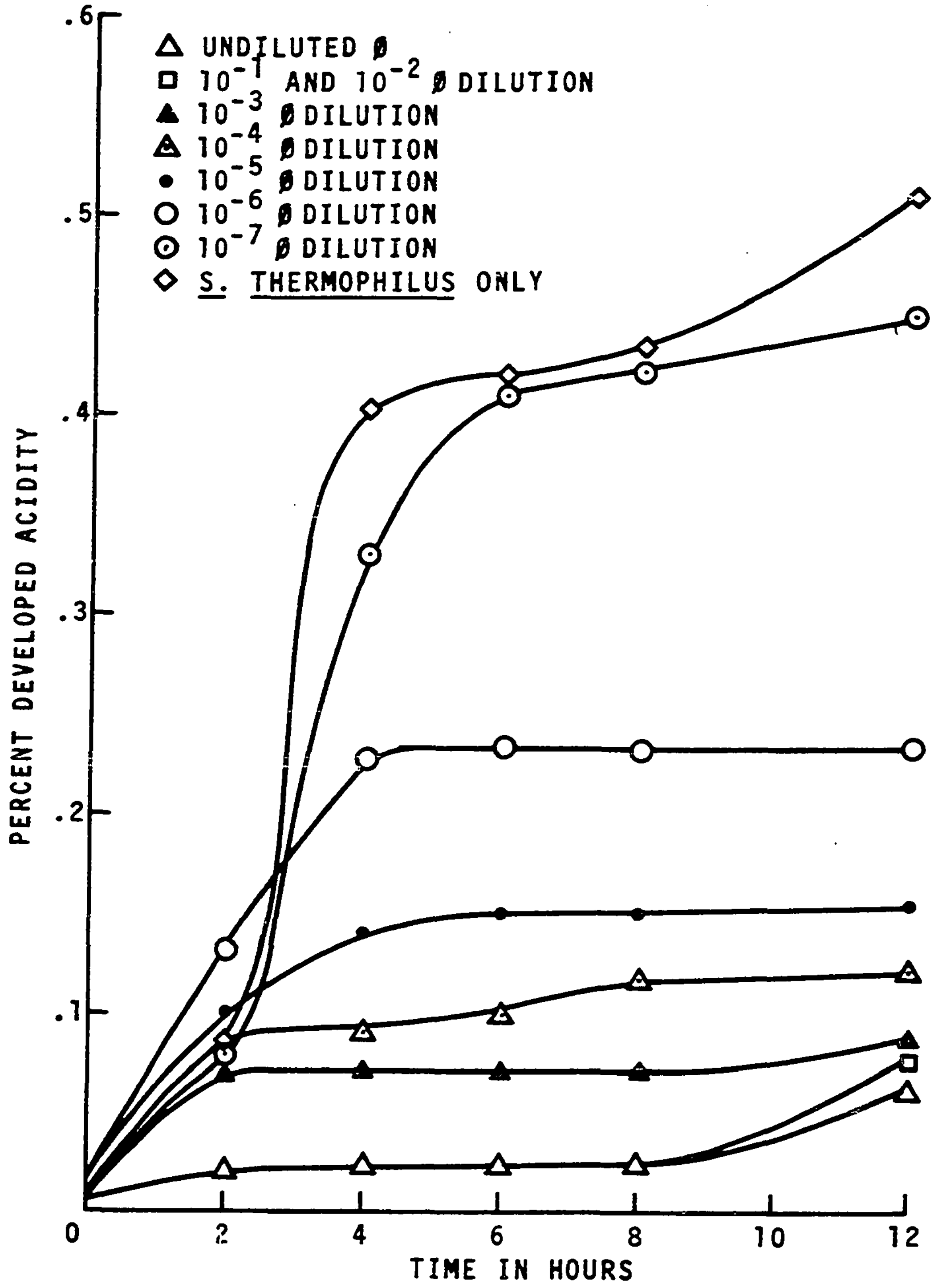
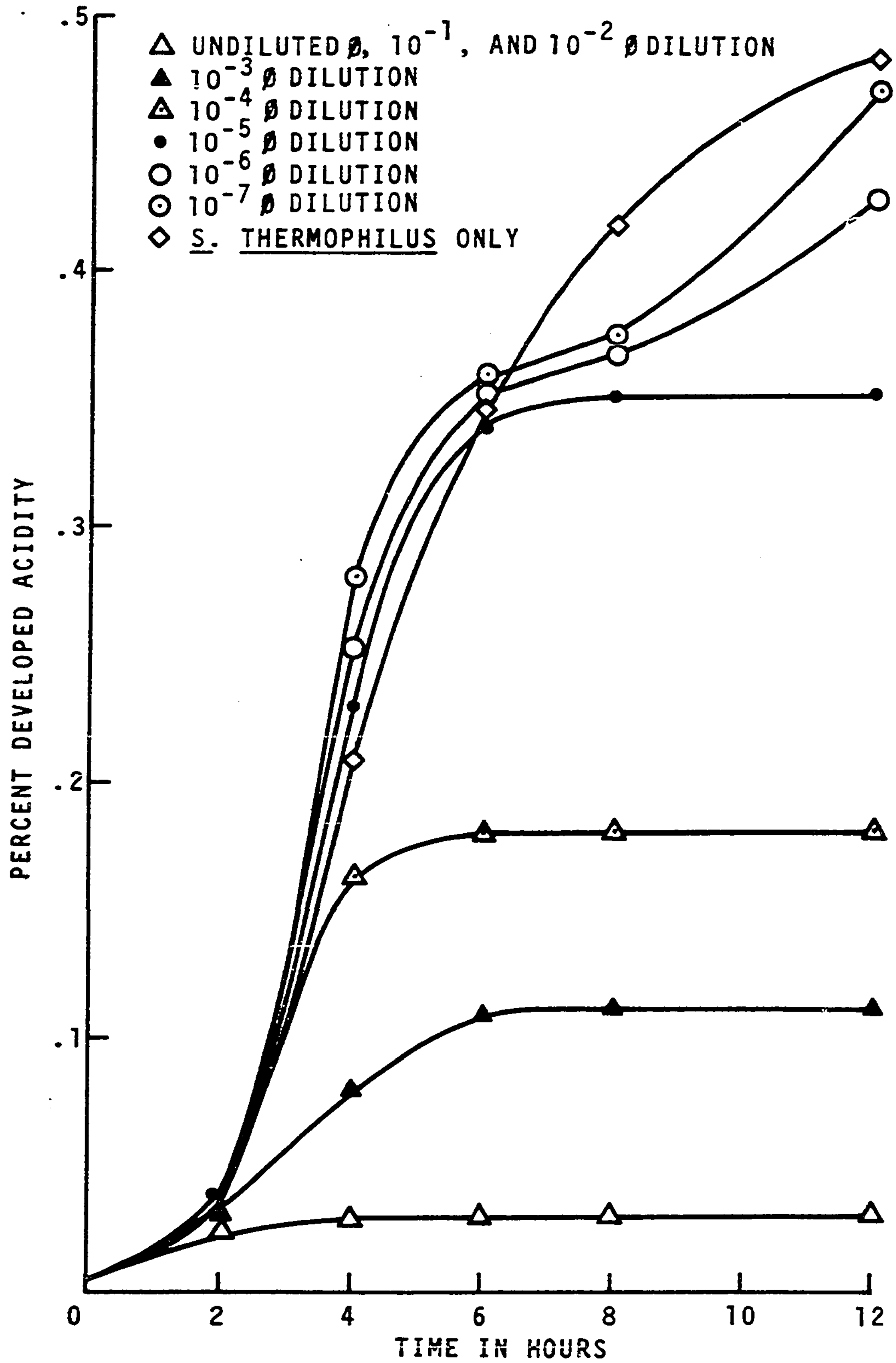

UNDILUTED Ø
10^{-1} AND 10^{-2} Ø DILUTION
10^{-3} Ø DILUTION
10^{-4} Ø DILUTION
10^{-5} Ø DILUTION
10^{-6} Ø DILUTION
10^{-7} Ø DILUTION
S. THERMOPHILUS ONLY
PERCENT DEVELOPED ACIDITY
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

Figure 16. Effect of varying concentrations of *S. thermophilus* phage (Ø) on acid production by *S. thermophilus* ST_4 in milk.


UNDILUTED Ø, 10^{-1}, AND 10^{-2} Ø DILUTION
10^{-3} Ø DILUTION
10^{-4} Ø DILUTION
10^{-5} Ø DILUTION
10^{-6} Ø DILUTION
10^{-7} Ø DILUTION
S. THERMOPHILUS ONLY
PERCENT DEVELOPED ACIDITY
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

Effect of cellular lysis by specific bacteriophage on associative growth of S. thermophilus and S. lactis strains as measured by acid production

The counts/ml of *S. thermophilus* ST_A and *S. lactis* C_2 and their specific phages at 0 h of incubation in milk were as follows: *S. thermophilus* ST_A - 24 X 10^6; *S. thermophilus* $ST_A\emptyset$ - 45 X 10^6; *S. lactis* C_2 - 110 X 10^5; *S. lactis* $C_2\emptyset$ - 53 X 10^4. The results of this experiment are presented in Figure 17. The amount of acid produced by the mixture of *S. thermophilus* ST_A and *S. lactic* C_2 was much higher than the acids produced individually by each culture. When phage active against *S. thermophilus* was added to the mixed culture of *S. thermophilus* ST_A and *S. lactis* C_2, as expected, acid productions were distinctly lower than the control. Still, this amount of acid produced was slightly higher than by *S. lactis* C_2 alone. This might indicate that the cell contents of *S. thermophilus*, liberated upon phage lysis, were slightly stimulatory to *S. lactis* C_2.

When *S. lactis* C_2 was phaged in a mixed culture, the amount of acid produced was lower than the mixed culture without any phage. Again, the amount of acid produced by *S. thermophilus* ST_A in the presence of phage-lysed *S. lactis* C_2 was much higher between 2 to 10 h of incubation, compared to *S. thermophilus* alone. This increase in acid during the early

Figure 17. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. lactis* C_2 (SL) and *S. thermophilus* ST_A (ST) strains as measured by acid production.

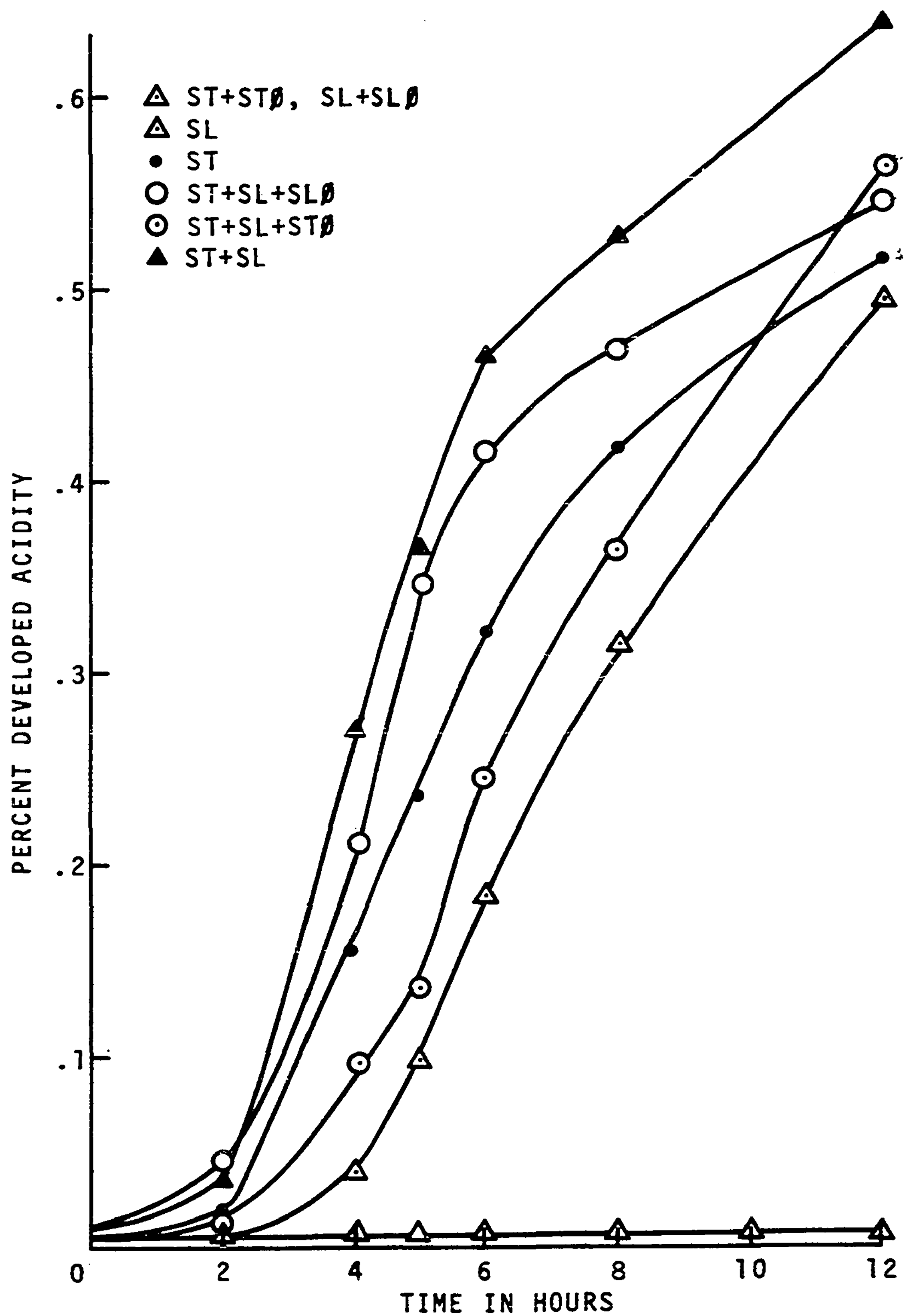
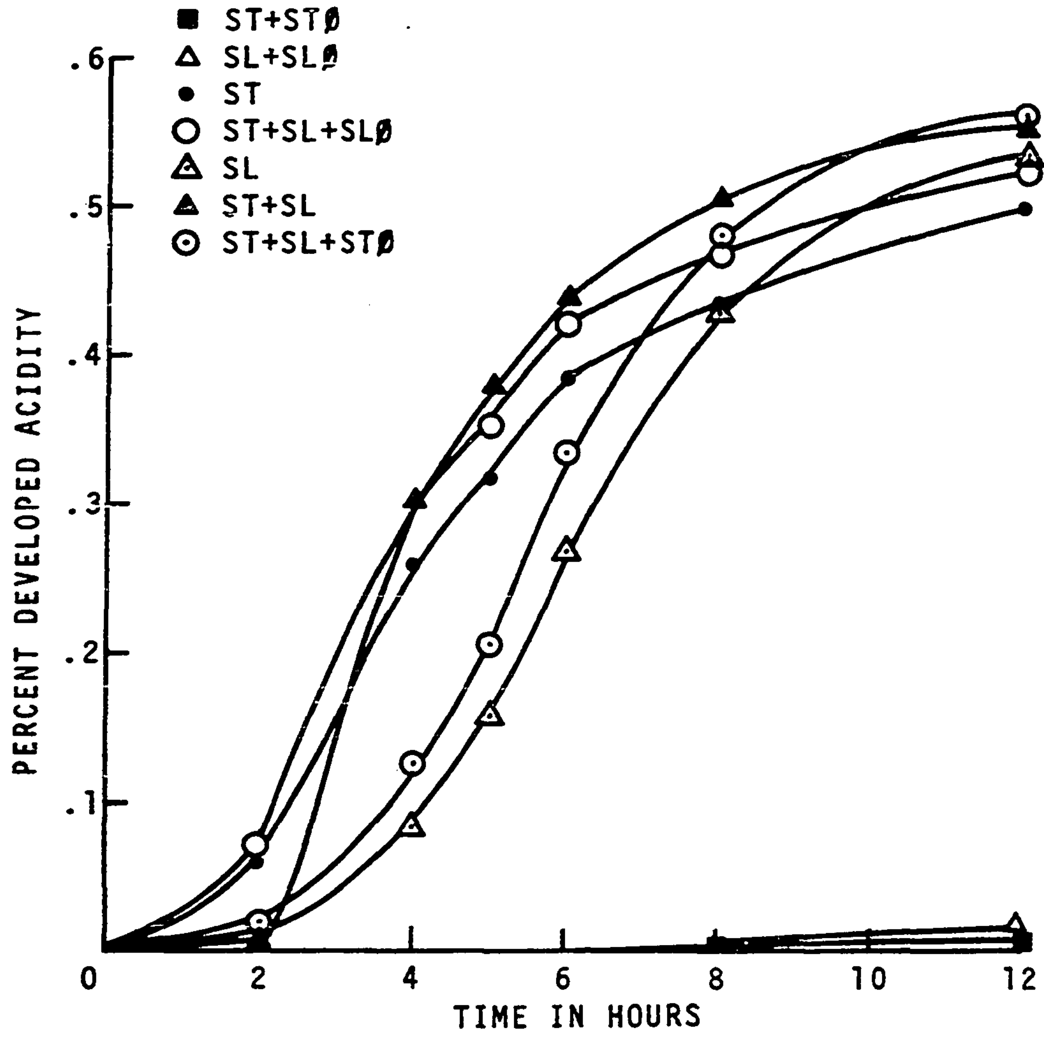

ST+STØ, SL+SLØ
SL
ST
ST+SL+SLØ
ST+SL+STØ
ST+SL
PERCENT DEVELOPED ACIDITY
.1
.2
.3
.4
.5
.6
0
2
4
6
8
10
12
TIME IN HOURS

Figure 18. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. lactis* MS (SL) and *S. thermophilus* ST_4 (ST) strains as measured by acid production.


ST+STØ
SL+SLØ
ST
ST+SL+SLØ
SL
ST+SL
ST+SL+STØ
PERCENT DEVELOPED ACIDITY
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

stages of growth may be due to both stimulation of *S. thermophilus* and or growth of *S. lactis* C_2 until it is completely lysed.

The acid production was completely stopped when phage active against *S. thermophilus* ST_A and *S. lactis* C_2 were added individually to their homologous hosts. Also, no reduction or increase in acid production was observed when *S. lactis* phage was added to *S. thermophilus* in skim milk and vice versa. This signifies that these phages were specific and did not lyse other species.

This experiment was repeated using two different strains: *S. lactis* MS and *S. thermophilus* ST_4. The counts/ml of *S. thermophilus* ST_4 and *S. lactis* MS and their specific phages, at 0 h of incubation in milk were as follows: *S. thermophilus* ST_4 - 24×10^5; *S. thermophilus* $ST_4Ø$ - 53×10^6; *S. lactis* MS - 100×10^5; *S. lactis* MSØ - 21×10^5.

Effect of lysis by specific bacteriophage on associative growth of Lactobacillus and S. lactis

To study the associative growth relationships of *Lactobacillus* and *S. lactis* in the presence and absence of their phage, strains of *S. lactis* were grown with a strain of *L. helveticus* and of *L. lactis* along with their specific phages in appropriate combinations.

The bacterial and phage counts/ml of *L. helveticus* and *S. lactis* C_2, at 0 h of incubation/ml in milk were as follows:

S. lactis C_2 - 120 X 10^5; *S. lactis* $C_2\emptyset$ - 72 X 10^4; *L. helveticus* - 95 X 10^5; *L. helveticus* ∅ - 20 X 10^5. The results are presented in Figures 19 and 20. The amount of acid produced by a mixed culture of *S. lactis* C_2 and *L. helveticus* was much higher than the individual cultures. When phage active against either *S. lactis* C_2 or *L. helveticus* was added to the mixture, the total amount of acid production was significantly lower than the mixed culture. Seemingly phage lysate of one species did not have any observable effect on the growth and acid production of the other species.

Similar trends in acid productions were obtained when the combination of *L. lactis* and *S. lactis* MS were used. The results of these experiments are presented in Figure 20. The bacterial and phage counts/ml of *L. lactis* and *S. lactis* MS, at 0 h of incubation in milk were as follows: *L. lactis* - 42 X 10^5; *L. lactis* ∅ - 52 X 10^3; *S. lactis* MS - 70 X 10^5; S. lactis MS ∅ - 28 X 10^4.

Effect of cellular lysis by specific bacteriophage on associative growth of S. thermophilus and Lactobacillus

Figure 21 presents the associative growth relationships between *S. thermophilus* ST_A and *L. helveticus*, in the presence and absence of their specific phage. The bacterial and phage counts/ml at 0 h of incubation in milk were as follows: *S. thermophilus* ST_A - 160 X 10^4; *S. thermophilus* $ST_A\emptyset$ -

Figure 19. Effect of cellular lysis by bacteriophage (Ø) on associative growth of S. lactis C_2 (SL) and L. helveticus (LH) strains as measured by acid production.

△ SL+SLØ
◇ LH+LHØ
SL
□ LH+SL+LHØ
LH+SL+SLØ
◆ LH
▼ LH+SL

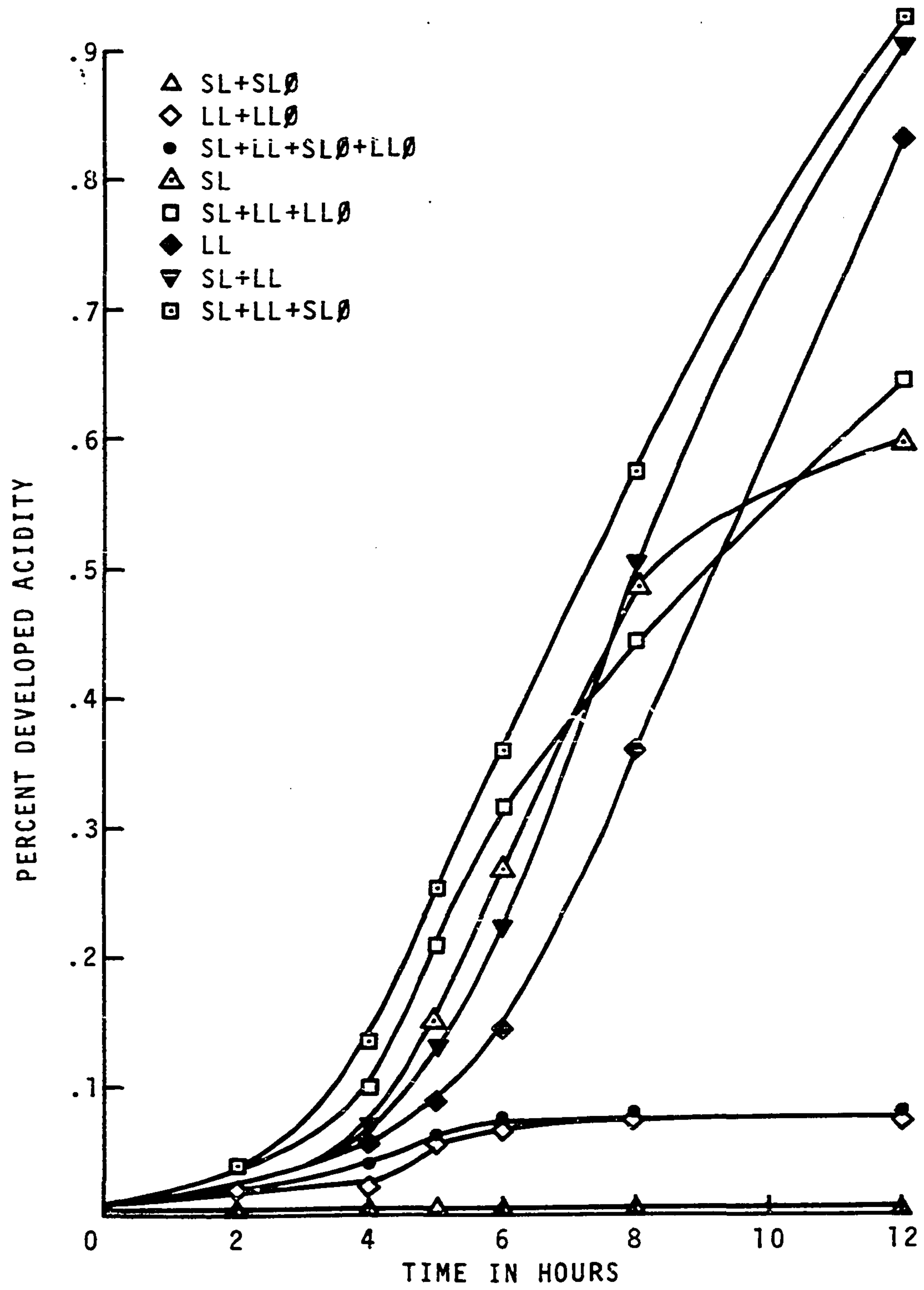
PERCENT DEVELOPED ACIDITY

1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0

2
4
6
8
10
12

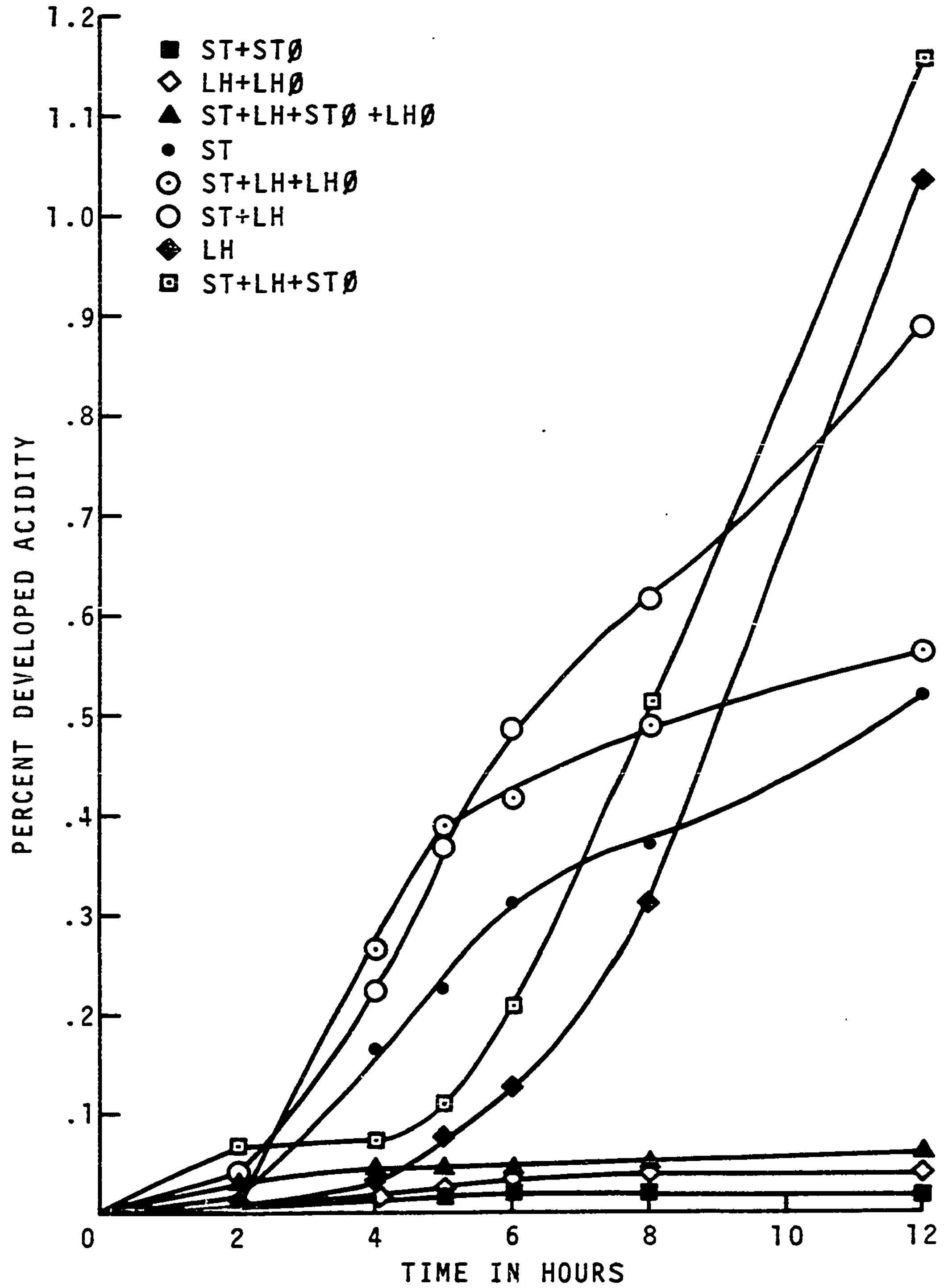
TIME IN HOURS

Figure 20. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *L. lactis* (LL) and *S. lactis* MS (SL) strains as measured by acid production.



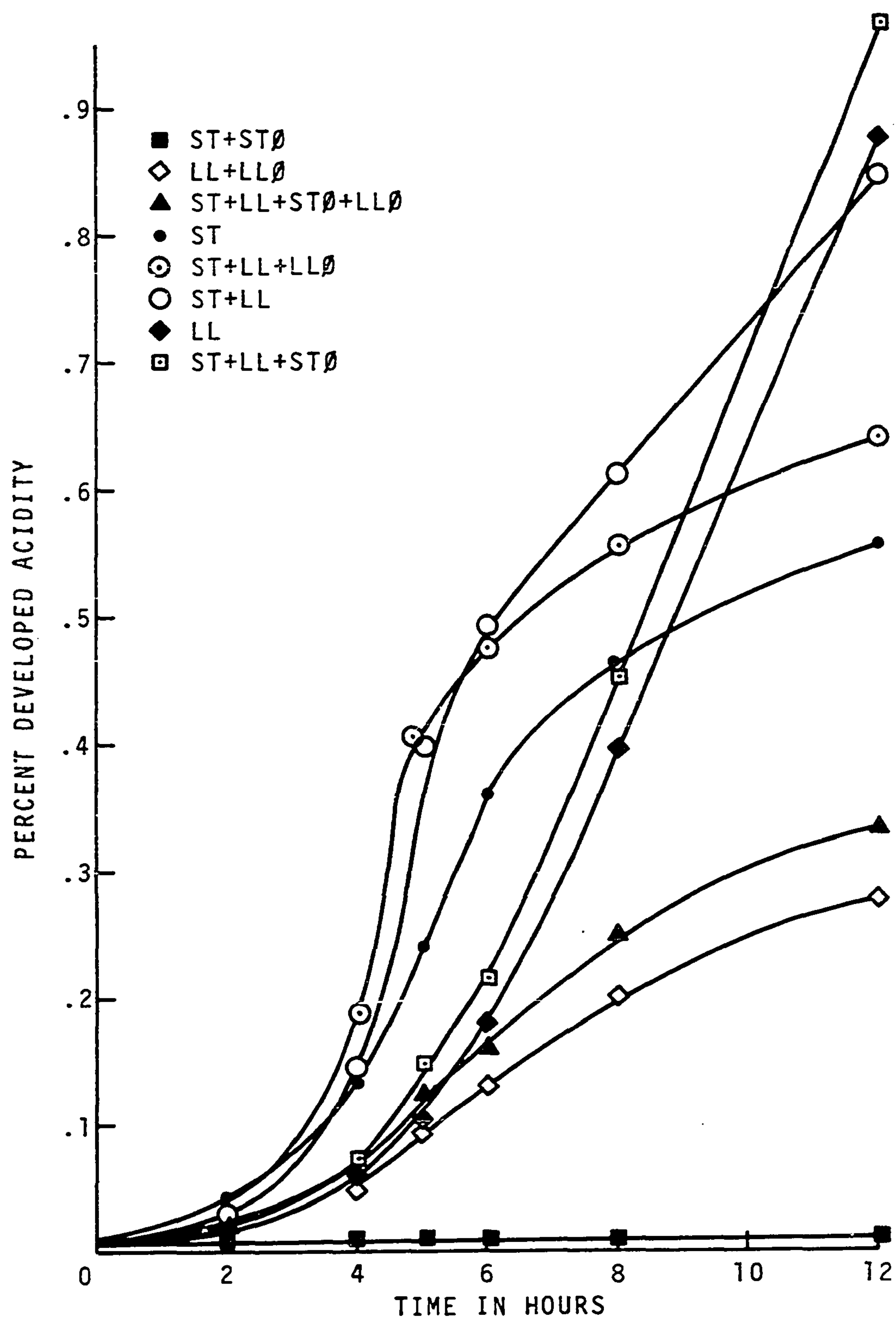
SL+SLØ
LL+LLØ
SL+LL+SLØ+LLØ
SL
SL+LL+LLØ
LL
SL+LL
SL+LL+SLØ
PERCENT DEVELOPED ACIDITY
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

Figure 21. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. thermophilus* ST_A (ST) and *L. helveticus* (LH) strains as measured by acid production.


1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
ST+STØ
LH+LHØ
ST+LH+STØ +LHØ
ST
ST+LH+LHØ
ST+LH
LH
ST+LH+STØ
PERCENT DEVELOPED ACIDITY
TIME IN HOURS

38 X 10^6; L. helveticus - 120 X 10^5; and L. helveticus Ø - 150 X 10^4. These data indicate that when specific S. thermophilus ST_A phage was added to a mixture of S. thermophilus ST_A and L. helveticus, the amount of acid production was much higher than the control to which no phage was added. This probably signifies that S. thermophilus competes with L. helveticus during the later part of the growth cycle, even though stimulation occurred at the early part of the growth phase. This was evidenced by the fact that L. helveticus alone produced more acid at 12 h of incubation than the mixture of S. thermophilus ST_A and L. helveticus. Also, the phage lysate of S. thermophilus ST_A, liberated while the mixed culture was growing at 37 C, appears to have a stimulatory effect on L. helveticus. When L. helveticus was lysed in a mixed culture, acid production was significantly low. Similar trends in acid productions were observed when L. lactis and S. thermophilus ST_4 were used. It is illustrated in Figure 22. The bacterial and phage counts/ml at 0 h of incubation in milk were as follows: S. thermophilus ST_4 - 32 X 10^5; S. thermophilus ST_4Ø - 38 X 10^6; L. lactis - 40 X 10^5; and L. lactis Ø - 32 X 10^3. This may be one reason S. thermophilus bacteriophage may not be suspected during the manufacture of Swiss cheese. No such increase in acid production, in the presence of bacteriophage was observed with two-strain mixtures involving different strains of S. lactis and species

Figure 22. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *L. lactis* (LL) and *S. thermophilus* ST_4 (ST) strains as measured by acid production.



ST+STØ
LL+LLØ
ST+LL+STØ+LLØ
ST
ST+LL+LLØ
ST+LL
LL
ST+LL+STØ
PERCENT DEVELOPED ACIDITY
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

of Lactobacillus.

Increased acid production when S. thermophilus was phaged was also found in triple-species mixtures of S. lactis C_2, L. helveticus, and S. thermophilus ST_A, and S. lactis MS, L. lactis, and S. thermophilus ST_4. These data are presented in Figures 23, 24, 25, and 26. In these experiments, the bacterial and phage counts/ml at 0 h of incubation were as follows: S. thermophilus ST_A - 30 X 10^5; S. thermophilus ST_4 - 30 X 10^5; S. lactis C_2 - 120 X 10^6; S. lactis MS - 80 X 10^5; L. helveticus - 190 X 10^5; L. lactis - 120 X 10^5; S. thermophilus ST_AØ - 56 X 10^6; S. thermophilus ST_4Ø - 58 X 10^5; S. lactis C_2Ø - 48 X 10^5; S. lactis MSØ - 32 X 10^5; L. helveticus Ø - 260 X 10^4; and L. lactis Ø - 40 X 10^4.

Effect of lysis of S. thermophilus on acid production of other S. thermophilus strains grown in association

In this experiment two phage-unrelated strains of S. thermophilus and their specific phages were included. The counts/ml of S. thermophilus strains ST_A and ST_4, and their specific phages at 0 h of incubation in milk were as follows: S. thermophilus ST_A - 33 X 10^5; S. thermophilus ST_AØ - 100 X 10^5; S. thermophilus ST_4 - 48 X 10^5; and S. thermophilus ST_4Ø - 45 X 10^5. The results of this experiment are presented in Figure 27. Lysis of one strain of S. thermophilus did not have any observable effect on the acid production of the

Figure 23. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. lactis* C_2 (SL), *S. thermophilus* ST_A (ST), and *L. helveticus* (LH) strains as measured by acid production.

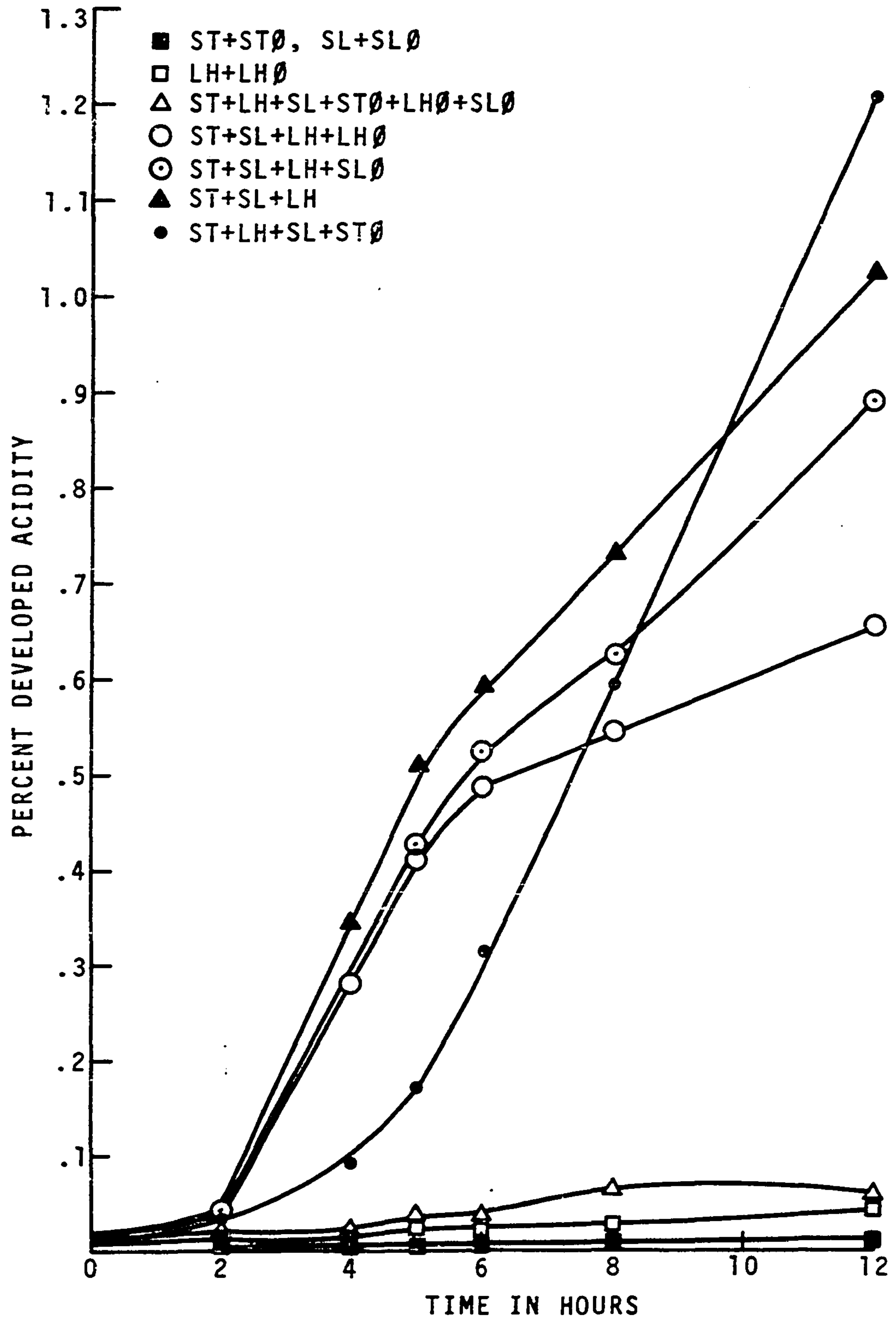
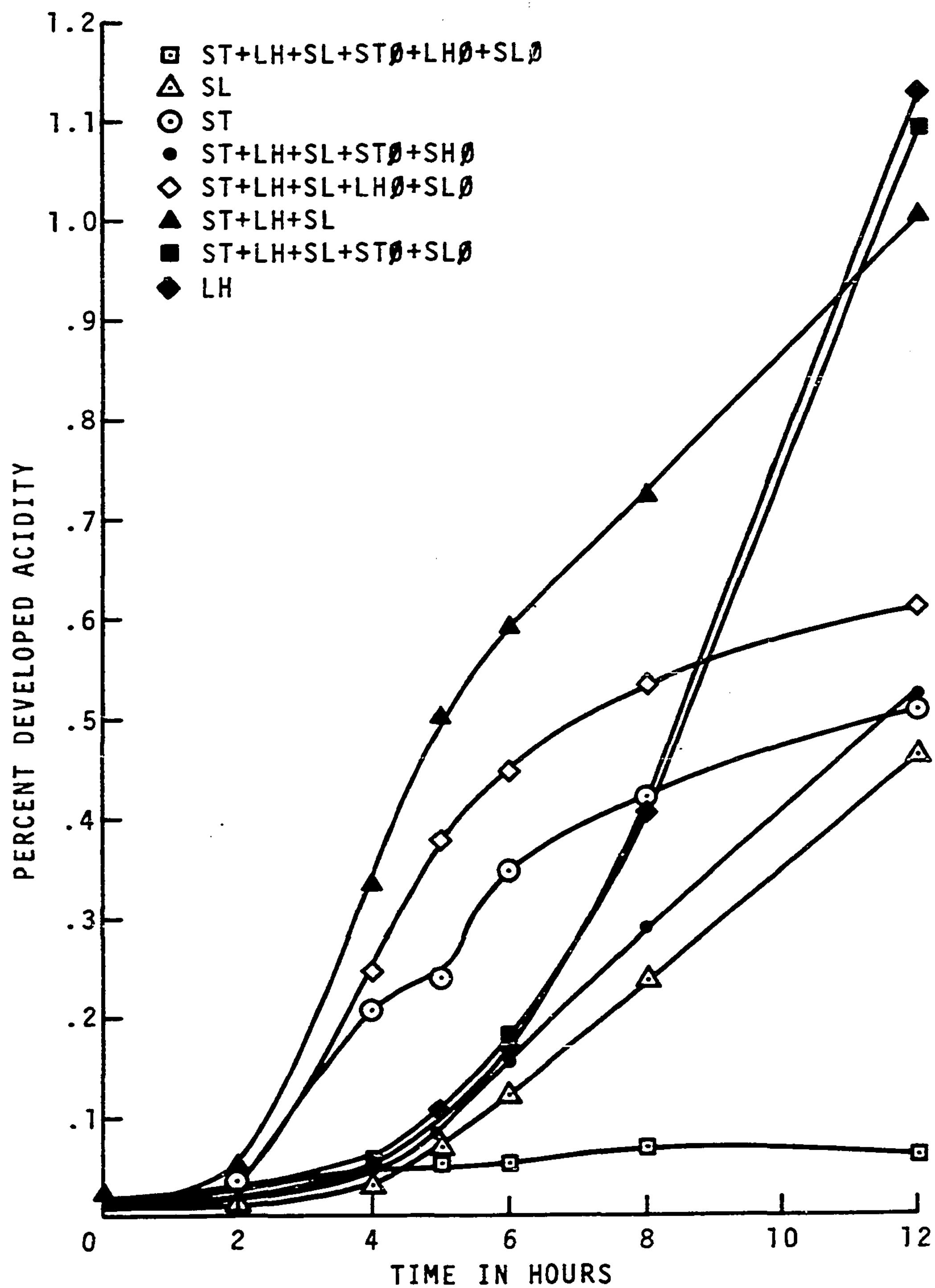
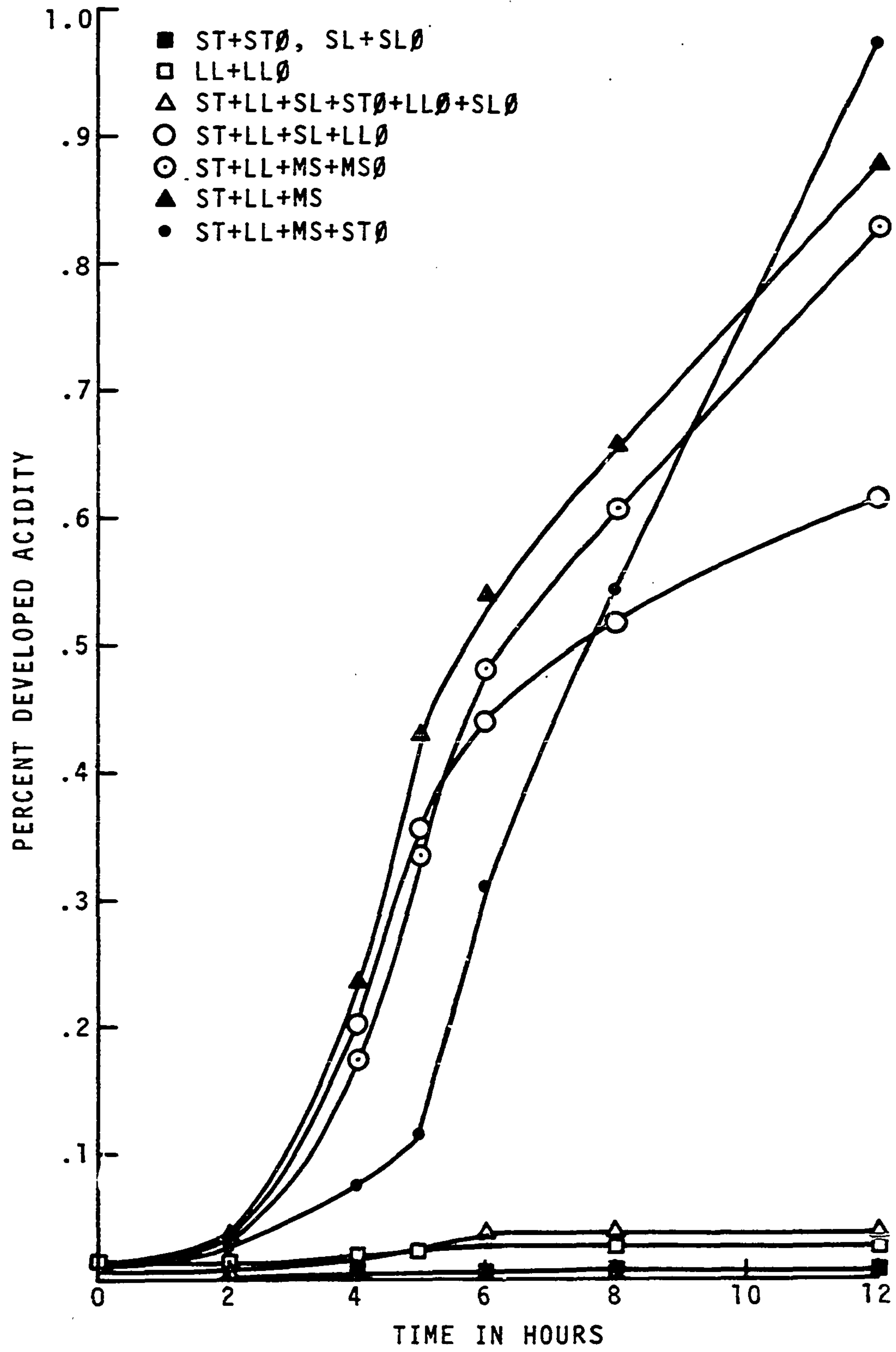

ST+STØ, SL+SLØ
LH+LHØ
ST+LH+SL+STØ+LHØ+SLØ
ST+SL+LH+LHØ
ST+SL+LH+SLØ
ST+SL+LH
ST+LH+SL+STØ
PERCENT DEVELOPED ACIDITY
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

Figure 24. Effect of cellular lysis by bacteriophage (Ø) on associative growth of _S. lactis_ C_2 (SL), _S. thermophilus_ ST_A (ST), and _L. helveticus_ (LH) strains as measured by acid production.



1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
PERCENT DEVELOPED ACIDITY
ST+LH+SL+STØ+LHØ+SLØ
SL
ST
ST+LH+SL+STØ+SHØ
ST+LH+SL+LHØ+SLØ
ST+LH+SL
ST+LH+SL+STØ+SLØ
LH
0
2
4
6
8
10
12
TIME IN HOURS

Figure 25. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. lactis* MS (SL), *S. thermophilus* ST_4 (ST), and *L. lactis* (LL) strains as measured by acid production.



1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
PERCENT DEVELOPED ACIDITY
ST+STØ, SL+SLØ
LL+LLØ
ST+LL+SL+STØ+LLØ+SLØ
ST+LL+SL+LLØ
ST+LL+MS+MSØ
ST+LL+MS
ST+LL+MS+STØ
0
2
4
6
8
10
12
TIME IN HOURS

Figure 26. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. lactis* MS (SL), *S. thermophilus* ST_4 (ST), and *L. lactis* (LL) strains as measured by acid production.

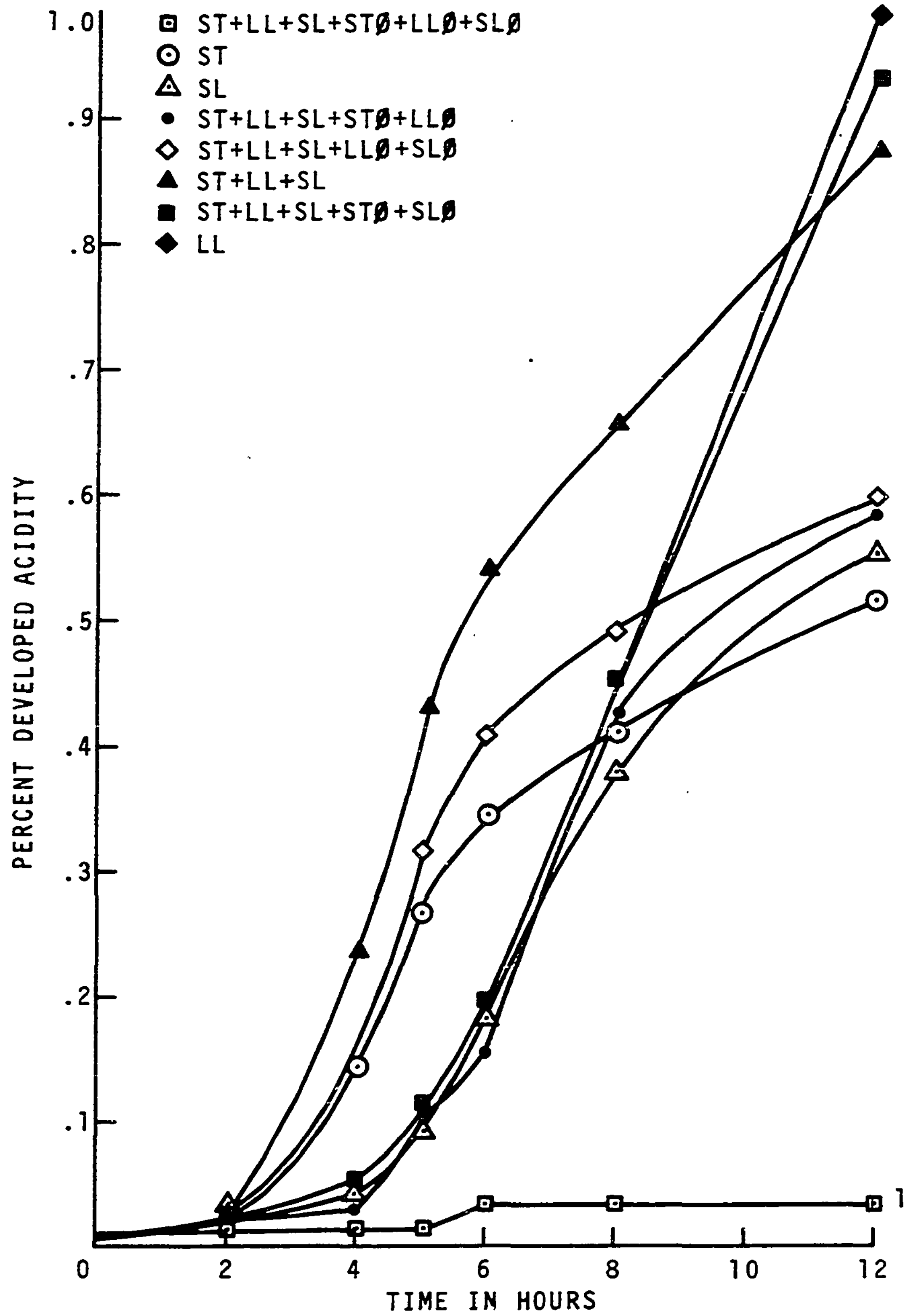
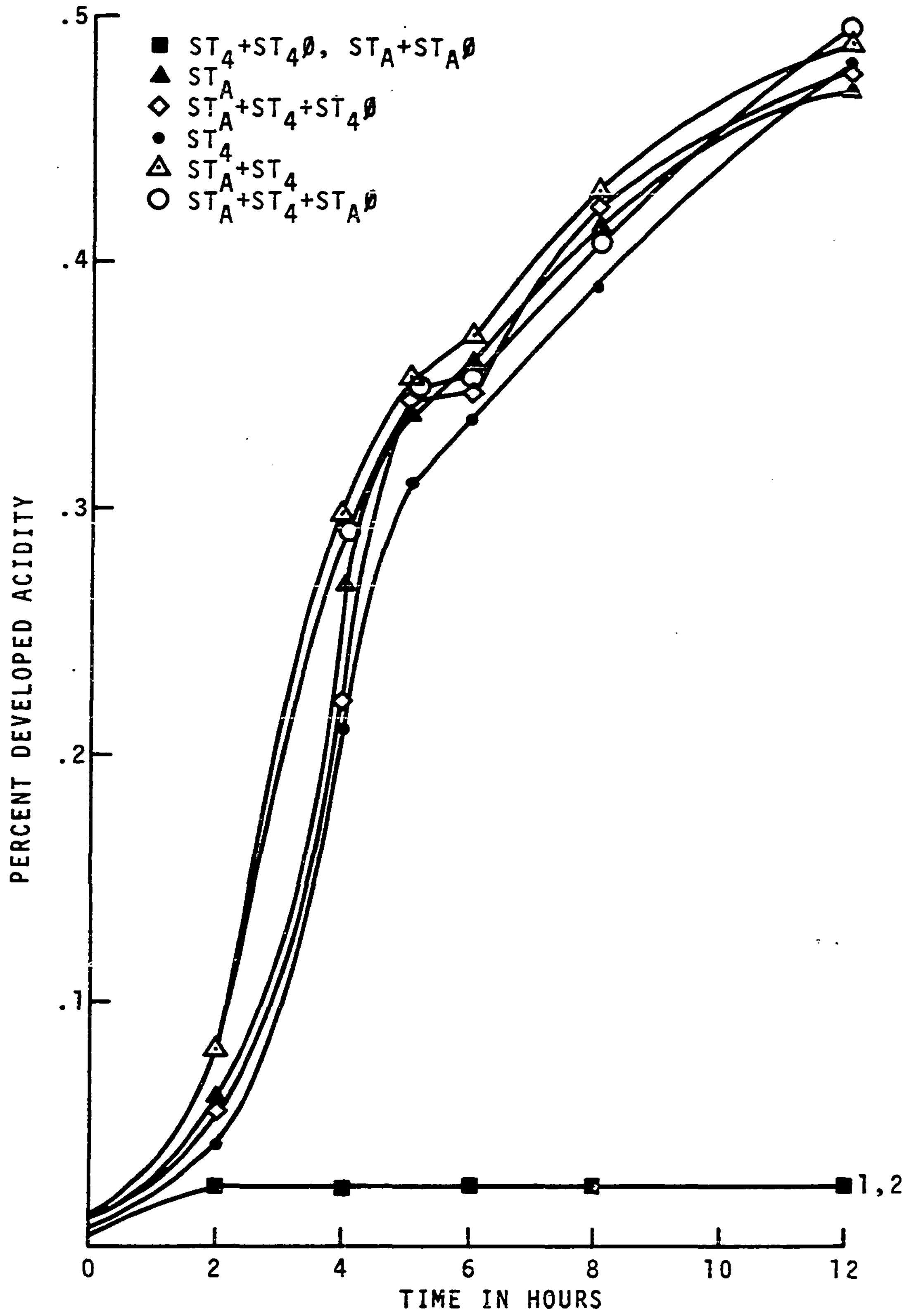

ST+LL+SL+STØ+LLØ+SLØ
ST
SL
ST+LL+SL+STØ+LLØ
ST+LL+SL+LLØ+SLØ
ST+LL+SL
ST+LL+SL+STØ+SLØ
LL
PERCENT DEVELOPED ACIDITY
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS
1

Figure 27. Effect of cellular lysis by bacteriophage (Ø) on associative growth of *S. thermophilus* strains (ST_4,ST_A) as measured by acid production.

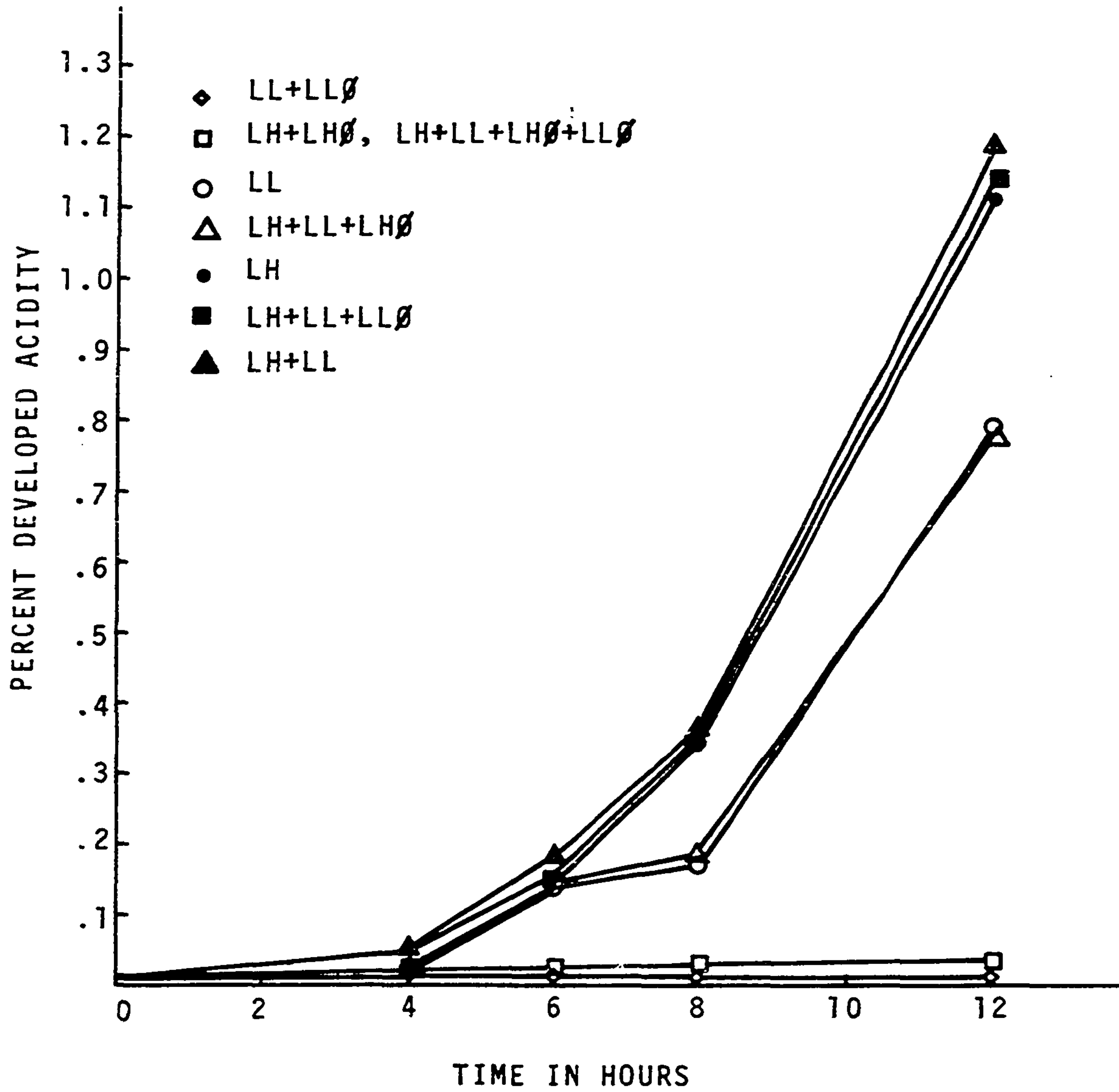

ST4+ST4Ø, STA+STAØ
STA
STA+ST4+ST4Ø
ST4
STA+ST4
STA+ST4+STAØ
PERCENT DEVELOPED ACIDITY
.1
.2
.3
.4
.5
0
2
4
6
8
10
12
1,2
TIME IN HOURS

other strain of *S. thermophilus* when they were grown together. So, mixed cultures of *S. thermophilus* could be effectively used to overcome phage problems during the manufacture of Swiss and Italian cheeses, and yogurt.

Effect of lysis of species of Lactobacillus on acid production of other species of Lactobacillus grown in association

Lactobacillus helveticus and *L. lactis* and their specific phages were included in this study. The counts/ml of *L. helveticus*, *L. lactis*, and their specific phages at 0 h of incubation in milk were as follows: *L. helveticus* - 80×10^5; *L. helveticus* Ø - 32×10^5; *L. lactis* - 65×10^5; and *L. lactis* Ø - 200×10^4. The results of this investigation are presented in Figure 28. When *L. helveticus* was phaged in a mixed culture of *L. helveticus* and *L. lactis*, the amount of acid produced was closer to the amount produced by *L. lactis* alone. A similar response was observed with *L. helveticus*. So, in a mixed culture even if one component is lysed by phage the other strain will still be active and produce enough acid. This observation will have practical application in the dairy industry to overcome problems associated with phages active against *Lactobacillus*.

Figure 28. Effect of cellular lysis by bacteriophage (Ø) on associative growth of species of *Lactobacillus* as measured by acid production.


LL+LLØ
LH+LHØ, LH+LL+LHØ+LLØ
LL
LH+LL+LHØ
LH
LH+LL+LLØ
LH+LL
PERCENT DEVELOPED ACIDITY
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
TIME IN HOURS

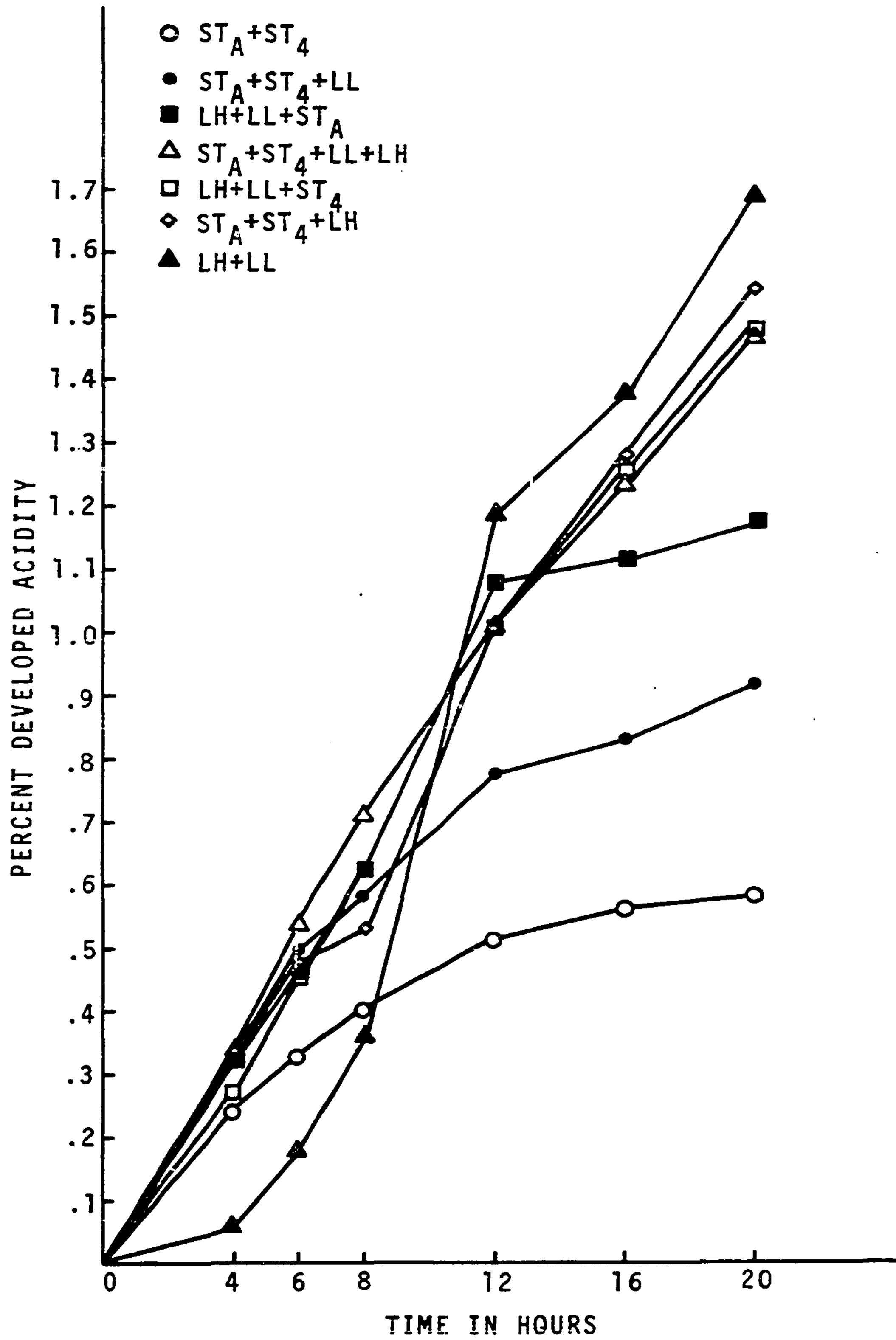
Effect of cellular lysis by bacteriophage on associative growth of strains of S. thermophilus and species of Lactobacillus

The results from earlier experiments clearly indicated that when S. thermophilus is lysed in a mixed culture of S. thermophilus and Lactobacillus, the resulting acid production was much higher than the control without bacteriophage. On the contrary, when Lactobacillus is lysed by it's bacteriophage in a mixed culture, the acid production was significantly low. Both the decreased acid production or abnormally high amounts of acid production are deleterious to cheese making. It is not customary to use mixed cultures of S. thermophilus and Lactobacillus. The aim of this experiment is to check the effect of lysis of one strain of S. thermophilus or Lactobacillus on the acid production by other phage unrelated strains growing in association. The counts/ml of S. thermophilus strains ST_A and ST_4, L. helveticus, L. lactis, and their specific phages at 0 h of incubation in milk were as follows: S. thermophilus ST_A - 36 X 10^5; S. thermophilus ST_AØ - 250 X 10^5; S. thermophilus ST_4 - 210 X 10^4; S. thermophilus ST_4Ø - 130 X 10^5; L. helveticus - 80 X 10^5; L. helveticus Ø - 32 X 10^5; L. lactis - 65 X 10^5; and L. lactis Ø - 200 X 10^4. The results of these experiments are presented in Figures 29, 30, and 31. When both the strains of S. thermophilus were phaged in a mixed culture (composed of two

Figure 29. Effect of cellular lysis by bacteriophage (Ø) on associative growth of strains of *S. thermophilus* (ST_A and ST_4) and species of *Lactobacillus* *L. helveticus* (LH) and *L. lactis* (LL) as measured by acid production.

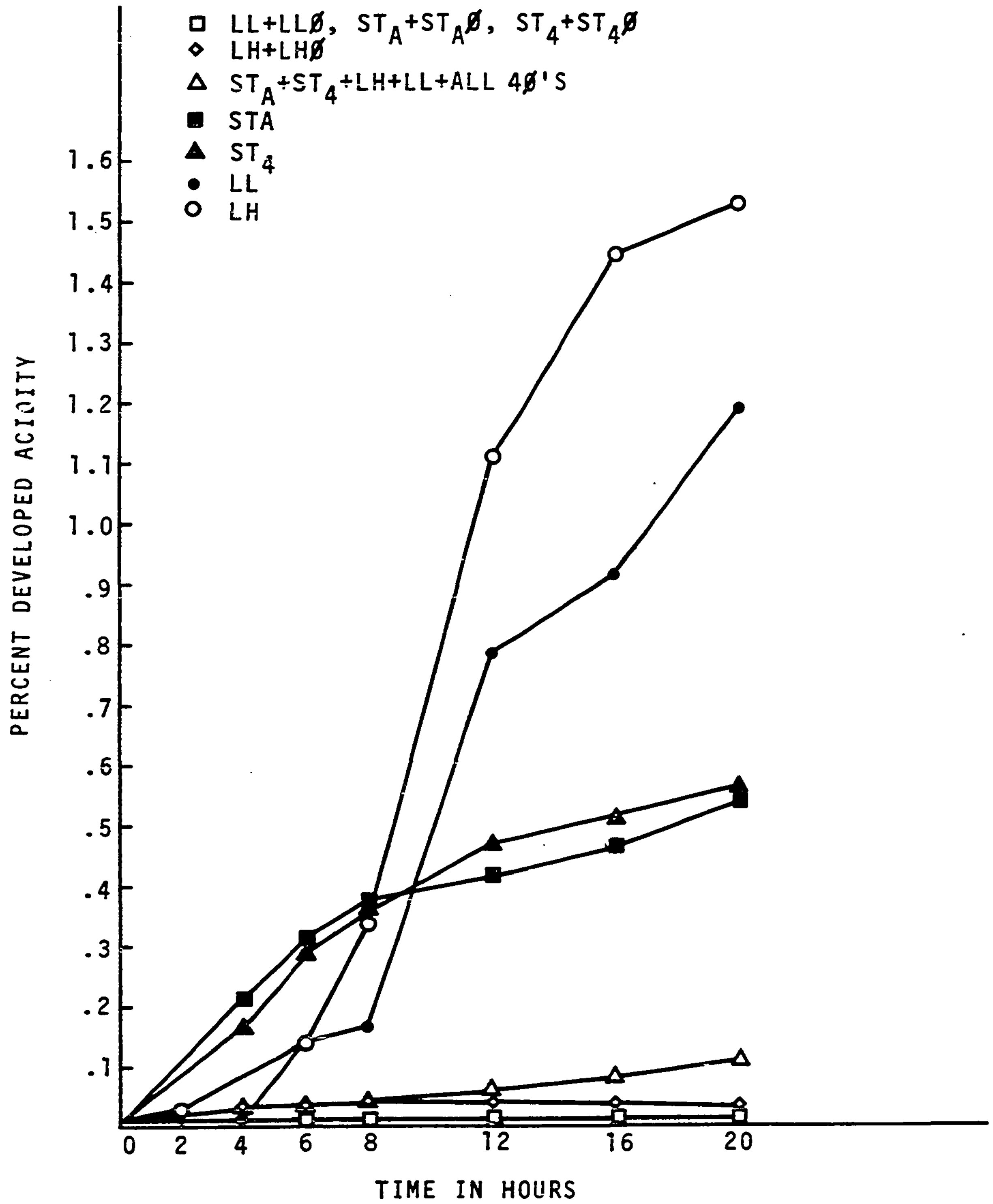
▲ $ST_A+ST_4+LH+LL$
● $ST_A+ST_4+LH+LL+ST_AØ$
■ $ST_A+ST_4+LH+LL+ST_4Ø$
◇ $ST_A+ST_4+LH+LL+ST_AØ+ST_4Ø$
○ $ST_A+ST_4+LH+LL+LHØ$
△ $ST_A+ST_4+LH+LL+LLØ$
◆ $ST_A+ST_4+LH+LL+LHØ+LLØ$
□ $ST_A+ST_4+LH+LL+ALL\ 4Ø'S$
PERCENT DEVELOPED ACIDITY
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
4
6
8
12
16
20
TIME IN HOURS

Figure 30. Effect of cellular lysis by bacteriophage (Ø) on associative growth of strains of *S. thermophilus* (ST_A and ST_4) and species of *Lactobacillus* *L. helveticus* (LH) and *L. lactis* (LL) as measured by acid production.



○ ST_A+ST_4
● ST_A+ST_4+LL
■ $LH+LL+ST_A$
△ $ST_A+ST_4+LL+LH$
□ $LH+LL+ST_4$
◇ ST_A+ST_4+LH
▲ LH+LL
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
PERCENT DEVELOPED ACIDITY
0
4
6
8
12
16
20
TIME IN HOURS

Figure 31. Effect of cellular lysis by bacteriophage (Ø) on associative growth of strains of *S. thermophilus* (ST_A and ST_4) and species of *Lactobacillus* *L. helveticus* (LH) and *L. lactis* (LL) as measured by acid production.



LL+LLØ, ST_A+ST_AØ, ST_4+ST_4Ø
LH+LHØ
ST_A+ST_4+LH+LL+ALL 4Ø'S
STA
ST_4
LL
LH
PERCENT DEVELOPED ACIDITY
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
12
16
20
TIME IN HOURS

strains of S. thermophilus, L. lactis and L. helveticus), the amount of acid produced between 8 to 20 h of incubation was significantly higher than the control, without any bacteriophage. Also, initially (until 8 h of incubation) there was a reduction in acid production when both the strains of S. thermophilus were phaged in a mixed culture. On the other hand, when only one strain of S. thermophilus (ST_A) was lysed by it's specific phage, acid productions were closer to the control, both at the initial (up to 8 h) and later (8 to 20 h) stages of incubation. Also, the pattern of acid production was quite similar to the acid production by a mixed culture of L. helveticus, L. lactis, and S. thermophilus ST_4. This indicates that using two different phage specific strains of S. thermophilus is one way of controlling bacteriophage problems in industry.

When L. helveticus and L. lactis were lysed by their bacteriophage in a mixed culture (involving L. helveticus, L. lactis, S. thermophilus ST_A and ST_4), the resulting acid production was significantly lower than the control without bacteriophage. Lysis of L. lactis alone did not affect the acid production by the mixed culture. Also, the amount of acid produced was closer to the amount produced by the mixed culture made of two strains of S. thermophilus and L. helveticus. The amount of acid produced by mixed culture when L. helveticus was phaged was less than the control. This decrease in acid production was not due to inhibition, since L. lactis

alone produced much less acid than the mixed culture.

When phages active against *S. thermophilus* ST_A and ST_4 *L. lactis* and *L. helveticus* were added to the mixed culture, there was a cessation in acid production. All single cultures failed to produce acid when their specific phages were added. The data obtained from these experiments clearly indicate the superiority of using mixed strains of *S. thermophilus* and *Lactobacillus* over single strain cultures, in controlling bacteriophage problems in the dairy industry.

Effect of S. thermophilus and L. bulgaricus bacteriophage(s) on acid production and flavor development of yogurt

As a practical demonstration of the possible commercial importance of these phages, the effects of *S. thermophilus* and *L. bulgaricus* bacteriophages on acid production and flavor development of yogurt was studied. The results of these experiments are depicted in Tables 44, 45, and 46. The strain of *L. bulgaricus* and its phage used in this experiment were isolated from commercial yogurt. The counts of *L. bulgaricus* bacteriophage, before inoculation, were rather low compared to the counts of its host. Part of the reason may be an inadequate plating technique used to enumerate *L. bulgaricus* bacteriophage. Even such a low concentration of *L. bulgaricus* bacteriophage distinctly affected the acid production by it's homologous host. Both *S. thermophilus*, and *L.*

Table 44. Effect of *S. thermophilus*[a] and *L. bulgaricus*[b] bacteriophages[c,d], added after 2 h of incubation, on acid production and flavor development of yogurt.

Culture(s) and phage(s) used	% titratable acidity[e] after 2 h at 45 C	% titratable acidity after 4.5 h at 45 C	Counts in product
			S. thermophilus
ST_4	ND[f]	.310	65 X 10^6
ST_4+Ø[g]	.235	.310	110 X 10^5
LB	ND	.350	-
LB+Ø[g]	.250	.370	-
ST_4+LB	ND	.800	150 X 10^6
ST_4+LB + ST_4Ø[g]	.315	.715	190 X 10^5
ST_4+LB + LBØ[g]	.310	.590	230 X 10^6
ST_4+LB + ST_4Ø[g]+LBØ[g]	.310	.630	89 X 10^5

[a,b,c,d]Same as described in Table 45.

[e]Titratable acidities determined at 0 h of incubation are presented in Table 45.

[f]Not determined.

[g]Phage preparation (2%) added after 2 h incubation at 45 C.

Counts in product		
L. bulgaricus	*S. thermophilus* ∅	*L. bulgaricus* ∅
-	-	-
-	83×10^5	-
30×10^6	-	-
41×10^5	-	94×10^3
48×10^7	-	-
140×10^6	110×10^5	-
180×10^4	-	220×10^3
44×10^5	32×10^6	180×10^3

Table 44. (Continued)

Culture(s) and phage(s) used	Final pH	Flavor Evaluation		Coccus:rod ratio at 4.5 h (microscopic)
		Consistency	Flavor	
ST_4	5.95	Fluid	No acid flavor	-
ST_4+Ø	5.95	Fluid	No acid flavor	-
LB	5.50	Fluid	No acid flavor	-
LB+Ø	5.30	Slightly viscous	No acid flavor	-
ST_4+LB	4.20	Custard	Yogurt flavor	1:1
ST_4+LB + ST_4Ø	4.50	Weak body	No yogurt flavor	0.33:1
ST_4+LB + LBØ	5.05	Very weak body	Low acid, lack of yogurt flavor	4:1
ST_4+LB + ST_4Ø+LBØ	5.00	Weak body	Objectionable flavor, low acid flavor	2:1 (very few)

Table 45. Effect of *S. thermophilus*[a] (ST_4) and *L. bulgaricus*[b] (LB) bacteriophages[c,d] on acid production and flavor development of yogurt.

Culture(s) and phage(s) used	% titratable acidity at 0 h	% titratable acidity after 4.5 h at 45 C	Counts in product (4.5 h)
			S. thermophilus
ST_4	.170	.310	65×10^6
ST_4+Ø[e]	.170	.170	<10/ml
LB	.175	.350	-
LB+Ø[e]	.180	.210	-
ST_4+LB	.185	.800	150×10^6
ST_4+LB + ST_4Ø[e]	.180	.400	<10/ml
ST_4+LB + LBØ[e]	.185	.550	42×10^7
ST_4+LB + ST_4Ø[e]+LBØ[e]	.185	.230	<10/ml

[a]Concentration of *S. thermophilus* at 0 h of incubation - 63×10^5/ml.

[b]Concentration of *L. bulgaricus* at 0 h of incubation - 65×10^5/ml.

[c]*S. thermophilus* phage at 0 h of incubation - 240×10^4 /ml.

[d]*L. bulgaricus* phage at 0 h of incubation - 130×10^2/ml.

[e]Phage added at 0 h of incubation.

Counts in product (4.5 h)		
L. bulgaricus	*S. thermophilus* Ø	*L. bulgaricus* Ø
-	-	-
-	52×10^7	-
30×10^6	-	-
92×10^4	-	52×10^4
48×10^7	-	-
45×10^7	45×10^7	-
52×10^7	-	70×10^4
75×10^4	36×10^7	44×10^4

Table 45. (Continued)

Culture(s) and phage(s) used	Final pH	Flavor evaluation		Coccus:rod ratio at 4.5 h (microscopic)
		Consistency	Flavor	
ST_4	5.95	Fluid	No acid flavor	-
ST_4+Ø	6.65	Fluid	No acid flavor	-
LB	5.50	Fluid	Slight acid lacks typical flavor	-
LB+Ø	6.20	Fluid	No acid flavor	-
ST_4+LB	4.20	Custard	Yogurt flavor	1:1
ST_4+LB + ST_4Ø	5.35	Pronounced weak body (liquid)	No acid flavor	0:13
ST_4+LB + LBØ	5.10	Slight weak body	Lacking acid, sweet flavor	150:1
ST_4+LB + ST_4Ø+LBØ	6.25	Fluid	Milky flavor	0:1

Table 46. Effect of *S. thermophilus*[a] (ST_4) and *L. bulgaricus*[b] (LB) bacteriophage[c,d], added after 1 h of incubation, on acid production and flavor development of yogurt.

Culture(s) and phage(s) used	% titratable acidity[e] after 1 h at 45 C	% titratable acidity after 4.5 h at 45 C	Counts in product (4.5 h)
			S. thermophilus
ST_4	ND[f]	.310	65 X 10^6
ST_4+Ø[g]	.180	.260	10 X 10^1
LB	ND	.350	-
LB+Ø[g]	.190	.310	-
ST_4+LB	ND	.800	150 X 10^6
ST_4+LB + ST_4Ø[g]	.210	.585	20 X 10^1
ST_4+LB + LBØ[g]	.220	.580	270 X 10^6
ST_4+LB + ST_4Ø[g]+LBØ[g]	.220	.330	14 X 10^1

[a,b,c,d]Same as described in Table 45.

[e]Titratable acidities determined at 0 h of incubation are presented in Table 45.

[f]Not determined.

[g]Phage preparation (2%) added after 1 h incubation at 45 C.

Counts in product (4.5 h)		
L. bulgaricus	*S. thermophilus* Ø	*L. bulgaricus* Ø
-	-	-
-	45×10^7	-
30×10^6	-	-
121×10^3	-	120×10^4
48×10^7	-	-
160×10^6	43×10^7	-
30×10^4	-	98×10^4
160×10^4	49×10^7	160×10^4

Table 46. (Continued)

Culture(s) and phage(s) used	Final pH	Flavor evaluation		Coccus:rod ratio at 4.5 h (microscopic)
		Consistency	Flavor	
ST_4	5.95	Fluid	No acid flavor	-
ST_4+Ø	6.35	Fluid	No acid flavor	-
LB	5.50	Fluid	Slight acid, lacks typical flavor	-
LB+Ø	5.80	Fluid	No acid flavor	-
ST_4+LB	4.20	Custard	Yogurt flavor	1:1
ST_4+LB + ST_4Ø	4.85	Weak body	Objectionable green flavor, no yogurt flavor	0:10
ST_4+LB + LBØ	4.90	Weak body, almost liquid	Slight acid flavor, lacks yogurt flavor	50:0
ST_4+LB + ST_4Ø+LBØ	5.90	Fluid	No acid flavor, sweet milky	0:1

bulgaricus bacteriophage(s) adversely affect the body, acid production, flavor development, and coccus to rod ratio of yogurt. So, phages active against S. thermophilus and L. bulgaricus are exceedingly important, in terms of their harmful effects, in the manufacture of yogurt. Even if phage contamination occurred after 2 h or incubation (during the manufacture of yogurt), both the flavor and acid development were retarded. When specific bacteriophage was added, as expected, the viable bacterial counts of the product were distinctly low. At the same time bacteriophage titers were distinctly high in the product.

SUMMARY AND CONCLUSION

The effect of various growth conditions on plaque formation by S. thermophilus bacteriophage was investigated. From these findings, a modified double-layer agar plate procedure was developed for the isolation and enumeration of S. thermophilus bacteriophage. This procedure was quite efficient in recovery of the maximum number of bacteriophage particles. Using this procedure, conditions influencing the stability, proliferation, and enumeration of S. thermophilus bacteriophage were determined. The results of this investigation showed that S. thermophilus bacteriophage was quite stable from pH 5.0 to 6.5 and that 5 C was a better storage temperature than 21 C when the suspending medium was acidic. Also, S. thermophilus bacteriophage proliferates at a much faster rate at pH 6.0 than at 6.5 or 7.0. This finding indicates that high phage titers are probably obtained in Swiss cheese whey at about 5 h after dipping.

During purification or enumeration, considerable reductions in phage numbers were observed after Millipore filtration. A simplified procedure using chloroform was developed to kill the bacterial cells without materially affecting the phage titer, thereby obviating filtration. This procedure is more efficient for recovery of phage particles and could be effectively used in the dairy industry.

A technique suitable for shipment of whey without sub-

stantial reduction of *S. thermophilus* and species of *Lactobacillus* bacteriophage particle numbers was developed. This was accomplished by adding 4% calcium carbonate, maleate buffer (7.6 ml/100 ml whey), and 2% specific *S. thermophilus* or *Lactobacillus* culture to the whey samples. This procedure served to enrich the bacteriophage during shipment.

Using all these techniques, the incidence of *S. thermophilus* and *Lactobacillus* bacteriophages in 45 Swiss and Italian cheese plants was determined. Twenty-four commercial *S. thermophilus* and 18 *Lactobacillus* strains were used as indicators to isolate phage, in addition to the cultures that were used in each individual cheese plant. Results of this study showed that 92% of the Swiss cheese plants and 65% of the Italian cheese plants surveyed had *S. thermophilus* bacteriophage. Phages active against *Lactobacillus* were recovered from 45% of the Italian cheese plants and 15% of the Swiss cheese plants included in this study.

All phages isolated from these sources were purified and were then used to study strain specificity of the different *S. thermophilus* and *Lactobacillus* strains. Extensive cross reactions were observed, signifying the relative nonspecificity of these phages or homology between host strains. The information obtained from this study should be of significant help both to culture manufacturers and cheese makers in developing a successful rotation scheme of phage-unrelated strains.

Electron microscopy of phages active against S. thermophilus and species of Lactobacillus revealed unusual morphological characteristics.

Associative growth studies revealed that when S. thermophilus was lysed in a mixed culture of S. thermophilus, species of Lactobacillus, and S. lactis, the ensuing acid production was much higher than occurred in the control culture. Species of Lactobacillus were the principal contributors to this increased acid production. This increase in acid production by species of Lactobacillus was attributed to the lack of competition from the lysed coccus culture and possibly to stimulation from the intracellular metabolic pool of S. thermophilus liberated by phage lysis. When Lactobacillus phage was present in a mixed culture, acid production was always significantly lowered. Finally, both S. thermophilus and L. bulgaricus bacteriophages distinctly affect the body, acid production, and flavor development of yogurt.

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