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Title: Development of an Internal pH-Controlled, Phage Inhibitory Bulk Starter Medium for the Propagation of Thermophilic Lactic Acid Bacteria Used in the Production of Mozzarella Cheese

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A starter medium (Insure-IT) was developed for growing the thermophilic lactic acid bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Insure-IT generated high numbers of cells (10^8-10^9/ml) with high acid-producing activity and in appropriate coccus:rod proportions without the need for additional neutralization. When compared to six other commercially manufactured bulk starter media used for the production of mozzarella cheese, Insure-IT produced cells with superior activity to those grown in all other media, even when challenged by bacteriophages. However, no improvement was made in ability to reduce final phage titers.

To provide a method for controlling the final coccus:rod ratios produced in the mature starter, changes in incubation temperature,

break pH and the initial culture inoculum ratios were examined. Adjustments in temperature and break pH did not produce dramatic changes in the final ratios of cell-types and altered inoculum ratios gave inconclusive results.

Attempts to inhibit phage proliferation through the addition of chelating agents, primarily ammonium phosphate and sodium citrate, to bind calcium and magnesium ions were ineffective. Other attempts at phage inhibition by limiting the movement of free phages within the medium by the addition of gelling compounds and the removal of phages from the medium by the adsorption onto heat-killed cells, were also unsuccessful. Although phages did adsorb to the dead cultures, they appeared to be able to detach themselves and remain infectious.

Experiments to determine pH ranges and cation (calcium and magnesium) concentrations inhibitory to the adsorption and replication of these phages were also conducted. Effects of pH on phage adsorption were generally minimal, as all but one of the thermophilic strains showed >70% adsorption under all of the pH conditions examined (4.0 - 7.0 in increments of .5), and appeared to be strain dependent since a pattern relating these factors was not apparent. Concentrations of calcium or magnesium (0 -100 mM) had no influence on adsorption of phages to thermophilic strains (>98% adsorption). Results of phage replication under these conditions were limited but showed inhibition below cation concentrations of 10 mM. However, this appeared to be due, at least in part, to decreased growth of the thermophilic cultures at these ion levels.

Development of an Internal pH-Controlled, Phage Inhibitory Bulk Starter Medium for the Propagation of Thermophilic Lactic Acid Bacteria Used in the Production of Mozzarella Cheese

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INTRODUCTION

The origin of mozzarella cheese has been traced to southern Italy and was traditionally a product made from water buffalo milk, fermented by *Streptococcus thermophilus* (*S. thermophilus*) and consumed fresh. This cheese variety is now produced from cow's milk, includes *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lb. bulgaricus*) and/or *Lactobacillus helveticus* in the culturing process and its use in pizza has made it one of the fastest growing and most popular cheeses produced in the United States. One modern US cheese plant alone processes nearly 700,000 lb. of milk into 60,000 lb. of mozzarella daily (Honer, 1992) and the total US production of Italian-type cheese, from nearly 200 operations, exceeds 1.5 billion pounds per year (Thunell, 1989).

Two factors that play influential roles in the successful production of mozzarella cheese, and of cheese in general, are bulk starter media and bacteriophage (phage). In cheese production, the standard practice is for bacterial cultures to be inoculated into a medium and grown in bulk quantities (300-1,000 gal.) for addition (approx. 1%) to vat milk for ripening. In order to decrease the length of the lag

period preceding growth and acid production, which would allow the cheesemaker to decrease the amount of time and/or starter needed for milk ripening, special starter media have been formulated that allow for optimum growth and holdover (keeping quality) of these cultures. The major innovation allowing this has been the use of pH control. This is achieved either by the addition of base ("external" neutralization) or the incorporation of buffers ("internal" neutralization) in order to neutralize the acid produced during starter ripening. The demand for these tailored media, along with the large production capacities of modern cheese plants, has made the production of bulk starter media a major part of the dairy industry.

Because of the ever-present threat to starter cultures of infection by bacteriophage, which can result in production slowdowns or product losses, manufacturers of starter media have also attempted to incorporate substances into their media which would inhibit phage replication. Although internally neutralized, phage inhibitory bulk starter media have been used successfully for mesophilic lactic cultures, particularly in cottage cheese production, only limited success has been observed using phage inhibitory media for the thermophilic lactic cultures used in mozzarella cheese.

Therefore, the goals of the research efforts for this thesis were to develop a growth medium for thermophilic lactic starter cultures that possessed the following characteristics:

1) components that would provide buffering capacity so that procedures, equipment and chemicals required for external neutralization could be eliminated.

2) growth of starter cultures to higher activities (rates of acid production in milk) than other commercially available media.
3) final concentrations of *S. thermophilus* and *Lb. bulgaricus* at or near ratios of 1:1.
4) components that would inhibit bacteriophage infection of the starter cultures.

It was felt that a medium possessing these qualities would be of great benefit to the mozzarella cheese industry and that the information learned through its development could also aid others working with lactic acid bacteria and cultured dairy products.

STARTER CULTURES, BULK STARTER MEDIA, BACTERIOPHAGES AND THEIR ASSOCATION WITH MOZZARELLA CHEESE: A REVIEW

The Discovery and Use of Starter Cultures

Fermented milk products have, for thousands of years, been a major staple and source of nutrition in the diet of mankind. In the early days of cheesemaking, the souring of milk and resulting curd formation was relied completely, and unknowingly, on its natural bacterial flora. Progress in understanding milk fermentations was slow to develop and it was not until the late 1800's that Joseph Lister isolated *Streptococcus lactis* (Lister, 1878), now classified as *Lactococcus lactis* ssp. *lactis*, from raw milk. A few years later, Conn (1889) in the United States, Storch (1890) in Denmark and Weigmann (1896) in Germany concluded that it was these naturally present bacteria that were responsible for the souring of cream. This initiated what could be considered the first starter culture system, wherein starter was prepared daily by transferring small amounts of soured cream into fresh cream that would be used to make butter. It would later be discovered that many other bacteria, including lactobacilli, leuconostoc, propionibacteria, streptococci and other lactococci, as well as certain yeasts and molds, were also involved in the vast number of fermented milk products now recognized.

Since their discovery, the maintenance and use of starter cultures has undergone many adaptations and improvements. Initially,

cheesemakers maintained mixed strain mother cultures, the bacterial content (i.e., species and numbers of strains) of which was unknown, by repeated transfers in milk. Cultures that performed better than others were often shared between cheese plants and this practice soon gave rise to independent companies that would produce fresh starter and supply it to cheese plants near their location. These were initially distributed as chilled, liquid cultures but, with the work of Rogers (1914), were later produced and supplied in the form of lyophilized powders. Today, based on the findings of Cowan and Speck (1963) which showed that lactic cultures still maintained high acid-producing ability after being frozen in liquid nitrogen, cultures are primarily supplied as frozen concentrates (Christensen, 1971; Farr, 1969). These cultures are supplied in cans, commonly in volumes of 70 or 125 ml, which are thawed and added directly to a growth medium in the bulk starter tank.

The most recent developments in culture storage and use, termed direct vat set (DVS) cultures, have seen limited success in certain fermented milk products. This approach involves the use of larger volumes of concentrated frozen or lyophilized (Speckman et al., 1974) cultures that are added directly to vat milk, eliminating the need for the production of bulk starter.

Adaptations and improvements in the use and maintenance of starter cultures has been the cornerstone to the manufacture of consistent, high quality fermented milk products. However, much of this success must also be attributed to subsequent developments in the design of starter media and advances in methods for combating bacteriophage infections of starters.

The Evolution of Starter Media

The use of starter media tailored specifically to the nutritional requirements of individual groups of lactic acid bacteria, has significantly enhanced the efficiency of production of cultured milk products. Originally, raw milk, and later pasteurized milk, was used to grow the starter strains that would be used in the day's cheesemaking. The eventual application of antibiotics to treat mastitis in dairy cattle, however, necessitated that cheesemakers obtain antibiotic free milk from selected producers. This provided a purer starting product and helped to maintain more reliable starter growth.

The development and use of spray-dried nonfat milk (NDM) for growing starter cultures, the springboard to the formulation of starter media, gave the cheesemaker much more flexibility in starter production. Unlike fluid milk, NDM was stable for extended storage, could be reconstituted when desired and was slightly more consistent than seasonal herd milk. The use of pretested NDM, which ensured the absence of antibiotics and further increased its uniformity, soon followed.

Attitudes by cheesemakers toward whey, the by-product of curd formation, had traditionally been as a food source in certain cultures while others thought of it only as a waste product. However, the abundance of nutrients contained therein, along with environmental problems which arose from its unregulated disposal, initiated efforts to find practical uses for this product and/or its components. Incorporation into starter media was one such area of research

(Anderson et al., 1974; 1977; Ausavanodom et al., 1973; Ogden, 1981). Because of the large economical advantage of using a product that previously had very little monetary value, whey-based media soon gained wide acceptance over the use of high priced NDM solids for starter production. Even individual components of whey such as whey permeate, a product high in lactose, have since gained use in media formulations (Parente et al., 1991).

This break from the traditional milk based starter medium was made possible through a great deal of research on the specific nutritional requirements of starter bacteria (Anderson et al., 1953; Collins et al, 1950a; Garvie et al., 1956; Wright, 1936). Results of these efforts provided information as to the optimum concentrations of the various constituents required for growth and, therefore, media which met these conditions could be produced. These nutrients are most commonly supplied to whey-based starter media in the form of lactose, glucose or sucrose as a carbohydrate source, milk and whey proteins for a source of nitrogen, and yeast extract to supply vitamins and minerals (Whitehead et al, 1992). Buffering compounds, such as phosphates, carbonates and hydroxides, as well as phage inhibitory substances (i.e., phosphates and citrates), have also been added to media (Whitehead et al, 1992).

Other major advances in starter production involved new methods for culturing starter bacteria. The first of these, termed external pH control, were developed by Ausavanodom et al. (1973) and Richardson et al. (1977; 1978) and involved the addition of ammonium hydroxide to a whey-based bulk starter medium in order to maintain a constant pH of 6.0. This allowed for extended growth

near the optimal pH of these organisms, prevented acid injury to the cells (Harvey, 1965) and extended the holdover time of the mature starter. The result provided an economic advantage to the cheesemaker since the highly active cultures that were produced, significantly reduced the volume of starter needed to inoculate milk in the cheese vat. Modifications of this technology have since been developed that involve the use of other neutralizers and various neutralization patterns. These include the use of sodium or potassium hydroxides in single or multiple step additions (Reddy, 1986) and/or the maintenance of the medium at various pH values for extended periods of time.

The approach that followed, termed internal pH control, was a major breakthrough in returning simplicity to the in-plant production of starter. "Phase 4," developed by Sandine and Ayres (1981; Willrett et al., 1979; 1981), incorporated buffering components (i.e., phosphate and citrate) directly into the starter medium which allowed a pH of 5.2 to be maintained after culture ripening . The end result was the same as that using external neutralization (i.e., higher numbers of viable bacteria, prevention of acid injury, extended holdover) without the need for additional machinery and hazardous chemicals.

During the development of pH-controlled starter media, progress was also made in the formulation of phage inhibitory media (PIM). These were constructed on the basis of research which showed the need for divalent ions, particularly calcium and magnesium, in the replication processes of bacteriophages for lactic acid bacteria (Collins et al., 1950b; Reiter, 1956; Shew, 1949; Watanabe and Takesue,

1972). Based on these early reports, Hargrove (1959; Hargrove et al., 1961) was one of the first to incorporate phosphate salts into whey-based bulk starter media in order to chelate calcium ions. Sozzi (1972), however, later reported that these cations were not always necessary for the replication of all lactic phages. Nevertheless, this approach has been used in numerous attempts to develop such media (Ausavanodom et al., 1973; Gulstrum et al., 1979; Khosravi et al., 1991; Rajagopal et al., 1990; Richardson, 1978; Richardson et al., 1977; Sandine et al., 1981; Whitehead et al., 1991; Willrett et al., 1979; 1981; 1982) and has attained limited success in preventing phage infection.

The Role of Bacteriophages in Cheesemaking and Methods for Their Control

Bacteriophages for lactic acid bacteria were first recognized by Whitehead in 1935 (Whitehead and Cox, 1935). Still considered to be the primary cause of slow and failed ("dead") vats of starter and cheese milk, their activity results in production and economic losses to cheesemakers worldwide.

The infection process may result in either lysis of the culture, through lytic infection, or incorporation of the phage genome into the host chromosome (lysogeny). Although lysogenic strains are extremely common (Huggins and Sandine, 1977; Park and McKay, 1975), they have not been shown to be significant contributors of lytic phage for other cultures (Reiter, 1973) as was initially

suspected. Nevertheless, this is still a factor that should not be overlooked when selecting strains for cheesemaking.

Lytic infections can be the most detrimental to starter cultures due to the replication rates of phages. These infections can produce bursts between 2 and 200 phage particles per infected cell (Keogh, 1973; Nicholls, 1962), which allows them to rapidly outgrow and decimate the culture population. Once the process begins, phages can be transferred by air currents and aerosols to all parts of the cheese plant and may remain a persistent problem. As a result, methods for inhibiting or preventing phage infection of starter strains have gained a great deal of attention over the last fifty years.

The more practical approaches to phage prevention have involved sanitation, the aseptic design of starter production facilities and modifications to the cheesemaking process. These areas have been reviewed by Lawrence and Pearce (1972), Sandine (1976; 1979) and Huggins (1984) and give specific attention to the following: 1) the use of chlorine solutions (200 ppm) for and sanitizing equipment and airspace, 2) the design of factories, starter rooms and bulk starter tanks to minimize opportunities for phage entry and persistence, 3) the use of higher starter inoculum levels and the process of early renneting to inhibit transfer of phage through vat milk during culture ripening, 4) the proper handling and disposal of whey, 5) the use and proper rotation of phage unrelated strains, 6) the use of phage inhibitory media.

From the standpoint of the starter culture, there are essentially two philosophies on how to approach the host/phage relationship. The first system involves the aseptic handling of mixed or defined

strain cultures in order to prevent contact with phage. Although cultures maintained in this fashion have been shown by Galesloot et al. (1966) to be susceptible to increased phage sensitivity with successive transfers, this is still the method of choice in the US and New Zealand and is very effective when the aforementioned preventative practices are applied. The second approach allows deliberate contact between mixed starters and phage in order to continually challenge the cultures and encourage the emergence of phage resistant cells. Successful use of this system has been reported by Galesloot et al. (1966) in the Netherlands and by Crawford and Galloway (1962) in Scotland. Consequently, the notion of exposing starter cultures to phage in an attempt to produce mutant strains which possess the same cheesemaking qualities, but are no longer sensitive to phage, has been exploited by numerous researchers (Heap and Lawrence, 1976; Huggins and Sandine, 1979; Limsowtin and Terzaghi, 1976; Marshall and Berridge, 1976).

The first defined strain program, introduced by Whitehead in New Zealand, involved the daily rotation of single or paired strains in an effort to improve consistency over the mixed strain cultures that were previously in use. This proved to be a model system for the application of phage insensitive cultures and still sees widespread use. Defined strain culture systems without rotation have also been developed. Limsowtin, Heap and Lawrence (1977) implemented the first such system in New Zealand, which involved the daily use of six compatible, separately grown, phage unrelated cultures. A similar approach was developed by Sandine and co-workers (1989; Thunell et al., 1981; 1984) which included daily testing of the strains against

whey composites from the day's cheesemaking. Strains which began to show phage sensitivity were removed, cultured to select for phage-insensitive, fast-acid producing mutants suitable for cheese manufacture, and then reintroduced into the starter system.

The most recent developments in providing phage resistance to starter cultures have been based on genetic approaches. To date, three general categories of naturally occurring phage defense mechanisms in lactic acid bacteria have been identified and reviews have been compiled by Davies and Gasson (1984), Daly and Fitzgerald (1987), Klaenhammer (1987), Sanders (1988) and Jarvis (1989). These defense systems consist of the prevention of phage adsorption to the cell (deVos et al., 1984; Sanders and Klaenhammer, 1983), host-controlled restriction/modification of phage DNA (Chopin et al., 1984; Gautier and Chopin, 1987; Hill et al., 1989; Sanders and Klaenhammer, 1981) and an abortive infection process (Gautier and Chopin, 1987; Hill et al., 1989; Sing and Klaenhammer, 1989; 1990), whereby the infected cell dies prior to the production and release of new virus particles. Culture rotation systems are now being implemented which involve the pairing of cultures that possess different defense mechanisms (Klaenhammer and Sing, 1991) or which include conjugatively transformed strains that contain multiple resistance systems (Sanders et al., 1986).

Batt and co-workers (Kim and Batt, 1991; Kim et al., 1992) have taken another approach and have engineered strains that possess plasmid constructs which express antisense RNA specific for conserved lactic phage gene sequences. The antisense mRNA binds to the complementary sense strand of phage mRNA and inhibits the

expression of the particular gene by interfering with translation of the mRNA. These constructs are stable and have been shown to inhibit infection by 40-99% depending on the targeted gene sequence.

Recent studies have also been reported in which specific receptor sites have been identified for certain lactococcal phages. Valyasevi et al. (1990), reported that certain carbohydrates found on the cell wall, galactose in all cases and sometimes rhamnose, were essential for phage binding. They also showed that the addition of rhamnose or purified cell walls could result in the inactivation of these phages. Later work (Valyasevi et al., 1991) with a single lactococcal phage/host system indicated that a membrane protein was required for phage infection of that strain. Information on the binding requirements of phages for starter strains may eventually allow the incorporation of components into starter media which would bind phages and prevent their contact with these cultures.

Thermophilic Lactic Cultures and the Design of Starter Media for Mozzarella Cheese Production

The group of lactic acid bacteria commonly referred to as thermophiles by the cheese industry and used to make products such as mozzarella and provolone cheeses, yogurt and Swiss cheese, essentially consists of the following organisms: *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus*. These fastidious, microaerophilic, Gram-positive rods and cocci produce lactic acid through the

homofermentative metabolism of lactose and were first described by Orla-Jensen (1919).

Although they only border on the classical definition of thermophilic bacteria (i.e., growth temperatures above 45-50^{0}C), they have acquired this designation due to their growth and use at higher temperatures than other lactic acid bacteria. These characteristics were studied by Radke-Mitchell and Sandine (1986) who found optimum growth at 35-42^{0}C for *S. thermophilus* and 43-46^{0}C for *Lb. bulgaricus*. When combined, however, these optimal temperature ranges were shown to have little impact on culture growth. They also showed that *S. thermophilus* exhibited an uncoupling of growth from acid production with the optimum temperature for the latter being 2-8^{0}C above the growth optimum. This supported previous work by Martley (1983) who showed optimum acid production occurring between 39.3-46.1^{0}C for *S. thermophilus* and 41.8-46.6^{0}C for *Lb. bulgaricus* and *Lb. helveticus*.

Many strains of these bacteria, referred to simply as rods (*Lb. bulgaricus, Lb. helveticus*) and cocci (*S. thermophilus*) by the dairy industry, show associative growth patterns that have made their combined, rather than individual use, the industry standard. However, it has been observed that not all strains are compatible (Moon and Reinbold, 1974;1976; Pette and Lolkema, 1950) and, therefore, each should be carefully screened prior to introduction into the cheesemaking process.

Some of the primary products contributing to this symbiotic relationship, particularly for *S. thermophilus*, are free amino acids and peptides whose presence are commonly attributed to the

proteolytic action of *Lb. bulgaricus* on milk proteins. Based in part on the work of Miller and Kandler (1967) and Shankar and Davies (1978), it has also been suggested that the strong proteinase activity of *Lb. bulgaricus* produces free peptides, compensating for the weak proteinase activity of *S. thermophilus*, and that these are then hydrolyzed by the stronger peptidase activity of *S. thermophilus* to amino acids, thereby making them available for use by both organisms (Radke-Mitchell and Sandine, 1984).

A by-product of *S. thermophilus* metabolism that is stimulatory to *Lb. bulgaricus* was found by Galesloot et al. (1968) to be formic acid. Later work by Higashio et al. (1977) showed that this effect could be enhanced either by the use of formate in combination with pyruvate, or with the substitution of oxaloacetate, fumerate, L-malate or α-ketogluterate for pyruvate in combination with formate. Dreissen et al. (1982) has shown that *S. thermophilus* also produces CO_2, from the urea present in milk (Tinsen et al., 1982), at levels which are stimulatory to *Lb. bulgaricus*.

In the design of starter media for growing these organisms in combination, these factors must be considered in order to obtain optimum yields of healthy cells in the desired coccus:rod proportions. Starting with a whey or milk base, which will supply an abundant source of protein (casein, α-lactalbumin, ß-lactoglobulin) and carbohydrate (lactose - which is preferable to other carbohydrates since it will be most abundant in the milk used for cheesemaking), it is also desirable to add an additional source of vitamins and amino acids (yeast extract, hydrolyzed milk and/or whey protein) in order to optimize growth of these fastidious organisms. The addition of

buffering components or the neutralization of media during culture ripening is preferred as well in order to prevent acid injury to the cells and lengthen the growth period to increase cell numbers.

Another area of concern when producing starter for mozzarella cheese is the final coccus:rod ratio. Many cheesemakers feel that this should be near 1:1 (though this may vary depending on the enumeration procedure) or at least in even proportions so that the ripened starter is not heavily dominated by either culture. The basis for this preference stems from observations that heavily imbalanced starter can alter properties of the cheese (i.e., melt, stretch, browning) that are important for its use in pizza and other cooked foodstuffs. Although mozzarella cheese is generally consumed fresh, increased proteolytic breakdown can be caused by excessive numbers of rods causing a soft body defect during storage. Also, excessive proportions of cocci can result in "short" cheese which has very little stretch. Because of these concerns a great deal of effort is expended in attempts to produce exact ratios. However, some recent experiments in mozzarella cheesemaking by Oberg (1990), starting with coccus:rod ratios ranging from 4:1 to 1:4, and Kindstedt and co-workers (Yun et al, 1992a,b; Larose et al., 1992; Effendi et al., 1992), starting with ratios ranging from 10:1 to 1:10, suggest that variance in ratios within these parameters are of little or no consequence to the final physical characteristics of the product.

One method to control these ratios has focused on patterns of neutralization. The basis for this lies in the difference in optimal pH ranges (Tayeb et al., 1984) for culture growth; *S. thermophilus* favors more neutral conditions (pH 6.5) and *Lb. bulgaricus* prefers

more acidity (pH 5.5). Therefore, maintenance of the medium near the optimal pH range for one strain or the other should, in theory, influence the ratio in the corresponding direction. A study by Beal et al. (1989), however, showed that maximum acid production actually occurred at pH values above the optimum for selected strains of *S. thermophilus* and *Lb. bulgaricus* grown in combination.

The same ratio control scenario has also been employed based on temperature adjustments near the different growth optimums for these cultures. A study by Radke-Mitchell and Sandine (1986), however, showed that growth at the optimum temperatures had little influence on their combined growth capabilities but that it may influence the production of certain stimulatory factors.

Conclusion

Recognition of bacterial involvement in milk fermentations and the subsequent isolation and identification of the specific organisms involved, primarily those grouped into the lactic acid bacteria, were the first major steps toward the practical use of these organisms as starter cultures and a truer understanding of the cheesemaking process. Research into the metabolism and growth requirements of these organisms enabled the production of starter media containing these growth factors. When combined with methods to neutralize the inhibitory effects of accumulating lactic acid, these media were further improved to allow both higher yields and extended use of the mature starter.

A complete understanding of the relationships between these bacteria and their interactions with elements such as bacteriophages, however, is only beginning to unfold. It is clear that the associative growth properties of some of these organisms, *S. thermophilus* and *Lb. bulgaricus* in particular, are quite complex and variable and much work still remains to be done in order to better understand their symbiosis so that more precise control of their combined growth can be obtained in products where specific proportions of these strains are desired. Also, although much progress has been made in physical approaches for controlling phages in the external processing environment, understanding how to inhibit or prevent their replication once they invade a product/culture (in a manner acceptable for food manufacture) has been slow and difficult. However, with the advances currently being made in understanding both phages and hosts at the molecular level, natural systems of phage inhibition are being recognized and, in turn, are being applied toward industrial needs. In the end, the information learned will benefit scientist, manufacturer and consumer alike.

FORMULATION OF AN INTERNAL pH-CONTROLLED STARTER MEDIUM FOR GROWING THERMOPHILIC LACTIC CULTURES TO PRODUCE MOZZARELLA CHEESE

Materials and Methods

Cultures, Equipment and Standard Test Procedures

Cultural procedures. The majority of the cultures used for these experiments were obtained as frozen concentrates from the commercial culture suppliers listed in Table 1. These strains were either thawed and used directly from the can or were divided, transferred to separate containers and kept frozen (-40^{0}C) until needed. All other strains were obtained from culture collections of persons in the Department of Microbiology at Oregon State University (Table 1). All cultures were grown in MRS (for the growth of lactobacilli; deMan et al., 1960) or M17 (for the growth of lactococci; Terzaghi and Sandine, 1975) media and aliquoted (.1 ml) into 5 ml cryovials (Corning Inc., Corning, NY) containing 3 ml of litmus milk (11% NDM, .075% litmus) and 1 ml of 20% glycerol for the maintenance of frozen (-40^{0}C) culture stocks.

For the enumeration and differentiation of samples taken from mature starter or thawed commercial concentrates, two plating media were utilized. Initially, cultures were streaked on Yogurt Lactic Agar (YLA; Matalon and Sandine, 1986) and incubated for 24-

Table 1. List of cultures used in this study

Source	Cultures
Culture Technology, Inc., Millville, UT	*Lactobacillus delbrueckii* ssp. *bulgaricus*: 10R, 60R *Streptococcus thermophilus*: 150C, 190C
Galloway-West Co., Fond du Lac, WI	Mixed *Lactobacillus* and *Streptococcus thermophilus* strains: 101, 111, 121, 131, 141, 151, 161, 171, 181, 191
Culture Collections, Microbiology Dept., OSU	*Lactobacillus delbrueckii* ssp. *bulgaricus*: 121-R[a], 181-R[a] *Lactococcus lactis* ssp. *cremoris*: 205[b], KH[b] *Lactococcus lactis* ssp.*lactis*: C2[c], ML3[b]
Rhône-Poulenc, Marschall Products, Madison, WI	*Lactobacillus delbrueckii* ssp. *bulgaricus*: R110, R160 *Streptococcus thermophilus*: C90, C120, C150, C160
Vivolac Cultures Corp., Indianapolis, IN	*Lactobacillus delbrueckii* ssp. *bulgaricus*: 210, 260, 270 *Streptococcus thermophilus*: 621, 651, 681, 690, 691

[a]W. Sandine.
[b]B. Geller.
[c]H. Wyckoff.

48 h at 37^{0}C in anaerobic jars (Difco Laboratories, Detroit, MI; Becton Dickinson and Co., Lockeysville, MD) using GasPak hydrogen/CO_2 generators (Becton Dickinson and Co.). This medium was later replaced with TPYE agar (Millard et al., 1989) for use with the ISO-GRID filtration system (QA Life Sciences Inc., San Diego, CA) as this system allowed for easier differentiation between *S. thermophilus* and *Lb. bulgaricus*. The procedure involved filtering a culture dilution through a hydrophobic grid membrane filter which was then placed on the agar medium. Incubation was for 72-96 h at 37^{0}C using the same anaerobic jar system. All dilutions were made in either 9 or 9.9 ml volumes of .1% peptone broth (Difco Laboratories) and were mixed by vortexing.

Coccus:rod ratios were determined by both microscopic examinations and viable cell counts. Microscopic comparisons were made by staining .01 ml starter and activity test samples with methylene blue and examining under 1,000x magnification using a light microscope (Zeis, West Germany) equipped with an ocular grid (CPL W10x, Zeis). Ratios were then determined by enumerating individual colony forming units of rods and cocci within a field of vision. Ratios determined by viable counts were made by comparing characteristic rod and coccus colonies on growth media. These appeared as dark blue (*Lb. bulgaricus*) or light blue (*S. thermophilus*) colonies on TPYE agar and showed irregular (*Lb. bulgaricus*) or smooth (*S. thermophilus*) morphologies on YLA agar.

Fermentation apparatus. A custom designed Fermentation Control System (Jewel In Line Systems, Longmont, CO) was used to evaluate the performance of the various starter media formulations

and fermentation conditions. The system is composed of six 5-L stainless steel tanks that can be thermally processed in-place and which are individually controlled for temperature, pH and agitation. This is accomplished through a programmable computer system which also keeps a constant record of temperature and pH which can later be processed and printed.

Unless indicated otherwise, all fermentations were conducted under the following conditions. All media were reconstituted to 7% solids with tap water in a final volume of 2 L. After thorough mixing, media were heated to 85^{0}C for 45 min, cooled to 42^{0}C (incubation temperature), inoculated with .1 or .2% culture, incubated until the break pH (4.6) was reached (approx. 5-7 h) and cooled to approximately 15^{0}C with circulating tap water. Ten ml samples were removed and refrigerated overnight for evaluation on the following day.

Activity test. To determine the acid-producing potential of cultures in mature starter, 10 ml aliquots of pasteurized (63^{0}C for 30 min) 9% NDM were inoculated with ripened starter at concentrations of .5 and 1%. After tempering to 42^{0}C, these samples, along with an uninoculated control, were incubated at this temperature for 2.5 h. The activity was then recorded as the change in pH (ΔpH) over this time period. This test allowed the comparison of media based on the ability of the ripened starter to rapidly produce acid in milk.

Development of a Base Medium

To begin formulating a buffered starter medium, the simplest approach was to start with a previously proven system. Working with mesophilic lactic starter cultures, Willrett (1982) showed that a whey-based medium containing 2% trimagnesium phosphate provided better buffering in the range of pH 5.5-6.0 than either NDM or a similar whey-based medium containing 1% dibasic ammonium phosphate. It was later theoretically derived that trimagnesium phosphate (40 mM) could be generated in situ from magnesium hydroxide (120 mM) and monobasic ammonium phosphate (80 mM), thus compensating for the expense and lack of availability of this compound. Rajagopal (1986) and Khosravi (1991) later used this approach to develop starter media for thermophilic lactic cultures. With one of these media, trimagnesium phosphate was generated through neutralization with a magnesium hydroxide slurry, in a one-step addition, to a medium containing both mono- and dibasic ammonium phosphates. The latter medium was chosen as a starting point for the experimental media formulations.

All of the media formulations were based on dried sweet whey (Tillamook County Creamery Assoc., Tillamook, OR) supplemented with NDM (Peake, Galloway-West Co.) and yeast extract (YE; Amberex 1003, Universal Foods Corp., Milwaukee, WI). In most cases, buffering was supplied by the addition of magnesium hydroxide [$Mg(OH)_2$] and monobasic ammonium phosphate ($NH_4H_2PO_4$) for the in situ generation of trimagnesium phosphate [$Mg_3(PO_4)_2$]. Some formulations also involved the substitution or addition of dibasic

ammonium phosphate [$(NH_4)_2HPO_4$], magnesium oxide, dibasic magnesium phosphate and GW 4-4 as buffering components, sodium carbonate for buffering and CO_2 liberation, malic and adipic acids to adjust the starting pH, formic acid for stimulating rod growth and glucose, maltrin and maltodextrin as extra carbohydrates. A list of media formulations is compiled in Table 2 and a summary of their constituents, excluding whey, NDM and YE, is given in Table 3. To provide a standard for comparison, a commercial medium (Italian Cul-Sure, Galloway-West Co.) was used alongside all of the other formulations as a control. Performance comparisons were conducted with the Fermentation Control System, involved the use of Galloway-West cultures and were made primarily in terms of starter activities and the final coccus:rod ratios.

Comparison of Commercial Bulk Starter Media

Medium 47 was compared to six other commercial bulk starter media with respect to the activities and coccus:rod ratios of the ripened starters. These media were Italian Cul-Sure (ICS; Galloway-West Co., Fond du Lac, WI), Biotherm (Auropech, Menomonee Falls, WI), Italiano (Chr. Hansen's Laboratory, Inc., Milwaukee, WI) CR, Thermolac and Thermostar II (Rhône-Poulenc, Marschall Products, Madison, WI). Using the Fermentation Control System, media were reconstituted and cultured according to the manufacturer's specifications. All media were heated (85^{0}C for 45 min), cooled to 42^{0}C, inoculated with .2% culture (*Lb. bulgaricus* 210, 260 and *S. thermophilus* 651 and 690 combined), incubated (with neutralization

Table 2. Media formulations

Medium	Ingredients	% (w/v)
1	whey	4.67
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.05
2	whey	4.42
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
3	whey	4.18
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	glucose	.23
4	whey	3.95
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	glucose	.47
5	whey	3.90
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	glucose	.47
	sodium formate	.05

Table 2, cont.

6	whey	4.12
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	glucose	.23
	malic acid	.07
7	whey	3.95
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	glucose	.23
	malic acid	.23
8	whey	4.35
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	malic acid	.07
9	whey	4.35
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.29
	adipic acid	.07
10	whey	4.0
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.5
	malic acid	.19

Table 2, cont.

11	whey	3.79
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.5
	malic acid	.19
	glucose	.23
12	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.5
	maltrin	.2
13	whey	3.4
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.5
	maltrin	.2
	malic acid	.25
14	whey	3.3
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.1
	$Mg(OH)_2$	.5
15	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1

Table 2, cont.

16	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	malic acid	.25
17	whey	3.58
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium formate	.025
18	whey	3.56
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium formate	.05
19	whey	3.53
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium formate	.075
20	whey	3.51
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium formate	.1

Table 2, cont.

21	whey	3.51
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium carbonate	.09
22	whey	3.42
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium carbonate	.19
23	whey	3.37
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium carbonate	.23
24	whey	3.33
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium carbonate	.28
25	whey	3.23
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.6
	maltrin	.1
	sodium carbonate	.37

Table 2, cont.

26	whey	3.4
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.2
	$Mg(OH)_2$	.7
	malic acid	.25
27	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.7
28	whey	3.1
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.1
	$Mg(OH)_2$	.7
29	whey	2.5
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.7
	$Mg(OH)_2$	.7
30	whey	3.0
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.1
	$Mg(OH)_2$	.8
31	whey	2.9
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.1
	$Mg(OH)_2$	.9

Table 2, cont.

32	whey	2.0
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.7
	$Mg(OH)_2$	1.2
33	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.3
	maltrin	.1
	GW 4-4	.3
34	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	$Mg(OH)_2$	.5
	maltrin	.1
	GW 4-4	.1
35	whey	3.2
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	2.1
	magnesium oxide	.6
36	whey	3.6
	NDM	.7
	YE	.4
	$NH_4H_2PO_4$	1.6
	magnesium oxide	.6
	maltrin	.1

Table 2, cont.

37	whey	4.48
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.63
	$(NH_4)_2HPO_4$	.14
	magnesium phosphate, dibasic	.28
	glucose	.3
38	whey	4.62
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.4
	$(NH_4)_2HPO_4$	.14
	magnesium phosphate, dibasic	.54
	glucose	.3
39	whey	4.76
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.4
	magnesium phosphate, dibasic	.54
	glucose	.3
40	whey	4.47
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.4
	$(NH_4)_2HPO_4$	.14
	magnesium phosphate, dibasic	.7
	glucose	.3

Table 2, cont.

41	whey	4.32
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.88
	$(NH_4)_2HPO_4$	.14
	$Mg(OH)_2$	.36
	glucose	.3
42	whey	4.32
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.88
	$(NH_4)_2HPO_4$	.14
	$Mg(OH)_2$	.36
	glucose	.67
43	whey	4.32
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.88
	$(NH_4)_2HPO_4$	.14
	$Mg(OH)_2$	.36
	glucose	.23
	sodium carbonate	.19
44	whey	4.32
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.88
	$(NH_4)_2HPO_4$	.14
	$Mg(OH)_2$	.36
	glucose	.23
	sodium carbonate	.19
	sodium formate	.05

Table 2, cont.

45	whey	4.32
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.88
	$(NH_4)_2HPO_4$	.14
	$Mg(OH)_2$	.36
	glucose	.23
	sodium carbonate	.28
	sodium formate	.05
46	whey	4.32
	NDM	.6
	YE	.4
	$NH_4H_2PO_4$	.88
	$(NH_4)_2HPO_4$	.14
	$Mg(OH)_2$	.36
	glucose	.23
	sodium formate	.05
47	Insure-IT premix	1.67
	whey	3.0
	NDM	.6
	YE	.35
	$NH_4H_2PO_4$	1.12
	maltodextrin	.27

Table 3. Summary of media formulations[a]

Medium	$Mg(OH)_2$[b]	$NH_4H_2PO_4$[b]	$(NH_4)_2HPO_4$[b]	Other constituients
1	.05	1.2	-	-
2	.29	1.2	-	-
3	.29	1.2	-	glucose
4	.29	1.2	-	glucose
5	.29	1.2	-	glucose, formate
6	.29	1.2	-	glucose, malic acid
7	.29	1.2	-	glucose, malic acid
8	.29	1.2	-	malic acid
9	.29	1.2	-	adipic acid
10	.5	1.2	-	malic acid
11	.5	1.2	-	malic acid, glucose
12	.5	1.6	-	maltrin
13	.5	1.6	-	maltrin, malic acid
14	.5	2.1	-	-
15	.6	1.6	-	maltrin
16	.6	1.6	-	maltrin, malic acid
17	.6	1.6	-	maltrin, formate
18	.6	1.6	-	maltrin, formate
19	.6	1.6	-	maltrin, formate
20	.6	1.6	-	maltrin, formate
21	.6	1.6	-	maltrin, carbonate
22	.6	1.6	-	maltrin, carbonate
23	.6	1.6	-	maltrin, carbonate
24	.6	1.6	-	maltrin, carbonate
25	.6	1.6	-	maltrin, carbonate
26	.7	1.2	-	malic acid
27	.7	1.6	-	-
28	.7	2.1	-	-
29	.7	2.7	-	-
30	.8	2.1	-	-
31	.9	2.1	-	-
32	1.2	2.7	-	-
33	.3	1.6	-	maltrin, GW 4-4
34	.5	1.6	-	maltrin, GW 4-4
35	-	1.6	-	Mg-oxide
36	-	2.1	-	Mg-oxide, maltrin
37	-	.63	.14	glucose, Mg-phosphate (diabsic)

Table 3, cont.

38	-	.4	.14	glucose, Mg-phosphate (diabsic)
39	-	.4	-	glucose, Mg-phosphate (diabsic)
40	-	.4	.14	glucose, Mg-phosphate (diabsic)
41	.36	.88	.14	glucose
42	.36	.88	.14	glucose
43	.36	.88	.14	glucose, carbonate
44	.36	.88	.14	glucose, carbonate, formate
45	.36	.88	.14	glucose, carbonate, formate
46	.36	.88	.14	glucose, formate

[a]Not including whey, NDM and YE.
[b]%(w/v)

if needed) until the suggested break pH was reached then cooled. Samples (10 ml) were removed, refrigerated overnight and evaluated the following day.

Optimizing the Concentration of Yeast Extract

Once the medium had been developed, various changes to the formulation and culturing process were evaluated to try to improve on culture performance and/or to reduce ingredient costs. Yeast extract is an expensive nutrient supplement and was evaluated to see what effect its reduction would have on culture performance. This was accomplished by decreasing the YE concentra tions in medium 46 from .4 to .3, .2, .1 and .05% while simultaneously increasing the amount of whey respectively in order to maintain a solids level of 7%. Using the Fermentation Control System, media were inoculated with .1% of multiple strain cultures 101, 111, 121, 131, 141, 151, 161, 171, 181 or 191 and then compared by the starter activities generated under these conditions.

In addition, six other yeast extracts (Amberex 695, Universal Foods Corp.; AYE 2200 and 2312, Gist-brocades Food Ingredients, Inc., King of Prussia, PA; Fidco 8005 and 8070, Food Ingredients Development Co., White Plains, NY; Veeprex B460, Champlain Ind. Ltd., Ontario, Canada) were compared to determine if the type or quality of this ingredient was influential. Yeast extracts were evaluated at concentrations of .25 and .35% in medium 47 using a .2% combined inoculum of cultures *Lb bulgaricus* 210, 260 and *S. thermophilus* 681 and 690. Media were prepared as before using the Fermentation Control System and evaluated in terms of the starter activities and coccus:rod ratios.

Use of Condensed Fish Solubles, Fish Peptones and Poultry Peptones as Substitutes for Yeast Extract

Alternate nutrient sources, condensed fish solubles (FS; Zapata Haynie Corp., Hammond, LA) and spray-dried fish and poultry peptones (FP and PP; California Spray Dry Co., Stockton CA) were evaluated in order to determine their usefulness as substitutes or supplements for YE. These products are comparable to YE in terms of protein and amino acid content and their use would provide considerable savings in ingredient costs. Formulations were based on medium 47 and consisted of the substitution of either FS, FP or PP for part or all of the YE with proportional increases in whey when necessary. Using the Fermentation Control System, media were inoculated with multiple cultures (Vivolac Cultures Corp.) at a total concentration of .2% and comparisons were made in terms of starter activities and the final coccus:rod ratios.

Controlling the Final Coccus:Rod Ratio

Although recent reports by Oberg (1990) and Kindstedt and coworkers (Yun et al, 1992a,b; Larose et al., 1992; Effendi et al., 1992) may suggest otherwise, cheesemakers have historically believed that subtle changes in mozzarella cheese properties can be produced by small changes in the coccus:rod ratios of the mature starter. For this reason, many cheesemakers desire to be able to manually adjust the fermentation conditions of a starter medium in order to alter the final culture ratio. Therefore, attempts were made to alter the final ratios in

either medium 46 or medium 47. Using the Fermentation Control System, media were inoculated with culture and evaluated for starter activities and the final coccus:rod ratios.

Temperature. Using a .2% inoculum in medium 47, combined cultures *Lb. bulgaricus* 10R, 60R and *S. thermophilus* 150C and 190C were incubated at the following temperatures (^{0}C): 36.7, 37.8, 38.9, 40, 41.1 and 43.4. In a similar experiment using medium 46 and mixed strain culture 101, growth temperatures of 42, 44, 46 and 48^{0}C were examined. Because of the organisms different optimum growth temperatures, lower temperatures would be expected to favor coccus growth and higher temperatures should promote rod growth.

Break pH. These experiments were based on the pH optima of the two culture types. Because the growth of *Lb. bulgaricus* should be favored at lower pH values, the effect of lowering the break pH was examined with respect to the resulting coccus:rod ratios. Samples were taken at various pH values (5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2, 4.1) and were immediately cooled to 4^{0}C for further evaluation. Experiments were conducted in medium 47 using cultures *Lb. bulgaricus* 210, 260 and *S. thermophilus* 651 and 690 in a combined inoculum of .2%.

Inoculum ratio. Three situations were examined to try to alter the final coccus:rod ratios by changing the initial ratios. The first involved a broad range (100:1, 10:1, 1:1, 1:10, 1:100) of starting inocula into medium 46, the second used a more narrow range (5:1, 2:1, 1:1, 1:2, 1:5) in medium 47 and the third employed the addition of the coccus strains at various intervals (.5 h, 1 h, 1.5 h,

and 2 h) after the start of the fermentation process in medium 46. All inoculum ratios, which involved both Marshall's and lab cultures, were based on volume.

Results and Discussion

Development of a Base Medium

The basis for the initial ingredient combinations used in media 1-32 was two-fold. First, the effect of increased concentrations of buffering components (i.e., magnesium hydroxide and monobasic ammonium phosphate) on culture performance was evaluated. The primary concerns in this respect were the sensitivity of *Lb. bulgaricus* to phosphate concentrations increased up to 2%, as was demonstrated by Wright and Klaenhammer (1984), along with the possibility of heavy sedimentation of the insoluble trimagnesium phosphate. Second, other compounds were added to some of these formulations either to adjust the initial pH (malic and adipic acid) or to try to stimulate culture growth. The latter was attempted by the addition of extra carbohydrates (glucose and maltrin) or compounds (i.e., CO_2 liberated from carbonate, formate) stimulatory to *Lb. bulgaricus.*

The remaining formulations in these initial experiments either involved the substitution of other buffering compounds for magnesium hydroxide (media 33-40) or the partial replacement of monobasic ammonium phosphate with dibasic ammonium phosphate (media 41-46). Some of the latter formulations also involved the addition of carbonate and/or formate to enhance lactobacillus growth.

The activities and coccus:rod ratios obtained from the mature starter produced by the various media formulations are given in Table 4. Since one of the primary objectives for this medium, at this stage of development, was to produce mature starter with higher activity than other commercial media, the former judgment was emphasized. In order to standardize these values, a numerical rating system was developed, whereby, the activity of the experimental medium was subtracted from that of the control medium. This system also helped minimize activity differences due to possible variations between cultures and culture production lots.

Examination of the activity ratings revealed nine media formulations (5, 17, 18, 19, 20, 23, 44, 45, 46) that provided activities above the control. Of the nine formulations, eight contained sodium formate; these were also the only initial formulations to contain formate. Sodium carbonate was involved in three of the formulations, including the one without formate, but problems with excessive foaming, due to CO_2 release, made its addition somewhat undesirable. Therefore, the activity test and coccus:rod ratio data, along with factors such as foaming and/or sediment formation in certain formulations, led to medium 46 being chosen for further evaluation. Figure 1 shows the growth patterns of combined strains of *Lb. bulgaricus* and *S. thermophilus* in this medium.

Due to problems and concerns with the large scale blending of this medium, a mixture containing whey, magnesium hydroxide and formic acid was combined and spray dried to form a single "premix" component that was then added to the remaining ingredients of medium 46. The drying of the initial formulations were performed

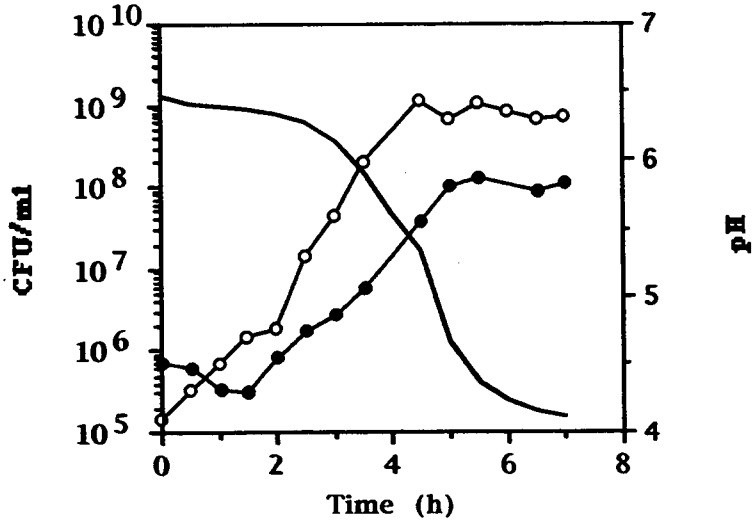

Figure 1. Growth patterns of *Lb. bulgaricus* and *S. thermophilus* strains in medium 46. Combined cultures *Lb. bulgaricus* 210 and 260.(•), *S. thermophilus* 651 and 690 (°), pH (-).

Table 4. Performance comparisons of mature starter produced in media formulations 1-46

Medium	Activity[a]	Rating[b]	C:R ratio[c]	Cultures
1	.75	-.31	20:1	191
2	.86	-.20	20:1	191
3	1.04[d]	-.16[d]	15:1[d]	101,191
4	1.03	-.30	5:1	101
5	1.43	+.10	20:1	101
6	.72	-.55	50:1	151
7	.86	-.40	50:1	181
8	1.02[d]	-.18[d]	10:1[d]	101,191
9	.87	-.19	20:1	191
10	1.06	-.20	15:1	181
11	1.00[d]	-.26[d]	50:1[d]	151,181
12	1.21[d]	-.20[d]	12:1[d]	181
13	1.11	-.06	10:1	181
14	1.15	-.27	10:1	181
15	1.16[d]	-.18[d]	11:1[d]	151,181
16	1.15	-.02	3:1	181
17	1.29[d]	+.05[d]	20:1[d]	111,181
18	1.40	+.09	5:1	181
19	1.37	+.06	20:1	181
20	1.36	+.05	5:1	181
21	1.18	-.07	5:1	181
22	1.20	-.05	10:1	181
23	1.35	+.18	15:1	111
24	1.21	-.04	10:1	181
25	1.23	-.02	50:1	181
26	1.08	-.09	3:1	181
27	1.31	-.08	10:1	181
28	1.05	-.37	10:1	181
29	1.13	-.29	15:1	181
30	1.22	-.32	5:1	181
31	1.30	-.09	2:1	181
32	1.26	-.13	10:1	181
33	.80	-.61	15:1	181
34	1.18	-.23	20:1	181
35	1.25	-.29	5:1	181
36	1.34[d]	-.14[d]	13:1[d]	181
37	1.04	-.33	50:1	181
38	.65	-.72	20:1	181

Table 4, cont.

39	.58	-.79	20:1	181
40	.73	-.64	15:1	181
41	1.10[d]	-.20[d]	27:1[d]	151,181
42	1.12	-.14	100:1	181
43	1.15	-.09	50:1	181
44	1.47	+.23	50:1	181
45	1.36	+.12	100:1	181
46	1.47[d]	+.19[d]	8:1[d]	101,181

[a]Using .1% inoculum.

[b]Activity comparison between the experimental formulation and the control: (formulation activity) - (control activity).

[c]Final ratio determined microscopically.

[d]Mean value.

by the School of Pharmacy at OSU and Table 5 compares the activities and coccus:rod ratios produced using these formulations. Since the results were acceptable, the composition was then adapted to allow the incorporation of a premix formulation into the bulk production of this medium. Therefore, in order to keep consistent with the production medium, formulations and comparisons involving premix (medium 47) were eventually used in place of medium 46. Buffering comparisons between these media and NDM are presented in Figure 2.

Comparison of Commercial Bulk Starter Media

Six commercial bulk starter media were compared to medium 47 and the results are presented in Table 6. Biotherm and Thermostar II were internally neutralized while the other four (CR, ICS, Italiano and Thermolac) received external neutralization. With the exception of CR, all media were very comparable in their production of acceptable coccus:rod ratios. In terms of starter activity, medium 47 produced the most active cultures with Biotherm a close second and CR, Italiano and Thermolac giving very poor activity. Therefore, the goal to develop an internally neutralized bulk starter medium that produced acceptable coccus:rod ratios and gave culture activities higher than other commercially available starter media was successfully accomplished.

Table 5. Performance comparisons of starter media containing premix

Medium	Activity[a]	Final coccus:rod ratio[b]
46[c,d]	1.75[e]	6:1[e]
46 + premix #1[c]	1.76	8:1
46 + premix #2[d]	1.81	4:1
46 + premix #3[d]	1.79	3:1
46 + premix #4[d]	1.82	3:1

[a]Using a 1% inoculum.
[b]Determined microscopically.
[c]Culture 161.
[d]Cultures 210, 260, 651, 690 combined.
[e]Mean value.

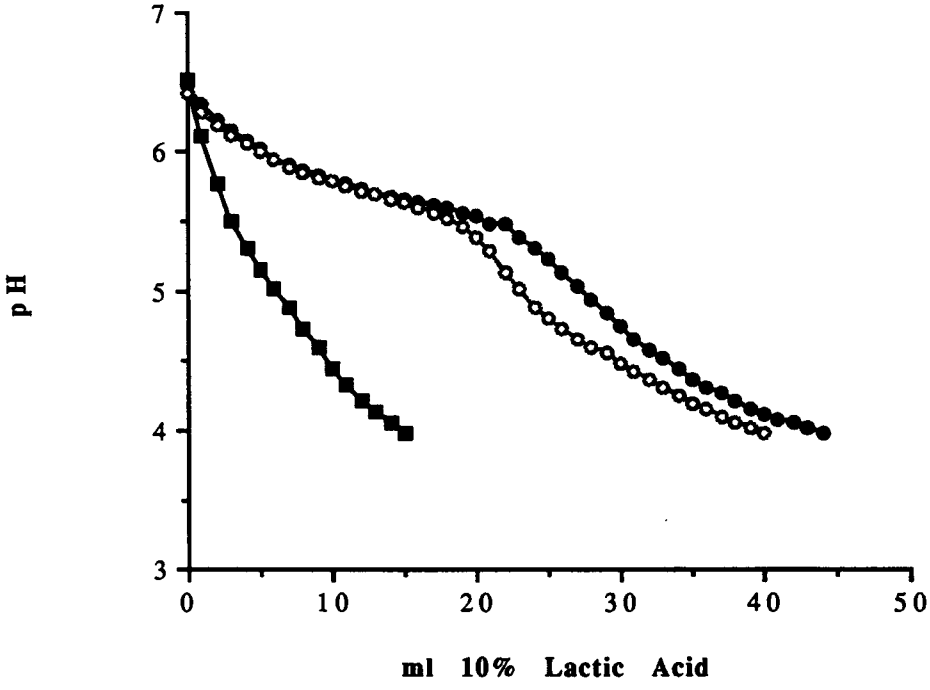

Figure 2. Buffering curves for 7% NDM (■), medium 46 (●) and medium 47 (○).

Table 6. Comparison of culture performance in medium 47 to other commercial bulk starter media

	Media						
	47	Biotherm	CR	ICS	Italiano	Thermolac	Thermostar II
Activity[a]	1.80[b]	1.73	.50	1.04	.72	.71	1.01[b]
Final C:R ratio[c]	4:1[b]	1:1	1:7	1:1	1:1	1:2	3:1[b]

[a]Using a 1% inoculum.
[b]Mean value.
[c]Determined microscopically.

Optimizing the Concentration of Yeast Extract

In general, the activities of cultures grown in medium 46, displayed in Figure 3, showed a tendency to decline slightly with decreasing concentrations of yeast extract. The results appeared to be somewhat culture dependent as well. Although multiple strain culture 121 showed an immediate and drastic decline in activity, most others displayed little variation through concentrations decreasing to .1%. Therefore, indications were that the YE concentration in the medium could be lowered from .4% (w/v) to possibly .3-.1% (w/v) without any serious loss in performance. This consideration would benefit the media manufacturer through the monetary savings incurred using lower levels of this costly ingredient.

Previous studies with yeast extracts in a medium for mesophilic lactic starters showed that the source of YE could have a significant effect on culture growth (A. Nooteboom, personal communication). For this reason, Amberex 1003, the YE found to be most stimulatory for these mesophilic cultures, was used in the development of this medium. Thermophilic lactic cultures, however, showed no discernible preference toward any of the yeast extracts evaluated. None of the brands at either of the concentrations tested showed significant reductions in activity or problematic changes in coccus:rod ratios. These data, compiled in Table 7, also support the previous results which showed that the concentration of YE could be decreased without hindering culture performance.

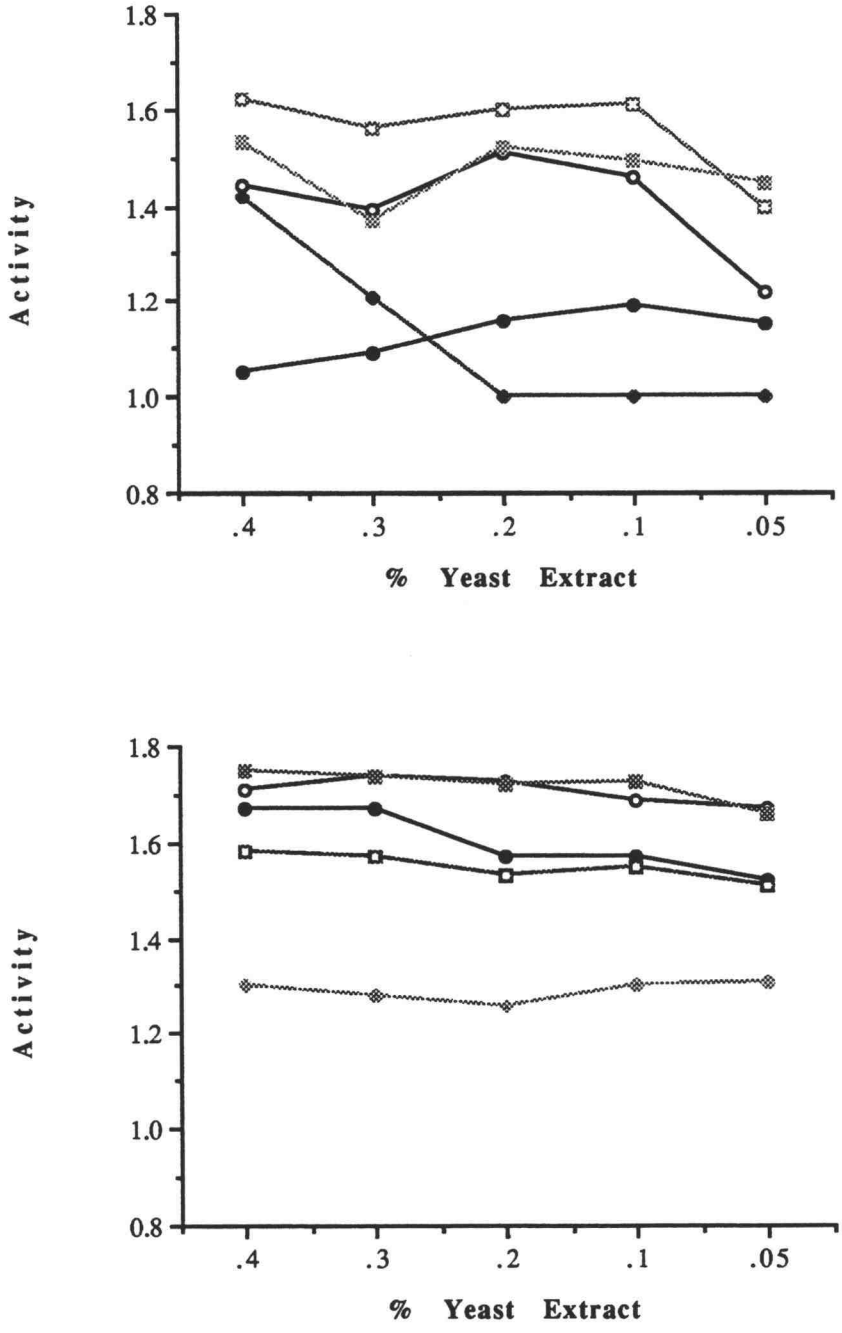

Figure 3. Activities of cultures grown with decreased concentrations of yeast extract in medium 46. Top: culture 101 (○), 111 (●), 121 (◆), 131 (□), 141 (▩). Bottom: culture 151 (●), 161 (○), 171 (□), 181 (▩), 191 (◆).

Table 7. Comparison of different yeast extracts (YE) for culture performance in medium 47

	Amberex		Gist-brocades		Fidco		Veeprex
	1003	695	2200	3212	8005	8070	B430
Activity[a] (.25% YE)	1.76[c]	1.76	1.72	1.81	1.79	1.80	1.83
Activity[a] (.35% YE)	1.78[c]	1.77	1.76	1.77	1.75	1.79	1.79
Final C:R ratio[b] (.25% YE)	4:1[c]	2:1	5:1	3:1	3:1	3:1	3:1
FinalC:R ratio[b] (.35% YE)	2:1[c]	2:1	4:1	3:1	4:1	3:1	2:1

[a]Using a 1% inoculum.
[b]Determined microcsopically.
[c]Mean value.

Use of Condensed Fish Solubles, Fish Peptone and Poultry Peptone as Substitutes for Yeast Extract

Although the odor was quite offensive, the use of fish solubles, either to supplement or replace YE, proved to be a suitable substitute in terms of the activity and coccus:rod ratios generated in the mature starter (Table 8). Since the data suggested that a deodorized and dried form of this ingredient might be an acceptable replacement, spray dried fish and poultry peptones were also evaluated. Starter produced using these substitutes also gave very acceptable activities and coccus:rod ratios (Table 9), even at half the usual concentration of YE in medium 47.

Currently, the primary markets for these products are as nutrient supplements in bacterial culture media. Since these are not involved in foodstuffs, they have not been conditioned to meet the standards of food quality ingredients. If these standards are met, these products could provide a very inexpensive replacement or supplement for yeast extract in starter media.

Controlling the Final Coccus:Rod Ratio

As indicated previously, the effects of small differences in coccus:rod ratios in mature starter are still largely speculative and are a topic of continual debate within the mozzarella cheese industry. Large variations in starter culture ratios, however, can have significant impact on the quality of the cheese. One example relates to its elasticity. Starter heavily dominated by the less proteolytic *S.*

Table 8. Effect of the addition of fish solubles to medium 47 on culture[a] performance

	% Yeast extract/fish solubles			
	.35/0	.18/.18	0/.35	0/1.3
Activity[b]	1.01	1.12	1.08	1.37
Final C:R ratio[c]	1:3	1:1	1:2	2:1

[a]*Lb. bulgaricus* 210, 270 and *S. thermophilus* 651 and 690 combined.
[b]Using a 1% inoculum.
[c]Determined microscopically.

Table 9. Comparison of yeast extract, fish peptone and poultry peptone for culture[a] performance in medium 47

	% Yeast extract		% Fish peptone		% Poultry peptone	
	.18	.35	.18	.35	.18	.35
Activity[b]	1.76	1.77	1.77	1.79	1.78	1.83
Final C:R ratio[c]	1:1	4:1	3:1	2:1	2:1	3:1

[a]*Lb. bulgaricus* 210, 260 and *S. thermophilus* 681 and 690 combined.
[b]Using a 1% inoculum.
[c]Determined microscopically.

thermophilus culture can produce "short" cheese which possesses very little stretch and poor meltability. Both qualities are undesirable in food products such a pizza. Conversely, too many lactobacilli can result in increased proteolysis of the cheese during storage and create a soft bodied cheese with decreased shelf life and bitter off flavors.

Similar problems have also been addressed (Johnson, 1992) in the production of Swiss cheese. Here again, starter culture proportions are important since multiple cultures are involved: *S. thermophilus*, *Lb. helveticus*, *Lactococcus lactis* and strains of propionibacteria. In this case, excessive numbers of lactobacilli can result in extensive protein breakdown during warm room ripening. This can produce brittle cheese which may crack and split with gas production by the propionibacteria instead of forming the characteristic rounded eyes.

Therefore, the ability to exercise some degree of control over the final proportion of culture strains is important for quality cheese production. For mozzarella, although the impact of subtle ratio changes is questionable, large ratio differences can be problematic and the ability to specifically alter these is desirable.

Temperature. The data presented in tables 10 and 11 indicate that the temperature of incubation, over the range of 37 to 48^{0}C, had very little influence on the final coccus rod ratios in media 46 and 47. Although it would be expected that the growth of pure cultures of *S. thermophilus* (optimum growth temperature between 35-42^{0}C) would be favored at lower incubation temperatures over that for *Lb. bulgaricus* (optimum growth between 43-46^{0}C) and vise versa, these results were consistent with those of Radke-Mitchell and Sandine

Table 10. Culture performance in medium 47 at varied incubation temperatures

	Temperature (^{0}C)					
	37	38	39	40	41	43
Activity[a]	1.77	1.82	1.88	1.92	1.95	1.97
Final C:R ratio[b]	3:1	3:1	3:1	3:1	4:1	7:1
Run time[c]	7:54	7:12	6:52	6:12	6:12	5:44

[a]Using a 1% inoculum.
[b]Determined microscopically.
[c]Time (h) from inoculation to break pH (4.6).

Table 11. Culture performance in medium 46 at varied incubation temperatures

	Temperature (^{0}C)			
	42	44	46	48
Activity[a]	1.40	1.37	1.29	1.40
Final C:R ratio[b]	5:1	5:1	5:1	5:1

[a]Using a 1% inoculum.
[b]Determined microscopically.

(1986) who showed that this factor was not influential when the cultures were grown together. However, growth was in some way affected by increasing temperatures (37 to 43^{0}C). Although ratios were not dramatically affected, the data in Table 11 show that the time required to reach the break pH was decreased and that starter activity was increased. Therefore, it appears that other factors (i.e., pH, media constituents, culture produced stimulatory factors) may either overshadow or compensate for less than ideal temperatures and allow for relatively equal growth of mixed cultures.

Break pH. Table 12 gives the results of decreased break pH in medium 47. One would expect the proportion of rods to increase since these conditions should favor the growth of *Lb. bulgaricus* (optimum pH around 5.5) over *S. thermophilus* (optimum pH around 6.5). However, the data suggest that because of the confines of this fermentation procedure, notable ratio changes may not result. Since only 52 min elapsed between the samples taken at pH 5.0 and pH 4.4 (the point before acid production began to rapidly decline), it would appear that the time frame may be too limited to allow for significant changes in culture proportions to occur.

Inoculum ratio. Attempts to accurately control the final coccus:rod ratios based on the alteration of the initial ratios were not entirely successful. The data presented in Table 13 show that large alterations in final ratios can be produced when large differences in the inoculum ratio is used. However, the increased proportions of lactobacilli also resulted in decreased starter activities.

Small initial ratio variations (Table 14) did not result in the desired subtle changes to the final ratios either. The practice of

Table 12. Culture performance at varied break points in medium 47

	Break pH									
	5.0	4.9	4.8	4.7	4.6	4.5	4.4	4.3	4.2	4.1
Activity[a]	1.49	1.49	1.52	1.53	1.57	1.58	1.59	1.56	1.54	1.41
Final C:R ratio[b]	21:1	9:1	9:1	10:1	6:1	9:1	5:1	9:1	5:1	7:1
Run time[c]	5:02	5:06	5:12	5:18	5:26	5:38	5.54	6:20	7:12	8:24

[a]Using a 1% inoculum.
[b]Determined microscopically.
[c]Time (h) from inoculation to break pH.

Table 13. Culture[a] performance using broad range coccus:rod inoculum ratios in medium 46

	Coccus:rod inoculum ratio[b]				
	100:1	10:1	1:1	1:10	1:100
Activity[c]	1.66	1.76	1.58	.87	.33
Final C:R ratio[d]	35:1	2:1	1:6	1:20	1:100

[a]*Lb. bulgaricus* 121-R and *S. thermophilus* C120 combined.
[b]Determined volumetrically.
[c]Mean activity values using a 1% inoculum.
[d]Mean C:R ratios determined viably.

Table 14. Culture[a] performance using narrow range coccus:rod inoculum ratios in medium 47

	Coccus:rod inoculum ratio[b]				
	5:1	2:1	1:1	1:2	1:5
Activity[c]	1.67	1.68	1.67	1.66	1.58
Final C:R ratio[d]	17:1	26:1	10:1	7:1	9:1

[a]*Lb. bulgaricus* R110, R160 and *S. thermophilus* C120 and C160 combined.
[b]Determined volumetrically.
[c]Using a 1% inoculum.
[d]Determined microscopically.

adding cocci later in the fermentation process (Table 15) was also of little benefit since the changes that did result were accompanied by large reductions in activity; possibly due to lower final cell numbers.

Table 15. Culture[a] performance resulting from the delayed addition of *S. thermophilus* to medium 46

	Time of coccus culture addition (h)				
	0	.5	1	1.5	2
Activity[b]	1.70	1.58	1.54	1.17	.52
Final C:R ratio[c]	2:1	1:1	1:2	1:3	1:12
Final coccus CFU/ml	$5.3x10^8$	$3.1x10^8$	$1.9x10^8$	$7.0x10^7$	$1.0x10^7$
Final rod CFU/ml	$2.8x10^8$	$3.2x10^8$	$3.6x10^8$	$2.4x10^8$	$1.2x10^8$

[a]*Lb. bulgaricus* 121-R and *S. thermophilus* C120 combined.
[b]Using a 1% inoculum.
[c]Determined viably.

STUDIES ON PHAGES FOR THERMOPHILIC LACTIC CULTURES AND APPROACHES TO INHIBIT THEIR GROWTH IN STARTER MEDIA

Materials and Methods

Cultures, Bacteriophages and Methods for their Isolation and Enumeration

Cultures. The cultures used are listed in Table 1 and the methods and media for the differentiation of *Lb. bulgaricus* from *S. thermophilus* were the same as those described previously (p. 14,16). Additional media used for the enumeration of these organisms were MRS agar (for lactobacilli) and M17 agar (for lactococci and *S. thermophilus*). Cultures were plated using the spread plate method whereby .1 ml of a culture dilution was spread evenly over an agar surface with a sterile glass rod. After allowing 15 min for the sample to absorb into the medium, plates were incubated for 24-48 h at 37^0C in anaerobic jars using GasPak hydrogen/CO_2 generators. Lactococci were treated in the same fashion using M17 medium and incubating at 30^0C.

Bacteriophages. The phages used in these experiments were either obtained from lab collections or isolated from whey samples. A list of these phages and their host strains is shown in Table 16.

Table 16. List of bacteriophages and host strains used in this study

Host strain	Phage	Phage origin
Lactobacillus delbrueckii ssp. *bulgaricus*:		
210	210	whey
Lactococcus lactis ssp. *cremoris*:		
205	205	OSU[a]
KH	kh	OSU[a]
Lactococcus lactis ssp. *lactis*:		
C2	c2	OSU[b]
ML3	ml3	OSU[a]
Streptococcus thermophilus:		
621	621	whey
651	651	whey
681	681	whey
690	690	whey
691	691	whey
C150	c150	whey

[a]B. Geller.
[b]H. Wyckoff.

Spot assays. To detect phages in samples of whey and starter, the samples were first screened using a spot assay. An agar medium in Petri dishes, either MRS or M17, was overlaid with molten (48^{0}C) top agar which consisted of 2.5 ml of the same medium (containing .6% agar), to which was added .05 ml of 1 M calcium chloride and .1 ml culture (grown overnight). After the top agar solidified, portions of whey samples (.025 or .01 ml) were spotted on the surface of the medium and allowed to absorb into the agar before incubation. Plates were incubated for 12 h at 37^{0}C and samples containing phages were identified by the presence of plaques or a clear zone within the spotted area.

Phage propagation and purification. Whey samples that tested positive for phage by the spot assay were inoculated (.05 ml) into a broth medium (10 ml), either MRS or M17, which had previously been seeded with culture and incubated at 42^{0}C until cell growth was visually observed. Calcium chloride, .05 ml of a 1 M solution, was also added at the point of phage addition. The phage-inoculated cultures were allowed to incubate until culture lysis occurred. The lysed cultures were adjusted to approximately pH 6.0 with 3 M sodium hydroxide, centrifuged at 4,500 rpm for 15 min and the supernatants collected and filtered (.45 µm pore diameter syringe filters). Phage stocks were maintained under refrigeration for daily use and were frozen (-40^{0}C) in MRS or M17 media containing 20% glycerol for extended storage.

Plaque assays. The bottom agar layer consisted of either MRS or M17 medium (1.5% agar) which was overlaid with 2.5 ml of molten (48^{0}C) homologous medium (.6% agar) containing .05 ml of 1

M calcium chloride, .1 ml of 6-12 h culture and .1 ml of phage dilution. After solidifying, the plates were incubated for 12 h at 37^{0}C (30^{0}C for mesophilic cultures). Phage titers were determined by counting the number of plaques that formed and multiplying by the dilution factor.

Phage Inhibition by Commercial Bulk Starter Media

Medium 47 was compared to six other commercial bulk starter media with respect to the activities and final PFU/ml of the ripened starters produced in the presence of phages. Using the Fermentation Control System, media were reconstituted and cultured according to the manufacturer's specifications. All media were inoculated with .2% culture (*Lb bulgaricus* 210, 260 and *S. thermophilus* 651 and 690 combined) and approximately 100 PFU/ml of phages 210 and 651. Dilutions (1/10) made from the samples were centrifuged (15 min at 4,500 rpm), the supernatants filtered (.45 µm pore diameter syringe filters) and plaque assays performed.

Phage Inhibition by Cation Chelating Agents

Since cations, particularly calcium, have been shown to be important for the replication of most lactococcal phages (Collins et al., 1950b; Reiter, 1956; Shew, 1949; Sozzi, 1972; Watanabe and Takesue, 1972), it was desired to learn how the addition of compounds capable of chelating these cations would influence phage replication in a medium designed for thermophilic starter cultures. Success has

been reported using this approach to reduce phage infection for mesophilic starters (Ausavanodom et al., 1973; Hargrove, 1959; Hargrove et al., 1961; Richardson, 1978; Richardson et al., 1977; Sandine et al., 1981; Willrett et al., 1979; 1981) but for the thermophilic cultures, the reported successes (Khosravi et al., 1991; Rajagopal et al., 1990) have not been as widespread.

Phosphates and citrate. To try to improve phage inhibition in medium 46, phosphate concentrations were increased from 1.0 up to 2.0% (w/v) using both mono- and diammonium phosphates. Whey concentrations were decreased proportionally in order to keep a total solids level of 7%. Media formulations, evaluated using the Fermentation Control System, were inoculated with .2% of combined cultures *Lb. bulgaricus* R110, R160 and *S. thermophilus* C90 and C150 and approximately 100 PFU/ml of phage c150. Dilutions (1/10), made from the samples, were centrifuged (15 min at 4,500 rpm), the supernatants filtered (.45 μm pore size syringe filters) and plaque assays performed. Comparisons were made in terms of starter activities and the final PFU/ml.

Sodium citrate, a chelating agent used in certain starter media for mesophilic cultures, was evaluated as well using concentrations of .1 and .3% (w/v) with equivalent reductions in whey. Media formulations and samples were treated and evaluated as before and involved the use of the same phage and cultures.

Other chelating agents. Five additional compounds (ammonium oxalate, 8-hydroxyquinoline-5-sulfonic acid, DL-α,ε-diaminopimelic acid, EDTA and EGTA; Sigma Chemical Co., St. Louis, MO), some of which were non-food grade, were evaluated for their

effectiveness in preventing phage infection through their chelating abilities. These were added at concentrations of .1% (w/v) to MRS and M17 media containing 10 mM calcium chloride (10 ml final volumes). Approximately $1x10^6$ CFU/ml of overnight cultures (*Lb. bulgaricus* 210 and *S. thermophilus* 651) and 10 PFU/ml of homologous phages (phage 210 and phage 651) were added and the samples incubated for 6 h at 42^0C. Plaque assays and viable cell counts were determined.

The chelating agents that appeared to successfully inhibit phage proliferation were added to medium 47 and evaluated with the Fermentation Control System using .2% of combined cultures *Lb. bulgaricus* 210, 270 and *S. thermophilus* 651 and 690 with approximately 100 PFU/ml of phages 210 and 651. Dilutions (1/10) made from the samples were centrifuged (15 min at 4,500 rpm), the supernatants filtered (.45 µm pore diameter syringe filters) and plaque assays performed. Comparisons were made in terms of starter activities and the final PFU/ml.

Cation Requirements for Culture Growth, Phage Adsorption and Phage Replication

Since binding free cations did not appear to provide additional phage protection to medium 46 and cation chelation has been shown to be an effective means of phage control in starter media for mesophilic lactic cultures, is was of interest to know if these organisms and their phages had different requirements for calcium and magnesium in their growth processes. Therefore, *Lb. bulgaricus*

and *S. thermophilus* strains and phages were examined and compared to *Lc. lactis* and *Lc. cremoris* strains and phages to determine the approximate levels of these elements required for culture and phage growth.

In order to remove these ions from the selected growth medium (PMN; Gilliland and Rich, 1990) a chelating resin (imminodiacetic acid; Sigma Chemical Co., St. Louis, MO) was utilized. PMN medium was passed through a column packed to a volume 5 times that required to chelate the estimated concentrations of calcium (11.8 mM) and magnesium (4.1 mM) ions (approximately 20 ml resin/100 ml media) and autoclaved. Ions could then be reintroduced to samples of the cation-chelated medium (ccPMN) at the desired concentrations through the addition of either calcium chloride or magnesium chloride.

Culture growth. Cultures were grown overnight in PMN broth, centrifuged for 15 min at 4,500 rpm, washed with phosphate buffer (pH 7.0), centrifuged, resuspended in .1% peptone broth and 1/100 dilutions were made. Samples (.01 ml) from culture dilutions were added to 1 ml aliquots of PMN, ccPMN and ccPMN broth that contained either 1, 10 or 100 mM calcium chloride or magnesium chloride. The samples were incubated at either 30^{0}C (*Lc. cremoris* and *Lc. lactis* cultures) or 42^{0}C (*Lb. bulgaricus* and *S. thermophilus* cultures) for 8 h. Viable cell counts were performed by spread plating.

Phage adsorption. Cultures were grown overnight in PMN broth, centrifuged for 15 min at 4,500 rpm, washed in phosphate buffer (pH 7.0), centrifuged again and resuspended in .1% peptone

broth. Approximately $1x10^6$-$1x10^7$ CFU/ml of culture and $1x10^4$ PFU/ml of homologous phage were added to 10 ml culture tubes containing 5 ml PMN, ccPMN or ccPMN broth supplemented with either 1, 10 or 100 mM calcium chloride or magnesium chloride. After allowing 15 min for adsorption at either 30 or 42^0C, samples were centrifuged (15 min at 4,500 rpm), supernatants were filtered through .45 μm pore size syringe filters and plaque assays were performed.

Phage replication. Cultures were grown overnight in PMN broth, centrifuged, washed and resuspended as before. Approximately $1x10^4$-$1x10^5$ CFU/ml of culture and 10 PFU/ml of homologous phage were added to 10 ml culture tubes containing 5 ml PMN, ccPMN or ccPMN broth supplemented with either 1, 10 or 100 mM calcium chloride or magnesium chloride. Samples were incubated for 8 h at either 30 or 42^0C after which they were centrifuged, supernatants were filtered (.45 μm pore size syringe filters) and plaque assays were performed.

pH Requirements for Phage Adsorption

These studies were conducted in order to determine if the adsorption of phages to host cultures is influenced by the pH of the growth medium. If so, media could be adjusted, through all or part of the fermentation cycle, to maintain pH ranges antagonistic to phage adsorption.

Cultures were grown for 8-12 h in either MRS or M17 broth and diluted into fresh medium (10 ml final volume) to an approximate

concentration of $1x10^6$-$1x10^7$ CFU/ml. The media were adjusted with either 10% lactic acid or 5% ammonium hydroxide to pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 (a range encompassing what would be encountered through the fermentation cycle of a typical starter medium) and homologous phages were added to final concentrations of approximately $1x10^4$ PFU/ml. After allowing an adsorption period of 20 min at ambient temperature, samples were centrifuged for 15 min at 5,000 rpm and the supernatants, which would contain unadsorbed phages, were filtered using syringe filters (.45 μm pore diameter). Plaque assays were conducted on the sample supernatants.

Use of Stabilizers and Rennet to Inhibit Phage Transfer Through Media

The process of renneting vat milk earlier in the cheesemaking process (<1 h after starter addition) in an attempt to inhibit bacteriophage infection has been an accepted practice in the cheese industry for many years (Lawrence and Pearce, 1972). The assumption is that the transfer of phages present in the milk (or starter) will be inhibited by the coagulated curd matrix. Thus, new phage particles that burst from lysed cells will not be able to flow freely through the milk vat to infect other starter bacteria.

With this in mind, the idea of thickening the starter medium in order to inhibit phage transfer was examined. To begin, various stabilizers were added to medium 47 in order to determine their effectiveness in keeping the insoluble components of the medium in

suspension. The medium was reconstituted (7% solids) in volumes of 500 ml in 1-L media bottles to which was added .1 or .25% of one of the following stabilizers: Carbopol 960, carboxymethlycellulose (high or low viscosities) carrageenan (iota or kappa forms), guar gum, methyl cellulose (15 or 4,000 centiposes) and xanthan gum. Samples were heated and held at 85^{0}C for 45 min, cooled to 37^{0}C and examined for sedimentation over a period of seven hours.

Those stabilizers that completely prevented sedimentation were examined at a concentration of .2% (w/v) for their ability to sustain culture growth (*Lb. bulgaricus* 210, 260 and *S. thermophilus* 651 and 690 combined) and inhibit phage proliferation (phage 651) in medium 47. These experiments were performed with the Fermentation Control System under standard conditions except that the media were left unstirred during the ripening period and were only mixed at the point of sample collection. Mature starter was examined for activity and final phage titers.

Experiments were also conducted to evaluate the actual contribution of the early renneting process to phage inhibition. Experiments were performed in 1-L culture bottles using 500 ml volumes of whole milk. After tempering the milk to 30 or 42^{0}C, the bottles were inoculated with either *Lb. bulgaricus* 210, *S. thermophilus* 651 or *Lc. lactis* C2 (30^{0}C milk). Rennet (single strength calf) was added in excess (.1% final concentration instead of .01%) to ensure rapid and complete milk coagulation. Dilutions of phages 210, 651 or c2 were sprayed over the milk surface and after either 6 (*Lb. bulgaricus* 210 and *S. thermophilus* 651) or 8 h (*Lc. lactis* C2) samples were taken from the top, middle and bottom of the

culture vessels, along with a mixed sample. These were compared for both the final CFU/ml (using TPYE medium for strains 210 and 651) and PFU/ml.

Adsorption of Phage to Heat Killed Cells

One idea recently suggested for inhibiting phage proliferation in starter media maintains that cultures could be added to hydrated bulk starter prior to heat treatment in order to adsorb phages that may be present. Both cultures and phages would then be destroyed during the heating process and any phage introduced after this point could still adsorb to the remaining dead cells. Assuming that the process was irreversible, the pre-adsorbed phages would not be a threat to viable cultures. This notion has been indirectly substantiated by Valyasevi et al. (1990) who showed that purified cell walls could be used to inactivate phage kh for *Lc. cremoris* KH.

This theory was tested in three different experiments. The first involved the exposure of cultures to various heat treatments in order to determine if adsorption was in any way affected by the condition of the cell after they were destroyed by heat. Cultures were grown overnight in either MRS or M17 broth and then exposed to one of the following conditions: a) pasteurization (63^{0}C for 30 min), b) thermal processing (85^{0}C for 45 min), c) autoclaving (121^{0}C for 15 min at 15 psi). Non-heat treated controls were also included. Samples were cooled to ambient temperature, inoculated with approximately 1x10^3 PFU/ml of homologous phage and allowed a 15 min adsorption period. The samples were centrifuged (15 min at 4,500 rpm) and the

supernatants were filtered (.45 μm pore size syringe filters) and plaque assayed.

The second experiment involved the addition of cultures to medium 46 prior to heat treatment in order to provide a substrate for phage attachment before the usual point of culture addition. Using the Fermentation Control System a .2% combined inoculum of cultures *Lb. bulgaricus* R110, R160 and *S. thermophilus* C150 and C160 was added and media were heated through the standard cycle. The tanks were then inoculated with approximately 100 PFU/ml of phage c150 and, after 15 min, reinoculated with .2% of live cultures. Fermentation was allowed to continue as usual and comparisons were made in terms of starter activities.

In the final experiment, cultures (*Lb. bulgaricus* 210 and *S. thermophilus* 651) were grown overnight in either MRS or M17 broth and then destroyed through heat exposure (85^0C for 45 min). Homologous phage were added and allowed 15 min for adsorption before centrifugation. The supernatants were discarded and the cell pellets were resuspended in .1% peptone broth (10 ml) to be used for the phage inoculum. This was done to determine if phages that initially adsorbed to dead cells could dissipate and still be infectious. Media (medium 47) were processed using the Fermentation Control System with an inoculum of .1% of combined cultures *Lb. bulgaricus* 210 and *S. thermophilus* 651 plus .005% of the resuspended cells. Evaluations were made in terms of starter activities and final phage titers.

Effect of the Initial Phage Concentration on the Performance of Cultures in Medium 47 and Activity Tests

Various phage inocula were added to both medium 47 and activity milk in order to gain an understanding of the level of phage contamination required to produce a decline in culture performance in starter media and vat milk for cheesemaking.

Using the Fermentation Control System, medium 47 was inoculated with .2% culture (*Lb. bulgaricus* 210, 260 and *S. thermophilus* 651 and 690 combined) and either 1, 10 or 100 PFU/ml of phages 210 and 651. Dilutions (1/10) made from the samples were centrifuged (15 min at 4,500 rpm), the supernatants filtered (.45 μm pore diameter syringe filters) and plaque assays performed. Comparisons were made in terms of starter activity and the final PFU/ml.

To test the influence of the initial phage concentration in activity milk, standard activity tests were conducted using combined cultures *Lb. bulgaricus* 210, 260 and *S. thermophilus* 651 and 690. Phages 210 and 651, either individual or combined, were added at concentrations of 1, 10, 100, $1x10^3$, $1x10^4$ or $1x10^5$ PFU/ml. Comparisons were made in terms of the culture activities under the different conditions.

Results and Discussion

Phage Inhibition by Commercial Bulk Starter Media

Of the six media compared with medium 47, three (Italiano, Thermolac and Thermostar II) gave lower final concentrations of either *Lb. bulgaricus* or *S. thermophilus* phages yet only one (Italiano) gave a lower total phage titer (Table 17). However, the activity of cultures subjected to phage and grown in medium 47 was still far superior to that of cultures grown and challenged with phage in any of the other media. It was also notable that only one of the six media (Biotherm) produced cultures in the control state (Table 6) that showed activity greater than that of cultures challenged with phage and grown in medium 47.

Although it was disappointing that a method or supplement to increase phage inhibition in medium 47 was not discovered, it still maintains several advantages over other starter media. The increased culture activity can allow for the production of smaller quantities of starter medium and the use of a lower percentage of starter in cheese milk. Also, less attention to the fermentation process and no additional neutralizing agents are required. Therefore, the cheesemaker will save both time and expense at these steps of the cheese making process.

Table 17. Comparison of culture activity and final phage titers produced in medium 47 to other commercial bulk starter media

	Media						
	47	Biotherm	CR	ICS	Italiano	Thermolac	Thermostar II
Activity[a]	1.18	.94	.30	1.04	.63	.71	1.01
Final PFU/ml:							
phage 210[b]	1.9×10^6	3.6×10^8	7.5×10^7	3.4×10^6	2.5×10^4	9.6×10^8	2.5×10^7
phage 651[c]	6.9×10^6	1.8×10^7	2.0×10^6	3.5×10^6	5.0×10^3	$<1 \times 10^3$	2.0×10^3

[a]Using a 1% inoculum.
[b]For *Lb. bulgaricus* 210.
[c]For *S. thermophilus* 651.

Phage Inhibition by Cation Chelating Agents

Phosphates and citrate. Increasing the phosphate concentration of medium 46 from 1.0 up to 2.0% (w/v) did not appear to inhibit phage proliferation. An upper limit of 2% total phosphate was used because previous studies with another starter medium showed inhibition of the cultures, particularly the lactobacilli, at or above this level (data not shown). The data presented in Table 18 show no substantial changes in culture activity or final phage titers with these formulation changes. The addition of citrate to medium 46 at concentrations of .1 and .3% (w/v) also had no effect on phage proliferation (Table 19) and produced a decline in culture activity as the concentration increased.

Other chelating agents. Of the five compounds examined, only three provided more than a two-log decrease in final phage numbers and only one of these, ammonium oxalate, was effective against both the *Lactobacillus* and *Streptococcus* phages (Figure 4). However, when ammonium oxalate was added to medium 46 at the same concentration, final phage titers were not reduced (Table 20).

These findings would suggest that either insufficient cation chelation occurs or that the phage/host interactions of these thermophilic lactic cultures have lower cation requirements for replication than their mesophilic counterparts. Whatever the case, this approach does not appear to be as effective in starter media for thermophilic cultures as it has been reported for mesophilic starter cultures.

Table 18. Culture performance and phage proliferation with increased phosphate concentrations in medium 46

	% Phosphate (w/v)					
	Control	1.0	1.25	1.5	1.75	2.0
Activity[a]	1.80	1.68	1.74	1.76	1.73	1.69
Final PFU/ml	-	2.0×10^5	3.9×10^5	2.5×10^5	5.1×10^5	4.7×10^5

[a]Using a 1% inoculum.

Table 19. Phage proliferation with citrate addition to medium 46

	% Citrate (w/v)					
	0		.1		.3	
Activity[a]	1.74	1.67	1.70	1.63	1.66	1.50
Final C:R ratio[b]	3:1	2:1	2:1	4:1	2:1	2:1
Final PFU/ml	-	$2x10^5$	-	$4x10^5$	-	$6x10^5$

[a]Using a 1% inoculum.
[b]Determined microscopically.

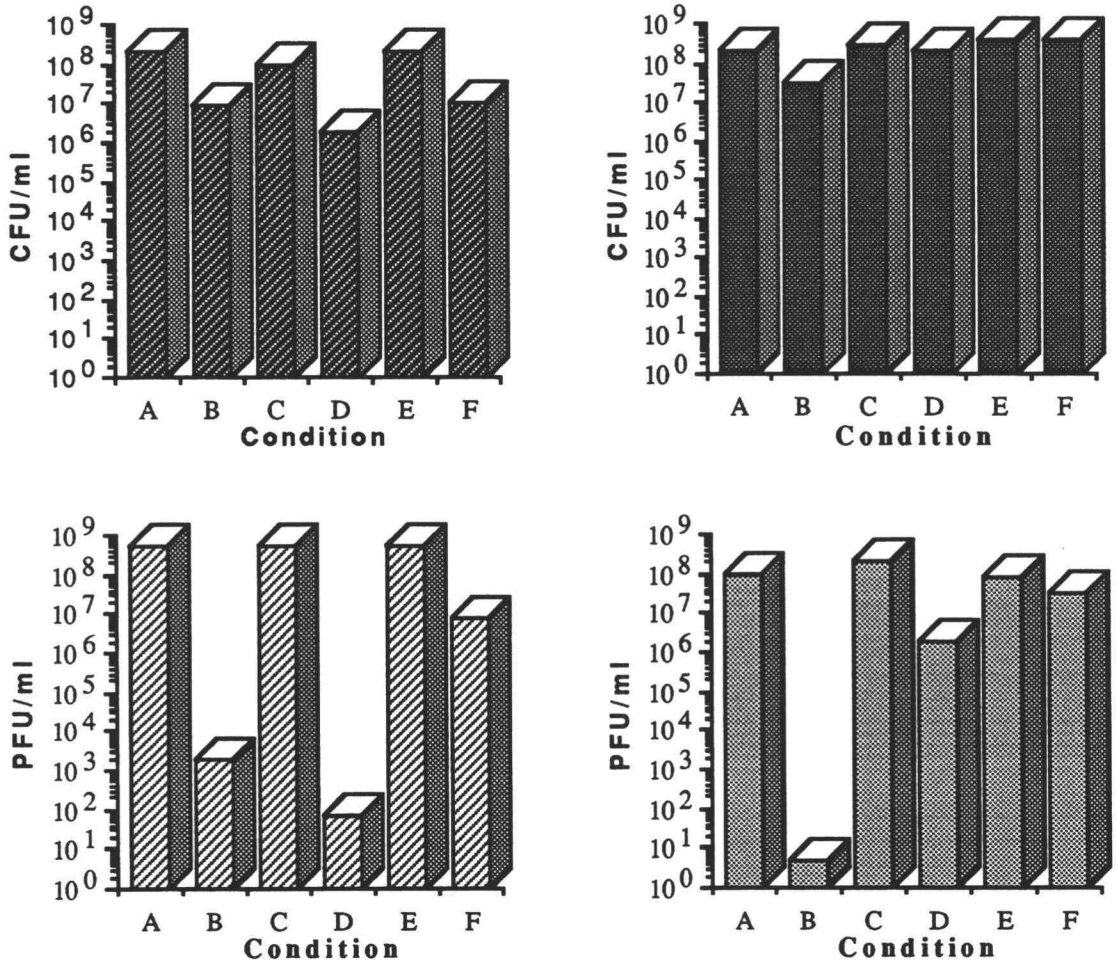

Figure 4. Culture and phage growth for *Lb. bulgaricus* and *S. thermophilus* grown in the presence of various chelating agents. Culture and phage *Lb. bulgaricus* 210 (left) and *S. thermophilus* 651 (right) grown with the following additives: control (A), ammonium oxalate (B), DL-α,ε-diaminopimelic acid (C), EDTA (D), EGTA (E), 8-hydroxyquinoline-5-sulfonic acid (F).

Table 20. Addition of ammonium oxalate to medium 46 and its effect on culture activity and phage proliferation

	Medium 46		Medium 46 + ammonium oxalate
Activity[a]	1.69	1.56	1.54
Final PFU/ml:			
phage 210[b]	-	$5x10^5$	$1x10^4$
phage 651[c]	-	$2x10^7$	$1x10^8$

[a]Using a 1% inoculum.
[b]For *Lb. bulgaricus* 210.
[c]For *S. thermophilus* 651.

Cation Requirements for Culture Growth, Phage Adsorption and Phage Replication

To ensure that cation removal was successful, samples were examined by instrumental neutron activation analysis (INAA) at the OSU Radiation Center. The results of analysis (Table 21) showed that standard PMN medium contained slightly more calcium and slightly less magnesium than was anticipated. However, the chelated medium (ccPMN) contained very little calcium (1.2mM) and magnesium was removed beyond detection by this analysis.

Culture growth. Although the lactobacilli and streptococci were completely inhibited by the removal of calcium and magnesium (Table 22; Figure 5, 6), growth of the lactococci appeared to be unaffected (Table 22; Figure 6),. When these elements were reintroduced to the medium, growth patterns were similar within but differed between genera. The *Lb. bulgaricus* strains appeared to grow best near 10 mM concentrations of either cation. Similar results for *Lb. bulgaricus* were also reported by Wright and Klaenhammer (1983) who showed essentially no growth in a cation-exchanged medium and increasing growth with magnesium concentrations of 1 and 10 mM. The *S. thermophilus* strains, however, appeared to show an inverted relationship between their needs for calcium and magnesium. Growth increased at the highest tested levels of magnesium but decreased with increasing concentrations of calcium. This information suggests that completely removing calcium and magnesium from media designed for thermophilic starter cultures in an attempt to prevent phage

Table 21. Instrumental neutron activation analysis (INAA) of PMN based media

Sample	Concentration (mM)	
	Calcium	Magnesium
PMN	15.9±0.5	3.6±0.7
ccPMN	1.2±0.3	<3.4[a]
ccPMN+10 mM $CaCl_2$	10.6±0.7	<4.6[a]
ccPMN+10 mM $MgCl_2$	1.2±.04	14.5±1.9

[a]No photopeak was evident, therefore, the upper limit value at a 99% confidence level was reported. This would represent the maximum possible concentration of the element that could exist in the sample yet still remain undetected.

Table 22. Effect of Calcium and Magnesium Concentrations on the Growth of *Lb. bulgaricus*, *S. thermophilus*, *Lc. cremoris* and *Lc. lactis*

Culture	Initial CFU/ml	Final CFU/ml							
				PMN + $CaCl_2$ (mM)			PMN + $MgCl_2$ (mM)		
		PMN	ccPMN	1	10	100	1	10	100
210[a]	$1.9x10^3$	$1.4x10^8$	$<1.0x10^4$	$1.0x10^5$	$5.6x10^7$	$9.0x10^4$	$1.2x10^6$	$3.8x10^8$	$7.5x10^7$
181-R[a]	690	$2.2x10^7$	$<1.0x10^4$	$1.5x10^5$	$8.1x10^7$	$5.2x10^6$	$1.0x10^5$	$3.7x10^7$	$3.0x10^5$
651[b]	660	$1.5x10^8$	$<1.0x10^4$	$2.6x10^7$	$3.0x10^4$	$<1.0x10^4$	$<1.0x10^4$	$2.4x10^8$	$1.5x10^8$
681[b]	$1.4x10^3$	$1.5x10^8$	$<1.0x10^4$	$2.1x10^8$	$5.0x10^6$	$1.0x10^4$	$2.1x10^5$	$1.8x10^8$	$1.3x10^8$
KH[c]	$9.0x10^3$	$6.5x10^8$	$2.4x10^9$	$2.9x10^5$	$8.7x10^8$	$2.9x10^8$	$2.9x10^8$	$6.3x10^8$	$6.0x10^8$
205[c]	$3.8x10^3$	$5.3x10^8$	$3.0x10^8$	$6.6x10^5$	$2.8x10^8$	$1.2x10^8$	$8.0x10^7$	$2.0x10^8$	$3.9x10^8$
C2[d]	$1.4x10^4$	$1.4x10^9$	$1.2x10^9$	$5.2x10^7$	$1.1x10^9$	$1.3x10^9$	$1.2x10^9$	$1.4x10^9$	$1.3x10^9$
ML3[d]	$1.3x10^4$	$1.9x10^9$	$1.4x10^9$	$2.7x10^8$	$1.4x10^9$	$1.7x10^9$	$1.1x10^9$	$1.2x10^9$	$1.3x10^9$

[a]*Lb. bulgaricus.*
[b]*S. thermophilus.*
[c]*Lc. cremoris.*
[d]*Lc. lactis.*

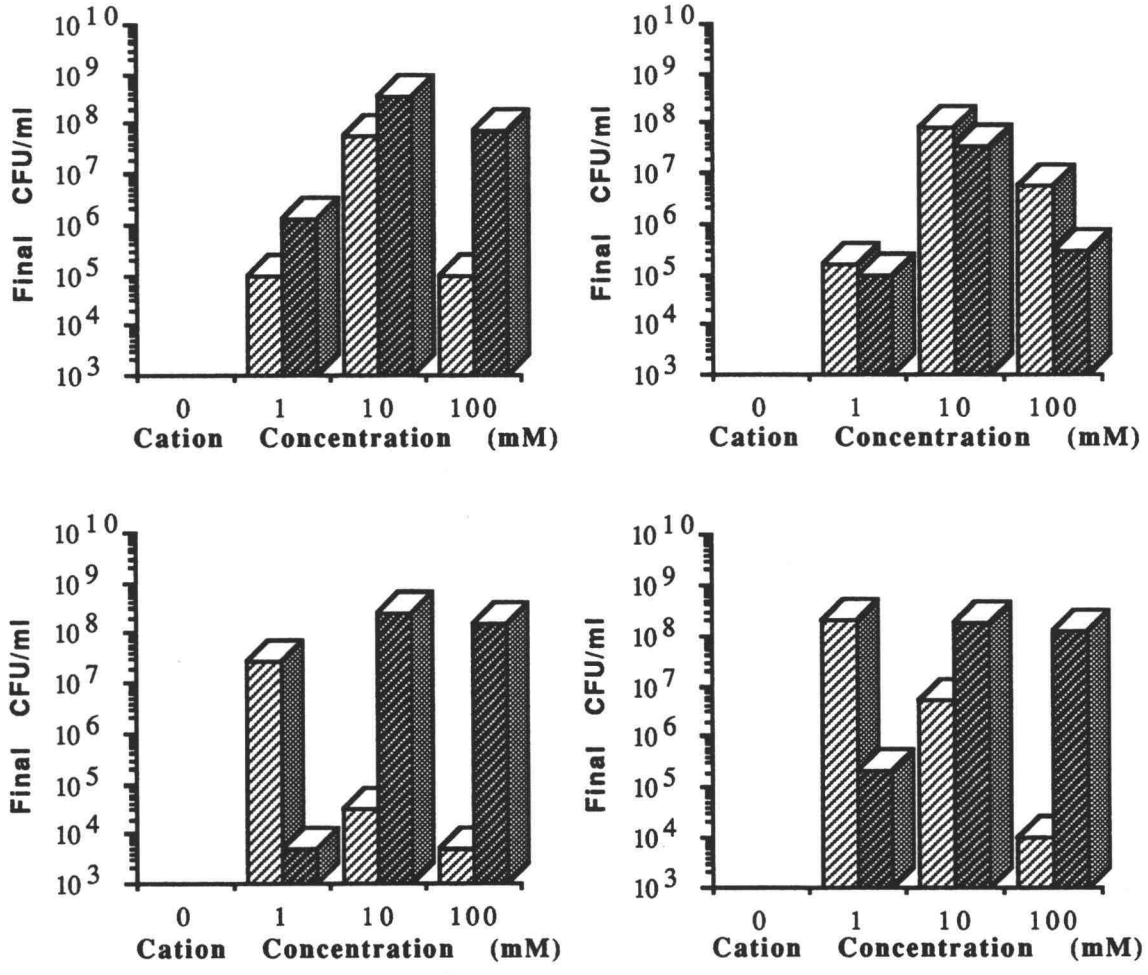

Figure 5. Growth of *Lb. bulgaricus* and *S. thermophilus* at various concentrations of calcium chloride (▨) and magnesium chloride (■). Cultures *Lb. bulgaricus* 210 (top left) and 181-R (top right) and *S. thermophilus* 651 (bottom left) and 681 (bottom right).

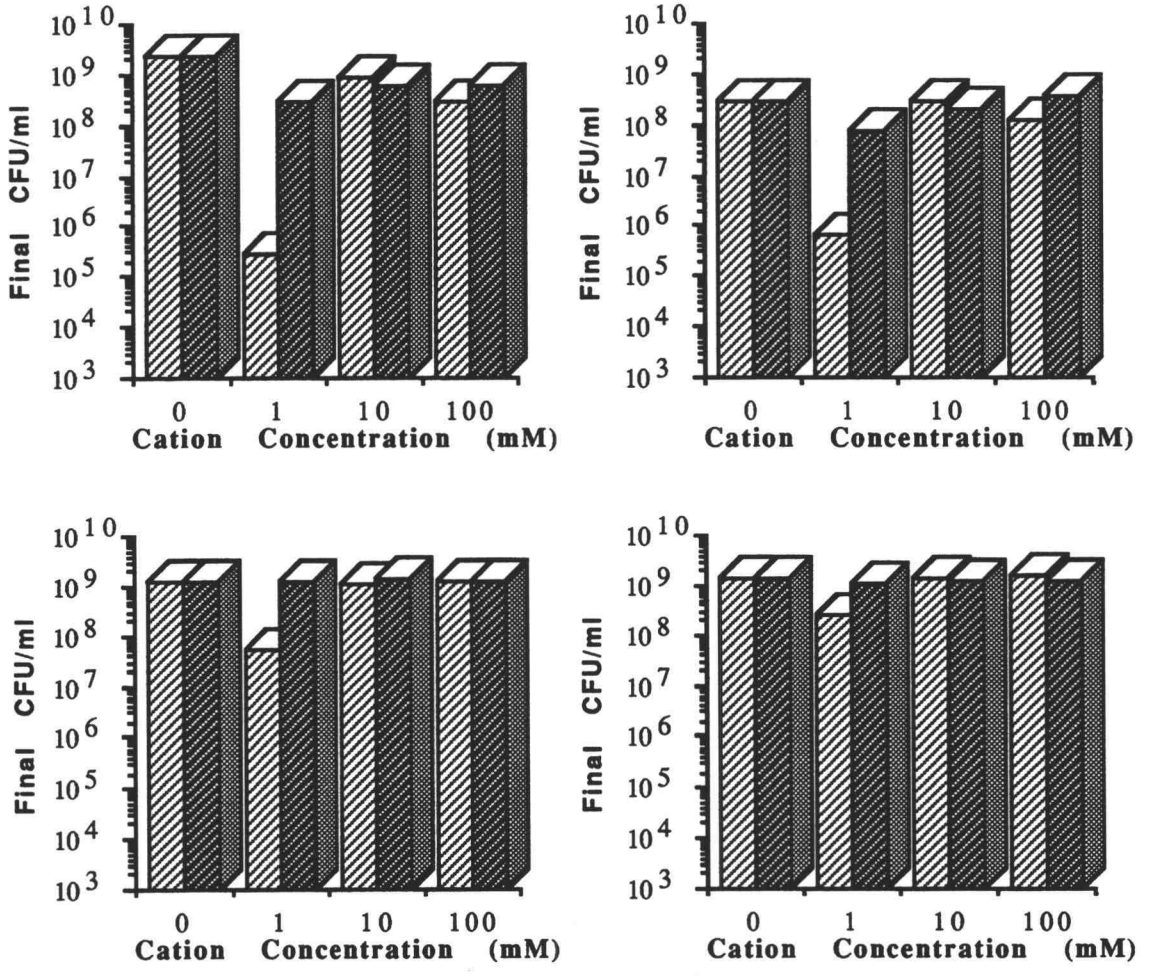

Figure 6. Growth of *Lc. cremoris* and *Lc. lactis* at various concentrations of calcium chloride (▨) or magnesium chloride (■). Cultures *Lc. cremoris* KH (top left) and 205 (top right) and *Lc. lactis* C2 (bottom left) and ML3 (bottom right).

infection may be self defeating due to the negative impact on culture growth.

Phage adsorption. The data in Table 23 and Figure 7 show that for the thermophilic starter strains that were tested, greater than 97% of the phages adsorbed to the cells regardless of the calcium or magnesium concentration. In contrast, the four lactococcal strains each showed different patterns of phage adsorption under the same conditions (Table 23; Figure 8). Both *Lc. cremoris* KH and *Lc. lactis* ML3 showed nearly 80% adsorption or more under all cation levels, *Lc. cremoris* 205 never recorded over 70% of the phages adsorbed and phage c2, for *Lc. lactis* C2, adsorbed fewer phages with increasing cation levels.

None of the cultures examined showed a correlation between lower cation concentrations and decreased phage adsorption. Even though the *Lb. bulgaricus* and *S. thermophilus* strains will not grow under cation depleted conditions, their phages are still able to adsorb. This supported the findings of Watanabe and Takesue (1972) who showed that calcium was only involved in the DNA penetration step.

Phage replication. Varied results were observed between the cultures that were examined (Table 24; Figure 9, 10). Three of the strains, *Lb. bulgaricus* 210, *S. thermophilus* 681, and *Lc. cremoris* KH, showed little or no increase in phage titer when grown in 0 or 1 mM concentrations of either calcium or magnesium while phages for all of the strains showed substantial or highest growth at 10 mM concentrations of one or both of these elements. The lack of phage growth that was observed for *Lb. bulgaricus* 210 and *S. thermophilus*

Table 23. Effect of calcium and magnesium concentrations on the adsorption of phages to *Lb. bulgaricus*, *S. thermophilus*, *Lc. cremoris* and *Lc. lactis*

Culture	Initial CFU/ml[a]	Final PFU/ml							
				PMN + $CaCl_2$ (mM)			PMN + $MgCl_2$ (mM)		
		PMN	ccPMN	1	10	100	1	10	100
210[b]	$1.3x10^7$	20	80	50	40	40	140	10	40
681[c]	$3.8x10^6$	80	70	40	100	20	40	130	30
KH[d]	$2.8x10^7$	510	$1.6x10^3$	$1.5x10^3$	790	620	$1.8x10^3$	$1.3x10^3$	860
205[d]	$2.0x10^7$	$3.3x10^3$	$4.8x10^3$	$3.9x10^3$	$4.1x10^3$	$4.1x10^3$	$4.2x10^3$	$3.3x10^3$	$7.3x10^3$
C2[e]	$3.0x10^7$	$1.7x10^3$	$1.5x10^3$	$1.2x10^3$	$2.3x10^3$	$3.8x10^3$	$1.3x10^3$	$1.9x10^3$	$5.8x10^3$
ML3[e]	$3.4x10^7$	120	240	200	240	230	130	240	$2.1x10^3$

[a]Initial PFU/ml of $1x10^4$.
[b]*Lb. bulgaricus.*
[c]*S. thermophilus.*
[d]*Lc. cremoris.*
[e]*Lc. lactis.*

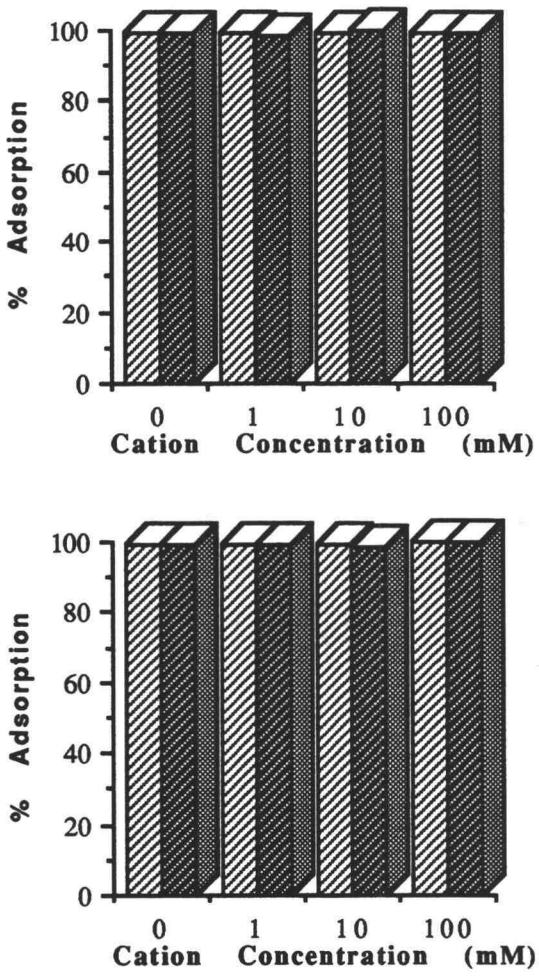

Figure 7. Percent adsorption of phages to *Lb. bulgaricus* and *S. thermophilus* at various concentrations of calcium chloride (▨) or magnesium chloride (■). Cultures *Lb. bulgaricus* 210 (top) and *S. thermophilus* 681 (bottom).

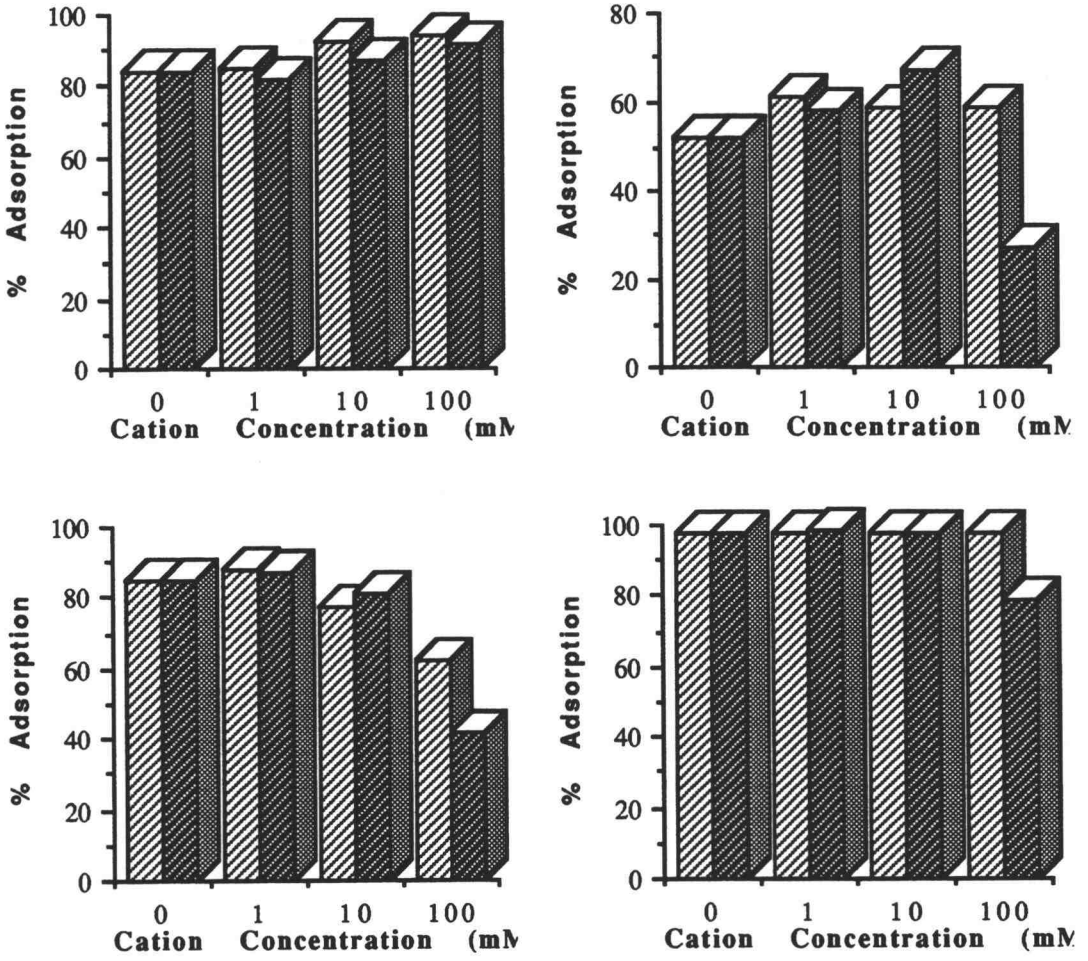

Figure 8. Percent adsorption of phages to *Lc. cremoris* and *Lc. lactis* at various concentrations of calcium chloride (▨) or magnesium chloride (■). Cultures *Lc. cremoris* KH (top left) and 205 (top right) and *Lc. lactis* C2 (bottom left) and ML3 (bottom right).

Table 24. Effect of calcium and magnesium concentrations on the replication of phages for *Lb. bulgaricus*, *S. thermophilus*, *Lc. cremoris* and *Lc. lactis*

Culture	Initial CFU/ml[a]	Final PFU/ml							
				PMN + $CaCl_2$ (mM)			PMN + $MgCl_2$ (mM)		
		PMN	ccPMN	1	10	100	1	10	100
210[b]	$1.1x10^5$	$2.5x10^7$	<10	10	$3.3x10^3$	<10	<10	<10	<10
681[c]	$3.8x10^4$	$1.0x10^9$	<10	<10	$1.6x10^6$	$1.0x10^9$	<10	$1.8x10^9$	<10
KH[d]	$1.6x10^5$	$1.2x10^9$	<10	<10	$5.7x10^9$	10	<10	$2.8x10^9$	$1.6x10^9$
205[d]	$1.2x10^5$	$1.6x10^{10}$	$1.8x10^6$	$1.3x10^9$	$5.6x10^9$	10	$8.9x10^8$	$1.7x10^9$	10
C2[e]	$2.8x10^5$	$2.4x10^9$	<10	$1.2x10^9$	$1.4x10^9$	30	130	$1.5x10^9$	$6.5x10^4$
ML3[e]	$3.2x10^5$	$1.0x10^9$	$1.2x10^6$	$7.1x10^8$	$1.5x10^9$	$4.3x10^3$	$3.7x10^5$	$2.7x10^8$	$6.0x10^9$

[a]Initial PFU/ml of 10.
[b]*Lb. bulgaricus.*
[c]*S. thermophilus.*
[d]*Lc. cremoris.*
[e]*Lc. lactis.*

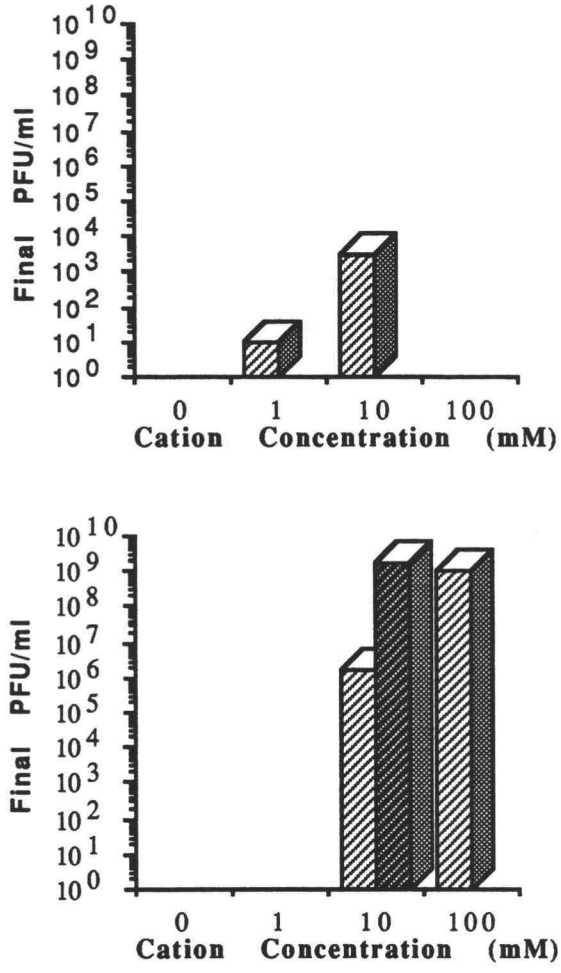

Figure 9. Growth of phages for *Lb. bulgaricus* and *S. thermophilus* at various concentrations of calcium chloride (▨) and magnesium chloride (■). Cultures and phages *Lb. bulgaricus* 210 (top) and *S. thermophilus* 681 (bottom).

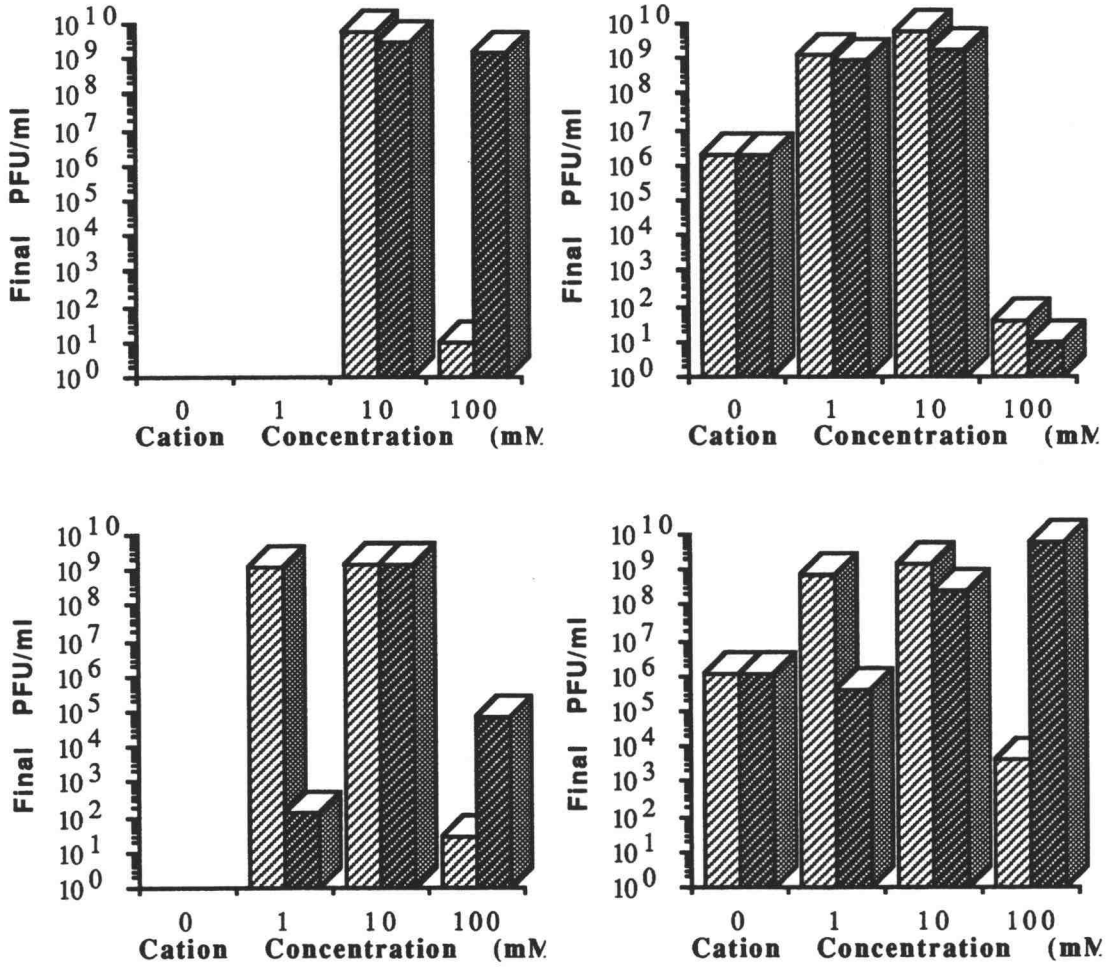

Figure 10. Replication of phages for *Lc. cremoris* and *Lc. lactis* at various concentrations of calcium chloride (▨) or magnesium chloride (■). Cultures and phages *Lc. cremoris* KH (top left) and 205 (top right) and *Lc. lactis* C2 (bottom left) and ML3 (bottom right).

681 in the cation free medium would reflect the lack of cell growth also seen for these strains under this condition. However, since the *Lactococcus* strains did grow in the absence of calcium and magnesium, the lack of phage growth seen under the same conditions for *Lc. cremoris* KH and *Lc. lactis* C2 would suggest that the replication processes of their phages were inhibited.

In summary, it appeared that there were differences between the requirements for calcium and magnesium in the growth and phage replication processes for these thermophilic and mesophilic starter cultures. The thermophiles were much more sensitive to low levels of these cations for culture growth and would not grow at all if they were completely removed, their phages appeared to adsorb regardless of the ion concentration and replication of their phages appeared to be inhibited at low cation levels (1 mM).

pH Requirements for Phage Adsorption

A trend reflecting phage adsorption patterns based on medium pH consistent for both genera examined was not observed. The data presented in Table 25 and Figures 11 and 12 show that for the *S. thermophilus* strains, the pH of the medium either had little or no effect on phage adsorption (strains 681, 691) or produced varied results (strains 621, 651, 690). A more pronounced influence was observed for the *Lb. bulgaricus* strains which showed a general decline in phage adsorption with increasing pH (Table 25; Figure 11). The lactococci each showed a related pattern of depressed phage

Table 25. Influence of pH on the adsorption of phages to *Lb. bulgaricus*, *S. thermophilus*, *Lc. cremoris* and *Lc. lactis*

Culture	CFU/ml	Initial PFU/ml	Unadsorbed phages (PFU/ml) in tested pH range							
			Control[a]	4.0	4.5	5.0	5.5	6.0	6.5	7.0
210[b]	$8.5x10^5$	$5.9x10^3$	$6.3x10^3$	30	110	$2.6x10^3$	$3.9x10^3$	$4.6x10^3$	$2.3x10^3$	$2.4x10^3$
621[c]	$3.2x10^7$	$1.4x10^4$	$1.2x10^4$	50	400	$1.7x10^3$	$2.8x10^3$	$3.0x10^3$	$3.8x10^3$	$4.2x10^3$
651[c]	$6.2x10^7$	$1.7x10^4$	$1.1x10^4$	830	$5.1x10^3$	960	$1.9x10^3$	40	20	50
681[c]	$5.0x10^7$	$3.9x10^3$	$4.1x10^3$	<10	20	40	120	160	90	180
690[c]	$3.0x10^6$	$5.2x10^3$	$5.0x10^3$	<10	610	$1.4x10^3$	60	440	480	440
691[c]	$7.1x10^7$	$3.8x10^3$	$2.1x10^3$	<10	10	<10	20	<10	60	190
KH[d]	$4.1x10^7$	$4.0x10^3$	$3.8x10^3$	90	550	800	130	60	70	130
C2[e]	$2.5x10^8$	$6.8x10^3$	$4.5x10^3$	340	$2.0x10^3$	$5.5x10^3$	$2.4x10^3$	200	150	150

[a]Phage added to MRS (pH 6.5) or M17 (pH 7.0) medium without culture.
[b]*Lb. bulgaricus.*
[c]*S. thermophilus.*
[d]*Lc. cremoris.*
[e]*Lc. lactis.*

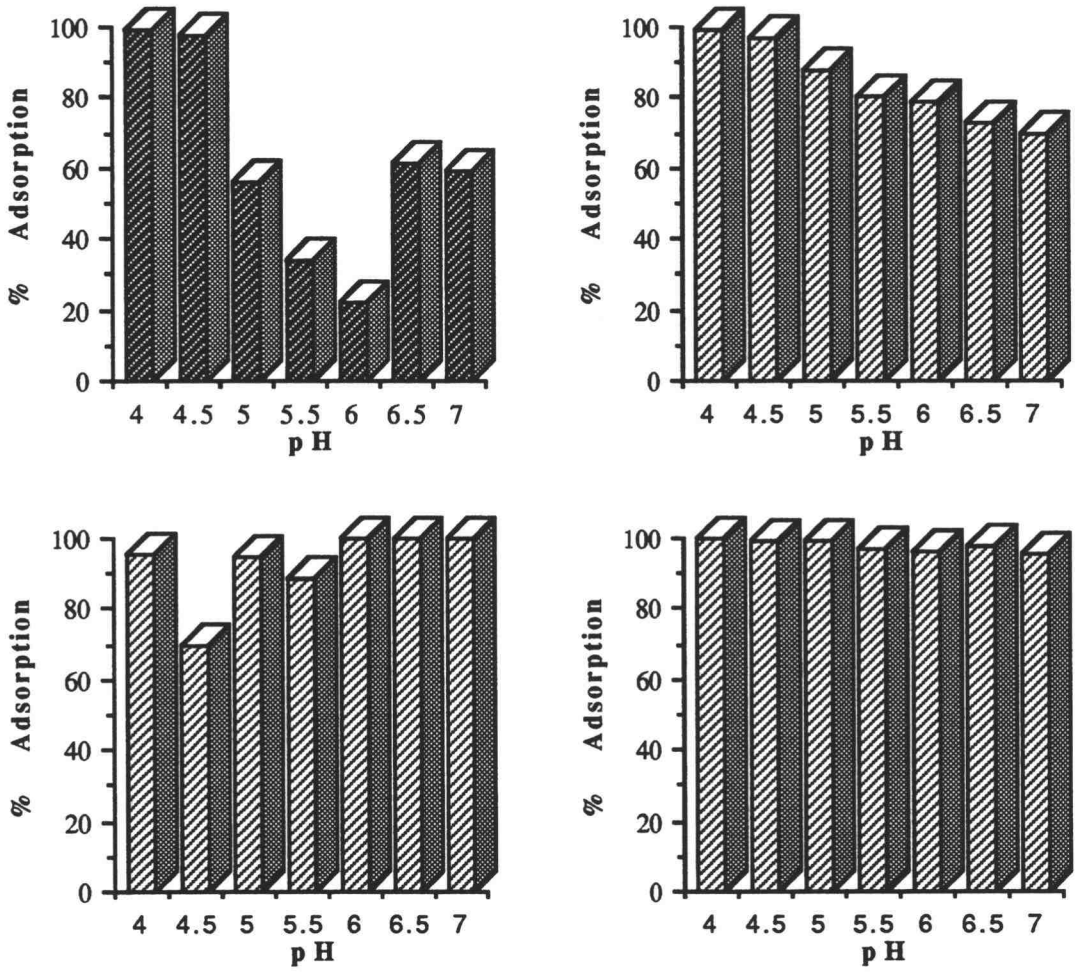

Figure 11. Percent adsorption of phages to *Lb. bulgaricus* or *S. thermophilus* at pH 4 to 7. Cultures *Lb. bulgaricus* 210 (top left) and *S. thermophilus* strains 621 (top right), 651 (bottom left) and *S. thermophilus* 681 (bottom right).

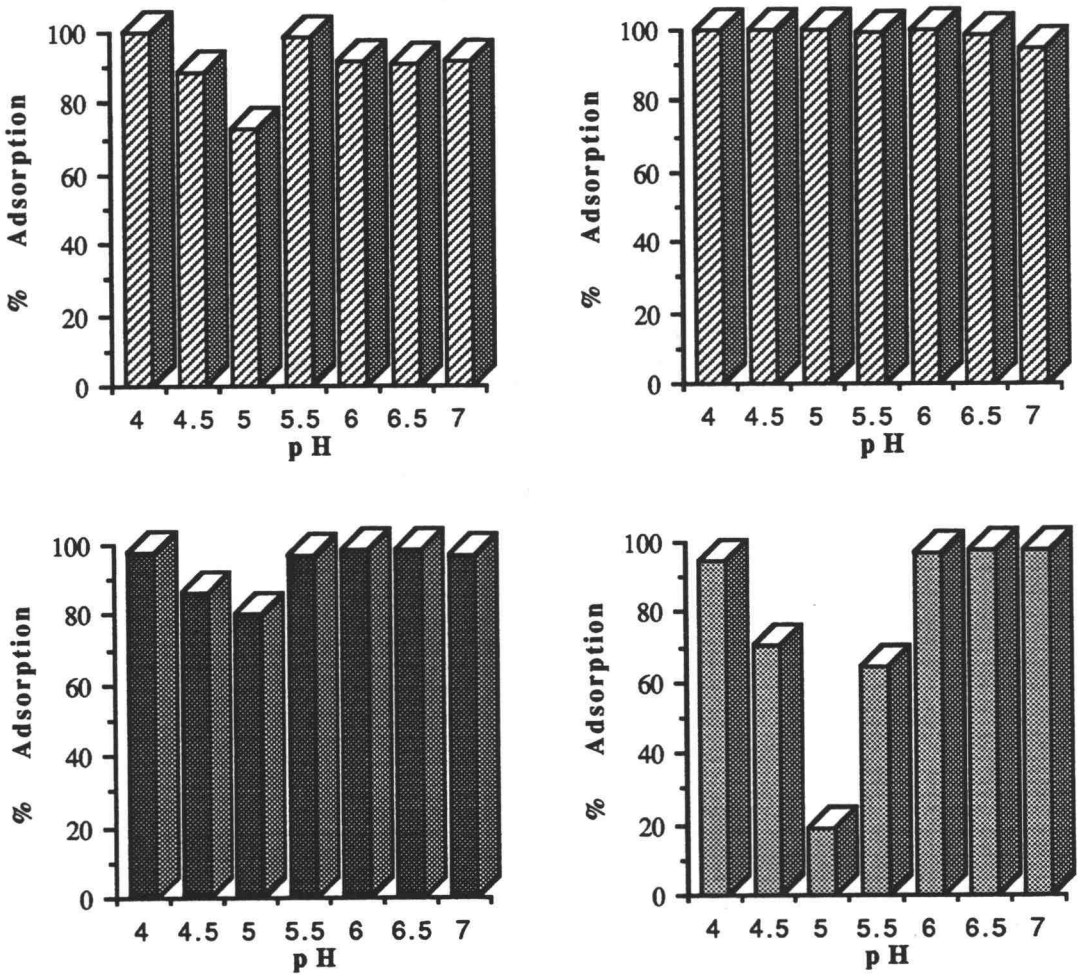

Figure 12. Percent adsorption of phages to *S. thermophilus*, *Lc. cremoris* or *Lc. lactis* at pH 4 to 7. Cultures *S. thermophilus* 690 (top left) and 691 (top right), *Lc. cremoris* KH (bottom left) and *Lc. lactis* C2 (bottom right).

adsorption occurring near pH 5.0 with *Lc. lactis* C2 being the most affected (Table 25; Figure 12).

The varied results that were observed for the *Lb. bulgaricus* and *S. thermophilus* strains indicate that the adsorption of phage to starter cultures may be dependent on the particular strain of culture or phage. Since these genera are used simultaneously for mozzarella cheese production, the data would suggest that the adjustment or holding of starter media at designated pH ranges would be of little benefit in terms of phage protection.

Use of Stabilizers and Rennet to Inhibit Phage Transfer Through Media

Of the nine stabilizers examined only two, xanthan gum and carrageenan (kappa form), completely prevented sedimentation of the insoluble components of medium 47. Therefore, further testing with cultures and phages was only done with these additives. When cultured, medium 47 with carrageenan developed into a solid gel that made it unacceptable for use as starter due to its inability to be remixed. Acceptable activity did develop when xanthan gum was added (1.57 with a control activity of 1.58), however, no benefit was found in terms of phage protection (final phage titer of $1x10^9$).

The initial indication that stabilizing the starter medium did not inhibit phage transfer prompted the examination of phage activity in milk set with rennet to test the early renneting theory. In all cases, phages that were initially sprayed over the surface of coagulated milk were recovered from all levels of the fermentation vessel at an

increase in number (Table 26). These results would suggest that early renneting may not have the phage prevention significance that it has traditionally been given.

Adsorption of Phages to Heat Killed Cells

Exposure of cells to varying degrees of heat treatment did not alter the cells enough to prevent phage adsorption. The data in Table 27 show that phages adsorbed to the surfaces of heat-killed cells to the same degree as they did to live non-heat treated cells.

Assuming that the phages bound irreversibly, the addition of heat inactivated cells to the starter medium prior to the actual culture addition was considered a possible way to remove any phage present in the medium before they could come in contact with the viable starter bacteria. However, since the control medium recorded a culture activity of 1.24 and the medium that contained the heat inactivated cultures only produced an activity of .82 when phages were added (the phage inoculated control was .79) it would appear that this procedure was ineffective and suggests that the binding of these phages is probably reversible.

Similar results were seen in the final experiment wherein the phage inoculum consisted of phages adsorbed to heat inactivated cells. The data in Table 28 show that the phages that were adsorbed to inactivated cells and then added to medium 47 produced comparable final phage titers to those of free phages added to media.

These results would indicate that these phages can adsorb onto and dissipate from dead cells and still remain infectious. Therefore,

Table 26. Recovery of phages from rennet coagulated milk

	Initial PFU/ml	Final PFU/ml		
		Top	Middle	Bottom
phage 210[a]	4.1	2.3×10^4	7.0×10^3	1.0×10^3
phage 651[b]	.7	100	100	<100
phage C2[c]	8.9	6.8×10^7	1.8×10^7	5.0×10^7

[a]For *Lb. bularicus* 210.
[b]For *S. thermophilus* 651.
[c]For *Lc. lactis* C2.

Table 27. Adsorption of phages to heat treated cells

Culture	Heat treatment	% Adsorption
Lb. bulgaricus 210	none	99.2
	pasteurization[a]	99.2
	thermal processing[c]	99.5
	autoclaving[d]	99.8
S. thermophilus 651	none	>99.8
	pasteurization[a]	>99.8
	thermal processing[c]	>99.8
	autoclaving[d]	>99.8

[a]63^{0}C for 30 min.
[b]85^{0}C for 45 min.
[c]121^{0}C for 15 min. at 15 psi.

Table 28. Effect of free phages and phages adsorbed to heat killed cultures on culture activity and final phage titers in medium 47

	Control	Free phage inoculum	Adsorbed phage inoculum
Activity[a]	1.79	.05	.06
Final PFU/ml[b]	-	1.6×10^8	5.0×10^8

[a]Using a 1% inoculum.
[b]Combined phage titers for both *Lb. bulgaricus* 210 and *S. thermophilus* 651.

attempts to inactivate phages by "adsorbing them out" of media with this approach would appear to be unproductive.

Effect of the Initial Phage Concentration on the Performance of Cultures in Medium 47 and Activity Tests

The results in Table 29 show that although the starter activities decreased in a stepwise manner with increased phage inoculum the final phage titers did not correlate. Final titers were comparable in that they differed by no more than two logs but the activities for all test conditions were all well below the control. Therefore, it would appear that even low level phage contamination can still produce noticeable results.

For the activity tests, the data in Figure 13 would indicate that phage contamination of non-infected starter cultures in vat milk at levels less than 1,000 PFU/ml may have little discernible effect on culture growth. For individual culture/phage combinations, the initial concentration of the *Lb. bulgaricus* phage had little impact on culture activity while the higher concentrations of the *S. thermophilus* phage resulted in noticeable activity reduction. The greatest activity drops, however, were observed when both strains and phages were used in association.

Table 29. Varied initial phage concentrations in medium 47 and their effect on culture activity and final phage titers

	Initial phage concentration (PFU/ml)			
	0	1	10	100
Activity[a]	1.86	1.43	1.33	1.13
Final PFU/ml:				
phage 210[b]	-	7.0×10^6	5.0×10^8	4.7×10^8
phage 651[c]	-	3.4×10^8	8.9×10^6	2.4×10^6

[a]For *Lb. bulgaricus* 210.
[b]For *S. thermophilus* 651.

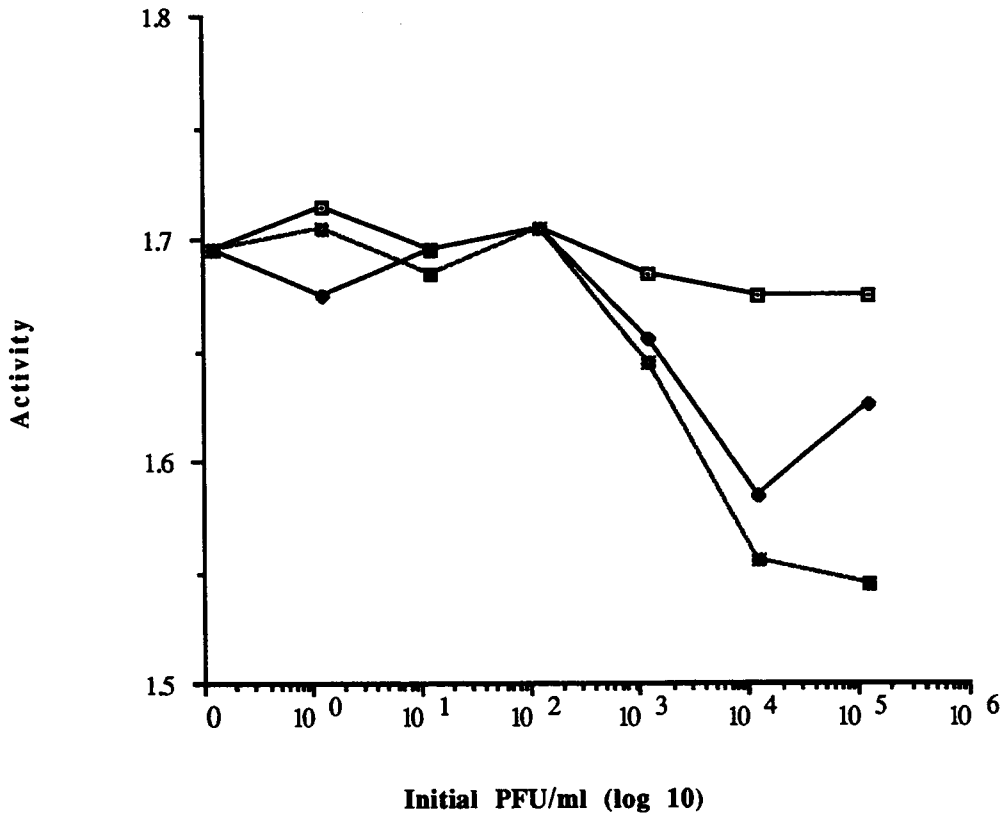

Figure 13. Activity of combined *Lb. bulgaricus* and *S. thermophilus* strains grown in milk containing various initial phage concentrations. Phage *Lb. bulgaricus* 210 ( ), phage *S. thermophilus* 651 ( ) and combined phages *Lb. bulgaricus* 210 and *S. thermophilus* 651( ).

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APPENDICES

APPENDIX I

Microtiter Plate Assay for the Detection of Bacteriophages in Whey and Starter Media

Introduction

The most common methods presently used to screen for the presence of bacteriophages in cheese whey involve the addition of a filtered whey sample to a test tube which contains sterile milk, a pH indicator (i.e., brom cresol purple) and a particular starter strain. The presence or absence of phages is then determined by the ability of the culture to grow and produce a color change in a given amount of time. This type of system is fairy rapid and simple to use but can become very cumbersome if large numbers of cultures and/or whey samples are to be examined.

Therefore, in an effort to speed the process of screening whey and starters samples for the isolation of phages, attempts were made to modify the microtiter plate assay system of Tortorello et al. (1991), used for measuring phage infection of lactococci, for use with the thermophilic lactic starter cultures *Lb. bulgaricus* and *S. thermophilus*. This application would provide a relatively simple and rapid method for screening large numbers of whey and/or

starter samples for the presence of bacteriophages along with an estimate of their numbers.

Materials and Methods

Cultures and Bacteriophages

Evaluations were made using cultures and phages for *Lb. bulgaricus* 210 and *S. thermophilus* 651 (Table 1). Prior to use in the assay, strains were grown in MRS (*Lb. bulgaricus*) or M17 (*S. thermophilus*) medium for approximately 12 h and diluted to an OD_{600} of .1 (approx. $1x10^7$ CFU/ml for both strains) and .2 in PMN medium. Phage stocks were serially diluted (1:10) in .1% peptone broth to give a range of decreasing inocula. When added to the microtiter wells this would produce initial culture concentrations of approximately $1x10^5$ CFU/ml with initial phage concentrations starting at around $1x10^7$ PFU/ml and decreasing to 10/ml. Viable cell counts and plaque assays were performed as described previously using MRS and M17 media.

Growth Media

Several medium/indicator combinations were examined in order to optimize conditions for culture growth and plate readability. A list of these combinations is compiled in Table 30. The test media (M17, MRS, PMN and NDM) were selected based on their history of use with these specific cultures. The color indicators consisted primarily of either pH dependent dyes chosen for their range of color change, namely brom cresol purple (purple → yellow color change between

Table 30. Media and indicators used in the microtiter plate phage assay

Indicator / reaction	Medium			
	M17[a]	MRS[b]	PMN[c]	11% NDM
blue-gal[d] (.04 & 08%): clear→blue with ß-galactosidase activity			X	
brom cresol purple (.004%): purple→yellow between pH 5.2-6.8	X	X	X	X[c,e]
chlor phenol red (.004%): red→yellow between pH 4.8-6.2	X	X	X	
methylene blue (.004%): reduced from blue→white	X	X	X	
resazurin (.004%): reduced from purple→red→white	X	X	X	

[a]Buffering components removed and adjusted to pH 7.2.
[b]Buffering components removed and adjusted to pH 6.8.
[c]Adjusted to pH 6.8.
[d]5-bromoindolyl ß-D-galactopyranoside.
[e].015% BCP.

pH 6.8-5.2) and chlor phenol red (red → yellow color change between pH 6.2-4.8) or reducing dyes (methylene blue and resazurin).

In order to increase the rate at which the pH of the media would decline, the major buffering components were removed from both M17 (ß-glycerol phosphate) and MRS (ammonium citrate, sodium acetate, di-potassium phosphate) media and the pH was adjusted to 7.2 and 6.8 respectively with 3 N NaOH. NDM and PMN medium were simply adjusted to a higher pH (6.8).

Microtiter Plate Assay

Assays were performed in sterile 96 well flat-bottom microtiter plates using a multichannel micropipette. Phage dilutions (50 µl) were added to each of the wells (except for a control row) and mixed with 50 µl of a culture dilution. After a 15 min adsorption period at ambient temperature, 150 µl of dye containing media was added and the plates incubated at 37^{0}C until the control wells showed complete color change. Figure 14 shows the set-up of a typical plate.

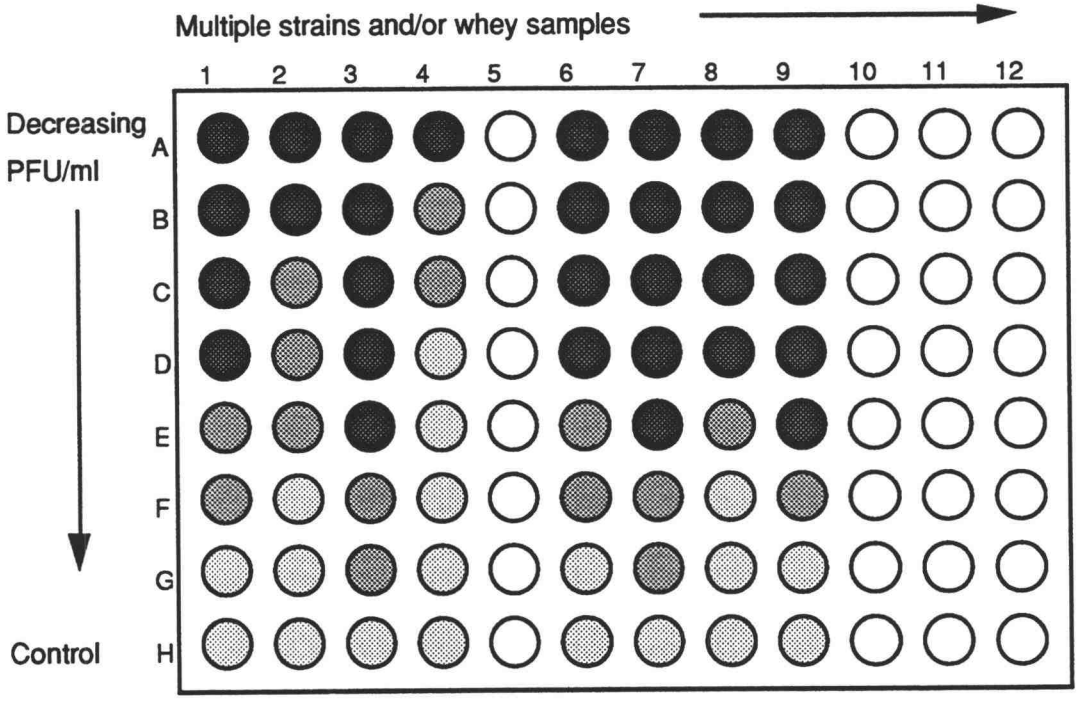

Figure 14. Typical setup and reaction of the microtiter plate phage assay. Shading represents color changes due to heavy growth (◎), light growth (◉) and no growth (●): (○) are unused.

Results and Discussion

Of the various indicators that were tested, the dark blue → green → yellow color change of BCP (.015%) in 11% NDM was the most pronounced and easiest to read. Part of this appeared to be due to the opaque background provided by the milk; the other media were all translucent. Although BCP and chlor phenol red used in the other growth media did prove to be acceptable, the areas of color change were not as defined as those observed in BCP-milk. Resazurin was found to be problematic due to an initial and irreversible reduction from purple to red during autoclaving (filter sterilization may be an alternative but was not attempted) and both methylene blue and blue-gal were ineffective. Figure 15 shows the color reactions observed using some of the medium/indicator combinations

As for the growth media themselves, PMN medium appeared to facilitate more rapid growth than either MRS or M17 media. Generally, all media began to show color changes by 3.5 to 4.5 h but, depending on the culture, could take up to 6 h for optimum readability. The rate of growth, however, was not substantially increased in PMN medium and did not compensate for the easier readability of milk containing BCP.

The initial cell concentration, using either a .1 or .2 OD_{600} culture appeared to have an effect on the outcome of the test. It can be seen in Figure 15 that more growth was observed with the higher cell concentrations, resulting in decreased sensitivity to lower initial phage concentrations. With the .1 OD_{600} culture the test appeared to

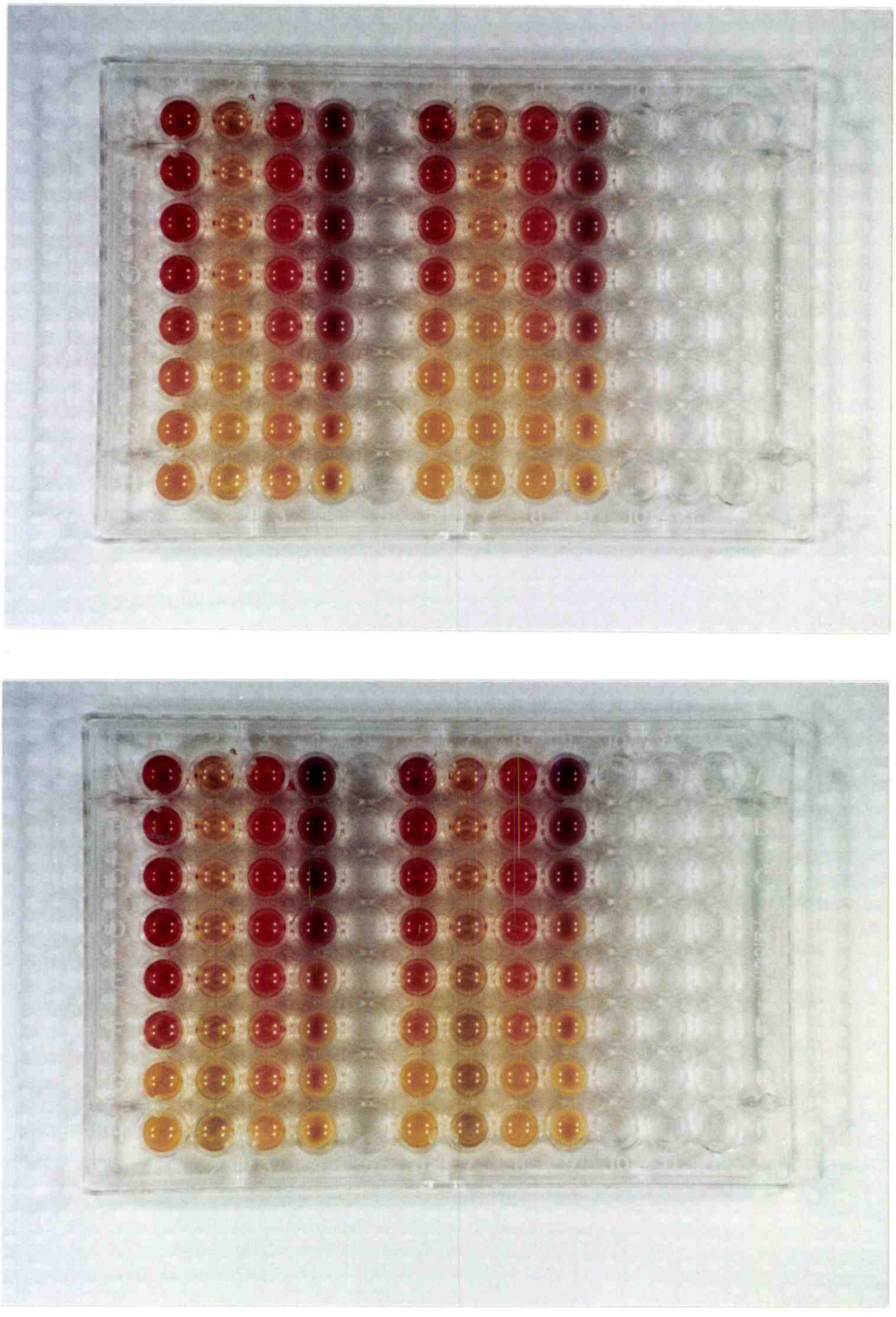

Figure 15. Microtiter plate phage assays. Phage activity is indicated by inhibition of color change in wells containing serially diluted phage preparations (top to bottom) into various strains of *L. bulgaricus* (top) or *S. thermophilus* (bottom). Different strains are left to right.

be quite sensitive as incomplete color changes were observed at phage concentrations as low as 10-100 PFU/ml.

Although the preliminary results showed that this method could be adequately adapted for use with thermophilic lactic starter cultures, there is still room for refinement. With continued work this could prove to be an excellent method to rapidly recognize and quantify phages in cheese whey and starter samples.

APPENDIX II

Evaluation of Commercial Thermophilic Coccus:Rod Cultures for Viability along with Performance Comparisons Between Various Commercial Bulk Starter Media

by

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Comparison of Commercial Thermophilic Starter Cultures

Many companies produce thermophilic cultures for the manufacture of mozzarella cheese. These cultures consist of strains of *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, some contain *Lactobacillus helveticus* and they are packaged either as single, mixed or multiple strains. The objective of this study was to compare the viable coccus/rod ratios in multiple strain frozen concentrates of thermophilic cultures by various manufacturers.

Cultures obtained from four different manufacturers (designated A-D) were examined and are listed in Table 31. Viable cell counts were performed using either yogurt lactic agar (incubated 24 h at 37^{0}C) or hydrophobic grid membranes with TPYE agar (incubated 72 h at 37^{0}C).

Figure 16 shows the percentage of viable rods in the nine cultures tested from manufacturer A. These ranged from 1% to 92% viable rods and multiple samples of given cultures were generally inconsistent. For the eight cultures examined from manufacturer B (Figure 17) viable rod percentages ranged from 1% to 61% and again, multiple samples of given cultures were generally inconsistent. The next figure (Figure 18) shows the percentage of viable rods, 1% to 54% recovered in the four cultures tested from manufacturer C. Cultures from the final manufacturer (D) are presented in Figure 19. These ranged from 1% to 74% viable rods and as with the other culture suppliers, multiple samples of given cultures were inconsistent.

Table 31. Number of cultures tested from each manufacturer

Manufacturer	No. of cultures
A	9
B	8
C	4
D	10

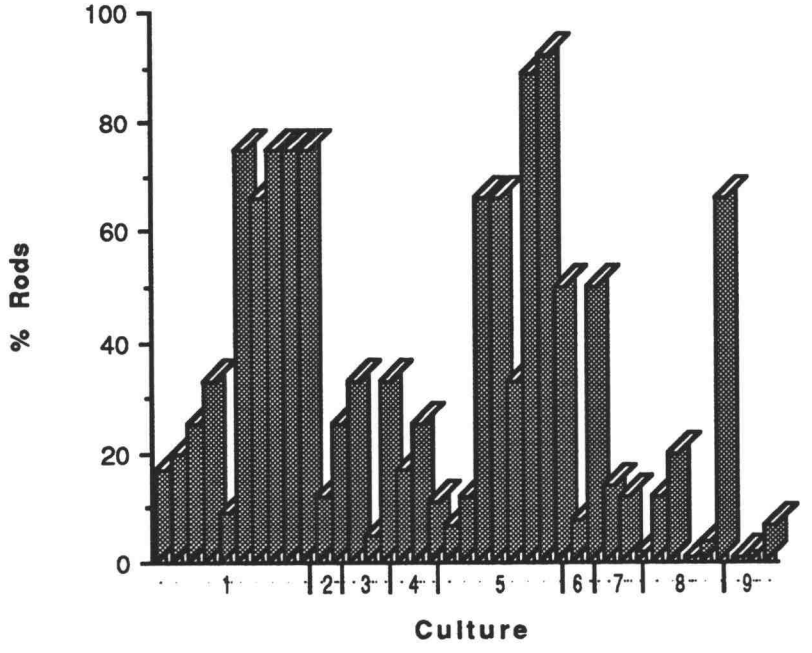

Figure 16. Viable rods recovered from cultures produced by manufacturer A. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

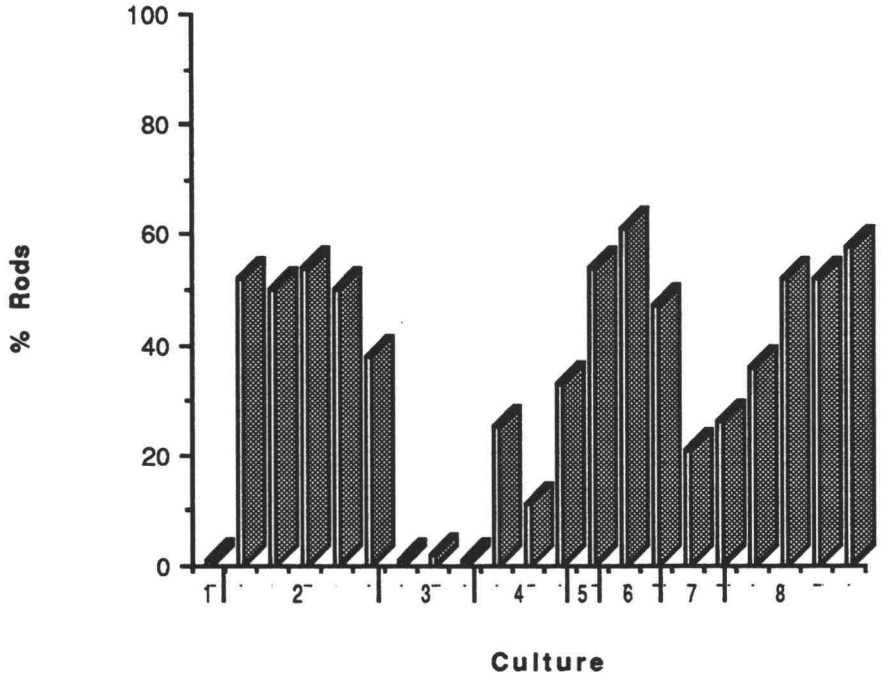

Figure 17. Viable rods recovered from cultures produced by manufacturer B. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

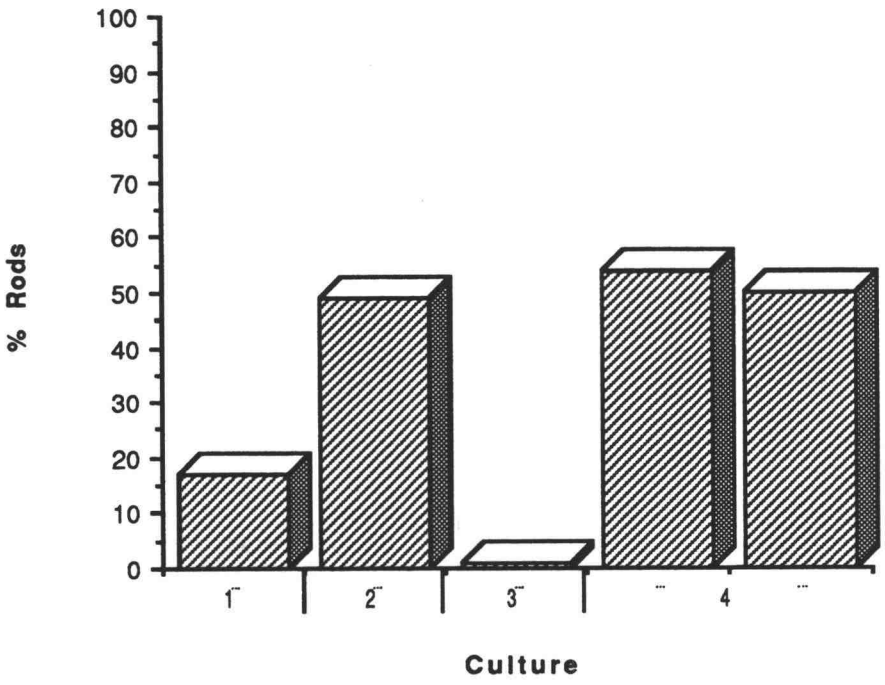

Figure 18. Viable rods recovered from cultures produced by manufacturer C. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

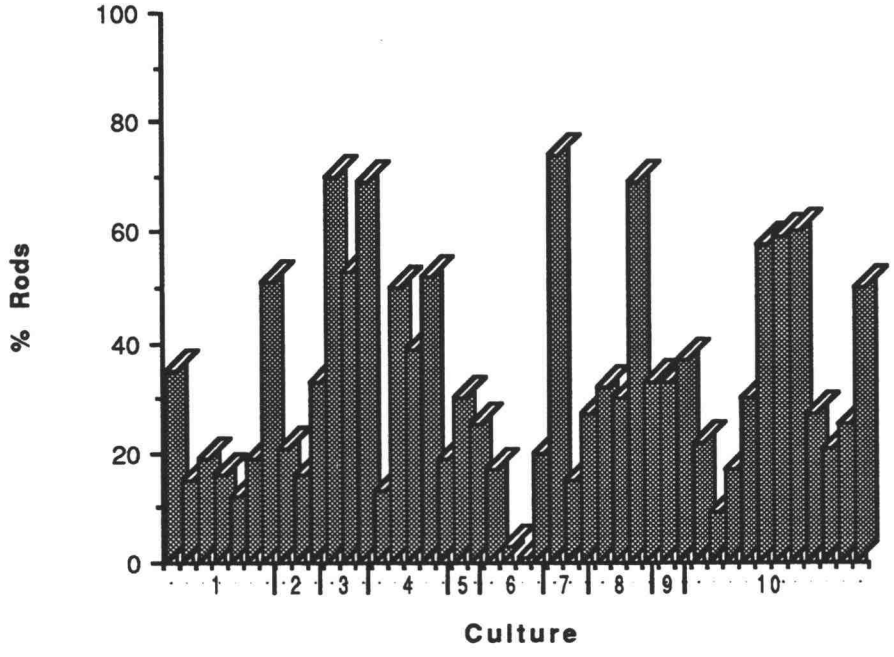

Figure 19. Viable rods recovered from cultures produced by manufacturer D. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

With some cultures, viable ratios were also compared to microscopic ratios since most cheese manufacturers use the later to evaluate the condition of their starter. Figure 20 shows the comparison of these two methods. Microscopic ratios were determined on the basis of colony forming units in order to correlate with viable cell counts. In all cases, the proportion of rods recovered using culture media was lower than that observed microscopically and would suggest that not all of the rods observed microscopically were viable.

In conclusion, there was a large variance in the proportion of viable rods between different cultures and within multiple samples of specific cultures. Also, fewer rods were viable than were present microscopically. This was observed for all of the cultures from all of the manufacturers that were tested and is likely to be a factor in the difficulty of producing consistent balanced coccus/rod ratios in starter. It appears that work still needs to be done to ensure consistency and uniformity in frozen culture concentrates.

Performance Comparisons Between Commercial Bulk Starter Media

In addition to cultures, there are also numerous types of media available for the production of thermophilic starters. In this study, seven different commercially available bulk starter media for the production of Italian type cheese were compared in terms of activity, phage protection and coccus/rod ratios produced in the mature starter.

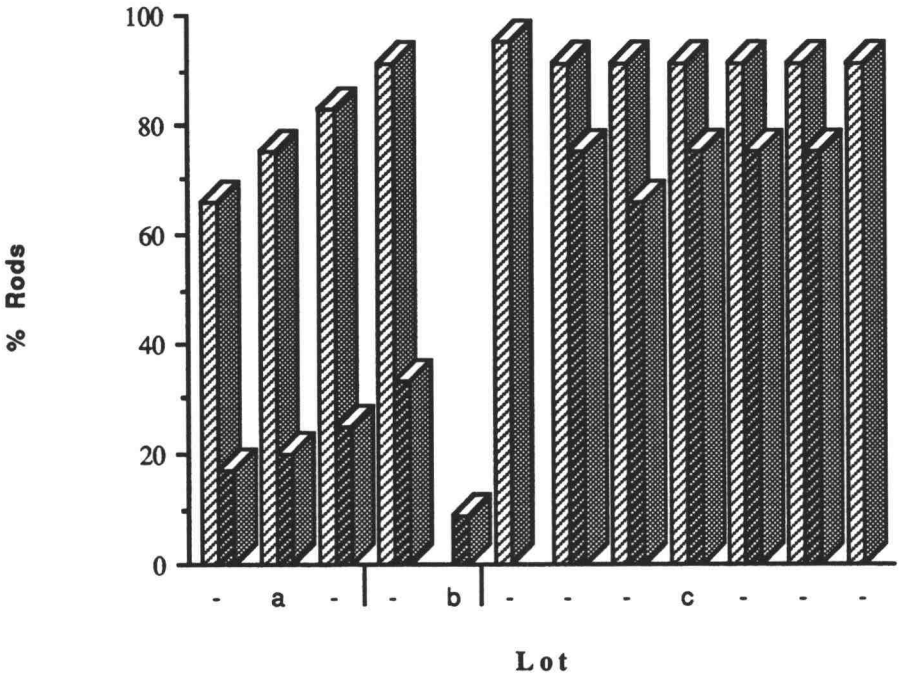

Figure 20. Viable rods recovered from different lots of culture 1 produced by manufacturer A as determined by microscopic (▨) and viable cell counts(■). For microscopic counts, cultures were stained with methylene blue and ratios were determined by counting colony forming units. Viable counts were performed using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

Figure 21 shows the six station fermenter used for these studies. The six tanks can be individually programmed for temperature, pH and agitation and the results are recorded continuously.

Three tests were used to evaluate each medium. Culture activity was determined by the change in pH in 9% NDM after a 2.5 h incubation at 42^{0}C. Plaque assays for phage were performed using an overlay that consisted of a top agar (MRS or M17) seeded with an actively growing culture, a given phage dilution and calcium chloride (to enhance adsorption). The mixture was then poured over a bottom layer of agar (MRS or M17) in a petri dish. Phages used were recently isolated from cheese plants. Viable cell counts were performed with hydrophobic grid membranes using MRS agar that contained the dye erioglaucine to help differentiate between the rod and coccus strains. These were incubated 72 h at 37^{0}C. Cultures used were supplied by a commercial manufacturer and were added to the starter directly from the can.

Figure 22 shows activity comparisons for cells grown in the seven media with and without phages. Medium I cells showed the highest activity under both conditions even though it also showed a larger change in activity than some of the other media.

The plaque forming units per ml (PFU/ml) of rod and coccus phages that were recovered in the mature starter from the seven media are shown in Figure 23. The media were inoculated with 100 PFU/ml of each phage and medium V showed the best overall phage protection (only 1-2 log increase). Media III and IV showed the best inhibition of coccus phages but gave very little protection from rod phages.

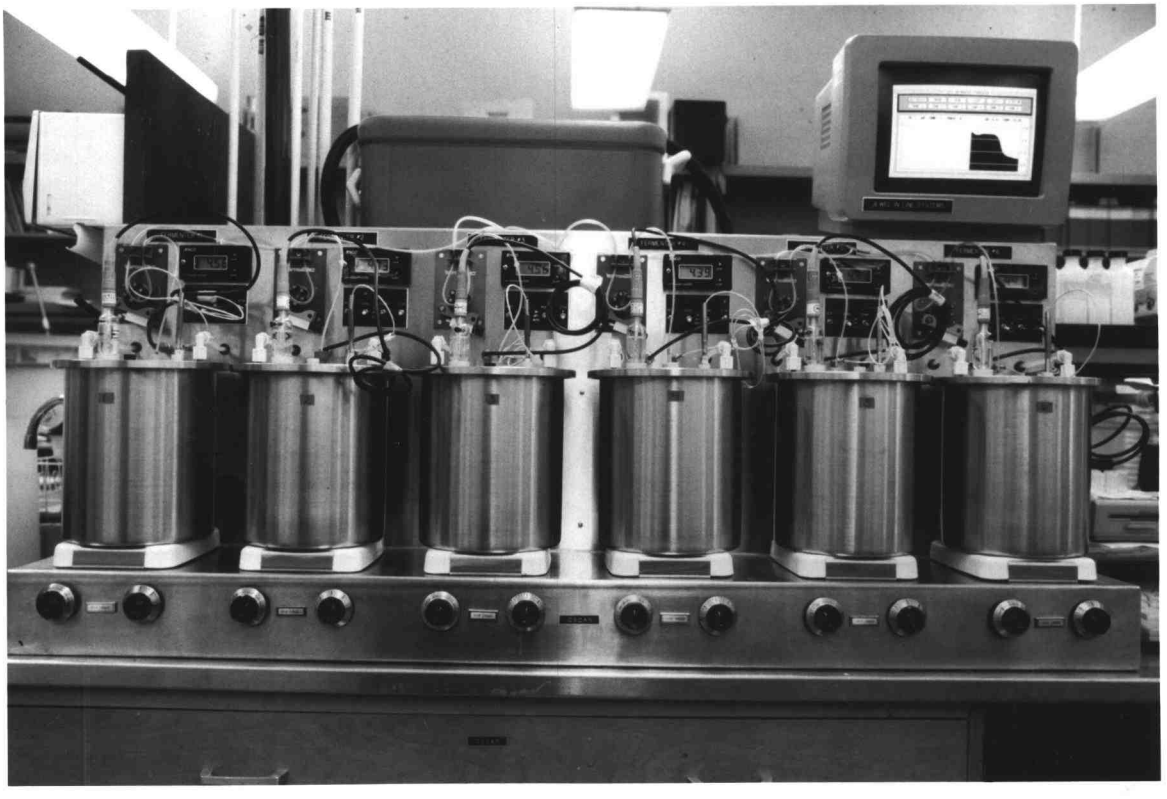

Figure 21. Fermentation Control System used for evaluating starter media and culture performance. Jewel In Line Systems, Longmont, CO.

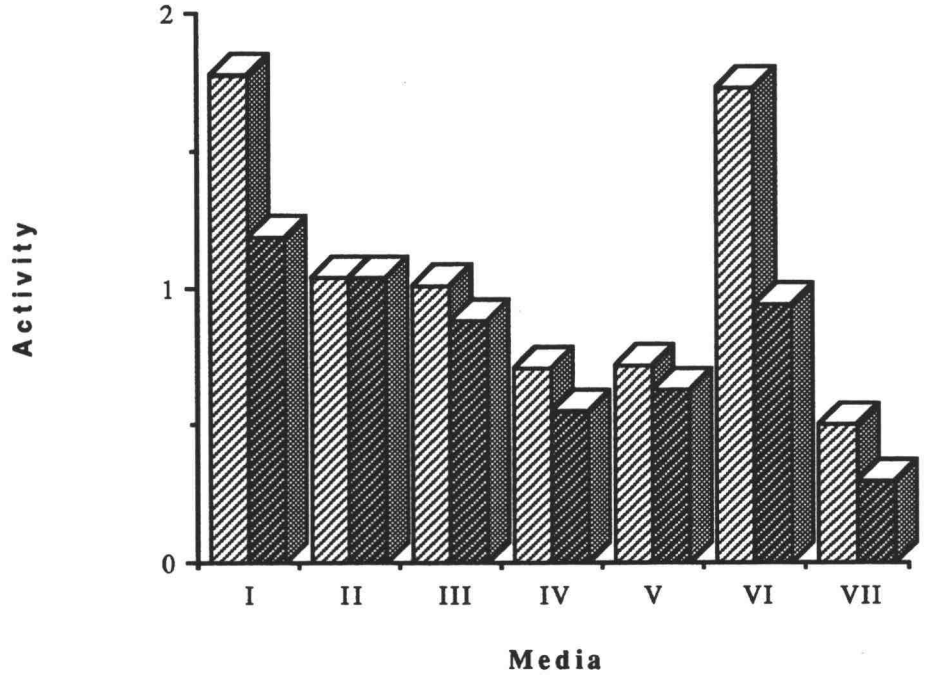

Figure 22. Comparison of acid-producing ability of cultures produced in seven commercial bulk starter media. Cultures were grown in the absence (▨) or presence of phages (■). Acid-production (activity) was determined by inoculating .1 ml mature starter into 10 ml of sterile 9% NDM and incubating for 2.5 h at 42^{0}C. Culture activity was recorded as the change in pH (ΔpH).

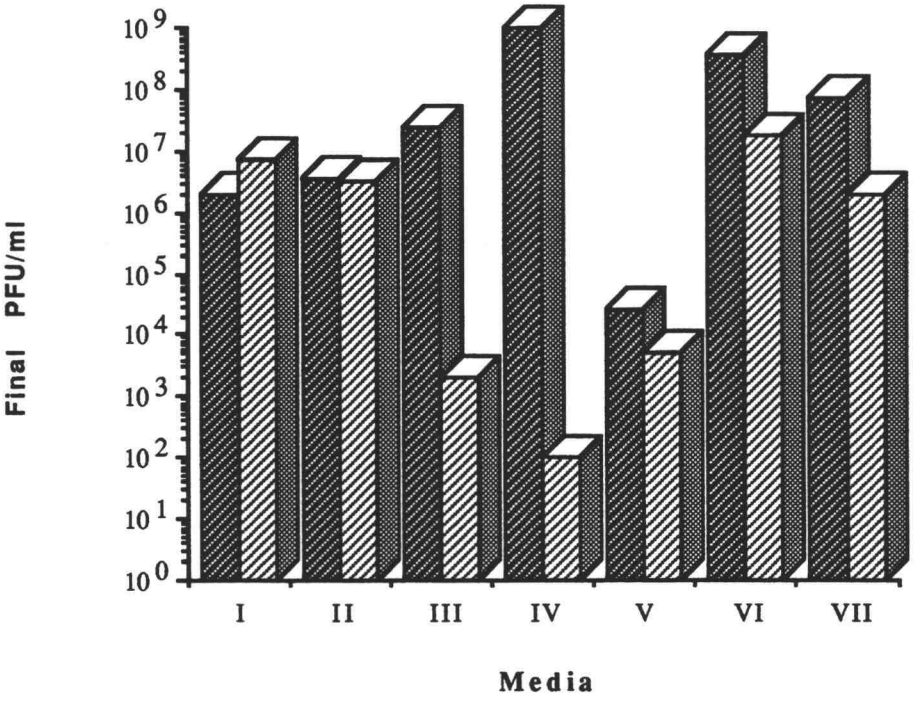

Figure 23. Comparison of phage replication in seven commercial bulk starter media. Final titers (PFU/ml) of phages for rod (▨) and coccus (▨) cultures. Media were inoculated with approximately 100 PFU/ml of phages and incubated at 42^{0}C under manufacturers specifications.

Figure 24 compares the seven media for the percentage of viable rods present in the mature starter. The ideal range would be between 20% and 60%. This was only reached by medium II in both the presence and absence of phages with the majority of the other media being low in rods.

Ratio comparisons using different cultures and media are shown in Figures 25 and 26. The first graph (Figure 25) shows the viable rods recovered using five different cultures in medium I. Results were quite variable both between cultures and within a given culture. The second graph (Figure 26) shows the percentage of rods recovered from starter grown in four different media using several different cultures. As with medium I, the other media also showed quite variable ratios. However, media II and IV generally produced higher numbers of rods.

To summarize, medium I produced cells with considerably higher acid-producing activity than all of the other media even in the presence of phage. Medium VI was the only other medium comparable in activity but produced higher phage titers than medium I. Medium V gave the best overall phage protection (only 1-2 log increase) even though cells generated therein had considerably lower culture activity than those generated in medium I or VI. Media III and IV showed lower coccus phage numbers but produced very high levels of rod phage. The best overall coccus/rod ratios, both with and without the presence of phages, were seen in medium II. Comparisons of media using multiple cultures, however, revealed that large variations in cell type ratios were produced within each medium.

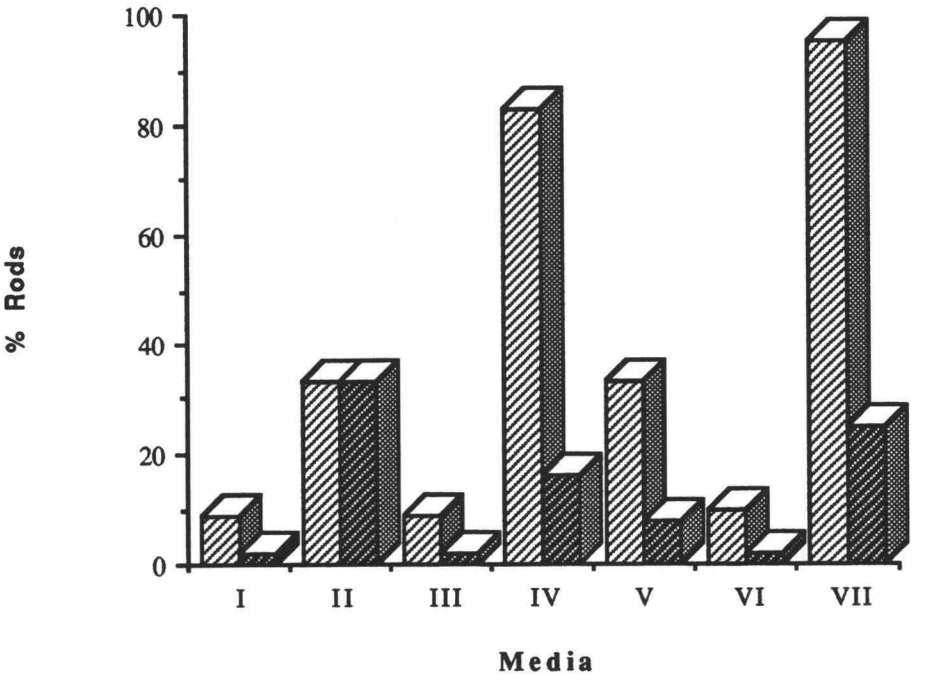

Figure 24. Comparison of seven commercial bulk starter media for their ability to support rod growth. Cultures grown in the absence (▨) and presence of phages (■). Media were inoculated with approximately 100 PFU/ml of phages and incubated at 42^{0}C under manufacturers specifications. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

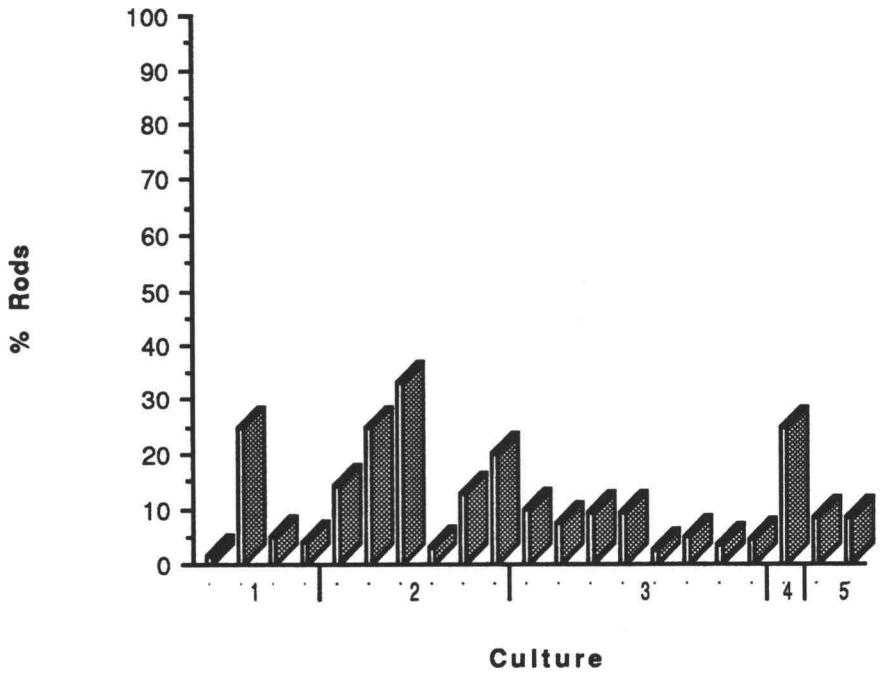

Figure 25. Proportion of rods recovered in medium I using five separate cultures. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).

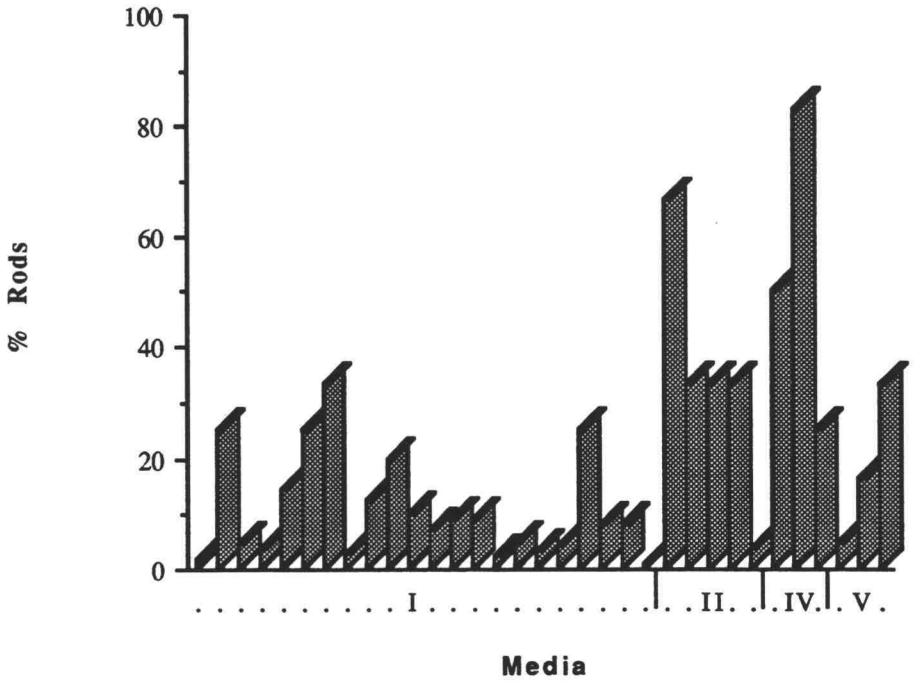

Figure 26. Proportion of rods recovered in four commercial bulk starter media. Cultures were enumerated and differentiated using either YLA (incubated 24 h at 37^{0}C) or TPYE agar (incubated 72 h at 37^{0}C).