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Factors Affecting Growth of Proteinase Positive and Proteinase Negative Streptococcus cremoris UC310 in Ultrafiltered Milk Retentate

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FACTORS AFFECTING GROWTH OF PROTEINASE POSITIVE AND PROTEINASE NEGATIVE *STREPTOCOCCUS CREMORIS* UC310 IN ULTRAFILTERED MILK RETENTATE

by

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Brent Karl Pope

TABLE OF CONTENTS

LIST OF TABLES

Table

Page

LIST OF FIGURES

Figure

ABSTRACT

Factors Affecting Growth of Proteinase Positive and Proteinase Negative *Streptococcus cremoris* UC310 in Ultrafiltered Milk Retentate

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Whole milks were adjusted to pH 5.8, 6.2, or 6.7 with HCl and batch pasteurized at 63° C for 30 min. Each was concentrated 5:1 (40% total solids) through a single tube polysulfone membrane Abcor ultrafiltration unit. Lactose (L), casein hydrolysate (CH), and one of two brands of yeast extract (YE1, YE2) were added into cooled retentates at 0.1, 0.3, 0.5, 0.7 or 0.9% and equilibrated overnight at 4° C. Five percent proteinase positive (Prt+) *Streptococcus cremoris* UC 310+ (v/w) milk based culture was added. Unfortified retentate was also inoculated with 0.1, 0.3, 0.5, 0.7 or 0.9% starter and pH readings were taken on all samples for 24 h during incubation at 23° C. Similar substrates were inoculated with proteinase negative (Prt-) *S. cremoris* UC 310-.

Lactose had no significant effect on acid production. Casein hydrolysate had a slight positive effect. Yeast extract had a significant effect at all preacidification levels and a significant difference was also noticed between the brands. Mean times required for the proteinase positive culture to reach pH 5.1 in 5x retentate from milk acidified to pH 5.8 were 24 , 12 , 10, 10, and 24 h for L, CH, YE1, YE2, and the control respectively. Proteinase negative variants of this strain had mean times of >24 h, 14 h, 11 h, 11 h, and >24 h respectively. These time differences were significantly different

between Prt+ and Prt- variants. A minimum concentration of 0.2% yeast extract produced the most stimulation while greater quantities provided no additional benefit. Taste panelists were unable to detect yeast extract in retentates fermented by either culture variant.

(75 pages)

INTRODUCTION

The use of ultrafiltration of whole milk has many significant benefits to cheese producers. These benefits include increased product yield, reduced rennet requirements, reduced labor and energy requirements, more uniform consistency, reduced amounts of whey for disposal, and the potential for continuous production (7, 9, 10, 17, 23, 25, 30, 38, 46, 48, 56).

The practicality for manufacturing all varieties of cheeses from highly concentrated retentates, however, has not yet been fully realized. High moisture soft cheese and semi-hard cheese have been produced (28, 30), but difficulty has been encountered in the production of hard cheese such as Cheddar (5, 12, 23, 33, 34, 46). Australian researchers now claim to manufacture Cheddar cheese which is normal in every respect using UF retentates through a continuous process, although the operating parameters are still secret and will be released only under licensing agreements (2).

Lactic cultures grow more slowly in retentate than in normal milk thus requiring longer incubation periods and they cannot often attain the desired pH (12, 33). This problem is not associated with low concentrations of milk, but when protein ratios are greater than about 3:1 (retentate protein: normal milk protein) the fermentation of retentates, compared to normal milk, places greater demands on starter bacteria for lactic acid production. This requirement significantly lengthens cheese manufacturing times (20, 32). Covacevich and Kosikowski (11, 12) attribute this time requirement to the buffering effect of the higher concentration of proteins (23, 24, 32, 33, 34). When milk is ultrafiltered, proteins, insoluble salts of calcium and phosphate are concentrated and cause an increase in buffer capacity greater than the concentration factor of the retentate. Milk concentrated to 1/5th its original

volume has a buffer capacity seven times higher than normal milk. As a result, pH reduction becomes difficult despite the presence of many active starter bacteria.

Narasimhan and Ernstrom (35, 36) also observed this slow acid production in cottage cheese production from retentate. They noted several possible factors that could inhibit growth of the *Streptococcus lactis* cultures they were using, but attributed the problem primarily to the high concentration of colloidal calcium phosphates. Above pH 5.2, phosphates in milk are insoluble and bound to the casein micelles. However, as the pH falls below 5.1, these phosphates become completely soluble. They showed that high concentrations of these phosphate salts inhibit growth of lactic cultures. Others have also reported phosphate inhibition of lactic cultures in the preparation of bulk cultures (55).

Brown (7), working with a process to make cheese curd continuously from ultrafiltered milk, found that he had to culture 5x retentate for 18 to 36 hours before it reached the pH of 5.1 to 5.2 necessary for his Cheddar cheese experiments. Others working with retentate concentrations greater than 2:1 have experienced delays in acid production by starter cultures (10, 12, 17, 20, 33, 34, 46).

Researchers have used various organic stimulants to encourage the growth and acid production of lactic streptococci (19, 21, 40, 42, 43, 44, 45, 53). Mistry and Kosikowski (32) suggested a larger inoculum level to compensate for the slow acid production in concentrated retentates. The direct introduction of stimulants into cheese vats has not been approved by the U.S. Food and Drug Administration, but regulations do not prohibit such components introduced as starter media (26, 49, 50). Stoddard and Richardson (45) proposed the introduction of high amounts of yeast extract

into the bulk culture substrate allowing sufficient carry-over into the cheese vat for acid production by proteinase negative bacteria such that high concentrations in the bulk starter preparation were not inhibitory to acid production (40, 44, 45).

The purpose of this work was to analyze the potential to reduce the time necessary for cheesemaking by adding stimulants to retentates from whole milk that had been concentrated five times (1/5th the normal volume) and to evaluate the time required to ferment retentates with different amounts of culture inocula. Retentates were cultured using both the proteinase positive (Prt+) and proteinase negative (Prt-) variants of *Streptococcus cremoris* strain UC310. This strain has been shown to have product yield potential for the Prt- variant by Heap and Richardson (19) in the manufacture of casein and by Stoddard and Richardson (44, 45) in the production of cottage cheese.

REVIEW OF LITERATURE

Ultrafiltration (UF) provides a method to concentrate some milk components at ambient temperatures without causing the off-flavors that can arise from methods using a heat treatment (16). During UF two components are obtained. These are an ultrafiltrate or permeate, which is a fluid with only soluble minerals, lactose, ions and small molecules; and a retentate, which is a concentration of milk proteins, fat, and insoluble minerals. All major components of milk except salts and some particles of small molecular weights are retained in the retentate as milk is forced through a porous membrane. Salts, lactose, and small particles collected in the permeate are discarded during or at the end of the UF process when the desired retentate composition is obtained. This final retentate (often called a precheese) can be adjusted to about the same composition as a finished cheese (15, 18, 30).

The pressure required to force the milk through the membrane is related to the pore size used (38). The rate at which permeate is produced (permeation rate or flux) declines from the beginning of filtration, rapidly at first and then eventually stabilizing at a rate that is dependent on the concentration of solids in the retentate (15, 16). The maximum milk concentration that is currently commercially feasible is 5:1 (original volume of milk to retentate), which results in about 60% moisture (46).

Advantages of Ultrafiltration

Many benefits can be realized from UF. The principle advantage is improved yield through incorporation of some fat normally lost in whey and whey proteins in the product. Between 8 and 30% yield increases have been reported depending on the type of cheese that is produced, primarily due to

the capture of whey proteins in the curd (3, 9, 29, 47, 56). Rennet requirements are reduced by 80% of the amount normally needed (30). Cheese plants can achieve greater productivity without buying more equipment because concentrated retentates will produce more product, pound for pound, than the original milk (3, 9, 19, 47, 56). Greater product consistency and quality is achieved since each batch of milk can be adjusted to a uniform protein, fat, and lactose content and all processing steps can be duplicated (3, 9, 47, 56). This could in turn increase consumption because if consumers can depend on a consistently high quality cheese they may eat more (2). Cheeses produced from UF also avoid the production of large quantities of whey and the problems associated with its disposal and pollution potential (30, 47, 56). Even when UF is used to produce hard cheeses requiring some whey expulsion, the volume of the whey is still lower (20). UF also provides the possibility for continuous production of cheeses (2, 9, 50).

Ultrafiltration can be carried out in the cheese plant or even on the dairy farm. Advantages to use on the dairy farm include the ability to use permeate for animal feed, less holding tank capacity required for retentate, less energy to keep raw retentate cold, and reduced cost for shipping retentate to cheese plants. UF systems are highly reliable for use on a day to day basis (24, 57). Several large factories in Europe now use UF to make a number of soft cheeses, (46) and the process is gaining popularity for use in the United States (47).

Feasibility of Cheesemaking

During cheesemaking casein micelles join in a three dimensional network to form a curd either because of the production of an acid or due to an

addition of protein stability by an enzyme. Syneresis results in the expulsion of liquid whey from the curd as it shrinks and causes the casein to form a tight bond. The UF process provides another mechanism to expel this liquid without the loss of the soluble proteins that are normally lost in the whey (30).

Soft Cheese

The UF process is an ideal technique to produce soft cheeses (9, 17). A product with the same basic composition as different finished cheeses can be obtained from UF of whole or skim milk (30). Soft fresh and ripened cheese have been prepared successfully from this precheese after culturing with proper bacteria and renneting. These cheeses have better adjustment of the weight and uniformity between makes since much of the heterogeneity of cheese from batch to batch can be eliminated by establishing proper protein/fat/lactose ratios in the retentate (30).

Medium-fat soft cheese prepared from UF whole milk retentate by Yan and co-workers (56) had a 41% increase in yield, and a 50% decrease in make time. Increased yields were partially attributed to higher moisture in the cheese and to higher solids not fat (SNF). No whey was expelled from the curd.

In another study (28) Domiati and Feta cheeses made from UF concentrates of pasteurized skim milk were of excellent quality. Protein loss was reduced from 37 to 13% when compared to conventional cheesemaking methods.

Yogurt made from UF contained 21% total solids (TS) and was a very acceptable product. Skim milk powder did not have to be added since proteins were concentrated by UF, nor did the mix need to be homogenized.

Yogurt made from UF milk was considered to be a very satisfying product and is possibly more suitable for a dairy dessert (10, 56).

Medium Hard Cheese

Diafiltration dilutes the soluble minerals and lactose in the retentate and removes greater amounts of solubles in the permeate (39). During UF the milk is partially concentrated and then a predetermined volume of water is added to the retentate at the same rate permeate is being removed. During diafiltration of preacidified milk colloidal calcium phosphate is solublized and removed. Removal of this calcium is necessary to prevent precipitation on the surface of cheese made from concentrated retentates (8, 14, 32, 35, 36).

Covacevich and Kosikowski (11) reported that mozzarella cheese made from diafiltered retentates have had good to excellent flavor and body. Stretching characteristics improved with up to four weeks of aging at 5^{o} C as did meltability. One plant in Wisconsin is processing 100,000 lb/day Mozzarella cheese with an 18% yield increase in a continuous process (47).

Hard Cheese

Barbano and Bynum (5) produced Cheddar cheese from whole milk reduced in volume by 20% by using UF. Total solids were 15.05% compared to 11.98% in untreated milk. Composition of cheese from UF milk was comparable to control cheese. Fat losses in the whey decreased and fat retention by the cheese during pressing increased. Fat retention was attributed to the mechanical homogenization of the milk during concentration by UF. Yield increases of 2 to 3% above normal calculated theoretical yields were noted.

Skim milk was concentrated to 12% solids and cream was added to the retentate to provide proper casein to fat ratios (0.7%) by Barbano and Malik (4). Conventional Cheddar making procedures were employed. Yields from all runs equaled or exceeded theoretical yields from the VanSlyke yield formula with about a 4% improvement over cheese made from unconcentrated, unstandardized whole milk.

Kealey and Kosikowski (23) produced concentrated retentates and used them to supplement normal milk producing concentrations about 1.3 times higher than unsupplemented milk. They experienced yield increases in the supplemented cheese over controls. Rennet requirements were reduced and the general quality of retentate Cheddar was equal to the control.

Cheddar produced by Chapman et al. (10) was produced from retentates of a two-fold concentrate through a membrane with a molecular cut off of 20,000 Daltons. The retentate had 7.6% fat, 7.01% protein, 4.66% lactose, and 80.73% water and was used directly for the manufacture of Cheddar and Cheshire cheeses. Traditional cheddaring methods encountered some fat losses during the wheying process, but less was lost during pressing. Less whey was produced by Cheddar from UF since the milk already had a lower water content. Yields were the same as Cheddar made from normal whole milk. Both cheeses had acceptable characteristics of texture and body, but the flavor was milder than that of high quality Cheddar or Cheshire.

Sutherland and Jameson (46) used milk concentrated to 1/5th its original volume (5x) for making Cheddar cheese by traditional methods. They reported producing some cheeses that were acceptable as Cheddar but with no increase in yield. Ernstrom et al. (14) produced a Cheddar type cheese from 5x retentate and used a vacuum pan evaporation to remove excess

moisture. This cheese base was blended with conventionally produced Cheddar to produce 16 to 18% yield increases in process cheese.

A new Australian process (registered by Australia Post. Publication No. VBP 6666. ISSN 0818-6456. Summer Issue 1987. Vol. 3, No. 2.) uses a continuous automated process to produce Cheddar cheese from 32,000 liters of milk per hour (2). Details of the methods used in this procedure are still secret and will only be released under licensing agreements. However, cheese made from this process could not be differentiated by cheese graders from cheese made in traditional vats.

Most others who have made hard cheese from retentates have not shown any yield increases because traditional methods of cheesemaking were used and greater solids losses in whey still existed (46). Cheeses generally exhibited a corky and crumbly body and lacked flavor characteristic of good Cheddar (11). Whey from cheese made from concentrated retentates normally contained at least twice as much protein and nearly five times as much fat as did whey from control cheese (1). Use of traditional cheesemaking methods loses many of the advantages inherent in the UF principle demonstrated by Maubois and Mocquot (30). Yields are reduced as protein and fat are lost in the whey. To eliminate moisture reduction by the expulsion of whey the protein would need to be concentrated approximately seven fold (11), or another method would need to be used to lower the moisture without losing whey proteins such as the technique described by Ernstrom et al (14).

Starter Cultures

Acid development in cheeses is normally accomplished through the addition of a bacterial culture--generally *Streptococcus cremoris* or *Streptococcus lactis* --or the hydrolysis of delta gluconolactone. Fresh soft cheeses such as cottage cheese may use either method to lower the pH to the desired level. However, bacterial growth is important to the production of characteristic flavors associated with aged medium hard and hard cheeses through their production of peptones, peptides, amino acids, and fatty acids (26).

Proteinase Activity

Lactic bacteria may be either fast or slow growing. One method for determining if bacteria are fast- (often called proteinase positive--Prt+) or slow-coagulating (proteinase negative--Prt-) bacteria is to autoclave milk, cool it to 22^{o} C, and inoculate it with 1% freshly coagulated inoculum. Fast cultures will coagulate sterile milk at 22^{o} C in less than 16 h. Slow cultures often require more than 48 h (31, 40).

On a glycerophosphate-milk-agar (GMA) (27) fast and slow cultures can be differentiated by colony size. The buffer in GMA agar allows the fast cultures to grow to greater cell densities, forming larger colonies, without being inhibited by their own metabolic by-products. Slow cultures form small colonies on GMA primarily due to their reduced proteolytic activity. Cell replication is slowed and halted because of their inability to break down milk protein (22, 31). The Prt- bacteria also stop growing at lower population densities than Prt+ bacteria in milk. This is probably due to a limitation of free amino acids and/or peptides required for their growth. Acid production by Prt-

bacteria does continue after cell growth stops and eventually is sufficient for coagulation (31, 48).

Proteinase activity has been determined by measuring the quantity of free nitrogenous matter in the whey and by UV spectroscopy. The Prt+ cells released more non-proteinaceous nitrogen (NPN) in whey than did Prt- cells. The higher NPN values indicated greater peptidase activity in the Prt+ culture (44). During growth Prt+ cells break down sufficient casein for their own metabolic needs (19, 31, 40) with excess peptides for the metabolic requirements of one to nine Prt- bacteria in the surrounding medium (52).

Acid Production

Under optimum conditions, acid production and growth are directly linked as the bacteria metabolize lactose to obtain energy. The maximum density of the cells is related to the availability or concentration of the carbohydrate source in the medium. Many researchers have used acid measurements as an indicator of bacterial growth. This indicator is not justified when the organisms are placed in a medium where nutrition is inadequate or under inhibitory stress. Under conditions of stress growth is uncoupled from the production of lactate. These stresses include temperature above 40^{o} C, salt concentrations above 4.5%, and high concentrations of H+, normally around pH 5.0. These stresses completely inhibited growth while the production of lactate continued. Initially, growth and acid production proceed at the same rate in milk, which indicates that growth and product formation are coupled. If growth and acid development are plotted on a curve, the lines are parallel. As the H+ concentration increases, these two lines begin to diverge. Lactate production continues while bacterial density slows and stabilizes (47).

Hickey et al. (20) noted that acid production did not uncouple from bacterial growth in 5x retentate. The uncoupling of growth and acid production by strains of *Streptococcus cremoris* was observed in normal milk. Retentate concentrated to 5x showed the least change in pH while accumulating the largest concentrations of lactic acid when compared to retentates of lower concentrations. The UF process caused an apparent stimulation of growth and acid production of *S. cremoris* and *S. lactis* above their normal levels in a milk substrate.

Buffer Capacity

Normal milk exhibits buffering capacity due to its proteins, insoluble calcium, and phosphate salts. During UF these are concentrated with a resultant increase in buffer capacity. As a result, pH reduction becomes more difficult despite high concentrations of active starter bacteria. Large amounts of acid are required to lower the pH in retentates due to their higher buffering capacity. The rate of acid production can be increased by using more starter bacteria. Larger inoculum size can partially compensate for the increased demand for lactic acid and reduce make time for cheese from UF milk (32).

Concentrated retentates produced and cultured by Mistry and Kosikowski (34) resisted pH change below 5.2 despite large numbers of cells. Even after 8.5 h the pH did not reach 4.6 while the control (unconcentrated milk) did in 6 h.

The greatest buffer capacity occurs at approximately pH 5.1-5.3 in UF milks. Milk concentrated to 5x required approximately 1.3% lactic acid to reach pH 5.1. Mistry and Kosikowski (33) noted a marked decrease in growth rate and lactose metabolism below pH 5.2 at which point bacterial population

was 10^9 cfu/ml. Brown (7) noted that reduction of pH to 5.1 in 5x retentate took between 18 and 36 h.

Stimulant Addition

Proteinase negative variants were unable to lower the pH sufficiently to make cottage cheese from normal milk within 6 h. The fastest pH change for either proteinase variant occurred at 35^{o} C (45).

Yeast extract (YE) was shown to stimulate proteinase negative bacteria sufficiently for use in cottage cheese manufacture. The use of 0.1 to 0.2% YE accelerated acid production sufficiently to attain pH 4.7 after 3.5 to 4 h (42, 53). Extracts of pancreas and liver also contain peptides that are stimulatory to lactic bacteria (43). Addition of these stimulants reduced coagulation times by 17-41% with concentrations of 0.015-1.0% (42).

Extracts of yeast contain principally nucleic acids, peptides, and amino acids while yeast autolysates also contain cell wall debris. Yeast extracts are generally more stimulatory than autolysates, but there is wide variation among brands and even between batches. Yeast extracts from autolysed yeast contain more peptides and amino acids because enzymes are released during cell lysis that hydrolyze proteins (55). Wright and co-workers (54, 55) found that casein hydrolysate was useful in the preparation of a media to grow lactic streptococci.

Since Prt- cultures do not attain high cell numbers in milk they break down less protein than Prt+ cultures (19, 22). Addition of yeast extract allows the Prt- cells to grow to high numbers without milk breakdown, provides the factors to allow rapid acid production without being linked to cell growth, or both (45). These stimulants added to nonfat dry milk (NDM) only slightly improved the performance of Prt+ bacteria; however, Prt- bacteria were

stimulated 6 to 11 times more than Prt+ bacteria (40, 43). With the addition of yeast extract, Prt- cells could produce pH changes near those made by Prt+ cells (40). Speck et al. (43) showed that these peptides do not provide any extra stimulus to coliforms.

Increased Yields

Heap and Richardson (19) undertook a study to determine yield differences between Prt+ and Prt- bacteria in the production of casein. Prt- cells were grown in high numbers using pH control and stimulatory media prior to inoculation. They found a yield increase of 5.6% from the Prt- culture over the Prt+. Stimulant was added to the substrate with both types of culture. Thus proteolytic activity in the Prt+ culture was not suppressed.

Stoddard and Richardson (44, 45) demonstrated a 2.26% yield increase in the manufacture of cottage cheese from the proteinase negative variant over the positive. Ekart et al. (13) reported that cottage cheese yields were increased almost 10% when non-agglutinating Prt- cultures were used. A similar result was noted by Hicks et al. (21) with Prt- bacteria producing greater yields in Cheddar cheese manufacture.

MATERIALS AND METHODS

Milk Preparation

Raw, whole milk was obtained from the Utah State University Dairy Products Laboratory after clarification. Milk was batch pasteurized at 63° C for 30 min and stored for less than three days at 4° C until ultrafiltered.

Acidification of Milk

Milk was adjusted to either pH 6.7, 6.2, or 5.8 with HCl prior to UF. Addition of 47 mL of concentrated HCl to each 39 kg of milk yielded a final pH of about 5.8. Addition of 27 mL HCl to each can of milk yielded a final pH of approximately 6.2. Acid was added to milk chilled below 4° C to avoid protein precipitation. Milk with no acid added had a pH of approximately 6.7.

Ultrafiltration

Batches of milk were ultrafiltered using an Abcor HFK-130 (Abcor Inc., Cambridge, MA, 02139), single stage, spiral wound, polysulfone membrane with a molecular weight cut-off of 10,000 Daltons and a total surface area of 5 m^2. Milk was added to a balance tank and recirculated through the membrane by a centrifugal pump until the desired concentration was obtained. An inlet pressure of 420 kPa (60 psi) and outlet pressure of 280 kPa (40 psi) were used throughout the process.

Milks were ultrafiltered at 54° C until the retentate reached a total solids of approximately 40%. This was determined by an Automatic Volatility Computer (AVC-80) microwave unit by CEM (CEM Corp, Indian Trail, NC 28079). Total solids were adjusted by removing or adding permeate to the retentates.

Diafiltration

During UF soluble minerals, ions, and lactose concentrations in the liquid phase remain constant (6). Addition of water to the retentate dilutes the concentration of these solubles and allows more of them to be removed by removal of this added water. The process of adding water to retentate during UF is called diafiltration. Percent diafiltration is the weight of water added based on the original weight of the milk (7, 14, 39). It is a necessary step in the formation of retentates for hard and semi-hard cheeses because the lactose content is generally above the accepted limit without some amount of diafiltration (1).

Diafiltration to remove lactose provides a precise control of the final pH in fermented retentate by governing the amount of lactic acid that may be formed (6, 14). Cheese made from acidified retentate by Ernstrom et al. (14) had a more normal body and texture principally due to a lower calcium content. No calcium lactate precipitated to the surface of the cheese as in cheeses from non-acidified milk. Diafiltration can be used to accurately control lactose content and the final pH, although some consideration must be made for buffer capacity in a specific batch. Diafiltration of 52-56% resulted in a post-fermentation pH of 5.1 to 5.2 (7).

In batches where diafiltration was used water addition began after approximately 60% of the original milk volume had been removed. Deionized water was added to the holding tank at the same rate as permeate was removed until the desired percentage of water had been added (based on the original milk weight). Milk was then concentrated to the final volume by removal of the remaining 20% permeate (7, 14, 39).

Cleaning

Membranes were cleaned by rinsing with water until all visible milk had been removed; washing with alkali (NaOH, pH 12) for 30 min; rinsing with water for 5 min; washing with acid (HNO_3, pH 2) for 30 min and rinsing with water for 5 min. During the cleaning the inlet pressure was 350 kPa (50 psi) and outlet pressure was 213 kPa (30 psi) (7). A solution of 100 ppm sodium metabisulfite was circulated for 5 min and the outlet corked to soak the membranes in this solution while not in use and prevent microbial growth (41).

Starter Culture

Nonfat dry milk (NDM) was reconstituted with deionized water by adding 100 g NDM to 900 mL water and stirring with a magnetic rod. Substrate for Prt- cells contained of 0.5% AYE-Light yeast extract (Busch Industrial Products, Inc., St. Louis, MO 63127) to stimulate growth. NDM and stimulated NDM (SNDM) were sterilized at 121° C for 15 min, cooled to 30° C for inoculation with the appropriate culture or stored at 4° C until needed for weekly propagation.

Culture Maintenance

Streptococcus cremoris UC310 Prt+ and Prt- variants were obtained from the culture bank in the Department of Nutrition and Food Sciences, Utah State University. The Prt+ bacteria were inoculated into sterile 16 mm test tubes containing 10 mL 10% (NDM) . The Prt- bacteria were inoculated into sterile test tubes containing SNDM containing 0.5% yeast extract (Becton, Dickinson, and Co, Cockeysville, MD 21030). Test tubes were inoculated with 1% freshly coagulated culture and incubated at 30° C for 3 h, frozen, and stored at -40° C until needed to minimize changes during this study (44).

Bulk Culture Preparation

Frozen cultures were thawed and incubated at 30° C until coagulated. Bacteria were inoculated into sterile glass bottles containing 100 mL of 10% NDM or SNDM and incubated 10 to 12 h to produce a bulk culture containing 10^8-10^9 cfu/mL.

Retentate Fermentation

Retentates were stored at 4° C for less than 1 wk prior to inoculation to avoid spoilage by psychotrophic organisms.

Inoculum Level

Using a 0.7% inoculum of commercial bulk starter Brown noted that acid development to pH 5.1 took from 18 to 36 hours (7). Although no problem was encountered with spoilage organisms, the potential exists (1). Mistry and Kosikowski (32) recommended that to speed acid production a higher inoculum could be used.

To determine a suitable inoculum level 100 mL beakers were sanitized with chlorine vapor (200 ppm) and 50 g retentate was weighed into each. Beakers were inoculated with 0.1, 0.3, 0.5, 0.7 or 0.9% (v/w) Prt+ cultures in increasing amounts and the pH recorded over 24 h. The procedure was repeated for Prt- cells.

Temperature

Brown found that when 5x retentate was inoculated at temperatures above 30° C a hard acid curd was formed within 10 h (7). This phenomenon was explained by the endothermic nature of protein hydrophobic bonding. As temperature increases water that is bound to the aliphatic protein side chains

is released with an increase in entropy. The protein side chains then form hydrophobic bonds with each other. Although this has an unfavorable enthalpy it is off-set by the entropy of the water being released. After further study it was found that at temperatures below 25° C the retentate stayed liquid. It was necessary that fermented retentate remain in a liquid form for use in a continuous cheesemaking process.

Studies by Wolk and Tittsler (53) have shown that the amount of lactic acid produced by lactic Streptococci is related to the incubation temperature. *Streptococcus cremoris* and *Streptococcus lactis* both performed best at 34.4° C. However, the present study used 23° C as an incubation temperature for all retentates to avoid the formation of acid curd that was demonstrated to be a problem for Brown (7).

Substrates

Substrates chosen for analysis were lactose (L), casein hydrolysate (CH), AYE-Light yeast extract (Busch Industrial Products, Inc., St. Louis, MO 63127) (YE1), and yeast extract from BBI™ (Becton, Dickinson, and Co, Cockeysville, MD 21030) (YE2).

Fifty grams of retentate and 0.05, 0.15, 0.25, 0.35, or 0.45 g of one of the substrates (corresponding to 0.1, 0.3, 0.5, 0.7, or 0.9%, w/w) were weighed on a Sartorius analytical balance (Sartorius GMBH Göttingen, Germany) and mixed in a 100 mL beaker that had been sanitized with 200 ppm chlorine sprayed through an atomizer bottle. Two beakers were prepared for each substrate concentration and one was used for inoculation with each proteinase culture variant. All other concentration percentages used were w/w unless otherwise noted.

Fifty grams of retentate was also weighed into five beakers without substrate for inoculation of 0.1, 0.3, 0.5, 0.7, or 0.9% culture. A second set of beakers was prepared for inoculating the other culture variant. Samples were stirred and allowed to equilibrate overnight at 4° C.

All experiments used whole milk retentates that had been ultrafiltered to 40% total solids using milks at pH 6.7, 6.2, or 5.8. Samples were inoculated with 5% (v/w) fresh milk base starter and incubated at 23° C. The pH was measured at the time of inoculation and throughout 24 h. Control samples (no additional substrates) were inoculated with increasing concentrations of starter (1, 3, 5, 7, and 9%, v/w) to show the effects of high initial cell concentrations.

pH

Values of pH were determined with a Ross™ Combination electrode (model 8103, Orion Research, Inc., Cambridge, MA 02139) on an Altex pH meter (model Φ60, Beckman Instruments, Inc., Fullerton, CA 902634).

Buffer Capacity

Buffer capacity was estimated by a method described by Sutherland and Jameson (46). Retentate was accurately diluted 3:25 with deionized water containing sodium azide (0.4 g/L). Aliquots (15 mL) were added to screw-capped vials containing increasing amounts from 0-1.1 mL of 1.20 M lactic acid in 0.1 mL increments. Sufficient water was added to bring the total volume to 16.1 mL. The tubes were shaken overnight at room temperature (23° C) and the pH determined. Buffer capacity was expressed as mM lactic acid/pH unit/g retentate.

Graphs of the buffer capacity were obtained by setting up a titration buret and setting a drip rate of 1 drop per 1-2 min. pH was plotted using a Ross™ Combination electrode (model 8103, Orion Research, Inc., Cambridge, MA 02139) and a Sargent-Welch pH recorder (model pHR, Sargent-Welch Scientific Co., Skokie, IL 60076).

Taste Panel

Retentate was prepared without diafiltration as described previously using milk acidified to pH 5.8. Six different levels of YE1 (from 0 to 0.5% in 0.1% increments) were added to separate batches of whole milk retentate (approximately 2 kg each) in duplicate. Five percent (v/w) Prt+ or Prt- cultures of *S. cremoris* UC 310 were inoculated into each container and incubated at 23° C. Upon reaching pH 5.1 (±0.05) the cultured retentates were refrigerated overnight.

A taste panel was set up to establish a threshold where the flavor of the yeast extract could be detected. Approximately 10 g of each sample (4°-8° C) were separately placed in a paper cup with a wooden popsicle stick. Booths with white lighting were used to decrease distractions. Panelists were provided a reference sample (no YE1) and asked to taste a small amount and compare it to each of the other samples (in increasing concentrations of YE1) for flavor difference only. Water was provided to drink between samples. A control was inserted midway into the test as well as at the end to identify guessing. This resulted in a total of eight samples tested by each person.

Twenty nine panelists tested samples produced by both culture variants. The retentate cultured with Prt+ was tested in the morning and panelists returned to test the Prt- retentates in the afternoon. Detection of YE1

was scored at the first point where the panelist consistently noticed a flavor difference. Those who missed the blank in the middle position were scored at the next level where they again noticed a flavor difference. Panelists who missed the blank in the last position were scored as not being able to detect a difference.

Inhibition by Acids

Fifty grams of 5x retentate were measured into 12 separate 100 mL beakers. Sodium hydroxide (1.0 N) was added to three beakers in 0.1 mL increments from 0.1 mL to 0.3 mL. Hydrochloric acid (1.0 N) was added to eight other beakers in 0.1 mL increments from 0.1 mL to 0.8 mL. Each set of acid and base additions was prepared in duplicate. Chemicals were mixed into the retentate and equilibrated overnight at 4° C. Each beaker was inoculated with 5% (v/w) Prt+ culture and incubated at 23° C. The pH was measured and recorded over 24 h.

Ten percent NDM was prepared by methods discussed previously. Ten mL were measured into each of 80 test tubes and autoclaved at 121° C for 15 min. Milk was then cooled to 4° C before adding acid or salt substrates. Substrates were calculated in a molar basis at $1x10^{-4}$, $2x10^{-4}$, and $3x10^{-4}$ M.

Twelve different substrates were chosen and mixed in duplicate for culture. These were HCl, NaCl, $CaCl_2$, lactic acid, sodium lactate, H_2SO_4, Na_2SO_4, citric acid, sodium citrate, calcium citrate, acetic acid, sodium acetate. Two tubes were used as a positive control for no added reagent.

After adding substrate the tubes were mixed on a VWR Vortex® Mixer (Scientific Industries, Inc. Bohemia, NY 11716) for 10 s. Tubes were then returned to 4° C and equilibrated overnight.

Tubes were inoculated with 5% (v/v) *S. cremoris* UC 310+ and again mixed. The pH was measured over 24 h. The procedure was repeated with Prt- bacteria.

Statistics

Statistical analysis was used to show a level of significance between different retentates, added substrates, levels of inocula, and between the proteinase variants used.

Interpolation of Time

The procedure used to determine pH called for measurements at specific time intervals. It did not allow determination of the time a specific pH was reached. Therefore, to compare the time that pH 5.1 was reached in a given beaker, interpolation was necessary.

Data were entered in Cricket™ Graph (Cricket Software, Philadelphia, PA 19104) for MacIntosh™ computer. The pH values were shown on the Y axis and time on the X axis. Graphs were plotted, enlarged up to 12 times, and the time of reaching 5.1 was interpolated from the graph. This gave good approximation to the second decimal place.

Analysis of Variance

Analysis of Variance (ANOVA) was figured separately for each experiment and a new design was drawn as necessary. One-way and two-way ANOVA were calculated using StatWorks™ (Heyden and Son, Inc., Philadelphia, PA 19104) for the MacIntosh™ computer.

Experiments with more than two factors required a more complex program. Data for multi-factorial ANOVA were coded for analysis by the

computer program FCT (ANOVA for IBM compatible PC, Dr. Rex Hurst, Utah State University).

RESULTS AND DISCUSSION

Ultrafiltration of Whole Milk

Whole milk took slightly longer to ultrafilter than did skim milk due to the milk fat reducing the permeate flux, but caused no membrane fouling as noted by Yan et al. (56). Process times for 300 lb milk averaged 2.5 h without diafiltration and 3.5 h with diafiltration. Temperatures between 50° and 55° C allowed a higher permeation rate, inhibited bacterial growth, and reduced UF time over lower temperatures (30, 56).

Preacidification

Acid may be directly added to milk without coagulation prior to UF if the temperature is below 4° C. Milk samples acidified below pH 5.65 tend to coagulate during UF and clog the membranes (14). No difficulty was experienced because these limits were not exceeded.

The pH of the retentate was slightly higher than the pH of the milk prior to UF. This was most likely a result of concentrating the milk proteins to a higher level with a resultant increase in buffer capacity and losing some of the acid into the permeate during UF.

Diafiltration

Both lactose content of the retentate and the milk pH prior to UF affect the pH in the final cheese. During the first 16 weeks a cheese matures about 95% of the lactose is converted to lactate by the bacteria (46). Lactose content in UF cheese is above the accepted limit if some amount of diafiltration is not used (1). Milk with a pH of 6.7 requires about 3.9% lactose in the retentate to stabilize at pH 5.2 at 16 weeks. For milk at pH 6.4 it is necessary to retain about 3.3% lactose in the retentate (46). At pH 5.8 less lactose would be

needed and greater amounts would need to be eliminated by diafiltration.

Peri et al. (39) used a method to reduce lactose and other solubles. This process called diafiltration adds water to the retentate at the same rate as permeate is removed and effectively dilutes the solubles found in the aqueous phase of the retentate. As UF continues this diluted lactose is removed, leaving a lower concentration of lactose in the retentate.

Brown recommended 52 to 54% diafiltration to reach a final pH of 5.1 in 5x retentates (7). However, this estimate was based on starter with no additional stimulant. When 0.2% yeast extract was added to the diafiltered retentates the final pH fell to 4.9 compared to the control (about pH 5.1). Addition of stimulants required greater amounts of diafiltration to control the final pH.

The pH of diafiltered retentates was considerably higher than the pH of the milk prior to UF. Milk acidified to pH 5.8 produced a 5x retentate with a pH of 6.0 to 6.1 when 50% diafiltration was used. This was most likely a result of the higher buffer capacity of the retentate and washing portions of the acid into the permeate during UF and diafiltration.

Retentate Fermentation

Concentrated, non-diafiltered retentates of milk were modified by addition of nutrients to compare their ability to support acid production by lactic cultures. Both Prt+ and Prt- variants of *Streptococcus cremoris* UC310 were used in the majority of the experiments. Graphs of acid production by Prt- variants generally exhibited more variation between good and bad nutrients because their metabolic requirements are more complex than Prt+ cells (42, 44, 45, 52).

Effect of Temperature

Brown (7) found that when 5x retentate was inoculated at temperatures above 30° C a hard acid curd was formed within 10 h. One of the objectives of this study was to obtain a precheese that remained liquid after fermentation. Although several researchers have shown that lactic streptococci perform best between 30° and 35° C (48, 53), this study used 23° C for all retentate fermentations so that retentates remained fluid, which is necessary for the continuous cheese production operation described by Brown (7). Using the lower temperature retentates remained liquid until about pH 5.0 after which the formation of a soft acid curd was observed.

Inoculum Level

Covacevich and Kosikowski (11) experienced a slow rate of acid production in their work with Cheddar cheese from concentrated retentates. They used a 1% inoculum (v/w) of *S. cremoris* at 23° C and reported a sluggish pH drop during 48 h. Brown (7) noted that acid development to pH 5.1 in concentrated retentate took from 18 to 36 h using a 0.7% inoculum (v/w) of frozen concentrated starter culture.

To compensate for slow acid production Mistry and Kosikowski (33) recommended that a higher level of inoculum be used. Concentrated retentate inoculated with increasing levels of Prt+ culture from 0.1% to 32% (v/w) is plotted in Figure 1.

A linear relationship is seen between the concentration of cells in the inoculum and the time required to reach pH 5.1. However, part of this linear appearance may be due to the reduction of total solids (TS) by addition of the starter culture. Milk-based bulk culture preparations had about 10% TS (w/w). Adding 32% (v/w) starter to retentates with 40% TS (w/w) would dilute them to

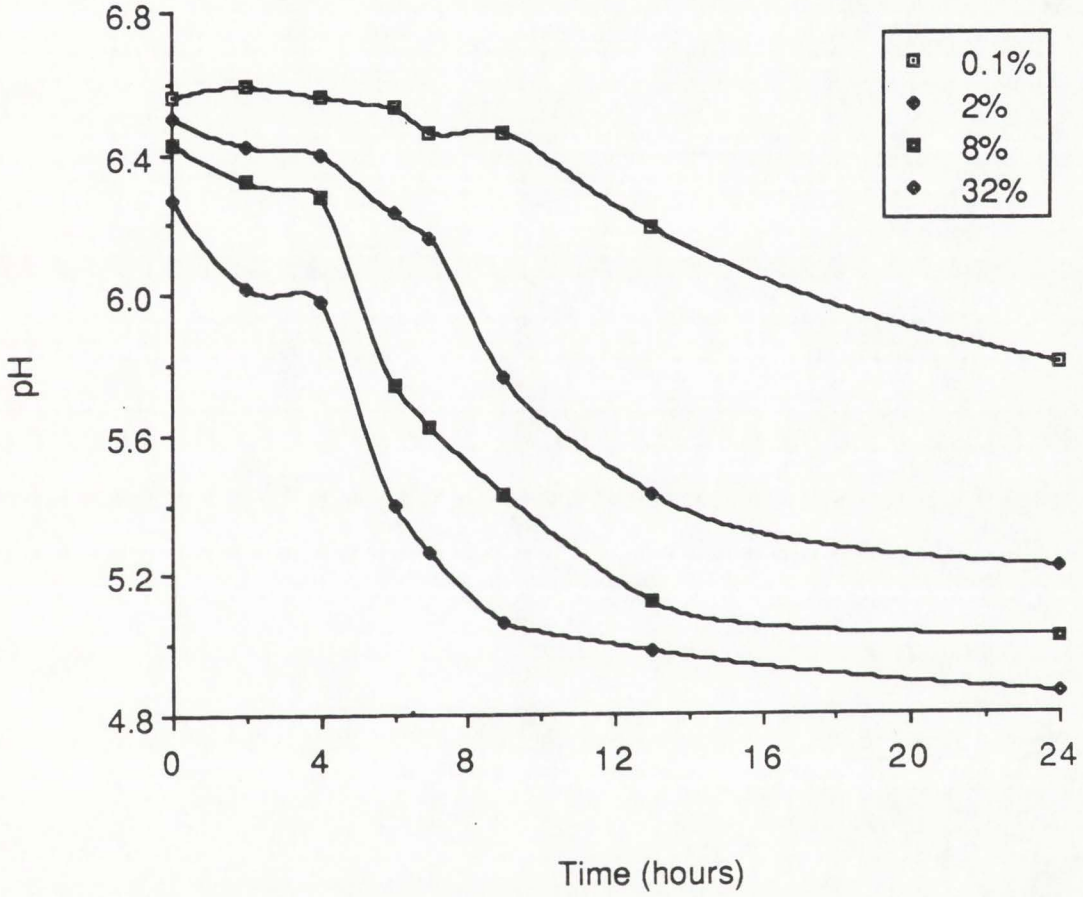

Figure 1. Effect of inoculum level (v/w) on pH during incubation of Prt+ *S. cremoris* in 5x retentate.

about 33% TS (w/w).

A second experiment with smaller gradients in inoculum level was performed using Prt+ bacteria. Inocula from one to nine percent were used. The results (Figure 2) show that one percent is too small, but little variation could be detected between the other levels.

The experiment was repeated with Prt- bacteria (Figure 3). Greater separation was seen between the 1% and 3% inoculum (v/w) level, but 5, 7, and 9% showed little difference. Therefore 5% (v/w) was used throughout the remainder of the experiments.

Effect of Preacidification

Normal milk exhibits some buffering capacity (BC) due to its proteins and insoluble calcium and phosphate salts. Mistry and Kosikowski (32) found that the greatest BC occurred between 5.1 and 5.2. During UF these components are concentrated and cause an increase in BC.

The BC was measured on retentates made from milks acidified to pH 6.7, 6.2, and 5.8. The pH changed more rapidly with the first addition of lactic acid in retentates with no acid added than those preacidified to pH 6.2 and 5.8 (Figure 4). From the graph of pH vs. lactic acid added it could be calculated that about 0.35, 0.29, and 0.25 mM lactic acid were required to lower the pH of one gram of retentates preacidified to pH 6.7, 6.2, and 5.8 respectively.

Sutherland and Jameson (46) showed that the curve of of BC fit a 4th-order polynomial by standard regression analysis. Standard regression techniques were applied to the curves of pH 6.7, 6.2, and 5.8 retentates using an application of Cricket™ Graph (Cricket Software, Philadelphia, PA 19104) on a MacIntosh™ computer. All curves fit the 4th-order polynomial with R greater than 0.99. Retentates from milks preacidified to pH 5.8 fit the equation

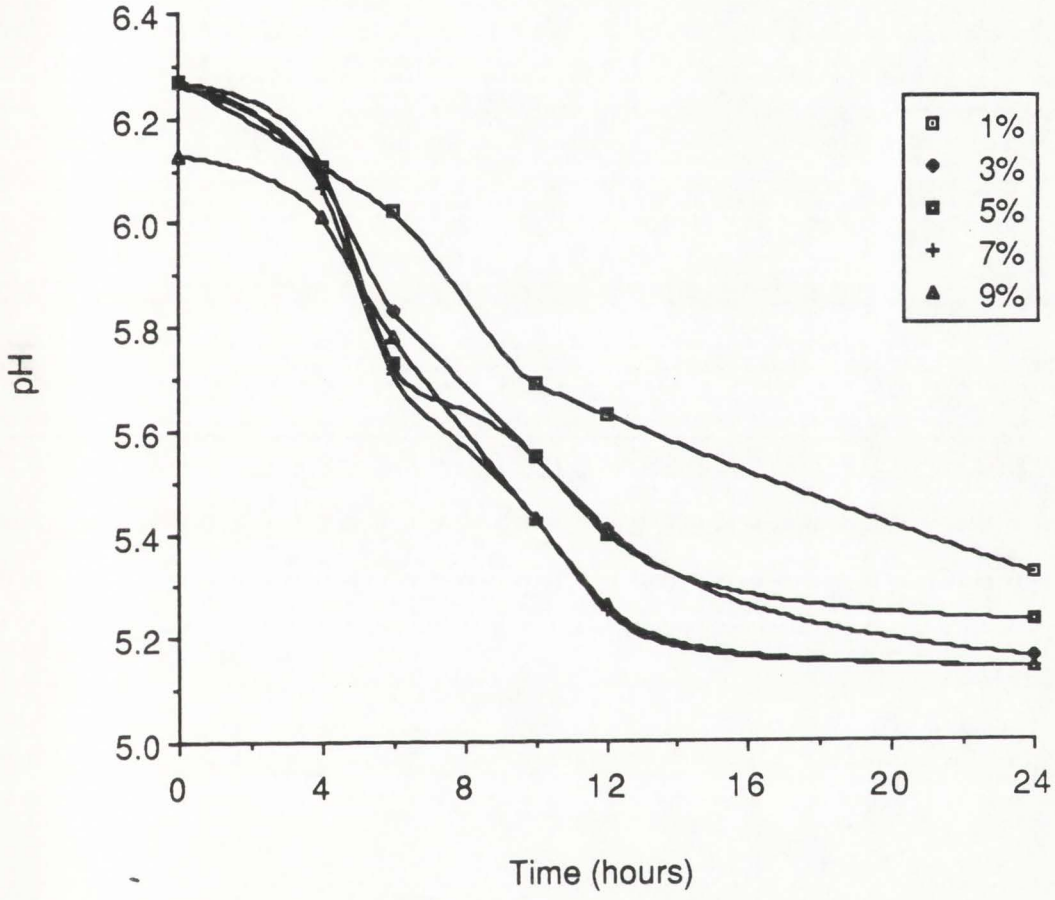

Figure 2. Effect of inoculum level (v/w) on pH during incubation of Prt+ *S. cremoris* in 5x retentate that was diafiltered 50%. Milk was acidified to pH 5.8 prior to UF.

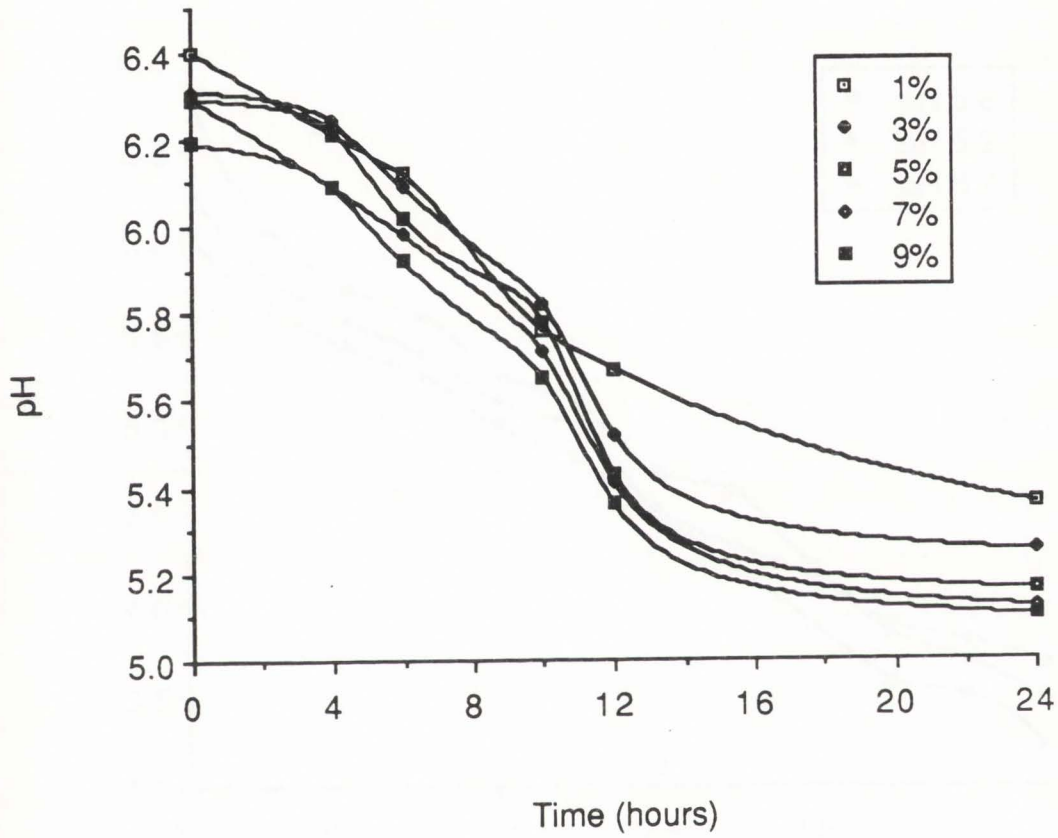

Figure 3. Effect of inoculum level (v/w) on pH during incubation of Prt- *S. cremoris* in 5x retentate that was diafiltered 50%. Milk was acidified to pH 5.8 prior to UF.

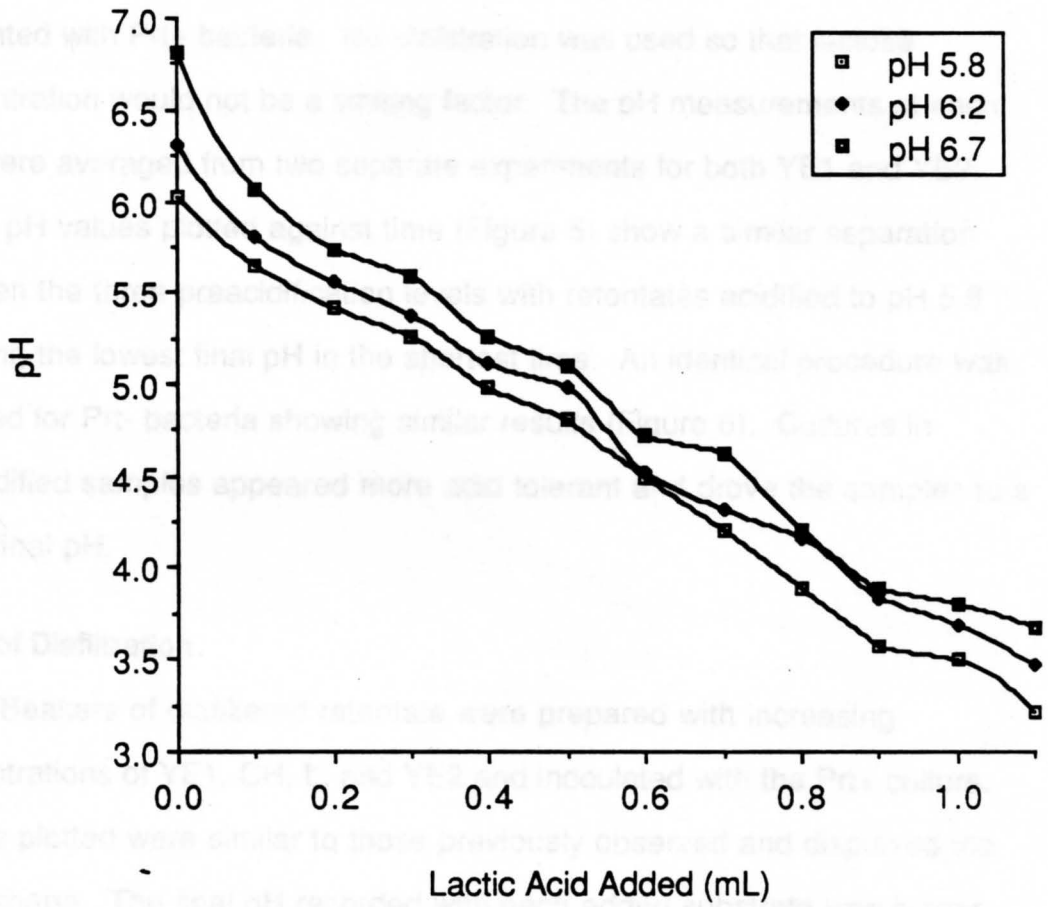

Figure 4. Effect of acidification of milk to pH 6.7, 6.2, or 5.8 prior to UF on pH reduction in 5x retentate upon the addition of lactic acid.

$y = 6.0 - 3.693\ x + 6.113\ x^2 - 9.618\ x^3 + 4.589\ x^4$. Retentate from milks preacidified to pH 6.2 fit the equation $y = 6.270 - 4.59\ x + 7.382\ x^2 - 9.647\ x^3 + 4.225\ x^4$. Retentate from milks with no acid added (pH 6.7) fit the equation $y = 6.774 - 7.870\ x + 18.114\ x^2 - 23.428\ x^3 + 10.147\ x^4$.

Retentates stimulated with yeast extract at each pH level were fermented with Prt+ bacteria. No diafiltration was used so that lactose concentration would not be a limiting factor. The pH measurements at each time were averaged from two separate experiments for both YE1 and YE2. These pH values plotted against time (Figure 5) show a similar separation between the three preacidification levels with retentates acidified to pH 5.8 attaining the lowest final pH in the shortest time. An identical procedure was followed for Prt- bacteria showing similar results (Figure 6). Cultures in preacidified samples appeared more acid tolerant and drove the samples to a lower final pH.

Effect of Diafiltration

Beakers of diafiltered retentate were prepared with increasing concentrations of YE1, CH, L, and YE2 and inoculated with the Prt+ culture. Graphs plotted were similar to those previously observed and displayed the same shape. The final pH recorded with each added substrate was higher than those of a retentate that had not been diafiltered except retentate fortified with lactose. Retentates with lactose added showed a lower final pH than the control. Similar graphs were observed using Prt- cultures. However, retentate samples fortified with lactose had the same final pH as the control.

Graphs of acid development over 24 h displayed the same shape whether the retentates were diafiltered or not. The only difference observed between the two treatments on retentates was the final pH achieved.

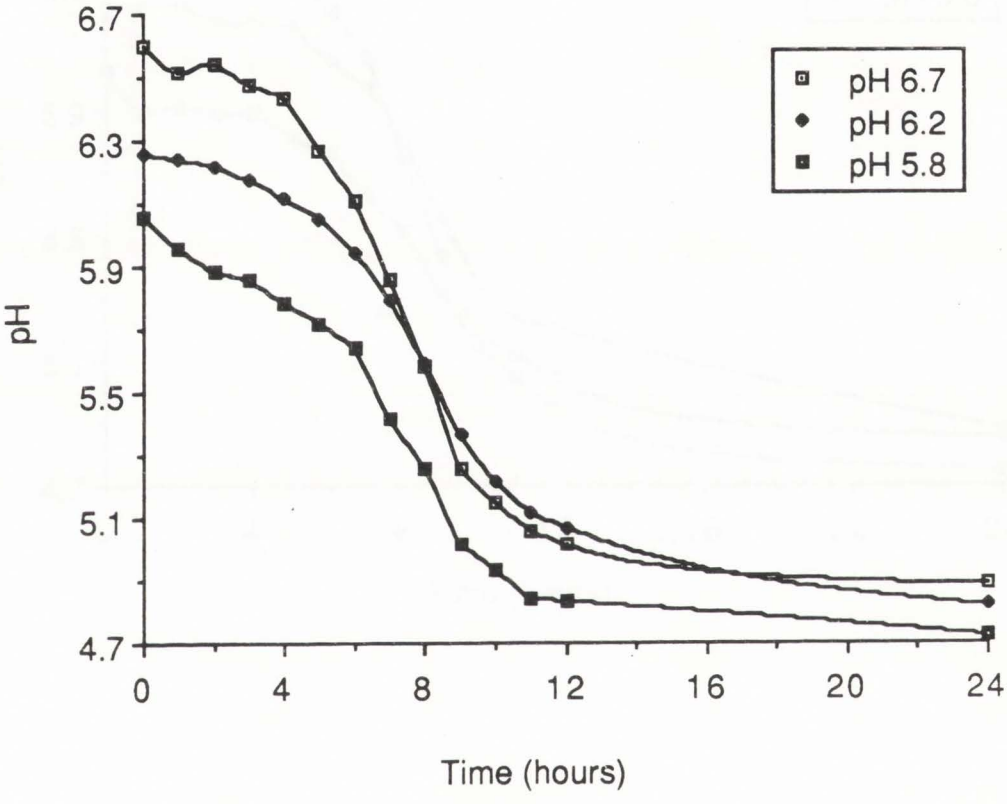

Figure 5. Effect of acidification of milk to pH 6.7, 6.2, or 5.8 prior to UF on pH of 5x retentate during fermentation with Prt+ *S. cremoris*.

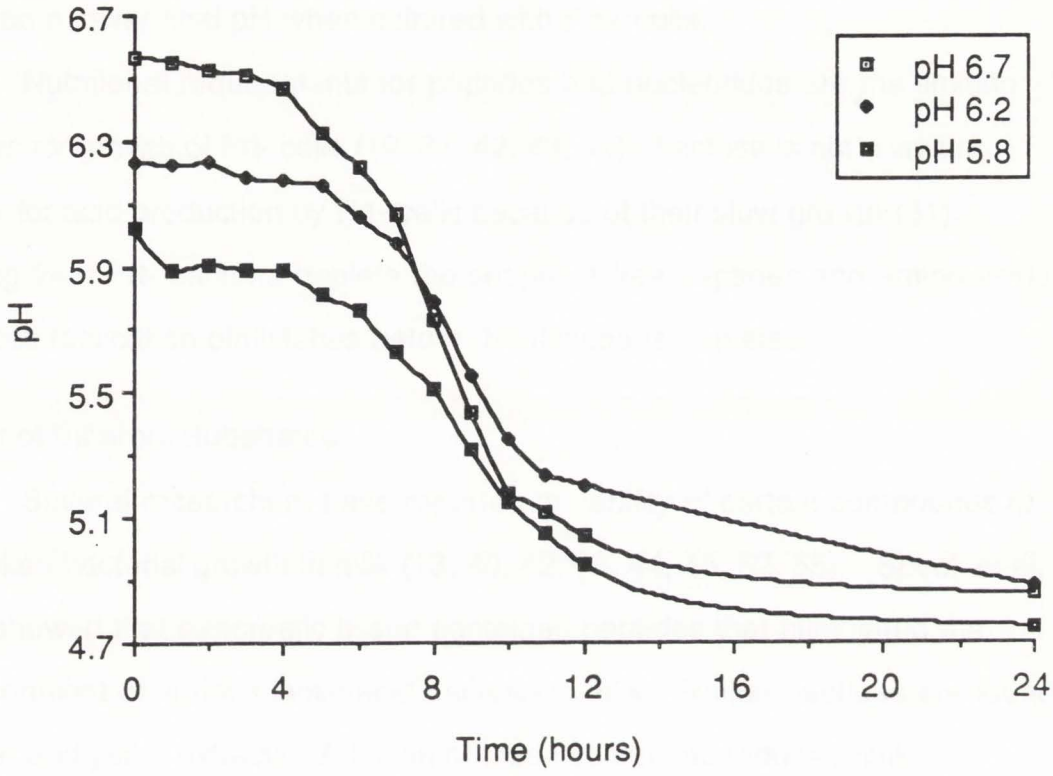

Figure 6. Effect of acidification of milk to pH 6.7, 6.2, or 5.8 prior to UF on pH of 5x retentate during fermentation with Prt- *S. cremoris*.

Diafiltered retentates depend on the lactose concentration for the amount of acid that may be produced because lactose is the limiting factor for bacterial growth (7, 14, 46). Supplementation of extra lactose in diafiltered retentates allowed a lower final pH when cultured with Prt+ cells.

Nutritional requirements for peptides and nucleotides are the limiting factors for growth of Prt- cells (19, 31, 42, 43, 52). Lactose is not a limiting factor for acid production by Prt- cells because of their slow growth (31). During 24 h Prt- bacteria deplete the supply of free peptides and amino acids and cell replication diminishes before the lactose is depleted.

Effect of Different Substrates

Several researchers have reported the ability of certain compounds to stimulate bacterial growth in milk (13, 40, 42, 43, 44, 45, 52, 55). Speck et al. (43) showed that pancreatic tissue contained peptides that stimulated the development of various lactic acid bacteria in milk. Similar peptides are found in liver and yeast extracts. Addition of these stimulants reduced milk coagulation times by 17-41% using stimulant concentrations of 0.015-1.0% (w/w). Casein hydrolysate was shown to be useful in the preparation of a media to grow lactic Streptococci (54, 55). Stoddard and Richardson (45) found that 0.1 to 0.2% (w/w) yeast extract added to NDM only slightly improved the performance of Prt+ cells, however cell mass of Prt- cells increased 6 to 11 times. With the addition of yeast extract Prt- cells could produce pH changes near those made by Prt+ cells.

Stoddard and Richardson (44, 45) working with proteinase negative variants in cottage cheese production showed that high levels of yeast extract could be incorporated into the bulk culture without any adverse effect. The high concentration of yeast extract from the bulk culture carried over into the

cottage cheese vat leaving sufficient levels of yeast extract to stimulate bacteria to accelerated growth and acid production.

Two brands of yeast extract, AYE-Light yeast extract (Busch Industrial Products, Inc., St. Louis, MO 63127) (YE1) and yeast extract (Becton, Dickinson, and Co, Cockeysville, MD 21030) (YE2), casein hydrolysate (CH) and lactose (L) were analyzed for their effect on the stimulation of acid production. Increasing concentrations from 0.1 to 0.9% (w/w) of each substrate were added to 50 g of retentate and cultured.

Figure 7 shows the effect of the highest concentration (0.9%, w/w) of all substrates on acid development by Prt+ bacteria. Both YE1 and YE2 were able to reduce the pH to 5.1 in about 10 h compared to 24 h for the control and lactose. Casein hydrolysate appears to have a slight positive effect reducing the pH in a shorter time. A similar result was seen by Prt- bacteria in Figure 8.

Yeast Extract

Wright (55) observed that there were differences between extracts and autolysates of yeast in their ability to stimulate acid production by lactic bacteria. There was even a difference noticed between brands of yeast extract in his media for pH control. Therefore, both brands of yeast extract (YE1 and YE2) were compared for a difference in their ability to increase acid production rates.

Brands

Beakers of retentate were fortified with 0.3 and 0.5% (w/w) of each brand of yeast extract and inoculated with Prt+ culture. A second set of beakers was prepared the same way, inoculated with Prt- culture, and pH was plotted over 24 h (Figures 9 and 10). Analysis of variance (ANOVA) was

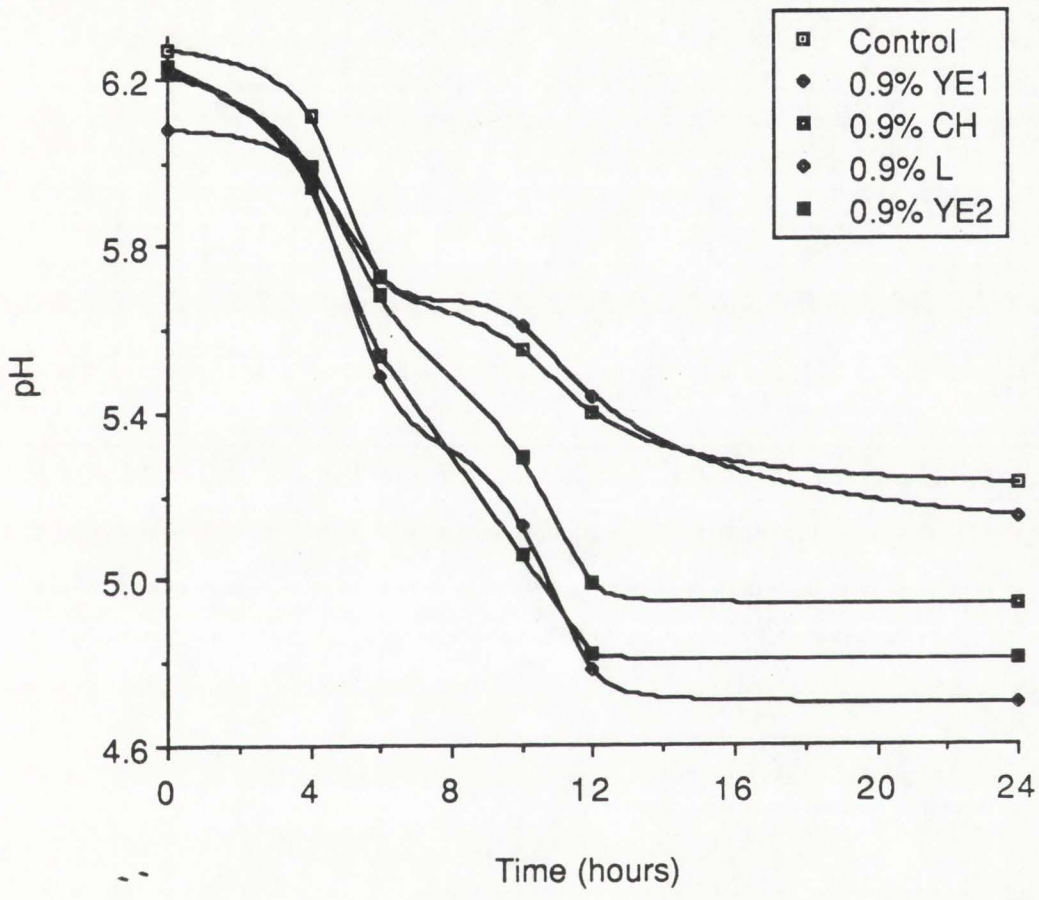

Figure 7. Effect of different additives (0.9%, w/w) on pH during fermentation with Prt+ *S. cremoris* of 5x retentate. Milk was acidified to pH 5.8 prior to UF and diafiltered 50% (YE1 denotes AYE-Light yeast extract, CH denoted casein hydrolysate, L denotes lactose, and YE2 denotes yeast extract from BBL™).

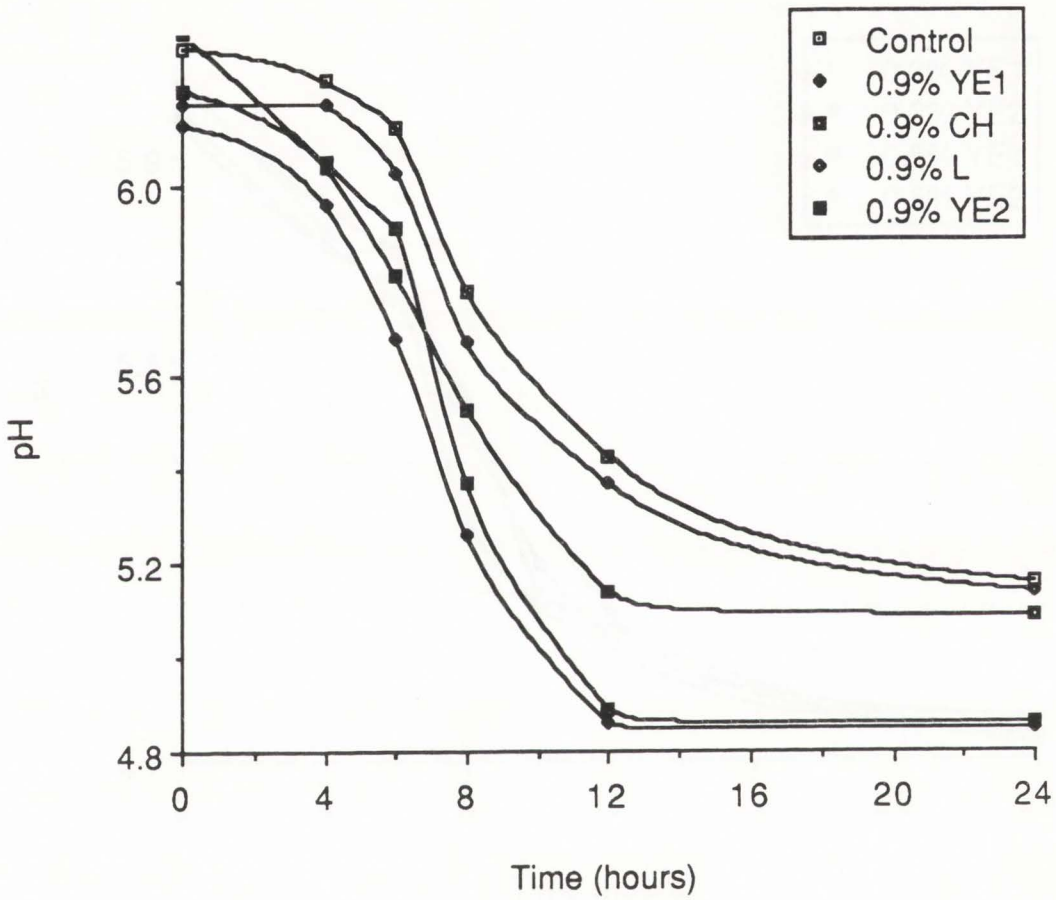

Figure 8. Effect of different additives (0.9%, w/w) on pH during fermentation with Prt- *S. cremoris* of 5x retentate. Milk was acidified to pH 5.8 prior to UF and diafiltered 50% (refer to Figure 7 for abbreviations).

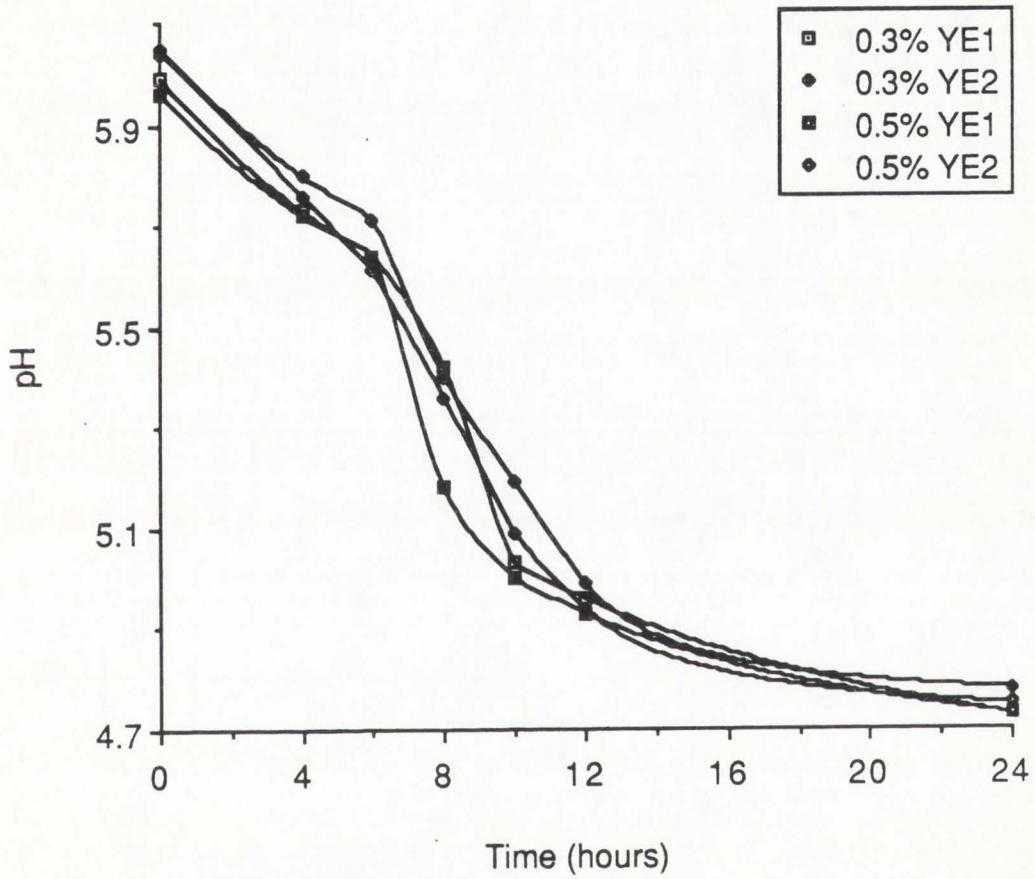

Figure 9. Effect of two brands of yeast extract (YE1 and YE2) on pH during fermentation of 5x retentate with Prt+ *S. cremoris*. Milk was acidified to pH 5.8 prior to UF and diafiltered 50%.

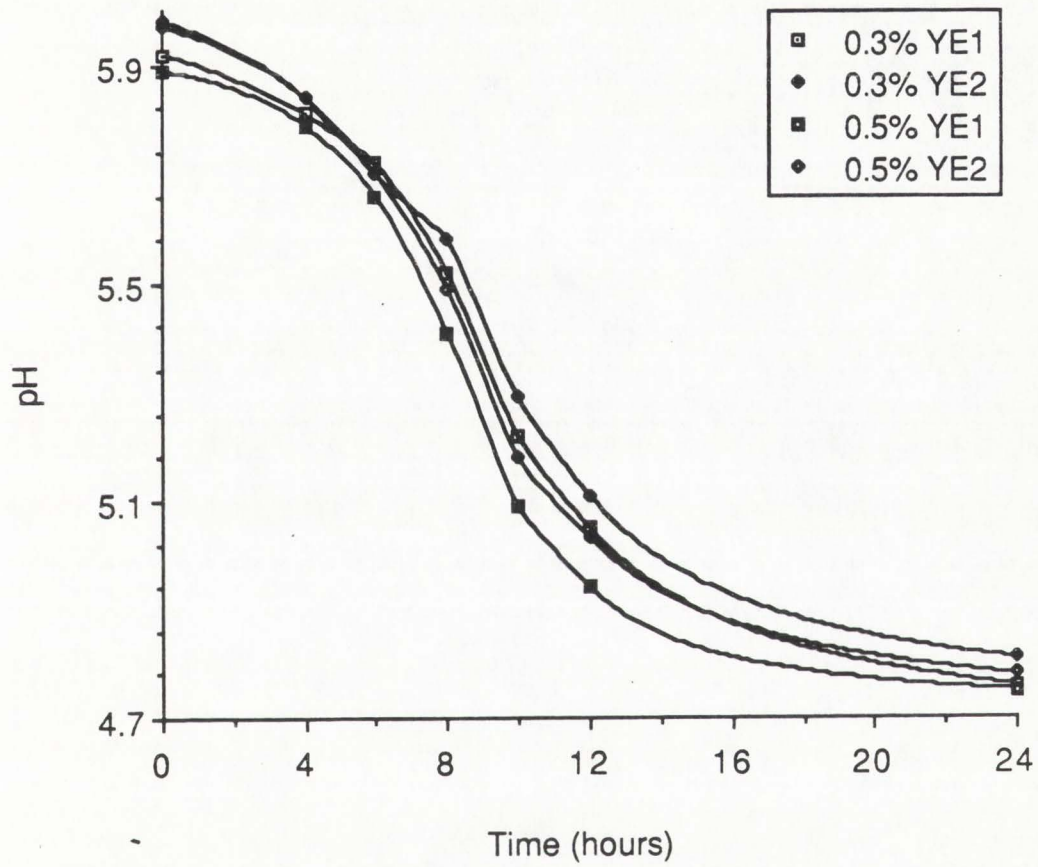

Figure 10. Effect of two brands of yeast extract (YE1 and YE2) on pH during fermentation of 5x retentate with Prt- *S. cremoris*. Milk was acidified to pH 5.8 prior to UF and diafiltered 50%.

performed using a three-way, split plot design (Table 1). Factors in the ANOVA were brands, concentration, and proteinase activity. Control beakers (no yeast added) were omitted from the ANOVA.

There was a significant difference noted between the brands used as demonstrated by an F-value of 123.53. The YE1 showed an average time for acid development of 9.98 h while YE2 averaged 11.16 h.

A difference was also seen between Prt+ and Prt- cultures with an F-value greater than 27 for the proteinase variants. The Prt+ culture performed better reaching pH 5.1 in an average of 9.83 h compared to 11.32 h for the Prt- bacteria.

No significant differences were noted between the two concentrations of YE used. Using 0.3% YE (both brands averaged) in retentates pH 5.1 was reached in 10.06 h compared to 11.08 h using 0.5% YE. One would expect that the higher concentration of YE would yield the lower time (verified by the following section).

TABLE 1. ANOVA table for differences between Prt+ and Prt - variants, concentration of yeast extract, and brands of yeast extract.

Source	Sum of Squares	Deg. of Freedom	Mean Squares	F-ratio	Critical F α=0.05
Prt+ vs. Prt-	22.0523	1	22.0523	27.0913	4.494
Concentration	12.6563	1	12.6563	15.5483	4.494
Prt x Con	1.4823	1	1.4823	1.8210	4.494
Error A	13.0240	16	0.8140		
Brand	11.7723	1	11.7723	123.5289	4.494
Prt x Brand	0.4623	1	0.4623	4.8510	4.494
Con x Brand	0.0563	1	0.0563	0.5908	4.494
P x C x B	0.0303	1	0.0303	0.3179	4.494
Error B	1.5240	16	0.0953		
Total	63.0598	39	1.6169		

Quantity

After determining that YE1 provided the most stimulus of those nutrients tested, different levels were tested to determine an optimum concentration for maximum acid production and low cost. Levels from 0 to 0.5% YE1 were prepared for fermentation using both Prt+ and Prt- cultures and pH was plotted over 24 h (Figures 11 and 12).

A one-way ANOVA was used to determine a difference and confidence intervals were used to determine the point where a difference could be seen (Table 2). Only data from Prt- cultures were used since differences in stimulation are only minor for Prt+ bacteria (40, 44, 52). There was a significant difference between the control and 0.1%. Also a significant difference was seen between 0.1% and 0.2%. However, no significant difference was observed between the higher levels, indicating that a concentration of 0.2% YE1 was sufficient to stimulate acid production.

A confidence interval was calculated (Table 3) using the means from each concentration level of YE1. To show significance a given concentration needed to decrease the final time by 2.25 h over the previous level. Retentates cultured with no YE1 showed a mean of 29.13 h to reach pH 5.1 while retentates cultured with 0.1% YE1 averaged 20.71 h. A concentration

TABLE 2. ANOVA table for amount of yeast extract necessary for maximum bacterial stimulation.

Source	Sum of Squares	Deg. of Freedom	Mean Squares	F-ratio	Prob>F
Amount YE1	976.1834	5	195.2367	113.2119	0.000
Error	31.0414	18	1.7245		
Total	1007.2248	23			

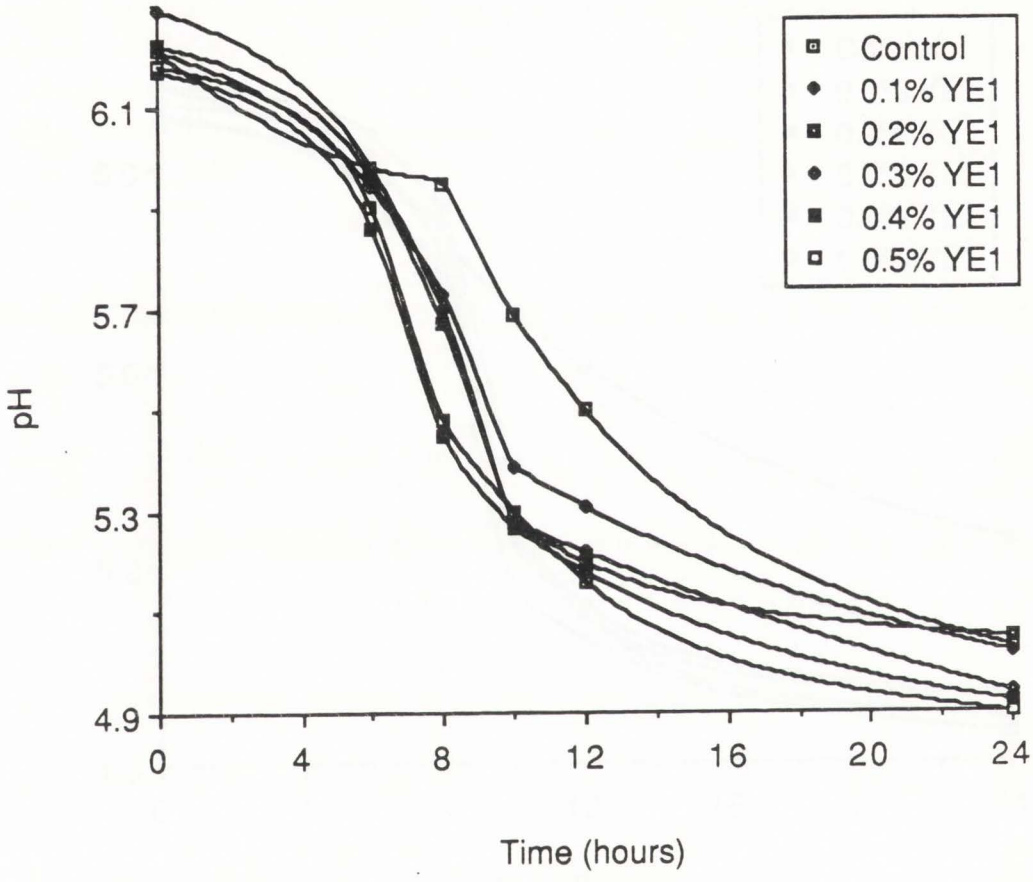

Figure 11. Effect of yeast extract concentration on pH during fermentation of 5x retentate with Prt+ *S. cremoris*. Milk was acidified to pH 5.8 prior to UF and diafiltered 50%.

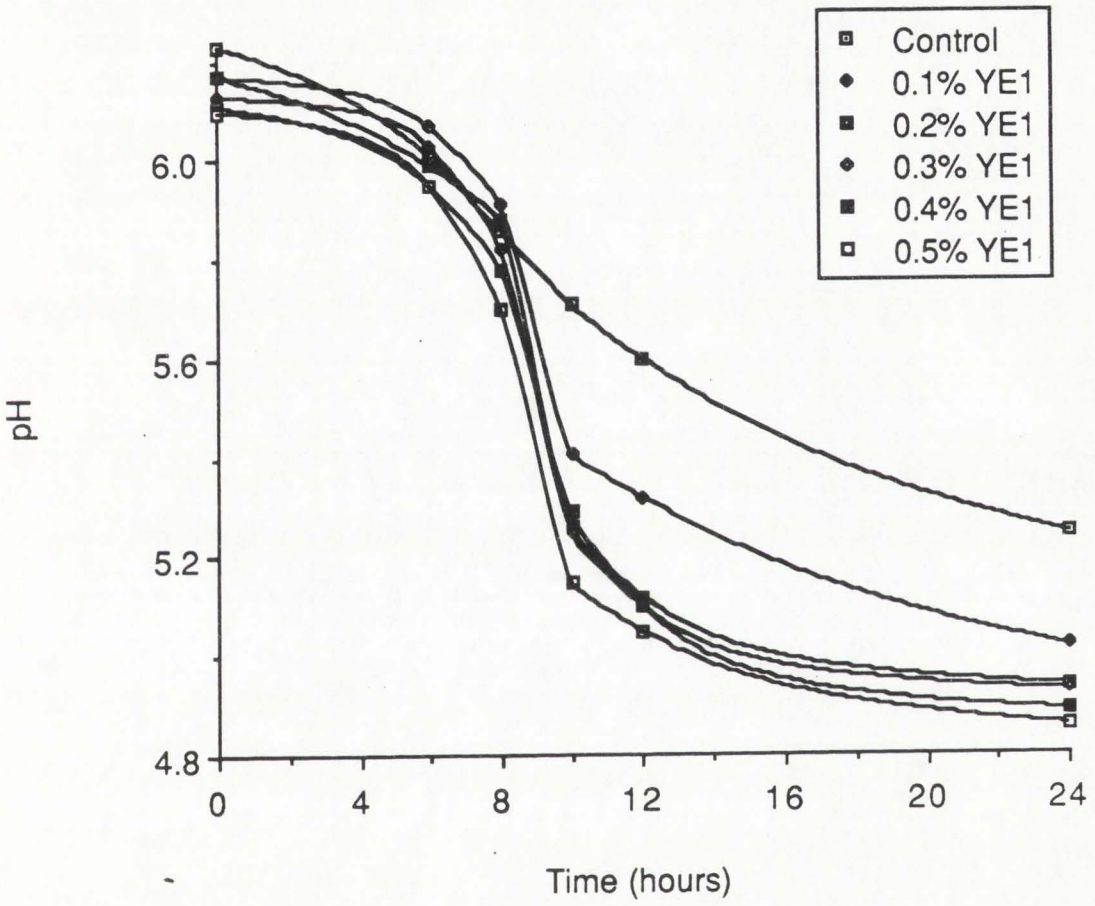

Figure 12. Effect of yeast extract concentration on pH during fermentation of 5x retentate with Prt- *S. cremoris*. Milk was acidified to pH 5.8 prior to UF and diafiltered 50%.

TABLE 3. Confidence interval for concentration of yeast extract necessary for maximum bacterial stimulation.

Mean (hours)	Confidence Interval
y_0[a] = 29.1250	
y_1 = 20.7125	$(y_i - y_j) \pm t\,[(MSE/df_i) + (MSE/df_j)]^{1/2}$
y_2 = 12.5250	
y_3 = 12.5250	$(y_i - y_j) \pm 2.101[2(1.7245/3)]^{1/2}$
y_4 = 12.0075	
y_5 = 10.7575	$(y_i - y_j) \pm 2.2527$

[a] y_0 denotes 0% yeast extract, y_1 denotes 0.1% yeast extract, etc.

of 0.2% YE1 reduced the necessary time to 12.53 h. Above that concentration no further significant time reductions were observed.

To further determine if the stimulatory effect of YE1 was linear or logarithmic on acid production, the time required to reach pH 5.1 was interpolated from the graph and plotted against concentration. The Prt+ bacteria showed relationship $y = 10.108 * x^{-0.079}$ and a fit of R = 0.96 (Figure 13) showing an excellent logarithmic relationship. A better fit was obtained with the Prt- culture. The equation was $y = 10.192 * x^{-0.072}$ and had a fit of R = 0.99 (Figure 14).

Level for Taste Detection

A taste panel established a threshold where the yeast flavor could be detected in non-diafiltered retentate. Panelists were provided a reference sample (no YE1) and asked to taste a small amount and compare it to each of the other samples (in increasing concentrations of YE1) for flavor difference. Each was instructed to taste each sample and compare it to the reference for flavor difference only.

Twenty nine panelists tested samples produced by both Prt+ and Prt-

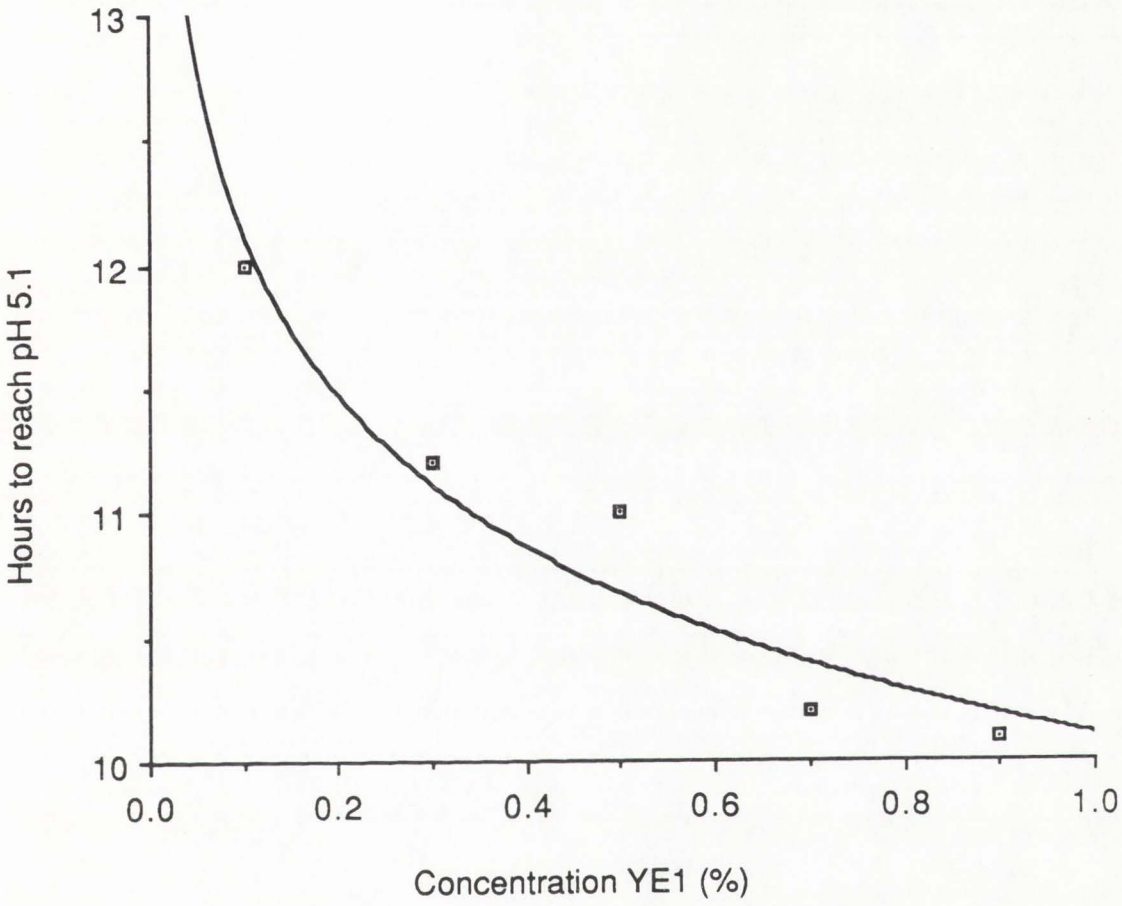

Figure 13. Effect of yeast extract concentration on time required to reach pH 5.1 in 5x retentate cultured with Prt+ *S. cremoris*.

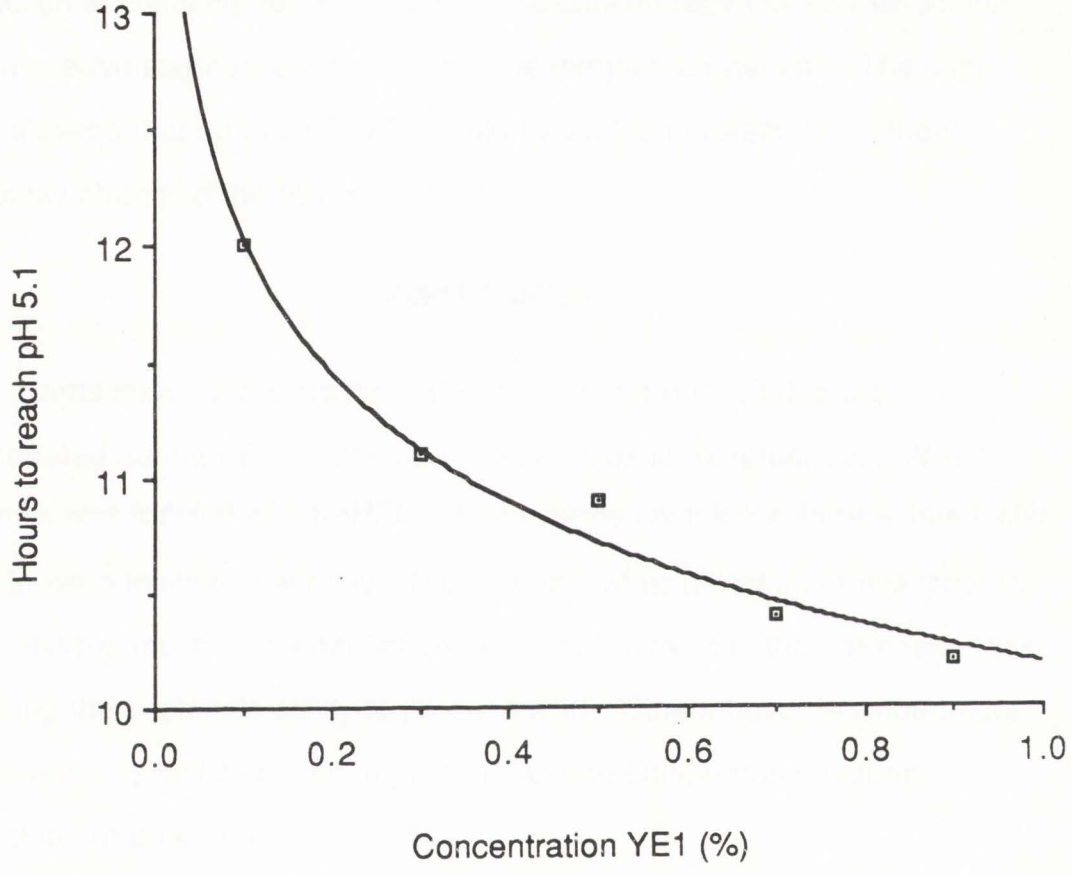

Figure 14. Effect of yeast extract concentration on time required to reach pH 5.1 in 5x retentate cultured with Prt- *S. cremoris*.

culture variants. At the highest concentration, less than half of the panelists could detect a difference between the reference and the sample for either culture variant (Figure 15).

A level of 0.2% YE1 is all that is required to provide optimum acid production in concentrated retentates. This concentration of YE1 would not have any adverse effect on the flavor of the fermented retentate. The taste panel showed that up to 0.5% YE1 could be added to retentates without noticeably changing the flavor.

Acid Inhibition

Narasimhan and Ernstrom (35) showed that phosphates are concentrated during UF approximately three times in 5x retentates. When skim milk was fortified with K_2HPO_4 to the same level found in retentate lactic bacteria were inhibited severely. They found that acidification of milk prior to UF solubilizes much of the phosphate and it is removed in the permeate, thus improving the bacteria's ability to produce acid. Others have described poor performance by lactics grown in high phosphate concentrations during preparation of bulk cultures (36, 55).

Mistry and Kosikowski (33) showed that lactic streptococci can produce up to 0.7% (w/w) lactic acid in milk (pH 4.2-4.4) without sustaining damage. However, below this pH the cytoplasmic pH drops and the cell stops functioning.

To test whether the concentration of lactate developed in retentate was inhibitory to the bacteria or if some other factor were involved the pH of two lots of retentate was adjusted with HCl and NaOH to pH 7.0 and 6.0, equilibrated overnight, and inoculated with Prt+ bacteria. To half of each pH

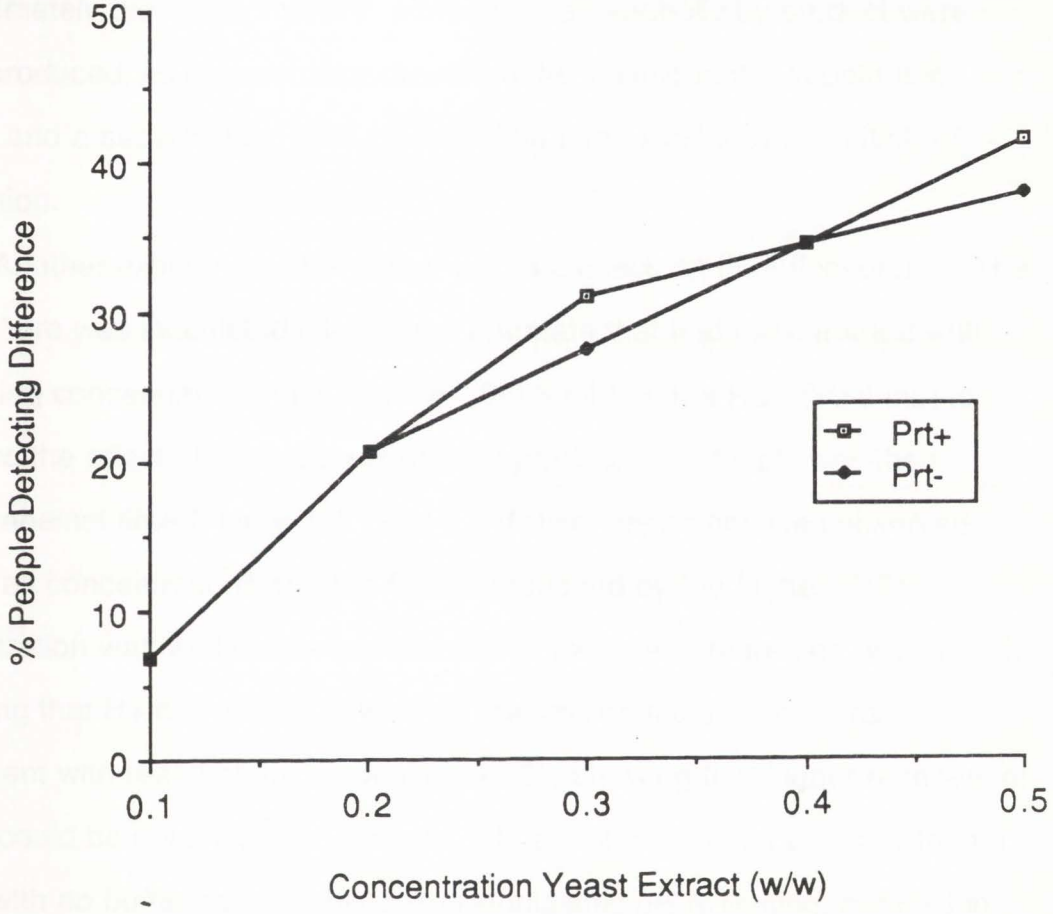

Figure 15. Effect of yeast extract concentration in 5x retentate on the ability of taste panelists to detect flavor differences between a control and yeast fortified samples cultured with Prt+ or Prt- *S. cremoris.*

group was added 0.2% (w/w) yeast extract. The pH was plotted over 24 h (Figure 16).

It was assumed that if the hydrogen ion content were the limiting factor, causing a low cytoplasmic pH in the cell, that both test groups would reach approximately the same final pH. However if a metabolic by-product were being produced, its concentration would be the limiting factor in both test groups and a separation in final pH would be seen similar to the initial pH separation.

Another experiment was performed as a check on the effect of pH. The Prt+ culture was inoculated into 50 g of retentate that had been treated with increasing concentrations of 1 N NaOH (0-0.3 mL) or 1 N HCl (0-0.8 mL) to measure the effect of the initial pH on acid production. The pH was then plotted against time (Figure 17). Again a distinct separation was observed among all concentrations and the final pH reached by the highest HCl concentration was well below that reached in the retentate treated with NaOH, indicating that H+ concentration was not the limiting factor. This was in agreement with research by Ustunol et al. (51) showing that higher numbers of cfu/mL could be obtained from a media with an internal buffer capacity than a media with no buffer capacity because cytoplasmic pH is limiting, not H+ ion concentration in the retentate.

According to Osborne (37) the most important inhibitory compound produced by lactic culture metabolism is lactic acid or lactate salts. High concentrations of lactate produced during bulk culture preparation by batch or continuous culture of cheese starter affect culture growth. He found that when lactate is removed by diffusion membrane cells can grow to concentrations as high as 10^{11} cfu/mL in bulk media preparation.

To test the effect of lactic acid on bacterial acid production, five different

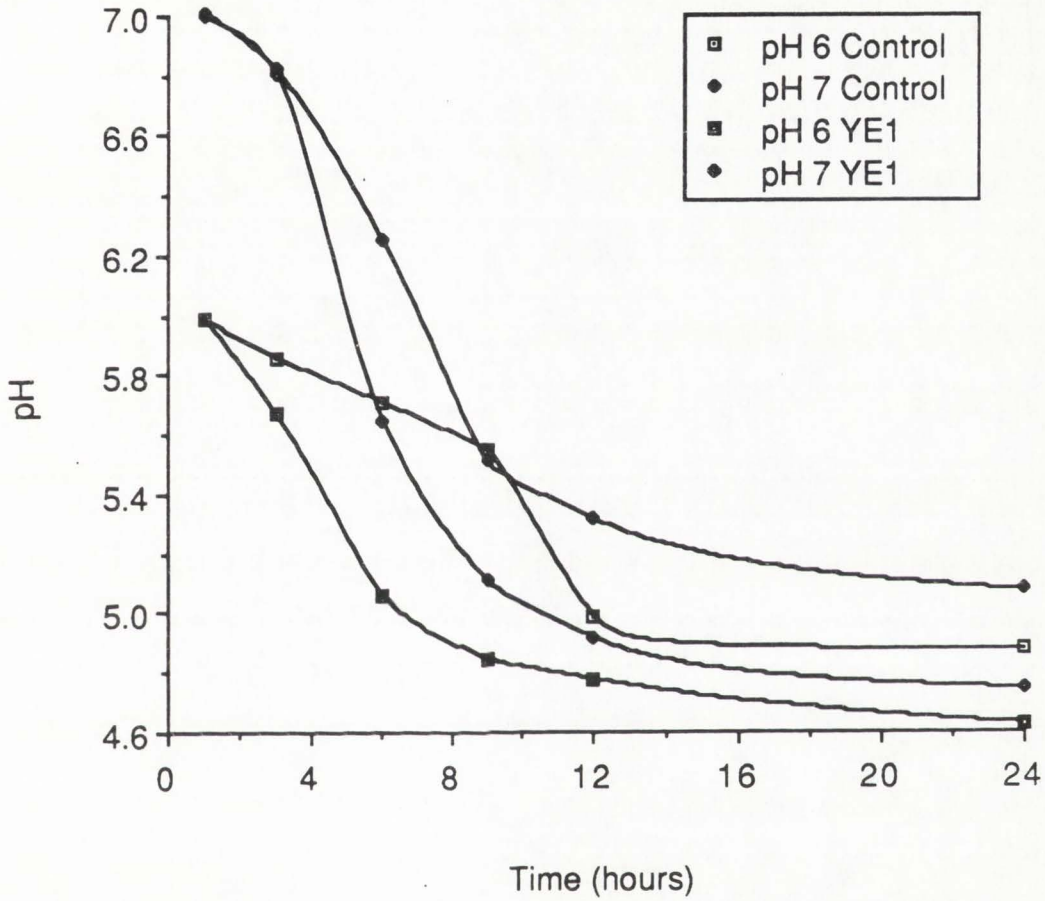

Figure 16. Effect of pH adjustment of 5x retentate to 6.0 or 7.0 on pH during fermentation with Prt+ *S. cremoris.*

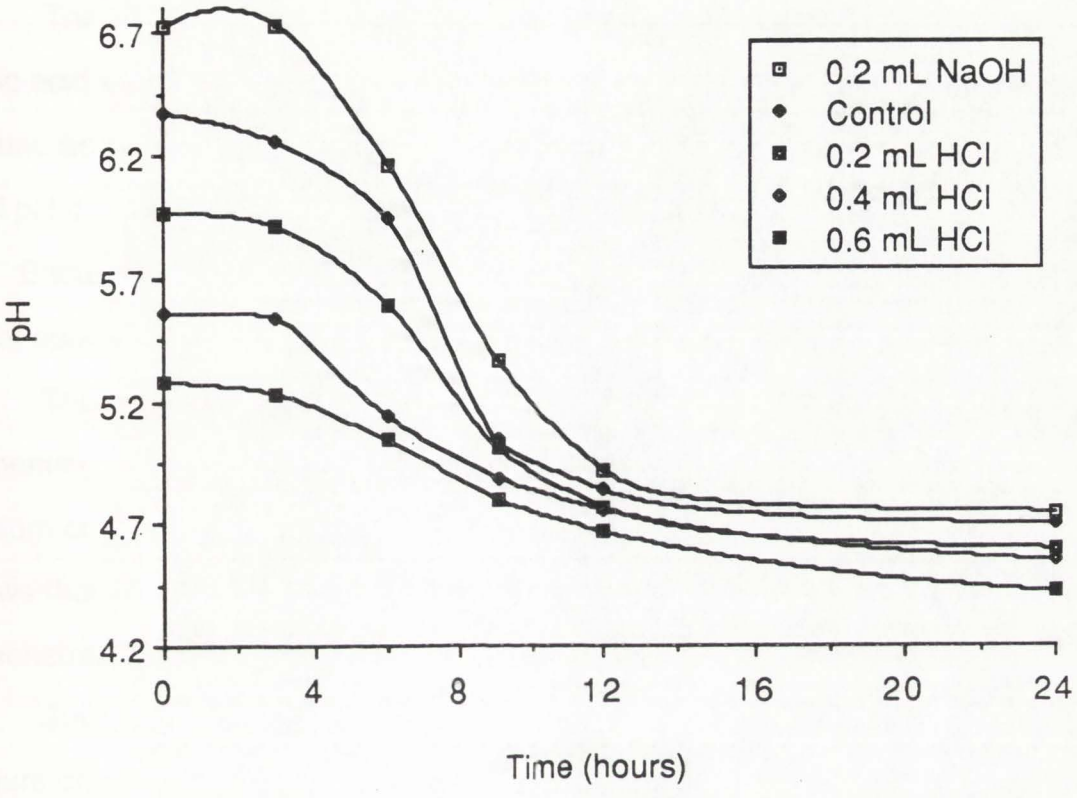

Figure 17. Effect of initial pH (chemically adjusted) of 5x retentate on the pH during fermentation with Prt+ *S. cremoris.*

acids were mixed to 1 N concentrations and mixed with NDM. The effect of those acids was observed by reduction in pH obtained by Prt+ and Prt- bacteria over 24 h.

The final pH reached by Prt+ or Prt- bacteria grown in acetic acid or lactic acid was much higher than the pH of bacteria in citric, hydrochloric, or sulfuric acids (Figures 18 and 19). Acetic acid was highly inhibitory yielding a final pH of only 4.6 while bacteria grown in lactic acid produced a final pH of 4.5. Bacteria grown in NDM acidified with the other three acids reached a final pH of approximately 4.2 (for Prt+ culture).

The inhibitory effects of lactate and acetate were again verified by fermenting NDM with the sodium salts of the same acids used previously. Sodium chloride was added as a control for the Na+ ion. Figure 20 clearly shows that lactate and acetate are inhibitory for Prt+ bacteria and Figure 21 demonstrates the effect on Prt- bacteria.

Substrates tested for inhibitory effects included hydrochloric acid, sodium chloride, calcium chloride, lactic acid, sodium lactate, sulfuric acid, sodium sulfate, acetic acid, sodium acetate, citric acid, sodium citrate, and calcium citrate. All sodium salts were compared for inhibitory effects. Calcium salts were also compared against sodium salts to insure that any inhibition noted was due to the anion, not the cation.

Using a two-way ANOVA, which analyzed the substrate, concentration, and the interaction between them, these results were statistically quantified (Table 4). The specific substrate had a significant effect, however the concentration (1×10^{-4}, 2×10^{-4}, or 3×10^{-4} M) did not make a significant difference. The interaction was significant, showing that the concentration was only important if the substance was already somewhat inhibitory.

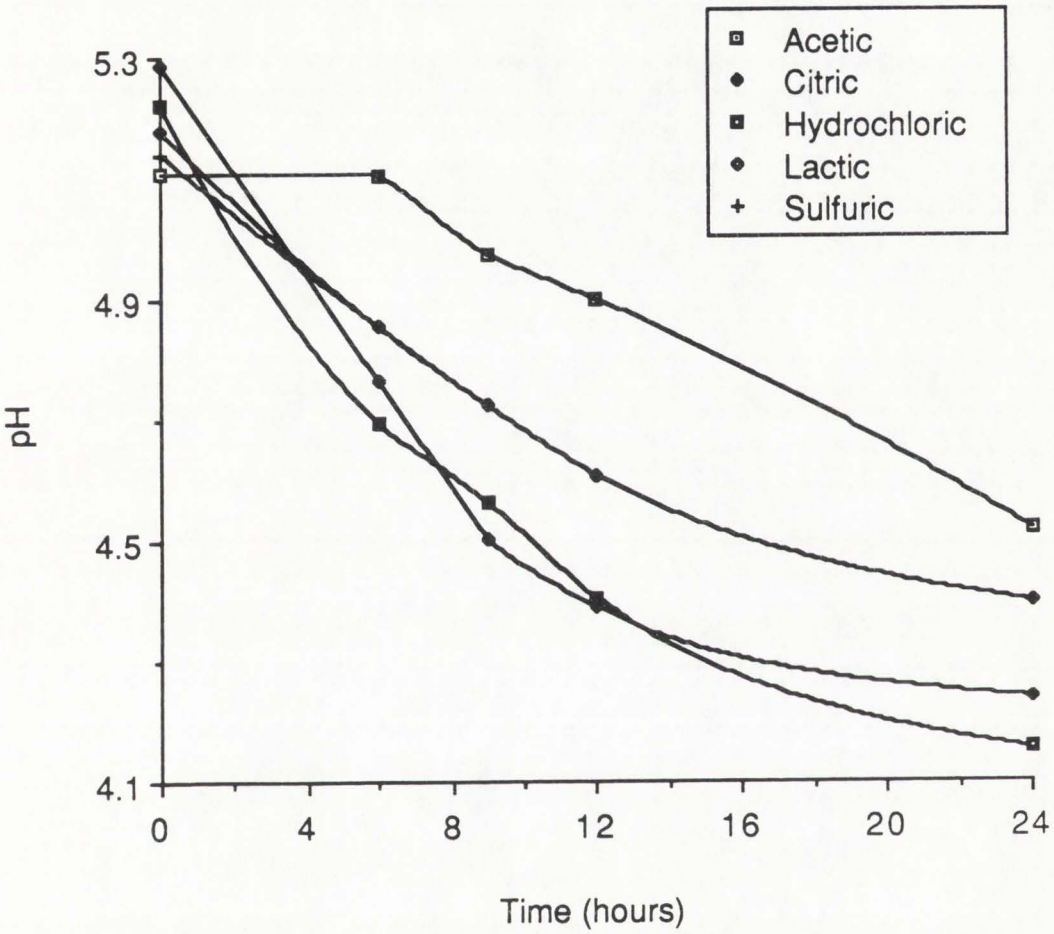

Figure 18. Effect of different acids added to 10% NDM on pH during fermentation with Prt+ *S. cremoris.* Initial acid concentration in milk was 3 x 10^{-4} M.

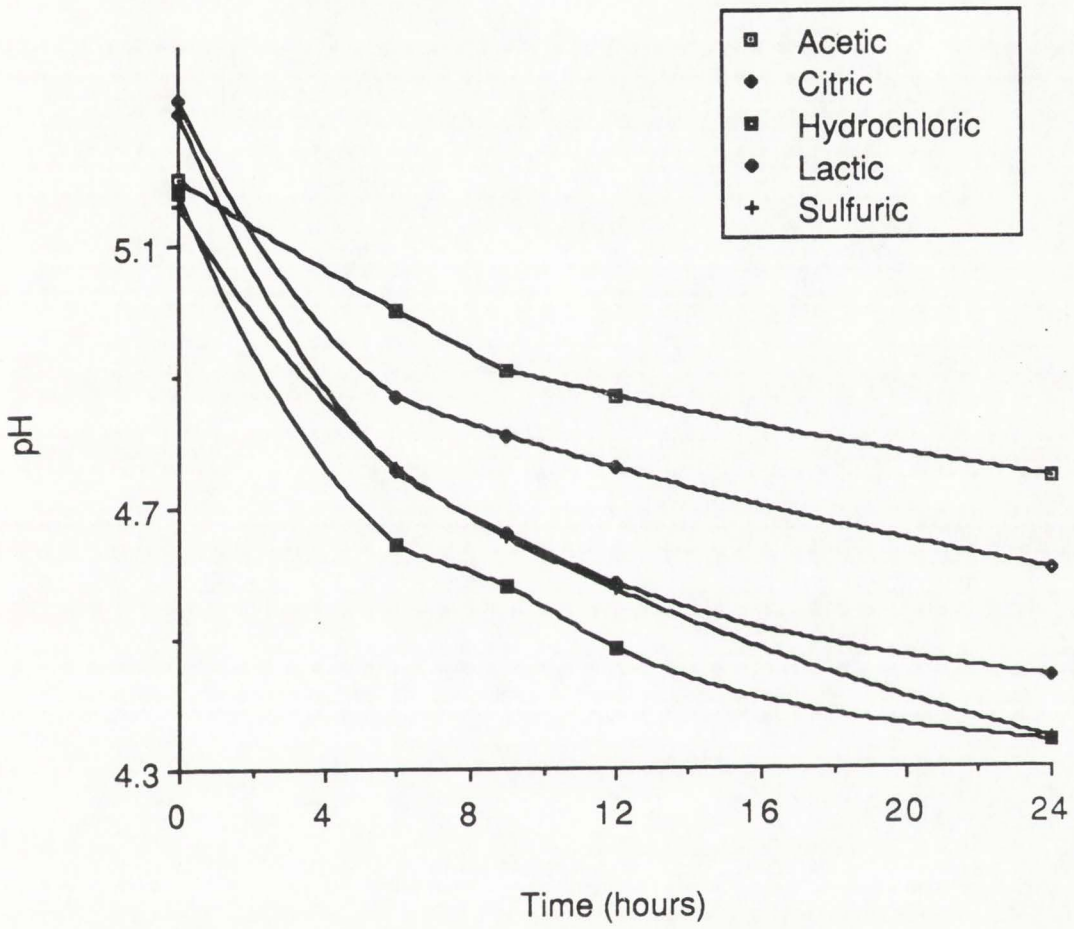

Figure 19. Effect of different acids added to 10% NDM on pH during fermentation with Prt- *S. cremoris.* Initial acid concentration in milk was 3 x 10^{-4} M.

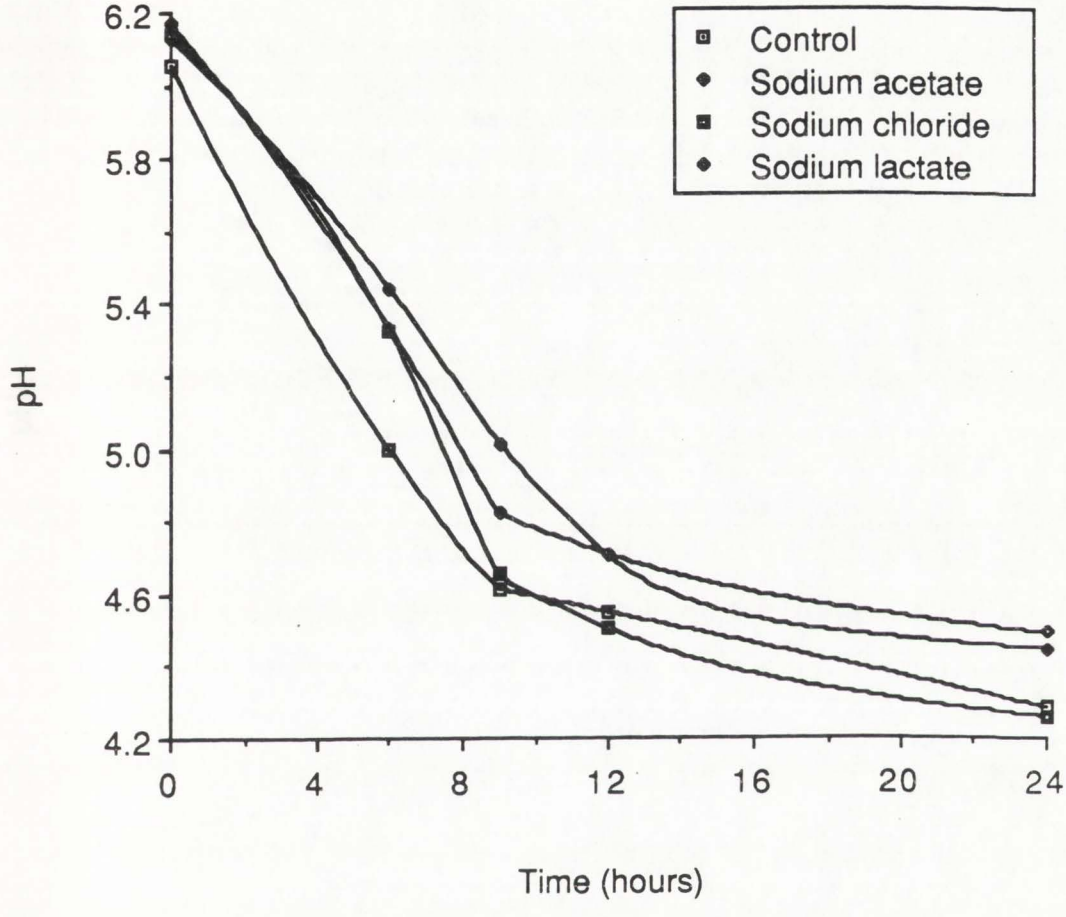

Figure 20. Effect of different salts added to 10% NDM on pH during fermentation with Prt+ *S. cremoris.* Initial salt concentration in milk was 3 x 10^{-4} M.

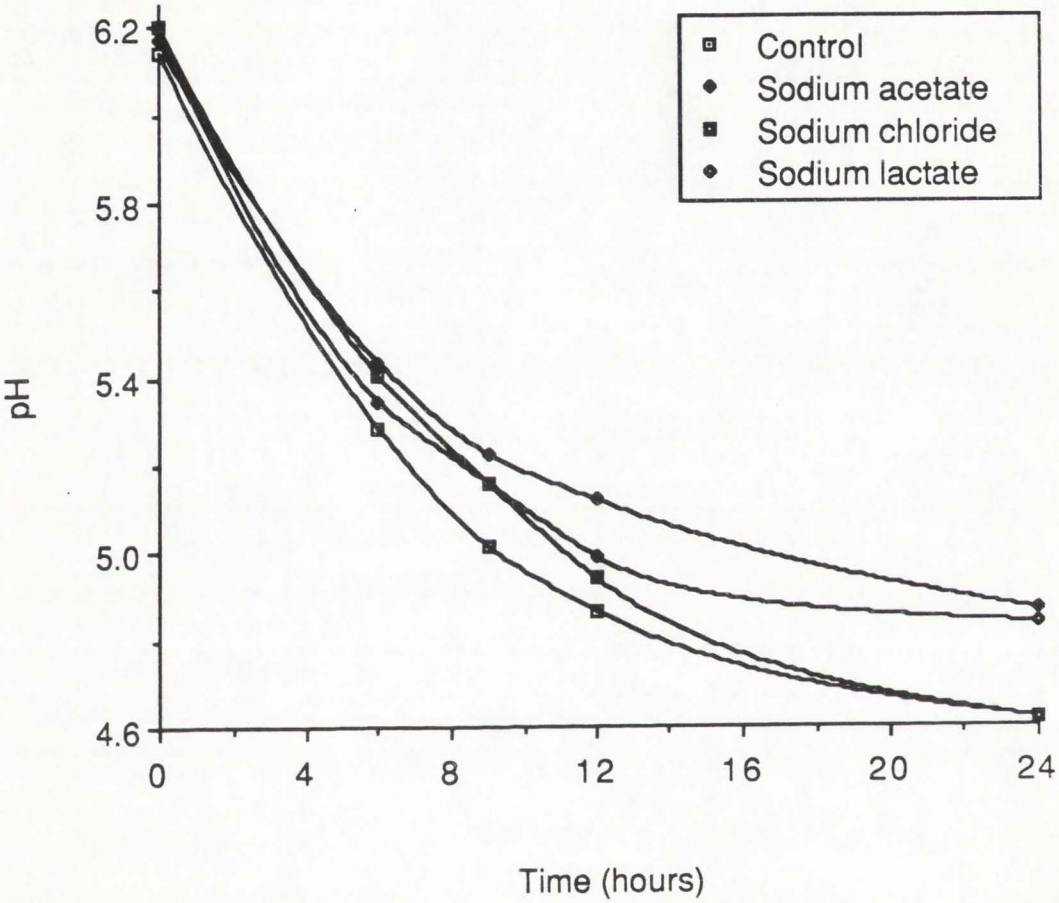

Figure 21. Effect of different salts added to 10% NDM on pH during fermentation with Prt- *S. cremoris*. Initial salt concentration in milk was 3 x 10^{-4} M.

TABLE 4. Effect of different acids and their conjugate salts on pH reduction of 5x retentates by Prt+ and Prt- *S. cremoris.*

Source	Sum of Squares	Deg. of Freedom	Mean Squares	F-ratio	Critical F α=0.05
Substrate	0.7331333	12	0.0610944	744.1462	2.425
Concentration	0.0000333	2	0.0000167	0.2034	3.634
Interaction	0.1639666	24	0.0068319	83.2144	2.235
Error	0.0032000	16	0.0000821		
Total	63.0598	39	1.6169		

A confidence interval was calculated (Table 5) indicating that the interval for a significance was 0.00414 pH units. Therefore, substrates that reached a pH above 4.289 were deemed inhibitory while substrates reaching a final pH below 4.281 were not inhibitory. Sodium lactate, acetic acid, sodium acetate and lactic acid were categorized as inhibitory to acid production by *S. cremoris* UC310.

TABLE 5. Confidence interval among final pH reached in test tubes of 10% NDM with added substrates (0.3 mM concentrations) that were cultured with Prt+ and Prt- *S. cremoris*.

Substrate	Means (pH)	Confidence Interval
Sodium Lactate	4.437	
Acetic Acid	4.417	MSE = .0000821
Sodium Acetate	4.397	
Lactic Acid	4.367	$df_{MSE} = 39$
Sodium Citrate	4.287	
Control	4.285	$t_{.025,39} = 2.021$
Sodium Sulfate	4.285	
Calcium Citrate	4.282	$CI = y \pm 2.021[2(.0000821)/39)]^{1/2}$
Citric Acid	4.277	
Sodium Chloride	4.273	$4.285 \pm .00414$
Calcium Chloride	4.245	
Hydrochloric Acid	4.222	
Sulfuric Acid	4.210	

CONCLUSIONS

1. Concentrated milk retentates have a very high buffer capacity and require higher than normal amounts of acid to reduce the pH to 5.1. This may be partially overcome by using five percent milk-base inoculum, or by using concentrated starter culture produced under pH control.

2. The addition of acid to milk prior to ultrafiltration (preacidification) reduces the demand for high acid production by cultures in cheesemaking.

3. Extracts of yeast provide a significant stimulus to proteinase-positive and negative variants of *Streptococcus cremoris* UC310. Time to reach pH 5.1 in 5x whole milk retentates can be reduced from 24 h to 10 h using Prt+ cultures and 0.2% (w/w) YE. The fermentation time can be reduced from 27 h to 11 h for Prt- cultures and 0.2% (w/w) YE. There was significant difference between brands of YE used, but both of those tested reduced fermentation time substantially. No flavor difference could be detected between retentates cultured with YE and without.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Fortifying retentates with yeast extract increased the rate of acid production by *Streptococcus cremoris* bacteria. Addition of yeast extract also lowered the final pH. Therefore, to control the final pH of the fermented retentates, further study is needed to determine the quantity of diafiltration necessary when yeast extracts are used in the media as stimulants.

2. The demand for acid from lactic cultures was reduced significantly by adding acid to milk prior to ultrafiltration. This demand could be further reduced in cottage cheese manufacture by adding acid directly to chilled retentates (0^{o}-4^{o} C) either prior to fermentation (refer to Figure 17) or by replacing fermentation with direct acidification. Research should be conducted to determine how much acid can be added to retentates and what effect the addition would have on the quality of the curd. By further lowering the demand for lactic acid produced by starter organisms, ultrafiltered retentates may show potential for continuous cottage cheese production.

3. It is known that temperatures above 25^{o} C cause the formation of a hard acid curd in 5x retentates. Methods to use higher temperatures (30^{o}-34^{o} C), which are optimal for lactic streptococci, while maintaining a liquid retentate should be studied to determine if fermentation times can be reduced further.

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