NUTRITIONAL FACTORS IN MILK AFFECTING GROWTH OF LACTIC ACID STREPTOCOCCI AND LACTOBACILLI

by

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INTRODUCTION

This thesis represents one of a series of studies on activity of the bacteria of starter cultures used in cheeses and fermented milks. Lack of acid production in cultured milk products represents a serious economic problem to the dairy industry. In order to properly control activity of starter cultures, it is necessary to understand fundamental factors which may influence acid production. Previous studies in the Department of Bacteriology at Oregon State College have demonstrated bacteriophage to be a primary cause of starter failure. However, numerous instances of retarded activity of starter cultures have been observed which appear to be reflections of nutritional conditions in the culture milk.

The primary purpose of these investigations, therefore, has been development of fundamental information on variation in growth promoting activity of certain milk constituents. Studies on variations in growth of starter culture bacteria in fresh milk and reconstituted non-fat milk solids were confined to the protein phase of the milk system. This approach was suggested by previous

work conducted in these laboratories. Also the complexity of the system dictated that the more obvious possibilities receive attention first. However, intact milk and chemically defined systems have been avoided wherever possible. While possessing some obvious advantages, these approaches are limited in that they are too complex to be properly studied or are too synthetic to be representative of the milk system. Wherever possible, purified milk constituents have been combined into simplified nutrient systems for study under specific isolated conditions. Such simplified nutrient systems have been of particular value in studies on influence of heat on the nutritional value of milk for the lactic acid bacteria.

As results of the first sections of the thesis indicated the importance of protein degradation products, an attempt was made to further study the nature of these materials. Therefore certain fundamental investigations into the structure, possible source, and relative stimulatory property of selected protein degradation products, were included in these studies.

HISTORICAL

Influence of variations in milk on activity of starter culture bacteria.

In recent years nutritional requirements of the lactic acid bacteria have received considerable study. This is due, in no small part, to the need by these organisms for many of the growth factors required by higher animals. The nutrition of such bacteria varies according to species and gross differences have been observed within strains of a single species. This variation is of practical significance and although the fastidious nature of the lactic acid bacteria provides a convenient tool for the study of metabolism and nutrition, it also results in certain difficulties when these organisms are employed in industrial lactic acid fermentations. In spite of the fact that milk is considered to be a highly nutritious food, growth of lactic organisms in this medium has been observed to vary with changes in the lactating animal or even in milk from different quarters of the same animal. For example, Baker and Hammer (7, pp.1-95) have reported considerable variation in acidities produced by a given starter growing in lots of milk from individual animals. Knudsen (50, pp.130-140) confirmed this observation and stated that one of two cows kept in the same stall and receiving identical

feed might produce excellent milk for starter culture while the adjacent cow might produce milk thoroughly unsatisfactory for these purposes. The problem therefore did not appear to be related to the immediate care of the cow, but rather it appeared to be related to the physiology of the specific animal concerned.

Kelly (42, pp.330-331) also reported that some cultures produced flat-yeasty flavors in milk from a certain herd while other starters did not. When inoculated into other milks, the formerly yeasty starter produced a satisfactory flavor. Prouty (68, pp.331-334) observed that mastitic milk with pH above 6.9 failed to support growth of starter and additions of as little as 10% of mastitic milk to normal milk inhibited normal development of lactic streptococci. Leitch (53, pp.534-538) found that cows suffering from mastitis excrete an inhibitory principle into the milk. Singh and Laxminarayana (78, p.85) also reported that mastitic milk reduced growth of lactic streptococci and suggested that an antibacterial agent is responsible for the inhibition. Davis and McClemont (19, pp.94-103) did not eliminate this possibility, but suggested that, in addition, certain growth factors may be deficient in milk from an abnormal udder. In a later paper, Davis (21, p.199) has reported that certain lactic streptococci are markedly inhibited by the addition of 5%

bovine serum to milk. As mastitic milk is known to be high in some serum components the inhibitory action may reside within such fractions.

Influence of heat on milk used as a medium.

Chambers (13, pp.531-538) noted a germicidal principle present in raw milk which is destroyed by heat. The germicidal action was reported to be specific, depending on the individual cow and the species of bacteria growing in the milk. Using Streptococcus lactis as a test organism, Sherman and Curran (77, p.17) found the antibacterial action of raw milk had its primary effect in prolonging the lag phase of early growth. The lag phase of S. lactis was observed to be one-half hour longer in raw milk than in identical pasteurized milk. Knudsen and Sorensen (49, p.88) reported that the effect of heating at low temperatures was due to destruction of bactericidal substances while the beneficial effect at higher termperatures was due to formation of stimulatory substances.

Jones and Sims (39, p.328) reported a six-hour lag in initiation of growth of mastitic streptococci in raw milk. The lag period in boiled milk was approximately one hour. The authors concluded that the effect was one of adaptation rather than the appearance of resistant forms. In a later report, these same authors (40, p.329)

established the name "lactenin" for the inhibitory principle. The factor was reported to destroy pyogenic streptococci and delay growth of mastitic bacteria. More recently Auclair and Hirsch (5, pp.45-59) and Auclair and Berridge (4, p.373) obtained high concentrations of factors identified as "lactenin 1" and "lactenin 2". Both lactenin fractions were reported to interfere with metabolic processes involving sulfhydryl groups. Heated milk and colostrum were found to possess properties which allowed S. agalactiae to overcome inhibition by lactenin. Sommer (85, p.541) suggested that the lack of inhibition in heated milk is due to the lowering of the oxidation-reduction potential to a point where prompt growth is possible. He considered that an Eh of minus 0.2 was satisfactory for the lactic streptococci of starter cultures. As sulfhydryl groups are known to lower oxidation-reduction potential, and as the inhibitory factors are reported to interfere with sulfhydryl group processes, it is possible that the work of Auclair, and the opinions of Sommer represent different expressions of the same fact.

Numerous other investigators have emphasized the importance of proper oxidation-reduction potential in initiation of bacterial growth. Dubos (24, p.566) has noted that a low oxidation-reduction potential is highly

important in initiation of growth of facultative anaerobes. Badger (6, pp.514-516) found that the reducing agent, ascorbic acid, would shorten the lag phase of Diplococcus pneumoniae. Elliker and Frazier (26, pp.822-824) found that the oxidation-reduction potential of reconstituted sterile skim milk became significantly elevated upon aging. Addition of thyoglycolic acid to aged milk stimulated growth of certain lactobacilli but failed to stimulate growth of S. lactis or Streptococcus thermophilus. The effect of addition of reducing substances to milk inoculated with lactic streptococci also was studied by Katznelson and Hood (41, pp.27-28). They observed that the addition of glutathione to milk stimulated subsequent activity of starter bacteria. The difference in starter activity in reduced milk as compared to milk to which no reducing agent had been added, was markedly increased when both milks were agitated. Cysteine produced stimulation only in agitated milks.

The oxidation-reduction potential of a medium also may have an influence on the requirements of organisms for certain growth factors. McNutt and Snell (56, pp. 565-566) using media not containing vitamin B_{12} have been able to obtain abundant growth of some organisms which normally require this vitamin by simply maintaining the medium in a reduced state through the addition of ascorbic

acid or glutathione. It was the opinion of these investigators that many organisms require the proper oxidation-reduction potential before vitamin B_{12}, or agents equally effective, can be synthesized.

Formations of stimulative factors by heating medium.

It seems well established, however, that heating of a given medium also produces factors which can not be attributed to poising of oxidation-reduction potential in a range favorable for growth. Orla-Jensen (59, pp. 374-379) was able to obtain increased growth only when certain constituents of the medium were heated with carbohydrates or products of carbohydrate metabolism. Heat activation involved such substances as glucose, methyl glyoxal or pentoses as one moity and yeast extract or protein as the other factor.

Davis (20, pp.188-194) was able to obtain better growth in a medium in which yeast, glucose and milk were heated together than in a similar medium in which these components were heat sterilized separately. Heating of milk also appears to produce certain factors not related to inhibitory substances. Hammer and Baker (34, p.9) in 1928 reported that milk heated to 71°C. for 30 minutes allowed more rapid acid production than did milk heated to 62.8°C. for the same length of time. They however

reported essentially the same rate of acid production in milk heated to 71°C. for 30 minutes as in milk heated to higher temperatures for this period. Bogdanoff (10, p. 682) reported increased growth with increases in heat treatment up to 15 minutes at 120°C. Davis (22, pp.215-217) obtained more rapid growth in milk heated to 82.2°C. by the flash method than in milk heated to 73.9°C. by the same process. Acid production in milk boiled for 24 hours was greater than in milk heated to 82.2°C. by the flash method. In general, however, it seems that there is a definite limit to the beneficial effect accruing from heating. Foster (27, pp.988-997) reported that autoclaving milk at 121°C. for periods of about 15 minutes resulted in more rapid growth of lactic streptococcus starter than heating at 80°C. for 10 minutes. Longer periods of heating than 15 minutes at 121°C., however, resulted in less rapid growth. Golding et al. (32, p.2) also reported diminished activity in milks heated to 100°C. or above for 30 minutes. Little information is available on reasons for decreased activity in grossly overheated milk. Singh and Laxminarayana (78, pp.82-85) postulated that the effect may be due to toxic products formed as a result of interaction of protein and lactose constituents. Foster (32, p.996) suggested also that heat destroys some essential growth substance present

in milk in limited quantities.

An interesting report by Snell et al. (81, pp.495-509) indicated that pyruvate will replace the need for heat activation of media used for cultivation of Lactobacillus bulgaricus. Rogers et al. (88, pp.140-144) were able to partially replace the need for heat activation of a basal medium for Lactobacillus gayoni through the addition of purified N-D glucosylglycine. It was considered that other factors might be involved and that glucosylglycine served as one of several intermediate forms. The stimulation of another organism by this growth factor has been reported in a subsequent paper (89, p.77). It was observed that only two cultures (L. acidophilus and L. gayoni) responded to glucosylglycine but at least ten species were inhibited by antagonists of glucosylglycine. This suggested to the authors that although glucosylglycine was required by only a few organisms, the metabolism of this substance or similar compounds may be common to a number of bacterial species.

Protein-sugar interactions in heated milk.

The interaction between reducing sugars and proteins was first characterized by Maillard (54, p.66; 55, p.258). More recently Lea and Hannan (52, pp.438-439) and Patton and Chism (63, p.167) suggested that there are definite

steps in the Milliard reaction which may influence the nutritive value of proteins heated in the presence of reducing sugars. Lea and Hannan found that as much as 7% of the final equilibrium concentration of sugar could be combined with protein without impairing solubility or color. On prolonged heating as much as 90% of the lysine, 70% of the arginine, 30% of the histidine, 50% of the methionine and 30% of the tyrosine present reacted with glucose in the medium. Acid hydrolysis liberated all of the methionine, one-third of the tyrosine and 70% of the lysine, but histidine and arginine were not recovered. The availability of combined sugar-amino compounds for biological purposes has been the subject of numerous investigations. Overby and Frost (61, pp.345-350) noted a significant decrease in rat repletion response when amino nitrogen decreased 6 to 8%. Microbiological assay of heat-browned solutions of dextrose and protein hydrolysate indicated loss of many individual amino acids. The greatest loss occurred with the amino acid, tryptophane. The amino acid composition as determined by microbiological assay did not give an accurate index of the nutritive value of the solutions for rat studies.

In a series of papers, Cook and co-workers (17, pp. 56-62; 18, pp.63-81; 16, pp.220-235) reported that heat used in the sterilization of evaporated milk and powdered

milk decreases the protein efficiency in proportion to the time and temperature of heating. It was observed that when the amino groups of casein were blocked by acetylation, browning could occur on subsequent heating. This was taken to mean that additional amino groups were made free in the heating process. Lactalbumin, however, did not have this property. These authors believed that heating brought about the formation of new cross linkages which were more resistant to biological attack than those present in native protein. It was found that heated mixtures of casein and lactose were more resistant to enzymatic hydrolysis than similar unheated systems. Kraft and Morgan (51, pp.567-581) report very drastic reductions in biological value of autoclaved skim milk. One sample which had been autoclaved for 25 minutes at 120°C. failed to support rat growth unless lysine was added to the diet. Schroeder et al. (73, pp.61-74) observed that all reducing sugars decrease nutritive values when heated with protein. A subsequent report (74, pp.553-560) noted that the rate of protein-sugar reaction was retarded in aqueous solutions. Schroeder et al. (75, pp.973-983) also reported that the browning reaction, per se. is not correlated with nutritional losses.

Although there are numerous areas of dispute concerning the various effects of heating, the literature

seems to be in general agreement that proteins heated for long periods of time at high temperatures in the presence of reducing sugar, lose some portion of their biological value. The loss seems especially pronounced in protein hydrolysate solutions. The decreased biological value has been attributed to inactivation of lysine, arginine, histidine and tryptophane. It must be emphasized, however, that studies with higher animals may not necessarily be applicable to studies in the microbiological field. Also studies with a given species of bacteria may not be an index of the nutritive value of the medium for other species.

Nitrogen metabolism of the lactic acid bacteria.

The first accurate characterization of nutritional requirements of the lactic streptococci was made by Niven (58, pp.343-350). The amino acids valine, isoleucine, methionine and aspartic acid were found essential for growth of S. lactis. In all, a total of 14 amino acids were necessary for complete development. Glutamine was also found to be essential for this organism. The report that S. lactis required glutamine confirmed the earlier observations of Pollack and Linder (66, pp.655-661) who found that glutamine plus asparagine were more than 25 times as effective as glutamic acid plus aspartic acid

in the nutrition of the lactic streptococci. It was noted that many lactobacilli required only glutamic acid. Collins et al. (15, pp.69-74) were unable to obtain growth of Streptococcus cremoris in the medium used by Niven, but found that the addition of sorbitan-monooleate-acetate or a crude product identified as reticulogen rendered the medium complete for S. cremoris.

Anderson and Elliker (1, pp.163-167; 2, pp.608-613) reported no effect when cystine, tryptophane, aspartic acid, and serine were omitted from an otherwise complete medium. Fourteen other amino acids were required for growth of one or more of the 35 strains of S. lactis and S. cremoris studied. It was noted that Wilson's liver fraction L and certain crude protein hydrolysates markedly stimulated growth of S. lactis and S. cremoris in synthetic medium and in milk.

Influence of protein hydrolysates on bacterial growth.

The preferential utilization of protein breakdown products in milk was first reported by Orla-Jensen (60, p.58). He observed that the addition of peptones from casein, markedly stimulated growth of starter in milk. In addition, it was indicated that growth of starter bacteria in milk might closely parallel the concentration of degraded protein. Eagles et al. (25, pp.364-369; 71, pp.

1534-1539) found that casein digests containing large concentrations of "sub-peptones" (peptides) were most effective in stimulating growth of milk cultures of lactic acid bacteria isolated from cheese. The importance of peptides and peptones to the lactic acid bacteria was investigated by Kluyver (47, pp.367-373), who stated that these substances were essential for the proliferation of butter starter organisms.

The molecular size of the most highly stimulatory peptide fractions remains in some dispute. Davis and Mattick (23, pp.83-89) stated that streptococci grow fastest in media containing the greatest amount of relatively long chain peptides. They also discussed the possibility of stimulation by S-H groups in certain peptides. Braz and Allen (11, pp.26-32) noted that yeast protein extract stimulates acid production by starter culture but does not necessarily result in an increase in cell numbers. They also noted that starter cultures showed decreased activity in normal milk after being cultured in milk supplemented with protein extracts. In the light of the later report, it must be considered that failure to find increased cell numbers might represent selection of more fastidious strains, which would not grow on the plating medium.

The first attempts to concentrate the stimulatory

factors found in crude protein digests were made by Smith (80, pp.369-371). The factor was not precipitable by lead, silver or zinc salts and was not adsorbed on charcoal or Fuller's earth. It was found to be water soluble and fat insoluble.

The importance of peptide-like factors for growth of starter in milk was also investigated by Stoors and Anderson (87, pp.605-611). Alcoholic extracts of fish protein hydrolysates were found to double growth of some strains of S. lactis. Slower growing strains were stimulated more than those producing acid at a rapid rate. The relationship of a growth factor, first observed to be required by the hemolytic streptococci, to growth phenomena in other streptococci was reported by Sprince and Woolley in 1944 (83, pp.213-217). The active factor was non-dializable, and could be partially purified by a series of solvent extractions. The term "strepogenin" was used to denote the growth-promoting factor found in protein hydrolysates. In the same year, Skeggs and Wright (79, pp.117-118) reported that trypsinized vitamin-free casein replaced the requirement of S. lactis for glutamine and asparagine. These investigators were of the opinion that glutamine is involved in the synthesis of factors contained in protein digests.

The relative growth-promoting activity of certain

protein hydrolysates has been studied in some detail. With the activity of Wilson's liver fraction "L" given an arbitrary rating of one, trypsin digests of casein were reported by Sprince and Woolley (84, p.1735) to have an activity of six and trypsin digests of insulin an activity of 40. Kodieck and Mistry (48, pp.109-116) found that casein hydrolysates were relatively inactive for Lactobacillus casei. Wright et al. (92, pp.687-695), on the other hand, reported that casein hydrolysates are very effective in stimulating growth of Lactobacillus bulgaricus. Scott et al. (76, pp.481-488) noted that trypsinized lactalbumen and trypsinized casein possessed "strepogenin" activity. A large portion of the activity of casein digest, however, was derived from trypsin used in the hydrolysis. Kennedy (43, p.337) found that enzymatically hydrolyzed lactalbumen stimulated growth of L. casei in a medium containing "strepogenin". Kizer et al. (46, pp.303-309) also reported that tryptic hydrolysates of lactalbumens possess growth stimulatory properties for certain lactic acid bacteria when added to milk, and to a nutritionally complete semi-synthetic medium. A major portion of the stimulation was shown to originate from impurities concomitant to the enzyme rather than protein hydrolysate per se.

Mechanism of stimulation by peptides.

Sprince and Woolley (84, pp.1734-1736) were of the opinion that growth promoting properties of "strepogenin" resided within some specific residue of protein hydrolysis. Following treatment with dinitroflurobenzene, insulin no longer possessed growth promoting properties. This was taken to be an indication that some of the free amino groups of insulin were present in an amino acid which was a component of the active factor. It was known that the free amino groups of insulin are due solely to glycine, phenylalanine and lysine. Glycine was considered to be the essential amino acid. Tests of synthetic peptides containing glycine were found to result in the following decreasing order of activity: serylglycylglutamic acid, glutamlytyrosylglutamic acid, glycylserylglutamic acid, glycylalanylglutamic acid and glycylglutamic acid. In the light of information developed in this thesis it is also important to consider that glutamic acid was present in each of the above peptides. The activity of the synthetic peptides was reported to be heat stable.

The mechanism of peptide utilization by lactic acid bacteria has been extensively investigated by Snell and co-workers. Kihara and Snell (45, pp.791-800) showed that peptides of l-alanine surpassed free l-alanine in

ability to support L. casei in the absence of vitamin B-6. The enhanced activity of the peptide was attributed to inhibition of utilization of the free amino acid, but not the peptide, by an antagonistic amino acid present in the medium. A second mechanism was reported by Kihara, Klatt and Snell (44, pp.801-807) and Gale (30, pp.227-229). It was shown that free amino acids may be decarboxylated by enzymes of the organism while a peptide-bound amino acid is not so affected. Peters and Snell (64, pp.521-531) were able to duplicate the growth-promoting properties of casein hydrolysates for Lactobacillus delbrueckii by the addition of large amounts of free histidine. Histidine peptides were much more active than the free amino acid. A third mechanism is thus observed in that the organism had the ability to concentrate histidine peptides from a dilute medium, but not free histidine. The first mechanism has also been observed for peptides of serine (67, pp.533-540). In this instance serine peptides were 15 to 90 times as effective as free serine in overcoming the antagonistic effects of alanine. This indicates that alanine prevents utilization of the structurally related amino acid serine. Most serine peptides, however, are not subject to this antagonistic effect and hence specific peptide structure containing the serine moiety is not required. The fact that

various mechanisms are responsible for increased growth in the presence of peptides or partial hydrolysates of protein casts serious doubt on the advisibility of ascribing such stimulation to a specific peptide or a specific group of peptides.

The transport and utilization of amino acids involve a series of exceedingly complex mechanisms. Gale (29, pp.53-76) showed that glutamic acid, glutamine, and histidine are unable to pass across the cell wall unless energy is supplied by some exergonic mechanism such as the simultaneous fermention of glucose. The passage of lysine has been reported to involve at least two factors: (1) the phosphate ion and (2) certain other amino acids (57, pp.81-84). The presence of phosphate favors the accumulation of lysine by streptococci growing in an acidic medium. Uptake of free lysine is reduced by the presence of arginine, histidine, glutamic acid and aspartic acid. Mixtures of neutral amino acids have no effect on the uptake of lysine or its subsequent migration out of the cell. Gale and Van Halteren (31, pp.34-36) showed that the formation of peptides by extracellular amino acids depends to some extent on the concentration of free amino acids in the medium. They observed an accumulation of free glutamic acid within cells of Staphylococcus aureus which occurred when cells were incubated with

glutamic acid and glucose. The accumulation was inhibited if aspartic acid, cysteine, alanine or glycine were present. The decrease in rate of accumulation of intemal glutamic acid was accompanied by the formation of combined glutamate in the medium. Chromatographic analyses were used to show the formation of peptides containing glutamic acid and the inhibitory amino acids. The presence of glucose was essential for the formation of the extracellular peptides.

Hydrolysis of milk protein by bacteria of starter cultures.

Although many peptides are quite stimulatory, they may be hydrolyzed before incorporation into the cell. Stone (86, pp.821-827) demonstrated that numerous peptides are hydrolyzed apparently on some site outside the cell wall. Growth was inhibited until peptide hydrolysis occurred. The presence of manganese ion and a sulfhydryl compound was required for proline peptide hydrolysis.

The utilization and hydrolysis of the more complex proteins of milk were of considerable interest to a number of early workers in the field of microbial nutrition. Sperry and Rettger (82, pp.445-459) and Berman and Rettger (9, pp.367-388) believed that bacteria were unable to decompose protein without the aid of a hydrolyzing agent. Hucker (38, pp.1-16) also stated that chemically

pure casein was not available to washed cells of S. lactis. By using a more complete basal medium, Peterson, Pruess, and Fred (65, pp.161-177) were able to demonstrate that a strain of S. lactis could carry out proteolysis of sodium caseinate. Frazier and Rupp (66, pp.655-661) did not consider S. lactis to be proteolytic because of the length of time required to show a significant increase in amino nitrogen in milk. Numerous reports since that time, however, have shown that strains of S. lactis carry out protein hydrolysis at varying rates. Barthal and Sadler (8, pp.654-656) noted that cultures of lactic acid bacteria of the Streptococcus genus often produce the same amount of soluble nitrogen from casein as mixed starters. The amount of amino nitrogen produced by the pure culture, however, was far less than the amount of amino nitrogen produced by the mixed microflora of butter starter. Harriman and Hammer (36, pp.40-49) and Collins and Nelson (14, pp.652-658) reported increases in soluble nitrogen after 24 hours growth. The most active increase occurred during the first three days of growth with a somewhat slower increase after this period through the fourteenth day. Hydrolysis was increased by buffering the medium with calcium carbonate.

SECTION I

VARIATION IN GROWTH-PROMOTING PROPERTIES OF FRESH WHOLE MILKS

Previous investigations by Anderson and Elliker (1, pp.161-167; 2, pp.608-613) demonstrated marked stimulation of lactic streptococci in synthetic media and milk after addition of liver fraction L and enzymatic digests of milk. Since liver L is considered an abundant source of peptides and similar compounds are liberated by enzymatic hydrolysis of milk proteins, the results suggested stimulation by peptides. It also was considered possible that a similar factor might be involved in the variation in rate of acid production frequently observed for lactic streptococcus starter cultures in different individual herd or cow samples of milk. This section, therefore, represents at attempt to correlate rate of activity of lactic streptococci with concentration in such milks of protein degradation products in the form of peptides, peptones, and proteoses. For the sake of brevity and to avoid misunderstanding in nomenclature in the following descriptions and discussion, the term peptides will be used to designate all the protein fragments occurring between amino acids and the complete proteins (caseins, albumins, and globulins).

Experimental

A simple experiment was carried out to demonstrate that the peptide fraction in milk from different cows may cause marked variation in the growth of mixed strain lactic starter cultures and single strains isolated from them. In the study, rate of growth was correlated with quantity of peptide in individual cow samples. As preliminary studies had indicated erratic results with abnormal milks, only normal milks were included in this experiment. Abnormal milks were considered those from animals with mastitis or animals on special rations, or in early or late stages of lactation (less than 30 or more than 300 days).

Pint quantities of representative samples were obtained from Holstein and Jersey cows in various stages of lactation. The milk from each animal was divided into two portions and immediately refrigerated. In order to minimize any microbiological or enzymatic action in the milk, it was chilled, skimmed, and tested as soon as possible. Five ml. quantities of the skimmed milk from each animal were tubed in triplicate and pasteurized by steaming for 10 minutes. This milk was used for rate of growth tests of starter culture or individual strains.

The method recommended by Shahani and Sommers (76a, pp.1003-1008) for the determination of proteose peptone

minus the nonprotein nitrogen was used to obtain peptide content of raw milks. This method made it possible to correlate rate of growth with a defined fraction of the milk and eliminated the error due to presence of fractions such as nonprotein nitrogen.

Cultures employed for determining growth-promoting properties of the various milk samples included representative mixed strain commercial lactic streptococcus starters commonly used in this country for buttermilk, cottage cheese, and Cheddar cheese manufacture and single strains of Streptococcus lactis (SLE) and S. cremoris (144F and H-6). Activity of the cultures was maintained by daily transfer in reconstituted skimmilk and incubation at 21.5°C. Active 18-hour cultures were inoculated into experimental milks at the rate of 1%. The results are presented as per cent lactic acid calculated from the actual acidities produced by the organisms in a 5-hour incubation period at 30°C. At the end of the incubation period they were quickly frozen to prevent further growth and subsequently thawed and titratable acidities were determined. The quantity of acid produced was used as an index of growth. This was correlated with peptide nitrogen level and results were plotted.

Figures 1 and 2 present correlation between peptide nitrogen in milk and acid production of two mixed

commercial cultures (B and C), and Figures 3, 4 and 5 show results obtained with three individual strains of lactic streptococci used in starters. Results indicate that normal milk from healthy cows varies in its growth-promoting property for the lactic streptococci according to its peptide content. There is a significant correlation for each of the cultures tested since the correlation coefficient r is significantly greater than 0.5 at the 1% level for peptides.

Discussion

Previous studies by Anderson and Elliker (1, pp.161-167) indicated that not all commercial cultures or individual strains react similarly to peptide stimulation. However, all that were investigated in this study showed a positive response when cultured in an otherwise nutritionally complete medium. If, in the normal milk, the response to peptide was positive but no correlation existed, the cause usually could be traced to some individual strain nutritional requirement.

Additional data not shown in Figure 1 to 5 indicate that high protein content in milk does not necessarily favor more rapid acid production by starter organisms. In general, starter cultures and individual strains grew equally well in high and low protein milk. Two animals

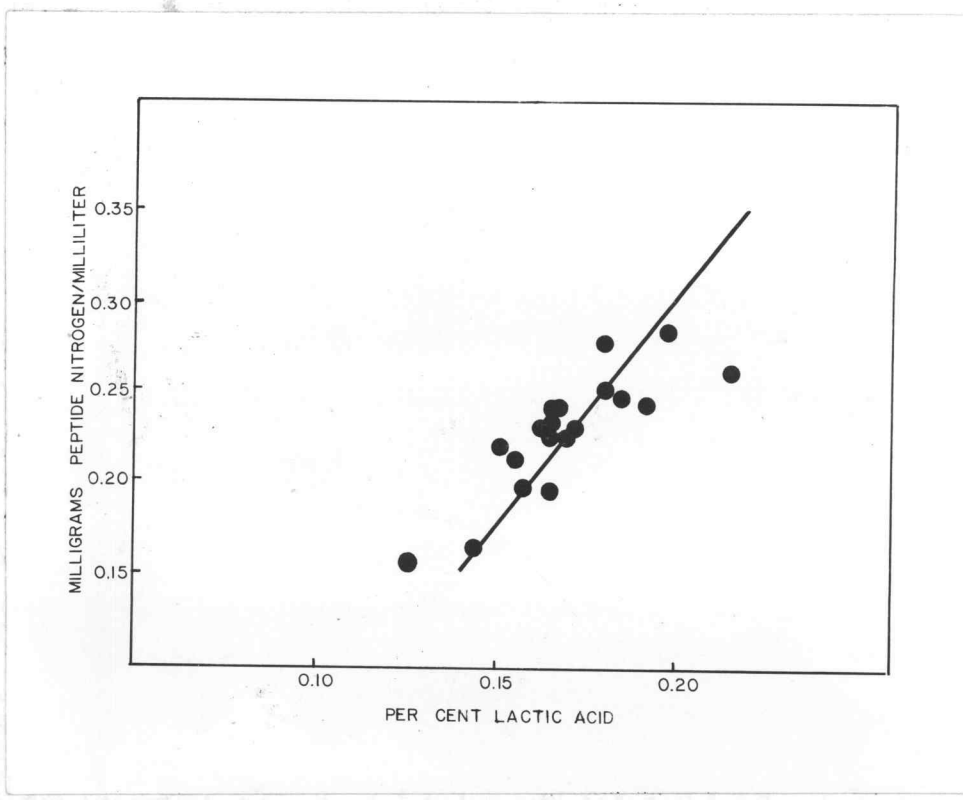

Figure 1. Relation between peptide nitrogen and acid produced by commercially available mixed strain lactic streptococcus starter culture B in individual cow samples of milk. Cultures were incubated at 30°C. for 5 hours. Regression equation is $y = 0.0824 + 0.382x$ where y is peptide nitrogen in mg/ml and x is per cent lactic acid.

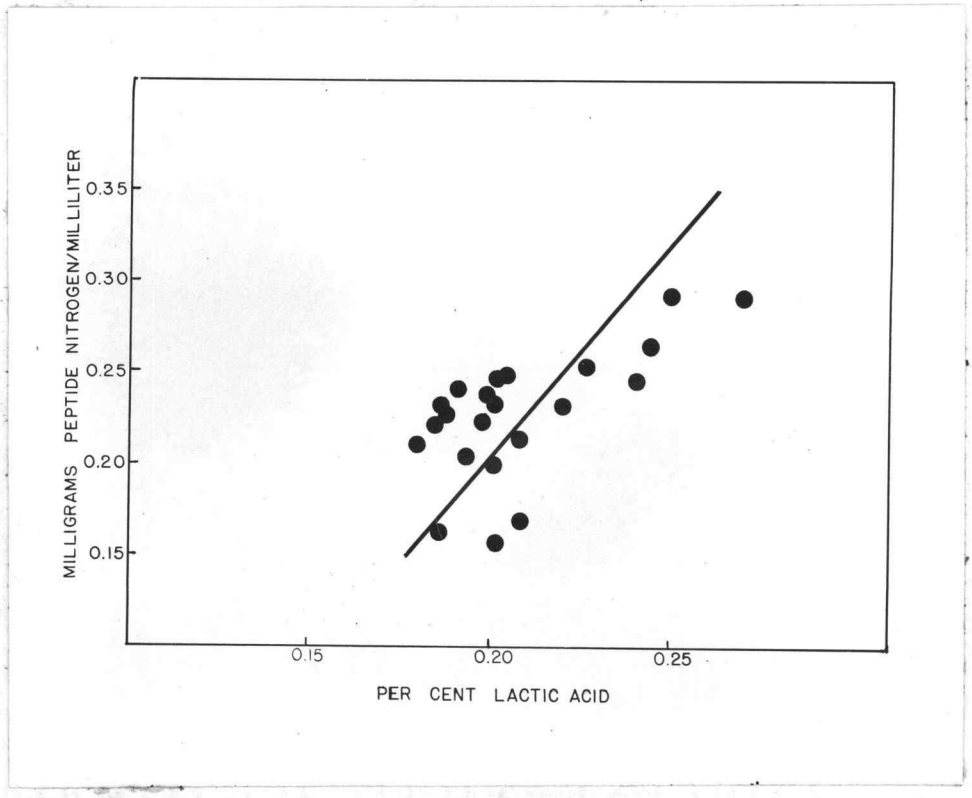

Figure 2. Relation between peptide nitrogen and acid produced by commercially available mixed strain lactic streptococcus starter culture C in individual cow samples of milk. Cultures were incubated at 30°C. for 5 hours. Regression equation is $y = 0.112 + 0.424x$ where y is peptide nitrogen in mg/ml and x is per cent lactic acid.

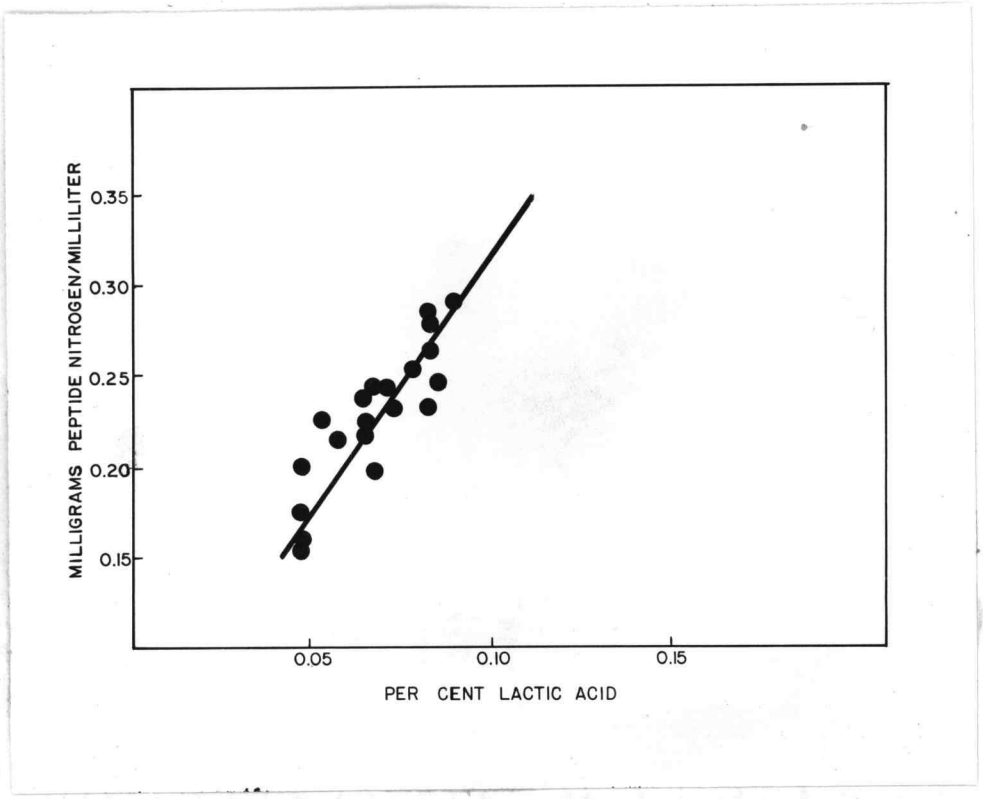

Figure 3. Relation between peptide nitrogen and acid produced by a single strain lactic streptococcus starter culture SLE in individual cow samples of milk. Cultures were incubated at 30°C. for 5 hours. Regression equation is $y = -0.00258 + 0.306x$ where y is peptide nitrogen in mg/ml and x is per cent lactic acid.

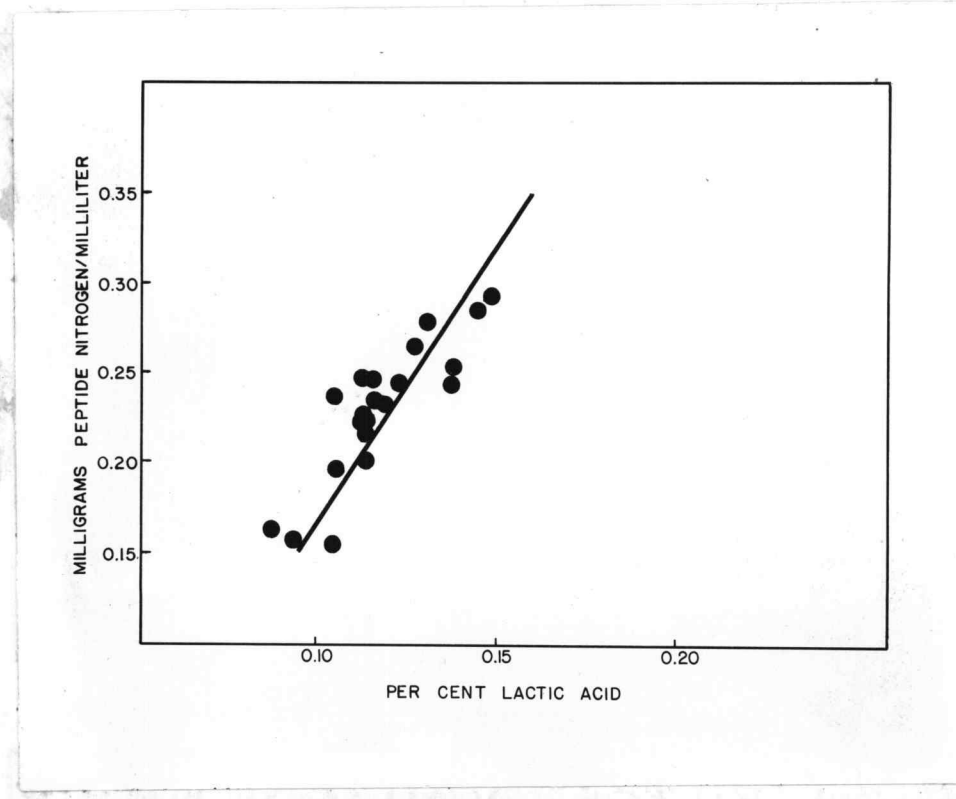

Figure 4. Relation between peptide nitrogen and acid produced by a single strain lactic streptococcus starter culture 144F in individual cow samples of milk. Cultures were incubated at 30°C. for 5 hours. Regression equation is $y = 0.0432 + 0.322x$ where y is peptide nitrogen in mg/ml and x is per cent lactic acid.

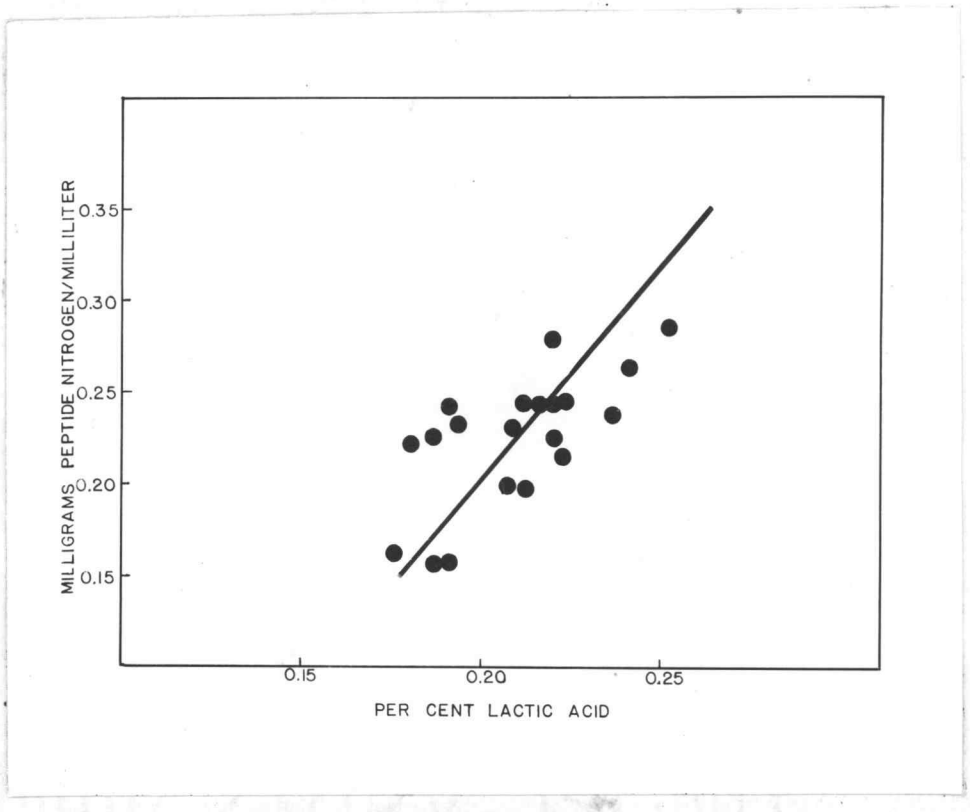

Figure 5. Relation between peptide nitrogen and acid produced by a single strain lactic streptococcus starter culture H6 in individual cow samples of milk. Cultures were incubated at 30°C. for 5 hours. Regression equation is $y = 0.121 + 0.389x$ where y is peptide nitrogen in mg/ml and x is per cent lactic acid.

on a ration deficient in certain essential nutrients (in this case, low carotene) produced milk which supported poor growth, although the protein as indicated by total nitrogen was normal. Other data also indicated no correlation between rate of acid production of lactic streptococci tested and content of nonprotein nitrogen in various samples of milk from individual cows.

The growth response of mixed commercial starter cultures to peptide variations was comparable to that of the individual strains. Some single and one mixed commercial culture did not show a direct correlation, although the response to peptide nitrogen was positive in almost all instances. A possible explanation for this observation may be found in previous studies on the nutrition of the individual strains of lactic streptococci (1, pp.161-167), in which some cultures were shown to require certain purine and pyrimidine bases for optimum growth. It is possible that milk may vary in these constituents. Other studies by Anderson and Elliker (2, pp.608-613) demonstrated that milk supplemented with adenine, guanine, or uracil accelerated the growth of the strains which previously had been shown to require one or more of the purine and pyrimidine bases. One fastgrowing mixed-strain commercial starter did not respond as markedly as the others. It is possible that the strains used in the

mixed culture in the present investigation initiate growth faster on less of the peptide fraction so that this does not become a limiting factor for growth in normal milk.

There may be variations in other substances in milk besides the peptides which influence growth of lactic streptococci. Milk sampled early in the lactation period and milk from some cows producing a high volume provided slower growth of cultures than normal milk from the middle stages of lactation. This might be due to some inhibitory substance in milk from cows in early lactation. In the milk from abnormally high-producing animals there may actually be a suboptimal level of certain metabolites in the milk which are required for fast starter culture growth.

There was no significant difference between Jersey and Holstein milk in the amount of acid produced by starter cultures when the initial acidities were taken into consideration. Therefore, the amount of protein appears to have no appreciable influence on the rapidity of growth. Additional evidence on the influence of total protein activity of starter culture is provided in Section VI of this thesis. Results of Sextion VI indicate that the lactic streptococci of starter cultures utilize intact native milk protein poorly if at all.

The variation in the peptide nitrogen appeared to be a characteristic of either the individual animal or the individual milking. There was no evidence that it was related to either of the breeds tested. The milk samples used were tested as soon as possible after being drawn to reduce natural enzymatic activity to a minimum. The presence of a natural proteolytic enzyme might conceivably increase the peptide content if the milk were stored. However, Shahani and Sommers (76a, pp.1003-1008) have shown such increases to be negligible over a 10-day period when stored at temperatures of 0° to 5°C.

SECTION II

VARIATION IN GROWTH OF LACTIC STREPTOCOCCI IN RECONSTITUTED SPRAY PROCESS NON-FAT MILK SOLIDS

The heat treatment of spray-dried skim milk varies according to the use for which the milk is intended. Dry milk destined for use by the baking industry customarily receives a pre-heat treatment at temperatures near 190°F. for periods of 30 to 45 minutes. Milk destined for reconstitution purposes, however, received pre-heat treatment of only 160°F. for 10 to 15 minutes or less. The above dry milks customarily are referred to as "high heat" and "low heat" powders, respectively. Horrall and Elliker demonstrated the advantages of using reconstituted spray dried non-fat milk solids as a medium for starter culture (37, pp.250-256). It is known, however, that not all such milks have equal ability to support growth of starter culture. The primary purposes of this section were to investigate the effects of heat treatment, season of production, and state of soluble nitrogen in such milks on activity of lactic streptococcus starter cultures.

Experimental

Source of Milks Tested. Spray process non-fat milk solids were obtained from a number of commercial

processing plants in the states of Wisconsin, Ohio, California, Washington, Idaho and Oregon. Sealed five-pound samples of low heat powder were obtained from a Caldwell, Idaho, drying plant at the rate of one each month for a total of 19 months. After receipt the sealed cans of milk were refrigerated at 39°F. and held in the original sealed cans until required for use in the study.

Analytical Methods. Trichloracetic acid soluble nitrogen (TCAN) was determined by adding 20 ml. of 10% total solids reconstituted skim milk to 30 ml. of 15% TCA. The mixture was allowed to stand for two hours at 4°C. and then was filtered through Whatman No. 40 filter paper. Total nitrogen in the filtrate was determined by micro-Kjeldahl procedure. Free amino nitrogen was estimated by formaldehyde titration according to the method developed by Pyne (70, pp.1006-1014). Starter activity in the various milks was determined by incubating the culture at 30°C. for 5 hours and titrating the acid developed in this period. Titration was carried out with N/20 sodium hydroxide to an electrometrically determined endpoint of pH 8.3.

Cultures Used. Strains of S. cremoris used included the following: 306, 11E, R6, 9596. Three strains of S. lactis used included: IS, SLE and 7963. Three commercial

mixed butter starter cultures were obtained and identified as H, F, and M. Strains 7963 and 9595 were obtained from the American Type Culture collection. Strains 306 and 11E were originally isolated and identified by Dr. A. W. Anderson. Strain IS was obtained from Dr. F. E. Nelson of Iowa State College. Strains R6 and SLE were obtained from the stock culture collection of the Oregon State College Department of Bacteriology.

Variation in Starter Culture Growth in Low Heat Reconstituted Non-Fat Milk Solids. After all milks used in the study were received, they were opened and an aliquot reconstituted to 10% total solids. Five ml. quantities were dispensed into tubes and autoclaved at 121°C. for 5 minutes. As quickly as the tubes could be cooled to 30°C., they were inoculated with approximately 0.05 ml. of the respective starter. The tubes were incubated in a water bath at 30°C. for 5 hours. Immediately after incubation, the tubes were quickly frozen in an isopropyl alcohol bath at -20°C. This procedure instantly stopped acid production and the tubes could be removed, warmed to 30°C. and titrated at the workers' convenience. Results expressed in Table 1 show the relationship between activity of starter and season of production, TCAN, and amino nitrogen of the milk. Inspection of Table 1

TABLE 1

Influence of Variation in Season of Production and Chemical Composition of Low Heat Spray Process Non-Fat Milk Solids on Activity of Starter Culture

Producing Firm	Location	Production Date	TCA Sol N	Amino N	Activity of Strain Number									
					R6	IS	306	9596	7963	IIE	SLE	H	F	M
Kraft	Wisc.	12-51	491	80	26[1]	44	36	22	28	43	47	--	--	--
"	"	3-52	532	86	25	42	28	17	21	37	40	--	--	--
"	"	6-52	525	84	23	43	27	17	19	41	44	--	--	--
"	"	9-52	580	84	23	40	28	24	25	39	45	--	--	--
Golden State	Calif.	Unknown	623	88	24	40	24	21	23	40	46	--	--	--
"	"	"	547	91	18	36	31	20	16	31	39	44	39	44
"	"	"	520	87	17	41	27	16	20	42	37	44	43	42
Prairie Farms	Ill.	5-53	542	82	14	34	33	23	18	42	39	39	39	42
Lady Smith	Wisc.	5-53	475	79	28	39	26	24	19	35	45	40	40	39
Borden	Unknown	Unknown	523	84	22	34	26	18	19	35	38	43	46	45
Flambeau	Minn.	4-53	520	88	19	41	28	18	20	40	33	35	39	42
Darigold	Oregon	4-53	571	87	21	38	31	20	28	31	34	35	44	36
Darigold	Idaho	11-51	505	83	17	42	--	12	16	--	44	39	37	34
"	"	12-51	542	85	22	37	--	16	20	--	39	37	37	41
"	"	1-52	613	88	19	41	--	20	21	--	41	37	40	39
"	"	2-52	537	89	17	44	--	17	20	40	38	40	39	36
"	"	3-52	573	84	18	44	--	13	21	46	40	39	42	33
"	"	4-52	595	91	22	38	--	16	22	41	37	40	37	39
"	"	5-52	541	85	17	41	--	12	26	44	39	37	36	36
"	"	6-52	524	83	16	33	--	18	19	43	36	43	40	37
"	"	7-52	620	87	24	41	--	21	19	46	35	36	44	40

(continued on next page)

Table 1, continued

Producing Firm	Location	Production Date	TCA Sol N	Amino N	Activity of Strain Number									
					R6	IS	306	9596	7963	11E	SLE	H	F	M
Darigold	Idaho	8-52	580	91	18	41	--	19	26	40	36	40	41	36
"	"	9-52	528	80	16	34	--	16	21	39	40	39	43	41
"	"	10-52	507	82	21	39	--	22	16	35	39	42	40	44
"	"	11-52	544	86	20	33	--	20	16	37	36	39	42	33
"	"	12-52	503	79	17	40	--	18	21	41	38	36	38	46
"	"	1-53	585	82	22	37	--	12	23	42	37	40	40	34
"	"	2-53	573	88	24	41	--	17	18	37	35	38	37	40
"	"	3-53	475	78	21	36	--	21	16	35	36	35	35	34

[1] Activity measured as counts of semi-automatic titrimeter needed to titrate to pH 8.3 5 ml. culture. All data minus blank. 100 counts = 4 ml. N/10 NaOH. Culture incubated at 30°C. for 5 hours.

indicates no apparent correlation between nitrogen state or season of production and activity of starter culture in milk. Sample plots have indicated the relationship to be exceedingly random. It also was noted that cultures were not uniform in their response to a given milk. For example, strains 7963 and SLE were of almost equal activity in milks 6 and 8 while strain 11E showed active development only in milk 8. Also acid production by strain 9596 was almost twice as great in milk 21 as in milk 10, although growth of strain IS was greater in milk 10. Likewise there appeared to be no apparent correlation between season of production and activity of starter culture in the milk. The most significant differences observed appeared to be between the various sources of milk. As processing procedures were not available, it was not possible to determine if the differences were due to variation in manufacturing treatment or to variations in the producing herds.

Effect of High and Low Forewarming Temperatures on Subsequent Activity of Starter Culture. "High" and "Low" heat dry skim milks were obtained from three commercial sources. In two instances it was possible to obtain low heat and high heat skim milk solids manufactured from the same lot of fresh skim milk. In another instance, high

and low heat samples were obtained from the same plant on consecutive days although no attempt was made to use identical milk for both processing procedures. Each milk was reconstituted to 10% total solids and divided into two equal portions. One portion was heated in an autoclave for 15 minutes at 121°C. and the other was pasteurized by heating to 68°C. for 10 minutes. After heating, 5 ml. quantities of each milk were dispensed aseptically into sterile tubes. Inoculation, incubation and titration of acid were carried out as outlined previously. Although certain exceptions may be observed, it would appear that reconstituted low heat milk is a better medium for starter culture when autoclaved than when pasteurized at 68°C. for 10 minutes (Table 2). This is in agreement with numerous reports of beneficial action of heat on raw fluid milk. Milk produced with high forewarming temperatures was found to permit more active growth of starter culture than low heat milk when both milks received identical pasteurization treatment. This effect was especially noticeable with the more actively growing cultures. For example, culture IS grown in the three high heat milks studied, produced activities of 40 - 44 and 35 respectively but activities of only 35 - 37 - 30 respectively when grown in low heat milk. Similarly commercial culture F produced activities of 40, 56, and 49 for an

TABLE 2

Influence of Pre-Heat Treatment and Subsequent Heat Processes of Reconstituted Spray Process Non-Fat Milk Solids on Activity of Starter Cultures

Starter Strain	Milk Source											
	Washington				Oregon				California			
	Pasteurized		Autoclaved		Pasteurized		Autoclaved		Pasteurized		Autoclaved	
	Hi[1]	Low[2]	Hi	Low	Hi	Low	Hi	Low	Hi	Low	Hi	Low
11E	5[3]	5	18	14	8	12	24	26	9	4	12	18
306	14	14	15	16	4	4	10	16	15	12	12	17
IS	40	35	33	46	44	37	40	42	35	30	38	47
SLE	23	15	30	31	2	3	18	20	25	16	31	34
F	40	36	36	42	56	39	39	45	49	39	41	51
M	13	16	25	25	10	10	18	14	18	16	24	27
H	36	30	34	35	40	39	14	28	27	24	31	42

1 Hi = High heat baker's grade non-fat milk solids.

2 Low = Low heat cottage cheese type non-fat milk solids.

3 Activity measured as counts of semi-automatic titrimeter required to titrate 5 ml. culture to pH 8.3. All data minus blank. 100 counts = 4 ml. N/10 NaOH.

average activity of 48 micro-moles per tube in pasteurized high-heat milk while the identical culture growing in pasteurized low-heat milk produced activities of 36 , 36 , and 39 for an average activity of 38 micro-moles per tube. Heating the reconstituted milk to autoclave temperatures, however, frequently reversed relative activities of starter culture growing in high- and low-heat reconstituted milk. In almost all instances autoclaving of reconstituted low-heat milk resulted in more active subsequent growth of starter culture. However, autoclaving of reconstituted high-heat milk frequently resulted in less activity than was observed in similar milk which had received only pasteurization treatment. Organisms which were most affected by additional strong heating of high-heat milk included strains IS, F, and H.

Discussion

Observations that the strains of starter bacteria used do not show uniform response to a given milk, indicate that the growth response of a given organism in reconstituted skim milk may be dependent on the individual nutritional requirements of the strain of organism involved as well as the condition of the medium. In general, the multiple strain cultures were more uniform in growth response than single strain cultures. It might

be assumed, therefore, that interaction of a number of strains growing together in milk would tend to level out the irregularities in response which could occur with each strain growing separately. Results of subsequent sections which show large variations in response of single strain cultures to added growth factors would tend to strengthen this supposition.

Results in Table 2 indicate that the effect of heat on milk is additive in nature. It was observed that milks reconstituted from low-heat non-fat milk solids were markedly improved as a medium by heating to autoclave temperatures. High-heat milks, however, were not improved by additional strong heat. In some instances high-heat milk supported less growth after being autoclaved. Although moderate heat treatment was observed to result in stimulation, and excessive heat in inhibition, no indication was given of the nature of inhibitory and stimulatory substances. As high-heat reconstituted skim milk is unsuitable for cottage cheese and cultured buttermilk but supports greater activity than low-heat reconstituted skim milk, isolation or identification of the heat stimulatory factors may be of considerable economic significance. The extreme variability in organism response, however, suggests that treatment of milk to stimulate activity would need to vary with the specific

starter culture used. The ideal would be one heat treatment that would provide optimum conditions for a variety of the cultures used under commercial conditions.

SECTION III

COMPARATIVE STIMULATION OF STARTER CULTURE BACTERIA BY HYDROLYSATES OF PURIFIED PROTEINS

Numerous early investigations have suggested the importance of hydrolyzed protein fractions in the stimulation of growth of the lactic acid bacteria. This section represents studies on the comparative stimulative properties of certain protein hydrolysates. In addition, fractionation procedures were used in attempts to identify constituent amino acids of active peptide chains.

Experimental

Preparation of Hydrolysate. Commercial casein, lactalbumin (Nutritional Biochemical) and insulin (Eli Lily Co.) were suspended in 1% concentration in 0.2% sodium bicarbonate buffer. Trypsin (Nutritional Biochemicals 1:300) was added at the rate of 0.0005 gram per ml. of suspension. The mixture was overlaid with tolvene and allowed to incubate for 24 hours at 37°C. After digestion, enzyme activity was terminated by autoclaving the mixture at 121°C. for 15 minutes and then rapidly reducing the steam pressure. The heating process was sufficient to remove the toluene and precipitated the small amount of intact protein remaining after hydrolysis.

Leuconostoc hydrolysate was obtained by culturing

strain D2 of Leuconostoc dextranicum in 10% reconstituted skim milk for one week at 30°C. At the end of this time, acid precipitable protein was removed by adjusting the pH to 4.6 and filtering. As the milk had been heat sterilized, such treatment removed a large part of the serum protein as well as casein. Sufficient 90% ethanol was added to bring the final suspension to 70% alcohol by volume. The alcohol-protein mixture was heated to 75°C. and material insoluble at this temperature was removed by centrifugation and discarded. Alcohol was then removed from the filtrate by vacuum distillation at temperatures below 58°C. Because of the presence of non-protein nitrogen compounds, it was not possible accurately to determine the concentration of protein nitrogen in milk hydrolysates. Therefore, an indirect estimation was made by subtracting nitrogen soluble in phosphotungstic acid from total nitrogen as determined by micro-Kjeldahl procedure. Phosphotungstic acid-soluble nitrogen was determined by mixing 20 ml. of alcohol-soluble hydrolysate with 30 ml. of distilled water, 30 ml. of 30% sulfuric acid, and 20 ml. of 15% sodium tungstate (Kelly, 42a, p.6). Two hours were allowed for the precipitate to form and filtration was carried out through Whatman No. 40 filter paper. Milk protein hydrolysate (MPH) (from Baltimore Biological Lab.), liver fraction "L", and liver

fraction "2" (from Nutritional Biochemicals, Inc.) were obtained from commercial sources.

Comparative Response of Starter Culture to Certain Protein Hydrolysates. Each of the previously-mentioned protein hydrolysates was added to skim milk in concentrations of 25 mg. per ml. Culture tubes of the variously supplemented milks were inoculated with 1/10 ml. of 24 hour culture and incubated for 4.5 hours at 30°C. The short period of incubation was required in order to detect maximum differences in stimulation of the rapidly growing cultures. Cultures used as well as methods of titration are discussed in Section I of this thesis. Results of the experiment consist of growth obtained in similar but unsupplemented milk. Of the 7 hydrolysates tested it may be seen that leuconostoc digest and insulin hydrolysate provide the most outstanding and constant stimulation of starter culture (Table 3). Commercial culture M was stimulated by all hydrolysates but the most pronounced effect occurred with the addition of leuconostoc digest which almost doubled the growth obtained in supplemented milk. Strain IS, however, was noticeably inhibited by the addition of leuconostoc digest as well as by both liver preparations. Slight inhibition also occurred when liver fraction "2" was added to milk

TABLE 3

Stimulation of Single Strain and Mixed Strain Commercial Starter Culture By Various Protein Hydrolysates

Culture	Hydrolysate						
	Milk Protein	Liver L	Liver 2	Casein	Lact-albumin	Insulin	Leuc. ext.
	Percent increase or decrease of activity as compared to unsupplemented control						
306	7	30	20	56	62	71	68
IS	0	-12	-1	12	21	29	-16
SLE	28	27	3	13	51	5	56
9596	-1	8	-8	20	24	28	28
11E	21	22	16	43	69	81	89
262	3	14	3	12	19	28	34
H	5	21	25	44	51	86	84
M	10	14	10	33	54	75	91
F	3	20	12	36	47	51	66
Average	9.5	16	8.9	30	44	50	56

Tubes were incubated at 30°C. for 4.5 hours. At the end of this time titratable acid was determined and compared with titratable acidity of control tube with no hydrolysate.

cultures of strain 9596. Strain 11E and the mixed commercial starters were effectively stimulated by all hydrolysates tested. Although strain SLE showed good response to other hydrolysates it was not stimulated noticeably by insulin hydrolysate. This effect was observed and results therefore substantiated in a second trial. In all instances, lactalbumin hydrolysate proved to be a more effective stimulant than casein hydrolysate, milk protein hydrolysate or either of the liver preparations. For example, strain SLE was stimulated only 13% above control growth by the addition of casein hydrolysate, but developed 51% additional growth in the presence of lactalbumin hydrolysate. Under conditions of the test, commercial milk protein hydrolysate, liver "L" and liver fraction "2" were not markedly effective in stimulating growth of a majority of the strains tested. Casein hydrolysate was noticeably less stimulative than lactalbumin but appreciably more effective than either of the liver fractions or commercial milk protein hydrolysate.

Identification of Stimulative Peptides in Leuconostoc Digest of Milk. The alcohol-soluble extract of leuconostoc culture in milk appeared to be the most active of the protein hydrolysates tested. Therefore an attempt was made to identify the particular stimulative

constituents involved. Paper chromatography was selected as the most convenient tool for separation of the various constituents. Solvent systems containing butanol, acetic acid and water (4:1:1), and tertiary butanol, formic acid and water (5:1:1), were used in preliminary studies but did not give clear cut separation. A phenol-water system appeared to give good separation, but the mechanical difficulties concurrent with removal of all traces of phenol rendered this system unsuitable. Successful separation was made with the 2-butanol, ammonia solvent suggested by Roland and Gross (90, pp.502-505). The original solvent was prepared from 2-butanol and 3% ammonia (3:1). As modified for this study, the ammonia concentration of the aqueous phase was increased to 4%. This addition permitted separation of some previously overlapping components. The technique employed permitted the solvent front to proceed beyond the dimensions of the paper, so no Rf values can be given.

It was found that substitution of Whatman No. 4 paper for the Whatman No. 1, originally suggested, permitted the addition of larger amounts of hydrolysate and substantially increased rate of solvent movement. Approximately 30 mg. of hydrolysate were deposited on a line drawn across the small dimension of a large (18x22) chromatographic paper. Addition of the large quantity

of hydrolysate required 6 or 7 separate applications each followed by drying with heated air. After about 30 hours descending resolution in the usual fashion, the chromatogram was removed from the chamber and air dried with the aid of infrared bulbs. One-inch strips parallel to the direction of solvent flow were cut from the edges and center of the solved chromatogram. The strips were developed by spraying with 0.25% ninhydrin freshly prepared in ethanol containing 10% pyridine. The developed strips showed 16 separate bands positive to ninhydrin.

Bioautographic techniques were then used to identify biologically-active areas in untreated sections of the chromatogram. Synthetic agar for the study consisted of nutrient originally suggested by Henderson and Snell (36a, pp.15-29) added to 1.5% Noble agar. The agar was prepared just before use and autoclaved for 3 minutes at 121°C. To avoid additional heat treatment the agar was inoculated with washed cells of strain 306 as quickly as the sterilized agar reached 45°C. The inoculated medium was poured into a 6x16 inch Pyrex baking dish. A 4x14 inch section of the chromatogram, cut parallel to the direction of solvent flow, was embedded into the agar surface while the agar was still in the semi-solid state. Prior to embedding, the chromatogram was autoclaved for 3 minutes at 121°C. Although neither of the above heat

treatments was sufficient to destroy all microorganisms present, the high lvel of inoculum and the short period of incubation prevented the appearance of noticeable contamination. Aseptic conditions in the pan were maintained with an aluminum foil cover. After 30 hours of incubation at 32°C. the paper strip was removed and the agar surface examined in oblique light. Areas showing stimulation were outlined in pencil. These marks could be used subsequently to locate stimulative areas on similar sections of the chromatogram. Two stimulative areas were noted. One remained quite near the point of origin while the other was roughly 2/3 the distance between the point of origin and the most actively moving fraction. Transverse sections of the two active areas were cut out with scissors and ground in a Waring blender. The ground pulp was eluted twice with hot water and twice with 2N HCl and filtered through a medium-grade sintered glass filter. All washings were combined and digested by refluxing in constant boiling HCl for 24 hours. The digested residue was then twice evaporated to dryness under vacuum at 60°C. Chromatograms of the digested area were performed with conventional procedure and butanol, ethanol, and water (4:1:1) solvent on Whatman No. 1 paper.

Chromatograms of the slowest moving active area

showed the presence of spots equivalent to glutamic acid, serine and leucine, with glutamic acid as the most active area. Chromatogram analysis of the more actively moving area produced spots compatible with glutamic acid, glycine and a third unknown area between tyrosine and valine.

Discussion

Stimulation of the lactic streptococci of starter cultures by addition of protein hydrolysates appeared to be dependent upon at least three variables including the strain of organism and the nature of the source and characteristics of the hydrolytic agent. As noted in the previous section, surprisingly large differences in response were observed within the various strains studied. Certain strains of culture were markedly stimulated by identical concentrations of the same hydrolysate which was inactive or even inhibitory for another strain. For example, growth of strain M was almost doubled by the addition of leuconostoc hydrolysate, while strain IS was inhibited. In this particular event, the inhibition of strain IS may be due in part to some specific antibiotic action on the part of the leuconostoc species. However, as strain IS was also inhibited by other enzymatic digests, it does not seem likely that the inhibition was due solely to antibiotic action.

The nature of the source protein in leuconostoc extracts could not be determined but such extracts were unusually effective in stimulating growth. As the leuconostoc extract was composed of all alcohol-soluble portions obtained from culture growing in milk, it must also be considered that certain growth factors produced some of the stimulation observed. Such factors might have been excreted by the growing culture and extracted along with alcohol-soluble protein degradation products.

As shown in bioautographic studies, at least two of the stimulative fractions appeared to be peptides. This information, however, does not exclude the existence of other forms of stimulative agents which would not necessarily be used in fulfilling the nitrogen requirements of the starter culture.

Trypsin-hydrolyzed casein was considerably less effective in producing stimulation than other trypsin-hydrolyzed milk proteins. It was also noted that stimulation proceeded according to the concentration of sulfhydryl-disulfide groups in the protein. The most effective pure protein, insulin, was followed by lactalbumin and casein, which is also the order of concentration of disulfide linkages present in the native protein. Assuming that most disulfide linkages would be reduced to other forms by autoclaving, it would appear possible that

some protein product with reduced sulfur as a moity could serve as an important growth stimulant.

As it is necessary that bioautographic studies be carried out in synthetic media there can be no certain correlation between such studies and stimulation in a complex media such as milk. Snell and co-workers (81, pp.495-509) have pointed out that stimulation by peptides depends largely on the nutritional balance of the medium. These workers as well as certain others have demonstrated at least three mechanisms of stimulation by peptides. These include: (a) assimilation of a free amino acid but not that of its peptides may be inhibited by the presence of an antagonistic amino acid; (b) a free essential amino acid but not appropriate peptides may be destroyed, i.e., decarboxylated or deaminated by bacterial enzymes elaborated into the medium; (c) the efficient absorption of a free amino acid but not appropriate peptides may require increased levels of structurally unrelated amino acids or other nutrients in the medium. Therefore, in a complex medium such as milk, the response to given peptides would not necessarily parallel the response obtained in a synthetic medium containing free amino acids at artificially determined levels. Unfortunately, mechanical difficulties make it impossible to obtain purified extracts in concentration sufficient to

study stimulation in milk.

SECTION IV

EFFECT OF HEAT ON MILK AS A MEDIUM FOR THE LACTIC STREPTOCOCCI OF STARTER CULTURE

The present study was undertaken in order to further investigate both beneficial and deleterious actions of strong heating of milk used for starter cultures.

Experimental

Cultures used in the study included strains of Streptococcus lactis (SLE and 262), Streptococcus cremoris (11E, 206, 22E and 144) and mixed strain commercial lactic cultures (A, B, and C). Calcium phosphate-caseinate complex (caseinate) was obtained by centrifugation of fresh skim milk at 50,000 RPM in a Sharples super centrifuge. Extraneous matter was first removed from the milk in a preliminary clarification at 25,000 RPM. After collection, the caseinate complex was suspended in ice water and recentrifuged at 50,000 RPM. After two such washings the preparation was free of reducing sugars. Whey proteins were obtained from acid precipitated whey by salting out with ammonium sulfate and then dialyzing until salt free. Amino nitrogen was determined colorimetrically with a Beckman B spectrophotometer at 400 m using a modification of the procedure originally described by Harding and MacLean (35, pp.217-238).

The defined nutrient system used to study individual milk components consisted of the respective purified milk protein and lactose together with buffer salts, minerals and growth factors in concentrations similar to those suggested by Henderson and Snell (36a, pp.15-29). Casein was employed at a level of 1%, whey protein, where used, was employed at a level of 0.3% and lactose at the rate of 1%. Although the concentrations employed were not identical with levels in milk, the lower content, particularly of casein, facilitated preparation of a homogeneous, reproducible nutrient system. Higher concentrations of casein presented mechanical difficulties such as foaming and sedimentation. Lactose which was present in all nutrient systems, was sterilized by heating separately, or in the presence of other components, according to the design of the experiment. Growth was estimated by titration of acid produced using an electrometrically determined endpoint of 8.3. Results were expressed in terms of micromoles of lactic acid developed per 5 ml. tube of medium. Results presented have been repeated at least twice and in some instances numerous times.

Hydrolytic Effects of Heat. Thermal effects on milk constituents in a defined system were studied by heating milk proteins in the presence and absence of lactose for

varying periods of time. Results are indicated in Table 4. A mild heat treatment at 60°C. for 10 minutes served as a control. It resulted in practically no demonstrable change in the protein and eliminated interfering lactic acid bacteria. The data show that autoclaving casein in the absence of lactose resulted in hydrolysis which continued progressively for as long as heating was maintained (60 minutes). Protein hydrolysis also occurred when casein was heated in the presence of lactose. Autoclaving a mixture of these components for 15 minutes resulted in a noticeable increase in free amino nitrogen. However, when the heating period was extended to 60 minutes at 121°C. there was a marked reduction in free amino nitrogen. It would appear, therefore, that hydrolysis continues during extended heating periods but that a critical time is reached after which protein degradation products presumably are combined with lactose more rapidly than stimulatory degraded products are formed from native protein. As would be expected, heating a mixture of caseinate complex, lactose and purified whey proteins also resulted in protein hydrolysis as indicated by increase of free amino groups during early stages of heating. However, the uptake of amino groups by lactose during prolonged heating was appreciably retarded in the presence of whey proteins.

TABLE 4

Effect of Heat on Free Amino Nitrogen Content of Simplified Milk Constituent Nutrient Systems

Constituents Heated[a]	Heat treatment		
	60°C. for 10 min.	121°C. for 15 min.	121°C. for 60 min.
	mgm/100 ml	mgm/100 ml	mgm/100 ml
Caseinate only	0.79	5.22	9.72
Caseinate + lactose	0.81	5.03	0.87
Caseinate + whey protein only	1.12	5.30	9.45
Caseinate + whey protein + lactose	1.07	5.16	2.10

[a] Medium consisted of mineral salts, vitamins, and added milk constituents.

Effect of Heating of Medium on Growth of Cultures in Defined Nutrient Systems. The observation that heating of casein results in change in amino nitrogen concentration led to further studies on effect of heating medium on subsequent growth of starter culture. Nutrient systems were prepared containing either 1% caseinate and 1% lactose or 1% caseinate, 1% lactose, and 0.3% purified whey protein. The preparations were sterilized in the following manner: (a) protein and lactose heated separately for periods of 10 minutes at 60°C., 15 minutes at 121°C. and 60 minutes at 121°C.; (b) protein and lactose compounds heated together for the periods indicated in (a). After treatment, the constituents were cooled and combined with vitamins and salts as necessary to make a complete nutrient medium. Five ml. quantities were dispensed aseptically in sterile tubes and inoculated with 0.1 ml. quantities of twice-washed cell suspension. Results of the experiment appear in Table 5.

Examination of results in Tables 4 and 5 show a relationship between amount of free amino nitrogen and extent of growth in the defined nutrient system. This is especially true with strains 11E and 22E. Using caseinate complex as the sole source of protein which was heated separately from lactose, strain 11E produced only 19 micromoles of lactic acid per 5 ml. in the mildly

TABLE 5

Effect of Heating Different Medium Constituents on Growth of Lactic Streptococci in Defined Milk Protein Systems

Heating time	Culture	Constituents heated together			
		Caseinate[a]	Caseinate + lactose	Caseinate + whey protein	Caseinate + lactose + whey protein
Past. at 60°C. for 10 minutes	SLE	61[b]	63	225	231
	262	47	51	196	187
	11E	19	19	151	150
	306	25	23	110	114
	22E	22	29	147	154
	144	59	55	191	197
	F	61	59	183	176
	H	47	51	191	198
	E	69	66	210	218
Past. at 121°C. for 15 minutes	SLE	162	172	325	331
	262	143	148	290	289
	11E	150	177	282	294
	306	105	105	180	191
	22E	123	151	231	357
	144	151	171	285	301
	F	120	130	250	250
	H	138	173	277	309
	E	145	185	287	331
Past. at 121°C. for 60 minutes	SLE	165	121	318	298
	262	142	113	295	284
	11E	154	61	280	221
	306	106	57	183	157
	22E	145	39	226	178
	144	159	135	291	279
	F	140	104	263	254
	H	144	35	268	202
	E	156	125	293	280

[a] Lactose added after heating.

[b] Results expressed in terms of micromoles lactic acid produced per 5 ml. tube.

heated system, 150 micromoles in media heated for 15 minutes at 121°C. and 154 micromoles in media heated for 60 minutes at 121°C. When casein was heated with lactose, the acid production in preparations autoclaved for 15 minutes was elevated to 177 micromoles per 5 ml. However, acid production dropped to only 61 micromoles in aliquots of the same system heated for 60 minutes at 121°C. Similar effects were noted with other species. Of considerable interest was the fact that casein and lactose heated together for short periods of time (15 minutes at 121°C.) allowed greater acid production than did preparations which were heated separately.

Mildly heated casein was a poor nitrogen source and supported only minimal growth of the particular organisms tested. The addition of purified whey proteins provided considerable stimulation in mildly heated systems. However, little additional effect by heating for 15 minutes at 121°C. was noted in preparations containing whey proteins. Of greater significance was the observation that the presence of whey protein partially counteracted the adverse effects of autoclaving when lactose and caseinate were heated together for one hour. It was not possible to use purified whey protein as the only nitrogen source

due to its tendency to coagulate if heated in the absence of a protective colloid.

The protective action of whey protein in preventing uptake of stimulatory protein degradation products by lactose suggested that certain linkages most common to such protein were responsible for the effect. As a reduction of protein disulfide to sulfhydryl was also known to be a product of heating milk, it seemed reasonable to continue study on the effects of certain substances rich in sulfur groups. Caseinate in 1% concentration was heated with 1% lactose at 121°C. for 30 minutes in pH 6.7 M/15 phosphate buffer. Compounds rich in various thiol linkages were added to certain of these preparations before heating was begun. Heating was carried out in 100 ml. Erlenmeyer flasks containing 50 ml. of preparations. Trichloracetic acid soluble nitrogen (TCA soluble N) on heated samples was determined by mixing 20 ml. of the heated system with an equal volume of cold TCA. After 2 hours of storage at refrigerator temperature the mixture was filtered through Whatman No. 40 paper and soluble nitrogen determined by conventional micro-Kjeldahl procedures. Thirty ml. portions of the heated systems were clarified by digestion with 50 mg. of 1 300 trypsin and intensity of the browning (Milliard reaction) determined with the aid of a Beckman B spectrophotometer at 510 m .

Casein heated in phosphate buffer without further additives was used as standard for 100% transmittance. The data presented in Table 6 summarize the results of these experiments. It may be seen that those substances containing disulfide linkages exert a protective action against the Milliard (browning) reaction as well as retarding uptake of trichloracetic acid soluble nitrogen. Insulin which contains large numbers of sulfur to sulfur cross linkages was more effective in retarding the Milliard and allied reactions than whey proteins which contain an intermediate number of such groups. Cystine was very effective in preventing browning while cysteine did not possess this capacity.

In order to investigate the possibility of vitamin inactivation or toxic product formation during prolonged heating at high temperatures, the following experiment was devised: Samples of raw skim milk were autoclaved at 121°C. for periods of 15 and 60 minutes. After cooling, the milk was fortified with vitamin-free casein hydrolysate (Nutritional Biochemicals, Inc.) in concentrations of: 0, 3, 6, 10, 20, 30, 60, 100 and 200 mgm./100 ml. The milk was aseptically tubed, inoculated with 1% starter culture and incubated 5 hours at 30°C. Three commercial starters, A, B, and C plus S. lactis SLE and S. cremoris 11E were employed in the test. Comparisons

of growth (final minus initial titratable acidity) are listed in Table 7.

It may be observed that milk autoclaved for one hour supported less growth than milk autoclaved for only 15 minutes and that small quantities of vitamin-free protein hydrolysate stimulated growth in both such milks. At levels of hydrolysate less than 60 mgm./100 ml. growth in overheated milk was appreciably less than growth in similarly supplemented milk heated for only 15 minutes. Quantities of hydrolysate greater than 60 mgm./100 ml. permitted about the same growth in milk heated for either 15 or 60 minutes.

Four cultures (A, B, C, and 11E) showed increased growth in proportion to the amount of hydrolysate added. Culture SLE reached maximum growth at a concentration of 100 mg./100 ml. but appeared to be inhibited at 200 mg./100 ml.

During the course of the study it was observed that heating to 121°C. for periods of 10 minutes, cooling the milk rapidly and reheating on subsequent days produced about the same effects on amino nitrogen content of milk as the equivalent exposure to continuous heating. In other words, continuous heating for an extended period has about the same effect as intermittent heating for short periods totaling the equivalent time and temperature.

TABLE 6

Effect of Presence of Certain Sulfur Containing Materials on TCA Soluble Nitrogen Content and Extent of Browning of Simplified Milk Component Systems During Heating at 121°C. for 30 Minutes

Material Added	TCA Soluble Nitrogen	Extent of Browning
None	mg/ml	% transmission
(Caseinate[a] only)	4.1	100
Caseinate + lactose[b]	1.9	24
Caseinate + lactose + cystine[c]	3.4	72
Caseinate + lactose + cysteine[c]	2.2	31
Caseinate + lactose + methionine[c]	2.0	30
Caseinate + lactose + insulin[d]	3.0	59
Caseinate + glutathione[c]	2.3	34
Caseinate + lactose + whey proteins[e]	2.7	48

[a]caseinate - 1%

[b]lactose - 1%

[c]cystine, cysteine, methionine, glutathione - 1 mg/ml

[d]insulin 5 mg/ml

[e]whey protein 25 mg/ml

TABLE 7

Response of Single and Multiple Strain Starter Cultures to Increasing Concentrations of Vitamin Free Hydrolysate Added to Milks Autoclaved for 15 and 60 Minutes, Respectively

Conc. of hydrolysate added	Acid Production by Various Cultures									
	A		B		C		SLE		11E	
mgm/ml	Min. at 121°C.		Min. at 121°C.		Min. at 121°C.		Min. at 121°C.		Min. at 121°C.	
0	30[a]	26	46	38	44	36	31	25	26	20
3	31	26	49	39	49	36	30	26	28	19
6	30	28	51	40	47	39	33	26	30	22
10	33	29	52	44	49	41	33	28	34	26
20	61	36	56	49	57	55	59	38	54	39
30	61	43	59	52	61	57	64	52	54	70
60	63	60	64	61	67	67	67	62	71	72
100	78	74	75	74	72	69	73	68	80	79
200	80	79	80	80	77	78	56	57	81	80

[a]Micromoles lactic acid produced per 5-ml. tube

Such action seemed to be related in part to the modification of disulfide linkages during heating (93, pp.427-434). It was noted that cystine inhibited uptake of amino groups by sugar while sulfhydryl of thiomethyl-ether (methionine) compounds did not. Whey proteins likewise inhibited the sugar-protein reaction during extended periods of strong heating.

Discussion

Heat activation of milk used for starter culture can be explained by at least two distinct reactions, most important of which is hydrolysis of the casein fraction. It is to be expected that this or any similar reaction which serves to break up the protein molecule into more utilizable fractions should stimulate starter growth.

Results of the present study suggest another stimulatory action resulting from heating milk. It was shown that protein and lactose heated together produce a stimulation of certain cultures that is not observed when these components are heated separately. This would suggest formation of a lactose-protein compound which is stimulative to lactic streptococci. Orla-Jensen (59, pp. 374-379) has reported heat activation of media on the basis of a similar metabolite involving carbohydrate and nitrogenous compounds. Pentoses or carbohydrate

degradation products such as methyl glyoxal were effective as stimulants only when heated in the presence of yeast extract. Unheated mixtures of the components, or components heated individually were not effective in producing increased growth.

The detrimental effect of excessive heat on milk used for starter may be explained on the basis of heat-stimulated uptake of available nitrogen by lactose. A deleterious action did not take place when lactose and casein were heated separately. Rather, the additional heat produced a continuing hydrolysis of casein and slight increased activity of the starter cultures. Other data have further substantiated the conclusion that the detrimental effect of overheating is due principally to uptake of available nitrogen by milk sugar. It was found that addition of small quantities of vitamin-free casein hydrolysate to overheated milk can be used to reverse the detrimental effects of overheating on starter activity.

The presence of the disulfide linkage appears to be one of great significance in limiting certain reactions during heating of milk. Heated synthetic systems containing only casein and lactose were more inclined to undergo browning and loss of available nitrogen than similar systems containing added whey protein, cystine or insulin. Added cysteine, however, allowed browning and nitrogen

loss reactions to proceed at a normal rate. Thus it would appear that the oxidized (disulfide) form inhibits certain protein-sugar interactions. The importance of whey-protein in preventing nutritional losses in milk due to heating is thus apparent when it is considered that such proteins are the primary source of the disulfide linkages in milk.

Of considerable interest was the observation that mildly heated casein complex was a poor protein source for lactic streptococci. Addition of twice precipitated whey protein produced some stimulation of these species but the results would indicate that intact milk proteins are not utilized as readily as partially hydrolyzed fractions. Anderson et al. (3, pp.1083-1088) have indirectly verified this by showing a direct relationship between concentration of low molecular weight protein derivatives and activity of starter cultures in milk.

SECTION V

STIMULATORY SUBSTANCES FOR LACTIC ACID BACTERIA PRODUCED BY HEATING TOGETHER CERTAIN COMBINATIONS OF AMINO ACIDS AND LACTOSE

Although workers as early as Orla-Jensen (59, pp. 374-379) reported heat activation of media for lactic acid bacteria, it is only in recent years that explanations on mechanism of activation have been provided. Snell *et al.* (81, pp.495-509) have reported pyruvate to replace substances formed upon heating of synthetic culture medium. Foster (27, pp.988-997) suggested that increased activity of starter culture in heated milk is the result of heat-stimulated hydrolysis of milk protein on corresponding increase in available nitrogen. Previous sections of this thesis have shown that substances stimulative to lactic streptococci are formed when milk protein is heated in the presence of lactose. Because the latter effect seemed to be different in nature from mechanisms suggested by either Foster or Snell *et al.*, it was investigated in greater detail.

Experimental

Amino acids of different specific configuration (glycine, cystine, glutamic acid, histidine, tryptophane, leucine and lysine) were mixed with equimolar quantities

of lactose and heated in an autoclave at 121°C. for 15 minutes. Quantities equal to 0.01 mg./ml. were added to fresh skim milk which had been pasteurized at 60°C. for 10 minutes.

A high quality raw milk was selected and the heat treatment given was sufficient to prevent development of acid in uninoculated control tubes. In addition, the high level of inoculation and short incubation period would appear to overshadow growth of contaminating organisms surviving the mild heat treatment. Additional heat treatment was avoided in order to minimize thermal activated reactions within the system.

Five-ml. portions of the supplemented milk were aseptically tubed and seeded with Streptococcus thermophilus strain C3. It was noted that the lactose-glycine mixture was slightly stimulative and the cystine-lactose mixture was highly effective in stimulating growth of the test organism. As other workers (Rogers et al., 88, p. 141; 89, p.77) had previously reported stimulation with glycine mixtures, investigations were confined to the more effective cystine-lactose system.

Three separate lots of lactose-cystine mixture were prepared and heated as described previously. Quantities of the mixture were then added in gradient dilution to milk pasteurized at 60°C. for 10 minutes. The milk was

tubed aseptically and inoculated with representative strains of S. thermophilus (C3, MC and S), L. bulgaricus (Ga), and S. lactis (7963).

The cultures of S. thermophilus and L. bulgaricus are representative of starter cultures used in domestic production of Swiss cheese and fermented milks. Strain 7963 is used as a representative strain of S. lactis of the National Type Culture collection. Composite results of the three trials are included in Figure 6. It may be seen that all strains tested were stimulated to some extent, but strains C3 and MC were the most responsive to lower concentrations of the mixture. Concentrations above 0.04 mg. per ml. were less effective than concentrations of 0.02 and 0.04 mg. per ml. while a concentration of 1 mg. per ml. was definitely inhibitory to these strains. Strain S of S. thermophilus was much less responsive to the added cystine-lactose combination but was limited at the same concentration as other strains of this species. L. bulgaricus was effectively stimulated at concentrations 10 to 50 times greater than those most effective for S. thermophilus. A culture of Lactobacillus acidophilus was included in one of the trials, but was stimulated only very slightly at the highest concentration of cystine-lactose mixture employed. At concentrations of 1.0 mg. per ml. S. lactis was stimulated to

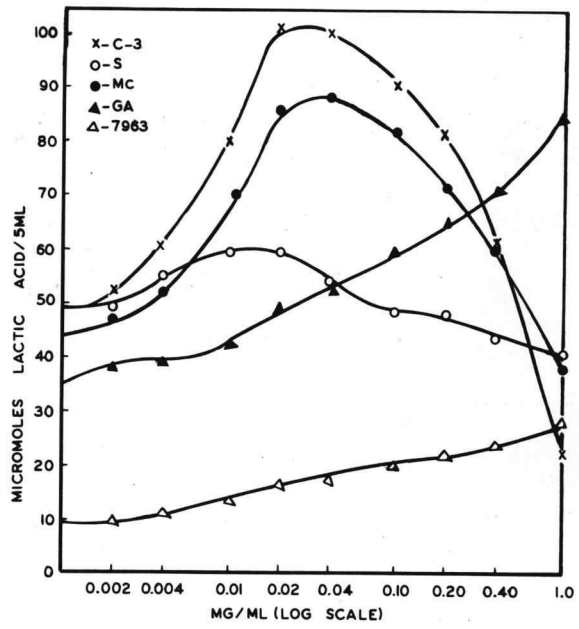

Figure 6. Rate of lactic acid production by lactic starter cultures in pasteurized milk containing various increments of heated lactose-cystine mixture ranging from 0 to 1.0 mg./ml. Cultures were S. thermophilus (C-3, S, and MC), L. bulgaricus (GA), and S. lactis (7963).

approximately three times the growth at zero concentration.

Because of the possibility of stimulation by cysteine formed through the reduction of cystine, it was considered necessary to include the reduced form in stimulation studies. Heated preparations consisting of cystine and lactose, cysteine and lactose, and unheated systems of glutathione, cysteine and cystine were added to pasteurized milk in concentrations of 0.02 mg. per ml. Results of the experiment are given in Table 8. The data given suggest that although cysteine is slightly effective in stimulating growth of S. thermophilus, the primary stimulative effect observed in earlier studies is not the result of lowered oxidation-reduction potential. Likewise heated systems of cysteine and lactose were no more effective than cysteine alone.

Discussion

Results of this section have provided a partial explanation for superior growth of some lactic acid organisms in heated milk. They suggest that certain lactose-protein combination products formed upon heating of the milk system are required for active growth of some strains of S. thermophilus. Some of the stimulation observed in cystine-lactose mixtures may have been due to cysteine

TABLE 8

Quantitative Response of S. thermophilus to Heated Cystine-Lactose Mixture Added to Pasteurized* Skim Milk

Concentration of cystine-lactose mixture added	Acid production** by following strains:		
	C3	S	MC
mg/ml			
0	52	48	44
0.002	55	51	44
0.004	60	51	51
0.01	79	58	66
0.02	104	60	90
0.04	101	51	90
0.10	90	50	84
0.20	89	50	80
0.40	50	47	49
1.0	22	36	36

* Laboratory pasteurized at 60°C. for 8 minutes.

** Micromoles lactic acid produced after 8 hours incubation at 30°C.

or other sulfhydryl groups formed through reduction of cystine.

However, in view of the relatively low activity of cysteine and complete inactivity of glutathione it would seem doubtful if much stimulation can be attributed to such action. As the heated mixture of cysteine and lactose was relatively ineffective compared to the heated cystine-lactose system, it would appear that a product formed during heating of the latter system is vital to formation of the unknown factor.

Results of this section also indicate fundamental differences in response to heated milk between species such as S. cremoris and S. lactis on the one hand and others such as S. thermophilus. Results of Section III indicate that S. lactis has primary response to heat activated increases in available nitrogen. However, results of this section indicate that primary stimulation of S. thermophilus may be the result of protein-lactose interaction products.

There are at least two explanations for inhibition of growth of S. thermophilus at high concentrations of heated cystine-lactose mixture. One possible reason is antagonism by free uncombined cystine. Another explanation would be competitive antagonism by the stimulatory factor when it is present in higher concentrations.

SECTION VI

UTILIZATION OF PURIFIED MILK PROTEINS BY SPECIES OF BACTERIA COMMON TO STARTER CULTURE

Sections I and III of this thesis have shown the importance of low molecular weight fractions of protein in stimulating growth of species of bacteria common to starter culture. The extent of utilization of native milk protein, however, has not been previously elaborated. Such information is of fundamental significance as well as practical importance to many fields of the dairy industry. The following section reports a study of the utilization of purified milk proteins by growing cultures of lactic streptococci and enzymatic studies with resting cells and cell free enzyme extracts.

Experimental

Washed calcium phosphate-calcium caseinate complex was obtained by centrifugation as described in Section III of this thesis. θ lactoglobulin was isolated in purified form using methods originally described by Palmer (62, pp.359-367). Alpha lactalbumin was precipitated from acid whey using the method developed by Gordon and Semmett (33, pp.223-224). The defined nutrient medium employed for testing utilization of protein was essentially the same as that employed by Anderson and Elliker

(3, pp.1083-1088), with the exception that purified proteins were substituted for the amino acids used in the original medium. Growth in the synthetic systems was determined by titration of acid using an electrometrically determined endpoint of pH 8.3. Cell-free proteolytic extracts as well as resting cells were obtained from a 24 hour culture grown in broth containing glucose 0.5%, lactose 0.5%, peptone 1.0%, yeast extract 0.5% and sodium acetate 0.1%. Cells intended for use in enzyme preparations were harvested in a Sharples supercentrifuge and immediately frozen. The frozen mixture was ground with alumina and then clarified in a Sorval centrifuge at approximately 10,000 r.p.m. The clarified cell extracts were held in the frozen state until just before use. Extent of protein hydrolysis by intact cells and cell-free enzymes was determined by measuring the increase in TCA-soluble nitrogen in the substrate. This fraction was estimated by mixing one volume of the protein suspension containing cells or enzyme with an equal volume of 10% cold trichloracetic acid and holding at 4°C. for three hours. Soluble nitrogen in the filtrate was determined by semi-microKjeldahl procedure.

Defined nutrient systems were prepared with one of the following substrates as the sole source of protein: caseinate complex, isoelectric casein, serum globulins,

β lactoglobulin and alpha lactalbumin. All systems were filter sterilized with the exception of colloidal caseinate. The latter material resisted filtration but, where necessary, it was pasteurized at 75°C. for 5 minutes. Such treatment was found sufficient to remove interfering lactics without apparent influence on subsequent growth of starter bacteria. Because of the prolonged incubation period required in this particular phase of the investigation, stronger heat treatment was required than employed in earlier sections of the study. Preparations used in investigations of enzymatic hydrolysis were not heated.

Growth in Purified Protein Substrates. Growth in the nutrient system was estimated by titration of the acid produced after 18 hours of incubation at 32°C. Results of the study are presented in Table 9. It may be seen that lactalbumin is the only native milk protein which is utilized by either single strain culture or mixed commercial starter culture. Minimal growth was observed in caseinate complex as well as other intact milk protein. Isoelectric casein, however, served as a reasonably good source of nitrogen and supported about twice as much growth as lactalbumin. Sizeable variations in growth were observed among the various strains employed in the study. Culture 11E seemed to be the most

TABLE 9

Utilization of Purified Protein by Starter Cultures in Defined Nutrient Systems

Culture	Nitrogen Source				
	Caseinate	Lactalbumin	Lactoglobulin	Whey globulins	Isoelectric casein
7963	12[a]	146	8	8	212
SLE	16	134	12	12	316
11E	12	88	4	8	286
H	20	184	8	12	292
F	24	168	12	20	280

[a] Micromoles lactic acid/5-ml. tube.

nutritionally demanding of the various strains studied. Isoelectric casein was utilized to a certain extent by this organism but utilization of the lactalbumin was extremely limited. Single strain cultures 262 and SLE as well as commercial culture H and F were similar in their patterns of milk protein utilization.

Hydrolysis of Milk Protein by Cell-Free Extracts. The activity of cell-free extracts of S. lactis (7963), S. cremoris (9596) and L. dextranicum (D2) was checked against caseinate complex, isoelectric casein and lactalbumin. Five ml. of approximately 1% protein solution was mixed with 4 ml. of pH 6.6 M/15 phosphate buffer (pH 6.5) and one ml. cell-free extract. After 6 hours of incubation at 32°C., TCA-soluble nitrogen was determined on the contents of the entire tube. The results are shown in Table 9 and are expresses as mgm. increase in TCA-soluble nitrogen per mgm. nitrogen of enzyme extract (QN/N). Blank values obtained in a substrate free system were subtracted before calculations were made. It may be seen that isoelectric casein was hydrolyzed more readily than the caseinate complex or alpha lactalbumin. All proteins tested, however, were hydrolyzed by cell-free enzymes of the three species involved. It was of interest that caseinate complex was hydrolyzed more effectively than

alpha lactalbumin by enzyme extracts although caseinate complex was not utilized by intact growing cells (Table 9). Enzymes of the particular strain of S. lactis involved were more effective in hydrolyzing lactalbumin than either L. dextranicum or S. cremoris. Caseinate, however, was more resistant to hydrolysis than isoelectric casein. For example, enzymes extracted from S. cremoris increased TCA-soluble nitrogen of isoelectric casein 8.1 mgm. for each mgm. of enzyme extract nitrogen. Only 4.8 mgm. increase was effected with caseinate complex as substrate.

Studies also were conducted on the relative proteolytic ability of intact cells and enzyme extracts (Table 10). Proteolytic activity of resting cell and cell-free preparations were compared at isonitrogenous levels on colloidal caseinate, isoelectric casein and lactalbumin substrates in phosphate buffer (pH 6 M/15) which included .01 per cent glucose. In this instance, no attempt was made to standardize intact cell and cell-free nitrogen. The primary purpose of this experiment was to compare substrates affected by intact cells and cell enzyme extracts. Cell-free extracts were again demonstrated capable of hydrolyzing caseinate complex, isoelectric casein and lactalbumin. Resting cells of L. dextranicum

produced very slight increases in TCA-soluble nitrogen with the caseinate complex substrate. The effect was quite small, however, when compared to increases in TCA-soluble nitrogen with isoelectric casein as substrate. In general, intact cells of these species did not hydrolyze caseinate complex. Conversely, resting cells of all three species were capable of hydrolyzing either lactalbumin or isoelectric casein. Rate of hydrolysis of lactalbumin by cell-free enzyme extract was not as rapid as hydrolysis of caseinate complex by the same preparation.

Uptake of TCA-Soluble Nitrogen by Growing Culture. Preliminary attempts to study utilization of TCA-soluble nitrogen were made with milk as the medium. Such studies were not successful because the presence of high concentrations of non-protein nitrogen masked the small changes occurring in degraded protein. A nutrient system was then devised which was purposely limited in available nitrogen and which was well suited to the analytical procedures already employed in other segments of the study. It consisted of caseinate complex 1%, yeast extract 0.5%, lactose 1%, and M/15 Phosphate-Acetate buffer (pH 6.7). One liter was dispersed into each of several 2-liter Erlenmeyer flasks and sterilized by autoclaving at 121°C. for 12 minutes. Cells used for inoculum were separated

TABLE 10

Hydrolysis of Certain Milk Proteins by Cell-Free Enzyme Extracts of Starter Culture Bacteria

Organism	Substrate		
	Caseinate complex	Isoelectric casein	Lactalbumin
	QN/N	QN/N	QN/N
S. cremoris 9596	4.8	8.1	1.5
L. dextranicum D2	3.9	6.0	1.8
S. lactis 7963	4.2	7.8	3.6

QN/N = mgm. increase of TCA-soluble nitrogen per mgm. nitrogen in enzyme extract.

by centrifugation but were not washed. The entire harvest of a 10-ml. broth culture served as inoculum for each liter of medium. Fifty-ml. aliquots were withdrawn at 0, 6, 12, 20, 24, 27, 30, 36, and 48 hours. TCA-soluble nitrogen was determined on duplicate 25-ml. portions at each withdrawal. Changes in pH and TCA-soluble nitrogen during certain ages of the culture are presented in Figures 7 and 8. As the system was low in available nutrients, initiation of growth was quite slow. A minimum of 12 hours was required before pH was lowered and at least 24 hours incubation at 32°C. was necessary for the single strain culture to produce sufficient acid to coagulate the casein. Inspection of the data indicates that both cultures removed TCA-soluble nitrogen during stages of growth immediately preceding coagulation. The mixed commercial lactic starter was faster growing, was more hydrolytic in later stages of growth and removed larger amounts of nitrogen from the medium in the early stages than the single strain culture. There was uptake of nitrogen by both cultures in the more rapid stages of growth preceding coagulation of casein near its isoelectric point. After pH 4.9 was reached by the growing culture, however, liberation of TCA-soluble nitrogen exceeded uptake of the soluble fraction of casein.

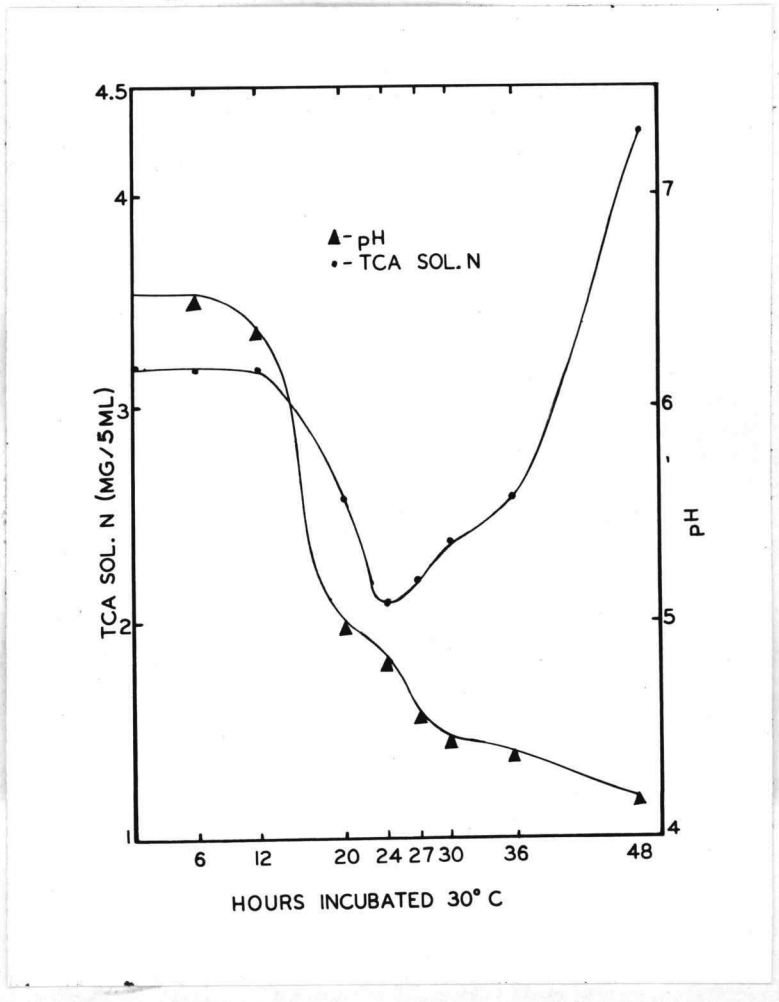

Figure 7. Influence of growth of mixed strain commercial starter culture on pH and soluble nitrogen of synthetic milk nutrient system.

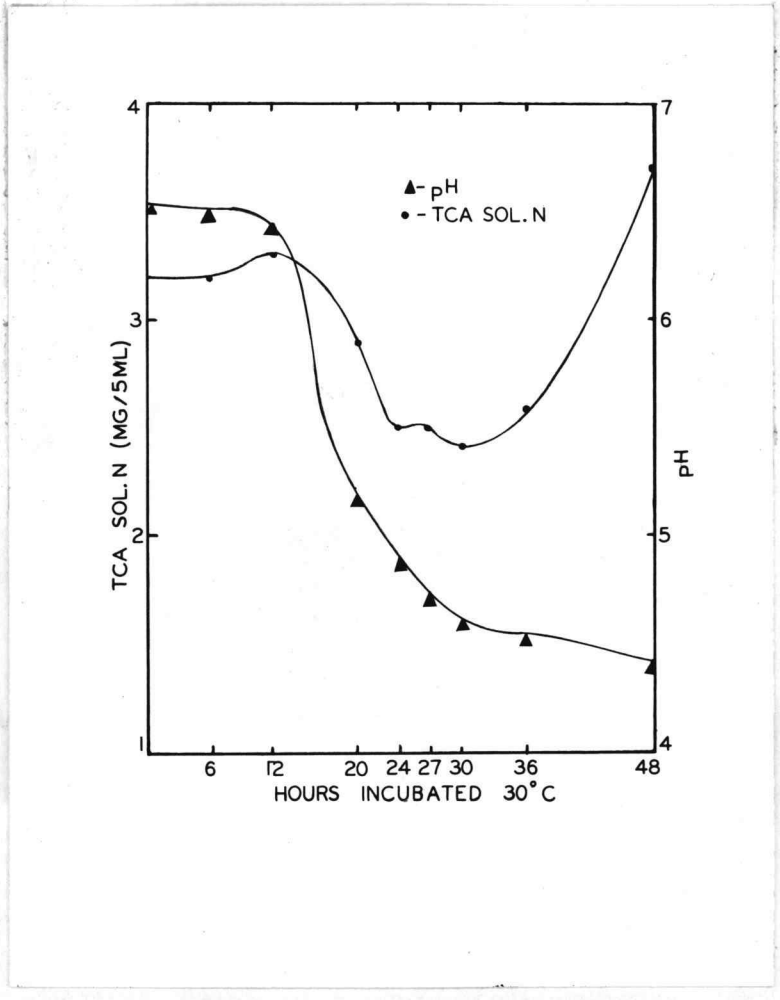

Figure 8. Influence of growth of single strain (9596) S. cremoris on pH and soluble nitrogen of a synthetic milk nutrient system.

Discussion

With the possible exception of lactalbumin, the native proteins in milk do not appear to be utilized as nitrogen sources by the lactic streptococci of starter. Although lactalbumin can be utilized as the sole source of protein by growing cultures it seems doubtful that lactalbumin serves as the primary source of nitrogen during phases of active acid production in milk. Methods used in precipitation and purification of this protein also may have altered it sufficiently to permit utilization. Earlier work by Anderson et al. (1, pp.161-167) and sections of this thesis which show a close relationship between level of low molecular weight protein degradation products and activity of starter culture in milk appear to verify the unavailability of intact milk protein to starter bacteria. It is not clear why caseinate complex is unavailable, although isoelectric casein is a good source of protein for lactic streptococci. In this regard it is possible that the size and shape of the particles involved may be important. Ford (28) has observed colloidal caseinate complex to have a minimum diameter of about 500 Angstroms, with a majority of the particles of larger diameter. Casein in solution, however, appears to be dispersed as fibular molecules with dimension 10 and 50 Angstroms. The latter molecule would

therefore expose many times as much surface area and provide greater opportunity for enzyme activity and orientation on a specific hydrolytic cell site. Another important difference between the caseinate complex and casein in solution is that casein is chemically combined with calcium phosphate. Thus the configuration of caseinate-complex micelle would be expected to differ from the configuration and exposure of specific groups of isoelectrically precipitated casein micelles. Results presented in Table 9 show that ruptured cells possess enzymes which will hydrolyze caseinate complex while results in Table 10 indicate that the enzymes are not available for extracellular proteolytic functions. Isoelectric casein is readily hydrolyzed by cell-free enzyme extracts and, in addition, it is also hydrlyzed by intact resting cells. It may be that hydrolysis of caseinate precedes stepwise with the loss of calcium phosphate complex and unfolding of the protein chain at the isoelectric point serving as the first step. Thus the multitude of enzymes present in cell extracts might carry out both processes while intact cells would be limited to the second or hydrolytic step. Such an action would be similar to that observed with trypsin which will not attack ovalbumin or serum globulins until they have been denatured by heat or other

means. In any event, it is apparent that cells of lactic streptococci contain enzymes which will hydrolyze caseinate complex, isoelectric casein and lactalbumin. However, caseinate complex is not readily utilized as a source of nitrogen by growing cells and is not hydrolyzed by heavy concentrations of resting cells. An interesting verification of other results was observed in experiments which show uptake of the TCA-soluble fraction by growing cells. Low molecular weight protein fractions decreased only during early phases of growth preceding coagulation of casein. Once the isoelectric point was approached, however, decrease of the soluble nitrogen fraction ceased and casein was hydrolyzed by growing cells of lactic streptococci. Apparently death and rupture of the cell was not required for hydrolysis of casein as acid continued to increase during first stages of hydrolysis. Also resting cells produced increases in TCA-fraction above that observed in control substrate-free systems.

TABLE 11

Comparison of Hydrolysis of Purified Milk Proteins by Intact Cells and Cell Free Enzyme Extracts of Starter Culture Bacteria

Organism	Substrate		
	Caseinate	Isoelectric casein	Lactalbumin
S. cremoris 9596			
resting cells	0.1	4.7	1.0
cell free	3.2	5.1	2.4
S. lactis 7963			
resting cells	0.2	7.8	2.6
cell free	5.6	10.0	5.2
L. dextranicum D2			
resting cells	0.4	5.1	2.6
cell free	3.2	4.5	6.1

Values shown are mgm. increase in TCA-soluble nitrogen per ml. of one percent protein solution.

SUMMARY AND CONCLUSIONS

Chemical analysis of individual cow samples of fresh milk indicated variation in peptide content. Rate of acid production by mixed strain commercial cultures and individual strains of lactic streptococci, in most instances, increased with increase in the peptide content of the milks from individual cows. Different cultures varied somewhat in their response to the peptide content of milk.

The peptide content of fresh milk appeared to exert greater effect on rate of growth of lactic streptococci than the protein content of milk. No significant difference was apparent between Jersey and Holstein milk from which the fat was removed. Preliminary experiments indicated the correlation between rate of growth and peptide content to be poor in a number of samples of milk from mastitic animals and from those in early or late stages of lactation.

Studies with low heat spray processed non-fat milk solids have indicated no observable relationship between activity of starter culture and season of production, level of trichloracetic acid soluble nitrogen or amino nitrogen. Analysis of individual lots of milk solids indicated definite variations in peptide level. However

attempts to correlate peptide level and activity proved the relationship to be extremely random. Response of different cultures to individual milks was also random. This would indicate that proper combinations of certain factors of metabolism of the organism on one part, and conditions within the milk medium on the other, are required for optimum activity of the lactic streptococci in reconstituted spray process milk.

Stimulation of lactic streptococci in starter culture by addition of peptide-rich fractions provided additional evidence of the importance of such fractions to starter activity.

Alcohol-soluble extracts of a milk culture of Leuconostoc sp. and tryptic digests of lactalbumin and insulin were exceptionally effective in stimulating growth of starter culture in milk. Tryptic digest of casein and commercial hydrolysates of milk and liver fractions also produced some stimulation, but of much less magnitude.

Large differences in response to protein hydrolysates were noted between strains; certain cultures were inhibited and others decidedly stimulated by identical concentrations of the same hydrolysate. One possible explanation of this effect deals with antagonists of amino acid metabolism by units within the protein

hydrolysate. As numerous investigators have reported differences within amino acids essential for lactic acid bacteria, one might also expect differences in response to competitive antagonists of amino acids.

Stimulatory factors which may have been peptides were isolated from the alcohol-soluble extract of leuconostoc culture. Specific amino acids included in one peptide were glutamic acid, serine and leucine. A second was found to contain glutamic acid, glycine and a unit of undetermined structure.

Studies suggest that beneficial effects of heat on milk used for a culture medium for lactic streptococci may be accounted for on the basis of (1) heat stimulated hydrolysis of casein and to a lesser extent (2) possible formation of a protein-lactose metabolite.

Effects of heat on milk constituents in a defined system were studied by heating milk protein in the presence and absence of lactose for varying periods of time. It was found that autoclaving casein at 121°C. resulted in hydrolysis which continued for as long as heating was maintained (60 min.). Autoclaving a mixture of these components for 15 minutes resulted in increase in free amino nitrogen but continued autoclaving resulted in a decrease in amino nitrogen.

Thus the detrimental effect of prolonged autoclaving

of milk protein nutrient systems was shown to be due to uptake of amino nitrogen in the presence of lactose. Addition of amino nitrogen in the form of protein hydrolysates to overheated milk was shown to restore activity of cheese and butter starters in such milk.

The detrimental effect of overheating was partially overcome or prevented by presence of whey proteins, insulin and cystine.

Low heat non-fat dry milk solids were usually improved as a medium for the lactic streptococci of starter culture by autoclaving at 121°C. for 15 minutes. Similar milk which had received high forewarming temperatures usually supported less activity after autoclaving. For this reason the heat activated changes in nutritive properties appeared to be additive in nature.

Studies with purified milk protein nutrient systems may provide an explanation of the direct relationship between level of peptide nitrogen and activity of starter culture. Of the milk proteins tested, lactalbumin was the only native protein which was readily utilized as the sole source of protein by growing cells of lactic streptococci.

Calcium phosphate-calcium caseinate complex did not support growth but isoelectrically precipitated casein served as a reasonably good source of protein for growing

cells.

Studies with cell free extracts have shown that lactic streptococci possess enzymes which will hydrolyze calcium phosphate-calcium caseinate complex. Resting cells, however, did not appear to have such enzymes available for extracellular functions or at least substrate and enzyme were unable to achieve contact in such a way as to bring about hydrolysis. It appears, therefore, that failure of starter culture bacteria can not be explained simply on a basis of enzyme specificity. These results also explain, in large measure, reasons for the dependence of lactic streptococci on lower molecular weight fractions in milk and the observed correlation between activity of starter culture and peptide level in milk.

Growing culture removed low molecular weight protein fractions from synthetic milk nutrient systems. Uptake of TCA-soluble nitrogen continued until acid produced by the growing culture reduced pH to near the isoelectric point. After the isoelectric point of casein was approached, level of TCA-soluble nitrogen increased and growing cells appeared able to hydrolyze cell coagulated casein.

Fundamental differences in response to heated milk between species common to starter culture and species

such as S. thermophilus indicated that the lactic streptococci respond primarily to increases in available nitrogen while S. thermophilus was stimulated in instances where protein was heated with lactose. Growth of this species in unheated milk was minimal but could be greatly stimulated by the addition of a heated mixture of cystine and lactose.

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