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RIPENING, TEXTURAL AND FLAVOR CHARACTERISTICS OF REDUCED FAT CHEDDAR CHEESE MADE WITH ADJUNCT CULTURES OF *Lactobacillus casei* L2A.

by

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ABSTRACT

RIPENING, TEXTURAL AND FLAVOR CHARACTERISTICS OF REDUCED FAT CHEDDAR CHEESE MADE WITH ADJUNCT CULTURES OF *Lactobacillus casei* L2A.

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Twenty-eight batches of cheese were made to evaluate the use of adjunct cultures of *Lactobacillus casei* L2A to increase flavor intensity, improve texture and decrease the incidence of bitterness in reduced fat Cheddar cheese. Cheeses were made with different levels of live and heat shocked cells of an adjunct culture of *Lactobacillus casei* L2A and a slow acid producing culture of *Lactococcus lactis* var. *lactis*. Cheese proteolysis was monitored for 20 weeks through determinations of pH 4.6 and 12% TCA-soluble nitrogen and SDS polyacrylamide gel electrophoresis. Adhesiveness, cohesiveness, springiness and hardness were measured by the Instron Universal Testing Machine. A trained panel evaluated the same cheese textural parameters, flavor intensity and development of bitterness after 8 months of ripening. Hydrophobic peptide extracts from

cheeses were analyzed by high performance liquid chromatography on a reverse phase column.

Reduced fat Cheddar cheeses had a high moisture content (average 49%), which probably enhanced *Lactobacillus casei* activity. Cheeses made with heat-shocked *Lactobacillus casei* presented the highest degree of proteolysis and the greatest amount of 12% TCA-soluble nitrogen. In all cheeses, α_{s1}-casein was more extensively degraded than β-casein, especially when heat shocked cells of *Lactobacillus casei* were used. Cheeses made with live cells of *Lactobacillus casei* were more acidic and, consequently, harder, less cohesive and less springy. Hardness decreased in all cheeses during the first 8 weeks of ripening as the result of the breakdown of the casein matrix. A lack of typical Cheddar cheese flavor was noticed by the sensory panel in all cheese samples. Increasing the inoculation concentration of heat shocked cells of *Lactobacillus casei* to the milk was the best approach to reduce bitterness in the cheese. Chromatographic data showed that the peptide fraction corresponding to a distinctly bitter cheese had a high concentration of low molecular weight and highly hydrophobic peptides. This peptide fraction was rich in hydrophobic amino acids like phenylalanine and tyrosine. The bitter taste intensity was somewhat reduced when heat shocked cells of *Lactobacillus casei* were used as an adjunct culture.

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Abbreviation key: WSN = water soluble nitrogen; **FDM** = fat in the dry matter; **TCA-N** = trichloroacetic acid soluble nitrogen; **LLC-0.5, LLC-1.0, LLC-1.5** = addition of 0.5, 1.0 and 1.5% of live *Lactobacillus casei* L2A; **HSLC-0.5, HSLC-1.0, HSLC-1.5** = addition of 0.5, 1.0 and 1.5% of heat shocked *Lactobacillus casei* L2A; **HPLC** = high performance liquid chromatography.

INTRODUCTION

The growing consumer interest in low calorie food has led the cheese industry to provide consumers with a large variety of reduced fat cheese. However, reduced-fat cheese has been frequently associated with lack of flavor and a tendency to develop excessive off-flavors. The off-flavors typically noted in reduced-fat cheese are bitter, brothy and meaty (Johnson and Chen, 1991; Olson and Johnson, 1990b), which are probably imparted by specific peptides (Edwards and Kosikowski, 1983; Harwalkar and Elliott, 1971; Olson and Johnson, 1990b; Richardson and Creamer, 1973; Stadhouders et al. 1983; Visser, 1977b and 1977c). In addition to off-flavor development, texture and body defects have also been associated with reduced-fat cheeses (Johnson and Chen, 1991; Olson, 1984; Simard, 1991). The partial removal of fat from cheese has been considered the main cause for the appearance of these defects (Banks et al., 1989; El-Neshawy et al. 1986; Hargrove, 1966b; McGregor and White, 1990a; Olson and Johnson, 1990a).

Cheese is described as a three dimensional network formed by casein in which the milk fat globules are embedded (Rosenberg, 1992). The rest of the matrix is filled by a

continuous phase which is comprised of soluble proteins, minerals, sugars, vitamins, minor components and microbial flora. The stabilization of this multi-component structure is sustained by numerous physical and chemical interactions. The physicochemical interactions and microbial activity that occur during cheesemaking and ripening determine the quality attributes of the cheese. The delicate balance of the specific concentrations of each of the cheese components is responsible for the type and extent of these interactions.

To produce reduced-fat cheese, the traditional manufacturing procedure must be changed (Banks et al., 1989; Hargrove et al., 1966b; Madsen et al., 1970; Olson and Johnson, 1990a). As milk fat is reduced, cheese moisture must be increased to improve flavor, body and texture properties of reduced fat cheese (Simard, 1991). The consequence of changes in the balance of the cheese components may lead to the occurrence of texture and flavor problems.

Fat is known to contribute to physical characteristics giving opacity and influencing cheese rheology, and helps solubilize many flavor compounds modifying their perception and volatility, especially in the mouth. Fat also enhances smoothness and mouthcoating and reduces cheese cohesiveness (Degouy, 1993). The removal of fat from cheese will cause the formation of a more compact mesh in the protein network, with great impact on several mechanical properties of the cheese (McGregor and White, 1990a; Rosenberg, 1992). The relatively

high water levels required for reduced fat cheese manufacturing reduces firmness of the curd (Rosenberg, 1992). Removal of fat, which is considered a precursor of many flavor components, will also lead to a lack of typical flavor in the final product. The oil/water interface in the cheese is of critical importance for microbial growth. The reduction in the fat level causes flavor changes by affecting the microbial flora. Increased water content in the cheese also alters the microbial growth patterns and enzyme activity, which may lead to enhanced proteolysis and consequently to off-flavor production (O'Donnell, 1993).

The choice of a suitable starter culture is of utmost importance for production of a quality reduced-fat cheese. Not all lactic starter cultures appear to be suitable for reduced fat cheese (Olson, 1991). Evidence has demonstrated that slow acid producing cultures with less proteolytic activity and possessing specific debittering activities should be preferable for low-fat cheese (Rosenberg, 1992). The use of starter culture adjuncts such as *Lactobacilli* spp. may reduce bitterness and improve flavor in reduced-fat cheese (Ardo and Mansson, 1990; Bartels et al. 1987; El-Abboudi et al. 1991; O'Donnell, 1993; Trepanier et al. 1992).

The objective of this paper is to verify the impact of an adjunct culture of *lactobacilli* on the texture and flavor of reduced fat Cheddar cheese. A softening of the curd, improvement in flavor, and elimination of the bitter flavor

commonly associated with reduced fat cheeses would increase the consumption in this cheese category.

LITERATURE REVIEW

1. REDUCED FAT CHEESE

In the last two decades we have seen dramatic shifts toward the consumption of low fat foods (Egbert et al., 1991). The wish to avoid high calorie food to control body weight and a possible connection between animal fats and circulatory disorders have been the main causes for this increased consumption of low fat foods (Hargrove et al. 1966a; Madsen et al. 1970; Simard, 1991).

According to Dexheimer (1992), a projection of sales of low fat foods showed that this food category will hit $41 billion by 1995. Reduced fat cheese will account for an estimated 9% of the retail cheese market valued at $6.7 billion. A survey conducted by Delta's New Products News showed that cheese accounted for 23% of the total new non-fat/low fat products introduced in the market in 1991. This number increased to 36% in 1992 (Rosenberg, 1992).

To service the growing consumer market in low energy food, several attempts have been made by cheese manufacturers to develop a palatable reduced fat cheese (Simard, 1991). Despite the increasing consumption in reduced fat cheeses, several problems have been encountered in their production. Lack of flavor, development of off-flavors (meaty-brothy, bitter, and unclean), and body defects (hardness or softness, weakness, and pasty) are the three main factors responsible for the low quality of reduced fat cheeses when compared to their full fat counterparts (Johnson and Chen, 1991). The lack of the flavor masking effect of fat which greatly contributes to cheese flavor makes the off-flavors more readily perceived in reduced fat cheeses. The meaty/brothy flavors are generated in cheese by the flavor precursors furanones and pyrazines, produced by the browning type reaction between α-dicarbonyls and amino acids. Unclean off-flavor is generated by Strecker-type reactions which produce aldehydes and alcohols (Lindsay, 1991). Bitterness is caused by the accumulation of bitter tasting peptides in cheese, formed by enzymatic attack on the caseins (Emmons et al. 1962b; Lowrie and Lawrence, 1972; Stadhouders, 1974; Sullivan and Jago, 1972; Sullivan et al. 1973).

Levels of fat in cheese have a direct impact on the acceptability by consumers. Fat is the precursor of many flavor compounds and influences the body and texture of cheese. Total or partial removal of fat from cheese leads to

hard, tough, dry body and low flavor levels of the cheese (McGregor and White, 1990a).

The use of traditional procedures to make reduced fat cheese has led to the production of a very hard, rubbery and tasteless cheese, even after extensive ripening (Banks et al. 1989; El-Neshawy et al. 1986; Hargrove et al. 1966b; Simard, 1991). To improve the flavor, body and texture of reduced fat cheese, the traditional manufacturing procedure must be changed (Banks et al. 1989; Hargrove et al. 1966b; Madsen et al. 1970; Olson and Johnson, 1990a) and slow acid producing starters associated with low proteolytic activity should be chosen (Olson, 1991). Several attempts have been made to improve the quality of reduced fat cheeses, including changes in the traditional manufacturing process (Banks et al. 1989, Chen, 1991; Chen et al. 1992), milk preparation prior to cheese manufacture (McGregor and White, 1990a and 1990b; Metzger and Mistry, 1993), culture selection (Chen et al. 1992; Simard, 1991), and use of fat replacers (El-Neshawy et al. 1986).

One way to improve the texture of reduced fat cheese is to increase the water content in the curd. The moisture content in reduced fat cheese must be higher than that normally employed in full fat cheese to improve the textural properties of the cheese (Banks et al. 1989; Simard, 1991). Milk fat globules fill the interstitial spaces within the protein network of the cheese (Rosenberg, 1992). Removal of

fat from cheese causes the formation of a more compact mesh in the protein network, consequently yielding a hard cheese. Increased water content occupies the space previously occupied by fat, loosening the protein network with positive effects on the textural properties of the cheese (Bryant, 1993). However, the relatively high moisture content required to promote the desired textural characteristics in reduced fat cheeses may also lead to excessive proteolysis and consequently to off-flavor production. If lactic acid production is not controlled during the manufacturing procedure, high moisture cheese develops a very acid flavor and the body becomes weak and pasty with age (Johnson and Chen, 1991). According to Degouy (1993), the key issues in reduced fat cheese manufacture consist of the replacement of fat properties, binding of water and control of the proteolytic activity to develop flavor and avoid bitterness formation.

Increased moisture retention in the curd (44-45%) can be achieved by cutting the curd using larger knives (1 cm instead of 0.6 cm), applying a lower cooking temperature and milling the curd at a higher pH (5.9 vs 5.2) than normally used for full fat Cheddar cheese (Chen, 1991). A modification of the standard Cheddar process to produce a low fat cheese with high moisture content was implemented by Banks et al. (1989). They developed a technique for production of a reduced fat Cheddar cheese containing intermediate and low fat content. A control Cheddar cheese with 37.9% moisture and 33.1% fat, an

intermediate fat Cheddar cheese with 42.9% moisture and 25.6% fat and a low fat cheese with 47.2% moisture and 16.8% fat were produced by decreasing cook temperature and reducing stirring and cheddaring time. The scalding temperature was reduced from 39°C (control cheese) to 37°C and 35°C in the intermediate and low fat cheeses, respectively. The stirring time for both the experimental cheeses was 30 minutes as compared to 60 minutes for the control cheese. The control cheese was cheddared for 90 minutes while both the intermediate and low fat cheeses were cheddared for 60 minutes. They found that the flavor and physical properties of the cheese with intermediate fat content was comparable to those of regular Cheddar cheese, but the cheese with low fat content was over-firm and did not develop an adequate Cheddar flavor. According to Olson and Johnson (1990a) reduction of the fat content by 33% yields acceptable cheese, but reduction of fat over 50% results in cheese with poor flavor quality and physical properties.

The effects of milk pasteurization temperatures, starter culture and rennet levels, and drain and mill pH on the quality of a 33% reduced fat Cheddar cheese were studied by Chen (1992). Their results showed that a pasteurization temperature of 77.7°C produced a cheese with higher moisture content than that made with milk pasteurized at 73.3°C. The cheese made with a 2% starter level produced higher Cheddar flavor intensity than cheese made with 0.5% of starter level,

but the cheese presented excessive acid off-flavors and inferior body. Improved Cheddar flavor and body were obtained when higher rennet levels were used, especially for young cheeses. But over 6 months of ripening lower rennet level cheeses were preferable because the cheeses presented fewer off-flavors and had higher Cheddar flavor quality. They concluded that pH control is a critical factor in producing a good quality 33% reduced fat Cheddar cheese. A pH of 6.37 at drain increased the moisture content, flavor intensity and body breakdown when compared with a drain pH of 6.13. Moisture content and flavor quality were also higher in cheeses with higher mill pH.

The use of a lower cooking temperature or simply elimination of cooking from the manufacturing process can also be used to achieve higher moisture content in the cheese. Elimination of the cooking temperature (45.9°C for 15 minutes), increased the moisture content in Mozzarella cheese, causing a softening of the curd with consequent improvement in the textural properties (Tunick et al. 1991).

According to Olson and Johnson (1990a), pH of the cheese can also be controlled by washing the curd or partially diluting the whey with water. Washing the curd before salting has the advantage of reducing the lactose and lactic acid concentration in the cheese. Reducing the concentration of lactose by washing the curd results in a higher pH at drainage, thus increasing the water retention in the cheese

(Simard, 1991). However, this process resulted in large quantities of salt-drippings being expelled during pressing (Olson and Johnson, 1990a).

Milk homogenization appears to contribute to improved texture and smoothness of reduced fat cheese. According to Olson and Johnson (1990b), a softer and less elastic Cheddar-type cheese was produced when the cheese milk containing 1.4% fat was homogenized prior to manufacture. This result was in accordance with Emmons et al. (1980) who, in addition to a softer and less elastic cheese, found a slightly higher moisture content in the cheese made with homogenized milk when compared to non-homogenized milk. In addition to improvement in texture, homogenization of cream or whole milk used to standardize milk for reduced fat cheese production can improve flavor, possibly due to the increased dispersion of fat globules, which make them more accessible for enzyme reactions (Hargrove et al. 1966b). Homogenization pressures higher than 2,500 psi are not recommended. Use of high homogenization pressures produces brittle and inferior quality cheese (Hargrove et al. 1966b; Olson and Johnson, 1990b). Tunick et al. (1991) also noticed adverse affects on the rheological and melting properties of reduced fat Mozzarella cheese when high homogenization pressure (17200 kPa) was applied to cheese milk, as opposed to low homogenization pressure (10300 kPa).

When studying the effect of homogenization on the quality of reduced-fat Cheddar cheese, Metzger and Mistry (1993) verified that homogenization of the cream prior to the standardization process improved the texture of the cheese. They observed that in all treatments where skim milk or standardized milk were submitted to homogenization, an extensive curd shattering occurred in the cheese, but when skim milk was standardized to 1.9% fat with the previously homogenized cream, curd shattering did not occur.

Ultrafiltration (UF) is another method that can be applied to enhance the quality of reduced fat cheese. According to McGregor and White (1990a), two advantages of UF process for making cheese are increased retention of whey proteins in the curd with positive effects on yield and reduction of calcium which favors the softness of the cheese (McGregor and White, 1990a). Acidification and diafiltration were performed along with the ultrafiltration process. The advantages of these two treatments were an enhancement in the removal of calcium from the protein matrix, reducing the firmness and improving the body and texture of cheese, and a reduction in the level of lactose in the retentate to a point that allows better control of the pH. The results showed a significant improvement in the flavor and body and texture of the cheese manufactured from acidified and diafiltered UF lowfat milk as compared with the cheese made from UF lowfat milk without acidification and diafiltration.

Condensation of the milk prior to cheese making can be another alternative to improve the quality of reduced fat cheeses. Anderson et al. (1992) made reduced fat Cheddar cheese from condensed skim milk standardized with cream. The treatments employed in the experiment were: non-condensed 10.3% total solids (TS) as a control; condensed, 15.4% TS; condensed, 18.3% TS and condensed, 22.2% TS. Cheeses made with condensed milk with the highest level of total solids were extremely firm and crumbly. The results suggested that the condensed milk with 15.4% TS and 18.3% TS were more suitable for reduced fat cheese making.

Fat replacers have been added to standardized milk to enhance the quality of reduced fat cheese. El-Neshawy et al. (1986) carried out an experiment to improve the body and organoleptical properties of low-fat Ras cheese. Carrageenan and carboxymethylcellulose (CMC) were added to cheese milk containing 1%, 1.5% and 2% fat to improve the water retention. These compounds form complexes with milk proteins increasing the water binding capacity, with positive effects on the moisture retention of the curd. They found that cheese made from milk containing no stabilizers was hard, tough and deficient in flavor. The addition of stabilizer to cheese milk enhanced the softness and smoothness of low fat cheese, improving body characteristics.

According to Olson and Johnson (1990a) the lactic starter cultures have a pronounced effect on all cheeses, but especially on reduced fat cheeses. In the manufacture of reduced fat cheese, starter cultures possessing lower rate of acid production, lower proteolytic activity (Olson, 1991; Rosenberg, 1992), and debittering activity (Rosenberg, 1992) should be preferable. Lactic starters commonly used for full fat cheeses are frequently associated with meaty/brothy flavors in reduced fat cheeses (Johnson and Chen, 1991).

The effects of four different starter cultures on the ripening of reduced-fat cheese were evaluated by Chen et al. (1992). They used *Lactococcus lactis* subsp. *lactis* and *cremoris*, and two strains *of Lactococcus salivarius* subsp. *thermophilus*. They verified that the cheese produced with *S. thermophilus* were acceptable but exhibited less lactic acid and flavor development, and also less protein breakdown. More typical Cheddar cheese was produced with the two strains of *Lactococcus*. The *Lactococcus lactis* subsp. *lactis* developed lower proteinase and higher peptidase activity, while the *Lactococcus lactis* subsp. *cremoris* developed higher proteinase and lower peptidase activity.

The use of *lactobacilli* as a starter culture adjunct has been successfully applied as a way to reduce bitterness and improve flavor in cheese (El-Neshawy et al. 1986; El-Soda et al. 1981; El-Soda, 1986; Hargrove et al. 1966b; Johnson et al. 1993; O'Donnell, 1993; Simard, 1991; Trepanier et al. 1991a,

1991b and 1992). Johnson et al. (1993) used *Lactobacillus helveticus* CNRZ attenuated by spray-drying, freeze-drying or freeze shocking in the manufacture of reduced fat cheese. They found that proteolysis as measured by trichloroacetic acid (TCA) and phosphotungstic acid (PTA) soluble nitrogen was higher in the cheeses made with attenuated *lactobacilli* adjuncts than that in the control cheese. They also observed that the addition of attenuated *lactobacilli* produced a cheese with enhanced flavor and body without causing bitterness or excessive softening of the curd.

El-Neshawy et al. (1986) demonstrated that the addition of *Lactobacillus casei* at the ratio of 1:1 with *S. lactis* subsp. *lactis* in combination with $MnCl_2$, enhanced the flavor of Ras cheese. Hargrove et al. (1966b) also observed improvement in the flavor quality of reduced fat Cheddar cheese when *Lactobacillus casei* used as supplemental starter was added to the cheese milk as a 0.5% inoculum.

2. TEXTURE DEVELOPMENT DURING RIPENING

The ripening of cheese is a complex process which involves several biochemical changes, including the fermentation of lactose, the degradation of proteins, and the hydrolysis of fat (Law et al. 1992; Schormuller, 1968). All these processes result in a gradual change in the cheese curd from a coarse structure to a more or less plastic homogeneous

substance (Schormuller, 1968) and in the development of the aroma and taste that together constitute the typical cheese flavor.

The casein comprises a group of phosphoproteins, composed of four different fractions, α_{s1}- , α_{s2}-, β- and κ-casein (Eigel et al. 1984), in a proportion of 3:1:3:1 (Grappin et al. 1985). In addition to the four principal gene products, there are several minor proteins which originate mainly from post-secretion proteolysis of the primary caseins by the action of plasmin. These include the λ-caseins and the proteose peptones 5 and 8-fast, all derived from the β-casein (Fox, 1992). The four primary caseins differ within each fraction according to the degree of phosphorylation and glycosylation (Grappin, 1985).

Proteolysis is considered the most important biochemical event during cheese ripening and affects both flavor and texture (Fox, 1989a). These biochemical changes are catalyzed by enzymes, which may have their origin in coagulating agents, starter cultures, the naturally occurring or contaminating microbes in milk, or possibly the enzymes present in the original milk (Desmazeaud and Gripon, 1977; Kamaly and Marth, 1989; Scolari et al. 1993). During this process, casein, the major structural component of curd, is slowly broken down to smaller compounds and the cheese loses its rubbery character, acquiring the desired texture (Kamaly and Marth, 1989). The rate, extent, and nature of these changes during cheese

ripening, as well as the amount and nature of the degradation products depends upon the enzyme involved in the process and the ripening conditions (Grappin et al., 1985).

2.1. Effect of proteolysis on texture

The pH and the ratio of intact casein to moisture are the two main factors responsible for texture changes during cheese ripening (Lawrence et al., 1987). Changes in textural properties in different cheese varieties are due to proteolytic activity on α_{s1}- and β-casein which constitute the cheese protein matrix (Bertola et al., 1992). Rennet is the first proteolytic agent involved in casein breakdown (Desmazeaud and Gripon, 1977). Proteolysis of α_{s1}-casein rendering the polypeptide α_{s1}-I is considered the first phase in texture development during ripening. This phase takes place during the first two weeks of ripening, when the rubbery texture of the young cheese is converted to a smoother body. The cheese matrix is greatly weakened when only a single bond in about 20% of the α_{s1}-casein is cleaved by the coagulant yielding the α_{s1}-I peptide. The second phase involves a slow degradation of the rest of the α_{s1}-casein and other caseins with more gradual changes in the texture (Lawrence et al. 1987).

Beta-casein undergoes very little proteolysis in cheese (Fox, 1989a and 1993; Phelan et al., 1973) but in solution it is readily hydrolysed by the action of chymosin and other rennets to yield β-I, β-II and β-III caseins (Visser and Slangen, 1977). The intermolecular hydrophobic interactions between the hydrophobic C-terminal region of β-casein, which contains the chymosin susceptible sites for cleavage, is the most plausible explanation for its high resistance to hydrolysis by rennet in cheese (Fox, 1989a).

The rate of proteolysis is affected by several factors. These include rennet and plasmin retention in the curd, salt to moisture ratio, temperature of ripening, type of coagulant used, and changes in pH (Lawrence et al. 1987).

Although most coagulant is lost in the whey at drainage, some remains entrapped in the curd. The amount entrapped depends upon the total quantity added to the cheese milk, the pH of cheese milk and curd, cooking temperature and rate of acid production by the starter culture (Visser, 1993). Holmes et al. (1977) verified that rennet retention in the curd is pH dependent. The rennet retention increased as the pH of the curd decreased. At lower pH, a greater proportion of α_{s1}-casein was hydrolysed when calf rennet was used. An important fact is that the retention of microbial rennet in the curd, unlike calf rennet, is not affected by changes in milk pH. The extent of degradation of α_{s1}-casein in the presence of microbial

rennet was the same irrespective of the initial pH of the milk (Lawrence et al. 1987).

The proportion of plasmin retained in the cheese curd is also important in proteolysis. Plasmin is a native milk proteinase which is associated with the casein micelles (Fox, 1989b; Visser, 1993). Plasmin activity in cheese is evidenced by the accumulation of γ-caseins (Creamer, 1975; Richardson and Pearce, 1981). Plasmin activity is very important in cheese with high pH, such as Swiss type cheeses and other high cook varieties in which the coagulant is denaturated (Matheson, 1981).

The effect of the salt to moisture ratio on the susceptibility of α_{s1}- and β-casein to hydrolysis was demonstrated in an experiment in which Cheddar cheese was made with linear S/M gradients across a single 20 kg block (Thomas and Pearce, 1981). They verified that the salt concentration had a large effect on the rate of proteolysis of both α_{s1}- and β-casein. In one-month old cheese containing 4% S/M (salt in moisture) approximately 5% of the α_{s1}-casein and 50% of the β-casein remained intact, whereas in the cheese containing 8% S/M, 60% of the α_{s1}-casein and 95% of the β-casein was unhydrolysed. A linear relationship between S/M and proteolysis was found between the two extremes.

The hydrolysis of α_{s1}- and β-casein is greatly affected by temperature. A Cheddar cheese ripened at 15°C for 8 days developed the same texture as a Cheddar cheese ripened at 10°C

for 15 days. When the temperature was increased to 20°C the cheese became more brittle and less springy. Cheese proteolysis correlated negatively with cheese firmness and springiness, indicating softening of the cheese as the protein framework was degraded (Lawrence et al. 1987).

Protein breakdown was reported to increase in cheese with low acidity (Noomen, 1978). At higher pH (6.2), β-casein was more quickly degraded than α_{s1}-casein whereas at lower pH (5.4), α_{s1}-casein was more extensively degraded than β-casein. At pH above 5.6, β-casein was degraded more than α_{s1}-casein, presumably due to the action of increased plasmin activity.

2.2. Measurements of textural properties

Rheology is defined as the study of deformation and flow of matter (Shoemaker et al. 1992). The rheological properties of cheese affect eating quality (consistency in the mouth), usage (cutting, grating, spreading, melting, etc), handling and packaging (Walstra and Peleg, 1991).

Cheese exhibits both fluid and solid like properties and is considered a viscolastic material (Konstance and Holsinger, 1992; Shoemaker et al., 1992). This implies that at short times of deformation, with low strain levels, cheese behaves as an elastic material, recovering its original shape when the stress is removed. At longer times, cheese behaves as viscous material, because most of the deformation remains after the

stress is removed (Walstra and Peleg, 1991). The yield stress, the point where permanent deformation occurs (van-Vliet, 1991), is very small for the majority of the cheeses. Even a small force applied to the test piece can cause some permanent deformation (Walstra and Peleg, 1991).

Caseins are the major structural component in cheese, but the milk fat greatly contributes to product quality (Konstance and Holsinger, 1992). The removal of milkfat by 50% or more in Cheddar-type cheeses has led to unacceptable flavor and physical properties (Olson and Johnson, 1990a). With the increased demand for a reduced fat Cheddar cheese that simulates its full fat counterpart, evaluation of textural properties has assumed even greater importance (Konstance and Holsinger, 1992).

The caseins, fat and moisture are responsible for the structure and rheological properties of the cheese (Prentice, 1992). Casein forms a continuous meshlike structure, giving the "solid" nature of cheese and entrapping fat and water. Fat acts mainly as a filler. At low temperatures, fat contributes to the rigidity of the casein matrix. When the temperature is 20°C or above, fat is in a liquid form and has little contribution to cheese firmness. Water moves freely in the space between fat and casein and acts mainly as a low-viscosity lubricant (Prentice, 1992). The viscoelastic properties of cheese arise from the interaction among the major components (Lee et al. 1992).

Most of studies on mechanical and textural properties of cheeses have been obtained through uniaxial compression tests performed by the Instron Universal Testing Machine (Instron Corporation, Canton, MA) (Ak and Gunasekaran, 1992). The use of the texture profile analysis (TPA) in two successive compression cycles is the method most often used for food analysis (Bertola et al., 1992).

The Instron Universal Testing Machine can be used to measure multiple textural parameters, such as hardness, fracturability, adhesiveness, cohesiveness, springiness, gumminess and chewiness (Tunick and Nolan, 1992). There are a wide array of test conditions to be considered when evaluating the rheological properties of cheese, including specimen dimensions, deformation rate, and surface friction and sample lubrication (Sherman, 1989).

Culioli and Sherman (1976) studied the influence of cheese maturity, test temperature, sample shape, sample height and surface area on the force-compression behavior of Gouda cheese in the Instron Universal Testing Machine. The force required to achieve a given percent compression increased from the center to the edge of the cheese block for both young and mature cheese. The force required to achieve any given compression correlated inversely with temperature. The force also decreased with increasing sample height. The sample shape also had influence on the compression force. Cubic samples

required a higher force to achieve a given percent compression when compared to cylindrical shapes.

The effect of sample lubrication and deformation rate on the rheological properties of Cheddar cheese under uniaxial compression test was investigated by Ak and Gunasekaran (1992). Six different deformation rates were applied to both lubricated and non-lubricated cheese samples. There was no significant effect of lubrication on the parameters studied, but the data from the non-lubricated tests had higher coefficients of variation (CV = 31%) than those from the lubricated test (CV = 13.5%), suggesting that lubrication gives more reproducible results. Chu and Peleg (1985) studied the compressive behavior of selected food materials, including process American cheese, with small height-to-diameter ratios. Their results showed that the dimensional effect was more pronounced when there was considerable friction at sample-machine contact surfaces. They noticed that thinner specimens appeared stiffer, as measured by deformability modulus.

Bertola et al. (1992) analyzed rheological parameters during ripening of Tybo Argentino cheese. They applied the compression test in two successive cycles to determine hardness, cohesiveness and adhesiveness. Two viscoelastic parameters (elastic moduli and relaxation times) were also determined. Hardness decreased and adhesiveness increased during ripening whereas cohesiveness did not show any relevant variation. Analysis showed degradation of α_{s1}-casein was more

rapid than β-casein. They concluded that high concentrations of intact α_{s1}-casein contributed to the rigidity of the protein framework whereas low concentrations contributed to the increased elasticity and softening of the cheese. They found good negative correlation between both water and TCA-soluble nitrogen and concentration of intact α_{s1}-casein with both correlation coefficients higher than -0.88.

Force-compression tests were also performed on Cheddar cheese to evaluate the starter culture effect on ripening characteristics and rheological parameters (Amantea et al., 1986). Six strains of bacteriophage-insensitive *Lactococcus cremoris* and conventional *Lactococcus cremoris* were used in the experiment. Rheological parameters were correlated to the degree of proteolysis as measured by trichloroacetic acid (TCA)-soluble nitrogen, age, pH and moisture content. They found that the firmness decreased with increased moisture content in the cheese. They also verified that the yield point of the force-compression curves, when permanent deformation occurs (van-Vliet, 1991), decreased as the free amino groups increased with age. Creamer and Olson (1982) also noted an inverse correlation between yield force and the concentration of intact α_{s1}-casein in the cheese, suggesting that the texture of the cheese softens during aging.

Based on the correlation between firmness and the quantity of intact α_{s1}-casein in the cheese (Walstra and van-Vliet, 1982), Lawrence et al. (1987) found an explanation for

the weakening of the cheese with age. The breakdown products formed during casein breakdown were essentially water soluble and therefore did not contribute to the protein matrix, causing the softening of the cheese.

Green et al. (1981) used the Instron Universal Testing Machine to study the texture of Cheddar cheese made from concentrated milk. Firmness and cohesiveness of the cheese increased as the concentration factor of the milk increased, suggesting a more rigid structure in the concentrated milk cheeses. Adhesiveness, which expresses the capacity of the cheese particles to stick to a steel plate, decreased with increased concentration of milk, probably due to the reduced level of fat and lower proteolysis in the protein matrix of the more concentrated milk cheeses. Elasticity was little affected by increased milk concentration, therefore being less affected by differences in composition, network structure and extent of proteolysis.

Stampanoni and Noble (1991a and 1991b) also used the Instron to study rheological parameters in cheese analogs made with varying amounts of fat (10, 17.5 and 25%), sodium chloride (0.5 and 2.0%) and citric acid (0.1 and 1.2%). They verified that the cheeses with higher fat level was softer, less springy and more cohesive and more adhesive than cheeses with lower fat level. When salt or acid content was increased, firmness also increased while cohesiveness and springiness decreased significantly.

The ultimate objective of rheological investigation on cheese is the correlation of the measured instrumental properties with sensory data (Konstance and Holsinger, 1992). Lee et al. (1978) evaluated rheological properties of commercial cheeses representing a wide variety of texture using both instrumental methods and sensory panels. The cheeses used in the experiment included Cream cheese, Camembert, Mozzarella, Muenster, Swiss cheese, American processed cheese, and mild, sharp and processed Cheddar cheese. Hardness, elasticity, adhesiveness and chewiness as assessed by the Instron correlated significantly with the same parameters evaluated by the sensory panel.

Chen et al. (1979) also used the Texture Profile Analysis (TPA) curve obtained by the Instron to evaluate the parameters of firmness, cohesiveness, chewiness and adhesiveness in 11 cheese varieties. They used a plunger (d = 6.4) at a crosshead speed of 2.5 cm/min. to penetrate the cheese samples to a depth of 1.91 cm. The sample height was 2.54 cm. The same samples were submitted to a trained panel and both instrumental and sensory results were correlated. Each of the four parameters evaluated by the trained panel correlated significantly with the corresponding parameters measured by the Instron, with the correlation coefficients ranging from 0.79 to 0.85.

3. FLAVOR DEVELOPMENT DURING RIPENING

Despite intensive research done on Cheddar cheese flavor over the past years, the agents responsible for flavor formation and how they carry out their functions are poorly understood. The reactions involved are very complex and there is a wide spectrum of products formed, some in very low concentration (Aston and Dulley, 1982).

More than 100 potential flavor compounds have been identified in over 800 different cheese varieties recognized in the world. These flavor compounds result from the action of microorganisms and enzymes on the carbohydrates, fat and proteins present in the cheese. These components are broken down to lactic acid, fatty acids and amino acids, respectively, generating what is termed the primary step of cheese ripening. These primary products, with exception of fatty acids, contribute little to cheese flavor. The characteristic cheese flavor is generated in the secondary step by the action of microorganisms and added enzymes on the primary products (Kristoffersen, 1973).

There appear to be only two facts on Cheddar cheese flavor formation that all investigators seem to agree: milkfat must be present in the cheese for flavor perception and lactic starters must be used to develop typical Cheddar cheese flavor (Lawrence and Gilles, 1987). The current concept which finds much support is that Cheddar cheese flavor results from the

combination of many compounds, which, when present in proper quantitative balance, give rise to a typical flavor. When an excess or lack of one or more of the component compounds is present, atypical flavor is produced (Aston and Dulley, 1982).

Harper (1959) separated the water soluble fraction containing the flavor from cheese into taste and aroma fractions. The chemical compounds included in the taste fraction included lactic acid, amino acids, non-volatile fatty acids, keto acids, non-volatile amines, salt, and fragments of proteins and fats. The aroma fraction included amines, fatty acids, aldehydes, ketones, alcohols, esters, H_2S and mercaptans.

3.1. Effect of proteolysis on Flavor

During proteolysis, the paracasein and the minor proteins are gradually converted to simpler nitrogenous compounds, namely, proteoses, peptones, polypeptides, amino acids, and ammonia (Singh and Ganguli, 1972). The rate of protein degradation varies from cheese to cheese, but protein hydrolysis to amino acids occurs to some extent in all cheese varieties (Harper and Kristoffersen, 1956). McGugan et al. (1979) suggested that highly degraded protein of aged cheese produces more flavor compounds than does the protein of a mild cheese. Sanders et al. (1946) found that proteolysis was much more pronounced in cheese made from high bacterial count milk

than in that made from low bacterial count milk, but the desirable flavor and quality of the cheese was directly related to the quality of the milk and not to the rate and extent of proteolysis.

Although the contribution of amino acids to cheese flavor is very controversial, they appeared to provide an important "background" flavor (Harper and Kristoffersen, 1956). Keeney and Day (1957) suggested that amino acids are involved in cheese flavor formation through the Strecker degradation reaction (a reaction between amino acids and dicarbonyl compounds). These reactions might occur in Cheddar cheese ripening with subsequent formation of aldehydes which could contribute to flavor. Griffith and Hammond (1989) also demonstrated that a wide variety of compounds were produced by reaction between dicarbonyls and amino acids with direct impact on Swiss cheese. Kosikowski (1951) found a direct relationship between the intensity of the flavor and the total concentration of free amino acids in cheese. Erekson (1949) reported a direct relation between cheese flavor and free tryptophan content. Glutamic acid was found to correlate well with flavor in cheese (Harper, 1959). Proline was found to impart considerable flavor in Swiss cheese as well as glutamic acid in provolone cheese (Harper and Kristoffersen, 1956).

Marked increases of amino acids coincide with optimum flavor and body (Kosikowski, 1951). This has led some investigators to use a specific amino acid as a measure of

ripening. Tyrosine results from the hydrolysis of low molecular weight peptides. An appreciable amount of tyrosine is liberated after the amount of soluble protein has reached its maximum. Therefore, total tyrosine is an important indicator of Cheddar cheese ripening (Silverman and Kosikowski, 1955). They claimed that measurement of tyrosine is a much more sensitive index of protein breakdown during cheese ripening than is soluble protein. Phosphotungstic acid (PTA)-soluble nitrogen levels were shown to be good estimates of the total free amino acid levels in Cheddar cheese (Jarrett et al. 1982).

Addition of *Lactobacillus casei* to enhance liberation of amino acids has been reported. Bullock and Irvine (1956) verified that amino acids were liberated more rapidly in cheese made with a supplemental culture of *Lactobacillus casei* than in that made with only the usual lactic starter. They found that the amino acid content of the cheese with added *Lactobacillus casei* and control cheese were similar at four months, but at 8.5 months a higher level of amino acids appeared in the inoculated cheese. Tittsler et al. (1948), added *Lactobacillus casei* to cheese milk and they observed that the resultant cheese was generally more acid, had a higher level of flavor, and a shorter body. Improvements in flavor of cheese made from milk inoculated with *Lactobacillus casei* were also noticed by Sherwood (1939).

4. BITTER PEPTIDES IN CHEESE

Bitterness is a flavor defect quite frequently encountered in Cheddar and Gouda cheese. The accumulation of bitter tasting peptides formed by the action of proteolytic enzymes from rennet and starter culture on caseins cause the defect (Emmons et al. 1962b; Lowrie and Lawrence, 1972; Stadhouders, 1974; Stadhouders and Hup, 1975; Sullivan and Jago, 1972; Sullivan et al. 1973). Czulak (1959) classified bitter flavor defects into three types, according to the time of first appearance and duration: Type I, Bitterness appears within a month or two after manufacture and disappears in 2-4 months of subsequent ripening; Type II, The defect appears as type 1 but persists indefinitely throughout the ripening; and Type III, Bitterness appears in 3-5 months after manufacturing and persists indefinitely throughout the ripening. In Cheddar cheese, bitterness has long been recognized as a major defect, although it can be found in other cheese varieties (Stadhouders, 1979). According to Czulak (1959), the first two flavor defects are the most frequently encountered in Cheddar type cheese.

4.1. Mechanism of bitter flavor formation and degradation

Formation and degradation of bitter peptides is accomplished by the proteolytic enzymes present in the cheese. The proteolytic system involved in the cheese ripening process is quite complex and may include enzymes from rennet (chymosin and pepsin), proteinases from rennet substitutes, proteinases and peptidases from starter culture, proteinases and peptidases from non-starter microorganisms and endogenous milk proteinases (Lemieux and Simard, 1992). Many studies have been conducted and several hypotheses have been established to elucidate the complex mechanism by which bitter peptides are formed and degraded in cheese.

The mechanism postulated by Czulak (1959) involves the combined action of rennet and bacterial proteolytic enzymes. The attack of proteinases from rennet on casein releases the bitter peptides which are believed to be further hydrolized by the peptidase system of non-bitter strains of lactic acid bacteria. Bitter strains of starter culture lack the ability to degrade bitter peptides which accumulate resulting in the bitter taste.

Czulak (1959) suggested that pH development plays an important role in the accumulation of bitter peptides in cheese. Experimental data (Czulak and Hammond, 1958) have shown that bitter flavor was most frequently found in cheese where the pH was 5 or less and was seldom found in cheeses

with pH higher than 5.2 during the first week after manufacture. Based on those data, Czulak (1959) postulated a mechanism (Figure 1) which would explain the transient bitterness in cheese.

The proteolytic activity of rennet enzymes is enhanced at low pH. Chymosin has its maximum activity at pH 4.9-5.5, whereas pepsin has its maximum activity at still lower pH. Enhanced proteolytic activity at low pH may be compared to that of a larger amount of rennet added to cheesemaking at normal or higher pH. A large pool of polypeptides and bitter tasting peptones are formed by this enzyme activity. The activity of the bacterial proteinases is stimulated by the breakdown of casein by rennet enzymes, but at low pH the polypeptides and peptones are not degraded at a sufficient rate and tend to accumulate in the cheese, thus generating the bitter taste.

As ripening progresses, more and more casein is broken down, causing an upward shift in pH, due to the increase in the number of amino groups formed. This increased pH favors the proteinases from the starter culture, which have their optimum at pH 6 or higher. Thus, the surplus of polypeptides and peptones is broken down to amino acids and the bitter flavor gradually disappears.

Czulak's hypothesis is supported by Emmons et al. (1962b), who explained the appearance of Type I and Type II bitterness. In Type I, bitter peptides accumulates as a result

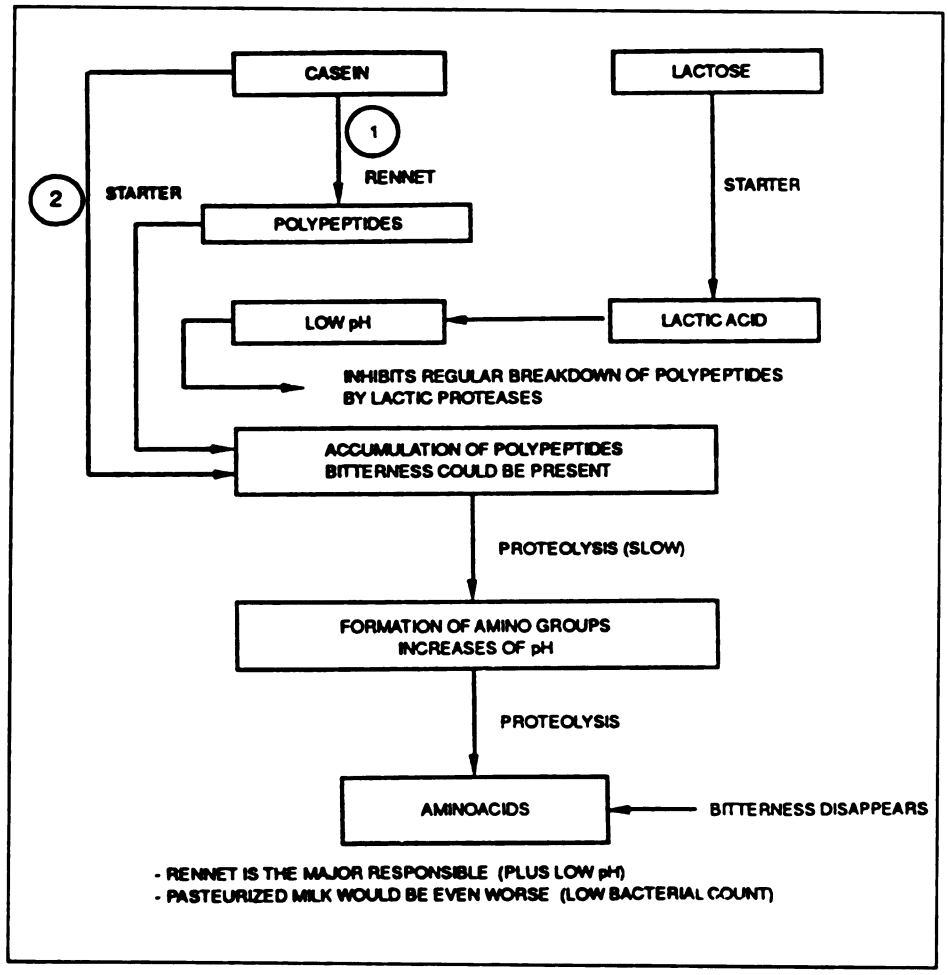

Figure 1 - Czulak's hypothesis for the formation and breakdown of bitter peptides in cheese. Source: Lemieux and Simard (1992)

of bitter strain activity. A secondary flora of *lactobacilli* may have provided a suitable peptidase activity for degradation of the bitter peptides to non-bitter peptides and amino acids, reducing the bitter flavor. In Type II, bitterness persists due to a lack of secondary peptidase activity. Although the mechanisms for Type I and Type II bitterness were supported by Czulack's hypothesis, the third type of bitter flavor, which appears later in the ripening process, was not readily explained.

In 1972, Lowrie and Lawrence (1972) formulated an alternative hypothesis to that proposed by Czulak (1959) for the formation/degradation of bitter peptides. In their hypothesis, rennet had a secondary role in bitterness formation and the starter proteinases were responsible for the defect. In this hypothesis, the major role of rennet in cheese ripening was the degradation of casein into high molecular weight non-bitter peptides. Increasing the concentration of rennet increased the concentration of those polypeptides in the cheese. Subsequent degradation of those peptides by starter proteinases led to an accumulation of low molecular weight bitter peptides. Subsequent degradation of those peptides by the peptidase system present in certain strains of lactic acid bacteria led to a disappearance of bitterness. The mechanism proposed by Lowrie and Lawrence (1972) is shown in Figure 2.

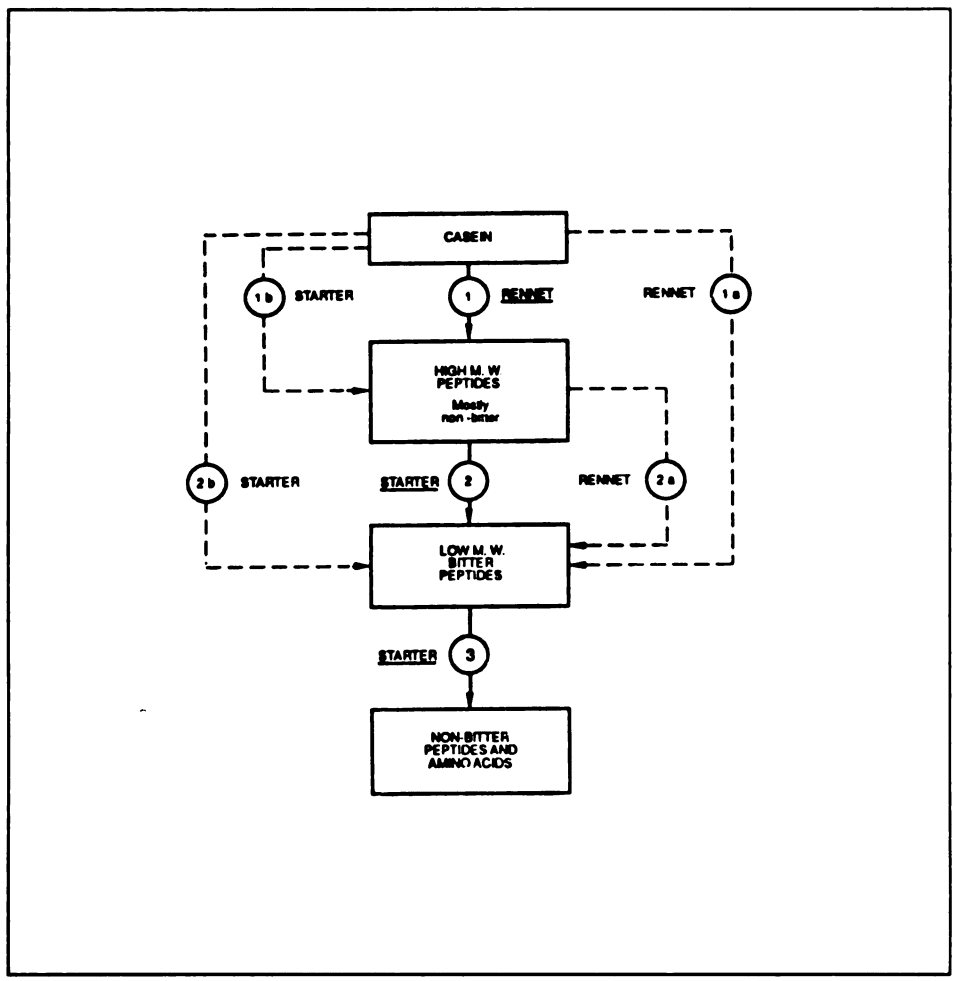

Figure 2 - Lowrie and Lawrence's hypothesis for the formation and breakdown of bitter peptides in cheese. Source: Lemieux and Simard (1992)

Findings from Czulak's (1959) and Lowrie and Lawrence's (1972) hypothesis on the existence of bitter flavor in cheese led Stadhouders and Hup (1975) to postulate a scheme for the formation/degradation of bitter peptides. They concentrated their studies on Gouda cheese and especially on the amount of rennet retained in the curd. They suggested that the development of bitter flavor may have been due to a synergistic effect of bitter peptides produced by rennet and those produced by the proteinase system of lactic acid bacteria. They suggested that the bitter flavor was caused by a disproportion between the production of bitter peptides from casein by rennet and their consequent breakdown by the proteolytic enzymes of the starter culture. This disproportion led to an accumulation of bitter peptides in cheese above the threshold value with consequent appearance of the defect.

Under this concept both rennet and starter bacteria may produce bitter peptides, alone or together. The ability of the starter to do so will depend on its proteolytic system, type (slow and fast variants) and the number of viable cells, which may be reduced by increasing cooking temperatures, phage infection or the presence of antibiotics. The type of rennet (animal or microbial) and its content (chymosin and pepsin) play a major role in the formation of bitterness, which is also affected by milk pH, cooking temperature and, of course, amount of rennet used for setting the milk. Salt content also plays an important role, as it affects proteolysis.

Although not proven, Stadhouders and Hup's concept has been supported by a large number of experimental results (Lemieux and Simard, 1992). The formation and breakdown of bitter peptides in cheese is shown in Figure 3.

The hypothesis of Lowrie and Lawrence (1972) was developed for Cheddar cheese and does not seem to fit the results obtained by Stadhouders and Hup's experiments on Gouda cheese (1975). Gouda cheese has composition and ripening conditions different from those of Cheddar cheese. Lemieux and Simard (1992) believe that the proteolytic properties of the starter bacteria may have been influenced by the high cooking temperature and early salt addition to the curd during Cheddar manufacturing.

Visser (1977a and 1977b) made aseptic Gouda cheese, in which the action of rennet and starter culture could be studied separately, to investigate the contribution of both enzymatic systems on bitterness formation. He concluded that rennet and bitter starter bacteria were independently able to produce bitterness in Gouda cheese, although the starter bacteria appeared to produce more bitterness than rennet. Based on his results and under the conditions in the cheese, non-bitter starter can degrade bitter peptides. The bitterness observed in normal and aseptic Gouda cheese was due to separate actions of rennet and bitter starter bacteria. A mechanism proposed by Visser (1977a) for the development of bitterness in Gouda-type cheese is given in Figure 4.

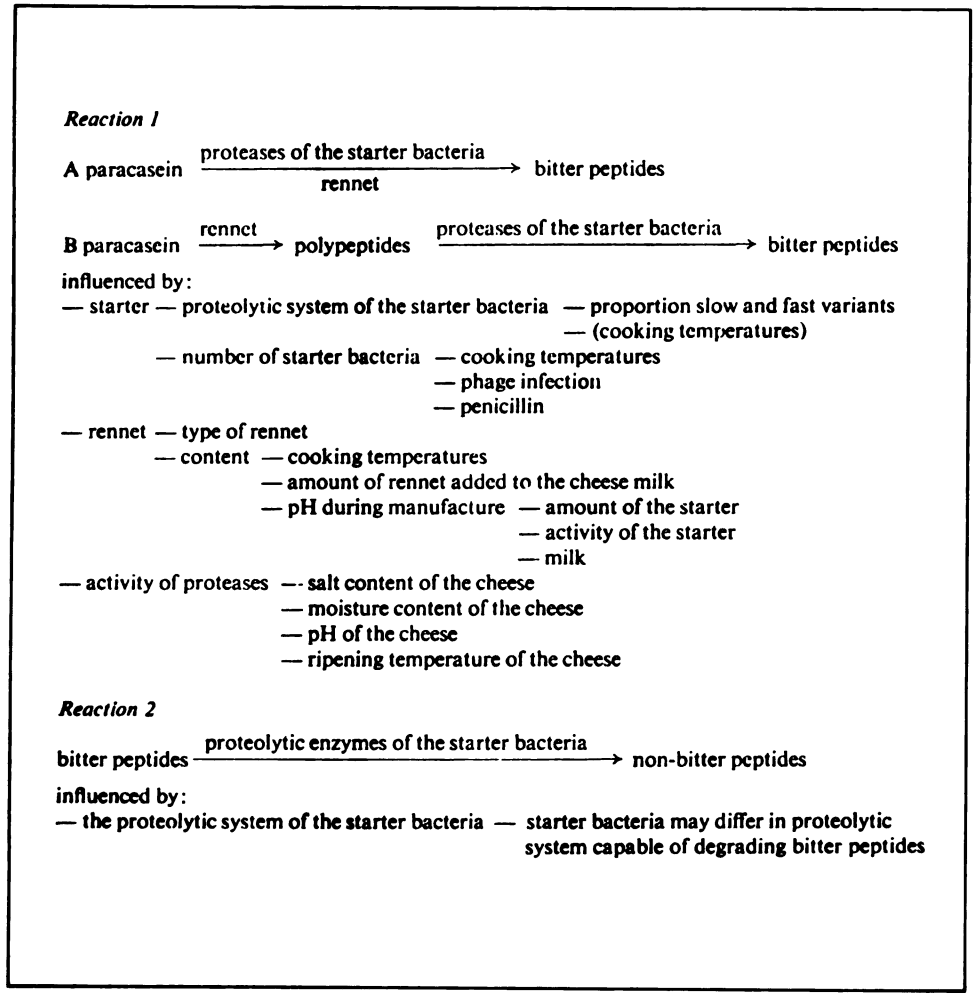

Figure 3 - Tentative scheme for the formation and breakdown of bitter peptides in cheese. Source: Stadhouders and Hup (1975)

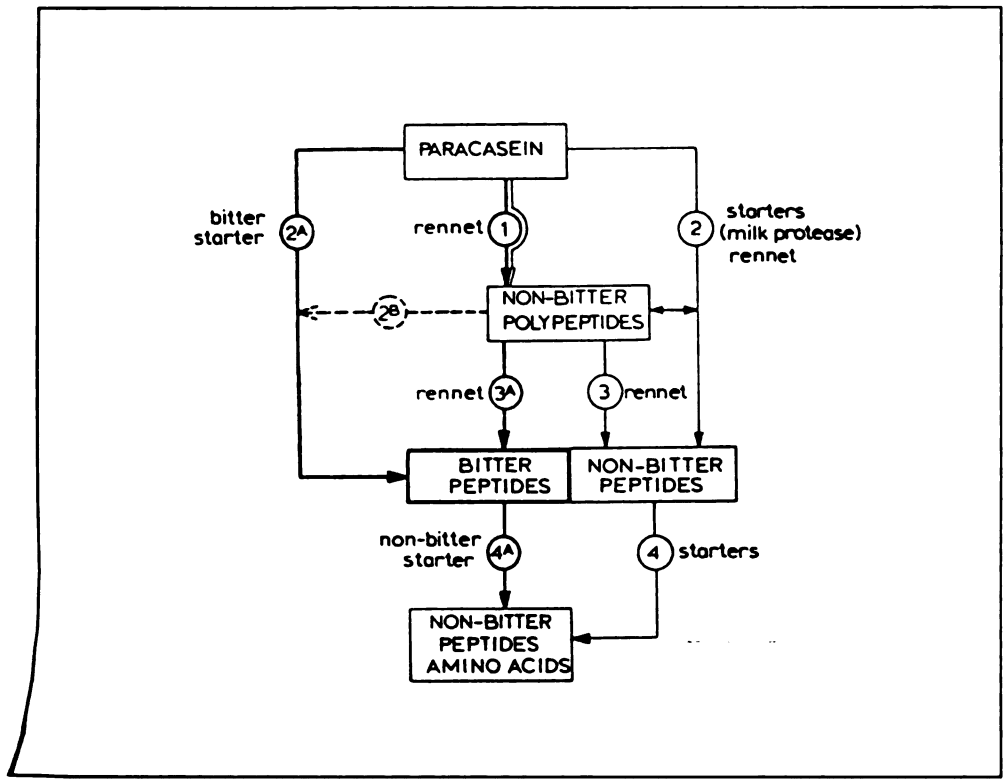

Figure 4 - Mechanism for the development of bitterness in Gouda-type Cheese. Source: Visser (1977).

4.1.1 - Role of rennet in cheese bitterness

Rennet is the first proteolytic agent involved in the overall mechanism of casein breakdown in cheeses. The primary function of rennet is to specifically cleave the Phe(105)-Met(106) peptide bond of κ-casein (Fox, 1988; Visser et al.; 1976). During this "primary" phase of milk clotting, the hydrophilic macropeptide, consisting of the fragment 106-169, is removed from κ-casein. The "secondary" phase includes the aggregation of the unsoluble para-κ-casein (fragment 1-105) with the other (destabilized) casein components under the influence of Ca^{2+}, resulting in the formation of a casein clot. The "tertiary" phase of rennet action relates to a subsequent slow general proteolysis of other peptide bonds in casein. The requirements for suitable milk clotting enzymes should combine a high and specific cleavage capacity towards κ-casein at the pH of milk with a low general proteolytic activity during cheese ripening (Visser, 1981).

In a study on aseptic and chemically acidified (Glucano-Delta-Lactone) Cheddar cheese, O'Keeffe et al. (1976) found that rennet was mainly responsible for the level of proteolysis detected by gel electrophoresis, soluble Nitrogen at pH 4.6 and gel permeation. They also found that rennet alone is capable of releasing free amino acids, but only a limited range of free amino acids was produced at quantifiable levels, which includes methionine, histidine, glycine, serine

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and glutamic acid. Similar results were reported by Visser (1977b) who studied the contribution of rennet enzymes on proteolysis and flavor development in Gouda cheese.

Rennet is of major importance in hydrolysis of α_{s1}-casein during the initial ripening period forming a large peptide α_{s1}-I which is further degraded by rennet and by other proteinases during subsequent ripening (Ledford et al., 1966). The α_{1s}-I peptide has been reported in all studies on rennet action and is the most easily formed product of rennet activity on α_{s1}-casein (Grappin et al., 1985).

The action of rennet on β-casein is more variable and more dependent upon manufacturing conditions such as pH and salt concentration. β-casein is hydrolyzed by rennet at pH 6.5 into three major products of increased electrophoretic mobilities, representing the large breakdown products β-I, β-II and β-IIIa (Grappin et al., 1985). According to Creamer (1976), the weak hydrolysis of β-casein in the curd might be explained by the association between the C terminal region of β-casein and α_{s1}-casein, which make the cleavage sites no longer accessible to rennet action. Visser and Slangen (1977), identified other bonds on β-casein that were cleaved by rennet. Those bonds were indentified as the residues Leu-163-Ser-164 and Leu-127-thr-128, with the latter giving rise to the β-IIIb peptide.

Proteinases other than rennet will also be able to coagulate milk under suitable conditions, but most are too proteolytic relative to their milk-clotting activity. Since many of these preparations are more generally proteolytic (less specific) than rennet, they could be important sources of bitterness in cheese (Fox, 1988).

Additional considerations relative to the involvement of rennet in cheese proteolysis and bitterness include the level used in cheese manufacture and the retention of the enzymes in the curd. Increased amount of calf rennet at cheesemaking may result in an increase of bitter flavor in the cheese (Stadhouders and Hup, 1975). The effects of rennet level on bitterness is shown in Table 1.

Lawrence et al. (1972) showed that bitter flavor was produced in cheese with high levels of rennet when bitter starters were used for cheesemaking. Their results have shown that when non-bitter starters are used in cheesemaking, a 3-fold increase over the normal concentration of calf rennet did not lead to such a high concentration of bitter peptides that the cheese proved to be bitter. They suggested, based on Czulak's hypothesis (Czulak, 1959), that the bitter peptides formed by rennet action were degraded by peptidases from these particular starters.

Rennet retention in the curd is pH and temperature dependent (Stadhouders and Hup, 1975). When the initial pH of cheese milk is decreased, more rennet remains in the cheese

Table 1. Effect of rennet level on bitterness of six month old cheese manufactured with bitter strain *Lactococcus cremoris*[a]

Rennet Level[b] (ml/100 l milk)	Bitterness[c]
66	4.6
44	3.9
22	2.8
13	2.0
6.5	1.8

Source: Lowrie, 1976
[b]A rennet level of 22 ml/100 l milk was considered to be normal level.
[c]Bitterness by sensory evaluation (1 = absent; 5 = extremely bitter)

and there is a great propensity for bitterness development (Table 2).

Stadhouders and Hup (1975) suggested that the increased rennet retention in the curd at lower pH may be caused by changes in the calcium phosphate/casein complex. Reduction of colloidal calcium phosphate causes weakening of the protein matrix which becomes more accessible for enzymatic attack, with consequent release of bitter peptides. Holmes et al. (1977) studied the distribution of milk clotting enzymes between curd and whey during Cheddar cheese making and found that the retention of rennet in the curd is pH dependent, with 86% of the rennet activity retained in curd formed at pH 5.2 compared to approximately 31% in curd formed at pH 6.6.

According to Stadhouders and Hup (1975) the cooking temperature also affected rennet retention and bitter flavor development. Their results showed that at higher cooking temperatures, less rennet was found in the cheese and the bitter flavor was less intense. Those observations are shown in Table 3. They also concluded that above cooking temperatures of 35°C (35-39°C), in addition to the reduced rennet content, the growth of conventionally bitter starters is restricted by temperature with a decrease in the content of their proteases and consequently in the reduced bitter flavor. They also found that curd retention of microbial coagulants is apparently lower than that of bovine rennet and is less pH dependent.

Table 2. Effect of the initial pH of the cheese milk on the rennet content and the bitter flavour of Gouda cheese[a]

pH of the cheese milk	Rennet content of the cheese (μl/kg cheese)	Bitter score[b] after a ripening time of 17 weeks
6.56	280	1.5
6.38	380	1.3
6.25	500	2.0

[a]Source: Stadhouders and Hup (1975).
[b]Bitterness by sensory evaluation (0 = absent; 4 = very strongly bitter).

Table 3. Effect of the cooking temperature in the manufacture of Gouda cheese on the rennet content and the bitterness[a]

Cooking temperature (°C)	Rennet content of the cheese µl/kg cheese)	Bitter score[b] of the cheese after a ripening time of 4 months
33.5	350	2.9
35.0	315	2.2
36.5	270	1.5
38.0	230	1.0

[a]Source: Stadhouders and Hup (1975).
[b]Bitterness by sensory evaluation (0 = absent; 4 = very strongly bitter).

Czulak (1959) proposed that rennet was directly involved in bitterness through production of bitter peptides in cheese which are rendered non-bitter to a varied degree by microbial peptidases from starter microorganisms. When rennet or other proteinases used as coagulants are used at excessive levels or retained in the curd at high levels, they could directly form bitter peptides in cheese. However, a more indirect involvement is the more generally accepted hypothesis (Visser, 1977b; Visser, 1981). Generally rennet proteolysis results in formation of large peptide moieties from casein which may be non-bitter but may become precursors to bitter peptides via further proteolytic cleavage (Lawrence et al. 1972).

4.1.2 - Role of starter microorganisms in cheese bitterness

The most important role in bitterness appears to be played by the starter bacteria (Lawrence and Gilles, 1969; Richardson and Creamer, 1973). Emmons et al. (1960a, 1960b and 1962a) investigated the effect of manufacturing conditions on the formation of bitterness in Cheddar cheese and found that the starter microorganism was the main factor responsible for the defect.

The relationship between the strain of starter bacteria used in cheesemaking and the bitterness of the cheese produced has been clearly established (Emmons et al., 1962a and 1962b).

The starter lactococci (formely known as streptococci) have been classified as bitter or non-bitter according to whether or not they produce bitter flavor in cheese. Lowrie et al. (1972), verified that during cheese ripening, bitterness appears to decrease in intensity very slowly in cheese made with a non-bitter strains and not at all in those made with a bitter strain.

The most commonly used starter microorganisms used in cheese making belong to the group N *lactococci*. The proteolytic enzyme system present in the lactic *lactococci* has been investigated (Law and Kolstad, 1983; Visser, 1993). According to Visser (1993) the proteolytic system of the starter bacteria is divided into extracellular and intracellular proteinases and peptidases. The enzymatic activity of proteinases is towards the hydrolysis of native proteins whereas the peptidase cleaves peptides produced by action of proteinases (Kamaly and Marth, 1989).

4.1.2.1 - The extracellular proteinase activity

The proteolytic action of extracellular proteinases of starter *lactococci* are of considerable significance for flavor development in cheese. They contribute to the initial fragmentation of the native milk caseins initiating substrate degradation for peptidases in the subsequent hydrolysis during cheese ripening (Laan et al., 1989; Umemoto and Itoh, 1981).

The location of the extracellular proteinase activity appears to be cell wall surface bound. Exterkate (1979) suggested that the proteinases are probably located at the periphery of the cell, based on the fact that size and charge of casein molecules restricts their diffusion into the cell wall. There were three different types of proteolytic activity related to the cell wall proteinases, designated PI, PII, and PIII. The PI, PII, and PIII proteinase activity had pH and temperature optima of 5.8 and 40°C, 6.5 and 30°C, and 5.4 and 30°C, respectively (Exterkate, 1976b).

Much of the evidence for the involvement of extracellular proteinase activity with bitterness comes from cheese making trials where proteinase negative (prt^-) and proteinase positive (prt^+) mutant strains were used (Mills and Thomas, 1980). Exterkate (1975 and 1976a) studied the proteolytic activity of a slow lactic acid producing variant of *Lactococcus cremoris*, so called prt^-, and verified the lack of PI and PII activities which were normally present in the prt^+ variants. These prt^- mutants are often considered to be non-bitter strains and generally reach lower total cell populations in cheese and thus have less total proteinase/peptidase activity compared to prt^+ strains. The lower total cell population of non-bitter strains was explained by Lowrie et al. (1972) who showed that bitter strains were relatively temperature insensitive at the normal cooking temperature of 38°C, whereas the non-bitter strains

were inhibited at this temperature. Increased cell densities in the cheese may lead to a high concentration of starter proteinase and elevated levels of bitter peptides (Lowrie and Lawrence, 1972). Lawrence and Gilles (1969) have demonstrated that "slow" starters never cause bitter cheese. Bitterness was absent even when conditions for bitterness formation were present, such as increased level of starter added to give a high rate of acid production or when the salt in moisture level and pH was such that bitterness would be certainly present if a "fast" starter is used (Lawrence and Gilles, 1969; Stadhouders and Hup, 1975). Non-bitter strains are less proteolytic than bitter strains and would be expected to degrade high molecular weight peptides at a slower rate (Gordon and Speck, 1965a and 1965b; Martley and Lawrence, 1972). Lowrie et al.(1972) observed that bitter strains grew more rapidly and reached higher cell population than non-bitter strains prior to salting during normal Cheddar cheesemaking.

4.1.2.2 - Peptidase activity

During the fermentation process in cheese making, the lactic acid bacteria grow and produce lactic acid and other flavor-related metabolites, as well as peptides. During cheese ripening the microbial cells autolyze with consequent release of proteinases and peptidases into the cheese environment.

These enzymes develop important roles in subsequent proteolysis, releasing amino acids and contributing to overall cheese flavor development (Schmidt, 1990).

The peptidase activity of lactic acid bacteria are predomintantly aminopeptidases with a wide range of specificity (Law and Kolstad, 1983). Although peptidase activity has been found in extracellular cell-free broth, on the cell surface, and in the intracellular fractions (Schmidt, 1990), the major portion of its activity is located near the outside surface of and in the membrane, which suggests that peptide hydrolysis is a function of the membrane (Exterkate, 1981 and 1984). Unlike cell wall-associated proteinases which are in direct contact with the substrate when bacterial cells are intact, the intracellular peptidase activity must be released by cell lysis for peptidase activity to take place (Mills and Thomas, 1980). A high cell density in the cheese does not necessarily mean that a high concentration of peptidase is available for degradation of bitter peptides since cells may remain intact and substrate will therefore be inaccessible to the intracellular enzymes (Thomas and Mills, 1981).

A scheme for the pathway of protein degradation by lactic acid bacteria is given in Figure 5 (Visser, 1993). The proteinases bound to the cell wall surface are thought to act on casein fragments generated by rennet and by indigenous proteinases. The possession of surface-bound proteinases

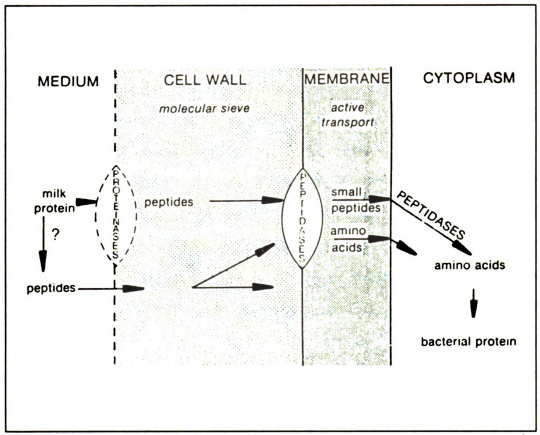

Figure 5 - Utilization of milk protein for synthesis of bacterial protein in lactic streptococci. Source: Thomas (1991)

allows lactic acid bacteria to hydrolyse large molecules, such as milk proteins, to peptides which are then small enough to diffuse into the cell wall. Those peptides are further degraded by extracellular and intracellular peptidases located at or outside the surface of the cytoplasmic membrance since the size limit for peptide transport through the membrane is four to 6 amino acid residues.

Sullivan et al. (1973) verified that the bitter peptides isolated from casein, contain a high content of proline and N-terminal pyrrolidonecarboxilic acid, formed by cyclization of glutamine. This high content of proline and the presence of an N-terminal pyrrolidonecarboxylyl residue would effectively block normal aminopeptidase activity. Thus, the resistance of these peptides to enzymic hydrolysis would tend to result in their accumulation in cheese. They suggested that bitter strains might differ from non-bitter strains on the basis of whether the organism contained a pyrrolidonecarboxylyl peptidase. The main conclusion from their study was that all starter cultures, including bitter and non-bitter strains, had cytoplasmatic aminopeptidases required for degradation of bitter peptides to non-biter peptides. These peptidase activities included an aminopeptidase of wide specificity, a proline iminopeptidase and an aminopeptidase-P. What made the difference in each strains ability to hydrolyze bitter peptides, was the possession of pyrrolidone carboxylyl peptidase by non-bitter strains, but not by bitter starters.

One may conclude that the cell wall-associated proteinase system developed an important role in the formation of bitter peptides and that the intracellular peptidases had an important role in the removal of bitter tasting peptides (Mills and Thomas, 1980).

4.2. The Source of Bitter Peptides

The source of bitter peptides in cheese has been the subject of several studies (Guigoz and Solms, 1976; Hamilton et al. 1974; Hodges et al. 1972; Huber and Klostermeyer, 1974; Richardson and Creamer, 1973; Visser et al., 1983a and 1983b) and α_s- and β-casein appear to be the major sources (Schmidt, 1990). According to Schmidt (1990), the role of minor caseins, whey proteins and the fat globule membrane-associated proteins in the formation of bitterness has not received as much attention as the major caseins fractions. Due to their globular conformation, the whey proteins are much more resistant to proteolysis than caseins (Fox, 1989b).

The casein micelles, formed in the milk system through the association of casein fractions, possess an amphiphilic structure in which discrete regions of their primary structure are comprised of mostly hydrophobic amino acid residues, whereas other regions contain hydrophilic amino acid residues (Schmidt, 1990; Swaisgood, 1982). All the bitter peptides have one common characteristic, that is, lipid solubility or

hydrophobicity (Lemieux and Simard, 1992). This finding has led to investigation of the hydrophobic portions of casein as the main source of bitter peptides in cheese (Schmidt, 1990; Swaisgood, 1982).

Schmidt (1990) pointed out three important points that should be mentioned about the formation of bitter peptides from caseins: 1) Most of the work done so far was based on the addition of proteinases to model systems containing purified casein fractions. The response of individual casein fractions to proteolysis differs from that of micellar caseins present in milk (Fox and Guiney, 1973; Phelan et al. 1973). Therefore, the results obtained from individual casein systems and milk or cheese systems may not be comparable; 2) During cheesemaking, most of the peptides are lost in the whey, thus, peptides responsible for bitterness in cheese may be different from those existing in a milk system; 3) A low level of bitterness may be essential for the overall flavor of cheese. The bitterness problem only appears when the concentration of bitter peptides in cheese exceeds certain threshold levels.

Alpha$_{s1}$- and β-casein are potential sources of bitter peptides, since they have high average hydrophobicities, 1.17 and 1.33 Kcal/residue, respectively (Visser, 1977b). According to Exterkate (1976b), the action of proteinases present in the cell wall of the starter culture on the β-casein may be the source of bitter peptides in cheese. Visser et al. (1983 and 1983a) concluded that the C-terminal portion of β-casein has

an extremely bitter taste and is the main source of bitter peptides in Gouda cheese.

Phelan et al. (1973) and Creamer (1975) found that β-casein is highly resistant to proteolysis in Cheddar cheese, while α_{s1}-casein is extensively degraded during ripening. The main reason for this resistance to proteolysis is due to polymerization of β-casein which makes hydrolysis difficult.

4.3. Chemical and Structural Aspects of Bitter Peptides

Bitter peptides are rich in hydrophobic amino acids and are released from the casein molecules primarily by the action of rennet and bacterial proteinases (Crawford and Zwaginga, 1977; Stadhouders, 1974 and 1979; Sullivan and Jago, 1972). Fractionation of an extract of bitter Cheddar cheese on gel filtration chromatography using Sephadex G-25 has shown that the most bitter fraction occurs in the molecular range of 2000-4000 da (Richardson and Creamer, 1973). Pelissier et al. (1974), Visser et al. (1975) and Wieser and Belitz (1976) demonstrated that, because of their solubility, only relatively small peptides have been found to be bitter.

Bitterness which can represent a problem for many other foods, is reasonably well correlated with the degree of molecule hydrophobicity. Matoba and Hata (1972) have related bitterness to hydrophobicity and concluded that the presence

of at least one hydrophobic side chain in a peptide molecule is an essential requirement for bitter taste.

Matoba and Hata (1972) concluded that bitterness is attributable to hydrophobic amino acids in peptides regardless of amino acid sequence and regardless of the state of the amino or carboxy terminal group. On the other hand, Shiraishi et al. (1973) concluded that the presence of hydrophobic groups, as well as the sequence of the amino acids, is important for taste. From the literature reviewed there was not a definitive statement as to which of these theories was most plausible. But considering the large varieties of peptides exhibiting bitterness, the overall hydrophobicity can be considered as the most important determinant of peptide bitterness.

Bitterness of peptides can be evaluated from the hydrophobic property of their amino acid side chain, according to the Q-rule proposed by Ney (1971). The Q-rule has been accepted as the most important predictor of peptide bitterness. According to the Q-rule, the average hydrophobicity, Q, is the ratio of the sum of the amino acid side chain hydrophobicities of a peptide to the number of amino acid residues:

$$Q = \frac{\sum \Delta f}{n}$$

where Δf = free energy of transfer of the side chains of the amino acid residues (cal.mol^{-1}), n = number of amino acid residues, Q = average hydrophobicity of a peptide.

Peptides with Q-values below 1300 cal. res^{-1} are not bitter, whereas peptides with Q-values higher than 1400 cal. res^{-1} are bitter. No prediction can be made about the peptide bitterness for Q-values between 1300 and 1400 cal. res^{-1}. The Q-rule is valid only for molecular weights up to approximately 6000 da; above this limit peptides with Q-values higher than 1400 cal.res^{-1} are not bitter. Prediction of bitterness threshold may be possible if the structure of a peptide molecule is known.

The contribution of L-proline residues to bitterness has become evident from the study of Shiraishi et al. (1973) and Shinoda et al. (1986), on synthetic di- and tripeptides. Amino acids such as leucine, phenylalanine or tyrosine, which present high hydrophobicity, cause marked peptide bitterness. The bitterness is more intense when leucine and phenylalanine are located at the C-terminus (Ishibashi et at. 1988).

Sullivan et al. (1973) verified that the bitter peptides which have been isolated from casein, contain a high content of proline and in one instance, N-terminal pyrrolidonecarboxilic acid, formed by cyclization of glutamine. This high content of proline along the peptide chain make the peptide resistant to normal endo- and exopeptidase attack. The presence of an N-terminal

pyrrolidonecarboxylyl residue would effectively block normal aminopeptidase activity.

4.4. Control of Bitterness Level in Cheese

According to Sullivan et al. (1973), bitterness can only be controlled by control of total proteinase activity in the cheese. The increase in peptidase activity must be adequate to prevent the initial formation of bitterness (accumulation of bitter peptides) by bacterial proteinase. According to Jago (1974), reduction of proteolytic activity can be achieved by culture selection, use of mixed cultures, proper selection and use of coagulants and adjusting manufacturing parameters (pH, temperature, salt-in-moisture, etc.).

4.4.1 - Control by manufacturing adjustments

The amount of proteases in the curd can be controlled by using minimal amounts of rennet and by limiting the starter cell population (Lawrence et al. 1972; Lowrie et al. 1972b). According to Jago (1974) the starter cell population in the curd should be limited by growth repression at the cooking temperature. At abnormally low cooking temperature (33°C) the non-bitter strains reach high cell populations in the curd, producing bitter cheese whereas the typical bitter strains are

more heat-resistant and reach higher cell populations at 37-38°C (Lowrie et al. 1972).

Bitterness is markedly reduced by limiting the amount of rennet in the curd. Lawrence and Pearce (1972) pointed out the amount of rennet added to cheese milk should be the minimum necessary for coagulation within 30-40 minutes. At increased setting temperature less rennet is spent for the coagulation process, thus the setting temperature should be increased by 1-2°C to reduce the amount of rennet in the curd, reducing consequently the chance of bitterness (Jago, 1974; Lawrence and Gilles, 1969 and 1971).

A high salt-in-moisture content reduces the incidence of bitterness in cheese manufactured with a bitter starter (Lawrence and Gilles, 1969). A possible explanation for this is given by Fox and Walley (1971) and Sullivan and Jago (1972). They found that a high salt-in-moisture may inhibit the degradation of β-casein by rennet and bacterial proteinases.

4.4.2 - Control by culture selection

Lawrence and Gilles (1969) found that cheese was seldom bitter when non-bitter strains were used as starter, regardless of the amount of rennet, salt or moisture present, or the rate of acid production in the curd, or the final pH of the cheese. On the other hand, they found that when bitter

strains were used, the cheese was invariably bitter except when the salt-in-moisture and the pH of the cheese were relatively high.

Bitter and non-bitter strains differ in their growth characteristics. According to Exterkate and Stadhouders (1971), bitter strains grow rapidly under normal cheese-making conditions and reach high cell populations in the cheese curd prior to salting while the non-bitter strains are inhibited at the normal cooking temperature in cheesemaking. Based on their results, one may conclude that all strains are potentially capable of contributing directly to the formation of bitter peptides in cheese.

Sullivan and Jago (1970) claimed that the major difference between bitter and non-bitter strains is the possession of a pyrrolidone carboxylyl peptidase by non-bitter strains, but not by the bitter strains. Therefore, non-bitter starters are suitable for degradation of the bitter peptides present in the cheese.

Certain starters resulted in bitter taste in cheese and pairing bitter starters with non-bitter starters markedly reduced bitter flavor development in Cheddar cheese (Lawrence and Pearce, 1968; Creamer et al., 1970). The same conclusion was achieved by Visser (1977a) when manufacturing Gouda cheese.

The choice of suitable starter is therefore of utmost importance to the control of bitter compounds formed in cheese during ripening. According to Olson and Johnson (1990b) lactic starters may have to be scrutinized for proteolytic and peptidolytic activities and profiles as they specifically impact on bitterness formation. Preliminary studies have shown that cultures with less proteolytic activity and slower acid production produced a better cheese, with less bitterness than did a more proteolytic culture (Olson, 1984 and 1991). The starter cultures should be chosen based on their ability to degrade the bitter peptides as rapidly as they are formed by rennet and other proteinases, so that they never exceed the threshold level for bitterness perception (Jago, 1974).

4.4.3 - The Use of Lactobacillus

The peptidolytic enzymes of *Lactobacillus casei*, used as adjunct culture and its possible use as an agent to improve the flavor and body and texture of cheeses have been studied (El-Neshawy et al. 1986; El-Soda et al. 1981; El-Soda, 1986; Hargrove, 1966b; Simard, 1991). According to Simard (1991) and Laleye (1989), *Lactobacillus casei* possesses three exopeptidases (a dipeptidase, an amino-peptidase, and a specific carboxypeptidase) and one endopeptidase which increase protein and peptide hydrolysis with positive effects on texture and flavor of cheese. The action of those

peptidases on the bitter peptides is a desirable characteristic of this microorganism and might improve considerably the texture, body and flavor of the cheese.

Several treatments have been applied to *Lactobacillus* species to improve body and flavor development in cheeses. El-Soda et. al. (1981) demonstrated that Cheddar cheese added with whole cells or cell free extracts from *Lactobacillus casei*, showed an acceleration of ripening, with high values for protein breakdown and volatile acid formation, but a rapid bitter flavor development was detected in cheese. Trepanier et al. (1991a and 1991b) studied the addition of live cells and cell homogenates of *Lactobacillus casei* to Cheddar cheese. Their results showed 40% increase in flavor development, but no difference in rheological properties was observed between the control and the experimental cheese.

The addition of heat and freeze treated cultures of Lactobacillus have shown promising results. Bartels et al. (1987) verified a reduced bitterness and great improvement in flavor intensity in Gouda cheese with freeze shocked *Lactobacillus helveticus* added. Trepanier et al. (1992) carried out an experiment for accelerating the maturation of Cheddar cheese by using heat treated *Lactobacillus casei* and neutrase. A good quality Cheddar cheese, presenting 50% increase in flavor, was produced when heat shocked cells were added to the cheese milk. However, high levels of bitterness

developed in the cheeses with heat shocked cells added in combination with neutrase.

A study to establish adequate conditions for heat-shocking cells of *lactobacilli* was carried out by El-Abboud et al. (1991). A treatment of 67°C/15 sec. was considered the best for inactivating the microorganism without detrimental effects to its enzymatic system. According to Law (1984), this treatment prevents the bacteria from producing acid while keeping intact their proteolytic system. El-Abboud et al. (1991), suggested that the increased proteolytic potential in the cheese with heat-shocked cells added may lead to a further degradation of peptides responsible for bitterness. According to Ardo and Mansson (1990), the heat treated cells are added to the cheese milk prior to renneting and the enzymes contribute to the development of a desirable flavor. The aminopeptidase from *Lactobacillus casei* responsible for the degradation of bitter peptides is highly heat resistant, allowing for continued activity during cheese ripening. If sufficient levels of such enzyme are present in the cheese, the peptides which cause bitter taste are broken down to amino acids.

4.5. Peptide Separation

The appearance of bitter flavor during the ripening of cheese has long been found as being attributable to the accumulation of bitter peptides. One way to gain information as to the kind of peptides which contribute to the cause of bitter taste, consists of isolation and chemical characterization of the bitter fraction from the cheese.

Several methods are available for isolation of bitter peptides from cheese. All follow similar patterns based on the hydrophobic nature of the bitter compounds. Due to their relatively large content of hydrophobic side chain amino acids, bitter peptides are lipid-soluble and easily extractable with organic solvents (Sullivan and Jago, 1970).

Peptide separation can be accomplished by several tehniques. The process usually consists of a primary separation by gel permeation chromatography, followed by separation by high performance liquid chromatography. Sephadex gels have been extensively used for preliminary separation of peptides from cheese extracts (Champion and Stanley, 1982; Edwards and Kosikowski, 1983; Harwalkar and Elliott, 1971; Hetting and Parmelee, 1965; Richardson and Creamer, 1970 and 1973; Schalinatus and Behnke, 1975; Visser, 1977c; Visser et al. 1983a). Richardson and Creamer (1970 and 1973) used gel filtration chromatography on Sephadex G-25 of an alcoholic extract of bitter Cheddar cheese to demonstrate

that most bitter fractions occurred in the molecular weight range of 2000-4000 daltons.

Separation by gel permeation has some disadvantages. It is a highly time consuming technique and suffers from lack of resolution. Numerous peaks may be included within each fractions and further separation is thus needed to separate the individual components (Rank et al. 1985). This separation can be accomplished by using paper and thin-layer chromatography, high-voltage paper electrophoresis, ion-exchange chromatography or by high-performance liquid chromatography (HPLC) on a reversed-phase type column (Champion and Stanley, 1982; Edwards and Kosikowski, 1983; Gordon and Speck, 1965b; Harwalkar and Elliott, 1971; Hetting and Parmelee, 1965; Huber and Klostermeyer, 1974; Schalinatus and Behnke, 1975; Visser et al. 1983a).

Reversed-phase high-performance liquid chromatography for profile studies of peptides produced during ripening of Cheddar cheese was initially proposed by Cliffe et al. (1989) and has been the method of choice for fractionation of bitter peptides. Despite the initial cost of the equipment and difficulty in the interpretation of the data, HPLC procedures allow speed of analysis and the resolution is comparable if not better than two-dimensional gels (Rank et al. 1985). Champion and Stanley (1982) obtained 71 peaks when a bitter extract from Cheddar cheese, previously fractionated on

Sephadex G-50, was applied to a reverse-phase HPLC column using a liner gradient from 0 to 90% methanol in water.

Chapter 1

Ripening Characteristics of Reduced Fat Cheddar Cheese Using Heat-Shocked and Live *Lactobacillus casei* L2A as Adjunct Culture

INTRODUCTION

The last two decades have seen shifts toward the consumption of low fat foods (Egbert et al., 1991). The wish to avoid high calorie foods to control body weight and a possible connection between animal fats and circulatory disorders has been the main cause for this increased consumption of low fat foods (Hargrove et al., 1966a; Madsen et al., 1970; Simard, 1991). The growing consumer interest in low calorie food has led the cheese industry to provide consumers with a large variety of reduced fat cheese. Levels of fat in cheese have a direct impact on the sensory acceptability by consumers. Fat is the precursor of many flavor compounds and influences body and texture of cheese (McGregor and White, 1990a). The use of traditional procedures to make reduced fat cheese has led to the production of hard,

rubbery and tasteless cheese, even after extensive ripening (Banks et al., 1989; El-Neshawy et al., 1976; Hargrove et al., 1966b; Simard, 1991). To improve the flavor, body and texture of reduced fat cheese, the traditional manufacturing procedure has been modified (Banks et al., 1989; Hargrove et al., 1966b; Madsen et al., 1970; Olson and Johnson, 1990), in addition to changes in milk preparation prior to manufacture (McGregor and White, 1990a and 1999b; Metzger and Mistry, 1993), culture selection (Chen et al. 1992; Simard, 1991), or use of fat replacers (El-Neshawy et al., 1986).

The lactic starter cultures greatly affect all cheeses, but especially reduced fat cheeses (Olson and Johnson, 1990). Due to the association of reduced fat cheese with high acid levels caused by increased lactose concentration and a range of off-flavors caused by proteolytic activity (Rosenberg, 1992), the choice of a suitable starter for making reduced fat cheese is an important factor to consider. Starter culture possessing lower rates of acid production, lower proteolytic activity (Olson, 1991; Rosenberg, 1992), and debittering activity (Rosenberg, 1992) is prefered. The use of *lactobacilli* as starter culture adjunct has been successfully applied as a way to reduce bitterness and improve flavor in cheese (El-Neshawy et al., 1986; El-Soda, 1986; El-Soda et al., 1981; Hargrove et al., 1966b; Johnson et al., 1993; O'Donnell, 1993; Simard, 1991; Trepanier et al., 1991a, 1991b and 1992). The peptidolytic enzymes of *Lactobacillus casei*,

used as adjunct culture and its possible use as an agent to improve the flavor and body and texture of cheeses have been studied (El-Neshawy et al. 1986; El-Soda, 1986; El-Soda et al., 1981; Hargrove et al., 1966b; Simard, 1991). According to Simard (1991), *Lactobacillus casei* possesses three exopeptidases (a dipeptidase, an amino-peptidase, and a specific carboxypeptidase) and one endopeptidase which increase protein and peptide hydrolysis resulting in positive effects on the texture and flavor of cheese.

During ripening, cheese body becomes weaker and smoother due to the effects of the proteolytic breakdown of the structural matrix. Increasing the peptidase activity during ripening without changes in the balance of acid production during the manufacturing process could be an interesting approach to improve flavor and soften the body of reduced fat cheese. *Lactobacillus casei* is a suitable microorganism for that purpose as it possesses highly heat resistant peptidolytic enzymes (Ardo and Mansson, 1990).

The objective of this work is to evaluate the use of live and heat shocked cells of *Lactobacillus casei* as an adjunct culture in the manufacture of reduced fat Cheddar cheese. Protein degradation (i.e., soluble nitrogen fractions, breakdown of α_s- and β-caseins) will be followed during ripening.

MATERIALS AND METHODS

Culture Preparation

The lactic starters used in this experiment were BIOLAC DSS LF slow acid producing culture (Marschall Products, Madison, WI) consisting of selected single strain of *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus casei* subsp. *casei* L2A (Food Research Institute, Ottawa, Canada). The culture was supplied in slant form and was subcultured daily in All Purpose Tween (APT) broth (37°C/24 h) for one week and then in 11.5% reconstituted non-fat dry milk (NFDM) daily, at the same conditions, during the week preceeding cheese manufacture.

The live *Lactobacillus casei* subsp. *casei* L2A was added to the cheese milk before rennet addition. The heat shock treatment was adapted from El-Abboudi et al. (1991). Following the normal incubation period, the culture was cooled to 4°C. The heat shock treatment was performed in a Spiratherm (Cherry-Burrel Corporation, Cedar Rapids, Iowa). The Spiratherm unit was properly sanitized immediately before using. The stainless steel coiled system (0.4 cm i.d.) consisted of a heater followed by a holding tube and a cooler. The holding tube had a total volume of 210 ml. The pH of the cooled culture was adjusted to 6.5 with sterile 1 M NaOH to avoid precipitation during the heat treatment. A positive, sanitary pump with variable speed (Waukesha Foundry Company

Inc., Waukesha, WI) was used to pump the bacterial suspension through the system. The flow rate through the apparatus was set to 9.5 ml/sec to give a process time of 22 seconds. The temperature applied to the culture was 67°C. Loss of heat was prevented by covering the holding tube with insulating material. Temperature was checked in both extremes of the holding tube of the apparatus. After heat treatment, the culture was stored at 4°C until use.

Cheesemaking

Twenty-eight batches of reduced fat Cheddar cheese were manufactured from milk received by the Dairy Plant, Department of Food Science and Human Nutrition at Michigan State University. The milk was standardized to 1.5% fat with skimmilk. All cheeses were manufactured from a single lot of milk. Milk of each vat received the following treatments: In the Control treatment, single strain of *Lactococcus cremoris* subsp. *cremoris* (1% inoculum) was used as the starter. In treatments LLC-0.5, LLC-1.0 and LLC-1.5, *Lactococcus cremoris* subsp. *cremoris* (1% inoculum) plus live *Lactobacillus casei* L2A, at 0.5%, 1.0% and 1.5% inoculum, respectively, were used. In treatments HSLC-0.5, HSLC-1.0 and HSLC-1.5, *Lactococcus cremoris* subsp. *cremoris* (1% inoculum) plus heat shocked *Lactobacillus casei* L2A, at 0.5%, 1.0% and 1.5% inoculum, respectively, were used. Four trials were conducted for each treatment.

Reduced fat Cheddar cheese was manufactured based on the process described by Wilster (1980) with modifications to produce a high moisture reduced fat cheese (Chen, 1991). The standardized milk was pasteurized at 75°C/18 s, cooled to 32.2°C, weighed (127.12 kg) and inoculated with starter culture, 40 min prior to rennet addition (12.7 ml Chymax-Double Strength, Pfizer, Milwaukee, WI). After 30 min of coagulation, the curd was cut with horizontal and vertical knives (1.7 cm). After 20 min of stirring the curd was gradually heated to 37.7°C in approximately 15 min, followed immediately by draining. Fifteen minutes later the curd was cut in blocks which were turned every 15 min until a curd pH of 5.6 was achieved. The curd was then milled, salted (0.3 kg/100 kg of milk), hooped and pressed (2.79 kg/cm^2) for 12 h. After vacuum packing the cheeses were ripened at 11°C for 20 weeks. A complete time schedule to make reduced fat Cheddar cheese of high moisture content is presented in Table I-1 (Appendix I).

Compositional Analysis of Cheese

Fat content of cheese samples were determined by the Babcock method, as described by Marshall (1992). Measurements of pH were determined using a CORNING 150 pH Meter (Halstead Essex, Medfield, MA). Moisture in cheese was determined by the vacuum oven method (Marshall, 1992).

Assessment of Proteolysis

Nitrogen Fractions.

Samples were taken from each block of fresh cheese and after 4, 8, 12, 16 and 20 weeks of ripening. The citrate-cheese extract described by Gripon et al. (1975), was used for determination of all nitrogen fractions (Total Nitrogen (TN), Water Soluble Nitrogen (WSN) and 12% Trichloroacetic Acid (TCA-N)).

Ripening Indexes

The Ripening Extension Index (REI) and Ripening Depth Index (RDI) represented the ratio of WSN to TN and TCA-N to TN, respectively, and were determined as described by Furtado and Partridge (1988).

Electrophoresis

Proteins were extracted from cheese according to Tieleman and Warthesen (1991) with modifications. A 0.2 g aliquot of grated cheese was dissolved in a buffer containing the following composition: 0.001M sodium phosphate (pH 7.2), 2% SDS, 0.06M Tris-HCl, 9.6% glycerol, 4.8% β-mercaptoethanol, and 0.01% bromophenol blue. The cheese buffer was heated for 40 min. at 100°C, and cooled before use.

Vertical SDS-polyacrylamide gel electrophoresis was carried out in a Hoefer Model SE600 gel cell (Hoefer Scientific Instruments, San Francisco, CA) following the

technique stated by Laemmli (1970). The slab gels measured 160 x 180 x 1.5 mm. The acrylamide concentration was 10% and 4% (w/v) in the separating and stacking gel, respectively.

The gels were scanned on the Shimadzu Dual-Wavelength TLC Scanner Model CS-930 (Kyoto, Japan), provided with a DR-2 Data Recorder. A zigzag scanning mode with a slit size of 6 (height) and 2 (width) was used to limit the beam size on the sample surface. The scan width was set to 13 mm. A sensitivity of 2 (medium) was used during scanning. Gels were scanned at a wavelength of 580 nm. The signal from the start point to the end point of each peak was determined by the area signal, integrated, and the relative areas of the individual protein peaks were recorded. The initial peak areas were considered 100% of the individual proteins. The peak areas of the main casein fractions are listed in Appendix I (Table I-8 and I-9). A complete list of parameters used during gel scanning are listed in Table I-5 (Appendix I).

Statistical Analysis

A randomized split plot design with repeated measurements was used in this experiment. The main plots consisted of the treatments (cheese made with different cultures and levels), split because each cheese characteristic was analyzed at different points in time. Analysis of variance and contrasts among treatment means were performed using the MSTATC computer

program (Crops and Soil Science, Michigan State University, MI).

The linear model (Gill, 1986) for response variable Y used in this experiment was:

$$Y_{ijk} = M + T_i + C_{(i)j} + P_k + (TP)_{ik} + E_{ijk},$$

$$(i = 1...t,\ j = 1...n \text{ per } i,\ k = 1...p),$$

where the terms are overall mean (M), treatment effect (T), effect of cheese within treatment (C), period effect (P), treatment x period interaction (TP), and residual (E). It was assumed that $\sum_i T = 0$, $\sum_k P = 0$, $\sum_i (TP) = 0$, and $\sum_k (TP) = 0$. The cheese term (C) represented error associated with treatment differences, and variability among cheeses ordinarily was substantial.

RESULTS AND DISCUSSION

Composition of Cheese

The average composition of the cheeses, determined immediately after manufacture, is shown in Table I-1. The legal limits in the U.S. for moisture and fat in dry matter (FDM) contents of Cheddar cheese are $\leq$ 39% and $\geq$ 50%, respectively (Kosikowski, 1978). The average moisture content of the cheeses (49.65%) was considerably above the legal limit, and the average FDM was lower (28.40%). There were no differences ($p<0.05$) in moisture content between treatments. The higher moisture content of reduced fat Cheddar cheese is

Table I-1 - Composition of reduced fat Cheddar cheese manufactured with adjunct culture of *Lactobacillus casei* L2A.

Cheese (treatment)	Fat (%)	Moisture (%)	Protein (%)
LLC-0.5	14.44[2] ab (0.24)	49.95 a[3] (0.56)	29.33 ab (0.51)
LLC-1.0	14.19 bc (0.24)	49.77 a (0.90)	29.12 a (0.90)
LLC-1.5	14.37 abc (0.32)	49.97 a (0.58)	29.04 ab (0.38)
HSLC-0.5	14.19 bc (0.12)	49.83 a (0.53)	28.98 ab (0.27)
HSLC-1.0	14.19 bc (0.24)	49.33 a (0.71)	28.95 ab (0.49)
HSLC-1.5	14.00 c (0.00)	50.06 a (1.20)	28.74 b (0.51)
Control	14.69 a (0.37)	49.16 a (0.50)	29.61 a (0.24)

[1]Treatments at different levels of addition of adjunct culture.
LLC = live cells of *Lactobacillus casei* L2A.
HSLC = heat-shocked cells of *Lactobacillus casei* L2A.
[2]Means with standard deviations in brackets.
n = 4 replications for all treatments.
[3]Means containing the same letter within a column do not differ significantly ($p<0.05$).

required to attain firmness approximating that of normal Cheddar cheese (Banks et al., 1989). Cheeses with lower fat content contain more protein per unit volume than full fat cheeses and require more water to present similar body and texture (Olson and Johnson, 1990). Higher water levels may enhance proteolysis during ripening if the cheese salt content is not increased. In the present study, all cheeses were salted at the same level.

Cheese pH

The changes in pH of cheeses during ripening showed similar patterns within treatments and no differences ($p<0.05$) were found among levels of addition of starter culture. Figure I-1 displays the changes in pH of cheeses made with addition of 1.5% of adjunct culture of *Lactobacillus casei* L2A. The complete set of pH changes during ripening is displayed in Table I-2 (Appendix I).

As a result of gradual fermentation of residual lactose by the starter culture, the pH of all cheese decreased during the first 12 weeks of ripening. A greater level of residual lactose was expected due to the high moisture content. A similar result was observed by Lee et al. (1992) on reduced fat Cheddar cheese. After 30 min of pressing, HSLC-1.5 cheeses had the highest pH (5.56), which was probably due to the reduced lactose fermenting ability of the heat shocked adjunct culture. The lowest pH was shown by the LLC-1.5

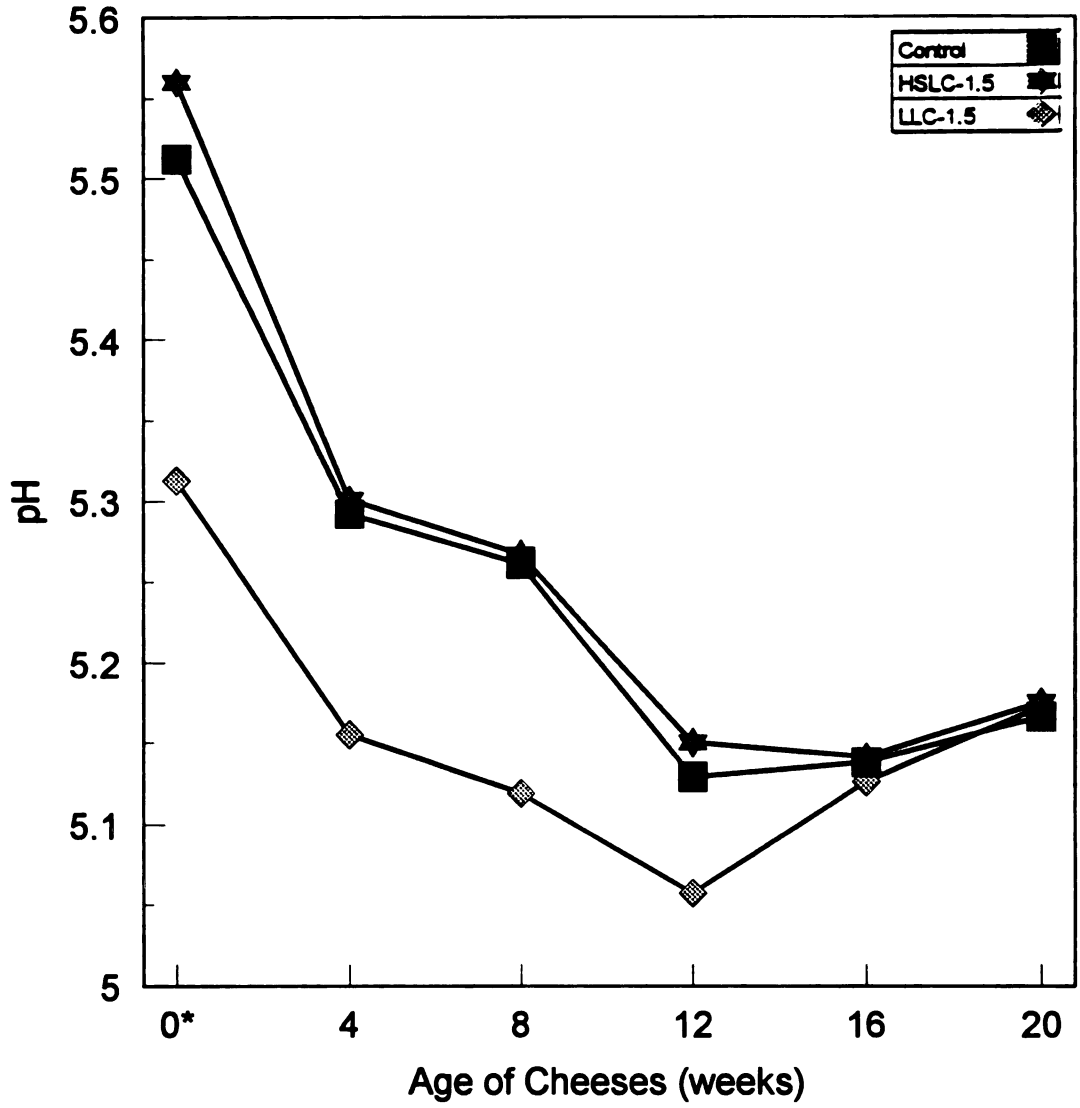

Figure I -1. Changes in cheese pH throughout ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A.
* 30 min. after beginning pressing.

cheese, which may be related to the greater acidification capacity caused by the presence of live cells of *Lactobacillus casei*. The pH of the LLC cheeses was lower ($p<0.05$) than the pH of the other two treatments during the first 12 weeks of ripening. After 12 weeks, the pH of all cheeses started increasing to reach 5.17 at 20 weeks. This increase in pH is probably related to the accumulation of basic compounds resulting from the protein breakdown during ripening.

Proteolysis

Protein hydrolysis is considered a good index of cheese ripening, as proteolysis of casein is the result of several proteinase and peptidase activities. The results of WSN and TCA-N analyses during the ripening of reduced fat Cheddar cheese are given in Figure I-2. No differences ($p<0.05$) were found among levels of addition of starter culture, thus only the 1.5% level was presented. The evolution of N fractions and ripening indices during ripening for all cheeses are displayed in Table I-3, I-4 and I-5 (Appendix I). There was a four fold increase in WSN values for all treatments (Control, LLC-1.5 and HSLC-1.5) after 20 weeks of ripening (Figure I-2A). The WSN for the HSLC-1.5 (0.93%) was higher ($P<0.05$) than that for the LLC-1.5 (0.86%) at 20 weeks. In cheese, WSN consists predominantly of medium and small peptides and free amino acids. Rennet is considered to be the main agent in producing medium peptides which are further degraded to small peptides

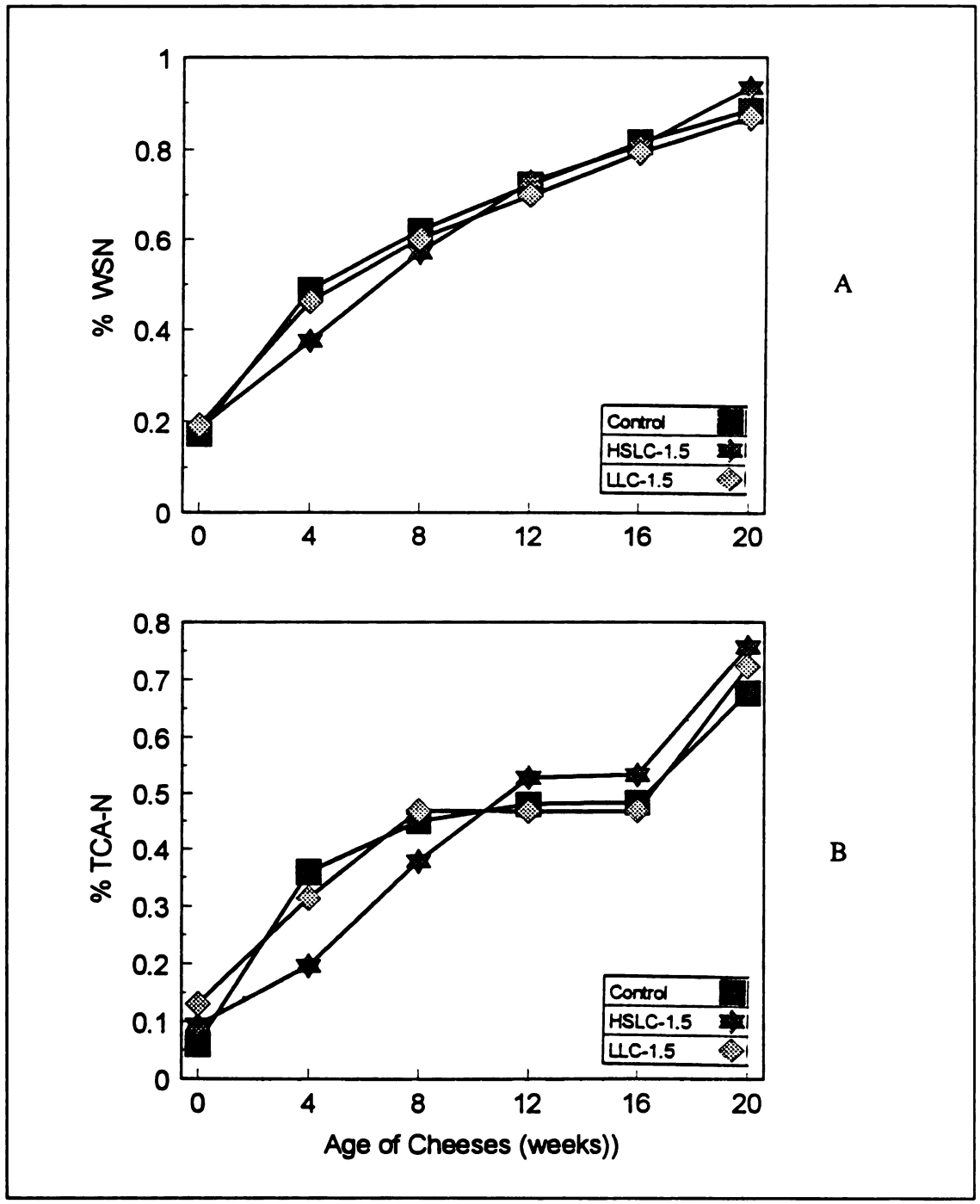

Figure I-2. Evolution of nitrogen fractions during the ripening of reduced fat Cheddar cheese made with 1.5% of live and heat shocked *Lactobacillus casei* L2A. A = WSN (water-soluble nitrogen); B = 12% TCA-N (TCA-soluble nitrogen).

and amino acids by starter peptidases (Adda et al., 1982; Fox, 1993; Fox and Law, 1991; O'Keeffe et al., 1978). According to Mulvihill and Fox (1977), the specificity of chymosin on α_{s1}-casein is pH-dependent, the protein being optimally hydrolysed to smaller peptides at pH 5.8. LLC-1.5 had a low pH (Figure I-1) throughout ripening and at 20 weeks had the least WSN. The liberation of TCA-N during ripening is shown graphically in Figure I-2B. The levels of TCA-N increased as the cheese aged. The most pronounced peptidolytic activity was shown by the HSLC-1.5 treatment. The TCA-N increased from 0.1 to 0.75% in 20 weeks and was higher ($P<0.05$) than that for the Control treatment, which increased from 0.1 to 0.68% in 20 weeks. These results confirm the findings of other studies (Ardo and Mansson, 1990; El-Abboudi et al., 1991; Law, 1984), indicating that the heat-shocking of *Lactobacillus casei* released aminopeptidases which were ultimately responsible for the greater proteolysis associated with TCA-N observed in the HSLC-1.5 treatment.

The results of the ripening indices at 20 weeks for the three levels of adjunct cultures are presented in Figure I-3. A Ripening Extension Index of 21 and 19% (Figure I-3A) were found for the HSLC and LLC treatments, respectively, independent of the level of inoculation with adjunct cultures of *Lactobacillus casei*. No differences ($p<0.05$) were found among the HSLC nor the LLC levels of treatments, but all HSLC results were higher ($P<0.05$) than those observed for the

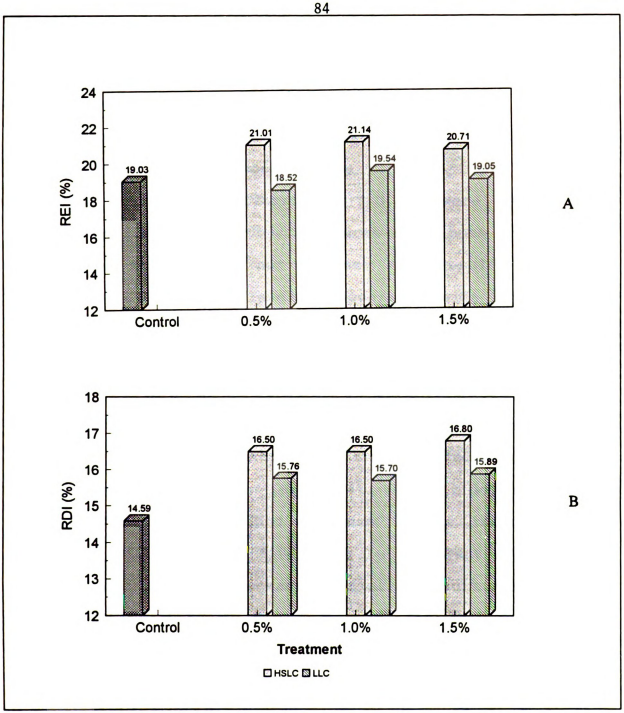

Figure I - 3. Changes in the ripening indices during the ripening of reduced fat Cheddar cheese made with different levels of adjunct culture of *Lactobacillus casei* L2A. A = REI (Ripening Extension Index); B = RDI (Ripening Depth Index).

Control and LLC treatments. These results are supported by the pH changes observed throughout ripening (Figure I-1). The LLC had the lowest pH which likely decreased the proteinase activity of the starter in the early stages of ripening. In Figure I-3B, the changes in the Ripening Depth Index are shown. Again, no differences ($P<0.05$) were found among different levels of adjunct cultures added both for the HSLC and LLC treatments, nor between the results of these treatments within each level of inoculation with *Lactobacillus casei*. However, the RDI for all of them were higher ($P<0.05$) than that for the control. These results confirm that the addition of heat-shocked cells of *Lactobacillus casei* increase the aminopeptidase activity and the liberation of amino acids from peptides, as indicated elsewhere (Ardo and Mansson, 1990; El-Abboudi et al., 1991; Law, 1984). The peptidolysis was not enhanced by a three-fold increase in the inoculation of adjunct cultures. This finding is in agreement with that of Law et al. (1993), who observed a similar result when studying the contribution of lactococcal proteinases to proteolysis in Cheddar cheese.

The results concerning the percentage of WSN corresponding to TCA-soluble nitrogen after 20 wk of ripening are presented in Figure I-4. The TCA-N of LLC-1.5 and HSLC-1.5 (81.11 and 83.39%, respectively) were similar ($P<0.05$), but both were higher ($P<0.05$) than that (76,67%) observed for the Control treatment. These results indicate that endogenous

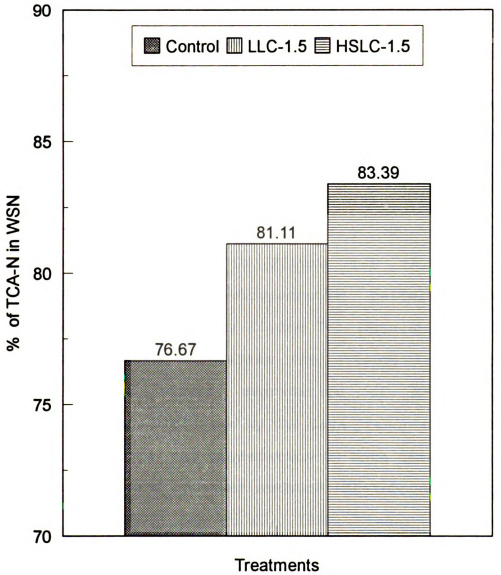

Figure I - 4 - Percentage of water soluble nitrogen (WSN) corresponding to TCA-soluble nitrogen (TCA-N) after 20 weeks of ripening.

enzymes from *Lactobacillus casei* have peptidase activity, which can be enhanced when the aminopeptidases are freed from the cell by the heat shock treatment.

These results are important considering that the degradation of casein implicates the corresponding breakdown of the protein matrix. The loosening of the cheese protein matrix causes texture changes and increases the water binding capacity, which are important to reduced fat cheeses.

Electrophoretic Patterns

Typical densitograms of the Control, HSLC-1.5 and LLC-1.5 treatments are shown in Figure I-5 (A, B, and C), respectively. The electrophoretic patterns throughout ripening are presented in Figure I-1 (Appendix I). After 20 weeks of ripening α_{s1}-casein was extensively degraded as opposed to a slight degradation of β-casein. These observations are confirmed by the data presented in Figure I-6 (A, B) which shows the degradation of both caseins throughout ripening. As found by other authors (El-Abboudi et al., 1991; Fox and Law, 1991; Law et al., 1992; Visser, 1993) β-casein was quite resistant to rennet and bacterial enzymes during ripening. As shown in Figure I-6A, β-casein was slowly broken down during 20 weeks of cheese ripening and at the end 79.11, 70.43 and 82.14% of original value were still intact for Control, HSLC-1.5 and LLC-1.5 treatments, respectively. The Control and LLC-1.5 values did not differ, but both were

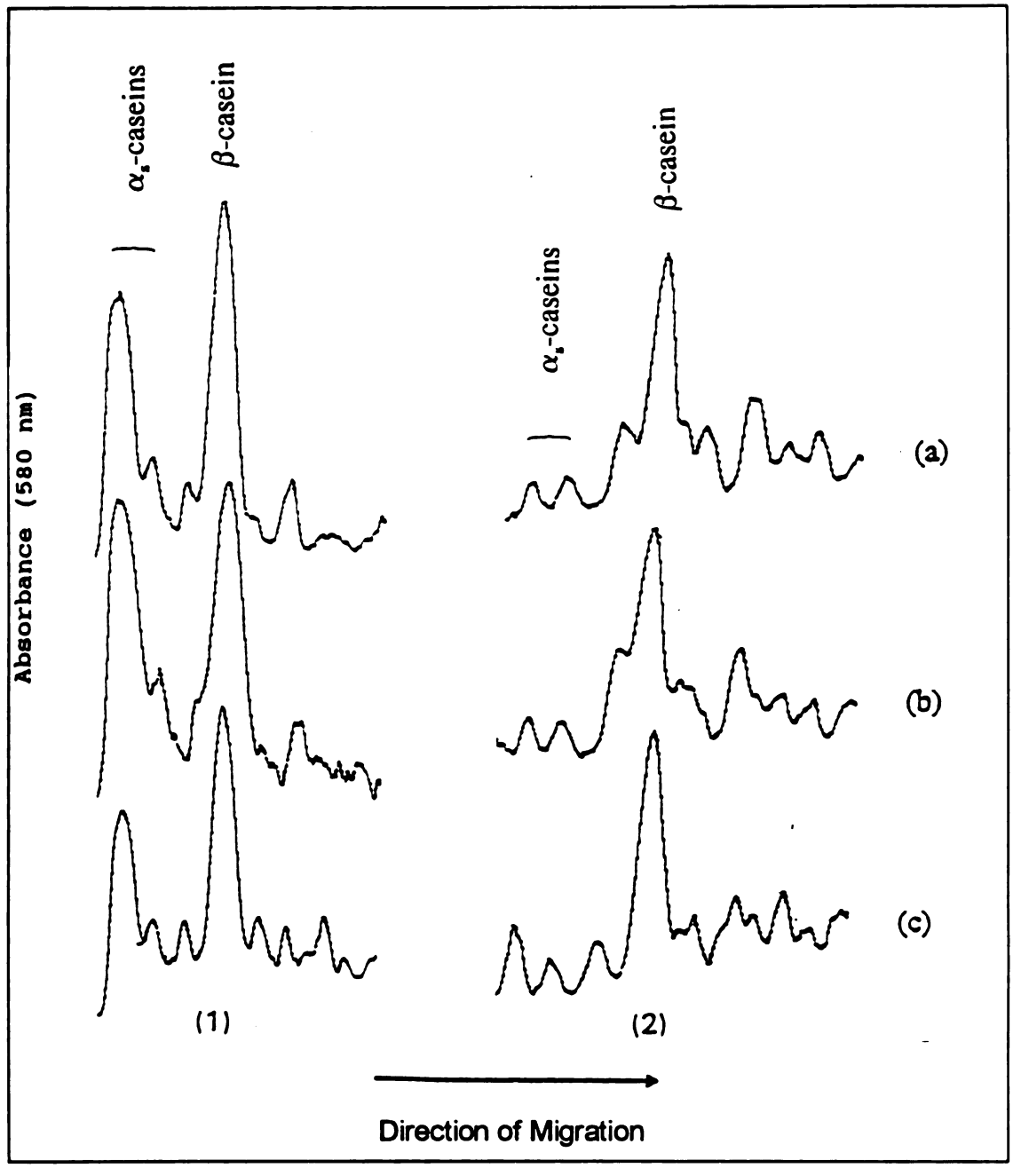

Figure I - 5 - SDS-Polyacrylamide gel electrophoretograms of caseins in reduced fat Cheddar cheese at 1 week and 20 weeks (2) of ripening. (a) Control cheese; (b) cheese made with heat shocked *Lactobacillus casei* L2A; (c) cheese made with live *Lactobacillus casei* L2A.

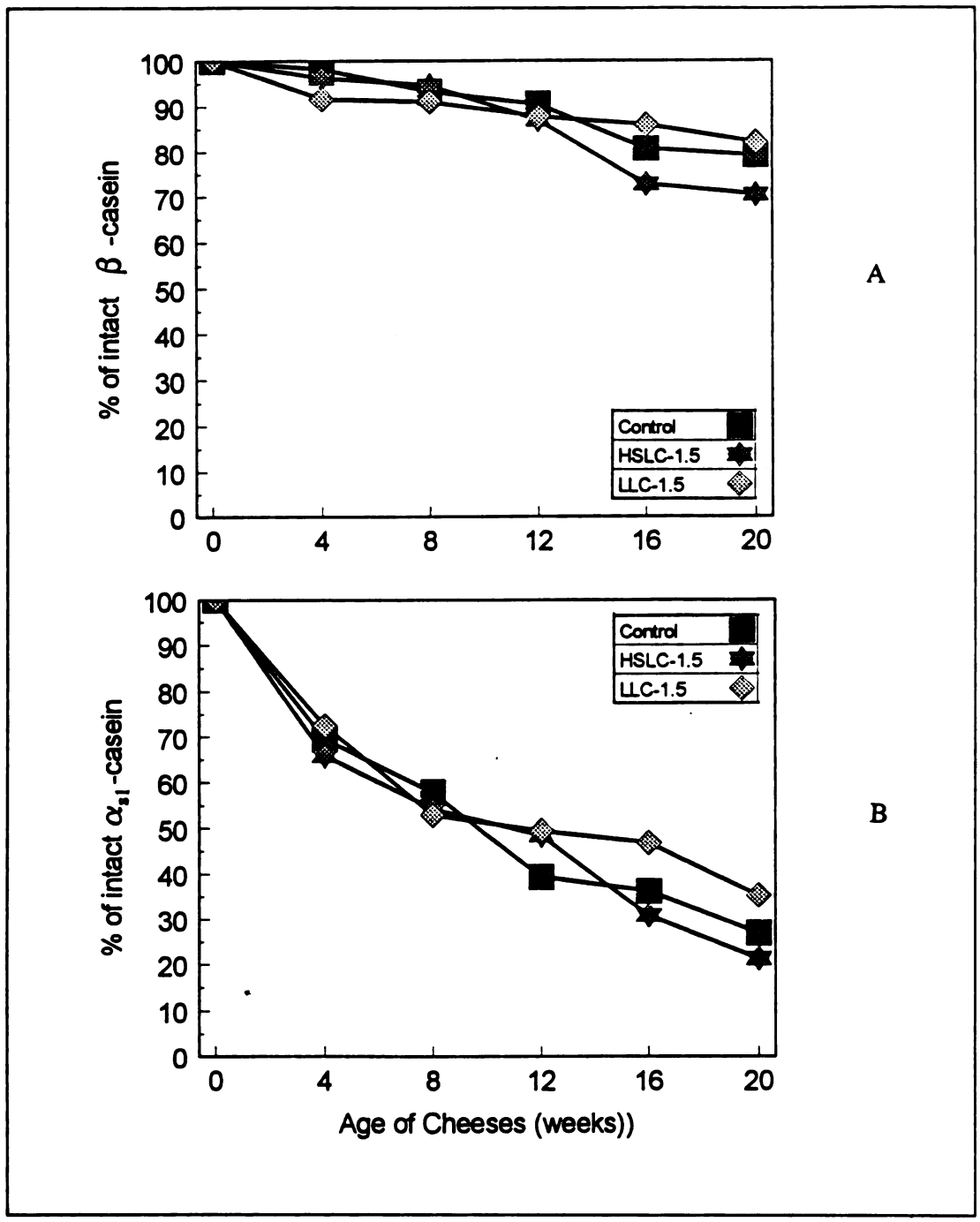

Figure I - 6 - Degradation of β-casein (A) and α_{s1}-casein (B) throughout the ripening of reduced fat Cheddar cheese made with 1.5% of live and heat shocked *Lactobacillus casei* L2A.

higher than that for the HSLC-1.5 cheeses ($P<0.05$). The values presented in Figure I-6B show that α_{s1}-casein was extensively broken down. Only 26.94, 21.31 and 35.30% of the original α_{s1}-casein remained intact after 20 weeks of ripening, in the Control, HSLC-1.5 and LLC-1.5 cheeses, respectively. These values were different ($p<0.05$) from each other. These results are in agreement with those found by Lau et al. (1991), Visser and Groot-Mostert (1977) and Kaminarides et al. (1990), who observed that 85, 90 and 77% of α_{s1}-casein, respectively, had been degraded at the end of the ripening of different cheeses.

As shown in Figure I-6 (A, B), both caseins were degraded more in the HSLC cheese. This was likely the result of the strong aminopeptidase activity of the heat shocked cells of *Lactobacillus casei* L2A.

Figure I-7 (A, B, C) shows the plots of progressive α_{s1}-casein degradation against the increase in WSN throughout reduced fat Cheddar cheese ripening. The production of soluble nitrogen correlated negatively (correlation coefficient of -0.98, -0.97 and -0.99 for LLC-1.5, HSLC-1.5 and Control treatments respectively) with the decrease in the amount of intact α_{s1}-casein.

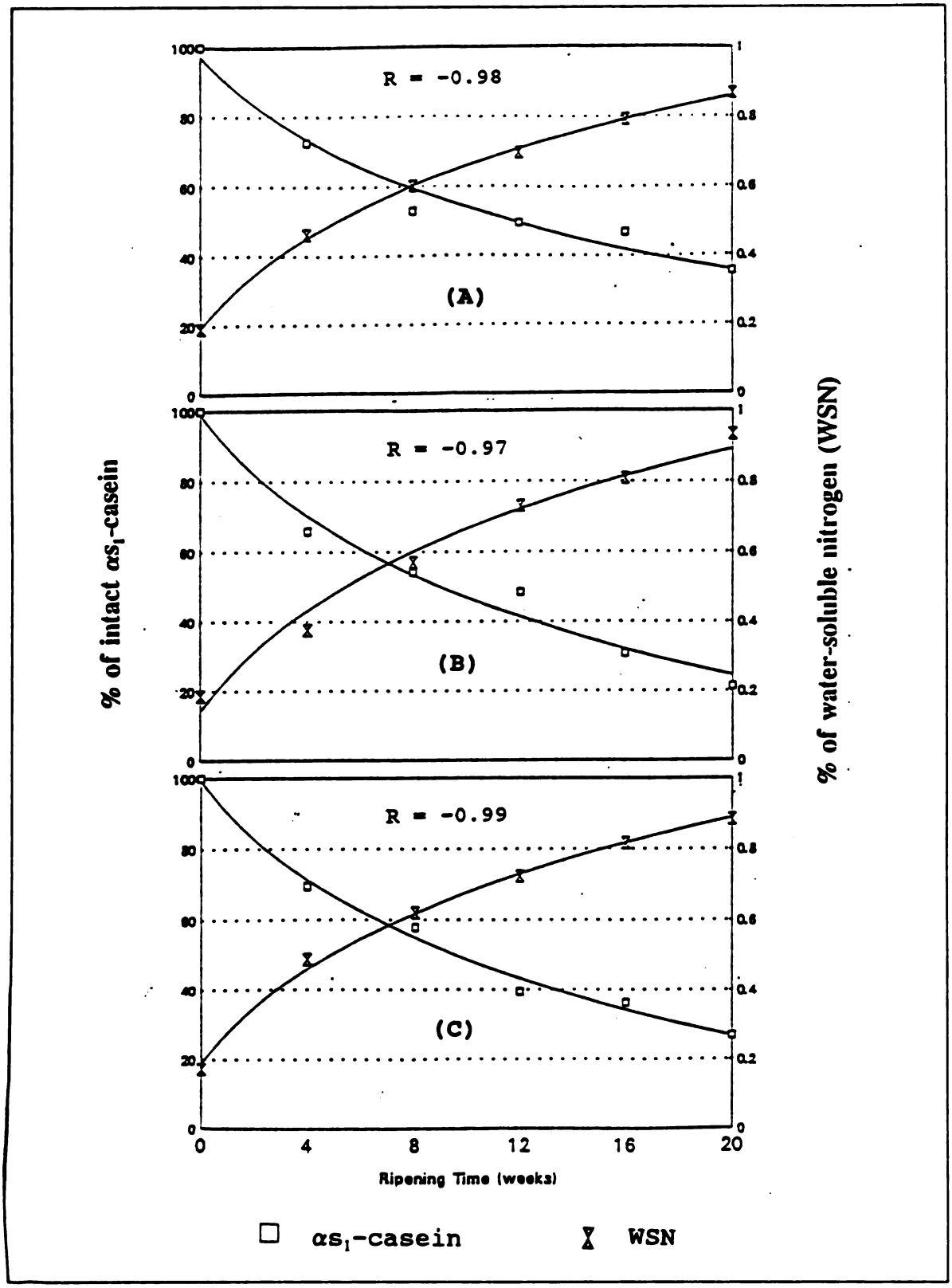

Figure I - 7. Correlation between degradation of α_{s1}-casein and increase in water soluble nitrogen (WSN) throughout the ripening of reduced fat Cheddar cheese. A = LLC-1.5 ; B = HSLC-1.5; C = Control. R = Correlation Coefficient.

CONCLUSIONS

The results of this experiment showed that the addition of adjunct cultures of live and heat shocked *Lactobacillus casei* L2A enhanced starter aminopeptidase activity in reduced fat Cheddar cheese. The addition of live cells lowered the pH of cheeses, resulting in inhibition of aminopeptidase activity when compared to the HSLC treatment. The heat shock treatment of *Lactobacillus casei* L2A cultures inhibited the acid producing ability of the microorganism without destroying the aminopeptidases. Therefore, higher levels of pH 4.6 and 12% TCA-N were produced in HSLC cheeses.

Chapter 2

The Use of *Lactobacillus casei* L2A as Adjunct Culture and its Effects on Flavor and Texture of Reduced Fat Cheddar Cheese.

INTRODUCTION

Milkfat affects the textural and structural properties of cheese. When fat is removed from the protein network, the cheese becomes more compact and tends to present different mechanical properties from a full fat cheese. Fat affects the mouthfeel and palatability of the cheese and the ratio of water to protein, which ultimately regulate firmness and elasticity. As there is a growing concern about the consumption of fat in the diet, much research has been devoted to the making of reduced fat cheeses (Banks et al. 1989; Lee et al., 1992; Lindsay, 1991; Olson and Johnson, 1990). However, the use of traditional processes to make reduced fat Cheddar results in cheeses with a variety of textural and flavor defects. It is well established (Lawrence et al. 1983) that the texture and the flavor characteristics of a mature cheese result mainly from the degradation of the cheese

proteins, which is in turn highly influenced by such factors as moisture, the ratio of moisture to casein, salt and the proteolytic and peptidolytic activities of the starter. Reduced fat Cheddar cheese tends to have higher moisture content than its regular counterpart (Simard, 1991). This is an important feature as the role of water in the cheese is quite broad: it influences enzymatic activity and enhances protein hydrolysis, and may react chemically with the fat or casein, and therefore affects the rheological properties of the cheese. Some water is bound to protein, but most of it occupies the space between the fat and the casein, acting as a lubricant between their surfaces (Prentice, 1992). The major structural component of a cheese is a continuous protein matrix, which is gradually broken down during ripening. This breakdown is important to cheese texture: as each peptide bond is cleaved, two new ionic groups are generated and each of them will compete for the available water in the system (Creamer and Olson, 1982) which is gradually bound to the newly formed peptides and amino acids. Proteolysis by rennet is believed to be responsible for the softening of cheese texture early during ripening via the hydrolysis of α_{s1}-casein (Fox, 1988; Noomen, 1983), a task which is accomplished later by peptidases and aminopeptidases from the starter. Therefore, the choice of starters plays a major role in determining the final characteristics of cheese. As the development of off-flavors (meaty-brothy, bitter and unclean) is a common problem

in reduced fat Cheddar cheese, the use of adjunct cultures with higher peptidolytic activities has been extensively studied. Live or heat-shocked cells of *Lactobacillus casei* have been a main source of peptidolytic enzymes for Cheddar cheese trials (Laleye, 1989; Simard, 1991; Trepanier et al. 1992), due to its recognized ability to promote extensive peptides degradation during cheese ripening.

The common textural and off-flavors problems of reduced fat cheese could be approached by the evaluation of changes in the cheese moisture and enhancement of peptidolytic activity during ripening. The objectives of the present work were to evaluate the effects of moisture content and the use of different levels of adjunct cultures of live and heat-shocked *Lactobacillus casei* on the textural and rheological properties and flavor characteristics of reduced fat Cheddar cheese, as well as to relate the rheological information to the degradation of α_{s1}-casein during cheese ripening.

MATERIALS AND METHODS

Rheological Measurements

Cheeses 4, 8, 12, and 16 weeks of age were analyzed for texture properties by the Instron Universal Testing Machine, model 1102, following the procedure described by Chen, A.H. et al. (1979), with modifications. Four textural

characteristics, hardness, cohesiveness, adhesiveness, and elasticity were measured.

Sample preparation

Cheese samples were kept in plastic bags at room temperature overnight and cut in cubes 2.54 cm (height) x 2.54 cm (width) x 3.5 cm (length), immediately prior to the test. The test was performed at a constant temperature of 22°C.

Setting of the Instron

An Instron Compression Load Cell (500 N), fixed to the crosshead, was used to make the compression tests. The parameters hardness, cohesiveness and adhesiveness were based on force-displacement measurements with the use of a plunger. A plunger (0.6 cm diameter) was attached to the moving crosshead, which was set to cycle mode at a constant speed of 2.5 cm/min in both upward and downward directions. A flat holding plate was used to keep the cheese sample held on the stationary horizontal bedplate of the machine, avoiding sticking of the cheese on the plunger in its upward movement. The penetration of the plunger into the cheese sample was set to 1.91 cm, which corresponded to 75% of the height of the sample.

A bite was considered as a complete cycle of one downward and one upward movement of the plunger, which penetrated and then was removed from the sample. To perform the tests, 2

bites were taken. The chart speed was set to 5 cm/min. A typical texture profile analysis (TPA) curve obtained by the Instron Universal Testing Machine is shown in Figure II-1.

Determination of the parameters

The parameters hardness, cohesiveness, and adhesiveness were defined and determined from the TPA curves, according to formulas described by Friedman et al. (1963). Hardness, defined as the maximum force exerted on the sample, was measured from the TPA as the height of the first bite. Cohesiveness was measured as the ratio of the area, in arbitrary units, under the second peak and the area under the first peak (A_2/A_1). Adhesiveness was measured as the area (A_3), in arbitrary units, of the negative peak beneath the base line of the TPA curve, which represented the work necessary to remove the plunger from the cheese sample.

Areas under the peaks were calculated by using the cut and weigh method. The chart paper was photocopied using the same type of paper to reduce errors due to variations in paper density. The peaks were cut and weighed in a digital analytical scale. The individual peak weights were recorded and then converted to area units.

The parameter springiness was calculated using the same settings described previously, except that the test was performed in a single compression step, with a flat plate with 4 cm diameter. In this procedure, springiness was expressed

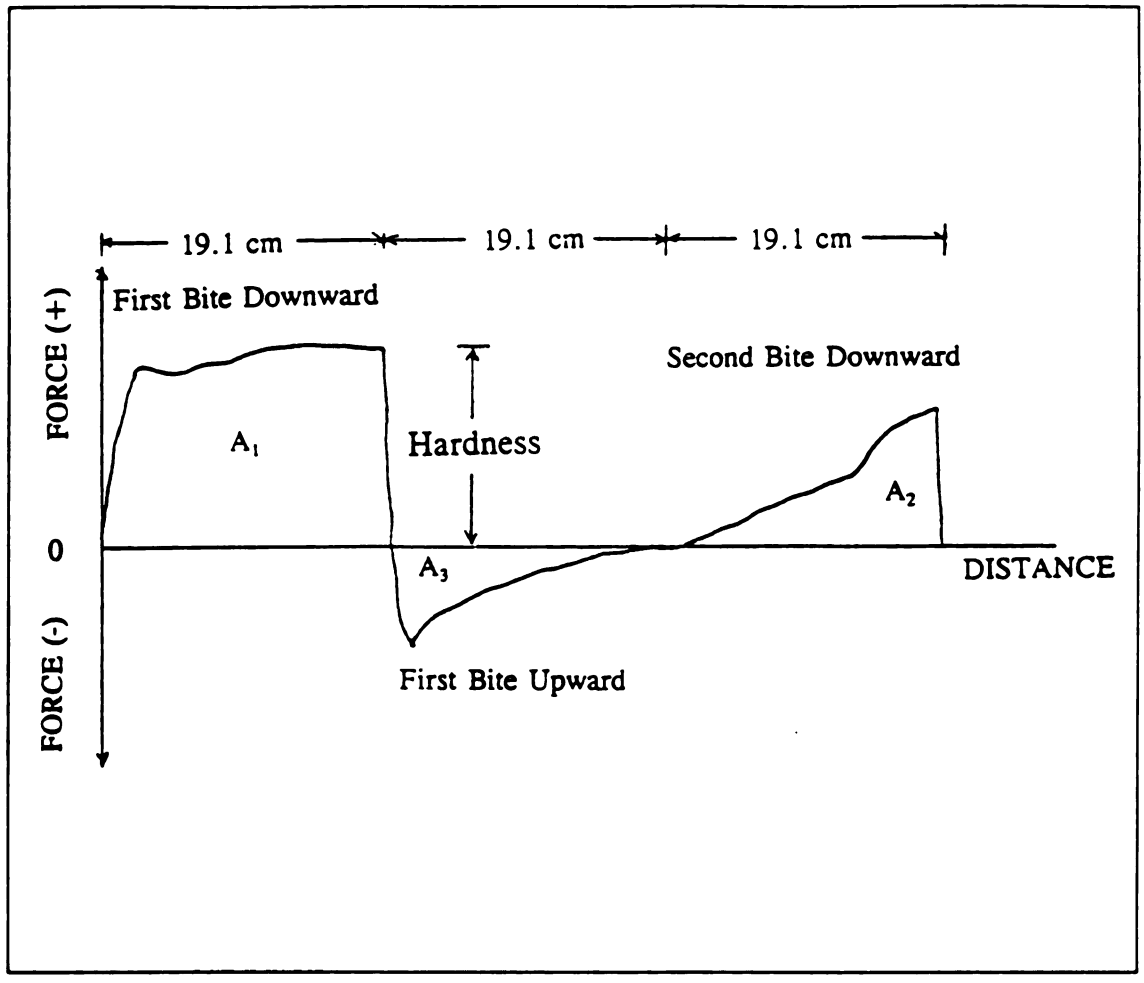

Figure II -1. Typical texture profile analysis (TPA) curve obtained by the Instron Universal Testing Machine.

as the ratio of the sample heights before and after the compression. An illustration of how springiness was calculated is shown in Figure II-2.

Organoleptic Assessment

The organoleptic evaluation of the cheese was carried out by a trained taste panel of 13 members, including faculty and graduate students from the Department of Food Science and Human Nutrition. Cheeses were sampled at 32 weeks of age, coded with three-digit numbers and four samples of each were presented simultaneously to the panelists.

Four textural characteristics, hardness, cohesiveness, adhesiveness, springiness were evaluated. A balanced Hedonic Scale of 1-9 was used for rating each textural attribute. Two flavor characteristics were evaluated: Cheddar cheese flavor intensity and bitterness. A diluted quinine sulfate solution and Cheddar cheeses with different degrees of bitterness were submitted to the panelists during the training sessions for characterization of bitter flavor. A balanced Hedonic Scale of 1-6 was used for rating the flavor intensity and bitterness attributes. A score of 6 was attributed to a sample exhibiting "very strong" flavor and a score of 1 to one exhibiting "not observed" flavor. Analysis of variance and contrasts among treatments means at the 95% level were performed using the MSTATC computer program (Crops and Soil Science, Michigan State University, MI). All sample forms of questionnaire

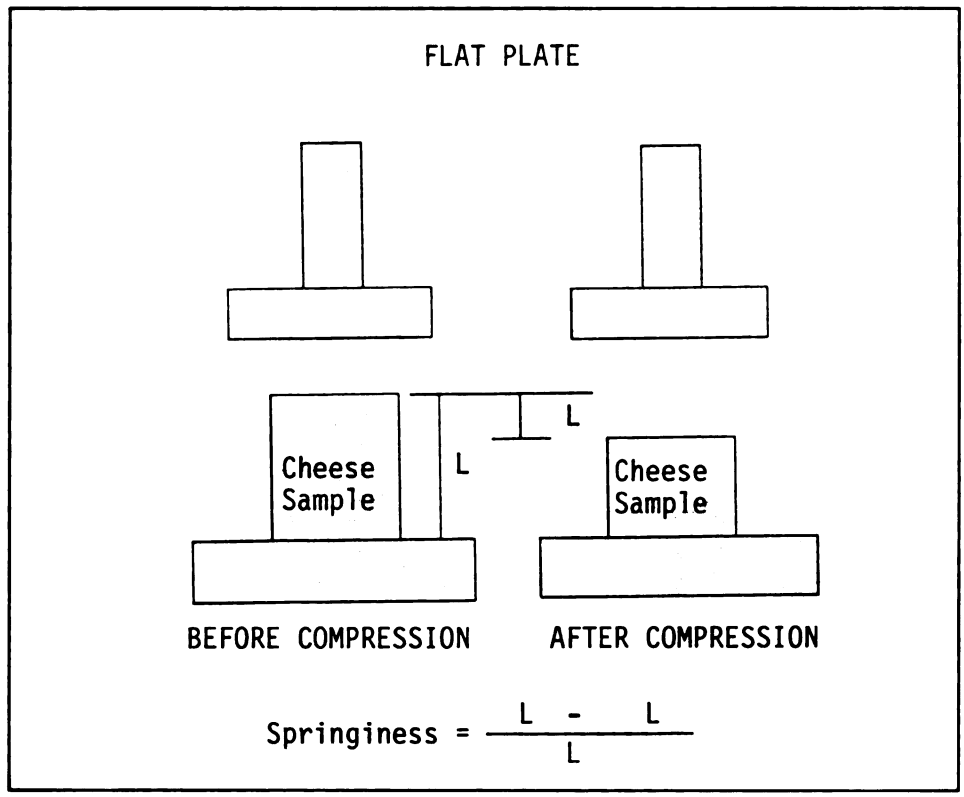

Figure II - 2. Textural definition of springiness.
Source: Chen et. al. (1979)

presented to the panelists during training and judgement sessions are in the Appendix II (Figures II-1 thru II-12).

RESULTS AND DISCUSSION

Rheological Evaluation

As previously reported in Chapter 1, only the results of the highest level of addition of adjunct culture of *Lactobacillus casei* L2A (1.5%) will be discussed. Complete data set for adhesiveness, cohesiveness, springiness and hardness are listed in the Appendix section, under Tables II-1, II-2, II-3 and II-4, respectively.

Adhesiveness

Adhesiveness is defined as the work required to overcome the attraction forces between the surface of the cheese and the surface of the compression plate, as the plunger begins its upstroke (van-Vliet, 1991; Zoon, 1991). The adhesiveness of all cheeses decreased during ripening. No differences ($p<0.05$) were found among the three treatments (Figure II-3). These results may be related to changes in the structural matrix of the cheese due to the breakdown observed in α_{s1}-casein.

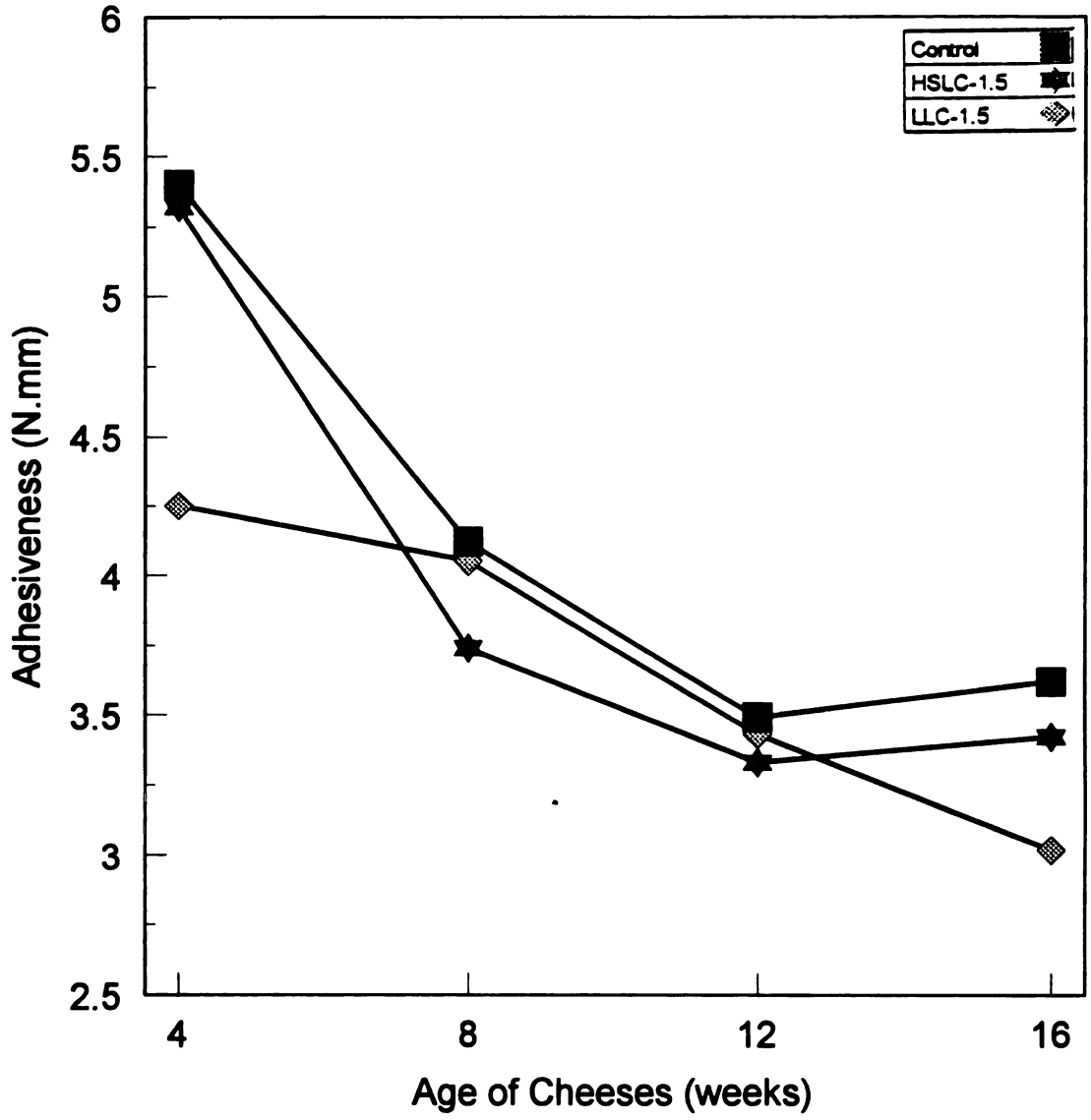

Figure II - 3 - Adhesiveness throughout the ripening of reduced fat Cheddar cheese made with 1.5% of live and heat shocked cells of *Lactobacillus casei* L2A.

Cohesiveness

Cohesiveness or crumbliness can be described as the extent to which a food stuff can be deformed before it ruptures (Zoon, 1991) or the resistance of a material to external forces, tending to separate parts of the body (van-Vliet, 1991). Figure II-4 depicts the results of cohesiveness throughout ripening. The scores showed variations among treatments, especially during the first eight weeks of ripening. As the LLC cheeses had the lowest pH throughout most of the ripening period, this may have affected the mineral content of the curd. According to Lawrence et al. (1983), the mineral content of a cheese serves as an indication of the extent to which the structure of the casein sub-micelles have been disrupted. The lower the pH of a cheese, less calcium is retained in curd, which makes it more crumbly, a characteristic that does not change much during ripening. The LLC cheeses, which had the lowest pH (Figure I-1, Chapter 1), were less cohesive ($p<0.05$) than both the HSLC-1.5 and Control cheeses over the first 8 weeks of ripening. No differences ($p<0.05$) were found among treatments after 12 weeks of ripening. The lower pH of the LLC-1.5 cheeses may have made them shorter with fracturing at smaller deformation. This is in agreement with the findings of Walstra and van-Vliet (1982) who reported that more acidic cheeses require less force to fracture.

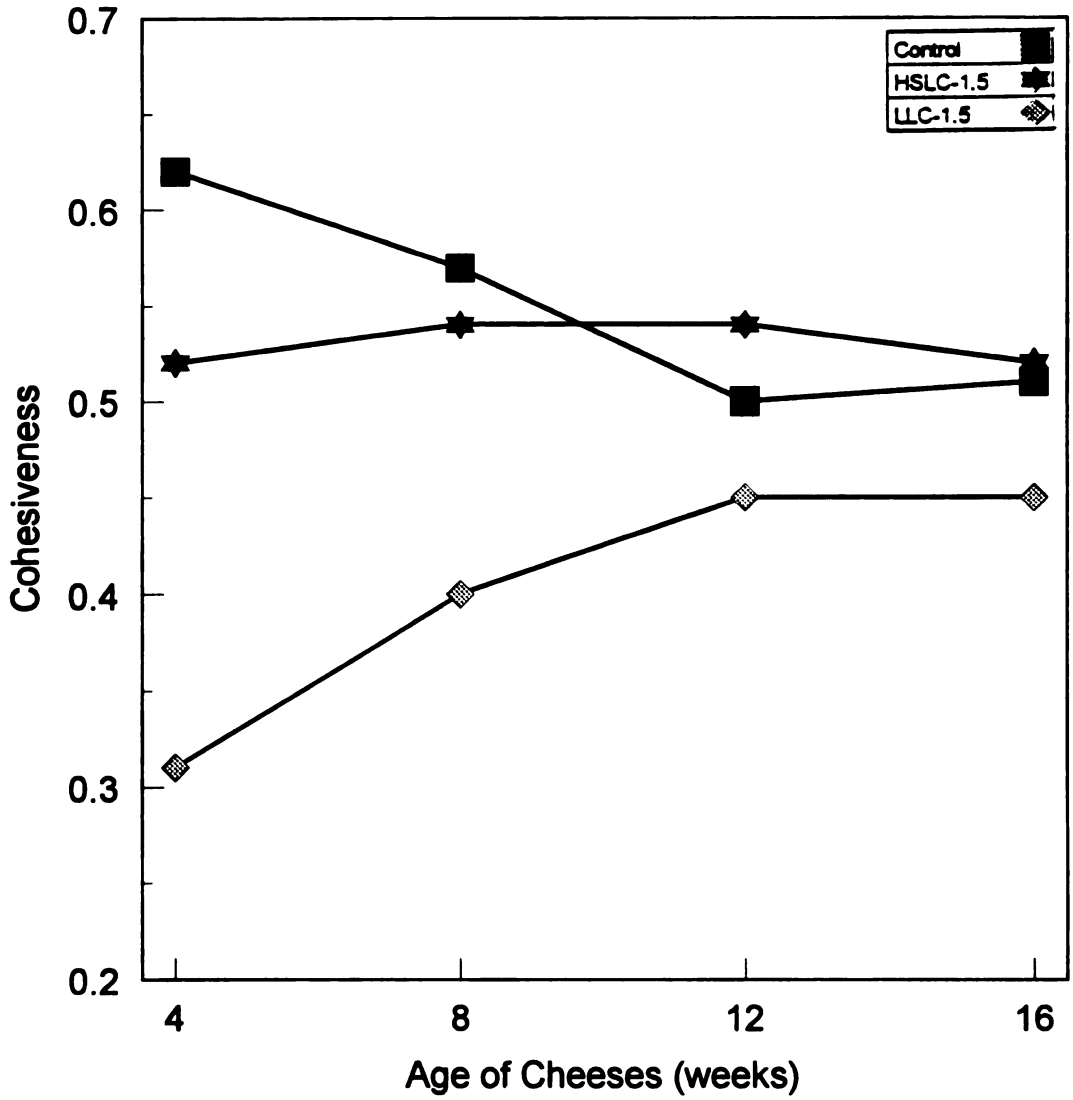

Figure II - 4 - Cohesiveness throughout the ripening of reduced fat Cheddar cheese made with 1.5% of live and heat shocked cells of *Lactobacillus casei* L2A.

Springiness

Springiness is defined as the textural property manifested by a tendency to recover from a large deformation after removal of the deforming force (van-Vliet, 1991). No significant changes were observed during ripening, but the mean scores attained by the LLC-1.5 cheeses were lower ($p<0.05$) than those observed for the other cheeses (Figure II-5). As discussed earlier, the pH of LLC-1.5 cheeses was the lowest from the beginning. This increased acidification decreased the retention of calcium in the curd, which is a critical factor in cheese texture. Springiness decreases during ripening as the original rubbery texture of fresh cheese is gradually changed to a smoother body, due to α_{s1}-casein breakdown.

Hardness

Hardness is defined as the force necessary to attain a given deformation (van-Vliet, 1991). The hardness of all treatments decreased rapidly until eight weeks of ripening and then the process slowed down (Figure II-6). Hardness of the Control and HSLC-1.5 treatments were lower ($p<0.05$) than those of the LLC treatment after 16 weeks of ripening. Again, this may be related to the lower pH of cheeses made with live cells of *Lactobacillus casei* L2A. The lactic acid produced probably removed calcium from the paracaseinate, in the form of calcium lactate, which is soluble in water and was lost in the whey.

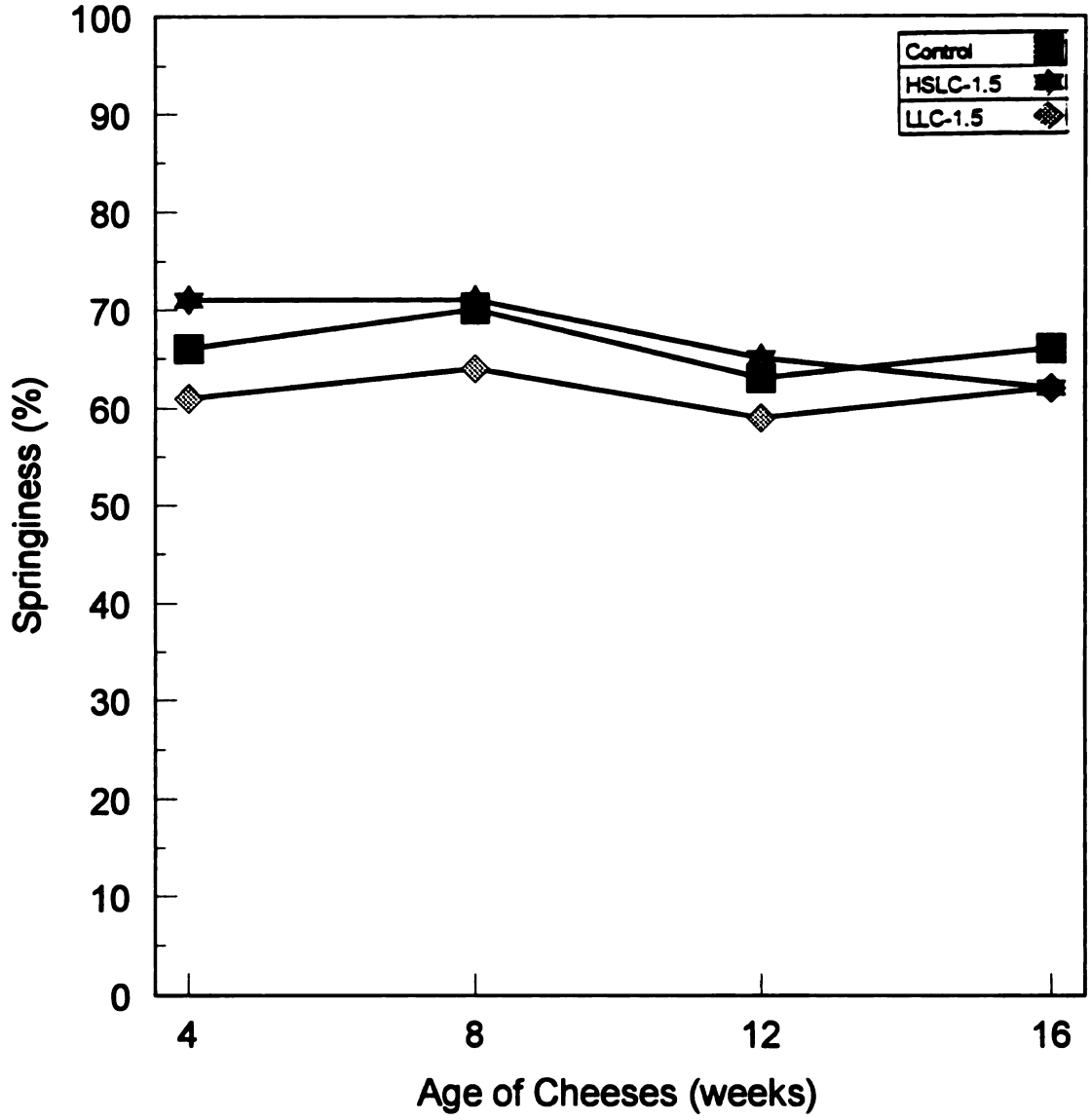

Figure II - 5 - Springiness throughout the ripening of reduced fat Cheddar cheese made with 1.5% of live and heat shocked cells of *Lactobacillus casei* L2A.

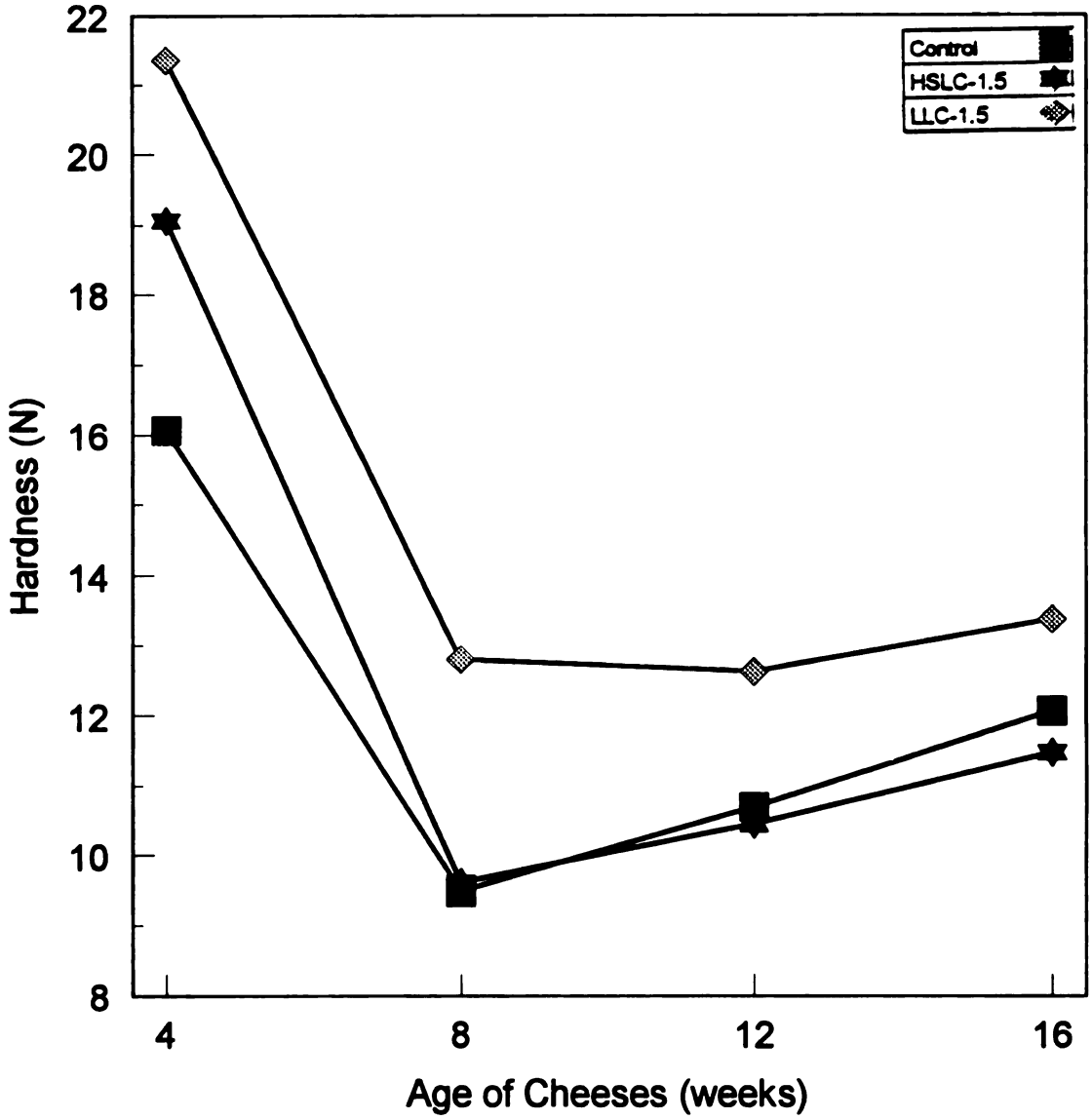

Figure II - 6 - Hardness throughout the ripening of reduced fat Cheddar cheese made with 1.5% of live and heat shocked cells of *Lactobacillus casei* L2A.

Therefore, these cheeses had a more demineralized casein backbone, which rendered the cheese structure less pliable and firmer. This is supported by Walstra and van Vliet (1982) who also reported increased firmness in cheeses with low pH values.

According to Lin et al. (1987), hardness is the most important parameter of cheese texture with respect to consumer preference and sensory perception. Moisture plays a decisive role in the softening of cheese texture by enhancing protein hydrolysis. All cheeses from these experiments presented moisture content around 49.65%, which is considerably higher than the values of traditional Cheddar cheese. The breakdown products of casein are largely water soluble and cannot contribute to the protein matrix. The electrophoretic results have shown a gradual degradation of α_{s1}-casein with a correspondent increase in TCA-N throughout ripening. These events seem to correlate with the observed decrease in cheese hardness, as found by other authors (Amantea et al., 1986; Bertola et al., 1992; Creamer and Olson, 1982; Lin et al., 1987; Walstra and van-Vliet, 1982). These results suggest that in the making of reduced fat Cheddar cheese, the degree of proteolysis is of utmost importance as it can affect hardness and partially overcome the lack of calcium in the structure of more acidic cheeses. The contribution of water is important, due not only to its physical role, but also because the presence of a greater amount of water facilitates the access

of microorganisms and peptidolytic enzymes to the casein (Prentice, 1992) stimulating the phenomenon of proteolysis.

Sensory Evaluation

Textural characteristics

The seven experimental reduced fat Cheddar cheeses, all fully ripened (eight months) were evaluated by a trained sensory panel. The results of four textural characteristics (adhesiveness, cohesiveness, springiness and hardness) are shown in Figure II-7 (A, B, C and D, respectively). The textural characteristics of all cheeses, as scored by the sensory evaluation panel are displayed in Table II-5 (Appendix II).

Adhesiveness in sensory evaluation is defined as the force necessary to remove the food sample that adheres to the mouth surface during the eating process (Chen et al., 1979). The scores (Figure II-7A) for the HSLC at the 0.5 and 1.0% levels of addition of adjunct culture, were slightly lower than those of HSLC-1.5 and the Control and LLC cheeses, but no differences ($p<0.05$) were found among all treatments. The average score of all treatments (4.9) indicate that cheeses were moderately adhesive.

Figure II-7B shows the scores of cohesiveness, which is defined as the degree to which the cheese sample deforms before rupturing (Chen et al., 1979). There were no differences ($p<0.05$) among levels of addition of adjunct

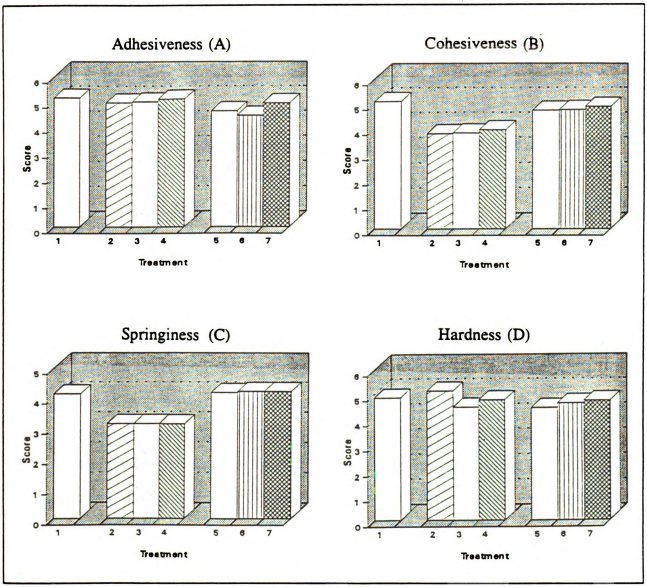

Figure II - 7 - Textural characteristics of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as scored by the sensory evaluation panel. 1 = Control; 2, 3, 4 = LLC at 0.5, 1.0 and 1.5% of addition, respectively; 5, 6, 7 = HSLC at 0.5, 1.0 and 1.5% of addition, respectively.

cultures in the HSLC trial nor between the latter and the Control cheeses, but both have shown cohesiveness scores higher ($p<0.05$) than those of all three LLC cheeses. The average score for the HSLC cheese was 4.8 which means a moderately cohesive cheese. Cohesiveness is related to crumbliness which means the tendency of a cheese to break down into small, irregular particles, that was a clear characteristic of LLC cheeses. As discussed earlier, these cheeses were more acidic and had a weaker structure due to a possible lower calcium content.

Springiness is understood as the rate at which the cheese returns to its undeformed condition after the compression force is removed (van-Vliet, 1991; Zoon, 1991). The results of the evaluation of springiness are shown in Figure II-7C. Again, there were no differences ($p<0.05$) among the levels of addition of adjunct culture of *Lactobacillus casei* L2A in the HSLC experiment nor between the latter and the Control cheese, but both have presented springiness scores higher ($p<0.05$) than those observed for the LLC cheeses. These results are probably related to the reduced degree of mineralization commonly found in more acidic cheeses, as it is the case of the LLC cheeses. These cheeses were made with the help of live *Lactobacillus casei* cells which kept their normal fermentation ability in addition to that of regular mesophile starter used in the manufacturing process. As a result, the pH of the LLC cheeses throughout ripening was lower than in both Control and

HSLC cheeses. The LLC cheeses springiness average score was 3.1, which rated them in between a moderately to a not springy cheese.

In Figure II-7D, the average scores of hardness detected by the panel, are shown. Hardness in sensory applications is defined as the force required to penetrate the cheese with the teeth or to compress it between the molar teeth (van-Vliet, 1991; Zoon, 1991). No differences ($p<0.05$) in hardness existed among the treatments after eight months of ripening.

Organoleptic Assessment

Scores resulted from the evaluation of Cheddar cheese flavor intensity and bitterness are shown in Figure II-8 (A and B, respectively) and II-6 (Appendix II). A traditionally made full fat Cheddar, aged for eight months, was used as a reference for the sensory evaluation. Figure II-8A, shows the scores for Cheddar cheese flavor intensity, as given by the trained panel. No differences ($p<0.05$) were detected among the Control, LLC or HSLC treatments, but panelists found more ($p<0.05$) Cheddar cheese flavor intensity in the full fat sample (score of 3.7, rated as "distinct Cheddar cheese flavor"). All the other cheeses presented an average score of about 2.6 correspondent to a rating of "moderate Cheddar cheese flavor." These results should be expected, as the cheeses were made with a substantial reduction in their fat content. It is widely accepted in the pertinent literature

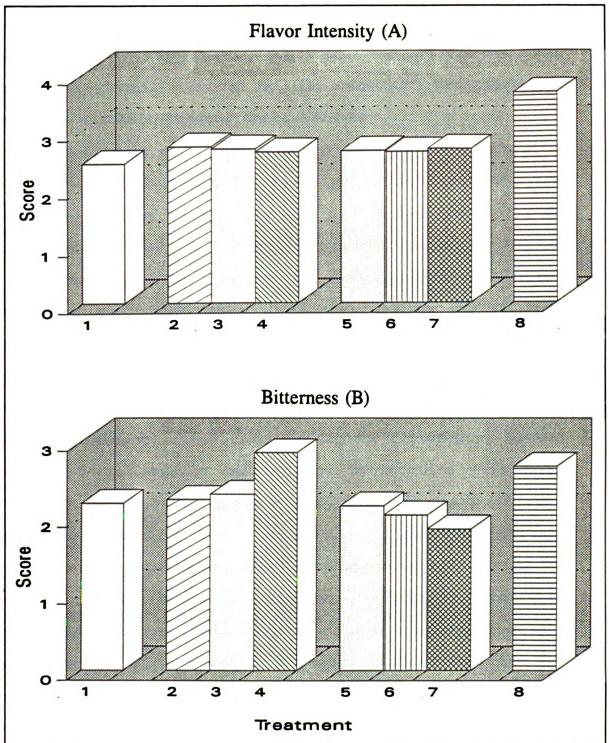

Figure II - 8 - Organoleptic characteristics of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as scored by the sensory evaluation panel. 1 = Control; 2, 3, 4 = LLC at 0.5, 1.0 and 1.5% of addition, respectively; 5, 6, 7 = HSLC at 0.5, 1.0 and 1.5% of addition, respectively; 8 = full fat Cheddar cheese.

(Banks et al., 1989; Jameson, 1990) that reduced fat cheeses, despite being well accepted by diet conscious consumers, do lack some distinct Cheddar cheese flavor.

Figure II-8B shows the scores for bitterness. Again, a full fat Cheddar cheese was taken as a reference for the panelists. Cheddar cheese made with 1.5% added live cells of *Lactobacillus casei* and the full fat cheese had scores higher ($p<0.05$) than the other cheeses. The results showed decreasing bitterness perception when increasing the amount of heat shocked cells of *Lactobacillus casei* were added to the milk. The lowest score was 1.85 (HSLC-1.5), rated as "slightly bitter cheese" as opposed to the highest score attributed to LLC-1.5 treatment, which corresponded to a "somewhat bitter cheese." These results confirm the debittering effect of intracellular aminopeptidases of *Lactobacillus casei* (Laleye, 1989; Simard, 1991; Trepanier et al., 1992), which are able to hydrolyse bitter peptides before they accumulate to a critical level in cheese. Cheese made with the addition of live cells of *Lactobacillus casei* scored poorly for bitterness, possibly due to the low pH observed in the manufacturing and ripening process. The low pH, which favors the proteolytic activity of rennet enzymes, may have caused an increase in the production of bitter peptides. The low pH also inhibits peptidase activity of the starter cultures allowing accumulation of bitter peptides. These bitter peptides are broken down to non-bitter compounds by *Lactobacillus casei* aminopeptidases,

dipeptidases and carboxypeptidases with an optima pH of 6.5, 7.6 and 7.2, respectively (Simard, 1991). When *Lactobacillus casei* is heat shocked, the disrupted cells liberate these enzymes which become entrapped in the cheese curd and intensify the peptidolytic activity during ripening.

CONCLUSIONS

Reduced fat cheese with moderate Cheddar flavor was attained. The results indicated that the level of addition of adjunct culture used did not influence the cheese texture. The addition of live cells of *Lactobacillus casei* L2A brought acidifying activity to the cheese, resulting in increased solubilization of the calcium-phosphate complex. The altered salt balance made the cheese harder, less cohesive and less springy than the other cheeses.

Chapter 3

High Performance Liquid Chromatography of Peptides and Amino Acids in Reduced Fat Cheddar Cheese With Added *Lactobacillus casei* L2A.

INTRODUCTION

Degradation of casein during cheese ripening results in the formation of a large variety of flavor compounds including the bitter tasting peptides. The manifestation of bitterness in cheese involves the accumulation of bitter peptides to an extent that exceeds the flavor threshold. It is generally assumed that bitter flavor is due to a disproportion between the formation of bitter peptides by the proteases of rennet and starters and their breakdown to smaller, non bitter compounds, by aminopeptidases secreted by the starter bacteria (Stadhouders and Hup, 1975). These bitter peptides have a high proportion of hydrophobic amino acid residues in their structure and a molecular weight in the range of 1,000 to 12,000 daltons (Kamaly and Marth, 1989; Lemieux and Simard, 1992). Due to its particular aminopeptidase system, *Lactobacillus casei* is believed to exhibit debittering

activity (Habibi-Najafi and Lee, 1994) which enables that microorganism to be used as an adjunct culture to reduce bitterness in cheese. In the making of reduced fat Cheddar cheese, the development of off-flavors, like bitter or meaty-brothy, is a common problem, which has been approached by the use of adjunct cultures possessing high peptidolytic activity like the heat shocked cells of *Lactobacilus casei* (Simard, 1991; Trepanier et al., 1992). When disrupted cells of *Lactobacillus casei* are entrapped in the cheese curd, aminopeptidases are easily released into the cheese matrix, enhancing the degradation of polypeptides, many of them bitter-tasting compounds.

Reverse-phase high performance liquid chromatography for profile studies of peptides produced during ripening of Cheddar cheese was initially proposed by Cliffe et al. (1989) and has been the method of choice for fractionation of bitter peptides. Since bitter peptides are relatively rich in hydrophobic amino acids, they exhibit higher retention on a reverse phase system compared to more hydrophilic peptides. Different fractions collected from the HPLC column can be hydrolyzed and submitted to amino acid analysis which will provide more information on the nature and amino acid composition of those peptides. This work was undertaken to evaluate the development of bitterness in reduced fat Cheddar cheese made with live and heat shocked cells of *Lactobacillus casei* and to study the use of high performance liquid

chromatography as a tool to characterize the main peptide fractions involved in the formation of bitter taste.

MATERIALS AND METHODS

Organoleptic Assessment

The organoleptic evaluation of the cheese was carried out by a trained taste panel of 5 members, including faculty and graduate students from the Department of Food Science and Human Nutrition. Slurries (66% moisture) of freeze-dried cheeses aged 4, 8, 12, 16 and 20 weeks were prepared, coded with three-digit numbers and four replications of each were presented simultaneously to the panelists. The temperature of the samples during the sensory evaluation was 7°C.

A balanced Hedonic Scale of 1-10 was used for rating the cheese for bitterness. A score of 10 was attributed to a sample exhibiting "very strong" bitter flavor and a score of one indicating "not observed" bitter flavor. A diluted quinine sulfate solution and Cheddar cheeses with different degrees of bitterness were submitted to the panelists during the training sessions for characterization of bitter flavor. Analysis of variance and contrasts among treatment means were performed using the MSTATC computer program (Crops and Soil Science, Michigan State University, MI). Sample forms of questionnaire presented to the panelists during training and judgement sessions are in the Appendix III (Figures III-1 and III-2).

Peptide Extraction

The peptides were extracted from the cheeses by the Chloroform-methanol extraction method of Harwalkar and Elliott (1971) as illustrated in Figure III-1, which extracts the more hydrophobic peptides of cheese. The freeze-dried cheese (55 grams) was blended for five minutes in 250 ml of 2:1 chloroform-methanol solution. The mixture was filtered (Whatman #4) under vacuum. The precipitate was re-extracted twice with 100 ml of chloroform-methanol solution and all the filtrates were pooled. Water equivalent to 20% of the pooled filtrate volume was added to form a biphasic mixture in a separatory funnel. After 12 hours, the bottom chloroform layer containing mostly lipids was discarded. The top aqueous-methanolic layer containing the peptides was filtered (Whatman # 4). The white precipitate formed at the interface of the two layers was discarded and the aqueous-methanolic phase was collected, rotary evaporated to remove the methanol, centrifuged and the clear supernatant containing the peptides was freeze-dried for HPLC analysis.

Peptide Analysis by High-Performance Liquid Chromatography

Sample Preparation for HPLC

The freeze-dried samples of the chloroform-methanol extract from the cheeses were dissolved at 4 mg/ml in HPLC grade water and filtered through a 0.45 μm Millipore filter

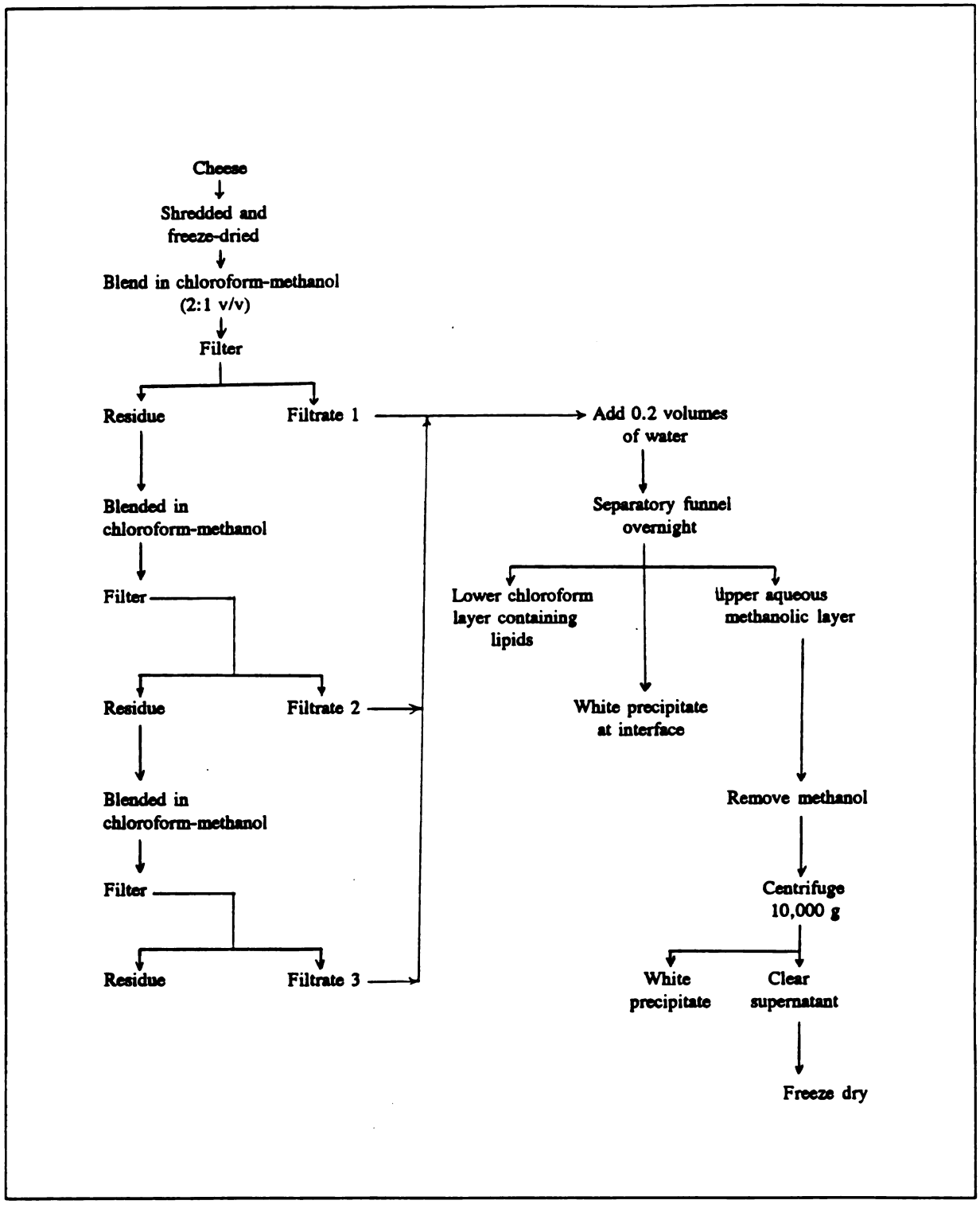

Figure III -1 - Extraction procedure used to separate the hydrophobic peptide extract from reduced fat Cheddar cheese.
Adapted from Harwalkar and Elliott (1971)

prior to injection on the column. The samples were prepared immediately before the runs to reduce any hydrolysis of the peptides.

Solvents

All solvents used during the experiment were HPLC grade. Water and acetonitrile were degassed for at least 30 minutes and 1.5 minutes, respectively. The solvents were mixed in the required volumes, degassed for 1 min and then equilibrated to room temperature.

HPLC Analysis

Chromatography was performed on a Waters liquid chromatograph (Millipore Corporation, Milford, MA), consisting of two M-6000 solvent delivery units, an M-660 Solvent Programmer and a Rheodyne injector Model 7125 (Rheodyne, Cotati, CA) coupled to a Waters 486 Tunable Absorbance Detector (Millipore Corporation, Milford, MA) interfaced with the SRI 8600-2000 Peaksimple II data system (SRI Instruments, Torrance, CA).

All separations were carried out on a 3.9 x 150 mm, 5 μm. 100 A° reverse phase Delta-Pak column (Millipore Corporation, Milford, MA), which was protected by a 1 mm ID x 2 cm guard-column (Upchurch Scientific, Inc., Oak Harbor, WA), packed with a reverse phase Perisorb RP-18, 30-40 μm (Upchurch

Scientific, Oak Harbor, Wa). The detector was set at a wavelength of 214 nm.

The peptide extract, filtered through a 0.45 μm Millipore filter, was injected into the 200 μl loading loop and then onto the column. Eight tenths mg of peptide extract was loaded onto the column for each analytical separation. A linear gradient system was used to elute the peptides from the column. Eluent A consisted of 0.1% trifluoroacetic acid in water and eluent B consisted of 0.1% trifluoroacetic acid in acetonitrile-water (90:10). The gradient of B increased from 10 to 45% over 93 minutes. A solvent flow rate of 1.0 ml per ml was used. All HPLC runs were performed at room temperature (22°C).

The chromatogram was divided into four fractions, with the purpose of separating more efficiently the eluted peptides according to their expected hydrophobicity. Four injections were made in order to collect sufficient material for amino acid analysis. The cuts were assigned based on the chromatographic area and upon the presence of certain characteristic peaks, which allowed a more efficient separation of the eluted peptides.

A solvent gradient of 10%-100% B over 10 minutes with 10 minutes holding at the final solvent volume ratio were performed between runs. This was designed to remove the last traces of the sample from the column and re-equilibrate with the initial aqueous solvent.

Amino Acid Analysis by High-Performance Liquid Chromatography

The four peptide fractions collected from the Delta-Pak reverse phase column were hydrolysed in 6 N HCl for 12 h prior to derivatization. Determination of amino acid composition of the peptide fractions was carried out by HPLC, by derivatizing the amino acids using phenyl-isothiocianate (PITC) to produce phenyl-thiocarbamyl amino acid derivative (PIC-AA). The derivatization reaction was performed as described in the Pico Tag TM Amino Acid Analysis System Operators Manual (Waters Chromatography Division, Millipore Corp., Manual # 88140, Milford, MA).

The chromatograph assembly (Waters Chromatography Division, Millipore Corp., Milford, MA) consisted of two Waters 510 pumps, a 720 System Controller, a 730 Data Module, a 440 Detector and a WISP-712 Injector. This equipment was fitted with a 15 cm Waters Pico-Tag AA Hydrolysates Reverse-Phase column. A binary gradient, as described in the manual above, was used to elute the amino acids from the column. The eluate system consisted of solvent A, 0.1 M NaAc/TEA pH 6.3 and solvent B, 60% acetonitrile/water. A flow rate of 1 ml/min was used. The temperature was kept at 38°C during elution. A sample volume of 15 μl of amino acid hydrolysate was injected and the PIC-AA derivatives were detected at 254 nm.

However, as this work dealt with ripened Cheddar cheese, one should expect a high content of free amino acids in the peptide extract, resulting from extensive degradation of casein. The presence of those amino acids would thus interfere with the results of the amino acid composition of the peptides eluted from the Delta-Pak column, leading to values higher than expected. Previous work (Champion and Stanley, 1982), has shown that the free amino acids elute in the very beginning of the chromatogram. Therefore, to identify and determine the retention time of these free amino acids, a pool of amino acid standards were injected in the Delta-Pak reverse phase column, under the same conditions applied for the cheese peptide extract. Four fractions were collected using the same cutting time as already mentioned, derivatized with PITC, injected in the PICO-TAG column, and eluted under 254 nm. This procedure allowed identification of the fraction containing free amino acids present in the peptide extract.

RESULTS AND DISCUSSION

For the same reason reported in Chapter 1, only the results of the highest level of addition of adjunct culture of *Lactobacillus casei* L2A (1.5%) will be discussed in this section.

Sensory Evaluation

The scores for bitterness are shown in Figure III-2. The slurry (66% moisture) from the cheese aged 20 weeks, treated with live cells of *Lactobacillus casei* (LLC), was rated between somewhat and distinctly bitter by the panelists, with an average score of 4.8, which was higher ($p<0.05$) than the scores given to the HSLC and Control treatments, both considered only slightly bitter (3.30 in both cases). The bitterness score for the LLC treatment increased gradually as the cheese aged. As postulated by different authors (Kamaly and Marth, 1989; Lemieux and Simard, 1992; Stadhouders and Hup, 1975), bitter taste comes from peptides released mainly by rennet chymosin, which is stimulated by lower pH. The bitter flavor would disappear only if sufficient amounts of starter aminopeptidases are present in the cheese to hydrolyze the bitter peptides at a rate faster than they are formed. The LLC cheese had a low pH throughout most of ripening (Figure I-1, Chapter 1). The increased rennet activity at lower pH may have accounted for an increase in the production of low molecular weight polypeptides, many of them hydrophobic and potentially bitterness precursors. The LLC cheese was made with a normal mesophilic culture plus the addition of live cells of *Lactobacillus casei* L2A and, therefore, had a higher starter population density as compared to the Control cheese. High cell numbers in the curd may contribute to lowering the pH and to an increased number of bacterial proteases (Law et

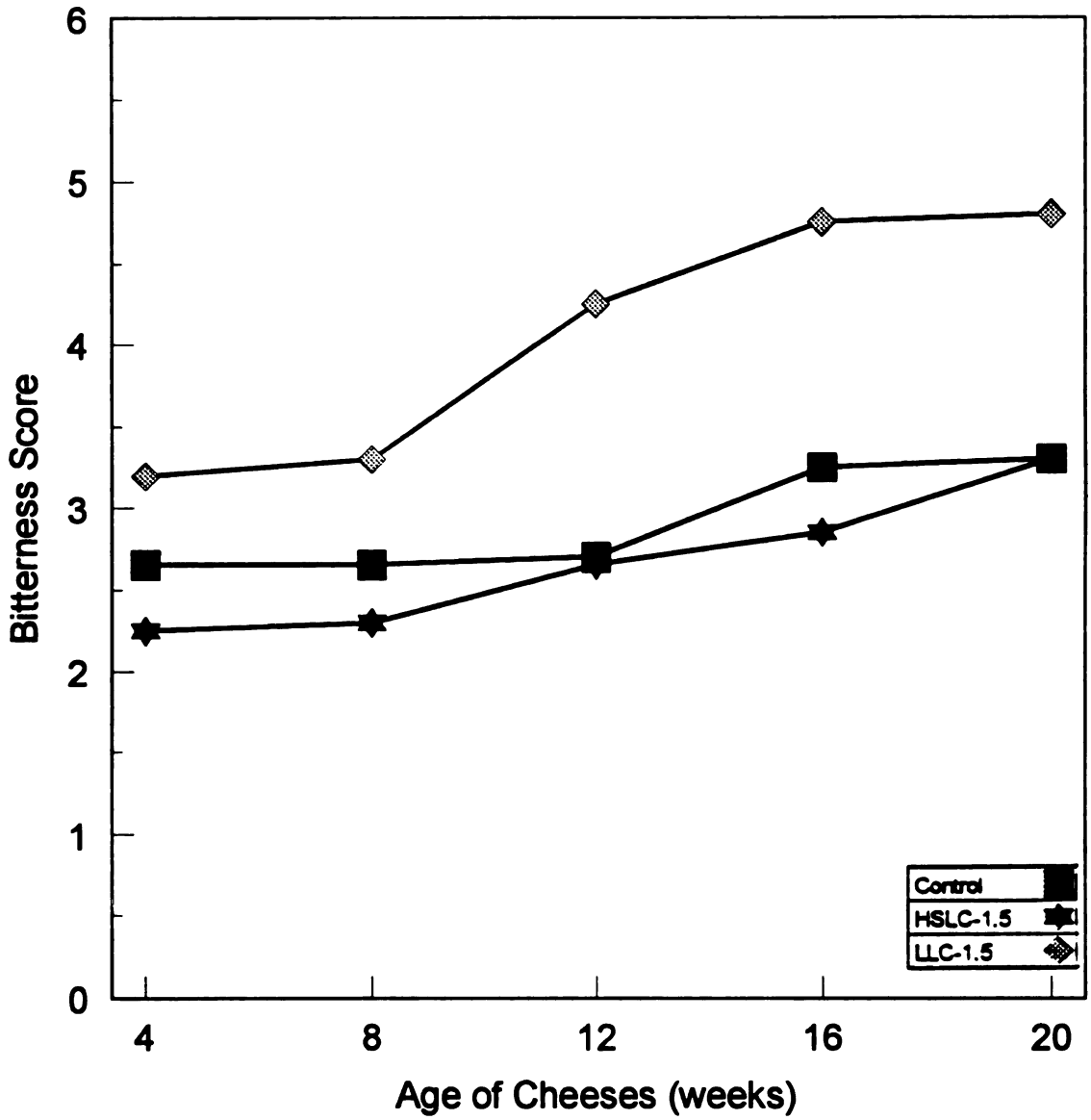

Figure III - 2 - Evolution of bitterness during the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as determined by trained sensory panel.

al., 1992; Stadhouders and Hup, 1975) which contribute to the initial degradation of α_{s1}-casein to medium size peptides, some of them possibly bitter (Thomas and Pritchard, 1987).

HPLC Analysis of Cheese Peptides

The HPLC chromatograms presented here are representative of each set of cheeses. The results were highly reproducible between trials.

As a reference, a HPLC peptide standard mixture containing five different peptides (Gly-Tyr, Val-Tyr-Val, Methionine-enkephalin, Leucine-enkephalin and angiotensin II (Sigma Chemical Co., St. Louis, MO) were run under the same conditions alone and together with the hydrophobic extract. The elution pattern of the peptide standard mixture is shown in Figure III-3. They had a hydrophobicity (Q) of 1,435, 2,083, 1,364, 1,588, and 1,821 cal. res^{-1}, respectively, as calculated by Ney's formula (Guigoz and Solms, 1976). It was observed that the standard peptides did not elute strictly in order of increasing hydrophobicity. Molecular weight (MW) may have played a role also. The peptides apparently eluted in order of increasing MW. Three of them (A = gly-tyr, MW 238 da, Q = 1,435 cal. res^{-1}, B = val-tyr-val, MW = 379 da, Q = 2,083 cal. res^{-1}, C = methionine-enkephalin, MW = 573 da, Q = 1,364 cal. res^{-1}) eluted between 4 and 32 min, in fraction 1, and the other two (D = leucine-enkephalin, MW = 555 da, Q = 1,588

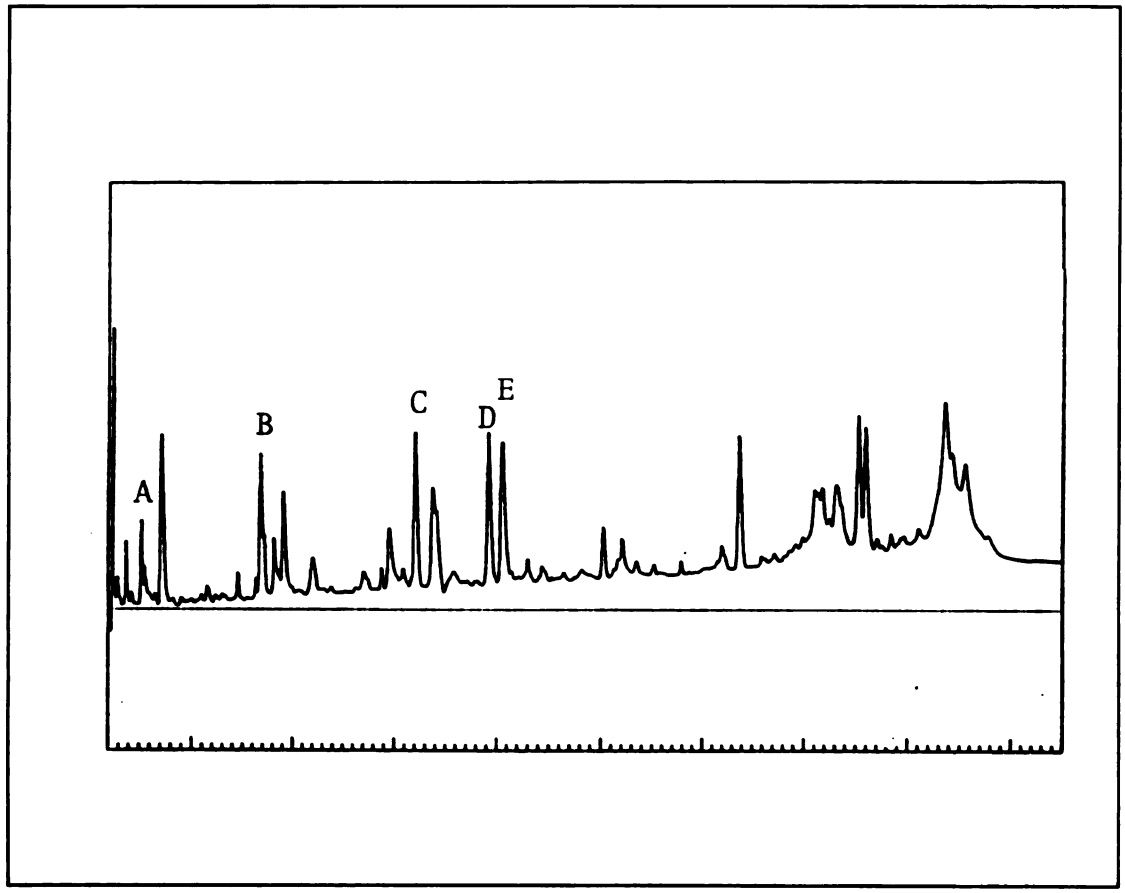

Figure III - 3. High performance liquid chromatography elution patterns of standard peptides along with hydrophobic extract of reduced fat Cheddar cheese. A = gly-tyr, B = val-tyr-val, C = methionine enkephalin, D = leucine enkephalin and E = angiotensin II.

cal. res^{-1}, and E = angiotensin II, MW = 1,046 da, Q = 1,821 cal. res^{-1}) eluted between 37 and 42 min., in the area corresponding to fraction two. It is interesting to notice that when a ratio MW/Q was calculated, with exception for methionine enkenphalin, the values found increased along with retention time (MW/Q = 0.17, 0.18, 0.42, 0.35, and 0.57 for peaks corresponding to gly-tyr, val-tyr-val, methionine enkenphalin, leucine enkenphalin, and angiotensin II, respectively, as in Figure III-4). As found by Champion and Stanley (1982), the HPLC separation of bitter peptides from Cheddar cheese has shown that, in general, compounds of higher molecular weight eluted later despite the positive relationship between retention time and hydrophobicity. The authors suggest that the retention of peptides from the hydrophobic extract is influenced by a combination of molecular weight and hydrophobicity factors.

The chromatograms related to the hydrophobic extracts of cheeses 4 and 20 weeks of age are shown in Figures III-4 and III-5, respectively. As cheeses aged and casein was broken down, great numbers of peptides were formed as seen by comparing the peak areas recorded in both chromatograms. The peak areas of selected peaks from the chromatograms are listed in Table III-2 (Appendix III). After 4 weeks of ripening no marked differences were observed among the peptide profiles of treatments Control, HSLC-1.5 and LLC-1.5, with the exception of peak G (Figure III-4). The area corresponding to peak G

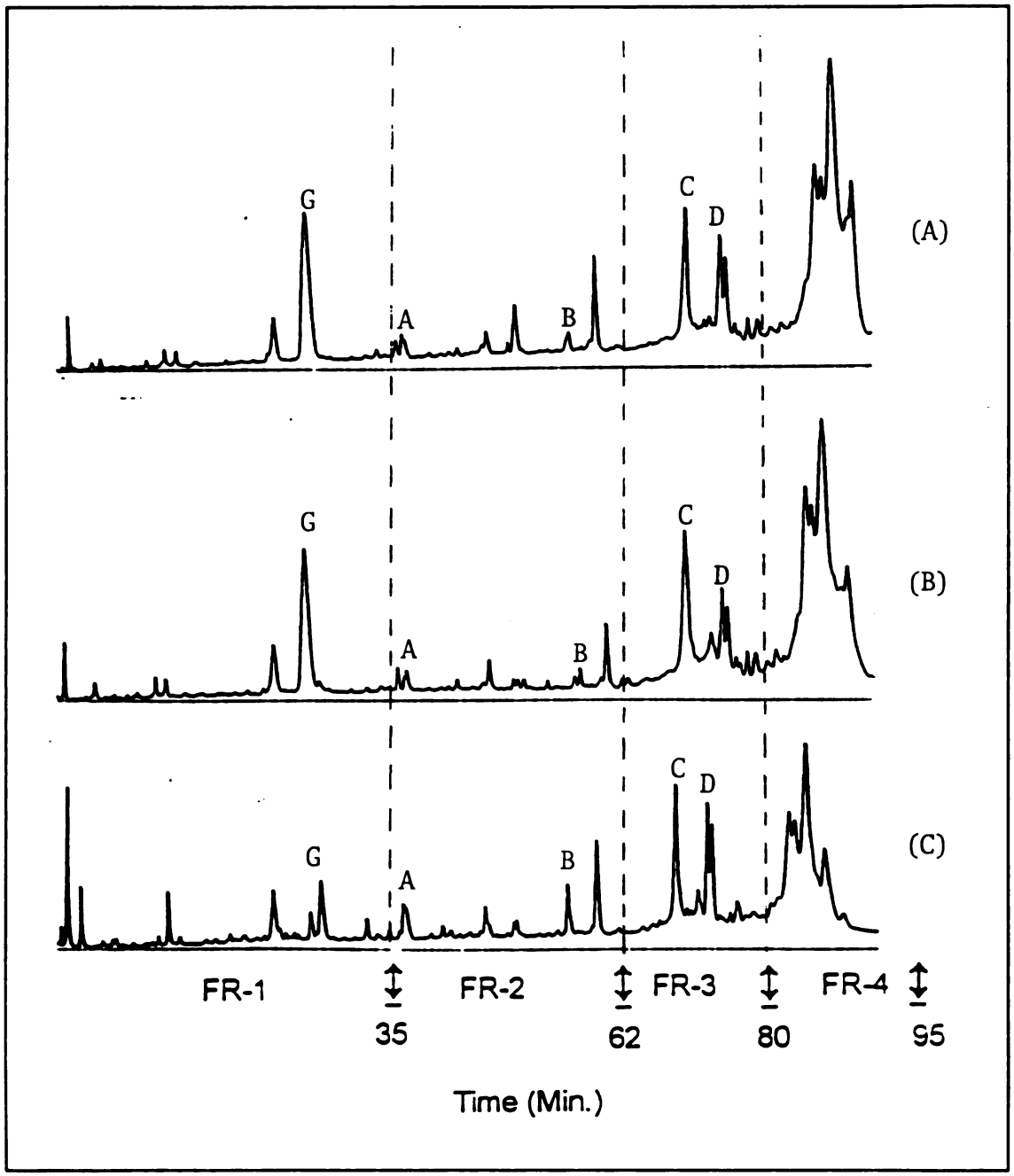

Figure III - 4 - High performance liquid chromatography elution patterns of hydrophobic extracts of Control (A), HSLC-1.5 (B), and LLC-1.5 (C) cheeses after 4 weeks of ripening.
FR = Fraction Number

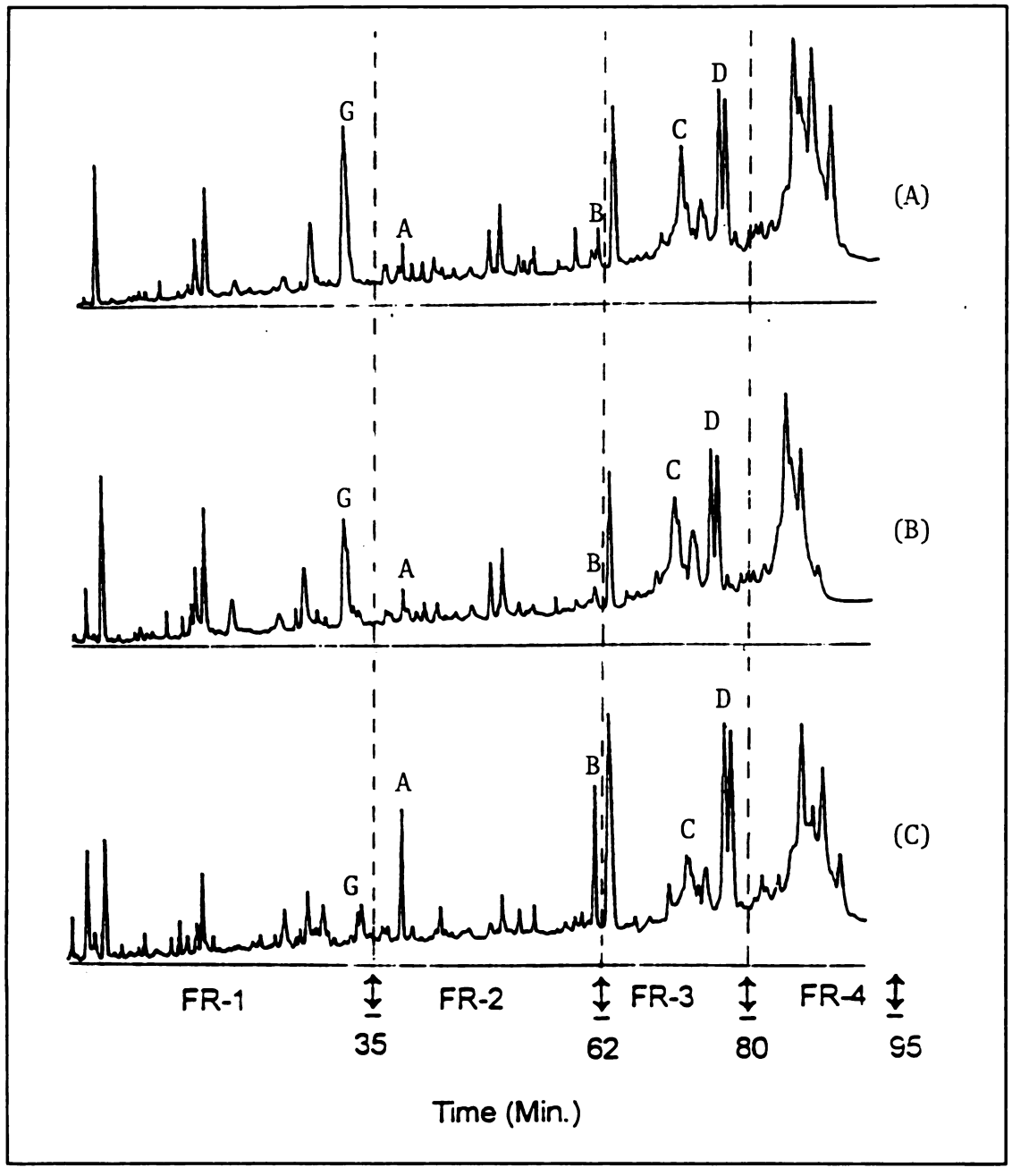

Figure III - 5 - High performance liquid chromatography elution patterns of hydrophobic extracts of Control (A), HSLC-1.5 (B), and LLC-1.5 (C) cheeses after 20 weeks of ripening.
FR = Fraction Number.

(13.1) for the LLC-1.5 was smaller ($p<0.05$) than those for the Control and HSLC-1.5 cheeses (44.9 and 42.6, respectively). As this cheese had a lower pH than the other two cheeses, one could expect a more intense casein breakdown in early ripening due to rennet proteases, as discussed earlier. After 20 weeks of ripening, more peaks were detected in the area corresponding to fraction 1. With the hydrophobic nature of the reverse phase column, low molecular weight and weakly hydrophobic peptides should elute first at the UV detector outlet (Champion and Stanley, 1982). No other relevant differences were observed between 4 and 20 weeks of ripening in the chromatograms of the HSLC-1.5 and Control cheese hydrophobic extracts, except for a slight decrease of peak C (from 29.5 to 26.8 in the Control and from 35.6 to 33.0 in the HSLC-1.5) and increase in the area of peak D (from 14.8 to 19.6 in the Control and from 11.3 to 19.4 in the HSLC-1.5), as the possible results of gradual breakdown of larger polypeptides to small units of varied hydrophobicity, which may have accounted for the appearance of several peaks at the beginning of the chromatograms in the region of fraction 1. Some marked differences were detectable between the peptide profiles of LLC-1.5 samples aged for 4 and 20 weeks. There was a net decrease ($p<0.05$) in the area of peak C (from 24.2 to 20.7). An increase ($p<0.05$) in the area of peaks A (from 4.8 to 15.3) and B (from 7.3 to 12.5) was noticed, in the area corresponding to fraction 2. These results pointed out a

possible accumulation of two peptides with molecular weight and Q values greater than 1046 da and 1821 cal. res^{-1}, based on the retention times of the standard peptides discussed earlier. These peptides did not accumulate in the Control nor in the HSLC-1.5 cheeses. It is well known that bitterness is most intense in hydrophobic medium sized peptides (tri- to hexa-) (Lemieux and Simard, 1991) with molecular weight of less than about 1,400 da (Visser, 1977). The peptide side chain hydrophobicity and, consequently, the total amino acid hydrophobicity plays the most significant role in creating a perception of bitter taste. Hydrophobicity from leucine, phenylalanine, tyrosine, valine, proline, lysine and tryptophan causes marked peptide bitterness (Lemieux and Simard, 1991). These amino acids have hydrophobicity Q values ranging from 1,500 to 3,000 cal.res^{-1}, and according to Ney's rule (Guigoz and Solms, 1976) all peptides having high average hydrophobicities, 1,400 cal.res^{-1} or higher, are bitter in taste.

As discussed earlier, the LLC cheeses had a more intense bitter taste (Figure III-2) than the other cheeses. When comparing the peaks in the area corresponding to fraction 2 of the chromatograms in Figure III-5, the areas of peaks A (15.3) and B (12.5) of the LLC-1.5 extract, are much larger ($p<0.05$) than their counterparts in the Control (8.8 and 5.6, respectively) and HSLC (3.9 and 1.6, respectively). The peptides with low molecular weight and medium to high

hydrophobicity, believed to be the more bitter tasting (Guigoz and Solms, 1976; Lemieux and Simard, 1991), seem to be concentrated in fraction 2, especially in the LLC-1.5 cheese. The higher cell density of this treatment has resulted in the accumulation of peptides A and B, which may have not been hydrolyzed quick enough to non-bitter compounds.

The total area under the peaks on the chromatograms is directly proportional to the number of peptide bonds present in the hydrophobic extracts of the reduced fat Cheddar cheese. The calculation of this area can shed some light on the results concerning the elution profile of the LLC-1.5 hydrophobic extract after 20 weeks of cheese ripening. Figure III-6 and Table III-1 (Appendix III) illustrates the relative chromatographic peak area for the four fractions. These areas represent the average of four injections of the hydrophobic extract to the HPLC column. No difference ($p<0.05$) in relative peak area was noticed in fractions 1, 3, and 4. Fraction 4 presented the highest relative peak area, which is explained by the presence of high molecular weight peptides in that region. In fraction 2, the relative peak area of the LLC-1.5 was larger ($p<0.05$) than those for the other two treatments. The LLC-1.5 extract showed the highest relative peak area (20.71), as opposed to the HSLC-1.5 and Control extracts which had both similar low relative peak areas (12.77 and 12.13, respectively). These results suggest that in the LLC-1.5 trial, there was a more intense breakdown of high molecular

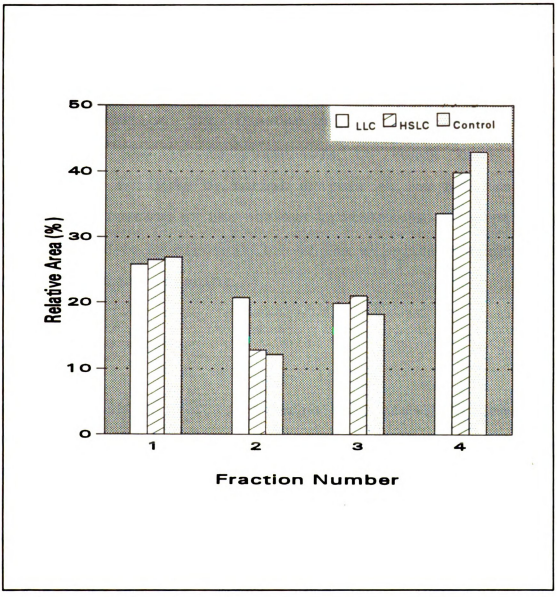

Figure III - 6 - Relative peak areas of peptide fractions eluted from the high performance liquid chromatography column.

weight peptides (fraction 4), possibly highly hydrophobic, to low molecular weight bitter tasting peptides of intermediate hydrophobicity (Fraction 2). These potentially bitter peptides may have accumulated in the cheese due to faster formation than degradation. This Cheddar cheese was more acidic than the others and it is reasonable to think that a more proteolytic activity by rennet enzymes at low pH, associated with an inhibition of the starter proteinases and peptidases, may have led to an accumulation of low molecular weight bitter peptides during ripening.

Cheese Amino Acids

The elution profile of amino acid standards present in Fraction 1, as they eluted from the Delta-Pak reverse phase column, is shown in Figure III-7A. The retention times of standard amino acids (Figure III-7B) and the fact that no free amino acids were found in fractions 2, 3 and 4, indicate that all free amino acids eluted in fraction 1. This confirms the results of Champion and Stanley (1982). Thus, the fractions 2, 3, and 4 collected from the elution of the hydrophobic extract did not contain free amino acids from cheese ripening.

Figure III-8 (A, B and C) shows the relative distribution of amino acids in the peptide fractions 2, 3 and 4, at 20 weeks of cheese ripening. According to the Ney's rule (Guigoz and Solms, 1976), the following amino acids can be listed in

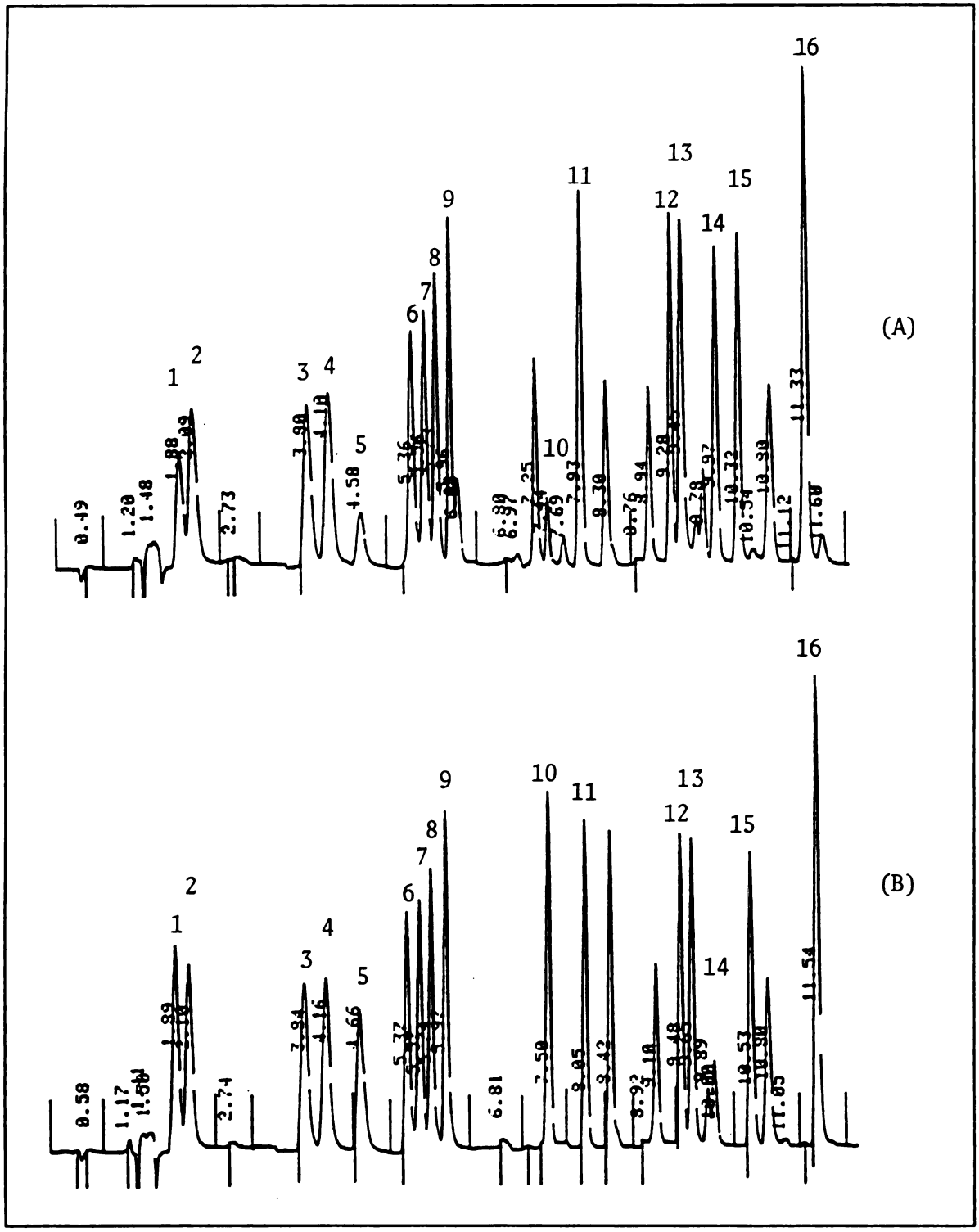

Figure III - 7 - Chromatographic profiles of amino acids standards. A = amino acid standards eluted in the first fraction. B = amino acid present in the standard mixture. 1 = asp, 2 = glu, 3 = Ser, 4 = gly, 5 = his, 6 = arg, 7 = thr, 8 = ala, 9 = pro, 10 = tyr, 11 = val, 12 = ile, 13 = leu, 14 = Nor-leu, 15 = phe and 16 = lys.

decreasing order of hydrophobicity: Try (3,000 cal. res^{-1}), Ile, Tyr, Phe, Pro, Leu, Val and Lys (1,500 $cal.res^{-1}$). In Figure III-8B, it is seen that the highest concentration of amino acids originated from the hydrophobic peptide extract of the cheese trials (fraction 3) were from glutamic acid, proline and leucine. Leucine was the only one with a high degree of hydrophobicity. A very similar pattern was observed in the amino acids from peptides in the fraction four, for all three cheeses (Figure III-8C).

As expected, based on the significant difference in relative peak area and peptide profile observed earlier, the main differences were observed in the distribution of amino acids related to the peptides collected in fraction 2 (Figure III-8A). As observed in the other two fractions there was an important concentration of glutamic acid, proline and leucine in the extract of all cheese trials. However, in the LLC-1.5 cheese extract, the amount of glutamic acid was lower ($p<0.05$) than in the other two extracts. There was a net increase in the concentration of highly hydrophobic amino acids like tyrosine, phenylalanine, and lysine in all cheese extracts. The concentration of phenylalanine and tyrosine in Fraction 2 was higher ($p<0.05$) in the peptide extract of the LLC-1.5 cheese than in those of the HSLC-1.5 and Control treatment. As shown in Figure III-2, the former cheese was considered somewhat or distinctly bitter by the sensory evaluation panel, as the other two cheeses (HSLC-1.5 and Control). These results

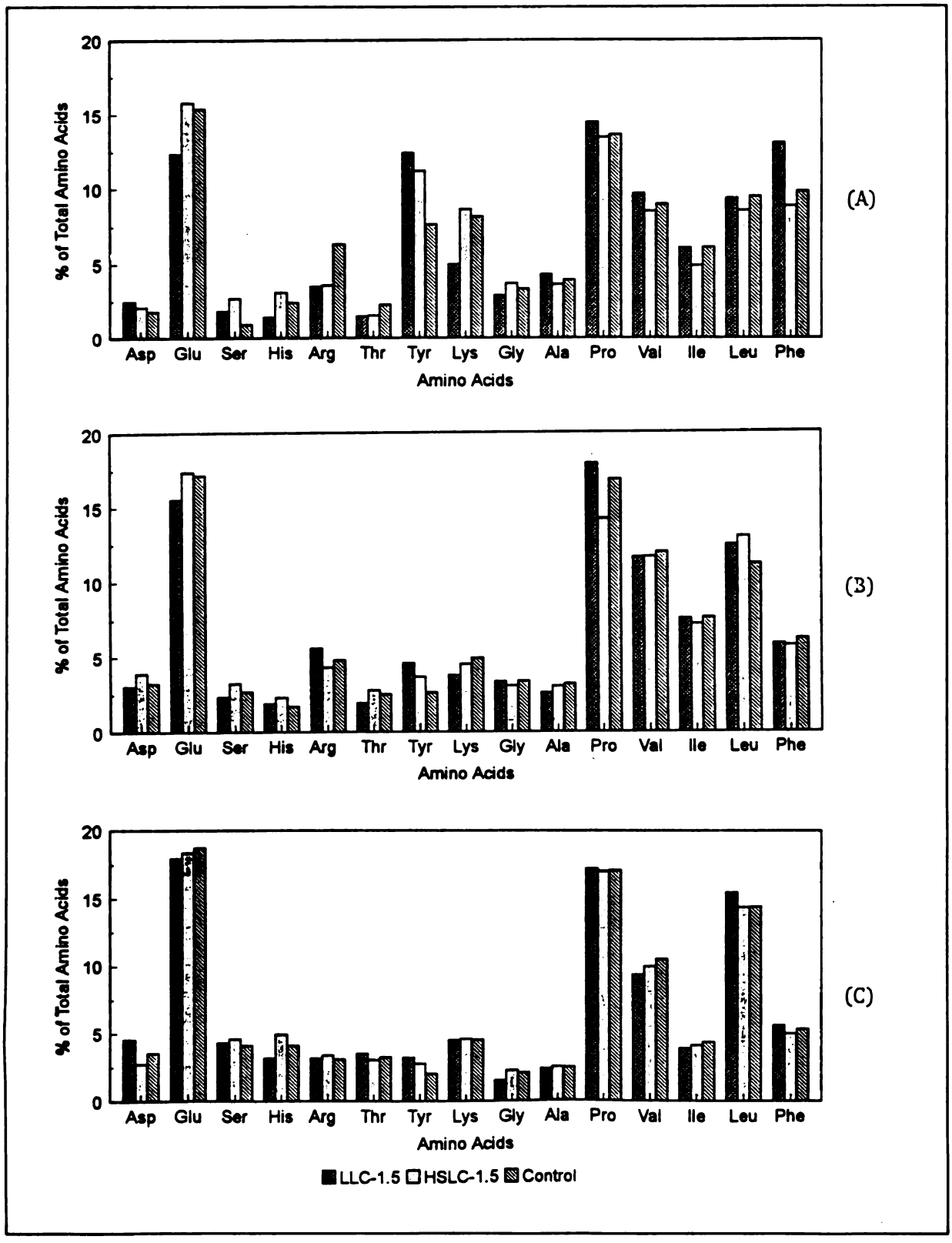

Figure III - 8 - Relative distribution of amino acids in peptide fractions of reduced fat Cheddar cheese at 20 weeks of ripening. A = Fraction 2, B = Fraction 3, and C = Fraction 4.

indicate that the development of bitter flavor in reduced fat Cheddar cheese is related to the presence of hydrophobic peptides at certain levels. The amino acids estimated to be involved in bitterness and related in this work, have been reported elsewhere (Edwards and Kosikowski, 1983) as precursors of bitter taste in Cheddar cheese.

CONCLUSIONS

The findings of this work demonstrate that reversed phase HPLC is an adequate tool to evaluate and identify the formation of bitter compounds in reduced fat Cheddar cheese. It has been shown that peptides with an average hydrophobicity above 1,400 cal.res^{-1} and a molecular weight higher than 1,046 were involved in the formation of bitter taste, as they were present in higher concentrations in the cheese which had a distinct bitter flavor. The pooled fraction pertinent to these peptides has shown a high concentration of hydrophobic and potentially bitter amino acids like tyrosine, phenylalanine, and lysine.

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SUMMARY AND CONCLUSIONS

All cheeses had a high moisture (49.35%) and a low fat (14.8%) content. Increasing the inoculation levels (0.5, 1.0 and 1.5%) of adjunct cultures did not seem to interfere with the ripening characteristics of the cheeses. Cheeses made with live cells of *Lactobacillus casei* L2A were more acidic and had a lower pH throughout ripening. Cheeses made with heat shocked adjunct cultures of *Lactobacillus casei* L2A presented the highest degree of proteolysis as measured by the greatest amount of pH 4.6 and 12% TCA-soluble nitrogen.

In all cheeses α_{s1}-casein was more extensively broken down than β-casein. In the cheese treated with heat shocked cells of *Lactobacillus casei* L2A, the aminopeptidases liberated from the disrupted cells seemed to have played an important role in proteolysis; in this treatment only 21.31% and 70.43% of α_{s1}-casein and β-casein, respectively, were left intact at the end of ripening. The liberation of nitrogen correlated significantly with the decrease in the amount of intact α_{s1}-casein.

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Cheeses made with live cells of *Lactobacillus casei* L2A were harder and less cohesive and less springy than the other cheeses. This was probably related to its low pH and, eventually, to its low degree of mineralization. Hardness decreased in all cheeses during ripening, especially in the cheeses made with heat shocked cells of *Lactobacillus casei* L2A. The softening of the cheese texture was related to the breakdown of the casein matrix during ripening.

A lack of distinct Cheddar cheese flavor was observed in all cheeses by the sensory evaluation panel; flavor was rated as "moderate Cheddar cheese flavor". Bitter flavor was related to a high concentration of low molecular weight and highly hydrophobic peptides. These peptides were found in greater concentration in a pooled fraction of the hydrophobic extract of cheese made with live cells of *Lactobacillus casei* L2A, eluted from the chromatographic column. This cheese was found to be "somewhat bitter". Chromatographic analysis of this peptide fraction following derivatization has shown it to be composed of higher concentration of hydrophobic amino acids like phenylalanine, tyrosine and lysine than found in non-bitter HSLC and Control fractions. These amino acids may increase the potential bitterness of low molecular weight peptides when present in their structure. It is finally concluded that the incorporation of heat shocked cells of *Lactobacillus casei* L2A to the manufacturing process of

reduced fat Cheddar cheese has potential as an approach to controll the intensity of bitter flavor.

FUTURE RESEARCH

1. Utilization of the same protocol of this dissertation using Cheddar cheese with varying degrees of fat and moisture content;

2. Increase the levels of heat shocked cells of *lactobacilli* added to the cheese milk in the manufacture of reduced fat Cheddar cheese.

3. Utilization of proteinase-negative mesophilic *lactococci* or thermophilic *streptococci* and *lactobacilli* to reduce the formation of bitter compounds in cheese;

4. Study of the utilization of non-bitter strains possessing pyrrolidone carboxylyl peptidase (PCP) activity in the manufacture of reduced-fat Cheddar cheese;

5. Study of new natural products (globular proteins) modified to act as fat-replacers in low fat cheeses (i.e. Simplesse);

6. Study of milk indigenous proteinases (plasmin, thrombin, etc.) in the development, accumulation and degradation of bitter peptides in cheese;

APPENDICES

APPENDIX I

APPENDIX I

Table I-1 - Time schedule for making reduced fat Cheddar cheese of high moisture content.

OPERATION	TIME	TEMPERATURE (°F)	ACIDITY		COMMENTS
			TA	pH	
Add starter	0:00	90	.15	6.62*	Marschall's Biolac DSS LF (1%) and L. casei (adjunct)
Add rennet	0:40	90	.16	6.60*	Recombinant Chymosin, 10cc/100 kg of Milk
Cut curd	1:15	90	.10	6.55*	1.7 cm knives, slow agitation
Start cooking	1:35	90	.10	6.50*	Raise heat slowly
End cooking and draining whey	1:50	100	.12	6.44*	
Cut & 1st Turn	2:05				
Milling	6:35			5.6**	
Salting	6:50				0.3 kg/100 kg milk 3 additions 10 min. apart.
Hooping	7:20				
Start pressing	7:30				9 kg/30 min. 18 kg/12 hr.

* = Whey pH
** = Curd pH

APPENDIX I

Table I-2 - Changes in cheese pH throughout the ripening of reduced fat Cheddar cheese made with different levels of adjunct culture of *Lactobacillus casei* L2A.

Cheese	Week 0[1]	Week 4	Week 8	Week 12	Week 16	Week 20
LLC-0.5	5.38[2a] (0.04)	5.22[b] (0.01)	5.19[b] (0.06)	5.06[b] (0.08)	5.14[a] (0.00)	5.20[a] (0.01)
LLC-1.0	5.33[a] (0.01)	5.19[b] (0.00)	5.16[b] (0.08)	5.05[b] (0.08)	5.13[a] (0.00)	5.18[a] (0.08)
LLC-1.5	5.31[a] (0.02)	5.15[b] (0.01)	5.12[b] (0.06)	5.06[b] (0.04)	5.13[a] (0.00)	5.17[a] (0.06)
HSLC-0.5	5.47[a] (0.02)	5.28[a] (0.00)	5.26[a] (0.04)	5.17[a] (0.04)	5.08[a] (0.05)	5.14[a] (0.03)
HSLC-1.0	5.45[a] (0.05)	5.28[a] (0.01)	5.26[a] (0.01)	5.17[a] (0.03)	5.11[a] (0.05)	5.17[a] (0.04)
HSLC-1.5	5.56[a] (0.16)	5.30[a] (0.00)	5.27[a] (0.03)	5.15[a] (0.02)	5.14[a] (0.04)	5.17[a] (0.03)
Control	5.51[a] (0.15)	5.29[a] (0.00)	5.26[a] (0.01)	5.13[a] (0.01)	5.14[a] (0.04)	5.17[a] (0.01)

[1]pH after 30 minutes pressing.
[2]Means with standard deviations in parentheses.
[abcde]Means in same column with different superscripts are different ($p<0.05$).
n = 4 replications.

APPENDIX I

Table I-3 - Soluble Nitrogen (SN) evolution throughout the ripening of reduced fat Cheddar cheese made with different levels of adjunct culture of *Lactobacillus casei* L2A.

Cheese	Week 0[1]	Week 4	Week 8	Week 12	Week 16	Week 20
LLC-0.5	0.16[2a] (0.01)	0.46[ab] (0.07)	0.59[a] (0.01)	0.68[a] (0.06)	0.82[a] (0.02)	0.85 [c] (0.03)
LLC-1.0	0.17[a] (0.01)	0.45[ab] (0.07)	0.60[a] (0.01)	0.68[a] (0.01)	0.82[a] (0.01)	0.89 [bc] (0.02)
LLC-1.5	0.19[a] (0.04)	0.46[ab] (0.06)	0.60[a] (0.04)	0.70[a] (0.02)	0.79[a] (0.03)	0.87 [c] (0.02)
HSLC-0.5	0.17[a] (0.02)	0.42[ab] (0.01)	0.57[a] (0.04)	0.68[a] (0.03)	0.78[a] (0.02)	0.95[a] (0.01)
HSLC-1.0	0.18[a] (0.02)	0.40[ab] (0.01)	0.59[a] (0.02)	0.72[a] (0.02)	0.79[a] (0.01)	0.96[a] (0.02)
HSLC-1.5	0.19[a] (0.03)	0.38 [b] (0.01)	0.57[a] (0.05)	0.73[a] (0.02)	0.81[a] (0.03)	0.93[ab] (0.02)
Control	0.17[a] (0.02)	0.49[a] (0.11)	0.62[a] (0.03)	0.72[a] (0.02)	0.82[a] (0.03)	0.88 [bc] 0.03)

[1]day following manufacture.
[2]Means with standard deviations in parentheses.
n = 4 replicates.
[abc]Means in same column with different superscripts are different ($p<0.05$).

Table I-4 - Non-Protein Nitrogen (NPN) evolution throughout the ripening of reduced fat Cheddar cheese made with different levels of adjunct culture of *Lactobacillus casei* L2A.

Cheese	Week 0[1]	Week 4	Week 8	Week 12	Week 16	Week 20
LLC-0.5	0.10[2ab] (0.01)	0.24 [bc] (0.03)	0.42[a] (0.03)	0.51[ab] (0.03)	0.54[a] (0.02)	0.72[ab] (0.02)
LLC-1.0	0.09[ab] (0.00)	0.26 [bc] (0.02)	0.46[a] (0.03)	0.49[ab] (0.01)	0.49[a] (0.00)	0.71[ab] (0.02)
LLC-1.5	0.13[a] (0.02)	0.31[ab] (0.00)	0.47[a] (0.02)	0.47 [b] (0.03)	0.47[a] (0.05)	0.72[ab] (0.04)
HSLC-0.5	0.06 [b] (0.05)	0.26 [bc] (0.02)	0.39[a] (0.05)	0.51[ab] (0.02)	0.53[a] (0.03)	0.75[a] (0.01)
HSLC-1.0	0.09[ab] (0.05)	0.18 [c] (0.01)	0.40[a] (0.07)	0.51[ab] (0.03)	0.52[a] (0.04)	0.75[a] (0.02)
HSLC-1.5	0.09[ab] (0.05)	0.20 [c] (0.00)	0.38[a] (0.10)	0.53[a] (0.03)	0.53[a] (0.02)	0.75[a] (0.04)
Control	0.06 [b] (0.01)	0.36[a] (0.12)	0.45[a] (0.07)	0.48[ab] (0.05)	0.48[a] (0.06)	0.68 [b] (0.02)

[1]Day following manufacture.
[2]Means with standard deviations in parentheses.
n = 4 replications.
[abc]Means in same column with different superscripts are different ($p<0.05$).

Table I-5 - Total Nitrogen, Ripening Extension Index and Ripening Depth Index after 20 weeks of ripening of reduced fat Cheddar cheese made with different levels of adjunct culture of *Lactobacillus casei* L2A.

CHEESE	TN	REI (SN/TN)	RDI (NPN/TN)
LLC-0.5	4.60[1AB]	18.53 [C]	15.77[A]
LLC-1.0	4.56[AB]	19.53 [B]	15.70[A]
LLC-1.5	4.55[AB]	19.05 [BC]	15.87[A]
HSLC-0.5	4.54[AB]	21.01[A]	16.49[A]
HSLC-1.0	4.54[AB]	21.16[A]	16.50[A]
HSLC-1.5	4.51 [B]	20.69[A]	16.79[A]
CONTROL	4.64[A]	19.02 [BC]	14.57 [B]

[1]Mean scores with standard deviations in brackets.
n = 4 replications
[AB]Means in same column with different superscripts are different ($p<0.05$).

APPENDIX I

Table I-6 -List of parameters used to scan the electrophoretic gels in the Shimadzu Dual-Wavelength Scanner.

Photo Mode Select	Abs. Transmission
Signal Proc.	
Linearizer	Off
Accum No.	1
Background correct	1
Signal Average	16
Output Mode Select	
Ordinate	X8
Abscissa	X1
Output Select	Curve & Print
Drift Line	On
Peak Detect	
Mode	2
Drift Line	0
Signal	Area
Sensitivity	Med.
Min. Area	10
Min. Width	0
Scan Width	
"X"	13
Delta "Y"	0.20
Program Scan Parameters	
Wavelength 1	580
Wavelength 2	580
"X" Start Position	Variable
"Y" Start Position	Variable
"X" End Position	Variable
"Y" End Position	Variable
Lane Dist.	1
Total Lane	Variable

APPENDIX I

Table I-7 - Degradation of α_{s1}-casein (peak area) throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A.

Weeks	Control	HSLC	LLC
0	43.00[1a] (1.13)	46.80[a] (1.00)	33.20[b] (3.25)
4	24.60[a] (1.41)	23.30[a] (0.42)	21.60[a] (1.00)
8	16.60[a] (0.14)	15.05[b] (0.21)	12.65[c] (0.07)
12	10.95[b] (0.64)	13.30[a] (0.00)	11.85[ab] (0.64)
16	9.95[b] (0.64)	8.55[c] (0.64)	11.10[a] (0.85)
20	6.80[c] (0.42)	6.45[b] (0.21)	9.40[a] (0.28)

[1]Means (peak area) with standard deviations in parentheses. n = 2 replications.
[abc]Means in same row with different superscripts are different ($p<0.05$).

APPENDIX I

Table I-8 - Degradation of β-casein (peak area) throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A.

Weeks	Control	HSLC	LLC
0	44.05[1a]	39.90[b]	43.40[a]
4	43.25[a]	38.40[a]	39.80[a]
8	41.05[a]	37.75[a]	39.50[a]
12	39.85[a]	34.70[a]	38.05[a]
16	35.50[ab]	29.00[b]	37.25[a]
20	34.85[b]	28.10[b]	35.65[a]

[1]Means (peak area) with standard deviations in parentheses. n = 2 replications.
[ab]Means in same row with different superscripts are different ($p<0.05$).

APPENDIX I

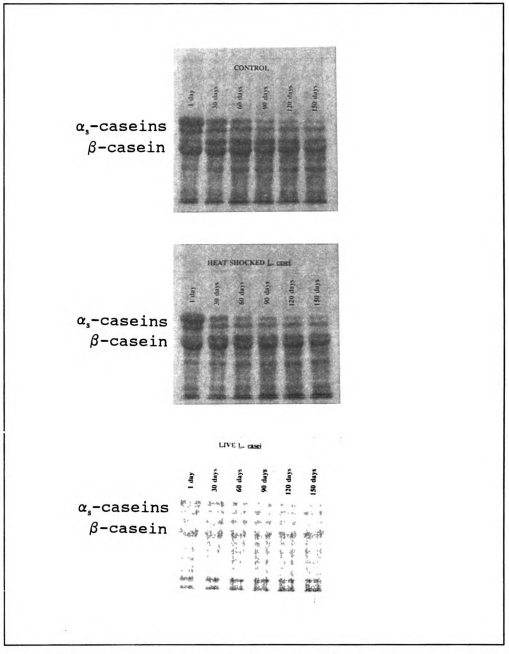

Figure I-1 - Electrophoretic patterns throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A.

APPENDIX II

APPENDIX II

Table II - 1 - Adhesiveness throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as measured by the Instron Universal Testing Machine.

CHEESE	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS
LLC-0.5	4.29[1AB] (0.76)	4.27[A] (0.42)	3.24[A] (0.25)	3.56[AB] (0.51)
LLC-1.0	3.14 [B] (0.93)	3.37 [BCD] (0.08)	3.48[A] (0.31)	4.23[A] (0.05)
LLC-1.5	4.26[AB] (0.45)	4.06[AB] (0.72)	3.43[A] (0.46)	3.02 [B] (0.49)
HSLC-0.5	4.16[AB] (0.55)	3.88[ABC] (0.24)	2.94[A] (0.13)	3.41[AB] (0.07)
HSLC=1.0	4.62[A] (0.79)	2.91 [D] (0.41)	3.20[A] (0.22)	3.36[AB] (0.67)
HSLC-1.5	5.33[A] (0.25)	3.15 [CD] (0.25)	3.34[A] (0.32)	3.42[AB] (0.41)
CONTROL	5.41[A] (0.82)	4.12[AB] (0.18)	3.50[A] (0.29)	3.62[AB] (0.28)

[1]Mean scores with standard deviations in brackets.
n = 3 replications.
[ABCD]Means in same column with different superscripts are different ($p<0.05$).

APPENDIX II

Table II - 2 - Cohesiveness throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as measured by the Instron Universal Testing Machine.

CHEESE	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS
LLC-0.5	0.45[1AB] (0.20)	0.55[AB] (0.04)	0.56[A] (0.03)	0.46[A] (0.11)
LLC-1.0	0.47[AB] (0.11)	0.47 [BC] (0.03)	0.53[A] (0.04)	0.49[A] (0.07)
LLC-1.5	0.31[B] (0.07)	0.40 [C] (0.04)	0.45 [B] (0.03)	0.45[A] (0.03)
HSLC-0.5	0.61[A] (0.07)	0.48 [BC] (0.03)	0.50[AB] (0.03)	0.49[A] (0.04)
HSLC=1.0	0.54[A] (0.05)	0.54 [BC] (0.06)	0.56[A] (0.04)	0.50[A] (0.03)
HSLC-1.5	0.52[A] (0.04)	0.54[AB] (0.01)	0.54[A] (0.02)	0.52[A] (0.05)
CONTROL	0.62[A] (0.07)	0.57[A] (0.02)	0.50[AB] (0.03)	0.51[A] 0.02)

[1]Mean scores with standard deviations in brackets.
n = 3 replications.
[ABC]Means in same column with different superscripts are different ($p<0.05$).

APPENDIX II

Table II - 3 - Springiness throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as measured by the Instron Universal Testing Machine.

CHEESE	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS
LLC-0.5	0.65[1] ^{BC} (0.03)	0.66 ^{BC} (0.02)	0.63 ^{C} (0.01)	0.65 ^{B} (0.02)
LLC-1.0	0.62 ^{C} (0.01)	0.66 ^{BC} (0.04)	0.63 ^{C} (0.05)	0.64 ^{B} (0.00)
LLC-1.5	0.62 ^{C} (0.02)	0.65 ^{C} (0.02)	0.59 ^{D} (0.01)	0.63 ^{B} (0.00)
HSLC-0.5	0.69^{AB} (0.03)	0.73^{A} (0.06)	0.68^{A} (0.02)	0.67 ^{C} (0.00)
HSLC=1.0	0.71^{A} (0.05)	0.72^{AB} (0.03)	0.67^{A} (0.01)	0.67^{A} (0.00)
HSLC-1.5	0.72^{A} (0.06)	0.71^{AB} (0.01)	0.66^{AB} (0.01)	0.67^{A} (0.00)
CONTROL	0.66^{ABC} (0.02)	0.71^{AB} (0.02)	0.64 ^{BC} (0.03)	0.67^{A} (0.00)

[1]Mean scores with standard deviations in brackets.
n = 4 replications.
^{ABCD}Means in same column with different superscripts are different ($p<0.05$).

APPENDIX II

Table II - 4 - Hardness throughout the ripening of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as measured by the Instron Universal Testing Machine.

CHEESE	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS
LLC-0.5	23.13[1]^{A} (0.70)	14.52^{A} (0.84)	12.65^{A} (0.62)	12.78^{AB} (0.53)
LLC-1.0	18.88^{BC} (0.98)	11.55^{C} (0.20)	11.56^{B} (1.10)	13.52^{A} (0.51)
LLC-1.5	21.36^{AB} (1.62)	12.80^{B} (0.74)	12.63^{A} (0.06)	13.38^{A} (0.58)
HSLC-0.5	18.67^{BCD} (2.64)	8.32^{E} (0.81)	10.52^{C} (0.45)	11.37^{CD} (0.36)
HSLC-1.0	18.35^{CD} (1.01)	9.77^{D} (0.53)	10.87^{BC} (0.37)	10.48^{D} (0.38)
HSLC-1.5	19.06^{BC} (0.33)	9.62^{D} (0.24)	10.45^{C} (0.15)	11.48^{C} (0.48)
CONTROL	16.07^{D} (0.40)	9.48^{D} (0.17)	10.70^{C} (0.61)	12.07^{BC} (0.81)

[1]Mean scores with standard deviations in brackets.
n = 3 replications
^{ABCDE}Means in same column with different superscripts are different ($p<0.05$).

APPENDIX II

Table II-5 - Textural characteristics of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as scored by the sensory evaluation panel.

CHEESE	ADHESIVENESS	COHESIVENESS	SPRINGINESS	HARDNESS
LLC-0.5	4.96[B] (0.30)	3.77[B] (0.07)	3.10[B] (0.07)	5.13[A] (0.24)
LLC-1.0	5.00[B] (0.21)	3.80[B] (0.66)	3.13[B] (0.77)	4.48[A] (0.48)
LLC-1.5	5.10[B] (0.36)	3.93[B] (0.58)	3.12[B] (0.24)	4.77[A] (0.50)
HSLC-0.5	4.62[B] (0.70)	4.71[A] (0.60)	4.17[A] (0.39)	4.45[A] (0.62)
HSLC-1.0	4.44[B] (0.81)	4.73[A] (0.27)	4.21[A] (0.11)	4.63[A] (0.71)
HSLC-1.5	4.94[B] (0.24)	4.86[A] (0.24)	4.21[A] (0.21)	4.73[A] (0.55)
Control	5.15[B] (0.54)	5.07[A] (0.27)	4.12[A] (0.37)	4.86[A] (0.46)

[1]Mean scores with standard deviations in brackets.
n = 4 replications
[A,B]Means in same column with different superscripts are different ($p<0.05$).

APPENDIX II

Table II-6 - Organoleptic characteristics of reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A, as scored by the sensory evaluation panel.

CHEESE	FLAVOR INTENSITY	BITTERNESS
Control	$2.43^{1,b}$ (0.14)	2.18^{c} (0.23)
LLC-0.5	2.72^{b} (0.14)	2.23^{c} (0.03)
LLC-1.0	2.68^{b} (0.08)	2.30^{bc} (0.12)
LLC-1.5	2.63^{b} (0.23)	2.85^{a} (0.38)
HSLC-0.5	2.65^{b} (0.13)	2.15^{c} (0.37)
HSLC-1.0	2.63^{b} (0.22)	2.03^{c} (0.25)
HSLC-1.5	2.68^{b} (0.23)	1.85^{c} (0.45)
Full Fat	3.67^{a} (0.12)	2.67^{ab} (0.45)

[1]Mean scores with standard deviations in brackets.
n = 4 replications
[a,b,c] Means in same column with different superscripts are different ($p<0.05$).

APPENDIX II

CONSENT FOR TASTE PANEL MEMBERS

Food Science and Human Nutrition Department
Michigan State University

Reduced-fat Cheddar cheese prepared from pasteurized low fat milk, cultures (**Lactococcus lactis** subsp. **cremoris** and **Lactobacillus casei**), rennet, calcium chloride, salt and natural color.

I_____have read the above list of ingredients and find none that I am allergic to. I have also been informed on the nature of the research (including experimental materials and procedures) which will be used during the tasting session. I understand that the taste panel will take 5-15 minutes each time. I agree to serve on the taste panel which will be conducted on _____, 1992. I understand that I am free to withdraw my consent and to discontinue participation in the panel at any time without penalty.

_____
Signature

_____
Date

Figure II-1 - Consent form presented to panel members.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Adhesiveness**

INSTRUCTIONS

Place sample between molars; chew seven times: press the sample to the roof of mouth with the tongue. Evaluate the force required to remove the sample from the roof of the mouth with tongue. Rate samples from least adhesive to most adhesive. Expectorate the sample; rinse mouth with water.

925 # 123 # 187

Least Adhesive _____

_____

Most Adhesive _____

Figure II-2 - Sample form of questionnaire presented to the panel to evaluate adhesiveness of reduced fat Cheddar cheese during training sessions.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Cohesiveness**

INSTRUCTIONS

Place sample between molars; compress fully; evaluate the degree to which the sample deforms rather that crumbles, breaks, or falls apart as cohesive. Rank the samples from least cohesive to most cohesive. Expectorate the sample: rinse mouth with water between samples.

963 # 789 # 435

Least Cohesive _____

_____

Most Cohesive _____

Figure II-3 - Sample form of questionnaire presented to the panel to evaluate cohesiveness of reduced fat Cheddar cheese during training sessions.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Springiness**

INSTRUCTIONS

Place sample between molars; compress partially without breaking the sample structure; release. Evaluate the degree to which the sample returns to its original shape as springiness. Rate the samples from least springy to most springy. Expectorate the sample; rinse mouth with water.

354 # 123 # 858

Least Springy _____

_____

Most Springy _____

Figure II-4 - Sample form of questionnaire presented to the panel to evaluate springiness of reduced fat Cheddar cheese during training sessions.

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APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Hardness**

INSTRUCTIONS

Place sample between molars; bite through once; evaluate for hardness. Rank the samples from least hard to most hard. Taste the samples in the following order. Expectorate the sample; rinse mouth with water.

925 # 789 # 187

Least hard _____

_____

Most hard _____

Figure II-5 - Sample form of questionnaire presented to the panel to evaluate hardness of reduced fat Cheddar cheese during training sessions.

APPENDIX II

NAME: _____ DATE: _____

TYPE OF SAMPLE: **Cheese**

CHARACTERISTIC STUDIED: **Bitterness**

INSTRUCTIONS

"Bitterness is somewhat distasteful, resembling the taste of quinine or caffeine. The bitter sensation is somewhat delayed in terms of its initial perception and tends to persist for sometime after sample expectoration. Bitterness is observed by a taste sensation that occurs at the base of the tongue"

Place sample into the mouth and chew it until a semiliquid stage is reached. Roll the macerated sample in the mouth for sufficient time to determine both taste and aroma. Rank the sample from non bitter to most bitter. Expectorate the sample; rinse mouth with water between samples.

Sample # 425	Sample # 421	Sample # 915
Non bitter		
Moderately bitter		
Most bitter		

COMMENTS:

Figure II-6 - Sample form of questionnaire presented to the panel to evaluate bitterness of reduced fat Cheddar cheese during training sessions.

APPENDIX II

NAME: _____ DATE: _____

TYPE OF SAMPLE: **Cheese**

CHARACTERISTIC STUDIED: **Cheddar Cheese Flavor Intensity**

INSTRUCTIONS

"High-quality Cheddar cheese should possess the characteristic Cheddar flavor, which is best described as clean, moderately aromatic, nutty-like, and pleasantly acidic".

Place sample into the mouth and chew it until a semiliquid stage is reached. Roll the macerated sample in the mouth for sufficient time to determine both taste and aroma. Rank the sample from imperceptible to extremely perceptible. Expectorate the sample; rinse mouth with water between samples.

Sample # 354 Sample # 789 Sample # 345

Imperceptible _____
Moderately perceptible _____
Extremely perceptible _____

COMMENTS:

Figure II-7 - Sample form of questionnaire presented to the panel to evaluate cheese flavor intensity of reduced fat Cheddar cheese during training sessions.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Adhesiveness**

INSTRUCTIONS

Place sample between molars; chew seven times: press the sample to the roof of mouth with the tongue. Evaluate the force required to remove the sample from the roof of the mouth with tongue. Place an X next to the value which best describes the adhesiveness of the sample. Expectorate the sample; rinse mouth with water.

Sample # 621

_____	1	Not Adhesive
_____	2	
_____	3	
_____	4	
_____	5	Moderately Adhesive
_____	6	
_____	7	
_____	8	
_____	9	Very Adhesive

COMMENTS:

Figure II-8 - Sample form of questionnaire presented to the panel to evaluate adhesiveness of reduced fat Cheddar cheese.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Cohesiveness**

INSTRUCTIONS

Place sample between molars; compress fully: evaluate the degree to which the sample deforms rather than crumbles, breaks, or falls apart as cohesive. Place an X next to the value which best describes the cohesiveness of the sample.

Note: When evaluating **cohesiveness**, remember, if the sample falls apart easily, the sample is not cohesive and should get a lower score.

Sample # 425

_____	1	Not Cohesive
_____	2	
_____	3	
_____	4	
_____	5	Moderately Cohesive
_____	6	
_____	7	
_____	8	
_____	9	Very Cohesive

COMMENTS:

Figure II-9 - Sample form of questionnaire presented to the panel to evaluate cohesiveness of reduced fat Cheddar cheese.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Springiness**

INSTRUCTIONS

Place sample between molars; compress partially without breaking the sample structure. Place an X next to the value which best describes the springiness of the sample. Expectorate the sample: rinse mouth with water.

Sample # 852

_____	1	Not Springy
_____	2	
_____	3	
_____	4	
_____	5	Moderately Springy
_____	6	
_____	7	
_____	8	
_____	9	Very Springy

COMMENTS:

Figure II-10 - Sample form of questionnaire presented to the panel to evaluate springiness of reduced fat cheddar cheese.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Hardness**

INSTRUCTIONS

Place sample between molars; bite through once; evaluate for hardness. Place an X next to the value which best describes the hardness of the sample. Expectorate the sample; rinse mouth with water.

Sample # 642

_____ 1 Not Hard
_____ 2
_____ 3
_____ 4
_____ 5 Moderately Hard
_____ 6
_____ 7
_____ 8
_____ 9 Very Hard

COMMENTS:

Figure II-11 - Sample form of questionnaire presented to the panel to evaluate hardness of reduced fat Cheddar cheese.

APPENDIX II

NAME: _____ DATE: _____
TYPE OF SAMPLE: **Cheese**
CHARACTERISTIC STUDIED: **Flavor**

INSTRUCTIONS

Place sample into the mouth and chew it until a semiliquid stage is reached. Roll the macerated sample about in the mouth for sufficient time to determine both taste and aroma. Expectorate the sample and determine the overall flavor judgment. Rate the sample for bitterness and Cheddar flavor intensity on the following scale:

Sample # 280

BITTERNESS:
___ 1 Not observed
___ 2 Slightly bitter
___ 3 Somewhat bitter
___ 4 Distinctly bitter
___ 5 Strongly bitter
___ 6 Very strongly bitter

CHEDDAR CHEESE FLAVOR INTENSITY:
___ 1 No Cheddar cheese flavor
___ 2 Slight Cheddar cheese flavor
___ 3 Moderate Cheddar cheese flavor
___ 4 Distinct Cheddar cheese flavor
___ 5 Strong Cheddar cheese flavor
___ 6 Very strong Cheddar cheese flavor

COMMENTS:

Figure II-12 - Sample form of questionnaire presented to the panel to evaluate flavor of reduced fat Cheddar cheese.

APPENDIX III

APPENDIX III

Table III-1 - Distribution of peptides by fractions (% area) after 20 weeks of ripening of reduced fat Cheddar cheese.

CHEESE	FRACTION I	FRACTION II	FRACTION III	FRACTION IV
LLC-1.5	25.74[A] (2.58)	20.71[B] (4.24)	19.85[A] (1.46)	33.70[A] (5.49)
HSLC-1.5	26.41[A] (5.40)	12.77[A] (2.71)	21.00[A] (2.58)	39.82[A] (8.91)
Control	26.81[A] (4.20)	12.13[A] (1.89)	18.18[A] (1.84)	42.88[A] (7.38)

[1]Means with standard deviations in brackets.
n = 4 replications
[A,B]Means in same column with different superscripts are different ($p<0.05$).

Table III-2 Areas (x 1000, arbitrary units) of selected peaks from chromatographic profiles of peptides extracted from reduced fat Cheddar cheese made with adjunct culture of *Lactobacillus casei* L2A.

Cheese	Peak G		Peak A		Peak B		Peak C		Peak D	
	4 wks.	20 wks.	4 wks.	20 wks.	4 wks.	20 wks.	4 wks.	20 wks.	4 wks.	20 wks.
Control	44.9[1a] (0.7)	34.9[b] (0.3)	3.8[a] (0.7)	8.8[b] (0.5)	3.8[a] (1.4)	5.6[b] (1.8)	29.5[a] (0.7)	26.8[b] (0.5)	14.8[a] (0.2)	19.6[b] (0.8)
HSLC	42.6[a] (0.7)	33.7[b] (1.5)	2.8[a] (1.1)	3.9[a] (0.5)	2.4[a] (0.8)	1.6[a] (0.4)	35.6[a] (0.8)	33.0[b] (0.6)	11.3[a] (1.4)	19.4[b] (1.9)
LLC	13.1[a] (1.6)	2.5[b] (0.6)	4.8[a] (1.4)	15.3[b] (1.0)	7.3[a] (1.4)	12.5[b] (1.8)	24.2[a] (2.1)	20.7[b] (1.3)	11.8[a] (0.5)	19.4[a] (1.7)

[1]Means with standard deviations in brackets.
n = 4 replications.
[a,b]Means in same row within same column with different superscripts are different ($p<0.05$).

APPENDIX III

NAME: _____ DATE: _____

TYPE OF SAMPLE: **Cheese**

CHARACTERISTIC STUDIED: **Bitterness**

INSTRUCTIONS

"Bitterness is somewhat distasteful, resembling the taste of quinine or caffeine. The bitter sensation is somewhat delayed in terms of its initial perception and tends to persist for sometime after sample expectoration. Bitterness is observed by a taste sensation that occurs at the base of the tongue"

Place sample into the mouth. Roll it about in the mouth for sufficient time to determine taste. Rank the sample from non bitter to most bitter. Expectorate the sample; rinse mouth with water between samples.

Sample # 280 Sample # 420 Sample # 580

Non bitter _____
Moderately bitter _____
Most bitter _____

COMMENTS:

Figure III-1 - Sample form of questionnaire presented to the panel to evaluate bitterness of reduced fat Cheddar cheese slurry during training sessions.

APPENDIX III

NAME: _____ DATE: _____

TYPE OF SAMPLE: **Cheese**

CHARACTERISTIC STUDIED: **Bitterness**

INSTRUCTIONS

Place sample into the mouth and chew it until a semiliquid state is reached. Roll the macerated sample about in the mouth for sufficient time to determine taste. Expectorate the sample and determine the overall flavor judgment. Rate the sample for bitterness on the following scale. Rinse mouth with water between samples.

OBS. (1 = not observed; 10 = very strongly bitter)

Sample # _____	1	2	3	4	5	6	7	8	9	10
Sample # _____	1	2	3	4	5	6	7	8	9	10
Sample # _____	1	2	3	4	5	6	7	8	9	10
Sample # _____	1	2	3	4	5	6	7	8	9	10

COMMENTS:

Figure III-2 - Sample form of questionnaire presented to the panel to evaluate bitterness of reduced fat Cheddar cheese slurry.

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