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Flavor development in lactic fermentation of ultrafiltered cottage cheese whey

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Flavor development in lactic fermentation of ultrafiltered cottage cheese whey

by

Anand Rao

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DEDICATION

This work is dedicated to my loving parents for their everlasting moral support.

INTRODUCTION

Traditionally, cheeses have been made from whole or skimmed milk, with the production of large amounts of whey as a byproduct. Cheese whey has abundant nutrients such as whey proteins and lactose, which are not incorporated into the cheese matrix during cheesemaking. The presence of these organic components in whey causes its biological oxygen demand to be very high, thus posing problems with disposal.

Ultrafiltration of cheesemilk has been suggested as a means to minimize the high costs of treatment or disposal of whey, and to retain a portion of nutrients of whey in cheese. However, researchers (47) have reported that when cheesemilk was concentrated by ultrafiltration, the final product had flavor problems. Harper et al. (35) found that whey proteins had an inhibitory effect on rennet enzymes in cheese slurries, and suggested that this inhibition of proteolytic enzymes may have contributed to atypical ripening patterns reported in cheeses.

Another possible reason for lack of flavor development in cheeses made from ultrafiltered milk was suggested by Di Palma (23), who found that during the process of concentration by ultrafiltration, some of the milk components necessary for flavor development were lost into the permeate.

The object of this project is to use cottage cheese whey as a model system to study the effect of ultrafiltration on flavor development during fermentation of whey retentate by lactic acid bacteria. The importance of whey components partitioned into permeate during ultrafiltration in flavor development will be examined.

LITERATURE REVIEW

Composition of Milk

Milk is a very complex system containing several hundred compounds, many of which are present in very low concentrations. Considerable research has been done in the past 75 years on the composition of milk and characteristics of milk components. Several authors have summarized these research findings (1, 7, 31, 43, 77, 85, 95). The chemical composition of milk is greatly influenced by several factors such as breed of the cow, period of lactation, seasonal changes, nutritional quality of feed, and state of health (3, 36, 72). Typical composition of cow's milk is: water, 87%; fat, 3.5–3.7%; protein, 3.5%; lactose, 4.9%; and mineral ash, 0.7% (49).

The lipid system in milk is composed of triglycerides, phospholipids, sterols, fat-soluble vitamins and other minor components. Milk lipids are quite saturated and contain approximately 60% saturated, 38% monounsaturated, and 2% polyunsaturated fatty acids (36, 48).

Milk proteins can be broadly divided into two groups: casein and whey proteins. Casein is considered the major milk protein and accounts for about 80% of the total protein (15, 30). Jenness et al. (46) defined whole casein as that portion of milk protein that precipitates when the pH of skim milk is adjusted to 4.6. Various fractions of casein have been isolated and identified, the main fractions being α, ß, κ, and γ caseins (13, 15, 66). Researchers showed that the caseins in milk exist as soluble complexes and as micelles, which are large, highly organized structures that contain colloidal milk salts (13, 66, 80). It is believed that the micellar structure is stabilized by hydrophobic bonds and a colloidal phosphate framework (80). Rose (80) reported that the colloidal phosphate framework contains about 66% of the total calcium, 33% of the magnesium, 50% of the

inorganic phosphorus, and 10% of the total citrate in skim milk.

Whey proteins are considered to be a minor group of milk proteins (approximately 20% of total protein) and include ß-lactoglobulin (ß-lg), α-lactalbumin (α–la), bovine serum albumin (BSA), immunoglobulins, and a proteose-peptone fraction (13, 30, 66). Other proteins that occur in trace amounts in milk include enzymes and milk fat globule membrane (MFGM) proteins. Many enzymes have been identified in milk; these include aldolase, amylase, catalase, cytochrome C reductase, esterase, lactase, lipase, peroxidase, phosphatase (acid and alkaline), protease, and xanthine oxidase (85, 92). It is generally believed that the enzymes enter milk by diffusion from mammary tissue or by lysis of somatic cells and leucocytes (85, 92).

Lactose is the major carbohydrate present in milk. It is a disaccharide that yields D-glucose and D-galactose on hydrolysis. The two monosaccharides are linked together by a ß-1-4 linkage through the aldehyde group of ß-D-galactose; thus the aldehyde portion of the glucose residue acts as the reducing group for the lactose molecule (73). The configuration of this aldehydic portion determines whether lactose exists in the alpha or the beta form (96).

The ash content of milk as determined by standard procedures (4) does not represent the actual salt content in milk. Most of the volatile minerals are lost because of the high temperature involved in ashing. Normal milk is generally considered to have an ash content of 0.7%. This value is equivalent to about 0.9% salt content (49). Salts, although present in low concentrations, significantly influence heat stability, rennet coagulation of milk, and age-thickening of sweetened condensed milk (49, 74). Salts in cow's milk are generally chlorides, phosphates, and citrates of potassium, sodium, calcium, and magnesium (43, 49). Potassium, sodium, and chlorine are in an ionized state in the serum

portion. Phosphates, calcium, magnesium, and citric acid exist partly in solution and partly as complexes with proteins (49). Milk and dairy products are recognized as excellent sources of calcium.

Milk contains trace amounts of aluminum, arsenic, barium, boron, bromide, cadmium, chromium, cobalt, copper, fluorine, iodine, iron, lead, manganese, molybdenum, nickel, rubidium, selenium, silicon, silver, tin, vanadium, and zinc. Nutritionally, milk is considered a poor source of chromium, copper, fluorine, iron, manganese, nickel, silicon, vanadium, and zinc (43).

Cheese

There is a vast amount of literature referring to almost 2,000 names applied to cheese (84). In general, cheese is perceived as the product obtained by coagulation of milk and drainage of whey. The two most common ways of coagulating the cheese-milk are by addition of an enzyme (rennet) or by the action of lactic acid produced by microorganisms. Combinations of these two methods may also be used in some cases. The different varieties of cheese are produced by varying factors such as the microorganisms used, the method of milk coagulation, and the later processing steps in cheesemaking. After the cheesemilk forms a firm curd, it is cut to facilitate drainage of whey. When the required amount of acid develops in the cheese vat, the whey is drained either partially or completely. After complete removal of whey, the cheese curds may be allowed to mat together in the vat or in a hoop. In the case of unripened cheeses like cottage cheese, the curds are not allowed to mat and are washed with water to remove lactose and stop further fermentation. For ripened cheeses, the hooped curds are allowed to undergo further fermentation in controlled atmospheric conditions.

Partitioning of milk components

The components of milk are partitioned into the cheese curds and whey during cheesemaking. The various casein fractions interact with each other and with calcium while forming the cheese curds. The serum proteins are enclosed in the curd matrix at coagulation, but a high proportion is released into the whey (84). Researchers (33, 64, 82) have shown that whey proteins, especially ß-lg, interact with casein at high processing temperatures. The serum proteins are also less heat-resistant than caseins and coat the casein micelles when denatured. Thus, part of the serum proteins might be retained in the cheese made from milk processed at high temperatures (84).

The amount of lactose present in milk is considerably more than the lactic acid-producing bacteria are capable of fermenting. The amount of lactose in skim milk drops from 5.1% to only 4.3% after 18 hours of incubation with lactic culture (5). Approximately 80 to 85% of the original lactose remains unfermented. Because lactose is present in the serum portion of the milk, it is released into the whey during cheese-making.

Partitioning of salts in milk during cheesemaking depends on the type of cheese and manufacturing procedures (25). It is well known that the minerals in milk are distributed partly as ions in serum and partly in colloidal state with milk proteins (15, 19, 30, 49). The distribution of these salts in either the serum phase or the colloidal phase is affected by the pH and temperature (49). Both temperature and pH can vary greatly depending on the variety of cheese and its manufacturing procedure.

Irvine et al. (42) found that cream and cottage cheeses are lower in mineral content than Cheddar, brick, and blue varieties. They reported that cottage cheese contained 85 mg calcium per 100 g cheese and 146 mg phosphorus per 100 g cheese. They concluded that a marked reduction in calcium content is to be expected in the manu-

facture of those varieties of cheeses in which high acidities are developed or in which the curds are washed with acidified water. Feeley et al. (25) reported the ratio of calcium to nitrogen as 0.247 for nonfat cheesemilk. After processing into cottage cheese, the ratio dropped to 0.012. A two-fold reduction in calcium over magnesium and about a three-fold reduction in calcium over phosphorus were observed.

Feeley et al. (25) reported that both cottage and Cheddar-type cheeses have similar amounts of sodium, potassium, and magnesium in whey samples. However, cottage cheese whey contained more than twice as much calcium and about 35% more phosphorus than cheddar cheese whey. They also reported that Ricotta cheese contained more than twice as much magnesium and about four times as much calcium as creamed cottage cheese. These differences were attributed to the higher acidity developed during processing of cottage cheese. Calcium and magnesium salts become more soluble in acidic conditions. No appreciable differences in the mineral contents of cottage cheese made by either the long-set lactic acid method without added rennet or the short-set method with added rennet were found.

Wong et al. (94) investigated the effect of manufacturing variables on the retention of minerals in cottage cheese. With the traditional manufacturing method of cottage cheese, 57 to 69% of the minerals in cheesemilk were lost into whey. When the curd size was increased by cutting with 1.28-cm knives instead of 0.64-cm knives, the calcium in the curd increased by about 60% and the phosphorus increased by 18%. The increased retention was mostly attributed to a reduction in losses of minerals in the wash water. The larger curd particles exposed less surface area from which the minerals could be leached.

No increase in retention of calcium or phosphorus in cottage cheese was observed when calcium chloride was added to milk (94). However, addition of certain phosphate salts increased the amount of calcium in the curd, by increasing the retention of calcium. The calcium ion formed a complex with polyphosphates thus increasing the bound or colloidal calcium.

These variations in the partitioning of minerals and proteins with different manufacturing techniques, even for a single variety of cheese, make tabulation of a standard, precise composition of whey impractical.

Flavor development

The formation and complexity of flavors in various types of cheeses have been subjects of investigation by several researchers (10, 20, 32, 63). Although each variety of cheese has its own characteristic flavor, extensive research has shown that all cheese flavors are generated mainly by the interaction of compounds produced by decomposition of milk constituents. The flavor unique to each variety of cheese is the result of the desired balance of flavor and aroma compounds (27). The complexity of the cheese flavor increases with age because the ratio and proportions of the individual compounds changes with time (20).

Microorganisms play a key role in the development of flavor in fermented products. Law and Sharpe (57) made three types of cheese under aseptic conditions: one made with single strain starter; one made with single strain starter with controlled addition of nonstarter bacteria previously isolated at commercial creameries; and one made with ∂-gluconic acid lactone (GAL) as an acidifying agent instead of bacteria. Development of typical balanced Cheddar flavor in starter-only cheese and lack of flavor in GAL-acidified cheese showed the significant contribution of starter bacteria to flavor develop-

ment. Although nonstarter bacteria produced some off-flavors, the basic flavor intensity was enhanced when these organisms were present.

Notwithstanding the established importance of starter cultures in cheese flavor development, since the 1970s researchers have questioned the direct correlation between bacterial numbers and flavor development. The production of methanethiol in Cheddar cheese is closely correlated with the development of typical flavor (59). However, Law and Sharpe (57) found that starter bacteria such as lactobacilli, streptococci, and micrococci did not produce methanethiol. They concluded that the methanethiol normally present in Cheddar cheese probably arises from nonenzymic decomposition of L–methionine.

Law and Sharpe (57) found that although typical Cheddar flavor cannot be produced in cheese coagulated by acid rather than by starter culture, those starters that gave the best flavored cheese died out rapidly in the cheese. Microbial metabolism ceased after the curd had been salted and pressed. The typical flavor of cheese developed only after the viable starter numbers decreased to about one-thousandth of the numbers at curd milling time. This correlation between decrease in cell numbers and increase in flavor suggested the possibility that intracellular starter enzymes are released into cheese matrix during early stages of maturation.

From their findings, Law and Sharpe (57) theorized that addition of starter enzymes during cheesemaking would increase the rate of maturation of normal cheese. They also hypothesized that such enzyme supplementation would produce normal flavors in cheese made from GAL-acidified milk. Normal cheesemilk or GAL-acidified cheesemilk were supplemented with lysozyme-treated starter cells. The lysozyme-sensitized cells would rupture immediately upon addition of salt and pressing and there-

fore would only contribute their intracellular enzymes. These enzymes would theoretically generate cheese flavors in GAL-acidified cheese and increase the rate of formation or intensity of flavor in normal cheese. However, they found that the taste panel scores for enzyme-supplemented cheese were similar to those for control cheese. There was no difference in the rate of flavor formation or the intensity of flavor produced between the enzyme-supplemented and control cheeses. The amount of enzyme supplementation did not have any effect on the intensity of cheese flavor. The GAL-acidified cheese did not develop Cheddar flavor. Free amino acid production was used as an index of enzyme activity that resulted in maturation of the cheeses. The amount of free amino acids in enzyme-supplemented cheeses was comparable to or higher than in the control, indicating that the enzymes were in an active state.

From their findings, Law and Sharpe (57) defined the "correct conditions" for proper flavor development in cheeses as: (a) a supply of flavor precursors (e.g., free amino acids) derived from enzymic breakdown of lactose, proteins, and fats; (b) a low pH which prevents most enzyme-catalyzed reactions from proceeding too quickly; (c) a low redox potential (–150 to –200 mV) to maintain flavor compounds such as methanethiol in their reduced form.

Beide and Hammond (10) analyzed the flavor compounds present in Swiss cheese. They placed these components into three groups: oil-soluble, water-soluble, and water-soluble-nonvolatile. Vangtal and Hammond (89) correlated many of these components with the terms used to describe flavor characteristics. They found that ethanol-soluble carbonyl compounds were correlated with a number of flavor notes. However, Kowalewska et al. (54) found that not all carbonyl compounds produced in cheese are aromatic by themselves. Their work indicated that some of the flavor notes are generated

by complexing of carbonyl compounds such as glyoxal, methylglyoxal, and dihydroxyacetone with free amino acids produced as a result of proteolysis.

Griffith and Hammond (32) showed that flavor notes similar to those identified in Swiss cheese could be produced *in vitro* by reacting carbonyl compounds with free amino acids at ambient temperature (25°C). They found that glyoxal, methylglyoxal, ethanal, and dihydroxyacetone generated odors with certain amino acids. In particular, glyoxal, methyl-glyoxal, and dihydroxyacetone generated more intense odors than ethanal. Methylglyoxal-phenylalanine mixtures produced compounds such as phenylacetaldehyde, benzaldehyde, and acetophenone, which had been identified as flavor compounds in various cheeses. In addition, products of glyoxal-proline, glyoxal-lysine, and dihydroxyacetone-proline mixtures were shown to be responsible for some flavor notes similar to those in Swiss cheese.

These studies clearly showed that during the maturation of cheese, the starter organisms or starter enzymes produced the flavor precursors. The acidic environment of the maturing cheese helped in actual formation of flavor compounds by nonenzymic or at least nonmicrobial reactions.

Whey

Whey is the liquid byproduct of cheesemaking. Approximately 83% of the volume of milk used for cheesemaking appears as whey (84). Clark (17) reported total cheese whey production in the U.S. to be 50.9 billion pounds in 1985. This volume translates to about 3.3 billion pounds of whey solids. Of this amount, 2.9 billion pounds are produced from sweet-type whey and 0.4 billion pounds from acid-type whey. Morr (67) speculated that an additional 99-110 million pounds of whey proteins are present in whey derived from casein manufacture.

Composition

Whey contains valuable nutrients such as whey proteins, lactose, minerals, and other minor constituents. The composition of whey varies according to the type of cheese from which it is derived. Whey obtained from rennet coagulation of milk is referred to as "sweet" whey to differentiate it from "acid" whey obtained from cottage cheese manufacture or from isoelectric precipitation of casein (37, 67). The approximate composition of sweet whey is as follows: protein, 0.8%; lactose, 4.9%; fat, 0.2%; minerals, 0.5%; lactic acid, 0.2%; and water, 93% (67). Typical composition of acid whey has been reported as: protein, 0.7%; lactose, 4.4%; fat, 0.04%; minerals, 0.8%; lactic acid, 0.5%; and water, 93.5% (37, 67).

The major proteins remaining in the serum or whey upon precipitation of caseins consist of ß-lactoglobulins (ß-lg), α-lactalbumins (α-la), bovine serum albumin (BSA), the immunoglobulins (Ig), and components of the proteose-peptone fraction.

The most abundant of the whey proteins is ß-lactoglobulin, present at 2.45 to 4.2 g/l (30). Four genetic variants of this protein have been identified in cow's milk from the western hemisphere (93). Based on their mobilities in polyacrylamide gel electrophoresis at pH 8.5, these variants have been characterized as ß-lactoglobulins A, B, C, and D (75). The estimated molecular weights of these protein species range from 18,275 daltons for ß-lg-D to 18,362 daltons for α-lg-A (93). Researchers (66, 82) have demonstrated that when milk is heated, ß-lg interacts with κ-casein by disulfide interchange. This improves the heat stability of casein micelles (80). This interaction may reduce the amount of ß-lg in whey obtained from cheesemaking.

The second most available whey protein is α-lactalbumin (30). The amount of α-la in milk has been estimated to be 0.7 to 1.75 g/l (30). Two genetic variants of α-la, A and B, have been identified. The B-variant, which is the slower moving variant in alkaline gel electrophoresis, is the only variant found in milk from cattle in the Western hemisphere (93). The molecular weight of α-la-B has been calculated as 14,174 daltons (93). Brodbeck and Ebner (11) showed that α-la plays an important role in formation of lactose from UDP-galactose and glucose in lactating mammary tissue. The importance of this protein in milk or whey processing is not well understood.

Serum albumin was first crystallized from whey by Polis et al. (76) and was shown to be identical to the albumin in bovine blood serum. The heterogeneity of this protein was demonstrated by its resolution into several protein bands by isoelectric focusing (86). The concentration of BSA in milk has been reported at 0.25 to 0.45 g/l (93). The molecular weight of BSA is 66,500 to 69,000 daltons (93).

The immunoglobulins are a minor group of whey proteins present at 0.67 to 1.15 g/l in milk (44). They may occur as polymers or monomers. The immunoglobulin monomer consists of two heavy polypeptide chains and two light polypeptide chains, bonded together by disulfide linkages and noncovalent bonds (93). The immunoglobulins are divided into four classes based on their molecular differences and antigenicity: IgG1, IgG2, IgA, and IgM. Immunoglobulin IgG1, the principal immunoglobulin of bovine milk, ranges in size from 161,000 to 163,000 daltons. The amount of IgG1 in milk has been reported to range from 0.52 to 1.15 g/l (93).

The proteose-peptone fraction in milk is generally described as a mixture of heat-stable acid-soluble (at pH 4.6) phosphoglycero proteins insoluble in 12% trichloroacetic acid (93). The main components of this fraction have been identified as components 3, 5,

and 8, according to their increasing mobilities in alkaline electrophoresis. Component 3 is found only in the whey fraction of skim milk (71). The monomer molecular weight of component 3 has been estimated as 22,000 daltons, but it may exist as a closely associated dimer (71). Component 5 is distributed between the serum and casein micelles in milk and is partially coprecipitated during isoelectric precipitation of casein (53). Component 8 has been separated by gel-permeation chromatography into 8-fast and 8-slow fractions with estimated molecular weights of 4,100 and 9,900 daltons, respectively (53).

Ultrafiltration

Anti-pollution legislation is forcing cheese manufacturers to seek ways to utilize rather than dispose of whey. Although whey has been used in feeds for livestock, it is not very economical to haul large volumes of whey from the cheese plant to distant farms. Whey retains up to 70% of the food value of the original milk (51), and its value can be enhanced by its separation into the major components of protein, lactose, and salts.

Ultrafiltration (UF) is a fractionation process that has received much attention in recent years for the recovery of whey components (21, 79). Michaels (cited in 29) suggested that the term UF be used to describe the process of separation of solutes that are greater than 10 solvent diameters in size. Richert (79) explained UF as a process that employs semipermeable membranes through which molecules are forced as the result of a pressure gradient. The solution components are separated largely on the basis of their molecular size and shape. The solvent and smaller solutes pass through the membrane pores and are collected as permeate. Larger solutes are retained by the membrane and are collected as retentate.

An extension of UF to increase the ratio of protein to lactose or salts is called diafiltration (DF). In this process, the protein is concentrated by normal UF procedures,

and is then diluted with water. Ultrafiltration is continued with replacement of the permeate with water until the required purity of protein is reached. The protein is then concentrated to produce high quality protein concentrate (29).

Ultrafiltration membranes for whey processing are designed to pass lactose and salts but retain proteins either by creating a sieving effect (79, 84) or by acting as diffusion barriers (65). Early membranes used for whey processing were cellulose nitrate films. They were nonporous at low humidities, but swelled and became porous after contact with water. Newer membranes are made of other materials such as cellulose acetate, polyamide, polysulfone, and acrylic copolymer. Synthetic copolymers that give higher fluxes and are more resistant to heat and detergent cleaning are now replacing the cellulose membranes (84). These synthetic materials have a very thin (0.1–1 μm) selective layer supported by a much thicker (20–100 μm) layer that contains large pores.

The rate of fractionation of whey by UF is generally increased by increasing the feed pressure. Filtration rate is limited by fouling of the membranes and concentration polarization (29). In concentration polarization, a layer of retained macromolecules builds up on the membrane and continues to thicken and form a gel. After a critical concentration has been reached, the back-diffusion of macromolecules from the gel equals the convective movement of the molecules towards the membrane. When this happens, the permeation rate becomes independent of pressure. Further increase in pressure at this stage does not improve the flux and only thickens the gel.

Acid whey and sweet whey differ in fouling UF membranes (37, 70). Permeation rates for acid whey were reported to be only 60% of those for sweet whey. This difference was attributed to the greater amount of calcium in acid whey. Increasing the amount of calcium in sweet whey from the normal level (0.4 mg/g) to 1.3 mg/g reduced the per-

meation rate to one-third the normal rate. Muller et al. (70) reported a reduction in the amount of material deposited on UF membranes when calcium in whey was chelated by EDTA. They suggested that the fouling from acid whey is caused by a complex formed by a casein component with calcium phosphate-citrate.

The effect of initial pH of whey on permeation of UF membranes was studied by Muller et al. (70). Sweet whey exhibited minimum permeation at pH 4.3, but permeation increased three-fold at pH 3 and pH 7. Acid whey also had low permeation at pH 4.3, but permeation was only doubled at pH 2.9, and no significant improvement in permeation was observed at pH 7.

Muller et al. (70) noted that membrane permeation decreased with time because of formation of a secondary layer. They observed the decrease in permeation rate for acid whey with initial pH 4.4. The initial permeation rate could be restored by adjusting the pH to 2.9 during ultrafiltration. However, the permeation rate obtained by adjusting to pH 2.9 was lower than the permeation rate of acid whey initially at pH 2.9. Similar improvement in permeation of acid whey (pH 4.3) was noted when the membrane surface was flushed at hourly intervals with water adjusted to pH 2. Flushing with water at neutral pH had little effect. Muller et al. (70) suggested that, at pH 2.8, the membrane deposits formed a secondary dynamic membrane of relatively high permeability. If the secondary membrane was allowed to form at pH 4.4, neither pH adjustment of whey nor flushing with acidified water had a large effect on its permeability.

Various methods have been studied to reduce membrane fouling and to improve the flux. The simplest pretreatment of whey is filtration or centrifugation to remove fine particles of casein (84). Pasteurization of whey helps to prevent precipitation of calcium salts during UF (29). Hayes et al. (38) reported a doubling of permeation rate of acid

whey that was heated at 80°C for 15 seconds and then adjusted to pH 5.2 to 5.9. Heating of whey caused aggregation of a complex of casein and ß-lg, which retained calcium phosphate (29). Formation of membrane deposits was thus retarded.

Kaiser and Glatz (50) tried to improve flux of cheese whey in UF by first precipitating a portion of the soluble proteins with carboxymethyl cellulose (CMC). Flux improved if the precipitation yielded large-sized particles, but was reduced when small aggregates of CMC-protein were formed.

Fractionation of whey components

Because UF membranes are extremely permeable to lactose, minerals, and short-chain polypeptides, the percentage of these components fractionated from whey roughly equals the percentage of permeate separated. Removal of 90% lactose approximately requires about 90% reduction in original volume of whey. Because of the high ratio of lactose to protein in whey, retentate with protein concentrations greater than 50-60% on a dry weight basis is difficult to obtain with normal UF (61). Higher concentrations of purer protein in retentate can be obtained by repeated cycles of DF.

Fenton-May et al. (26) assumed 100% retention of proteins and zero retention of all other components and predicted protein concentrations of 36, 53, and 69% for 80, 90, and 95% volume reduction, respectively, for whey during UF. However, they realized only 30, 42, and 45% protein for whey concentrated to 20, 10, and 5% of initial volume. These differences were observed because neither 100% retention of protein nor zero retention of other components is possible under practical conditions. Barbano et al. (6) detected 0.25 g/l of protein in permeate when milk was ultrafiltered. Approximately 90% of the permeate protein was identified as α-la.

The process of ultrafiltration acts as a partial demineralizer. Fenton-May et al. (26) reported that whey contained 11.2% ash on a dry weight basis. With 80% volume reduction, the ash content was reduced to 8.3% of dry weight. With 95% volume reduction, the retentate contained 4% ash on a dry weight basis.

The pH of whey during ultrafiltration or diafiltration plays a significant role in the demineralization of whey (22, 39). If whey is ultrafiltered at pH 6.6 (above the isoelectric point of whey proteins), anions such as Cl^- and NO_3^- are lost into the permeate. If the pH of whey is below the isoelectric point of whey proteins (e.g., pH 3.5), a significant portion of cations is removed into permeate. Such preferable removal of ions based on pH can be used to produce whey protein concentrate with extremely low ash content. Hiddink et al. (39) suggested ultrafiltering whey at pH 6.6 and then applying diafiltration at pH 3 to 3.5 to produce high protein whey concentrate which is low in ash.

Amino acids in whey are retained during concentration (26, 60). Mavropoulou and Kosikowski (60) reported an average of 0.59% free amino acids in whey powders. Fenton-May et al. (26) did not find any major difference in the amino acid profile of whey and whey protein concentrate. A five-fold increase in amino acid concentration corresponding to a similar increase in protein concentration during ultrafiltration was observed. These researchers suggested that all the amino acids in whey are either bound to or are part of the protein.

Ultrafiltration of whey removes most of the salts, lactose, and water from the protein and yields an undenatured, high-protein concentrate with many applications in other foods. The most common use of UF for whey processing is the production of spray-dried whey protein concentrate (WPC) (21). In this process, whey is concentrated by UF to 18-22% protein, and the retentate is pasteurized and spray dried under mild temperature con-

ditions (67). Specialized WPCs such as those with hydrolyzed lactose, no lactose, or a low sodium content are produced for use in special dietary food products (2). In contrast to casein products such as nonfat dry milk, WPC is highly soluble in acidic conditions (69). This unique property facilitates its use in acidic food products such as carbonated drinks and fruit juices, thereby improving the nutritional quality of these foods (67). Other properties of WPC that are important in food products include their whipping properties (16, 69, 68), emulsion capacity (69), viscosity (68, 83), foaming (68), and gelation (52, 90).

Heat denaturation and gelation

Whey proteins form heat-induced gels under appropriate conditions. The protein gel is a three-dimensional matrix formed when denatured proteins are held together by ionic, hydrophobic, and disulfide bonds.

The heat-induced gelation of whey proteins is a two-stage process (22). First, denaturation occurs as the monomers combine into oligomers by disulfide bonding. Extensive polymerization that results in decreased solubility then takes place upon cooling. Temperature (24), ions (52, 90), and pH (90) are important factors affecting the formation of a strong whey protein gel. In most studies of gelation, protein denaturation was defined as reduced protein solubility at the pH of interest. Some researchers considered changes in calcium binding, content of sulfhydryl (SH) groups, or antigenic activity as indicators of denaturation.

Dannenberg and Kessler (18) studied the reaction kinetics of heat-induced denaturation of ß-lactoglobulins A and B and of α-lactalbumin in milk over a wide temperature/time range. Denaturation of ß-lg was best described by an apparent reaction order of 1.5, and α-la denaturation followed first-order kinetics.

Hillier et al. (40) observed the effects of temperature and concentration of total solids on the denaturation rates of α-la and ß-lg in cheese whey. They noted that the rate constants of denaturation changed at about 90°C. The denaturation of α-la was slower above 90°C for cheese whey concentrated three-fold. The rate of denaturation of α-la was not affected by the solids concentration. A similar change in the reaction mechanism was observed for the genetic variants of ß-lg at temperatures closer to 100°C. It was also noted that the two genetic variants, ß-lgA and ß-lgB, exhibited temperature-dependent heat sensitivity: below 90°C, ß-lgA was more stable than ß-lgB, but the situation was reversed at higher temperatures. However, this difference in relative heat stability disappeared as the total solids concentration increased.

The relative susceptibility of whey proteins to denaturation is affected by the total solids concentration. McKenna and O'Sullivan (64) reported that the percent denaturation of whey proteins in skim milk was independent of concentration at heating times of 5 min or less. If skim milk was heated for more than 5 min, whey proteins were more heat-stable in concentrated milk than in unconcentrated milk.

Hillier et al. (40) noted the differences in susceptibility of α-la and ß-lg to thermal denaturation. Lactalbumin was more susceptible to thermal denaturation in concentrated whey than in normal cheese whey. In contrast, ß-lg denaturation was retarded when the total solids concentration increased to about 20%. Guy et al. (33) reported minimal protein denaturation in cottage cheese whey concentrated to 20% total solids.

Valdicelli et al. (88) investigated the heat sensitivity of isolated whey proteins at temperatures between 60°C and 95°C at different pH levels. Serum albumin was most sensitive to heat, followed by ß-lgA, ß-lgB, α-la, and proteose-peptone. Only ß-lg was affected by pH; the most extensive denaturation of ß-lg occurred at pH 5.8 at 80 to 85°C.

However, the maximum total protein denaturation occurred at pH 6.2. Hillier et al. (40) showed that the rate of denaturation of both α-la and ß-lg was slower at pH 4 than pH 6 or 9, and probably slowest at the isoelectric point. Further investigation (41) demonstrated that the formation of gels was slower at alkaline pH, and suggested that at high pH, gel formation was inhibited by electrostatic repulsion between protein molecules with like charges. Dunkerley and Hayes (24) reported that the gel strength of whey protein gels was poor if heat treatment was below 80°C. Gel strength increased significantly as pH was decreased from 7.86 to 4.69.

In contrast to these findings, Langley et al. (56) reported that it was impossible to produce whey protein gels (15% w/v) at pH 5.0 at 80°C. Gels produced at pH 8.0 were much stronger than those produced at pH 6.5. These researchers used either pure α-la or ß-lg protein solutions and a short heating time of 5 min. When they heated a mixture of α-la and ß-lg, the gel strength increased greatly. This suggests that the interaction of individual proteins to form complexes contributes greatly to gel strength. Extensive formation of such complexes might explain why others obtained gel formation of protein mixtures at acidic pH values.

The importance of SH groups in heat-induced gelation of whey proteins was demonstrated by Hillier et al. (41) with isolated whey proteins. Aqueous solutions of whey proteins heated to 80°C formed opaque gels only when the protein powder contained relatively high amounts of SH groups, and clear gels formed from those with fewer SH groups. The formation of disulfide cross-links between polypeptide chains was retarded in the presence of compounds that reacted with SH groups.

The effects of dialyzable constituents of whey, such as calcium and lactose, on protein denaturation were also investigated. Varunsatian et al. (90) compared the effects

of calcium chloride, sodium chloride, and magnesium chloride on the aggregation of whey proteins. Calcium ions affected heat aggregation the most, especially above the isoelectric pH 5.5. The whey protein most sensitive to calcium was ß-lg.

Kohnhorst and Mangino (52) studied the involvement of calcium in ionic bonding of proteins and in gel formation and reported that the calcium content was negatively correlated with gel strength. They concluded that calcium effects are concentration-dependent, and calcium levels up to 11 mM (0.44 mg/ml) improve gel strength.

Hillier et al. (40) showed that salts and lactose stabilize α-la and ß-lg against denaturation. At a constant protein level, increased amounts of calcium and lactose decreased the rate of denaturation of individual proteins. If calcium was increased above 0.4 mg/ml, no significant effect on thermal denaturation resulted. However, increased lactose concentration in the range of 34 to 55 mg/ml decreased the rates of denaturation.

Buchheim and Jelen (14) studied the microstructure of heat-coagulated whey protein under different conditions of pH and temperature. They concluded that upon heating, coagulable whey proteins formed compact structures similar to those of rennet- or acid-coagulated caseins.

Growth of lactic cultures in whey

Fermentation of whey or whey components fractionated by UF for production of food products has been extensively investigated in the USSR and in some European countries (97). In the United States, research has been directed mostly towards yeast fermentation of whey for the production of alcoholic beverages and food additives such as food acids, enzymes, food gums, and amino acids (55, 97).

Researchers have studied the applicability of whey as a growth medium for lactic cultures (16, 78). However, literature dealing with growth of bacteria in whey or its ultrafiltrates could not be found. Most researchers have used whey-based media to propagate cheese starter cultures or to prepare phage resistant media, and have studied the activity of these starter cultures in cheese milk or in other synthetic media.

Richardson et al. (78) compared the activity of lactic acid bacteria in whey, modified whey, and milk. Acid development was poor in unmodified Cheddar cheese whey, but good acid production in phosphated whey medium was observed. When milk was inoculated with cultures grown in various media, the cultures from phosphated whey-based medium had greater activity than those propagated in nonfat dry milk.

Chen and Richardson (16) studied the application of a phosphated whey medium formulated from acid whey (APWM) for cottage cheese manufacture. Compared to the cultures grown in nonfat dry milk medium or in commercial phage inhibitory medium, the cultures propagated in APWM were very active in cheese milk. Even slow single-strain cultures were capable of rapid acid production in the cheese vat with less lag time upon transfer into milk.

MATERIALS AND METHODS

Preparation of Retentate

Acid whey from manufacture of cottage cheese was obtained from Anderson-Erickson Dairy (Des Moines, IA). Whey was clarified using a De Laval model 100-AE centrifugal separator (The De Laval Separator Co., Poughkeepsie, NY) to remove any suspended curd fines. Approximately 182 kg (400 lbs) of clarified whey were held in a double-jacketed cheese vat and ultrafiltered by passage through an Amicon UF unit (Amicon Corp., Danvers, MA), fitted with two hollow fiber cartridges. Each cartridge contained approximately 500 hollow fibers (type H26P; 30,000 MW cutoff), providing a total filtration surface of about 500 m^2. The unit was operated with inlet pressure of 25 psi and outlet pressure of 18-20 psi. The retentate was circulated back into the cheese vat, and the permeate was collected in sanitized milk cans. During ultrafiltration, the temperature of whey was maintained below 20°C by circulating cold water in the jacket of the cheese vat.

Ultrafiltration was continued until the volume of retentate in the vat was approximately 10% of the initial whey volume. At this stage, the retentate was diluted with an equal volume of distilled water. The diluted retentate was diafiltered until its volume was halved, or until foaming made further processing impossible. If foaming was excessive before the diafiltration step, food grade antifoam FG-10 emulsion (Dow Corning Corp., Midland, MI) was added at 2.5 ml per 45.5 kg (100 lbs) whey-retentate mixture. The ultra-filtration procedure is shown in Figure 1.

The final retentate was transferred into 500-ml polyethylene bottles (Fisher Scientific, Pittsburgh, PA) and stored at -20°C until further use. Permeate was mixed well to insure homogeneity and was stored frozen in 500-ml polyethylene bottles. Before use,

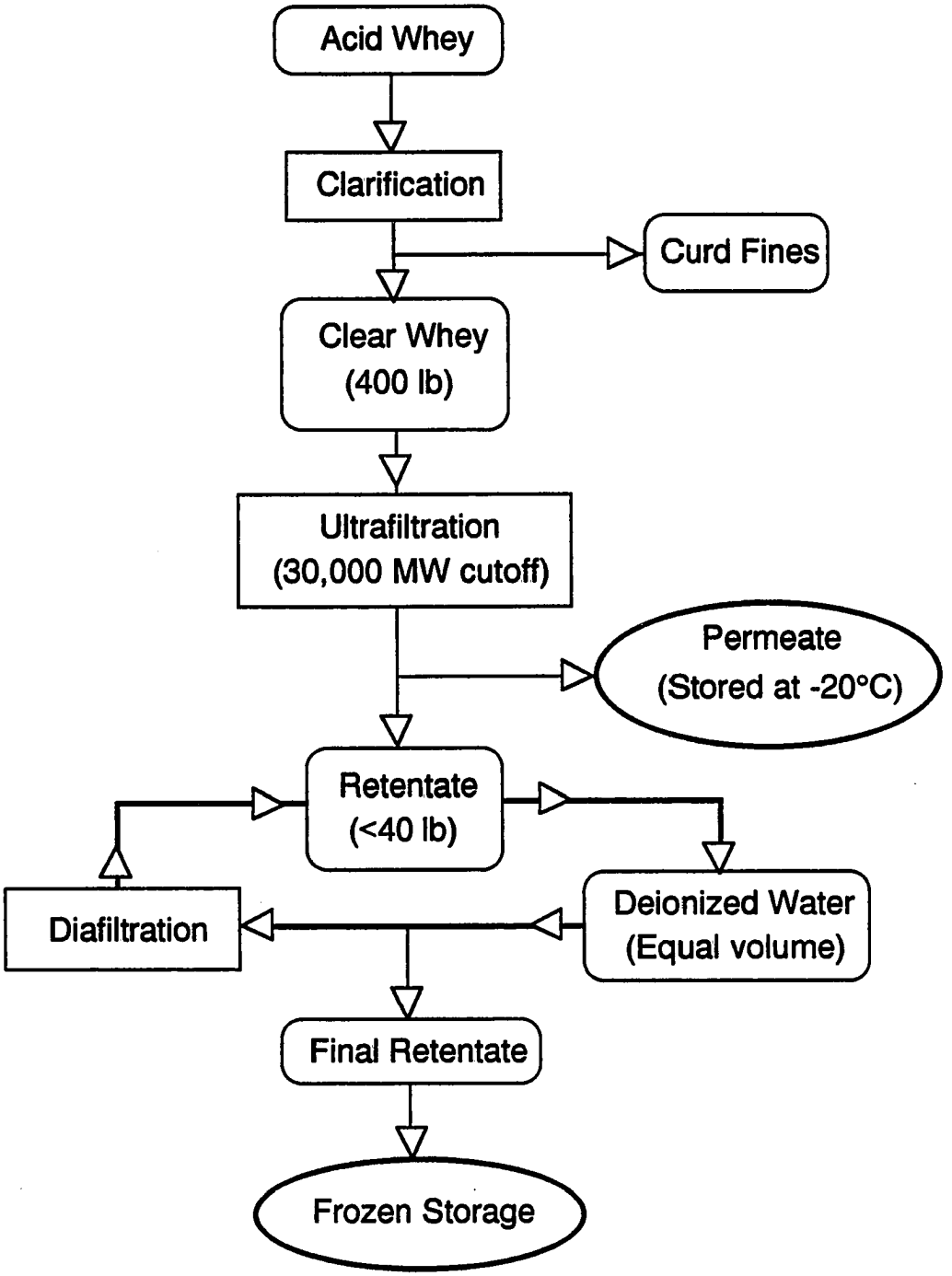

Figure 1. Flow diagram for preparation of whey retentate

individual bottles of retentate and permeate were thawed at 4°C and tempered to room temperature in a water bath.

Compositional Analyses

Total solids

Total solids of acid whey, retentate, and permeate were determined by the oven method for milk (4) on duplicate samples. Three grams of sample were weighed into tared aluminum flat-bottom dishes. Samples were evaporated to dryness over a steam bath, oven dried at 100°C for 3 h, and weighed after cooling to room temperature in a desiccator. The total solids were expressed as percent of the initial sample weight.

Protein

Samples of whey, retentate, and permeate were analyzed in duplicate for nitrogen content according to Kjeldahl nitrogen assay (81). Samples were digested with concentrated sulfuric acid in the presence of Kjeldahl catalyst mixture. Ammonia from the digested samples was released by distillation after reaction with 40% sodium hydroxide solution. The evolving nitrogen was collected by dissolution into dilute boric acid solution. The amount of ammonia was estimated by titration with standard 0.1 *N* sulfuric acid. The grams of nitrogen in the sample was converted to grams protein by multiplying with the protein factor of 6.32.

Lactose

Lactose content of retentate and permeate samples was determined by the colorimetric method described by Lawrence (58). Duplicate diluted samples of retentate and permeate were reacted with phenol (5% aqueous solution) and concentrated (37*N*) sulfuric acid. The absorbence of the samples was read at 490 nm with a Spectronic 601 spec-

trophotometer (Milton Roy Company/Analytical Products Division, Rochester, NY). The percent lactose in the samples was estimated from a standard curve prepared with lactose solutions in distilled water.

Ash

The ash content of retentate and permeate samples was determined in duplicate by the gravimetric procedure (4). Five-milliliter samples were weighed into tared crucibles, dried on a steam bath, and ashed overnight in a muffle furnace at 550°C. The crucibles were allowed to cool to room temperature in a desiccator before weighing. The ash content of the samples was calculated as percent of initial sample weight.

Starter Culture

Propagation and maintenance

Pure cultures of a lactic acid-producer (*Streptococcus lactis*, D44) and a citric acid-fermenter (*Leuconostoc dextranicum*) were isolated from a commercially available mixed culture (Chr. Hansen's Laboratory, Inc., Milwaukee, WI). Cultures were stored frozen at –75°C in sterile reconstituted nonfat dry milk (NFDM) at 11% solids with 5% glycerol. Working cultures were propagated in 10 ml sterile reconstituted NFDM at 32°C. Cultures were transferred (1% inoculum) two to three times per week. Cultures that were about 18 h old and that had coagulated the milk were used for inoculation of retentate samples.

Growth in retentate samples

Growth of the culture in samples of retentate diluted with permeate, mineral solution, or distilled water was monitored by measuring the pH and the number of viable cells. Whey retentate was diluted to 3.5% protein, the pH was adjusted to 6.0±0.2 by

dropwise addition of 0.1*N* KOH, and the preparation was dispensed in 100-ml volumes in sterile 250-ml Earlenmeyer flasks. The preparations were inoculated with mixed culture containing 1% (vol/vol) each of *Streptococcus lactis* and *Leuconostoc dextranicum* or with a single culture of *Leuconostoc dextranicum* at 1% (vol/vol). Samples were taken immediately after inoculation and at 2-h intervals until 12 to 14 h of total incubation time. The inoculated samples were incubated at 32°C.

The pH of the samples was measured with a Radiometer PHM61 laboratory pH meter (Radiometer, Copenhagen, Denmark). Samples were also serially diluted and plated by the pour plate method onto Bacto Lactobacilli MRS and Bacto Plate Count agars (Difco Laboratories, Detroit, MI). Both cultures grew well on MRS agar, but *Leuconostoc dextranicum* grew slowly on the plate count agar. Colony forming units (CFU) of *Streptococcus lactis* were obtained on the plate count agar plates after 48 h of incubation. This value was subtracted from the colony count obtained on MRS agar to obtain the CFU for *Leuconostoc dextranicum*. When cultures were grown singly, colony counts were performed on MRS agar. The entire experiment was repeated three times to obtain average colony counts. The growth curves in different preparations were statistically compared by general linear model (GLM) to test for variations in the slopes of the curves.

Sample Preparation

Dilution of retentate

Generally, experiments were performed on retentate preparations that contained 3.5% protein. The amount of thawed retentate required to make 100 ml of final material with 3.5% protein was measured into a clean 250-ml Erlenmeyer flask, and was diluted with distilled water, permeate, or aqueous solutions of known composition in different combinations.

To prepare samples with ashed permeate, the volume of permeate required to dilute the retentate was ashed for about 12 h and redissolved in a small amount of reagent grade concentrated nitric acid. This was added to the retentate with sterile distilled deionized (SDD) water to achieve the desired dilution.

The pH of all samples was adjusted to 6.0±0.1 by dropwise addition of 0.1*N* NaOH. The samples were inoculated with 18 h old starter culture, either singly (2% inoculum) or as mixed culture (1% inoculum of each), and were incubated at 32°C for 16-18 h or until pH 4.5 was reached.

Heat coagulation

Samples were coagulated at pH values ranging from 3.5 to 6.0 (increments of 0.5 units) to establish the ideal heat coagulation temperature and pH needed to obtain protein gels with desirable strength. Retentate was diluted with SDD water to 3.5% protein and pH was adjusted by dropwise addition of either 1*N* NaOH or 0.1*N* HCl. When the effect of pH on flavor as well as gel strength was being measured, samples were fermented with starter culture to the desired pH. Slight adjustment of the fermented samples' pH was done by addition of either base or acid when necessary. Samples were then heated at 70, 80, or 90°C in a water bath for 10 min and cooled to 25°C. Strength of the gels was measured with a Brookfield Viscometer Model LVF equipped with a Helipath stand and a T-bar spindle (Brookfield Eng. Laboratories, Stoughton, MA). The penetration depth was set to 30 mm from the surface of the gel. Dial readings were noted when the spindle had penetrated about 20 to 25 mm into the sample. In general, the dial readings are converted into centipoise values to reflect the viscosity of the solution being tested. Because only the relative gel strength of the heat-coagulated protein matrix was of concern in this study, the dial reading was used as a gel strength indicator.

Once the parameters for heat coagulation were established, all samples used in the sensory analysis were coagulated according to the following procedure. After the samples reached pH 4.5, 25-ml aliquots of the fermented retentate were dispensed into 50-ml beakers. The beakers were covered with aluminum foil and were placed in a water bath set to 90°C. One beaker was used to monitor the samples' internal temperature. Samples were held at 90°C for 10 min, immediately cooled to about 10°C in ice water, and stored at 5°C until used.

Identification of Components Affecting Flavor

Fatty acids

The free fatty acids in whey permeate were analyzed by a combination of column, thin layer, and gas-liquid chromatography techniques. The permeate was first concentrated ten-fold in a vacuum rotary evaporator (Rota Vapor-R, Buchi, Switzerland). The concentrated permeate was seeded with lactose crystals and stored at 5°C for 7 days, at which time the lactose crystals were removed from the serum by filtration through Whatman No. 41 filter paper.

Column chromatography Celite was added to the concentrated delactosed permeate at a ratio of 1.5 g celite per gram permeate solids. The mixture was evaporated to dryness in a rotary evaporator, and 50 g of this preparation were packed in a glass column (30 cm x 11 mm id). The column was eluted first with 50 ml ethyl ether, then with 50 ml ethanol, and flushed thoroughly with nitrogen between elutions.

The eluates from the column were dried in a rotary evaporator. A portion of the dry material was dissolved in SDD water and used to dilute retentate for fermentation. Some of the dry material was esterified for fatty acid analysis.

Esterification Portions (90 mg) of the dry material were placed in screw-capped bottles and reacted overnight at 55°C with 500 µl acidified (15 µl H_2SO_4) decyl alcohol. The esterified mixture was diluted with 600 µl hexane before separation on TLC plates.

Thin layer chromatography The esterified samples (100 µl) were streaked onto silica type-G (Sigma Chemical Co., St. Louis, MO) TLC plates of 0.75 mm thickness. Standard decyl ester preparations of lactic, acetic, and butyric acids were placed as 10 µl spots on either side of the unknown sample streak. The plates were developed in hexane:ether (85:15) mixture for 45 min. After air drying, the developed plates were sprayed with 0.2% 2',7'–dichlorofluorescein in 95% ethanol. The plates were viewed under ultraviolet light and the separated streak components were marked.

The separated streaks were scraped off the TLC plate and placed in a Buchner type filtering funnel of 10 to 15 µm porosity. The esters were eluted from the material with 15 ml hexane in three steps of 5 ml per step. The eluates were evaporated to dryness under a slow stream of nitrogen and dissolved in 2 ml distilled ether for gas chromatographic analysis.

Gas-liquid chromatography Samples (5 µl) were injected in triplicate into a Varian Model 3700 gas chromatograph (Varian Associates, Inc., Palo Alto, CA) equipped with a SPB1-DB nonpolar capillary column and a flame ionization detector. The injector and detector temperatures were set at 250°C. The oven temperature was controlled by a linear temperature programmer. The initial oven temperature was set at 50°C. After a 4-min solvent delay, the temperature was increased to 200°C at 10°C/min and was held at 200°C for 10 min.

Unknown peaks were identified by using the retention times of decyl esters of acetate and butyrate as standards. The amount of a fatty acid in a sample was calculated as follows:

$$\text{Amount of fatty acid (mg)} = \frac{\text{Sample peak height}}{\text{Standard peak height}} \times \text{Amount of standard (mg)}$$

Amino acids

The free amino acids in permeate were determined at the Iowa State University Protein Facility with an automated high performance liquid chromatographic (HPLC) system. The free amino acids in the permeate were first derivatized with phenyl isothiocyanate under basic conditions to produce phenylthiocarbamyl (PTC) derivatives. The derivatives were separated by reverse-phase chromatography on a C18-silica column with a gradient buffer system consisting of Buffer A (.05 M Sodium acetate, pH 5) and Buffer B (17.05 M acetonitrile, pH 6). The separation was started with a Buffer A:B ratio of 7:93 and was ended after 20 minutes with a Buffer A:B ratio of 40:60. Peaks were detected at 254 nm and peak areas were measured using a Hewlett-Packard 3392 integrator. A PTC-derivative of norleucine was injected along with the sample as a standard.

The amount of each amino acid in the sample was calculated by dividing each peak area (corrected for the different molar absorptivities of the various amino acids) by the peak area of norleucine, and multiplying this ratio by the total amount of norleucine added to the sample.

Diacetyl, glyoxal, and methylglyoxal

These compounds were detected in fermented retentate preparations by an HPLC procedure developed by Bednarski et al. (9). Samples were centrifuged in a Beckman model J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 14,000 x *g* for 10 min at 25°C. Supernates were collected as 25-ml portions in screw-capped test tubes and adjusted to pH 8.0±.2 with 1 *N* NaOH. Approximately 1.3 ml of an aqueous 1% solution of o-phenylenediamine were added to each sample to give a final concentration of 0.05% o-phenylenediamine. A 340-µl portion of skatol solution (1 mg/ml in methanol) was added as an internal standard.

The mixture was allowed to stand undisturbed for 4 hr at room temperature, at which time the pH was adjusted to 3.0 by dropwise addition of 1*N* HCl. Approximately 2 ml chloroform were added and mixed thoroughly. The chloroform (bottom) layer was separated by centrifugation at 11,000 x *g* for 10 min and transferred to a screw-capped vial with a transfer pipette. This extraction process was conducted three times and the chloroform fractions were pooled.

The chloroform was allowed to evaporate at room temperature and the residue dissolved in 1 ml methanol. The mixture was filtered through Cameo HPLC 3 mm nylon filters of 0.22 µ pore size (Micron Separations, Inc., Honeoye Falls, NY).

The samples were then analyzed on a Waters' model ALC-201 HPLC system equipped with a 100 A STYRAGEL gel permeation chromatography column (30 cm x 7.8 mm id) (Waters Associates, Inc., Framingham, MA). The column packaging material was fully porous, highly cross-linked styrene-divinylbenzene copolymer. A mixture of HPLC-grade methanol and water (68:32) was used as the mobile phase. The flow rate was maintained at 0.7 ml/min. Sample injection size varied between 10 and 20 µl. The

separated peaks were detected by a Varian model 2050 detector (Varian Associates, Walnut Creek, CA) at 254 nm. The output was integrated with a Hewlett-Packard (Palo Alto, CA) Model 3392A integrator.

The glyoxal, methylglyoxal, and diacetyl peaks in the samples were identified by comparing their retention times to those of standards. Their amounts were calculated as follows:

$$\text{Amount of unknown } (\mu g) = \frac{\text{Unknown peak height}}{\text{Skatol peak height}} \times \text{Amount of skatol } (\mu g)$$

Dihydroxyacetone

The enzymic assay described by Weiland and Witt (91) was adapted to determine the amount of dihydroxyacetone (DHA) in permeate. Samples were first deproteinized by adding 12.5% (w/v) trichloroacetic acid and removing the precipitate by centrifugation at 11,000 x *g* for 15 min. The supernate was adjusted to pH 7 with 0.1*N* NaOH and assayed.

In the assay system, dihydroxyacetone (DHA) in the sample was phosphorylated with ATP to dihydroxyacetone phosphate by the action of glycerokinase. Dihydroxyacetone phosphate was then reduced with NADH to glycerol-3-phospate by the action of glycerophosphate dehydrogenase. The decrease in NADH, measured at 339 nm in a Spectronic 601 spectrophotometer (Milton Roy Company, Rochester, NY), was proportional to the initial amount of dihydroxyacetone. The dilution that occurred during deproteination was taken into account when calculating the amount of dihydroxyacetone in permeate.

Organoleptic Evaluation

Preliminary Screening

Three experienced panelists tasted samples during the initial trial period. These trials screened components detected in permeate and other compounds suspected to be precursor compounds for cheese flavor for their influence on flavor development. The compounds were added in various combinations to the solution used to dilute retentate. Four terms, acidity, astringency, sweetness, and complexity, were defined by the panelists to describe the flavor profile of fermented samples. The terms acidity, astringency, and sweetness were selected to describe the degree of the respective flavors detected in the sample. Complexity was defined as a flavor note that described the fullness of fermented flavor sensed in the retentate-permeate preparation. The flavor effects of components (or their combinations) identified during the preliminary screening were retested for validity by a formal sensory panel.

Formal Sensory Panel

The final organoleptic analyses were done using a trained panel of 12 members. The panelists were trained by providing them with samples prepared to generate the individual flavors that could be described by the terms previously defined. Once the panelists were able to identify the individual flavor characteristics, isolation booths with controlled lighting conditions were used for further training. In the isolation booths, the panelists were trained to detect the individual flavor characteristics in samples with a complex flavor profile and to assign a numerical score to measure the intensity of the flavor. All scores were given on a linear scale of 0 (no detectable flavor) to 6 (high flavor intensity). An example of the score card used in the evaluation of samples is shown in Figure 2.

Score Card

Name _____

Taste the samples and assign a score on a scale of 0–6, for the flavor attributes listed below. Standards are provided as positive and negative references. Thank you.

0 1 2 3 4 5 6
Not Present **Moderate** **High**

SAMPLE # _____

Flavor:
Acidity _____
Astringency _____
Sweetness _____
Complexity _____

SAMPLE # _____

Flavor:
Acidity _____
Astringency _____
Sweetness _____
Complexity _____

SAMPLE # _____

Flavor:
Acidity _____
Astringency _____
Sweetness _____
Complexity _____

SAMPLE # _____

Flavor:
Acidity _____
Astringency _____
Sweetness _____
Complexity _____

Remarks:

Figure 2. Score card used in the organoleptic analyses of heat-coagulated fermented ultrafiltered whey

The panelists were presented with sample set 1 shown in Table 1. Three replications of these treatments were judged in a random order by the panelists. Based on the results of statistical analysis of set 1 and to establish the effect of lactate and dilution with water on flavor development, the panelists were presented with samples from treatment set shown in Table 2. The treatment set 2 was replicated two times for flavor analysis.

Statistical Analysis

The final organoleptic evaluations were conducted as a split plot design. The different compositions of the retentate mixes before fermentation were considered as the statistical treatments (Tables 1 and 2). Treatment set 1 was replicated three times and set 2 was replicated twice. The scores given by the panel members were statistically analyzed using the SAS computer package (SAS Institute, Inc., Gary, NC). The main effects of the treatments were analyzed by the ANOVA procedure. The individual treatments were then contrasted to see the effects of interaction of the components. The treatment differences were analyzed using Waller-Duncan's K-ratio T-test, with K-ratio of 100 ($\alpha \approx 0.05$).

Table 1. Composition of the diluents used in preparation of sample set 1 for organoleptic analyses

	Components in the diluents[a]				
Treatment	Mineral salts[b]	DHA[c]	Lactose[d]	Acetate[c]	Amino acids[e]
1	●	●	●	●	●
2	●	●	●	●	○
3	●	●	●	○	○
4	●	●	○	○	○
5	●	○	●	○	○
6	●	○	○	●	○
7	●	○	○	○	○
8	Whey Permeate only				
9[f]	●	●	●	●	●

[a] ● Component is present in the treatment; ○ Component is not present in the treatment.

[b] The mineral salts used and their concentrations are listed in Table 4.

[c] Amounts of these components were calculated so that the final concentration of the component in diluent would be equal to that supplied by the same volume of whey permeate. Concentrations of the individual components in permeate are listed in Table 6.

[d] Amount of lactose added was equal to the amount supplied by permeate in rentetate-permeate mixture of treatment 2.

[e] Stock solution supplying amino acids in the concentrations listed in Table 5 was used.

[f] The treatment was not fermented prior to heat coagulation.

Table 2. Composition of the diluents used in preparation of sample set 2 for organoleptic analyses

Treatment	Components in the diluents[a]				
	Mineral salts[b]	DHA[c]	Lactose[d]	Acetate[c]	Lactic acid[e]
1	●	○	○	○	○
2	Whey Permeate only				
3	Distilled Sterile water only				
4	●	●	●	●	●
5	●	○	●	●	●

[a] ● Component is present in the treatment; ○ Component is not present in the treatment.

[b] The mineral salts used and their concentrations are listed in Table 4.

[c] Amounts of these components were calculated so that the final concentration of the component in diluent would be equal to that supplied by the same volume of whey permeate. Concentrations of the individual components in permeate are listed in Table 6.

[d] Amount of lactose added was equal to the amount supplied by permeate in retentate-permeate mixture of treatment 2.

[e] Aqueous solution (85%) of lactic acid was added after fermentation so that the total percent lactic acid in the treatment would be equal to the amount of lactic acid in retentate-permeate mixture of treatment 2.

RESULTS AND DISCUSSION

Composition

General Composition

The proximate compositions of whey, retentate, and permeate used in the study are shown in Table 3. Approximately 400 lbs of acid whey were concentrated to about 20 lbs of retentate, giving a 95% volume reduction.

The composition of the whey used in this study was similar to that used by McDonough et al. (62), except for a slightly higher mineral content. The total volume reduction was 5% greater in our study. However, McDonough et al. (62) reported 22.36% total solids in the retentate after 90% volume reduction, compared to 13.55% total solids obtained after 95% volume reduction in our study. A major portion of the total solids of the retentate in their study was lactose. Retentate obtained in our study contained a higher proportion of protein.

Table 3. Proximate composition of acid whey, retentate, and permeate. The values shown are mean values of three replications

Component	Whey		Retentate		Permeate	
	X	SD	X	SD	X	SD
	Concentration (%)					
Total Solids	6.81	0.22	13.55	3.75	6.22	0.05
Protein	0.97	0.10	11.63	2.45	0.39	0.05
Lactose	4.79	0.79	2.72	0.94	4.89	0.17
Ash	0.77	0.03	0.31	0.14	0.76	0.05

Table 3 illustrates that the process of ultrafiltration/diafiltration resulted in approximately twelve-fold concentration of the whey proteins. The process increased the ratio of protein to lactose from 0.2 in whey to 4.28 in retentate. McDonough et al. (62) reported that when cottage cheese whey was concentrated by ultrafiltration to 10% of the initial volume (90% volume reduction), 78% of the lactose was removed. In our study, 95% volume reduction resulted in removal of 97% of the initial lactose. This relatively high proportion of lactose removal can be attributed to diafiltration.

It is known that a majority of milk salts exists in ionic form (25). These salts would, therefore, freely permeate through the ultrafiltration membrane. Table 3 shows that the ash content of retentate is considerably lower than that of permeate. The ash to protein ratio of 0.79 in whey is reduced to 0.03 in retentate after ultrafiltration/diafiltration. Compared to the results of McDonough et al. (62), the retentate obtained in this study had a lower concentration of minerals as a result of diafiltration.

Mineral Components of Permeate

During the initial trial studies, it was noted that the flavor and mouthfeel of heat-coagulated retentate samples improved considerably when ashed permeate was added to the sample before fermentation. This observation lead us to believe that the mineral components in the permeate had a significant role in development of desirable flavor and texture.

Researchers (49, 94) have shown that although some minerals are bound to proteins in milk, a major portion of the milk minerals is released into whey during cheesemaking. This is especially true for acid whey (25), in which more calcium and phosphorus salts are present than in sweet whey. During ultrafiltration and diafiltration of whey, most of these minerals are partitioned into permeate. Mineral composition of acid whey

permeate is not available in the literature at this time, but is expected to be very similar to that of the permeate of milk. Therefore, minerals in skim milk permeate listed by other researchers were selected for this study (12, 19, 39). Salts of minerals identified in milk and milk permeate were added to whey retentate in various combinations during dilution. Taste panel members screened these combinations, identifying the salt combinations that improved the flavor of retentate over that of retentate-water mixture. After all combinations of salts were screened, a list of salts having a positive influence on flavor was prepared. The final amounts of salts to be used to dilute retentate in this study (Table 4) were determined based on the formulation of milk salt solution reported by Jenness and Koops (45).

Table 4. Composition of mineral solution that simulates the mineral profile of permeate. This solution was used as a diluent in preparation of samples for flavor analysis

	Amount	Ionic Contribution							
Compound	(g/l)	Na	K	Ca	P	Cl	SO_4	CO_2	Citrate
					(m moles)				
KH_2PO_4	1.58		11.60		11.60				
$K_3Citr{\cdot}H_2O$	1.02		9.39						3.13
$Na_3Citr{\cdot}2H_2O$	2.12	21.62							7.21
K_2SO_4	0.18		2.07				1.03		
$CaCl_2{\cdot}2H_2O$	1.32			8.98		17.95			
K_2CO_3	0.30		4.34					2.17	
KCl	1.08		14.45			14.45			
TOTAL	7.60	21.62	41.85	8.98	11.60	32.40	1.03	2.17	10.34

The major difference between the mineral solution used in this study and the one suggested by Jenness and Koops (45) is the absence of magnesium in our formulation. Initial tasting of samples indicated that neither magnesium chloride nor magnesium citrate had a favorable effect on the flavor of fermented retentate and therefore these salts were not included in the formulation. It has been shown that acid whey contains significantly more magnesium than does sweet whey (90, 94). It has also been shown that magnesium is retained to a greater degree during ultrafiltration/diafiltration if the pH of whey is above the isoelectric point of whey proteins (39). Because the pH of whey used in this study was around 4.5, it can be assumed that a major portion of magnesium was present in the retentate. The amount of magnesium retained was probably sufficient to support, even after dilution, its need in microbial metabolism. Further addition of magnesium salts, therefore, did not improve the flavor of fermented retentate.

Amino Acid Profile of Permeate

The results of free amino acid analysis of whey permeate are shown in Table 5. The values for the concentrations of amino acids in whey are comparable to those reported for skim milk (30, 36), with a few exceptions. Proline in milk is reported to range from 2.5 to 5.4 mg/l, but 5.56 mg/l proline in whey permeate were found in the current study. Similarly, tyrosine in milk has been reported from 0.1 to 0.5 mg/l, but 3.53 mg/l were found in whey permeate. In general, the concentrations of amino acids shown in Table 5 are in the lower range of those reported as normal for milk. This phenomenon was expected since studies have shown that amino acids are retained during ultrafiltration (26, 60).

Each of the amino acids, in the concentrations shown in Table 5, was tested for its effect on flavor development in fermented retentate. After screening various combina-

Table 5. Free amino acid composition of whey permeate

Amino Acid	Concentration (mg/l)
Alanine	0.08
Arginine	2.53
Aspartic acid	1.37
Glutamic acid	9.05
Glycine	0.55
Histidine	2.31
Isoleucine	1.08
Leucine	1.48
Lysine	3.83
Methionine	1.16
Phenylalanine	1.92
Proline	5.56
Serine	0.85
Threonine	0.96
Tyrosine	3.53
Valine	1.15

tions of the amino acids, the panelists concurred that the effect of amino acids on flavor was subtle and that amino acids seemed to have an effect on flavor only when other components such as minerals were present in the diluent. Specifically, four amino acids, glutamic acid, proline, tyrosine, and lysine, seemed to have a positive effect on the flavor. Because the effect of the rest of the amino acids was very subtle, it was decided that diluents containing all the amino acids in the concentrations shown in Table 5 would be used in further analyses.

Other Organic Components in Permeate

Organic components that were detected in whey permeate and seemed to have a favorable effect on flavor development are listed in Table 6. Gas chromatographic analysis of permeate for fatty acids showed high proportions of acetic and butyric acids: acetic

acid was detected at 474.5 mg/l, and butyric acid was found at 134.6 mg/l. When fermented retentate preparations to which these acids were added singly were tasted, only acetic acid seemed to improve the flavor. Butyric acid had a negative effect on flavor development.

Table 6. Organic components in whey permeate identified as having a positive influence on flavor development in retentate

Component	Concentration (mg/l)
Dihydroxyacetone	8.7
Acetic acid	474.5
Amino acids	37.41
Glutamic acid	9.05
Proline	5.56
Tyrosine	3.53
Lysine	3.83

Dihydroxyacetone was detected in whey permeate at 8.7 mg/l. Detection of dihydroxyacetone in commercially available skim milk posed problems because the higher concentration of protein in milk interfered with the assay. The amount of dihydroxyacetone detected in milk samples varied between 8.5 and 14.6 mg/l, with an average value of 10.2 mg/l. Permeate from ultrafiltration of skim milk contained 9.3 mg/l dihydroxyacetone. A major portion of this compound permeated through the membrane during ultrafiltration. Dihydroxyacetone could not be detected in whey retentate. It was probably below the limit of detection of the assay.

Production of Carbonyl Compounds

The amounts of glyoxal, methylglyoxal, and diacetyl detected in permeate, retentate and some samples used in flavor analyses are shown in Table 7. It can be seen that the amounts of glyoxal and methylglyoxal increased considerably in samples after fermentation. These increases might result from metabolism of some precursor compounds by the starter culture. Glyoxal, methylglyoxal, and diacetyl have been shown to contribute to the flavor profile of cultured dairy products (20, 32, 34).

Table 7. Carbonyl compounds detected in acid whey permeate, retentate, and their mixtures. Values are means of four replications

Sample	Glyoxal		Methylglyoxal		Diacetyl	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
	(μg/ml)					
Pure permeate	3.18	1.03	1.99	0.56	3.82	1.34
Pure retentate	1.03	0.15	0.98	0.19	1.38	0.19
Retentate+Permeate[a] (28% + 72%)	2.79	1.14	2.40	0.41	2.42	0.43
Retentate+Permeate[b] (28% + 72%)	4.60	1.39	5.40	2.58	2.74	0.90
Retentate+Water[a] (30% + 70%)	0.77	0.06	0.65	0.06	1.13	0.78
Retentate+Water[b] (30% + 70%)	3.16	0.80	3.77	1.07	1.60	0.39

[a]The samples were not fermented.
[b]The samples were fermented.

Various possible precursor compounds were tested for their effect on production of glyoxal, methylglyoxal, and diacetyl in diluted retentate samples. The results are

shown in Table 8 and Figure 3. Relatively high amounts of glyoxal, methylglyoxal, and diacetyl were detected in retentate samples diluted with permeate (treatment 1). Only the diluents that contained dihydroxyacetone (treatments 8 and 10) allowed production of significant amounts of methylglyoxal and glyoxal. However, when these samples were tasted during the screening process, panelists did not detect great improvements in flavor.

Bednarski et al. (8) found that addition of dihydroxyacetone to lactic culture media increased the amounts of glyoxal and methylglyoxal after fermentation. Researchers (32, 54) have shown that while glyoxal and methylglyoxal may not directly contribute to aroma of cheese, they may react with free amino acids to produce flavor compounds. These observations may explain the relative abundance of these carbonyl compounds in treatments 8 and 10 and the reduced amount of glyoxal in treatment 10. The dihydroxyacetone added to treatment 8 may have been converted to glyoxal and methylglyoxal, but the threonine added to treatment 10 may have reacted only with glyoxal. Treatment 1, which contains free amino acids contributed by permeate, contained less methylglyoxal and glyoxal than did treatment 8.

Unlike glyoxal and methylglyoxal, diacetyl is known to be an aromatic compound that contributes to the flavor of fermented dairy products (34). *Leuconostoc dextranicum* is known to produce diacetyl (28). The possible precursor compounds used in treatments 2–10 did not have a significant effect on the production of diacetyl in the fermented samples. Retentate preparation containing permeate (treatment 1) had the highest concentration of diacetyl. A portion of this diacetyl may have been contributed by the permeate.

Growth of Cultures

Acid production by the mixed culture containing *Streptococcus lactis* and *Leuconostoc dextranicum* in samples of retentate diluted with permeate, mineral solution, or

Table 8. Possible precursor compounds used in the diluents of whey retentate and their effect on concentration of glyoxal, methylglyoxal, and diacetyl in the samples after fermentation. Values are means of three replications

Treatment No.	Diluent	Glyoxal $\bar{X}$	Glyoxal SD	Methylglyoxal $\bar{X}$	Methylglyoxal SD	Diacetyl $\bar{X}$	Diacetyl SD
		(μg/ml)					
1.	Permeate	4.60	1.39	5.40	2.58	2.74	0.90
2.	Mineral Solution[a]	1.52	0.43	1.63	0.11	1.12	0.35
3.	Mineral Solution + Ethanol[b]	1.56	0.19	1.96	0.40	1.70	0.19
4.	Mineral Solution + Glycine[b]	1.50	0.03	2.04	0.09	1.53	0.0
5.	Mineral Solution + Serine[b]	1.26	0.26	1.63	0.25	0.78	0.62
6.	Mineral Solution + Threonine[b]	1.99	0.06	1.95	0.18	1.44	0.14
7.	Mineral Solution + Acetone[b]	2.11	0.06	2.03	0.05	1.42	0.20
8.	Mineral Solution + Dihydroxyacetone[b]	6.92	1.93	7.57	1.02	2.37	0.45
9.	Mineral Solution + Rhamnose[b]	2.21	0.04	1.75	0.05	1.33	0.05
10.	Mineral Solution + Threonine[b] + Dihydroxyacetone[b]	3.77	2.25	7.30	0.71	1.82	0.82

[a]Composition of the mineral solution is shown in Table 4.

[b]Concentration of the component in the final mixture before fermentation was about 100 x the concentration of methylglyoxal detected in retentate-permeate mixture.

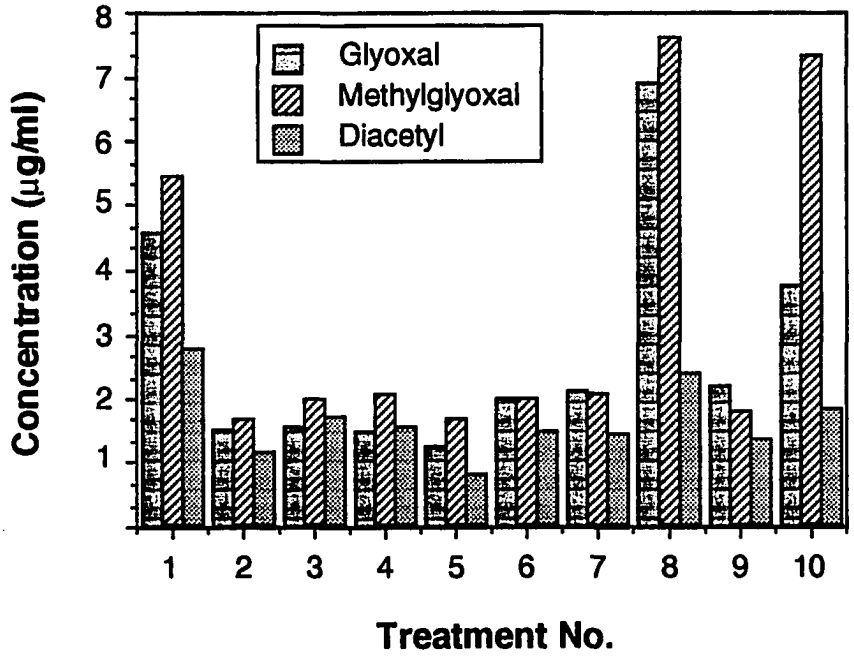

Figure 3. Production of glyoxal, methylglyoxal, and diacetyl in retentate samples containing various possible precursor compounds. The compositions of the treatments are listed in Table 8

distilled water is shown in Table 9 and Figure 4. As can be seen from Figure 4, the decrease of sample pH was relatively rapid through about 8 h of incubation. Retentate diluted with distilled water was at the lowest pH at the end of incubation. The samples diluted with permeate or mineral solution were at the same final pH. When samples at the same pH were tested for percent lactic acid by titration with a standard base, samples diluted with permeate or mineral solution were found to contain more lactic acid than samples diluted with distilled water. This suggests that the mineral salts present in permeate or mineral solution might be providing partial buffering capacity to the samples.

Table 9. pH profile of retentate samples diluted with permeate, mineral solution, or distilled water and fermented with a mixed culture of 1% *Streptococcus lactis* and 1% *Leuconostoc dextranicum*

Time (h)	Permeate	Mineral Sol.	Distilled Water
		(pH)	
0.0	5.98	5.92	5.91
3.8	5.75	5.65	5.55
3.9	5.75	5.75	5.65
5.3	5.85	5.55	5.45
5.8	5.65	5.38	5.43
7.8	5.65	4.75	5.35
8.8	4.85	5.05	4.85
9.8	4.70	4.70	4.50
10.8	4.60	4.70	4.40
13.3	4.50	4.50	4.20

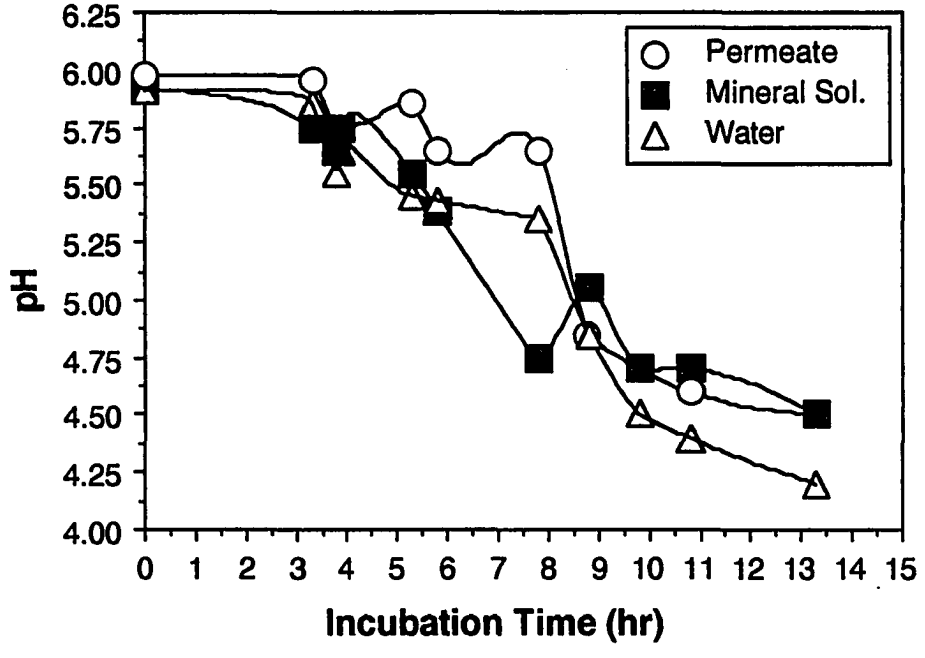

Figure 4. pH profile of retentate samples diluted with permeate, mineral solution, or distilled water and fermented with a mixed culture of 1% *Streptococcus lactis* and 1% *Leuconostoc dextranicum*

Growth of the mixed cultures in retentate preparations was reasonably consistent. Colony counts of *Leuconostoc dextranicum* are presented in Table 10 and Figure 5. Growth of *Streptococcus lactis* is shown in Table 11 and Figure 6. Numbers of *Leuconostoc dextranicum* increased steadily by about 2 log units during the incubation period. Numbers of *Streptococcus lactis* increased by less than 1 log unit by the end of incubation. The final counts of *Streptococcus* cells were lower than the counts of *Leuconostoc* cells in samples prepared with permeate or mineral solution. Species of *Streptococcus* are known to be slightly less acid-tolerant than are lactobacilli (87). The low final pH values of the samples may have inhibited the growth of *Streptococcus lactis*. The major contributor to acid production in fermentation seemed to be *Leuconostoc dextranicum*.

Table 12 and Figure 7 show the pH profiles of samples fermented with *Leuconostoc dextranicum* only. In contrast to the pH profiles of the mixed cultures (Figure 4), the pH decline is more linear and stops at a higher final pH. Enhanced acid production by mixed cultures over that produced by single cultures is typical of starter culture interaction. Typically, retentate-permeate mixtures fermented with the single culture required 18-20 h to reach pH 4.5.

Growth of *Leuconostoc dextranicum* as a single culture in retentate diluted with permeate, mineral solution, or distilled water is shown in Table 13 and Figure 8. Growth in the sample containing permeate was slower and the final cell numbers obtained were significantly lower than in the other samples ($P<0.01$). Reasons for this retarded growth could not be found. Better growth of *Leuconostoc dextranicum* in mixed culture (Figure 5) may have been due to a symbiotic interaction with the *Streptococcus* culture.

Table 10. Growth of *Leuconostoc dextranicum* in mixed culture grown in retentate diluted with permeate, mineral solution, or distilled water

Time (h)	Permeate	Mineral Sol.	Distilled Water
	Log (CFU/ml)		
0.0	6.1	5.9	6.48
3.3	6.2	N.A.[a]	N.A.
3.8	6.7	N.A.	6.7
5.8	6.7	N.A.	6.7
7.8	7.23	7.3	7.23
8.8	7.41	7.57	7.7
10.8	7.68	8.11	8.11
13.3	8.0	7.53	8.38

[a]Not available.

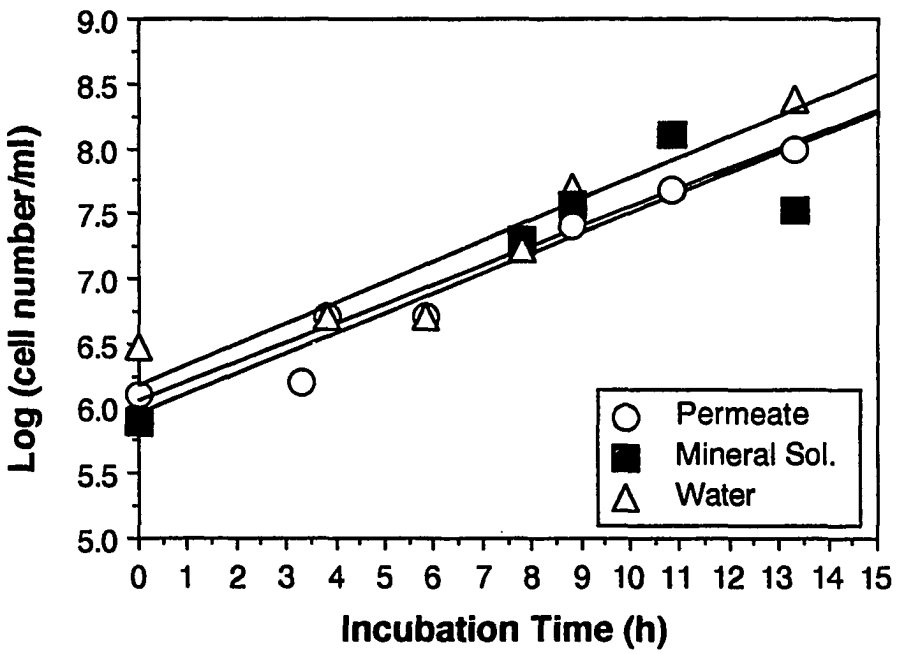

Figure 5. Growth of *Leuconostoc dextranicum* in mixed culture grown in retentate diluted with permeate, mineral solution, or distilled water

Table 11. Growth of *Streptococcus lactis* in mixed culture grown in retentate diluted with permeate, mineral solution, or distilled water

Time (h)	Permeate	Mineral Sol.	Distilled Water
		Log (CFU/ml)	
0.0	6.58	6.87	6.79
3.3	6.49	6.72	6.58
3.8	7.12	7.51	7.11
5.3	6.72	6.75	6.75
5.8	7.26	7.18	6.94
7.8	7.23	7.30	7.23
8.8	7.92	7.23	7.58
10.8	7.86	7.36	8.08
13.3	7.48	7.56	7.48

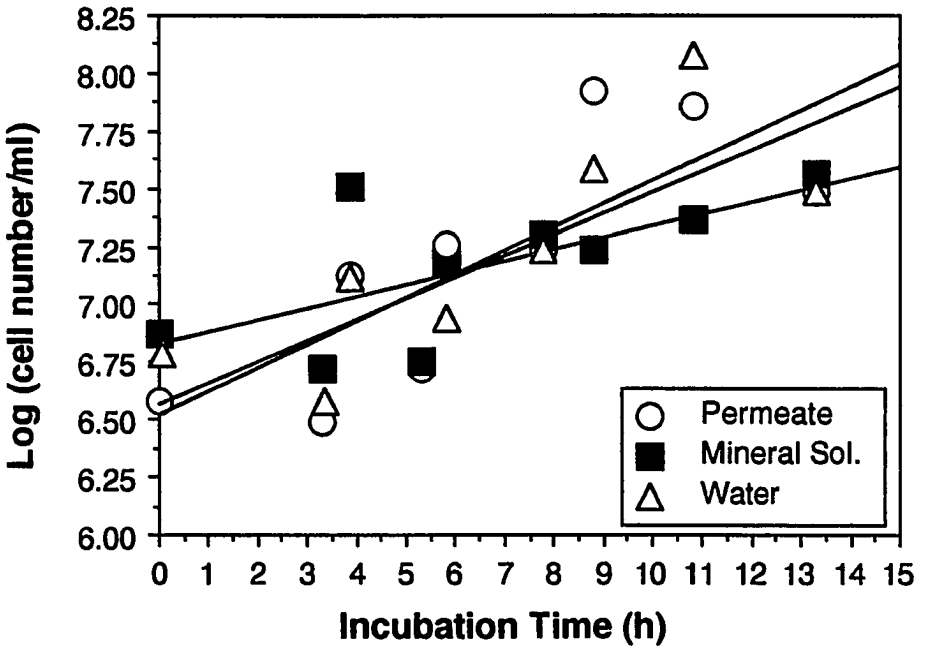

Figure 6. Growth of *Streptococcus lactis* in mixed culture grown in retentate diluted with permeate, mineral solution, or distilled water

Table 12. pH profile of pure culture of *Leuconostoc dextranicum* grown in retentate diluted with permeate, mineral solution, or distilled water

Time (h)	Permeate	Mineral Sol.	Distilled Water
		(pH)	
0	5.93	5.98	5.93
4	5.75	5.72	5.60
6	5.68	5.57	5.40
8	5.58	5.38	5.13
10	5.45	5.15	4.93
12	5.33	5.00	4.75

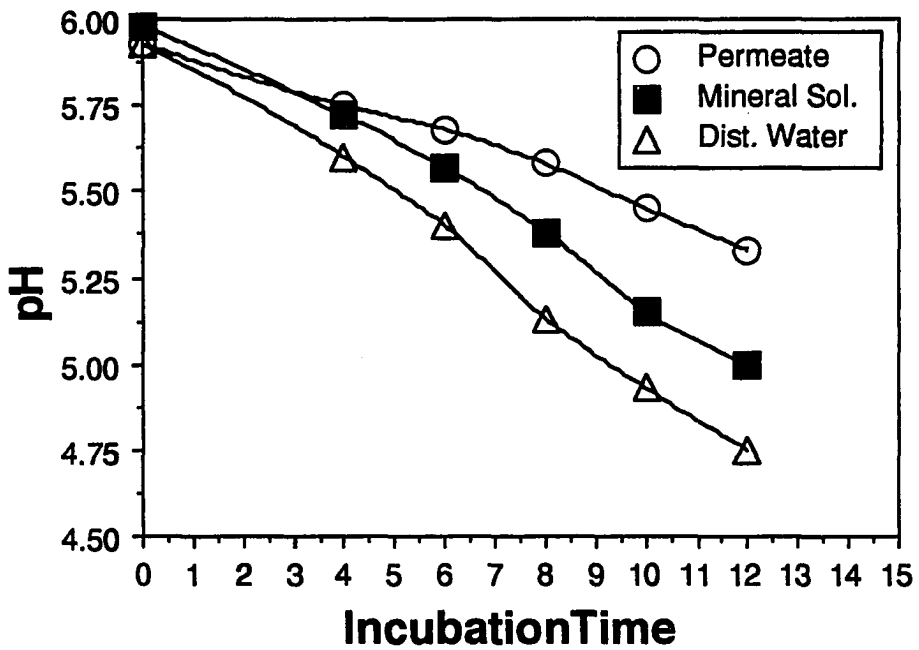

Figure 7. pH profile of pure culture of *Leuconostoc dextranicum* grown in retentate diluted with permeate, mineral solution, or distilled water

Table 13. Growth of *Leuconostoc dextranicum* as pure culture grown in retentate diluted with permeate, mineral solution, or distilled water

Time (h)	Permeate	Mineral Sol.	Distilled Water
		Log (CFU/ml)	
0	6.85	6.96	7.04
4	7.24	7.43	7.40
6	7.39	7.90	8.23
8	7.55	8.04	8.10
10	7.72	8.30	8.25
12	7.69	8.24	8.43

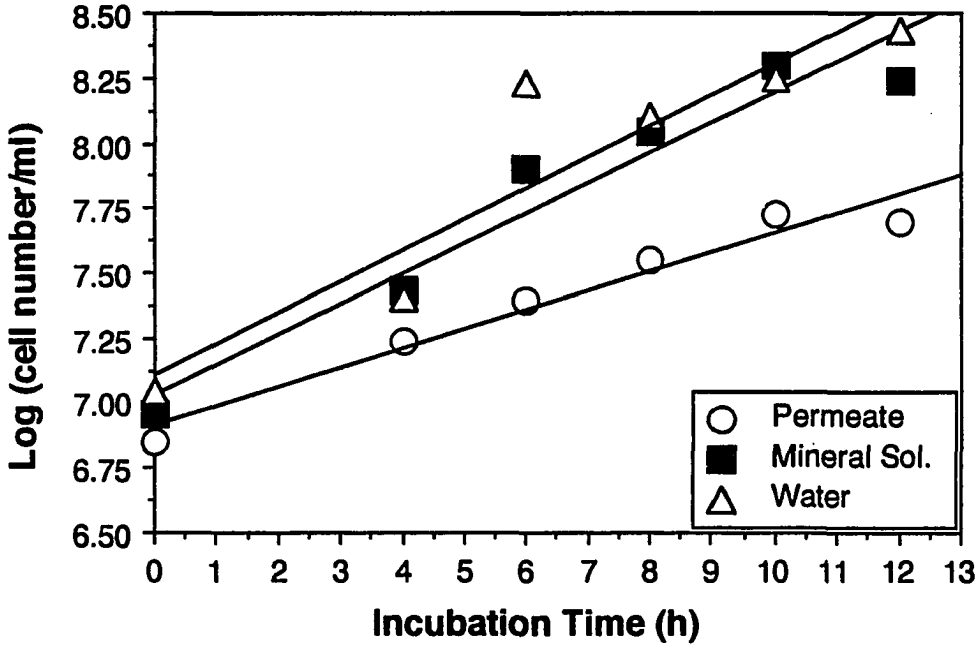

Figure 8. Growth of *Leuconostoc dextranicum* as pure culture grown in retentate diluted with permeate, mineral solution, or distilled water

Although comparison of Figures 4 and 7 shows that acid production in samples inoculated with *Leuconostoc dextranicum* alone was slower, the panelists agreed during the initial trial period that the flavor profile of these samples was similar to those inoculated with mixed culture, once the final pH reached 4.5. Because it was easier to obtain consistent growth with a single culture, for the rest of this study samples were fermented with 18 h-old pure cultures of *Leuconostoc dextranicum.*

Heat Gelation of Protein Mixes

The results of heat coagulation of pH-adjusted retentate samples are shown in Table 14 and Figure 9. The dial readings of the viscometer were used as indicators of relative gel strength. As pH decreased below 5.0, heating temperature had a greater effect on gel strength. The greatest gel strength was obtained by heating the protein mixes at 90°C for 10 min.

Hillier et al. (40) found that ß-lg A was more heat-tolerant below 90°C and at pH 6.0. The heat tolerance decreased at pH 4.0. Contrary to these findings, De Rham and Chanton (22) reported that the insolubilization of whey proteins in ultrafiltrate concentrate did not change between pH 4.5 and 6.0, but increased from pH 7.2 to 6.0. Because proteins must be in a denatured state and insoluble for proper gel formation, the current observation of stronger gels produced at lower pH values and higher heating temperatures agrees with the findings of Hillier et al. (40), but disagrees with the findings of De Rham and Chanton (22). It should be noted that De Rham and Chanton (22) heat-treated the whey protein concentrate at 95°C for 5 min, but 90°C for 10 min heat treatment was used in this study.

Table 14. Dial readings of Brookfield-Helipath viscometer (Model LVF) equipped with a T-bar spindle. All samples were cooled to 25°C before measuring the curd strength. Values are means of three replications

pH	Heat gelation temperature 70°C	80°C	90°C
	(Dial Readings)		
3.5	6.5	9.5	17.5
4.0	6.0	9.0	15.75
4.5	5.5	8.0	11.5
5.0	5.4	6.25	7.25
5.5	5.2	6.2	6.0
6.0	4.8	5.5	5.25

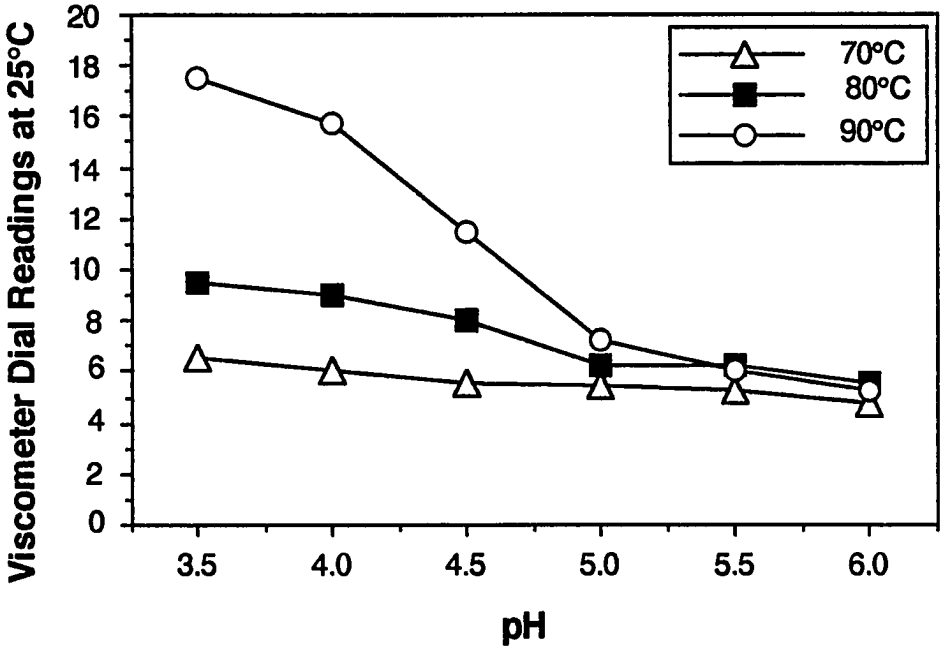

Figure 9. Heat gelation profile of pH-adjusted whey protein concentrates at three temperatures, as measured by Brookfield-Helipath Viscometer (Model LVF) equipped with T-bar spindle. All readings were taken after the samples were cooled to 25°C

Figure 9 shows that protein gels with the greatest gel strength were obtained at pH 3.5. However, when the samples were tasted during the initial trial period, the acid flavor at this pH was too intense to detect any other flavors developed during fermentation. The panel members agreed that the lowest pH acceptable for tasting was 4.5. As a result, all samples prepared for flavor analysis were fermented to a final pH of 4.5 and heat-coagulated at 90°C for 10 min.

Statistical Analysis of Sensory Panel Results

Statistical Model

Statistical analysis was performed on two sets of treatments (Tables 1 and 2) to substantiate the findings from the preliminary screening of components influencing flavor. The taste panel scores were first analyzed for variance to see if the treatment mean differences were statistically significant. The treatment means were compared to examine differences. The treatments were then contrasted to analyze the effect of specific components in the diluents on the flavor characteristics of the product and to answer specific questions posed by individual contrasts.

Whereas comparison of treatment means highlights the similarities or differences between all possible pairs of treatment means, a properly set up contrast analyzes the significance of individual factors in a set of treatments to the observed response. In setting up a contrast, a set of treatments related to the effect being tested is selected and assigned coefficients of either negative or positive values. The sum of the coefficients is always zero. The hypothesis that the difference between the average values of the treatment scores assigned positive or negative coefficients is zero is tested by an F test.

An example of the expected mean squares for the first set of treatments (Table 1) is shown in Table 15. The table shows the sources of variance and the corresponding

Table 15. An example of the analysis of variance table showing the sources of variation and the expected mean squares

Source	Degrees of Freedom	Expected Mean Squares
TREATMENT	(t-1) = 8	$\sigma_b^2 + s\,\sigma_a^2 + rs\,\sigma_T^2$
REP(TRT)	t x (r-1) = 18	$\sigma_b^2 + s\,\sigma_a^2$
JUDGE	(s-1) = 11	$\sigma_b^2 + tr\,\sigma_J^2$
TRT*JUD	(t-1) x (s-1) = 88	$\sigma_b^2 + r\,\sigma_{TJ}^2$
Residual Error	t x (r-1) x (s-1) = 198	σ_b^2

expected mean squares for the set containing 9 treatments (t) replicated 3 times (r) and having 12 judges (s). It can be seen from this table that to test for the significance of the treatment variance ($rs\,\sigma_T^2$), the denominator should be the variance of treatments within a replication ($\sigma_b^2 + s\,\sigma_a^2$) and not the residual (experimental) error. If the variance of treatments within a replication is not significant, then it is very unlikely that the variance due to treatment differences would be significant. To check for the validity of using REP(TRT) as an error term, an F test is performed using the residual error as the denominator.

Analysis of Treatment Set 1

The ANOVA table for treatment set 1 is shown in Table 16. The means of these treatments are compared in Table 17. The treatment effects on acidity, sweetness, and complexity were significant at the probability level of 0.001. Scores for the flavor note astringency were affected by treatment differences at 0.05 probability level.

Comparison of treatment means shows that the degree of acidity was highest in

Table 16. Table of ANOVA for treatment set 1

Response Variable	Source	df	Sum of Squares	F
Acidity	TRT	8	191.49	9.98***
	REP(TRT)	18	43.17	
Astringency	TRT	8	51.32	3.45*
	REP(TRT)	18	33.44	
Sweetness	TRT	8	217.01	7.09***
	REP(TRT)	18	68.83	
Complexity	TRT	8	126.02	7.70***
	REP(TRT)	18	36.83	

* Significant at 0.05 level.

*** Significant at 0.001 level.

Table 17. Means comparisons of treatments in set 1. The treatments are diluents used to dilute samples of retentate. Values are means of 36 observations per treatment

Treatment	Acidity $\bar{X}$	Astringency $\bar{X}$	Sweetness $\bar{X}$	Complexity $\bar{X}$
	(Taste Panel Score)			
1. Mineral Sol. + DHA + Lactose + Acetate + Amino acids	$3.47^{a,b}$	$1.86^{b,c,d}$	$3.83^{a,b,c}$	$3.50^{b,c}$
2. Mineral Sol. + DHA + Lactose + Acetate	$3.58^{a,b}$	$1.75^{c,d}$	$4.19^{a,b}$	$3.94^{a,b}$
3. Mineral Sol. + DHA + Lactose	2.25^{c}	$2.25^{a,b,c,d}$	$3.86^{a,b,c}$	$3.14^{c,d,e}$
4. Mineral Sol. + DHA	$2.89^{b,c}$	$2.36^{a,b,d}$	2.25^{e}	2.61^{e}
5. Mineral Sol. + Lactose	2.19^{c}	1.53^{c}	4.75^{a}	$3.42^{b,c,d}$
6. Mineral Sol. + Acetate	3.81^{a}	$2.08^{a,b,c,d}$	$2.75^{d,e}$	$3.14^{c,d,e}$
7. Mineral Solution	$2.89^{b,c}$	$2.55^{a,b}$	2.30^{e}	2.58^{e}
8. Permeate	4.75^{d}	$1.75^{c,d}$	$3.67^{b,c,d}$	4.61^{a}
9. Mineral Sol. + DHA + Lactose + Acetate + Amino acids[1]	2.72^{c}	2.80^{a}	$3.0^{c,d,e}$	$2.78^{d,e}$
ESTIMATE OF STANDARD ERROR[2]	1.55	1.36	1.96	1.43

[1]Treatment was not fermented.

[2]Calculated as square root of [REP(TRT) ÷ df].

[a,b,c,d,e]Values in the same column with the same superscript are not significantly different ($P<0.05$).

the retentate-permeate mixture ($P<0.05$). Treatments 1, 2, and 6, all of which contained acetic acid, were given equivalent flavor scores for acidity. Treatment samples 3, 4, 5, and 7, all of which were fermented, did not differ significantly from unfermented sample containing minerals, dihydroxyacetone, lactose, acetic acid, and amino acids (treatment 9). However, treatment 1, which had the same composition as treatment 9, but was fermented before tasting, was judged as having a higher degree of acidity.

Scores assigned to sample astringency were generally at the low range of the scale. The degree of astringency of the sample diluted with mineral solution (treatment 7) and the one diluted with permeate (treatment 8) differed significantly ($P<0.05$). The non-significant differences between treatments 1 and 7 and treatments 7 and 9 indicate that simultaneous addition of DHA, lactose, acetate, and amino acids does not lower the astringency without fermentation. The degree of astringency was similar in sample fermented with permeate and most other fermented samples (treatments 1–6).

All the fermented samples containing added lactose (treatments 1-3 and 5) were assigned similar scores for sweetness. Except for treatment 5, fermented samples containing lactose were judged to be similar in sweetness to the retentate preparation containing permeate (treatment 8). Treatment 5, which contained mineral solution and lactose, had the highest score for sweetness. Sweetness scores were not affected when the diluents contained mineral salts and dihydroxyacetone or acetic acid, individually.

Panel members assigned the highest score for the complexity of flavor to the retentate-permeate sample. Treatment 2, which contained retentate diluted with a mixture of mineral solution, dihydroxyacetone, lactose, and acetic acid, was judged to have flavor complexity similar to that of the sample diluted with permeate. Treatment 1, which contained amino acids in addition to all the components of treatment 2, had flavor complexity

similar to that of treatment 2 but less than that of the retentate-permeate sample. This finding was unexpected, given the anticipated interaction of DHA and amino acids to yield flavor compounds. The sample containing both DHA and amino acids was expected to develop greater complexity than the sample that contained DHA without amino acids.

To analyze further the effect of individual components of diluents on flavor notes, the treatments were contrasted. The contrasts are shown in Table 18. Contrasts 1, 2, and 3 test the variance of main effects of treatments containing DHA and lactose, in addition to mineral salts (treatments 3, 4, 5, and 7). Contrast 1 examines the influence of DHA on the flavor scores when lactose is present in the diluent along with mineral salts. Contrast 2, a reciprocal of contrast 1, tests the effect of lactose on flavor scores as related to presence of DHA. Contrast 3 examines the interaction of DHA and lactose and the effect of their interaction, if any, on the flavor scores.

Contrasts 4, 5, and 6 test the effects of acetic acid on flavor scores as related to concurrent presence of DHA, lactose, and minerals. Contrast 4 tests the variance in responses due to presence of acetic acid in the diluent containing DHA, lactose, and mineral salts. Contrast 5 examines the response of flavor notes to presence of DHA and lactose at the same time. Contrast 6 tests the interaction between acetic acid and DHA-lactose combinations in the diluents.

The effect of fermentation on flavor profile of samples is tested by comparing two samples of similar composition (treatments 1 and 9) in contrast 7. The variations in flavor scores as affected by amino acids is examined by contrast 8. The general effect of "synthetic" diluents against permeate is examined in contrast 9. Sample diluted with permeate is compared with sample diluted with mineral solution in contrast 10.

Table 18. Statistical treatment contrasts for treatment set 1. The treatments are diluents used to dilute retentate

	Treatment	Contrast [a]									
		1	2	3	4	5	6	7	8	9	10
1.	Minerals + DHA + Lactose + Acetate + Amino acids							●	●	○	
2.	Minerals + DHA + Lactose + Acetate				●	●	●		○	○	
3.	Minerals + DHA + Lactose	●	●	●	○	●	○			○	
4.	Minerals + DHA	●	○	○						○	
5.	Minerals + Lactose	○	●	○						○	
6.	Minerals + Acetate				●	○	○			○	
7.	Mineral solution	○	○	●	○	○	●			○	○
8.	Permeate									●	●
9.	Minerals + DHA + Lactose + Acetate + Amino acids [b]							○			

[a] Means of treatments containing the components (●) were compared against those without the components (○).

[b] The treatment was not fermented.

The results of analysis of variance on treatment contrasts are shown in Table 19. The results are also shown in a more meaningful form in Table 20. Acidity scores were influenced by the presence of lactose and acetic acid. The contrasts show that flavor scores for acidity were not affected by the presence of DHA (contrast 1) or amino acids (contrast 8). Although the effect of DHA-lactose interaction was not significant on scores for acidity, the contrasts indicate that lactose alone affected acidity scores ($P<0.05$). Presence of acetic acid in the diluents influenced the acidity scores to a greater degree than presence of lactose ($P<0.001$). As expected, the acidity scores were greatly influenced by whether the diluent was natural permeate or synthetic mixtures.

Of interest is the low significance ($P<0.1$) of effect of fermentation on acidity scores (contrast 7). Comparison of treatment means (Table 17) shows that the acidity scores for treatments 1 and 9 were significantly different ($P<0.05$). However, the probability that fermentation affected the acidity scores is shown to be lower according to contrasts. A closer examination of treatment means shows that the acidity scores for treatment 9 were not significantly different from those of the treatments containing DHA (treatment 4) and lactose (treatment 5), individually, and from that of the preparation containing mineral solution alone (treatment 7). Because the mean acidity scores for treatments 4, 5, and 7 were lower than for treatment 1, it can be surmised that lactose and DHA lowered the perception of acidity.

Using mineral solution instead of permeate as diluent increased the astringency scores. This finding suggests that the low astringency scores of retentate-permeate mixture are not entirely due to the mineral salts contributed by permeate. Contrasts 2 and 4 indicate that the astringency scores were also influenced by lactose and by acetic acid in the presence of DHA-lactose. Flavor scores for sample astringency were strongly influ-

Table 19. ANOVA table for treatment contrasts for set 1

Response Variable	Source	df	Sum of Squares	F
Acidity	Contrast 1	1	0.03	0.01
	Contrast 2	1	16.00	6.67*
	Contrast 3	1	0.03	0.01
	Contrast 4	1	45.56	19.00***
	Contrast 5	1	6.67	2.78
	Contrast 6	1	1.56	0.65
	Contrast 7	1	10.12	4.22†
	Contrast 8	1	0.22	0.09
	Contrast 9	1	95.16	39.68***
	Contrast 10	1	62.35	26.00***
	REP(TRT)	18	43.17	
Astringency	Contrast 1	1	2.51	1.35
	Contrast 2	1	11.67	6.28*
	Contrast 3	1	7.56	4.07
	Contrast 4	1	8.51	4.58*
	Contrast 5	1	3.67	1.98
	Contrast 6	1	0.01	0.00
	Contrast 7	1	16.05	8.64**
	Contrast 8	1	0.22	0.12
	Contrast 9	1	2.94	1.58
	Contrast 10	1	11.68	6.29*
	REP(TRT)	18	33.44	

† Significant at 0.1 level.

* Significant at 0.05 level.

** Significant at 0.01 level.

*** Significant at 0.001 level.

Table 19. (Continued)

Response Variable	Source	df	Sum of Squares	F
Sweetness	Contrast 1	1	8.03	2.10
	Contrast 2	1	148.03	38.71***
	Contrast 3	1	6.25	1.63
	Contrast 4	1	5.44	1.42
	Contrast 5	1	81.0	21.18***
	Contrast 6	1	0.11	0.03
	Contrast 7	1	12.5	3.27
	Contrast 8	1	2.35	0.61
	Contrast 9	1	1.91	0.50
	Contrast 10	1	33.35	8.72**
	REP(TRT)	18	68.83	
Complexity	Contrast 1	1	0.56	0.27
	Contrast 2	1	16.67	8.15**
	Contrast 3	1	0.84	0.41
	Contrast 4	1	16.67	8.15**
	Contrast 5	1	16.67	8.15**
	Contrast 6	1	0.56	0.27
	Contrast 7	1	9.39	4.59*
	Contrast 8	1	3.56	1.74
	Contrast 9	1	63.57	31.07***
	Contrast 10	1	74.01	36.17***
	REP(TRT)	18	36.83	

Table 20. Summary of treatment contrasts for treatment set 1

Response Variable	Contrast	$\mu 1$[a]	$\mu 2$[b]	$\mu 1-\mu 2$	P[c]
Acidity	1. Effect of DHA	2.57	2.54	0.03	0.9155
	2. Effect of lactose	2.22	2.89	–0.67	0.0188
	3. Effect of DHA-lactose interaction	2.57	2.54	0.03	0.9155
	4. Effect of acetate	3.70	2.57	1.13	0.0004
	5. Effect of DHA/lactose when acetate is present	2.92	3.35	–0.44	0.1126
	6. Effect of acetate-DHA/lactose interaction	3.24	3.03	0.21	0.4301
	7. Effect of fermentation	3.47	2.72	0.75	0.0547
	8. Effect of amino acids	3.47	3.58	–0.11	0.7643
	9. Natural permeate vs synthetic mixtures	3.01	4.75	–1.74	0.0001
	10. Permeate vs mineral solution	2.89	4.75	–1.86	0.0001
Astringency	1. Effect of DHA	2.31	2.04	0.26	0.2606
	2. Effect of lactose	1.89	2.46	–0.57	0.0220
	3. Effect of DHA-lactose interaction	2.40	1.95	0.46	0.0588
	4. Effect of acetate	1.92	2.40	–0.49	0.0463
	5. Effect of DHA/lactose when acetate is present	2.00	2.32	–0.32	0.1767
	6. Effect of acetate-DHA/lactose interaction	2.15	2.17	–0.01	0.9519
	7. Effect of fermentation	1.86	2.80	–0.94	0.0088
	8. Effect of amino acids	1.86	1.75	0.11	0.7335
	9. Natural permeate vs synthetic mixtures	2.05	1.75	0.30	0.2244
	10. Permeate vs mineral solution	2.55	1.75	0.80	0.0220

[a]Average score of the treatments that contain the component (Table 18).

[b]Average score of the treatments that do not contain the component (Table 18).

[c]Probability that the value of $\mu 1-\mu 2=0$.

Table 20. (Continued)

Response Variable	Contrast	μ1[a]	μ2[b]	μ1–μ2	P[c]
Sweetness	1. Effect of DHA	3.06	3.53	–0.47	0.1646
	2. Effect of lactose	4.31	2.28	2.03	0.0001
	3. Effect of DHA-lactose interaction	3.08	3.50	–0.42	0.2173
	4. Effect of acetate	3.47	3.08	0.39	0.2483
	5. Effect of DHA/lactose when acetate is present	4.03	2.53	1.50	0.0002
	6. Effect of acetate-DHA/lactose interaction	3.25	3.31	–0.06	0.8666
	7. Effect of fermentation	3.83	3.00	0.83	0.0874
	8. Effect of amino acids	3.83	4.19	–0.36	0.4435
	9. Natural permeate vs synthetic mixtures	3.42	3.67	–0.25	0.4891
	10. Permeate vs mineral solution	2.30	3.67	–1.37	0.0085
Complexity	1. Effect of DHA	2.88	3.00	–0.13	0.6065
	2. Effect of lactose	3.28	2.60	0.69	0.0105
	3. Effect of DHA-lactose interaction	2.86	3.02	–0.15	0.5297
	4. Effect of acetate	3.54	2.86	0.68	0.0105
	5. Effect of DHA/lactose when acetate is present	3.54	2.86	0.68	0.0105
	6. Effect of acetate-DHA/lactose interaction	3.26	3.14	0.12	0.6065
	7. Effect of fermentation	3.50	2.78	0.72	0.0461
	8. Effect of amino acids	3.50	3.94	–0.44	0.2040
	9. Natural permeate vs synthetic mixtures	3.19	4.61	–1.42	0.0001
	10. Permeate vs mineral solution	2.58	4.61	–2.03	0.0001

enced by fermentation. Fermentation of samples of similar composition decreased the degree of astringency perceived by the judges ($P<0.01$). The effect on astringency of using either permeate or synthetic mixtures as diluents is not significant.

As expected, scores for sweetness were affected by lactose. Although presence of DHA and lactose together in the diluent did not influence the scores, this combination became significant if acetic acid was present in the diluent ($P<0.001$). The sweetness scores of the retentate-permeate sample and the samples containing other diluents were not significantly different from each other ($P<0.5$), which indicates that some of the other diluents imparted at least as much sweetness to the sample as did permeate. However, the difference between retentate-permeate and retentate-mineral solution was significant.

The overall complexity of samples was affected greatly by the type of diluent used and by fermentation. The differences between permeate and synthetic mixtures, and between permeate and mineral solution, were highly significant ($P<0.001$). Lactose and acetic acid, individually, influenced the complexity of samples. Samples were given a higher score for complexity when lactose was present in the diluent. When acetic acid was present in the diluent, the combined presence of DHA and lactose became significant. From the data available, the individual contribution of DHA to the complexity could not be determined. As expected, fermentation of the sample improved the complexity of the flavor.

Analysis of Treatment Set 2

The analysis of variance (ANOVA) for treatment set 2 is summarized in Table 21. The general variances of the response variables due to treatment differences were shown to be highly significant. The actual differences in the magnitude of responses according to the treatments are tabulated in Table 22

Table 21. Table of ANOVA for treatment set 2

Response Variable	Source	df	Sum of Squares	F
Acidity	TRT	4	225.62	19.97**
	REP(TRT)	10	28.25	
Astringency	TRT	4	101.25	13.21**
	REP(TRT)	10	19.17	
Sweetness	TRT	4	138.33	20.85**
	REP(TRT)	10	16.58	
Complexity	TRT	4	252.28	31.80**
	REP(TRT)	10	19.83	

** Significant at 0.01 level.

Table 22. Means comparisons of treatments in set 2. The treatments are diluents used to dilute samples of retentate. Values are means of 24 observations per treatment

Treatment	Acidity $\bar{X}$	Astringency $\bar{X}$	Sweetness $\bar{X}$	Complexity $\bar{X}$
	(Taste Panel Score)			
1. Mineral Sol.	2.29^{a}	2.42^{a}	2.12^{a}	2.29^{a}
2. Permeate	3.50^{b}	1.42^{b}	3.46^{b}	4.54^{b}
3. SDD Water	0.91^{c}	4.04^{c}	0.79^{c}	0.83^{c}
4. Min + DHA + Acetate + Lactose + Lact. acid	$4.38^{b,d}$	2.08^{b}	3.42^{b}	4.25^{b}
5. Min + Lactose + Acetate + Lact. acid	4.58^{d}	1.71^{b}	3.54^{b}	4.29^{b}
STANDARD ERROR[e]	1.68	1.38	1.29	1.41

[a,b,c,d] Values in the same column with the same superscript are not significantly different (P>0.05).

[e]Calculated as square-root of [REP(TRT) ÷ df].

The average scores given to samples of retentate-permeate mixture, for all the variables except astringency, were significantly higher than the scores given to retentate samples diluted with either mineral solution or distilled water. Of all the samples in this set, the degree of astringency was the lowest in retentate-permeate mixture. According to the taste panel scores, the retentate sample diluted with distilled water had the highest degree of astringency. The scores for this flavor note were not significantly different between the sample diluted with mineral solution and the ones diluted with mixtures of minerals, lactose, acetic acid, lactic acid, and dihydroxyacetone.

The panelists assigned the highest acidity score to the sample without DHA (Treatment 5). The sample that contained DHA developed the same perceived acidity as did the retentate-permeate mixture. Lactic acid was added to both synthetic mixtures to adjust their total lactate content to that of retentate-permeate mixture. Sweetness and the complexity of flavor perceived in treatments 4 and 5, which contained added lactose, were similar to the sweetness and complexity of retentate-permeate mixture. Samples prepared with water or mineral solution had lower scores in all categories.

To answer specific questions regarding the effect of components of the diluents on the flavor variables, contrasts were set up (Table 23). Contrast 1 compares the treatments that either contain or are devoid of DHA, i.e., treatments 4 and 5, respectively. This contrast examines the significance of DHA to the differences observed in flavor notes. Contrast 2 examines the contribution of minerals to the flavor characteristics of the sample. The influence of having either permeate (treatment 2) or synthetic mixture with DHA (treatment 4) is examined by contrast 3. Contrast 4 analyzes the differences between using the synthetic mixture with DHA (treatment 5) and permeate (treatment 2).

Table 23. Statistical treatment contrasts for treatment set 2. The treatments are diluents used to dilute retentate

	Treatment	Contrast[a]			
		1	2	3	4
1.	Mineral Solution		●		
2.	Permeate			◉	◉
3.	SDD Water		○		
4.	Minerals + DHA + Lactose + Acetate + Lactic acid	◉		○	
5.	Minerals + Lactose + Acetate + Lactic acid	○			○

[a]Means of treatments containing the component (◉) were compared against those not containing the component (○).

The results of analysis of variance of the treatment contrasts are listed and the significances shown in Table 24. A summary of the results is shown in Table 25. Lack of significance for the variance of contrast 1 indicates that DHA had no effect on the flavor scores of treatments 4 and 5. Presence of minerals significantly improved the scores for acidity, sweetness, and complexity, and decreased the astringency scores. This finding was in accordance with the preliminary screening which showed a marked degree of difference in astringency of retentate-permeate and retentate-water mixtures.

These results substantiated earlier findings that the minerals played a key role in flavor development.

Table 24. ANOVA table for treatment contrasts for set 2

Response Variable	Source	df	Sum of Squares	F
Acidity	Contrast 1	1	0.52	0.18
	Contrast 2	1	22.69	8.03*
	Contrast 3	1	9.19	3.25
	Contrast 4	1	14.08	4.99*
	REP(TRT)	10	28.25	
Astringency	Contrast 1	1	1.69	0.88
	Contrast 2	1	31.69	16.53**
	Contrast 3	1	5.33	2.78
	Contrast 4	1	1.02	0.53
	REP(TRT)	10	19.17	
Sweetness	Contrast 1	1	0.19	0.11
	Contrast 2	1	21.33	12.86***
	Contrast 3	1	0.02	0.01
	Contrast 4	1	0.08	0.05
	REP(TRT)	10	16.58	
Complexity	Contrast 1	1	0.02	0.01
	Contrast 2	1	25.52	2.87***
	Contrast 3	1	1.02	0.51
	Contrast 4	1	0.75	0.38
	REP(TRT)	10	19.83	

* Significant at 0.05 level.

** Significant at 0.01 level.

*** Significant at 0.001 level.

Table 25. Summary of treatment contrasts for treatment set 2

Response Variable	Contrast	μ1[a]	μ2[b]	μ1–μ2	P[c]
Acidity	1. DHA vs non-DHA samples	4.38	4.58	–0.20	0.6768
	2. Water vs mineral solution	2.29	0.91	1.38	0.0177
	3. Permeate vs DHA	3.50	4.38	–0.88	0.1015
	4. Permeate vs non-DHA	3.50	4.58	–1.08	0.0496
Astringency	1. DHA vs non-DHA samples	2.08	1.71	0.37	0.3702
	2. Water vs mineral solution	2.42	4.04	–1.62	0.0023
	3. Permeate vs DHA	1.42	2.08	–0.66	0.1263
	4. Permeate vs non-DHA	1.42	1.71	–0.29	0.4823
Sweetness	1. DHA vs non-DHA samples	3.42	3.54	–0.12	0.7436
	2. Water vs mineral solution	2.12	0.79	1.33	0.0050
	3. Permeate vs DHA	3.46	3.42	0.04	0.9130
	4. Permeate vs non-DHA	3.46	3.54	–0.08	0.8271
Complexity	1. DHA vs non-DHA samples	4.25	4.29	–0.04	0.9204
	2. Water vs mineral solution	2.29	0.83	1.46	0.0050
	3. Permeate vs DHA	4.54	4.25	0.29	0.4895
	4. Permeate vs non-DHA	4.54	4.29	0.25	0.5523

[a]Average score of the treatments that contain the component (Table 23).

[b]Average score of the treatments that do not contain the component (Table 23).

[c]Probability that the value of μ1–μ2=0.

CONCLUSIONS

Acid whey retentate was diluted to 3.5% protein with various diluents and fermented with 1% active culture of *Leuconostoc dextranicum* to a final pH of 4.5. The gel strength of heat-coagulated samples of retentate diluted with sterile distilled water was measured with a viscometer at various temperatures. The protein mix formed a relatively strong gel when heated at 90°C for 10 minutes.

The results indicate that milk minerals that are normally partitioned into permeate during ultrafiltration and diafiltration play a key role in flavor development. Preliminary screening of fermented samples showed that the major minerals such as sodium, potassium, and phosphorus have a significant role in development of flavor.

Calcium ions play a key role in aggregation and gel formation of milk proteins (22). Proper aggregation and gel formation reduces the degree of astringency associated with mouthfeel of samples. The preliminary screening of salts showed that gel-forming ability of retentate-water mixtures was not improved by addition of calcium chloride. The degree of astringency of retentate-water mixture was similar to that of retentate-water mixture containing calcium chloride. De Rham and Chanton (22) showed that the amount of calcium ions required for proper heat-induced gel formation of whey proteins is approximately constant at various pH values. Excess amounts of calcium in the mix did not improve gel formation. Studies have shown that a major portion of calcium is retained during ultrafiltration and diafiltration (12, 19). It is possible that the retentate in the current study had the minimum amount of calcium required for gel formation, but did not have other ions such as potassium and phosphorus for gel formation. The synergistic effect of the minerals listed in Table 4 helped in improving the flavor generation in the fermented retentate preparations.

During the preliminary screening of components affecting flavor, the panelists did not perceive any improvement in flavor although high amounts of glyoxal and methylglyoxal were present in the samples. It was felt that amino acids would be required in the preparations to generate aroma-bearing compounds from methylglyoxal and glyoxal. However, the results of the formal taste panels indicated that addition of amino acids along with other components did not affect the flavor scores. This discrepancy might be attributed to differences in the compositions of the diluents used in preliminary screening and formal analysis. During the preliminary screening, the components were added to the diluent either singly or in combinations of two to three. In the formal taste panels, those treatments (treatment 2, Table 17; treatment 4, Table 22) that attained scores similar to retentate-permeate preparations had several components at once in the diluents. This simultaneous presence of components might have enhanced the proteolytic ability of the starter organisms, which resulted in generation of flavor components without any added free amino acids.

Although the composition of cheese whey varies according to the manufacturing practices, the results of this study can be used to understand flavor generation during fermentation of ultrafiltered acid whey retentates with lactic acid bacteria. Mineral components that are lost into permeate during ultrafiltration play a key role in lessening of astringency of heat-induced whey protein gels. The complexity of flavor of retentate-permeate mixtures can be simulated by using DHA, acetic acid, lactose, and lactic acid in the diluents of retentate. The amount of lactate, and not the final pH, is important for flavor perception.

The results of this study may not be entirely extrapolated to fermentation of ultrafiltered milk or sweet whey, but can be used to explain the basic nature of flavor pro-

duction in those systems. Because of the presence of casein-bound minerals in milk, the mineral salt profile of the retentate will differ from that observed in the current study. However, it should be noted that the mineral profile of the retentate needs to be very similar to that of milk serum for proper flavor formation. Because some minerals will be lost to the permeate during ultrafiltration of milk, the milk retentates will have to be supplemented with some minerals before fermentation. The supplementation of minerals will be of greater importance if diafiltration is used.

Dihydroxyacetone, which is important in production of flavor precursor compounds by lactic acid bacteria, also enters the permeate during ultrafiltration of whey. It can be assumed that similar permeation will occur during ultrafiltration of milk. The sensory analysis results in this study could not be used to substantiate the extent of contribution of dihydroxyacetone to improvement of the flavor complexity of retentate preparations. However, the results suggested that DHA, in combination with other components, improved the flavor profile of the samples. Therefore the flavor of fermented milk retentates might be improved by supplementation with DHA. The presence of milk fat in whole milk or partially skimmed milk retentates will change the flavor profile of the fermented product.

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APPENDIX: POLYACRYLAMIDE GEL ELECTROPHORESIS

To determine the interactions between whey proteins after fermentation and/or heating of the retentate preparations, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used. Samples were prepared such that only the proteins that were not precipitated by centrifugal precipitation would be detected in the gels.

Sample Preparation

The retentate mixtures used were prepared as were those used in organoleptic analysis. All the samples were heat–treated at 90°C for 10 min, except for the samples that were used without heat-coagulation.

After thorough mixing, a portion (approximately 25 g) of the retentate preparation was placed in a test tube and centrifuged at 12,000 *g* for 20 min. The clear supernate was decanted into a 50 ml-beaker. To denature the proteins present in the supernate, 1.25 g crystalline trichloroacetic acid (TCA) were added to 10 ml of the supernate, and mixed thoroughly. In the case of pure permeate, 40–50 ml of supernate was used. The mixture was centrifuged at 12,000 *g* for 30 min to precipitate the denatured proteins. The pellet was collected and resuspended in acetone. This wash was repeated three times to remove residual TCA. The protein preparation was then allowed to air–dry overnight and resuspended in distilled water to obtain a final concentration of approximately 400 µg/ml.

Protein standards (α-lactalbumin, ß-lactoglobulin, immunoglobulin G, and bovine serum albumin) were obtained either in crystalline or solution form from Sigma Chemical Company (St. Louis, MO). The standards were also diluted in dis-

tilled water to obtain protein concentrations of 400 µg/ml. The standards and the protein isolates were diluted 1:4 (0.75 ml sample + 2.25 ml buffer) in pH 6.8 sample buffer (0.0625 *M* Tris-HCl, 2% SDS, 10% glycerol, 1.0% 2–mercaptoethanol, 0.002% bromophenol blue and 0.001% phenol red tracking dyes). The samples were heated in a boiling water bath for 2 min to promote a complete SDS–protein interaction.

Gel Preparation

Thickness of the gel used in the study was 1.2 mm. The compositions of the stacking and separating gels are shown in Table 26. AcrylAide™ cross-linker was obtained from FMC Corporation, Marine Colloids Division (Rockland, ME). Gel-Bond PAG plastic support film (FMC, Rockland, ME) was used to support gels that would not crack or shrink. The gels were cast in a vertical slab MiniProtein II system (BioRad Laboratories, Richmond, CA) according to the directions supplied by FMC Corporation for AcrylAide™ cross-linker.

Electrophoresis

Procedure

After setting up the MiniProtein II system with the cast gels, the buffer chambers were filled with pH 8.3 electrophoresis buffer (0.025 *M* Tris–HCl, 0.192 *M* glycine, and 0.1% SDS). The samples and standards were loaded into the wells in 50 µl volumes.

The samples were electrophoresed at 250 volts, constant voltage, until the tracking dye completely crossed the stacking gel. The current was then set to 120 volts, constant voltage, until the bromophenol blue dye crossed the lower edge of the

Table 26. Compositions of separating and stacking gels used in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whey retentate preparations

Gel Type	Acrylamide (%)	Stock Solution (ml)	Dist. Water (ml)	Buffer Solution (ml)	TEMED[a] (µl)	APS[b] (µl)
Separating	12.0	3.75[c]	3.7	2.5[d]	2.5	5.0
Stacking	5.0	5.00[e]	0.0	5.0[f]	5.0	100.0

[a]N,N,N',N'–tetramethylethylenediamine.

[b]10% ammonium persulfate solution made fresh in distilled water.

[c]33% total solids containing acrylamide and AcrylAide at a 32:1 ratio.

[d]4x Separating Gel Buffer: 1.5*M* Tris–HCl, 0.4% sodium dodecyl sulfate (SDS), pH 8.8; 181.5 g Tris adjusted to pH 8.8 with 1 *M* HCl and 4.0 g SDS, made to 1 liter with distilled water.

[e]31.75 ml of 33% acrylamide/AcrylAide cross-linker stock solution diluted to 100 ml final volume with distilled water.

[f]2x Stacking Gel Buffer: 0.25 *M* Tris–HCl, 0.2% SDS, pH 6.8; 15.137 g Tris adjusted to pH 6.8 with 1 *M* HCl and 0.1 g SDS, made to 500 ml with distilled water.

gel (approximately 45 min).

Processing of Gels

The gels were allowed to fix and stain with mild agitation in fix/stain buffer (0.05% Coomassie Brilliant Blue R-250, 25% isopropyl alcohol, 10% glacial acetic acid) for 5 h. They were then destained in a mixture of 40% isopropanol, 10% glacial acetic acid, 5% glycerol, and distilled water. Destain solution was changed 3–4 times to expedite the process.

The gels were allowed to dry overnight at room temperature. Dry gels were wrapped in plastic wrap for extended preservation.

Observations

Photographs of SDS–PAGE gels are shown in figures 10 and 11. Although it is not possible to draw extensive conclusions from these two gels, the heating conditions used in this study seemed to increase the solubility of ß–lg. A portion of the whey proteins, especially α–la, was partitioned into permeate during ultrafiltration.

The ß–lg band in lane 6 of Figure 10, which contains a sample of heat–treated retentate–permeate mixture, is darker than the corresponding band in lane 5 (unheated). Similarly, the ß–lg band is darker for heat–treated retentate–water mixture (lane 8) than for unheated retentate–water mixture (lane 7). Figure 11 shows that heat–treated samples of retentate diluted with various diluents (lanes 5 and 7) exhibit electrophoretic patterns similar to those of heat–treated retentate–permeate mixture (lane 6). Heat-treated retentate samples diluted with either permeate or synthetic mixtures, contained a portion of ß–lg in soluble form in the supernate. These samples also exhibited a lower degree of astringency compared to samples diluted with distilled water. Possibly the smoothness of the whey protein gels prepared with per-

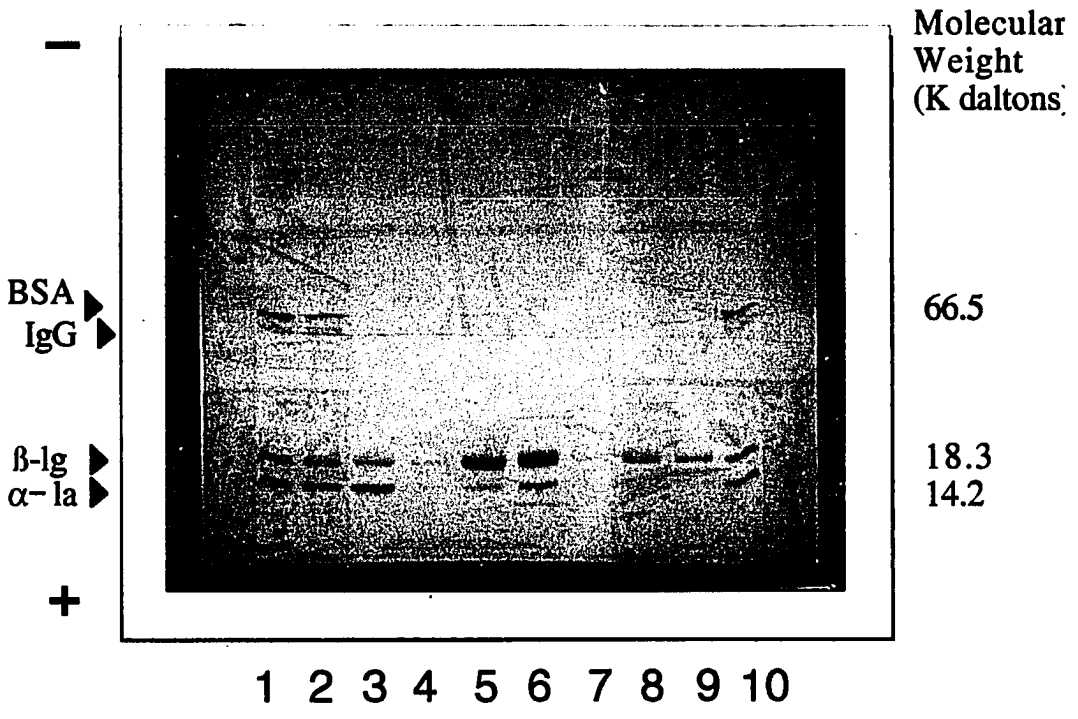

Figure 10. SDS–PAGE patterns of proteins isolated from supernates of permeate and retentate samples. Lanes 1, 2, and 10 contain protein standards. Lane 3, pure permeate (unfermented/unheated); lane 4, pure retentate (unfermented/unheated); lane 5, retentate+permeate (fermented/unheated); lane 6, retentate+permeate (fermented/heated); lane 7, retentate+water (fermented/unheated); lane 8, retentate+water (fermented/heated); lane 9, retentate+minerals+DHA+lactose+acetate+amino acids (unfermented/ heated)

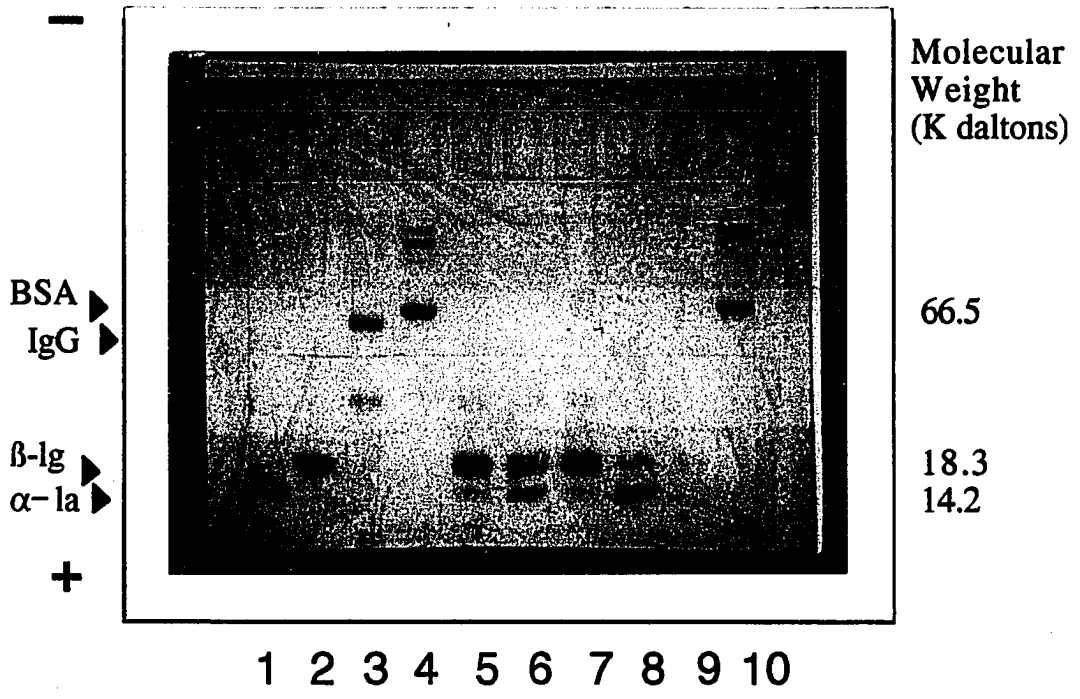

Figure 11. SDS–PAGE patterns of proteins isolated from supernates of permeate and retentate samples. Lane 1, α–lactalbumin; lane 2, ß–lactoglobulin; lane 3, immunoglobulin G; lane 4, bovine serum albumin; lane 5, retentate+ minerals+DHA+lactose+acetate+amino acids (fermented/heated); lane 6, retentate+permeate (fermented/heated); lane 7, retentate+minerals+DHA+ lactose+acetate (fermented/heated); lane 8, pure permeate (unfermented/ unheated); lane 9, pure retentate (unfermented/unheated); lane 10, bovine serum albumin

meate or synthetic mixtures may be partially related to the amount of ß–lg available in the serum portion.

Because of the pore size (30,000 MW cutoff) of the UF membranes used in this study, it can be expected that whey proteins of low molecular weight would pass through the membrane. Although the molecular weight of ß–lg monomers is about 18,000 daltons, a high proportion of this protein was not found in the permeate (Figure 10, lane 3; Figure 11, lane 8). This may be because ß–lg exists mostly in dimer or polymer form. The same samples show a relatively higher proportions of α–la in permeate than in retentate. The molecular weight of the B-variant of α–la is around 14,000 daltons. Because α–la exists mostly as a monomer, it can be expected to pass through the UF membrane used in this study.

Comparison of retentate samples diluted with synthetic mixtures (Figure 11, lanes 5 and 7) to that diluted with permeate (lane 6) shows that the latter contains a higher amount of α–la in the serum portion. This suggests that a significant portion of α–la in retentate–permeate mixtures was contributed by the permeate.