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Critical Issues Affecting the Future of Dairy Industry: Individual Contributions in the Scope of a Global Approach

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ABSTRACT

Several constraints that have been affecting the dairy industry are identified in a critical fashion, and directions are given with an emphasis on food processing implemented at the postproduction level. The rationale for modifications aimed at enhancing the appeal of condensed dairy products should be consubstantiated in strengthening of organoleptic characteristics, improvement of nutraceutical impact, and reduction of polluting power. This enumeration follows an order of increasing time scale required for consumer perception and increasing size scale associated with expected impact. Pursuance of such streamlines should lead to manufacture of dairy products that resemble nature more closely in terms of milk coagulation, milk fat modification, milk fermentation, whey fermentation, and starter culture addition. Directions for research and development anticipated as useful and effective in this endeavor, and which have been previously and consistently adopted in the development of an individual research program, are characterization and development of alternative rennets from plant sources, development of starter and nonstarter cultures from adventitious microflora, utilization of probiotic strains as starter cultures, upgrading of whey via physical or fermentation routes, and modification of milk fat via lipase-mediated interesterification reactions.
(**Key words**: milk fat, probiotics, rennet, starter)

Abbreviation key: **PNS** = pasteurized milk without addition of starter, **PWS** = pasteurized milk with addition of a commercial starter, **PPTA** = phosphotungstic acid, **R&D** = research and development.

The tendency for Man to consider little things as important has led to the development of many big things.

Georg Lichtenberg (German physicist, 1742–1799)

CRITICAL ISSUES AFFECTING THE FUTURE OF DAIRY INDUSTRY

Milk has for ages been recognized as the richest liquid food nature has ever produced owing to its high and balanced contents of protein, fat, sugar, vitamins, and mineral salts. However, utilization of milk produced by the females of a given species to feed members of another species makes a few inadequacies apparent because the requirements (from both the nutritional and organoleptic points of view) of the later species may differ considerably from those of the former species. The problems raised acquire greater complexity as the age of the consumer species progresses. The most dramatic example encompasses utilization of milk produced by ruminants as a major supplement of the diet of human adults.

To reduce the extent of inadequacies, modification of milk is often necessary. Such modification can conceptually be implemented upstream or downstream of the production process [i.e., at the preproduction level (via modification of the feeding regime of the animal or via genetic manipulation of the animal itself) or at the postproduction level (via physical or chemical modification of the components of milk)]. Such modifications are summarized in Figure 1.

Because of the herbivore nature of most commercial milk-producing species and their associated unique digestive tracts, alternative foods for ruminants are relatively limited, and excessive supplementation of such foods with additives is prone to create imbalances that would jeopardize healthy and regular growth of females. However, seasonal variations of the qualitative and quantitative composition of milk depend not only on the composition of the feed but also on the prevailing environmental conditions of temperature and humidity and the physiological state of the animal. Their interactive roles often make it difficult for one to predict the exact composition of the milk. Therefore, feed modification is not particularly attractive.

Genetic engineering is a difficult process due to the complexity of the genome of ruminants. Identification of genes that code for specific biosynthetic pathways is difficult and often demands intensive working

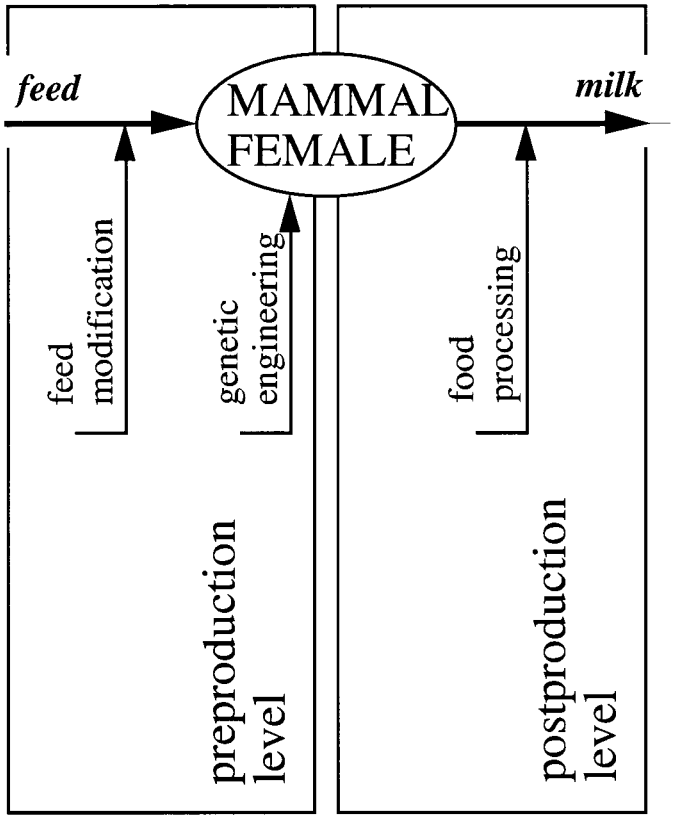

Figure 1. Strategies for modification of milk and vectors for the implementation of these strategies.

periods. Attempts to modify it are, in turn, accompanied by a great component of pure chance because of the stochastic nature of several of the genetic tools available. In addition, modification of such pathway will always interfere with other metabolic routes because of the histologically integrated nature of cell operation, so the outcome is often somewhat unpredictable. On the other hand, reproduction via mandatory sexual routes may lead to hybridization with loss of expression of modified genes. Use of inbreeding would restrict such effect but would cause degeneration of good intrinsic characteristics of the species. Finally, any such modification is a long-term effort due to the generation time (pregnancy plus growth until sexual maturity is attained), which is longer for larger ruminants. Therefore, genetic engineering is not particularly efficient.

Modification of milk once this material has been produced is not subject to such tight constraints as those arising from metabolic considerations. Nor does modification require artificial modification of the animal and, hence, deliberate acceleration of the processes of natural evolution undergone by the earth biota. Furthermore, modification allows a wide range of physical and chemical transformations. It takes place outside the animal in an environment devoid of bioethical restrictions and where no back disturbance of the mechanisms of genetic expression and metabolic operation is permitted. In addition, control strategies are simpler and more effective, their implementation is less expensive and more accurate, and results can be obtained on a short time scale over reduced background noise. Finally, if plain physical processes or, alternatively, biocatalytic processes that mimick natural routes are considered, no environmental risks are, in principle, expected, and, hence, persuasion of the final consumer to accept them is easier. Therefore, food processing is particularly useful.

When postproduction modification of milk is considered, two issues are of major importance and should be effectively addressed: 1) the added value of milk (i.e., considerations from the point of view of the producer, which are driven by cost) and 2) the effect of milk or a derived dairy product upon the consumer (i.e., considerations from the point of view of the consumer, which drive price).

With respect to the added value of milk, the extremely high water content of milk makes its handling (e.g., transportation, packaging, and storage) rather expensive because of the negligible opportunity cost of water. The easiest way to overcome this difficulty is through bulk concentration of milk (or some of their components). Such concentration can be effected via processes driven by heat, pressure, or chemical potential. Heat-driven processes include evaporation (as in the production of evaporated milk) and precipitation (as in the production of whey cheese). Pressure-driven processes include centrifugation (as in the production of butter) and ultrafiltration (as in the production of ultrafiltered cheese and whey protein concentrates). Chemical potential-driven processes include precipitation (as in cheese making via acidification or rennet action and in the production of yogurt via acidification). In all of these cases the water content is decreased so the resulting product earns value because of reduction of a posteriori handling costs.

With respect to the effect of a dairy product upon the consumer, three types of consequences are perceived thereby after ingestion of a dairy product: 1) immediate consequences, which are associated with sensory responses; 2) short- and medium-term consequences, which are associated with direct physiological responses; and 3) medium- and long-term consequences, which are associated with indirect, cumulative effects of said product upon the environ-

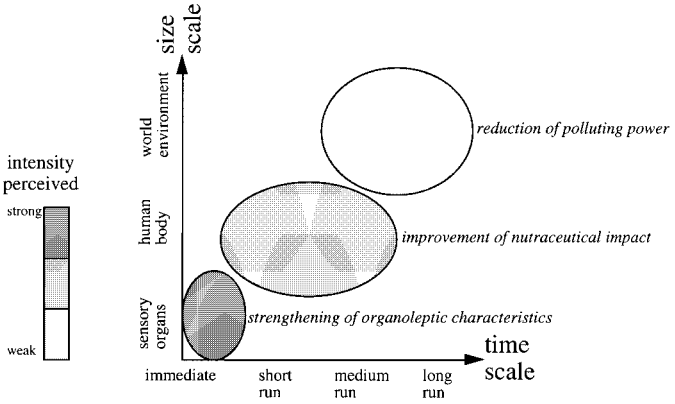

Figure 2. Rationale for modifications aimed at enhancing the appeal of a dairy product to the consumer with respect to time and size scales affected and the intensity of the related effect.

ment. Therefore, modifications aimed at enhancing the appeal of a dairy product to a prospective consumer (and concomitant enhancement in demand and increased price) should focus on 1) strengthening its organoleptic characteristics, 2) improving its nutraceutical impact, or 3) reducing the polluting power of the by-products associated with its manufacture, respectively (see Figure 2).

In view of that reasoning, the most important and lasting contributions of research and development (**R&D**) to the dairy industry are expected to be on condensed dairy products (rather than on plain milk). These contributions will be achieved at the expense of enhancing the sensory and nutraceutical profile of said dairy products and developing environment friendly manufacture processes that allow sustained production. The routes to achieve such goals derive from use of dairy products as a vehicle for incorporation of a desired extraneous compound or microorganism or modification of the dairy product itself. These routes, which can take place once or twice, are depicted in Figure 3.

One of the most effective ways to proceed according to this rationale is through R&D efforts focused on the various steps of the process of manufacture of cheese, which is a dairy product of excellence in terms of intrinsic value and consumer appeal. The use of dairy products as vectors for transport of probiotic cultures of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the absence of significant changes arising from their presence was probably the most important breakthrough in dairy science and technology during the latest decades. This development will likely continue to make its effects apparent during at least one more decade. It should be emphasized that this mode of action has attempted to make dairy

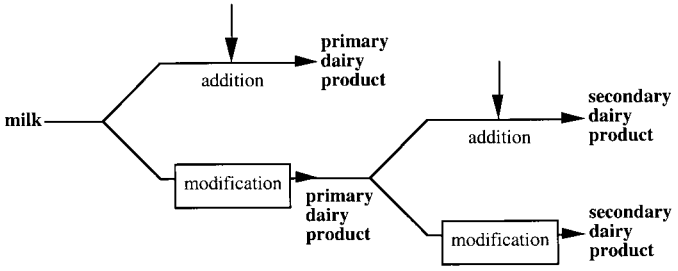

Figure 3. Reasoning that underlies transformation of milk into primary dairy products and primary into secondary dairy products.

products resemble nature in that the microbiological composition was deliberately made more similar to that prevailing in the gut of the newly born. The coming years will, in my opinion, have more and more frequent and successful attempts to make the manufacture processes of dairy products resemble nature more closely. Such a trend will be based on the more extensive and imaginative use of natural cell-free enzymes or of microorganisms that nature has designed to bring about reactions that are mimicked in several nuclear steps in dairy processing. These trends can be summarized in Figure 4. Of particular relevance here is the consistent loss of biodiversity observed in the dairy industry, which has derived from utilization of selected microbial rennets and starter and nonstarter cultures. This trend also has

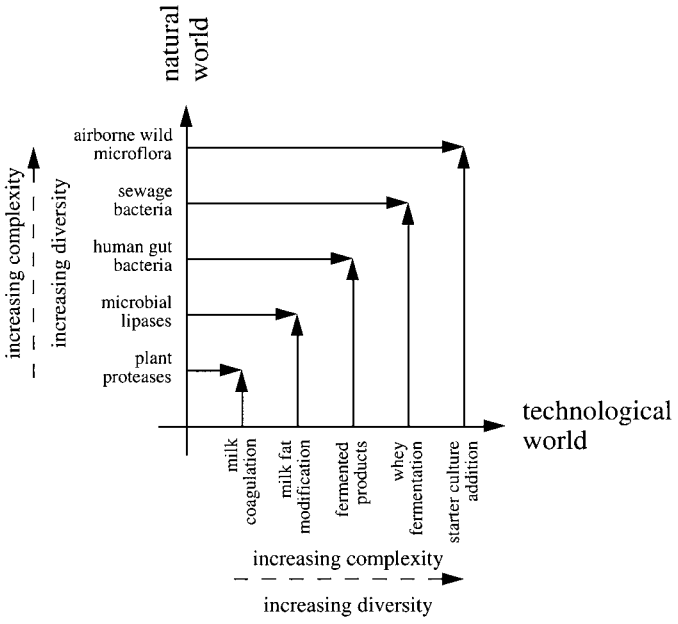

Figure 4. Trends in dairy science and technology for the coming decade.

paralleled the worldwide trend toward intensive exploitation of univarietal plants and animals via preferential (sexual and assexual) multiplication of improved plant and animal breeds.

In the microbial world, cloning of bacteria, yeasts, and fungi with defined and suitably balanced media leads to fast and controlled increase in biomass or to production of desired enzymes with given characteristics suitable for the manufacture of several dairy products. However, sequential generations in the absence of disturbance factors (e.g., phages or extreme temperature, pH, and ionic strength) that are usually present in outer and wild environments decrease the pressure toward transmission and expression of genes encoding for resistance to such factors. Elimination of the natural Darwinistic selection in the utilization of commercial starter and nonstarter cultures will eventually make those excessively labile for widespread use. Furthermore, if only a few selected strains from a few selected species are considered, the richness of keynote flavors and the possibility for unknown probiotic roles that characterize traditional dairy products (and arise from equivalent, yet alternative metabolic routes) will be lost, and, hence, standardization will eventually lead to poorer dairy foods. This conclusion can be extended to dairy food by-products. The intrinsic capacity of some wild strains to utilize disaccharide carbon sources in a particularly efficient way could be an advantage utilized in upgrading whey (with concomitant production of unique exopolysaccharides). The use of microbial enzymes, which effect in vivo the extracellular, stereospecific breakdown of fats (the first step of energy release from such energy-rich compounds), can be employed advantageously in engineering milk fat for tailor-made purposes. Both of these processes mimic nature (and so are efficient and balanced) and are available at low cost provided that they are objectively applied. Finally, consideration of enzymes produced by alternative varieties of plants should also be considered for dairy processing to take advantage of the diversity of compounds produced by plants, as has been practiced in the pharmaceutical industry.

Major accomplishments with respect to the progress of dairy science are expected to be consubstantiated along the following: 1) characterization and development of alternative rennets from plant sources, 2) development of starter and nonstarter cultures from adventitious microflora, 3) utilization of probiotic strains as starter cultures, 4) upgrading whey via physical or fermentation routes, and 5) modification of milkfat via lipase-mediated interesterification reactions. These topics will be discussed in detail in the next section. The products that were studied by the R&D team supervised by the author (and which are depicted in Figure 5 after overlaying on the typical cheese-making process) encompassed such biocatalysts as 1) plant rennet (from *Cynara cardunculus*), 2) probiotic starter (*Lb. acidophilus* and *Bifidobacterium lactis*), 3) wild starter or nonstarter (*Lactococcus lactis, Leuconostoc* spp., *Debaromyces hansenii, Yarrowia lipolytica*, and *Enterococcus faecium*), 4) bacterium inocula (*Rhanella aquatilis*), and 5) lipase (*Mucor javanicus*). Such intermediate and final products studied were 1) fresh cheese and ripened cheese, 2) whey and whey cheese, 3) exopolysaccharide (lactan), and 4) milk fat and modified milk fat. Included bioprocesses were 1) proteolysis, lipolysis, and glycolysis (prevailing during cheese ripening; 2) fermentation (of lactose to exopolysaccharide); 3) precipitation (of soluble whey proteins); and 4) interesterification (of milk fat with olive oil).

CONTRIBUTIONS TO ADDRESS CRITICAL ISSUES AFFECTING THE FUTURE OF DAIRY INDUSTRY

Characterization and Development of Alternative Rennets from Plant Sources

Foreword. The focus of this research effort was the qualitative and quantitative characterization of the aqueous extraction process of cyprosins from thistle

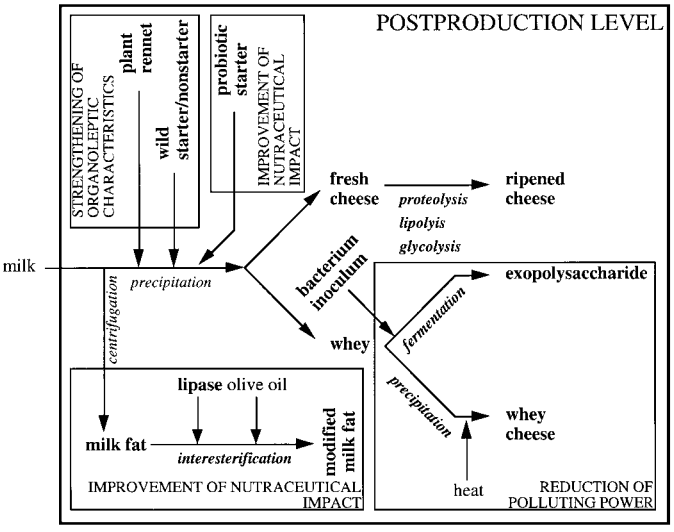

Figure 5. Various steps of cheese making with indication of processes (in italic type) and products (in plain type). Processes and products studied by the research and development team supervised by the author are indicated in bold type.

flowers (and related plants); the determination of the peptide bonds in bovine, ovine, and caprine caseins labile to these enzymes; and the comparison between such plant and commercial animal rennets in terms of cheese making with bovine, ovine, and caprine milks independently. These results have provided insight into the actual performance of plant rennets. This pilot has also enabled production of such rennets from commonly available varieties, following optimized processing conditions, to permit easy technology transfer to industrial cheese making with various milk types.

Overview. Cheese making is initiated by a process of destabilization of casein micelles followed by the formation of a protein network and sequential expression of whey caused by a spontaneous process termed syneresis, which is often complemented by application of external pressure. According to the most commonly accepted models, casein micelles comprise spherical submicelles with an approximate molecular mass of 5 MDa, connected via colloidal calcium phosphate bonds. The bulk of the submicelles comprises α_{S1}-CN and β-CN stabilized by hydrophobic interactions. κ-Casein is located preferentially on the surface with the (more hydrophobic) aminoterminus interacting with the bulk of the submicelle and the (more hydrophilic) carboxyterminus interacting with the surrounding aqueous medium. The submicelles poorer in κ-CN are located more internally in the micelle. Stabilization of this overall complex structure is ensured by the surface change (ζ potential of –15 to –20 mV), the steric hindrance associated with the protruding hydrophilic segment of κ-CN, the existence of glycosylated residues, and the level of hydration. Destabilization of micelles can be effected by physicochemical factors (e.g., lower pH values or higher temperatures) or by biochemical factors (i.e., protease-mediated cleavage of peptide bonds). The latter is the most common in cheese making, and usually the Phe_{105}-Met_{106} bond of κ-CN is hydrolyzed, hence releasing its more hydrophilic domain, which, in turn, facilitates interaction and aggregation of micelles. A good rennet for cheese making should possess a high clotting power but a low proteolytic activity. That is, it should readily attack the aforementioned peptide bond but be much less active upon other peptide bonds of that and of the other caseins because of risk of low yields, or development of bitter flavors, or both.

Commercial rennets, containing mainly chymosin and some pepsin, were originally obtained from the abomasa of recently born ruminant calves, but shortage of supply and ethical concerns have generalized the use of rennets produced via microbial routes using genetically engineered bacteria and fungi. Preparations possessing proteolytic activity have also been obtained from plant sources, but their utilization in cheese making has consistently proven unsuitable either because of low yields or excessively bitter flavors of the final product. A major exception to this rule is proteases, termed cyprosins (or more recently, cardosins), that are present in the violet part of the flower (the stylets and stigmae) of *Cynara cardunculus* and such related species as *Cynara humilis* and *Cynara scolimus*. These prickly varieties of thistle, a plant of the Compositae family and a cousin of the common artichoke, have been traditionally employed in the manufacture of Portuguese cheeses such as Serra and Azeitão, and successfully employed in the manufacture of French cheeses such as Camembert and Gruyère and Italian cheeses like Bel Paese, Grana, and Provolone. *Cynara* spp. grow wild and abundantly in some Mediterranean regions. After collection of the mature plants, the flowers are dried under shade in the open air and stored in dry places. Extraction of cyprosins via aqueous infusion of the macerated, previously dried flowers is fast and simple. Such unique enzymes are aspartic proteases, unlike papain, ficin, and bromelain, which are sulfhydryl proteases. Crude extracts of the thistle flowers display more intense proteolytic activity than do animal or microbial rennets and in bovine milk may lead to the development of bitter flavors in semi-hard and hard cheeses. Conversely, excellent cheese characterized by unique flavors can be obtained from ovine and caprine milks and soft cheeses from bovine milk.

Specific results and conclusions. The effects of four processing parameters encompassing the liquid extraction of proteinases from flowers of the wild thistle (viz. time of grinding, pH of the aqueous buffer, salt concentration of the aqueous buffer, and homogenization time of the aqueous suspension) on their final proteolytic activity (assayed via absorbance of the *o*-phthaldialdehyde derivative of terminal amino residues of peptides and free amino acids) were studied using a response surface methodology (46). Fitting an empiric quadratic model to experimental data pertaining to the average enzymatic activity yielded equations describing the loci of the optima. Simultaneous solutions of these equations for the local maxima indicated that the maximum specific caseinolytic activity (i.e., 9.5 μmol of equivalent leucine/min and per g of thistle flower) is obtained via grinding flowers for ca. 36 s, use of pH 5.9 and absence of salt in the buffer, and homogenization

of the suspension for appoximately 15 min. Increased pH values and time of grinding of stigmae and stylets positively affected the proteolytic activity of the extracts. Homogenization time affected activity negatively, and salt had virtually no effect.

The effect of storage and lyophilization on the clotting and proteolytic activities of extracts of *C. cardunculus* were also empirically studied because the former are potential sources of variability in ready-to-use rennets (F. K. Tavaria, M. J. Sousa, and F. X. Malcata, 1998, unpublished data). Fresh extracts displayed lower clotting activity and lower overall proteolytic activity than did lyophilized ones. Both fresh and lyophilized extracts were stored at 4°C up to 4 wk, and the latter were reconstituted either in water or in citrate buffer and subsequently stored. Storage significantly decreased the clotting power of the extracts, and preliminary lyophilization was less detrimental than direct storage. Fresh extracts lost 65% of their clotting power by 4 wk; lyophilized extracts lost 34% when reconstituted in water and 38% when reconstituted in citrate during the same time frame. Proteolytic activity (combined degradation of α_S-CN and β-CN) increased with storage time, and lyophilized extracts reconstituted in citrate buffer exhibited the lowest activity. Use of lyophilized extracts is an appropriate alternative to fresh, crude extracts provided that appropriate buffers are used for reconstitution.

The proteolytic activities of cardosins A and B from *C. cardunculus* on ovine α_S-CN and β-CN (either separated or as sodium caseinate) were assessed during 10 h of hydrolysis, and significant differences were detected in the action of each cardosin on ovine caseins (44, 51). With respect to cardosin A, β-CN was more susceptible to proteolysis than α_S-CN. In sodium caseinate, α_S-CN and β-CN were degraded up to 46 and 76%, respectively; as isolated caseins, hydrolysis of α_S-CN was 67% and of β-CN was 76%. During treatment with cardosin B, β-CN were more prone to hydrolysis than were α_S-CN (degradations of 100 and 81%, respectively) when treated as isolated caseins. The reverse was observed in sodium caseinate (with 100 and 87% degradation for α_S-CN and β-CN, respectively).

The physicochemical characteristics of sodium ovine and caprine caseinates were varied between pH 6.5 (to parallel milk), pH 5.5 (to parallel fresh cheese), and pH 5.2 and 5% NaCl (to parallel ripened cheese) (47). Caprine caseinate underwent more extensive degradation than did ovine caseinate, and proteolysis of both α_S-CN and β-CN was inhibited by presence of salt, likely as consequence of conformational changes of cardosins. A broader specificity of cardosins toward caprine than ovine caseinate was detected. The first cleavage site for ovine κ-CN was Phe_{105}-Met_{106}; caprine κ-CN was first cleaved at Lys_{116}-Thr_{117}. β-Casein was cleaved at Leu_{127}-Thr_{128} and Leu_{190}-Tyr_{191} in both ovine and caprine caseinates and in caprine caseinate also at Glu_{100}-Thr_{101} and Leu_{136}-Pro_{137}. α_{S_1}-Casein was cleaved at Phe_{23}-Val_{24} in both ovine and caprine caseinates and at Trp_{164}-Tyr_{165} and Tyr_{173}-Thr_{174} in caprine caseinate. Finally, α_{S_2}-CN was cleaved at Phe_{88}-Tyr_{89} in both caseinates and at Ser_9-Ser_{10} and Tyr_{179}-Leu_{180} in caprine caseinate.

When similar cheeses were manufactured with standard animal rennet and with plant rennet extracted from *C. cardunculus* (30, 32, 45, 48), it became apparent that the latter exhibited higher values for the ripening extension index (i.e., ratio of water-soluble nitrogen to total nitrogen) than did the former. Standard animal rennet displayed higher values for the ripening depth indices (i.e., ratio of TCA-soluble nitrogen and phosphotungstic acid (**PPTA**)-soluble nitrogen to total nitrogen). Partial sequencing of the peptides indicated that β-CN (f 1–190) or β-I-CN and α_S-CN (f 24–191) or α_S-I-CN were the primary products of hydrolysis of β-CN and α_S-CN, respectively. The Phe_{23}-Val_{24} in α_S-CN was cleaved earlier in cheese manufactured with plant rennet than that with animal rennet, which may explain the smoother texture of the former cheese. The presence of salt did not produce a significant effect. Peptides β- (f 128–*), β- (f 166–*), β- (f 191–*), and α_{S_1}- (f 24–165) were produced only by the plant rennet. Peptides β- (f 164–*), β- (f 191–*), and α_{S_1}- (f 120–199) were produced only by the animal rennet, and the complementary peptides α_{S_1}- (f 1–23) and α_{S_1}- (f 24–191) were produced by both rennets. Broader specificity of plant rennet was also shown in cheese than in suspensions of caseinate, probably as a consequence of the triphasic gel (water, protein, and fat) nature of the former that is no longer present in the latter.

Changes in the major physicochemical parameters and biochemical characteristics of bovine, ovine, and caprine milk cheeses manufactured with *Cynara* spp. aqueous extracts were studied during a 68-d ripening period (50). By the end of the ripening time, protein contents were 29, 23, and 30% of total solids for bovine, ovine, and caprine milk cheeses, respectively; nitrogen contents soluble in water were 33, 46, and 49% total nitrogen, respectively. The nitrogen contents soluble in TCA were 10, 11, and 7% total nitrogen, respectively. The nitrogen contents soluble in

PPTA were 1.5, 1.9, and 1.4% total nitrogen, respectively. The free amino acid contents were 21, 18, and 24 μM of equivalent leucine, respectively. Urea-PAGE of water-insoluble fractions indicated high levels of proteolysis of β-CN (especially in caprine cheeses) but less proteolysis of α_S-CN (with an initial time lag, especially in ovine cheese, probably a result of unfavorable pH during that period). Therefore, the bitter peptides resulting from the action of cardosins are less abundant in cheeses manufactured from milk of small ruminants (in which the activity upon α_{S1}-CN is much lower, although the higher fat contents of the former may also be implicated in these effects).

Degradation patterns of β- and α_S-CN from bovine, ovine, and caprine caseinates produced by aqueous extracts of *Centaurea calcitrapa*, a plant of the Compositae family, were monitored by urea-PAGE and compared with those of a commercial rennet (53). The plant proteases degraded both types of casein more extensively than did the commercial rennet on the same protein content basis and exhibited higher specificity toward ovine and caprine caseinates than the latter.

Experimental cheeses manufactured from ovine milk using cyprosins were also compared with cheeses manufactured under the same conditions but using commercial animal rennet (49). The type of rennet had no effect on cheese composition (bulk and surface pH and moisture, fat, protein, and salt contents) at the 5% level of significance, but cheeses manufactured with plant rennet exhibited higher levels of water-soluble nitrogen (as percentage of total nitrogen) and lower levels of TCA- and PPTA-soluble nitrogen than did those manufactured with animal rennet. The peptide profiles obtained by reverse-phase HPLC were rather different, especially at intermediate stages of ripening. Plant rennet led to profiles that were much richer both in quality and quantity than those obtained with animal rennet, and the degree of hydrophobicity of such peptides decreased consistently with time. Therefore, bitter peptides resulting from the action of cyprosins disappear gradually as the ripening period elapses, and softer cheeses do not contain them to a significant extent.

To determine whether differences observed in the experiments reported above were mainly due to the action of rennet and not to the action of the cheese microflora, controls were prepared with sterilized milk without addition of any starter or nonstarter culture. It was concluded via ANOVA that removal of microflora from the cheeses led to no statistical differences, at the 5% level of significance, between cheeses, although pairwise comparisons indicated that the degree of significance of such conclusions increased slightly for longer ripening times.

Development of Starter and Nonstarter Cultures from Adventitious Microflora

Foreword. This research effort enumerated and identified adventitious strains in traditional cheeses manufactured according to artisanal practices, characterized their biochemical roles during cheese ripening, defined a mixed culture possessing starter and nonstarter activities that could be used as an alternative to commercial starters, and determined the sites of injury during the commonly followed starter preservation process of spray-drying. The program has furthered the knowledge on the actual performance of wild microflora and has allowed production of an alternative starter or nonstarter mixture in pilot scale, exhibiting higher environmental and better ecological resistance and more intense and unique flavor-generation capacity.

Overview. Cheese made from pasteurized milk generally does not develop mature flavor as does cheese made from raw milk. However, reliance on wild microflora only for large-scale manufacture of cheese is risky because of their variability coupled with the possible presence of potential pathogens. Consequently, pasteurization is a crucial step toward standardization of milk and, hence, consistent cheese production. However, rennet proteases cleave the Phe_{105}-Met_{106} bond of κ-CN and several others of both β-CN and α_{S1}-CN with concomitant production of large peptides. Further degradation into smaller peptides and release of free amino acids is mainly brought about by peptidases produced by starter and nonstarter bacteria that are released following lysis. Therefore, development of a balanced and full flavor makes it necessary to add, postpasteurization, microorganisms with desired peptidolytic properties and lipolytic properties. These microorganisms should be able to effect the specific release of free fatty acids from the triglycerides of milk fat to compensate for the loss of enzymatic activities that is brought about by pasteurization. As a consequence, a considerable variety of commercial cultures is now available. On the other hand, observations of many experimental cheeses manufactured with defined-strain lactic starters and addition of other cultures (leuconostocs, lactobacilli, and pediococci) have provided sufficiently comprehensive evidence of many unique interactions between such added cultures and adventitious nonstarter lactic acid bacteria during cheese ripening. Evidence has also been provided for those interactions

playing important roles in the process of flavor generation.

The extent of the aforementioned interactions is particularly important when traditional cheeses are considered in view of the ill-defined contaminating microflora, especially if raw milk is used. One such cheese is Serra, the best representative of the Portuguese farm cheeses, which is manufactured according to traditional practices from raw ovine milk using plant rennet (thistle flower). It is the most prized cheese on the Portuguese market and is an important source of income for the local farmers. Depending on the manufacture techniques employed during coagulation, whey draining, pressing, and salting and the environmental conditions prevailing during ripening, different characteristics can be imparted to the cheese.

Specific results and conclusions. With respect to the characterization of the adventitious cheese microflora, two model cheeses were considered: ovine raw milk cheese manufactured with plant rennet (Serra cheese) and ovine plus caprine raw milk cheese manufactured with animal rennet (Picante cheese).

For Serra cheese (35, 36), four independent variables were considered (27, 28, 31, 33, 52): geographical location of the cheese maker (representative of the whole region of artisanal cheese production), ripening time (representative of major trends throughout maturation), position within the lactation season (representative of major differences in milk composition and weather conditions), and axial position within the cheese (between the rind and the bulk of the cheese, representative of various degrees of anaerobiosis). Microorganisms isolated from cheese were grown on a variety of selective media and duly identified using morphological and biochemical tests. The number of staphylococci and enterococci found in raw milk was 4.2×10^3 and 1.5×10^4 cfu/ml and accounted for more than 60% of the whole milk microflora. It was found that 1) lactic acid bacteria and *Enterobacteriaceae* predominated throughout ripening, 2) the number of lactic acid bacteria reached 10^7 cfu/g by 21 d and stabilized thereafter, 3) the number of *Enterobacteriaceae* reached the maximum number by 7 d (ca. 10^7 cfu/g), 4) staphylococci were less abundant, and their maximum viable numbers (ca. 10^3 cfu/g) were attained by 7 d (the number of coliforms decreased more slowly than staphylococci), and 5) yeasts attained levels of 10^3 cfu/g by 21 d and stabilized thereafter. For 35-d cheeses two major species of *Leuconostoc* were identified, *Leuconostoc lactis* and *Leuconostoc mesenteroides*, and the former was the more abundant lactic acid bacterium. *Lactococcus lactis* ssp. *lactis* and *Lactobacillus paracasei* ssp. *paracasei* were the next most prevalent genera. Lactic acid bacteria detected at the lowest levels were *Ent. faecium*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillus brevis*. *Hafnia alvei* was the dominant enterobacterium by 35 d, but *Serratia liquefaciens*, *Serratia odorifera*, *Escherichia coli*, *Citrobacter freundii*, and *Erwinia* spp. could also be detected. The dominating staphylococcus by 35 d was *Staphylococcus xylosus*, and *Staphylococcus aureus* was only detected at very low levels. One could also find *Staphylococcus epidermidis* and *Staphylococcus simulans*. The range of yeasts found in cheese by 35 d of ripening was wide, although with apparent predominance of *Sporobolomyces roseus*, followed by *Leucosporidium scottii* and *D. hansenii* (35). The high degrees of correlation between total bacteria and lactobacilli and lactococci and between these latter two groups and proteolytic bacteria suggested that lactic acid bacteria were the major microflora and accounted for most microbially driven proteolysis in cheese (37). At all stages of ripening, cheeses manufactured in spring exhibited the lowest numbers of lactic acid bacteria and yeasts; those manufactured in winter showed the lowest numbers of coliforms and staphylococci. In general, cheeses produced throughout winter are organoleptically classified as better than those from spring, probably owing to the numbers of lactic acid bacteria and yeasts (known to exhibit proteolytic and peptidolytic properties) and, to a lesser extent, to the prevailing environmental conditions (i.e., lower temperatures and higher relative humidities).

For Picante cheese, the evolutions of lactic acid bacteria, enterobacteria, staphylococci, and yeasts were studied throughout a 6-mo ripening period (12, 13, 14, 17, 18). Lactic acid bacteria were the dominant constituents of the microbial population during the whole ripening period. The high proportion of enterococci suggested their possible role in the ripening process, especially by 6 mo, when *Ent. faecium* was the most abundant (57% of all isolates). Dominant lactic acid bacteria by 9 d were *Leuc. mesenteroides* and *Lc. lactis*, which could not be detected after 40 d. Conversely, *Lb. plantarum* and *Lb. paracasei* survived until 140 d. The most common species of *Enterobacteriaceae* in fresh cheese was *S. liquefaciens* (55% of all isolates) followed by *Enterobacter cloacae* and *Serratia rubidae*. Although *E. coli* and *H. alvei* were not detected in the curd, they dominated after 80 d. Staphylocci were detected in 6-mo-old cheeses, and the most abundant species

were *Staphylococcus hominis* and *Staph. xylosus*; *Staph. aureus* accounted for only 6% of all isolates. High levels of yeasts were found in the curd (ca. 10^6 cfu/g of cheese) with *D. hansenii* present throughout the whole period. *Yarrowia lipolytica*, which accounted for half of the isolates by 110 d of ripening, was likely responsible for some of the most unique organoleptic characteristics.

The microbiological quality of Serra cheese was assessed throughout ripening and geographical origin (25, 26, 34, 51). Several cheeses manufactured from distinct milk batches of a few dairy farms scattered over its Appélation d'Origine Protegée (AOP) region were qualitatively and quantitatively assessed in terms of their microbiological composition. Members of the *Enterobacteriaceae* and lactic acid bacteria were the predominant microbial groups in all dairies throughout maturation. Numbers of *Enterobacteriaceae*, staphylococci, and enterococci were significantly affected by the geographical location of the dairy, which suggested that a tighter control in hygiene practices should be enforced. The qualitative changes in the microbial profile throughout the ripening period reflect prevailing physicochemical characteristics. At the beginning of ripening, when pH is relatively high, proteolytic *Enterobacteriaceae*, yeasts, and homofermentative lactic acid bacteria dominate; acid-tolerant yeasts and heterofermentative strains become more and more important as the onset of ripening is approached.

Experimental cheeses were manufactured from raw milk, pasteurized milk without starter (**PNS**) or pasteurized milk with addition of a commercial starter (**PWS**), and the traditional manufacture process was mimicked as closely as possible (47). The microbial counts were higher for raw milk cheeses than for PNS cheeses, as expected, and physicochemical characteristics such as moisture, fat, protein, and NaCl contents and pH of the three types of cheeses were similar. Pasteurization had no significant effect on proteolysis as measured by the water-soluble nitrogen, the TCA-soluble nitrogen, or the PPTA-soluble nitrogen fractions. However, the TCA-soluble and the PPTA-soluble fractions for the PWS milk cheeses ripened for more than 28 d were higher than were those for the raw or PNS milk cheeses of similar ripening times. The cheeses and their water-soluble extracts could not be distinguished by urea-PAGE for up to 14 d, but clear differences were apparent at 68 d of ripening for the PWS milk cheeses with respect to the raw and PNS milk cheeses. Therefore, presence of a commercial starter accelerated and intensified the biochemical processes associated with cheese ripening, but their effect did not generate the complete traditional flavor profile. Hence, evidence was made available on the important role of microorganisms in the manufacture of some of the most unique traditional cheeses and the necessity for including some of the wild, native microflora of cheese in the manufacture of improved commercial cultures.

Ten different microorganisms isolated from 35-d-old Serra cheese (which included lactic acid bacteria, coliforms, staphylococci and yeasts) were assayed for growth rates and capacity to lower pH, promote acid coagulation, and produce gas in ovine milk over a 5-d period (29). The microorganisms were also tested for peptidase and lipase activities via detection of clearing zones after incubation of inoculated skim milk agar or tributyrin agar, respectively. Analytical assays were also performed by colorimetry and HPLC of free amino acids and free fatty acids in experimental small cheeses manufactured from sterilized ovine milk, coagulated with a plant rennet and ripened for 21 d at 5°C and 95% relative humidity. *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum*, *Lc. lactis* ssp. *lactis*, and *L. scottii* or *D. hansenii* displayed significant proteinase and peptidase activities. *Leuconostoc lactis*, *Ent. faecium*, and *Y. lipolytica* exhibited only peptidase activity. *Yarrowia lipolytica* was also able to hydrolyze both tributyrin and milk fat; *Leuc. mesenteroides* ssp. *mesenteroides/dextranicum*, *Lc. lactis* ssp. *lactis*, and *Ent. faecium* showed lipase activity only on milk fat after a long incubation period. Short- and medium-chain fatty acid residues were released preferentially by microbial lipases, although the lipases from *Leuc. mesenteroids* ssp. *mesenteroides/dextranicum* and *Y. lipolytica* could also hydrolyze long-chain fatty acids. In view of their hydrolase activities *Lc. lactis* ssp. *lactis* and *Leuconostoc* ssp. were considered as potential starters for Serra cheese making; *D. hansenii* and *Y. lipolytica* were considered as potential adjunct starters, and *Ent. faecium* has potential as secondary microflora.

Four species of bacteria (*Ent. faecium*, *Enterococcus faecalis*, *Lb. plantarum*, and *Lb. paracasei*) and three species of yeasts (*D. hansenii*, *Y. lipolytica*, and *Cryptococcus laurentii*), previously isolated from Picante cheese, were assayed, both independently and as a set, for glycolysis, proteolysis, and lipolysis of caprine and ovine milks in the presence and absence of salt for a 2-mo ripening period (15, 16). Production of lactic acid was highest for *Lb. paracasei* followed by *Ent. faecium*. Citrate metabolism was observed for *Ent. faecalis*. *Yarrowia lipolytica* exhibited extensive proteolysis and lipolysis; *C. laurentii* also released

large quantities of free fatty acids. Ripening time was a significant processing parameter for lipolysis, but milk source was not. Sodium chloride content was critical for rates of proteolysis and lipolysis. A mixed-strain starter for Picante including *Lb. plantarum*, *Ent. faecium* (or *Ent. faecalis*), and *D. hansenii* (or *Y. lipolytica* or both) is of potential interest and worthy of further studies.

Finally, survival of cell concentrates of lactobacilli following spray-drying of skim milk with and without ascorbic acid and monosodium glutamate was evaluated during storage at different conditions of temperature and water activity (55). High temperature and high water activities were detrimental to survival of dried cells. The death rate was higher in the presence of ascorbic acid and monosodium glutamate during storage at 20°C. After being dried, cells showed sensitivity to NaCl, lysozyme, penicillin, and pyronin Y, indicating damage to the cell membrane, cell wall, and DNA, respectively. These results provided fundamental information on why lower microbial productivity is observed when cells are spray-dried.

Utilization of Probiotic Strains as Starter Cultures

Foreword. This research utilized two probiotic strains, *Lb. acidophilus* and *B. lactis*, as starter for the manufacture of cheese. Their viability during refrigerated storage was also studied (*B. lactis* is a strain with unique probiotic properties, which has been isolated recently and made available commercially by NIZO, Ede, The Netherlands). This pilot study used commercially available microbial preparations that were selected for their acid and oxygen tolerance. These preparations might transfer to industrial cheese making with bovine milk or milk from small ruminants (caprine and ovine). The research provided insight into the performance of probiotic bacteria.

Overview. Supplementation of milk with *Lb. acidophilus*, *Bifidobacterium* spp., or both has been very popular in recent years because these species are resistant to gastric juice and intestinal bile salts. These species are frequently associated with health-promoting and therapeutic properties, which encompass anticarcinogenic activity, reduction of cholesterol, antimicrobial activity, and improvement of protein metabolism. Their capacities for acid production during fermentation of milk and reduced acidification rate during postprocessing refrigerated storage enables them to be used in industrial manufacture of various fermented dairy products. Examples include fermented milk, yogurt, and ice cream. Such dietary cultures will be effective in fermented dairy products if they are present in high concentrations that are sufficiently viable at the time of consumption. However, incorporation of bifidobacteria into the food chain can be difficult because these microorganisms are anaerobes and prefer to grow at temperatures of 37 to 41°C and pH values of 6.5 to 7.9. Hence, large initial inocula often need to be provided. Another constraint associated with the use of such dietary cultures in fermented dairy products is the lack of acid tolerance of some species, which leads to rapid decline of their viability unless refrigerated. These problems will be eventually overcome via strain engineering and modified culturing techniques. However, an easier and more feasible alternative is incorporation into cheese (to be consumed in fresh or matured form). In addition to keeping most nutritional components of milk, cheese does not usually require low-temperature storage and possesses pH conditions that are more suitable for the long term preservation of these microorganisms. Such incorporation is not a completely new idea. Actual use of a starter composed solely of a mixture of *Lb. acidophilus* and *Bifidobacterium* spp. had not been tried previously and so was selected as the goal for this research effort.

Cultivation of bifidobacteria in milk is relatively difficult when compared with cultivation of conventional starters because milk is not, in a sense, a natural medium for growth of these nutritionally fastidious microorganisms. Although milk contains all essential nutrients for growth, these are not always in acceptable forms or in optimal concentrations. Several amino acids that are either stimulatory or essential for growth of bifidobacteria and lactobacilli, such as Arg, Glu, Ile, Leu, Try, Tyr, Cys, and Val, are generally present in too low levels as free amino acids or as low molecular weight peptides. Available nitrogen is, therefore, growth limiting unless specific milk protein hydrolysates are added to the milk (or are generated in situ). Associated growth rate enhancement has been attributed to the increase in peptides and free amino acids derived from degradation of κ-CN. Casein hydrolysates obtained via enzymatic action stimulate the growth capacity of various strains of *Bifidobacterium*. Such enhancement is much greater than that obtained by addition of free amino acids. Because probiotic strains are slow acid producers, either more initial starter bacteria or addition of a protein hydrolysate generated via preliminary incubation of (part of the) cheese milk with a protein hydrolysate is necessary. For practical convenience, the latter was selected for further consideration.

Specific results and conclusions. In our research effort, growth and acidification rates of and maintenance of viability in pure cultures and cocultures of *Lb. acidophilus* and *B. lactis* were experimentally determined. The broths utilized were reconstituted bovine skim milk and whole ovine and caprine milks (and cheeses manufactured therefrom) (19, 20) after addition to milk samples previously incubated with commercial proteases. Those strains were used as starter inocula at various concentrations and were maintained at various temperatures, relative humidities, NaCl concentrations, and storage times. Microbiological monitoring at different locations inside the cheese was by absorbance, viable counts, titratable acidity, and pH. These assays were complemented with biochemical assessments (viz. degree of proteolytic breakdown measured by the total amounts of nitrogen soluble in water, TCA, and PPTA) and sensorial assessments (viz. flavor and texture). The technologies of one of the cheeses produced in largest quantities worldwide (Gouda) and one of the cheeses with largest social and economic regional importance in the Mediterranean basin (Cabra) were adapted in order to use a combination of *B. lactis* and *Lb. acidophilus* as the sole starter. That is scalding temperature was set to 38°C to promote growth in the presence and absence of milk protein hydrolysates.

Higher degrees of enzymatic hydrolysis of milk proteins exert a stimulatory effect on growth of and metabolic activity by *B. lactis*. Thus its poor growth in milk might be due to lack of peptides and free amino acids (21). Conversely, the rates of growth of and acid production by *Lb. acidophilus* are not significantly affected by addition of milk hydrolysates. Although this microorganism intrinsically grows more slowly, its proteolytic system is apparently able to generate its own nitrogen source. Studies of bovine, ovine, and caprine milks confirmed that *B. lactis* required more low- or medium-sized peptides (molecular mass below 2 kDa) than free amino acids. Interestingly, *B. lactis* was able to grow better in untreated ovine and caprine milks than in bovine milk; however, maximum growth rates (for clear probiotic effect) could not be attained, even with the higher protein and vitamin contents of these milks. The enhanced rates of growth of and acidification by *B. lactis* when cocultured with *Lb. acidophilus* compared with its single-strain counterpart, especially in the presence of milk hydrolysates, suggested some degree of commensalism between the strains. The rates indicated that mixed thermophilic starter of *B. lactis* and *Lb. acidophilus* coupled with milk hydrolysate has the advantage of rapid growth and production of lactic (and acetic) acids. Therefore, good probiotic properties can be associated with good functional properties of cheese.

Cultures of *B. lactis* exhibited no significant loss of viability either with increased NaCl concentration or increased storage temperature. Conversely, viability of *Lb. acidophilus* decreased with increased storage temperature and increased salt concentration (24). When cocultured with *Lb. acidophilus*, *B. lactis* was significantly less tolerant to high salt levels and temperatures than was the single-strain culture. However, viable numbers after up to 8 wk of refrigerated storage were still greater than the threshold required for commercial application. Coculture with *B. lactis* led to no detrimental effects on the viability of *Lb. acidophilus*.

After 1 wk of storage of Gouda cheese at 9°C, *B. lactis* reached 3 to 4×10^9 and 2×10^9 cfu/g for initial inocula of 7 and 3.5% (wt/vol), respectively. By 9 wk, *Lb. acidophilus* decreased by two log cycles to 0.2 to 5 $\times 10^7$ cfu/g; *B. lactis* decreased by less than one log cycle to 6 to 18×10^8 cfu/g (22). The presence of milk hydrolysate as a bifidogenic factor had no detrimental effect on bacterial count but had to be carefully handled to avoid development of off-flavors, which were more severe in Gouda cheese than in Cabra cheese. In cheeses with 4% (wt/wt) salt, survival of *B. lactis* during 9 wk of storage ranged from 55% in the center region to 35% in the outer region. Survival of *Lb. acidophilus* was 1.5% in the rind and 27% in the innermost bulk. The best compromise between sensorial, physicochemical, and probiotic attributes of the final cheese was obtained with addition of 0.3% (vol/vol) milk hydrolyzate, 3.5% (wt/wt) salt, and *B. lactis* and *Lb. acidophilus* at 3×10^7 and 7×10^6 cfu/ml, respectively. The concentration of *Lb. acidophilus* observed in cheese was 2 to 4×10^8 cfu/g, which corresponded to the increase of more than one log cycle. *Bifidobacterium lactis* displayed no growth even in the presence of milk hydrolysates.

Because time was found to be a crucial parameter of survival and viability of incorporated bacterial strains, acceleration of ripening by increasing temperature and relative humidity was investigated (23). Postulated empiric models have provided a good fit to the experimental data generated. Such models have been able to predict a decrease of 25 d in ripening time with no impairment of either biochemical (proteolysis and lipolysis indices) or sensorial (flavor and texture) factors when cheeses were ripened at 10°C and 95% relative humidity. All indices of proteolysis and lipolysis, which correlate with flavor develop-

ment, increased with increasing storage temperature to a much greater extent than with increasing relative humidity. The increase in PPTA-soluble nitrogen was the most significant. Proteolysis in probiotic cheese differed slightly when compared with reference cheeses, especially concentrations of PPTA-soluble nitrogen and TCA-soluble nitrogen. Thermophilic starter proteinases and peptidases were apparently more active than were their mesophilic counterparts. Storage time was statistically the most important factor in accelerating extent and depth of proteolysis and lipolysis.

Upgrading of Whey via Physical or Fermentation Routes

Foreword. This research effort assessed the technical and economic feasibilities of reducing the biological oxygen demand (BOD) of whey through sequential steps of protein recovery. Protein was thermally precipitated for a posteriori use as food or for lactose utilization by fermentation to produce a polysaccharide gum for a posteriori use as food or nonfood additive. The results provided insight into the possibility for selective precipitation of whey proteins and the performance of a wild microorganism in metabolizing lactose and converting it to a gum. The pilot-scale study has defined processing conditions that will transfer to industrial cheese making with milk from a variety of sources.

Generic overview. Whey utilization has plagued the dairy industry for the past several decades, and increasingly whey is utilized as a consequence of the steadily increasing demand for cheese. More than 25% of all milk produced in the US is converted to cheese, and 9 kg of whey result from every 1 kg of cheese manufactured. The market value of whey derives solely from its solids, which sell for ca. US$250/tonne of dry solids. Although ultrafiltration enables removal of valuable proteins from whey (which account for ca. 1.5% of the total whey content and are known to possess a high nutritional capacity and a balanced amino acid content, second only to egg proteins). Removal by thermal precipitation is more effective from a thermodynamic point of view because heat is a more degraded, and hence less expensive, form of energy than work necessary for convective percolation. However, both of these processes utilize little of the majority of solids of whey. Lactose (ca. 5.5% of the total whey content) is responsible for its extremely high biological oxygen demand of ca. 50,000 mg/L. For example, 1 tonne of whey requires for complete oxidation of its carbonated material, via biological routes, all oxygen contained in ca. 1100 m^3 of water saturated with molecular oxygen. Removal of the oxygen makes the water unable to support aerobic life and, thus, labile to all sorts of contaminating anaerobes including several pathogens). In developing countries, approximately 40 billion kg of whey annually (roughly 47% of the total worldwide whey production) is disposed of in waste treatment facilities and on farm fields at a significant environmental cost to the dairy industry. Although several attempts at whey utilization have been successful, the primary limitation of most proposed processes is the lack of economic feasibility (owing to the dilute nature of lactose in whey and the unavailability of large quantities of whey at a single site) rather than the lack of technical feasibility.

Precipitation by heat processing still remains the simplest method to recover proteins from sweet and acid wheys. Such processes encompass aggregation phenomena, which are preceded by unfolding and denaturation of protein. Several whey cheeses exist throughout Europe [e.g., Requeijão (Portugal); Ricotta (Italy); and Serac, Brousse, Broccio, and Grueil (France)]. All of them are suitable dietetic products because of their high protein contents and low fat contents. The traditional manufacture of Requeijão consists of heating whey or mixtures of whey with ovine or caprine milk. The temperature ranges from 90 to 100°C for ca. 30 min under smooth stirring conditions, and curd that has spontaneously risen to the surface is scooped out and drained. Precipitated whey proteins have also been used as additives to Cheddar cheese for improvement of yield, to fermented fresh cheeses such as Quarg and Cottage for improvement of consistency, and to yogurt for improvement of viscosity and water-holding capacity.

The small size of the lactose molecule obviates its recovery by ultrafiltration; the dilute concentrations of lactose render concentration processes based on evaporation excessively expensive. Therefore, the most efficient ways of upgrading lactose (and also soluble whey proteins or hydrolyzates) are based on fermentation. Such processes possess, with respect to their chemical synthesis counterparts, the advantage of extremely high specificity, and consequent low levels of by-products, and the possibility of production of high value-added products depending on the strain selected. Exopolysaccharide gums from whey lactose are attractive products because they can be produced efficiently from substrates at relatively low concentrations and in the presence of high carbon to nitrogen ratios (as in whey). They also are intermediate specialty chemicals with sufficient value to offset part

of the costs of whey transportation and concentration, and an internal market for gums exists in the dairy industry. Such gums are valuable to the food industry, primarily owing to their ability to modify the rheology of aqueous systems, as thickening, suspending, emulsifying, stabilizing, lubricating, film-forming, water-retaining, and chelating agents. They are also useful to nonfood industries as adhesives, pastes, building materials, cleaners, polishes, seed coatings, binders, paper products, petroleum- and water-well drilling muds, wastewater treatment, and pharmaceuticals.

Specific results and conclusions. The independent and combined effects of temperature, heating period, and stirring rate on the extent of precipitation of each of the major proteins in acid wheys at various stages of lactation were studied using two-level, replicated complete factorial designs (38, 39, 43). Statistical analyses indicated that temperature is the most important factor in the precipitation of α-LA and β-LG from whey. The magnitude of the independent effects of heating time and stirring rate on the precipitation of these two proteins from ovine whey reach local maxima as lactation time elapses. The yield of α-LA and β-LG via thermal precipitation was maximized using again temperature, heating time, and stirring rate as independently manipulated variables. Loci of the values for each manipulated variable (when the remaining two were fixed), which lead to critical points, were obtained for both proteins and for the nature of such points. Comparative calculation of the various loci has indicated that there were maxima for the precipitation of α-LA and β-LG from the whey. However, the maximum value for the selectivity of precipitation of β-LG relative to that of α-LA (within physically realizable conditions and without extrapolation from the experimental range covered) existed only for caprine whey (at a temperature of 92°C, heating time of 46 min and stirring rate of 79 rpm). The genetic variants of fractions of α-LA and β-LG of whey from Portuguese native breeds of ewes and goats were characterized by isoelectric focusing and differential scanning calorimetry. Ovine and caprine α-LA and caprine β-LG appear as a single variant. Ovine β-LG is accounted for by two variants with a good correlation of pI and temperature of onset of denaturation when NaCl is present.

In attempts to optimize manufacture of Requeijão, several whey cheeses were produced according to a factorial design. Combinations were used of various levels of the processing variables heating time, heating temperature, and fractional addition of ovine or caprine milk (43). Chemical analyses were carried out for all cheeses. Sensorial and rheological analyses were carried out for selected whey cheeses and a reference (i.e., a whey cheese produced locally according to traditional procedures). A true local maximum existed for moisture content (at a temperature of ca. 93°C, heating time of ca. 30 min and addition of ca. 17% ovine milk), which lay well within the range chosen for experimentation. Fat content of Requeijão was affected positively by heating temperature (especially via its quadratic effect) and, to a lesser extent, by heating time (especially via its linear effect). Nitrogen content was affected especially by heating temperature (via its quadratic effect); and moisture content was affected especially by heating temperature (via its quadratic effect). The sensorial analyses showed that the experimental whey cheeses produced were preferred over the reference whey cheese. For rheological analyses, the most significant observations pertained to the high strain dependence of the dynamic moduli, absence of a true equilibrium storage modulus, and relatively low difference between the loss and the storage moduli (42). Meanwhile, the qualitative and quantitative evolution of the microflora of Requeijão was monitored in unpackaged form or packaged under a vacuum, under plain CO_2 or under plain N_2, to assess potential extension of shelf-life. The dominant families throughout storage were *Pseudomonadae* and lactic acid bacteria with inner pH being independent of type of modified atmosphere packaging. Use of only CO_2 made quantities of viable yeasts, staphylococci, spore-forming clostridia, and *Enterobacteriaceae* negligible for at least 15 d. The total free fatty acid concentration was virtually constant for 1 wk, but major differences became apparent by 2 wk between the unpackaged form and the vacuum-packaged form. When compared with typical cheeses manufactured from the same milk, Requeijão was richer in free butyric, caproic, caprylic, lauric, and linoleic acids and was poorer in palmitic and oleic acids.

Soil samples from farm fields regularly treated with whey were screened for bacteria able to produce polysaccharide gums from lactose. One of the most promising microorganisms, *Rhanella aquatilis*, a facultative anaerobe, produces a polysaccharide gum from lactose composed of mannose, galactose, and galacturonic acid. The process conditions during fermentation (inoculum size, temperature, degree of aeration, pH, and ion strength) were statistically optimized using maximum biomass production or maximum gum productivity as alternative objective functions (40). Aqueous solutions of this gum exhibited shear-thinning and elastic flow behaviors

with an estimated power law model flow index of 0.26 at 1% (wt/wt) gum. Lactan solutions exhibited a shear-thinning behavior, but postharvest addition of chloride salts and pH changes affected the observed apparent viscosity only slightly over the wide pH range 2 to 11. These solutions showed excellent thermostability (with retention of more than 80% of their original viscosity after treatment to 121°C for 15 min). Such flow properties indicated potential industrial applications for food and nonfood products requiring a moderate degree of thickening, wet-end additives, and coating agents for paper products, ceramics, detergents, and binders for building materials.

The conditions of recovery of gum by precipitation with ethanol, followed by centrifugation and drying of the gum, and recovery of ethanol by distillation were also optimized (41). Preliminary economic and market assessments using marginal costs have indicated that, per tonne of whey, the fermentation process costs US$1.13 for evaporation of water and US$8.40 for evaporation of ethanol, whereas the traditional reference process of whey solid drying costs US$3.33 and US$0.00, respectively. Conversely, the products of fermentation process cost US$105.00, and those of the traditional reference process of whey solid drying cost US$21.60. Therefore, the net profit of the fermentation process was ca. US$100.47/tonne of whey; the net profit of the traditional process was only US$18.33. If the investment in a plant devised to treat whey is ca. US$63/tonne of whey treated yearly, and if only 30% of the net profit is actually attained, then the pay-out time of the capital investment will be 2.5 yr, which has associated a rate of return of investment of ca. 40%. These assessments suggest industrial implementation of the new process provided that the transportation costs of whey do not excessively add to the total.

Modification of Milkfat via Lipase Mediated Interesterification Reactions

Foreword. This research qualitatively and quantitatively characterized the lipase-catalyzed incorporation of mono- and polyunsaturated fatty acid residues into milk fat at the expense of removal of medium- and long-chain fatty acid residues using olive oil (or hydrolysates thereof) and oils extracted from microalgae. The physicochemical properties of such engineered fats and the processing conditions utilized to bring about the interesterification reactions were assessed. This research has provided insight into the actual performance of immobilized lipases. It has also allowed design of a process that efficiently effects tailored exchange of fatty acid residues of milk fat by other residues with higher nutraceutical interest.

Overview. During the last two decades, enhanced consumer interest in the relationship of food to good health has led to extensive criticism of milk fat. In terms of flavor and texture, consumers view milk fat favorably, but they also tend to view milk fat negatively because of its saturated fat and cholesterol content. The resultant demand for low fat dairy products has led to a surplus of milk fat and a concomitant interest on the part of the dairy industry to find profitable uses for this material.

The fundamental units of milk fat are triglycerides (i.e., glycerol molecules esterified by three fatty acids of the same or different types). The relative amounts of the various fatty acid residues determine the nutritional and functional properties of milk fat.

During digestion of long-chain triglycerides, they become part of chylomicrons, which penetrate through the mucosal wall to the lymphatic system and travel to the liver where they are finally oxidized. Triglycerides of this type that are not used are deposited as fat in fat cells throughout the body. Conversely, short-chain triglycerides are not metabolized through the gut but in the liver, like carbohydrates. Because they are not incorporated in chylomicrons, they are not likely to be stored in the adipose tissues of the body. Milk fat possesses, unlike most oils and fats from natural origin, the unique advantage of being particularly rich in short- and medium-chain triglycerides. Long-chain triglycerides (such as those accounted for by mono- and polyunsaturated fatty acids) are needed in the human diet because of their beneficial effects on health. Short-chain triglycerides are also beneficial because of their easy absorption by the organism and facility of being used as a fast energy source (in addition to their unique and favorable organoleptic properties). For these reasons, physical mixtures of both types of triglycerides have been used in the past. However, structural lipids have proven to be a better alternative than plain mixtures. In fact, each triglyceride in a physical mixture is absorbed at its own rate (and those with longer chains may cause negative symptoms in people suffering from colitis, cystic fibrosis, or Crohn's disease). Those triglycerides containing both long- and short-chain triglycerides are absorbed at intermediate rates (which are not prone to causing symptoms of fat malabsorption) (11).

Of particular interest are unsaturated fatty acid residues, which derive from a common biosynthetic

pathway and are usually subdivided into three families: ω-3 (α-linolenic, eicosapentaenoic, and docosahexaenoic acids), ω-6 (linoleic acid), and ω-9 (oleic acid). Unsaturated fatty acids are obtained from saturated fatty acids in activated form via aerobic and stereospecific dehydrogenation by enzymatic action in plant and animal tissues. Humans require, but do not have the capacity to synthesize, some of the ω-3 and ω-6 fatty acids (e.g., linolenic acid), so they must rely only on dietary sources. Other unsaturated fatty acids (such as eicosapentaenoic and docosahexaenoic acids) can be synthesized by the human body, but because of age and poor habits with regard to exercise, smoking, and alcohol intake, the enzyme system in the metabolic pathways may not be functioning at an optimum level, thus resulting in inadequate synthesis. This factor is more critical for such groups as premature newborns and ill people; hence, a direct intake of some essential fatty acids is generally recommended. The proportion of some ω-3 polyunsaturated fatty acids in the diet has attracted increasing interest because of their unique biological activities. For example, eicosapentaenoic acid is a natural precursor of a large group of lipid mediators, the eicosanoids, which includes such families of compounds as prostaglandins, thromboxanes, leukotrienes, and hydroxy fatty acids. These lipid mediators are associated with a number of different physiological actions and counteractions in various organs of the body and have been claimed to provide beneficial effects in prevention or treatment of diseases like cancer, diabetes, coronary heart disease, high blood pressure, and autoimmune diseases. Docosahexaenoic acid has been shown to be an important structural fatty acid in nervous tissues such as the brain or the retina. Because it is the predominant structural fatty acid in the gray matter of the brain, and because it is poorly synthesized from dietary precursors, it is not surprising that this acid is the most abundant ω-3 polyunsaturated fatty acid present in human breast milk. γ-Linolenic acid is an isomer of α-linolenic acid and is an intermediate in the conversion of linoleic acid into arachidonic acid (which is claimed to have important effects in the treatment of diseases such as multiple sclerosis, arthritis, eczema, and premenstrual syndrome). Food sources rich in unsaturated fatty acid residues are limited to a few seeds and fruits and fish oils. Hence, by chemically manipulating the profile of the fatty acid residues of milk fat at the expense of oils and fats obtained from such sources, one can enhance the nutritional image or improve the functional properties of milk fat.

The composition of milk fat can be chemically modified by interesterification reactions effected by inorganic bases or by lipases. Lipases are enzymes that are highly selective catalysts, which operate under relatively mild processing conditions. Milk fat modified via lipase-catalyzed processes is likely to be readily accepted by consumers because lipases are obtained from living organisms, and their action parallels metabolic pathways (as opposed to classical bulk chemical syntheses). In addition, it is possible to manufacture fats with desired characteristics by taking advantage of the distinct specificities of different lipases. Lipase-catalyzed processes are particularly interesting if microbial extracellular lipases are employed because they are less expensive and intrinsically more thermostable than animal or plant lipases.

Traditionally, the process of modifying milk fat via lipase technology has involved the time-consuming and expensive steps of vigorously stirring the mixture of oils or melted fats with a lipase, incubating, and thermally treating to inactivate the residual lipase. The immobilization of lipases by adsorption on a suitable carrier for the modification of melted milk fat permits efficient use of lipases, enhances their thermal stability, allows regeneration of the support with fresh enzyme, and decreases the potential for contamination of the product via residual lipase, thus avoiding the need for downstream thermal treatment. Although carrying out the reaction in a hydrocarbon solvent lowers the viscosity of the reaction medium with obvious pumping advantages, the reaction product has then to be bleached and deodorized for nutritional safety. The refinement process usually destroys, or at least masks, the unique buttery flavor. Therefore, solvent-free systems have been selected recently with the extra advantage that concentrations of substrate can be raised to maximum. In addition, it has been shown that if the lipase is immobilized in the micropores of a bundle of hollow fibers with hydrophobic characteristics, the reaction liquid(s) will undergo small pressure drops when flowing through the lumen and shell sides of the fibers. This phenomenon occurs because the oil flows tangentially through the reactor instead of percolating through a packed (or fluidized) bed of beads containing the immobilized lipase. Also, the high mechanical power input required by perfectly stirred reactors is avoided. This type of hollow fiber device is currently manufactured for industrial-scale operation encompassing such activities as recovery of organic solutes with a liquid membrane entrapped in the micropores of the fibers, dialysis in artificial kidneys, and oxygenation of blood during open-heart surgery.

Specific results and conclusions. Production of engineered milk fat via enzyme-mediated processes

was via characterization of the physicochemical, organoleptic, and nutritional modifications derived from changes in the fatty acid profile of milk fat in the presence (or absence) of other fats and oils. The lipases employed were obtained from various sources and immobilized by adsorption on a hollow fiber reactor. The kinetics of these processes were simulated by statistically fitting mathematical models based on postulated multiple Ping Pong Bi Bi mechanisms to experimental data on the rates of interchange of various fatty acid moieties (3, 4, 5, 6, 7, 8, 9, 10). The extent of the interesterification reactions was evaluated by assays for water content, total free fatty acids, each type of free fatty acid, total triglycerides and each type of triglyceride (1, 2). The triglycerides of modified milk fat (via acidolysis with oleic acid) with a lipase from *M. javanicus* had ca. 27% more oleic acid residues and 7.5% less lauric, 5.7% less myristic and 6.1% less palmitic acid residues than milk fat. Triglycerides of engineered milk fat also displayed a 26.6% net reduction in the total saturated fraction, a 20.5% net increase in the total monounsaturated fraction, and a 16.6% net increase in the total polyunsaturated fraction. Monoene triglycerides such as CoCoO, BLaO, BMO, CiCiO, CyLaO/CoMO, BPO, CiLaO, CyMO/CoPO, BSO, LaPO/MMO, LaSO/MPO, PPO/MSO, PSO, and SSO increased by 53.5, 39.9, 34.7, 88.9, 170.5, 21.9, 73.1, 29.2, 11.2, 33.2, 18.7, 19.5, 17.1, and 9.3%, respectively, relative to their initial amounts. Polyene triglycerides such as BOO, OBO, CoOO/OCoO, CyOO, LaOO, MOO, POO, SOO and OOO increased by 35.8, 23.1, 41.6, 53.6, 69.1, 25.3, 22.1, 13.6, and 41.7%, respectively (where B = $C_{4:0}$, Co = $C_{6:0}$, Cy = $C_{8:0}$, Ci = $C_{10:0}$, La = $C_{12:0}$, M = $C_{14:0}$, P = $C_{16:0}$, S = $C_{18:0}$, and O = $C_{18:1}$). Although a certain degree of net hydrolysis was observed (especially with respect to medium- and long-chain fatty acids), the lipase-mediated process was, in general, feasible and led to a spreading of the melting range.

The process developed is more environmentally friendly, technically competitive, and economically feasible than alternative processes based on chemical syntheses or even lipases in free form. The process likely will become commercially viable and assist in solving some of the current problems associated with surplus milk fat.

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