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CHARACTERIZATION OF THE PROTEOLYTIC SYSTEM IN  
*LACTOCOCCUS LACTIS* STARTER CULTURES

by

Christina Beer

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

1998

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## ABSTRACT

Characterization of the Proteolytic System in  
*Lactococcus lactis* Starter Cultures

by

Christina Beer, Doctor of Philosophy  
Utah State University, 1998

Major Professor: Dr. Rodney J. Brown  
Department: Nutrition and Food Sciences

The proteolytic system of *Lactococcus lactis* starter cultures influences both flavor and the characteristic body and texture of cheese. The ability to further understand and control how different components of this proteolytic system work together to hydrolyze milk proteins would be of immense importance to the dairy industry.

The goal of this research was to characterize *Lactococcus lactis* subsp. *lactis* starter bacteria with varying *prt* operon compositions by proteinase specificity, aminopeptidase and lipase activities, growth, and influence on cheese flavor. By using a cheese slurry system, a statistical model to predict milk protein hydrolysis patterns was developed.

*Lactococcus lactis* subsp. *lactis* C20 has five plasmids of 55 (pJK550), 48 (pJK480), 43 (pJK430), 3.7 (pJK037), and 2.1 (pJK021) kilo bases. Two of these plasmids (pJK550 and pJK430) are necessary for full proteolytic capability, i.e., clotting milk in 16 h at 20°C. Plasmid pJK550 codes for a proteinase that catalyses the first step in casein degradation. Plasmid pJK430 codes for an oligopeptide

transport system, which further transports peptides across the membrane for bacterial metabolism. Strains were constructed containing twelve different combinations of proteolytic phenotypes, such as Lac<sup>+</sup>PrtP<sup>+</sup>Opp<sup>+</sup>, Lac<sup>+</sup>PrtP<sup>+</sup>Opp<sup>-</sup>, Lac<sup>+</sup>PrtP<sup>-</sup>Opp<sup>+</sup>, Lac<sup>+</sup>PrtP<sup>-</sup>Opp<sup>-</sup>, Lac<sup>-</sup>PrtP<sup>+</sup>Opp<sup>+</sup>, Lac<sup>-</sup>PrtP<sup>+</sup>Opp<sup>-</sup>, Lac<sup>-</sup>PrtP<sup>-</sup>Opp<sup>+</sup>, and Lac<sup>-</sup>PrtP<sup>-</sup>Opp<sup>-</sup>. The proteinase specificities of these strains toward milk proteins were dependent on the genotypes present. Genetically all strains showed a P<sub>1</sub>-type proteinase. Enzymatically C2O had group g proteinase specificity, whereas the rest of the strains containing the proteinase gene showed mixed group specificity.  $\alpha_{s1}$ -Casein was only slightly hydrolyzed by all strains.  $\beta$ -Casein had a variable pattern, as did mixed casein and milk.  $\kappa$ -Casein hydrolysis showed similar degradation patterns in all strains except CB06, which varied in its profile from the other strains.

Sensory evaluation showed that culture had a significant effect on rancidity but not on acidity or bitterness. It also showed that the proteolytic system was associated with lipase activity in these strains.

A statistical prediction model was developed that allowed strains to be classified according to their amino acid hydrolysis patterns. Mixed casein solution proved to be the best substrate for this analysis. Relationships among strains were seen more easily with canonical analysis and distance tables than by looking only at amino acid hydrolysis patterns.

(216 pages)

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Christina Beer

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## LIST OF SYMBOLS, NOTATION, DEFINITIONS

**Abbreviation key**

**AAA** = amino acid analysis

**ADI** = arginine deiminase

**ANOVA** = analysis of variance

**AP** = aminopeptidase

**BCP** = bromocresol purple

**CFE** = cell free extract

**DFA** = discriminant function analysis

**GSH** = reduced glutathione

**JK** = Jenness-Koops

**kb** = kilobases

**LAB** = lactic acid bacteria

**Lac** = lactose utilizing phenotype

*lac* = lactose gene

**LSD** = least square difference

**NSLAB** = non starter lactic acid bacteria

**NOP** = neutral oligopeptidase

**Opp** = oligopeptide transport phenotype

*opp* = oligopeptide transport gene

**PCR** = polymerase chain reaction

**PMF** = proton motive force

*pNA* = *p*-Nitroanalide

**Prt** = proteinase activity

*prt* = proteinase gene

**PrtM** = maturation lipoprotein

*prtM* = lipoprotein gene

**PrtP** = proteinase phenotype

*prtP* = proteinase gene

**TCA** = trichloroacetic acid

**TFA** = trifluoroacetic acid

**TTC** = 2, 3, 5 triphenyl tetrazolium chloride

**SDS-PAGE** = sodium dodecyl sulfate polyacryl gel electrophoresis

**SEM** = standard error of the mean

**+** = contains

**-** = lacks

## CHAPTER I

### GENERAL INTRODUCTION

The focus of many research groups is to control the proteolytic breakdown of caseins in cheese products. Without the proteolytic system in bacteria, the cheese varieties that exist today would be very limited. Our purpose was to (a) construct strains with different proteolytic capabilities, (b) characterize their acid production and ability to grow in milk, (c) observe how these strains hydrolyzed and utilized milk proteins and how the proteolytic genes interact with each other, (d) characterize the strains on Cheddar cheese flavor using a slurry system, and (e) develop a prediction model that allows the researcher to better understand the relationship between strains.

The proteolytic system is responsible for breakdown of milk proteins (caseins) into shorter chain peptides or individual amino acids that can then be taken up by bacteria. Concomitantly, the cheese matrix made up of these caseins is broken down, causing softening of the cheese body. In some cheeses this may be a defect. Other factors such as desired cheese flavor and off-flavors also develop from metabolism of these peptides and amino acids. Fatty acid metabolism also plays a role in obtaining desired cheese flavor.

Our approach to find out how the proteolytic system influences breakdown of caseins was to make genetically modified strains of *Lactococcus lactis* subsp. *lactis* and incubate them with solutions of individual caseins, mixtures of more than one casein, and milk. Pure dipeptides, generated from  $\beta$ -casein hydrolysis, were also used as substrates. Breakdown results were monitored using an amino acid analyzer. We further characterized these bacteria for aminopeptidase activity. To study the effect of modified strains on cheese flavor, we made Cheddar cheese slurries. Slurries were analyzed for

aminopeptidase and lipase activity. A trained taste panel evaluated slurries for acidity, bitterness, and rancidity. The slurries were monitored for starter, non-starter lactic acid bacteria, and microbial contamination. A statistical prediction model was developed to classify strains according to their amino acid hydrolysis patterns. Canonical analysis of amino acid analysis data and distance tables made relationships among strains clear.

## CHAPTER II

### LITERATURE REVIEW

#### LACTIC ACID BACTERIA IN FERMENTED DAIRY FOODS

Lactic acid bacteria (LAB) are microorganisms that can produce lactic acid from hexoses. They are further divided into two groups depending on their fermentation products. The first are homofermentative LAB, which ferment hexoses through the Emden-Meyerhoff-Parras pathway to yield lactic acid as the only product. Alternatively, heterofermentative LAB yield CO<sub>2</sub>, acetic acid, ethanol, and lactic acid from hexose fermentation (68). Most LAB are beneficial microbes because they do not produce putrefaction products.

The most common LAB of interest to the dairy industry belong to the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, or one species of *Streptococcus* (5, 14, 65, 104). Other bacteria used in the dairy industry include *Brevibacterium*, *Bifidobacterium*, and *Propionibacterium* (Table 1). The most important group is the lactobacilli. Lactobacilli are found in fermented dairy products but they can also be found in a variety of other food fermentations (104). These bacteria are often found in acidic fermented foods because of their acid resistance and ability to grow at pH 5.0 (63). Lactobacilli are usually used as starters together with other LAB, e.g., in acidophilus milk, cheeses, yogurt, and kefir fermentations (83).

Lactococci are usually used alone or in conjunction with other LAB in milk fermentation. Cheddar cheese manufacture uses *Lactococcus lactis* strains as its starter (49). *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* are two widely used starters in today's cheese industry (54). They are primarily used in Cheddar

TABLE 1. List of bacteria used in dairy manufacture (49, 77).

Fermented foods	Strains used <sup>1</sup>
Cheddar cheese	<i>Lc. lactis</i> ssp. <i>lactis</i>
Gouda cheese	<i>Lc. lactis</i> ssp. <i>cremoris</i>
	<i>Lc. lactis</i> ssp. <i>lactis</i> <i>Lc. lactis</i> ssp. <i>cremoris</i>
Swiss and Emmentaler cheese	<i>Leuconostoc</i> species
	<i>S. thermophilus</i>
	<i>Lb. helveticus</i> , <i>Lb. lactis</i>
	<i>Lb. bulgaricus</i>
Gruyère cheese	<i>P. freudenreichii</i> ssp. <i>shermanii</i>
	<i>Lb. lactis</i> , <i>Lb. helveticus</i>
	<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i>
	<i>Lc. lactis</i> ssp. <i>cremoris</i> <i>Lc. lactis</i> ssp. <i>lactis</i>
	<i>P. freudenreichii</i> ssp. <i>shermanii</i>
Limburger cheese	<i>B. linens</i>
Parmesan cheese	<i>Lb. helveticus</i>
	<i>Lb. delbrueckii</i> ssp. <i>lactis</i>
Mozzarella cheese, Yogurt	<i>Lb. fermentum</i>
	<i>S. thermophilus</i>
	<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i>
Havarti and Danbo cheese	<i>Lc. lactis</i> ssp. <i>cremoris</i>
Prästost and Herrgård's cheese	<i>Lc. lactis</i> ssp. <i>lactis</i> bv. <i>diacetylactis</i>
	<i>Ln. cremoris</i>
Acidophilus milk	<i>Lb. acidophilus</i>
Cultured buttermilk	<i>Leuconostoc</i> species

<sup>1</sup> Lc. = *Lactococcus*, Ln. = *Leuconostoc*, Lb. = *Lactobacillus*,  
S. = *Streptococcus*, P. = *Propionibacterium*, B. = *Brevibacterium*

and Cheddar-type cheeses but they are also used as starters in Swiss-type cheeses together with CO<sub>2</sub> forming *Propionibacterium freudenreichii* subsp. *shermanii*.

Pediococci are not used as starters in the dairy industry but they may be

found in non-starter LAB (NSLAB) involved in cheese (50, 114). *Pediococci* are found as starters in sausage fermentation and as silage inoculates (60, 132). Genetically modified *Pediococci* have also been proposed for starters in Mozzarella cheese manufacture (18).

Diacetyl is a major product from citrate fermentation by *Leuconostoc* species and *Ln. mesenteroides* subsp. *cremoris* is used in the dairy industry for this particular purpose. Other *Leuconostoc* species are used in the starter mix for Scandinavian- and Swiss-type cheese varieties (Table 1). They are also involved in buttermilk and sour cream formation.

The only *Streptococcus* species associated with dairy fermentation is *Streptococcus thermophilus*. This strain is used in Swiss and Italian cheeses and yogurt manufacture in conjunction with *Lactobacillus delbrueckii* subsp. *bulgaricus*, where it contributes to the special yogurt flavor.

### ACCELERATION OF CHEESE RIPENING

Cheese ripening describes the process in which fresh curd is converted into a flavorful cheese that has a characteristic body and texture. During this process, numerous proteolytic, lipolytic, and other enzymes act upon proteins in the cheese matrix (114, 133). These enzymes come from starters, non-starter bacteria, milk, and rennet. The traditional ripening process, in which no external source of enzyme is added, is relatively slow and can take up to 24 mo before the cheese is considered mature (45, 47). During maturation, some cheeses are stored under refrigeration (usually 4-8°C) and thus maturation time is an economic issue (43).

In 1978, an expert group (F16) was established by the International Dairy Federation to investigate the possibility of accelerated cheese ripening (86, 87, 88,

89). Most research on accelerated cheese ripening focused on proteolysis in Cheddar cheese and Dutch-type cheeses (45). These cheese varieties are made in larger volumes and their ripening is slow and thus can more easily be manipulated. Their flavor change is not as dramatically influenced by proteolysis as the flavors of Brie, Roquefort, and Gorgonzola are by lipolysis.

### **Methods of Accelerated Cheese Ripening**

Before the mid 1970's there was a notion that to accelerate cheese ripening, lipolysis and proteolysis had to be increased. It was understood that these two factors play a significant role in flavor acceleration, but means to control them were limited. Law and other researchers proposed that caseins are broken down into peptides and subsequently into amino acids (88, 90, 147). Recent attention has been given to proteolytic and peptidolytic systems since the role of lipases is already well established. To increase the concentration of peptides in the final cheese, direct addition of enzyme or modified bacterial starters to the milk or to the curd has been given much attention (43).

*Direct enzyme addition.* The first enzyme known to be added to cheese milk is rennet (47). Rennet is an enzyme usually extracted from stomachs of calves or kids. In the past, rennets were crude enzyme preparations of selected proteinases. Today, the cheese industry uses chymosin or pepsin, two enzymes that have been purified and genetically engineered to replace rennet. About 6% of the rennet stays in the curd. During early ripening this residual rennet breaks down  $\alpha_{s1}$ -casein, thus softening the texture. The texture is further modified by plasmin and other bacterial proteinases.

These coagulated-type cheeses usually ripen in between 3 wk and 2 y (45, 47). The length of ripening is inversely proportional to the percent moisture in



the pressed cheese. Proteolysis is essential in development of flavor in all cheese varieties, and influences flavor in five ways (20, 25, 46, 47, 48, 55, 67, 90, 123):

1. Proteins are broken down into potential bitter flavor compounds.
2. Released amino acids are further degraded into amines, thiols, thioesters, acids, etc., where they may contribute to beneficial flavors.
3. Good tasting compounds are released during mastication.
4. Ammonium is formed and thus pH increases.
5. Texture changes because of breakdown of the protein matrix, change in pH, and increased capacities of newly formed amino and carboxyl groups to bind water.

Secondary proteolysis further influences cheese flavor in three ways (47):

1. Rennet-derived peptides, if kept at a low concentration, may contribute to good flavor. However, an imbalance or an excess of certain peptides can lead to bitterness.
2. Rennet-derived peptides can be used by secondary microflora but also by starter bacteria. This contributes to background flavor, but if too excessive it causes breakdown of amino acids into other acids, thiols, thioesters, amines, and other compounds.
3. Flavorful and aromatic compounds are released due to cheese texture modifications. This may be the most important contribution of proteolysis to cheese flavor (47).

Different methods have been approached to accelerate cheese ripening. Addition of enzymes has been used by many research groups (111). Use of bacterial and fungal proteinases increases proteolysis but flavor intensity and quality of cheese do not match proteolysis patterns. In addition, bitterness is encountered (46, 48, 159). Several research groups show promising sensory

results using microbial proteinases (94, 95, 100, 140, 141) but cheese body and texture are adversely affected (94). Law and Wigmore (96) investigated use of Neutrase, a neutral metallo-proteinase isolated from *Bacillus subtilis*, and showed that it intensifies the flavor in Cheddar cheese. Treatment with Neutrase also increases gross proteolysis (44, 53, 96, 129). Law and King (93) and Piard et al. (117) further investigated addition of proteinase using liposomes as vehicles. Liposome-encapsulated proteinase is largely retained in the curd (2, 3, 36, 51) and released into the cheese matrix since an increase in rate of  $\beta$ -casein hydrolysis is observed (93). Hayashi et al. (64) investigated addition of aminopeptidases derived from *Brevibacterium linens* and found that this peptidase increases proteolysis and, together with Neutrase, scores even higher on sensory analysis of Cheddar cheese.

Addition of plasmin, an enzyme found in milk, was found to accelerate cheese ripening by Farkye and Fox (40, 41). The advantage of using plasmin as an enzyme is that it is indigenous to milk in association with the caseins, giving an even distribution throughout the milk. Plasmin also shows a narrow specificity to casein hydrolysis. The major disadvantage, however, is that the enzyme is very expensive.

**Modified starters.** Another approach to increase proteinase (Prt) and peptidase activity in cheese is use of modified starter systems. Attention has been given to combinations of proteinases together with lactose and proteinase negative ( $\text{Lac}^- \text{Prt}^-$ ) strains as sources of peptidases (48, 159). Much research has been done to characterize both  $\text{Prt}^+$  and  $\text{Prt}^-$  mutants, and their possible use as starters or as adjuncts in cheese procedures (15, 16, 78). Kempler et al. (70) and Mills and Thomas (105) both suggested exclusive use of  $\text{Prt}^-$  strains in Cheddar cheese manufacture. Richardson et al. (128) investigated use of *L. lactis* subsp.

*cremoris* HP and 104 as potential starters in Cheddar cheese.  $\text{Prt}^-$  mutants produce enough acid in commercial cheese manufacturing conditions to qualify them as starters. Oberg et al. (113) manufactured Cheddar cheese with  $\text{Prt}^-$  mutants of *L. lactis* subsp. *cremoris* UC73. After 90 d of storage at 7°C, cheese made with  $\text{Prt}^-$  strains shows better body and texture scores but blander overall flavor and flavor intensity scores than controls. Stadhouders et al. (142) showed that Gouda cheese made with  $\text{Prt}^-$  mutants has very little flavor and suggested that  $\text{Prt}^-$  strains not be used in this cheese manufacture. Farkye et al. (42) made Cheddar cheese exclusively with *L. lactis* subsp. *cremoris* UC317 ( $\text{Prt}^+$ ) or *L. lactis* subsp. *cremoris* UC041 (the  $\text{Prt}^-$  mutant of UC317). They also showed, together with other research groups, that it takes a higher inoculum of  $\text{Prt}^-$  starters and a longer cheese-making time compared to  $\text{Prt}^+$  starters (42, 105, 113, 115). Other approaches to increase enzyme concentration in cheese include heat-shocked or freeze-shocked bacterial cells (19, 52, 56, 72, 91, 150), fast-lysing starters, use of adjunct starters (17, 18), or starters genetically engineered in their proteolytic systems (103).

**Temperature.** Cheddar cheeses are ripened under refrigeration between 4-8°C. The simplest approach to accelerating the ripening process is to increase the storage temperature. This is of no extra cost to the manufacturer and may save time and money.

During the ripening process, numerous complex biochemical reactions occur. These reactions might not accelerate at the same relative time as they occur under normal storage conditions, thus creating off-flavors (9, 45). Law et al. (92) found that it takes 50% less time to reach the same maturation stage at 13°C than it takes at 6°C to 13°C. They also concluded that ripening temperature is the most important factor in determining flavor intensity. Aston et al. (4)

investigated the effect of increasing storage temperature, from 8°C to 20°C for 1 mo, together with a *lac<sup>-</sup>pvt<sup>-</sup>* mutant. The results showed that cheeses stored at 20°C and then at 8°C, containing the *lac<sup>-</sup>pvt<sup>-</sup>* starter mix supplement, show a higher rate of maturation. Cromie et al. (22) also showed that with increased storage temperature, total bacterial counts, lactic acid bacteria, lactobacilli, and off-flavor-producing lactobacilli increase. Increase in NSLAB raises the potential of defects in the cheese since most NSLAB are heterofermentative and thus can produce off-flavors.

**Slurries.** Ripening can also be accomplished using a slurry system to simulate cheese making and aging (47). These systems usually contain 60% moisture (3-6% salt-in-moisture), compared to not more than 39% in traditional Cheddar cheese. In the 1960's, Kristoffersen et al. (79) developed a method that allowed accelerated development of Cheddar cheese flavor. They showed that flavor develops within days after storage at 30°C in slurries containing about 40% solids, 21% fat, and 3.5% salt. After 9 d, the slurries develop a characteristic sharp Cheddar taste. Further research was done to find the mechanism of Cheddar flavor production (61, 135, 136).

Addition of potassium sorbate produces a clean Cheddar flavor as reported by Dulley and Taylor (35). Dulley (34) investigated use of 7-d-old Cheddar slurries added to cheese. This method does not increase proteolysis, though flavor intensity does increase. A high number of lactobacilli are detected ( $10^5$ - $10^7$  CFU/g), which could contribute to accelerated ripening. The same observation was made by Von Boeckelman and Lodin (156) where lactobacilli concentration increased from  $10^6$  to  $10^9$  CFU/g when slurries were added to Prästost (a Swedish hard cheese variety) cheese milk.

Singh and Kristoffersen (135) observed that addition of 100 ppm reduced

glutathione (GSH) and incubation at 30°C or 35°C is necessary to obtain proper Cheddar flavor intensity after 7 d. Flavor is slightly improved by addition of 500 ppm sodium citrate, 5 ppm MnSO<sub>4</sub>, and 2 ppm riboflavin together with daily agitation. Dulay (33) reported higher tyrosine concentration and higher pH in slurries treated with GSH. The treatment, however, has no effect on aerobic plate count, coliform, yeast, mold, or staphylococci counts in curd or slurry. Lin (97) showed that with addition of GSH to slurries, diacetyl production is enhanced but concentrations of acetaldehyde, formic, acetic, butyric, and longer chain fatty acids are reduced. Other researchers reported that addition of GSH does not influence redox potential (130), but instead hydrogen sulfide and ethanethiol production increase (99, 130), and to some degree carbonyl and dimethyl sulfide concentrations increase (130).

Samples et al. (131) showed that presence of the enzyme  $\gamma$ -glutamyl transferase (E.C. 2.3.2.2), an enzyme associated with the milk fat globule membrane, is necessary to produce hydrogen sulfide and methanethiol. Harper et al. (62) showed that GSH-treated slurries release higher concentrations of peptides and reduce the rate of  $\beta$ -casein degradation. This observation confirmed the possible explanation of Harper and Kristoffersen (61) that GSH is responsible for dissociation of peptides, thus making them more accessible to proteolytic attack, protection of enzyme groups, and feedback-control relationships.

## PROTEOLYTIC SYSTEM OF LACTIC ACID BACTERIA

Lactic acid bacteria play an important part in much of the food industry but extensive studies have been carried out only on proteolytic systems of those bacteria involved in milk fermentations.

The best understood system is that of *L. lactis* (10, 84, 139, 145). A

proteinase found on the surface of the cell catalyzes partial hydrolysis of milk caseins into a wide range of oligopeptides (Figure 1). Several transport systems are available that can transport peptides of various sizes across the membrane. Once inside the cell, intracellular peptidases break down peptides into individual amino acids.

### Lactococcal Cell-Envelope Proteinase System

The proteinase catalyzing the first step in casein degradation is encoded by a single gene (*prtP*) generally carried on a plasmid. This gene has been cloned,

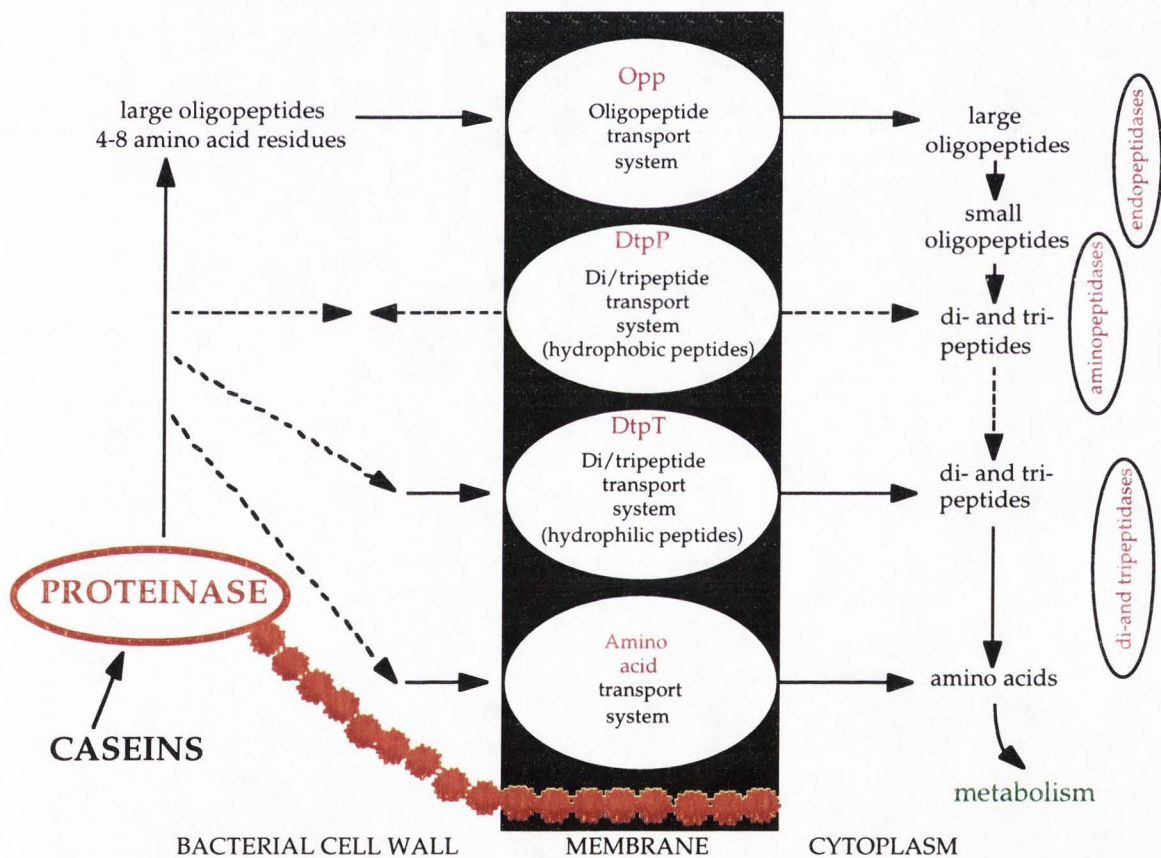


Figure 1. Possible pathway in degradation of milk caseins into individual amino acids to support growth for lactococcal strains in milk (81).

sequenced, expressed in other bacteria, and modified by recombination (74). It belongs to the subtilase subfamily of serine proteases (134). This protein has a long C-terminal domain whose only known function is to anchor the mature proteinase to the cell wall (57, 158). The N-terminal part of the mature enzyme contains the catalytic domain. The gene *prtP* is closely linked to a membrane-associated maturation lipo-protein (*prtM*) gene (58, 59, 73, 75, 157, 158). After the proteinase is synthesized by the cell, it is translocated across the cell membrane. During or after the translocation step, the N-terminal 154 amino acid residue pro-region, together with a 33 amino acid residue of the proteinase, is auto-proteolytically removed with the help of PrtM. The mature proteinase of approximately 180 kd is anchored to the cell wall (58, 59, 73, 75, 157, 158).

Visser et al. (153) compared the action of cell-envelope proteinases from a variety of *L. lactis* subsp. *cremoris* strains for their ability to degrade caseins. From sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), thin-layer chromatography, and starch gel electrophoresis, two distinct proteinase types, P<sub>I</sub> and P<sub>III</sub>, were identified (154, 155). Since then, the proteinase types have been further classified into seven distinct groups a-g (14, 81). The different specificities of proteinases are based on ability to hydrolyze  $\alpha_{s1}$ -casein (Table 2). The specificities are also reflected in point mutations, which result in variations in the structural gene of PrtP (81). P<sub>I</sub>-type proteinase, reference strain *L. lactis* subsp. *cremoris* HP (154), is now reclassified as a group g proteinase. The P<sub>III</sub>-type proteinase of reference strain *L. lactis* subsp. *cremoris* AM1 (155) is now classified as having a group a proteinase (81). For simplicity, P<sub>I</sub> and P<sub>III</sub> will still be used throughout the dissertation.

Reid et al. (124) investigated the action of cell wall proteinase isolated from *L. lactis* subsp. *cremoris* SK11, a P<sub>III</sub>-type proteinase, on  $\alpha_{s1}$ -casein. Sixteen

TABLE 2. Proteinase specificities on cleavage of  $\alpha_{s1}$ -casein f1-23 (14, 81)<sup>1</sup>.

Group	Main fragments generated from $\alpha_{s1}$ -casein (f1-23)
a	1-16, 1-17, 1-21 <sup>2</sup>
b	1-16 <sup>2</sup> , 1-17, 1-21 <sup>2</sup>
c	1-16 <sup>2</sup> , 1-17 <sup>2</sup> , 1-21 <sup>2</sup>
d	1-21
e	1-8, 1-9, 1-13 <sup>2</sup> , 1-16 <sup>2</sup> , 1-21 <sup>2</sup>
f	1-8 <sup>2</sup> , 1-9 <sup>2</sup> , 1-13, 1-16 <sup>2</sup> , 1-17 <sup>2</sup> , 1-21 <sup>2</sup>
g	1-8, 1-9, 1-13 <sup>2</sup>
h	1-9, 1-13, 1-16

<sup>1</sup> Numbering is according to *Lactococcus lactis* subsp. *cremoris* SK11 proteinase sequence (158).

<sup>2</sup> Cleavage rates to produce main fragments vary compared to the other groups.

trifluoroacetic acid (TFA) soluble oligopeptides have been identified and sequenced (Table 3). The earliest peptides (1-h digestion) are generated from the C-terminal end (62-169, 170-199, 143-148, 122-130, 131-142, 149-156, and 157-161). These seven peptides comprise the complete C-terminal of  $\alpha_{s1}$ -casein (Figure 2). Three peptides from the N-terminus are also formed in the early digestion, though in smaller quantities (1-17, 17-23, and 34-37). No early peptides were isolated from regions 38-74 and 99-121, which contain most of the phosphorylated amino acid residues. Exterkate et al. (37) investigated the effect of proteinases from reference strains on  $\alpha_{s1}$ -casein fragment 1-23. Both enzymes produce different digestion patterns. The P<sub>I</sub>-type proteinase cleaves bonds His<sub>8</sub>-Glu<sub>9</sub>, Glu<sub>9</sub>-Gly<sub>10</sub>, and Gln<sub>13</sub>-Glu<sub>14</sub>, generating peptides 1-9, 1-8, 1-13, 14-23, 9-23, and 10-23. Cleavage by P<sub>III</sub>, however, generates peptides 1-17, 1-16, 17-23, 18-23,



TABLE 3. TFA soluble fragments of  $\alpha_{s1}$ -casein digested with  $P_{III}$ -type proteinase from *L. lactis* subsp. *cremoris* SK11 (124).

$\alpha_{s1}$ -Casein fragment	Amino acid sequence
1-17	Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn
17-23	Asn-Glu-Asn-Leu-Leu-Arg-Phe
34-37	Lys-Glu-Lys-Val
75-84	Ser-Val-Glu-Gln-Lys-His-Ile-Gln-Lys-Glu
89-98	Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu
122-142	Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-Glu-Leu
122-130	Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln
131-142	Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-Glu-Leu
140-148	Gln-Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu
143-148	Ala-Tyr-Phe-Tyr-Pro-Glu
149-156	Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu
149-157	Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp
151-156	Arg-Gln-Phe-Tyr-Gln-Leu
157-161	Asp-Ala-Tyr-Pro-Ser
162-169	Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu
170-199	Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp-COOH

and 1-21.

Reid et al. (125) also investigated the action of  $P_I$  and  $P_{III}$  from *L. lactis* subsp. *cremoris* H2 and SK112 on  $\beta$ -casein. Six peptides are generated after 2 h (194-209, 183-193, 176-182, 166-175, 167-175, and 169-175) upon digestion with the  $P_I$ -type proteinase (Table 4). Only four peptides are generated when digested with  $P_{III}$  (193-209, 194-209, 47-52, and 47-52) after 4 h incubation (Table 5). Only peptide 194-209 is produced by both proteinase types (Figure 3, 4). The major differences is precipitation of protein after cleavage of fragment 43-46 with  $P_{III}$ .

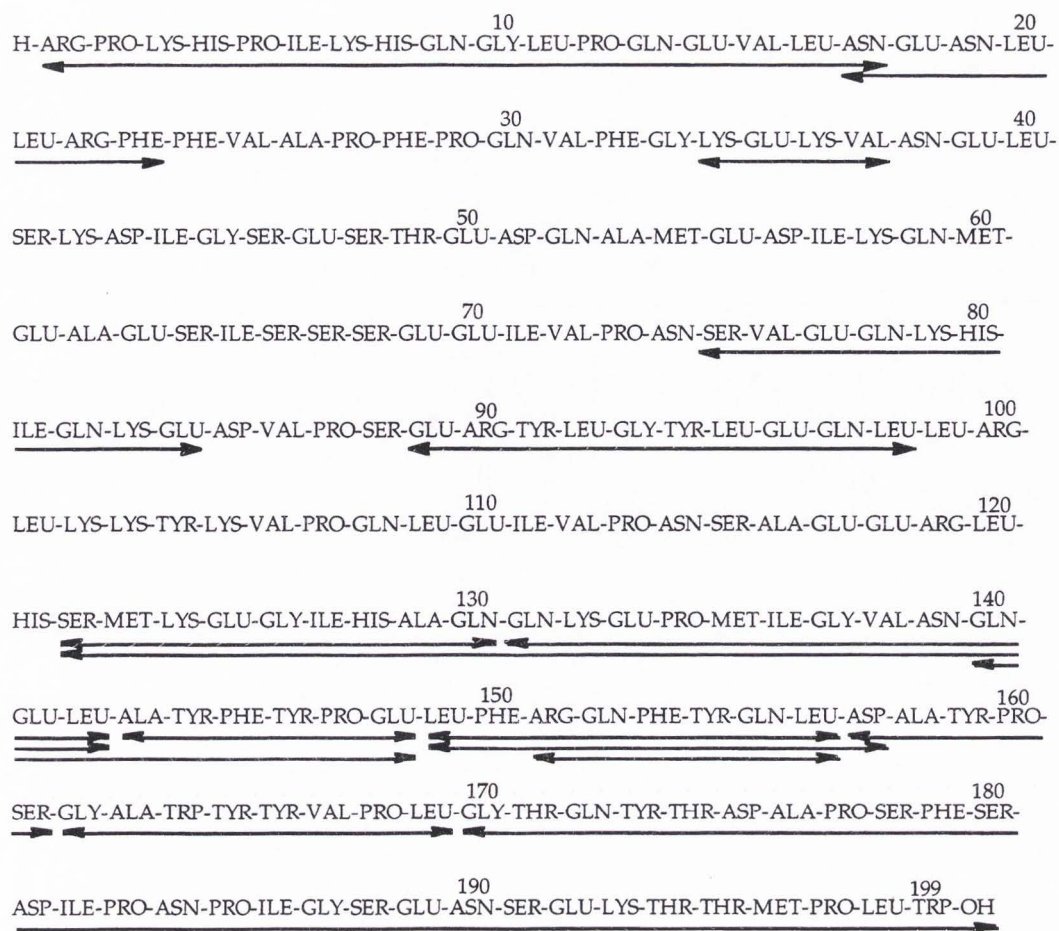


Figure 2.  $\alpha_{s1}$ -Casein sequence and cut sites for the  $P_{III}$ -type proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 (124).

This precipitate slowly digests and disappears after several hours.

Monnet et al. (106, 107, 108) also identified the same early peptides 194-209, 176-182, 183-193, and 167-175 ( $P_I$ -type proteinase) when looking at the proteinase effect of *L. lactis* subsp. *lactis* NCDO763.

Tables 6 and 7 show the peptides obtained from  $P_I$  and  $P_{III}$  digestion of  $\kappa$ -casein (124). Digestion with  $P_I$ -type proteinase from *L. lactis* subsp. *cremoris* H2 shows peptides 72-79, 96-106, and 103-106 after only 5-10 min incubation (Figure 5). Figure 6 shows the  $P_{III}$ -type digestion pattern from *L. lactis* subsp.

TABLE 4. TFA soluble fragments of  $\beta$ -casein digested with  $P_1$ -type proteinase from *L. lactis* subsp. *cremoris* HP (125).

$\beta$ -Casein fragment	Amino acid sequence
1-7	Arg-Glu-Leu-Glu-Glu-Leu-Asn
47-52	Asp-Lys-Ile-His-Pro-Phe
57-	Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-....
57-	Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-....
94-105	Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-Met-Ala-Pro-Lys
94-	Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-Met-....
102-	Met-Ala-Pro-Lys-His-....
106-	His-Lys-Glu-Met-Pro-Phe-....
133-	Leu-His-Leu-Pro-....
142-151	Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu
142-148	Ser-Trp-Met-His-Gln-Pro-His
142-146	Ser-Trp-Met-His-Gln
161-166	Ser-Val-Leu-Ser-Leu-Ser
167-175	Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln
164-168	Ser-Leu-Ser-Gln-Ser
169-175	Lys-Val-Leu-Pro-Val-Pro-Gln
164-	Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-....
166-175	Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln
176-192	Lys-Ala-Val-Pro-Tyr-Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu
176-182	Lys-Ala-Val-Pro-Tyr-Pro-Gln
176-181	Lys-Ala-Val-Pro-Tyr-Pro
183-193	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr
183-192	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu
183-190	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe
183-189	Arg-Asp-Met-Pro-Ile-Gln-Ala
190-193	Phe-Leu-Leu-Tyr
194-209	Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val

*cremoris* SK11. The first three peptides (66-79, 96-106, and 161-169) are seen

TABLE 5. TFA soluble fragments of  $\beta$ -casein digested with  $P_{III}$ -type proteinase from *L. lactis* subsp. *cremoris* SK112 (124).

$\beta$ -Casein fragment	Amino acid sequence
1-16	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser-Leu
1-6	Arg-Glu-Leu-Glu-Glu-Leu
44-52	Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe
47-52	Asp-Lys-Ile-His-Pro-Phe
53-60	Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr
53-58	Ala-Gln-Thr-Gln-Ser-Leu
69-74	Ser-Leu-Pro-Gln-Asn-Ile
94-	Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-....
120-128	Thr-Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr
124-133	Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu
124-132	Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn
124-131	Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu
133-143	Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-Gln-Ser-Trp
133-	Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-Gln-Ser-Trp-Met-....
134-143	His-Leu-Pro-Leu-Pro-Leu-Leu-Gln-Ser-Trp
142-148	Ser-Trp-Met-His-Gln-Pro-His
164-	Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-....
169-175	Lys-Val-Leu-Pro-Val-Pro-Gln
176-182	Lys-Ala-Val-Pro-Tyr-Pro-Gln
183-192	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu
183-193	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr
193-209	Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
194-209	Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val

relatively early. Only peptide 161-169 matches  $P_I$ -type patterns. After 5 h of incubation, three peptides are identical in both proteinase digestion patterns (24-

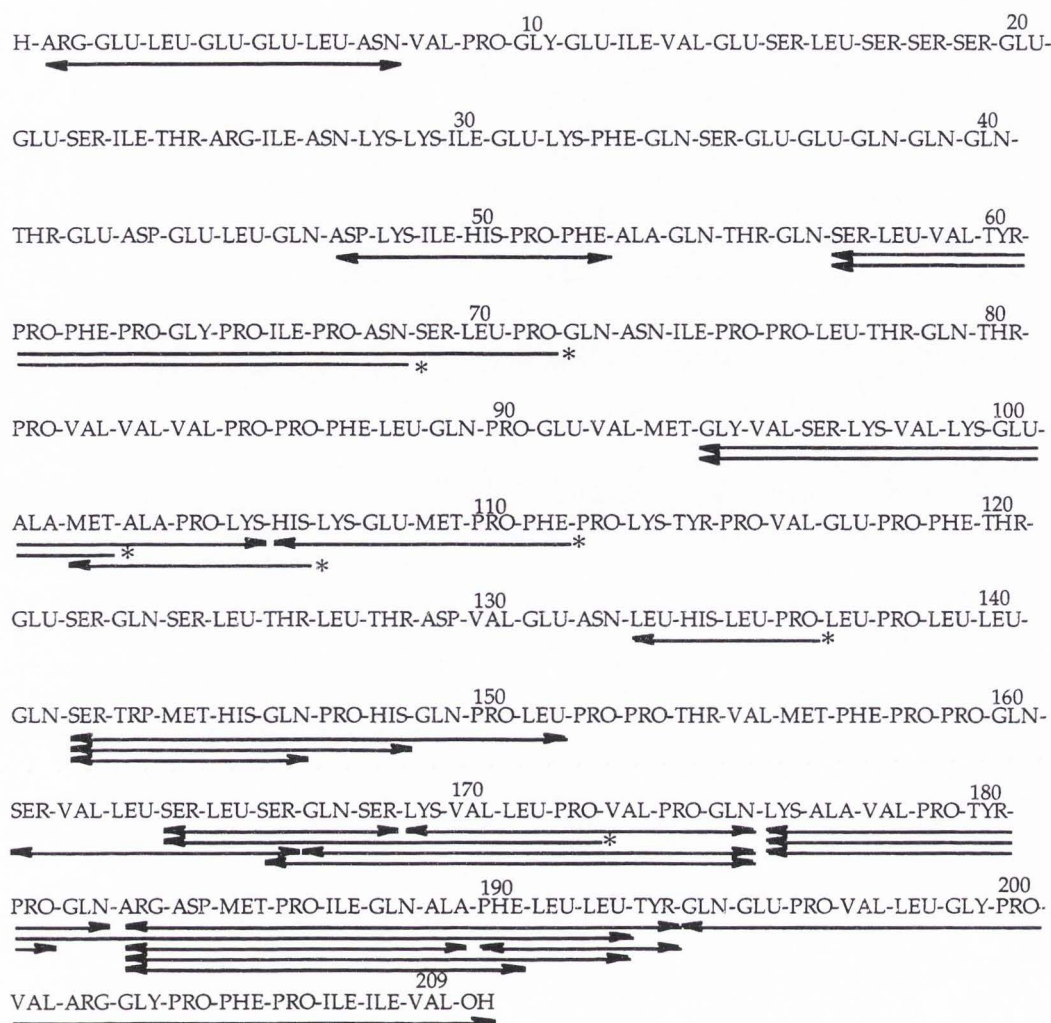


Figure 3.  $\beta$ -Casein sequence and cut sites for the  $P_I$ -type proteinase from *L. lactis* subsp. *cremoris* HP (125). (\*) means that the ends were not determined.

32, 72-79, and 96-106). The peptides produced in the earlier stages contain rich regions of histidine, which provides the cells with essential amino acid. This could explain why these peptides are generated first. Exterkate et al. (39) also show that there are strains which show a mixed ( $P_I/P_{III}$  or  $P_{III}/P_I$ ) proteinase type.

Nucleotide sequences for *L. lactis* subsp. *cremoris* Wg2 ( $P_I$ -type) and *L. lactis* subsp. *cremoris* SK11 ( $P_{III}$ -type) have been determined (158). These

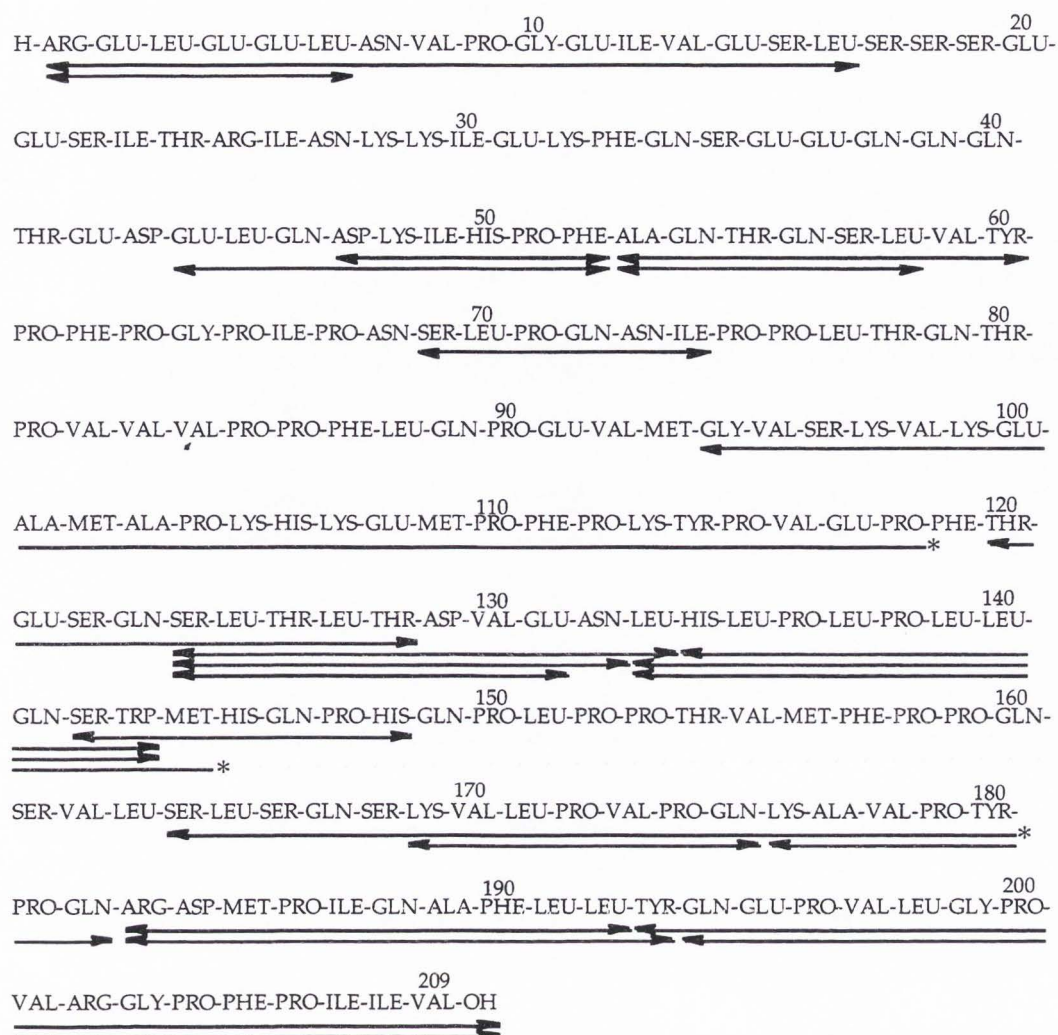


Figure 4.  $\beta$ -Casein sequence and cut sites for the  $P_{III}$ -type proteinase from *L. lactis* subsp. *cremoris* SK112 (125).(\*) means that the ends were not determined.

proteinases are similar in amino acid composition, have approximately the same molecular weight, and are immunologically and genetically related. The caseinolytic cleavage specificity, however, is different under optimum conditions.

The specificity differences of proteinases is believed to be a result of five amino acid substitutions in the subtilisin-like binding region (Table 8) (39, 158).

TABLE 6. TFA soluble fragments of  $\kappa$ -casein digested with  $P_I$ -type proteinase from *L. lactis* subsp. *cremoris* H2 (126).

$\kappa$ -Casein fragment	Amino acid sequence
24-32	Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu
33-41	Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu-Asn
72-79	Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu
96-106	Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met
96-100	Ala-Arg-His-Pro-His
96-102	Ala-Arg-His-Pro-His-Pro-His
103-106	Leu-Ser-Phe-Met

TABLE 7. TFA soluble fragments of  $\kappa$ -casein digested with  $P_{III}$ -type proteinase from *L. lactis* subsp. *cremoris* SK11 (126).

$\kappa$ -Casein fragment	Amino acid sequence
24-32	Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu
66-79	Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu
72-79	Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu
96-106	Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met
152-160	Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn
161-169	Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val

Exterkate et al. (39) also found a fragment C that contains nine amino acid substitutions between the proteinases. Two of these substitutions, Leu<sub>747</sub>  $\rightarrow$  Arg and Thr<sub>748</sub>  $\rightarrow$  Lys, are associated with specificity of the proteinases. Some strains in Table 8 show a mixed proteinase pattern ( $P_I/P_{III}$ ). *Lactococcus lactis* subsp. *cremoris* E8 and FD27 show the same amino acid substitutions in their subtilisin-like binding region. On the other hand, substitutions in the C fragment follow

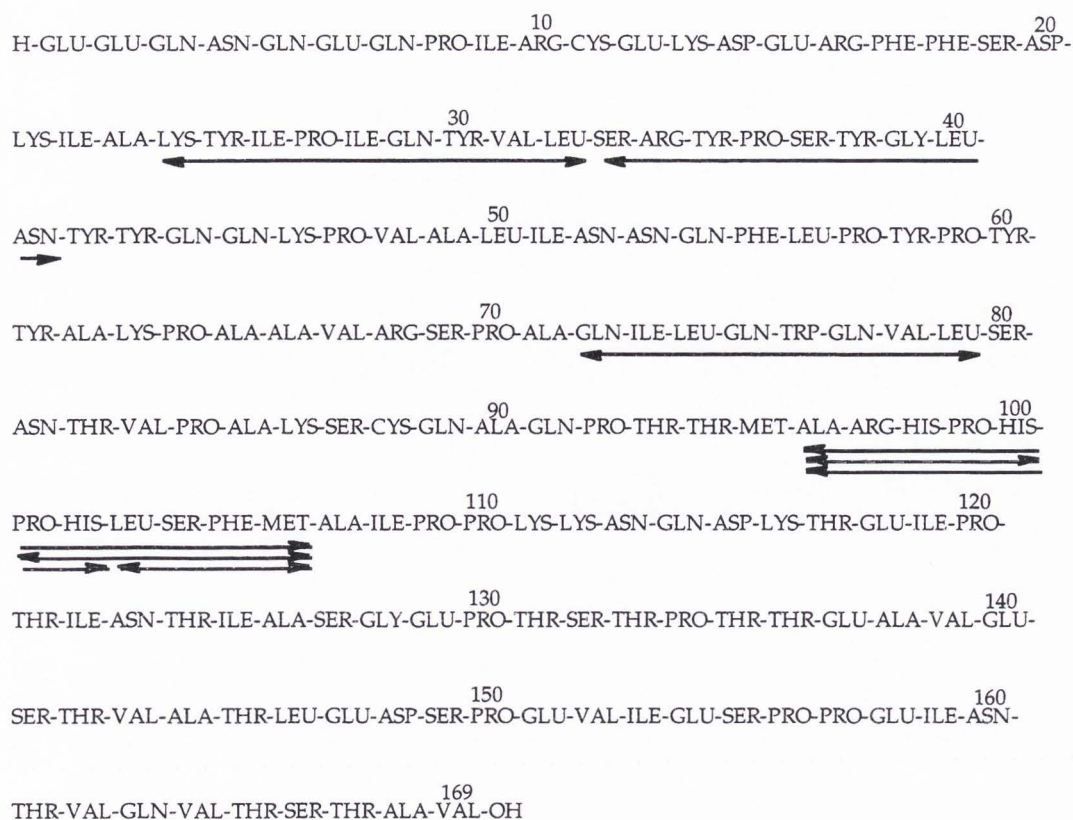


Figure 5.  $\kappa$ -Casein sequence and cut sites for the  $P_I$ -type proteinase from *L. lactis* subsp. *cremoris* H2 (126).

either a  $P_I$  or a  $P_{III}$  substitution compared with SK11 ( $P_{III}$ ) and HP ( $P_I$ ). This leads to the mixed proteinase characteristics of E8 and FD27. These discrepancies led to the new classification of proteinase types into groups a-g. Broadbent et al. (14) recently found a new proteinase type (group h), which had not been reported before. They found that strain *L. lactis* subsp. *cremoris* S3 showed an identical distal binding region to groups f and g. However, the subtilisin-like binding region showed yet another amino acid substitution (Table 8). Its specificity toward  $\alpha_{s1}$  (f1-23)-casein under cheese-like conditions shows a different pattern on reverse-phase HPLC than has been reported for proteinases in any of the other groups (14).



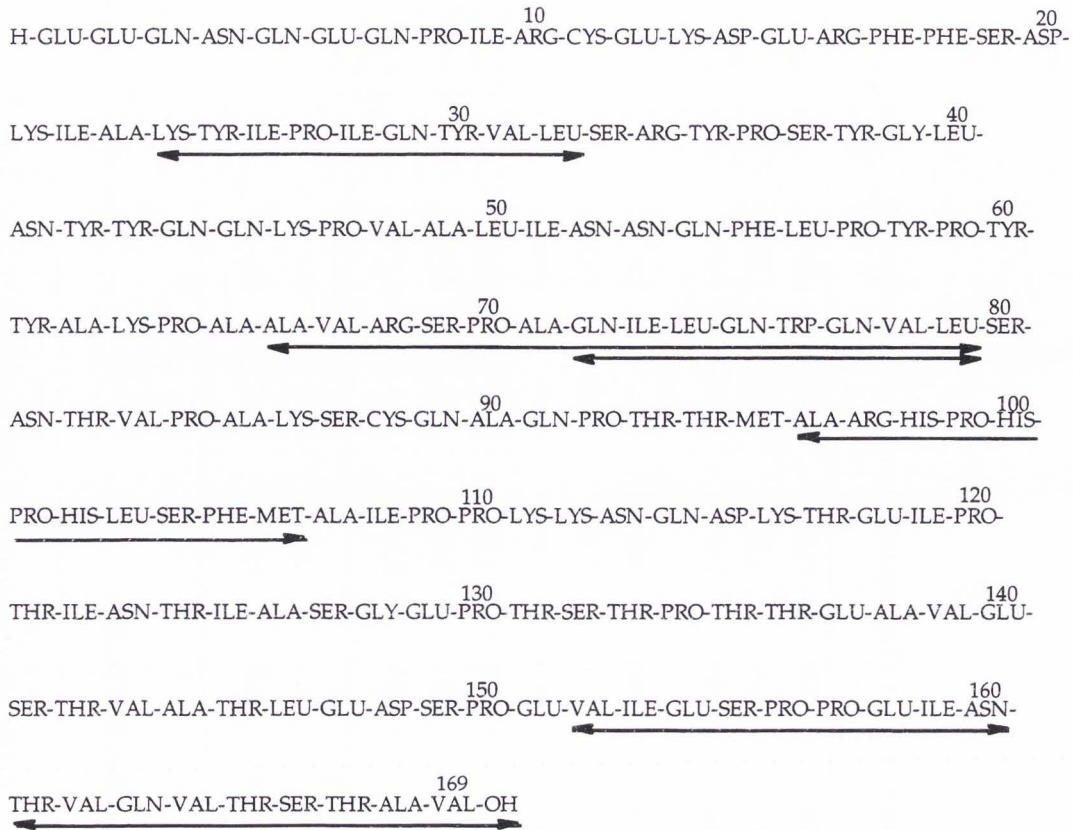


Figure 6.  $\kappa$ -Casein sequence and cut sites for the  $P_{III}$ -type proteinase from *L. lactis* subsp. *cremoris* SK11(126).

### Proteinase Regulation

Laan et al. (85) studied the regulation of proteinase synthesis of *L. lactis* subsp. *cremoris* Wg2. They showed that strains grown in the presence of amino acids as the only N-source have a higher specific growth rate than strains grown in milk. This is surprising because growth on a medium containing caseins as the only source of amino acids is dependent on proteinase activity, which means that there are some inhibitory factors in milk. Laan et al. (85) also found that proteinase production is inhibited by addition of casein, tryptic casein digest, or dipeptide Leu-Pro and they suggested that exogenously supplied peptides

TABLE 8. Amino acid substitutions for P<sub>I</sub> - and P<sub>III</sub> -type proteinases in various *L. lactis* strains (14, 39, 81).

Region	Amino acid residue <sup>1</sup>	Proteinase group							
		a	b	c	d	e	f	g	h
		SK11 (P <sub>III</sub> )	AM2 (P <sub>I</sub> )	E8 (P <sub>III</sub> /P <sub>I</sub> )	NCDO 763 (P <sub>I</sub> )	Wg2 (P <sub>I</sub> )	FD27 (P <sub>I</sub> /P <sub>III</sub> )	HP (P <sub>I</sub> )	S3 (?)
Subtilisin-like binding region	131	Ser	Thr	Thr	Thr	Thr	Thr	Thr	Thr
	138	Lys	Thr	Thr	Thr	Thr	Thr	Thr	Thr
	142	Ala	Asp	Ala	Ala	Ser	Ala	Asp	Ala
	144	Val	Leu	Leu	Leu	Leu	Leu	Leu	Leu
	166	Asn	Asp	Asp	Asp	Asp	Asp	Asp	Gly
	177	Leu	Ile	Ile	Leu	Leu	Leu	Ile	Ile
C fragment	747	Arg	Leu	Arg	Leu	Leu	Leu	Leu	Leu
	748	Lys	Thr	Lys	Thr	Thr	Thr	Thr	Thr
	763	Asn	His	Asn	His	Asn	His	His	His

<sup>1</sup> Amino acid numbering is identical to *L. lactis* subsp. *cremoris* SK11 proteinase amino acid sequence.

control regulation of proteinase synthesis.

### Lactococcal Peptidase System

Two potentially different pathways for degradation of large oligopeptide can be visualized: (a) cleavage by exopeptidase activity, where amino acids or dipeptides are removed from the N-terminal end, and (b) endopeptidase activity, where oligopeptides are degraded into smaller peptides and further degraded by aminopeptidases.

*Exopeptidase activity.* Four different aminopeptidases with the potential to cleave relatively large oligopeptides have been purified from lactococci. The aminopeptidase PepX, or X-prolyl dipeptidyl aminopeptidase, has been characterized extensively (71, 98, 101, 110, 162). This enzyme catalyses the cleavage of the N-terminal X-prolyl dipeptidyl residue. The primary products are X-Pro dipeptides, where X represents a wide range of amino acids such as Leu, Met, Gly, Ala, etc. (102). X-Pro dipeptidases are then further degraded into individual amino acids by prolidases (11, 69). With the high concentration of proline in caseins, especially  $\beta$ -casein, this could be one mechanism of action for the bacteria to obtain free amino acids and the growth-stimulating amino acid Pro. Another aminopeptidase, PepN, shows high activity toward substrates that contain Lys, Leu, or Arg at their N-terminus. Tan (143) observed that PepN cleaves more than one amino acid residue from  $\beta$ -casein digests. Baankreis and Exterkate (7), however, proposed that PepN is involved in metabolism of small oligopeptides, since PepN deficient strains have impaired ability to degrade tetra-, penta-, and heptapeptide substrates. A third aminopeptidase, PepA, is specific for the N-terminal Asp and Glu residues, thus complementing PepN, which does not use Glu residues (38, 144). The final aminopeptidase, PepC, shows high activity toward Ala, Lys, His, and Glu. The peptide has so far only

been purified from *L. lactis* subsp. *cremoris* AM2 (112). All four aminopeptidases (PepX, PepN, PepA, and PepC) may potentially work together and degrade a variety of oligopeptides for further transport into the cell.

**Endopeptidase activity.** A more probable route for cleavage of oligopeptides is the action of endopeptidases on oligopeptides to produce smaller peptides (122). Knowledge of endopeptidases is not as extensive as it is for exopeptidases, because of poor assays (122). Most studies have used casein fragments as substrates. The only well characterized endopeptidase system is that of the cell-envelope proteinase system, and the number of other endopeptidases in lactococci is not as clear (122).

Yan et al. (160, 161) isolated two different endopeptidases from *L. lactis* subsp. *cremoris* H61, LEPI and LEPII, which could degrade oligopeptides in the range of 7 to 30 amino acid residues long. However, the enzymes were not effective on whole caseins. Tan et al. (146) isolated an endopeptidase from *L. lactis* subsp. *cremoris* Wg2, called PepO, that also degrades a variety of oligopeptide sizes but not whole caseins. A similar enzyme, neutral oligopeptidase (NOP), was isolated by Baankreis (6) from *L. lactis* subsp. *cremoris* C13.

Intracellular endopeptidases have also been purified from *L. lactis* subsp. *lactis* NCDO763 (109, 110) and *L. lactis* subsp. *lactis* bv. *diacetyllactis* (27), and they both show similarities to LEPII and NOP specificities but they differ in molecular weight and their ability to degrade whole caseins. A metalloendopeptidase was isolated from *L. lactis* subsp. *lactis* HP by Baankreis (6) and it was similar in nature to the enzyme LEPI isolated by Yan et al. (160).

Other amino-, di-, and tri-peptidases have been isolated (8, 12, 66, 152). Also prolidases, a proline dipeptidase capable of cleaving proline-containing

dipeptides, and proline iminopeptidases have been purified and characterized (7, 10, 69).

## LACTOCOCCAL PROTEIN TRANSPORT SYSTEMS

### **Arginine/Ornithine Amino Acid Transport Systems**

Various lactococci can metabolize Arg to Orn, CO<sub>2</sub>, and ammonia by the arginine deiminase (ADI) pathway (1, 23, 148). The pathway consists of three intracellular enzymes and one antiport. An antiport allows the stoichiometric exchange between extracellular and intracellular material. This antiport catalyzes the exchange between extracellular Arg and intracellular Orn (24, 31, 118). The driving force for this exchange is supplied by the Arg and Orn concentration gradient. Thus, no energy is required for transport of Arg across the membrane.

### **Proton-Motive-Force-Coupled Amino Acid Transport Systems**

Lactococci cannot generate a proton-motive force (PMF) by electron flow as found in the electron-transfer system of *Clostridium* species and other heme-containing bacteria, since they are unable to synthesize cytochromes (76). In lactococci, however, the ATP formed through substrate-level phosphorylation is consumed on the membrane-bound ATPase complex to generate an electrochemical gradient for protons across the membrane. The driving force for H<sup>+</sup> dislocation is supplied by the free energy for ATP hydrolysis (76).

*L. lactis* subsp. *cremoris* Wg2 facilitates the translocation of Leu, Ile, and Val together with one proton (21, 28, 29, 30). Ala, Gly, Ser, and Thr are also translocated across the membrane using a PMF-coupled system. Driessen (27) suggested that Ser and Thr share a common transport system and that Ala

together with Gly share a distinctively different transport system in *L. lactis* subsp. *cremoris*.

A Lys carrier, which also has specificity for Orn, has been studied in *L. lactis* subsp. *cremoris* Wg2 (32). This carrier has a strong substrate specificity that is distinct from the Arg/Orn antiport, though together they maintain the Arg concentration needed in the cell, since some Arg is used up during biosynthesis (76).

Limited studies have been done on other amino acids, although these studies show that uptake of His, Pro, Met, Cys, Tyr, and Phe is linked to a PMF in *L. lactis* species (27, 29, 116, 120).

### **Phosphate-Bond-Driven Amino Acid Transport**

The phosphate-bond-driven (ATP-driven) system is a unidirectional, transinhibitional, and internal pH regulated system but the driving force of the system has not yet been established (76). Production of energy by glycolysis, ATP, or through the ADI pathway is required (121). Glutamate, Gln, Asp, and Asn use this transport system. In lactococci, Glu and Asp are both taken up in their acidic forms, though they possess different mechanisms of transport across the membrane (119, 121).

### **Di/Tripeptide and Oligopeptide Transport**

The oligopeptides derived from caseins must further be degraded into smaller peptide sizes that can be transported across the membrane. Free amino acids are also formed and these are transported across the membrane as described above.

Carboxypeptidase activity has not been found in any lactococci strains

and thus, lactococci can only degrade casein-derived peptides from the N-terminal end. With the initial breakdown of  $\beta$ -casein by general aminopeptidases, free amino acids and smaller peptides are produced (139). However, none of the amino acids essential for lactococci (Ile, His) or growth stimulating amino acids (Pro, Phe) are produced (127), and thus the microorganism has to obtain these essential amino acids through di-/tripeptide and oligopeptide transport. The  $\beta$ -casein derived fragment containing  $\beta$ -casomorphin is broken into dipeptides by PepX (71). The lactococcal di- and tripeptide transport system (DtpP and DtpT) shows high affinity for PepX-derived substrates. Since free Pro only enters the cell via passive diffusion, the dipeptides entering this way provide the cell with the essential amino acids Pro, Phe, and Ile (138).

The process of transporting dipeptides across the membrane requires energy. Van Boven and Konings (151) studied uptake of the dipeptide Leu-Leu in *L. lactis* subsp. *cremoris* and suggested that it is ATP-driven. Smid et al. (137) studied the uptake mechanism for Ala-Glu and suggested that it is PMF-driven. This system also has affinity for dipolar ionic and negatively charged peptides. Kunji et al. (82) described two different transport systems, one which preferentially translocates di- and tripeptides and a second which transports up to six amino acid residue-long peptides. However, they did not know whether the di- and tripeptides can be translocated via the oligopeptide transport system or if they use two different transport systems.

Oligopeptide transport is coupled to ATP hydrolysis (82). An oligopeptide transport system (OPP) was found in *L. lactis* subsp. *lactis* SL135 that is encoded on a plasmid pSV8 (149). This plasmid encodes all the genes necessary for the oligopeptide transport system (*opp*) and for an endopeptidase (*pepO*).

Oligopeptide transport is essential for uptake of tripeptides or peptides up to eight amino acid residues long. Kunji et al. (80) also showed that the Opp system is essential for *L. lactis* growth on  $\beta$ -casein-derived peptides and the transport system provided high growth rates, if Leu and His were added as free amino acids. It does not appear, however, that PepO is essential for growth in milk (149). Table 9 shows a summary of the different transport systems and their specificities (76, 81, 139).

TABLE 9. Transport systems found in *Lactococcus* with their specificities and their energy driving mechanisms (76).

Transport system	Mechanism <sup>1</sup>	Specificity
Branched-chain amino acids	PMF	Leu, Ile, Val
Neutral amino acids I	PMF	Ala, Gly
Neutral amino acids II	PMF	Ser, Thr
Basic amino acids I	PMF	Lys, Orn
Basic amino acids II	PMF	His <sup>2</sup>
Aromatic amino acids	PMF	Phe, Tyr, Trp <sup>3</sup>
Glutamate	ATP	Glu, Gln
Asparagine	ATP	Asn
Aspartate	exchange	Asp, Glu <sup>4</sup>
Arg-Orn antiport	antiport	Arg, Orn, Lys
Proline	diffusion	Pro
Di- and Tripeptides	PMF/ATP	Di- and tripeptides <sup>5</sup>
Oligopeptides	ATP	Tri- and hexapeptides <sup>6</sup>

<sup>1</sup>PMF = proton-motive-force, ATP = ATP-driven

<sup>2</sup>Substrate specificity has not been studied

<sup>3</sup>Substrate specificity has not been studied in detail

<sup>4</sup>System with low affinity for acidic amino acids

<sup>5</sup>Arginine containing peptides are not transported

<sup>6</sup>Neutral oligopeptides not containing proline residues



## CONCLUSION

Understanding the proteolytic system of cheese starter bacteria will benefit the dairy industry. Our research focused on characterizing the proteolytic system of *Lactococcus lactis* ssp. *lactis* with the help of Amino Acid Analysis. Strains with various proteolytic make-up were constructed and their ability to hydrolyze milk proteins was investigated.

Bitterness, acidity, and rancidity flavors were evaluated by trained sensory panels to see the influence of these bacteria on Cheddar cheese flavors. Tremendous work is required to investigate all parameters of these starter cultures. Thus, another of our goals was to develop a statistical prediction model to see the ability of a starter strain to hydrolyze milk proteins. Based on dendograms, the industry can thus choose a strain that will act the same or similarly on a certain milk protein. This strain can thus be used in the fermentation process regardless of its proteolytic genotype.

## REFERENCES

- 1 Abdelal, A. T. 1979. Arginine catabolism by microorganisms. *Annu. Rev. Microbiol.* 33:139.
- 2 Alkhalad, W., M. El Soda, J. C. Gripon, and L. Vassal. 1989. Acceleration of cheese ripening with liposome-entrapped proteinase: influence of liposomes net charge. *J. Dairy Sci.* 72:2233.
- 3 Alkhalad, W., J. C. Piard, M. El Soda, J. C. Gripon, M. Desmazeaud, and L. Vassal. 1988. Liposomes as proteinase carriers for the accelerated ripening of Saint Paulin-type cheese. *J. Food Sci.* 53:1674.

- 4 Aston, J. W., P. A. Grieve, I. G. Durward, and J. R. Dullely. 1983. Proteolysis and flavour development in Cheddar cheese subjected to accelerated ripening treatments. *Aust. J. Dairy Technol.* 38:59.
- 5 Axelsson, L. T. 1993. Lactic acid bacteria. Page 3 *in* Lactic Acid Bacteria: Classification and Physiology. S. Salminen and A. von Wright, eds. Marcel Dekker, Inc., New York, NY.
- 6 Baankreis, R. 1992. The role of lactococcal peptidases in cheese ripening. Ph.D. thesis. Univ. Amsterdam, Netherlands.
- 7 Baankreis, R., and F. A. Exterkate. 1991. Characterization of a peptidase from *Lactococcus lactis* subsp. *cremoris* HP that hydrolyzes di- and tripeptides containing proline or hydrophobic residues as the aminoterminal amino acid. *Syst. Appl. Microbiol.* 14:317.
- 8 Bacon, C. L., M. Wilkinson, P. V. Jennings, I. Ni Fhaolain, and G. O'Cuinn. 1993. Purification and characterization of an aminotripeptidase from cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2. *Int. Dairy J.* 3:163.
- 9 Birkeland, S. E., and R. K. Abrahamsen. 1987. Accelerated cheese ripening. II. Review of methods. *Meieriposten* 76:601.
- 10 Boeckelmann, W., B. Kiefer, A. Geis, and M. Teuber. 1988. Degradation of  $\beta$ -casein by mesophilic starter bacteria. Page 225 *in* Milk Proteins. Nutritional, Clinical, Functional and Technological Aspects. C. A. Barth and E. Schlimme, eds. Springer Verlag, New York, NY.
- 11 Booth, M., V. Jennings, I. Ni Fhaolain, and G. O'Cuinn. 1990. Prolidase activity of *Lactococcus lactis* subsp. *cremoris* AM2: partial purification and characterization. *J. Dairy Res.* 57:245.
- 12 Bosman, B. W., P. S. T. Tan, and W. N. Konings. 1990. Purification and characterization of a tripeptidase from *Lactococcus lactis* subsp. *cremoris*

- Wg2. Appl. Environ. Microbiol. 56:1839.
- 13 Broadbent, J. R., and J. K. Kondo. 1993. Biotechnology of dairy starter cultures. Page 77 in Dairy Science and Technology Handbook. Vol. 2. Y. H. Hui, ed. VHC Publishers Ltd., Cambridge, UK.
  - 14 Broadbent, J. R., M. Strickland, B. C. Weimer, M. E. Johnson, and J. L. Steele. 1997. Peptide accumulation and bitterness in Cheddar cheese made using *Lactococcus lactis* single-starters with distinct proteinase specificities. J. Dairy Sci. 81:327.
  - 15 Broome, M. C., D. A. Krause, and M. W. Hickey. 1991. The use of proteinase negative starter and lactobacilli in Cheddar cheese manufacture. Aust. J. Dairy Technol. 46:6.
  - 16 Broome, M. C., and M. W. Hickey. 1991. Proteinase activity of non-starter lactobacilli. Aust. J. Dairy Technol. 46:12.
  - 17 Broome, M. C., and M. W. Hickey. 1991. Peptidase activity of non-starter lactobacilli. Aust. J. Dairy Technol. 46:19.
  - 18 Caldwell, S. L., D. J. McMahon, C. J. Oberg, and J. R. Broadbent. 1996. Development and characterization of lactose-positive *Pediococcus* species for milk fermentation. Appl. Environ. Microbiol. 62:936.
  - 19 Castaneda, R., L. Vassal, J. C. Gripon, and M. Rousseau. 1990. Accelerated ripening of Saint Paulin cheese variant by addition of heat-shocked lactobacillus suspension. Neth. Milk Dairy J. 44:49.
  - 20 Castberg, H. B., and H. A. Morris. 1976. Degradation of milk proteins by enzymes from lactic acid bacteria used in cheese making. A review. Milchwiss. 31:85.
  - 21 Crielaard, W., A. J. M. Driessen, D. Molenaar, K. J. Hellingwerf, and W. N. Konings. 1988. Light-induced amino acid uptake in membrane vesicles of

- Streptococcus cremoris* and *Clostridium acetobutylicum* fused with reaction center containing proteoliposomes. J. Bacteriol. 170:1820.
- 22 Cromie, S. J., J. E. Giles, and J. R. Dulley. 1987. Effect of elevated ripening temperature on microflora of Cheddar cheese. J. Dairy Res. 54:69.
- 23 Crow, V. L., and T. D. Thomas. 1982. Arginine metabolism in lactic streptococci. J. Bacteriol. 150:1024.
- 24 Cunin, R., N. Glansdorff, A. Pierrd, and V. Stalon. 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. 50:314.
- 25 Desmazeaud, M. J., and J. C. Gripon. 1977. General mechanism of protein breakdown during cheese ripening. Milchwiss. 32:731.
- 26 Desmazeaud, M. J., and C. Zevaco. 1976. General properties and substrate specificity of an intracellular neutral protease from *Streptococcus diacetylactis*. Ann. Biol. Anim. Biochim. Biophys. 16:851.
- 27 Driessen, A. J. M. 1987. Amino acid transport in lactic streptococci. Ph.D. thesis, Univ. Groningen, Groningen, Netherlands.
- 28 Driessen, A. J. M., K. J. Hellingwerf, and W. N. Konings. 1987. Mechanism of energy coupling to entry and exit of neutral and branched chain amino acids in membrane vesicles of *Streptococcus cremoris*. J. Biol. Chem. 262:12438.
- 29 Driessen, A. J. M., S. De Jong, and W. N. Konings. 1987. Transport of branched chain amino acids in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 169:5193.
- 30 Driessen, A. J. M., J. Kodde, S. De Jong, and W. N. Konings. 1987. Neutral amino acid transport by membrane vesicles of *Streptococcus cremoris* is subjected to regulation by internal pH. J. Bacteriol. 169:2748.

- 31 Driessen, A. J. M., B. Poolman, R. Kiewiet, and W. N. Konings. 1987. Arginine transport in *Streptococcus lactis* is catalyzed by a cation exchanger. Proc. Natl. Acad. Sci. USA 84:6093.
- 32 Driessen, B., C. Van Leeuwen, and W. N. Konings. 1989. Transport of basic amino acids by membrane vesicles of *Lactococcus lactis*. J. Bacteriol. 171:1453.
- 33 Dulay, T. A. 1980. Studies on accelerated cheese ripening for process cheese spread manufacture. Dairy Sci. Abstr. 43:5540.
- 34 Dulley, J. R. 1976. The utilization of cheese slurries to accelerate the ripening of Cheddar cheese. Aust. J. Dairy Technol. 31:143.
- 35 Dulley, J. R., and G. C. Taylor. 1972. Proc. Aust. Biochem. Soc. 5:52.
- 36 El Soda, M., M. Johnson, and N. F. Olson. 1989. Temperature sensitive liposomes: a controlled release system for the acceleration of cheese ripening. Milchwiss. 44:213.
- 37 Exterkate, F. A., A. C. Alting, and C. J. Slangen. 1991. Specificity of two genetically related cell-envelope proteinases of *Lactococcus lactis* subsp. *cremoris* towards  $\alpha_{s1}$ -casein (1-23)-fragment. Biochem. J. 273:135.
- 38 Exterkate, F. A., M. De Jong, G. J. C. M. DeVeer, and R. Baankreis. 1992. Location and characterization of aminopeptidase N in *Lactococcus lactis* subsp. *cremoris* HP. Appl. Microbiol. Biotechnol. 37:46.
- 39 Exterkate, F. A., A. C. Alting, and P. G. Bruinenberg. 1993. Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. Appl. Environ. Microbiol. 59:3640.
- 40 Farkye, N. Y., and P. F. Fox, 1991. Preliminary study on the contribution of plasmin to proteolysis in Cheddar cheese: cheese containing plasmin

- inhibitor, 6-aminohexanoic acid. *J. Agric. Food Chem.* 39:786.
- 41 Farkye, N. Y., and P. F. Fox, 1992. Contribution of plasmin to Cheddar cheese ripening: effect of added plasmin. *J. Dairy Res.* 59:209.
- 42 Farkye, N. Y., P. F. Fox, and G. F. Fitzgerald. 1990. Proteolysis and flavor development in Cheddar cheese made exclusively with single strain proteinase -positive or proteinase-negative starters. *J. Dairy Sci.* 73:874.
- 43 Fedrick, I. 1987. Technology and economics of the accelerated ripening of Cheddar cheese. *Aust. J. Dairy Technol.* March/June:33.
- 44 Fedrick, I. A., S. J. Cromie, J. R. Dulley, and J. E. Giles. 1986. The effects of increased starter populations, added neutral proteinase and elevated temperature storage on Cheddar cheese manufacture and maturation. *N. Z. J. Dairy Sci. Technol.* 21:191.
- 45 Fox, P. F. 1989. Acceleration of cheese ripening. *Food Biotechnol.* 2:133.
- 46 Fox, P. F. 1989. Proteolysis in cheese during manufacture and ripening. *J. Dairy Sci.* 72:1379.
- 47 Fox, P. F. 1993. Exogenous enzymes in dairy technology. A review. *J. Food Biochem.* 17:173.
- 48 Fox, P. F. and M. B. Grufferty. 1991. Exogenous enzymes in dairy technology. Page 219 *in* *Food Enzymology*. Vol. 1. P. F. Fox, ed. Elsevier Appl. Sci., London.
- 49 Fox, P. F., J. Law, P. L. H. McSweeney, and J. Wallace. 1993. Biochemistry of cheese ripening. Page 389 *in* *Cheese: Chemistry, Physics and Microbiology*. 2nd ed. P. F. Fox, ed. Chapman & Hall, London.
- 50 Fox, P. F., J. A. Lucey, and T. M. Cogan. 1990. Glycolysis and related reactions during cheese manufacture and ripening. *Crit. Rev. Food Sci. Nutr.* 29:237.

- 51 Fresta, M., E. Wehrli, and G. Puglisi. 1995. Neutrase entrapment in stable multilamellar and large unilamellar vesicles for acceleration of cheese ripening. *J. Microencapsulation* 12:307.
- 52 Frey, J. P., E. H. Marth, M. E. Johnson, and N. F. Olson. 1986. Heat- and freeze-shocking cause change in peptides and protease activity of *Lactobacillus helveticus*. *Milchwiss.* 41:681.
- 53 Garcia, F. E., H. Reuter, D. Prokopek, A. Olano, and M. Ramos. 1993. Effect of enzyme addition on the manufacture of Spanish hard cheese from milk concentrated by ultrafiltration. II. Ripening of cheeses. *Kieler Milchwirtschaftliche Forschungsberichte* 45:301.
- 54 Garvie, E. I. 1984. Taxonomy and identification of dairy bacteria important in cheese and fermented dairy products. Page 56 in *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davies and B. A. Law, eds. Elsevier Appl. Sci., London.
- 55 Grappin, R., T. C. Rank, and N. F. Olson. 1985. Primary proteolysis of cheese proteins during ripening. *J. Dairy Sci.* 68:531.
- 56 Guinee, T. P., M. G. Wilkinson, E. O. Mulholland, and P. F. Fox. 1991. Influence of ripening temperature, added commercial enzyme preparations and attenuated mutant ( $Lac^-$ ) *Lactococcus lactis* starter on the proteolysis and maturation of Cheddar cheese. *Irish J. Food Sci. Technol.* 15:27.
- 57 Haandrikman, A. J., J. Kok, H. Laan, S. Soemito, A. M. Ledebøer, W. N. Konings, and G. Venema. 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. *J. Bacteriol.* 171:2789.
- 58 Haandrikman, A. J., J. Kok, and G. Venema. 1991. Lactococcal proteinase

- maturation protein PrtM is a lipoprotein. *J. Bacteriol.* 173:4517.
- 59 Haandrikman, A. J., R. Meesters, H. Laan, W. N. Konings, J. Kok, and G. Venema. 1991. Processing of the lactococcal extracellular serine proteinase. *Appl. Environ. Microbiol.* 57:1899.
- 60 Hammes, W. P., A. Bantleon, and S. Min. 1990. Lactic acid bacteria in meat fermentation. *FEMS Microbiol. Rev.* 87:165.
- 61 Harper, W. J., and T. Kristoffersen. 1970. Biochemical aspect of flavor development in Cheddar cheese slurries. *J. Agric. Food. Chem.* 18:563.
- 62 Harper, W. J., A. Carmona, and T. Kristoffersen. 1971. Protein degradation in Cheddar cheese slurries. *J. Food Sci.* 36:503.
- 63 Hayakawa, K. 1992. Classification and actions of food microorganisms with particular reference to fermented foods and lactic acid bacteria. Page 139 *in* Functions of Fermented Milk. Y. Nakarawa and A. Hosomo, eds. Elsevier Appl. Sci., London.
- 64 Hayashi, K., D. F. Revell, and B. A. Law. 1990. Accelerated ripening of Cheddar cheese with the aminopeptidase of *Brevibacterium linens* and a commercial neutral proteinase. *J. Dairy Res.* 57:571.
- 65 Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Stanley, and S. T. Williams. *Bergey's Manual of Determinative Bacteriology*. Vol. 2. Williams & Wilkins, Baltimore, MD.
- 66 Hwang, I. K., S. Kaminogawa, and K. Yamauchi. 1981. Purification and properties of a dipeptidase from *Streptococcus cremoris*. *Agric. Biol. Chem.* 45:159.
- 67 IDF 1991. Chemical methods for evaluating proteolysis in cheese maturation. Bulletin 216, International Dairy Federation, Brussels.



- 68 Jay, J. M. 1986. Fermented foods and related products of fermentation. Page 364 in *Modern Food Microbiology*. 3rd ed. J. M. Jay, ed. Van Nostrand Reinhold Co., New York, NY.
- 69 Kaminogawa, S., N. Azuma, I. K. Hwang, Y. Susuki, and K. Yamauchi. 1984. Isolation and characterization of a prolidase from *Streptococcus cremoris* H61. *Agric. Biol. Chem.* 48:3035.
- 70 Kempler, G. M., K. A. Baldwin, L. L. McKay, H. A. Morris, S. Halambeck, and G. Thorsen. 1979. Use of genetic alterations to improve *Streptococcus lactis* CZ as a potential Cheddar cheese starter. *J. Dairy Sci.* 62 (Suppl.1): 42 Abstract.
- 71 Kiefer-Partsch, B., W. Boeckelmann, A. Geiss, and M. Teuber. 1989. Purification of an X-prolyl-dipeptidyl aminopeptidase from the cell wall proteolytic system of *Lactococcus lactis* subsp. *cremoris*. *Appl. Microbiol. Biotechnol.* 31:75.
- 72 Kim, M. S., S. C. Kim, and N. F. Olson. 1994. Effect of commercial fungal protease and freeze-shocked *Lactobacillus helveticus* ODR101 on accelerating cheese fermentation: I. Composition. *Milchwiss.* 49:256.
- 73 Kiwaki, M., H. Ikemura, M. Shimidzu-Kadota, and A. Hirashima. 1989. Molecular characterization of a cell wall-associated proteinase gene from *Streptococcus lactis* NCDO763. *Mol. Microbiol.* 3:359.
- 74 Kok, J. 1990. Genetics of the proteolytic system of the lactic acid bacteria. *FEMS Microbiol. Rev.* 87:15.
- 75 Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Ledeboer, and G. Venema. 1988. Nucleotide sequence of the cell wall proteinase gene from *Streptococcus cremoris* Wg2. *Appl. Environ. Microbiol.* 54:231.
- 76 Konings, W. N., B. Poolman, and A. J. M. Driessen. 1989. Bioenergetics

- and solute transport in lactococci. *CRC Crit. Rev. Microbiol.* 16:419.
- 77 Kosikowski, F. V. 1977. Cultures and starters. Page 16 *in* Cheese and Fermented Milk Foods. 2<sup>nd</sup> ed. F. V. Kosikowski, ed. F. V. Kosikowski and Assoc., Brooktondale, NY.
- 78 Krause, D. A., M. C. Broome, and M. W. Hickey. 1991. The isolation and characterization of a proteinase negative cheese starter. *Aust. J. Dairy Sci. Technol.* 46:1.
- 79 Kristoffersen, T., E. M. Mikolajcik, and I. A. Gould. 1967. Cheddar cheese flavor. IV. Direct and accelerated ripening process. *J. Dairy Sci.* 50:292.
- 80 Kunji, E. R. S., A. Hagting, C. J. De Vries, V. Juillard, A. J. Haandrikman, B. Poolman, and W. N. Konings. 1995. Transport of  $\beta$ -casein derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. *J. Biol. Chem.* 270:1569.
- 81 Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoeck* 70:187.
- 82 Kunji, E. R. S., E. J. Smid, R. Plapp, B. Poolman, and W. N. Konings. 1993. Di-tripeptides and oligopeptides are taken up via distinct transport mechanisms in *Lactococcus lactis*. *J. Bacteriol.* 175:2052.
- 83 Kurman, J. A. J. L. Rasic, and M. Kroger. 1992. *Encyclopedia of Fermented Fresh Milk Products*. Van Nostrand Reinhold, New York, NY.
- 84 Laan, H., E. J. Smid, P. S. T. Tan, and W. N. Konings. 1989. Enzymes involved in the degradation and utilization of casein in *Lactococcus lactis*. *Neth. Milk Dairy J.* 43:327.
- 85 Laan, H., H. Bolhuis, B. Poolman, T. Abee, and W. N. Konings. 1993. Regulation of the proteinase synthesis in *Lactococcus lactis*. *Acta*

Biotechnol. 13:95.

- 86 Law, B. A. 1978. The accelerated ripening of cheese by use of non-commercial starter and enzymes—a preliminary assessment. International Dairy Federation, Document 108, Brussels, Belgium.
- 87 Law, B. A. 1980. Accelerated ripening of cheese. *Dairy Ind. Intern.* 45:15.
- 88 Law, B. A. 1982. Cheeses. Page 147 *in* *Economic Microbiology*. Vol. 7. Fermented Foods. A. H. Rose, ed. Academic Press, London.
- 89 Law, B. A. 1983. Accelerated ripening of cheese and cheese products. IDF Document 157:33.
- 90 Law, B. A. 1987. Proteolysis in relation to normal and accelerated cheese ripening. Page 365 *in* *Cheese: Chemistry, Physics and Microbiology* Vol. 1. P. F. Fox, ed. Elsevier Appl. Sci., London.
- 91 Law, B. A. 1987. Accelerated cheese ripening of non-Cheddar cheese. Bulletin 209, International Dairy Federation, Brussels.
- 92 Law, B. A., Z. D. Hosking, and H. R. Chapman. 1979. The effect of some manufacturing conditions on the development of flavour in Cheddar cheese. *J. Soc. Dairy Technol.* 32:87.
- 93 Law, B. A., and J. S. King. 1985. Use of liposomes for proteinase addition to Cheddar cheese. *J. Dairy Res.* 52:183.
- 94 Law, B. A., and A. S. Wigmore. 1982. Accelerated cheese ripening with food grade proteinases. *J. Dairy Res.* 49:137.
- 95 Law, B. A., and A. S. Wigmore. 1982. Microbial proteinases as agents for accelerated cheese ripening. *J. Soc. Dairy Technol.* 35:75.
- 96 Law, B. A., and A. S. Wigmore. 1983. Accelerated ripening of Cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. *J. Dairy Res.* 50:519.

- 97 Lin, Y. C. 1971. Carbohydrate fermentation in Cheddar curd ripening. Dairy Sci. Abstr. 34:2335.
- 98 Lloyd, R. J., and G. G. Pritchard. 1991. Characterization of X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *lactis*. J. Gen. Microbiol. 137:49.
- 99 Manning, D. J. 1979. Chemical production of essential Cheddar flavour compounds. J. Dairy Res. 46:531.
- 100 Marschke, R. J., D. E. J. Nickerson, W. D. Jarrett, and J. R. Dullely. 1980. A cause of increased proteolysis in Cheddar cheese manufactured from milk containing Maxilact. Aust. J. Dairy Sci. Technol. 35:84.
- 101 Mayo, B., J. Kok, K. Venema, W. Boeckelmann, M. Teuber, H. Reinke, and G. Venema. 1991. Molecular cloning and sequence analysis of the X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. 57:38.
- 102 Mayo, B., J. Kok, W. Boeckelmann, A. Haandrikman, K. J. Leenhouts, and G. Venema. 1993. Effect of X-prolyl dipeptidyl aminopeptidase deficiency on *Lactococcus lactis*. Appl. Environ. Microbiol. 59:2049.
- 103 McGarry, A., J. Law, A. Coffey, C. Daly, P. F. Fox, and G. F. Fitzgerald. 1994. Effect of genetically modifying the lactococcal proteolytic system on ripening and flavour development in Cheddar cheese. Appl. Environ. Microbiol. 60:4226.
- 104 McKay, L. L., and K. A. Baldwin. 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol. Rev. 87:3.
- 105 Mills, O. E., and T. D. Thomas. 1980. Bitterness development in Cheddar cheese: effect of level of starter proteinase. N. Z. J. Dairy Sci. Technol.

- 15:131.
- 106 Monnet, V., W. Boeckelmann, J. C. Gripon, and M. Teuber. 1989. Comparison of cell wall proteinases from *Lactococcus lactis* subsp. *cremoris* AC1 and *Lactococcus lactis* subsp. *lactis* NCDO763. II. Specificity towards bovine  $\beta$ -casein. *Appl. Microbiol. Biotechnol.* 31:112.
- 107 Monnet, V., D. LeBars, and J. C. Gripon, 1986. Specificity of a cell wall proteinase from *Streptococcus lactis* NCDO763 towards bovine  $\beta$ -casein. *FEMS Microbiol. Lett.* 36:127.
- 108 Monnet, V., J. P. Ley, and S. Gonzales. 1992. Substrate specificity of the cell envelope-located proteinase of *Lactococcus lactis* subsp. *lactis* NCDO763. *Int. J. Biochem* 24:707.
- 109 Muset, G., V. Monnet, and J. C. Gripon. 1989. Intracellular proteinases of *Lactococcus lactis* NCDO763. *J. Dairy Res.* 56:765.
- 110 Nardi, M., M. C. Chopin, A. Chopin, M. M. Cals, and J. C. Gripon. 1991. Cloning and DNA sequence analysis of X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *lactis* NCDO763. *Appl. Environ. Microbiol.* 57:45.
- 111 Nasr, M. M., M. M. El Sayed, and Y. A. El Samragy. 1991. Acceleration of Edam cheese ripening using acid fungal protease. *Nahrung* 35:143.
- 112 Neviani, E., C. Y. Boquien, V. Monnet, L. P. Thanh, and J. C. Gripon. 1989. Purification and characterization of an aminopeptidase from *Lactococcus lactis* subsp. *cremoris* AM2. *Appl. Environ. Microbiol.* 55:2308.
- 113 Oberg, C. J., L. H. Davis, G. H. Richardson, and C. A. Ernstrom. 1986. Manufacture of Cheddar cheese using proteinase-negative mutants of *Streptococcus cremoris*. *J. Dairy Sci.* 69:2975.
- 114 Olson, N. F. 1990. The impact of lactic acid bacteria on cheese flavor.

- FEMS Microbiol. Rev. 87:131.
- 115 Otto, R., W. M. DeVos, and J. Garvielli. 1982. Plasmid DNA in *Streptococcus cremoris* Wg2: influence of pH on selection of chemostats of variants lacking a protease plasmid. Appl. Environ. Microbiol. 43:1272.
- 116 Otto, R., R. G. Lageveen, H. Veldkamp, and W. N. Konings. 1982. Lactate efflux induced electrical potential in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 146:733.
- 117 Piard J. C., M. El Soda, W. Alkhalaf, M. Desmazeaud, L. Vassal, and J. C. Gripon. 1986. Acceleration of cheese ripening with liposome-entrapped proteinase. Biotechnol. Letters 8:241.
- 118 Poolman, B., A. J. M. Driessen, and W. N. Konings. 1987. Regulation of arginine/ornithine exchange and the arginine deiminase pathway in *Streptococcus lactis*. J. Bacteriol. 169:5597.
- 119 Poolman, B., A. J. M. Driessen, and W. N. Konings. 1987. Regulation of solute transport in streptococci by external and internal pH values. Microbiol. Rev. 51:489.
- 120 Poolman, B., and W. N. Konings. 1988. Growth of *Streptococcus lactis* and *Streptococcus cremoris* in relation to amino acid transport. J. Bacteriol. 170:700.
- 121 Poolman, B., E. J. Smid, and W. N. Konings. 1987. Kinetic properties of a phosphate-bond-driven glutamate-glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*. J. Bacteriol. 169:2755.
- 122 Pritchard, G. G., and T. Coolbear. 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. 12:179.
- 123 Rank, T. C., R. Grappin, and N. F. Olson. 1985. Secondary proteolysis of

- cheese during ripening. A review. *J. Dairy Sci.* 68:801.
- 124 Reid, J. R., C. H. Moore, G. G. Midwinter, and G. G. Pritchard. 1991. Action of a cell wall proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 on bovine  $\alpha_{s1}$ -casein. *Appl. Microbiol. Biotechnol.* 35:222.
- 125 Reid, J. R., K. H. Ng, C. H. Moore, T. Coolbear, and G. G. Pritchard. 1991. Comparison of bovine  $\beta$ -casein hydrolysis by P<sub>I</sub> and P<sub>III</sub>-type proteinases from *Lactococcus lactis* subsp. *cremoris*. *Appl. Microbiol. Biotechnol.* 36:344.
- 126 Reid, J. R., T. Coolbear, C. J. Pillidge, and G. G. Pritchard. 1994. Specificity of hydrolysis of bovine  $\kappa$ -casein by cell envelope-associated proteinase from *Lactococcus lactis* strains. *Appl. Environ. Microbiol.* 60:801.
- 127 Reiter, B., and J. D. Oram. 1962. Nutritional studies on cheese starters. I. Vitamins and amino acid requirements of single strain starters. *J. Dairy Res.* 29:63.
- 128 Richardson, G. H., C. A. Ernstrom, J. M. Kim, and C. Daly. 1983. Proteinase negative variants of *Streptococcus cremoris* for cheese starters. *J. Dairy Sci.* 66:2278.
- 129 Ridha, S. H., J. J. M. Crawford, and A. Y. Tamine. 1984. The use of food grade neutral proteins to accelerate Cheddar cheese ripening. *Egypt. J. Dairy Sci.* 12:63.
- 130 Samples, D. R. 1985. Some factors affecting the production of volatile sulfhydryl compounds in Cheddar cheese slurries. *Diss. Abstr. Int. B*, 46:1402.
- 131 Samples, D. R., S. L. Dill, R. L. Richter, and C. W. Dill. 1986. A mechanism for volatile sulfhydryl production in Cheddar cheese slurries. *J. Dairy Sci.* 69 (Suppl. 1):62.
- 132 Seale, D. R. 1986. Bacteria inoculants as silage additives. *J. Appl. Bacteriol.*

- Symp. Suppl. 61:9S.
- 133 Seitz, E. W. 1990. Microbial and enzyme-induced flavors in dairy foods. *J. Dairy Sci.* 73:3664.
- 134 Seizen, R. J., W. M. De Vos, J. A. M. Leunissen, and B. W. Dijkstra. 1991. Homology modeling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteases. *Prot. Engin.* 4:719.
- 135 Singh, S., and T. Kristoffersen. 1970. Factors affecting flavor development in Cheddar cheese slurries. *J. Dairy Sci.* 53:533.
- 136 Singh, S., and T. Kristoffersen. 1971. Influence of lactic cultures and curd milling acidity on flavor of Cheddar cheese slurries. *J. Dairy Sci.* 54:1589.
- 137 Smid, E. J., A. J. M. Driessen, and W. N. Konings. 1989. Mechanism and energetics of dipeptide transport in membrane vesicles of *Lactococcus lactis*. *J. Bacteriol.* 171:292.
- 138 Smid, E. J., and W. N. Konings. 1990. Relationship between utilization of proline and proline-containing peptides and growth of *Lactococcus lactis*. *J. Bacteriol.* 172:5286.
- 139 Smid, E. J., B. Poolman, and W. N. Konings. 1991. Casein utilization by lactococci. *Appl. Environ. Microbiol.* 57:2447.
- 140 Sood, V. K., and F. V. Kosikowski. 1979. Accelerated Cheddar cheese ripening by added microbial enzymes. *J. Dairy Sci.* 62:1865.
- 141 Sood, V. K., and F. V. Kosikowski. 1979. Ripening changes and flavor development in microbial enzyme treated Cheddar cheese slurries. *J. Food Sci.* 44:1690.
- 142 Stadhouders, J., L. Toepoel, and J. T. M. Wouters. 1988. Cheese making with Prt<sup>-</sup> and Prt<sup>+</sup> variants of N-streptococci and their mixture. Phage sensitivity, proteolysis and flavour development during ripening. *Neth.*



- Milk Dairy J. 42:183.
- 143 Tan, P. S. T. 1992. The biochemical, genetic and physiological properties of aminopeptidase N from *Lactococcus lactis*. Ph.D. thesis, Univ. Groningen, Groningen, Netherlands.
- 144 Tan, P. S. T., and W. N. Konings. 1990. Purification and characterization of a post-proline dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 56:526.
- 145 Tan, P. S. T., B. Poolman, and W. N. Konings. 1993. The proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. 60:269.
- 146 Tan, P. S. T., K. M. Pos, and W. N. Konings. 1991. Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 57:3593.
- 147 Thomas, T. D., and G. G. Pritchard. 1987. Proteolytic enzymes of dairy cultures. FEMS Microbiol. Rev. 46:245.
- 148 Thompson, J. 1987. Ornithine transport and exchange in *Streptococcus lactis*. J. Bacteriol. 169:4147.
- 149 Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman. 1993. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. J. Bacteriol. 175:7523.
- 150 Vafopoulou, A., E. Alichanidis, and G. Zerfiridis. 1989. Accelerated ripening in Feta cheese, with heat-shocked cultures or microbial proteinases. J. Dairy Res. 56:285.
- 151 Van Boven, A., and W. N. Konings. 1987. A phosphate-bond-driven dipeptide transport system in *Streptococcus cremoris* is regulated by the internal pH. Appl. Environ. Microbiol. 53:2897.

- 152 Van Boven, A., P. S. T. Tan, and W. N. Konings. 1988. Purification and characterization of a dipeptidase from *Streptococcus cremoris* Wg2. *Appl. Environ. Microbiol.* 54:43.
- 153 Visser, S., F. A. Exterkate, C. J. Slangen, and G. J. C. M. DeVeer. 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine  $\alpha_{s1}$ -,  $\beta$ -, and  $\kappa$ -casein. *Appl. Environ. Microbiol.* 52:1162.
- 154 Visser, S., C. J. Slangen, F. A. Exterkate, and G. J. C. M. DeVeer. 1988. Action of a cell wall proteinase ( $P_I$ ) from *Streptococcus cremoris* HP on bovine  $\beta$ -casein. *Appl. Microbiol. Biotechnol.* 29:61.
- 155 Visser, S., A. J. P. M. Robben, and C. J. Slangen. 1991. Specificity of cell-envelope-located proteinase ( $P_{III}$ -type) from *Lactococcus lactis* subsp. *cremoris* AM1 in its action on bovine  $\beta$ -casein. *Appl. Microbiol. Biotechnol.* 35:477.
- 156 Von Boeckelman, I., and L. O. Lodin. 1974. Recent advances in the microbiology of handling processing and manufacture and storage of milk and milk products. 19th International Dairy Congress 1E:441.
- 157 Vos, P., M. V. Asseldonk, F. V. Jeneren, R. J. Siezen, G. Simons, and W. M. De Vos. 1989. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. *J. Bacteriol.* 171:2795.
- 158 Vos, P., G. Simons, R. J. Siezen, and W. M. De Vos. 1989. Primary structure and organization of the gene for a prokaryotic cell envelope-located serine proteinase. *J. Biol. Chem.* 264:13579.
- 159 Wilkinson, M. 1992. Studies on the acceleration of Cheddar cheese ripening. Ph. D. thesis, National Univ. Ireland, Cork.

- 160 Yan, T. R., N. Azuma, S. Kaminogawa, and K. Yamauchi. 1987. Purification and characterization of a substrate-size recognizing metalloendopeptidase from *Streptococcus cremoris* H61. *Appl. Environ. Microbiol.* 53:2296.
- 161 Yan, T. R., N. Azuma, S. Kaminogawa, and K. Yamauchi. 1987. Purification and characterization of a novel metalloendopeptidase from *Streptococcus cremoris* H61. *Eur. J. Biochem.* 163:259.
- 162 Zevaco, C., V. Monnet, and J. C. Gripon. 1990. Intracellular X-prolyl dipeptidyl peptidase from *Lactococcus lactis* ssp. *lactis* purification and properties. *J. Appl. Bacteriol.* 68:357.

## CHAPTER III

CHARACTERIZATION OF *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* STRAINS  
WITH DIFFERENT PRTP/PRTM AND OPP SYSTEMS

## ABSTRACT

Much research has explored proteinase and oligopeptide transport systems in *Lactococcus lactis* subsp. *lactis* and *cremoris* because it influences cheese flavor and body development. The proteinase system consists of enzymes that cleave caseins into peptides of various sizes (PrtP), a protein maturation lipoprotein (PrtM), and an oligopeptide transport system (Opp). After membrane transport, peptides produced by PrtP are further hydrolyzed by various peptidases.

Twelve strains were constructed which differed in their *lac*, *prtP*, and *opp* genotypes. Individual milk proteins, a mixture of these proteins, and milk were used as substrates to study the interaction of PrtP and Opp in constructed strains. Amino acid analysis was determined by measuring the trichloroacetic acid soluble peptides generated. Total proteolysis was measured. The data were normalized and represented in bar graphs. Generation time, acid production, and reduction-oxidation generation were also measured. Biochemical characterization, aminopeptidase and dipeptidase assays were performed to further investigate and explain hydrolysis patterns. Specificity of proteinases present in PrtP containing strains was also investigated, since the specificity will influence the ability of the strains and specific peptides generated from hydrolysis of caseins.

Strains with the same phenotype behaved differently when grown under the same conditions. Biochemical analyses further support the results by the

differences observed in the biochemical profiles. Though the proteolytic system is active all the time, an available carbohydrate source, i.e., lactose, will be used first for energy conversion. Once the carbohydrate source has been depleted, the proteolytic system becomes important. Together with the oligopeptide transport system, the strains will now use amino acids for energy. Thus, lactose positive strains use lactose as their primary energy source, whereas lactose negative strains metabolize amino acids for their energy. Specificity of the proteinase showed an influence in breakdown of  $\alpha_{s1}$ -casein hydrolysis patterns. The strains hydrolyzed  $\alpha_{s1}$ -casein only to small amounts, which could be explained by proteinase type. The other substrates showed more hydrolysis occurring, which is also consistent with the proteinase type the strains possess. At the same time, factors such as aminopeptidases and other transport systems, which were not accounted for in this study, influenced the growth rates of the strains.

## INTRODUCTION

The proteolytic enzymes of mesophilic lactococci are essential for rapid growth in milk and production of flavor components in Cheddar-style cheeses. Because of its importance in cheese flavor and body development, genetic and biochemical characterization of the lactococcal proteinase system's lactose utilization (Lac) and proteinase activity (Prt) has been studied extensively (19, 21, 28, 29, 30, 37, 48, 49).

Lactose utilization and proteinase activity in lactic acid bacteria (LAB) are unstable, plasmid-encoded traits that can lead to unpredictable and failed milk fermentations (21). The loss of plasmids explains the instability of these traits. Strategies to stabilize and amplify the proteinase genes to alter proteinase specificity and activity have been developed to manufacture more flavorful

products, increase cheese yield, and accelerate cheese ripening (21, 28, 29, 30, 48, 49).

*Lactococcus lactis* subsp. *lactis* C20 and its proteolytic capabilities have been studied extensively (11). This strain contains five plasmids of 55 (pJK550), 48 (pJK480), 43 (pJK430), 3.7 (pJK037), and 2.1 (pJK021) kilo bases (kb). Plasmid pJK550 codes for lactose utilization (*lac*) and the proteinase system (*prtP*). The *prtP* gene is closely linked to a maturation lipoprotein *prtM* gene. The *prtM* gene codes for a membrane-associated lipoprotein. As the proteinase is translocated across the cell membrane, or shortly thereafter, the lipoprotein is involved in removing an N-terminal pro-region of the proteinase (12, 13) and thus rendering a fully active serine proteinase (PrtP) anchored to the cell membrane. This serine proteinase hydrolyzes the milk caseins into various oligopeptides (12).

Leenhouts et al. (31) successfully integrated the *prtP* and *prtM* genes from *L. lactis* subsp. *cremoris* Wg2 into the *L. lactis* subsp. *lactis* MG1363 chromosome. The mutants contained different numbers of integrated proteinase gene copies, which influenced the growth in milk. The mutants all produced more proteinase than the parent strain, though mutants differed in proteinase activity depending on the integrated copy numbers.

Plasmid pJK430 together with pJK550 is needed to acquire full proteolytic capabilities and maximum growth in milk. The plasmid pJK430 is homologous with a region on plasmid pVS8 found in *L. lactis* subsp. *cremoris* SL135, which is associated with oligopeptide transport (*Opp*) (54). The *opp* gene is found in other lactococci and in the strains used in this study. Plasmid pJK430 has been sequenced and stabilized into the chromosome of some *Lactococcus* species (31). This system is important in fermented products that contain little or no residual carbohydrate for energy. Thus, amino acids become an important source of

metabolic energy in fermented dairy products (15, 24).

The plasmid pJK480 is thought to be involved in conjugal transfer of the pJK550 plasmid. Together these two plasmids form stable cointegrates and aid each other in the conjugation event. Plasmids pJK037 and pJK021 are cryptic plasmids and so far no functional identification has been made (K. Gillies and J. Kondo, personal communications).

The oligopeptide transport system is only one kind of transport system available to microorganisms for amino acid residues longer than three amino acids. Free amino acids in milk can be taken up via three different transport mechanisms. The first is coupled to the proton-motive driven transport that primarily takes up Met, Leu, Ile, Val, Ser, Thr, Ala, Gly, and Lys, among others (7). A second is driven by the high-energy phosphate bond in ATP and takes up Glu, Gln, Asn, and Asp, among others. Another transport mechanism uses an antiport, which catalyses the stoichiometric exchange between extracellular Arg and intracellular Orn; the driving force is the concentration gradient across the membrane (7, 20). Proline enters the cell by passive diffusion or as part of a di- or tripeptide (44, 45). Tynkkynen et al. (50) characterized an oligopeptide transport system in *L. lactis* strains; the transport system contains a cluster of genes encoded by the *oppDFBCA* and *pepO* genes. Smid et al. (46) described the transport mechanism and the energetics involved in peptide uptake by *L. lactis*. The dipeptide Ala-Glu is taken up with two other peptides via a symport, which allows simultaneous uptake of two peptides. They developed mutants that were unable to transport alanine, di-, and trialanine. The alanine-deficient mutant *L. lactis* MG 1363, however, was still able to show uptake of larger peptides of tetra- and penta-alanine, thus demonstrating another transport system. Kunji et al. (26)

described an ATP-dependent oligopeptide transport system that permits the uptake of four to six amino acid residue oligopeptides into the cell.

The proteinase and peptidase systems mentioned above differ in their specificity toward the major caseins. There are different classes of serine proteinases ( $P_V$ ,  $P_{III}$ ,  $P_I/P_{III}$ , and  $P_{III}/P_I$  intermediate now classified into groups a-h) (25). They are strain dependent and differ in their specificity and rate of hydrolysis toward  $\alpha_{s1}$ - and  $\kappa$ -casein, thus producing different oligopeptides, the majority of which contain His residues (38). Reid et al. (38) described hydrolysis of  $\kappa$ -casein by proteinases produced by *L. lactis* strains. The hydrolysis of  $\beta$ -casein is also strain-dependent (9), which is associated with proteinase specificity toward  $\beta$ -casein. All peptides from  $\beta$ -casein degradation originate from amino acid residues Ala<sub>53</sub>-Met<sub>93</sub> and Asp<sub>129</sub>-Val<sub>209</sub> (35), where the fragment Gln<sub>194</sub>-Val<sub>209</sub> is a major source of bitter tasting peptides (53).  $\alpha_{s1}$ -Casein is the third major casein of interest. Most peptides produced by action of *L. lactis* subsp. *cremoris* SK11 on  $\alpha_{s1}$ -casein in vivo come from the C-terminal (39). The action of the different proteinase types on the N-terminal f1-23 fragment of  $\alpha_{s1}$ -casein resulted in completely different hydrolysis patterns (8).

This study was designed to characterize the influence of the proteinase system on lactococci growth in milk. To further delineate the role of PrtP/PrtM and Opp, isogenic strains were constructed with C2O as the common genetic background. Specificity of the proteinase of the strains involved in this study was also investigated.



## MATERIALS AND METHODS

### Strain Construction

Strains of *L. lactis* subsp. *lactis* used in this study were constructed to express all possible combinations of Lac, PrtP, and Opp (Table 10). Mutant CB06 was constructed by transducing LM2306 with an UV-induced (15 s) prophage from C2O. Mutants from this transduction were isolated by plating the mixture on bromocresol purple (BCP) (2) media containing lactose (BCP-lac) and were screened for large yellow colonies, indicating lactose utilization. Lac<sup>+</sup> colonies were further characterized for proteinase activity using Fast Slow Differential Agar II (2) by selecting faint yellow colonies with halos.

Mutant CB01 was constructed by inducing the prophage in CB06 (contained in the LM2306 background) and transfecting LM2301 with the resulting particles. Mutants were plated directly on BCP-lac and 4 to 5 yellow colonies were randomly selected. Both CB01 and CB06 now possessed a transductionally shortened pJK550 plasmid containing only *lac*. The *prtP/prtM* region was cut out and the resulting plasmid was about 23 kb shorter than the original pJK550 plasmid.

Mutant CB16 was obtained by isolating pGK13 (which contains a Bgl II fragment containing *prtP*) from JK062 and electroporating at 8.3 ms, 400  $\Omega$ , and 25  $\mu$ F (BioRad, Redwood, CA) it into LM2301. Strain JK062 was obtained from Oregon State University (Kevin Gillies, Marshall Products) and the rest of the strains used were obtained from Utah State University (Jeff R. Broadbent, Dept of Nutrition and Food Sciences). To see if stabilization of *opp* into the chromosome had an effect, two strains C2O and JK225 were included in our study. Three strains with the same phenotype obtained via different cloning methods.

TABLE 10. Strains and mutants used in this study.

Phenotype			Strains	Strain derivation	Medium	Reference
Lac	PrtP	Opp				
+ <sup>1</sup>	+	+	C2O	pJK550 ( <i>lac</i> <sup>+</sup> <i>prtP</i> <sup>+</sup> ); pJK430 ( <i>opp</i> <sup>+</sup> )	M17-lac	34
+	+	+	JK225	pJK550 electroporated into LM2306	M17-lac	54
+	- <sup>2</sup>	+	CB06	transduced LM2306 with prophage from C2O	M17-lac	this study
+	+	-	JK522	pJK550 electroporated into LM2301	M17-lac	54
+	-	-	CB01	transduced LM2301 with prophage from CB06	M17-lac	this study
-	+	+	JK062	clone containing <i>prtP<sup>d</sup></i> insert into LM2306	M17-glu	54
-	-	+	LM0230 LM2302 LM2306	no plasmids; chromosomally encoded <i>opp</i> <sup>+</sup>	M17-glu	47, 55
-	+	-	CB16	clone containing insert <i>prtP</i> <sup>+</sup> in LM2301	M17-glu	this study
-	-	-	LM2301 JK2048	plasmid-cured	M17-glu	38, 55

<sup>1</sup> strain contains phenotype

<sup>2</sup> strain does not contain phenotype

(LM0230, LM2302, and LM2306) were also included to observe any influence of strain construction.

All strains were maintained in M17 broth containing either lactose or glucose as their carbohydrate source. The strains were maintained by transferring them into fresh appropriate selection media every 2 wk. Strains were also frozen by suspending them in 10% (wt/vol) reconstituted non-fat milk together with 30% (vol/vol) glycerol and subsequently stored at  $-70^{\circ}\text{C}$ .

### **Proteinase, Aminopeptidase, and Dipeptidase Assays**

*General proteinase analysis.* Individual caseins prepared from milk as described by Hollar et al. (14) were used as the substrates for general proteinase assay. Pure caseins (obtained from Carol Hollar) were reconstituted in water and fractionated using fast protein liquid chromatography to isolate individual caseins. Each casein was added to Jenness-Koops buffer (JK) (pH 6.5) (16) in the concentration found in milk:  $\alpha_{s1}$ -Casein, 10 mg/ml;  $\beta$ -casein, 9.3 mg/ml; and  $\kappa$ -casein, 3.3 mg/ml. Each strain was grown overnight at  $30^{\circ}\text{C}$  in appropriate M17 broth and cells were collected by centrifugation ( $3,300 \times g$  for 10 min at  $4^{\circ}\text{C}$ ), washed three times with 0.85% (wt/vol) sterile saline, and dissolved in 0.85% (wt/vol) saline to an  $\text{OD}_{600}$  of 0.05. This suspension was considered the standardized cell suspension. Each casein substrate solution was inoculated with 1% (vol/vol) of each standardized cell suspension, incubated at  $30^{\circ}\text{C}$  for 6 h and prepared for AAA as described by Kaiser et al. (18). Casein substrates without bacteria were also included as controls. After incubation, the cell/substrate suspension was precipitated with 6% (vol/vol) trichloroacetic acid (TCA) to precipitate out larger peptides and undigested proteins. The sample was filtered through Whatman No. 1 (Whatman International Ltd., Maidstone, England). The

filtrate was collected and aliquots (2.5 ml) were freeze-dried.

The freeze-dried filtrate was reconstituted with 1 ml of 6N HCl, sonicated and vacuum sealed with exclusion of O<sub>2</sub>, and hydrolyzed at 110°C for 20 h. The hydrolysate was subsequently filtered through a 0.2- $\mu$ m syringe filter (Fisher Scientific, Pittsburgh, PA). Fifteen microliters were transferred to a test tube and gently dried under N<sub>2</sub>-gas. Each dried sample was rehydrated with 250  $\mu$ l of Beckman Na-S dilution buffer (Beckman Instruments, Fullerton, CA), filtered through another 0.2- $\mu$ m syringe filter, and loaded onto an Amino Acid Analyzer 6300 (Beckman Instruments, Fullerton, CA) sample cartridge in triplicates. Fifty microliters was injected to determine the general proteolytic capabilities of the strains and their phenotypes (36, 56).

***Aminopeptidase (AP) assay.*** Stock solutions of chromogenic substrates (10 mM) of Arg-, Leu-, Lys-, Val-, Met-, Pro-,  $\gamma$ -Glu-, and Ala- *p*-Nitroanalide (*p*-NA)-L-amino acid derivatives (Sigma Chemicals, St. Louis, MO) were dissolved in sterile 0.05 M sodium phosphate buffer (pH 7.2). Aliquots (1 ml) of 10 mM stock solutions were stored at -20°C, thawed, and dilutions (1mM) were made immediately before use. Cells were prepared for AP analysis by the method of Dias and Weimer (6), except cultures were grown overnight in M17 broth at 30°C. Cells were collected by centrifugation (3,300  $\times$  g for 10 min at 4°C) and washed three times with 0.05 M sodium phosphate buffer (pH 7.2). A volume of glass beads (Sigma Chemicals, St. Louis, MO) equal to the volume of the slurry was added and vortexed at full speed for 1 min. Cell suspensions were subsequently frozen (-70°C) and rapidly thawed three times to ensure cell lysis. The broken cells were centrifuged at 3,300  $\times$  g for 5 min and the supernatant was collected. This supernatant was considered the cell free extract (CFE) and was assayed for AP activity. Each assay mixture contained 100  $\mu$ l of 1 mM

chromogenic substrate in 0.05 M sodium phosphate buffer (pH 7.2) and 100  $\mu$ l CFE. Assays were carried out at 30°C in 96-well microtiter plates (Baxter Diagnostics, Inc., Deerfield, IL) with sterile tape as covering to prevent evaporation. Hydrolysis of the chromogenic substrates was measured by colorimetrically monitoring an increase in yellowness ( $b^*$ ) using Omnispec™ 4000 reflectance colorimeter (Wescor, Inc., Logan, UT). Readings were taken every 30 min for 6 h and assays were done in triplicate. Controls contained 100  $\mu$ l 0.05 M sodium phosphate buffer (pH 7.2) and 100  $\mu$ l of 0.5 M *p*-NA in 0.05 M sodium phosphate buffer (pH 7.2). Protein concentrations were measured using bicinchonic acid assay according to the manufacturer's manual (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used to obtain a standard curve.

***Dipeptidase assays.*** Dipeptides of Leu-Pro, Gly-Pro, and Ser-Leu were each suspended in JK buffer (pH 6.5) to a final concentration of 0.05 mM. Strains and mutants in Table 10 were incubated into appropriate M17 broth overnight at 30°C. Cultures were subsequently collected as described above, washed three times in JK buffer (pH 6.5), and suspended in JK buffer to a final  $OD_{600} = 0.05$ , and each dipeptide solution was inoculated with 1% (vol/vol) of standardized culture solution. These samples were incubated at 30°C for 6 h and then prepared for AAA as described above and run in triplicates. Controls without bacteria were also included.

### **Growth Rates and Generation Times**

Growth curves were done using automated colorimetry as described by Yuan (58) in pasteurized 10% (wt/vol) reconstituted nonfat-dried-milk containing 0.024% (wt/vol) 2, 3, 5 triphenyl tetrazolium chloride (TTC) to measure change in redox potential. This redox generation comes from the ability

of strains to convert NADH to NAD upon metabolism of amino acids and thus cause a color change in the TTC. Acid production rate was measured in reconstituted nonfat-dried-milk containing 0.02% (wt/vol) BCP for 24 h at 30°C with measurements taken every 1 h. Additionally, plate counts were done using Elliker agar (Difco Laboratories, Detroit, MI) at 0, 5, 10, and 24 h to ensure cell densities increased as the reaction changed colors ( $a^*$  for TTC and  $b^*$  for BCP) and to calculate generation times. Acid production estimates were done in triplicates, redox potential production and thus amino acid metabolism estimates were done in duplicates, and data are expressed as means  $\pm$  standard error of the mean (SEM).

### Proteinase Specificity Assay

Specificity assays for proteinase type were done on the strains containing the *prtP* gene (9). Strains were grown in 100 ml M17 containing the appropriate selective media (M17-Lac for Lac<sup>+</sup> strains and M17-Glu for Lac<sup>-</sup> strains) and incubated at 30°C for 12 h. Cultures were centrifuged at 2,000  $\times$  g for 15 min at 4°C in a Sorvall<sup>®</sup> RC-5B centrifuge (DuPont Instruments, Wilmington, DE). The pellet was further washed twice in 50 mM Tris-HCl (pH 7.0) containing 25 mM CaCl<sub>2</sub> (called wash buffer). After the final centrifugation step, the pellet was resuspended in 1.5 ml wash buffer and centrifuged again at 2,000  $\times$  g for 15 min at 4°C. The pellet was subsequently resuspended in 200  $\mu$ l of 50 mM acetate with 5 mM CaCl<sub>2</sub>, pH 6.8, with or without 4.5% (wt/vol) NaCl. This suspension was used to detect for enzyme activity. Succinyl-alanyl-glutamyl-prolyl-phenylalanyl-*p*-Nitroanalide (S-Glu) (Bachem Bioscience Inc., King of Prussia, PA) and 3-carbomethoxypropionyl-arginyl-prolyl-tyrosine-*p*Nitroanalide HCl (MS-Arg) (Chromogenix, Mölndal, Sweden) were used as chromogenic

substrates. Hydrolysis of the substrates (1 mM) was measured using an Omnispec™ 4000 reflectance colorimeter by monitoring the increase in yellow color ( $b^*$ ). Controls contained 100  $\mu$ l of wash buffer and 100  $\mu$ l of 0.05 M pNA in 100  $\mu$ l imidazole buffer (pH 6.5). Control strain *L. lactis* subsp. *cremoris* D11 ( $P_I$ -type) was also included. Assays were run in duplicates and readings taken every 5 min.

### Genetic and Phenotypic Analyses

**Plasmid.** All strains were characterized for their plasmid content by the method of Anderson and McKay (1) using 0.6% (wt./vol) agarose gels at 35 V for 9 h and subsequently staining them in ethidium bromide (0.5  $\mu$ g/ml) for 30 min.

**Gene sequence.** To further distinguish  $P_I$  and  $P_{III}$  specificity, polymerase chain reaction (PCR) amplification and nucleotide sequence analysis was done. The template was isolated by the method of Furrer et al. (10). Strains with the PrtP phenotype were grown overnight in 5 ml M17 broth at 30°C. Strains (250  $\mu$ l) were transferred into 1.5 ml Eppendorf centrifuge tubes and spun in a microcentrifuge at maximum speed for 4 min. The pellets were resuspended into 100  $\mu$ l phosphate buffered saline (pH 7.4) and centrifuged for 4 min at maximum speed. The pellets were subsequently resuspended into 85  $\mu$ l sterile distilled water, 10  $\mu$ l Amplitac Buffer II (Perkin Elmer, Norwalk, CT), and 4  $\mu$ l of freshly prepared lysozyme solution (10 mg/ml stock). The mixes were incubated at 30°C for 15 min, 1  $\mu$ l proteinase K (50 mg/ml stock) was added and further incubated at 50-55°C for 1 h. Thereafter, the tubes were placed in a boiling water bath for 10 min to inactivate the enzymes. The lysates were used as the template DNA for PCR.

Primers for PCR and the segments of the coding regions for *priP* are shown in Table 11 (9). PCR amplification was done by the method of Kuipers et al. (23) using a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, CT) and the amplification was done using 30 cycles. DNA was denatured at 94°C for 1 min, annealed at 54°C for 1.5 min, and extended at 72°C for 2.5 min. A fraction of the PCR (10 µl) products was run on a 2% (wt/vol) agarose gel (Metaphor agarose, FMC Bioproduct, Rockland, ME) to identify the fragments. The rest of the PCR products were cleaned up using the Prep-A-Gene DNA Purification Systems (Bio-Rad, Hercules, CA) according to the manufacturer's manual. Nucleic acid sequence was performed using the dideoxy chain termination method with the primers (41), translated into protein sequences, and compared to *L. lactis* subsp. *cremoris* SK11.

**Biolog analyses.** Biochemical profiles of strains were characterized using Biolog plates (Biolog, Inc., Hayward, CA). Strains were grown on BUGM-G media (Biolog, Inc., Hayward, CA) overnight at 30°C and transferred from plates using sterile cotton swabs into 0.85% (wt./vol) saline solution and adjusted to OD<sub>590</sub> = 0.2. Biolog microtiter wells were inoculated with 150 µl of the adjusted

TABLE 11. Primers used in cloning of *L. lactis* subsp. *lactis priP* segments (9).

Primer	Target DNA <sup>1</sup>	Sequence (5' to 3')
288 <sup>a</sup>	1268-1293	TGACATCCGTGTTTTTCAGCGGAAGC
289 <sup>b</sup>	732-756	ATTCACTGATACCGTTAAGCATGGC
286 <sup>a</sup>	2884-2907	AGCCGTCTTGATGTTGCCATCACG
287 <sup>b</sup>	2701-2724	AGCATGAAGTATTATCTATTGCGC

<sup>1</sup> nucleic acid sequence corresponds to that of *L. lactis* subsp. *cremoris* SK11

<sup>a</sup> antisense sequence

<sup>b</sup> sense sequence



bacterial suspension and incubated at 30°C. Color changes were observed and recorded after 6 and 24 h. The data were entered into a Biolog lactic acid bacteria database and strains were identified (3).

### Statistical Analysis

A correlation matrix for the Biolog data was constructed using SAS JMP (42). All substrates (Appendix A, Table A1) were included in the analyses.

## RESULTS

### Genetic Identification

*Plasmid identification.* All strains showed the expected plasmid profile. Strains JK062 and CB16 both showed the *prtP* clone at 9.75 kb. The rest of the lactose positive mutants showed a chromosomal band as well as the lactose plasmid band. C2O showed all five plasmids pJK550, pJK480, pJK430, pJK037, and pJK021. Strains JK225 and JK522 both contained the pJK550 plasmid, whereas both CB01 and CB06 contained the transductional shortened pJK550 (*lac<sup>+</sup>prtP<sup>-</sup>*) plasmid (Figure 7).

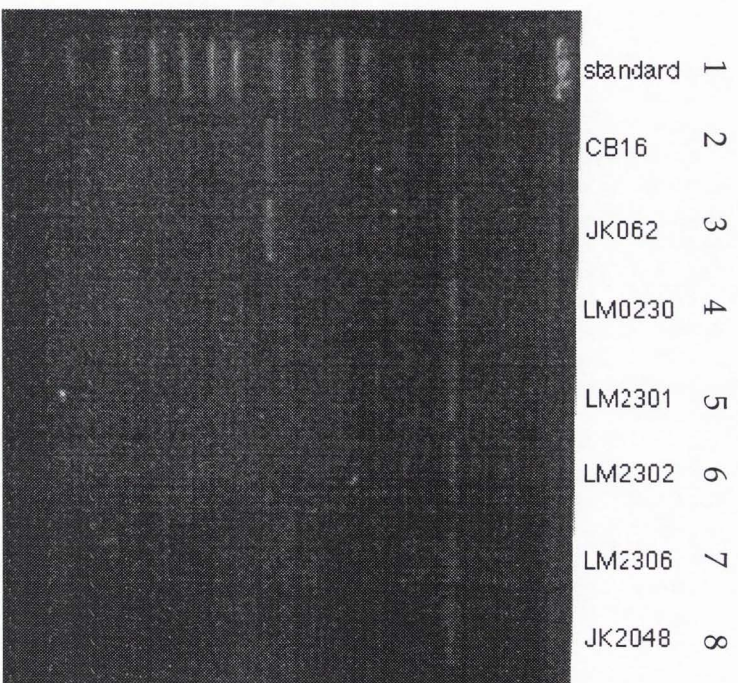
*Gene sequence.* Table 12 shows the amino acid substitutions of the strains in PrtP. All strains showed a group d type amino acid substitution compared to the control of *L. lactis* subsp. *cremoris* SK11 (group a proteinase).

### Biochemical Characterization

Biochemical analyses on Biolog GP MicroPlates were conducted to characterize the phenotypic differences between constructs. A variety of carbohydrates, organic acids, amino acids and their derivatives, and nucleic acids were tested as substrates (Appendix A, Table A1) (3). SAS JMP (42) was employed to estimate the correlation between all possible pairs of strains

Figure 7. Plasmid gel of strains used in this study. A 0.6% (wt/vol) agarose gel was run at 35V for 9 h and subsequently stained in fresh ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ) for 30 min.

Gel A. Lanes:



Gel B. Lanes:

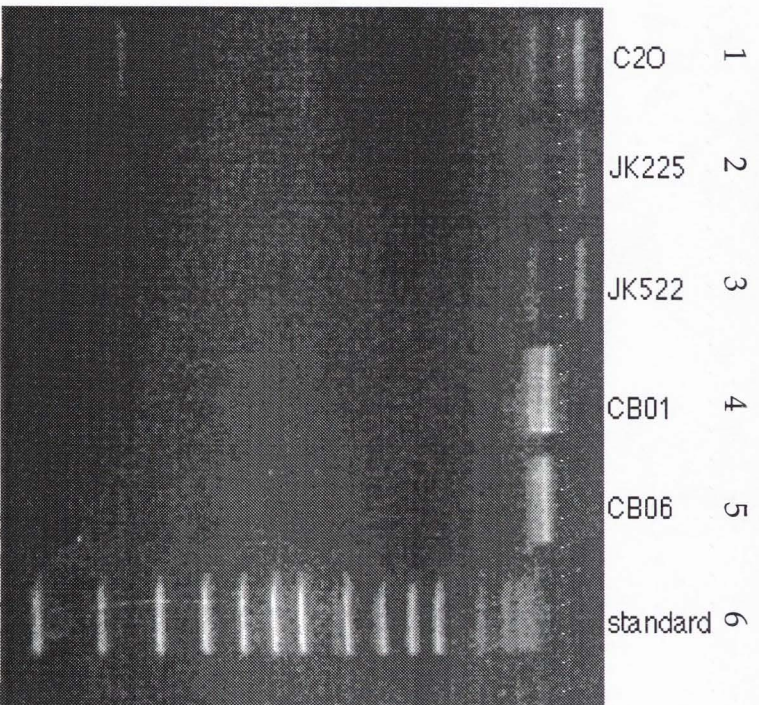


TABLE 12. Amino acid substitutions in *L. lactis* subsp. *lactis* strains used in this study.

Substrate-binding region	Residue	Amino acid substitution for cell-enveloped proteinase of the following strains				
		C2O (d)	JK225 (d)	JK522 (d)	JK062 (d)	CB16 (d)
Subtilisin-like-binding region	131	Thr	Thr	Thr	Thr	Thr
	138	Thr	Thr	Thr	Thr	Thr
	142	Ala	Ala	Ala	Ala	Ala
	144	Leu	Leu	Leu	Leu	Leu
	166	Asp	Asp	Asp	Asp	Asp
	177	Leu	Leu	Leu	Leu	Leu
Remote segment in fragment C	747	Arg	Arg	N/D	Arg	Arg
	748	Lys	Lys	N/D	Lys	Lys
	763	His	His	N/D	His	His

(Appendix A, Table A2). Not all Lac<sup>-</sup> mutants correlated significantly with the Lac<sup>+</sup> strains with R<sup>2</sup> ranging from 0.21 to 0.49. There was a stronger correlation (R<sup>2</sup> = 0.88) between CB16 and its parent strain LM2301, but not between CB01 and the same parent (R<sup>2</sup> = 0.57), or between CB06 and its parent strain LM2306 (R<sup>2</sup> = 0.23). This indicates that further differences will be expected in growth due to phenotypic differences not related to PrtP, Opp, or proteinase type.

### **Growth Characterization**

*Generation time.* All Lac<sup>+</sup> constructs had similar generation times (52 to 55 min), while all Lac<sup>-</sup> strains had slower generation times (64 to 73 min) (Table 13). Lactose positive strains with either PrtP, Opp, or a combination of both showed no difference in generation times. Lactose negative strains, however, showed larger differences. Strain JK062 possessing both PrtP and Opp had a faster generation time than CB16, which possesses only PrtP. On the other hand, JK062 showed similar generation time to strains with only Opp (64 to 65 min), except LM2302 (71 min). Strain CB16, possessing only PrtP, showed slower generation time (71 min) than strains with only Opp (64 min), again with the exception of LM2302. At the same time, CB16 had similar generation time to LM2301 and JK2048, two PrtP<sup>-</sup>Opp<sup>-</sup> strains. These observations suggest that Lac overrides the proteinase system in Lac<sup>+</sup> strains, whereas the proteinase system becomes more important in Lac<sup>-</sup> strains.

*Acid production.* The development of acid did not follow a trend though culture had a significant effect (Appendix A, Table A3). Three Lac<sup>+</sup> strains (JK225, JK522, and CB06) produced acid significantly faster than the rest of the strains. However, strain LM2302, with a Lac<sup>-</sup> phenotype, showed an even higher acid production rate than the three Lac<sup>+</sup> strains and it showed to be of

TABLE 13. Generation times, acid production, and redox-potential for *L. lactis* subsp. *lactis* strains grown in milk.

Strain	Phenotype			Generation time <sup>1</sup> (g) (min ± SD)	Acid production x 10 <sup>-8</sup> (Δb*/min/CFU) (mean ± SEM)	TTC change x 10 <sup>-8</sup> (Δa*/min/CFU) (mean ± SEM)
	Lac <sup>2</sup>	PrtP <sup>2</sup>	Opp <sup>2</sup>			
C2O	+	+	+	52 ± 2	9.8 ± 2.8 <sup>a</sup>	21.9 ± 11.2
JK225	+	+	+	53 ± 3	21.0 ± 2.8 <sup>d</sup>	63.9 ± 11.2
CB06	+	-	+	53 ± 3	25.5 ± 2.8	98.7 ± 11.2 <sup>d</sup>
JK522	+	+	-	53 ± 10	27.3 ± 2.8	105.5 ± 11.2 <sup>d</sup>
CB01	+	-	-	55 ± 5	8.5 ± 2.8 <sup>a</sup>	9.6 ± 11.2 <sup>a</sup>
JK062	-	+	+	65 ± 4	8.6 ± 2.8	81.4 ± 11.2 <sup>c, d</sup>
LM0230	-	-	+	64 ± 3	12.4 ± 2.8	64.7 ± 11.2
LM2306	-	-	+	64 ± 5	9.5 ± 2.8	4.2 ± 11.2
LM2302	-	-	+	71 ± 2	34.7 ± 2.8 <sup>b, d</sup>	27.9 ± 11.2
CB16	-	+	-	71 ± 4	5.9 ± 2.8	7.9 ± 11.2
LM2301	-	-	-	72 ± 1	6.7 ± 2.8	5.4 ± 11.2
JK2048	-	-	-	73 ± 2	11.5 ± 2.8	7.2 ± 11.2

<sup>1</sup> determined by plate count method

<sup>2</sup> + = contains phenotype, - = lacks phenotype

<sup>a</sup> not significantly different from Lac<sup>+</sup> strains

<sup>b</sup> significantly different from Lac<sup>+</sup> strains

<sup>c</sup> significantly different from Lac<sup>-</sup> strains

<sup>d</sup> significantly different from all strains

significance compared to Lac<sup>+</sup> strains. The other two Lac<sup>+</sup> strains (C2O and CB01) were not significantly different from some of the Lac<sup>-</sup> strains (Appendix A, Table A4). Strains with the same phenotype showed significantly different acid rates. Strains LM0230, LM2306, and LM2302 had an acid production rate of 12.4, 9.5, and 34.7 Δb\*/min/CFU respectively. These last three strains are phenotypically identical, though they metabolize various sources still available in the substrate with different activities and thus produce acid at different rates.

*TTC change.* A trend was evident with strains grown in the presence of the redox dye TTC. This redox dye is a colorless, water-soluble redox indicator which reduces to a red, water-insoluble triphenyl formazan with formation of  $\text{NAD}^+$ . The rate at which this reduction occurs provides an index of the rate of substrate oxidation and therefore of fermentation activity (17) of the microorganisms due to metabolism of nutrients that produce  $\text{NAD}^+$  or  $\text{NADH}$  (32).

The cultures showed a significant effect on redox potential (Appendix A, Table A5). The redox-potential change of CB01 was not significantly different from CB16, LM2301, LM2306, or JK2048 (Appendix A, Table A6) with  $\text{LSD}_{0.05}$  of 1.66, 4.21, 5.32, and 2.38 respectively. The strains CB16, LM2301, and JK2048 do not possess the Opp phenotype and thus cannot transport oligopeptides into the cell and metabolize them. Strain LM2306, however, has an Opp system but did not seem to have the right affinity for the substrates, since its redox-potential only measured  $4.2 \pm 11.2 \Delta a^*/\text{min}/\text{CFU}$ . The other  $\text{Lac}^-$  strains with a  $\text{PrtP}^-$  Opp<sup>+</sup> phenotype (LM0230 and LM2306) generated a greater change in redox-potential. Strain JK062, with a  $\text{Lac}^-$   $\text{PrtP}^+$  Opp<sup>+</sup> phenotype, showed the highest redox-potential change among the  $\text{Lac}^-$  strains. Lactose positive strains, except for CB01 and JK522, did not show the influence of the proteinase system on redox-potential, suggesting that the lactose system overrides the proteinase system.

*$\alpha_{s1}$ -Casein hydrolysis.* Figure 8 shows the relative amino acid concentrations for individual caseins, milk, and mixed casein solutions. The data are adjusted for controls. Positive bars represent an accumulation of amino acids or peptides, whereas negative bars represent utilization of liberated amino acids or peptides (also see discussion section).

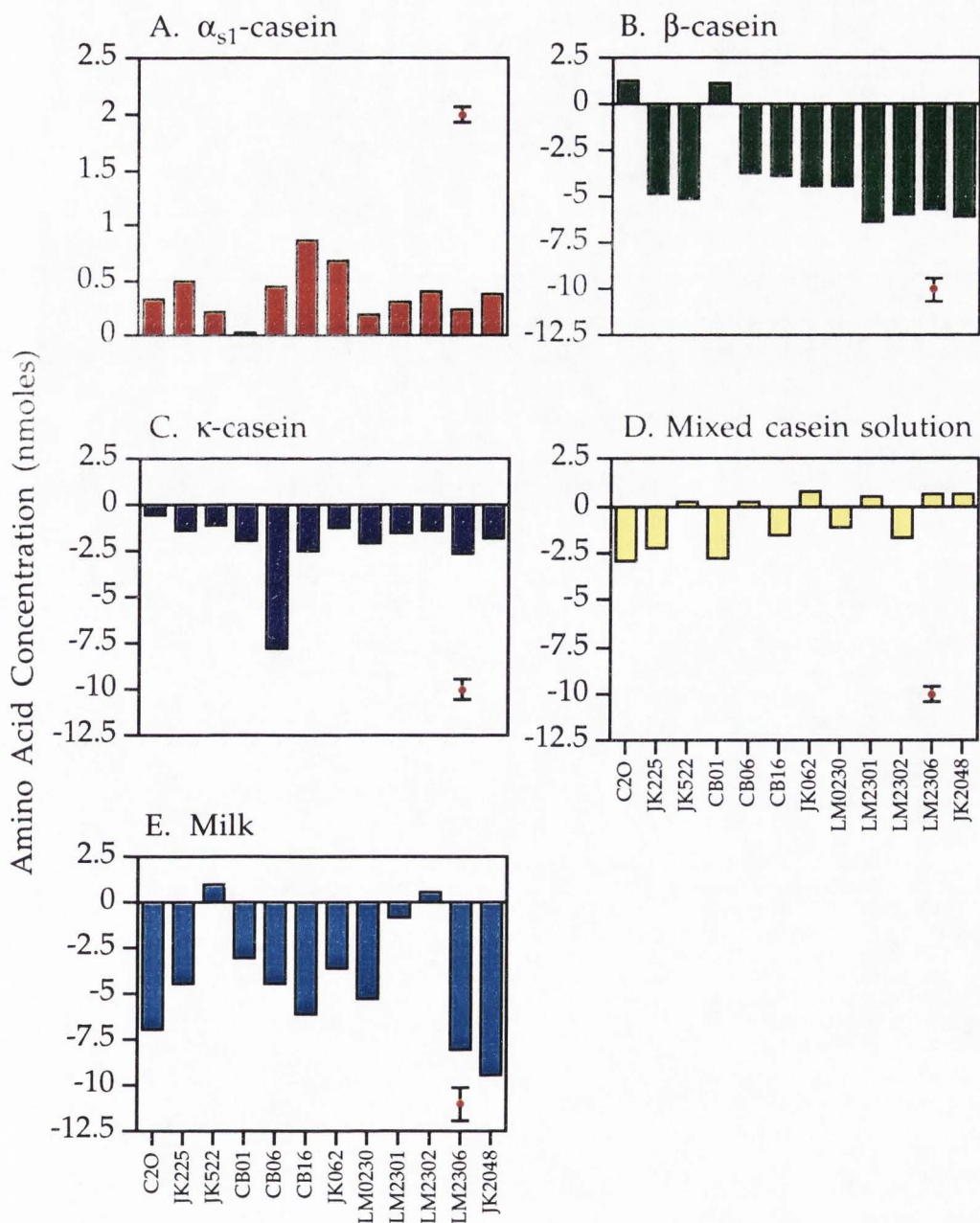


Figure 8. Cumulative amino acid concentrations of 6 % TCA-soluble fraction of growth media after 6 h incubation in (A)  $\alpha_{s1}$ -casein, (B)  $\beta$ -casein, (C)  $\kappa$ -casein, (D) mixed casein solution, and (E) milk. Error bars represent standard error of the mean (SEM).



$\alpha_{s1}$ -Casein fragments were only used to a minimum degree or not at all (Figure 8A). Lactose negative strains not possessing PrtP showed lower accumulation levels than Lac<sup>-</sup>PrtP<sup>+</sup> strains (JK062 and CB16), suggesting that fewer peptides are generated.

***$\beta$ -Casein hydrolysis.*** Figure 8B shows the relative amino acid concentration after incubation in  $\beta$ -casein. All lactose positive strains showed two metabolism patterns, again suggesting that the lactose system overrides the proteinase system. Strains C2O and CB01 showed the same concentration pattern, though they differed in PrtP and Opp (Table 13). This discrepancy suggests that both strains lack some specificity toward  $\beta$ -casein and at the same time have different transport systems. A combined effect of transport and specificity could influence the behavior of the strains toward the substrate. Strain CB01 does not possess a proteolytic system, which explains why it did not show hydrolysis/utilization of casein. Strain C2O, however, does have the full proteolytic system but still did not show any hydrolysis/utilization. Strains JK225, JK522, and CB06 also showed similar hydrolysis patterns. All three strains utilized the peptide fragments. No trend was seen regarding PrtP or Opp.

Lactose negative strains with a high redox-potential change (JK062, LM0230, and LM2302) efficiently utilized  $\beta$ -casein (Figure 8B, Table 13). All three of these strains possess the Opp phenotype, which explained how the various peptides produced were utilized. Strain LM2306, however, possesses the Opp phenotype and utilized the peptide fragments but its redox-potential was significantly lower. Though its Opp system is specific for the peptides generated, the rate at which they are taken up is very low. Although LM2306 contains Opp and its AP activity is high (Figure 9), this strain showed a low TTC change. This might suggest that the amino acid degradation pathways do not exist. Six hours

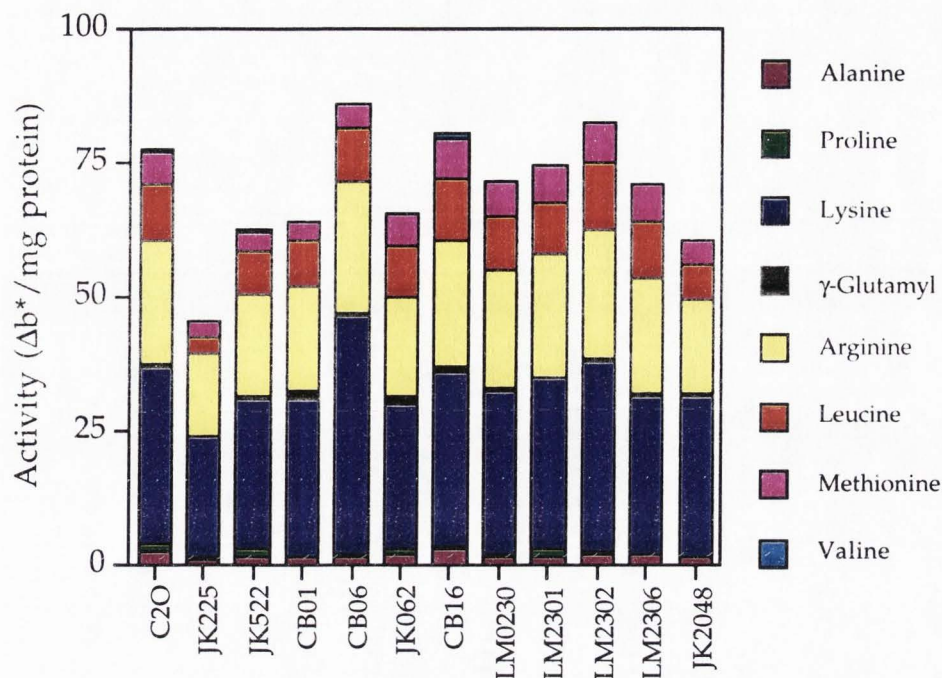


Figure 9. Aminopeptidase profiles of cell free extracts of various *L. lactis* strains.

of incubation gave the strains enough time to hydrolyze and utilize all the peptides of right sizes. Strains LM2301 and JK2048 did not fit the pattern. Both strains are  $\text{Lac}^- \text{PrtP}^- \text{Opp}^-$ , which explains the slow rates in Table 13 but does not explain why they utilize the peptide fragments. The data from  $\beta$ -casein hydrolysis patterns suggest that other transport systems are available in the uptake of  $\beta$ -casein derived fragments.

***$\kappa$ -Casein hydrolysis.***  $\kappa$ -Casein was not utilized to the same extent as  $\beta$ -casein except for CB06 (Figure 8C). All strains need the essential amino acid histidine, which is available from  $\kappa$ -casein hydrolysis. Strain CB06 readily metabolized the peptides generated.

*Mixed casein hydrolysis.* Figure 8D shows the amino acid concentration for the mixed casein solution. Strains C2O and JK225 both possess the PrtP and Opp, which resulted in a breakdown of the substrate. The peptide fragments were transported into the cell and metabolized. Strain JK522 only possessed PrtP, which established itself in the increase in bar size (Figure 8D). Strain CB01 did not possess any parts of the proteolytic system. The amino acid pattern, however, did not match the phenotype of this strain. Strain CB06 only possessed the Opp phenotype and thus should transport peptides of the right size for metabolism. However, this strain does not possess PrtP, so either the peptides were generated via another proteinase system or they were generated due to lysis of cells during starvation.

Both LM2301 and JK2048 do not possess a proteolytic system. This result was observed in the increase of hydrolysis bars. Strains LM0230 and LM2302 have the Opp phenotype and showed similar bar sizes and the peptides were transported by the cells. Strains CB16 and JK062 both possessed the PrtP phenotype. Though JK062 also had the Opp phenotype, it did not seem to metabolize the peptides. Strain LM2306 only possessed the Opp phenotype, but no or few peptides were transported and metabolized by the cell. This observation was also seen in the low TTC change ( $4.2 \pm 11.2$ ) (Table 13).

*Milk hydrolysis.* Figure 8E shows the hydrolysis pattern of milk. All Lac<sup>+</sup> strains, except for CB01, showed the same hydrolysis pattern as for mixed caseins, though the total hydrolysis amount increased. However, strain CB01, a Lac<sup>+</sup>PrtP<sup>-</sup>Opp<sup>-</sup> strain, utilized milk better than the mixed casein solution, suggesting that other factors are involved in utilization of milk.

Only two of the Lac<sup>-</sup> strains, CB16 and LM0230, showed the same hydrolysis pattern as in Figure 8D. Strain JK062 hydrolyzed and metabolized

milk more efficiently than mixed casein solution. The same was observed with LM2301, LM2306, and JK2048. These observations suggest that other factors, i.e., protein-protein interactions in micelles, other transport systems, other proteinase and peptidase systems, minerals, and vitamins, play a role in growth and metabolism of bacteria.

### **Aminopeptidase**

Aminopeptidase (AP) activity was determined for each strain to study differences not measured in the assay for presence of PrtP (Figure 9). All strains showed high activity against Lys, Arg, and Leu, suggesting the dominant AP is PepN (22). There was little activity against Val and Ala. All strains showed similar degrees of activity against Met. Proline and  $\gamma$ -Glu activity was very low or absent in all strains, suggesting that PepX and some other AP are not active or only active to a limited amount.

### **Dipeptidase**

To further explain differences observed in the proteinase and aminopeptidase study, dipeptidase patterns were investigated. Figure 10 shows the hydrolysis patterns obtained. Figure 10A shows the dipeptide Leu-Pro hydrolysis pattern. The levels of the individual components of the dipeptide were about the same in all strains except LM2306. Strains CB06, LM2306, and JK2048 differed in their profile compared to the control. Figures 10B and 10C show greater variety compared to the controls. In Figure 10B, levels of the individual components of the Gly-Pro dipeptide are within standard error of the mean and thus can be considered to be about the same. Strains C2O, JK225, JK522, and LM0230 transport the dipeptide components into the cell, whereas strains CB01, CB06, JK062, and LM2302 transported these components into the

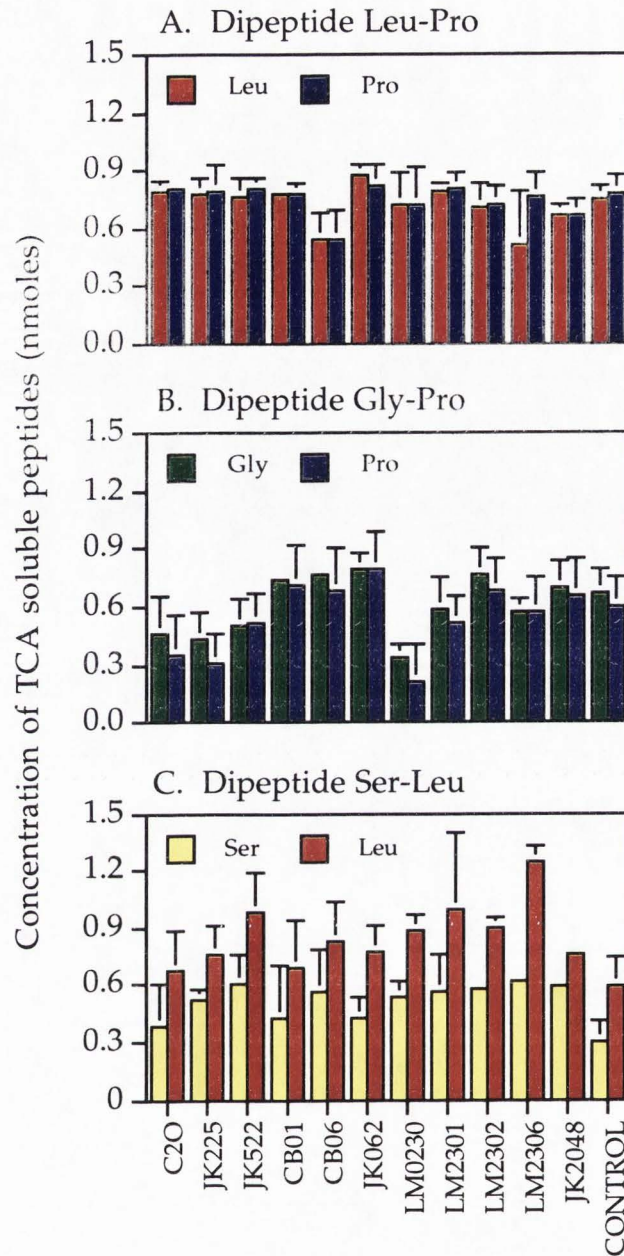


Figure 10. Dipeptide hydrolysis patterns for Leu-Pro (A), Gly-Pro (B), and Ser-Leu (C) using amino acid analysis. Error bars not visible are too small to be seen. Error bars represent standard error of the mean (SEM).

media. The Ser-Leu hydrolysis pattern (Figure 10C) shows a more distinct picture. All the strains secreted Ser-Leu as a dipeptide or its amino acids into the media compared to the control.

### Proteinase Specificity

Table 14 shows the data obtained from breakdown of the substrates MS-Arg and S-Glu as a measure of proteinase type. The control strain SK11 (P<sub>III</sub>-type strain) and S1 (P<sub>I</sub>-type strain) showed the characteristics for their proteinase type. *Lactococcus lactis* subsp. *lactis* C2O showed a P<sub>I</sub>-type pattern. The rest of the strains showed a mixed P<sub>I</sub> and P<sub>III</sub>-type pattern.

## DISCUSSION

*Lactococcus lactis* subsp. *lactis* C2O contains five plasmids, two of which confer full proteolytic capability. These two plasmids are pJK550, which contains *prtP* and *lac*, and pJK430, which contains *oppDFBCA*. *PrtP* codes for production of proteinase (PrpP) (36). *Opp* shows homology with pVS8, a plasmid coding for peptidase-peptide transport function across cell membrane in *L. lactis* subsp. *cremoris* SL135 (51, 52). Some constructs used in this study contained pJK550 (with a phenotype of Lac<sup>+</sup>PrpP<sup>+</sup>) and others contained transductional shortened pJK550, thus rendering only the Lac<sup>+</sup> phenotype (Table 11). Others contained the *Opp* system, either on the pJK430 plasmid or in the chromosome (Table 11) (57). The constructs did not correlate in their phenotypic composition to the parent background.

*Lactococcus lactis* subsp. *lactis* strains obtain their primary energy from carbohydrate metabolism. Lactose metabolism generates more energy than amino acid metabolism and thus strains with lactose-utilizing capabilities had a

TABLE 14. Relative initial activities of proteinases from lactococci strains toward MS-Arg and S-Glu in the presence (+) or absence (-) of 4.5% NaCl.

Strain	MS-Arg <sup>a</sup>		S-Glu <sup>a</sup>		Classification
	4.5% NaCl				
	-	+	-	+	
%					
C2O	122	100	14	52	P <sub>I</sub>
JK225	58	100	9	54	mixed
JK522	206	100	90	5550	mixed
CB16	171	100	92	428	mixed
JK062	2	100	4	15	mixed
S1	109	100	9	34	P <sub>I</sub> control
SK11 <sup>b</sup>	7	100	79	196	P <sub>III</sub> control

<sup>a</sup> activities are expressed as percentage of the activity against MS-Arg containing 4.5% salt.

<sup>b</sup> data from reference 9

faster generation time when grown in milk than their Lac<sup>-</sup> counterparts (Table 13).

Though *prtP* codes for the same proteinase, strains show various amino acid accumulation and utilization patterns. There are several possible explanations for variations in amino acid concentration patterns (Figure 8). Strains with identical phenotypes are not isogenic in their behavior toward caseins and peptide substrates. Strains C2O and JK225, both Lac<sup>+</sup>Prt<sup>+</sup>Opp<sup>+</sup>, show an opposite pattern for  $\beta$ -casein. The same is seen with the phenotypic identical LM0230, LM2302, and LM2306 strains. We can also explain the variation by looking at the substrate specificities of the strains (Table 13). Strain C2O shows a P<sub>I</sub>-type proteinase, whereas JK225 shows a mixed type. The local

environment of the proteinase might also change the specificity of the proteinase. Since JK buffer was used in the effort to simulate milk environment (it contains  $\text{Ca}^{++}$  as  $\text{Ca}_3(\text{CO}_3)_2$ ), the proteinase solubilizes off the bacterial membrane. This has an effect on the activity of the enzyme. The third explanation is the involvement of the oligopeptide transport system.

Strain CB16 could not metabolize peptides because it did not possess an oligopeptide transport system. This was evident in the similarities of generation time to plasmid-cured strains LM2301 and JK2048. The similar generation times of LM2302 and LM2306 (both  $\text{Opp}^+$ ) to JK062 ( $\text{PrtP}^+\text{Opp}^+$ ) suggest that there are peptides small enough to be transported by Opp by these mutants. Strain LM2302, though possessing an Opp system, could not utilize these peptides as well as LM2306, although change in redox potential was high compared to other strains.

To explain the significant difference in acid production rate (Table 13) between all lactose positive and the lactose negative strain LM2302, we took the biologic data into account (Appendix A, Table A1). Strain LM2302 utilized some sugars better than the  $\text{Lac}^+$  strains (D-mannitol,  $\alpha$ -methyl-D-glucoside, inulin, and D-sorbitol), thus generating a higher acid change. Though the strains had the same parental background, small discrepancies could be seen in the biologic data. Strains showing a high correlation in their biologic data also show similarities in their substrate utilization patterns (Appendix A, Table A1). The differences among phenotypically identical strains (C2O and JK225; LM0230, LM2302, and LM2306) were also found in their biologic utilization patterns.

Casein hydrolysis patterns showed various trends, which could not be explained by the sole involvement of the PrtP and Opp systems (Figure 8). The  $\alpha_{s1}$ -casein hydrolysis pattern can be explained by the involvement of the



proteinase type. All strains, except for C2O ( $P_I$ -type), showed a mixed proteinase specificity ( $P_I/P_{III}$ ), which helps explain the limited hydrolysis of this casein.  $\beta$ -Casein, on the other hand, was readily hydrolyzed by both proteinase types, which was seen in Figure 8B. At the same time, hydrolysis of  $\beta$ -casein was strain dependent and no proteolytic trend was seen in the hydrolysis patterns, suggesting that other transport systems are available and being used.

Other differences were observed that Lac, PrtP, Opp, and AP data could not explain. Some explanation could be that smaller peptides, resulting from hydrolysis of milk proteins, are degraded and taken up by the cell via different transport mechanisms than Opp (26, 43, 50). The proton-motive-force drives uptake of di/tripeptides in lactococci (43, 46), whereas ATP hydrolysis is coupled to transport of oligopeptides (26). Kunji et al. (26) also found that the oligopeptide transport system has a lower activity than the di/tripeptide system. Peptides containing at least 9-12 amino acid residues can be transported across the membrane (4). Following release of free amino acids or di- and tripeptides inside the cell, these peptides are further hydrolyzed by intracellular aminopeptidases to free amino acids, which are metabolized to other end products resulting in generation of lower redox potential. Strain JK062 ( $Lac^-$ ) changed the redox potential more rapidly than the strains without the proteolytic system, presumably because this strain, JK062, better utilizes peptides from  $\kappa$ -casein and  $\beta$ -casein degradation.

The peptide fragments formed by the milk proteins ( $\alpha_{s1}$ -casein,  $\beta$ -casein, and  $\kappa$ -casein) upon the action of  $P_I$ - and  $P_{III}$ -type proteinases have been identified (38, 44). The initial breakdown of  $\beta$ -casein by *L. lactis* subsp. *cremoris* AC1 leads to an average peptide size of 11-12 amino acid residues (44). Monnet et al. (35) separated early liberated peptides from  $\beta$ -casein in milk by reverse-

phase HPLC. These peptides have an average size of 10 amino acid residues, and since these peptides may be too long to be transported across the membrane, they must be broken down further by general and specific aminopeptidases. Smid et al. (45) and Reid et al. (40) suggested that only dipolar ionic oligopeptides are transported by the Opp system, thus leaving other transport systems available.

The milk protein  $\kappa$ -casein and  $\alpha_{s1}$ -(f1-9) is required by all strains because it provides the essential amino acid histidine. This protein is degraded into oligopeptides with sizes ranging from 3 to 10 amino acid residues (38). Some of these peptides can be transported by the cell right away, whereas the longer oligopeptides further have to be degraded.  $\alpha_{s1}$ -Casein also shows various oligopeptide sizes upon digestion with proteinase from *L. lactis* subsp. *cremoris* SK11 (39). Oligopeptides with sizes ranging from 5 to 30 amino acid residues have been identified. Some of these peptides must therefore be degraded before uptake into the cell.

To explain some of the variability seen in the hydrolysis patterns obtained from casein utilization (Figure 8), and not explained by Opp, dipeptide utilization patterns of three dipeptides were investigated (Figure 10). All three dipeptides (Leu-Pro, Gly-Pro, and Ser-Leu) can be obtained from  $\beta$ -casein fragments Ala<sub>53</sub>-Asn<sub>68</sub>, Ser<sub>164</sub>-Gln<sub>175</sub>, and Gln<sub>194</sub>-Val<sub>209</sub> (41). Leu-Pro can also be obtained from the  $\alpha_{s1}$ -(1-23) fragment (8) and from  $\kappa$ -casein (38). The dipeptide X-Pro, produced by the action of aminopeptidase PepX, has high affinity for the lactococcal di- and tripeptide transport system (44). Figure 10 shows, however, that only three strains (CB06, JK062, and LM2306) utilized Leu-Pro, whereas Gly-Pro was utilized by half of the constructs. This suggests that the di- and tripeptide transport system is strain dependent. Meijer et al. (33) showed that the

serine proteinase PrtP is inhibited, or its activity is largely reduced, in the presence of Pro-Leu or Leu-Pro. This helps explain some of the variability seen in Figure 8, since a fair amount of these dipeptides is obtained from casein hydrolysis.

Dipeptides competitively inhibit uptake of other dipeptides (22), e.g., Leu-Leu competitively inhibits uptake of Ala-Glu in *L. lactis* subsp. *cremoris*, and there are transport mechanisms utilized by several dipeptides (43). The constructs used in this study differed from *L. lactis* subsp. *cremoris* in utilization of peptides from various milk proteins. Laan et al. (27) reported that the growth rate of *L. lactis* MG1820 is influenced and limited by the presence of PrtP. In addition, the activity and other factors not accounted for influenced growth rate in our study (5, 27).

The specificity of the  $P_I$  and  $P_{III}$  enzyme assay showed that all but C2O had a mixed  $P_I/P_{III}$  proteinase enzyme activity. However, the genetic data revealed that the strains all contained the  $P_I$ -type proteinase (it is to expect that JK522 also possessed a  $P_I$ -type proteinase since it came from the same parent as the other strains). This  $P_I$ -type proteinase belongs to group d proteinase as classified in Kunji et al. (25). This also helps to explain why hydrolysis patterns of the caseins are different.

There are a few other possibilities that explain the amino acid accumulation patterns in Figure 8. If the strains contain the whole functional proteolytic system, the caseins will be broken up, transported into the cell, and thus the accumulated amino acid concentration will decrease and we have a negative bar. There will also be a negative bar with a  $Prt^-Opp^+$  phenotype, since there are no small TCA-soluble peptides being generated and thus all the non-digested fractions will precipitate with the TCA. The same will occur with the

PrtP<sup>-</sup>Opp<sup>-</sup> phenotype. The PrtP<sup>+</sup>Opp<sup>-</sup> phenotype, however, has the ability to generate TCA-soluble peptides and thus the amino acid concentration will increase and a positive bar will be visible in the graph.

### CONCLUSION

The results of this study show that *L. lactis* subsp. *lactis* strains possessing the same phenotype for Lac, PrtP, and Opp behave differently when grown under the same conditions. Utilization of milk proteins is strain dependent. Presence of both proteinase genes and lack of lactose utilization resulted in a 5-fold increase in  $\kappa$ -casein utilization.

### REFERENCES

- 1 Anderson, D. G., and L. L. McKay. 1983. A simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* 46:549.
- 2 Atlas, R. M. 1993. *Handbook of Microbiological Media*. L. C. Parks, ed. CRC Press, London, UK.
- 3 Biolog Inc. Manual. 1993. Micro Station System, Hayward, CA.
- 4 Broadbent, J. R., M. Strickland, B. C. Weimer, M. E. Johnson, and J. L. Steele. 1998. Peptide accumulation and bitterness in Cheddar cheese made using *Lactococcus lactis* single strain starters with distinct proteinase specificities. *J. Dairy Sci.* 81:327.
- 5 Bruinenberg, P. G., P. Vos, and W. M. de Vos. 1992. Proteinase overproduction in *Lactococcus lactis* strains: Regulation and effect on growth and acidification in milk. *Appl. Environ. Microbiol.* 58:78.

- 6 Dias, B., and B. Weimer. 1995. A semi-automated colorimetric method for determination of aminopeptidase activity in turbid solutions. *J. Rapid Meth. Automation Microbiol.* 3:223.
- 7 Driessen, A. J. M., B. Poolman, R. Kiewiet, and W. N. Konings. 1987. Arginine transport in *Streptococcus lactis* is catalyzed by a cation exchanger. *Proc. Natl. Acad. Sci.* 84:6093.
- 8 Exterkate, F. A., A. C. Alting, and C. J. Slangen. 1991. Specificity of two genetically related cell envelope proteinases of *Lactococcus lactis* subsp. *cremoris* towards  $\alpha_{s1}$ -casein-(1-23)-fragment. *Biochem. J.* 273:135.
- 9 Exterkate, F. A., A. C. Alting, and P. G. Bruinenberg. 1993. Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. *Appl. Environ. Microbiol.* 59: 3640.
- 10 Furrer, B., U. Candrian, C. Hoefelein, and J. Luethy. 1991. Detection and identification of *Listeria monocytogenes* in cooked sausage products and in milk by *in vitro* amplification of haemolysin gene fragments. *J. Appl. Bacteriol.* 70:372.
- 11 Gillies, K., and J. K. Kondo. 1990. Plasmid encoded elements required for the proteolysis of milk proteins in *Lactococcus lactis* subsp. *lactis* C2O. *FEMS Microbiol. Rev.* 87:P49.
- 12 Haandrikman, A. J., J. Kok, and G. Venema. 1991. Lactococcal proteinase maturation protein PrtM is a lipoprotein. *J. Bacteriol.* 173:4517.
- 13 Haandrikman, A. J., R. Meester, H. Laan, W. N. Konings, J. Kok, and G. Venema. 1991. Processing of the lactococcal extracellular serine proteinase. *Appl. Environ. Microbiol.* 57:1899.
- 14 Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown. 1991.

- Separation of major casein fraction using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:2403.
- 15 Hugenholtz, J., M. Dijkstra, and H. Veldkamp. 1987. Amino acid limited growth of starter cultures in milk. *FEMS Microbiol. Ecology* 45:191.
  - 16 Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Neth. Milk & Dairy J.* 16:153.
  - 17 Jones, G. A., and B. A. Humphrey. 1978. Evaluation of a dehydrogenase assay based on tetrazolium reduction for rapid in vitro estimation of fermentation activity in rumen contents. *Can. J. Anim. Sci.* 58:501.
  - 18 Kaiser, F. E., C. W. Gehrke, R. W. Zumwalt, and K. C. Kuo. 1974. Amino acid analysis: Hydrolysis, ion-exchange clean-up, derivatization, and quantitation by gas liquid chromatography. *J. Chrom.* 94:113.
  - 19 Kok, J. 1990. Genetics of the proteolytic system of lactic acid bacteria. *FEMS* 87:15.
  - 20 Kok, J., and G. Venema. 1988. Genetics of proteinases of lactic acid bacteria. *Biochimie* 70:475.
  - 21 Kondo, J. K., and L. L. McKay. 1985. Gene transfer systems and molecular cloning in group N streptococci: A review. *J. Dairy Sci.* 68:2143.
  - 22 Konings, W. N., B. Poolman, and A. J. M. Driessen. 1989. Bioenergetics and solute transport in lactococci. *CRC Crit. Rev. Microbiol.* 16:419.
  - 23 Kuipers, O. P., H. J. Boot, and W. M. de Vos. 1991. Improved site-directed mutagenesis method using PCR. *Nucleic Acid Res.* 19:4558.
  - 24 Kunji, E. R. S., A. Hagting, C. J. De Vries, V. Juillard, A. J. Haandrikman, B. Poolman, and W. N. Konings. 1995. Transport of  $\beta$ -casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. *J. Biol. Chem.* 270:1569

- 25 Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek* 70:187.
- 26 Kunji, E. R. S., E. J. Smid, R. Plapp, B. Poolman, and W. Konings. 1993. Dipeptides and oligopeptides are taken up via distinct transport mechanisms in *Lactococcus lactis*. *J. Bacteriol.* 175:2052.
- 27 Laan, H., H. Bolhuis, B. Poolman, T. Abee, and W. N. Konings. 1993. Regulation of proteinase synthesis in *Lactococcus lactis*. *Acta Biotechnol.* 13:95.
- 28 Law, B. A. 1984. Flavour development in cheeses. Page 187 in *Advances in Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davoes and B. A. Law, eds. Elsevier Applied Science Publishers, New York, NY.
- 29 Law, B. A. 1984. The accelerated ripening of cheese. Page 209 in *Advances in Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davoes and B. A. Law, eds. Elsevier Applied Science Publishers, New York, NY.
- 30 Law, B. A., and J. Kolstad. 1983. Proteolytic systems in lactic acid bacteria. *Antonie van Leeuwenhoek* 49:225.
- 31 Leenhouts, K. J., J. Gielema, J. Kok, and G. Venema. 1991. Chromosomal stabilization of the proteinase genes in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 57:2568.
- 32 Maiorova, I. P. 1971. Sugar determination in culture liquids using triphenyl tetrazolium chloride. *Appl. Biochem. Microbiol.* 7:423.
- 33 Meijer, W. C., J. D. Marugg, and J. Hugenholtz. 1996. Regulation of

- proteolytic enzyme activity in *Lactococcus lactis*. Appl. Environ. Microbiol. 62:156.
- 34 McKay, L. L., K. A. Baldwin, and P. M. Walsh. 1980. Conjugal transfer of genetic information in group N streptococci. Appl. Environ. Microbiol. 40:84.
- 35 Monnet, V., W. Boeckelmann, C. J. Gripon, and M. Teuber. 1989. Comparison of cell wall proteinases from *Lactococcus lactis* subsp. *cremoris* AC1 and *Lactococcus lactis* subsp. *lactis* NCDO 763: Specificity towards bovine  $\beta$ -casein. Appl. Microbiol. Biotechnol. 31:112.
- 36 Oberg, C. J., B. C. Weimer, L. V. Moyes, R. J. Brown, and G. H. Richardson. 1991. Proteolytic characterization of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains by the *o*-phthaldialdehyde test and amino acid analysis. J. Dairy Sci. 74:398.
- 37 Patel, K. N., F. M. Bartlett, and J. Hamid. 1983. Extracellular heat-resistant proteases of psychrotrophic *Pseudomonas*. J. Food Prot. 46:90.
- 38 Reid, J. R., T. Coolbear, C. J. Pillidge, and G. G. Pritchard. 1994. Specificity of hydrolysis of bovine  $\kappa$ -casein by cell envelope-associated proteinases from *Lactococcus lactis* strains. Appl. Environ. Microbiol. 60:801.
- 39 Reid, J. R., C. H. Moore, G. G. Midwinter, and G. G. Pritchard. 1991. Action of cell wall proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 on  $\alpha_{s1}$ -casein. Appl. Microbiol. Biotechnol. 33:222.
- 40 Reid, J. R., K. H. Ng, C. H. Moore, T. Coolbear, and G. G. Pritchard. 1991. Comparison of bovine  $\beta$ -casein hydrolysis by P<sub>I</sub> and P<sub>III</sub>-type proteinases from *Lactobacillus lactis* subsp. *cremoris*. Appl. Microbiol. Biotechnol. 36: 344.
- 41 Sanger F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with



- chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463.
- 42 SAS JMP. 1992 . Software for statistical visualization on the Apple  
Macintosh. SAS Institute Inc., Cary, NC.
- 43 Smid, E. J., A. J. M. Driessen, and W. N. Konings. 1989. Mechanism and  
energetics of dipeptide transport in membrane vesicles of *Lactococcus*  
*lactis*. J. Bacteriol. 171:292.
- 44 Smid, E. J. and W. N. Konings. 1990. Relationship between the utilization  
of proline and proline-containing peptides and growth of *Lactococcus*  
*lactis*. J. Bacteriol. 172:5286.
- 45 Smid, E. J., B. Poolman, and W. N. Konings. 1991. Minireview: Casein  
utilization by lactococci. Appl. Environ. Microbiol. 57:2447.
- 46 Smid, E. J., R. Plapp, and W. N. Konings. 1989. Peptide uptake is essential  
for growth of *Lactococcus lactis* on the milk protein casein. J. Bacteriol.  
171:6135.
- 47 Steele, J., and L. L. McKay. 1986. Partial characterization of the genetic  
basis for sucrose metabolism and nisin production in *Streptococcus lactis*.  
Appl. Environ. Microbiol. 51:57.
- 48 Thomas, T. D., and O. E. Mills. 1987. Proteolytic enzymes of dairy starter  
bacteria. Neth. Milk Dairy J. 35:255.
- 49 Thomas, T. D., and G. G. Pritchard. 1987. Proteolytic enzymes of starter  
cultures. FEMS Microbiol. Reviews 46:245.
- 50 Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A.  
Haandrikman. 1993. Genetic and biochemical characterization of the  
oligopeptide transport system of *Lactococcus lactis*. J. Bacteriol. 175:7523.
- 51 Tynkkynen, S., and A. von Wright. 1988. Characterization of a cloned  
chromosomal fragment affecting the proteinase activity of *Streptococcus*

- lactis* subsp. *lactis*. Biochimie 70:531.
- 52 Tynkkynen, S., A. von Wright, and E. L. Syvaoja. 1989. Peptide utilization encoded by *Lactococcus lactis* subsp. *lactis* SSL135 chromosomal DNA. Appl. Environ. Microbiol. 55:2690.
- 53 Visser, S., C. J. Slangen, G. Hup, and J. Stadhouders. 1983. Bitter flavour in cheese. 3. Comparative gel-chromatographic analysis of hydrophobic peptide fractions from twelve Gouda-type cheeses and identification of bitter peptides isolated from a cheese made with *Streptococcus cremoris* strain HP. Neth. Milk Dairy J. 37:181.
- 54 Von Wright, A., S. Tynkkynen, and M. Suominen. 1987. Cloning of a *Streptococcus lactis* subsp. *lactis* chromosomal fragment associated with the ability to grow in milk. Appl. Environ. Microbiol. 53:1584.
- 55 Walsh, P. M. and L. L. McKay. 1981. Recombinant plasmid associated with cell aggregation and high-frequency conjugation of *Streptococcus lactis* ML3. J. Bacteriol. 146:937.
- 56 Weimer, B. C., C. J. Oberg, L. V. Moyes, R. J. Brown, and G. H. Richardson. 1989. Comparison of classical ion exchange amino acid analysis and o-phthaldialdehyde methods to characterize proteolysis by *Lactobacillus bulgaricus*. J. Dairy Sci. 72:2873.
- 57 Yu, W., K. Gillies, J. K. Kondo, J. R. Broadbent, and L. L. McKay. 1995. Plasmid-mediated oligopeptide transport system in Lactococci. Page 509 in Genetics of Streptococci, Enterococci, and Lactococci. Vol. 85. J. J. Ferretti, M. S. Gilmore, T. R. Klaenhammer, and F. Brown, eds. Dev. Biol. Stand. Basel, Karger, Switzerland.
- 58 Yuan, T. C. 1991. An automated reflectance color meter instrument for

microbiological and enzymic assays. Ph. D. Dissertation, Utah State Univ.  
Logan, UT.

CHAPTER IV  
SENSORY EVALUATION AND CHARACTERIZATION OF CHEDDAR  
CHEESE SLURRIES MADE FROM *LACTOCOCCUS LACTIS* SSP. *LACTIS*  
MUTANTS DIFFERING IN THEIR PRTP/PRTM AND OPP SYSTEMS

**ABSTRACT**

Twelve Cheddar cheese slurries from *Lactococcus lactis* ssp. *lactis* starter cultures differing in their proteolytic system were made to investigate the effect of the proteolytic system on slurry flavor. Sensory panels were performed using seven trained judges to characterize the slurries for acidity, bitterness, and rancidity. Slurries were analyzed for contaminating microflora such as coliform, staphylococci, and non-starter lactic acid bacteria. None of the slurries showed coliform contamination. Staphylococcus contamination was observed after 21 d but no *Staphylococcus aureus* was found. Total aerobic plate counts showed a decrease in starter and an increase in non-starter lactic acid bacteria in all slurries incubated over time. Analysis of variance showed a significant culture effect on rancidity scores, whereas there was no significance related to acidity or bitterness scores. To further explain these results, aminopeptidase and lipase assays were done in the slurries during aging. Lipase activities showed similarities with rancidity scores. The data suggest a link between the lactococcal proteolytic system and the lipase system of these starters.

**INTRODUCTION**

Much research has been devoted to develop accelerated Cheddar cheese ripening methods. A mature Cheddar cheese has to be ripened for  $\geq 24$  mo at 7°C before it can be sold. Enormous economical profits would be made if ripening

time can be reduced. Since Cheddar cheese and Dutch-type cheeses can be easily manipulated and their flavor changes are not as drastically influenced by lipolysis as Brie, most research has evolved around these varieties (6).

Several methods have been developed to achieve this goal. Addition of enzymes directly to cheese milk, mainly as proteases and lipases, has been investigated by many research groups. Bacterial and fungal proteinases increase total proteolysis, but flavor intensity and quality of the cheese do not increase to the same extent as proteolysis does (7, 9), or flavor results are better but body and texture are inferior (12).

Modification of the proteolytic system of starters has also been investigated to shorten ripening time (9, 17, 19). This method allows an increase of proteolytic enzymes in the cheese. Proteinase negative ( $\text{Prt}^-$ ) strains have been used to control the amount of enzyme in cheese (13, 15). Farkye et al. (5) showed that a higher inoculum and longer make procedures are required when using  $\text{Prt}^-$  starters.

Increase in temperature is a third method to decrease ripening time. This method decreases cost of refrigeration. Law et al. (11) investigated the results of increasing temperature from 6°C to 13°C and found that it takes half the time to reach the same maturation stage as during refrigeration.

The last method is use of slurry systems (8). Kristoffersen et al. (10) developed this method by increasing cheese moisture and increasing ripening temperature to 30°C. They successfully developed Cheddar cheese flavor after only 9 d of storage at this elevated temperature. They further investigated the effect on flavor after addition of different chemicals, such as reduced glutathione and sodium citrate, and developed a cheese with characteristic Cheddar cheese flavor (18).

We have focused on how *L. lactis* subsp. *lactis* starter strains with modified proteolytic and oligopeptidase transport systems (PrtP/PrtM and Opp) influence slurry quality.

## MATERIALS AND METHODS

### Bacterial Strains

*Lactococcus lactis* subsp. *lactis* strains used in this study, each containing a different combination of the proteolytic system (PrtP/PrtM) and the oligopeptide transport system (Opp), were obtained from Jeff Broadbent (Utah State University, Department of Nutrition & Food Sciences) (Table 15). Mutant CB01, CB06, and CB16 were constructed as described in Chapter III. All strains were maintained in frozen state until needed as inoculum for milk. The strains were then transferred into M17 broth containing either lactose or glucose as the sole carbohydrate source and subsequently transferred three times into fresh selection broth before use as starter inoculi.

### Cheese Milk

Pasteurized skim milk (80°C for 25 s) obtained from the Utah State University Dairy Plant was standardized to 3.5% (wt/wt) fat with 35% cream. A split-plot experiment was designed and the 12 starter cultures were randomly appointed to a 20 lb (9.1 kg) vat. Those vats chosen for lactose negative starters were lactase (EC 3.2.1.23) treated (0.02% vol/wt) for better utilization of lactose by the starters. The lactase-treated milk was incubated at 4°C overnight. Before inoculation, the cheese milk was pre-acidified to pH 6.0 with 10% (vol/vol) acetic acid and vat pasteurized at 64°C for 30 min.

TABLE 15. Strains of *L. lactis* used in this study.

Strains	Phenotype <sup>1</sup>		
	Lac	PrtP	Opp
C2O	+	+	+
JK225	+	+	+
JK522	+	+	-
CB01	+	-	-
CB06	+	-	+
CB16	-	+	-
JK062	-	+	+
LM0230	-	-	+
LM2301	-	-	-
LM2302	-	-	+
LM2306	-	-	+
JK2048	-	-	-

<sup>1</sup> + = contains phenotype, - = lacks phenotype

### Slurries

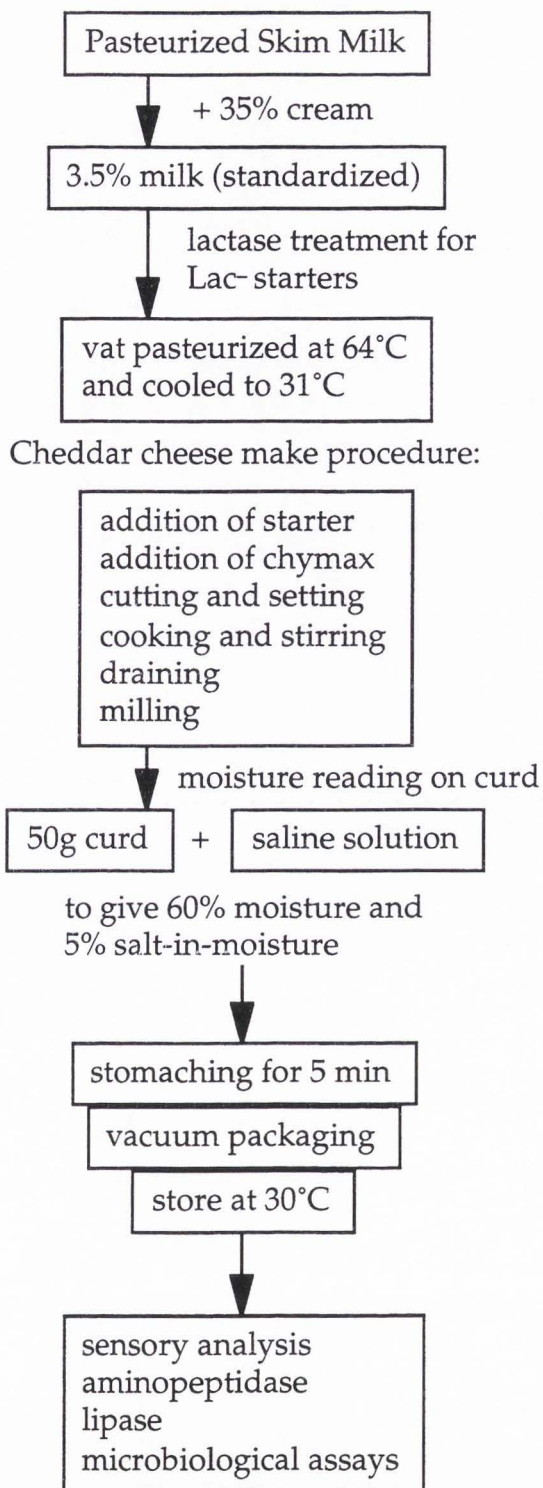
The flowchart in Figure 11 shows the cheese-making procedure used. The procedure through the milling step was as described by Samples (15). Following the milling step, the curd was analyzed for moisture content (1). An AVC 80 (CEM Corporation, Matthews, NC) microwave was used at 90% power for 5 min to measure the percent moisture in 4-5 g curd particles. A sterile NaCl solution was then added to give the curd a final concentration of 60% moisture and 5% salt-in-moisture (15). The formulas used to calculate the NaCl and water content needed are given in equations 1 and 2.

$$X = (a/0.4)(0.6-b/100)$$

Eq. 1

Figure 11. Flow chart of Cheddar slurry make procedure. The curd was analyzed for moisture and the correct saline solution added to 50 g of curd. The curd was made into slurries and further used for sensory analysis, bacterial counts, aminopeptidase, and lipase analyses.





$$Y = (ab/100 + X)(0.05\text{g NaCl}/1\text{ ml Water}) - (ac/100) \quad \text{Eq. 2}$$

X = ml sterile water needed

Y = g NaCl needed

a = g curd

b = percent moisture in curd

c = percent salt in curd

The curd was divided into 50-g portions and put into sterile stomacher bags. The saline solution was added to these bags and stomached into slurries using a Stomacher 400 lab blender (Seward Medical Ltd., London, UK) for 5 min at high speed. The bags were then vacuum packaged in a Multivac M885F (Multivac Inc., Kansas City, MO) and stored at 30°C.

### Microbiological Assays

Slurry samples were taken after 5, 7, 14, and 21 d. Table 16 shows the microbial characterization using selective media. Dilutions ranging from  $10^0$  to  $10^8$  were plated on selective media and incubated at appropriate temperatures for the appropriate amount of time (Table 16).

### Aminoamidase Assay

Each slurry was prepared for aminoamidase analysis (AP) by the method of Weimer et al. (18). Twenty grams of slurry was diluted with 180 ml of sterile double deionized water and blended in a Stomacher 400 lab blender (Seward Medical Ltd., London, UK) at 4°C for 5 min at high speed. The slurry was centrifuged at 10,000 rpm ( $10,300 \times g$ ) using a GSA rotor in a Sorvall<sup>®</sup> RC-5B centrifuge (DuPont Instruments, Wilmington, DE) for 30 min at 4°C then filtered through a GF/S filter (Whatman International Ltd., Maidstone, England) to separate fat from the slurry. The supernatant was further filtered through Whatman No. 2 and No. 5 (Whatman International Ltd., Maidstone, England) to

TABLE 16. Selective media used for isolation of potential microorganisms found in slurries.

Organism	Medium	Incubation	Reference
Total plate count	Elliker	aerobic, 30°C	2, p.330
NSLAB <sup>1</sup>	Rogosa	anaerobic, 37°C	2, p. 777
Coliforms	VRB <sup>2</sup>	anaerobic, 37°C	2, p. 977
Staphylococci	Baird Parker	aerobic, 37°C	2, p. 112
Pseudomonas	Kings B	aerobic, 4°C	2, p. 472

<sup>1</sup> non-starter lactic acid bacteria

<sup>2</sup> violet red bile agar

make sure that all the fat had been separated. During filtering the slurry solutions were kept on ice. Centriprep-30 concentrators (Amicon, Inc., Beverly, MA) with a cut-off point of 30,000 d were used to concentrate the extracts 2.5 times. All equipment had previously been sterilized. Assays were done as described by Dias and Weimer (4) at 37°C in 96-well microtiter plates (Baxter Diagnostic, Inc., Deerfield, IL) and covered with sterile tape to prevent evaporation. Hydrolysis of the substrates (1mM) was measured by monitoring the increase in yellowness ( $b^*$ ) using an Omnispec™ 4000 reflectance colorimeter (Wescor, Inc., Logan, UT). Assays were done in duplicate and readings taken every 5 min. Controls contained 100 µl of 0.1 M imidazole buffer together with 100 µl amino peptidase substrate. A blank control of 0.05 M *p*-Nitroanalide (*p*-NA) in 100 µl imidazole buffer was also included.

### Lipase Assay

Lipase activity was as described by Blake et al. (3). Six chromogenic substrate (5mM) Pal, Myr, Cap, But, Lau, and Ste-*p*-NA fatty acid derivatives

(Sigma Chemicals, St. Louis, MO) were dissolved in dimethyl sulfoxide and stored at  $-20^{\circ}\text{C}$ . Stock lipase solutions were diluted to 1 mM with 0.05 M sodium phosphate, pH 6.5 and 100  $\mu\text{l}$  pipetted onto 96-microtiter plates. Further, 100  $\mu\text{l}$  of extract was added and the change in yellowness ( $b^*$ ) monitored at  $37^{\circ}\text{C}$  in an Omnispec™ 4000 reflectance colorimeter (Wescor Inc., Logan, UT). These assays were also done in duplicates and readings taken every 10 min. Controls contained 100  $\mu\text{l}$  of 50-mM sodium phosphate buffer (pH 6.5) together with 100  $\mu\text{l}$  of lipase substrate. A 50-mM sodium phosphate buffer blank (pH 6.5) and a 50-mM *p*-NA in 100  $\mu\text{l}$  imidazole buffer blank were also included as controls.

### Sensory Analysis

*Selecting and training of judges.* Students, faculty, and staff of Utah State University were recruited as potential sensory panelists for evaluation of bitterness, acidity, and rancidity in Cheddar cheese slurries. A pool of 15 panelists with interest and time tasted slurries fortified with known levels of quinine-sulfate, lactic acid, and butyric acid (17). Extreme flavors (Table 17) were presented to each trainee to find those who had the ability to detect these flavors. Three different concentrations were made available for the trainees to taste and discuss. At the end of eight training sessions, the same set was given to the trainees as unknown samples. They evaluated the slurries for bitterness, acidity, and rancidity on a line scale from 0 to 16 (0 = no flavor perceptible and 16 = extreme flavor). Seven panelists were chosen based on their sensitivity and ability to recognize bitterness, acidity, and rancidity in cheese slurries.

*Sample preparation, serving, and taste panel.* Cheddar cheese slurries were made and packaged as described in the flowchart (Figure 11). Each of the seven panelists received 2.0-g slurry samples, which were put into plastic cups

TABLE 17. Chemicals and their concentrations used to simulate bitterness, acidity, and rancidity.

Flavor	Chemical	Concentrations
Bitterness	quinine-sulfate	0.1, 0.2, 0.3% (wt/wt)
Acidity	lactic acid	0.3, 0.6, 1.2% (vol/wt)
Rancidity	butyric acid	0.25, 0.5, 1.0% (vol/wt)

coded with random 3-digit numbers. To avoid positional bias, samples were served in different randomized orders among judges. Samples were evaluated in individual booths under white fluorescent light. Judges were asked to rinse their mouths with water between samples to avoid sensory overlap between samples. Slurry samples were evaluated by the judges after 5, 7, 14 and 21 d for acidity, bitterness, and rancidity. Six samples were provided at each session. The same liner scale used in the training was used throughout the study.

### Statistical Analysis

The distances on the line scale for each sample and flavor were measured, given a numerical value, and analyzed with a split-plot design using Minitab statistical software (14). The main effects were judge, culture used in the slurry, storage time, and which half of the split plot was used (called run). Two-way interactions of the main effects were also included as shown in the model (Equation 3).

$$Y = \text{judge} + \text{time} + \text{run} + \text{culture}(\text{run}) + \text{judge} * \text{run} + \text{judge} * \text{culture}(\text{run}) + \text{run} * \text{time} + \text{time} * \text{culture}(\text{run}) + \text{ErrorA} + \text{ErrorB}$$

Eq. 3

Statistical analyses (ANOVA) on the strains with and without Opp, PrtP, and Lac as their phenotypes were also performed using SAS as the statistical software (16) to see if any of the traits were important in rancidity flavor. Main effects included Lac (L), PrtP/PrtM (P), Opp (O), and Time (T). Their two-way, three-way, and four-way interactions were also included (Equation 4).

$$Y = L + O + P + T + L*P + L*O + L*T + P*O + P*T + O*T + L*P*O + L*P*T + L*O*T + P*O*T + L*P*O*T + \epsilon \quad \text{Eq. 4}$$

## RESULTS

### Cheddar Cheese Slurries

Moisture in the slurries before adjusting them to 60% ranged from 41 to 50%. After adjusting with sterile saline solution and blending, satisfactory slurry products were obtained and vacuum packaged.

### Microbiological Assays

After 1 wk at 30°C, some of the slurry packages showed various degrees of swelling, and gas was released upon opening. This bloatedness occurred randomly throughout the slurries made from the different starter strains. Possible correlation of swelling to non-starter lactic acid bacteria (NSLAB) was not determined. The aroma of these gas-containing slurry packages was somewhat pleasant and had no hint of an off aroma, which could be associated with rancidity, fruity, or barny.

Figure 12 shows the plate counts obtained for starters and NSLAB after 5, 7, 14, and 21 d. The data are represented as overall mean of microbial counts over time. General plate counts increased very slowly over time, though most individual slurries showed an increase in starter counts ( Figure 12). After a slow

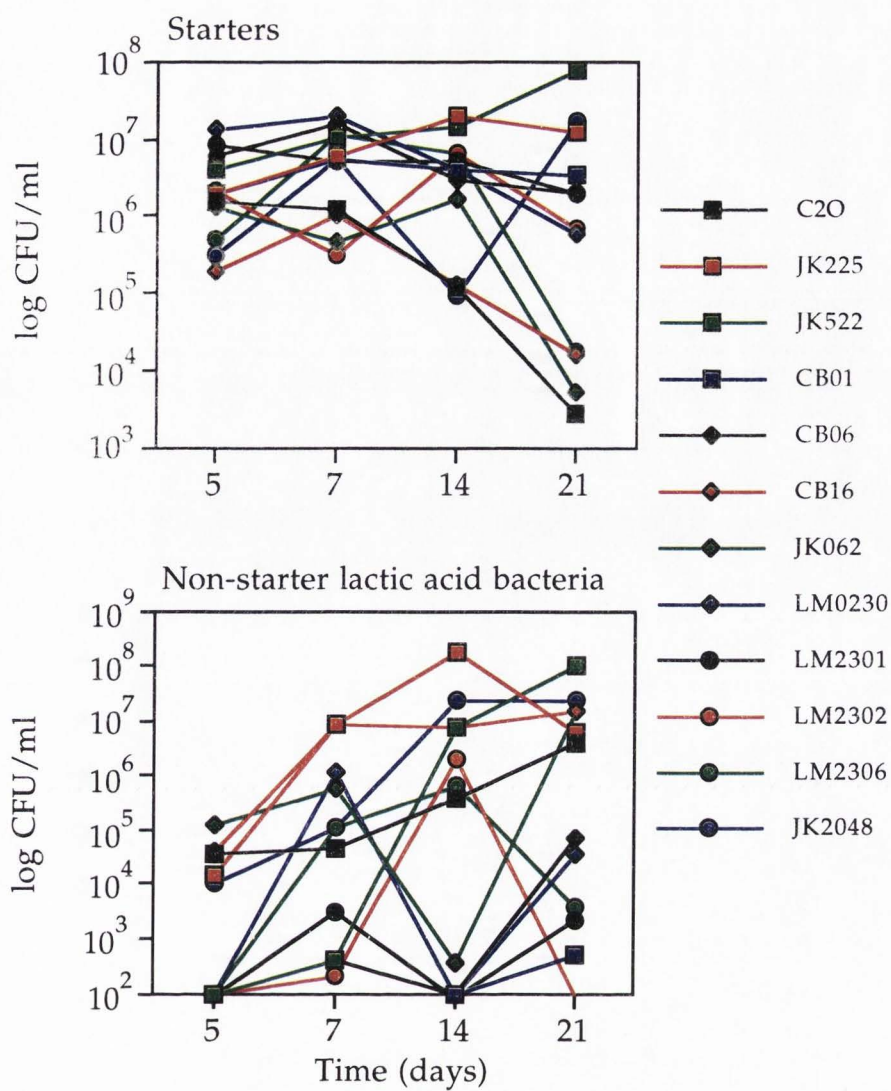


Figure 12. Microbial counts of starters and NSLAB from slurries made from various *L. lactis* subsp. *lactis* strains.

start, non-starter lactic acid bacteria increased rapidly to day 7 and leveled off after 14 d to an average population of  $10^7$  CFU/ml.

To control the degree of contamination and to make sure that the slurries were of no harm to the taste panelists, quality control for coliform, pseudomonas, and staphylococci was performed prior to panel activity. No coliforms or pseudomonas were detected at the end of the test period. *Staphylococcus* contamination was apparent at the end of the 21 d indicated by black colonies on Baird-Parker medium, but no *S. aureus* contamination was detected.

### **Aminopeptidase and Lipase Activity**

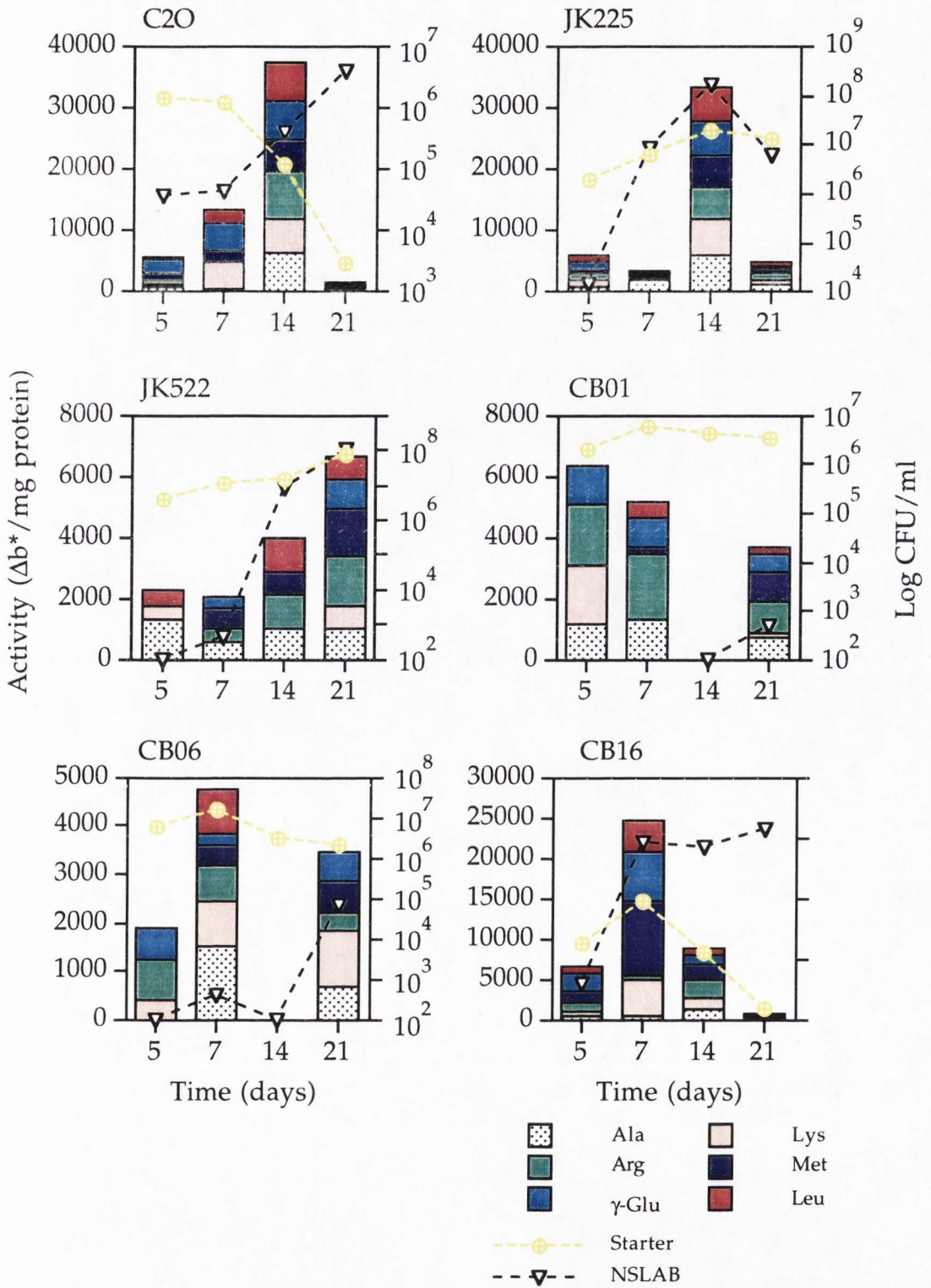
Figure 13 presents the individual AP data overlaid with the starter and NSLAB counts. Slurries made with C2O, JK225, and JK522 showed an increase in AP activity during the test period. At the same time, NSLAB counts increased but total starter counts either decreased or reached a stationary phase. Slurries made with CB06, CB16, JK062, LM0230, LM2302, LM2306, and JK2048 had increased AP activities including day 7, after which their AP activities decreased. These slurries also showed an increase in NSLAB counts, whereas the starter counts slowly decreased and even increased in JK2048. Slurries made with CB01 and LM2301 had an initially high activity, which later decreased. Their plate counts showed a slow decrease in starter and a slow increase in NSLAB.

Figure 14 shows the individual lipase activities obtained from the slurries. Those slurries made with JK225, JK522, CB06, and LM0230 showed an initially high lipase activity, which decreased on day 7 and then increased again on day 14. The starter counts in these slurries increased very slowly or not at all.

Slurries made with C2O, CB16, JK062, LM2302, LM2306, and JK2048 showed a low activity on day 5, and an increase on day 7, after which activities decreased throughout the rest of the period. The total starter counts on these all



Figure 13. Aminopeptidase activity profiles of slurries made with individual *Lactococcus lactis* subsp. *lactis* strains overlaid with general plate counts for starters and plate counts for NSLAB.



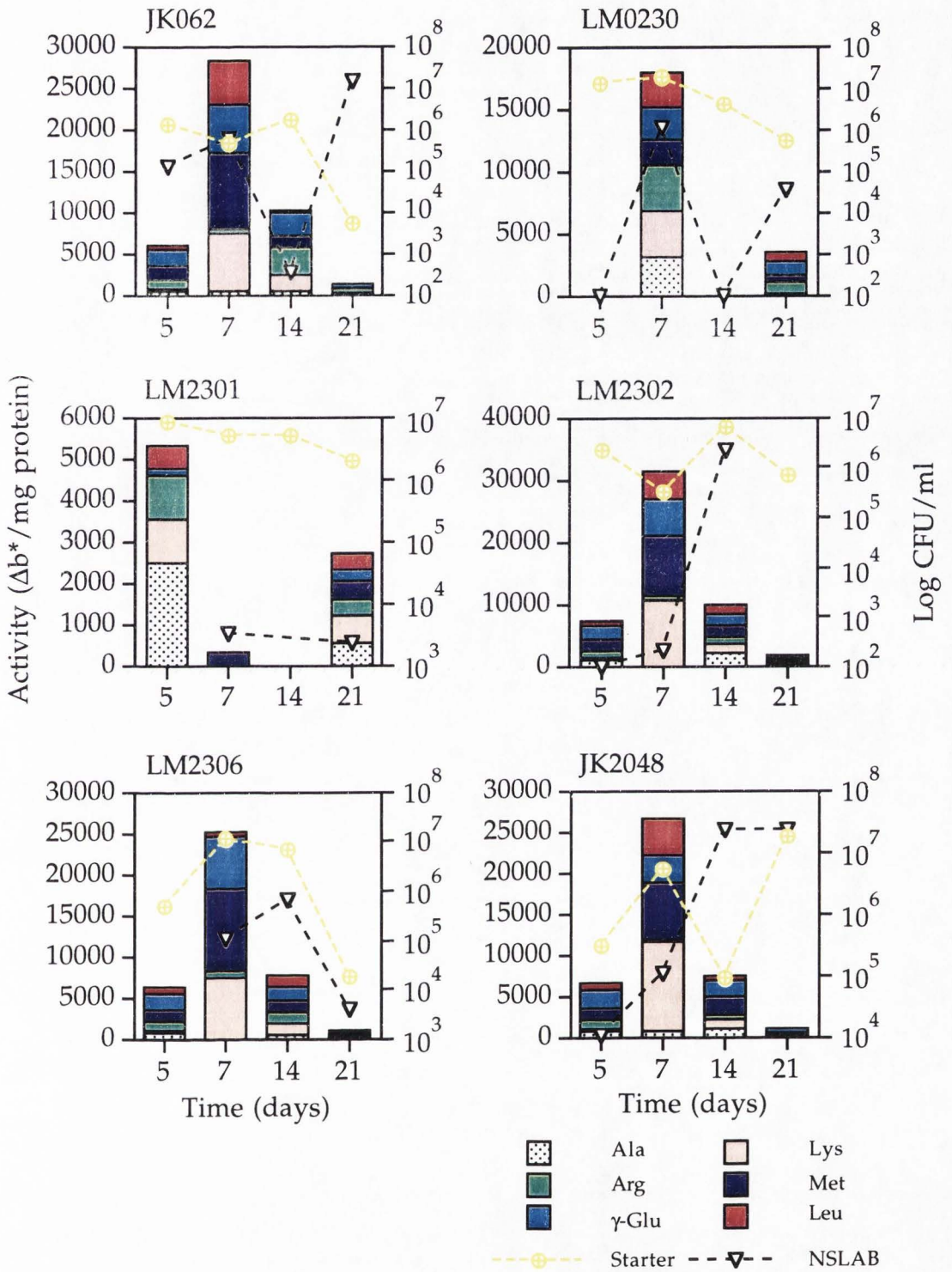
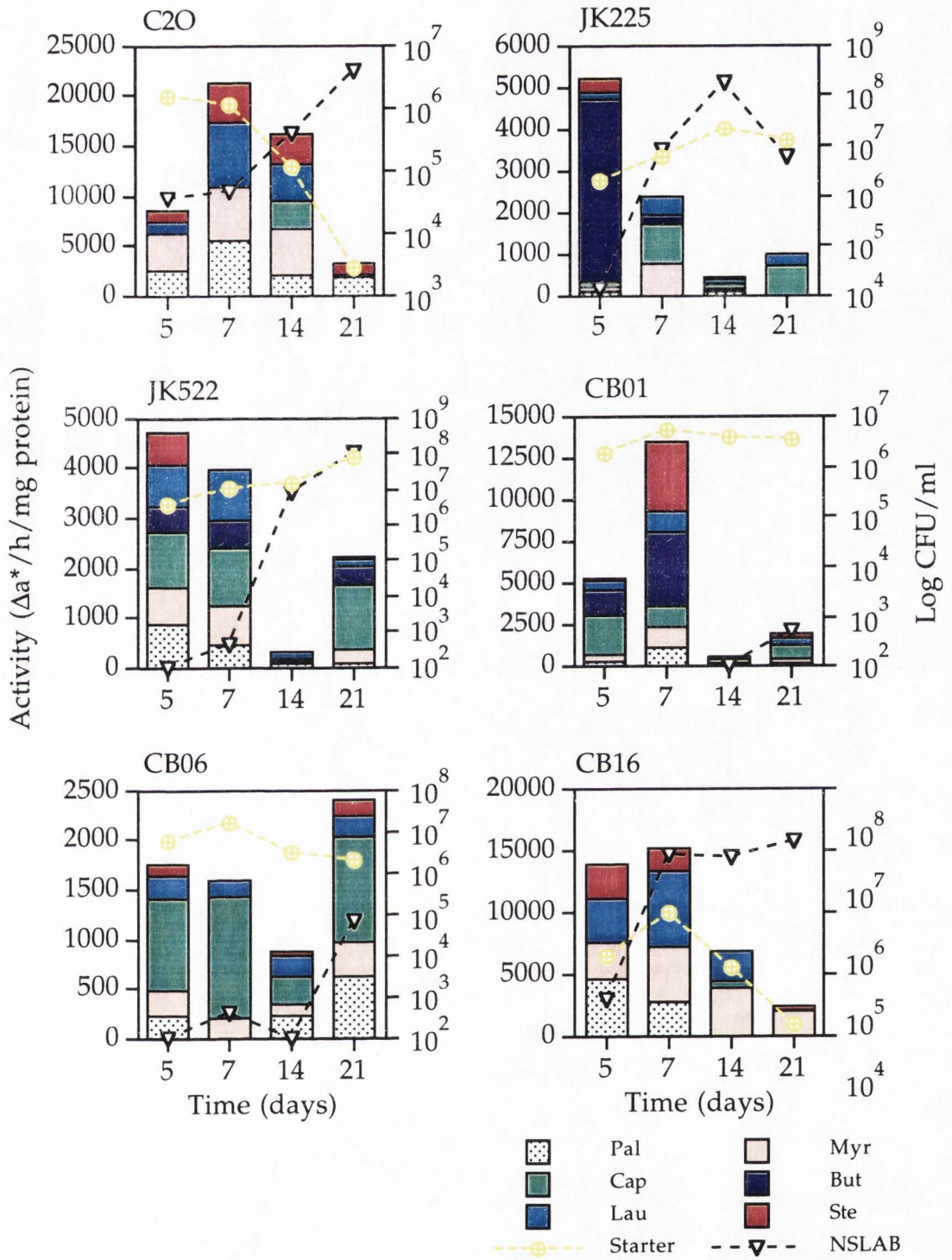


Figure 13 continued.

Figure 14. Lipase activity of slurries made with individual *Lactococcus lactis* subsp. *lactis* strains overlaid with general plate counts for starters and plate counts for NSLAB.



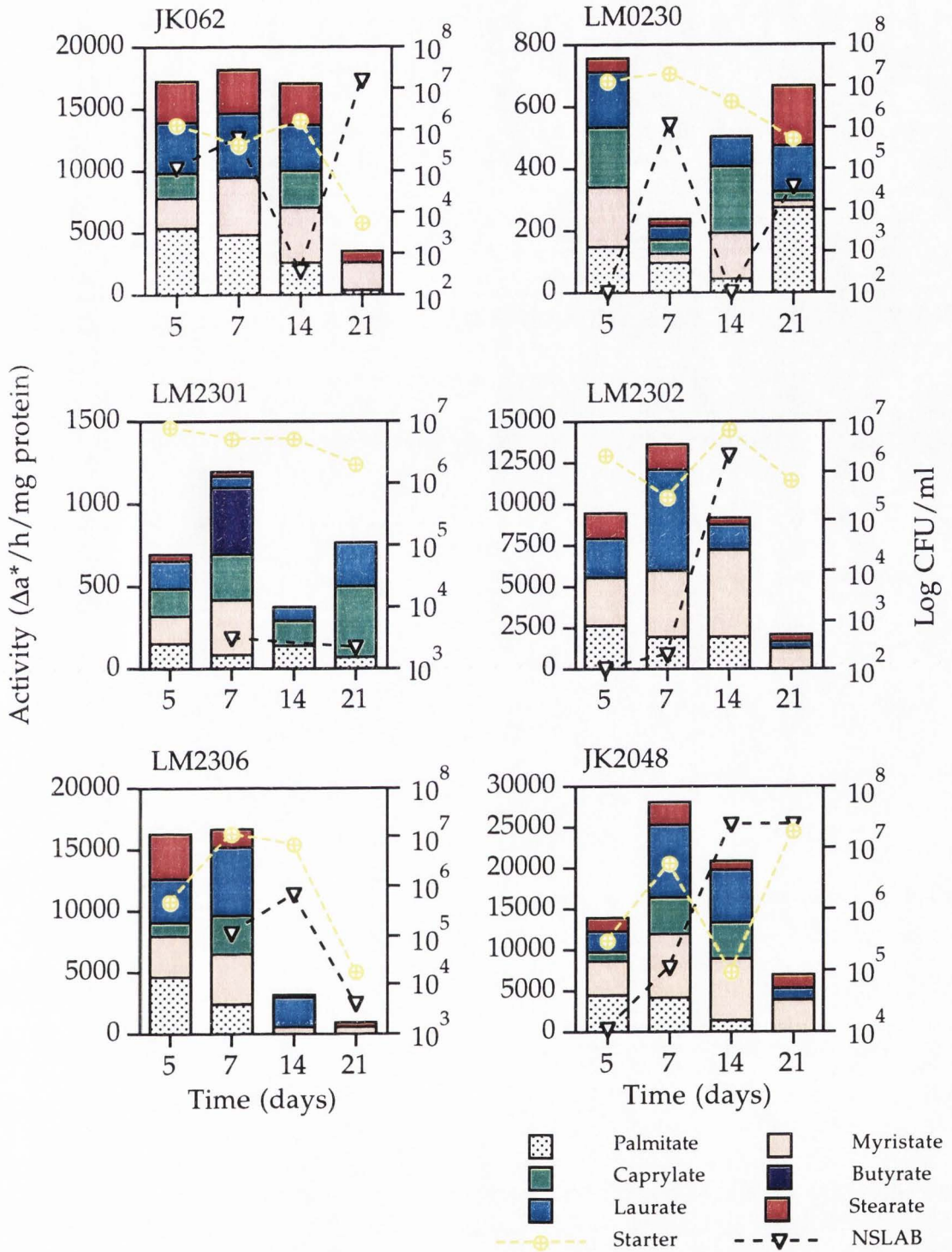


Figure 14 continued.

decreased, except for JK2048. Total NSLAB counts increased in C2O, CB16, JK062, LM2302, and JK2048. However, NSLAB decreased in slurry made with LM2306.

Slurries CB01 and LM2301 both have an initially low lipase activity, which increases by day 14 but does not reach the levels of day 5. The activity slowly increases again through day 21. The total starter counts are slowly decreasing in both slurries and the NSLAB counts are low as well.

### Sensory Evaluation

Repeated measures analysis of variance (ANOVA) was performed on all sensory panel data ( $\alpha = 0.05$ ) (14). Seven trained and screened judges participated in this study. There were variations among the judges in acidity, bitterness, and rancidity (Appendix A, Tables A7, A8, and A9). No culture effect was seen in the acidity or bitterness scores ( $P < 0.879$  and  $P < 0.992$ ). A significant culture effect was seen in the rancidity scores ( $P < 0.001$ ) (Appendix A, Table A9). Time effect and two-way time\*culture interaction were also significant in their acidity and rancidity scores ( $P < 0.001$  for both).

Table 18 shows the average rancidity scores over time. Since culture was a significant contributor to rancidity, we also investigated which one of the three traits (Lac, PrtP/PrtM, and Opp) contributed to rancid flavor. Figure 15 shows the rancidity scores over time when comparing strains with and without the trait of interest. Only Opp as a main effect showed significance ( $P < 0.046$ ). No significance was seen when comparing strains with or without PrtP/PrtM ( $P < 0.934$ ) or Lac ( $P < 0.347$ ) (Table 19). This result was surprising since Opp is related to peptide transport and not to rancidity. This suggests that rancidity is associated with the Opp system but it is not clear how it is related.

TABLE 18. Average rancidity scores<sup>1</sup> summarized over time and judges.

Culture	Time (d)				Phenotype		
	5	7	14	21	Lac	PrtP/PrtM	Opp
C2O	2.04	0.85	3.67	1.96	+	+	+
JK225	1.29	2.91	9.79	2.09	+	+	+
JK522	1.11	3.81	3.5	2.70	+	+	-
CB01	0.74	1.33	2.24	3.36	+	-	-
CB06	0.99	10.43	10.04	4.26	+	-	+
CB16	0.87	2.85	2.40	1.90	-	+	-
JK062	4.16	4.08	3.36	2.25	-	+	+
LM0230	3.40	2.29	7.74	4.54	-	-	+
LM2301	6.64	1.86	3.29	3.13	-	-	-
LM2302	2.80	0.78	3.40	6.10	-	-	+
LM2306	3.55	11.1	1.60	3.16	-	-	+
JK2048	1.50	2.38	0.59	3.54	-	-	-

<sup>1</sup> 0 = no flavor, 16 = extreme flavor

Table 20 shows the ANOVA performed to see if cultures had an effect on lipase activity, since rancidity scores showed significance in their culture effect. Both culture ( $P < 0.000$ ) and time\*culture interaction ( $P < 0.001$ ) were significant. This result was expected since lipase activity is associated with rancidity. To further investigate the similarities of rancidity to lipase Figure 16 was constructed. These graphs show the total rancidity scores of the judges overlaid with total lipase activity of cultures used in the slurries.

Figures B1 through B12 (Appendix B) show taste panel data (acidity,



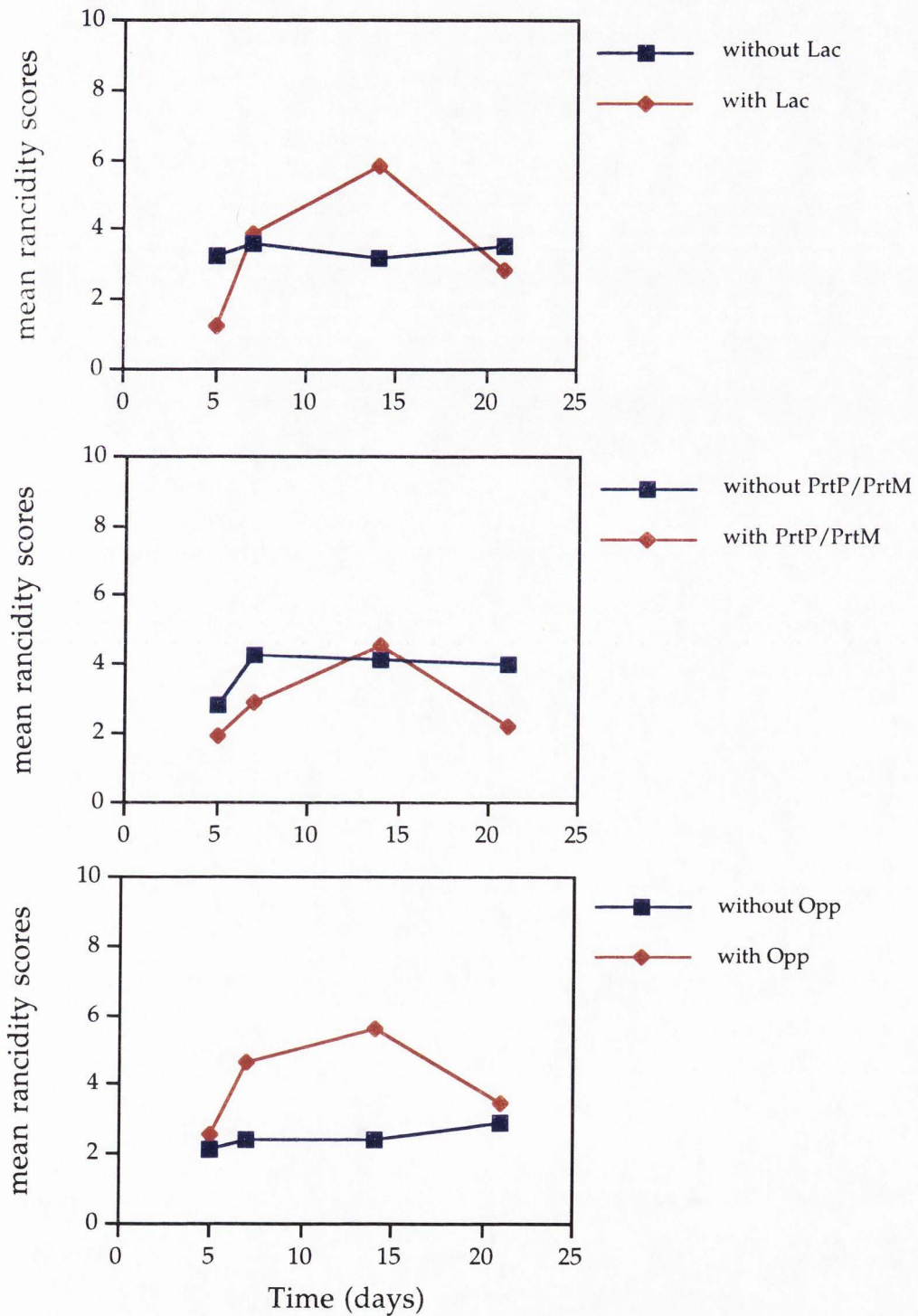


Figure 15. Rancidity scores over time when comparing cultures with and without a) Lac, b) PrtP/PrtM, and c) Opp as their traits.

TABLE 19. ANOVA for rancidity scores looking at cultures with and without Lac, PrtP/PrtM, or Opp.

Source	df	SS	MS	F	P > t <sup>1</sup>
Lac (L)	1	1.748	1.748	0.22	0.642
PrtP/PrtM (P)	1	10.597	10.597	1.36	0.261
Opp (O)	1	36.516	36.516	4.68	0.046
Time (T)	3	28.847	9.616	1.23	0.331
L*P	1	0.499	0.499	0.06	0.804
L*O	1	2.555	2.555	0.33	0.575
L*T	3	27.733	9.244	1.18	0.347
P*O	1	10.610	10.610	1.36	0.261
P*T	3	3.314	1.105	0.14	0.934
O*T	3	17.102	5.701	0.73	0.549
L*P*O	1	11.876	11.876	1.52	0.235
L*P*T	3	7.783	2.594	0.33	0.802
L*O*T	3	9.462	3.154	0.40	0.752
P*O*T	3	23.265	7.755	0.99	0.421
L*P*O*T	3	6.844	2.281	0.29	0.830
Error	16	124.913	7.807		
Total	47				

<sup>1</sup>  $\alpha$ -value = 0.05

bitterness, and rancidity) for each individual slurry made with the various strains of *L. lactis* subsp. *lactis*.

Bitterness stayed about the same in all the slurries throughout the test period. Acid scores were not significantly different in their culture effect, which was a result of lactase treatment of the milk before inoculation with starter and

TABLE 20. ANOVA for lipase activity.

Source of Variation	df	SS	MS	F	P > t <sup>1</sup>
Rep	1	17.43	17.43	0.36	0.561
Culture	11	8075.78	734.16	15.37	0.000
Run	1	7251.83	7251.83	151.84	0.000
Culture/Run	10	823.95	82.40	1.23	0.191
Error A	11	525.38	47.76	1.46	0.214
Time	2	216.62	108.31	3.10	0.063
Time*Culture	22	6649.74	302.21	8.66	0.001
Time*Run	2	281.73	109.37	3.13	0.062
Time*Culture/Run	20	6368.01	318.40	9.12	0.000
Error B	24	837.88	34.91		
TOTAL	71	16259.83			

<sup>1</sup>  $\alpha$ -value = 0.05

slurry making.

Rancidity scores showed various patterns. Slurries made with *L. lactis* subsp. *lactis* JK225, CB06, and LM0230 increased rapidly after 7 d. Slurries C2O, CB01, and CB16 showed a decreasing trend up to day 7, after which rancidity scores increased. Slurries JK062 and JK2048 showed decrease in rancidity over time. Slurries JK522, LM2301, and LM2306 showed various rancidity patterns, and slurry LM2302 increased in rancidity over the test period.

## DISCUSSION AND CONCLUSIONS

Slurry systems have been used since the late 1960's to study the effects of different aspects, such as bitterness and influence of starters, on cheese ripening (10). Our interest was to observe the ability of strains with different proteolytic

Figure 16. Individual lipase activity compared to average rancidity scores for each slurry over time. Rancidity scores are shown as a line graph, whereas lipase activity is represented in a stacked bar graph. Rancidity scores are averaged over judges.



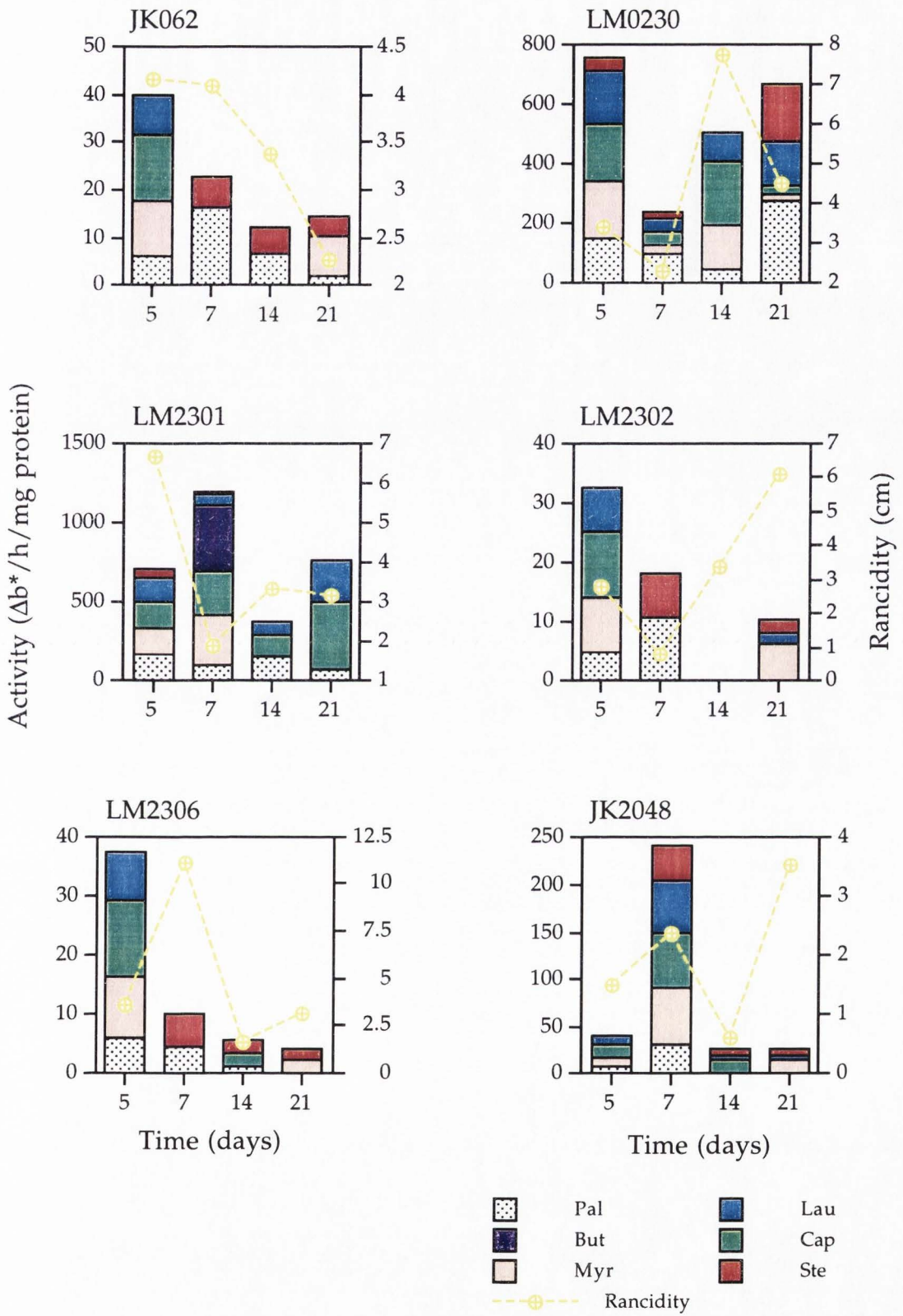


Figure 16 continued.

phenotypic combinations on their ability to influence acidity, bitterness, and rancidity. Acidity measurements were included to monitor that the lactase used to treat cheese milk prior to inoculation with starter was degrading lactose into galactose and glucose so that Lac<sup>-</sup> strains were not limited in their carbohydrate source. The enzyme worked satisfactorily since there was no culture effect on the acidity scores from the sensory analyses.

Bitterness was included because we hoped to see the influence of the proteolytic system on flavor, especially bitter flavor, development when using starters that had different proteolytic capabilities and different phenotypic makeup. The statistical analysis, however, showed that starter (culture) had no significant effect on bitterness scores. Thus, we could draw no conclusions about the influence of these starters on bitterness.

However, an interesting observation was made with the rancidity scores and lipase data. Both lipase taste panel data for rancidity had a significant culture and culture\*time effect (Tables 20 and A9). This suggests that there might be an association between lipase and rancidity. Figures 16A and 16B show individual slurry lipase activities together with average rancidity scores. Most rancidity scores have similar patterns as their corresponding lipase activity. However, rancidity scores appear to lag a couple of days behind lipase activity. This suggests that rancidity by-products need to reach a threshold before rancidity taste can be detected.

Since lipase activity patterns are similar to AP patterns, it raises the question, are lipase and aminopeptidase activity linked? The flavor change seems to be linked to the increase in enzymes degrading long chain fatty acids. Pasteurized whole milk contains short chain fatty acids that are part of the normal taste of milk. When the longer chain fatty acids are removed from the

triglyceride, they are made available to be made into shorter chain length fatty acids. These fatty acids contribute to rancid flavor directly or there is an accumulation of short chain fatty acids, which allows the taste panel's threshold for rancid flavor to be exceeded.

The Opp trait showed an influence on rancidity scores (Figure 15). An interesting question arises. Since the graphs of the Lac and Opp traits are very similar, do they share a common intermediate in the glycolysis pathway that together with AP activity leads to rancidity? All data suggest that AP is involved, but how is still uncertain.

## REFERENCES

- 1 Association of Official Analytical Chemists International. 1990. Official Methods of Analysis. Vol. 2. 15<sup>th</sup> ed. AOAC Inc., Arlington, VA.
- 2 Atlas, R. M. 1993. Pages 112, 330, 472, 777, and 977 *In Handbook of Microbiological Media*. L. C. Parks ed. CRC Press Inc., London, UK.
- 3 Blake, M. R., R. Koka, and B. C. Weimer. 1996. A semiautomated reflectance colorimetric method for the determination of lipase activity in milk. *J. Dairy Sci.* 79:1164.
- 4 Dias, B., and B. Weimer. 1995. A semi-automated colorimetric method for determination of aminopeptidase activity in turbid solutions. *J. Rapid Meth. Automation Microbiol.* 3:223.
- 5 Farkye, N. Y., P. F. Fox, and G. F. Fitzgerald. 1990. Proteolysis and flavor development in Cheddar cheese made exclusively with single strain proteinase -positive or proteinase-negative starters. *J. Dairy Sci.* 73:874.
- 6 Fox, P. F. 1989. Acceleration of cheese ripening. *Food Biotechnol.* 2:133.
- 7 Fox, P. F. 1989. Proteolysis in cheese during manufacture and ripening. *J.*



- Dairy Sci. 72:1379.
- 8 Fox, P. F. 1993. Exogenous enzymes in dairy technology. A review. J. Food Biochem. 17:173.
- 9 Fox, P. F., and M. B. Gruffey. 1991. Exogenous enzymes in dairy technology. Page 219 *in* Food Enzymology Vol. 1. P. F. Fox, ed. Elsevier Appl. Sci., London.
- 10 Kristoffersen, T., E. M. Mikolajcik, and I. A. Gould. 1967. Cheddar cheese flavor. IV. Direct and accelerated ripening process. J. Dairy Sci. 50:292.
- 11 Law, B. A., Z. D. Hosking, and H. R. Chapman. 1979. The effect of some manufacturing conditions on the development of flavour in Cheddar cheese. J. Soc. Dairy Technol. 32:87.
- 12 Law, B. A., and A. S. Wigmore. 1982. Accelerated cheese ripening with food grade proteinases. J. Dairy Res. 49:137.
- 13 Oberg, C. J., I. H. Davis, G. H. Richardson, and C. A. Ernstrom. 1986. Manufacture of Cheddar cheese using proteinase-negative mutants of *Streptococcus cremoris*. J. Dairy Sci. 69:2975.
- 14 Minitab Statistical Software. 1989. Minitab Inc., State College, PA.
- 15 Samples, D. R. 1985. Some factors affecting the production of volatile sulfhydryl compounds in Cheddar cheese slurries. Diss. Abstr. Int. B, 46:1402.
- 16 SAS/STAT User's Guide. 1992. Vol. 1. Version 6. 4<sup>th</sup> ed. Sas Institute Inc., Cary, NC.
- 17 Stadhouders, J., L. Toepoel, and J. T. M. Wouters. 1988. Cheese making with Prt<sup>-</sup> and Prt<sup>+</sup> variants of N-streptococci and their mixture. Phage sensitivity, proteolysis and flavour development during ripening. Neth. Milk Dairy J. 42:183.

- 18 Weimer, B. C., B. Dias, M. Ummadi, J. Broadbent, J. Jaegi, M. Johnson, F. Milani, J. Steele, and D. V. Sisson. 1997. Influence of NaCl and pH on intracellular enzymes that influence Cheddar cheese ripening. *Le Lait* 77:783.
- 19 Wilkinson, M. 1992. Studies on the acceleration of Cheddar cheese ripening. Ph.D. thesis, National Univ. of Ireland, Cork.

## CHAPTER V

DEVELOPMENT OF A STATISTICAL PREDICTION MODEL USING  
LACTOCOCCAL STARTER STRAINS VARYING IN  
THEIR PROTEOLYTIC PHENOTYPE

## ABSTRACT

Twelve strains of *Lactococcus lactis* subsp. *lactis* with different proteolytic compositions and oligopeptide transport systems were analyzed for their ability to hydrolyze bovine caseins as substrates. The amino acid profiles were converted to two-dimensional graphs with the aid of canonical discrimination function analysis. To investigate phenotypical trends of the strains, cluster analysis on canonical variables was performed. The cluster analysis showed no trend based on the phenotypical grouping of strains. A prediction model was developed to determine the probability of a strain showing a certain proteolytic phenotype based on its bovine casein hydrolysis profile. The best substrate, a mixture of  $\alpha_{s1}$ -,  $\beta$ -, and  $\kappa$ -caseins, correctly estimated all strains into their respective groups.

## INTRODUCTION

The proteolytic system of *Lactococcus lactis* has been thoroughly studied by many research groups (6, 8, 9, 10, 12, 15, 16). Their fermentation behavior has been studied extensively (9) to reduce the probability of failure in the cheese industry. The genetics of *Lactococcus lactis* subsp. *lactis* is well understood. The proteolytic system of lactococci consists of PrtP/PrtM, a proteinase PrtP that is rendered mature by PrtM, and Opp, an oligopeptide transport system. Both mechanisms, together with other transport systems for shorter chain peptides

and individual amino acids, are required for lactococci to utilize required nutrients for growth. Transport systems have been identified and characterized (7). The amount of laboratory work required for each of these characterizations is enormous.

Amino acid analysis coupled with discriminant function analysis can drastically reduce the time necessary to characterize proteolytic phenotypes of strains. These statistical methods can reduce large amounts of data from amino acid profiles to provide information about relatedness among strains (11, 13, 14). Probability models can be used to determine phenotypical groups associated with strains. Use of statistical models can reduce analysis time needed to predict phenotypes of strains by eliminating the necessity of genetic isolation and enzyme characterization.

## MATERIALS AND METHODS

### Bacterial Strains

Nine strains of *Lactococcus lactis* subsp. *lactis* with various proteolytic (PrtP) and oligopeptide transport (Opp) phenotypes were obtained from Jeff Broadbent (Utah State University, Department of Nutrition and Food Sciences) (Table 21).

Mutant CB06 was constructed by transducing LM2306 with a UV-induced (15 s) prophage from C2O. Mutants from this transduction were isolated by plating the mixture on bromocresol purple medium (2) containing lactose (BCP-lac) and were screened for large yellow colonies, indicating lactose utilization. Lactose positive colonies were further characterized for proteinase activity using Fast Slow Differential Agar II (2) by selecting faint yellow colonies with halos.

TABLE 21. Phenotype traits for Lac, PrtP, and Opp of *Lactococcus lactis* subsp. *lactis* strains used in this study.

Strain	Phenotype <sup>1</sup>		
	Lac <sup>2</sup>	PrtP <sup>2</sup>	Opp <sup>2</sup>
C2O	+	+	+
JK225	+	+	+
JK522	+	+	-
CB01	+	-	-
CB06	+	-	+
CB16	-	+	-
JK062	-	+	+
LM0230	-	-	+
LM2301	-	-	-
LM2302	-	-	+
LM2306	-	-	+
JK2048	-	-	-

<sup>1</sup> + contains, - lacks

<sup>2</sup> Lac = lactose utilization, PrtP = proteinase, and Opp = oligopeptide transport

Mutant CB01 was constructed by inducing the prophage (same as in C2O) in CB06 (contained in the LM2306 background) and transfecting LM2301 with the resulting particles. Mutants were plated directly on BCP-lac and four to five yellow colonies were randomly selected.

Mutant CB16 was obtained by isolating pGK13 (which contains a Bgl II fragment containing *prtP*) from JK062, and electroporating pGK13 into LM2301 at 8.3 ms, 400  $\Omega$ , 25  $\mu$ F (BioRad, Redwood, CA).

All strains were maintained frozen (-70°C) until needed as inoculum in

pure casein solutions. Strains were then transferred into fresh M17 broth containing glucose or lactose as the sole carbohydrate source and subsequently transferred into fresh selection broth before use.

### **Proteolysis Test**

**Substrate.** Individual caseins were prepared from milk by the method of Hollar et al. (3). Pure whole caseins (obtained from Carol Hollar) were reconstituted in water and fractionated using fast protein liquid chromatography to isolate individual caseins. These individual casein fractions were freeze dried and stored at  $-20^{\circ}\text{C}$  until needed. Each casein was added to Jenness-Koops buffer (4) (pH 6.5) in the concentration found in milk of 10 mg of  $\alpha_{s1}$ -casein, 9.3 mg of  $\beta$ -casein, and 3.3 mg/ml buffer of  $\kappa$ -casein.

**Incubation.** Each strain was grown overnight at  $30^{\circ}\text{C}$  in appropriate M17 broth, and cells were collected by centrifugation ( $3,300 \times g$  for 10 min at  $4^{\circ}\text{C}$ ). They were then washed three times with sterile 0.85% (wt/vol) saline solution and dissolved in fresh sterile 0.85% saline to an  $\text{OD}_{600}$  of 0.05 (to produce a standardized cell suspension).

Each casein-substrate solution was inoculated with 1% (vol/vol) of each standardized cell suspension and incubated at  $30^{\circ}\text{C}$  for 6 h. Controls containing no bacteria were also included. The samples were precipitated with trichloroacetic acid (TCA) to a final concentration of 6% (vol/vol) and filtered through Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). Aliquots of filtrates (2.5 ml) were freeze-dried. These lyophilized 6% TCA-soluble fractions obtained after 6 h incubation of the culture in casein solutions were then prepared for amino acid analysis (AAA) by the method of Kaiser et al. (5).

*Amino acid analysis.* The freeze-dried filtrates were reconstituted with 1 ml of 6N HCl, sonicated and vacuum sealed to exclude O<sub>2</sub>, and hydrolyzed at 110°C for 20 h. The hydrolysates were filtered through 0.2 µm syringe filters. Fifteen microliters of each sample was dried by gently blowing N<sub>2</sub> gas over the surface. Samples were rehydrated with 250 µl of Beckman NaS dilution buffer, loaded onto an Amino Acid Analyzer 6300 (Beckman Instruments, Fullerton, CA) in triplicates, and 50 µl was injected and analyzed.

### **Statistical Analyses**

*Discriminant function analysis.* Discriminant function analysis (DFA) is a technique that allows classification of individual samples into one, two, or more alternative groups based on a set of measurements. It is also used to identify which variables contribute to making these classifications (1, 13). Through DFA, a probability model was derived to predict the phenotypic composition of strains based on casein hydrolysis patterns (1, 13).

*Canonical analysis.* Canonical discriminant analysis was performed on all 15 individual amino acid concentrations of all 12 strains to reduce the results to two dimensions (12, 13). This technique derives the linear combination of variables that has the highest possible correlation with the group. It is a technique that reduces a multidimensional image to a two-dimensional plot (1). It combines canonical correlation, which examines the relationship between two sets of variables, with principal component analysis, which examines the relationship within a set of variables. A matrix of amino acid analysis data (n=15, df=14) was analyzed for three replicates of each strain (n=12, df=11) to determine canonical variables.

*Clustering analysis.* Clustering was also performed on the amino acid hydrolysis results to investigate if the strains would cluster according to their proteolytic profiles. Ward's hierarchical clustering method was used (12, 13).

## RESULTS

### Amino Acid Analyses

Amino acid analysis patterns of the various casein solutions were normalized and the control was subtracted. Negative concentration of an amino acid, i.e., relatively lower concentrations of that amino acid in 6% TCA-soluble fraction, indicates the cell utilized that amino acid. Positive concentrations suggest either secretion of the amino acid into the media or lack of transport of the amino acid.

Figure 17 presents the amino acid profiles for growth of the strains in  $\alpha_{s1}$ -casein substrate. Strains with PrtP and Opp phenotypes (C2O, JK225, and JK062) show lower Glu. This implies that other strains use less Glu or that the cell secretes more Glu, thus hiding the "true" uptake amounts. Both Gly and Asp are found in a higher concentration, suggesting that they are secreted by the bacteria as individual amino acids or as part of peptides. Proteolytic negative strains (JK2048, LM2301, and CB01) showed little difference in their amino acid profiles. There was no trend visible when PrtP<sup>-</sup> strains and Opp<sup>-</sup> strains were compared to their positive counterparts. This could be caused by low specificity of the proteinase system for the peptides generated.

The 6% TCA-soluble fractions from  $\beta$ -casein is shown in Figure 18. Proteolytic negative strains (JK2048 and LM2301) had similar profiles. Full proteolytic strains (JK062, C2O, and JK225) differed in four amino acids (Ser, Leu,



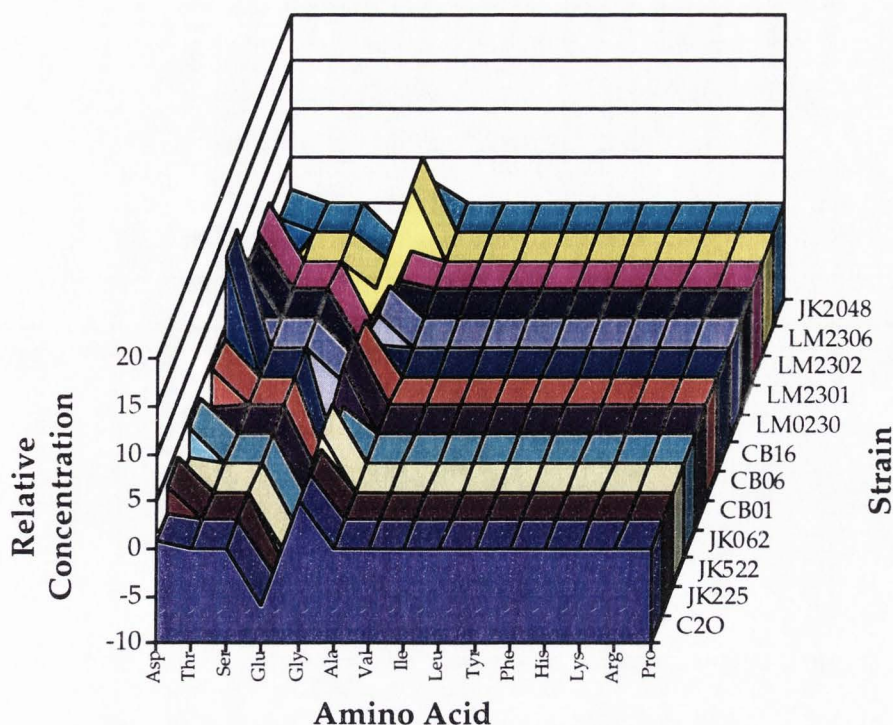


Figure 17. Amino acid concentration of TCA-soluble fractions (6%) of  $\alpha_{s1}$ -casein after incubation with *L. lactis* subsp. *lactis* strains for 6 h at 30°C.

His, and Lys). Strain C2O showed a very positive Lys peak, whereas the other two strains have a very negative peak. This suggests that C2O secretes Lys into the media. C2O also showed a less positive His peak than JK062 and JK225. Strain JK062 had a less negative Leu peak than C2O and JK225.

Strains with only PrtP (JK522 and CB16) showed various amino acid patterns, whereas Opp<sup>+</sup> strains (LM2302, LM2306, and CB06) had similar patterns. All PrtP<sup>-</sup>Opp<sup>+</sup> strains had amino acid patterns similar to that of C2O (PrtP<sup>+</sup>Opp<sup>+</sup>). This suggests that other transport systems are involved in the utilization of amino acids derived from  $\beta$ -casein hydrolysis. It may also suggest that the proteinase specificity of the strain is different.

Figure 19 shows the amino acid profile for  $\kappa$ -casein. The majority of

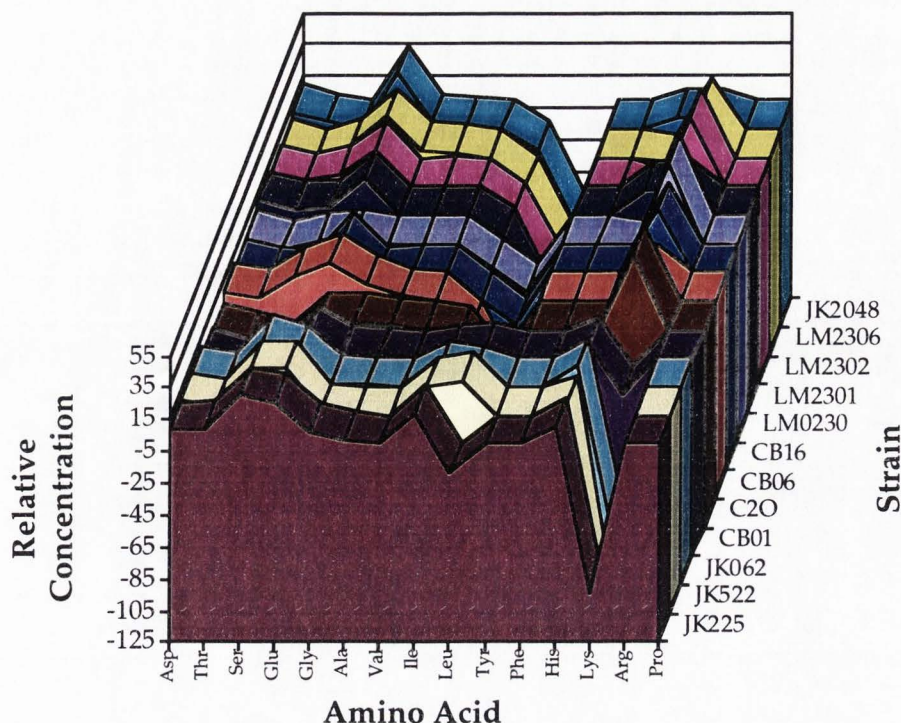


Figure 18. Amino acid concentration of TCA-soluble (6%) of  $\beta$ -casein after incubation with *L. lactis subsp. lactis* strains for 6 h at 30°C.

differences can be seen in the Glu, Ile, Phe, His, Lys, Arg, and Pro peaks. In the PrtP<sup>+</sup> strains (C2O, JK225, and JK062), the three amino acids of interest are Arg, Glu, and His. Strain C2O shows a lower accumulation of His than JK225 and JK062, whereas strain JK225 shows a lower level in Arg than C2O and JK062. Strain JK062 also shows a lower level in Glu concentration than C2O and JK225. All three strains have full proteolytic capabilities but they show different amino acid profiles. This again suggests that other transport systems are active together with Opp or the proteinase specificity is different. All proteolytic negative strains (LM2301, JK2048, and CB01) show very little or no differences except in the Ile peak. Strains with a combination of PrtP and Opp show a variety of profiles. Strains LM2306, LM2302, LM0230, and CB06 (PrtP<sup>-</sup>Opp<sup>+</sup>) all show similar

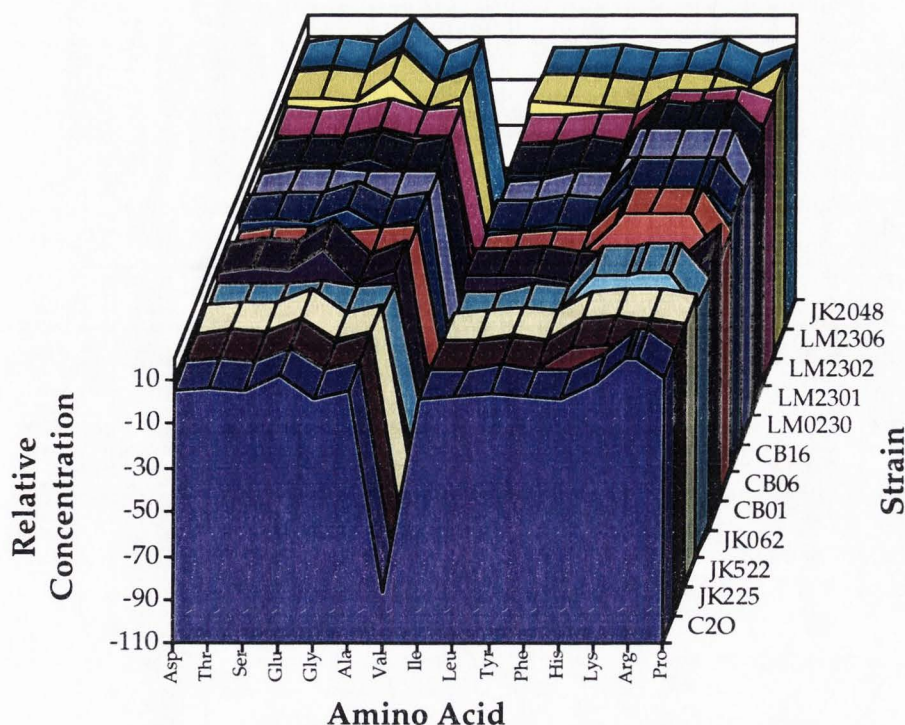


Figure 19. Amino acid concentration of TCA-soluble fractions (6%) of  $\kappa$ -casein after incubation with *L. lactis* subsp. *lactis* strains for 6 h at 30°C.

concentrations except in Glu, which is higher in LM2306, and in Lys, which is higher in CB06. Strains JK522 and CB16 (PrtP<sup>+</sup>Opp<sup>-</sup>) also are very similar except in the Arg peak, which is higher in CB16.

Figure 20 shows the amino acid hydrolysis pattern for mixed casein solution. Full proteolytic strains differ in the four amino acid profiles for Ser, Ile, His, and Lys. Strain C2O showed a higher Lys peak than Ser and His peaks. The Ile peak was highest in JK225. Strains containing no PrtP had similar concentration levels except in Asp, Glu, and Lys. No trends between strains containing only Opp<sup>+</sup> or Opp<sup>-</sup> strains were obvious nor between strains containing either PrtP<sup>+</sup> or PrtP<sup>-</sup>.

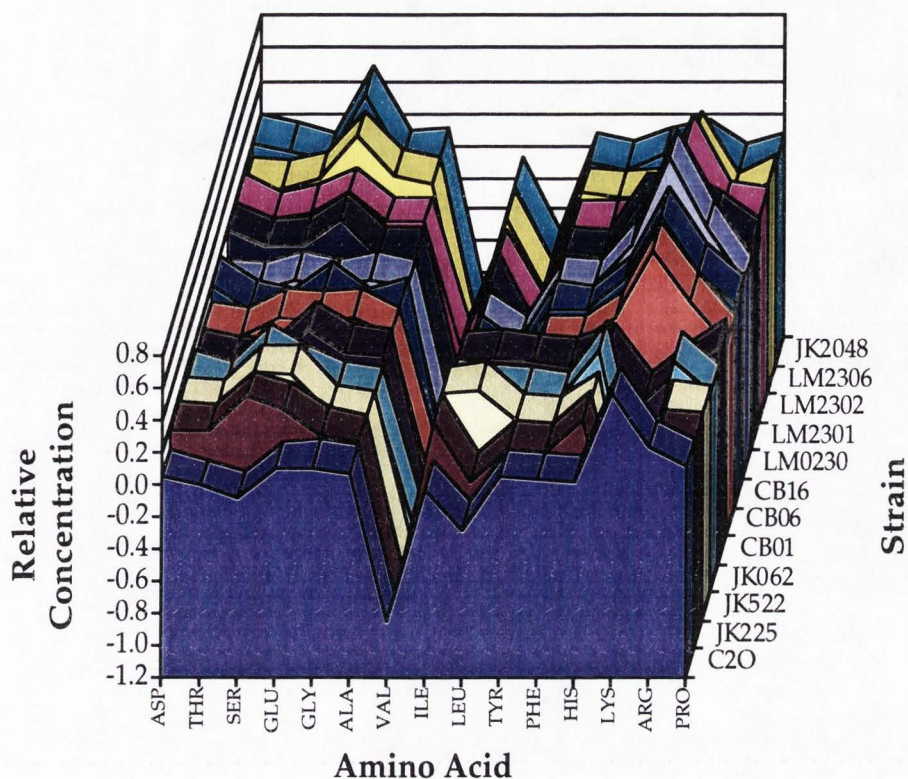


Figure 20. Amino acid concentration TCA-soluble (6%) fractions of mixed caseins after incubation with *L. lactis* subsp. *lactis* strains for 6 h at 30°C.

Figure 21 shows the amino acid hydrolysis pattern for milk. Glutamine showed various levels for all strains. The Leu concentration was highest in LM2302. Strain LM2302 stood out in the whole profile. It differed in all its amino acids except Asp, Thr, Ala, Arg, and Pro. Full proteolytic negative strains differed only in their His peak, being highest in JK2048, but otherwise showed a similar pattern. Prt<sup>-</sup> Opp<sup>+</sup> strains showed similar trends except for LM2302, which showed a higher Leu and Lys peak, and lower Glu and Gly peaks. Proteolytic negative strains showed no major differences. No differences could be seen between strains possessing either Opp<sup>+</sup> or Opp<sup>-</sup> (except LM2302), or

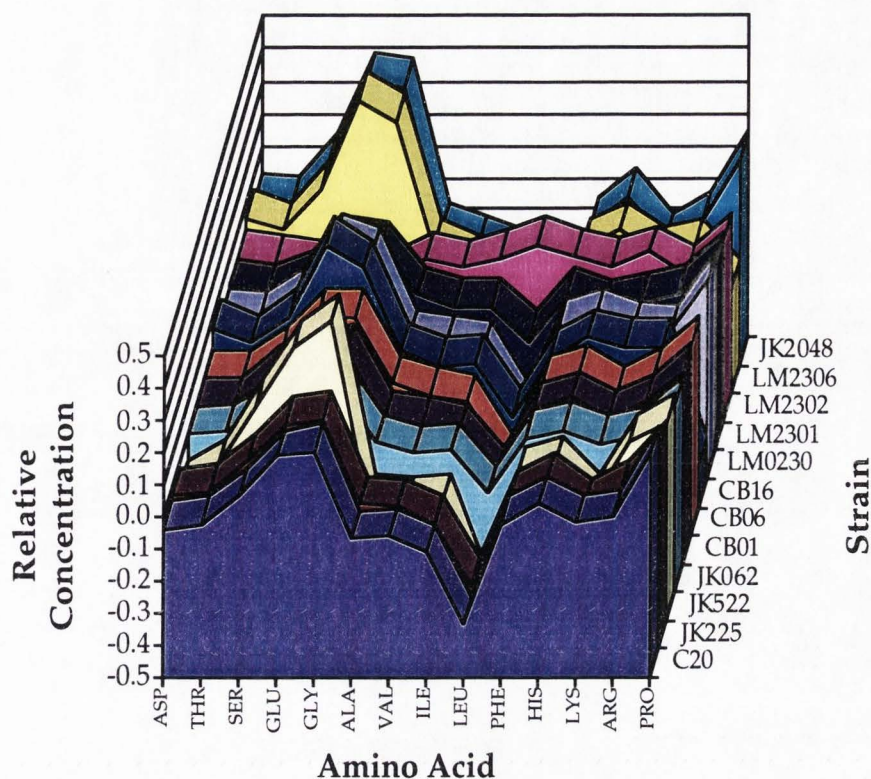


Figure 21. Amino acid concentrations of TCA-soluble fractions (6%) of milk incubated with *L. lactis* subsp. *lactis* strains for 6 h at 30°C.

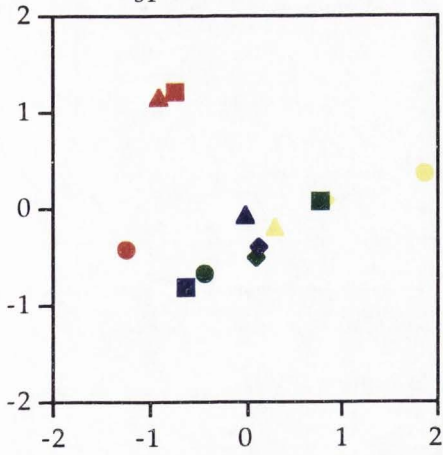
between strains possessing either PrtP<sup>+</sup> or PrtP<sup>-</sup>.

### Canonical Analyses

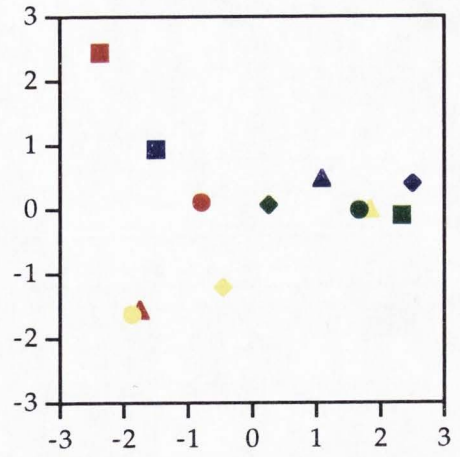
Canonical discriminant analysis (Figure 22) was performed on all TCA-soluble fractions obtained from the individual casein solutions of all strains to reduce the results to two dimensions (13). These plots further aid in identifying differences in the hydrolysis patterns shown in Figures 17, 18, 19, 20, and 21. For example, from Figure 17 we cannot distinguish between the hydrolysis patterns of strains C20, JK522, CB01, and LM0230 and thus might think that they are very closely related. However, Figure 22A shows us that C20 is more similar to

Figure 22. Canonical discriminant analysis for *Lactococcus lactis* subsp. *lactis* strains based on amino acid profiles following (A)  $\alpha_{s1}$ -casein, (B)  $\beta$ -casein, (C)  $\kappa$ -casein, (D) mixed casein solution, and (E) milk digestion. Each point was obtained from the mean of three TCA precipitated aliquots analyzed in duplicate on the AAA.

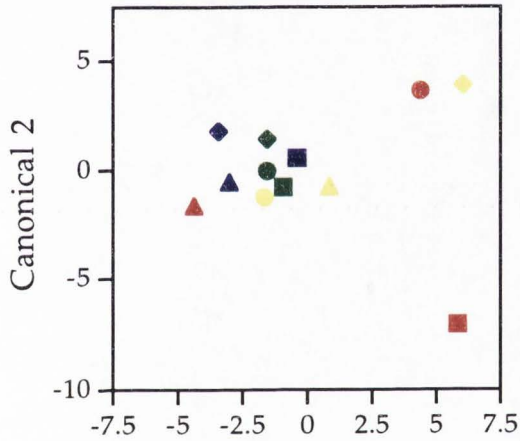
A.  $\alpha_{s1}$ -casein



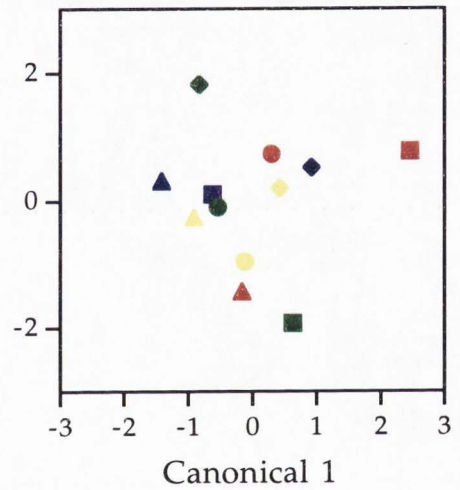
B.  $\beta$ -casein



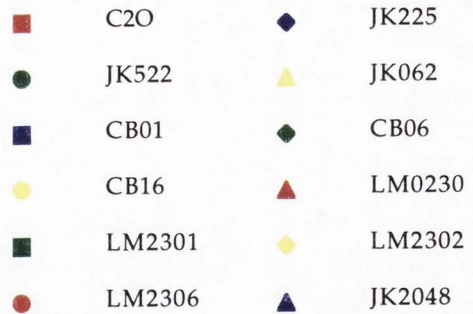
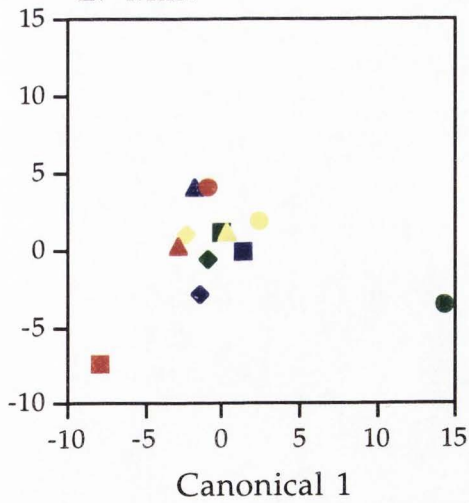
C.  $\kappa$ -casein



D. Mixed casein solution



E. Milk



LM0230 and JK522 than it is to CB01. Table A10 (Appendix A) aids in this interpretation by showing the distances between points in the figure. Strains JK522 and LM0230 show distances from C20 of 3.7 and 0, whereas CB01 has a distance of 4.1.

Figure 22B shows the canonical discriminant analysis for  $\beta$ -casein hydrolysis patterns of all strains. From Figure 18, C2O and LM0230 have hydrolysis patterns similar to that of LM2306. Figure 22B and Table A11 (Appendix A), however, tell us that strain C2O is most similar to CB01.

In Figure 19 the amino acid profile of  $\kappa$ -casein hydrolysis made by strains JK225 and JK522 is more similar than that made by LM0230. Figure 22C and Table A12 (Appendix A) also show that JK225 is closer to JK522 and almost as close to JK2048, even though JK2048 shows a different hydrolysis pattern.

Figure 20 shows the amino acid profile for the mixed casein solution. All strains show various patterns and thus it is hard to see which strains are closer. Figure 22D and Table 22 tells us that, though LM2301 and LM2302 show similar profiles, LM2302 is more related to JK522 and JK062 than it is to LM2301.

In Figure 21, the milk hydrolysis pattern can be seen. The profiles of JK062, CB01, CB06, and LM2301 look very similar. From Figure 22E and Table A13 (Appendix A), the distance from LM2301 to JK062 is closer than to the other strains and thus they are more closely related based on their amino acid hydrolysis patterns.

### **Discriminant Function Analysis**

The amino acid results were analyzed using discriminant function analysis to develop a prediction model of strains to be classified into phenotype groups. Error count estimates (Appendix A, Table A14), which are probabilities



TABLE 22. Generalized squared distance between strains of *L. lactis* subsp. *lactis* based on TCA-soluble amino acid fractions obtained from mixed casein-solution digestion.

Strain	C20	JK225	JK522	JK062	CB01	CB06	CB16	LM0230	LM2301	LM2302	LM2306	JK2048
C20	0											
JK225	171	0										
JK522	93	17	0									
JK062	655	30	3	0								
CB01	57	56	12	8	0							
CB06	80	40	14	100	13	0						
CB16	94	135	67	65	44	98	0					
LM0230	111	154	83	82	57	119	1	0				
LM2301	89	57	21	24	21	55	23	30	0			
LM2302	94	21	1	6	11	18	55	68	14	0		
LM2306	74	34	7	5	6	5	77	94	33	8	0	
JK2048	95	22	6	6	16	4	101	122	46	12	4	0

of misclassifying strains, were done for all substrates. Classes included  $\alpha_{s1}$ -,  $\beta$ -,  $\kappa$ -casein, mixed casein solution, and milk. Only the mixed casein solution gave an error count of zero based on this classification system. The other substrates gave error counts of 75% ( $\alpha_{s1}$ -casein), 28% ( $\beta$ -casein), 8% ( $\kappa$ -casein), and 11% (milk). This suggests that using a mixed casein solution as substrates would lead to the least amount of misclassification. The equations leading to this statement are shown below.

Generalized squared distance function:

$$D_j^2 (X) = (X - X_j)' \text{COV}^{-1} (X - X_j) \quad \text{Eq. 5}$$

Posterior probability of membership in each stain:

$$\text{Pr} (j/X) = \exp (-0.5 D_j^2(X)) / \sum_k \exp (-0.5 D_k^2 (X)) \quad \text{Eq. 6}$$

D = distance

X = sample measure

X = sample mean

COV = covariance, measure of linear association between two lines ( $y_1$  and  $y_2$ )

Pr = posterior probability of X belonging to strain j

j = strain

k = number of samples

With the use of these formula and a computer spreadsheet, it is easy to calculate the probability of a strain belonging to a certain group.

### Cluster Analysis

Ward's minimum cluster analysis was used to investigate if the strains would cluster together according to their proteolytic phenotypes. Ward's method was used because it allows the use of large data sets.

Figures 23 and B13 through B16 (Appendix B) show cluster dendograms

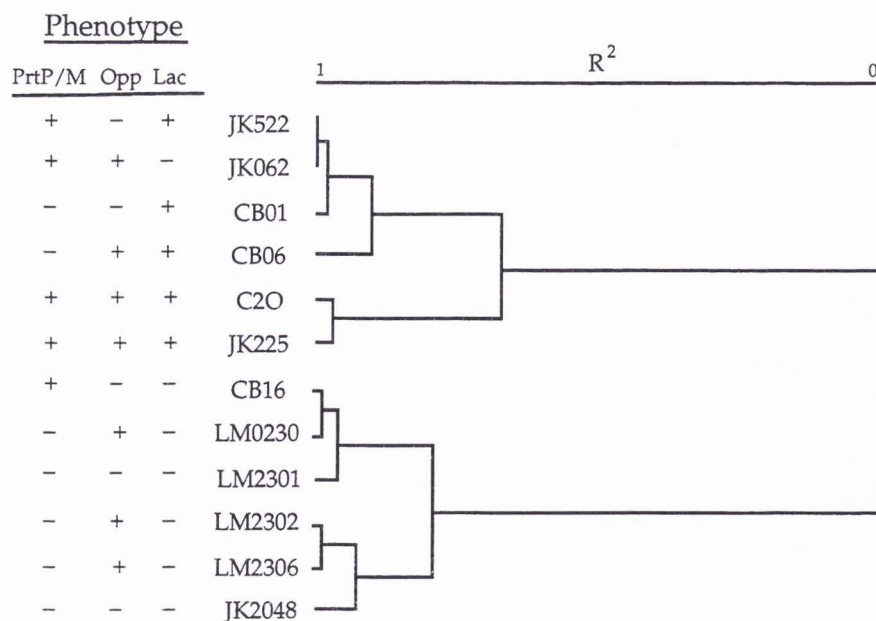


Figure 23. Cluster dendrogram of *L. lactis* subsp. *lactis* strains on mixed casein solution.

obtained for each substrate. The lengths of lines represent correlation coefficients ( $R^2$ ). Shorter lines represent higher correlation coefficients. In all dendrograms, except Figure B16, the  $Lac^+$  strains cluster together before joining the  $Lac^-$  cluster. In Figure B16, using milk as substrate, strains C2O and JK522 are the very last strains to join the clusters.

Fully proteolytic strains also cluster together before joining clusters containing either PrtP or Opp. Again, milk as the substrate does not follow this trend; instead, C2O joins the groups at a later  $R^2$ . Since mixed caseins showed to be the best substrate to work with, we investigated the dendrogram outcome with Figure 8D (Chapter III).

As strains join the dendrogram, they have completely different amino acid accumulation patterns as seen in Figure 8D. Strains JK522 and JK062 join at the same time and their proteolytic pattern is similar. Strain CB01, however, joins

next but has a completely different amino acid accumulation pattern. The amino acid pattern is explained by its lack in the proteolytic system but it does not explain why the strain would join before any other Lac<sup>+</sup> strains join the cluster. Strains C2O and JK225 join the cluster before they join the rest of the Lac<sup>+</sup> strains. Their amino acid accumulation patterns are also similar. However, the pattern for CB01 looks similar to C2O, yet it does not join with C2O until much later. The Lac<sup>-</sup> strains also show variability when comparing the two figures. The dendrogram shows LM2302 and LM2306 join at the same time even though their amino acid accumulation patterns in Figure 8D are opposite. Fully proteolytic negative strains, JK2048 and LM2301, show similar amino acid accumulation patterns, whereas they only show 0.78 similarities in the dendrograms.

We further used cluster analysis to see how the strains would cluster according to their flavor profiles (Figure 24). We used rancidity as flavor of choice, since Opp showed to influence rancidity in some way. All strains with both PrtP and Opp cluster together followed by all Lac<sup>+</sup> strains. Strain CB16, however, also joins the Lac<sup>+</sup> strains before joining the Lac<sup>-</sup> strains. This dendrogram shows similarities to Figure B13, where strains JK225 and JK522 first join before strains JK062, C2O, CB01, and CB06 join successively. The rest of the strains then join in various combinations.

## DISCUSSION AND CONCLUSION

Amino acid analysis of the 6% TCA-soluble fractions has been used to study the interaction between *L. lactis* subsp. *lactis* strains with various

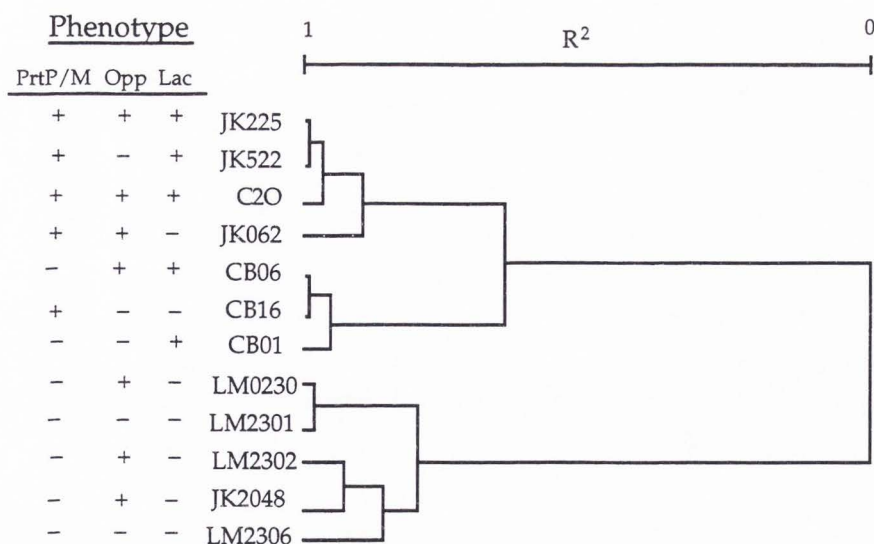


Figure 24. Cluster dendrogram of *L. lactis* subsp. *lactis* strains on rancidity flavor.

proteolytic phenotypes. The profiles of the amino acid hydrolysis patterns can be somewhat ambiguous and hard to interpret. Therefore, we developed a statistical model that allows us to predict the phenotype of the strains based on their proteolysis patterns. This allows us to minimize the analysis of the hydrolysis patterns and instead concentrate on the model itself.

The model we used shows us that the mixed casein solution will be the best substrate to use for this analysis. No misclassification is made on this substrate, whereas 8.3 to 75% mismatches were done with the other substrates. Together with the canonicals and distance tables, we can also see how related the strains are to one another. This cannot be done from the amino acid hydrolysis patterns alone.

Cluster analysis further aided in the interpretation of how related the strains were. An interesting observation was that all Lac<sup>+</sup> strains clustered together before they joined with the Lac<sup>-</sup> clusters. At the same time, fully

proteolytic strains behaved the same way as Lac<sup>+</sup> strains, suggesting that the lactose overrides the proteolytic system.

The knowledge on relatedness of bacteria is an important aspect in the dairy industry. However, it is a tedious job to determine how a strain behaves under various conditions. Looking at hydrolysis patterns becomes complicated as more strains and parameters, i.e., substrates, are involved and being investigated. Comparing genetic make-up of strains may not always be the most advantageous way. Though bacterial strains may be genetically related, they may not behave the same when hydrolyzing proteins and producing flavor and other products.

With the help of discriminant function and cluster analysis, strains can be grouped according to their desired products and not depending on their genetic make-up. Strains with different genetic make-up can produce the same or similar flavor. This statistical approach is of valuable interest to the dairy industry when evaluating potential starters for flavor contribution.

## REFERENCES

- 1 Afifi, A. A. and V. Clark. 1990. Canonical correlation analysis. Page 252 *in* Computer-Aided Multivariate Analysis. 2<sup>nd</sup> ed. A. Afifi and V. Clark, ed. Von Nostrand Reinhold, New York, NY.
- 2 Atlas, R. M. 1993. Page 564 *in* Handbook of Microbial Media. L. C. Parks, ed. CRC Press Inc., London, UK.
- 3 Hollar, C. M., A. J. R. Law, D. G. Dagleish, and R. J. Brown. 1991. Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:2403.

- 4 Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which stimulates milk ultrafiltrate. *Neth. Milk & Dairy J.* 16:153.
- 5 Kaiser, F. E., C. W. Gehrke, R. W. Zumwalt, and K. C. Kuo. 1974. Amino acid analysis: hydrolysis, ion-exchange clean-up, derivatization, and quantitation by gas liquid chromatography. *J. Chrom.* 94:113.
- 6 Kok, J., and G. Venema. 1988. Genetics of proteinases of lactic acid bacteria. *Biochimie* 70:475.
- 7 Konings, W. N., B. Poolman, and A. J. M. Driessen. 1989. Bioenergetics and solute transport in lactococci. *CRC Crit. Rev. Microbiol.* 16:419.
- 8 Law, B. A. 1984. Flavour development in cheeses. Page 187 *in* *Advances in Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davoes and B. A. Law, eds. Elsevier Applied Science Publishers, New York, NY.
- 9 Law, B. A. 1984. The accelerated ripening of cheese. Page 187 *in* *Advances in Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davoes and B. A. Law, eds. Elsevier Applied Science Publishers, New York, NY.
- 10 Law, B. A., and J. Kolstad. 1983. Proteolytic systems in lactic acid bacteria. *Antonie van Leeuwenhoek* 49:225.
- 11 Oberg, C. J., B. C. Weimer, L. V. Moyes, R. J. Brown, and G. H. Richardson. 1991. Proteolytic characterization of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains by the *o*-phthaldialdehyde test and amino acid analysis. *J. Dairy Sci.* 74:398.
- 12 Patel, K. N., F. M. Bartlett, and J. Hamid. 1983. Extracellular heat-resistant proteases of psychrotrophic *Pseudomonas*. *J. Food Prot.* 46:90.

- 13 SAS JMP. 1992. Macintosh Statistical Software. Cary, NC.
- 14 SAS/STAT User's Guide. 1992. Vol. 1. Version 6. 4<sup>th</sup> ed. Cary, NC.
- 15 Thomas, T. D., and O. E. Mills. 1987. Proteolytic enzymes of dairy starter bacteria. *Neth. Milk Dairy J.* 35:255.
- 16 Thomas, T. D., and G. G. Pritchard. 1987. Proteolytic enzymes of starter cultures. *FEMS Microbiol. Reviews* 46:245.



## CHAPTER VI

### GENERAL SUMMARY

Twelve strains of *Lactococcus lactis* subsp. *lactis* with various combinations of lactose utilization (Lac), proteolytic system (PrtP/PrtM), and oligopeptide transport system (Opp) were successfully constructed by various recombinant DNA techniques.

The phenotypes of the strains were verified by polymerase chain reaction and compared to *Lactococcus lactis* subsp. *cremoris* SK11. They all showed group d amino acid substitutions, which explain the variation in amino acid accumulation patterns.

All strains were analyzed for their proteinase specificity against various substrates, including  $\alpha_{s1}$ - ,  $\beta$ - ,  $\kappa$ -casein, mixed casein solution, and milk. Amino acid accumulation patterns varied among strains and substrate. Peptides and amino acids from  $\alpha_{s1}$ -casein degradation were only used to a small amount.  $\beta$ -Casein patterns were similar in all strains except in C2O and CB01. This suggests that the peptides generated by these two strains were not specific enough for C2O or CB01 to transport peptides into the cell.  $\kappa$ -Casein showed CB06 using a larger quantity of this protein, whereas the other strains had lower but similar amino acid accumulation patterns. Mixed casein solution and milk showed larger variations in their patterns.

Biochemical profiles showed no correlation between Lac<sup>-</sup> and Lac<sup>+</sup>. There was a strong correlation between CB16 and its parent strain LM2306 but not between CB06 and its parent strain LM2306. All Lac<sup>+</sup> strains had similar generation times, whereas Lac<sup>-</sup> strains had slower generation times. Strains showed a significant effect ( $P < 0.0001$ ) on acid production. Lactose positive strains had a faster acid production rate. Strain LM2302, however, a Lac<sup>-</sup> mutant,

showed a significant faster acid production rate than the Lac<sup>+</sup> strains. Strains also showed a significant effect ( $P < 0.0001$ ) in their redox potential development, although a trend between the strains was not noticeable.

The aminopeptidase activity was predominantly PepN in all strains. Dipeptidase activity showed some strains used and transported the dipeptides into the cell, whereas other strains seemed to secrete the dipeptide into the medium.

Cheese slurries were made using an accelerated ripening system. A satisfactory slurry, with 60% moisture and 5% salt-in-moisture, was made. General plate counts increased slowly over time. Non-starter lactic acid bacteria increased rapidly for the first week and then leveled off to an average population of 107 CFU/ml. Aminopeptidase activity slowly increased to day 7, except for LM2301. Lipase activity showed a more varied picture. Strains showed to be significant in their lipase activity ( $P < 0.0001$ ) as did the two-way interaction time\*culture ( $P < 0.001$ ).

No culture effect was seen in acidity or bitterness when analyzing sensory scores over a 3-wk period. Rancidity was significant in its culture effect ( $P < 0.001$ ), as well as in its time effect ( $P < 0.001$ ) and time\*culture interaction ( $P < 0.001$ ). Strains were also compared with and without the traits Lac, PrtP, and Opp on their contribution to rancidity flavor. Only Opp showed significance ( $P < 0.045$ ). An interesting question arises since the graphs of Lac and Opp influence are very similar. Do the strains share a common intermediate in the glycolysis pathway that together with aminopeptidase activity leads to rancidity? The data suggest that aminopeptidases are involved, but how is still uncertain.

Characterization of 12 strains is a tedious job and results are often hard to

interpret. Canonical discriminant analysis was performed on all amino acid accumulation patterns and plotted. These plots further aid in the identifying differences in relatedness of the patterns not seen by amino acid analysis. Together with distance tables, the relationship among strains is better understood.

Discriminant function analysis was also performed and a prediction model of strains to be classified into phenotype groups was developed. Mixed casein proved to be the most suited substrate for this analysis, since other substrates gave higher misclassifications.

Through construction of dendograms, a final picture was visible that told us the relatedness among all the strains on each substrate used. Lactose positive strains clustered together before joining with the Lac<sup>-</sup> cluster. Fully proteolytic strains also joined together before joining with strains containing only one of the traits, PrtP or Opp. Strains were also analyzed according to their rancidity flavor profile. Again, Lac<sup>+</sup> strains and fully proteolytic strains clustered together before joining the other strains. The question arises whether lactose utilization and parts of the proteolytic system share a common pathway intermediate at some point that leads to a rancid flavor.

Though bacteria may be genetically related, their products, whether it is a flavor profile or an amino acid profile, may be different. With the help of discriminant function and cluster analysis, a convenient tool to analyze and characterize potential starter strains is made available to the dairy industry. Instead of tedious laboratory characterization methods, the accumulated amino acid profile of a mixed casein solution can be used in our statistical prediction model. The relatedness among strains is clearly seen and thus the industry can make faster decisions.

APPENDICES

**APPENDIX A**  
**STATISTICAL TABLES**

TABLE A1. Activity of *L. lactis* strains on Biolog substrates.

Substrate	C2O	JK225	JK522	CB01	CB06	CB16	JK062	LM0230	LM2301	LM2302	LM2306	JK2048
$\alpha$ -cyclodextrin	++	++	++	++	//	++	//	++	++	++	++	++
$\beta$ -cyclodextrin	++	++	++	++	//	++	//	++	++	++	++	++
dextrin	++	++	++	++	++	++	++	++	++	++	++	++
glycogen	++	++	++	++	++	++	++	++	++	++	++	++
inulin	//	//	//	++	//	++	—	//	++	++	—	—
N-acetyl glucosamine	++	++	++	++	++	++	++	++	++	++	++	++
N-acetyl mannosamine	++	++	++	++	++	++	++	++	++	++	++	++
amygdalin	—	—	//	//	—	//	—	—	//	—	//	—
L-arabinose	//	//	//	//	//	//	//	//	—	—	//	//
arbutin	—	—	—	//	—	//	//	—	//	//	//	//
cellobiose	++	//	++	++	++	++	++	//	++	++	++	++
D-fructose	++	++	++	++	++	++	++	++	++	++	++	++
D-galactose	++	++	++	++	++	//	++	++	//	++	++	++
D-galacturonic acid	++	//	//	//	//	//	//	++	//	//	++	++
gentiobiose	++	//	++	++	++	++	++	++	++	++	++	++
D-gluconic acid	—	—	—	//	—	//	—	—	//	—	//	—
$\alpha$ -D-glucose	++	++	++	++	++	++	++	++	++	++	++	++
$\alpha$ -D-lactose	++	//	++	++	++	//	—	—	—	—	—	//
lactulose	++	//	++	++	++	//	—	—	—	—	—	//
maltose	++	++	++	++	++	++	++	++	++	++	++	++
maltotriose	++	++	++	++	++	++	++	++	++	++	++	++
D-mannitol	—	—	—	//	—	//	—	//	//	//	//	—
D-mannose	++	++	++	++	++	++	++	++	++	++	++	++
D-melezitose	—	—	//	//	//	//	—	//	//	—	—	—
D-melibiose	//	—	—	—	—	—	//	//	—	—	//	//
$\alpha$ -methyl-D galactoside	//	//	//	++	//	—	—	—	—	—	—	—
$\beta$ -methyl-D galactoside	++	//	++	++	++	//	—	—	—	—	—	//
3-methyl glucose	//	//	++	++	++	++	++	++	++	++	++	++

1 ++ = strong reaction, // = weak reaction, — = no reaction

TABLE A1 Cont.

Substrate	C2O	JK225	JK522	CB01	CB06	CB16	JK062	LM0230	LM2301	LM2302	LM2306	JK2048
α-methyl-D glucoside	//	//	//	++	//	++	—	++	++	++	++	++
β-methyl-D- glucoside	++	//	++	++	++	++	++	++	++	++	++	++
palatinose	//	//	++	//	//	//	//	//	//	//	//	//
D-psicose	++	++	++	++	++	++	++	++	++	++	++	++
salicin	++	++	++	++	++	++	++	++	++	++	++	++
sedoheptulosan	—	//	//	//	//	//	//	//	//	//	—	//
D-sorbitol	//	//	//	//	//	//	//	//	//	++	//	//
sucrose	—	—	//	//	—	—	—	—	—	—	—	//
D-tagatose	—	—	//	//	—	//	//	//	—	—	//	++
D-trehalose	++	++	++	++	++	++	++	++	++	++	++	++
turanose	//	//	++	//	//	//	++	//	//	//	//	—
xylitol	—	—	—	—	—	—	—	—	—	—	—	++
D-xylose	//	//	//	//	//	//	++	//	//	//	++	//
α-keto valeric acid	//	//	//	//	//	//	//	//	//	//	//	++
methyl pyruvate	++	++	++	++	++	++	++	++	++	++	++	++
pyruvic acid	++	++	++	++	++	++	++	++	++	++	++	//
N-acetyl-L- glutamic acid	//	//	//	—	—	—	//	//	—	//	//	//
glycerol	//	//	//	—	//	—	//	//	—	//	//	//
adenosine	++	++	++	++	++	++	++	++	++	++	++	++
2'-deoxyadenosine	++	++	++	++	++	++	++	++	++	++	++	++
inosine	++	++	//	++	—	++	//	++	++	//	++	//
thymidine	++	++	++	++	++	++	++	++	++	++	++	++
uridine	++	++	++	++	++	++	++	++	++	++	++	++
adenosine-5' monophosphate	—	//	//	//	—	//	//	//	++	—	//	//
uridine-5' monophosphate	//	//	//	//	—	//	//	++	//	//	//	//
fructose-6- phosphate	//	//	//	//	//	//	//	++	//	//	//	//
glucose-1- phosphate	—	—	//	//	—	—	//	—	—	—	//	//
glucose-6- phosphate	//	//	//	//	//	//	//	//	//	//	//	//

1 ++ = strong reaction, // = weak reaction, — = no reaction

TABLE A2. Biolog correlation matrix (x 100).

Strain	C2O	JK225	JK522	JK062	CB01	CB06	CB16	LM0230	LM2301	LM2302	LM2306	JK2048
	R <sup>2</sup>											
C2O	100											
JK225	69	100										
JK522	61	65	100									
JK062	43	54	44	100								
CB01	57	61	62	21	100							
CB06	67	58	77	41	67	100						
CB16	42	57	47	41	76	48	100					
LM0230	35	59	27	64	35	27	64	100				
LM2301	27	52	30	46	57	27	88	69	100			
LM2302	35	62	35	60	43	44	65	69	71	100		
LM2306	40	54	27	69	35	23	59	77	65	65	100	
JK2048	59	67	60	76	45	57	55	65	49	63	79	100



TABLE A3. ANOVA for acid development in *L. lactis* strains.

Source	df	SS	MS	F	P>t
Rep	2	$6.60 \times 10^{-15}$	$3.30 \times 10^{-15}$	2.1621	0.1389
Culture	11	$2.91 \times 10^{-13}$	$2.65 \times 10^{-14}$	17.3426	0.0000
Error	22	$3.36 \times 10^{-14}$	$1.53 \times 10^{-15}$		
Total	35	$3.31 \times 10^{-13}$			

TABLE A4. All possible differences between means for acidity scores.<sup>1</sup>

	C2O	JK225	JK522	CB01	CB06	CB16	JK062	LM0230	LM2301	LM2302	LM2306	JK2048
C2O	0											
JK225	11.2	0										
JK522	17.5	6.30	0									
CB01	1.25	12.5	18.8	0								
CB06	15.7	4.50	1.80	17.0	0							
CB16	3.91	15.1	21.4	2.66	19.6	0						
JK062	1.22	12.4	18.7	0.03	16.9	2.69	0					
LM0230	2.60	8.60	14.9	3.85	13.1	6.51	3.82	0				
LM2301	3.14	14.3	20.6	1.89	18.8	0.77	1.92	5.74	0			
LM2302	24.9	13.7	7.40	26.2	9.20	28.8	26.1	22.3	28.4	0		
LM2306	0.33	11.5	17.8	0.92	16.0	3.58	0.89	2.93	2.81	25.2	0	
JK2048	1.70	9.50	15.8	2.95	14.0	5.61	2.92	0.90	4.84	23.2	2.03	0

<sup>1</sup> LSD<sub>0.05</sub> critical value = 1.91

TABLE A5. ANOVA for TTC change in *L. lactis* strains.

Source	df	SS	MS	F	P>t
Rep	1	$4.72 \times 10^{-15}$	$4.72 \times 10^{-15}$	0.4409	0.5204
Culture	11	$3.32 \times 10^{-12}$	$3.02 \times 10^{-13}$	28.1589	0.0000
Error	11	$1.18 \times 10^{-13}$	$1.07 \times 10^{-14}$		
Total	23	$3.44 \times 10^{-12}$			

TABLE A6. All possible differences between the means of TTC change.<sup>1</sup>

	C2O	JK225	JK522	CB01	CB06	CB16	JK062	LM0230	LM2301	LM2302	LM2306	JK2048
C2O	0											
JK225	42.0	0										
JK522	83.7	41.7	0									
CB01	12.3	54.3	95.9	0								
CB06	76.9	34.9	6.75	89.1	0							
CB16	14.0	56.0	97.6	1.66	90.9	0						
JK062	59.5	17.5	24.2	71.8	17.4	73.5	0					
LM0230	42.9	0.85	40.8	55.1	34.1	56.8	16.7	0				
LM2301	16.5	58.7	1000	4.21	93.4	2.55	76.0	59.4	0			
LM2302	6.05	36.0	776	18.3	70.9	20.0	53.5	36.8	22.6	0		
LM2306	17.6	59.6	1010	5.32	94.5	3.66	77.1	60.5	1.11	23.7	0	
JK2048	14.7	56.7	98.3	2.38	91.6	0.72	74.2	57.5	1.83	20.7	2.94	0

<sup>1</sup> LSD<sub>0.05</sub> critical value = 6.57

TABLE A7. ANOVA for acidity scores.

Source of variation	df	SS	MS	F	P-value
Judge	6	144.37	24.06	11.80	0.000
Culture	11	11.80	1.07	0.53	0.876
Run	1	5.10	5.10	2.50	0.119
Culture/Run	10	6.70	0.67	0.33	0.000
Error (a)	66	134.58	2.04		
Time	6	6.10	1.02	2.94	0.000
Time*Culture	66	70.13	1.06	3.07	0.000
Time*Run	6	2.89	0.48	1.39	0.000
Time*Culture/Run	60	67.24	1.12	3.24	0.000
Error (b)	400	138.51	0.35		
TOTAL	555	505.49			

TABLE A8. ANOVA for bitterness scores.

Source of variation	df	SS	MS	F	P-value
Judge	6	83.34	13.89	10.42	0.000
Culture	11	3.66	0.33	0.25	0.992
Run	1	1.94	0.94	1.46	0.233
Culture/Run	10	1.72	0.17	0.13	0.999
Error (a)	66	87.94	1.33		
Time	6	4.74	0.79	1.90	0.080
Time*Culture	66	24.50	0.37	0.89	0.708
Time*Run	6	0.78	0.13	0.31	0.931
Time*Culture/Run	60	23.72	0.40	0.95	0.582
Error (b)	400	166.74	0.42		
TOTAL	555	370.93			

TABLE A9. ANOVA for rancidity scores.

Source of variation	df	SS	MS	F	P-value
Judge	6	44.87	7.48	6.96	0.000
Culture	11	42.63	3.88	3.61	0.001
Run	1	0.70	0.70	0.65	0.423
Culture/Run	10	41.93	4.19	3.90	0.000
Error (a)	66	70.94	1.07		
Time	6	31.22	5.20	8.59	0.000
Time*Culture	66	180.64	2.74	4.52	0.000
Time*Run	6	22.06	3.68	6.07	0.000
Time*Culture/Run	60	158.58	2.64	4.37	0.000
Error (b)	400	242.20	0.61		
TOTAL	555	612.50			

TABLE A10. Generalized squared distance between strains of *L. lactis* subsp. *lactis* based on TCA-soluble amino acid fractions of obtained from  $\alpha_{s1}$ -casein digestion.

Strain	C20	JK225	JK522	JK062	CB01	CB06	CB16	LM0230	LM2301	LM2302	LM2306
C20	0										
JK225	3.4	0									
JK522	3.7	0.4	0								
JK062	3.2	0.1	0.8	0							
CB01	4.1	0.8	0.1	1.3	0						
CB06	3.8	0.6	0.4	0.3	0.6	0					
CB16	7.6	3.6	6.4	3.0	7.6	3.9	0				
LM0230	0.0	3.5	3.6	3.5	4.0	3.9	8.4	0			
LM2301	3.8	0.7	2.1	0.3	2.9	1.1	1.6	1.2	0		
LM2302	3.9	0.8	2.3	0.4	3.1	1.1	1.4	4.3	0.0	0	
LM2306	3.0	2.0	0.7	2.5	0.5	1.9	10.5	2.7	4.5	4.7	0
JK2048	2.5	0.4	0.8	0.2	1.3	0.7	4.2	2.6	0.7	0.8	1.9

TABLE A11. Generalized squared distance between strains of *L. lactis* subsp. *lactis* based on TCA-soluble amino acid fractions obtained from  $\beta$ -casein digestion.

Strain	C20	JK225	JK522	JK062	CB01	CB06	CB16	LM0230	LM2301	LM2302	LM2306
C20	0										
JK225	33	0									
JK522	26	12	0								
JK062	27	13	2	0							
CB01	7	27	13	13	0						
CB06	20	15	11	10	12	0					
CB16	19	29	19	20	8	17	0				
LM0230	17	27	16	18	9	11	3	0			
LM2301	30	9	6	4	19	14	22	22	0		
LM2302	18	16	11	10	9	10	4	4	10	0	
LM2306	13	19	14	11	5	6	8	9	14	4	0
JK2048	20	8	8	6	11	11	15	18	6	7	6



TABLE A12. Generalized squared distance between strains of *L. lactis* subsp. *lactis* based on TCA-soluble amino acid fractions obtained from  $\kappa$ -casein digestion.

Strain	C20	JK225	JK522	JK062	CB01	CB06	CB16	LM0230	LM2301	LM2302	LM2306	JK2048
C20	0											
JK225	167	0										
JK522	109	16	0									
JK062	83	43	32	0								
CB01	105	24	13	29	0							
CB06	140	26	34	31	21	0						
CB16	96	20	12	14	21	29	0					
LM0230	141	22	18	38	36	43	10	0				
LM2301	90	24	9	20	10	22	9	20	0			
LM2302	128	100	80	61	72	94	89	140	82	0		
LM2306	1200	73	57	51	38	52	70	114	49	19	0	
JK2048	128	17	15	47	25	31	20	25	18	115	82	0

TABLE A13. Generalized squared distance between strains of *L. lactis* subsp. *lactis* based on TCA-soluble amino acid fractions obtained from milk digestion.

Strain	C20	JK225	JK522	JK062	CB01	CB06	CB16	LM0230	LM2301	LM2302	LM2306	JK2048
C20	0											
JK225	76	0										
JK522	509	266	0									
JK062	160	31	245	0								
CB01	164	24	215	9	0							
CB06	119	18	276	10	11	0						
CB16	205	53	203	23	23	31	0					
LM0230	94	58	315	38	52	39	59	0				
LM2301	158	26	356	9	13	13	27	32	0			
LM2302	113	30	308	25	35	28	50	7	22	0		
LM2306	185	73	297	55	75	74	52	30	56	34	0	
JK2048	168	64	324	34	57	51	48	28	41	25	9	0

TABLE A14. Error counts estimates for *Lactococcus lactis* subsp. *lactis* strains on various substrates

Strain	Rate					Priors
	$\alpha_{s1}$ -cn <sup>1</sup>	$\beta$ -cn	$\kappa$ -cn	mixed cn	milk	
C2O	1.0000	0.3333	0	0	0	0.0833
JK225	1.0000	0	0.3333	0	0	0.0833
JK522	0.6667	0	0.3333	0	0	0.0833
CB01	0.6667	0.3333	0	0	0.3333	0.0833
CB06	0.6667	0	0	0	0	0.0833
CB16	0.3333	0.6667	0.3333	0	0.3333	0.0833
JK062	1.0000	0.6667	0	0	0	0.0833
LM0230	0.6667	0.3333	0	0	0.3333	0.0833
LM2301	1.0000	0.3333	0	0	0.3333	0.0833
LM2302	0.6667	0	0	0	0	0.0833
LM2306	0.6667	0.6667	0	0	0	0.0833
JK2048	0.6667	0	0	0	0	0.0833
TOTAL	0.7500	0.2778	0.0833	0	0.1111	

<sup>1</sup> cn = casein

**APPENDIX B**  
**TASTE PANEL FIGURES**

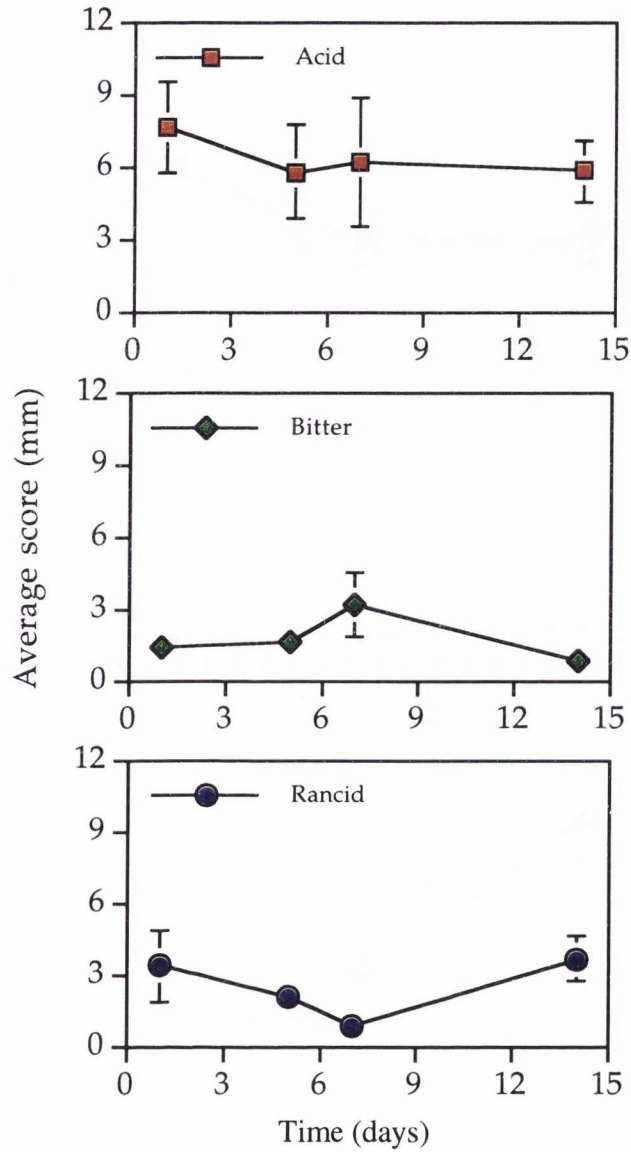


Figure B1. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* C2O. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

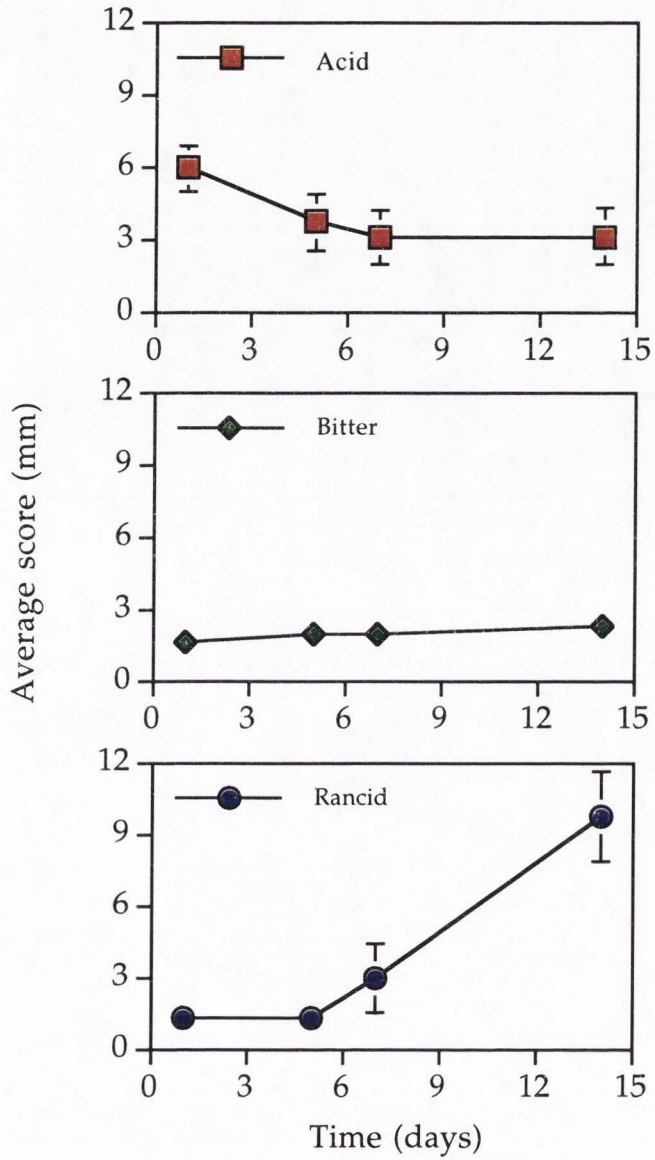


Figure B2. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* JK225. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

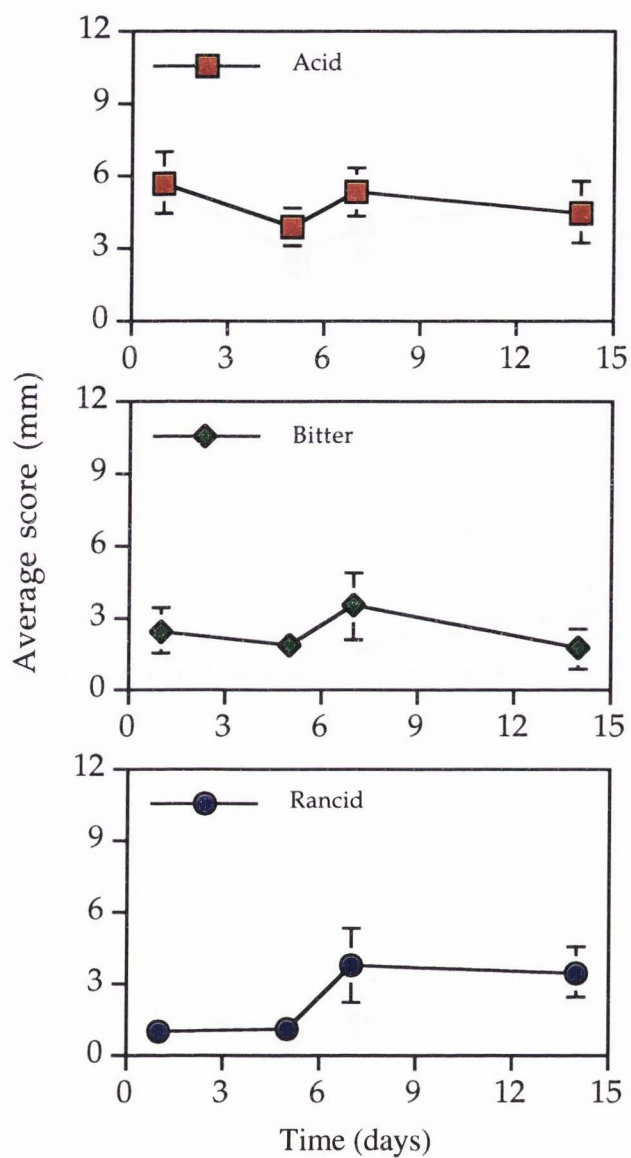


Figure B3. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* JK522. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

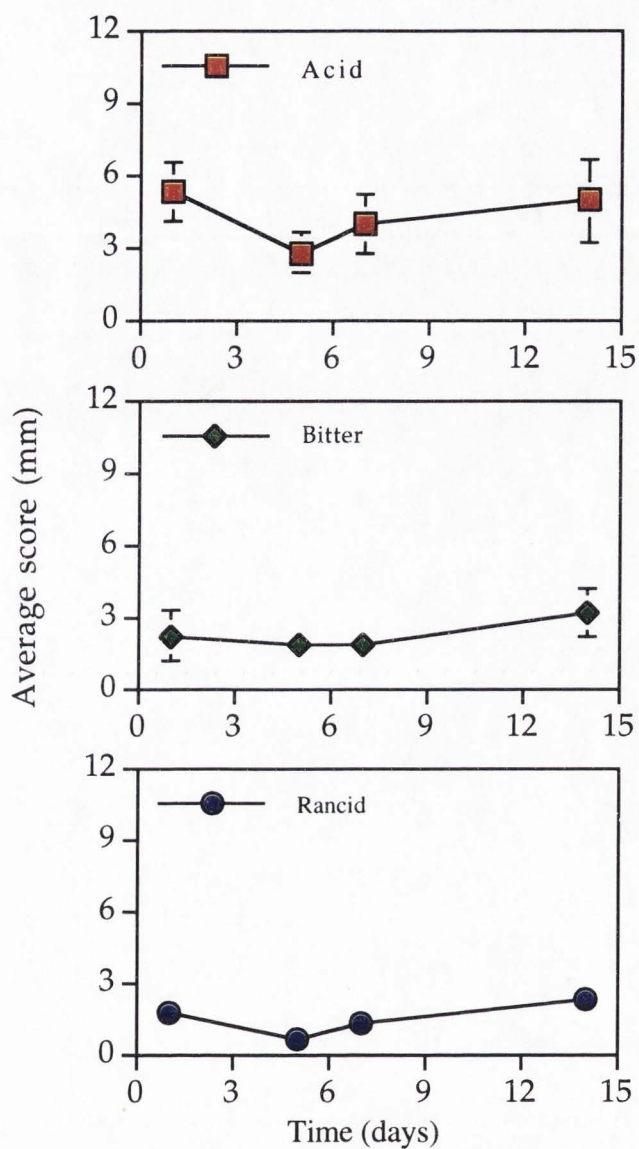


Figure B4. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* CB01. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.



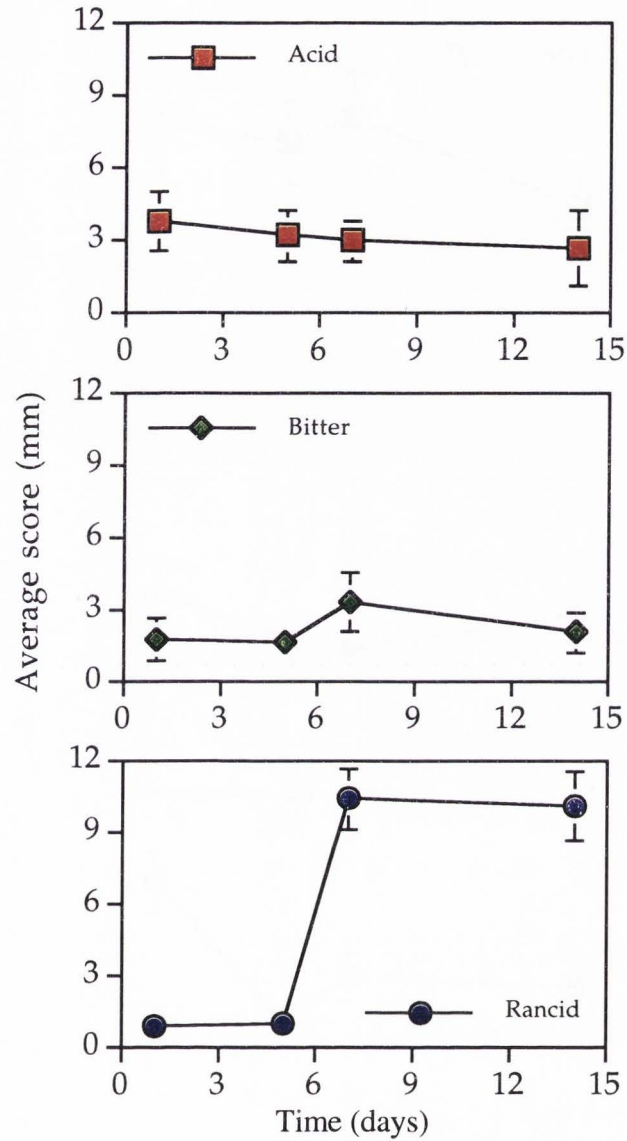


Figure B5. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* CB06. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

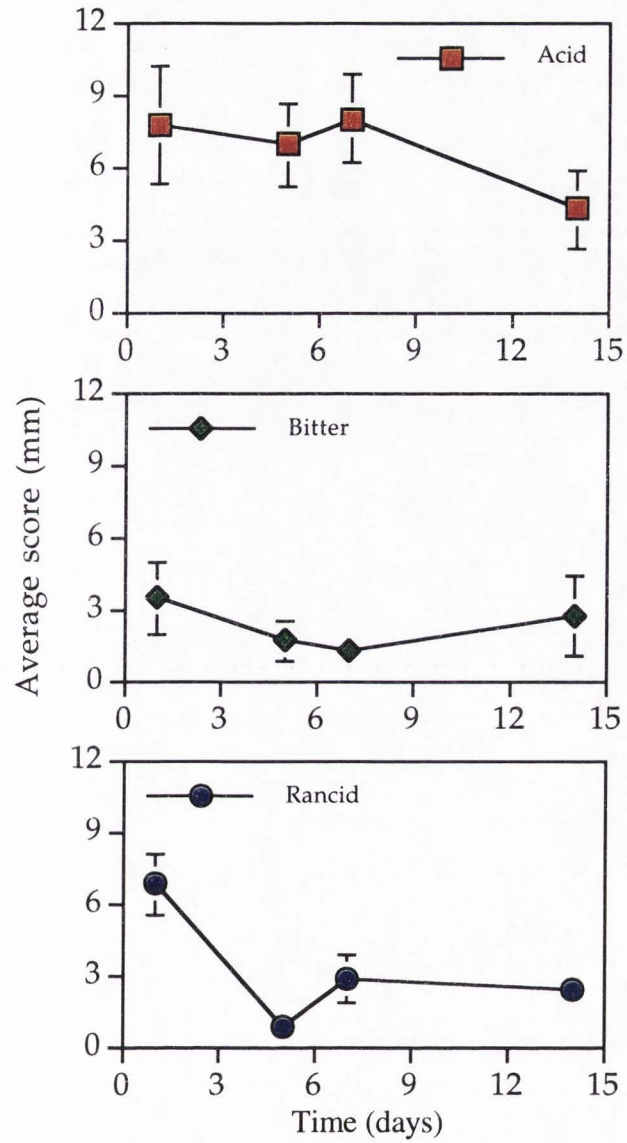


Figure B6. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* CB16. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

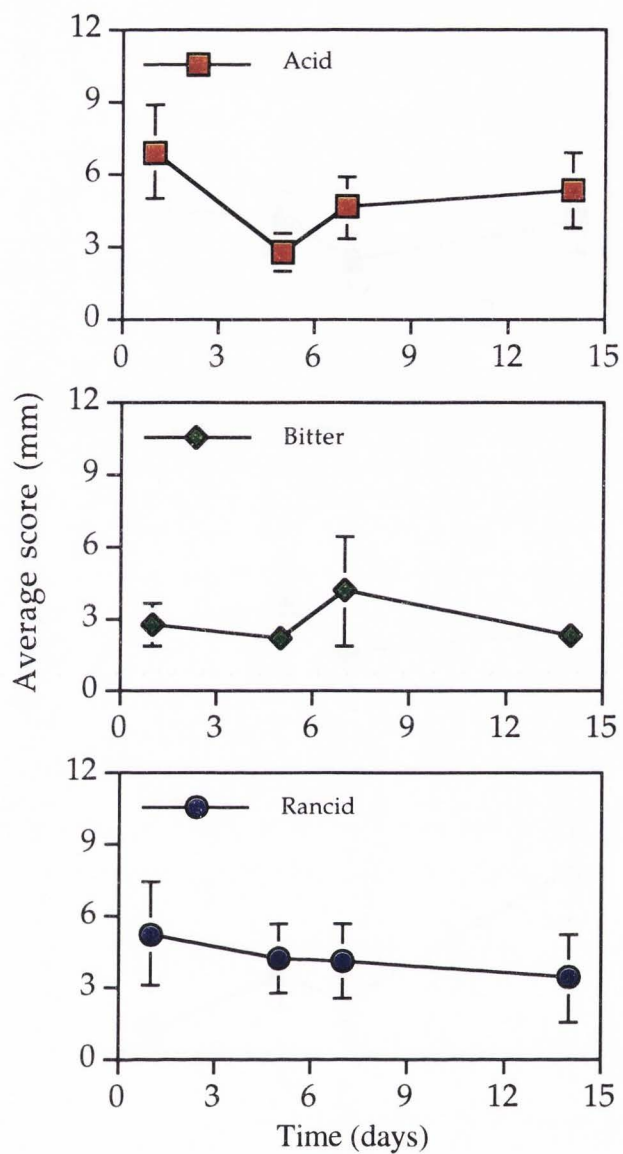


Figure B7. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* JK062. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

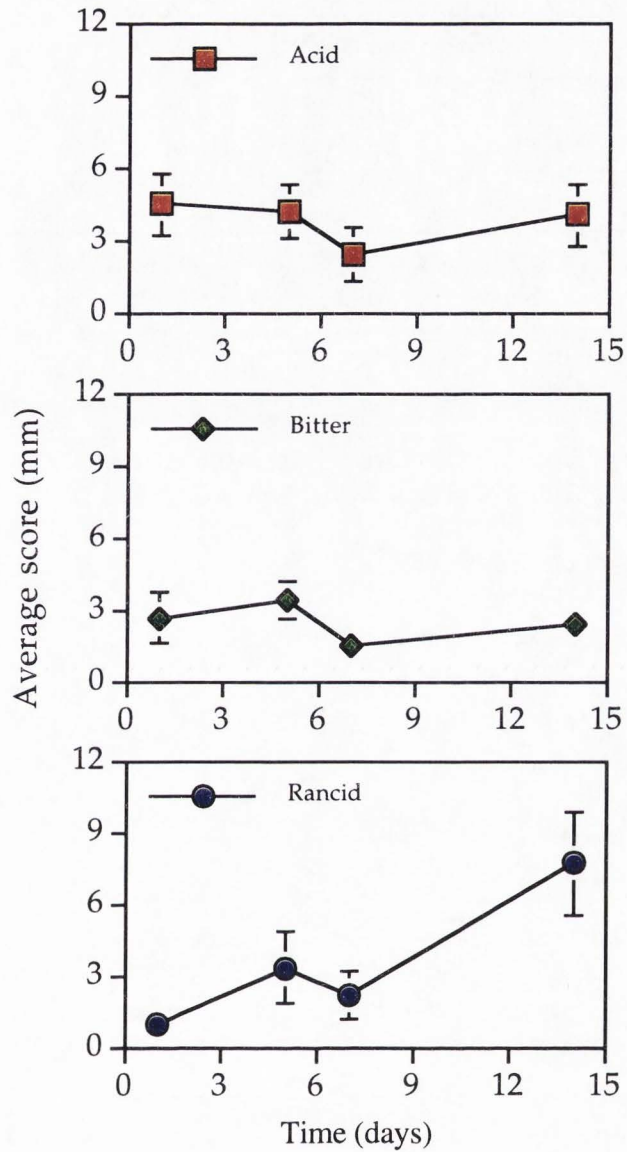


Figure B8. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* LM0230. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

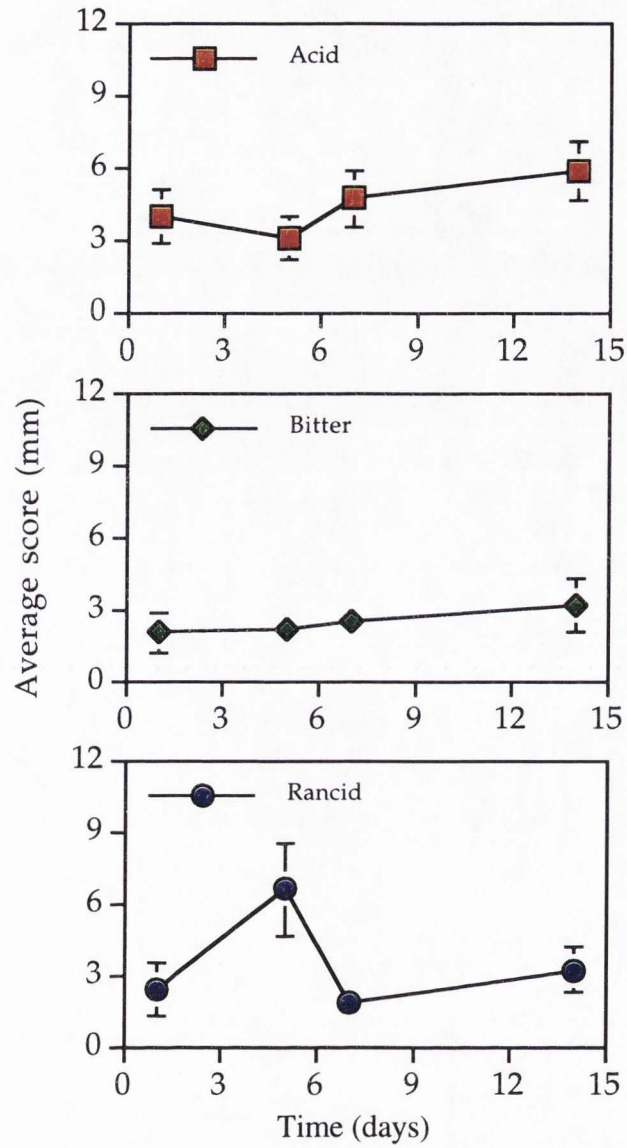


Figure B9. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* LM2301. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

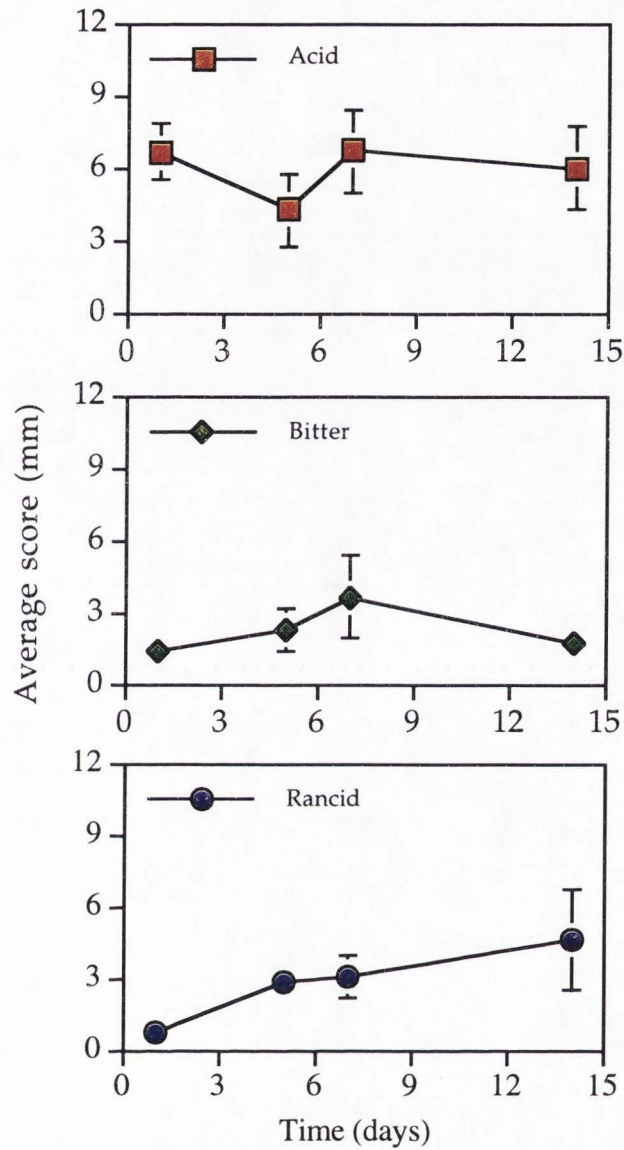


Figure B10. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* LM2302. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

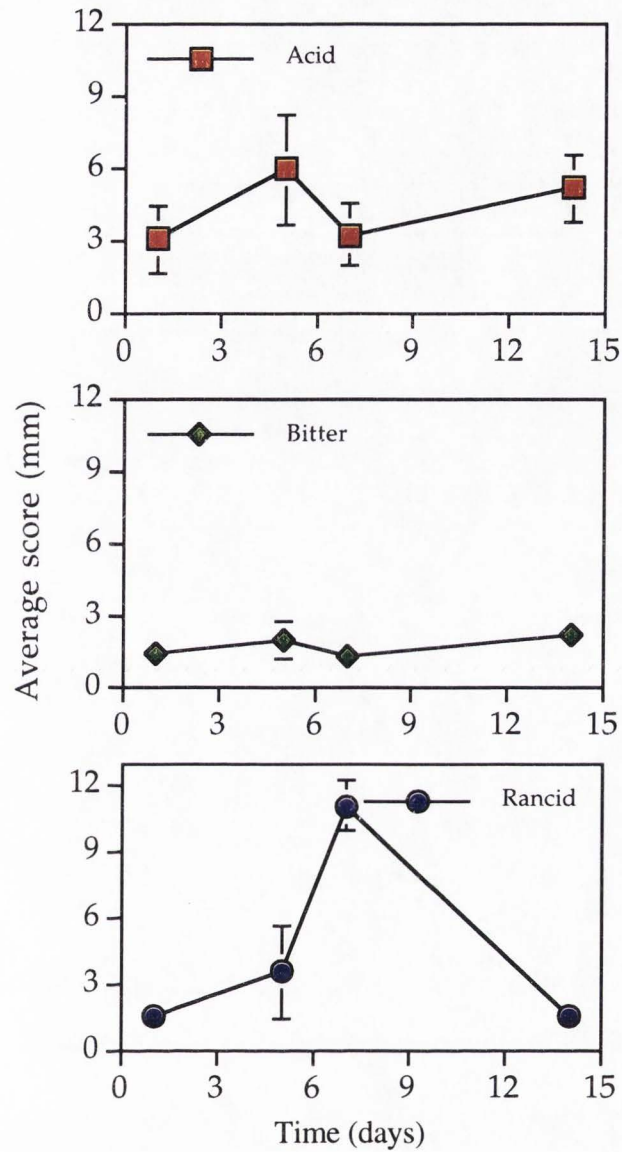


Figure B11. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* LM2306. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.

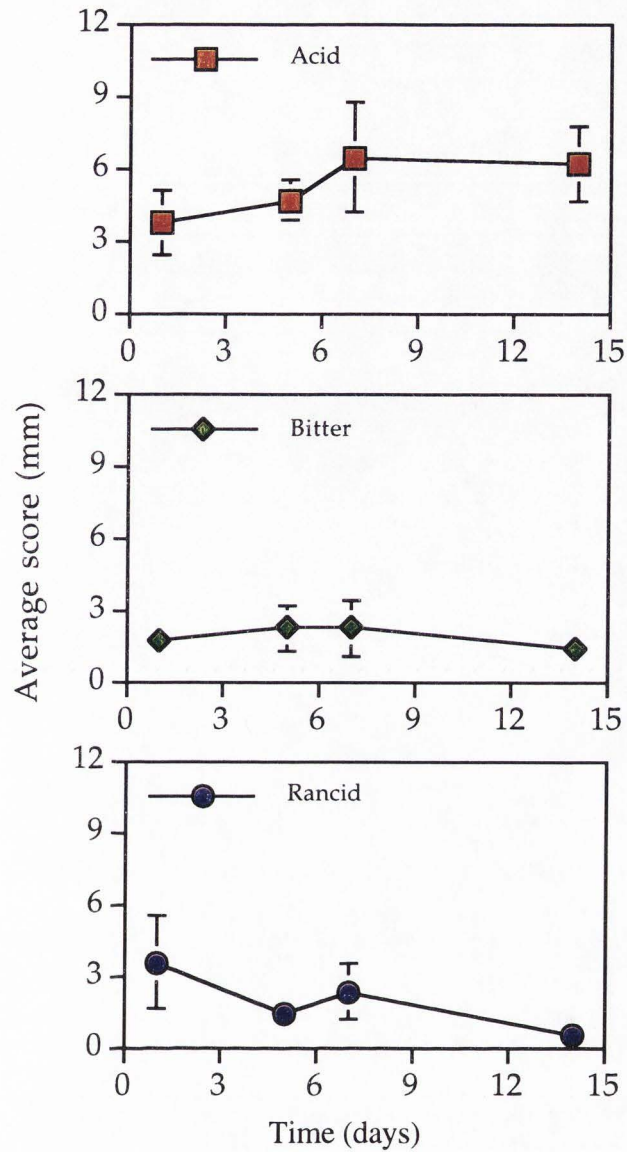


Figure B12. Taste panel data for Cheddar cheese slurry made with *L. lactis* subsp. *lactis* JK2048. Error bars represent standard error of the mean (SEM). Points not showing error bars are too small to be seen.



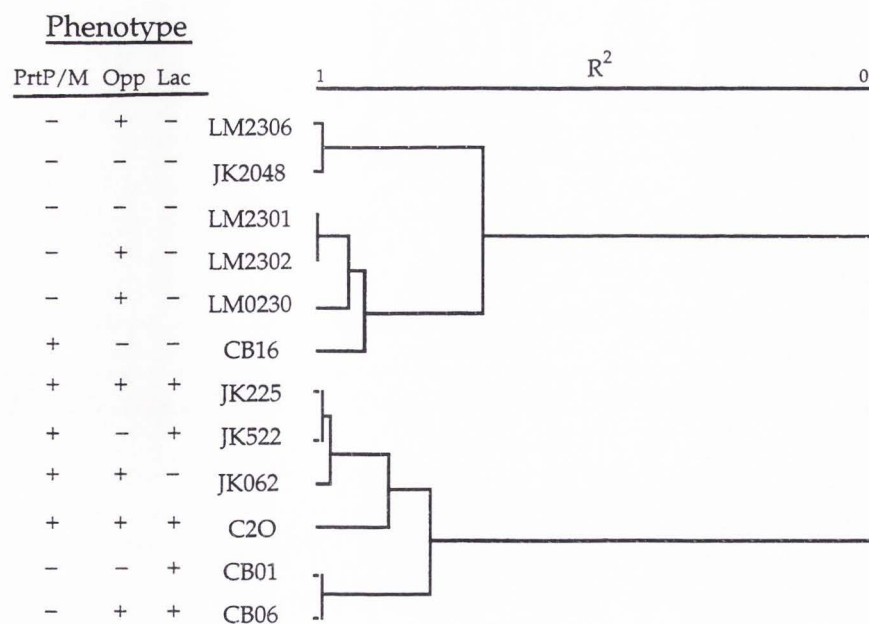


Figure B13. Cluster dendrogram of *L. lactis* subsp. *lactis* strains on  $\alpha_{s1}$ -casein.

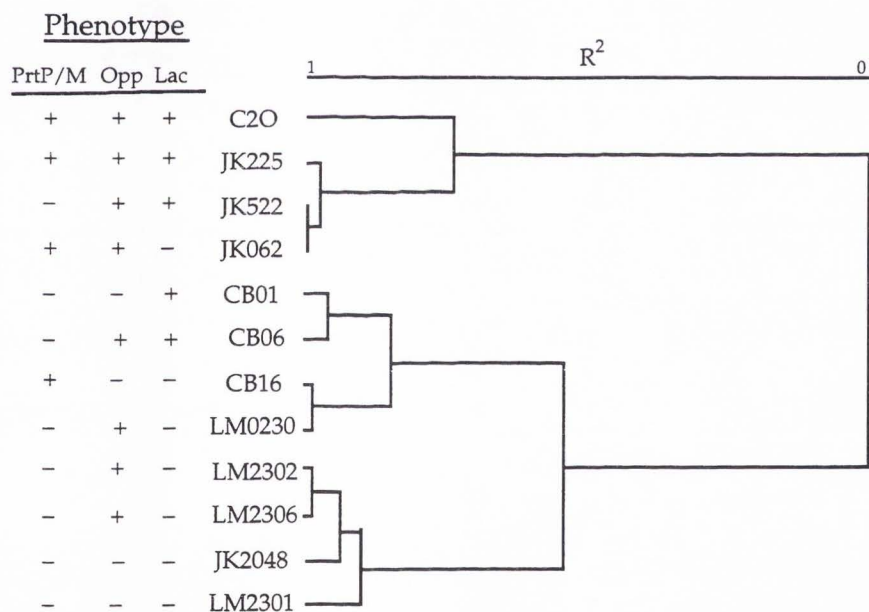


Figure B14. Cluster dendrogram of *L. lactis* subsp. *lactis* strains on  $\beta$ -casein.



**APPENDIX C**  
**BIBLIOGRAPHY**

## BIBLIOGRAPHY

- Abdelal, A. T. 1979. Arginine catabolism by microorganisms. *Annu. Rev. Microbiol.* 33:139.
- Afifi, A. A. and V. Clark. 1990. Canonical Correlation Analysis. Page 252 *in* Computer-Aided Multivariate Analysis. 2<sup>nd</sup> ed. A. Afifi and V. Clark, eds. Von Nostrand Reinhold, New York, NY.
- Alkhalad, W., J. C. Piard, M. El Soda, J. C. Gripon, M. Desmazeaud, and L. Vassal. 1988. Liposomes as proteinase carriers for the accelerated ripening of Saint Paulin-type cheese. *J. Food Sci.* 53:1674.
- Alkhalad, W., M. El Soda, J. C. Gripon, and L. Vassal. 1989. Acceleration of cheese ripening with liposome-entrapped proteinase: influence of liposomes net charge. *J. Dairy Sci.* 72:2233.
- Anderson, D. G., and L. L. McKay. 1983. A simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* 46:549.
- Association of Official Analytical Chemists International. 1990. Official Methods of Analysis. Vol. 2. 15<sup>th</sup> ed. AOAC Inc., Arlington, VA.
- Aston, J. W., P. A. Grieve, I. G. Durward, and J. R. Dulley. 1983. Proteolysis and flavour development in Cheddar cheese subjected to accelerated ripening treatments. *Aust. J. Dairy Technol.* 38:59.
- Atlas, R. M. 1993. Pages 112, 330, 472, 564, and 777, and 977 *In* Handbook of Microbiological Media. L. C. Parks ed. CRC Press Inc., London, UK.
- Axelsson, L. T. 1993. Lactic acid bacteria. Page 3 *in* Lactic Acid Bacteria: Classification and Physiology. S. Salminen and A. von Wright, Eds. Marcel Dekker, Inc., New York, NY.
- Baankreis, R. 1992. The role of lactococcal peptidases in cheese ripening. Ph.D.

thesis. Univ. Amsterdam, Netherlands.

Baankreis, R. and F. A. Exterkate. 1991. Characterization of a peptidase from *Lactococcus lactis* subsp. *cremoris* HP that hydrolyzes di- and tripeptides containing proline or hydrophobic residues as the aminoterminal amino acid. *Syst. Appl. Microbiol.* 14:317.

Bacon, C. L., M. Wilkinson, P. V. Jennings, I. Ni Fhaolain, and G. O'Cuinn. 1993. Purification and characterization of an aminotripeptidase from cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2. *Int. Dairy J.* 3:163.

Biolog Inc. Manual. 1993. Micro Station System, Hayward, CA.

Birkeland, S. E., and R. K. Abrahamsen. 1987. Accelerated cheese ripening. II. Review of methods. *Meieriposten* 76:601.

Blake, M. R., R. Koka, and B. C. Weimer. 1996. A semiautomated reflectance colorimetric method for the determination of lipase activity in milk. *J. Dairy Sci.* 79:1164.

Boeckelmann, W., B. Kiefer, A. Geis, and M. Teuber. 1988. Degradation of  $\beta$ -casein by mesophilic starter bacteria. Page 225 in *Milk Proteins. Nutritional, Clinical, Functional and Technological Aspects*. C. A. Barth and E. Schlimme, eds. Springer Verlag, New York, NY.

Booth, M., V. Jennings, I. Ni Fhaolain, and G. O'Cuinn. 1990. Prolidase activity of *Lactococcus lactis* subsp. *cremoris* AM2: partial purification and characterization. *J. Dairy Res.* 57:245.

Bosman, B. W., P. S. T. Tan, and W. N. Konings. 1990. Purification and characterization of a tripeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. *Appl. Environ. Microbiol.* 56:1839.

Broadbent, J. R., and J. K. Kondo. 1993. Biotechnology of dairy starter cultures. Page 77 in *Dairy Science and Technology Handbook*. Vol. 2. Y. H. Hui, ed. VHC

Publishers Ltd., Cambridge, UK.

Broadbent, J. R., M. Strickland, B. C. Weimer, M. E. Johnson, and J. L. Steele. 1998. Peptide accumulation and bitterness in Cheddar cheese made using *Lactococcus lactis* single strain starters with distinct proteinase specificities. *J. Dairy Sci.* 81:327.

Broome, M. C., and M. W. Hickey. 1991. Peptidase activity of non-starter lactobacilli. *Aust. J. Dairy Technol.* 46:19.

Broome, M. C., D. A. Krause, and M. W. Hickey. 1991. The use of proteinase negative starter and lactobacilli in Cheddar cheese manufacture. *Aust. J. Dairy Technol.* 46:6.

Bruinenberg, P. G., P. Vos, and W. M. de Vos. 1992. Proteinase overproduction in *Lactococcus lactis* strains: Regulation and effect on growth and acidification in milk. *Appl. Environ. Microbiol.* 58:78.

Caldwell, S. L., D. J. McMahon, C. J. Oberg, and J. R. Broadbent. 1996. Development and characterization of lactose-positive *Pediococcus* species for milk fermentation. *Appl. Environ. Microbiol.* 62:936.

Castaneda, R., L. Vassal, J. C. Gripon, and M. Rousseau. 1990. Accelerated ripening of Saint Paulin cheese variant by addition of heat-shocked lactobacillus suspension. *Neth. Milk Dairy J.* 44:49.

Castberg, H. B., and H. A. Morris. 1976. Degradation of milk proteins by enzymes from lactic acid bacteria used in cheese making. A review. *Milchwiss.* 31:85.

Crielaard, W., A. J. M. Driessen, D. Molenaar, K. J. Hellingwerf, and W. N. Konings. 1988. Light-induced amino acid uptake in membrane vesicles of *Streptococcus cremoris* and *Clostridium acetobutylicum* fused with reaction center containing proteoliposomes. *J. Bacteriol.* 170:1820.

- Cromie, S. J., J. E. Giles, and J. R. Dulley. 1987. Effect of elevated ripening temperature on microflora of Cheddar cheese. *J. Dairy Res.* 54:69.
- Crow, V. L., and T. D. Thomas. 1982. Arginine metabolism in lactic streptococci. *J. Bacteriol.* 150:1024.
- Cunin, R., N. Glansdorff, A. Pierrd, and V. Stalon. 1986. Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* 50:314.
- Desmazeaud, M. J., and C. Zevaco. 1976. General properties and substrate specificity of an intracellular neutral protease from *Streptococcus diacetylactis*. *Ann. Biol. Anim. Biochim. Biophys.* 16:851.
- Desmazeaud, M. J., and J. C. Gripon. 1977. General mechanism of protein breakdown during cheese ripening. *Milchwiss.* 32:731.
- Dias, B., and B. Weimer. 1995. A semi-automated colorimetric method for determination of aminopeptidase activity in turbid solutions. *J. Rapid Meth. Automation Microbiol.* 3:223.
- Driessen, A. J. M. 1987. Amino acid transport in lactic streptococci. Ph.D. thesis, Univ. Groningen, Groningen, Netherlands.
- Driessen, A. J. M., B. Poolman, R. Kiewiet, and W. N. Konings. 1987. Arginine transport in *Streptococcus lactis* is catalyzed by a cation exchanger. *Proc. Natl. Acad. Sci.* 84:6093.
- Driessen, A. J. M., J. Kodde, S. De Jong, and W. N. Konings. 1987. Neutral amino acid transport by membrane vesicles of *Streptococcus cremoris* is subjected to regulation by internal pH. *J. Bacteriol.* 169:2748.
- Driessen, A. J. M., K. J. Hellingwerf, and W. N. Konings. 1987. Mechanism of energy coupling to entry and exit of neutral and branched chain amino acids in membrane vesicles of *Streptococcus cremoris*. *J. Biol. Chem.* 262:12438.

- Driessen, A. J. M., S. De Jong, and W. N. Konings. 1987. Transport of branched chain amino acids in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 169:5193.
- Driessen, B., C. Van Leeuwen, and W. N. Konings. 1989. Transport of basic amino acids by membrane vesicles of *Lactococcus lactis*. J. Bacteriol. 171:1453.
- Dulay, T. A. 1980. Studies on accelerated cheese ripening for process cheese spread manufacture. Dairy Sci. Abstr. 43:5540.
- Dulley, J. R. 1976. The utilization of cheese slurries to accelerate the ripening of Cheddar cheese. Aust. J. Dairy Technol. 31:143.
- Dulley, J. R., and G. C. Taylor. 1972. Proc. Aust. Biochem. Soc. 5:52.
- El Soda, M., M. Johnson, and N. F. Olson. 1989. Temperature sensitive liposomes: a controlled release system for the acceleration of cheese ripening. Milchwiss. 44:213.
- Exterkate, F. A., A. C. Alting, and C. J. Slangen. 1991. Specificity of two genetically related cell-envelope proteinases of *Lactococcus lactis* subsp. *cremoris* towards  $\alpha_{s1}$ -casein (1-23)-fragment. Biochem. J. 273:135.
- Exterkate, F. A., A. C. Alting, and P. G. Bruinenberg. 1993. Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. Appl. Environ. Microbiol. 59: 3640.
- Exterkate, F. A., M. De Jong, G. J. C. M. DeVeer, and R. Baankreis. 1992. Location and characterization of aminopeptidase N in *Lactococcus lactis* subsp. *cremoris* HP. Appl. Microbiol. Biotechnol. 37:46.
- Farkye, N. Y., and P. F. Fox, 1991. Preliminary study on the contribution of plasmin to proteolysis in Cheddar cheese: cheese containing plasmin inhibitor, 6-aminohexanoic acid. J. Agric. Food Chem. 39:786.



- Farkye, N. Y., and P. F. Fox, 1992. Contribution of plasmin to Cheddar cheese ripening: effect of added plasmin. *J. Dairy Res.* 59:209.
- Farkye, N. Y., P. F. Fox, and G. F. Fitzgerald. 1990. Proteolysis and flavor development in Cheddar cheese made exclusively with single strain proteinase - positive or proteinase-negative starters. *J. Dairy Sci.* 73:874.
- Fedrick, I. 1987. Technology and economics of the accelerated ripening of Cheddar cheese. *Aust. J. Dairy Technol.* March/June:33.
- Fedrick, I. A., S. J. Cromie, J. R. Dulley, and J. E. Giles. 1986. The effects of increased starter populations, added neutral proteinase and elevated temperature storage on Cheddar cheese manufacture and maturation. *N. Z. J. Dairy Sci. Technol.* 21:191.
- Fox, P. F. 1989. Acceleration of cheese ripening. *Food Biotechnol.* 2:133.
- Fox, P. F. 1989. Proteolysis in cheese during manufacture and ripening. *J. Dairy Sci.* 72:1379.
- Fox, P. F. 1993. Exogenous enzymes in dairy technology. A review. *J. Food Biochem.* 17:173.
- Fox, P. F., and M. B. Gruffey. 1991. Exogenous enzymes in dairy technology. Page 219 *in* *Food Enzymology Vol. 1.* P. F. Fox, ed. Elsevier Appl. Sci., London.
- Fox, P. F., J. A. Lucey, and T. M. Cogan. 1990. Glycolysis and related reactions during cheese manufacture and ripening. *Crit. Rev. Food Sci. Nutr.* 29:237.
- Fox, P. F., J. Law, P. L. H. McSweeney, and J. Wallace. 1993. Biochemistry of cheese ripening. Page 389 *in* *Cheese: Chemistry, Physics and Microbiology.* 2nd ed. P. F. Fox, ed. Chapman & Hall, London.
- Fresta, M., E. Wehrli, and G. Puglisi. 1995. Neutrase entrapment in stable multilamellar and large unilamellar vesicles for acceleration of cheese ripening. *J. Microencapsulation* 12:307.

- Frey, J. P., E. H. Marth, M. E. Johnson, and N. F. Olson. 1986. Heat- and freeze-shocking cause change in peptides and protease activity of *Lactobacillus helveticus*. *Milchwiss.* 41:681.
- Furrer, B., U. Candrian, C. Hoefelein, and J. Luethy. 1991. Detection and identification of *Listeria monocytogenes* in cooked sausage products and in milk by *in vitro* amplification of haemolysin gene fragments. *J. Appl. Bacteriol.* 70:372.
- Garcia, F. E., H. Reuter, D. Prokopek, A. Olano, and M. Ramos. 1993. Effect of enzyme addition on the manufacture of Spanish hard cheese from milk concentrated by ultrafiltration. II. Ripening of cheeses. *Kieler Milchwirtschaftliche Forschungsberichte* 45:301.
- Garvie, E. I. 1984. Taxonomy and identification of dairy bacteria important in cheese and fermented dairy products. Page 56 *in* *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davies and B. A. Law Eds. Elsevier Appl. Sci., London.
- Gillies, K., and J. K. Kondo. 1990. Plasmid encoded elements required for the proteolysis of milk proteins in *Lactococcus lactis* subsp. *lactis* C2O. *FEMS Microbiol. Rev.* 87:P49.
- Grappin, R., T. C. Rank, and N. F. Olson. 1985. Primary proteolysis of cheese proteins during ripening. *J. Dairy Sci.* 68:531.
- Guinee, T. P., M. G. Wilkinson, E. O. Mulholland, and P. F. Fox. 1991. Influence of ripening temperature, added commercial enzyme preparations and attenuated mutant ( $Lac^-$ ) *Lactococcus lactis* starter on the proteolysis and maturation of Cheddar cheese. *Irish J. Food Sci. Technol.* 15:27.
- Haandrikman, A. J., J. Kok, and G. Venema. 1991. Lactococcal proteinase maturation protein PrtM is a lipoprotein. *J. Bacteriol.* 173:4517.

- Haandrikman, A. J., J. Kok, H. Laan, S. Soemito, A. M. Ledeboer, W. N. Konings, and G. Venema. 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. *J. Bacteriol.* 171:2789.
- Haandrikman, A. J., R. Meester, H. Laan, W. N. Konings, J. Kok, and G. Venema. 1991. Processing of the lactococcal extracellular serine proteinase. *Appl. Environ. Microbiol.* 57:1899.
- Hammes, W. P., A. Bantleon, and S. Min. 1990. Lactic acid bacteria in meat fermentation. *FEMS Microbiol. Rev.* 87:165.
- Harper, W. J., A. Carmona, and T. Kristoffersen. 1971. Protein degradation in Cheddar cheese slurries. *J. Food Sci.* 36:503.
- Harper, W. J., and T. Kristoffersen. 1970. Biochemical aspect of flavor development in Cheddar cheese slurries. *J. Agric. Food. Chem.* 18:563.
- Hayakawa, K. 1992. Classification and actions of food microorganisms with particular reference to fermented foods and lactic acid bacteria. Page 139 in *Functions of Fermented Milk*. Y. Nakarawa and A. Hosomo, eds. Elsevier Appl. Sci., London.
- Hayashi, K., D. F. Revell, and B. A. Law. 1990. Accelerated ripening of Cheddar cheese with the aminopeptidase of *Brevibacterium linens* and a commercial neutral proteinase. *J. Dairy Res.* 57:571.
- Hollar, C. M., A. J. R. Law, D. G. Dagleish, and R. J. Brown. 1991. Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:2403.
- Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Stanley, and S. T. Williams. *Bergey's Manual of Determinative Bacteriology*. Vol. 2. Williams & Wilkins, Baltimore, MD.

- Hugenholtz, J., M. Dijkstra, and H. Veldkamp. 1987. Amino acid limited growth of starter cultures in milk. *FEMS Microbiol. Ecology* 45:191.
- Hwang, I. K., S. Kaminogawa, and K. Yamauchi. 1981. Purification and properties of a dipeptidase from *Streptococcus cremoris*. *Agric. Biol. Chem.* 45:159.
- IDF 1991. Chemical methods for evaluating proteolysis in cheese maturation. Bulletin 216, International Dairy Federation, Brussels.
- Jay, J. M. 1986. Fermented foods and related products of fermentation. Page 364 in *Modern Food Microbiology*. , ed. 3rd ed. Van Nostrand Reinhold Co., New York, NY.
- Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Neth. Milk & Dairy J.* 16:153.
- Jones, G. A., and B. A. Humphrey. 1978. Evaluation of a dehydrogenase assay based on tetrazolium reduction for rapid in vitro estimation of fermentation activity in rumen contents. *Can. J. Anim. Sci.* 58:501.
- Kaiser, F. E., C. W. Gehrke, R. W. Zumwalt, and K. C. Kuo. 1974. Amino acid analysis: hydrolysis, ion-exchange clean-up, derivatization, and quantitation by gas liquid chromatography. *J. Chrom.* 94:113.
- Kaminogawa, S., N. Azuma, I. K. Hwang, Y. Susuki, and K. Yamauchi. 1984. Isolation and characterization of a prolidase from *Streptococcus cremoris* H61. *Agric. Biol. Chem.* 48:3035.
- Kempler, G. M., K. A. Baldwin, L. L. McKay, H. A. Morris, S. Halambeck, and G. Thorsen. 1979. Use of genetic alterations to improve *Streptococcus lactis* CZ as a potential Cheddar cheese starter. *J. Dairy Sci.* 62 (Suppl.1): 42 Abstract.
- Kiefer-Partsch, B., W. Boeckelmann, A. Geiss, and M. Teuber. 1989. Purification of an X-prolyl-dipeptidyl aminopeptidase from the cell wall proteolytic system of *Lactococcus lactis* subsp. *cremoris*. *Appl. Microbiol. Biotechnol.* 31:75.

- Kim, M. S., S. C. Kim, and N. F. Olson. 1994. Effect of commercial fungal protease and freeze-shocked *Lactobacillus helveticus* ODR101 on accelerating cheese fermentation: I. Composition. *Milchwiss.* 49:256.
- Kok, J. 1990. Genetics of the proteolytic system of the lactic acid bacteria. *FEMS Microbiol. Rev.* 87:15.
- Kok, J., and G. Venema. 1988. Genetics of proteinases of lactic acid bacteria. *Biochimie* 70:475.
- Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Ledebøer, and G. Venema. 1988. Nucleotide sequence of the cell wall proteinase gene from *Streptococcus cremoris* Wg2. *Appl. Environ. Microbiol.* 54:231.
- Kondo, J. K., and L. L. McKay. 1985. Gene transfer systems and molecular cloning in group N streptococci: A review. *J. Dairy Sci.* 68:2143.
- Konings, W. N., B. Poolman, and A. J. M. Driessen. 1989. Bioenergetics and solute transport in lactococci. *CRC Crit. Rev. Microbiol.* 16:419.
- Kosikowski, F. V. 1977. Cultures and starters. Page 16 *in* Cheese and Fermented Milk Foods. 2<sup>nd</sup> ed. F. V. Kosikowski and Assoc., Brooktondale, NY.
- Krause, D. A., M. C. Broome, and M. W. Hickey. 1991. The isolation and characterization of a proteinase negative cheese starter. *Aust. J. Dairy Sci. Technol.* 46:1.
- Kristoffersen, T., E. M. Mikolajcik, and I. A. Gould. 1967. Cheddar cheese flavor. IV. Direct and accelerated ripening process. *J. Dairy Sci.* 50:292.
- Kuipers, O. P., H. J. Boot, and W. M. de Vos. 1991. Improved site-directed mutagenesis method using PCR. *Nucleic Acid Res.* 19:4558.
- Kunji, E. R. S., A. Hagting, C. J. De Vries, V. Juillard, A. J. Haandrikman, B. Poolman, and W. N. Konings. 1995. Transport of  $\beta$ -casein-derived peptides by

the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. J. Biolog. Chem. 270:1569

Kunji, E. R. S., A. Hagting, C. J. De Vries, V. Juillard, A. J. Haandrikman, B. Poolman, and W. N. Konings. 1995. Transport of  $\beta$ -casein derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. J. Biol. Chem. 270:1569.

Kunji, E. R. S., E. J. Smid, R. Plapp, B. Poolman, and W. Konings. 1993. Di-tripeptides and oligopeptides are taken up via distinct transport mechanisms in *Lactococcus lactis*. J. Bacteriol. 175:2052.

Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70:187.

Kurman, J. A. J. L. Rasic, and M. Kroger. 1992. Encyclopedia of Fermented Fresh Milk Products. Van Nostrand Reinhold, New York, NY.

Laan, H., E. J. Smid, P. S. T. Tan, and W. N. Konings. 1989. Enzymes involved in the degradation and utilization of casein in *Lactococcus lactis*. Neth. Milk Dairy J. 43:327.

Laan, H., H. Bolhuis, B. Poolman, T. Abee, and W. N. Konings. 1993. Regulation of proteinase synthesis in *Lactococcus lactis*. Acta Biotechnol. 13:95.

Law, B. A. 1978. The accelerated ripening of cheese by use of non-commercial starter and enzymes – a preliminary assessment. International Dairy Federation, Document 108, Brussels, Belgium.

Law, B. A. 1980. Accelerated ripening of cheese. Dairy Ind. Intern. 45:15.

Law, B. A. 1982. Cheeses. Page 147 in Economic Microbiology. Vol. 7. Fermented Foods. A. H. Rose, ed. Academic Press, London.

Law, B. A. 1983. Accelerated ripening of cheese and cheese products. IDF Document 157:33.

- Law, B. A. 1984. Flavour development in cheeses. Page 187 *in* *Advances in Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davoes and B. A. Law, eds. Elsevier Applied Science Publishers, New York, NY.
- Law, B. A. 1984. The accelerated ripening of cheese. Page 209 *in* *Advances in Microbiology and Biochemistry of Cheese and Fermented Milk*. F. L. Davoes and B. A. Law, eds. Elsevier Applied Science Publishers, New York, NY.
- Law, B. A. 1987. Accelerated cheese ripening of non-Cheddar cheese. Bulletin 209, International Dairy Federation, Brussels.
- Law, B. A. 1987. Proteolysis in relation to normal and accelerated cheese ripening. Page 365 *in* *Cheese: Chemistry, Physics and Microbiology* Vol. 1. P. F. Fox, ed. Elsevier Appl. Sci., London.
- Law, B. A. and A. S. Wigmore. 1982. Accelerated cheese ripening with food grade proteinases. *J. Dairy Res.* 49:137.
- Law, B. A., and A. S. Wigmore. 1982. Microbial proteinases as agents for accelerated cheese ripening. *J. Soc. Dairy Technol.* 35:75.
- Law, B. A., and A. S. Wigmore. 1983. Accelerated ripening of Cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. *J. Dairy Res.* 50:519.
- Law, B. A., and J. Kolstad. 1983. Proteolytic systems in lactic acid bacteria. *Antonie van Leeuwenhoek* 49:225.
- Law, B. A., and J. S. King. 1985. Use of liposomes for proteinase addition to Cheddar cheese. *J. Dairy Res.* 52:183.
- Law, B. A., Z. D. Hosking, and H. R. Chapman. 1979. The effect of some manufacturing conditions on the development of flavour in Cheddar cheese. *J. Soc. Dairy Technol.* 32:87.

- Leenhouts, K. J., J. Gielema, J. Kok, and G. Venema. 1991. Chromosomal stabilization of the proteinase genes in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 57:2568.
- Lin, Y. C. 1971. Carbohydrate fermentation in Cheddar curd ripening. *Dairy Sci. Abstr.* 34:2335.
- Lloyd, R. J., and G. G. Pritchard. 1991. Characterization of X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *lactis*. *J. Gen. Microbiol.* 137:49.
- Maiorova, I. P. 1971. Sugar determination in culture liquids using triphenyl tetrazolium chloride. *Appl. Biochem. Microbiol.* 7:423.
- Manning, D. J. 1979. Chemical production of essential Cheddar flavour compounds. *J. Dairy Res.* 46:531.
- Marschke, R. J., D. E. J. Nickerson, W. D. Jarrett, and J. R. Dulley. 1980. A cause of increased proteolysis in Cheddar cheese manufactured from milk containing Maxilact. *Aust. J. Dairy Sci. Technol.* 35:84.
- Mayo, B., J. Kok, K. Venema, W. Boeckelmann, M. Teuber, H. Reinke, and G. Venema. 1991. Molecular cloning and sequence analysis of the X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris*. *Appl. Environ. Microbiol.* 57:38.
- Mayo, B., J. Kok, W. Boeckelmann, A. Haandrikman, K. J. Leenhouts, and G. Venema. 1993. Effect of X-prolyl dipeptidyl aminopeptidase deficiency on *Lactococcus lactis*. *Appl. Environ. Microbiol.* 59:2049.
- McGarry, A., J. Law, A. Coffey, C. Daly, P. F. Fox, and G. F. Fitzgerald. 1994. Effect of genetically modifying the lactococcal proteolytic system on ripening and flavour development in Cheddar cheese. *Appl. Environ. Microbiol.* 60:4226.
- McKay, L. L., and K. A. Baldwin. 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. *FEMS Microbiol. Rev.* 87:3.



- McKay, L. L., K. A. Baldwin, and P. M. Walsh. 1980. Conjugal transfer of genetic information in group N streptococci. *Appl. Environ. Microbiol.* 40:84.
- Meijer, W. C., J. D. Marugg, and J. Hugenholtz. 1996. Regulation of proteolytic enzyme activity in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 62:156.
- Mills, O. E., and T. D. Thomas. 1980. Bitterness development in Cheddar cheese: effect of level of starter proteinase. *N. Z. J. Dairy Sci. Technol.* 15:131.
- Minitab Statistical Software. 1989. Minitab Inc., State College, PA.
- Monnet, V., D. LeBars, and J. C. Gripon, 1986. Specificity of a cell wall proteinase from *Streptococcus lactis* NCDO763 towards bovine  $\beta$ -casein. *FEMS Microbiol. Lett.* 36:127.
- Monnet, V., J. P. Ley, and S. Gonzales. 1992. Substrate specificity of the cell envelope-located proteinase of *Lactococcus lactis* subsp. *lactis* NCDO763. *Int. J. Biochem* 24:707.
- Monnet, V., W. Boeckelmann, C. J. Gripon, and M. Teuber. 1989. Comparison of cell wall proteinases from *Lactococcus lactis* subsp. *cremoris* AC1 and *Lactococcus lactis* subsp. *lactis* NCDO 763: Specificity towards bovine  $\beta$ -casein. *Appl. Microbiol. Biotechnol.* 31:112.
- Muset, G., V. Monnet, and J. C. Gripon. 1989. Intracellular proteinases of *Lactococcus lactis* NCDO763. *J. Dairy Res.* 56:765.
- Nardi, M., M. C. Chopin, A. Chopin, M. M. Cals, and J. C. Gripon. 1991. Cloning and DNA sequence analysis of X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *lactis* NCDO763. *Appl. Environ. Microbiol.* 57:45.
- Nasr, M. M., M. M. El Sayed, and Y. A. El Samragy. 1991. Acceleration of Edam cheese ripening using acid fungal protease. *Nahrung* 35:143.
- Neviani, E., C. Y. Boquien, V. Monnet, L. P. Thanh, and J. C. Gripon. 1989. Purification and characterization of an aminopeptidase from *Lactococcus lactis*

- subsp. *cremoris* AM2. Appl. Environ. Microbiol. 55:2308.
- Oberg, C. J., B. C. Weimer, L. V. Moyes, R. J. Brown, and G. H. Richardson. 1991. Proteolytic characterization of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains by the *o*-phthaldialdehyde test and amino acid analysis. J. Dairy Sci. 74:398.
- Oberg, C. J., L. H. Davis, G. H. Richardson, and C. A. Ernstrom. 1986. Manufacture of Cheddar cheese using proteinase-negative mutants of *Streptococcus cremoris*. J. Dairy Sci. 69:2975.
- Olson, N. F. 1990. The impact of lactic acid bacteria on cheese flavor. FEMS Microbiol. Rev. 87:131.
- Otto, R., R. G. Lageveen, H. Veldkamp, and W. N. Konings. 1982. Lactate efflux induced electrical potential in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 146:733.
- Otto, R., W. M. DeVos, and J. Garvielli. 1982. Plasmid DNA in *Streptococcus cremoris* Wg2: influence of pH on selection of chemostats of variants lacking a protease plasmid. Appl. Environ. Microbiol. 43:1272.
- Patel, K. N., F. M. Bartlett, and J. Hamid. 1983. Extracellular heat-resistant proteases of psychrotrophic *Pseudomonas*. J. Food Prot. 46:90.
- Piard J. C., M. El Soda, W. Alkhalaf, M. Desmazeaud, L. Vassal, and J. C. Gripon. 1986. Acceleration of cheese ripening with liposome-entrapped proteinase. Biotechnol. Letters 8:241.
- Poolman, B., A. J. M. Driessen, and W. N. Konings. 1987. Regulation of arginine/ornithine exchange and the arginine deiminase pathway in *Streptococcus lactis*. J. Bacteriol. 169:5597.
- Poolman, B., A. J. M. Driessen, and W. N. Konings. 1987. Regulation of solute transport in streptococci by external and internal pH values. Microbiol. Rev. 51:489.

- Poolman, B., and W. N. Konings. 1988. Growth of *Streptococcus lactis* and *Streptococcus cremoris* in relation to amino acid transport. *J. Bacteriol.* 170:700.
- Poolman, B., E. J. Smid, and W. N. Konings. 1987. Kinetic properties of a phosphate-bond-driven glutamate-glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*. *J. Bacteriol.* 169:2755.
- Pritchard, G. G., and T. Coolbear. 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiol. Rev.* 12:179.
- Rank, T. C., R. Grappin, and N. F. Olson. 1985. Secondary proteolysis of cheese during ripening. A review. *J. Dairy Sci.* 68:801.
- Reid, J. R., C. H. Moore, G. G. Midwinter, and G. G. Pritchard. 1991. Action of a cell wall proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 on bovine  $\alpha_{s1}$ -casein. *Appl. Microbiol. Biotechnol.* 35:222.
- Reid, J. R., K. H. Ng, C. H. Moore, T. Coolbear, and G. G. Pritchard. 1991. Comparison of bovine  $\beta$ -casein hydrolysis by P<sub>I</sub> and P<sub>III</sub>-type proteinases from *Lactobacillus lactis* subsp. *cremoris*. *Appl. Microbiol. Biotechnol.* 36: 344.
- Reid, J. R., T. Coolbear, C. J. Pillidge, and G. G. Pritchard. 1994. Specificity of hydrolysis of bovine  $\kappa$ -casein by cell envelope-associated proteinases from *Lactococcus lactis* strains. *Appl. Environ. Microbiol.* 60:801.
- Reiter, B., and J. D. Oram. 1962. Nutritional studies on cheese starters. I. Vitamins and amino acid requirements of single strain starters. *J. Dairy Res.* 29:63.
- Richardson, G. H., C. A. Ernstrom, J. M. Kim, and C. Daly. 1983. Proteinase negative variants of *Streptococcus cremoris* for cheese starters. *J. Dairy Sci.* 66:2278.
- Ridha, S. H., J. J. M. Crawford, and A. Y. Tamine. 1984. The use of food grade neutral proteins to accelerate Cheddar cheese ripening. *Egypt. J. Dairy Sci.* 12:63.
- Samples, D. R. 1985. Some factors affecting the production of volatile sulfhydryl compounds in Cheddar cheese slurries. *Diss. Abstr. Int. B*, 46:1402.

- Samples, D. R., S. L. Dill, R. L. Richter, and C. W. Dill. 1986. A mechanism for volatile sulfhydryl production in Cheddar cheese slurries. *J. Dairy Sci.* 69 (Suppl. 1):62.
- Sanger F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463.
- SAS JMP. 1992 . Software for statistical visualization on the Apple Macintosh. SAS Institute Inc., Cary, NC.
- SAS/STAT User's Guide. 1992. Vol. 1. Version 6. 4<sup>th</sup> ed. Sas Institute Inc., Cary, NC.
- Seale, D. R. 1986. Bacteria inoculants as silage additives. *J. Appl. Bacteriol. Symp. Suppl.* 61:9S.
- Seitz, E. W. 1990. Microbial and enzyme-induced flavors in dairy foods. *J. Dairy Sci.* 73:3664.
- Seizen, R. J., W. M. De Vos, J. A. M. Leunissen, and B. W. Dijkstra. 1991. Homology modeling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteases. *Prot. Engin.* 4:719.
- Singh, S., and T. Kristoffersen. 1970. Factors affecting flavor development in Cheddar cheese slurries. *J. Dairy Sci.* 53:533.
- Singh, S., and T. Kristoffersen. 1971. Influence of lactic cultures and curd milling acidity on flavor of Cheddar cheese slurries. *J. Dairy Sci.* 54:1589.
- Smid, E. J. and W. N. Konings. 1990. Relationship between the utilization of proline and proline-containing peptides and growth of *Lactococcus lactis*. *J. Bacteriol.* 172:5286.
- Smid, E. J., A. J. M. Driessen, and W. N. Konings. 1989. Mechanism and energetics of dipeptide transport in membrane vesicles of *Lactococcus lactis*. *J. Bacteriol.* 171:292.

- Smid, E. J., and W. N. Konings. 1990. Relationship between utilization of proline and proline-containing peptides and growth of *Lactococcus lactis*. J. Bacteriol. 172:5286.
- Smid, E. J., B. Poolman, and W. N. Konings. 1991. Casein utilization by lactococci. Appl. Environ. Microbiol. 57:2447.
- Smid, E. J., B. Poolman, and W. N. Konings. 1991. Minireview: Casein utilization by lactococci. Appl. Environ. Microbiol. 57:2447.
- Smid, E. J., R. Plapp, and W. N. Konings. 1989. Peptide uptake is essential for growth of *Lactococcus lactis* on the milk protein casein. J. Bacteriol. 171:6135.
- Sood, V. K., and F. V. Kosikowski. 1979. Accelerated Cheddar cheese ripening by added microbial enzymes. J. Dairy Sci. 62:1865.
- Sood, V. K., and F. V. Kosikowski. 1979. Ripening changes and flavor development in microbial enzyme treated Cheddar cheese slurries. J. Food Sci. 44:1690.
- Stadhouders, J., L. Toepoel, and J. T. M. Wouters. 1988. Cheese making with Prt<sup>-</sup> and Prt<sup>+</sup> variants of N-streptococci and their mixture. Phage sensitivity, proteolysis and flavour development during ripening. Neth. Milk Dairy J. 42:183.
- Steele, J., and L. L. McKay. 1986. Partial characterization of the genetic basis for sucrose metabolism and nisin production in *Streptococcus lactis*. Appl. Environ. Microbiol. 51:57.
- Tan, P. S. T. 1992. The biochemical, genetic and physiological properties of aminopeptidase N from *Lactococcus lactis*. Ph.D. thesis, Univ. Groningen, Groningen, Netherlands.
- Tan, P. S. T., and W. N. Konings. 1990. Purification and characterization of a post-proline dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 56:526.

- Tan, P. S. T., B. Poolman, and W. N. Konings. 1993. The proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. 60:269.
- Tan, P. S. T., K. M. Pos, and W. N. Konings. 1991. Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 57:3593.
- Thomas, T. D., and G. G. Pritchard. 1987. Proteolytic enzymes of dairy cultures. FEMS Microbiol. Rev. 46:245.
- Thomas, T. D., and O. E. Mills. 1987. Proteolytic enzymes of dairy starter bacteria. Neth. Milk Dairy J. 35:255.
- Thompson, J. 1987. Ornithine transport and exchange in *Streptococcus lactis*. J. Bacteriol. 169:4147.
- Tynkkynen, S., A. von Wright, and E. L. Syvaaja. 1989. Peptide utilization encoded by *Lactococcus lactis* subsp. *lactis* SSL135 chromosomal DNA. Appl. Environ. Microbiol. 55:2690.
- Tynkkynen, S., and A. von Wright. 1988. Characterization of a cloned chromosomal fragment affecting the proteinase activity of *Streptococcus lactis* subsp. *lactis*. Biochimie 70:531.
- Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman. 1993. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. J. Bacteriol. 175:7523.
- Vafopoulou, A., E. Alichanidis, and G. Zerfiridis. 1989. Accelerated ripening in Feta cheese, with heat-shocked cultures or microbial proteinases. J. Dairy Res. 56:285.
- Van Boven, A., and W. N. Konings. 1987. A phosphate-bond-driven dipeptide transport system in *Streptococcus cremoris* is regulated by the internal pH. Appl. Environ. Microbiol. 53:2897.

- Van Boven, A., P. S. T. Tan, and W. N. Konings. 1988. Purification and characterization of a dipeptidase from *Streptococcus cremoris* Wg2. *Appl. Environ. Microbiol.* 54:43.
- Visser, S., A. J. P. M. Robben, and C. J. Slangen. 1991. Specificity of cell-envelope-located proteinase (P<sub>III</sub>-type) from *Lactococcus lactis* subsp. *cremoris* AM1 in its action on bovine  $\beta$ -casein. *Appl. Microbiol. Biotechnol.* 35:477.
- Visser, S., C. J. Slangen, F. A. Exterkate, and G. J. C. M. DeVeer. 1988. Action of a cell wall proteinase (P<sub>I</sub>) from *Streptococcus cremoris* HP on bovine  $\beta$ -casein. *Appl. Microbiol. Biotechnol.* 29:61.
- Visser, S., C. J. Slangen, G. Hup, and J. Stadhouders. 1983. Bitter flavour in cheese. 3. Comparative gel-chromatographic analysis of hydrophobic peptide fractions from twelve Gouda-type cheeses and identification of bitter peptides isolated from a cheese made with *Streptococcus cremoris* strain HP. *Neth. Milk Dairy J.* 37:181.
- Visser, S., F. A. Exterkate, C. J. Slangen, and G. J. C. M. DeVeer. 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine  $\alpha_{s1}$ -,  $\beta$ -, and  $\kappa$ -casein. *Appl. Environ. Microbiol.* 52:1162.
- Von Boeckelman, I., and L. O. Lodin. 1974. 19th International Dairy Congress 1E:441.
- Von Wright, A., S. Tynkkynen, and M. Suominen. 1987. Cloning of a *Streptococcus lactis* subsp. *lactis* chromosomal fragment associated with the ability to grow in milk. *Appl. Environ. Microbiol.* 53:1584.
- Vos, P., G. Simons, R. J. Siezen, and W. M. De Vos. 1989. Primary structure and organization of the gene for a prokaryotic cell envelope-located serine proteinase. *J. Biol. Chem.* 264:13579.

- Vos, P., M. V. Asseldonk, F. V. Jeneren, R. J. Siezen, G. Simons, and W. M. De Vos. 1989. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. *J. Bacteriol.* 171:2795.
- Walsh, P. M. and L. L. McKay. 1981. Recombinant plasmid associated with cell aggregation and high-frequency conjugation of *Streptococcus lactis* ML3. *J. Bacteriol.* 146:937.
- Weimer, B. C., B. Dias, M. Ummadi, J. Broadbent, J. Jaegi, M. Johnson, F. Milani, J. Steele, and D. V. Sisson. 1997. Influence of NaCl and pH on intracellular enzymes that influence Cheddar cheese ripening. *Le Lait* 77:783.
- Weimer, B. C., C. J. Oberg, L. V. Moyes, R. J. Brown, and G. H. Richardson. 1989. Comparison of classical ion exchange amino acid analysis and *o*-phthaldialdehyde methods to characterize proteolysis by *Lactobacillus bulgaricus*. *J. Dairy Sci.* 72:2873.
- Wilkinson, M. 1992. Studies on the acceleration of Cheddar cheese ripening. Ph.D. thesis, National Univ. of Ireland, Cork.
- Yan, T. R., N. Azuma, S. Kaminogawa, and K. Yamauchi. 1987. Purification and characterization of a novel metalloendopeptidase from *Streptococcus cremoris* H61. *Eur. J. Biochem.* 163:259.
- Yu, W., K. Gillies, J. K. Kondo, J. R. Broadbent, and L. L. McKay. 1995. Plasmid-mediated oligopeptide transport system in Lactococci. Page 509 in *Genetics of Streptococci, Enterococci, and Lactococci*. Vol. 85. J. J. Ferretti, M. S. Gilmore, T. R. Klaenhammer, and F. Brown, eds. Dev. Biol. Stand. Basel, Karger, Switzerland.
- Yuan, T. C. 1991. An automated reflectance color meter instrument for microbiological and enzymic assays. Ph. D. Dissertation, Utah State Univ. Logan, UT.



Zevaco, C., V. Monnet, and J. C. Gripon. 1990. Intracellular X-prolyl dipeptidyl peptidase from *Lactococcus lactis* ssp. *lactis* purification and properties. J. Appl. Bacteriol. 68:357.