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Proteolytic systems in lactic acid bacteria

BARRY A. LAW and JENS KOLSTAD[1]

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, UK

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The proteolytic systems of lactic acid bacteria are important as a means of making protein and peptide N available for growth and as part of the curing or maturation processes which give foods their characteristic rheological and organoleptic properties. The proteolytic systems of lactic acid bacteria are described in relation to their growth and their functions in protein-rich foods. Their role in the manufacture of milk products is discussed.

INTRODUCTION

When food microbiologists refer to the lactic acid bacteria they generally include the genera *Streptococcus* (faecal and lactic), *Leuconostoc, Lactobacillus* (*Lb.*) and *Pediococcus*. Taxonomically, such a group presents some contradictions (Hurst and Collins-Thompson, 1979) but this definition is functionally sensible and necessary. The lactic acid bacteria are all nutritionally fastidious, yet they compete remarkably well with other groups of microorganisms in foods, partially because their fermentative metabolism produces inhibitory conditions and compounds (low pH, low E_h, organic acids, H_2O_2, antibiotics) but also because they are well equipped to utilize the wide range of nutrients available in such habitats. They are found as important components of the microflora of a wide variety of fermented foods, including soy sauce, sausages, vegetables and milk products, where their main function is one of acidification. Their proteolytic systems are important, both as a means of making protein and peptide N availabe for growth and, fortuitously, as part of the curing or maturation processes which give the foods their characteristic rheological and organoleptic properties. This paper describes the proteolytic systems of lactic acid bacteria in

[1] British Council Scholar; present address, Dairy Research Institute, Agricultural University of Norway, N-1432, Ås-NLH, Norway.

relation to their growth and functions in protein-rich foods. The discussion is inevitably weighted towards their role in the manufacture of milk products since this area has been most intensively investigated. For descriptions of the lactic floras of fermented foods the reader is referred to articles by the following authors: Reuter (1975; meats), Fleming (1982; vegetables), Law (1982; cheeses), Vedamuthu (1982; fermented milks), Wood (1982; soy sauce).

Amino acids and peptides in nutrition

Pre-formed amino acids are an absolute requirement or a growth stimulant to all lactic acid bacteria. Detailed experimental data about these requirements are only available for some of the group, the most definitive studies having been done with group N streptococci (Reiter and Oram, 1962; Law et al., 1976) and *S. thermophilus* (Shankar, 1977; Bracquart and Lorient, 1979). Qualitative data on lactobacilli (Morishita et al., 1981) indicate that this group has most extensive requirements. A comparison of the amounts of the essential amino acids which the lactic streptococci need for maximum growth, with the concentrations of free amino acids in milk (Table 1) emphasizes the importance of proteolytic enzymes as mediators in releasing further amino acids from milk proteins to allow maximum growth.

Like other bacteria, the lactic acid bacteria can actively transport amino acids and peptides across the cell membrane into the cell against a concentration gradient (Leach and Snell, 1960; Mora and Snell, 1963; Brock and Wooley, 1964;

Table 1. Minimum concentrations of amino acids required by group N streptococci and *Streptococcus thermophilus* for maximum growth in the Ford (1962) defined medium + lactose after 17 h at 24 °C

	Concentration required in medium for maximum growth (μg/ml)				Concentration of free amino acids in milk
	S. lactis	*S. lactis* subsp. *diacetylactis*	*S. cremoris*	*S. thermophilus*	
Glu	77	87	70	150	35.9
Leu	41	37	32	n.e.	1.2
Ile	33	30	32	n.e.	0.8
Val	27	30	41	n.e.	2.6
Arg	37	36	39	n.e.	1.6
Cys	s[1]	s	27	80	n.d
Pro	n.e.	n.e.	38	n.e.	8.8
His	23	24	14	60	2.8
Phe	21	n.e.	6	n.e.	n.e.
Met	22	21	11	n.e.	n.d.

Data from Law et al. (1976), Shankar (1977) and Mills and Thomas (1981).
[1] n.d. = not detected; n.e. = not estimated; s = stimulatory.

Shelton and Nutter, 1964; Law, 1978; Rice et al., 1978). The free amino acids normally present in fresh milk are sufficient to support the growth of *S. cremoris* to cell densities corresponding to 8–16% of those found in coagulated (fully-grown) milk cultures (Mills and Thomas, 1980). Peptides of mol wt < 1500 provide a further source of amino acids, though milk proteins become important at high cell densities. Amino acid and peptide uptake in lactobacilli, leuconostocs and group N streptococci are mediated by separate systems, and the streptococci also have distinct dipeptide and oligopeptide transport (Law, 1978). The size exclusion limit for uptake by the latter system corresponds to peptides containing between three and seven residues (estimated average size in mixed peptides; Law et al., 1976) or five residues (estimated with a single peptide series; Rice et al., 1978). There is strong presumptive evidence that *S. lactis* can utilize peptides via cell wall-bound peptidases as well as transporting them intact. For example, although uptake competition between peptides can be demonstrated in *S. lactis* it is invariably weaker than that measured in *S. cremoris* (Law, 1978) and is not seen at all in longer-term growth response experiments (Law, 1977). These observations are consistent with the findings that whole cells of *S. lactis* hydrolyse peptides under non-transporting conditions and that peptidases are released when the cells are protoplasted or osmotically shocked under a variety of conditions (Law, 1979; Kolstad, Cliffe and Law, unpublished data; also see section Proteolytic enzymes in the lactic acid bacteria).

The significance of the uptake and utilization of amino acids and peptides in *S. thermophilus* is probably more complex than is the case with group N streptococci. The thermophilic streptococci not only have an absolute requirement for some amino acids but are also stimulated by non-essential amino acids (valine, methionine, leucine, tryptophan) to produce more acid than normal in milk (Shankar, 1977; Bracquart and Lorient, 1979). Presumably, their rates of cellular synthesis are rate-limiting for growth in the presence of "normal" amounts in milk. If these amino acids are supplied as peptides, similar stimulation can be observed (Desmazeaud and Hermier, 1972; Shankar, 1977). Stimulatory peptides isolated from milk cultures or casein digests by these authors would be too large to be transported by any of the known uptake systems in lactic acid bacteria but *S. thermophilus* probably has surface-bound peptidases which reduce them to a more manageable size (Shankar, 1977).

PROTEOLYTIC ENZYMES IN THE LACTIC ACID BACTERIA

The lactic acid bacteria are weakly proteolytic compared with many other groups of bacteria (e.g. *Bacillus, Proteus, Pseudomonas,* coliforms). However, the detailed studies of the last decade have given an insight into the complexity of the proteolytic "equipment" of the lactic acid bacteria. This complexity is seen not only in the numbers and types of different proteinases and peptidases,

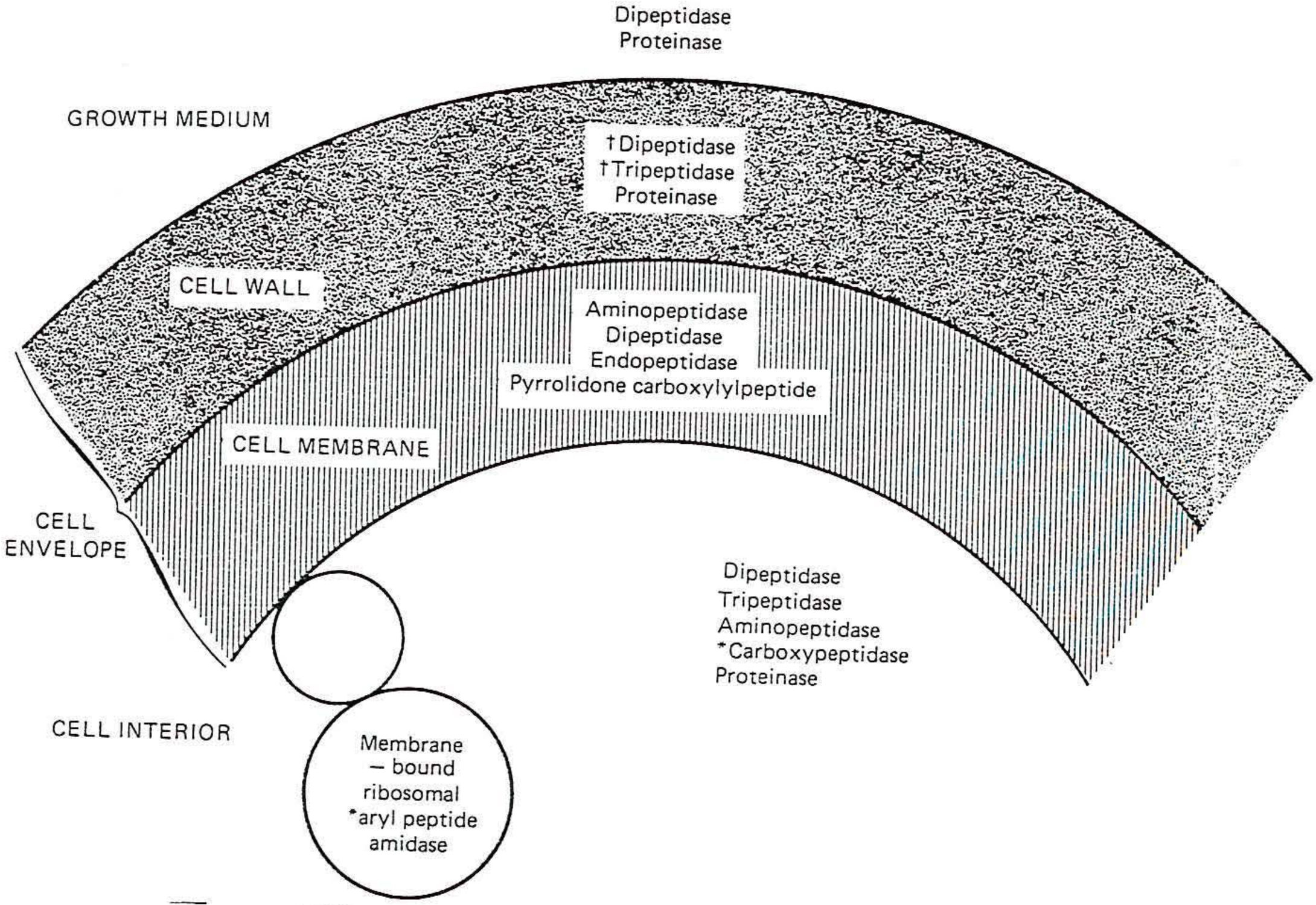

Fig. 1. Schematic representation of the possible cellular locations of proteinase and peptidase activities in lactic acid bacteria; see text for discussion.
* Only lactobacilli
† Only *Streptococcus lactis*

but also in their cellular distribution (Fig. 1). Not all of the proposed locations are established with certainty and in the following discussion we shall attempt to interpret the data on which they are based, as well as describing the properties of the enzymes themselves.

In much of the literature to be discussed, the distinction between proteinases and peptidases is made on the basis of their ability to degrade either intact proteins or peptides. This serves a useful purpose in that the enzymes can be related to their perceived cellular functions. We therefore propose to use the term "proteinase" for enzymes which are detected only by their action on native proteins, and peptidases for enzymes detected with peptides or peptide derivatives, irrespective of whether or not they also degrade native proteins.

Proteinases

1. *Extracellular*. Proteinases are secreted as "free" enzymes outside the cell by many bacterial genera and species (Pollock, 1962; Law, 1980) and one such enzyme was reported by Williamson et al. (1964) as a product of *S. lactis*. However, the criteria for conferring true extracellular status on such enzymes (Pollock,

1962) were not applied, especially in relation to the lack of any assessment of cell lysis or leakage. Also, a later report by Cowman and Speck (1967) showed that the same organism also produced an intracellular proteinase with some properties in common with the supposed extracellular enzyme. A further source of cell-free extracellular proteolytic activity (especially in cultures grown in non-milk media low in Ca^{2+} (Mills and Thomas, 1978)) could be the loosely-bound cell wall-located proteinase(s) first described by Thomas et al., (1974). Only one report (Exterkate, 1979*a*) suggests that group N streptococci may secrete a distinct cell-free extracellular proteinase and this is based on the different Ca^{2+} requirements of the cell-bound and cell-free proteolytic activities.

The cell wall location of a high proportion of the group N streptococcal proteinase activity is well established in that it can be detected in spheroplast supernatants containing lysozyme- or lysin-released cell wall components in the absence of intracellular marker enzymes (Thomas et al., 1974; Exterkate, 1975). The amount of activity recovered in this way is approximately equal to that measurable with whole cell suspensions incubated with casein. No purification and characterization of a cell wall proteinase has been reported, but inhibitor studies on crude preparations suggest that the *S. lactis* enzyme is a metalloproteinase. Exterkate (1976) distinguished three types of proteinase activity according to their temperature and pH optima and showed that there are wide strain variations in the numbers and combinations of these enzymes. Multiple proteinases in *S. lactis* have recently been demonstrated by zymograms of lysozyme extracts from milk-grown cells. At least four bands of caseinolytic activity were separated and shown to be distinct from the intracellular activity which is apparently due to only one proteinase (Cliffe and Law, unpublished data).

Although relatively little is known about the proteinases themselves, the factors controlling their synthesis, activity and attachment to the cell wall have been described. For example, *S. cremoris* and *S. lactis* release part of their proteolytic activity without lysing if they are suspended in buffer (Mills and Thomas, 1978). This phenomenon is temperature- and pH-dependent, and suppressed by added Ca^{2+}, suggesting an involvement of this ion in enzyme attachment. However, these results were obtained under artificial experimental conditions which have little relevance for understanding the normal function of Ca^{2+} in cells when they are actively producing proteinases under the influence of constituents in their growth medium. Perhaps this is why the later study of Exterkate (1979*a*) produced a different conclusion concerning the role of Ca^{2+}; a dependence on Ca^{2+} for accumulation of active proteinase in cell walls was observed in growing cultures but the ion stabilized the enzyme molecules in an active configuration, rather than binding them to the cell wall. In contrast to Mills and Thomas (1978), Exterkate (1979*a*) observed that actively growing or metabolizing cells released a constant amount of proteinase into the medium, irrespective of its Ca^{2+} content; only the enzyme in the cell wall was affected by the Ca^{2+}.

The de novo synthesis of cell wall proteinase is subject to regulation by amino acids or peptides at the level of mRNA translation (Exterkate, 1979*a*). Proteinase-specific mRNA appears to be either inherently long-lived or stabilized by the proximity of the cell membrane to the ribosomes (cf. Shires et al., 1974). Low concentrations of casein-derived amino acids do not prevent proteinase synthesis but cells respond to high extracellular concentrations by reducing the rate of enzyme synthesis until cellular metabolism has used up this external N source; proteinase synthesis then resumes in order to ensure a further supply of amino acids from proteins in the medium. Both the effect of Ca^{2+} on proteinase stability and the amino acid/peptide effect on its synthesis may explain the well-documented failure of cells which have been grown in non-milk media to grow rapidly in milk on sub-culture. Zymograms of cell wall proteinases from *S. lactis* cultures harvested from a complex, nutritionally rich medium (M17; Terzaghi and Sandine, 1975) illustrate the cause of this phenomenon when it is compared with one from milk-grown cells. Both media yield proteolytically active cells but the M17-grown cells are lacking two of the proteinase bands. This suggests differential repression or destabilization and warrants further study.

Populations of group N streptococci undergo spontaneous proteinase loss at high frequency, suggesting that the enzymes are encoded on extrachromosomal DNA. Plasmid linkage has long been suspected from evidence gained in curing experiments (see review by Davies and Gasson, 1981). Unequivocal, direct evidence to show that the proteinase gene(s) is carried on a plasmid has only recently been published after many confusing and conflicting reports assigning it to plasmids ranging from 10–30 megadaltons (Mdal). Gasson (1983) used protoplast regeneration to produce variants of *S. lactis* which only carried a 33 Mdal plasmid, yet retained proteinase activity. Loss of this plasmid resulted in the loss of all four bands of cell wall-bound proteinase activity previously detected in the zymograms of cell wall proteinases. A more detailed account of this plasmid study appears elsewhere in this issue (see the paper by M. Gasson).

Of the other lactic acid bacteria, lactobacilli and leuconostocs probably have cell wall-bound proteinases as evidenced by the hydrolysis of milk proteins by whole cell suspensions (Searles et al., 1970). Argyle et al. (1976) and Chandan et al. (1982) showed that the activity of *Lb. bulgaricus* was released from the cells by treatment with lytic enzymes or by osmotic shock. However, the precise location of the proteinase cannot be deduced from these studies; no estimate of intracellular enzyme leakage was reported, and its recorded properties may have been those of a mixture of proteinases from various other cellular locations. Despite these reservations there is little doubt that *Lb. bulgaricus* produces an extracellular proteinase and it is understood that the peptides which it releases from milk proteins act as stimuli to the growth of *S. thermophilus* in yogurt cultures (Shankar and Davies, 1978; Hemme et al., 1981).

A cell wall-bound proteinase has been demonstrated in *Lb. helveticus* and its location is supported by evidence that it is released from whole cells without any leakage of intracellular enzymes (Vescovo and Bottazzi, 1979). However, the enzyme was not purified or characterized. Other evidence of extracellular proteinase activity in lactic acid bacteria (including *S. faecalis*) was reviewed by Castberg and Morris (1976) but in the absence of proper localization data it is of little use in the present discussion. Some of the data may offer a comparative guide to the likely effects of these organisms on food proteins.

2. *Intracellular*. Although extracellular proteinases are a vital part of the mechanism by which bacteria make external proteins available for growth, their intracellular equivalents are equally important in the turnover of denatured or defective proteins, the activation of zymogens, and the termination of newly synthesized proteins. Opinions differ as to the relative distribution and precise intracellular location of the proteinases in the group N streptococci (Thomas and Mills, 1981) but it is clear that both *S. cremoris* and *S. lactis* have "soluble" enzymes of this type since proteolytic activity can be demonstrated in particle-free cell sap of osmotically lysed spheroplasts (Pearce et al., 1974; Exterkate, 1975, 1976). It could reasonably be expected that such different enzymes as cell wall extracellular and unbound intracellular proteinases would be encoded on genes located in separate DNA species, but evidence on this is scarce and contradictory. Exterkate (1976) found that a proteinase-deficient variant of *S. cremoris* HP had lost two cell wall proteinases and the intracellular proteinase, whereas Pearce et al., (1974) had shown that although equivalent variants of *S. lactis* had also lost cell wall proteinases, they retained all of their intracellular activity. Proteinase-negative variants of *S. lactis* NCDO 712 which have lost the four proteinase bands of the cell wall fraction, retained their intracellular activity (Cliffe and Law, unpublished data) confirming the observation of Pearce et al. (1974) and suggesting separate gene locations. Moreover, it would seem logical to have intracellular proteinases encoded on stable chromosomal DNA since their loss would be potentially more embarrassing to cellular metabolism than that of the plasmid-encoded surface-bound proteinases.

It is impossible to generalize about the properties of intracellular proteinases in group N streptococci since there are few reports dealing with purified enzymes, and the little information which is available suggests that different species have enzymes with widely varying characteristics. The situation is further complicated by the fact that most studies were done with mechanically disintegrated cells which may have contained proteinases whose original location was in membranes, ribosomes or cell walls in vivo. The *S. lactis* proteinase described by Cowman et al. (1968) was apparently an -SH enzyme of low mol wt but although the authors claimed to have purified it to homogeneity, it had dipeptidase and aminopeptidase activity. This strongly suggests that more than one enzyme protein was present in the preparation. Ohmiya and Sato (1975) described a high

mol wt (140000) proteinase from *S. cremoris* which hydrolysed α-, β- and κ-caseins, was inhibited by EDTA (ethylenediaminotetraacetate) and activated by Ca^{2+}. The most detailed study was done by Desmazeaud and Zevaco (1976), and Zevaco and Desmazeaud (1980) on a *S. diacetylactis* neutral metallo-proteinase (Mn^{2+} and Co^{2+}-activated) of 49500 mol wt. It showed specificity for Pro-Ile, Ala-Phe, Lys-Ala and Lys-Val bonds of β-casein.

In their earlier studies of the proteolytic system of *S. lactis,* Cowman and Speck (1967) reported that cell-free extracts from sonically disrupted cells contained not only cytoplasmic proteinases but also a "particulate" proteinase, sedimented at 27000 × *g*. Because this enzyme remained particle-bound after lysozyme treatment the authors assigned to it a membrane location. It formed inactive aggregates (prevented by -SH-blocking reagents) after removal from the particulate material by NaCl. This suggests that it required a particular spatial localization to be active and that it was capable of binding to sub-structures. However, there is insufficient supporting evidence to confirm it as a membrane-bound enzyme. In addition, it is difficult to reconcile its claimed role in the N nutrition of the cell (Cowman et al., 1967) with its position inside the cell wall, which would prevent proteins in the medium from reaching it. Further doubt is cast on the homogeneity of the "particulate" fraction and of the enzyme itself by the presence of dipeptidase activity together with the proteinase. Thomas et al. (1974) suggested that this fraction may have been contaminated with proteinase-bearing cell wall material even though it had been treated with lysozyme, because *S. lactis* walls were thought to be insensitive to this lytic enzyme. However, the situation is further confused by subsequent studies on spheroplasting methods for *S. lactis* (Gasson, 1980) which have shown that lysozyme can be used successfully to digest its cell wall. Unfortunately Cowman and Speck (1967) did not subject their preparation to electron microscopic examination so that it is impossible to decide if cell wall enzyme remained after lysozyme treatment. It should be noted though, that the cell wall enzyme described by Thomas et al. (1974) did not exhibit the low temperature oxidative deactivation which was observed by Cowman and Speck (1967), suggesting that the two enzymes may not have been the same type. Finally, the possibility remains that the "membrane-bound" enzyme represented several ribosomal or polysomal proteolytic activities involved in the extrusion of newly formed proteins through the membrane. The authors failed to treat their particulate fraction with RNAase to test this possibility, perhaps because at that time membrane-bound ribosomes were not thought to be significant in bacterial cells. Since the work of Smith et al. (1977) was published, however, it has become clear that such structures are intimately involved in the synthesis/extrusion of extracellular enzymes.

Although other lactic acid bacteria are known to contain intracellular proteinases, detailed studies are lacking. Lactobacilli generally produce neutral proteinases active on α-, β- and κ-casein but the intensity of their activity is extremely variable from strain to strain (Castberg and Morris, 1976). El Soda and Desma-

zeaud (1982) and El Soda et al. (1982) demonstrated proteolytic activity in lactobacilli of both the thermobacterium and betabacterium groups. In general, β-casein was the preferred substrate but whey proteins were also degraded. *S. thermophilus* strains show varying degrees of caseinolytic action (Desmazeaud and Juge, 1976) distributed between ill-defined particulate and soluble cell fractions. Unlike most of the lactobacillus proteinases, the streptococcal enzyme did not degrade α-casein.

Peptidases

1. *Extracellular*. Many classes of peptidases have been reported in every conceivable location in or on the cells of lactic acid bacteria but this part of the discussion refers to those enzymes whose activity manifests itself outside the cell membrane. The importance of obtaining the appropriate evidence before assigning extracellular status to enzymes applies equally well to the lactic acid bacteria peptidases. Such enzymes have been recovered from culture supernatants but they have probably, on present evidence, leaked from dead or moribund cells. It is known that group N streptococci autolyse to varying degrees during culture on different media (Vegarud and Langsrud, 1982; Vegarud et al., 1982). Indeed, the cell-free dipeptidase of *S. cremoris* and *S. lactis* closely resembles one of the intracellular dipeptidases of each organism (Law, 1979). though the question of why only one of many intracellular enzymes should 'leak out' remains to be answered.

Evidence for the existence of a distinct cell wall-bound peptidase in *S. lactis* (but not *S. cremoris*) is more convincing: Sørhaug and Solberg (1973) treated acetone-dried whole cells with trypsin and released a peptidase whose substrate profile suggested that it was a different enzyme from those released by mechanical cell disruption. It was not decided whether this peptidase was cell wall or membrane-bound and no data on intracellular enzyme leakage were reported. Law (1977, 1978) noted that although *S. lactis* utilized peptides containing essential amino acids, its growth was not inhibited by other peptides containing non-essential amino acids competing for the same transport system. Also, uptake competition between ^{14}C-labelled and unlabelled peptides was weak in *S. lactis* compared with *S. cremoris*, suggesting that a proportion of the peptide was hydrolysed outside the cell. Taken together with the observation that starved, non-transporting whole cells of *S. lactis* hydrolysed peptides, this presumptive evidence supports the idea of a cell wall peptidase. In addition, a low mol wt (26000) EDTA-sensitive di-/tripeptidase is released from *S. lactis* after treatment with lysozyme or suspension in Tris-buffer, under conditions in which the release of intracellular marker enzymes is only 1–2% (Law, 1979; Kolstad and Law, unpublished data). To data there have been no other reports of similar enzymes in other strains of group N streptococci, but other workers have not tested their preparations with the appropriate unsubstituted dipeptide substrates (cf. Thomas et al., 1974; Exterkate, 1975; Mills and Thomas, 1978).

The report of surface-bound peptidases of *S. thermophilus* (Shankar and Davies, 1978) was not supported by tests for the possibility of cell leakage or lysis, but the existence of such enzymes would help to explain the stimulation of this organism by *Lb. bulgaricus* in yogurt cultures. El Soda et al. (1978*b*) did not find peptidases in the solubilized cell wall fraction of *Lb. casei*, but Eggimann and Bachmann (1980) have purified a surface-bound aminopeptidase from *Lb. lactis*. This enzyme has a broad specificity, hydrolysing di- and tripeptides as well as aminopeptidase substrates. It shares this and many other properties with intracellular aminopeptidases of lactic acid bacteria and therefore convincing evidence is needed to distinguish it unequivocally as a truly extracellular enzyme.

2. *Intracellular*. Both the lactic streptococci and the lactobacilli are well endowed with peptidases, which can be demonstrated in various intracellular locations. Most of the available data are derived from cell-free extracts of mechanically disrupted cells within which the original in vivo localization may have been altered during the extraction and fractionation process. Nevertheless, the information can be useful because although it is of limited value in deciding the function of the peptidases in cellular metabolism, a detailed knowledge of their properties can be used to evaluate their influence in foods, either as spoilage organisms or part of the added microflora.

The group N streptococci contain a range of peptidases whose spectrum of bond specificities is probably wide enough to ensure the complete release of all amino acids from casein-derived peptides (Mou et al., 1975). Although these authors did not characterize any of the enzymes, they concluded that crude cell-free extracts of *S. lactis* subsp. *diacetylactis* and *S. cremoris* contained five different peptidases, based on the substrates which were hydrolysed; they found di- and tripeptidases, aminopeptidase-P, proline iminopeptidase and general aminopeptidase activities. Electrophoretic zymogram studies of crude sonic extracts of the three species of group N streptococci revealed between four and nine different peptidases (Sørhaug and Solberg, 1973; Sørhaug and Kolstad, 1981). Law (1979) found only three readily distinguishable peptidases in *S. lactis* and *S. cremoris* after the cells had been osmotically lysed and the extract centrifuged at a sufficiently high speed to ensure that all particulate fractions had been sedimented. This suggests that in vivo there are fewer cytoplasmic peptidases than previous studies of sonicates or homogenates would indicate. However, this supposition requires confirmation with a wider range of peptides and peptide derivatives as substrates.

Although many peptidases have been named according to the substrates which they hydrolyse, few have been purified and characterized. However, even those few reveal a remarkable degree of diversity in pH and temperature optima and mol wts (Table 2) even though they all appear to be EDTA-sensitive metalloenzymes like so many peptidases of microbial and animal origin. If the partially characterized peptidases described by Law (1979) are added to this list, the

Table 2. Comparison of intracellular peptide hydrolases from group N streptococci

Enzyme	Optimum pH	Optimum temperature	Inhibitor	Substrate	Molecular weight	Purification fold	Reference
Aminopeptidase I	6,5	35 °C	EDTA pCMB	tripeptides aminoacid-β-naphthylamides	85000	165	Desmazeaud and Zevaco (1979)
Aminopeptidase II	7,0	35 °C	EDTA pCMB	tripeptides	75000	165	Desmazeaud and Zevaco (1979)
Dipeptidase	7,5–8,0		EDTA pCMB	dipeptides	51000	73	Desmazeaud and Zevaco (1977)
Dipeptidase	10,3	60 °C	EDTA pHMB	dipeptides	240000 (32000)[1]	361	Sørhaug and Kolstad (1981); Kolstad and Sørhaug (1982)
Dipeptidase	8,0		1,10-phenanthroline EDTA	dipeptides	100000	192	Hwang et al. (1981)

[1] Monomer.

EDTA: ethylenediaminotetraacetate; pCMB: *p*-chloromercuribenzoate; pHMB: *p*-hydroxy-mercuribenzoate.

diversity, especially of mol wts, becomes even greater since these range from approximately 25000 mol wt for the true dipeptidase, to 50000 mol wt for the aminopeptidase.

Some of the intracellular peptidases of the group N streptococci are associated with subcellular structures. For example, the endo- and aminopeptidase activities of osmotically lysed *S. cremoris* HP are found in the cell fraction sedimenting at approximately 48000 × *g* and may be involved in peptide utilization by this organism if this fraction is assumed to contain cell membranes (Exterkate, 1975). The "particulate" fraction was, however, ill-defined with no confirmation of structure or homogeneity by electron microscopy, and no RNAase treatment to eliminate ribosomes. Exterkate (1977, 1979*b*) later showed that the kinetic properties and the interactions of the aminopeptidases with solvents and detergents were consistent with a membrane location. The physiological function of these "membrane-bound" peptidases remains unclear, partly because they were not tested against unsubstituted peptide substrates which the organism could be expected to encounter. Indeed, it is possible that the "endopeptidases" (P_{37} and P_{50}) described by Exterkate (1975) are aryl-peptide amidases (E.C. 3.5.1.) and have no general peptidolytic nor proteolytic activity. Such enzymes are present in the ribosomal fraction of *Lb. casei* (El Soda and Desmazeaud, 1981) and may function to cleave specific bonds in the signal sequence of newly synthesized proteins. These sequences are a vital part of the extrusion mechanism for cell envelope proteins emerging from membrane-bound ribosomes (Garnier et al., 1980), and the nature of the fraction bearing the P_{37} and P_{50} activities is not inconsistent with such a location and function for these enzymes. Although Exterkate (1975) had assumed that the amino- and endopeptidases were located on the outer surface of the membrane, this conclusion was only based on the ability of whole cells to degrade the appropriate substrates. While this may be valid, the possibility that the derivatized peptides could cross the cell envelope passively was not investigated. It is unlikely that the strains of *S. cremoris* investigated by Law (1977, 1978) for peptide utilization had peptidases on the outside of their membranes since competition experiments, both in terms of growth response and short-term uptake kinetics, proved that peptides were transported intact into cells by energy-dependent systems. If any hydrolysis occurred, it must have done so within the membrane, on its inside surface, or completely intracellularly.

Law (1979) used unsubstituted peptides to show that di- and tripeptidases were present in both the "particulate" (100000 × *g* pellet) and "cytoplasmic" fractions of osmotically lysed cells but, contrary to Exterkate's findings, the leucine aminopeptidase was largely cytoplasmic in *S. cremoris* NCDO 1196 and 2016, and completely so in *S. lactis* NCDO 2017. In addition the use of dipeptide substrates revealed that a peptidase(s) was released from *S. cremoris* NCDO 1196 by lysozyme or by osmotic shock, in the absence of intracellular marker enzyme leakage (Law, 1979; Kolstad and Law, unpublished data). The in vivo

location of this enzyme has not been established but studies with whole cells suggest that it does not have access to peptides in the growth medium, unlike the surface-bound peptidase of *S. lactis*.

Like the group N streptococci, *S. thermophilus* has intracellular peptidases capable of hydrolysing a wide range of substrates. Desmazeaud and Juge (1976) used electrophoretic zymograms to demonstrate the presence of two aminopeptidases and three dipeptidases, though some of the latter activity was attributable to one of the aminopeptidases. There are at present no reliable data on peptidase localization; Desmazeaud and Juge (1976) estimated their relative distribution in "particulate" and "soluble" fractions of mechanically disrupted cells but such information is of limited value for reasons already outlined.

Rabier and Desmazeaud (1973) purified an EDTA-sensitive dipeptidase and aminopeptidase from *S. thermophilus*; the dipeptidase (mol wt 50 000) was only active on unsubstituted dipeptides, whereas the aminopeptidase hydrolysed α-aminoacyl peptides, amino acid amides, oligopeptides and dipeptides.

Peptidases in lactobacilli have been studied in detail recently and they appear to have a wider range of activities than the lactic streptococci. *Lb. casei*, for example, has a broad-specificity true dipeptidase, an aminopeptidase, a narrow-specificity carboxypeptidase and an apparent endopeptidase, later identified as an aryl peptide amidase (El Soda et al., 1978*a*; El Soda and Desmazeaud, 1981). A carboxypeptidase was also found in one strain of *Lb. helveticus* but not in *Lb. lactis*, *Lb. bulgaricus* and lactobacilli of the betabacterium group (El Soda and Desmazeaud, 1982; El Soda et al., 1982). Amidase activity similar to that in *Lb. casei* was subsequently detected in *Lb. brevis* and *Lb. fermentum*, but not *Lb. helveticus*, *Lb. bulgaricus*, *Lb. lactis*, *Lb. acidophilus*, *Lb. buchneri* or *Lb. cellobiosus*.

A study of peptide localization in *Lb. casei*, using osmotically lysed cells, showed that most of them were in the cytoplasmic fraction, but the amidase sedimented at 150 000 × *g* (El Soda et al., 1978*b*). The authors assigned it a ribosomal location on this basis but did not attempt to confirm it by other criteria.

PROTEOLYSIS BY LACTIC ACID BACTERIA IN FOODS

Although lactic acid bacteria are present in many foods the significance of their proteolytic enzymes is not understood in alle cases. For example, lactic acid bacteria can be isolated from vacuum-packed beef and bacon, and from refrigerated poultry meat but they are not known to contribute to storage changes (Kitchell and Shaw, 1975; Barnes et al., 1979; Dainty et al., 1979). Reuter (1975) pointed out that lactic acid bacteria in fermented meat products are weakly proteolytic and may be significant for flavour but direct evidence for this is lacking. Muscle proteins are highly structured so that proteolysis is

limited by steric factors and such degradation that occurs is probably due to native enzymes. For example, fish muscle proteinases produce free amino acids in marinated herrings and the influence of lactic acid bacteria (betabacteria) is confined to decarboxylation which induces a blowing defect (Blood, 1975).

In contrast to the paucity of information on lactic acid bacteria proteinases in meat and vegetable foods, their significance in dairy products is well documented. The function of proteinases and peptidases in the yogurt fermentation has already been cited in relation to the growth of the starter culture. In addition, the release of threonine by peptidases is important for yogurt flavour since much of the acetaldehyde is derived from this amino acid via the threonine aldolase of *S. thermophilus* (Lees and Jago, 1976*a*, *b*; Shankar, 1977).

Proteolysis by cheese starters is not only important for their growth in milk but also for their role in the maturation of cheese. This process is essential for the conversion of the springy curd to typically inelastic textures of mature cheeses. Body/texture changes are chiefly attributable to α_{s1}-casein breakdown because this protein forms strong interactions with other caseins to form a structural network, which is weakened as the α_{s1}-casein bonds are broken (Creamer and Olson, 1982). General casein breakdown can also be linked with increased brittleness in maturing hard cheese because cleaved peptide bonds expose new ionic groups which bind water and render the cheese proteins less soluble. The later stages of proteolysis produce ~~small~~ peptides and amino acids which are involved in the development of cheese flavours, the buffering of cheese and the nutrition and growth of secondary floras. The contribution to cheese proteolysis by enzymes of starter bacteria varies in significance, depending on the extent to which these secondary floras produce proteinases and peptidases during cheese ripening. Microbial proteolysis is also superimposed on the action of chymosin, the coagulant used in the cheese vat to destabilize casein micelles and form the gel from which the cheese curds are made. In the short term, chymosin is very bond-specific, but during the following weeks and months of maturation this enzyme contributes to gross proteolysis in cheese. It is thought to produce a large proportion of the larger peptides to a lower limit of 1400 mol wt. Early rennet proteolysis is typified by the hydrolysis of the Phe_{23}-Phe_{24} bond (Hill et al., 1974; Gripon et al., 1975) or the Phe_{24}-Val_{25} bond (Creamer and Richardson, 1974) of α_{s1}-casein. Beta-casein degradation occurs only slowly in cheese and its products appear later in cheese maturation; the most sensitive bonds are Ala_{189}-Phe_{190} and Leu_{191}-Tyr_{192}. Endopeptidases from the starter bacteria also contribute to gross casein hydrolysis (i.e. hydrolysis leading to increased pH 4.6-soluble N). For example, an intracellular endopeptidase from *S. diacetylactis* rapidly hydrolyses α_{s1}-casein (but not whey proteins) and appears to be specific for peptide bonds involving the α-amino group of hydrophobic residues (e.g. X-Leu or X-Phe). This proteinase also hydrolyses Pro_{186}-Ile_{187} and Ala_{189}-Phe_{190} in β-casein although activity was low (Zevaco and Desmazeaud, 1980). On the other hand, the enzyme efficiently degraded peptides derived from

β-casein by chymosin action, by attacking the Lys_{176}-Ala_{177}, Lys_{119}-Val_{120} and Pro_{206}-Ile_{207} bonds. These authors concluded that the starter proteinases functioned in cheese chiefly by degrading those peptides released from casein by chymosin. This is consistent with reports by several research groups that cheeses made with chymosin alone contain pH 4.6-soluble N but very little peptide and amino acid N, whereas cheese made with starter and chymosin contain relatively large amounts of free amino N. Several independent studies have established that in both Gouda and Cheddar cheeses. the proteinases of starter bacteria can slowly degrade whole casein to low molecular mass (<1400) peptides and amino acids (Reiter et al., 1969; O'Keeffe et al., 1976; Visser, 1977). In normal cheese, chymosin-mediated gross proteolysis is so rapid (especially of α_{s1}-casein) that it is doubtful whether this activity is significant. The most important role of starter proteinases and peptidases appears to be the degradation of large rennet-derived peptides to small peptides and amino acids. Recent evidence from studies with *prt*⁻ variants suggests that the cell-bound extracellular proteinases of starters are significant in cheese proteolysis (Mills and Thomas, 1980) but a significant proportion of the proteinase activity, and most of the peptidase activity, is intracellular and only released into the cheese matrix during the early ripening stage when the cells lyse (Law et al., 1974). The contribution of the proteinases and peptidases of the thermophilic starters to cheese proteolysis has not been studied in great detail.

The proteinases of the group N streptococci have been closely implicated in the formation of the bitter defect in hard and semi-hard cheese. Bitter taste is caused by an accumulation of peptides with molecular weights ranging from around 1000 to 12000 which characteristically contain a high proportion of hydrophobic amino-acid residues (e.g. leucyl. prolyl, phenylalanyl). Richardson and Creamer (1973) showed that bitter peptides in New Zealand Cheddar cheese originated from near the chymosin-sensitive bond of α_{s1}-casein, supporting the idea that peptides released by rennet were the substrate for starter endopeptidases. These hydrophobic bitter peptides tend to accumulate in cheese, probably because they are degraded only slowly by the peptidases of starter bacteria. Their rates of degradation are likely to be influenced by the peptidase activity in the starter cells, which is strain-dependent (Sullivan et al., 1973) and by the rate of release of peptidases from lysing cells.

Opinions differ as to the importance of proteolysis by mesophilic starters in the production of bitter defects in cheese. Early hypotheses suggested that bitter peptides were produced by chymosin and that the so-called "bitter" starters were those which had insufficient peptidase activity to break down the bitter peptides to non-bitter peptides and amino acids (Czulak, 1959). However, the situation is more complex than this. While it is true that chymosin produces bitter peptides from casein, the starter proteinases can also do this and, indeed, can produce small bitter peptides from non-bitter, casein-derived peptides. Lowrie and Lawrence (1972) and Lowrie et al. (1974) suggested that this latter pro-

cess is the single most important determinant in bitterness development and that starters which multiplied at relatively high cooking temperatures during Cheddar manufacture (the "fast" starters) were the most likely to give bitter cheese, simply because the resultant high cell numbers contributed large quantities of bitter peptide-producing proteinases. This hypothesis was supported with experimental evidence showing that "bitter" starters could be made to produce non-bitter cheese if their number in curds was restricted by controlled bacteriophage infections or by higher cooking temperatures. Conversely, the slow "non-bitter" starters made bitter cheese if they were allowed to multiply to high cell numbers by altering the manufacturing process. Direct evidence for the involvement of starter cell wall proteinases in the development of bitterness was provided recently by the observation that proteinase-deficient variants of "fast" starters produce less bitterness in cheese than their parent strains, even when total starter cell populations are high (Mills and Thomas, 1981).

The factors controlling bitter defects in Gouda cheese appear to be more complex since the starters generally reach high populations in curd at the relatively low cooking temperatures used for this variety. Stadhouders and Hup (1975) showed that factors influencing the retention of chymosin in Gouda curd (e.g. cooking temperature, initial milk pH) also influence the tendency of the cheese to become bitter. They emphasized ~~that~~ some starter strains produce more bitter peptide-degrading peptidases than others. It is not known whether these are specific peptidases confined to non-bitter strains or general peptidases present at different levels. Chiba and Sato (1980) identified both dipeptidase and aminopeptidase activity in fractions of cell-free extracts from starter streptococci capable of reducing bitterness but individual enzymes were not isolated. It appears, then, that proteolysis by mesophilic starters is important in producing the bitter defect in cheese but its contribution depends on the cheese variety in question.

Amino acid catabolism

The group N streptococci do not appear to be active in amino acid degradation but lactobacilli can catalyse a number of conversions. For example, Sharpe and Franklin (1962) reported that lactobacilli isolated from Cheddar cheese would desulphurylate cysteine to produce hydrogen sulphide, a potent aroma volatile. Group D streptococci are particularly active in decarboxylating amino acids to their corresponding amines. These compounds have been cited as flavour compounds (Dahlberg and Kosikowsky, 1948) but they are also significant as toxins. They can combine with nitrogen oxides (especially in nitrate-treated foods) to form carcinogenic nitrosamines or they can act directly by producing symptoms of hypo- and hypertension (Castegnaro and Walker, 1980; Pedersen, 1980; Edwards and Sandine, 1981). Tyramine (from tyrosine), histamine (from histidine) and tryptamine (from tryptophan) are the food-related biogenic

amines. The amounts of amines which can safely be ingested before the onset of toxic symptoms varies from person to person depending on their efficiency of detoxification. It should be noted that other amine-producing bacteria have been isolated from cheese, including *Propionibacterium* spp., streptococci of the *viridans* group, *Lb. bulgaricus* and *Lb. plantarium*. Cheese starter streptococci are not active amine producers. Cheese appears to be a good substrate for amine production but the low incidence of cheese-related poisoning suggests that good cheese-making practice minimizes contamination by producer organisms.

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