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ASPECTS OF MILK PROTEIN CATABOLISM BY LACTOBACILLI

by

MALCOLM CHARLES BROOME
B AgrSc(Hons), M AgrSc(Melb), DipEd

A thesis submitted in fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

The School of Sciences
Deakin University

October, 1988
DEAKIN UNIVERSITY

CANDIDATES CERTIFICATE

I certify that the thesis entitled 'Aspects of Milk Protein Catabolism by Lactobacilli' and submitted for the degree of Doctor of Philosophy is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

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ACKNOWLEDGEMENTS

I would like to thank Dr. M. W. Hickey for supervision and encouragement throughout the course of this project. The assistance and advice given by Associate Professor D. R. Briggs and Drs. G. P. Jones and A. J. Hillier are gratefully acknowledged.

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ABSTRACT
ABSTRACT

Lactobacillus plantarum and subspecies of Lactobacillus casei were isolated from good quality mature Cheddar cheese and characterized with respect to metabolic functions that would allow their use in cheesemaking. In this way microbiological control of the maturation process with particular emphasis on protein catabolism was achieved. The lactobacilli isolated were selected for low growth rates (and acid production) in milk, and low proteinase activity to allow for their addition in high numbers to cheesemilk together with the normal starter flora (group N streptococci). The growth and acid production of the starter bacteria were unaffected by the presence of the lactobacilli during cheese manufacture and it was found that the added lactobacilli were able to grow and function under the conditions prevalent in Cheddar cheese during maturation. It was also demonstrated that the lactobacilli could be grown in an artificial medium to high numbers under controlled conditions and could be harvested for the preparation of cell concentrates, a necessary characteristic for commercialization. The lactobacilli also metabolized citrate, a potential problem in cheese maturation associated with CO₂ production but this did not adversely affect the maturation process under the conditions used.

Compared to the group N streptococci the non-starter lactobacilli possessed a proteinase system that had a higher temperature optimum and was less affected by heat and sodium chloride. They also possessed a more active peptidase system although both the lactobacilli and the starter organisms possessed a similar range of peptidases.

Non-starter lactobacilli were added to normal cheese and cheese made with proteinase negative starter. The added organisms did not adversely affect manufacturing parameters and did not metabolize citrate or lead to the formation of biogenic amines. However protein catabolism rates, particularly with respect to peptide degradation,
were increased, as was flavour development and intensity. It was observed that the body and texture of the cheeses was unaffected by the treatment. By controlling both the starter and non-starter microflora in the cheeses a practical system for favourably influencing cheese maturation was possible.

The investigation has demonstrated that carefully selected and characterized non-starter lactobacilli can be incorporated into Cheddar cheese manufacture in order to influence flavour development during maturation. Moreover the organisms can be added to the vat stage of manufacture without causing problems to the manufacturing process. This approach is a simple cost effective means of improving the cost of Cheddar cheese production and provides an unique opportunity to improve and control quality of all Cheddar cheese produced.
CHAPTER 1

GENERAL INTRODUCTION
GENERAL INTRODUCTION

The production of cheese in Australia in 1986/87 reached 177,000 tonnes with Cheddar and stirred curd accounting for 155,000 tonnes or 65% of total production. The manufacture of mature Cheddar cheese involves storage for up to 12 months at low temperatures (8 to 12°C) resulting in a significant storage cost (about $50 per tonne per month). Therefore any reduction in storage time achieved through accelerated maturation of the cheese would be of direct benefit to the dairy industry. In addition there is considerable variability in the flavour characteristics of cheeses made in different factory environments. This makes it difficult for very large multi-factory companies to service overseas markets with a uniform product. Therefore better control of the maturation process would enhance the quality and hence the marketability of Australian cheese in international markets.

1. Cheddar Cheese

In the manufacture of Cheddar cheese, rennet (chymosin) is used to coagulate the casein micelles and starter bacteria are used to ferment the milk lactose to lactic acid. The processes of coagulation and acid production, together with the application of heat and stirring, expels water and water-soluble components (lactose and whey proteins) from the coagulum. In this way the casein and lipid fractions of the milk are concentrated to form an acid curd, which is salted and allowed to mature over several weeks or months in order to produce a digestible product with the desired flavour and texture.

While the respective primary functions of the starter bacteria and rennet are the conversion of the milk lactose to lactic acid and the coagulation of the casein micelles, they also provide a source of enzymes which, with the natural milk enzymes and those originating from adventitious bacteria, play a large role in the maturation
process.

2. The Role of the Starter Bacteria

The traditional starter bacteria (group N streptococci) used in Cheddar cheese manufacture consist of the species *Streptococcus lactis* subsp. *lactis*, *S. lactis* subsp. *diacetylactis* and *S. lactis* subsp. *cremoris*. *S. lactis* subsp. *cremoris* is the species most commonly used since *S. lactis* subsp. *lactis* may produce flavour defects (Perry, 1961; Perry and McGillivray, 1964) and *S. lactis* subsp. *diacetylactis*, CO₂ from citrate (Harvey and Collins, 1961, 1963).

The role of starter bacteria in the development of typical Cheddar cheese flavour has been demonstrated by a number of workers (Mabbitt, Chapman and Sharpe, 1959; Reiter et al. 1967; Lowrie, Lawrence and Peberdy, 1974) and it appears that the contribution of starters to the maturation process is due principally to the release of enzymes from the autolysed cells (Law, Sharpe and Reiter, 1974). However, autolysis of the starter cells is a slow and complex process. Electron-microscopic examination of 5-month old Cheddar cheese showed extensive degradation of the bacterial cell wall but the spheroplasts remained intact (Umemoto, Sato and Kito, 1978). This may be due to the semi-solid gel structure of the cheese in which the cells are embedded and the osmotic stability provided by the high solute concentrations in the moisture phase (Thomas and Pritchard, 1987). Consequently, while the proteolytic enzymes associated with the cell wall of starter bacteria are available for protein degradation in the cheese, the intracellular enzyme systems may not be fully utilized because of limited substrate access.

Whereas the products of protein, fat and lactose degradation in Cheddar cheese make a contribution to Cheddar flavour (Ohren and Tuckey, 1969; McGugan, Emmons and Larmond, 1979) typical Cheddar cheese flavour may be due to rate limiting non-microbial chemical reactions (Lowrie, Lawrence and Peberdy, 1974; Law, Castanon and Sharpe, 1976b; Sharpe, 1979; Manning, 1979). These
reactions are apparently initiated by the development of correct conditions of pH and redox potential. Low redox potential is necessary to form active sulphydryl groups which may be related to optimum flavour development (Kristoffersen, 1967). The starter bacteria can also produce flavour compounds in Cheddar cheese as they possess a pyruvate dehydrogenase system (Broome et al. 1980) which can convert pyruvate formed from amino acids (i.e. sorino and alanino) to acetoain, butano-2,3-diol, diacetyl, acetic acid, acetaldehyde and ethanol. In cheese, when the lactose has been fully utilised fructose-1,6-biphosphate is no longer available to activate lactate dehydrogenase and any pyruvate formed would be metabolized by the pyruvate dehydrogenase system (Figure 1.1).

Thus the role of starter bacteria with respect to flavour development may simply be to:

1. Supply flavour precursors from the enzymic breakdown of carbohydrates, protein and fat;
2. Produce a low pH to suppress the growth of spoilage organisms;
3. Produce a low redox potential to keep sulphydryl compounds such as methanethiol in a reduced form.

3. The Role of the Non-Starter Bacteria

The role of the non-starter or adventitious microflora in Cheddar cheese maturation is less defined. The most dominant of these organisms are the lactobacilli (Lactobacillus plantarum, Lactobacillus casei, Lactobacillus brevis) and pediococci (Pediococcus pentosaceus) which are the only lactic acid bacteria to multiply in the maturing cheese (Chapman and Sharpe, 1981). Lactobacilli are almost always found in significant numbers in cheese whereas the pediococci, which can multiply at a similar rate to the lactobacilli and may even reach similar levels, occur much less frequently (Chapman and Sharpe, 1981).

It has been shown that while starter bacteria initially lower the redox potential in Cheddar cheese, their
**Figure 1.1. Pyruvate metabolism in lactic acid bacteria**
reductive effect is not maintained once the lactose is exhausted and/or the organisms lyse (Kristoffersen, 1967; Thomas, 1986). It would then be up to the non-starter bacteria to maintain the low redox potential necessary for flavour development (Thomas, 1985) by oxidizing a number of substrates found in Cheddar cheese, especially lactate (Thomas, 1986).

In addition to maintaining a low redox potential the lactobacilli, like the starter bacteria may influence the flavour of Cheddar cheese by supplying flavour precursors such as amino acids (Law and Kolstad, 1983). For example threonine can be converted by L. casei and L. plantarum to glycino and acetaldehyde (Hickey, Hillier and Jago, 1983b) while serine and alanine can form pyruvate (Nakac and Elliot, 1965). The typical non-starter lactobacilli in cheese (L. casei and L. plantarum) are known to possess a pyruvate oxidase system (Hickey, Hillier and Jago, 1983a) which can convert pyruvate to acetoin, diacetyl and acetate (Figure 1.2) when lactate dehydrogenase is inactivated (Figure 1.1). Either oxygen is required for these reactions or there must be some mechanism such as that found with the E. coli pyruvate dehydrogenase system whereby the enzyme cofactors are reoxidized (Mussey, 1963).

Adventitious lactobacilli present in Cheddar cheese are the result of post-pasteurization contamination of the cheese milk and curd and are representative of the natural microflora in the factory. They are able to multiply in maturing cheese up to about $10^6$ to $10^8$ g$^{-1}$ (Perry and Sharpe, 1960; Chapman and Sharpe, 1981). Nitrogen for growth is presumably available from the free amino acids, peptides or proteins that are present in abundance in the cheese but the carbon and energy source(s) for growth of the non-starter microflora in cheese have not been defined (Freyer, 1969). The lactobacilli also appear to produce a greater range of end products than the group N streptococci and are less susceptible to bacteriophage (Lawrence and Thomas, 1979).

Within the dairy industry thermophilic lactobacilli are
Figure 1.2. Pathways of pyruvate and citrate metabolism in lactobacilli (Hickey, Hillier and Jago, 1983a)

Bold numerals indicate the enzyme systems as follows:
1. lactate dehydrogenase.
2. pyruvate oxidase.
3. α-acetolactate synthase.
4. α-acetolactate decarboxylase.
5. acetoin dehydrogenase.
6. acetate kinase.
7. citrate (pro-35) lyase.
8. pyruvate decarboxylase.
9. oxaloacetate decarboxylase.

Square brackets indicate enzyme-bound compounds.
----- Indicates non-enzymic reaction.
used extensively as starter bacteria in cheeses where high
mako temperatures are used (ie. Swiss and Italian types).
In addition they are utilized in a diverse range of
fermented milk products including Bulgarian buttermilk,
Acidophilus milk, Yakult, Yoghurt, Kefir and Koumiss
(Marshall, 1984). In these products they ferment the
lactose to lactic acid and some organisms produce minor
metabolites vital for product quality and identity
(Marshall, 1984). The great diversity of lactobacilli
suggests that there may be strains that possess desirable
characteristics with respect to the maturation of Cheddar
cheese and possess metabolic pathways that could
complement the starter organisms normally used in
manufacture. Clearly, the degradation of milk protein is a
principal factor to be considered.

In the present study, several species of lactobacilli
were isolated from commercial Cheddar cheese and aspects
of their metabolism investigated and compared with that of
the group N streptococci. The strains were then identified
and characterized with respect to key metabolic functions
in order to select a group of organisms capable of being
included in a normal cheesemaking process (Chapter 2). The
potential role for lactobacilli as non-starter microflora
in cheese was then examined with respect to milk protein
catabolism with a view to influence flavour without
affecting other cheese characteristics (Chapter 3).
Furthermore, the application of the selected organisms in
Cheddar cheesemaking to control and/or accelerate the
maturation process was studied (Chapter 4).
CHAPTER 2

ISOLATION AND CHARACTERIZATION OF LACTOBACILLI
ISOLATION AND CHARACTERIZATION OF LACTOBACILLI

INTRODUCTION

1. The Microbiology of Cheddar Cheese

Non-starter organisms can enter the cheese manufacturing system at any point as a result of contamination from the atmosphere, equipment or dairy personnel. Normally pathogenic species such as salmonellae and staphylococci are suppressed by the added starter bacteria through competition for nutrients, a decrease in pH and the formation of specific inhibitory compounds such as organic acids, hydrogen peroxide and antibiotics (Lawrence, Thomas and Terzaghi, 1976). However, a number of non-pathogenic organisms survive Cheddar cheese manufacture (Chapman and Sharpe, 1981). Leuconostocs can be present at levels of up to $10^3$ g$^{-1}$ but do not multiply and slowly die out during maturation (Reiter et al., 1967). Similarly, the group D streptococci, commonly Streptococcus faecium and Streptococcus bovis, can be present in variable numbers ranging from 0 to $10^6$ g$^{-1}$ but die out either over a few weeks or months depending upon the strain. Initial numbers of micrococci at levels of $10^2$ to $10^6$ g$^{-1}$ slowly decrease to about 10 to $10^2$ g$^{-1}$ over approximately 6 months while the pediococci, if present, may reach levels of $10^7$ g$^{-1}$ (Chapman and Sharpe, 1981). Corynebacteria and aerobic spore formers can occur in small numbers in the curd and survive many months without multiplying or decreasing (Chapman and Sharpe, 1981).

Lactobacilli are present in the curd at levels of 10 to $10^4$ g$^{-1}$ and multiply up to about $10^6$ to $10^8$ in 10 to 60 days and then decline very slowly after 4 to 6 months (Perry and Sharpe, 1960; Chapman and Sharpe, 1981). The rapid growth of the lactobacilli in Cheddar cheese has led researchers to consider their role in cheese maturation.

The starter bacteria may reach levels of approximately $10^9$ g$^{-1}$ at the ex-press stage of Cheddar cheese manufacture but their numbers decline steadily during
cheese maturation at a rate which is dependent on the species and strain (Dawson and Feagan, 1957; Perry, 1961). It has been suggested that the lactobacilli (and pediococci) grow on the products released during the autolysis of starter cells (Thomas, 1987b). However the lactobacilli reach maximum levels (6-8 weeks) before significant numbers of starter organisms have autolysed (Cromie, Giles and Dulley, 1987). It has also been reported that many starter cells do not fully lyse even though there has been extensive degradation of their cell walls (Umemo, Sato and Kito, 1978). Therefore the carbon source for growth of the lactobacilli may not be entirely dependent on the autolysis of the starter cells.

2. A Role for Lactobacilli in Cheddar Cheese

As the lactobacilli have complex proteolytic systems (Hickey, Hillier and Jago, 1983c) and can produce greater amounts of amino acids than group N streptococci (Dolezalek, 1966; Miller and Kandler, 1967; Schmidt et al., 1976) they probably have a significant role in the catabolism of milk proteins in Cheddar cheese as well as their role in maintaining a low redox potential (Thomas, 1986; Thomas, 1987a). If lactobacilli with well defined proteolytic systems could be used to dominate the non-starter microflora it may be possible to influence maturation. Ideally these organisms would be added at the vat stage of manufacture to ensure an even distribution throughout the curd mass. For this to occur the lactobacilli added must complement the role and function of the starter organism. That is, they must not adversely affect:

1. Cheese yield by causing excessive protein hydrolysis during manufacture.

2. The rates of growth and acid production of the starter organisms by competing for carbon, nitrogen or other growth factors.

It would also be important in automated cheese manufacturing processes, where overall plant performance may be affected, that any organisms or enzymes added to
the cheesemilk must not alter the rate and conditions of curd formation. Clearly the use of non-starter lactobacilli must only influence the maturation of cheese and have little or no effect on manufacture (Figure 1.1). In order to achieve this they must:

1. Multiply at temperatures normally used in cheese storage (8 to 12°C) over a long period of time.
2. Grow and/or metabolize at salt concentrations of about 5% (w/w) in the moisture phase.
3. Utilize energy sources other than lactose (note they may also assist in utilizing residual lactose as long as it is present).

In addition the lactobacilli must not possess metabolic pathways that would produce flavour or texture defects in the final cheese (ie. lipolytic activity, citrate utilization, decarboxylation of amino acids).

It was considered that isolates of lactobacilli with the required characteristics may be found in the natural microflora of good quality commercial Cheddar cheeses. The aim of the present investigation was, therefore, to isolate and identify suitable organisms from Cheddar cheese and to characterize these organisms with respect to key metabolic functions that would enable use in normal cheesemaking and maturation.
MATERIALS AND METHODS

1. Bacteria

(a)Typed strains. The organisms used in this investigation were *L. casei* 151 and *L. plantarum* 343 obtained from the National Collection of Dairy Organisms (NCDO) at the National Institute for Research in Dairying, Reading, England and *S. lactis* subsp. cremoris BK5 was obtained from the Commonwealth Scientific and Industrial Research Organization (C.S.I.R.O.). Dairy Research Laboratory, Highett, Victoria.

(b) Lactobacilli from Cheddar cheese. Five commercial Cheddar cheeses classified as tasty, extra tasty or vintage were used as sources of lactobacilli. Cheese samples (10 g) were emulsified in 50 mL of 2% (w/v) sodium citrate at 45°C for 2 min using a Stomacher Laboratory Blender 400 (Model BA6021). Peptone water (40 mL of a pH 7.0, 0.1% solution) was added at 45°C and the solution macerated for a further 5 s. After serial dilution up to 10^{-8} in peptone water, aliquots (1 mL) of the dilutions were added to sterile Petri dishes. Either 15 mL of molten MRS agar (De Man, Rogosa and Sharpe, 1960), MRS agar plus 4% w/v NaCl, Rogosa agar (Rogosa, Mitchell and Wiseman, 1951) or Rogosa agar plus 4% w/v NaCl was added at 45°C. The contents were mixed as outlined in "Methods of Microbiological Examination of Dairy Products and for Dairy Purposes" (Australian Standard 1095.1.2-1971) and the plates incubated anaerobically in a Gas Pak 100 Anaerobic System (BBL Microbiology Systems, Cockeysville, USA) at 37°C for 3 days. Single colonies, picked off the plates, were subcultured in MRS broth at 37°C before microscopic examination using a Gram stain. Organisms which appeared as gram positive rods were selected for further characterization.

(c) Strain maintenance. The cultures used for
Inoculation were maintained as stock in MRS broth (De Man, Rogosa and Sharpe, 1960) at 37°C for the lactobacilli and in M17 broth (Terzaghi and Sandine, 1975) at 30°C for *S. lactis* subsp. *cromorise* BK5. Cells were stored at -72°C in an Ultra-Cold refrigerated cabinet.

2. Identification

(a) **Biochemical tests.**

(i) Growth in the presence of Teepol. Tubes containing MRS broth (10 mL) and Teepol (0.1 or 0.4% v/v) were inoculated with an overnight culture (1% v/v) of lactobacilli. The tubes were incubated at 30°C for up to 3 days and cell concentrations were determined by direct measurement of the optical density at 610 nm using a colorimeter (CHEMTRIX 20A).

(ii) Catalase activity. A 1.5% (v/v) solution of hydrogen peroxide was inoculated (1% v/v) with an overnight culture of lactobacilli and the solution observed for the evolution of gas.

(iii) A.P.I. Test (Appareils et Procédés d'Identification, La Balme Les Grottes, 38390 Montalieu Vercieu, France). An API 50 CHL Lactobacillus system was used for the biochemical identification of lactobacilli. Each isolate was added to an A.P.I. gallery containing 49 different carbohydrates and incubated at 30°C. The galleries were checked for acidification at 3, 6, 24 and 48 h and the fermentation profile compared to the profiles of known lactobacilli species.

(b) Colony and Morphological Examination. Cultures of lactobacilli were streaked on MRS agar and isolates were examined on the basis of colony and cell morphology.

(c) **Plasmid profile.** The plasmid content of lactobacilli cultures was screened as described by
Anderson and McKay (1983).

3. Growth Characteristics

(a) **Determination of growth.**

(i) Plate counts. Lactobacilli cell numbers in growth media were estimated by plate counts. After serial dilution to $10^{-8}$ in peptone water aliquots (1 mL) of the dilutions were counted on Rogosa's agar as described in 1 (b).

(ii) Broth media. Growth of lactobacilli in MRS broth was monitored at 610 nm as described in 2(a)(i) or at 650 nm using a spectrophotometer (Varian SuporScan 3).

(iii) Skim milk media. Growth of lactobacilli in reconstituted skim milk (10% w/v) was determined by the method of Kanasaki et al. (1975) as modified by Broome et al. (1982).

(iv) Dry weight of cells was determined by reference to a standard curve relating dry weight with optical density at 650 nm as described in (ii) above.

(b) **Essential components for growth in MRS broth.**

Solutions of peptone (1% w/v), beef extract (1% w/v), yeast extract (0.5% w/v), peptone (1% w/v) plus beef extract (1% w/v), beef extract (1% w/v) plus yeast extract (0.5% w/v), peptone (1% w/v) plus yeast extract (1% w/v), citrate (1% w/v), casamino acids (1% w/v) and lactate (1% w/v) were made up at pH 6.0 in a base MRS broth medium. The base MRS broth medium contained in 1 litre; Tween 80 (1 mL), $K_2HPO_4$ (2 g), sodium acetate (5 g), triammonium citrate (2 g), MgSO$_4$ 7H$_2$O (0.2 g) and MnSO$_4$ 4H$_2$O (0.2 g). Tubes containing 10 mL of each solution were inoculated (1% v/v) with an overnight culture of lactobacilli and incubated at 30°C for up to 3 days. Cell concentrations were monitored at 650 nm as described in 3(a)(ii).
(c) **Growth temperature.** MRS broth (10 mL) was inoculated (1\% v/v) with an overnight culture of lactobacilli and incubated over a temperature range of 20 to 50°C for up to 16 h in a Temperature Gradient Incubator (Toyo Kagaku Sangyo Co. Ltd., Model TN-3). Cell concentrations were monitored at 610 nm as described in 2(a)(i).

Growth at 15°C was also measured in MRS broth (10 mL) inoculated (5\% v/v) with an overnight culture of lactobacilli and incubated for up to 4 days. Cell concentrations were monitored at 610 nm as described in 2(a)(i).

(d) **Salt tolerance.** MRS broth (10 mL) containing NaCl (0.5 to 5\% w/v) was inoculated (2\% v/v) with an overnight culture of lactobacilli and incubated at 30°C for up to 24 h. Cell concentrations were monitored at 610 nm as described in 2(a)(i).

(e) **Growth at controlled pH.** Lactobacilli were grown in either MRS broth, a medium containing 10\% (w/v) skim milk permeate (the protein free fraction from ultrafiltration of skim milk and dried) and 1\% (w/v) yeast extract or a skim milk media (10\% w/v) at 37°C. Streptococci were grown in M17 broth or skim milk media (10\% w/v) at 30°C. The pH was maintained at 6.2 by the addition of 2.5 M NaOH using a peristaltic pump (Watson Marlow 101 FS/R) connected to a pH controller (JENCO Model 3671). Cell concentrations were monitored at 650 nm as described in 3(a)(ii).

(f) **Harvesting of cells.** When large quantities of cells were required (i.e. the preparation of cell free extracts) cells grown under pH control were harvested in mid log phase of growth by centrifugation at 5,000g for 10 min at 4°C (MSE High Speed 18) (see Appendix 1). Cells were then washed twice in 0.9\% (w/v) NaCl (0.5 x culture volume) at 4°C and once in 0.2 M KH₂PO₄ (adjusted to pH 5.5 with 0.2
M Na₂HPO₄) (0.5 x culture volume) before being resuspended in the same buffer to a final cell concentration of 15 mg (dry weight/mL) as described in 3 (a)(iv).

(g) Preparation of cell free extracts. The cell suspension (50 mL), prepared as described in (f) above except that 0.1 M phosphate buffer was used and the cells were resuspended at 20 mg (dry weight)/mL was sonicated in a MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, Sussex, England) at 4°C. Sonication was carried out at a probe amplitude of 18 microns for 16 cycles (each cycle 60 s on and 15 s off). Unbroken cells and cell debris were removed by centrifugation at 10,000g for 20 min at 4°C. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

4. Cellular Activity

(a) Oxidative activity. Lactobacilli were grown in MRS broth containing 15 mM glucose at 37°C and streptococci in M17 broth containing 14 mM lactose at 30°C without pH control. After 16 h or when the carbohydrate was exhausted the cells were harvested, washed and resuspended as described in 3 (f) except that 0.1 M KH₂PO₄ (adjusted to pH 5.5 with 0.1 M Na₂HPO₄) was used.

The cell suspension (0.5 mL) mixed with 1.0 mL of 0.1 M KH₂PO₄ (pH 5.5) containing MgCl₂ (2.5 mM) was added to the oxygen electrode of a Spectroplus-D (MSP Scientific Instruments, Crawley, England) at 30°C. The endogenous oxygen consumption was recorded on a chart recorder before the addition of 100 µL of substrate. Either sodium acetate (0.32 M), tri-sodium citrate (0.16 M), D(-)-lactate (0.32 M), L(+)lactate (0.32 M), lactose (0.32 M), sodium pyruvate (0.32 M), casein hydrolysate (3.2% w/v) or tryptone (3.2% w/v) were used as substrates. Casein hydrolysate and tryptone were obtained from Oxoid (Oxoid Australia Pty. Ltd., Melbourne).

The Spectroplus-D was calibrated with 0.1 M Tris-HCl
buffer (pH 8.0) at 30°C and the rate of oxygen uptake was calculated from the difference between the endogenous rate and the rate in the presence of substrate.

(b) Lipase activity. Bacterial cells were grown in skim milk media (10% w/v) under pH control and harvested, washed and resuspended as described in 3(f) except that 0.1 M KH₂PO₄ (adjusted to pH 6.2 with 0.1 M Na₂HPO₄) was used and the cells were resuspended at 30 mg (dry weight)/mL.

The cell suspension (0.5 mL) was added to 1.5 mL of 30% (w/w) milk fat in the milk salt solution of Jenness and Koops (1962) and 1.0 mL of 0.25 M KH₂PO₄ (adjusted to pH 6.2 with 0.25 M Na₂HPO₄) containing 0.12% sodium azide. After mixing in a stoppered 12 x 75 mm polypropylene tube a 1.0 mL sample was taken for the initial free fatty acid determination and the tubes were sealed and incubated at 37°C (lactobacilli) or 30°C (S. lactis subsp. cremoris BK5) for 7 days. Blank samples without milk fat were run simultaneously.

Titratable fatty acids in the incubation mixture were determined by the method of Umamoto, Umeda and Sato (1968).

(c) Citrate metabolism. Bacteria were grown in MRS broth (lactobacilli) or M17 broth (S. lactis subsp. cremoris BK5) under pH control and harvested, washed and resuspended as described in 3(f). except that 0.1 M KH₂PO₄ (adjusted to pH 5.5 with 0.1 M Na₂HPO₄) was used and the cells were resuspended at 20 mg (dry weight)/mL.

For studies using resting cells the reaction mixture (3 mL) contained in μmoles: phosphate buffer (0.1 M KH₂PO₄ adjusted to pH 5.5 with 0.1 M Na₂HPO₄), 300; MgCl₂, 5; thiamine pyrophosphate (TPP), 0.1; tri-sodium citrate, 100 and 20 mg (dry weight) of cells. For studies using cell free extract, the reaction mixture (3 mL) contained in μmoles: phosphate buffer, (0.1 M KH₂PO₄ adjusted to pH 5.5 with 0.1 M Na₂HPO₄), 300; MgCl₂, 10; TPP, 0.2; tri-sodium citrate, 100 and cell free extract (3 mg
protein). Incubations were carried out at 37°C (lactobacilli) and 30°C (S. lactis subsp. cremoris BK5) for 2 h and the reactions stopped by rapidly cooling the incubation mixtures in ice-water (whole cells) or by the addition of 0.1 mL of 10 M HCl (cell-free extracts). Incubation mixtures were then centrifuged at 10,000g for 10 min and the supernatants were assayed citrate as described in 5(a).

5. Analytical Methods

(a) **Citrate** was determined using a Boehringer Mannheim kit (Cat. No. 139 076).

(b) **L-lactic acid** was determined using a Boehringer Mannheim kit (Cat. No. 139 084).

(c) **Trichloroacetic acid (TCA)-soluble tyrosine** was determined as described by Hickey et al. (1983).

(d) **Phosphotungstic acid (PTA)-soluble amino nitrogen** was determined as described by Jarrett, Aston and Dulley (1982).
RESULTS

1. Lactobacilli From Cheddar Cheese

Sixteen organisms were isolated on selective media from commercial Cheddar cheeses as described under Methods. All of the organisms were gram positive and appeared as either rods or cocci (Table 2.1). Some lactobacilli can exist as short coccibacilli (Kandler and Weiss, 1986), however only those isolates readily identified as gram positive rods were included for further study.

2. Identification

The gram positive rods isolated from Cheddar cheese were maintained in MRS broth and identified using an A.P.I. 50 CHL Lactobacillus system as described under Methods. As shown in Figures 2.1 and 2.2 all the isolates were able to ferment a range of carbohydrates including ribose, sorbitol and mannitol which suggested they were strains of \textit{L. casei} or \textit{L. plantarum} (Kandler and Weiss, 1986). Within species, identification based on carbohydrate fermentation was inconclusive. However this group of organisms are known to produce variable results even at the subspecies level (Kandler and Weiss, 1986). For example the isolate MIL2A clearly resembles \textit{L. casei} subsp. \textit{casei} (Figure 2.2) even though it was not identical to either of the typed strains used for comparison. Also, the isolate ML1A although similar to \textit{L. plantarum} (Figure 2.2) and able to grow in the presence of 0.4% Teepol (Table 2.2) (unlike \textit{L. casei}), did not ferment L-arabinose which is normally a characteristic of \textit{L. plantarum}. All of the isolates tested were confirmed as catalase negative (Table 2.2) which is typical of all lactobacilli (Kandler and Weiss, 1986).

On the basis of the results obtained using the A.P.I. system and the ability to grow in the presence of Teepol the isolates used in this investigation were identified as \textit{L. casei} subsp. \textit{casei} CV1A, CV1B, FU1A, MIL2A, \textit{L. casei} subsp. \textit{rhamnosus} ML1B, \textit{L. casei} subsp. \textit{pseudoplantarum}
<table>
<thead>
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<th>Cheese source</th>
<th>Identification number</th>
<th>Shape</th>
<th>Gram stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mainland Vintage</td>
<td>ML1A</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Tasty, Mainland</td>
<td>ML1B</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Dairy Products,</td>
<td>ML2A</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td>New Zealand</td>
<td>ML2B</td>
<td>rod/cocci</td>
<td>+</td>
</tr>
<tr>
<td>Ballantyne Vintage</td>
<td>BALL1A</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td>Tasty, Ballantyne</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pty Ltd, South</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melbourne</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cradle Valley</td>
<td>CV1A</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Extra Tasty, Lactos</td>
<td>CV1B</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Pty Ltd, Tasmania</td>
<td>CV2A</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CV2B</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td>Farmers Union Vintage,</td>
<td>FU1A</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Farmers Union,</td>
<td>FU1B</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td>Adelaide, South</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Millel Extra Tasty</td>
<td>MIL1</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td>Vintage, Kraft</td>
<td>MIL2A</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Foods, South</td>
<td>MIL2B</td>
<td>rod</td>
<td>+</td>
</tr>
<tr>
<td>Melbourne</td>
<td>MIL3</td>
<td>cocci</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>MIL4</td>
<td>cocci</td>
<td>+</td>
</tr>
</tbody>
</table>

The organisms were isolated from Cheddar cheese and examined as described under Methods.
Figure 2.1. A.P.I. strain profiles of lactobacilli isolates

The lactobacilli isolates were tested by an A.P.I. 50 CHL Lactobacillus system as described under Methods. Shaded areas represent substrate utilization at 3, 6, 24 or 48 h.

A. ML1B
B. CV1A
C. CV1B
D. FUA
E. MIL2B
Figure 2.2. A.P.I. identification of isolates MIL2A and ML1A

The lactobacilli isolates MIL2A and ML1A were tested by an A.P.I. 50 CHL Lactobacillus system as described under Methods and the isolate profiles were compared to the A.P.I. profiles of known organisms provided with the Lactobacillus system. The A.P.I. profiles are reproduced for comparison. Shaded areas represent substrate utilization at 3, 6, 24 or 48 h.

A. Isolate MIL2A
B. L. casei casei ATCC 393-1 (A.P.I. Profile)
C. L. casei casei DSM 20312 (A.P.I. Profile)
D. Isolate ML1A
E. L. plantarum NCDO 1752 (A.P.I. Profile)
Table 2.2.  *Characteristics of lactobacilli isolates*

<table>
<thead>
<tr>
<th>Identification number</th>
<th>Catalase presence</th>
<th>Growth* in Teepol (0.1% (v/v) 24 h)</th>
<th>Growth* in Teepol (0.4% (v/v) 72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML1A</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>ML1B</td>
<td>-</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>CV1A</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>CV1B</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>FU1A</td>
<td>-</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>MIL2A</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>MIL2B</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>L. casei</em> 151</td>
<td>-</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> 343</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

* - No growth; +, ++, ++++, ++++, growth from weak to very strong.

Catalase activity and growth in Teepol were determined as described under Methods.
MIL2B and L. plantarum ML1A (Figures 2.1 and 2.2).

The plasmid profiles of lactic acid bacteria enable them to be used as a means of strain differentiation (Davies, Underwood and Gasson, 1981; Davies and Gasson, 1983). The lactobacilli also contain plasmid deoxyribonucleic acid (DNA) (Vescovo et al., 1981) and as shown in Figure 2.3 different profiles were evident for each of the isolates. In lactic acid bacteria the association of phenotypic properties (e.g. lactose utilization, proteinase activity, citrate metabolism) with plasmid DNA has been demonstrated (McKay, 1983). For lactobacilli complex plasmid profiles were observed in this study and it has been reported that lactose utilization is encoded on a plasmid in L. casei (Chassy, 1987). The diverse plasmid profiles of the isolates may indicate differing capabilities with respect to key metabolic functions as for other lactic acid bacteria. It is then possible that plasmid related attributes could effect differences in the end products observed during maturation of cheese even though the majority of the isolates are of the same species.

3. Growth Characteristics

(a) The effect of temperature and salt. The use of non-starter lactobacilli in normal cheesemaking and maturation requires them to function under the conditions prevalent in maturing cheese, that is they must be able to multiply at lower temperatures and grow at salt concentrations of up to 5% (w/w) in the moisture phase. As shown in Figure 2.4, L. plantarum 343 and ML1A demonstrate optimum growth from 28 to 37°C and L. casei 151 and MIL2A from 32 to 37°C. Similar results were evident for L. casei ML1B, CV1A, CV1B, FU1A and MIL2B (data not shown). Each of the organisms was also able to grow at 15°C (Table 2.3) and in the presence of 4% (w/v) NaCl at 30°C. The results suggest that of the organisms tested L. casei CV1A, CV1B and MIL2A and L. plantarum ML1A and 343 were best able to grow at the lower temperatures and higher salt.
Figure 2.3. Schematic representation of plasmid profiles of lactobacilli

Bacterial cells were grown in MRS broth and plasmid profiles prepared as described under Methods.

A. L. casei 151
B. " " ML1B
C. " " CV1A
D. " " CV1B
E. " " FU1A
F. " " MIL2A
G. " " MIL2B
H. L. plantarum 343
I. " " ML1A
Figure 2.4. The effect of temperature on the growth of lactobacilli

Tubes containing 10 mL of MRS broth were inoculated (1% v/v) with an overnight culture of lactobacilli and incubated in a Temperature Gradient Incubator at temperatures between 20 and 50°C for up to 16 h. Growth was determined as described under Methods.

- ○ L. casei MIL2A
- • " " 151
- △ L. plantarum ML1A
- ▲ " " 343
Table 2.3. Growth characteristics of lactobacilli isolates

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Growth* in MRS broth (15°C)</th>
<th>Growth* in MRS broth plus 4% NaCl (30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML1B</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>CV1A</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CV1B</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FU1A</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MIL2A</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>MIL2B</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>151</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML1A</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>343</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

* -, No growth; +, ++, ++++, ++++, growth from weak to very strong in 24 h.

Growth was determined as described under Methods.
concentrations. Further, the effect of increasing salt concentrations on the organisms indicates that the most tolerant were \textit{L. plantarum} ML1A and \textit{L. casei} 151 followed by \textit{L. casei} MIL2A and \textit{L. plantarum} 343 (Figuro 2.5).

(b) Alternative growth media. Normally the nutritional demands of the lactobacilli require them to be cultivated in complex media (ie. MRS broth). However, \textit{L. casei} and \textit{L. plantarum} tend to be more widely distributed in nature than other lactobacilli species and their nutritional requirements may be simpler. The growth of \textit{L. casei} 151 and \textit{L. plantarum} 343 (selected as representative of each species likely to be found in cheese) in less complex media was therefore investigated with respect to the components of MRS broth and some substrates (eg. lactate) that may be present in cheese. Yeast extract alone or in combination with other nutrients had the most positive effect on growth of \textit{L. casei} 151 and \textit{L. plantarum} 343 (Table 2.4) indicating that any media used for large scale production of these organisms must contain yeast extract. Both organisms also demonstrated an ability to utilize lactate and to a lesser extent citrate, indicating they would be able to access an energy source in cheese during maturation as Cheddar cheese normally contains 0.14% (w/w) citrate (Thomas, 1987a) and 1.2 to 1.5% (w/w) (DL) lactate at 3 months of age. No growth was observed in the base MRS broth media despite the presence of 0.2% (w/v) triammonium citrate.

Growth of \textit{L. casei} MIL2A was compared in MRS broth and a Permeate/Yeast Extract media prepared as described under Methods to determine whether a simple, less expensive growth media could be used for large scale cell production. As shown in Figure 2.6 the specific growth rates (\(\mu^1\)) in MRS broth and Permeate/Yeast Extract media were 0.53 h\(^{-1}\) and 0.39 h\(^{-1}\) respectively indicating that effective growth in a simpler media was possible but

\[\mu^1 = \log_{10}N_2 - \log_{10}N_1/(t_2-t_1)(0.301)\]
Figure 2.5. The effect of NaCl on the growth of lactobacilli

Tubes containing 10 mL of MRS broth were inoculated (2% v/v) with an overnight culture of lactobacilli and incubated at 30°C for 8 h. Growth was determined as described under Methods.

- L. casei MIL2A
- " 151
- L. plantarum ML1A
- " 343
Table 2.4. The effect of MRS broth components on the growth of L. casei 151 and L. plantarum 343

<table>
<thead>
<tr>
<th>Component</th>
<th>Relative growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. casei 151</td>
</tr>
<tr>
<td>Base MRS broth media**</td>
<td>-</td>
</tr>
<tr>
<td>Peptone (1% w/v)</td>
<td>+</td>
</tr>
<tr>
<td>Beef extract (1% w/v)</td>
<td>+</td>
</tr>
<tr>
<td>Yeast extract (0.5% w/v)</td>
<td>+++</td>
</tr>
<tr>
<td>Peptone (1% w/v)/Beef extract (1% w/v)</td>
<td>++</td>
</tr>
<tr>
<td>Beef extract (1% w/v)/Yeast extract (0.5% w/v)</td>
<td>+++</td>
</tr>
<tr>
<td>Peptone (1% w/v)/Yeast extract (0.5% w/v)</td>
<td>+++</td>
</tr>
<tr>
<td>Casamino acids (1% w/v)</td>
<td>++</td>
</tr>
<tr>
<td>Citrate (1% w/v)</td>
<td>+</td>
</tr>
<tr>
<td>Lactate (1% w/v)</td>
<td>++</td>
</tr>
</tbody>
</table>

*-, No growth; +, ++, ++++, ++++, growth from weak to very strong.
**Base MRS media was prepared as described previously (p. 15).

Tubes containing 10 mL of base MRS broth medium plus MRS broth components were inoculated (1% v/v) with an overnight culture of lactobacilli grown in MRS broth and incubated at 30°C for 3 days. Growth was determined as described under Methods.
Figure 2.6. Growth of *L. casei* MIL2A in permeate/yeast extract media

*L. casei* MIL2A was subcultured in MRS and Permeate/Yeast Extract media and inoculated (1% v/v) in 500 mL of the respective medium. The organisms were grown under pH control (6.2) at 37°C and aliquots were taken at intervals and plate counts determined as described under Methods.

- MRS broth
- Permeate/yeast extract
further work is required to optimize the nutrient requirements.

Non-starter lactobacilli added at the vat stage of manufacture must also complement the role and function of the normal starter organisms. They should not (i) adversely affect cheese yield by causing excessive protein hydrolysis, (ii) produce lactic acid or (iii) effectively compete with the starter organisms for essential nutrients (affecting growth and acid production kinetics). As shown in Figure 2.7 L. casei MIL2A (initial cell concentration $10^6 \text{mL}^{-1}$) demonstrated slow growth in skim milk media ($\mu = 0.15 \text{h}^{-1}$) and in the first 20 h the lactic acid concentration only increased by 4.3 mM. In addition there was little or no proteolytic activity, as measured by TCA-soluble tyrosine in the growth media, during the first 20 h of incubation. The decrease in PTA-soluble amino nitrogen, evident until the stationary phase of growth (60 h) suggested that any PTA-soluble material formed was immediately used by L. casei MIL2A for cell synthesis. Even though PTA-soluble material was apparently used for growth of L. casei MIL2A, the very slow growth rate in milk suggests that the presence of the organism would not affect the overall growth and acid kinetics of a starter organism during the 4 h period of cheese manufacture. During the stationary phase, PTA-soluble amino nitrogen concentrations increased steadily, indicating that the proteolytic system of the organism remained active and this characteristic would be essential for use of non-starter lactobacilli in cheese maturation. Similar results were obtained for L. casei ML1B, CV1A, CV1B, FUL1A, MIL2B and L. plantarum ML1A (data not shown).

4. Cellular Activity

Non-starter lactobacilli used in Cheddar cheesemaking must not possess metabolic pathways that would produce flavour or texture defects in the final cheese. For example high lipase activity can lead to unacceptable flavours (Forss, 1979) while citrate metabolism can result in $\text{CO}_2$ production and possibly an open textured cheese
Figure 2.7. Growth of *L. casei* MIL2A in skim milk medium

The lactobacilli isolate was inoculated (1% v/v of a skim milk culture) into 1 L of sterile skim milk media and grown under pH control (6.2) at 37°C. Aliquots were taken at intervals and cell dry weights, L-lactic acid, TCA-soluble tyrosine and PTA-soluble amino nitrogen were determined as described under Methods.

- Cell dry weight
- L-lactic acid
- TCA-soluble tyrosine
- PTA-soluble amino nitrogen
(Coventry, Hillier and Jago, 1978). Excessive free fatty acid formation in cheese can result in rancid flavour development but the group N streptococci are normally weakly lipolytic (Chapman and Sharpe, 1981). In contrast some lactobacilli can liberate, upon autolysis, intracellular lipases which may account for much of the lipolytic activity in hard cheese (Chapman and Sharpe, 1981). As shown in Table 2.5 *L. casei* MIL2A and 151 and *L. plantarum* MIL1A appeared to possess higher lipase activity as measured by titratable acidity than *S. lactis* subsp. *cremoris* BK5.

Citrate metabolism by *L. casei* and *L. plantarum* has been reported (Hickey, Hillier and Jago, 1983a) and the present study (Table 2.6) confirmed that *L. casei* CV1A, MIL2A, MIL2B and 151 and *L. plantarum* 343 were able to utilize citrate from the incubation medium. Cell free extracts of *L. casei* CV1A, MIL2A and MIL2B were also able to metabolize citrate more readily than the equivalent whole cells, which may be of significance in maturing cheese should cell lysis occur.

Citrate is metabolized by the reaction:

$$\text{citrate} + \frac{1}{2} \text{O}_2 \rightarrow 2 \text{acetate} + 2\text{CO}_2$$

This presumably involves the hydrolysis of citrate to acetate and oxaloacetate, the decarboxylation of oxaloacetate to pyruvate and the conversion of pyruvate to acetate via pyruvate oxidase (Figure 1.2) (Hickey, Hillier and Jago, 1983a)

Other metabolic pathways can favourably influence flavour development, especially those associated with O\textsubscript{2} utilization, by reducing the redox potential and thus stabilizing reduced sulphur compounds (Thomas, 1986). *L. casei* MIL2A and 151 and *L. plantarum* MIL1A and 343 were therefore examined with respect to their ability to oxidize a number of substrates (acetate, D-lactate, L-lactate, lactose, pyruvate, casein hydrolysate and tryptone) that may be present in cheese. As shown in Table 2.7 none of the organisms oxidized acetate and only *L.*
Table 2.5. Lipase activity of lactic acid bacteria

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Titratable fatty acids released (mL 0.02 M NaOH x 10/mg dry wt. cells/7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei</td>
<td></td>
</tr>
<tr>
<td>MIL2A</td>
<td>5.3</td>
</tr>
<tr>
<td>151</td>
<td>4.9</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
</tr>
<tr>
<td>ML1A</td>
<td>4.7</td>
</tr>
<tr>
<td>S. lactis subsp. cremoris</td>
<td></td>
</tr>
<tr>
<td>BK5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Cells were grown in skim milk media (10% w/v) under pH control (6.2) at 37°C, harvested, washed and resuspended at 30 mg (dry weight)/mL as described under Methods. The cell suspension (0.5 mL) was added to 1.5 mL of 30% (w/v) cream in the milk salt solution of Jonness and Koops (1962) and 1.0 mL of 0.25 M phosphate buffer (pH 6.2) containing 0.12% sodium azide and incubated at 37°C for 7 days. Titratable fatty acids were determined as described under Methods.
### Table 2.6. Citrate metabolism by lactic acid bacteria

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Citrate utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole cells</td>
</tr>
<tr>
<td></td>
<td>(μmol/mg dry</td>
</tr>
<tr>
<td></td>
<td>weight cells/h)</td>
</tr>
<tr>
<td>L. casei</td>
<td></td>
</tr>
<tr>
<td>CV1A</td>
<td>0.32</td>
</tr>
<tr>
<td>MIL2A</td>
<td>0.51</td>
</tr>
<tr>
<td>MIL2B</td>
<td>0.17</td>
</tr>
<tr>
<td>151</td>
<td>1.46</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
</tr>
<tr>
<td>343</td>
<td>1.37</td>
</tr>
</tbody>
</table>

Cells were grown in MRS broth at 37°C under pH control (6.2), harvested, washed and resuspended at 20 mg (dry weight)/mL as described under Methods. The incubation mixture for whole cells (3.0 mL) contained in μmoles: phosphate buffer (0.1 M KH₂PO₄ adjusted to pH 5.5 with 0.1 M Na₂HPO₄), 300; MgCl₂, 5; TPP, 0.1; tri-sodium citrate, 100 and 20 mg (dry weight) of cells. The incubation mixture for cell-free extracts (3.0 mL) contained in μmoles: phosphate buffer (0.1 M KH₂PO₄ adjusted to pH 5.5 with 0.1 M Na₂HPO₄), 300; MgCl₂, 10; TPP, 0.2; tri-sodium citrate, 100 and cell free extract (3 mg protein). Reactions were carried out for 2 h at 37°C and citrate was determined as described under Methods.
Table 2.7.  

**Oxidative activity of lactic acid bacteria**

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Oxygen uptake (nmole O₂/mg dry weight cells/min)</th>
<th>Acetate</th>
<th>D(-)-Lactate</th>
<th>L(+)-Lactate</th>
<th>Lactose</th>
<th>Pyruvate</th>
<th>Casein hydrolysate</th>
<th>Tryptone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(10 mM)</td>
<td>(20 mM)</td>
<td>(20 mM)</td>
<td>(20 mM)</td>
<td>(20 mM)</td>
<td>(0.2%)</td>
<td>(0.2%)</td>
</tr>
<tr>
<td>L. casei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIL2A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.9</td>
<td>0.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>151</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML1A</td>
<td>ND</td>
<td>0.2</td>
<td>0.2</td>
<td>ND</td>
<td>0.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>343</td>
<td>ND</td>
<td>0.3</td>
<td>0.9</td>
<td>1.0</td>
<td>11.9</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

ND, not detectable.

Cells were grown in MRS broth containing 15 mM glucose (lactobacilli) or M17 broth containing 14 mM lactose (streptococci) without pH control, harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.1 M KH₂PO₄ (adjusted to pH 5.5 with 0.1 X Na₂HPO₄) as described under Methods. Oxygen uptake was determined as described under Methods.
*L. plantarum* 343 oxidized all of the other substrates tested. All of the organisms were able to oxidize pyruvate under the conditions used. Of the organisms tested *L. plantarum* ML1A and 343 were able to oxidize D- and L-lactate, *L. casei* MIL2A and *L. plantarum* 343 oxidized lactose and *L. plantarum* 343 oxidized casein hydrolysate and tryptone. These results are consistent with those reported by Thomas (1986) where *L. plantarum* was the only organism able to oxidize both D- and L-lactate and casein hydrolysate.
DISCUSSION

1. Application in Cheesemaking

The lactobacilli isolated and characterized in this study were either *L. plantarum* or subspecies of *L. casei* and are representative of the dominant species of non-starter lactobacilli commonly found in mature Cheddar cheese (Chapman and Sharpe, 1981). Despite the majority of the isolates being *L. casei* differing plasmid profiles were evident. As plasmids encode phenotypic properties such as lactose utilization in lactobacilli (Chassy, 1987) it is possible that the organisms may possess diverse capabilities with respect to key metabolic functions. This in turn could result in the formation of different end products in a maturing cheese.

The non-starter lactobacilli grew and functioned under the conditions prevalent in maturing cheese as demonstrated by their ability to grow at lower temperatures and in NaCl concentrations normally present in Cheddar cheese (5% w/v in the moisture phase). *L. casei* and *L. plantarum* were also found to utilize citrate and lactate over an extended incubation time (Table 2.4) even though *L. casei* did not utilize lactate under changed test conditions (Table 2.7). As lactate is present in the moisture phase of cheese at concentrations of 400 to 500 mM (Thomas and Crow, 1983) the organisms would have an important energy source available to them over the lengthy cheese maturation period.

For non-starter lactobacilli to be used during cheese manufacture it would be an advantage to add the organisms to the cheesemilk to ensure an even distribution throughout the curd mass. This assumes the organisms would be entrapped in the curd and not lost in the whey. The organisms must also not affect starter performance or cheese yields as discussed previously (p. 11). It is unlikely that the non-starter organism *L. casei* MIL2A would adversely affect the growth and acid production kinetics of the starter organism in that (i) the time from
starter addition to milling of the curd is approximately 4 to 4.5h and for L. _casei_ MIL2A (generation time of 6.25 h) this would represent less than one doubling of the cell mass and (ii) only 2 mM lactic acid would be produced. A typical starter organism such as _S. lactis_ subsp. _cremoris_ BK5 would undergo a 5.7 fold increase in cell numbers and produce 55 mM lactic acid over the same period (Scott, 1986).

The use of additional organisms during manufacture requires that excessive protein degradation does not occur as this would adversely affect cheese yield (Law, 1987). _L. casei_ MIL2A demonstrated very low proteolytic activity in skim milk media and it was only during the stationary phase (i.e. growth had ceased) that the organism released increasing levels of PTA-soluble amino nitrogen into the growth medium. The results indicate that _L. casei_ MIL2A would only grow very slowly in the vat stage of manufacture and would not degrade significant amounts of protein at that time. However high numbers of stationary cells in cheese would probably degrade peptide material throughout maturation. It is this characteristic that is central to their controlled use in cheese maturation.

2. Growth Characteristics

If non-starter lactobacilli are to be added to cheesemilk in high numbers it must also be possible to grow and harvest them from artificial media in large quantities. For economic reasons, growth in complex media (i.e. MRS broth) may not always be desirable. In this study yeast extract proved to be an essential component for growth and when used with skim milk permeate, _L. casei_ MIL2A, although demonstrating a reduced specific growth rate compared to MRS broth, showed that the development of an alternate medium was feasible. Further investigation is necessary to optimize the nutrient requirements.

3. Cellular Activities

The ability of non-starter lactobacilli to metabolize compounds in maturing cheese other than proteins is
necessary to promote conditions suitable for optimum flavour development (i.e. reduced redox potential) and at the same time not lead to flavour or texture defects in the cheese during maturation. *L. casei* MIL2A and *L. plantarum* ML1A possessed some lipase activity, a potential source of unacceptable flavours, but the activities remained low (i.e. less than 6.0 mL 0.02 M NaOH x 10/mg dry wt. cells/7 days) (Umemoto, Umeda and Sato, 1968) and would be unlikely to significantly influence the development of rancid flavours in cheese. However, this characteristic should be monitored by graders if lactobacilli were to be used in cheesemaking. Both organisms were also able to metabolize citrate which has been associated with CO$_2$ production and possibly open texture problems in cheese (Czulak, 1953; Hoglund, Fryer and Gilles, 1972). The practical implications of using lactobacilli with an ability to utilize citrate, albeit at a low level, was therefore investigated with respect to cheese production (see Chapter 4).

The ability of the non-starter lactobacilli to oxidize substrates present in cheese would be an advantage in that they may be responsible for maintaining the low redox potential in maturing cheese necessary to stabilize reduced sulphur compounds (Thomas, 1986). Both *L. casei* and *L. plantarum* were able to oxidize a number of substrates including citrate, D- and L-lactate and pyruvate. Pyruvate is found in Cheddar cheese only at low concentrations (approximately 0.3 mM in the cheese moisture) (Kristoffersen and Gould, 1959) and therefore its oxidation would not greatly influence the redox potential of the cheese. However it must be noted that pyruvate is a key intermediary in glycolysis and in pathways associated with the development of flavour compounds (Figure 1.1) and any pyruvate formed may not be detected if it undergoes immediate oxidation (given that sufficient O$_2$ is available). The ability of both *L. casei* and *L. plantarum* to oxidize pyruvate by the pyruvate oxidase system (Figure 1.2) has been reported (Hickey, Hillier and Jago, 1983a).
4. Conclusion

In this study it would appear that the organism identified as *L. casei* MIL2A and to a lesser extent the isolates *L. casei* CV1A, MIL2B, ML1B and *L. plantarum* ML1A would have the metabolic characteristics suitable for addition to Cheddar cheese. They do not grow or produce significant levels of lactic acid and are not excessively proteolytic under conditions similar to those occurring during the vat stage of manufacture. In addition they grow at low temperatures and in the presence of NaCl. They also oxidize substrates (i.e. pyruvate, D- and L-lactate) present in Cheddar cheese and continue to produce PTA-soluble amino nitrogen as stationary cells. The organisms can also be grown in large numbers and are readily harvested from artificial media. However both organisms have the ability to metabolize citrate a potential problem with respect to cheese manufacture an aspect which was investigated further (Chapter 4).
CHAPTER 3

MILK PROTEIN METABOLISM BY LACTOBACILLI
MILK PROTEIN METABOLISM BY LACTOBACILLI

INTRODUCTION

1. The Proteolytic System of Lactic Acid Bacteria

The generalized scheme for the degradation of proteins by lactic acid bacteria (Figure 3.1) includes cell wall bound proteinases (and possibly extracellular proteinases) which reduce milk proteins to peptides small enough to penetrate the cell wall (Thomas and Mills, 1981; Thomas and Pritchard, 1987). These peptides may be further reduced in size by membrane endo- and exopeptidases which degrade the peptides to smaller peptides and amino acids either before or after transport into the cell (Thomas and Pritchard, 1987). The peptides are then cleaved to free amino acids by intracellular peptidases with wide specificity (Mou, Sullivan and Jago, 1975; Exterkate, 1975; Kolstad and Law, 1985).

The group N streptococci possess loosely bound cell wall proteinases (Exterkate, 1975; Mills and Thomas, 1978; Cliffe and Law, 1985) which are not normally excreted into the growth medium (Thomas and Pritchard, 1987). Within the cell wall of S. lactis subsp. cremoris up to four different proteinases have been differentiated (Exterkate, 1975; Exterkate, 1976b; Hugenholtz, Exterkate and Konings, 1984) but studies of the cell wall of S. lactis subsp. lactis suggest that only a single proteinase is present (Thomas and Pritchard, 1987). There is very little proteolytic activity elsewhere in the cell apart from one intracellular proteinase probably associated with intracellular protein metabolism rather than the caseinolytic activity of the cell (Exterkate, 1975). In contrast, peptidases are distributed throughout the cell wall, cell membrane and intracellular fractions of these organisms (Law, 1979; Exterkate, 1984; Kaminogawa, Ninomiya and Yamauchi, 1984; Kolstad and Law, 1985). The peptidases include aminopeptidases, dipeptidases,
**Figure 3.1.** Generalized scheme for protein degradation by lactic acid bacteria (Thomas and Pritchard, 1987)
tripeptidases and arylamidases (Thomas and Pritchard, 1987) but no carboxypeptidases have been detected.

2. The Proteolytic System of Lactobacilli.

(a) Proteinases. The proteolytic system of lactobacilli and the precise location of the proteinases in the cell is less defined than for the group N streptococci. Nevertheless the proteolytic activity of *L. bulgaricus*, *L. helveticus* and *L. lactis* appears to be associated with a single loosely bound surface proteinase (Argyle, Mathison and Chandan, 1976) as evidenced by the release of the proteinase from whole cells without any leakage of intracellular enzymes (Vescovo and Bottazzi, 1979; Ezzat et al., 1985). There is less information on the proteinases of the predominant lactobacilli in Cheddar cheese (*L. casei* and *L. plantarum*) although a recent report indicates two proteinases in the cell wall of *L. casei* possibly associated with hydrolysis of milk proteins (El Soda, Ezzat and El Shafei, 1987).

The location and function of the intracellular proteinases of the lactobacilli remains unclear even though a range of these organisms have been examined with respect to crude preparations of the cell components. (Baribo and Foster, 1952; Ohmiya and Sato, 1972; El Soda and Desmazeaud, 1982; Akuzawa, Ito and Yokoyama, 1983). Purified proteinases from lactobacilli have not been fully characterized but crude and partially purified cell wall proteinases of *L. bulgaricus* (Argyle, Mathison and Chandan, 1976) and *L. casei* (El Soda, Ezzat and El Shafei, 1987) have been studied indicating temperature optima of between 40°C and 50°C and pH optima of between 5.2 and 7.5.

While the group N streptococci preferentially degrade β-cascin *in vitro*, the lactobacilli are less specific and hydrolyse β, αs1 and κ-caseins (Chandan, Argyle and Mathison, 1982; Ezzat et al., 1985; Hegazi, 1987). The intracellular proteinases of some lactobacilli can also hydrolyse whey proteins (α-lactalbumin and
\(\beta\)-lactoglobulin). However as the whey proteins represent only 1% of the proteins in Cheddar cheese their hydrolysis would not contribute significantly to protein catabolites in the cheese (O’Keeffe, Fox and Daly, 1978). If ultrafiltration has been used for manufacture, the percentage of whey proteins in the curd increases significantly (2 to 2.5%) (B.J. Sutherland personal communication) and their role in cheese maturation may also increase.

In Cheddar cheese, \(\alpha_{s1}\)-casein can be completely degraded during maturation and it is clear that chymosin contributes significantly to the initial hydrolysis of this protein (Hill, Lahav and Givol, 1974; Gripon et al., 1975; Creamer and Richardson, 1974). \(\beta\)-casein degradation occurs slowly and its products (i.e. \(\gamma\)-caseins) appear later at a rate which is probably determined by the low moisture content and pH of the cheese (Creamer, 1975) and/or a modification of \(\beta\)-casein by the NaCl that renders it resistant to proteolytic attack (Fox and Walley, 1971; Creamer, 1971; Phelan, Guiney and Fox, 1973). It is therefore the breakdown of \(\alpha_{s1}\)-casein which most influences the body and texture changes in cheese. As proteolysis proceeds the small peptides and amino acids which are formed, contribute to the flavour and buffering of cheese and the maintenance of non-starter microflora.

(b) Peptidases. The peptidases of lactobacilli have been studied in more detail, due mainly to their important role in cheese maturation (Figure 1.1), and it is clear that the types and location of peptidases present in the cell differ between species and strains. No peptidase activity has been associated with the cell wall of \(L.\ casei\), but the soluble cytoplasmic fraction contains three exopeptidases (an aminopeptidase, a broad specificity dipeptidase and a narrow specificity carboxypeptidase) and the ribosomal fraction contains an aryl peptide amidase (El Soda, Bergère and Desmazeaud, 1978; El Soda and Desmazeaud, 1981). The peptidase system of \(L.\ plantarum\) consists of one non-specific aminopeptidase and three
dipeptidases but there is no carboxypeptidase or arylpeptide amidase activity (El Soda et al., 1983). The peptidases in the thermobacterium group of lactobacilli often used as starter organisms (L. helveticus, L. acidophilus, L. lactis and L. bulgaricus) have also been examined. Aminopeptidases and dipeptidases are present but no carboxypeptidase or endopeptidase activities have been detected (El Soda and Desmazeaud, 1982; Ezzat et al., 1982). The peptidase activities of L. helveticus and L. bulgaricus have been compared to S. lactis subsp. cremoris (Hickey, Hillier and Jago, 1983c) and a L. helveticus strain, that could release amino acids more rapidly from casein, was used as a starter organism in the manufacture of Cheddar cheese to influence maturation (Hickey, et al., 1983).

Peptidases specific for peptide bonds involving proline could also be important in cheese maturation as proline is an amino acid which produces a distinct nutty sweet flavour in cheese (Hintz, Slatter and Harper, 1956; Hickey et al., 1983). L. helveticus and L. bulgaricus possess proline iminopeptidase and aminopeptidase-P activity (Hickey, Hillier and Jago, 1983c) while prolyldipeptidyl-peptidase activity is present in a number of lactobacilli including L. casei and L. plantarum (Casey and Meyer, 1985).

(c) **Protein catabolites.** Small peptides and amino acids are associated with changes in body and texture and flavour development in cheese. Peptides also have been used as indices of maturation (Pham and Nakai, 1984), as a possible means of understanding differences in starter strains (Rank, Grappin and Olson, 1985) and as an indicator for differentiating cheeses made from milk of different sources (Tobler, Windemann and Baumgartner, 1983). Peptides from β-casein (Sullivan and Jago, 1972) and αs1-casein have both been implicated in the bitter flavour defect in cheese. The peptides usually have molecular weights ranging from 1,000 to 12,000 and characteristically contain a high proportion of
hydrophobic amino acid residues (Law and Kolstad, 1983). These peptides would accumulate if their degradation by peptidases in cheese is slow. Amino acid formation in Cheddar cheese has been found to correlate well with flavour development (Aston, Durwood and Dulley, 1983) and leucine, methionine and glutamic acid appear to be the best predictors of the extent of proteolysis (Marsili, 1985). The role of amino acids in flavour is not fully understood but it is generally considered that amino acids per se are not responsible for Cheddar flavour. They merely provide an essential background component and it is the numerous degradation products that contribute to flavour.

The group N streptococci do not appear to degrade amino acids but lactobacilli can catalyse a number of reactions involving amino acids (Law and Kolstad, 1983). For example, a lactobacilli isolated from Cheddar cheese was able to desulphurylate cysteine to produce hydrogen sulphide (Sharpe and Franklin, 1962). The enzymic decarboxylation of amino acids can also result in the formation of biologically active compounds (amines) which although implicated in flavour development (Dahlberg and Kosikowski, 1948) are now recognized as food toxins. They may combine with nitrogen oxides to form carcinogenic nitrosamines or they can act directly producing symptoms of hypo- and hypertension in individuals whose natural mechanisms for catabolism are inhibited or genetically deficient (Rice, Eitenmiller and Koehler, 1976). Tyramine (from tyrosine) and histamine (from histidine) are the most likely amines to be found in cheese and although there have been a number of reports indicating problems with these compounds in some cheese types (Voigt et al., 1974; Edwards and Sandine, 1981) the amine levels in Cheddar cheese are generally below those considered dangerous to susceptible individuals (Rice, Eitenmiller and Koehler, 1976).

The group N streptococci are not able to form tyramine and histamine (Voigt and Eitenmiller, 1977) but a number of species of non-starter bacteria from Cheddar cheese can
(Edwards and Sandine, 1981). These include strains of S. faecium, S. mitis, L. bulgaricus, L. plantarum and streptococci from the viridans group. Therefore amine formation is an important parameter to consider in selecting organisms for use in a cheese manufacturing process.

3. Proteolysis and Maturation

The general proteolytic system present in cheese is comprised of residual rennet activity, the enzymes associated with starter and non-starter organisms and naturally occurring milk proteinases. Such a complex array of proteinases and peptidases would result in a large number of compounds which together may contribute to Cheddar cheese flavour. This is the basis of the 'Component Balance Theory' of Mulder (1952) which also suggests that if this balance of compounds is upset, an atypical flavour is produced. From the evidence presented it would appear that proteinase activity cannot be increased substantially without adversely affecting body and texture of the cheese and may result in an accumulation of peptides some of which could lead to bitterness. However it is possible that an increase in (and control of) peptidase activity could directly influence flavour without affecting other cheese characteristics. Lactobacilli selected for this purpose must therefore:

1. Have a low proteinase activity so as not to degrade milk proteins in the vat stage of manufacture which could result in reduced cheese yield.

2. Degrade $\alpha_{s1}$-casein preferentially to be consistent with normal protein catabolism in maturing cheese.

3. Possess a broad range of accessible peptidases that function under the conditions of pH, moisture and salt concentrations present in cheese.

4. Possess a level of peptidase activity comparable to the group N streptococci in order to prevent a too rapid or unbalanced end product in terms of peptides and amino acids formed.
(5) Not possess amino acid decarboxylase activity resulting in the formation of biogenic amines.

(6) Maintain high numbers and even multiply in cheese throughout maturation in order to dominate the non-starter microflora of the cheese.

(7) Possess a proteolytic system that is not adversely affected by the temperatures encountered in the vat stage of manufacture.

The aim of the present study was to characterize the general proteinase system in lactobacilli with respect to the above parameters and to provide comparative data to the group N streptococci.
MATERIALS AND METHODS

1. Bacteria

(a) T**yped** strains. The organisms used in this investigation have been described previously (p. 13). These include *L. casei* strains ML1B, CV1A, MIL2A and MIL2B; and *L. plantarum* ML1A isolated from Cheddar cheese and identified as described in Chapter 2. *S. faecium* SD1, obtained from the C.S.I.R.O. Dairy Research Laboratory, Highton, Victoria was maintained as described for *S. lactis* subsp. cremoris BK5. Cells were grown at controlled pH in either a MRS or M17 broth, a medium containing 10% (w/v) skim milk permeate and 1% (w/v) sodium caseinate or a skim milk media (10% w/v) and harvested and resuspended as described previously (p. 16) except that the phosphate buffer was adjusted to pH 6.0.

(b) Preparation of cell free extracts. The cell suspension was prepared and sonicated as described previously (p. 17) except that 0.05 M Na₂HPO₄ (adjusted to pH 7.0 with 0.05 M KH₂PO₄) was used.

(c) Preparation of spheroplasts. Cells, suspended in phosphate buffer (0.05 M Na₂HPO₄ adjusted to pH 7.0 with 0.05 M KH₂PO₄) at approximately 20 mg (dry weight)/mL were diluted 1:1 with the same phosphate buffer containing 2 M sucrose and 4 mM MgCl₂ and incubated at 37°C for 2 h in the presence of egg white lysozyme (1 mg/mL at 40,000 U/mg protein, Sigma Chemical Company). Spheroplast formation was confirmed by suspending a sample (0.1 mL) of the incubation mixture in excess 0.1 M NaOH (2.5 mL) as described by Extorkate (1975). The incubation mixture was then centrifuged at 5,000g for 10 min and the sediment (containing spheroplasts) was washed twice (1.0 x suspension volume) in the phosphate buffer containing 1 M sucrose and 4 mM MgCl₂ before resuspension in the same buffer (1.0 x suspension volume).
During the preparation, intracellular material must not be released through lysis of spheroplasts and the extent to which lysis occurred was estimated by the release of aldolase (EC 4.1.2.13) into the digestion medium and compared to total aldolase activity of the cell after complete disruption (i.e. sonication). Aldolase was assayed as described by Bergmeyer, Gawehn and Grassel (1974).

2. Milk proteins

(a) Preparation of whole casein. Fresh raw milk obtained from an individual cow (genetically typed \( \alpha_{\text{S1}} \)-casein B, \( \beta \)-casein A and \( \kappa \)-casein A) was centrifuged at 1,000g for 20 min at 20°C (MSE High Speed 18). The milk was then cooled to 4°C and the skim milk fraction was collected from below the separated milk fat layer. The skim milk was adjusted to pH 4.6 with 1 M HCl and the precipitate collected by centrifugation at 1,000g for 10 min. The precipitate (casein) was redissolved in a volume of distilled H\(_2\)O equal to the initial volume of skim milk by adjusting the pH to 7.0 with 1 M NaOH. The precipitation and resuspension steps were repeated twice before the precipitated casein was stored at -20°C until fractionation.

(b) Casein fractionation and concentration. Partially purified fractions of \( \alpha_{\text{S1}} \)-casein, \( \kappa \)-casein, \( \beta \)-casein and \( \alpha_{\text{S2}} \)-casein were obtained by the urea precipitation procedure of Hipp et al. (1952) and Zittle and Custer (1963) as outlined by Fox and Guiney (1972). The protein concentration of each fraction was determined at 280 nm with reference to a standard curve. These casein fractions were further purified by chromatography on a DEAE cellulose Whatman DE52 column (3.2 x 28 cm). The column was prepared in 0.02 M Tris-HCl chromatographic buffer (pH 7.0) containing \( 10^{-4} \) M EDTA and 6.6 M urea as described by Andrews and Cheeseman (1971). The fractions containing \( \alpha_{\text{S1}} \)- and \( \beta \)-casein were dissolved separately in the chromatographic buffer and an aliquot containing
approximately 1.5 g protein in not more than 30 mL was applied to the column. The crude fractions were eluted at 4°C with 100 to 200 mL of Tris- HCl-urea buffer followed by 250 mL of the same buffer containing 0.075 M NaCl. A linearly-increasing concentration gradient of NaCl supplied from two connected reservoirs was then applied. The first reservoir contained 500 mL of Tris-HCl-urea buffer with 0.075 M NaCl and the second 500 mL of Tris-HCl-urea buffer with 0.175 M NaCl. The column was finally washed with 250 mL of Tris-HCl-urea buffer containing 0.175 M NaCl.

Column eluates were collected in 10 mL volumes (at a flow rate of 175 mL/h) using a LKB 2211 SuperRac fraction collector. The absorbance of the fractions was measured at 280 nm relative to the chromatographic buffer and the conductivity monitored by a conductivity meter (TPS LC81). The NaCl concentration was determined by reference to a standard curve which related conductivity to NaCl concentration in the chromatographic buffer.

The peak fractions containing the caseins were pooled and concentrated to approximately 3% (w/v) protein using an ultrafiltration cell (Amicon Model 402) fitted with Amicon PM10 membranes. The concentrated caseins were then dialysed twice against 100 volumes of deionized H₂O at 4°C for two 24 hour periods and stored at -20°C.

(c) Whey proteins. Commercial preparations (Sigma Chemical Company) of α-lactalbumin, β-lactoglobulin and bovine serum albumin were used in this investigation. Where heat treated proteins were required, samples (4 mL) were heated at 65°C, 80°C and 100°C for 30 min or 37°C for 90 min. The extent of denaturation was determined by a modification of the method of Harwalker (1980). The protein solution (0.5 mL) was added to 10 mL of H₂O adjusted to pH 4.5 with 1 M HCl and made up to 25 mL and centrifuged at 9,000g for 15 min. The supernatant (0.5 mL) was diluted 1:1 with H₂O and the protein content determined by the method of Lowry et al., (1951).
3. Proteinase Activity

(a) Proteinase assay. Bacterial cells of *S. lactis* subsp. *cremoris* and lactobacilli were grown, harvested and resuspended as described under 1(a) (see Appendix 2). The cell suspension (1.0 mL) was added to 2.0 mL of protein solution (0.75% w/v of sodium caseinate, \(a \text{g} \) or \(\beta \)-casein in the 0.2 M phosphate buffer at pH 6.0 and 30 \(\mu\)L of chloroform as a bacterial growth inhibitor. The assay mixture was incubated in a sealed 12 x 75 mm polypropylene tube at 37°C (lactobacilli) or 25°C (*S. lactis* subsp. *cremoris* BK5) for up to 10 days. Zero time samples were frozen immediately and cell blank samples without substrate were run simultaneously (see Appendix 3). TCA-soluble protein was determined as described below.

(b) Determination of protein degradation. Protein in the incubation sample (0.5 mL) was precipitated by the addition of 2.0 mL 10% (w/v) TCA. The precipitate was removed by centrifugation at 10,000g for 15 min and the supernatant was filtered (Whatman 542 filter paper) and assayed for protein by the trinitrobenzene sulfonic acid (TNBS) method (Habeeb, 1966; Jarrett, Aston and Dulley, 1982). The filtrate (0.1 mL) was mixed with 2.0 mL of 4% (w/v) NaHCO₃ (pH 8.5) and 1.0 mL of 0.1% (w/v) TNBS solution and incubated at 37°C for 2 h. The solution was then mixed vigorously with 1.5 mL of 4 M HCl and the absorbance read at 340 nm.

(c) Extracellular proteinases. Bacterial cells of lactobacilli were grown, harvested and resuspended as described under 1(a) except that 0.05 M Na₂HPO₄ (adjusted to pH 7.8 with 0.05 M KH₂PO₄) was used and the cells were resuspended at 7.0 mg (dry weight)/mL. The suspension was held at 30°C for 2 h and the cells were removed by centrifugation at 5,000g for 10 min (a control supernatant was prepared by centrifuging immediately). The supernatants were filtered through a 0.45 \(\mu\)m membrane (Sartorius, Minisart NML), adjusted to pH 6.0 with 0.2 M
HCl and concentrated (5:1) using a stirred ultrafiltration cell (Amicon Model 402) fitted with an Amicon PM10 membrane.

4. **Peptidase Activity**

(a) **Aminopeptidase.** (EC 3.4.11.11). Aminopeptidase activity was measured by the method of Exterkate (1975) using the substrates L-leucine-p-nitroanilide and L-alanine-p-nitroanilide. Cell free extract (1.0 mL), prepared as described in 1(b) was added to 1.0 mL of substrate (1 mM) in 0.05 M Na₂HPO₄ (adjusted to pH 7.0 with 0.05 M KH₂PO₄) and the mixture incubated at 37°C for 60 min. The reaction was stopped by the addition of 2 mL acetic acid solution (30% v/v) and the mixture centrifuged at 10,000g for 15 min. The supernatant was removed and the absorbance determined at 410 nm. Control samples were prepared by the addition of acetic acid solution before cell free extract addition to the substrate.

Activity was calculated from the molar coefficient of extinction, $E = 9600 \text{ M}^{-1}\text{cm}^{-1}$ (Pfeiderer, 1970) and expressed as umoles nitroaniline released/mg protein/h.

(b) **Proline iminopeptidase.** (EC 3.4.11.5). Activities were measured as described in (a) above using L-proline-p-nitroanilide as substrate.

(c) **Endopeptidase (EC 3.4.21.24).** Activities were measured as described in (a) above using N-glutaryl-L-phenylalanine p-nitroanilide and N-succinyl-L-phenylalanine p-nitroanilide as substrates.

(d) **Aminopeptidase-P (EC 3.4.11.9).** Activity was measured by the method of Yaron and Mlynar (1968) as described by Hickey, Hillier and Jago (1983c) using poly-L-proline as substrate.

(e) **Dipeptidase (EC 3.4.13.11).** Dipeptidase activity was measured by a modification of the method of El Soda,
Desmazeaud and Bergère (1978) using the substrates glycyl-L-tyrosine, glycyl-L-phenylalanine and L-leucyl-glycine. Cell free extract (1.0 mL) prepared as described in 1(b), was added to 1.0 mL of substrate (1 mM) in 0.05 M phosphate buffer (pH 7.0) and the mixture incubated at 37°C for 4 h. The reaction was stopped by the addition of 2 mL TCA solution (24% w/v) and the precipitate removed by centrifugation at 10,000g for 15 min. TCA-soluble protein was measured by the modified Yemm and Cocking ninhydrin method (0.1 mL sample volume) of Matheson and Tatttie (1964). Control samples consisted of 1.0 mL each of cell free extract and substrate incubated separately and mixed immediately before assay. Enzyme activity was expressed as μmoles x 10⁻² glycine equivalent released/mg protein/h.

(f) Tripeptidase (EC 3.4.11.4). Activities were measured as described in 4(e) above using DL-alanyl-DL-leucylglycine as substrate.

(g) Carboxypeptidase A (EC 3.4.16.1). Activities were measured as described in 4(e) above using N-carbobenzoxy-glycyl-L-proline and hippuryl-L-arginine as substrate.

5. High Performance Liquid Chromatography (HPLC) Analyses

(a) Preparation of protein digests. Proteinase assays were performed as described in 3(a) and the cells removed by centrifugation for 1 min in a microcentrifuge (Hettich, Mikroliter).

(i) For amino acid analysis, a sample (0.8 mL) of supernatant was diluted with 6.4 mL of high purity H₂O (Milli-Q water purification system. Millipore Corporation, Bedford, MA, U.S.A.) and 0.8 mL of internal standard (1 mM 2-aminobutyric acid). The solution was concentrated using an ultrafiltration cell (Amicon model 8010) fitted with an Amicon YM2 membrane (nominal molecular weight cut-off of 1,000). Five mL of the ultrafiltrate was freeze dried,
resuspended in 1.0 mL of high purity H₂O and filtered through a 0.22 μm Durapore filter (Millipore Corporation) and stored at -20°C until HPLC analysis.

(ii) For peptide analysis 0.2 mL of supernatant was added to 0.6 mL of a solution of Solvent A and Solvent B (90:10) as described below (5c) and filtered through a 0.22 μm Durapore filter (Millipore Corporation).

(b) Amino acids. HPLC was carried out using a LKB system (LKB-Produtkter AB, Bromma, Sweden) which consisted of two 2150 pumps, a 2152 controller and a 2151 variable wavelength monitor. A LKB Ultrapac Lichrosorb RP-18 (5 μm) 250 mm x 4 mm column preceded by a Lichrosorb RP 18 (7 μm) guard column was used for chromatographic separations at ambient temperature. The column eluate was monitored at 340 nm and peaks were quantitated with a Shimadzu C-R3A Chromatopac (Shimadzu Corporation, Kyoto, Japan) computing integrator (see Appendix 5).

Gradients were formed using 2 filtered (0.45 μm filters, Millipore Corporation) and degassed solutions as outlined by Jones, Pääbo and Stein (1981). Solvent A was tetrahydrofuran:methanol:0.05 M sodium acetate (pH 5.9), 1:19:80 and Solvent B was methanol:0.05 M sodium acetate (pH 5.9), 8:2. The gradient program used 0% B for 1 min from the time of sample injection, a linear step to 20% B in 14 min, an isocratic step at 20% B for 4 min, a linear step to 52% B in 8 min, an isocratic step at 52% for 5 min, a linear step to 62% B in 10 min, a linear step to 100% B in 8 min and finally an isocratic step at 100% B for 10 min. The flow rate throughout the gradient program was 1.0 mL/min.

Before injection, the samples were derivatized using a modification of the method of Jones, Pääbo and Stein (1981). The derivatizing solution was prepared daily by dissolving o-phthalaldehyde (10 mg) in absolute methanol (250 μL) with 2-mercaptoethanol (10 μL) and 0.4 M sodium borate (pH 9.5) (2.24 mL) and the solution was stored in the dark. Derivatization was carried out by mixing 100 μL
of sample with 100 µL of 0.25% (w/v) Brij 35 in 0.4 M sodium borate (pH 9.5) followed by 100 µL of the derivatizing solution. After 1 min 200 µL of 0.1 M KH₂PO₄ (pH 4.0) was added and after 3 min 20 µL was injected onto the HPLC column.

(c) Peptides. HPLC was carried out using a LKB system (LKB-Produkter AB) as described in (b) above with a Brownlee Aquapore RP-300 (7 µm) 220 x 4.6 mm column preceded by a Aquapore RP-300 (7 µm) 30 x 4.6 mm guard column. Chromatographic separations were carried out at ambient temperature and the column eluate was monitored at 210 nm. The peaks were quantitated with a Shimadzu C-R3A Chromatopac computing integrator.

Gradients were formed using 2 filtered (0.45 µm filters Millipore Corporation) and degassed solutions as described by Tobler, Windemann and Baumgartner (1983). Solvent A was an aqueous solution of sodium dodecyl sulfate (0.02% w/v) and trifluoroacetic acid (0.02% v/v) adjusted to pH 5.5. Solvent B was a mixture of acetonitrile and Solvent A (50:50). The gradient program used 10% B for 5 min from the time of sample injection, a linear step to 87% B in 30 min and an isocratic step at 87% B for 10 min. The flow rate throughout the gradient program was 0.8 mL/min. A 20 µL sample was injected onto the HPLC column.

(d) Proline. Proline was derivatized using the method of Cooper, Lewis and Turnell (1984). Chloramine T solution (20 mM) was prepared in dimethyl sulfoxide (2.0 mL) and 0.2 M sodium borate buffer (pH 9.5) (8.0 mL). Sodium borohydride (180 mM) was made up in 10 mL of 0.6 M lithium hydroxide solution and the o-phthaldialdehyde/2-mercaptoethanol solution was prepared as described in (b) above.

Derivatization was carried out by adding a 100 µL sample to 100 µL of chloramine T solution at 60°C. After 1 min 100 µL of sodium borohydride solution was added and the mixture was incubated for a further 10 min at 60°C and cooled to room temperature. o-Phthaldialdehyde/2-mercaptoethanol solution (100 µL) was added to 100 µL of
the sample solution and after 3 min 20 μL was injected onto the HPLC column.

Chromatographic separations were carried using the same conditions described for amino acids in 5 (b).

(e) **Biogenic amines.** Cells were incubated in a mixture (3.0 mL) containing in μmoles, phosphate buffer (0.1 M KH₂PO₄ adjusted to pH 5.5 with 0.1 M Na₂HPO₄) (300), pyridoxal phosphate (0.2) and one of the following: histidine, tryptophan, arginine, phenylalanine or lysine (6) or tyrosine (3) and 30 μL of chloroform. The assay mixture was incubated in a sealed 12 x 75 mm polypropylene tube at 25°C (S. lactis subsp. cremoris BK5) or 37°C (lactobacilli) for 24 h. Control incubation mixtures were frozen immediately after the addition of cells.

Cells were removed from the incubation mixture by centrifugation for 1 min in a microcentrifuge (Hettich, Mikrolitor) and a 1.0 mL sample diluted (1:1) with an internal standard (1 mM 2-aminobutyric acid) and filtered through a 0.22 μm, Durapore filter (Millipore Corporation) before HPLC analysis for amino acids as described in 5 (b).

6. **Electrophoresis**

(a) **Gel electrophoresis.** Electrophoresis was carried out in a vertical slab gel apparatus using a modification of the method of Laemmli (1970) as outlined in the LKB2001 Vertical Electrophoresis Laboratory Manual (LKB Produkter AB, Bromma, Sweden).

The resolving gel (15% (w/v) acrylamide) and the stacking gel (5% (w/v) acrylamide) were prepared from a stock solution of 38.95% (w/v) acrylamide and 1.05% (w/v) N,N-bismethylene acrylamide (McPherson and Kitchen, 1981). Samples of the incubation mixture (100 μL) were prepared for electrophoresis by the addition of 300 μL of Laemmli’s (1970) sample buffer and 10 μL of mercaptoethanol and immersed in boiling H₂O for 2 min. Samples (15 μL) were loaded onto the stacking gel and electrophoresis was
carried out at a constant current of 20 mA for 1 h followed by 30 mA for 4 h using bromphenol blue as a marker. The gels were stained for 30 min at room temperature in a solution of 0.06% (w/v) Coomassie Brilliant Blue R, 10% (v/v) methanol and 10% (v/v) acetic acid. Destaining was carried out by washing in methanol:acetic acid:water (1:2:17 v/v) for 2 h followed by an overnight wash in the same solution. Gels were scanned at 590 nm using a Shimadzu Dual-Wavelength TLC Scanner (Shimadzu Corporation, Kyoto, Japan. Model CS-910).

(b) Isoelectric focusing. Isoelectric focusing was performed in a 1 mm gel containing 5% (w/v) acrylamide prepared from a stock solution of 29.1% (w/v) acrylamide and 0.9% (w/v) N, N-bismethylene acrylamide, 2% (v/v) ampholyte (Bio-Lyte 3/10, Bio-Rad, Richmond, California, U.S.A.) and 7 M urea according to the LKB Application Note 250 (LKB-Produkter AB, Bromma, Sweden) and the method of Trieu-Cuot and Gripon (1981).

A LKB Multiphor 2117 apparatus (LKB-Produkter AB, Bromma, Sweden) cooled to 10°C was used for focusing. After prefocus for 1 h at 10 W 15 µL samples were applied to filter paper at the anodic side. The filter paper was removed after 1 h and focusing continued for a further 2.5 h.

pH measurements were made on each 10 mm of the gel using a surface electrode (LKB Multiphor Electrode 2117-11) at 10°C before the gel was stained according to the method of Blakesley and Boezi (1977).
RESULTS

1. Proteinase Activity

Both starter bacteria and the non-starter lactobacilli are weakly proteolytic in comparison to other bacterial groups but they are able to degrade milk proteins and actively transport amino acids and peptidases across the cell membrane against a concentration gradient (Figure 3.1) (Thomas and Pritchard, 1987). The proteolytic system of non-starter lactobacilli is less defined than for the group N streptococci even though these organisms appear to be proteolytically important in Cheddar cheese maturation.

The effect of rennet on proteinase activity was compared at the optimum growth temperature of L. casei 151, L. plantarum 343 and S. lactis subsp. cremoris BK5 (Table 3.1). L. casei 151 was more active than L. plantarum 343 but S. lactis subsp. cremoris BK5 had the most active proteolytic system (29.4 μmol x 10 gly equiv./mg dry wt. cells/7 days) and its prt− derivative least activity under the conditions used. Whatever the level of proteolytic activity produced by the cells the proteolytic activity associated with the presence of rennet was uniform (i.e. 5.3 to 7.2 μmol x 10 gly equiv./mg dry wt. cells/7 days) (Table 3.1). This suggests that the action of rennet is independent of the cellular proteinase activity when sodium caseinate is the substrate.

Many bacterial genera and species secrete proteinases as free enzymes into a growth media, depending on the salt concentration, although the lactic acid bacteria generally do not (Thomas and Pritchard, 1987). When cells of L. casei MIL2A were suspended in 0.05 M phosphate buffer (pH 7.8) at 30°C for 2 h (Table 3.2) 155 μg mL−1 of protein was released into the supernatant over the unincubated control. Some of the released proteins were proteinases as the activity in the supernatant increased from 0.09 to 0.17 μmol x 10 gly equiv./mg dry wt. cells/7 days. However a corresponding increase in the intracellular marker enzyme aldolase suggests that the increase may be
Table 3.1. **Proteinase activity of lactic acid bacteria**

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Proteinase activity (μmol x 10 gly equiv./mg dry wt. cells/7 days)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Cells + rennet</td>
<td></td>
</tr>
<tr>
<td><strong>L. casei</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>151</td>
<td>19.5</td>
<td>24.7</td>
<td></td>
</tr>
<tr>
<td><strong>L. plantarum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>343</td>
<td>2.0</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td><strong>S. lactis subsp. cremoris</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK5</td>
<td>29.4</td>
<td>35.6</td>
<td></td>
</tr>
<tr>
<td>BK5 prt−</td>
<td>0.9</td>
<td>8.3</td>
<td></td>
</tr>
</tbody>
</table>

Cells were grown in MRS broth (lactobacilli) or M17 broth (S. lactis subsp. cremoris BK5), harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 6.0 with 0.2 M Na₂HPO₄) as described under Methods. The cell suspension (1.0 mL) was added to a solution containing 2.0 mL of sodium caseinate (1.5% w/v) in 0.2 M phosphate buffer at pH 6.0, 30 μL of chloroform and where indicated 30 μL of rennet (1/40 dilution). The assay mixture was incubated at 37°C (lactobacilli) or 30°C (S. lactis subsp. cremoris BK5) for 7 days and the TCA-soluble protein released by the organisms alone was determined as described under Methods.
Table 3.2. The release of cell wall proteinases from *L. casei MIL2A*

<table>
<thead>
<tr>
<th>Fraction assayed</th>
<th>Proteinase activity (μmol x 10 gGly equiv./mg dry wt. cells/7 days)</th>
<th>Aldolase activity (ΔE/h)</th>
<th>Protein concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell suspension</td>
<td>8.44</td>
<td>10.56*</td>
<td>2380*</td>
</tr>
<tr>
<td>Supernatant (0 h)</td>
<td>0.09</td>
<td>0.12</td>
<td>21</td>
</tr>
<tr>
<td>Supernatant (2 h)</td>
<td>0.17</td>
<td>0.24</td>
<td>176</td>
</tr>
</tbody>
</table>

*Activity determined on disrupted cells.*

Cells were grown in MRS broth, harvested by centrifugation, washed and resuspended at 7.0 mg (dry weight)/mL in 0.05 M Na₂HPO₄ (adjusted to pH 7.8 with 0.05 M KH₂PO₄) as described under Methods. The whole cell suspension was tested directly and compared to the supernatants (prepared by centrifugation) obtained immediately or after incubation at 30°C for 2 h. Proteinase and aldolase activities of the whole cell suspension and the supernatants were determined as described under Methods.
due to proteinases released upon cell lysis.

The cell wall proteinase(s) of *L. casei* MIL2A were investigated further using lysozyme to release proteinase enzymes. As shown in Table 3.3 incubation with lysozyme released 28% of the total cell proteinase activity into the suspending medium while 33% of the activity remained associated with spheroplasts (39% of activity was not accounted for). The failure of the lysozyme to release all of the cell wall proteolytic activity may be due to the fact that the lactobacilli are more resistant to lysozyme (Neujahr, Borstad and Logardt, 1973; Klaenhammer, 1984) in contrast to the group *N. streptococci* where 100% of cell wall proteolytic activity can be released with lysozyme treatment (Exterkate, 1975). The spheroplasts formed did not release proteinases as an insignificant level of aldolase (less than 5% of total intracellular aldolase activity) was measured in the suspending medium (aldolase activity for whole cells and suspending media after treatment were 0.204 and 0.010 ΔE/min respectively). The sonication treatment resulted in the release of 51% of cell proteinase activity while 16% remained with the cell debris (Table 3.3). Again a similar proportion (33%) of the total activity was not detected suggesting that either the optimum proteolytic activity was dependant on the structural integrity of the cell wall or there was enzyme denaturation during cell lysis. The fact that cell digestion by lysozyme, a relatively gentle method of cell lysis, destroyed some proteolytic activity infers the former is more likely. That is, proteolytic activity is associated with the cell wall and is dependent on the structural integrity of the cell wall for optimum activity.

2. Factors Affecting Proteinase Activity

(a) pH. As shown in Figure 3.2a proteinase activity was observed over the pH range 5.0 to 7.0 with a maximum activity at pH 5.6 for *L. casei* MIL2A, pH 6.4 for *L. casei* 151 and pH 5.6 to 6.0 for *L. plantarum* ML1A. *L. plantarum*
Table 3.3. Localization of proteinase activity in *L. casei MIL2A*

<table>
<thead>
<tr>
<th>Fraction assayed</th>
<th>Proteinase activity (μmol gly equiv./mL incubation media/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell suspension</td>
<td>1.45</td>
</tr>
<tr>
<td>Cell free extract</td>
<td>0.74</td>
</tr>
<tr>
<td>Cell debris</td>
<td>0.23</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>0.48</td>
</tr>
<tr>
<td>Soluble cell wall material</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Cells were grown in MRS broth, harvested by centrifugation, washed and resuspended at 20 mg (dry weight)/mL in 0.05 M Na₂HPO₄ (adjusted to pH 7.0 with 0.05 M KH₂PO₄) as described under Methods. The cell fractions were prepared and assayed for proteinase activity as described under Methods. All results were corrected to account for dilution during fractionation.
Figure 3.2. The effect of pH on proteinase activity of resting cells of lactobacilli and 
S. lactis subsp. cremoris BK5

Cells were grown in MRS broth (lactobacilli) or M17 broth (S. lactis subsp. cremoris BK5), 
harvested by centrifugation, washed and resuspended in distilled water to give a 
concentration of 30 mg (dry weight)/mL as described under Methods. The cell suspensions 
(0.5 mL) were added to a solution containing 1 mL of aqueous sodium caseinate (3.0% w/v), 
1.5 mL of 0.4 M KH₂PO₄ (adjusted to the appropriate pH with 0.4 M Na₂HPO₄, and 30 µL of 
chloroform. The assay mixture was incubated at 37°C (lactobacilli) or 30°C (S. lactis 
subsp. cremoris BK5) for 7 days and the TCA-soluble protein released by the organisms alone 
was determined as described under Methods.

(a) Lactobacilli
- L. casei MIL2A
- " 151
- L. plantarum MIL1A
- " 343
(b) S. lactis subsp. cremoris BK5
- 30°C
- 37°C
- pret 30°C
- " 37°C
343 exhibited very low levels of proteolytic activity although some activity was detected at pH 6.2 and 7.0. The starter organism *S. lactis* subsp. *cremoris* BK5 (Figure 3.2b) possessed maximum proteolytic activity at pH 5.8 but a second smaller peak of activity was observed at pH 6.8. The proteinase activity at pH 5.8 was reduced by 60% at 37°C.

(b) **Temperature.** The effect of temperature on the proteinase activity of *L. casei* MIL2A was investigated. This assay was conducted only after it was decided to use *L. casei* MIL2A in the cheesemaking trials (Chapter 4) and it was the only organism tested in this way. *L. casei* MIL2A had optimum proteinase activity at 37°C (Figure 3.3). The organism exhibited increasing proteinase activity from 8°C and maintained good activity at the cooking temperatures encountered during cheese manufacture (37-42°C). At 8°C *S. lactis* subsp. *cremoris* BK5 had the same proteinase activity as *L. casei* MIL2A (1.8 μmol x 10 gly. equiv./ mg dry wt. cells/7 days) (data not shown).

(c) **Sodium and potassium.** Cheddar cheese contains approximately 1.9% (w/w) NaCl (equivalent to 5% w/w in the moisture phase) and it is also possible to use KCl as a partial replacement for NaCl as a means of reducing dietary sodium intake (Jameson, 1985). The effect of both Na⁺ and K⁺ on the proteinase activity of non-starter lactobacilli was therefore studied. As shown in Figure 3.4a, Na⁺ and K⁺ had a similar effect on the proteinase activity of *L. casei* MIL2A. At 5% (w/v) NaCl and 3.8% (w/v) KCl (equivalent to respective cation concentrations of 2% w/v) proteinase activities were reduced by approximately 40%. Under the same conditions Na⁺ reduced the proteinase activity of *S. lactis* subsp. *cremoris* BK5 60% (Figure 3.4b). The results are consistent with those reported previously indicating that the non-starter organisms are more salt tolerant than the group N streptococci (Turner and Thomas, 1980).
Figure 3.3. The effect of temperature on the proteinase activity of *L. casei* MIL2A

Cells were grown in MRS broth, harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH$_2$PO$_4$ (adjusted to pH 6.0 with 0.2 M Na$_2$HPO$_4$) as described under Methods. The cell suspension (170 mL) was added to 2.0 mL of sodium caseinate solution (1.5% w/v) in 0.2 M phosphate buffer at pH 6.0 and 30 µL of chloroform. The assay mixture was incubated at temperatures from 3 to 55°C for 7 days and the TCA-soluble protein released by *L. casei* MIL2A alone was determined as described under Methods.
Figure 3.4. The effect of sodium and potassium on the proteinase activity of L. casei MIL2A and S. lactis subsp. cremoris BK5

Cells were grown in MRS broth (L. casei MIL2A) or M17 broth (S. lactis subsp. cremoris BK5), harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 6.0 with 0.2 M Na₂HPO₄) as described under Methods. Cell suspensions (1.0 mL) were added to a solution containing 1.0 mL of sodium caseinate (3.0% w/v) in 0.4 M phosphate buffer at pH 6.0, 1.0 mL of either a 0, 3, 6, 9 or 12% (w/v) solution of K⁺ or Na⁺ (as NaCl or KCl) and 30 µL chloroform. The assay mixture was incubated at 37°C (L. casei MIL2A) or 30°C (S. lactis subsp. cremoris BK5) for 7 days and the TCA-soluble protein released by the organisms alone was determined as described under Methods.

(a) L. casei MIL2A
- Proteinase activity in the presence of Na⁺
  □ " " " " " K⁺

(b) Effect of Na⁺
- Proteinase activity of S. lactis subsp. cremoris BK5
  □ " " " " L. casei MIL2A
(d) **Growth media.** Group N streptococci and lactobacilli grown in complex broth media have lower proteinase activities than cells grown in milk (Exterkate, 1976b; Thomas and Mills, 1981; Bolcato, Spottoli and Dal Belin Peruffo, 1973; Hegazi, 1987). As expected, *L. casei MIL2A* grown in MRS broth had 15% less proteinase activity than cells grown in skim milk or 30% less than cells grown in skim milk permeate/sodium caseinate (Table 3.4). Therefore where it is necessary to maximize proteinase activity organisms should be grown in less complex media (e.g. skim milk).

(e) **Heat stability.** The proteinases of lactic acid bacteria added at the beginning of the cheese manufacturing process must be heat tolerant to survive cooking temperatures of 37°C for 90 min or higher depending on the cheese type. As shown in Table 3.5 the proteinase(s) of *L. casei MIL2A* were less affected by heat than those of *S. lactis* subsp. *cremoris* BK5. After a heating regime of 55°C for 10 min *L. casei MIL2A* and *S. lactis* subsp. *cremoris* BK5 retained 52% and 2% respectively of their original proteinase activity. At a heating regime likely to occur in Cheddar cheese manufacture (37°C for 90 min) *L. casei MIL2A* and *S. lactis* subsp. *cremoris* BK5 retained 93% and 59% respectively of their original activities.

3. **Protein Hydrolysis**

(a) **Caseins.** The lactobacilli have the ability to hydrolyse $a_{s1}^-$, $\beta^-$ and $\kappa$-caseins unlike the group N streptococci which preferentially degrade $\beta$-casein (Thomas and Pritchard, 1987). In this study the hydrolysis of the caseins in sodium caseinate by non-starter lactobacilli and the starter *S. lactis* subsp. *cremoris* BK5 were investigated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. As shown in Figure 3.5, *L. casei MIL2A* and 151 and *L. plantarum MIL1A* were able to hydrolyse $a_{s1}^-$ and $\beta$-caseins. *L. casei MIL2A* also appeared to
Table 3.4. The effect of growth media on the proteinase activity of *L. casei* MIL2A

<table>
<thead>
<tr>
<th>Growth media</th>
<th>Proteinase activity (µmol x 10 gly equiv./mg dry weight cells/7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS broth</td>
<td>12.6</td>
</tr>
<tr>
<td>Skim milk</td>
<td>14.9</td>
</tr>
<tr>
<td>Skim milk permeate/sodium caseinate</td>
<td>17.1</td>
</tr>
</tbody>
</table>

*L. casei* MIL2A was grown in either MRS broth, skim milk medium or skim milk permeate/sodium caseinate medium, harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH$_2$PO$_4$ (adjusted to pH 5.8 with 0.2 M Na$_2$HPO$_4$) as described under Methods. The cell suspension (1.0 mL) was added to 2.0 mL of sodium caseinate solution (1.5% w/v) in 0.2 M phosphate buffer at pH 5.8 and 30 µL of chloroform. The assay mixture was incubated at 37°C for 7 days and the TCA-soluble protein released by *L. casei* MIL2A alone was determined as described under Methods.
Table 3.5. The effect of heating on the proteinase activity of *S. lactis* subsp. *cremoris* BK5 and *L. casei* MIL2A

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Relative proteinase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. lactis</em> subsp. <em>cremoris</em> BK5</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>35</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>55</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>37</td>
<td>90</td>
<td>59</td>
</tr>
</tbody>
</table>

Cells were grown in MRS broth (lactobacilli) or M17 broth (*S. lactis* subsp. *cremoris* BK5), harvested by centrifugation and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 5.8 with 0.2 M Na₂HPO₄) as described under Methods. The cell suspensions (5.0 mL) were heated in water baths at the temperature and times indicated and samples (1.0 mL) were added to 2.0 mL of sodium caseinate solution (1.5% w/v) in 0.2 M phosphate buffer at pH 5.8 and 30 μL of chloroform. The assay mixture was incubated at 37°C (*L. casei* MIL2A) or 30°C (*S. lactis* subsp. *cremoris* BK5) for 7 days and the TCA-soluble protein released by the organisms alone was determined as described under Methods.
Figure 3.5. The hydrolysis of sodium caseinate by lactic acid bacteria

Cells were grown in MRS broth (lactobacilli) or M17 broth (S. lactis subsp. cremoris BK5), harvested, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 6.0 with 0.2 M Na₂HPO₄) as described under Methods. The cell suspension (1.0 mL) was added to 2.0 mL of sodium caseinate solution (1.5% w/v) in 0.2 M phosphate buffer at pH 6.0 and 30 µL of chloroform. The assay mixture was incubated at 37°C (lactobacilli) or 30°C (S. lactis subsp. cremoris BK5) for 7 days and samples (100 µL) were prepared and electrophoresed on sodium dodecyl sulphate polyacrylamide gels as described under Methods.

A. L. casei MIL2A (control)
B. " " " (7 days)
C. " 151 (control)
D. " " (7 days)
E. L. plantarum MIL1A (control)
F. " " " (7 days)
G. " 343 (control)
H. " " (7 days)
I. S. lactis subsp. cremoris BK5 (control)
J. " " " (7 days)
hydrolyse \( \kappa \)-casein while \( S. \text{ lactis} \) subsp. \( \text{ cremoris} \) BK5 hyrolysed \( \beta \)-casein and \( \kappa \)-casein. No detectable hydrolysis of casein occurred with \( L. \text{ plantarum} \) 343, probably due to the low proteolytic activity associated with this organism (Table 3.1). A densitometer scan of the acrylamide gel of the \( L. \text{ casei} \) MIL2A digest of sodium caseinate clearly showed \( \alpha_{\text{s}1} \)-, \( \beta \)- and \( \kappa \)-casein hydrolysis (Figure 3.6).

Both \( L. \text{ casei} \) 151 and \( L. \text{ plantarum} \) MIL1A hydrolysed \( \alpha_{\text{s}1} \)- and \( \beta \)-caseins (Table 3.6). \( L. \text{ casei} \) MIL2A preferentially hydrolysed \( \alpha_{\text{s}1} \)-casein although significant levels of \( \beta \)-casein were also degraded. \( \beta \)-casein was preferentially degraded by \( S. \text{ lactis} \) subsp. \( \text{ cremoris} \) BK5 although some \( \alpha_{\text{s}1} \)-casein was used. In this experiment the apparent proteolytic activity of \( S. \text{ lactis} \) subsp. \( \text{ cremoris} \) BK5 was lower than previously observed (Figure 3.2), probably due to the higher pH of the assay mixture (pH 6.2).

(b) Whey proteins. The intracellular proteinases of some lactobacilli can hydrolyse whey proteins (El Soda et al., 1982) however this is not an important characteristic in relation to normal cheese maturation as whey proteins represent only 1% of the proteins in cheese. The introduction of ultrafiltration in cheese manufacture (Sutherland and Jameson, 1981) would increase the proportion of whey proteins in the product and therefore it may be an advantage if the non-starter lactobacilli could hydrolyse these proteins. Furthermore heat denatured whey proteins are insoluble and have been reincorporated into cheese milk in order to increase overall cheese yield (Brown and Ernstrom, 1982). As shown in Table 3.7 there was some degradation of \( \alpha \)-lactalbumin by whole cells of \( L. \text{ casei} \) MIL2A but degradation of \( \beta \)-lactoglobulin only occurred after partial denaturation (82 to 95% soluble protein, data not shown) of the protein by heat. The heat treatments had little effect on the degradation of \( \alpha \)-lactalbumin or bovine serum albumin.

4. The Release of Peptides

Isoelectric focusing (IEF) has been used to separate
Figure 3.6. The hydrolysis of sodium caseinate by L. casei MIL.2A

Cells were grown, harvested, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 6.0 with 0.2 M Na₂HPO₄) as described under Methods. The cell suspension (1.0 mL) was added to 2.0 mL of sodium caseinate solution (1.5% w/v) in 0.2 M phosphate buffer at pH 6.0 and 30 μL of chloroform. The assay mixture was incubated at 37°C for 7 days and samples (100 μL) were prepared, electrophoresed on sodium dodecyl sulphate gels and scanned as described under Methods.

(a) control
(b) 7 days
Table 3.6. The degradation of $\alpha_{s1}$- and $\beta$-casein by lactic acid bacteria

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>TCA-soluble protein released ((\mu)mol x 10 gly equiv./mg dry wt. cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_{s1}$-casein</td>
</tr>
<tr>
<td><strong>L. casei</strong></td>
<td></td>
</tr>
<tr>
<td>MIL2A</td>
<td>18.3</td>
</tr>
<tr>
<td>151</td>
<td>14.6</td>
</tr>
<tr>
<td><strong>L. plantarum</strong></td>
<td></td>
</tr>
<tr>
<td>ML1A</td>
<td>15.9</td>
</tr>
<tr>
<td><strong>S. lactis subsp. cremoris</strong></td>
<td></td>
</tr>
<tr>
<td>BK5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Cells were grown in skim milk medium (10% w/v), harvested by centrifugation, washed and resuspended at 30 mg (dry weight)/mL in 0.1 M $\text{KH}_2\text{PO}_4$ (adjusted to pH 6.2 with 0.1 M $\text{Na}_2\text{HPO}_4$) as described under Methods. The cell suspension (1.0 mL) was added to a solution containing 2.0 mL of aqueous $\alpha_{s1}$- (1.3%) or $\beta$-casein (1.5% w/v), 1.0 mL of 0.2 M phosphate buffer at pH 6.2 and 30 µL of chloroform. The assay mixture was incubated at 37°C (lactobacilli) or 30°C (S. lactis subsp. cremoris BK5) for 7 days and the TCA-soluble protein released by the organisms alone was determined as described under Methods.
Table 3.7. The effect of heating on the degradation of whey proteins by L. casei MIL2A

<table>
<thead>
<tr>
<th>Protein</th>
<th>TCA-soluble protein released (μmol x 10 gly equiv./mg dry wt. cells)</th>
<th>Protein heat treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unheated control</td>
<td>65°C</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>3.6</td>
<td>1.9</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>0</td>
<td>3.6</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium caseinate</td>
<td>14.2</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd, not determined.

*L. casei MIL2A* was grown in skim milk medium, harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 5.8 with 0.2 M Na₂HPO₄) as described under Methods. The cell suspension (1.0 mL) was added to 2.0 mL of the appropriate protein (1.5% w/v solution heated at 65°C, 80°C and 100°C for 30 min) in 0.2 M phosphate buffer at pH 5.8 and 30 μL of chloroform. The assay mixture was incubated at 37°C for 7 days and the TCA-soluble protein released by *L. casei* MIL2A alone was determined as described under Methods.
the peptides released from casein by lactobacilli and consequently provide a means of finger printing different organisms (Righetti, et al., 1980). In this study IEF was used to separate the peptides released after \( \alpha_{s1} \)-casein digestion by \textit{L. casei} MIL2A and \textit{S. lactis} subsp. cremoris BK5 and as shown in Figure 3.7 only a limited number of peptides could be detected. The peptide patterns were also distorted on the gel, presumably by the presence of the phosphate buffer in the casein digest (Pharmacia Fine Chemicals, 1982). Generally \( \alpha_{s1} \)-casein digestion was accompanied by the appearance of a number of bands with isoelectric points (pI) between 5.0 and 6.0. No bands were evident above pI 6.0 although it has been shown that some major bands can have pI values in this range after digestion of sodium caseinate by lactobacilli (Righetti et al., 1980).

No major differences were evident in the peptide patterns produced by \textit{L. casei} MIL2A and \textit{S. lactis} subsp. cremoris BK5 which suggests that a common mechanism for the initial breakdown of \( \alpha_{s1} \)-casein may exist in these organisms. However further degradation by the various peptidases of the organisms may lead to the formation of differing end products.

Analysis of the peptides released after casein hydrolysis was also investigated using HPLC. As shown in Figure 3.8 HPLC can be used to detect differences in the peptide patterns of control and digested \( \alpha_{s1} \)-casein samples. In the \( \alpha_{s1} \)-casein control sample (Figure 3.8a and f) only 2 major hydrophilic peaks were evident and cells of \textit{L. casei} MIL2A, ML1B and CV1A and \textit{S. lactis} subsp. cremoris BK5 incubated without \( \alpha_{s1} \)-casein produced differing peptide patterns in terms of number of peaks and their relative sizes (Figure 3.8b, d, g and i). The presence of peptides in this medium suggests that either residual casein was still attached to the cells after harvesting from the skim milk growth medium or there was degradation of the cells themselves during the long incubation period. After an 8 day incubation of the same cells with \( \alpha_{s1} \)-casein there was an increase in the peak
Figure 3.7. Isoelectric focusing of αs1-casein digests

αs1-casein digests were obtained by the incubation of L. cæbei MIL2A and S. lactis subsp. cremoris BK5 with αs1-casein for up to 10 days as described under Methods. Cell free digests were added to the sample buffer and samples (15 μL applied to the anodic side) were isoelectrically focused on a 1 mm thick gel containing 5% (W/V) acrylamide, 2% (V/V) ampholyte (Bio-Lyte 3/10, Bio-Rad, Richmond, California, USA) and 7 M urea as described under Methods.

A. Sodium caseinate control
B. S. lactis subsp. cremoris BK5, 0 days
C. " " " " , 2 days
D. " " " " , 4 days
E. " " " " , 6 days
F. " " " " , 8 days
G. " " " " , 10 days
H. L. casei MIL2A, 0 days
I. " " " , 4 days
J. " " " , 6 days
K. " " " , 8 days
Figure 3.8. HPLC peptide pattern of αs1-casein after hydrolysis by milk
grown cells of lactic acid bacteria

Cells were grown in skim milk
medium (10% w/v), harvested by
centrifugation, washed and
resuspended at approximately 30 mg
(dry weight)/mL in distilled water
as described under Methods. The
cell suspensions (1.0 mL) were
added to a solution containing 1.0
mL of aqueous αs1-casein (1.5% w/v),
1.0 mL of 0.15 M ammonium
acetate (adjusted to pH 5.8 with
0.15 M acetic acid) and 30 μL of
chloroform. The assay mixture was
incubated at 37°C (lactobacilli) or
25°C (S. lactis subsp. cremoris
BM4) for 8 days and samples
prepared for HPLC analysis as
described under Methods.
Figure 3.8. (continued) HPLC peptide pattern of αs1-casein after hydrolysis by milk grown cells of lactic acid bacteria

(f) αs1-casein control

(g) L. casei CV1A

(h) L. casei CV1A plus αs1-casein

(i) S. lactis subsp. cremoris BK5

(j) S. lactis subsp. cremoris BK5 plus αs1-casein
area of the peptides but the increases were not uniform 
(Figure 3.8c, e, h and i). Further, the initial peak 
eluted by cells of L. casei MIL2A alone could not be 
detected after incubation with \( a_{s1}\)-casein (Figure 3.8b and 
c). L. casei CV1A produced a number of additional peptides 
20 and 35 min after sample injection indicating a possible 
difference in the proteolytic system of this organism.

In a further comparison, the incubation of L. casei 
CV1A and S. lactis subsp. cremoris BK5 with sodium 
caseinate (Figure 3.9) resulted in a peptide pattern very 
similar to that produced with \( a_{s1}\)-casein (Figure 3.8). 
This suggests that \( a_{s1}\)-casein may be preferentially 
degraded by these organisms as shown previously for L. 
casei MIL2A (see Table 3.6).

5. Peptidase Activities

Peptidase enzyme activities were measured for L. casei 
CV1A, MIL2A, MIL2B, 151, L. plantarum 343 and S. lactis 
subsp. cremoris BK5 and its' prt\(^{-}\) derivative. The mode of 
action of the peptidases investigated is presented in 
Section 4 of the Appendices.

(a) Aminopeptidase. The aminopeptidase activities of 
all the organisms tested exhibited greater specificity for 
L-h-ocinc-p-nitroanilide and L. casei strains had the more 
active aminopeptidases (Table 3.8). The level of 
aminopeptidase activity in S. lactis subsp. cremoris BK5 
and its' prt\(^{-}\) derivative were similar indicating that the 
derivatization procedure had not affected aminopeptidase 
enzyme activity.

(b)  Proline iminopeptidase \( \text{Pro}^-_{i}(X)n \), where \( x \) 
represents an amino-acyl group. As shown in Table 3.8 all 
the organisms tested exhibited proline iminopeptidase 
activity against the substrate L-proline-p-nitroanilide. 
However all strains of L. casei exhibited greater activity 
than either L. plantarum 343 or S. lactis subsp. cremoris 
BK5 and its' prt\(^{-}\) derivative.
Figure 3.2. HPLC peptide pattern of sodium caseinate after hydrolysis by L. casei CV1A and S. lactis subsp. cremoris BK5.

The experimental conditions were as described in Figure 3.1 except that sodium caseinate solution (1.5% w/v) was used as substrate.
### Peptidase activities of lactic acid bacteria

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Aminopeptidase*</th>
<th>Proline iminopeptidase*</th>
<th>Endopeptidase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-leu-p-nitroanilide</td>
<td>L-ala-p-nitroanilide</td>
<td>L-pro-p-nitroanilide</td>
</tr>
<tr>
<td>L. casei CV1A</td>
<td>35.3</td>
<td>5.1</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>38.4</td>
<td>5.8</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>41.3</td>
<td>5.9</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>25.9</td>
<td>5.0</td>
<td>0.81</td>
</tr>
<tr>
<td>L. plantarum 343</td>
<td>5.4</td>
<td>2.8</td>
<td>0.25</td>
</tr>
<tr>
<td>S. lactis subsp. cremoris BK5</td>
<td>17.1</td>
<td>1.9</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>18.9</td>
<td>2.2</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*μmol x 10 nitroaniline released/mg protein/h

Cells were grown in MRS broth (lactobacilli) or M17 broth (S. lactis subsp. cremoris), harvested by centrifugation, washed, resuspended and cell free extracts were prepared as described under Methods. Aminopeptidase activity (using the substrates L-leucine-p-nitroanilide and L-alanine-p-nitroanilide), proline iminopeptidase activity (using the substrate L-proline-p-nitroanilide) and endopeptidase activity (using the substrates N-glutaryl-l-phenylalanine p-nitroanilide and N-succinyl-l-phenylalanine p-nitroanilide) were determined as described under Methods.
(c) **Endopeptidase.** Endopeptidase activities against the substrates N-glutaryl-L-phenylalanine and N-succinyl-L-phenylalanine are presented in Table 3.8. The endopeptidase activity for all of the organisms was very low and as hydrolysis of the substrates can also be carried out by arylamidases (El Soda and Desmazeaud, 1981) true endopeptidase activity cannot be assumed. Nevertheless low activities were evident, particularly with *L. casei* C11A and *S. lactis* subsp. *cremoris* BK5 and its' **prt**-derivative.

(d) **Aminopeptidase-P X-Pro-(X)n where** X **represents any amino acid.** Aminopeptidase-P activity against the substrate poly-L-proline was not detected for any of the organisms tested under the conditions used.

(e) **Dipeptidase.** The dipeptides glycyl-L-tyrosine, glycyl-L-phenylalanine and L-leucyl-glycine were used to assay for dipeptidase activity. As shown in Table 3.9 all substrates were hydrolysed by the organisms although L-leucyl-glycine was the preferred substrate for most organisms except *L. casei* MIL28 and 151 which preferentially hydrolysed glycyl-L-phenylalanine.

(f) **Tripeptidase.** As shown in Table 3.9 all the organisms tested exhibited tripeptidase activity against the substrate DL-alanyl-DL-leucylglycine. The level of tripeptidase activity in strains of *L. casei* was generally higher than *L. plantarum* 343 and the starter organisms tested.

(g) **Carboxypeptidase.** The synthetic peptides N-carbobenzoxy-glycyl-L-proline and hippuryl-L-arginine were used to assay for carboxypeptidase activity. However no hydrolysis products could be detected indicating that the organisms did not possess carboxypeptidase activity towards these substrates.
### Table 3.9. Dipeptidase and tripeptidase activities of lactic acid bacteria

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Dipeptidase activity*</th>
<th>Tripeptidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycyl-L-tyrosine</td>
<td>Glycyl-L-phenylalanine</td>
</tr>
<tr>
<td><strong>L. casei</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV1A</td>
<td>3.0</td>
<td>3.8</td>
</tr>
<tr>
<td>MIL2A</td>
<td>4.0</td>
<td>6.2</td>
</tr>
<tr>
<td>MIL2B</td>
<td>1.3</td>
<td>11.7</td>
</tr>
<tr>
<td>151</td>
<td>7.1</td>
<td>15.7</td>
</tr>
<tr>
<td><strong>L. plantarum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>343</td>
<td>8.6</td>
<td>8.8</td>
</tr>
<tr>
<td><strong>S. lactis subsp. cremoris</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK5</td>
<td>9.4</td>
<td>6.6</td>
</tr>
<tr>
<td>BK5 prt</td>
<td>7.2</td>
<td>13.4</td>
</tr>
</tbody>
</table>

*μmol x 10 gly equiv. released/mg protein/h

Cells were grown in MRS broth (lactobacilli) or M17 broth (S. lactis subsp. cremoris BK5), harvested by centrifugation, washed, resuspended and cell free extracts were prepared as described under Methods. Dipeptidase activity (using the substrates glycyl-L-tyrosine, glycyl-L-phenylalanine and L-leucylglycine) and tripeptidase activity (using the substrate DL-alanyl-DL-leucylglycine) were determined as described under Methods.
6. The Release of Amino Acids

As shown in Figure 3.10 L. casei MIL2A, ML1B and CV1A and S. lactis subsp. cremoris BK5 released amino acids from $\alpha_s1$-casein. L. casei MIL2A and ML1B were the more active with glutamic acid, histidine, tyrosine, phenylalanine, leucine and lysine the predominant amino acids released. However there were differences between organisms in the levels released. For example glutamic acid, tyrosine, phenylalanine and leucine were released by L. casei MIL2A at 275, 130, 92 and 159 nmol/mg dry wt. cells/8 days respectively compared to 77, 67, 42, and 88 nmol/mg dry wt. cells/8 days for S. lactis subsp. cremoris BK5.

The incubation of L. casei CV1A with either $\alpha_s1$-casein or sodium caseinate did not greatly alter the levels of amino acids released although there was a small increase for most amino acids (Figure 3.11). This is consistent with the peptide analysis (Figure 3.9) and further supports the observation that $\alpha_s1$-casein is the preferred substrate when casein is hydrolysed by the non-starter lactobacilli.

Proline is an important amino acid in flavour of cheese particularly Swiss (Hintz, Slatter and Harper, 1956), and cannot be detected by the method used for the amino acid analysis as shown in Figure 3.10 as it is unable to form an o-phthalaldehyde/2 mercaptoethanol derivative. Therefore the method of Cooper, Lewis and Turnbull, (1984) was used to detect proline in the $\alpha_s1$-casein digests and as shown in Table 3.10 L. casei MIL2A released about 62% more proline than S. lactis subsp. cremoris BK5.

7. Decarboxylation of Amino Acids

The formation of amines in Cheddar cheese may represent a hazard to certain individuals when large amounts are ingested or the natural mechanisms for their degradation are inhibited or genetically deficient. As shown in Table 3.11 the only amino acids decarboxylated by the organisms tested were tyrosine and arginine. Tyrosine was decarboxylated to tyramine (chromatogram not shown) but only by S. lactis subsp. cremoris BK5 and S. faecium SD1
Cells were grown in skim milk medium (10% w/v), harvested by centrifugation, washed and resuspended at approximately 30 mg (dry weight)/mL in distilled water as described under Methods. The cell suspension (1.0 mL) was added to a solution containing 1.0 mL of aqueous casein (1.5% w/v), 1.0 mL of 0.15 M ammonium acetate (adjusted to pH 5.8 with 0.15 M acetic acid) and 30 mL chloroform. The assay mixture was incubated at 37°C (lactobacilli) or 25°C (S. lactis subsp. cremoris BK5) for 8 days. Cell blank samples without substrate were run simultaneously and the individual free amino acids present in the cell free medium were determined by HPLC as described under Methods.

(a) L. casei MIL2A
(b) " ML1B
Figure 3.10. (continued) Release of amino acids from $\alpha_{s1}$-casein by lactic acid bacteria

(c) L. casei CV1A
(d) S. lactis subsp. cremoris BK5
Figure 3.11. Release of amino acids from sodium caseinate by L. casei CV1A

The experimental conditions were as described in Figure 3.10 except that sodium caseinate solution (1.5% w/v) was also used as substrate.

- L. casei CV1A plus αs1-casein
- "   "   " sodium caseinate
Table 3.10. Proline release from α_{s1}-casein by lactic acid bacteria

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Proline released (nmol/mg dry wt. cells/8 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei MIL2A</td>
<td>128.4</td>
</tr>
<tr>
<td>S. lactis subsp. cremoris BK5</td>
<td>79.3</td>
</tr>
</tbody>
</table>

Cells were grown in skim milk medium (10% w/v), harvested by centrifugation, washed and resuspended at approximately 30 mg (dry weight)/mL in distilled water as described under Methods. The cell suspension (1.0 mL) was added to a solution containing 1.0 mL of aqueous α_{s1}-casein (1.5% w/v), 1.0 mL of 0.15 M ammonium acetate (adjusted to pH 5.8 with 0.15 M acetic acid) and 30 μL chloroform. The assay mixture was incubated at 37°C (L. casei MIL2A) or 30°C (S. lactis subsp. cremoris BK5) for 8 days and the proline present in the cell free medium determined by HPLC as described under Methods.
<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Tyr</th>
<th>His</th>
<th>Trp</th>
<th>Arg</th>
<th>Phe</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. casei</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CV1A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>MIL2A</td>
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<tr>
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</tr>
<tr>
<td>151</td>
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<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td><em>L. plantarum</em></td>
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<tr>
<td>343</td>
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<td>-</td>
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<tr>
<td><em>S. lactis</em> subsp <em>cremoris</em></td>
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<tr>
<td>BK5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>S. faecium</em></td>
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<td></td>
</tr>
<tr>
<td>SD1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Cells were grown in MRS broth (*lactobacilli*) or ML7 broth (*S. lactis* subsp. *cremoris* BK5 and *S. faecium* SD1) harvested by centrifugation, washed and resuspended at 20 mg (dry weight)/mL in 0.1 M KH₂PO₄ (adjusted to pH 5.5 with 0.1 M Na₂HPO₄) as described under Methods. The presence or absence of decarboxylase activity was determined as described under Methods.
which is known to have tyrosine decarboxylase activity. Arginine was degraded by *S. lactis* subsp. *cremoris*, *S. faecium* and *L. casei* presumably through a decarboxylation reaction to form urea and ornithine (Dulley, 1974).
DISCUSSION

1. Proteinase Activity

The degradation of $\alpha_{s1}$-casein in Cheddar cheese can go to completion and although hydrolysis by starter bacteria (to a limited extent) and non-starter lactobacilli is possible, chymosin is the principal catalytic enzyme (O’Keeffe, Fox and Daly, 1976). The non-starter lactobacilli L. casei MIL2A, 151 and L. plantarum MIL1A possess a proteolytic system capable of degrading both $\alpha_{s1}$- and $\beta$-casein. L. casei MIL2A is also able to hydrolyse $\kappa$-casein and to a limited extent $\alpha$-lactalbumin. Some hydrolysis of $\beta$-lactoglobulin by L. casei MIL2A occurred but only after partial denaturation of the protein by heat. The ability of L. casei MIL2A to hydrolyse $\alpha_{s1}$-, $\beta$- and $\kappa$-casein is in contrast to S. lactis subsp. cremoris BK5 which degrades $\beta$-casein and very little $\alpha_{s1}$-casein. Therefore high numbers of non-starter lactobacilli in cheese would probably assist in the hydrolysis of $\alpha_{s1}$-casein, consistent with normal protein catabolism in maturing cheese.

In Cheddar cheese $\alpha_{s1}$-casein undergoes extensive degradation during maturation while $\beta$-casein degradation is limited by the moisture content, pH and NaCl concentration of the cheese (Fox and Walley, 1971; Creamer, 1975). It appears that the action of chymosin and natural milk proteinases account for most of the $\beta$-casein degradation that occurs and studies on Gouda cheese indicate that starters (S. lactis subsp. cremoris) are accountable for about 10% of the $\beta$-casein degraded (Visser and de Groot-Mostert, 1977). In the present study non-starter lactobacilli hydrolysed $\beta$-casein in vitro suggesting that if the organisms were present in cheese they may also contribute to the degradation of this protein. Again if the levels of lactobacilli in cheese were increased the proportion of $\beta$-casein degraded may also increase and must be considered if atypical flavours or texture arise.
Protein degradation in the manufacture of Cheddar cheese must not be excessive in the vat stage of manufacture or reduced yields may result. *L. casei* MIL2A, 151 and *L. plantarum* MIL1A exhibited lower or similar proteinase activities to *S. lactis* subsp. *cremoris* BK5 when compared under optimum pH and temperature conditions. These results indicated that if selected lactobacilli were added to cheesemilk at the same time as the starter organism total proteinase activity would be of the same order of magnitude. However the conditions during cheese manufacture and maturation are not optimal and other factors must be considered (Figure 3.12 outlines the pH and temperatures encountered during manufacture and maturation of cheese).

The proteolytic system of *S. lactis* subsp. *cremoris* BK5 is adversely affected by the cooking temperature of cheese manufacture (37°C) the NaCl in cheese (equivalent to 5% w/w in the moisture phase) and by the final pH in the cheese (pH 5.2). These results were confirmed in the present investigation. In contrast, the proteolytic system of a typical lactobacilli (*L. casei* MIL2A) had an optimum at 37°C and was more tolerant to NaCl. *L. casei* MIL2A and *S. lactis* subsp. *cremoris* BK5 also have similar proteinase activities at 37°C and pH 5.2 (Figure 3.2) again indicating that proteinases from either starter or non-starter microorganisms could be of the same order in the cheese during storage if their numbers are similar. These organisms also demonstrated similar proteinase activities at 8°C indicating that the relative contribution of the non-starter lactobacilli to proteolysis would be maintained during cheese maturation. This would be desirable if no bitter peptides accumulated.

The ability of the non-starter lactobacilli to hydrolyse the major peptides resulting from the initial degradation of proteins is necessary to minimize peptide accumulation and to further promote flavour development (Figure 1.1). While active peptidases are required for this purpose, a range of peptidases comparable to that of the group N streptococci is desirable to prevent an
Figure 3.12. Temperature and pH changes during the manufacture and maturation of Cheddar Cheese (Scott, 1986)

--- Temperature
---- pH
unbalanced spectrum of small peptides and amino acids. The non-starter lactobacilli investigated in this study all possessed good aminopeptidase, dipeptidase and tripeptidase activities and some iminopeptidase activity was evident. A small amount of apparent endopeptidase activity was also evident in strains of _L. casei_ but virtually no activity was found in _L. plantarum_. However, as endopeptidase activity was determined using N-glutaryl-l-phenylalanine-p-nitroanilide and N-succinyl-l-phenylalanine-p-nitroanilide the activity detected could also be an arylamidase type enzyme normally associated with the ribosomes which is present in _L. casei_ but not in _L. plantarum_ (El Soda, Desmazeaud and Bergère, 1978; El Soda et al., 1983). No true endopeptidase activity has thus been demonstrated and the activity found in this study may be due to an aryl-peptidyl amidase. Aminopeptidase-P and carboxypeptidase activity could not be detected under the assay conditions used although a narrow specificity carboxypeptidase has been reported previously for _L. casei_ (El Soda, Desmazeaud and Bergère, 1978) and aminopeptidase-P activities are present in a number of other species of lactobacilli (Hickey, Hillier and Jago, 1983c).

In comparison to _S. lactis_ subsp. _cremoris_ BK5, the _L. casei_ strains had a more active aminopeptidase, proline iminopeptidase and tripeptidase systems. Dipeptidase activities were similar although variations occurred depending on the substrate used. Interestingly a prt-derivative of _S. lactis_ subsp. _cremoris_ BK5 exhibited a similar range of peptidase activities to the parent organism indicating that the loss of proteinase activity may have little effect on the peptidase system of this organism.

In general the non-starter lactobacilli can be described as having a complex peptidase system similar to _S. lactis_ subsp. _cremoris_ BK5 but exhibiting greater activity under conditions arising in cheese and therefore they may not produce an unbalanced range of peptides or amino acids if used in cheese manufacturing.
2. Protein Catabolites

While peptides containing a high proportion of hydrophobic amino acid residues are responsible for the bitter flavour defect in Cheddar cheese (Thomas and Pritchard, 1987) it is not unreasonable to expect other peptides to play a positive role in flavour development (McGugan, Emmons and Larmond, 1979). Qualitative analysis by IEF of the peptides formed after digestion of αs1-casein suggested that L. casei MIL2A and S. lactis subsp. cremoris BK5 had a common mechanism for the initial breakdown of the protein. Further analysis using HPLC revealed slight differences in the peptide patterns for both the non-starter lactobacilli (i.e. L. casei MIL2A, ML1B and CV1A) and S. lactis subsp. cremoris BK5 in terms of the number of peptides and their relative sizes. However these variations may reflect the relative differences in the proteinase and peptidase activities of the organisms and raises the possibility that flavour balance may be influenced (favourably or unfavourably) if the organisms were used in Cheddar cheese manufacture. Differences in the levels and proportions of amino acids released from αs1-casein by the non-starter lactobacilli and S. lactis subsp. cremoris BK5 again suggested that there may be variations in the flavour compounds ultimately formed by these organisms. L. casei MIL2A and ML1B both released greater amounts of amino acids from αs1-casein than S. lactis subsp. cremoris BK5 and therefore may be suitable organisms for influencing flavour development during cheese maturation.

3. Conclusion

In this study the proteolytic system of non-starter lactobacilli was investigated in order to select a suitable organism for influencing cheese flavour. These organisms all possessed relatively low proteinase activity which tolerated the cheese manufacturing process and were able to degrade milk proteins, particularly αs1-casein, more efficiently under cheese maturation conditions than
the starter organism *S. lactis* subsp. *cremoris* BK5. They also possessed a broad range of peptidases comparable to *S. lactis* subsp. *cremoris* BK5 although the peptide patterns and the relative proportions of amino acids produced differed. Generally, increased levels of amino acids were formed and no decarboxylase activity was evident.
CHAPTER 4

THE USE OF LACTOBACILLI IN THE MANUFACTURE OF CHEDDAR CHEESE
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INTRODUCTION

1. Accelerated Maturation

The maturation of Cheddar cheese and the development of full flavour is a time-consuming process necessitating storage for six to twelve months. Therefore any method which can accelerate maturation and reduce storage time will provide substantial savings. Methods to improve maturation have been based on increases in proteolysis and lipolysis in cheese resulting in increased flavour development (Law, 1984). In view of this, accelerated maturation has involved the use of exogenous enzymes, attenuated starters and modified manufacturing conditions (Law, 1980).

It is generally recognized that protein degradation is the major process influencing body and texture changes and the formation of flavour in cheese. In order to promote protein hydrolysis exogenous enzymes such as fungal rennets, neutral and acid proteinases and peptidases have been added to cheese (Kosikowski and Iwasaki, 1975; Law and Wigmore, 1982a,b; Fedrick et al., 1986). However the use of single or narrow spectrum proteolytic enzymes appears to have resulted in strong and unbalanced flavours (Kosikowski and Iwasaki, 1975) and it has been suggested that more complex enzyme mixtures may overcome this problem. Nevertheless trials using proteinase/peptidase mixtures of broad specificity from a Pseudomonas species also resulted in off-flavour development particularly after eight weeks maturation (Law, 1980).

Group N streptococci (starter organisms) possess the range of proteinases and peptidases most suitable for accelerated maturation of Cheddar cheese but high numbers added to the vat during manufacture would generate excessive acid production. Attempts have therefore been made to modify lactic acid bacteria and prevent or reduce
acid production without affecting proteolytic activity. For example, cell free extracts of starter bacteria used in combination with a commercial neutral proteinase accelerated the maturation of Cheddar cheese (Law and Wigmore, 1983) but too many of the peptidases from the cell free extract were lost in the whey (Law and Sharpe, 1975).

Whole cells which are physically entrapped in the cheese curd have also been modified to prevent acid production. These include treatment with lysozyme (Law, Castanon and Sharpe, 1976b), treatment with organic solvents (Exterkate, 1979), the production of x-ray mutants (Dilanian, Makarian and Chuprina, 1976; Singh and Ranganathan, 1977) and the use of mutants unable to hydrolyse lactose (lac⁻) or proteins (prt⁻) (Grieve and Dulley, 1983). Further, whole cells of lactobacilli subjected to sublethal heat treatments and used with normal starter have been shown to increase proteolysis and flavour scores of Swedish 'Household chees' making it possible to reduce storage time by one month (Pettersson and Sjöström, 1975). Increased proteolysis also occurred in Gouda cheese to which heat and freeze shocked strains of _L. helveticus_ were added but off flavours developed (Bartels, Johnson and Olson, 1987a,b).

The use of unmodified cells would only be possible where they do not produce excessive acid in the vat stage or where starter numbers can be reduced to compensate for any acid formed by the additional organisms (Hickey et al., 1983).

Using non-starter organisms Sherwood (1939) found that _L. casei_ and _L. plantarum_ could improve Cheddar cheese flavour although _L. casei_ was shown to produce an acid flavour and 'short' body as maturation proceedd (Tittsler et al., 1948). In contrast the addition of whole cells of _L. casei_ at the salting stage of Cheddar cheese manufacture did not result in the formation of adverse flavours but there was only a slight increase in proteolysis products relative to the control cheese (El Soda et al., 1981). Increased levels of _L. casei_ may have
had a more pronounced effect.

The promising results obtained with unmodified cells of non-starter bacteria, particularly the lactobacilli, suggests that a controllable and accelerated maturation system may be possible for Cheddar cheese. Moreover, in selecting suitable organisms for inclusion in Cheddar cheese manufacture the role and function of the starter organism must be considered (p. 5) and the added organisms must possess an appropriate proteolytic system (p. 50). In past investigations these selection criteria may not have been fully considered which would account for the variable results obtained.

2. Alternate Applications For Lactobacilli in Cheddar Cheese

In addition to the role for lactobacilli in accelerated maturation of normal Cheddar cheese there are two other potential applications. These relate to new procedures for manufacturing Cheddar cheese where either the composition of the cheese is modified using ultrafiltration (Sutherland and Jameson, 1981) or proteinase negative (prt⁻) organisms have been used as starters to effect gains in bacteriophage control (Richardson et al., 1983).

(a) Ultrafiltered milk. In conventional Cheddar cheese manufacture, chymosin coagulates only the casein fraction of the milk protein and the whey proteins, which remain in solution, are lost with the whey as it drains from the curd particles. As the whey proteins comprise approximately 15% of the total milk protein (Gordon and Kalan, 1974), there would be a considerable increase in cheese yield if these proteins were retained within the curd.

Ultrafiltration of the cheese milk, before the addition of starter and rennet, is the method currently favoured for incorporatingundenatured whey proteins into Cheddar cheese curd. The method requires that the whey volume be reduced as much as possible in order to minimize the loss of whey proteins. The technology required to consistently
produce good quality Cheddar cheese from ultrafiltered milk has reached commercial practice with the introduction of the APV-SiroCurd (APV Baker Pty. Ltd., Melbourne, Australia). However the development of flavour in this cheese may vary slightly from cheese produced by the traditional method if the microbial environment of the final cheese is allowed to differ significantly from 'normal'. The role of non-starter microflora in Cheddar cheese made from ultrafiltered milk has not yet been reported.

(b) Proteinase Negative Starter Bacteria. The loss of proteinase activity in starter bacteria occurs with the spontaneous loss of plasmid deoxyribonucleic acid (DNA) which encodes a proteinase gene(s) (McKay, 1983). Peptidase activities are unaffected (Exerkate, 1976a). In manufacturing, it has been shown that Cheddar cheese made using 45 to 75% prt⁻ organisms produced less bitterness than cheese made from normal starter (prt⁺) (Mills and Thomas, 1980). Proteinase negative cells also produce less acid per cell and hence greater numbers are required in cheesemaking and it was considered that the extra starter might compensate for the loss of proteinase activity as it affects maturation (Richardson et al., 1983).

Proteinase negative cells are also unable to multiply significantly in the vat due to the limited availability of non-protein nitrogen. This results in 1 or 2 generations of growth thereby reducing the opportunity for bacteriophage proliferation (Gamay et al., 1982). Normal starter cells must experience 5 to 6 generations in the vat for sufficient acid and susceptibility to bacteriophage is increased accordingly. A greater resistance to antibiotics has also been evident for prt⁻ cells (Richardson, 1984; Hafez, Brown and Richardson, 1985).

Further it has been claimed that the exclusive use of prt⁻ cells for Cheddar cheese manufacture results in greater product yield (O'Leary and Hicks, 1982), less bitterness (Lowrie et al., 1972) and more consistent
manufacturing times (Shelaih et al., 1983).

As expected the lack of cell wall proteinase also affects maturation in cheese made using prt\(^{-}\) starter in that flavour developed more slowly than in the control cheeses even though body and texture scores were similar (Oberg et al., 1986). Clearly the inclusion of non-starter lactobacilli in cheese made with prt\(^{-}\) starter cells may assist flavour development. Lactobacilli selected for use in normal Cheddar cheese manufacture were therefore also applied to cheese made with prt\(^{-}\) cultures in the present investigation.

3. Indices of Maturation

Amino acids were among the first compounds to be investigated for their contribution to cheese flavour. While many workers (Harper and Swanson, 1949; Kosikowski, 1951; Mulder, 1952; Kristofferson and Gould, 1960) observed a correlation between the level of free amino acids and cheese flavour, others (Dacre, 1953; Mabbot, 1955; Law, Castanon and Sharpe, 1976a) did not. Thus amino acids were generally considered to make little or no direct contribution to Cheddar cheese flavour. The amino acids, however, did produce an essential background component and their numerous degradation products may be all important in the formation of Cheddar cheese flavour. McGugan, Emmons and Larmond (1979) showed that a water-soluble fraction containing salts, amino acids and peptides contributed most to the intensity of Cheddar cheese flavour.

Aston and Douglas (1983) attempted to relate the concentrations of the volatile sulphur compounds (hydrogen sulphide, carbonyl sulphide, dimethyl sulphide and methanethiol), PTA-soluble nitrogen and TCA-soluble tyrosine to the flavour of Cheddar cheeses. Significant correlations between methanethiol levels and flavour assessments were observed from data obtained over the entire maturation period. However, Aston and Douglas (1983) concluded that the methanethiol levels were not suitable indicators of maturity in normal cheese because
(i) the variations in flavour scores for cheeses with similar methanethiol levels were large, (ii) the levels of methanethiol decreased after approximately six months of ripening, and (iii) the levels of methanethiol did not correlate with flavour assessments at individual sampling ages. No significant correlations between the level of hydrogen sulphide, carbonyl sulphide or dimethyl sulphide and Cheddar flavour were observed.

The relationships between PTA-soluble nitrogen and TCA-soluble tyrosine levels and flavour assessments were stronger than those of methanethiol (Aston, Durwood and Dulley, 1983). The correlation coefficients calculated for both PTA-soluble nitrogen and TCA-soluble tyrosine with total flavour, mature flavour and the estimated age were all significant at the $P=0.001$ level and are in agreement with the earlier results of Kosikowski (1951), Kristofferson and Gould (1960) and Pettersson and Sjöström (1975). Thus PTA-soluble nitrogen and TCA-soluble tyrosine were used for the quantitative estimation of maturation in cheeses.

4. Cheesemaking Parameters

In assessing the effectiveness of lactobacilli as a means of dominating the non-starter microflora and influencing Cheddar cheese maturation a number of factors other than protein degradation rates and flavour development must be considered in order to obtain meaningful results. These include the relative moieties, salt contents, fat in dry matter, ex-press pH and the number of the starter organisms in the cheeses. Each factor must be within normal limits to avoid a confounding of the results. For example, if the salt-in-moisture, normally 4% to 6%, approaches the upper limit, lactose utilization by the starter organisms will be inhibited (Turner and Thomas, 1980). This will increase the possibility of the lactose being converted to undesirable heterolactic products by the naturally occurring non-starter organisms (Turner and Thomas, 1980). High moisture levels resulting from poor manufacturing
technique are also associated with high lactose levels in the curd and may produce similar effects.

Starter growth and acid production in Cheddar cheese is another factor in determining the moisture content of the final product and the form in which the casein exists in the cheese curd. Acid development controls the conversion of the calcium paracasein of the rennet curd to the more elastic protonated paracasein (Chapman and Sharpe, 1981). Thus the growth of the starter bacteria and the extent of acid production (reflected by the ex-press pH) during cheesemaking will have a significant effect on the chemical and physical composition of the curd.

In addition the fat content of the cheese must be controlled (through standardization) to ensure a constant fat to protein ratio in the cheese. Regulations specify a minimum fat content and it is well established that variation in the fat in cheese will influence the body, texture and flavour perceptions of the cheese. Clearly the cheese manufacturing process is a dynamic one which must be effectively controlled to identify the role of non-starter lactobacilli in the process and maturation of the cheese.

The aim of the present study was to establish whether non-starter lactobacilli, previously isolated from Cheddar cheese could be used effectively in the cheesemaking process with a view to increasing the PTA-soluble nitrogen and amino acids without adversely affecting the body and texture and to determine the effect on the rate of maturation.
MATERIALS AND METHODS

1. **Bacteria**

   (a) **Typed strains.** The organisms used in this investigation have been described previously (p. 13 and 52).

   (b) **Isolation of proteinase negative (prt−) starter.** A culture of *S. lactis* subsp. *cremoris* BK5 grown in skim milk media (10% w/v) at 25°C for 16 h was diluted 10⁻³, 10⁻⁴ and 10⁻⁵ with sterile phosphate buffer (0.5 M Na₂HPO₄ adjusted to pH 7.0 with 0.5 M KH₂PO₄) and 1.0 mL aliquots of the dilutions spread on glycerophosphate-milk agar (GMA) (Limsowtin and Terzaghi, 1976). The GMA plates were incubated at 25°C for 3 to 5 days and the smaller colonies (assumed to be prt−) were picked off and subcultured in M17 broth at 25°C for 16 h. The cultures were confirmed as prt− by incubation in skim milk media (0.5% w/v) at 25°C for 16 h. Where no growth occurred, the organisms were taken to be prt−.

   (c) **Derivation of antibiotic resistant lactobacilli.** Organisms were grown in MRS broth to an optical density of 0.5 at 660 nm using a colorimeter (CHEMTRIX 20A), harvested by centrifugation at 5,000g for 10 min at 4°C (MSE High Speed 18) and resuspended in 1/50 the volume of MRS broth (without glucose). Samples (0.1 mL) were then spread plated onto Rogosa agar containing rifamycin (10 μg/mL). Rifamycin stock solution (5 mg/mL) was filter sterilized (Sartorius Minisart NML, 0.45 μm) prior to addition to the Rogosa agar.

   The plates were incubated in a GasPak 100 Anaerobic System at 37°C for 3 days and the colonies which grew were picked off and maintained in MRS broth containing rifamycin (50 μg/mL).
(d) Bulk culture preparation.

(i) Prt\textsuperscript{−} starter. When large quantities of prt\textsuperscript{−} starter were required the organism was grown in permeate medium (4.5% w/v dried whey permeate; 0.71% w/v yeast extract; 0.43% w/v casein hydrolysate; 0.375% w/v NaH\textsubscript{2}PO\textsubscript{4}·2H\textsubscript{2}O; 0.375% w/v Na\textsubscript{2}HPO\textsubscript{4}) at 32\degree C. Growth at controlled pH was carried out as described previously (p. 16) except that the pH was maintained at 6.0 by the addition of 20% NH\textsubscript{4}OH.

(ii) Lactobacilli were grown in 1 L of MRS broth under pH control to approximately 10\textsuperscript{10} colony forming units/mL and harvested by centrifugation as described previously (p. 16) except that the cells were resuspended in 200 mL of skim milk media (10% w/v).

2. Manufacture of Cheddar Cheese

Cheese was made from normal milk in 400 L vats as described in 'A Pocket Book of Cheddar Cheese Manufacture' prepared by the Australian Society of Dairy Technology (1970) using either 2.0% S. lactis subsp. cremoris BK5 or 2.25% S. lactis subsp. cremoris BK5 prt\textsuperscript{−} as starter. When included in the manufacture of cheese resuspended lactobacilli (200 mL), prepared as described in 1(d) were added together with the starter organisms.

3. Compositional Analysis of Cheese

(a) Moisture was determined according to the method outlined in 'The Sampling and Chemical Analysis of Cheese' (Australian Standard N75-1970).

(b) pH was determined in a slurry after 1:1 dilution of grated cheese with water.

(c) Salt was determined by a modified potentiometric method as described in the Analytical Services Group Laboratory Manual (Food Research Institute, Werribee, Victoria).
(d) Fat was determined by a modified Babcock method as described in the Analytical Services Group Laboratory Methods Manual (Food Research Institute, Werribee, Victoria).

(e) Lactose was determined using a Boehringer Mannheim kit (Cat. No. 176 303).

(f) L- and D-lactic acid were determined using a Boehringer Mannheim kit (Cat. No. 139 084).

(g) Citrate was determined using a Boehringer Mannheim kit (Cat. No. 139 076).

(h) Estimation of bacterial cell numbers.

(i) Lactobacilli. Cheese samples (10g) were emulsified, serial diluted in peptone water and 1 mL aliquots incubated on Rogosa agar or Rogosa agar plus 50 μg/mL of rifamycin (for samples from cheese manufactured using rifamycin resistant lactobacilli) as described previously (p. 13). The colonies which grew were assumed to be lactobacilli.

(ii) Streptococci. Aliquots (1 mL) of cheese sample prepared as described in (i) above were incubated on M17 agar at 30°C for 3 days and the colonies which grew were assumed to be streptococci.

4. Flavour and Grading

Cheeses were graded using the procedure for export cheeses in Australia (Department of Primary Industry, Export Inspection Manual, Dairy Produce Part ii).

5. Hydrolysis of Protein in Cheese

(a) Proteolytic activity. The proteolytic activity in cheese was estimated by a modified method of Dulley
(1974). A sample (10.0 mL) of a cheese homogenate (prepared by homogenizing 1.0 g of cheese with 5 mL 0.1 M citrate buffer, pH 5.5) was incubated with 2 mL of a 1.5% (w/v) solution of sodium caseinate in 0.2 M Na₂HPO₄ (adjusted to pH 5.5 with 0.1 M citric acid) and 30 µL chloroform in a stoppered 12 x 75 mm polypropylene tube at 32°C for 7 days. Samples (1.0 mL) were taken at the beginning and end of incubation and the TCA-soluble tyrosine determined as described by Hickey et al. (1983). The increase in TCA-soluble tyrosine equivalents over the 7 day incubation period was taken as a measure of proteolytic activity.

(b) TCA-soluble protein in cheese was determined as described by Hickey et al., (1983).

(c) PTA-soluble amino nitrogen in cheese was determined as described by Jarret, Aston and Dulley (1982).

6. HPLC Analyses of Cheese

(a) Preparation of the water soluble fraction (WSF).
Cheese samples (1 g) were homogenized with 3 mL of high purity H₂O (Milli-Q water purification system) and 1 mL of internal standard (10 mM, 2-aminobutyric acid) using a Sorvall Omni-Mixer with Micro Attachment at setting 6 for 1 min. The homogenate was transferred to a centrifuge tube and the Sorvall mixing chamber washed with 5 mL of high purity H₂O for 15 sec. The washings were transferred to the homogenate which was then held at 40°C for 30 min before centrifugation at 10,000g for 30 min at 4°C. The aqueous layer was removed, heated and centrifuged as described above then filtered (Whatman 542 filter paper) and stored at -20°C until analysis.

(b) Amino acids. The WSF obtained as described in (a) above was diluted 1:1 with high purity H₂O and ultrafiltered using an ultrafiltration cell (Amicon Model 8010) fitted with an Amicon YM2 membrane. The filtered
samples were derivatized and HPLC carried out as described previously (p. 58).

(c) **Peptides.** HPLC of the WSF obtained as described in (a) above was carried out as described previously (p. 59).

(d) **Proline.** HPLC of the WSF obtained and prepared as described in (b) above was carried out as described previously (p. 59).

7. **Electrophoresis of Cheese**

(a) **Gel electrophoresis.** Cheese samples were prepared by the method of Ledford, O’Sullivan and Nath (1966) and electrophoresis was carried out as described previously (p. 60).

(b) **Isoelectric focusing.** Cheddar Cheese samples (1.0 g) were homogenized with 10 mL of 7 M urea, warmed to 37°C and centrifuged at 1,000g for 10 min. A fat free sample was removed (200 µL) and mixed with 20 µL of a solution of 7 M urea (1.0 mL) and 2-mercaptoethanol (20 µL) before isoelectric focusing as described previously (p. 61).
RESULTS

1. The Role of Non-Starter Lactobacilli in Cheddar Cheese

(a) Cheese manufacture. If non-starter lactobacilli are added to milk for cheesemaking they must not interfere with the growth and acid production of the starter bacteria thereby affecting the overall conditions of curd formation. Manufacturing data (Table 4.1) shows that the non-starter lactobacilli (L. casei CV1A and MIL2B) did not affect the development of acidity during cheesemaking and as a result no alteration to the process was necessary. Furthermore, both the control cheese and the cheese containing lactobacilli had similar ex-press analytical data (Table 4.2) again indicating that the non-starter lactobacilli had little or no effect on normal cheesemaking parameters.

(b) Growth of lactic acid bacteria. During Cheddar cheese maturation it is well established that the numbers of starter streptococci decrease while the naturally occurring non-starter microflora (mainly lactobacilli) increase over the maturation period (Chapman and Sharpe, 1981). Similarly, in the present study, the control cheese showed a gradual decrease in starter streptococci numbers over 48 weeks while the lactobacilli increased rapidly to $>10^7$ g$^{-1}$ cheese in the first 12 weeks and then maintained these numbers (Figure 4.1). The presence of the non-starter lactobacilli L. casei CV1A and MIL2B in high numbers in the cheese resulted in a more rapid decrease in the starter streptococci population during maturation and the added lactobacilli organisms decreased only slightly over the same period. It was not until 48 weeks that similar numbers of lactobacilli were evident in the two cheeses (Figure 4.1).

It was possible to ascertain the ability of L. casei to dominate the non-starter lactobacilli population by using an antibiotic resistant strain. In a separate experiment
Table 4.1. Cheddar cheese manufactured with added lactobacilli

<table>
<thead>
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<th>Manufacturing details</th>
<th>Vat 1 Control</th>
<th>Vat 2 Lactobacilli added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk volume</td>
<td>400 L</td>
<td>400 L</td>
</tr>
<tr>
<td>Starter inocula</td>
<td>2.25% (w/v)</td>
<td>2.25% (w/v)</td>
</tr>
<tr>
<td>Lactobacilli numbers in vat</td>
<td>0</td>
<td>1.3 $\times 10^6$ mL$^{-1}$</td>
</tr>
<tr>
<td>Rennet</td>
<td>100 g</td>
<td>100 g</td>
</tr>
<tr>
<td>Salt (% of curd)</td>
<td>2.5% (w/w)</td>
<td>2.5% (w/w)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temp ($^\circ$C)</th>
<th>Lactic acid (% w/v)</th>
<th>Time (min)</th>
<th>Temp ($^\circ$C)</th>
<th>Lactic acid (% w/v)</th>
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</thead>
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<tr>
<td>Vat filled</td>
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<td>0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Rennet added</td>
<td>10</td>
<td>31.5</td>
<td>10</td>
<td>32.0</td>
<td>-</td>
</tr>
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<td>Cut</td>
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<td>32.0</td>
<td>41</td>
<td>32.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Heat on</td>
<td>50</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heat off</td>
<td>90</td>
<td>36.0</td>
<td>90</td>
<td>36.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Half whey off</td>
<td>130</td>
<td>36.5</td>
<td>130</td>
<td>36.0</td>
<td>0.12</td>
</tr>
<tr>
<td>Whey off</td>
<td>185</td>
<td>36.0</td>
<td>185</td>
<td>36.0</td>
<td>0.15</td>
</tr>
<tr>
<td>Dry</td>
<td>190</td>
<td>-</td>
<td>190</td>
<td>-</td>
<td>0.16</td>
</tr>
<tr>
<td>Milled</td>
<td>280</td>
<td>-</td>
<td>280</td>
<td>-</td>
<td>0.42</td>
</tr>
<tr>
<td>Salted</td>
<td>282</td>
<td>-</td>
<td>282</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hooped</td>
<td>285</td>
<td>-</td>
<td>290</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The Cheddar cheese was manufactured and lactobacilli (L. casei CV1A and MIL2B) added together where indicated as described under Methods.
Table 4.2. Analysis of Cheddar cheese manufactured with added lactobacilli

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Ex-press pH</th>
<th>Ex-press lactose (mM)</th>
<th>Moisture (% w/w)</th>
<th>Fat (% w/w)</th>
<th>Salt (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.21</td>
<td>7</td>
<td>38.58</td>
<td>32.0</td>
<td>1.90</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>5.18</td>
<td>6</td>
<td>38.57</td>
<td>31.5</td>
<td>1.91</td>
</tr>
</tbody>
</table>

The Cheddar cheese was manufactured, lactobacilli (*L. casei* CV1A and MIL2B) added together where indicated and analyses carried out as described under Methods.
Figure 4.1. Growth of lactic acid bacteria in Cheddar cheese manufactured with added lactobacilli

The Cheddar cheese was manufactured, stored at 8°C and the viable cell numbers estimated as described under Methods. Lactobacilli (L. casei CV1A and MIL2B) were added to the milk for cheesemaking where indicated.

Streptococci
○ Control
● Lactobacilli added

Lactobacilli
▲ Control
▲ Lactobacilli added
L. casei MIL2A was made resistant to rifamycin (Rif\textsuperscript{R}), thereby making it possible to measure the relative numbers of natural and added non-starter lactobacilli in cheese. As shown in Figure 4.2 when L. casei MIL2A (Rif\textsuperscript{R}) was added to give \(>10^8\) g\(^{-1}\) cheese no rifamycin sensitive (Rif\textsuperscript{S}) lactobacilli could be detected on the Rogosa agar plates up to 24 weeks maturation. This indicated that the added organisms had dominated the non-starter lactobacilli population. It was noted that a very small number (<0.1\% or \(10^4\) g\(^{-1}\) cheese) of the natural non-starter lactobacilli in the control cheese appeared resistant to rifamycin.

(c) Lactate formation. The initial pH of the control cheese and cheese with added lactobacilli were similar (pH 5.21 and pH 5.18 respectively) and both cheeses exhibited a decrease in pH in the first 4 weeks of maturation, presumably related to the utilization of residual lactose in the cheese (Figure 4.3a). The pH decreased again at 36 weeks but generally the pH of both cheeses was similar throughout maturation.

The starter streptococci used in Cheddar cheese manufacture normally produce only the L(+) isomer of lactic acid in cheese and it has been shown that a number of non-starter bacteria such as the pediococci and lactobacilli are able to convert L-lactate to D-lactate (Thomas and Crow, 1983). As shown in Figure 4.3b, L-lactic acid was the predominant isomer present in the ex-press cheeses and there was an increase in L-lactic acid during the first 4 weeks of maturation corresponding to a decrease in lactose from 7 mM to nil over the same period (data not shown). The racemization of lactate then proceeded until at 36 weeks the concentration of both the L(+) and D(−) isomers was approximately the same (80 mM) in both cheeses.

(d) Citrate utilization. As shown previously (Table 2.6) L. casei CV1A, MIL2A and MIL2B were able to metabolize citrate, a property which has been associated with \(\text{CO}_2\) production and possibly open texture problems in
Figure 4.2. Growth of rifamycin resistant L. casei MIL2A in Cheddar cheese

The Cheddar cheese was manufactured, stored at 8°C and the viable lactobacilli cell numbers estimated as described under Methods. L. casei MIL2A (Rif^R) was added to the milk for cheesemaking where indicated.

Cheddar cheese plus L. casei MIL2A (Rif^R)
- Total lactobacilli
- Rif^R lactobacilli

Control Cheddar cheese
- Total lactobacilli
- Rif^R lactobacilli
Figure 4.3. The effect of lactobacilli on the pH and lactic acid formation in Cheddar cheese

The Cheddar cheese was manufactured, stored at 8°C and assayed for L- and D-lactate and the pH determined as described under Methods. Lactobacilli (L. casei CV1A and MIL2B) were added to the milk for cheesemaking where indicated.

(a) pH
- Control
- Lactobacilli added

(b) Lactic acid
- L-lactic acid
  - Control
  - Lactobacilli added
- D-lactic acid
  - Control
  - Lactobacilli added
cheese. When cheese was made with added lactobacilli (L. casei CV1A and MIL2B) the ex-press citrate levels were lower than in the control cheese (0.06 and 0.10% w/w respectively) (Table 4.3), indicating that some citrate present in the cheesemilk was utilized during manufacture. The levels of citrate in both cheeses changed little during maturation and the amount utilized was small suggesting that with respect to citrate metabolism there would be similar amounts of CO₂ produced in the control cheese and cheese made with added lactobacilli.

(e) Protein hydrolysis. Proteinase activity, particularly with respect to α₅-casein degradation, is a major factor responsible for body and texture changes in maturing cheese, while the formation of small peptides and amino acids correlate well with flavour intensity. As shown in Table 4.4 proteinase activities in both the control cheese and cheese with added lactobacilli (L. casei CV1A and MIL2B) decreased similarly over the maturation period tested. Further the levels of TCA-soluble protein (an indicator of peptides and amino acids) released in both cheeses throughout maturation were the same (Figure 4.4a). In contrast PTA-soluble amino nitrogen (an indicator of small peptides and amino acids) differed markedly between the cheeses suggesting that the cheese made with added lactobacilli had the more active peptidase system (Figure 4.4b).

(f) Peptides in cheese. HPLC analysis of peptides can be used as an indicator of maturation and flavour development (Rank, Grappin and Olson, 1985). In the present study peptides from water soluble extracts of both a control cheese and a cheese made with lactobacilli added were separated by HPLC. Similar patterns were obtained for both cheeses ex-press and at 48 weeks the overall patterns were again very similar although differences in the number of peaks and their relative sizes were evident, (eg. 13 min after sample injection) (Figure 4.5). As no internal standard was included a quantitative comparison cannot be
### Table 4.3  The utilization of citrate in Cheddar cheese manufactured with added lactobacilli

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Citrate concentration (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Ex-press</td>
<td>0.10</td>
</tr>
<tr>
<td>12</td>
<td>0.10</td>
</tr>
<tr>
<td>24</td>
<td>0.12</td>
</tr>
<tr>
<td>36</td>
<td>0.11</td>
</tr>
<tr>
<td>48</td>
<td>0.07</td>
</tr>
</tbody>
</table>

The Cheddar cheese was manufactured, stored at 8°C and assayed for citrate as described under Methods. Lactobacilli (*L. casei* CV1A and MIL2B) were added to the milk for cheesemaking where indicated.
Table 4.4. The effect of lactobacilli on the level of proteinase activity in Cheddar cheese

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Proteinase activity (mg tyr equiv./g cheese/ 7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ex-press</td>
</tr>
<tr>
<td>Control</td>
<td>0.287</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>0.279</td>
</tr>
<tr>
<td>added</td>
<td></td>
</tr>
</tbody>
</table>

The Cheddar cheese was manufactured, stored at 8°C and assayed for proteinase activity as described under Methods. Lactobacilli (L. casei CV1A and ML2B) were added to the milk for cheesemaking where indicated.
Figure 4.4. The effect of lactobacilli on the release of TCA-soluble protein and PTA-soluble amino nitrogen in Cheddar cheese

The Cheddar cheese was manufactured, stored at 8°C and assayed for TCA-soluble protein and PTA-soluble amino nitrogen as described under Methods. Lactobacilli (L. casei CV1A and MIL2B) were added to the milk for cheesemaking where indicated.

(a) TCA-soluble protein
(b) PTA-soluble amino nitrogen

- Control
- Lactobacilli added
Figure 4.5. The effect of lactobacilli on the HPLC peptide pattern of Cheddar cheese at 48 weeks

The Cheddar cheese was manufactured, stored at 8°C and the water soluble fraction prepared as described under Methods. Lactobacilli (L. casei CVIA and MIL2B) were added to the milk for cheesemaking where indicated. Peptides were analysed on a Brownlee Aquapore RP-300 (7 μm) 220 x 4.6 mm column using a gradient program as described under Methods. Peaks were quantitated with a computing integrator.

(a) Control (ex-press)
(b) Lactobacilli added (ex-press)
(c) Control (48 weeks)
(d) Lactobacilli added (48 weeks)
made. Nevertheless analysis of the total peak areas suggested that more peptide material was present in the water soluble extract from the control cheese (data not shown). This would be consistent with the previous observations (Figure 4.4) in that with similar proteinase activity in the two cheeses (Table 4.4) and evidence of greater peptidase activity in the cheese made with added lactobacilli (Figure 4.4) any peptides formed would be more rapidly degraded in this cheese.

(g) **Amino acids in cheese.** The accumulation in cheese of amino acids correlates well with flavour intensity even though they have little influence on typical 'Cheddar' flavour. As shown in Figure 4.6 amino acid levels were higher in cheese made with added lactobacilli, again consistent with the evidence of higher peptidase activities in this cheese (Figure 4.4). In both cheeses the major amino acids present were glutamic acid, asparagine, histidine, alanine, valine, phenylalanine, leucine and lysine and the levels in the cheese made with added lactobacilli were approximately 50 to 140% higher than in the control cheese.

Tyrosine can be a source of problems in Cheddar cheese as it can be converted to the biogenic amine tyramine. Tyramine was detected in the control cheese and the cheese made with added lactobacilli at levels of 2.6 and 4.1 μmol g⁻¹ respectively which suggests that *L. casei* CV1A and MIL2B may be able to degrade tyrosine under the conditions found in maturing cheese. However the levels are similar to those previously reported for Cheddar cheese (0.3 to 5.4 μmol g⁻¹) (Chang, Ayres and Sandine, 1985). In addition tyrosine was not degraded by *L. casei* CV1A or MIL2B in vitro (Table 3.11) and therefore the tyramine formed in the cheese may also be due to the starter or other non-starter organisms.

(h) **Organoleptic assessment.** Cheeses were graded by two professional graders and a trained assessment panel of four people. In general both the control cheese and
Figure 4.6. The effect of lactobacilli on the release of free amino acids in Cheddar cheese at 48 weeks

The Cheddar cheese was manufactured, stored at 8°C and the free amino acids present in the water soluble fraction determined by HPLC as described under Methods. Lactobacilli (L. casei CV1A and MIL2B) were added to the milk for cheesemaking where indicated.

- Control cheese
- Lactobacilli added
cheese made with added lactobacilli exhibited good body and texture throughout maturation and typical 'Cheddar' flavour development occurred in both cheeses at the same rate up to 24 weeks. At 36 and 48 weeks flavour development was more enhanced in cheese made with added lactobacilli. No statistical evaluation was undertaken and further studies would be necessary to fully assess the effect of the added lactobacilli on Cheddar cheese flavour. However in a blind evaluation of the cheeses the professional graders consistently graded the cheese with added lactobacilli one grade point higher than the control from 36 weeks on using the standard grading procedure for export cheeses in Australia (Department of Primary Industry, Export Inspection Manual, Dairy Produce Part ii). A full grade point is highly significant in terms of cheese quality.

2. Proteinase Negative Starter Cultures in Cheese and the Effect of Added Lactobacilli

The use of prt− starter in Cheddar cheese manufacture has a number of potential advantages, including reduction of bacteriophage proliferation in the vat, greater product yield, less bitterness and more consistent manufacturing times (Gamay et al., 1982; O'Leary and Hicks, 1982; Lowrie et al., 1972; Shelaiah et al., 1983). However the lack of cell wall proteinase affects maturation in cheese made using prt− starter in that flavour development is slower. In the present study selected non-starter lactobacilli were added to cheese made with prt− starter and their effect on cheese maturation investigated.

(a) Cheese manufacture. As prt− starter bacteria produce less acid per cell than normal starter (Richardson et al., 1983) greater numbers are required in cheesemaking. These numbers are achieved by growing the organisms in an enriched media under pH control prior to addition to the cheesemilk. In the present study, even though higher numbers of S. lactis subsp. cremoris BK5 (prt−) were used (approximately $1.2 \times 10^8 \text{ mL}^{-1}$) the
acidity at milling was lower than in the 'prr' control cheese (Table 4.5). This indicates that either acid production per cell was too low or higher numbers of the prr starter would be necessary to achieve similar acidities at milling of the curd. This is despite the initial rates of acid production for the prr starter which appeared to be faster than for the control (Table 4.5). The addition of L. casei MIL2A (10^6 mL^-1 cheesemilk) with the S. lactis subsp. cremoris BK5 (prr) starter had no effect on acid development (Table 4.5).

The moisture, fat and salt levels were also similar in all cheeses (Table 4.6) but as expected lactose levels were higher in the 'prr' cheeses due to the lower acidity. Normally, higher lactose levels in cheese can lead to problems associated with the growth of undesirable adventitious organisms (eg. CO_2 production, off flavours, splitting of the curd) (Chapman and Sharpe, 1981) and therefore the observed levels would be unacceptable. The pH values of the 'prr' cheeses were also slightly higher than the control cheese (Table 4.6).

(b) Growth of lactic acid bacteria. As shown in Figure 4.7a the number of starter streptococci decreased during maturation in all cheeses and the decline was slower in the cheese made with S. lactis subsp. cremoris BK5 (prr) alone. The presence of L. casei MIL2A in the cheese was again accompanied by a more rapid decrease in starter streptococci numbers (see also Figure 4.1).

The lactobacilli counts increased in the control cheese and the cheese made with prr starter alone (particularly in the first 24 weeks) and the lactobacilli in the cheese where L. casei MIL2A had been added remained constant (Figure 4.7b). At 48 weeks the levels of lactobacilli in all three cheeses were comparable (approximately 10^8 g^-1 cheese).

(c) Lactate formation. The residual lactose levels in the prr starter cheeses were 8 mM higher than in the control cheese (Table 4.6). As expected these cheeses also
<table>
<thead>
<tr>
<th>Manufacturing details</th>
<th>Vat 1 Control</th>
<th>Vat 2 Prt</th>
<th>Vat 3 Prt plus L. casei MIL2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk volume (L)</td>
<td>400</td>
<td>360</td>
<td>360</td>
</tr>
<tr>
<td>Starter inocula (w/v)</td>
<td>2.25%</td>
<td>2.25%</td>
<td>2.25%</td>
</tr>
<tr>
<td>Starter numbers in vat</td>
<td>$2.4 \times 10^7$</td>
<td>$1.1 \times 10^8$</td>
<td>$1.4 \times 10^8$</td>
</tr>
<tr>
<td>Lactobacilli numbers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in vat</td>
<td>0</td>
<td>$10^1$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Rennet (g)</td>
<td>100</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Salt (% of curd)</td>
<td>2.5% (w/v)</td>
<td>2.5% (w/v)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temp (°C)</th>
<th>Lactic acid (% w/v)</th>
<th>Time (min)</th>
<th>Temp (°C)</th>
<th>Lactic acid (% w/v)</th>
<th>Time (min)</th>
<th>Temp (°C)</th>
<th>Lactic acid (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vat filled</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starter added</td>
<td>5</td>
<td>32.5</td>
<td>-</td>
<td>5</td>
<td>32.0</td>
<td>-</td>
<td>5</td>
<td>32.0</td>
</tr>
<tr>
<td>Rennet added</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Cut</td>
<td>40</td>
<td>32.5</td>
<td>0.11</td>
<td>41</td>
<td>32.0</td>
<td>0.11</td>
<td>45</td>
<td>32.0</td>
</tr>
<tr>
<td>Heat on</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>55</td>
<td>-</td>
</tr>
<tr>
<td>Heat off</td>
<td>90</td>
<td>36.5</td>
<td>0.12</td>
<td>85</td>
<td>38.0</td>
<td>0.125</td>
<td>95</td>
<td>38.5</td>
</tr>
<tr>
<td>Half whey off</td>
<td>143</td>
<td>37.0</td>
<td>0.135</td>
<td>115</td>
<td>38.0</td>
<td>0.14</td>
<td>125</td>
<td>38.5</td>
</tr>
<tr>
<td>Whey off</td>
<td>188</td>
<td>36.5</td>
<td>0.16</td>
<td>165</td>
<td>38.0</td>
<td>0.16</td>
<td>175</td>
<td>38.5</td>
</tr>
<tr>
<td>Dry</td>
<td>193</td>
<td>-</td>
<td>0.20</td>
<td>170</td>
<td>-</td>
<td>0.19</td>
<td>180</td>
<td>-</td>
</tr>
<tr>
<td>Milled</td>
<td>283</td>
<td>-</td>
<td>0.47</td>
<td>260</td>
<td>-</td>
<td>0.38</td>
<td>270</td>
<td>-</td>
</tr>
<tr>
<td>Salted</td>
<td>285</td>
<td>-</td>
<td>-</td>
<td>262</td>
<td>-</td>
<td>-</td>
<td>272</td>
<td>-</td>
</tr>
<tr>
<td>Hooped</td>
<td>293</td>
<td>-</td>
<td>-</td>
<td>267</td>
<td>-</td>
<td>-</td>
<td>280</td>
<td>-</td>
</tr>
</tbody>
</table>

Cheese was manufactured and *L. casei* MIL2A added where indicated as described under Methods.
Table 4.6. Analysis of Cheddar cheese manufactured with \textit{prt}^{-}\textit{ starter and L. casei MIL2A}

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Ex-press pH</th>
<th>Ex-press lactose (mM)</th>
<th>Moisture (% w/w)</th>
<th>Fat (% w/w)</th>
<th>Salt (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.12</td>
<td>6</td>
<td>37.31</td>
<td>31.5</td>
<td>1.89</td>
</tr>
<tr>
<td>Prt\textsuperscript{-}</td>
<td>5.15</td>
<td>14</td>
<td>37.99</td>
<td>32.0</td>
<td>1.90</td>
</tr>
<tr>
<td>Prt\textsuperscript{-} plus L. casei MIL2A</td>
<td>5.22</td>
<td>15</td>
<td>37.71</td>
<td>32.0</td>
<td>1.90</td>
</tr>
</tbody>
</table>

Cheddar cheese was manufactured using normal and proteinase negative starter and the analyses carried out as described under Methods. \textit{L. casei MIL2A} was added to the milk for cheesemaking where indicated.
Figure 4.7. Growth of lactic acid bacteria in Cheddar cheese manufactured with prt\textsuperscript{−} starter and L. casei MIL2A.

The Cheddar cheese was manufactured, stored at 8°C and the viable cell numbers estimated as described under Methods. L. casei MIL2A was added to the milk for cheesemaking where indicated.

(a) Streptococci
(b) Lactobacilli

○ Control
● Prt\textsuperscript{−}
△ Prt\textsuperscript{−} plus L. casei MIL2A
had a lower L-lactate concentration (ex-press) than the
control cheese (100 versus 150 mM respectively) (Figure
4.8). However the L-lactate concentration increased
rapidly in these cheeses in the first 4 weeks to be the
same as the control cheese (170 mM). No lactose was
evident in any of the cheeses at 4 weeks and the pH values
were all between 5.00 and 5.04.

The racemization of L-lactate to D-lactate was more
rapid in the control cheese and the addition of L. casei
MIL2A did not affect the rate of racemization in the
'prt-' cheeses (Figure 4.8).

(d) Protein hydrolysis. Proteinase activity in cheese
made with S. lactis subsp. cremoris BK5 (prt−) was much
lower than in the control cheese. Proteinase activity was
increased by the presence of L. casei MIL2A, although not
to the levels in the control cheese (Figure 4.9a). Again
there was a general decrease in proteinase activity in all
three cheeses over the maturation period (see also Table
4.4).

The amount of TCA-soluble protein released was also
reduced in the cheese made with S. lactis subsp. cremoris
BK5 (prt−) and the presence of L. casei MIL2A increased
the TCA-soluble protein released. The difference in
TCA-soluble protein released in the control and 'prt−'
cheeses (which contained similar chymosin levels) also
indicates that starter bacteria may play a more important
role in the initial degradation of milk proteins than
previously thought (O'Keeffe, Fox and Daly, 1978). Chymosin
has been considered the principal catalytic
enzyme but in the present study the loss of proteinase
activity in the starter reduced overall proteolysis by
approximately 50% (Figure 4.9a).

The greatest effect L. casei MIL2A had on protein
metabolism in the prt− starter cheese was the increase in
PTA-soluble amino nitrogen (Figure 4.9c). The release of
PTA-soluble amino nitrogen reached the levels observed in
the control cheese suggesting peptidase activities
associated with L. casei MIL2A were responsible.
Figure 4.8. The effect of *L. casei* MIL2A on lactic acid formation in Cheddar cheese manufactured with Prt starter.

The Cheddar cheese was manufactured, stored at 8°C and assayed for L- and D- lactate as described under Methods. *L. casei* MIL2A was added to the milk for cheesemaking where indicated.

**L-lactic acid**
- ○ Control
- △ Prt
- □ Prt plus *L. casei* MIL2A

**D-lactic acid**
- ● Control
- △ Prt
- ■ Prt plus *L. casei* MIL2A
Figure 4.9. The effect of L. casei MIL2A on the proteolytic activity in Cheddar cheese manufactured with Prt- starter

The Cheddar cheese was manufactured, stored at 8°C and assayed for proteolytic activity, TCA-soluble protein and PTA-soluble amino nitrogen as described under Methods. *L. casei* MIL2A was added to the milk for cheesemaking where indicated.

(a) Proteinase activity  
(b) TCA-soluble protein  
(c) PTA-soluble amino nitrogen

○ Control  
● Prt-  
△ Prt- plus *L. casei* MIL2A
(e) Peptides in cheese. The IEF pattern of control and 'prt−' cheeses, determined at 3 month intervals, is shown in Figure 4.10. A limited number of bands were evident in the cheese ex-press and the number and intensity of the bands increased during maturation. However no difference between the cheeses could be detected suggesting that the initial degradation of milk proteins in the cheeses was the same.

Further investigation of peptide formation was carried out using HPLC and it was found that the peptide patterns of the control and 'prt−' cheese at 48 weeks differed in terms of peak numbers and sizes particularly in the region 13 to 46 min after sample injection (Figure 4.11). Except in the region 13 to 25 min after sample injection the total peptide material was lower in the 'prt−' cheese reflecting the lower proteinase activity in this cheese. The presence of L. casei MIL2A in the 'prt−' cheese altered the peptide pattern in that it more closely resembled the control cheese. Although a quantitative comparison of data is not possible due to the absence of an internal standard the results do suggest that the level of peptide material was increased in the 'prt−' cheese when L. casei MIL2A was present but not to the level of the control (prt+) cheese (Figure 4.11). Clearly L. casei MIL2A has provided additional proteinase activity in the cheese.

(f) Amino acids in cheese. The concentration of free amino acids in the water soluble extract of 'prt−' cheese was less than in the control cheese (Figure 4.12). The addition of L. casei MIL2A with the S. lactis subsp. cremoris BK5 (prt−) starter increased the levels of free amino acids to that of the control cheese or higher.

The concentration of all amino acids increased throughout maturation (see Figure 4.13a for tyrosine) except arginine which decreased after 24 to 36 weeks maturation (Figure 4.13b). Arginine can be decarboxylated by a number of pathways and the decrease in free arginine is normally accompanied by an increase in ornithine (Dulley, 1974).
Figure 4.10. The effect of *L. casei* MIL2A on the isoelectric focusing pattern of Cheddar cheese manufactured with prt^- starter.

The Cheddar cheese was manufactured, stored at 8°C and prepared for IEF as described under Methods. *L. casei* MIL2A was added to the milk for cheesemaking where indicated. For IEF, cell free digests were added to sample buffer and samples (15 µL applied to the anodic side) were isoelectrically focused on a 1 mm thick gel containing 5% (w/v) acrylamide, 2% (v/v) ampholyte (Bio-Lyte 3/10 Bio-Rad, Richmond, California, USA) and 7 M urea as described under Methods.

A. Sodium caseinate (control)
B. Ex-press control cheese
C. Ex-press prt^- cheese
D. Ex-press prt^- cheese plus *L. casei* MIL2A
E. 3 month control cheese
F. 3 month prt^- cheese
G. 3 month prt^- cheese plus *L. casei* MIL2A
H. 6 month control cheese
I. 6 month prt^- cheese
J. 6 month prt^- cheese plus *L. casei* MIL2A
K. 12 month cheese
L. 12 month prt^- cheese
M. 12 month prt^- cheese plus *L. casei* MIL2A
Figure 4.11. The effect of L. casei MIL2A on the HPLC pattern of Cheddar cheese manufactured with Prt+ starter

The Cheddar cheese was manufactured, stored at 8°C and at 48 weeks the water soluble fraction prepared as described under Methods. L. casei MIL2A was added to the milk for cheesemaking where indicated. Peptides were analysed on a Brownlee Aquapore RP-300 (7 µm) 220 x 4.6 mm column using a gradient program as described under Methods. Peaks were quantitated with a computing integrator.
Figure 4.12. The effect of \textit{L. casei} MIL2A on the release of free amino acids from Cheddar cheese manufactured with \textit{prt}^{-} starter.

The Cheddar cheese was manufactured, stored at $8^\circ$C and at 48 weeks the free amino acids present in the water soluble fraction determined by HPLC as described under Methods. \textit{L. casei} MIL2A was added to the milk for cheesemaking where indicated.

- Control
- \textit{Prt}^{-}
- \textit{Prt}^{-} plus \textit{L. casei} MIL2A
Figure 4.13. The effect of *L. casei* MIL2A on the release of arginine and tyrosine during the maturation of Cheddar cheese manufactured with *prt*\(^{-}\) starter.

The Cheddar cheese was manufactured, stored at 8°C and the free amino acids present in the water soluble fraction determined by HPLC as described under Methods. *L. casei* MIL2A was added to the milk for cheesemaking where indicated.

(a) Tyrosine
(b) Arginine

- Control
- *Prt*
- *Prt*\(^{-}\) plus *L. casei* MIL2A
(1) arginine + H₂O $\rightarrow$ urea + ornithine
(2) urea $\rightarrow$ CO₂ + 2NH₃

Urea as a product of reaction (1) can be converted to NH₃ and CO₂ by the enzyme urease in reaction (2) which if present in either the starter or non-starter microflora of cheese could contribute to open texture. As shown previously (Table 3.11) S. lactis subsp. cremoris BK5 could degrade arginine to a limited extent but L. casei MIL2A was unable to do so. This suggests that the decrease in arginine levels observed in the present study was due to the starter or non-starter bacteria other than L. casei MIL2A.

The accumulation of tyrosine in cheese can also be of concern as it can be converted to tyramine. As shown in Figure 4.13 there was no evidence that tyrosine was being converted to tyramine although at 48 weeks levels of 0.8, 0.5 and 0.5 μmol tyramine g⁻¹ cheese were detected by HPLC in the control, 'prt⁻' and the 'prt⁻' with L. casei MIL2A cheeses respectively. Again this reaction was probably catalysed by the starter organism or a non-starter organism other than L. casei MIL2A (see also Table 3.11).

(g) Organoleptic assessment. Flavour development in cheese made with with prt⁻ starter was clearly slower than in the 'prt⁺' cheese and the addition of L. casei MIL2A improved flavour. However in all cases the prt⁻ cheeses demonstrated poor body and texture. Again no statistical evaluation was undertaken and further studies would be necessary to evaluate the contribution of L. casei MIL2A to flavour in 'prt⁻' in cheese.
DISCUSSION

1. The Role of Non-Starter Lactobacilli in Cheddar Cheese

(a) Cheese manufacture. Attempts to control cheese maturation, particularly flavour development have included the use of unmodified non-starter lactobacilli. In the present study the addition of non-starter lactobacilli to Cheddar cheese in the vat stage of manufacture was investigated and organisms were identified that would (i) not interfere with starter growth and acid kinetics and (ii) were not proteolytic to the extent that cheese yields may be affected.

It was shown that the addition of high numbers (>10⁶ g⁻¹) of selected non-starter lactobacilli (L. casei CV1A and MIL2B) to milk for cheesemaking was possible without affecting the rates of growth and acid production of the starter organisms and other cheesemaking parameters. The added non-starter lactobacilli remained at relatively high levels (10⁸ g⁻¹ cheese) throughout maturation and were able to dominate the non-starter microflora of the cheese to the exclusion of naturally occurring organisms. The only apparent effect on the starter organism was to bring about a more rapid decline in starter cell numbers in the cheese during maturation.

During the maturation stage the added lactobacilli had little effect on the pH or the rates of lactate racemization in cheese. A potential problem did arise in that the added lactobacilli were found to metabolize citrate which could lead to the production of CO₂ in cheese (see Chapter 2). Some citrate, normally present in cheesemilk, was utilized by the lactobacilli during the vat stage of manufacture but under the storage conditions used (8°C) there was no evidence of gas production and no more citrate was utilized in the cheese with added lactobacilli than in the control cheese. A citrate concentration of 0.1% (w/w) in cheese would produce approximately 2.3 mL CO₂ g⁻¹ should it be completely
metabolized. However it must be noted the theoretical CO₂ levels may be influenced by the solubility of the gas, its ability to bind with other compounds, the rate at which it diffuses from the cheese and the gas permeability of the packaging material.

(b) Protein metabolism. The initial degradation of protein in cheese made with added lactobacilli followed a similar pattern to that of the control cheese. Proteinase activities were similar and HPLC analysis of the peptides in the cheeses showed little difference. There were minor differences in the number of peaks and their relative sizes and the level of peptide material was apparently less in cheese made with the added lactobacilli. These differences were probably due to greater peptidase activity in this cheese (evidenced by the release of PTA-soluble amino nitrogen and individual amino acids) and by differences in the activities of various peptidases associated with the starter organism and non-starter lactobacilli (Table 3.8 and 3.9). It was possible that some of the increase in peptide material was the result of intracellular peptidases from the starter organisms which became more accessible as the organisms died out (and lysed). This occurred more rapidly in the presence of the added lactobacilli. Presumably the decline in starter numbers in the cheese was the result of autolysis but as this does not necessarily mean disruption of the spheroplast (Umemoto, Sato and Kito, 1978) the actual contribution of the starter peptidases to the degradation of peptides was not identified. The apparent lower levels of peptide material in cheese made with the added lactobacilli also suggested that problems associated with bitter peptide formation would be minimized in a cheese where peptidase levels were higher.

Individual amino acid levels were also much greater in cheese where lactobacilli had been added. Amino acids correlate well with flavour development although per se they mainly provide the essential background component and it is their numerous degradation products which contribute
to flavour (Aston Durward and Dulley, 1983). One group of degradation products from amino acids that would be of concern are the biogenic amines of which tyramine and possibly histamine are the most common in cheese. Tyramine was detected at higher levels in cheese made with added lactobacilli but the levels were still within normal limits. Furthermore it was shown that the non-starter lactobacilli used in this study did not degrade tyrosine to tyramine and therefore it would be unlikely that high levels of biogenic amines would form in the cheese.

2. Proteinase Negative Starter Cultures in Cheese and the Effect of Added Lactobacilli

(a) Cheese manufacture. The manufacture of Cheddar cheese with prt⁻ starter requires that the cell mass in the inoculum is increased in order to obtain normal acid production in the vat. In the present study the rate of acid development in cheesemilk inoculated with \textit{S. lactis} subsp. \textit{cremoris} BK5 (prt⁻) was different to the control and the milling acidities of the 'prt⁻' cheeses were lower which in turn reflected higher lactose levels in the ex-press cheese. However the excess lactose was completely converted to L-lactato and the pH values of the control and 'prt⁻' cheeses were similar within 4 weeks maturation.

The addition of \textit{L. casei} MIL2A to the cheesemilk did not affect growth and acid production of the prt⁻ starter and cheesemaking appeared normal. The added lactobacilli remained at high levels ($10^8$ g⁻¹) throughout maturation and again the only apparent effect on the starter organism was to cause a more rapid decline in starter cell numbers in the maturing cheese.

(b) Protein metabolism. Proteinase activity in cheese manufactured with prt⁻ starter was approximately half that found in the control cheese made with the prt⁺ starter. This observation indicates that the contribution of the starter to cheese proteolysis may be more significant than previous reports would suggest (O'Keeffe, Fox and Daly,
1978). However even though both cheeses were made with the same level of chymosin no measure of its retention in the curd was made and further work would be required to confirm the result. The addition of \textit{L. casei} MIL2A to 'prt{'\textsuperscript{-}}' cheese increased proteinase activity in the cheese but the level remained less than in the 'prt{'\textsuperscript{+}}' control cheese. HPLC analysis of peptides from the two cheeses indicated that a similar mechanism for the initial degradation of proteins may be operating.

The main effect of adding \textit{L. casei} MIL2A to 'prt{'\textsuperscript{-}}' cheese was to increase peptidase activity which resulted in PTA-soluble amino nitrogen and amino acids levels similar to the prt{'\textsuperscript{+}} control cheese. The increase in amino acids was also accompanied by a general improvement in flavour development in 'prt{'\textsuperscript{-}}' cheese and the addition of \textit{L. casei} MIL2A did not increase undesirable amino acid degradation products (eg. urea, tyramine) in the cheese.

The cheesemaking experiments demonstrated that a characterized non-starter lactobacillus (\textit{L. casei} MIL2A) could be inoculated with a prt{'\textsuperscript{-}} starter in the vat stage of manufacture and not affect normal manufacturing parameters. Furthermore \textit{L. casei} MIL2A improved protein catabolism (particularly peptide degradation) and flavour development during maturation of 'prt{'\textsuperscript{-}}' cheese, a problem that is evident in cheese made exclusively with prt{'\textsuperscript{-}} starter.

3. Conclusion

It has been demonstrated that non-starter lactobacilli isolated from commercial Cheddar cheeses could be characterized with respect to key selection criteria thereby enabling their use in the manufacture of Cheddar cheese. The lactobacilli selected for this purpose did not affect the growth and acid production kinetics of the starter or alter normal cheese parameters. They were able to dominate the non-starter microflora and did not increase proteolytic activity to the detriment of the body and texture of the cheese and peptides did not accumulate to levels where bitterness was evident. At the same time
peptidase activity in the cheese was increased there was a significant increase in the levels of amino acids released which was accompanied by an increase in flavour development and intensity. No problems in relation to citrate metabolism and biogenic amine formation were observed.
CHAPTER 5

GENERAL DISCUSSION
GENERAL DISCUSSION

1. Non-Starter Lactobacilli

*L. casei* and *L. plantarum* are the most prevalent of the non-starter bacteria in Cheddar cheese and can multiply up to levels of $10^8$ g$^{-1}$, having entered the manufacturing system as post-pasteurization contaminants. There are a great diversity of species and strains and they are capable of producing a greater range of end products, including many Cheddar cheese flavour and flavour precursor compounds, than the normal starter organisms (i.e. group N streptococci).

While it is clear that non-starter lactobacilli have an important function in Cheddar cheese maturation their specific role is less defined. They may either:

1. Contribute to the development of correct conditions of pH and redox potential thus allowing the formation of flavour compounds by way of rate limiting non-microbial chemical reactions.
2. Supply flavour precursors principally by way of proteolytic activity.
3. Directly form flavour compounds, particularly as a result of amino acid degradation.

It is likely that the non-starter lactobacilli influence flavour development through a combination of these functions.

The use of non-starter organisms to influence cheese maturation has been attempted with limited success in the past. Despite this, the results obtained indicated that unmodified cells of non-starter bacteria (especially lactobacilli) might be effectively used to influence maturation if a suitable system could be developed. The principal aim of this study was to select suitable non-starter lactobacilli for addition to Cheddar cheese at the vat stage of manufacture and to evaluate the effect of the organisms on the maturation of the cheese. Organisms used in this way were selected to enhance flavour development without affecting starter function or normal
 cheesemaking parameters.

Both *L. plantarum* or subspecies of *L. casei* isolated from mature Cheddar cheeses were selected for their ability to grow and function under the temperature, pH and salt concentrations prevalent in maturing Cheddar cheese and strains were identified that did not produce excessive levels of lactic acid in milk. This characteristic was significant in that it was considered necessary to add the non-starter lactobacilli to the cheesemilk to ensure an even distribution of the organisms throughout the curd and at the same time not interfere with the acid and growth kinetics of the starter organism.

The lactobacilli added in this way did not adversely affect cheese maturation in terms of flavour and texture and were able to oxidize substrates present in cheese, an advantage in contributing to and maintaining a low redox potential. The lactobacilli were also able to metabolize citrate, an undesirable characteristic because of associated CO₂ production, but during cheese maturation citrate utilization was not increased by the non-starter lactobacilli.

The use of alternate growth media for growth of the lactobacilli organisms was also investigated. Normally MRS broth was used for preparation of the large quantities of cells added to the cheesemilk whereas under commercial conditions the MRS growth media may be uneconomical. It was shown that a less complex media could be used but specific growth rates were reduced and further studies are necessary to optimize the conditions of cell production.

2. Protein Catabolism

The degradation of proteins is the major factor contributing to changes in body and texture of cheese as well as the development of flavour. The use of non-starter lactobacilli influenced protein catabolism during cheese maturation but it was found that a correct balance of proteinase and peptidase activities in the organisms was necessary. During the vat stage of cheese manufacture excessive proteinase activity could lead to reduced yields
while during maturation the body and texture of the cheese could be affected. At the same time it is desirable that a high level of peptidase activity exist in maturing cheese in order to degrade bitter peptides and promote amino acid formation. A broad range of peptidases should also be present to prevent a too unbalanced range of end products.

The lactobacilli in this study exhibited proteinase activities lower or similar to the starter organism (S. lactis subsp. cremoris BK5) but their proteolytic system was more resilient to the conditions prevalent in the manufacture and maturation of Cheddar cheese. They catabolized $\alpha_s$-casein, the major protein degraded in Cheddar cheese as well as $\beta$-casein and the rates of degradation were similar to the group N streptococci.

The non-starter lactobacilli also possessed a range of peptidases similar to but generally more active than those present in the group N streptococci. These peptidases also appeared to function effectively in the stationary phase of growth (note that the lactobacilli maintain viability in cheese throughout maturation). Minor differences were evident in the HPLC peptide patterns produced after digestion of $\alpha_s$-casein by the starter and non-starter organisms but higher levels of amino acids were produced by the non-starter lactobacilli. Such variations in the relative levels of the peptides and amino acids produced suggested that differences in the balance and intensity of flavour compounds formed by the different organisms may arise. Further there was no evidence that the non-starter lactobacilli were able to form undesirable amino acid degradation products such as biogenic amines.

From these results it appeared that some species/strains of non-starter lactobacilli would be suitable for use in cheesemaking as a means of controlling the maturation process if they could effectively dominate the non-starter microflora of the cheese and complement starter function.

3. Cheddar Cheese and the Effect of Added Lactobacilli

Traditionally the production of Cheddar cheese has
involved long storage times at low temperatures (six to twelve months at 8°C) to achieve full flavour and texture. The flavour that develops can vary according to the region and time of year of manufacture and presumably is brought about in part by differences in the natural non-starter microflora prevalent in the factory environment. An increase in the rates of maturation and flavour development would be a considerable advantage in terms of cost while control of the microbiology involved may lead to a more uniform product across differing manufacturing situations.

More recently two new procedures for manufacturing Cheddar cheese have appeared. One includes the use of ultrafiltered milk (Sutherland and Jameson, 1981) and the other the use of proteinase negative starter organisms (Richardson et al., 1983). Where cheese is made from ultrafiltered milk flavour development may vary slightly if the adventitious microbial population is altered from normal and it has been shown that flavour development in cheese made with proteinase negative starters is slower. In both cases the introduction of known non-starter organisms to dominate the microflora may favourably influence flavour development.

Controlled use of non-starter lactobacilli in Cheddar cheese manufacture was investigated in normal Cheddar cheese by adding lactobacilli in high numbers together with the starter organism. The lactobacilli did not interfere with the growth and acid kinetics of the starter nor did they alter normal cheesemaking parameters or final cheese composition. However throughout maturation there was a marked increase in amino acid formation and flavour development (relative to a control cheese) particularly in the later stages of maturation. At the same time proteolytic activity was not excessive in that body and texture were unaffected and bitterness did not develop. In addition citrate was not metabolized and biogenic amines did not form in the cheese.

Similarly, the addition of non-starter lactobacilli to cheese made with proteinase negative starter did not
affect manufacturing procedures but protein degradation rates were increased and flavour development and intensity improved. However proteinase activities were not improved to the levels exhibited in the control cheese, even though peptidase activity and the levels of amino acids were similar. Again there was no bitter flavour, citrate was not metabolized and biogenic amines did not form.

The present study has shown that non-starter lactobacilli can be incorporated into Cheddar cheese manufacture in order to influence flavour development during maturation. The organisms were isolated for this purpose from their natural environment (mature cheese) and were characterized with respect to their role in a complete microbiological control system. Moreover the organisms were able to be added at the vat stage of manufacture in very high numbers thereby dominating the non-starter microflora of the cheese. In this way a defined and complementary starter and non-starter system has been achieved in cheesemaking for the first time.
APPENDIX

1. Harvesting of Lactobacilli from Skim Milk Media

Bacterial cells can be harvested from skim milk media by centrifugation (Thomas and Turner, 1977) but not all strains sediment readily. This characteristic may be due to the presence of an acidic carbohydrate layer that forms on certain lactic acid bacteria when grown in autoclaved skim milk, which may adhere to casein micelles, thus inhibiting separation by centrifugation (Brooker, 1976; Thomas and Turner, 1977).

Lactobacilli from the present investigation together with L. bulgaricus 1243 and 1489 and L. helveticus 1829 (obtained from the NCDO at the National Institute for Research in Dairying, Reading, England) were compared for the ease with which they could be centrifuged from skim milk media (10% w/v). Cells were grown under pH control (pH 6.2) at 37°C to mid log phase of growth and the cell density, determined as described previously (p. 15), was measured before and after centrifugation (5,000g for 10 min and 10,000g for 20 min). The effect of sodium citrate (1% w/v final concentration) on cell sedimentation was also investigated by addition to the incubation medium prior to centrifugation. Sodium citrate would disrupt the casein micelle and should allow for a more efficient sedimentation.

As shown in Table A.1 most lactobacilli, with the exception of L. bulgaricus 1489 and L. helveticus 1829, sedimented readily at 5,000g. However the addition of citrate to the media improved sedimentation and at 10,000 g virtually all the cells were recoverable.

Throughout this investigation the organisms grown in skim milk media were able to be harvested effectively by centrifugation thereby allowing more meaningful comparisons to the milk/cheese environment to be made.
Table A.1. Sedimentation of lactobacilli from milk

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Percentage of sedimnted cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5,000g, 10 min</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
</tr>
<tr>
<td>L. casei</td>
<td></td>
</tr>
<tr>
<td>151</td>
<td>100</td>
</tr>
<tr>
<td>MIL2A</td>
<td>90</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
</tr>
<tr>
<td>343</td>
<td>100</td>
</tr>
<tr>
<td>ML1A</td>
<td>100</td>
</tr>
<tr>
<td>L. bulgaricus</td>
<td></td>
</tr>
<tr>
<td>1243</td>
<td>100</td>
</tr>
<tr>
<td>1489</td>
<td>62</td>
</tr>
<tr>
<td>L. helveticus</td>
<td></td>
</tr>
<tr>
<td>1829</td>
<td>30</td>
</tr>
</tbody>
</table>

Cells were grown in skim milk media (10% w/v) under pH control (pH 6.2) at 37°C to mid log phase of growth and the cell density measured before and after centrifugation. Sodium citrate was added, where indicated, to give a final concentration of 1% (w/v).
2. **Ultra-Cold Storage of Non-Starter Lactobacilli and the Effect on Proteinase Activity**

Non-starter lactobacilli grown up under pH control, harvested and resuspended in phosphate buffer were often stored at -72°C until used. The effect of ultra-cold storage on the proteolytic activity of *L. casei* MIL2A was investigated using cells grown in MRS broth at pH 6.2 and harvested as described previously (p. 16). Cells were suspended in either 0.2 M KH₂PO₄ (adjusted to pH 6.0 with 0.2 M Na₂HPO₄) or 0.2 M sucrose at 15 mg (dry weight)/mL before assaying for proteolytic activity as described previously (p. 55). After storage at -72°C for 2 weeks the proteolytic activities were again determined and compared to the original activities. As shown in Table A.2 the freezing of cells and the suspending medium had little effect on the proteolytic activity of *L. casei* MIL2A. This indicated that as a means of storage, suspension in phosphate buffer and freezing at -72°C would be unlikely to affect cell proteolytic activity in the short term.
Table A.2. The effect of ultra-cold storage on the proteinase activity of *L. casei* MIL2A

<table>
<thead>
<tr>
<th>Suspending medium</th>
<th>Proteinase activity (µmol x 10 gly equiv./mg dry wt. cells/7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before storage</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>4.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*L. casei* MIL2A was grown in MRS broth, harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.2 M KH₂PO₄ (adjusted to pH 6.0 with 0.2 M Na₂HPO₄) or 0.2 M sucrose as described under Methods (p. 16) and samples stored at -72°C for 2 weeks. Before and after storage cell suspensions (1.0 mL) were added to a solution containing 2.0 mL of sodium caseinate (1.5% w/v) in 0.2 M phosphate buffer at pH 6.0 and 30 µL of chloroform. The assay mixture was incubated at 37°C for 7 days and TCA-soluble protein released by *L. casei* MIL2A alone was determined as described under Methods (p. 55).
3. The Degradation of Sodium Caseinate by L. casei MIL2A

The low proteolytic activity of the non-starter lactobacilli used in the present study would require an assay procedure where high concentrations of organisms are incubated for a long period of time (7 days) in the presence of the substrate. In order to determine the minimum cell concentration required for the assay (p. 55) differing concentrations (0.01 to 30 mg dry weight cells/mL) of L. casei MIL2A, grown in MRS broth and harvested as described previously (p. 16), were incubated with a sodium caseinate solution (1% w/v) at 37°C for 7 days.

As shown in Figure A.1 detectable levels of TCA-soluble protein were released at cell concentrations of 1.0 mg (dry weight) cells/mL. However at higher concentrations (15 to 30 mg dry weight cells/mL) maximum levels of TCA-soluble protein were obtained. In the present study 15 mg (dry weight) cells/mL was used.

Further investigation of sodium caseinate degradation over time by L. casei MIL2A (15 mg dry weight cells/mL) indicated that a minimum period of 3 days was desirable for the assay procedure (Figure A.2). Up to this point cells of L. casei MIL2A, incubated alone, still formed TCA-soluble protein presumably through the degradation of associated proteins not removed during washing of the cells. After approximately 3 days there was no further degradation of protein except where the cells were incubated with the substrate. This result also emphasises the need to run a blank sample containing cells alone when the proteinase assay is performed. The incubation of substrate alone did not result in any release of TCA-soluble protein.
Figure A.1. The effect of cell concentration on the release of TCA-soluble protein from sodium caseinate

*L. casei MIL2A* was grown in MRS broth, harvested by centrifugation, washed and resuspended to give a range of concentrations from 0.01 to 30 mg (dry weight)/mL in 0.2 M KH$_2$PO$_4$ (adjusted to pH 6.0 with 0.2 M Na$_2$PO$_4$) as described previously under Methods (p. 16). The cell suspensions (1.0) were added to 2.0 mL of sodium caseinate solution (1.5% w/v) in 0.2 M phosphate buffer at pH 6.0 and 30 mL of chloroform. The assay mixture was incubated at 37°C for 7 days and TCA-soluble protein was determined as described previously under Methods (p. 55).
Figure A.2. The degradation of sodium caseinate by L. casei MIL2A

*L. casei* MIL2A was grown in MRS broth, harvested by centrifugation, washed and resuspended at 15 mg (dry weight)/mL in 0.1 M KH₂PO₄ (adjusted to pH 5.5 with 0.1 M Na₂PO₄) as described previously under Methods (p. 16). The cell suspension (7.5 mL) was added to 15 mL of sodium caseinate solution (3.0% w/v) in 0.3 M phosphate buffer at pH 5.5 and sodium azide (15 mg/mL of assay mixture). The assay mixture was incubated at 37°C and samples (1.0 mL) were taken at regular intervals for up to 10 days and TCA-soluble protein was determined as described previously under Methods (p. 55).

○ *L. casei* MIL2A plus sodium caseinate
● " " " alone
▲ Sodium caseinate alone
4. Peptidase Specificity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Catalytic specificity</th>
<th>Characterization substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopeptidase (EC 3.4.11.11)</td>
<td>NH₂-X\textsuperscript{↓}X-X-X-</td>
<td>L-leucine-p-nitroanilide L-alanine-p-nitroanilide</td>
</tr>
<tr>
<td>Proline imino-peptidase</td>
<td>NH₂-Pro\textsuperscript{↓}X-X-</td>
<td>L-proline-p-nitroanilide</td>
</tr>
<tr>
<td>(EC 3.4.11.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endopeptidase (EC 3.4.21.24)</td>
<td>-X-X\textsuperscript{↓}X-X-X-</td>
<td>N-glutaryl-L-phe p-nitroanilide N-succinyl-L-phe p-nitroanilide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase-P (EC 3.4.11.9)</td>
<td>NH₂-X\textsuperscript{↓}Pro-X-</td>
<td>Poly-L-proline</td>
</tr>
<tr>
<td>Dipeptidase (EC 3.4.13.11)</td>
<td>NH₂-X\textsuperscript{↓}X-COOH</td>
<td>Glycyl-L-tyrosine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycyl-L-phenylalanine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-leucyl-glycine</td>
</tr>
<tr>
<td>Tripeptidase (EC 3.4.11.4)</td>
<td>NH₂-X\textsuperscript{↓}X-COOH</td>
<td>DL-alanyl-DL-leucylglycine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase (EC 3.4.16.1)</td>
<td>-X-X\textsuperscript{↓}X-COOH</td>
<td>N-carbobenzoxy-glycyl-L-proline Hippuryl-L-arginine</td>
</tr>
</tbody>
</table>
5. Determination of Amino Acids by HPLC

HPLC precolumn derivatization of amino acids with o-phthalaldehyde/2-mercaptoethanol enables detection either with a fluorimeter or spectrophotometer but as spectrophotometric detection is about an order of magnitude less sensitive (Rowlett and Murphy, 1981; Church et al., 1983) fluorometric detection is preferred. Amino acid separation and detection by HPLC is also influenced by the type of column used for chromatographic separations (Hancock and Harding, 1984) and results based on a published method, can vary according to the equipment available.

In this investigation HPLC analysis of amino acids used the method of Jones, Pääbo and Stein (1981) except that a LKB Ultropac Lichrosorb RP-18 (5 μm) column (250 x 4 mm) preceded by a Lichrosorb RP-18 (7 μm) guard column was used for chromatographic separations and the eluate was monitored spectrophotometrically at 340 nm.

As shown in Figure A.3 good separation of most amino acids was achieved using the gradient program described under Methods (p. 58) although there was co-elution of glycine with threonine and methionine with tryptophan. This is a function of column type as other studies have shown that separation of these amino acids is possible (Jones, Pääbo and Stein, 1981; Umagat, Kucera and Wen, 1982; Cooper et al., 1984). However, the remaining amino acids exhibited good linearity of response when different amounts (0.2 to 3.0 nmoles) were analysed using the modified method of Jones, Pääbo and Stein (1981) (Table A.3). This indicated that the method used in the present study was sensitive and accurate for the quantitative determination of amino acids released after hydrolysis of casein by lactobacilli and in maturing Cheddar cheese.

The addition of known quantities of amino acids (5 μmoles) to Cheddar cheese samples (1.0 g) before the preparation of the water-soluble fraction as described previously (p. 111) resulted in recoveries of between 70 and 86% (Table A.4). The recovery was relatively uniform suggesting that while the quantitative determination of
Figure A.3. Chromatogram of amino acid standards

Amino acids were determined by HPLC as described previously under methods (p. 58). Each peak represents 2 nmols and the peak areas were quantitated with a computing integrator. Non standard abbreviations used are: γ-ABA, 4-aminobutyric acid and α-ABA, 2-aminobutyric acid.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.000</td>
</tr>
<tr>
<td>Glu</td>
<td>1.000</td>
</tr>
<tr>
<td>Asn</td>
<td>1.000</td>
</tr>
<tr>
<td>Ser</td>
<td>0.998</td>
</tr>
<tr>
<td>His</td>
<td>1.000</td>
</tr>
<tr>
<td>Gln</td>
<td>0.995</td>
</tr>
<tr>
<td>*Gly/Thr</td>
<td>1.000</td>
</tr>
<tr>
<td>Arg</td>
<td>0.999</td>
</tr>
<tr>
<td>Ala</td>
<td>0.999</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.998</td>
</tr>
<tr>
<td>γ-ABA</td>
<td>0.999</td>
</tr>
<tr>
<td>ε-ABA</td>
<td>0.999</td>
</tr>
<tr>
<td>*Met/Trp</td>
<td>0.999</td>
</tr>
<tr>
<td>Val</td>
<td>0.999</td>
</tr>
<tr>
<td>Phe</td>
<td>0.998</td>
</tr>
<tr>
<td>Ileu</td>
<td>1.000</td>
</tr>
<tr>
<td>Leu</td>
<td>1.000</td>
</tr>
<tr>
<td>Lys</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* Co-elution of amino acids

Amino acids were determined by HPLC as described previously under Methods (p. 58). Each amino acid was injected at 5 levels (0.2, 0.4, 1.0, 2.0, and 3.0 μmoles) and the peak areas were quantitated with a computing integrator. Non standard abbreviations used are: γ-ABA, 4-aminobutyric acid and ε-ABA, 2-aminobutyric acid.
Table A.4. Recovery of amino acids added to Cheddar cheese

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>70</td>
</tr>
<tr>
<td>Asn</td>
<td>74</td>
</tr>
<tr>
<td>Ser</td>
<td>78</td>
</tr>
<tr>
<td>His</td>
<td>78</td>
</tr>
<tr>
<td>Gln</td>
<td>86</td>
</tr>
<tr>
<td>*Gly/Thr</td>
<td>80</td>
</tr>
<tr>
<td>Arg</td>
<td>78</td>
</tr>
<tr>
<td>Ala</td>
<td>82</td>
</tr>
<tr>
<td>Tyr</td>
<td>82</td>
</tr>
<tr>
<td>*Met/Trp</td>
<td>80</td>
</tr>
<tr>
<td>Phe</td>
<td>74</td>
</tr>
<tr>
<td>Ileu</td>
<td>76</td>
</tr>
<tr>
<td>Leu</td>
<td>74</td>
</tr>
</tbody>
</table>

* Co-elution of amino acids

Amino acids (5 µmoles) were added to cheese (1 g) and water soluble extracts of control and amino acid supplemented cheeses were prepared as described previously under Methods (p. 113). Amino acids were determined by HPLC as described previously under Methods (p. 58).
amino acids in cheese by HPLC is reliable there may be some losses during the preparation of the water-soluble extract of the cheese.
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