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Review

# Enzymatic ability of *Lactobacillus casei* subsp. *casei* IFPL731 for flavour development in cheese

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**Abstract**

*Lactobacillus casei* subsp. *casei* IFPL731 is a wild strain isolated from artisanal goats' milk cheese. The strain shows a multiple enzymatic system that involves esterase, cell-envelope proteinase, aminopeptidases, dipeptidases, specialized peptidases for proline-containing peptides, and amino acid converting enzymes. The broad enzymatic system of *Lb. casei* IFPL731 is responsible for its hydrolyzing activity towards a number of peptides, including bitter and methionine-containing peptides. Both characteristics are of great interest as regards the use of the strain as a starter culture adjunct to influence the development of cheese flavour, which has been demonstrated in the manufacture of goats' milk and low fat cheeses. Moreover, *Lb. casei* IFPL731 shows methionine aminotransferase activity that leads to the production of the typical cheese aroma. The different enzymes from *Lb. casei* IFPL731 described in this review and its utilization as a starter culture adjunct in cheese manufacture make the strain one of the best characterized lactobacilli in the literature. 

*Keywords:* *Lactobacillus casei*; Peptidases; Proteolysis; Cheese flavour

## 1. Introduction

Cheese flavour is the result of the breakdown of milk components by enzymes from milk, rennet and micro-organisms, which produce a series of volatile and non-volatile compounds. Moreover, the enzymes from cheese-related microorganisms, particularly lactic acid bacteria, are the chief factors responsible for the formation of many such compounds that are essential for cheese flavour (El Soda, Madkor, & Tong, 2000).

Proteolysis is one of the major biochemical events in flavour development occurring during the maturation of most cheese varieties. Other enzymatic processes are also involved, such as lipolysis and lactic and propionic acid fermentation, leading to cheese flavour compounds (Fox et al., 1996).

Cheese proteolysis involves the concerted action of proteolytic enzymes, such as residual chymosin and plasmin, on intact casein in the cheese curd (primary phase of ripening) and the further breakdown of large peptides and oligopeptides into small peptides and amino acids by the cell-envelope proteinase (CEP) and peptidases of lactic acid bacteria (secondary phase of ripening) (Visser, 1993). Proteolysis can also lead to bitter peptides, which accumulate in cheese, causing bitterness. Ney (1979) was the first to establish a correlation between bitterness and the hydrophobicity of the peptides involved. However, more recent authors have associated the bitterness of a peptide with the presence of basic residues like arginine in the N-terminal region, and hydrophobic residues in the C-terminal region (Habibi-Najafi & Lee, 1996).

Proteolytic enzymes from lactic acid bacteria play an important role in the degradation of casein and peptides leading to the production of free amino acids. Microbial peptidases can also reduce bitterness by hydrolysing bitter peptides formed in the cheese. The amino acids that are produced contribute directly to the basic taste of the cheese and indirectly to cheese flavour since they are precursors of other catabolic reactions which produce volatile aroma compounds (Fox & Wallace, 1997). Although amino acid degradation, mainly due to

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the action of microbial enzymes, amino acid converting enzymes (AACE's), seems to be a rate limiting factor in flavour development, the increased formation of these precursors is an obligatory previous step (McSweeney & Sousa, 2000; Smit et al., 2000). It is definitely established that a well-balanced process of breakdown of milk proteins is essential for the development of a good cheese flavour (Fox, 1989).

The role of lactic acid bacteria in the development of textural and organoleptic characteristics of cheese depends on the enzymatic potential of the strains used in the cheese-making process. There are wide differences in the expression of proteinases, peptidases, AACE's and esterases among genera, species and strains of LAB (Pritchard & Coolbear, 1993; Amárita, Requena, Taborda, Amigo, & Peláez, 2001). Hence, it is important to study the enzymatic system in cheese-related bacteria in order to select an appropriate starter for cheese manufacture. Starter lactococci are usually the dominant flora during the initial weeks of cheese ripening. However, non-starter lactic acid bacteria belonging to genus *Lactobacillus* reach high densities during the late stages of ripening and also contribute to proteolysis and flavour development (Peterson & Marshall, 1990). Adjunct cultures of lactobacilli have been reported to possess higher intracellular enzyme activity than lactococci with high potential to degrade hydrophobic peptides and reduce bitter off-flavour (Habibi-Najafi & Lee, 1996; Laan, Tan, Bruinenberg, Limsowtin, & Broome, 1998).

Lipolysis occurs to a small degree in most cheese varieties and is only especially marked in mould-ripened varieties and in hard Italian cheeses made with rennet paste containing pregastric lipase and esterases (Fox & Stepaniak, 1993). Lactic acid bacteria are weakly lipolytic compared to other bacteria and moulds and little is known about their contribution to cheese lipolysis and flavour development. The fat fraction of cheese contributes to the accumulation of free fatty acids (FFAs). In addition to their direct impact on cheese flavour, FFAs also act as precursor molecules which lead to the production of other flavour compounds such as methylketones, esters, fatty acid lactones and alcohols (McSweeney & Sousa, 2000).

The native microflora of semihard goat's cheese (Majorero) has been characterized in our laboratory (Fontecha et al., 1990). Several isolates of lactococci and lactobacilli were studied for titratable acidity, proteolysis in milk and enzymatic activities in an attempt to develop an appropriate starter for the production of this type of cheese (Requena, Peláez, & Desmazeaud, 1991; Requena, Peláez, & Fox, 1993). As a result, a specific starter for the manufacture of semi-hard goats' milk cheese was developed (IFPL starter) and contained the isolate, *Lactococcus lactis* subsp. *lactis* IFPL359, and the adjuncts *Lactobacillus casei* subsp. *casei* IFPL731, *Lb. plantarum* IFPL935, *Leuconostoc mesenteroides* subsp. *dextranicum* IFPL709 and *Ln. paramesenteroides* IFPL705. The IFPL starter and its adjuncts were used successfully in the manufacture of this type of cheese (Requena, de la Fuente, Fernández de Palencia, Juárez, & Peláez, 1992). The results suggested that these strains contributed in different ways to proteolysis and lipolysis and they seemed to complement each other when used as adjuncts to the starter culture. The use of the IFPL starter in the manufacture of low-fat cheeses from ultrafiltered milk resulted in higher contents of free amino acids and volatile components in the cheeses during ripening and provided the cheeses with the best scores for aroma and flavour development (Rodríguez, Requena, Goudédranche, Maubois, & Juárez, 1996; Rodríguez et al., 1997).

In this paper we present an overview of the work done at our laboratory on the enzymatic system of *Lb. casei* subsp. *casei* IFPL731 and its influence on cheese ripening and flavour development.

## 2. Microorganism and culture conditions

*Lactobacillus casei* subsp. *casei* IFPL731 was isolated originally from Spanish goats' milk cheese (Majorero) (Fontecha et al., 1990) and is now in the culture collection (IFPL) of the Instituto del Frío (CSIC), Madrid, Spain. It was maintained in frozen stock cultures in MRS broth (Oxoid Co., Basingstoke, UK) containing 25% glycerol, at $-80^{\circ}C$.

Unless otherwise stated, the microorganism was grown in 10% skimmed milk supplemented with 0.1% Tween 20, 0.002% manganese sulphate and 0.004% sodium formate. It was subcultured twice overnight before being used to inoculate (1%) the batch culture and was grown at $30^{\circ}C$ until the pH fell to 5.0–5.2.

## 3. Characterization of the lipolytic system of *Lactobacillus casei* IFPL731

### 3.1. Esterase

Lactic acid bacteria are weakly lipolytic, but they may be responsible for the release of high levels of FFA when present in high cell numbers or over an extended ripening period. Several intracellular lipase and esterase activities have been described for lactobacilli (El Soda, Abd El Wahab, Ezzat, Desmazeaud, & Ismail, 1986; Lee & Lee, 1990) and they are usually higher than in lactococci (Requena et al., 1991; Crow, Holland, Pritchard, & Coolbear, 1994).

Comparison of esterolytic and lipolytic activities of several strains of lactobacilli and enterococci showed that *Lb. casei* IFPL731 expressed the highest esterolytic

Table 1
Characteristics of the enzymes purified from *Lactobacillus casei* subsp. *casei* IFPL 731

| | Substrate[a] | Molecular mass (kDa) | Quaternary structure | Type | pH optimum | Temperature optimum (°C) | Reference |
|---|---|---|---|---|---|---|---|
| Proteinase (CEP) | | 150 | mono | serine | 6.0 | 40 | Fernández de Palencia et al. (1997f) |
| Dipeptidase | Leu–Leu<br>His–Ala | 46 | mono | metallo | 7.5 | 60–70 | Fernández-Esplá and Martín-Hernández (1997) |
| Prolidase | Leu–Pro<br>Ile–Pro | 41 | mono | metallo | 6.5–7.5 | 55 | Fernández-Esplá et al. (1997b) |
| Serine aminopeptidase | Phe–Met<br>Leu–Gly<br>Arg–Pro–*p*NA | 67 | mono | serine | 7.0 | 40 | Fernández-Esplá et al. (1997a) |
| Aminopeptidase N (ApIII) | Lys–*p*NA<br>Leu–*p*NA | 95 | mono | metallo | 6.5 | 30 | Fernández de Palencia et al. (1997d) |
| Aminopeptidase C (ApII) | Leu–*p*NA<br>Ala–Met–*p*NA | 50 | tetra | thiol | 7.5 | 55 | Fernández de Palencia et al. (1997e) |
| Aminopeptidase (ApIV) | Lys–*p*NA<br>Pro–*p*NA | 30 | tetra | metallo | 7.0 | 35 | Fernández de Palencia et al. (1997d) |
| Esterase | $\beta$-naphtyl caprylate | 38 | tri | serine | 7.5 | 25–30 | Castillo et al. (1999) |

[a] Substrates preferentially degraded; *p*NA, *p*-nitroanilide derivatives substrates.

activity, being maximal using $\beta$-naphthyl caprylate or $\beta$-naphthyl butyrate as substrate (unpublished data). Following these studies, Castillo, Requena, Fernández de Palencia, Fontecha, and Gobbetti (1999) purified and characterized an intracellular esterase from *Lb. casei* IFPL731 which is active over a broad range of pH and temperature values, and which exhibits preference for $\beta$-naphthyl esters of short-chain fatty acids (C4-C8) (Table 1) and considerable activity on tributyrin. Short-chain fatty acids, even at low concentrations, are important flavour components of cheese (Urbach, 1993), and for that reason the esterase of *Lb. casei* IFPL731 may contribute positively to cheese flavour development.

## 4. Characterization of the proteolytic system of *Lactobacillus casei* IFPL731

### 4.1. Cell-envelope proteinase (CEP)

Lactic acid bacteria possess a CEP which is involved in the degradation of caseins and large peptides, produced from casein by the action of rennet, and the resultant formation of a large number of different oligopeptides. Two major proteinase types, PI and PIII, were initially recognized among lactococci, differing in their specificity towards caseins (Visser, Exterkate, Slangen, & de Veer, 1986). Subsequent studies with lactococcal CEP (Lc-CEP) have shown variants intermediate between types PI and PIII, based on their specificity towards the $\alpha_{S1}$-casein (1–23) fragment and two different charged chromophoric peptides (Exterkate, Alting, & Bruinenberg, 1993).

The CEP of *Lb. casei* IFPL731 was released by different methods according to Fernández de Palencia, Peláez, Requena, and Martín-Hernández (1995). The proteinase was strongly attached to the membrane and hence not released in $Ca^{2+}$-free buffer; it was necessary to use a chelating agent (EDTA) for its extraction. Solubilizing the cell wall using lysozyme and mutanolysin improved the yield of CEP released, even in the presence of $Ca^{2+}$. The enzyme was purified and characterized by Fernández de Palencia, Peláez, Romero, and Martín-Hernández (1997f). It belongs to the serine group of proteinases and its molecular mass was estimated to be about 150 kDa. Maximum activity was at pH 6.0 and 40°C (Table 1). Unlike lactococcal proteinases, the CEP from *Lb. casei* IFPL731 exhibits high instability at 4°C due to autoproteolysis. $Ca^{2+}$ molecules are ineffective as stabilizing agents for the enzyme.

Studies on substrate specificity of the proteinase towards $\alpha_{S1}$- and $\beta$-caseins, at high and low ionic strength, and the $\alpha_{S1}$-CN(f1–23) peptide were carried out by electrophoretic and HPLC analysis (Fernández de Palencia et al., 1997f; Fernández de Palencia, Peláez, & Martín-Hernández, 1997c). Hydrolysis was higher on $\beta$-casein than on $\alpha_{S1}$-casein, and lower degradation of both caseins was observed at high ionic strength (4% NaCl). The proteinase cleaved mainly bond $Gln^9$–$Gly^{10}$ of the $\alpha_{S1}$-CN(f1–23) peptide, resulting in two major fragments, f(1–9) and f(10–23); no further conversion of these fragments was observed. Specificity for bond $Gln^9$-$Glu^{10}$ has also been described by mixed-type proteinases of lactococci (Exterkate & Alting, 1993) and the CEP of *Lb. helveticus* (Martín-Hernández, Alting, & Exterkate, 1994). Differences were observed in specificity of the CEP from *Lb. casei* IFPL731 towards the $\alpha_{S1}$-CN(f1–23) peptide depending on the procedure of release from the cell (Fernández de Palencia et al., 1997c) suggesting the existence of changes in the conformational structure of

the proteinase as a consequence of the method of release. Differences in specificity between the bound and calcium-free released forms of the lactococcal CEP proteinase were also reported by Exterkate and Alting (1993).

Based on its specificity towards $\alpha_{S1}$- and $\beta$-caseins, $\alpha_{S1}$-CN(f1–23) and two charged chromophoric peptides, the proteinase from *Lb. casei* IFPL731 was classified as a PI/III mixed-type variant, different from lactococcal proteinases (Fernández de Palencia et al., 1997f).

### 4.2. Peptidases

Preliminary studies on the peptidase system of *Lb. casei* IFPL731 showed a high level of aminopeptidase, X-prolyl-dipeptidyl aminopeptidase (PepX) and dipeptidase activity in the cell-free extract of this microorganism (Requena et al., 1993). The aminopeptidase system of the strain was subsequently characterized (Fernández de Palencia, Peláez, & Martín-Hernández, 1997d). It consists of six active fractions (ApI–ApVI) with high specificity towards lysine- and leucine-*p*-nitroanilide. Three of these fractions were purified to homogeneity and characterized (Table 1). ApIII was identified as PepN with a molecular mass of 95 kDa, ApIV seems to be tetrameric with a subunit molecular mass of 30 kDa. Fraction ApII was identified as a PepC-like aminopeptidase with broad substrate specificity (Table 2), active on *p*-nitroaniline derivatives, dipeptides and tripeptides, particularly those containing a hydrophobic amino acid residue at the N-terminal of the molecule (Fernández de Palencia, Peláez, & Martín-Hernández, 1997e). This enzyme exhibits unique specificity, being active on peptides containing a proline residue at the N-terminal, and possesses high activity towards methionine-containing peptides. Methionine is assumed to be precursor for the formation of specific aroma compounds in cheese (Urbach, 1995).

Degradation of proline-rich oligopeptides released from caseins by proteinases requires the activity of proline-specific peptidases (Kok & de Vos, 1994). These enzymes may play an important role in cheese ripening, either by degrading proline-containing peptides which are often bitter, or by making peptides accessible to the action of other peptidases by removing proline (Baankreis & Exterkate, 1991). A serine aminopeptidase that specifically hydrolyses peptides with the structure X-Pro-Y and is also able to hydrolyse tri- and dipeptides, which do not contain proline, was purified from *Lb. casei* IFPL731 (Fernández-Esplá, Fox, & Martín-Hernández, 1997a). The enzyme shows different properties from those reported for PepX (Table 1). A prolidase, with a high level of specificity towards dipeptides of the X-Pro type, was also purified from the cell extract of *Lb. casei* IFPL731, suggesting a cooperative interaction between this enzyme and PepX (Fernández-Esplá, Martín-Hernández, & Fox, 1997b; Table 1). This is very important since dipeptides containing proline have been reported to be bitter (Shiraishi, Kazuo, Sato, Yamaoka, & Tuzimura, 1973), whereas free proline imparts a sweet flavour to cheese (Biede & Hammond, 1979).

The study of the aminopeptidase system of *Lb. casei* IFPL731 was completed with the purification of a dipeptidase which was active on dipeptides with a hydrophobic N-terminal amino acid (Fernández-Esplá & Martín-Hernández, 1997). The enzyme differs from other dipeptidases from lactic acid bacteria in that it shows a high temperature optimum and a complex kinetic behaviour (Table 1).

Table 2
Substrate specificity of the aminopeptidase PepC-like from *Lactobacillus casei* subsp. *casei* IFPL 731

| Substrate | Relative activity[a] (%) | Substrate | Relative activity[b] (%) |
|---|---|---|---|
| Leu–*p*NA | 100 | Ala–Met | 100 |
| Ala–*p*NA | 22 | Ala–Val | 55 |
| Met–*p*NA | 40 | Met–Ala | 90 |
| Val–*p*NA | 26 | Pro–Leu | 39 |
| Ala–Ala–*p*NA | 57 | Ala–Pro–Gly | 22 |

[a] Expressed as a percentage of maximal activity measured on *p*-nitroanilide (*p*NA)-derived amino acids and dipeptides.

[b] Expressed as a percentage of maximal activity measured on Ala-Met.

### 4.3. Proteinase and aminopeptidase deficient mutants of *Lb. casei* IFPL731

The role played by the CEP and peptidase activities of *Lb. casei* IFPL731 was studied after construction of proteinase ($Prt^-$) and aminopeptidase ($Ap^-$) deficient variants by treatment with ethyl methane sulphonate (Fernández de Palencia, Martín-Hernández, Hoosten, & Peláez, 1997a). Characterization of these variants demonstrated that their growth rates in MRS broth did not differ greatly from that of the parental strain. In milk, the $Prt^-$ mutant developed poorly and its maximal cell density was much lower than that of the parental strain. However, the $Ap^-$ variant grew at about the same rate as the parental strain, indicating that the deficiency in aminopeptidase activity of this mutant did not prevent it from growing rapidly in milk.

### 4.4. Hydrolysis of the β-casein (193–209) fragment (debittering effect)

The broad specificity of action of the aminopeptidase system of *Lb. casei* IFPL731 suggested that this microorganism may play an important role in cheese flavour development. Besides, it could play a role in

debittering bitter peptides with a high content of hydrophobic amino acids including proline, and promoting further action by other peptidases. The bitter tasting C-terminal part of β-casein, the so-called C-peptide, formed by the action of rennet and starter organisms, is a major cause of bitterness (Visser, Hup, Exterkate, & Stadhouders, 1983; Lemieux, Puchades, & Simard, 1989). Several starter strains and thermophilic lactobacilli, used as adjuncts, have been tested for their ability to degrade the C-peptide as related to growth conditions (Smit, Kruyswijk, Weerkamp, de Jong, & Neeter, 1996). The peptide is too long to be transported into the cells (Kunji, 1997) and therefore, differences among strains were the result of differences of sensitivity to lysis under cheesemaking conditions.

The debittering activity of *Lb. casei* IFPL731 was studied by measuring its ability to hydrolyse the C-peptide (Parra, Fernández de Palencia, Casal, Requena, & Peláez, 1999). Hydrolysis of the peptide was mainly by the intracellular fractions of *Lb. casei* IFPL731 containing endopeptidase and PepN aminopeptidase activities. Major products of hydrolysis were β-CN(f193-205) and β-CN(f194-209). Besides, unlysed whole cells and the purified CEP proteinase of *Lb. casei* IFPL731 also hydrolysed the peptide, with the preferential cleavage site being at bond $Leu^{198}$–$Gly^{199}$. This was the first time that hydrolysis of this peptide was also attributed to the lactic acid bacteria CEP proteinase, conflicting with published results for the purified CEP from *Lc. lactis* Wg2 (Juillard et al., 1995).

## 5. Enzymatic activity in cheese slurries

The role played by the CEP and the peptidase system of *Lb. casei* IFPL731 under cheese-like conditions was elucidated by adding the cell wall extract and the intracellular fraction of the $Prt^-$ and $Ap^-$ mutants of this strain to cheese slurries in which residual rennet was inactivated (Fernández de Palencia, Martín-Hernández, López-Fandiño, & Peláez, 1997b). Primary proteolysis of casein was produced mainly by rennet during cheesemaking, but once it was inactivated the main factor responsible for casein hydrolysis was the CEP, followed by plasmin and microbial intracellular proteinases. Amino acid nitrogen fraction was formed to a large extent by the aminopeptidase activity, but it was demonstrated that the CEP is important as a rate-limiting factor for such formation.

Parra, Requena, Casal, and Gómez (1996) reported the hydrolysis of hydrophobic peptides during incubation of cheese slurries with added whole cells, cell-free extracts (CFE) and cell lysates of different mesophilic lactobacilli. The ratio of hydrophobic to hydrophilic peptides decreased during incubation and was lower in the slurries inoculated with cell lysates of *Lb. casei* IFPL731, which indicates a high level of debittering activity for this strain. At the same time, the rate of conversion of water-soluble nitrogen to non-protein nitrogen and amino acid nitrogen was higher when cell lysates were added, confirming the assumption that the concerted action of the CEP and free peptidases provides ideal conditions for the generation and subsequent hydrolysis of peptides from casein.

The results concerning the debittering activity of *Lb. casei* IFPL731 towards both the C-peptide of β-casein and hydrophobic peptides in cheese, suggest that the use of this strain as a starter culture adjunct could be an effective means of reducing bitterness in cheese.

## 6. Induction of cell lysis to accelerate cheese ripening

The intracellular location of the esterase and peptidase activities of *Lb. casei* IFPL731 suggests that the potential of this strain to contribute enzymatically to flavour development could be enhanced by the early release of intracellular enzymes to the cheese matrix during maturation through bacterial lysis. The amino acids which are produced are essential precursors of compounds responsible for aroma (Fox & Wallace, 1997) and hence are a limiting factor in aroma development. Increasing their presence in the medium is therefore considered a fundamental step for development of the sensory characteristics of the cheese.

Different strategies have been used to induce early cell lysis of strains from the IFPL starter and adjuncts. One of these was to expose the microbial cells to a sub-lethal heat treatment ranging from 50°C to 54°C for 15 s (Asensio, Gómez, & Peláez, 1995). Another was to apply a high hydrostatic pressure from 100 to 400 MPa at 20°C for 20 min (Casal & Gómez, 1999). In both cases, substantial reductions were achieved in the viability and acidifying activity of the strains, including *Lb. casei* IFPL731, without adversely affecting their peptidase enzymes. Optimum results were found for *Lb. casei* IFPL731 by heating for 15 s at 50°C or pressurizing at 300 MPa for 20 min at 20°C. Heat-treated cultures of *Lb. casei* IFPL 731 were added as an adjunct to the IFPL starter in the manufacture of low-fat goats' milk cheese (Asensio, Parra, Peláez, & Gómez, 1996). Addition of heat-shocked cells did not affect the cheesemaking process but led to an increase in aminopeptidase activity and the increased amino acid nitrogen content correlated with enhancement of flavour and reduction of bitterness.

Another strategy recently proposed to induce lysis of the starter and adjuncts is the use of bacteriocins with a lytic effect (Fox et al., 1996). Although increasing attention has recently been focused on bacteriocins

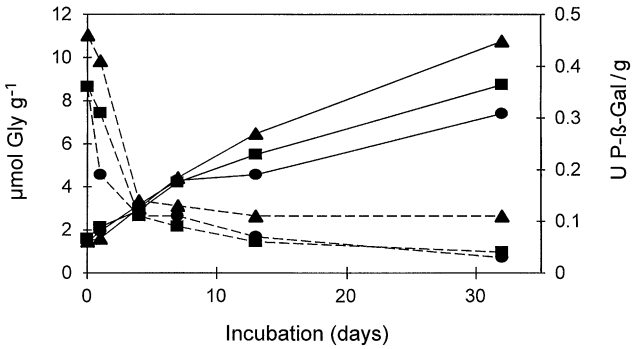


Fig. 1. Evolution of amino nitrogen (—) and phospho-$\beta$-galactosidase activity (– – –) in cheese-curd slurries supplemented with *Lc. lactis* IFPL105 Bac$^+$(▲), its variant Bac$^-$ (■), and control (●).

produced by lactic acid bacteria as inhibitors of spoilage and food-borne pathogenic microorganisms (Ray & Daeschel, 1992; de Vuyst & Vandamme, 1994; Schillinger, Geisen, & Holzapfel, 1996), there has been much less published concerning their use as potential cell lysis inducing agents in cheese manufacture.

Studies at our laboratory have shown that *Lc. lactis* IFPL105 isolated from raw goats' milk produces a bacteriocin with a broad inhibitory spectrum, including other LAB and Gram positive pathogens (Casla, Requena, & Gómez, 1996). The purification and molecular characterization of the genetic region that encodes its biosynthesis, resident in a plasmid of 46 kb, showed that it is a two-peptide lantibiotic identical to lacticin 3147 (Martínez-Cuesta et al., 2000a). This compound showed a bacteriolytic effect on the IFPL starter and *Lb. casei* IFPL731, resulting in the release of intracellular material (Martínez-Cuesta, Peláez, Juárez, & Requena, 1997; Martínez-Cuesta et al., 2000b). The bacteriocin producer, *Lc. lactis* IFPL105, when used in cheese slurries along with *Lb. casei* IFPL731 as starter adjuncts, has been demonstrated to produce a lytic effect on sensitive cells and the subsequent acceleration of proteolysis during incubation of slurries (Martínez-Cuesta, Fernández de Palencia, Requena, & Peláez, 1998). In these studies, it was observed that addition of *Lc. lactis* IFPL105 did not interfere with proper curd acidification by the starter and increased the level of amino nitrogen. This increase in amino nitrogen correlated with the early release of intracellular enzymes, such as phospho-$\beta$-galactosidase from the starter adjunct, due to the presence of the bacteriocin (Fig. 1).

## 7. Amino acid converting activity

It has been established that while the end products of proteolysis, i.e. peptides and amino acids, contribute directly to the background taste of cheese, other ripening compounds also appear to be essential for cheese flavour development. These include degradation products of free amino acids and FFAs, but also lactic acid, formed *via* enzymatic and/or chemical pathways (third phase of ripening) (Crow, Coolbear, Holland, Pritchard, & Martley, 1993). Among these pathways, the enzymatic conversion of amino acids to aroma compounds such as aldehydes, acids, alcohols, esters and thiols, plays a major role in cheese flavour development (Fox & Wallace, 1997). Indeed, degradation products from aromatic and branched-chain amino acids and methionine have been identified in various cheeses and contribute greatly to their typical flavour (Bosset & Gauch, 1993; Engels, Dekker, de Jong, Neeter, & Visser, 1997; Milo & Reineccius, 1997) but also tend to cause off-flavour (Dunn & Lindsay, 1985). Enzymes from microorganisms present in cheese that are involved in the degradation of amino acids include deaminases, decarboxylases, transaminases and enzymes which convert amino acid side chains. Moreover, as suggested by Smit et al. (2000), diversification of flavours can be obtained depending on the enzymes present in the cultures.

Since *Lb. casei* IFPL731 has been shown to enhance flavour development in semi-hard cheese when added as an adjunct (Requena et al., 1992; Rodríguez et al., 1996), it was reasonable to suppose that this strain would probably contain effective AACE's. The amino acid utilization ability of *Lb. casei* IFPL731 was, therefore, studied (Parra et al., 1998). Single amino acids and mixtures of amino acids were added with 1 g/kg NaCl and CFE of the strain. The pH was adjusted at 5.4. After incubation of the reaction mixtures, volatile components were measured by gas chromatography, amino acid utilization by HPLC and sensory analysis was performed using as reference the water-soluble nitrogen fraction of a 4-month raw ewes' milk cheese (Manchego). Volatile sulphur components were found in the mixture containing the CFE and 20 amino acids. In samples containing individual amino acids, volatile sulphur components were only found when S-containing amino acids were present. These samples received the highest flavour scores (Fig. 2). Therefore, S-containing amino acids seem to be a substrate for the enzymatic activity of *Lb. casei* IFPL731 in the production of the typical cheese flavour. This is consistent with the high level of PepC-like activity towards methionine-containing peptides in this microorganism (Fernández de Palencia et al., 1997e).

Thirty strains of lactic acid bacteria, including *Lb. casei* IFPL731, were screened for aminotransferase activity using methionine as substrate (MetAT) and different amino group acceptors. Strains were also tested for lyase and amino acid decarboxylase activities (Amárita et al., 2001). Of all the strains examined, only two strains of *Lb. casei* and five of *Lb. plantarum* (25%

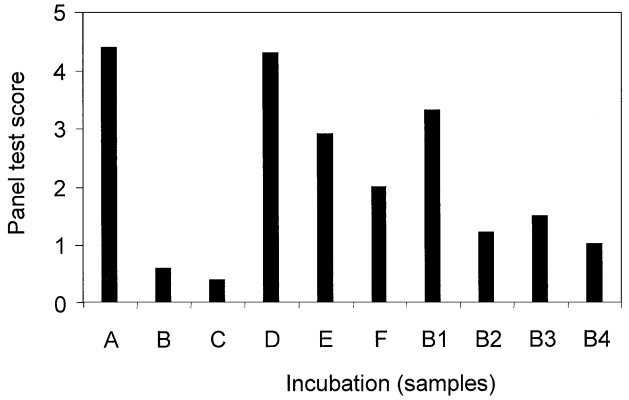


Fig. 2. Average aroma scores of incubations of the cell-free extract from *Lb. casei* subsp. *casei* IFPL731 and mixtures of amino acids (2 mM final concentration). (A) Total amino acids mixture (Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, Val); (B) Ala, Gly, Pro, His, Arg, Lys, Orn; (C) Ala, Gly, Pro, Gln, Glu, Asp, Asn; (D) Ala, Gly, Pro, Ser, Thr, Cys; (E) Met; (F) Cys. Controls: (B1) total amino acids mixture incubated with CFE heat-treated (110°C for 10 min); (B2) CFE; (B3) Met; (B4) Cys.

of strains assayed) showed MetAT. *Lb. casei* IFPL731 showed the highest MetAT when using pyruvate as amino group acceptor and showed no methionine lyase or glutamate and methionine decarboxylase activity.

While some authors suggest that cell lysis may not be important for amino acid catabolism since dead unlysed cells of lactic acid bacteria are able to degrade aromatic amino acids (Gao et al., 1997), others suggest that only minor alteration of the cytoplasmic membrane is needed to increase penetration of amino acids in the cells (Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997). The AACE's are co-factor dependent and their activity is reduced considerably at low pH. Consequently, they are likely to function better in intact cells than when they are released into the cheese medium and, therefore, a balance of both intact and lysed starter or adjunct cells in cheese appears to be of great importance in flavour development. Nevertheless, Yvon et al. (1997) reported that after cell lysis and release of the enzymes into the cheese matrix, these could still be active since the co-factor appeared to be quite well bound to the enzyme. The same authors reported that the aromatic amino-transferase (AraT) from *Lc. lactis* NCDO763 was still active at the pH of the cheese and high NaCl concentrations (4%).

At present, we are seeking to gain a better understanding of the effect of cell lysis on the activity of AACE's, using the bacteriocin produced by *Lc. lactis* IFPL105 in order to create the ideal balance of intact/lysed cells which is necessary for amino acid catabolism and flavour production in cheese. Furthermore, the contribution of the activity of the enzymes which further degrade the α-ketoacids, produced by transamination, into aroma compounds, remains to be elucidated.

## 8. Conclusion

*Lactobacillus casei* IFPL731 is being used as an adjunct for the manufacture of semi-hard cheese. Its enzymatic system, described in this paper, has considerable potential to influence the development of cheese flavour, as has been demonstrated in the manufacture of goats' milk and low-fat cheese. Its' esterase activity yields short-chain fatty acids which, even at low concentrations, are important flavour components in cheese. Owing to the broad specificity of its peptidase system, this microorganism is able to degrade a number of peptides, including bitter peptides with a high content of hydrophobic amino acids such as proline, and hence, is thereby able to promote further action by other peptidases. It can also degrade the bitter β-casein (193–209) fragment. Its high activity on methionine-containing peptides may contribute to flavour development, since this is one of the amino acids assumed to be a precursor of the formation of specific aroma compounds in cheese. Moreover, it can utilize this amino acid, initiated by a methionine aminotransferase activity, to produce typical cheese aroma.

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