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Enhancement of 2-methylbutanal formation in cheese by using a fluorescently tagged Lacticin 3147 producing *Lactococcus lactis* strain

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Abstract

The amino acid conversion to volatile compounds by lactic acid bacteria is important for aroma formation in cheese. In this work, we analyzed the effect of the lytic bacteriocin Lacticin 3147 on transamination of isoleucine and further formation of the volatile compound 2-methylbutanal in cheese. The Lacticin 3147 producing strain *Lactococcus lactis* IFPL3593 was fluorescently tagged (IFPL3593-GFP) by conjugative transfer of the plasmid pMV158GFP from *Streptococcus pneumoniae*, and used as starter in cheese manufacture. Starter adjuncts were the bacteriocin-sensitive strains *L. lactis* T1 and *L. lactis* IFPL730, showing branched chain amino acid aminotransferase and α-keto acid decarboxylase activity, respectively. Adjunct strains were selected to complete the isoleucine conversion pathway and, hence, increase formation of 2-methylbutanal conferring aroma to the cheese. The non-bacteriocin-producing strain *L. lactis* IFPL359-GFP was included as starter in the control batch. Fluorescent tagging of the starter strains allowed their tracing in cheese during ripening by fluorescence microscopy and confocal scanning laser microscopy. The bacteriocin produced by *L. lactis* IFPL3593-GFP enhanced lysis of the adjuncts with a concomitant increase in isoleucine transamination and about a two-fold increase of the derived volatile compound 2-methylbutanal. This led to an enhancement of the cheese aroma detected by a sensory panel. The improvement of cheese flavour and aroma may be of significant importance for the dairy industry.

Keywords: Cheese aroma; Lacticin 3147; Green fluorescent protein

1. Introduction

Free amino acids formed by proteolysis of casein contribute to the taste of ripened cheeses or can be converted into cheese flavour compounds (Christensen et al., 1999; McSweeney and Sousa, 2000). To

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achieve a good cheese flavour, it is necessary to have a balanced concentration of a wide range of aromatic compounds. In lactococci, branched chain amino acids (BCAAs) are primarily converted into α-keto acids by transamination (Yvon et al., 1997, 2000; Atiles et al., 2000) which are further converted into hydroxyacids or into flavour compounds such as carboxylic acids by an oxidative decarboxylation, or the methylaldehydes 2- and 3-methylbutanal and 2-methylpropanal by a non-oxidative decarboxylation (Yvon and Rijnen, 2001). The aldehydes can be further reduced to alcohols or oxidized to carboxylic acids. Enzymatic decarboxylation of α-keto acids is not a common feature in cheese microorganisms. However, conversion of α-keto acids to aldehydes may be chemically feasible (Koga et al., 1992; Nierop Groot and de Bont, 1998) and α-keto acid decarboxylating activity has been characterized in bacteria *Enterobacter cloacae* (Koga et al., 1992) and yeasts, *Saccharomyces cerevisiae* (Schure et al., 1998). Concerning the lactococci, decarboxylation of α-keto-β-methyl-*n*-valeric acid (KMVA) to methyl aldehydes was first reported in *Lactococcus lactis* var. *maltigenes* (Morgan, 1976). Later work has shown that most lactococci produce only small amounts of aldehydes (Yvon and Rijnen, 2001) and, in recent years, the ability of some particular lactococcal strains to produce aldehydes by decarboxylation of α-keto acids has reactivated the subject (Ayad et al., 1999; Amárita et al., 2001). Although formation of 2- and 3-methylaldehydes is considered responsible for malty off-flavours in Cheddar cheese (Morgan, 1976), these aldehydes are a major part of the volatile fraction of several cheeses such as Cheddar, Camembert, Emmental, and Parmesan (Barbieri et al., 1994; Yvon and Rijnen, 2001; Thierry and Maillard, 2002), suggesting that the overall acceptance of these compounds depends on the final balance of volatiles in the cheese.

Since the ability of lactic acid bacteria to degrade amino acids is strain-dependent, the selection of starter or adjuncts with unique amino acid catabolic activities has been proposed (Ayad et al., 2001). Additionally, cell lysis favours accessibility of the intracellular enzymes to their substrates and potentially enhances bacterial enzymatic activity. In this regard, one of the most interesting approaches is the use of bacteriocins (Martínez-Cuesta et al., 1997, 1998; Morgan et al., 1995, 1997). We have demonstrated that Lacticin 3147 has a lytic effect by causing pores in the cytoplasmic membrane of lactococcal-sensitive cells and, hence, promoting uncontrolled degradation of the cell walls by the lactococcal autolysin AcmA (Martínez-Cuesta et al., 2000). In a previous work, we constructed the *L. lactis* IFPL3593 transconjugant that produces Lacticin 3147, by transfer of the bacteriocin coding plasmid pBAC105 from *L. lactis* IFPL105 (Martínez-Cuesta et al., 2001). The bacteriocin producer *L. lactis* IFPL3593 accelerated cheese proteolysis during ripening by causing lysis of peptidolytic adjunct strains. The starter strain *L. lactis* IFPL359 (parental of the transconjugant IFPL3593) is also sensitive to the lytic action of the Lacticin 3147 (Martínez-Cuesta et al., 2000). Recent work has shown that the bacteriocin caused membrane permeabilization of *L. lactis* IFPL359, which facilitated free diffusion of amino acids into the cell, and further cell lysis (Martínez-Cuesta et al., 2002). Both facts, cell permeabilization and lysis, render the amino acid converting enzymes more accessible to their substrates and hence, an increased isoleucine transamination was observed. These results suggested the possibility to enhance flavour formation in cheese by combination of the constructed strain *L. lactis* IFPL3593 that produces Lacticin 3147, and strains sensitive to the bacteriocin having complementary action on the isoleucine metabolism.

Fluorescent labelling of a constructed strain can provide useful methods to allow its detection during cheese ripening. In previous research we have reported the detection of lactococcal cells carrying the *gfp* (gene encoding green fluorescent protein, GFP) from the jellyfish *A. victoria* by fluorescent microscopy during its growth in milk (Fernández de Palencia et al., 2000; Nieto et al., 2000). This preliminary result suggested that GFP could be used as a fluorescent-tag for monitoring the growth of the Lacticin 3147 producer *L. lactis* IFPL3593 during cheese ripening.

In the present work, the effect of Lacticin 3147 on transamination of isoleucine and further formation of the volatile compound 2-methylbutanal in cheese was studied. The Lacticin 3147 producer *L. lactis* IFPL3593 was used as starter and two other lactococcal strains, sensitive to the bacteriocin and showing complementary action on isoleucine catabolism, were used as starter adjuncts. *L. lactis* IFPL3593 and *L.*

lactis IFPL359 (non-producing bacteriocin), were tagged with the fluorescent protein GFP to monitor them during cheese ripening.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The *L. lactis* strains used in this study were: the Lacticin 3147-producing transconjugant IFPL3593 (Martínez-Cuesta et al., 2001); its wild-type parental strain IFPL359; strain T1, which is a Lac^- Prt^- (lactose- and proteinase-deficient) variant of IFPL359 (Requena and McKay, 1993); strain IFPL730 with high α-keto acid decarboxylase activity (Amárita et al., 2001), and the tagged strains IFPL 3593-GFP and IFPL359-GFP constructed in this work. Lactococci were grown (up to 9 log cfu/ml) at 30 °C in M17 broth (Oxoid, Basingstoke, UK), containing 8 g/l glucose (GM17).

Streptococcus pneumoniae R61 (Lacks, 1968), containing pMV158GFP and pAMβ1 plasmids, was used as donor bacteria in conjugation experiments. *S. pneumoniae* was grown to 9.12 log cfu/ml in AGCH medium (Lacks, 1968) supplemented with 2.5 g/l yeast extract and 8 g/l sucrose (AGCHYS). When required, the media contained tetracycline (1 μg/ml) and/or erythromycin (5 or 1 μg/ml for *L. lactis* or *S. pneumoniae*, respectively). Plasmid pMV158GFP is a derivative of the mobilizable plasmid pMV158 (Burdet, 1980), which contains the P_M-*gfp* cassette of pLS1RGFP (Nieto et al., 2000). Construction of the plasmid was described elsewhere (Nieto and Espinosa, 2003).

Growth of the cells was monitored by measuring the change in optical density (OD) of the cultures at 660 or 650 nm for *L. lactis* or *S. pneumoniae*, respectively, in a spectrophotometer (Spectronic 20D, Milton Roy, Rochester, USA).

2.2. Conjugation experiments

Intergeneric matings between *S. pneumoniae*, containing pAMβ1 and pMV158GFP, and *L. lactis* IFPL359 or IFPL3593 strains, were performed as follows: *S. pneumoniae* was grown at 37 °C in AGCHYS, supplemented with erythromycin and tetracycline, to an OD_{650} of 0.5 (9.12 log cfu/ml) and *L. lactis* was grown at 30 °C in GM17 to an OD_{660} of 0.5 (9.63 log cfu/ml). Then, donor and recipient cultures were independently sedimented by centrifugation at $6000 \times g$ for 10 min and resuspended in AGCH medium, adjusted to contain 10 mM $MgCl_2$ and bovine serum albumin at 2 mg/ml (AGCHMB). Donor (0.2 ml) and recipient (2 ml) bacteria were gently mixed with a pipette in an eppendorf tube and filtered onto sterile 13 mm diameter nitrocellulose filters (0.22 μm; Millipore, Bedford, USA), placed cell-side up on AGCHMB-agar plates supplemented with glucose (2 g/l) and DNase I (Roche, Mannheim, Germany) at 10 μg/ml, and incubated for 4 h at 37 °C. Cells were recovered from the filter by washing with 1 ml AGCHMB medium supplemented with 0.1 g/ml glycerol. The washings were plated on agar GM17 containing tetracycline, erythromycin or both antibiotics to test for the presence of pMV158GFP, pAMβ1, or both plasmids. Transconjugants were allowed to grow on the agar for 48 h at 30 °C. The presence of plasmids was confirmed by electrophoretical analysis on 0.6% agarose gels of alkaline lysates prepared as described (Birnboin and Doly, 1979). The expression of GFP by the transconjugants was tested by measuring the fluorescence of the cells on a LS-50B spectrophotometer (Perkin Elmer, Shelton, USA) by excitation at 488 nm with a slit of 2.5 nm, and detection of emission at 511 nm with slit of 10 nm, as previously described (Fernández de Palencia et al., 2000). Cultures were grown to an OD_{660} of 0.5, cells were sedimented by centrifugation at $6000 \times g$ for 10 min, washed and resuspended to the original volume in PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl), pH 8.0, prior to measurement of fluorescence. As a background, cells of the parental strains lacking pMV158GFP were used and their values of fluorescence were subtracted from cells harbouring pMV158GFP. The transconjugants *L. lactis* IFPL359-GFP and IFPL3593-GFP, both expressing GFP, were selected for further analysis prior usage as starters in cheese-making trials.

2.3. Cheese manufacture

Laboratory-scale cheese-making trials were carried out using commercial pasteurized cow's milk. Milk

(10 l per batch) was heated to 32 °C, supplemented with 0.2 g/l $CaCl_2$, and inoculated with 7.3 log cfu/ml of an overnight culture of *L. lactis* IFPL359-GFP (batch C) or its Lacticin 3147-producing transconjugant *L. lactis* IFPL3593-GFP (batch Bac), in skimmed milk. After 30 min incubation, the cheese vat milk was inoculated with an exponentially growing culture (OD_{660} of 0.7) of the adjuncts *L. lactis* T1 (8 log cfu/ml) (Lac^- Prt^-) and *L. lactis* IFPL730 (7.3 log cfu/ml) grown in GM17 broth, centrifuged at 6000 × *g* for 10 min, washed with autoclaved distilled water, and resuspended in the milk. Renneting was initiated by adding 0.025 g/l of chymosin (CHY-MAX 2080 IMCU/g, Chr. Hansen, Hørsholm, Denmark). Curd was cut, heated to 37 °C, scooped into cylindrical moulds and pressed for 90 min. When the curds reached pH 5.4, they were immersed for 2 h at room temperature in a sterile brine containing 100 g/l NaCl, 10 g/l α-ketoglutarate, and 10 g/l isoleucine. The obtained cheeses of 200 g were drained overnight at 4 °C and ripened at 10–12 °C and 85–90% relative humidity. The cheeses were analyzed in triplicate at 1, 7, 15, 30, and 45 days of ripening.

2.4. Microbiological analysis and detection of the bacteriocin

Cheeses were analyzed in accordance with the standards of the International Dairy Federation (1985). Lactococcal colony forming units were determined using bromocresol-purple lactose indicator agar (BCPL; McKay et al., 1970). After 24 h of incubation at 30 °C, lactococci formed yellow colonies due to their lactose-positive phenotype with the exception of *L. lactis* T1 (Lac^- Prt^-) colonies, which were white. *L. lactis* IFPL359-GFP. *L. lactis* IFPL3593-GFP were enumerated by transfer of yellow colonies onto BCPL-agar containing tetracycline at 1 μg/ml.

Bacteriocin activity in cheese was determined in samples of 0.5 g of cheese homogenized with 500 μl of 40% isopropanol in a vortex for 5 min. After centrifugation (10,000 × *g* for 10 min), 50 μl of supernatant was dispensed into wells of uniform diameter (7 mm) bored in M17-Lactose (M17-L) agar plates and sealed with tempered soft agar (45 °C). Plates were overlayed with a lawn of molten M17-L agar seeded with the indicator strain (*L. lactis* IFPL359) and incubated overnight at 30 °C. Bacteriocin activity was expressed as the area (mm^2) of inhibition surrounding each agar well. Control samples were cheeses manufactured with the isogenic strain *L. lactis* IFPL359GFP that no produces the bacteriocin.

2.5. Detection of GFP-fluorescently tagged lactococci by fluorescence and confocal scanning laser microscopy

For detection of GFP-fluorescently tagged bacteria, the lactococcal cells grown in GM17 broth to an OD_{660} of 0.4 were sedimented by centrifugation at 6000 × *g* for 10 min, washed, and resuspended to original volume in PBS buffer, pH 8.0. Cells were analyzed directly without fixing by phase-contrast or fluorescence microscopy with a Zeiss Axioplan Universal microscope (Zeiss, Oberkochen, Germany) with an excitation Standard FITC set D480/30 and emission TBP 460/530/610 fluorescent filters. GFP was detected by excitation at 495 nm and detection of emission at 530 nm. Representative areas of each sample, such as those depicted in Figs. 2 and 4, were imaged using a × 100 magnification objective with a numerical aperture of 1.25.

The GFP fluorescence expressed by lactococcal cells is impaired at external pH below 5.2, although it can be recovered by suspension of the cells in PBS buffer (pH 8.0) (Fernández de Palencia et al., 2000). Therefore, to improve detection of fluorescently tagged bacteria due to the low pH values reached in the cheeses, 0.4 g of cheese were homogenized with 1 ml of PBS buffer (pH 8.0), and incubated for 5 min at 30 °C. Cells and cheese samples were sedimented by centrifugation at 6000 × *g* for 10 min and washed twice in PBS buffer. After increasing the pH of the samples, and for detection of tagged and non-tagged bacteria, the genome of the bacteria was stained with 4′,6-diamidino-2-phenylindole dihydrochloride:hydrate (DAPI) reactive (Sigma-Genosys, Cambridge, UK) during 15 min at 20 °C, prior to cell-detection by microscopy. Analysis was performed by conventional fluorescence microscopy (as described above), selecting the appropriate excitation and emission filters for detection of GFP (495 and 530 nm) or DNA (400 and 460 nm). In addition, cheese sample areas were imaged for detection of GFP by a confocal scanning laser microscope Zeiss Axiovert 135 using a × 100 magnification objective with a numerical aperture of

2.5. Confocal illumination was provided by an Ar laser (488 nm laser excitation) with an OG 515 nm filter (green fluorescence signal). Image analysis was performed on CSLM images using a confocal system MRC 1024 Bio-Rad (Bio-Rad, Hercules, USA) for image acquisition and processing.

2.6. Physicochemical and enzyme analyses

The pH of cheeses was determined using a Metrohm Model 691 pH meter (Metrohm, Herisau, Switzerland) using 1 g of cheese homogenized with 5 ml of distilled water. Total solids were dried to constant weight at 102 ± 2 °C for gravimetric determination (International Dairy Federation, 1982). Amino nitrogen was determined from the non-protein nitrogen fraction as described by Church et al. (1983) by reaction with *o*-phthaldialdehyde (OPA). Determination of NaCl content was carried out as described by Yvon et al. (1998) by using the Sigma diagnostics chloride reagent (Sigma-Aldrich, St. Louis, USA).

Release of X-prolyl-dipeptidyl aminopeptidase (PepX) into the cheese matrix was used as cellular lysis marker. The enzyme extract was obtained by vortexing 5 g of cheese with 5 ml of 50 mM Tris–HCl buffer (pH 7.0) as previously described (Martínez-Cuesta et al., 2001). PepX activity was measured in 100 μl of extract using 100 μl of 1 mM Gly–Pro-*p*-nitroanilide (Sigma) solution in 50 mM Tris–HCl buffer (pH 7.0) as substrate and 300 μl of the Tris–HCl buffer. The incubation was carried out at 37 °C in a Peltier CPS-240A temperature controller in a model UV-1601 spectrophotometer (Shimadzu, Columbia, USA). Release of *p*-nitroaniline was measured as the increase in absorbance at 410 nm ($E_{410} = 8.800$ l mol^{-1} cm^{-1}) and PepX activity was expressed as micromoles of product released per minute, per milliliter of extract (U/ml).

2.7. Analysis of α-keto-β-methyl-n-valeric acid and 2-methylbutanal

The α-keto-β-methyl-*n*-valeric acid (KMVA) was obtained from 1.25 g of cheese vortexed for 2 min with 5 ml of 0.2% H_3PO_4, centrifuged at $14{,}000 \times g$ for 15 min at 4 °C and the supernatant filtered through a 0.45 μm pore size filter (Millipore). 2-Methylbutanal was extracted from cheese by homogenizing with ethyl acetate (1:1; wt/vol) and treated by derivatisation with 2,4-dinitrophenylhydrazine to form the corresponding dinitrophenyl hydrazone derivative, as described by Kuntz et al. (1998). KMVA and 2-methylbutanal were analyzed by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) using a Jasco RP-HPLC system (Jasco, Tokyo, Japan). Reaction products were separated using a Luna C18-2 analytical column (250×4.6 mm, 5 μm) (Phenomenex, Torrance, USA) held at 40 °C. Analysis of KMVA was performed according to Olalla-Herrera et al. (1993) by means of an isocratic elution of compounds at a flow rate of 1 ml/min using 0.2% H_3PO_4 in Milli-Q water (Millipore) as mobile phase. Optical density was measured at 210 nm. Chromatographic analysis of 2-methylbutanal was performed according to Schmidt et al. (1983) by means of an isocratic elution using as mobile phase 60% acetonitrile in Milli-Q water at a flow rate of 1.5 ml/min at 40 °C and measuring the optical density at 365 nm. KMVA and 2-methylbutanal were identified by comparing retention times with those of appropriate standard compounds. Data acquisition and processing were performed using Borwin chromatography software (JBMS Développements, Grenoble, France).

Table 1
Summary of conjugal transfers

Strain		Counts (cfu/ml)[a]			
Donor	Recipient	Donor	Recipient	Transconjugants	
				Tet^R	Erm^R
S. pneumoniae PMV158GFP/pAMβ1	*L. lactis* 3593	$(3.74 \pm 0.81) \times 10^7$	$(3.73 \pm 1.9) \times 10^9$	$(2.30 \pm 0.65) \times 10^2$	$(1.45 \pm 0.74) \times 10^3$
S. pneumoniae PMV158GFP/pAMβ1	*L. lactis* 359	$(4.90 \pm 2.05) \times 10^7$	$(4.12 \pm 2.08) \times 10^9$	$(2.53 \pm 0.62) \times 10^2$	$(9.6 \pm 4.10) \times 10^2$

Tet^R, tetracycline-resistant; Erm^R, erythromycin-resistant.
Frequency data are the averages of three separate experiments.
[a] Total number of donor, recipient or transconjugant cfu recovered per milliliter of cell suspension.

2.8. Sensory analysis

Six panelists were previously trained for detection of 2-methylbutanal by sniffing homogenates of cheese samples in water at 0 days of ripening, containing increased amounts of the aldehyde (up to 250 μg/g). At different intervals during ripening, samples of experimental cheeses were frozen at − 20 °C in screw cap tubes. For sensorial evaluation, the samples were thawed overnight at 4 °C. Panelists were requested to score aroma detection in homogenates of cheese samples in water (1:1 wt/vol) at room temperature. Aroma evaluation scale was from 0 (absent) to 10 (very strong).

2.9. Statistical analysis

Statistical analysis of results was performed using one-way analysis of variance (Statgraphics Plus 2.1; Statistical Graphics, Rockville, USA) to determine significant differences ($P < 0.05$) between batches in cheese composition, KMVA, 2-methylbutanal and aroma evaluation.

3. Results and discussion

3.1. Construction and detection of fluorescently labelled starter strains

The expression of the autofluorescent protein GFP in the bacteriocin-producing starter *L. lactis* IFPL3593 was carried out to discriminate this microorganism from the lactococcal adjuncts T1 and IFPL730 during cheese ripening. For this, we developed a method for the mobilization conjugative transfer of plasmid pMV158GFP from *S. pneumoniae* to *L. lactis* (see details in Materials and methods). This method includes the use of GM17 plating medium, which allows growth of the transconjugants but not of *S. pneumoniae* and, therefore, it does not require a selective marker in the recipient strain. The summary of conjugal matings is shown in Table 1. The lactococcal strains IFPL3593 and its parental IFL359 (bacteriocin non-producer) were the recipients for the plasmids pMV158GFP and pAMβ1 harboured by the donor strain *S. pneumoniae* R61. The viability of *L. lactis* recipient strains did not decrease during the mating period, while viable counts of the donor after conjugal transfer were approximately 35% of the initial count (data not shown). The mobilization of the non-conjugative pMV158GFP (tetracycline-resistant) by the plasmid pAMβ1 was similar in both strains IFPL359 and IFPL3593, and six-fold lower than the frequency of erythromycin-resistant transconjugants (Table 1). Plasmid analysis of IFPL3593 and one of the tetracycline transconjugants is shown in Fig. 1. As expected from previous work (Requena and McKay, 1993), several plasmids were detected in strain IFPL3593 (Fig. 1, lane 1). In addition to those plasmids, bands migrating to the same position as covalently closed and open circle forms of pMV158GFP (Fig. 1, lane 3) were detected in the IFPL3593 transconjugant (Fig. 1, lane 2). Analysis of 12 tetracycline-resistant transconjugants showed

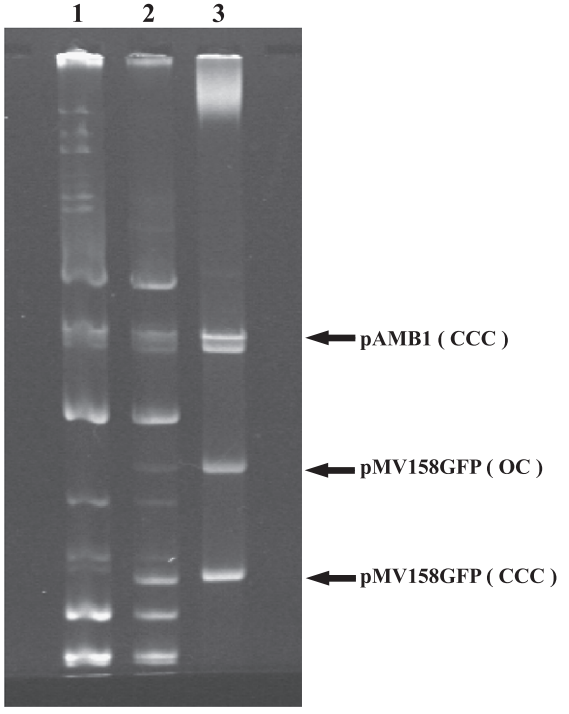

Fig. 1. Detection of pMV158GFP in *L. lactis* IFPL3593-GFP transconjugant. Agarose gel analysis of plasmid content of *L. lactis* IFPL3593 recipient strain (lane 1), *L. lactis* IFPL3593-GFP transconjugant (lane 2) and *S pneumoniae* R61/pMV158GFP, pAMβ1 donor strain (lane 3). Positions of covalently closed forms of the plasmids (CCC) and open circle forms of the pMV158GFP plasmid (OC) are indicated.

that 60% were also erythromycin-resistant (results not shown). This result reveals that in these cells, both plasmids were co-transferred. One transconjugant of *L. lactis* IFPL3593 (IFPL3593-GFP) and another of IFPL359 (IFPL359-GFP), harbouring pMV158GFP but not pAMβ1, were selected for further analysis, prior to their use as starters in cheese-making trials. Expression of GFP did not affect the growth of IFPL3593-GFP and IFPL359-GFP in GM17 medium compared with their parental strains, having both a doubling time of 36 ± 9.2 min during the exponential phase of growth. Lactococcal cells grown in GM17 medium were directly analyzed by phase-contrast and fluorescence microscopy (Fig. 2). The GFP synthesized by IFPL3593-GFP allowed its discrimination from T1 and IFPL730 (Fig. 2D) or from its parental strain IFPL3593 (Fig. 2E).

In conclusion, pMV158GFP was shown to be suitable for the fluorescent-tagging of lactococci. Moreover, the method developed for conjugative transfer from *S. pneumoniae* to *L. lactis* could be of general use for transfer of conjugative and mobilizable plasmids to lactococcal strains of industrial interest refringent to electroporation. The general application of intergeneric conjugation from *S. pneumoniae* to other lactic acid bacteria is currently under investigation.

3.2. Cheese composition and microbiology

Cheese manufacture was carried out with a defined bacterial system consisting of the Lacticin 3147 producer *L. lactis* IFPL3593-GFP, as a starter culture to cause cell lysis, and the adjuncts *L. lactis* T1 and *L. lactis* IFPL730 that are sensitive to the bacteriocin. Adjuncts were added to the cheese vat after being grown up to the mid-log phase, which is the stage of maximum sensitivity to the bacteriocin (Martínez-Cuesta et al., 1997). The starter used in the control batch was the bacteriocin non-producer *L. lactis* IFPL359-GFP.

Use of the bacteriocin producer IFPL3593-GFP as cheese starter did allow proper acidification during the manufacturing process. After pressing, the composi-

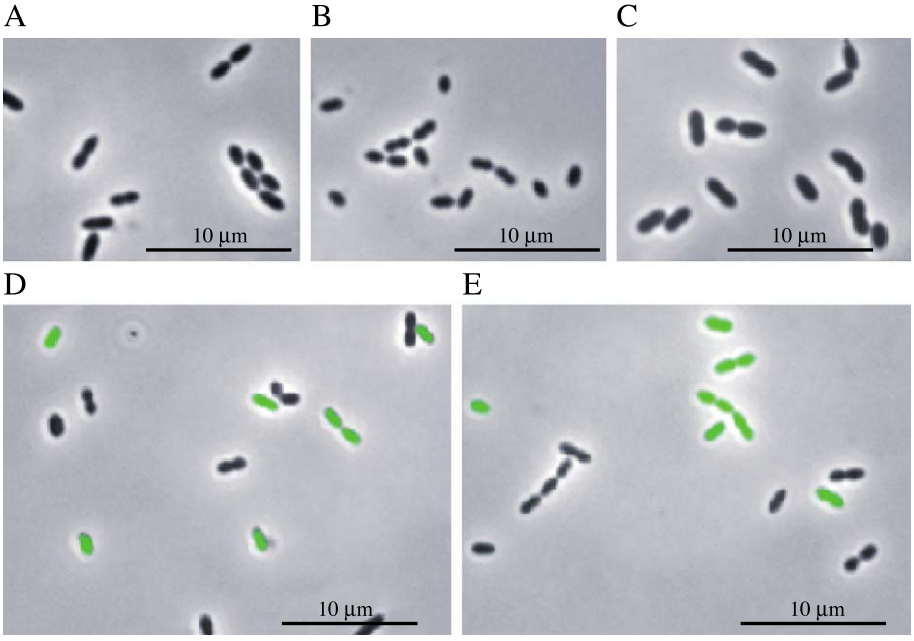

Fig. 2. Detection of expression of GFP by fluorescence microscopy. Exponential cultures of lactococcal cells grown in M17 medium were directly analyzed by phase-contrast and fluorescence microscopy. *L. lactis* IFPL3593-GFP (A), *L. lactis* T1 (B) and *L. lactis* IFPL730 (C) strains were detected by phase-contrast. Digital impose (overlay) of phase-contrast and fluorescent images of mixed lactococcal cultures containing IFPL3593-GFP, T1 and IFPL730 strains (D) or IFPL3593-GFP and IFPL3593 (E).

tion of the cheeses did not differ significantly ($P > 0.05$) from the control; the average pH was 5.4 (± 0.10) and the average value of dry matter was 47.7% (± 1.92).

Fig. 3 shows that IFPL3593-GFP and IFPL359-GFP had a similar viability throughout cheese ripening. Evolution of both adjuncts, *L. lactis* T1 and IFPL730, varied depending on the cheese. Adjuncts viability in cheeses manufactured with IFPL3593-GFP was lower than that in the control cheese from the beginning of ripening, indicating that the bacteriocin effect had already started during cheese manufacture.

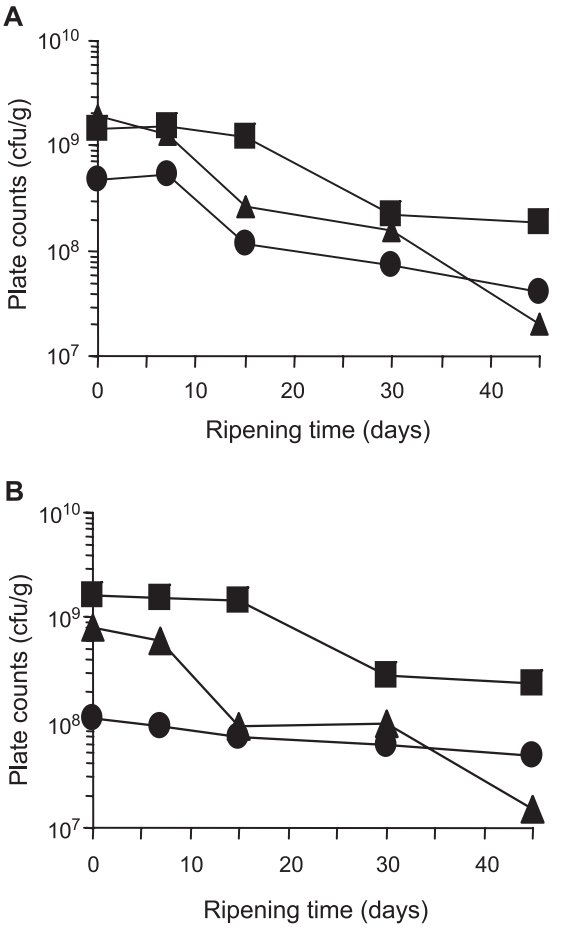

Fig. 3. Evolution of *L. lactis* IFPL359-GFP or *L. lactis* IFPL3593-GFP (■), *L. lactis* IFPL730 (●), and *L. lactis* T1 (▲) during ripening of cheeses manufactured with the non-bacteriocin-producer starter (A) and the Lacticin 3147-producer starter (B).

Visualization in situ of the GFP-labelled strains in cheese was possible using the optical sectioning capability of confocal scanning laser microscopy, which has the advantage of increased sensitivity, thus enabling observations of the subsurface structures of foods (Auty et al., 2001) and by conventional fluorescence microscopy. As an example, Fig. 4 shows detection of bacteria during cheese ripening. GFP fluorescence allowed identification of starter bacteria by confocal microscopy (IFPL3593-GFP in Fig. 4A and IFPL359-GFP in Fig. 4B, at 0 days of ripening). After staining with DAPI, the bacterial genome was visualized by conventional fluorescence microscopy during the ripening period (Fig. 4C, E, G and I). Also, with this technique, GFP-tagged starters could be detected at 0 days of ripening by the fluorescence conferred by GFP (IFPL3593-GFP in Fig. 4D). After 15 days of ripening, when the pH of the cheese decreased to 4.8, the fluorescence of GFP was impaired, as expected from previous results (Fernández de Palencia et al., 2000). To overcome this problem, the pH of the samples was increased and fluorescence was recovered. This method allowed the monitoring of the GPF-labelled bacteria during the entire ripening period (IFPL3593-GFP in Fig. 4F, H and J). Moreover, comparison of the images of a cheese sample for detection of DAPI (Fig. 4C, E, G and I) or GFP (Fig. 4F, H and J) allowed discrimination of GFP-tagged starter from other bacteria. These results show for the first time that strains of industrial interest harbouring plasmids encoding *gfp* gene could be directly monitored in cheese by conventional fluorescence and confocal scanning laser microscopy.

3.3. Release of intracellular enzymes, bacteriocin detection and proteolysis in cheese

Fig. 5A shows release of the intracellular PepX activity and bacteriocin detection in cheeses during ripening. Measurement of PepX activity in cheese matrix is considered an indicator of cell lysis (Chapot-Chartier et al., 1994). Values of PepX were significantly higher ($P < 0.05$) in cheeses made with the bacteriocin producer due to the lytic effect of the bacteriocin. The enzymatic activity peaked at 7 days of ripening. At this point, the maximum levels of bacteriocin were observed in cheeses manufactured with IFPL3593-GFP. Detection of bacteriocin and higher PepX activity in these cheeses was simultaneous

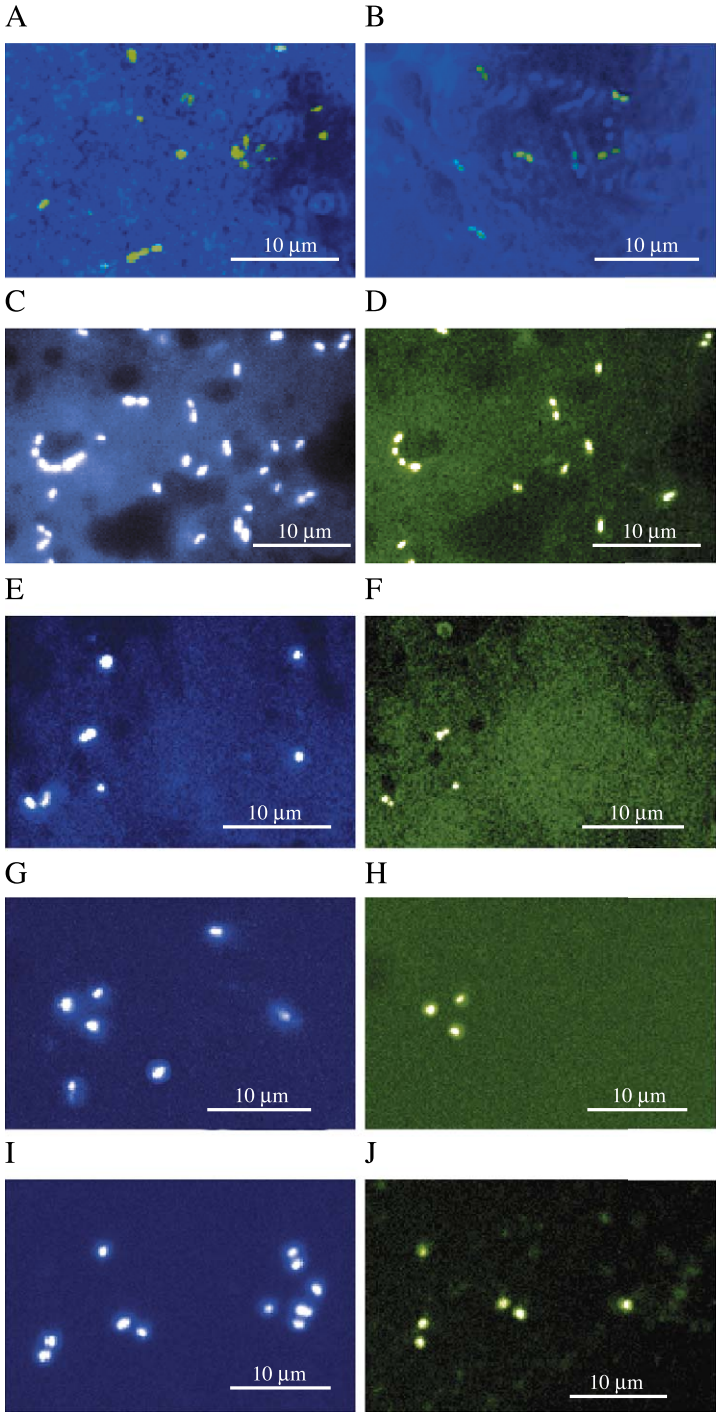

Fig. 4. Analysis by confocal scanning laser microscopy (A and B) and conventional fluorescence microscopy (C through J) during cheese ripening. Images of *L. lactis* IFPL3593-GFP and *L. lactis* IFPL359-GFP cells (A and B, respectively) in cheese matrixes at 1 day of ripening. Images of DAPI stained bacteria (C, E, G, I), or IFLP3593-GFP cells (D, F, H, J) in a batch Bac+ at 1, 15, 30 and 45 days of ripening.

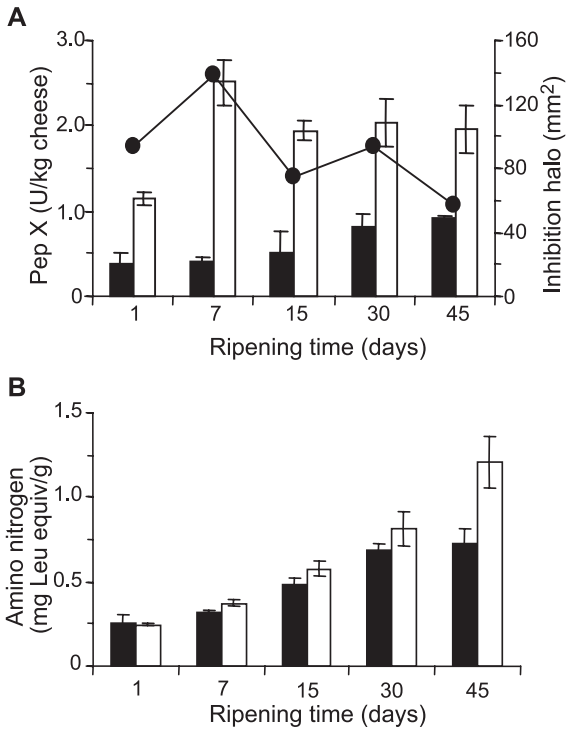

Fig. 5. (A) Release of X-prolyl dipeptidyl aminopeptidase (PepX) activity during ripening of cheeses manufactured with *L. lactis* IFPL359-GFP (solid bars; non-producing bacteriocin starter) and *L. lactis* IFPL3593-GFP (open bars; Lacticin 3147 producing starter) and Lacticin 3147 activity in cheeses made with *L. lactis* IFPL3593-GFP (●). (B) Amino nitrogen content (expressed as equivalents of leucine per g of cheese) of cheeses manufactured with *L. lactis* IFPL359-GFP (solid bars) and *L. lactis* IFPL3593-GFP (open bars).

with a lower viability of both adjuncts, T1 and IFPL730 (Fig. 3). The higher PepX activity found in cheeses manufactured with IFPL3593-GFP corresponded with a higher content ($P < 0.05$) of amino nitrogen (Fig. 5B). This result is consistent with previous work on acceleration of proteolysis by IFPL3593 in cheese using T1 as adjunct (Martínez-Cuesta et al., 2001).

3.4. Isoleucine catabolism in cheese

To complete the metabolic routes leading to the formation of volatiles in cheese, the adjuncts T1 and IFPL730 were selected based on their complementary action on the catabolism of isoleucine. T1, like its parental strain IFPL359, shows branched chain aminotransferase activity (unpublished results) and is sensitive to Lacticin 3147 (Martínez-Cuesta et al., 1997). IFPL730, is also sensitive to the bacteriocin and is able to decarboxylate α-keto acids formed by transamination of amino acids into aldehydes (Amárita et al., 2001). Branched chain amino acid catabolism by lactococci is primarily initiated by transamination, since the degradation occurs only in the presence of an α-keto acid as the amino group acceptor (Yvon et al., 1997). Transamination of isoleucine leads to formation of KMVA. Adding α-ketoglutarate to the cheese curd enhances conversion of amino acids into aroma compounds in St. Paulin (Yvon et al., 1998) and Cheddar cheeses (Banks et al., 2001). In the present study, isoleucine and the amino group acceptor α-ketoglutarate were added to the cheese brine, and the isoleucine

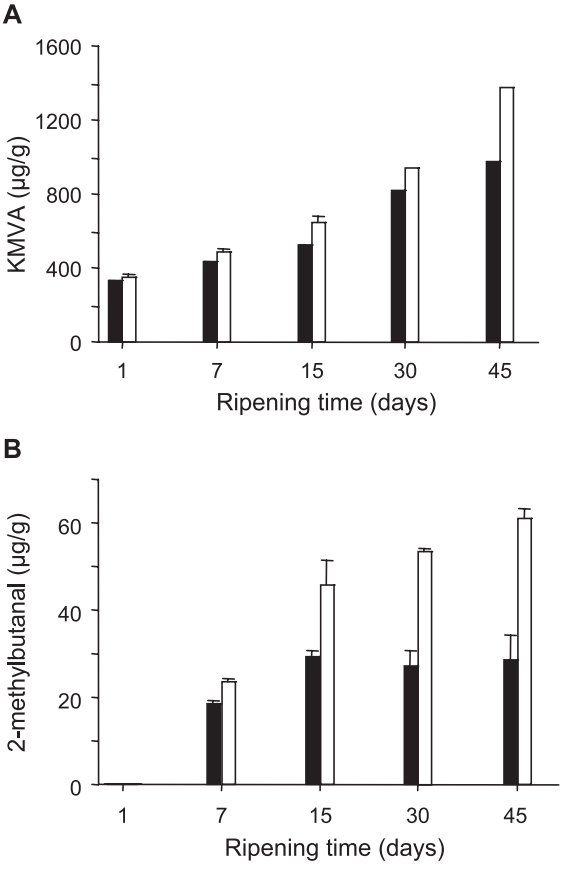

Fig. 6. Production of α-keto-β-methyl-*n*-valeric acid (KMVA; A) and 2-methylbutanal (B) during ripening of cheeses manufactured with *L. lactis* IFPL359-GFP (solid bars; non-producing bacteriocin starter) and *L. lactis* IFPL3593-GFP (open bars; Lacticin 3147 producing starter).

transamination during ripening was measured by formation of KMVA from isoleucine. Results obtained by HPLC are shown in Fig. 6A. An increase of KMVA values was detected throughout the ripening period, being higher ($P<0.05$) during ripening of cheeses manufactured with the bacteriocin producer IFPL3593-GFP. This result could be due to the effect of Lacticin 3147 on lactococci, since we have shown that pure cultures of IFPL359 incubated with Lacticin 3147 increased isoleucine transamination due to both membrane permeabilization and cell lysis caused by the bacteriocin (Martínez-Cuesta et al., 2002).

Once KMVA is produced, accumulation of this product turns out to be the limiting factor for complete amino acid conversion. Non-oxidative decarboxylation of KMVA leads to the formation of 2-methylbutanal. In this study, we used as adjunct IFPL730, a strain sensitive to the bacteriocin, which has been shown to produce aldehydes from BCAAs and methionine-derived α-keto acids (Amárita et al., 2001, 2002). The keto acid decarboxylating activity is not present in the other adjunct strain T1 (not shown). Production of 2-methylbutanal after 7 days of ripening was detected in both batches of cheese (Fig. 6B), and it was higher ($P<0.05$) in cheeses manufactured with IFPL3593-GFP. Higher formation of KMVA and its subsequent decarboxylation by IFPL730 is considered to be responsible for the higher values of 2-methylbutanal found in the experimental cheeses.

The overall results indicate that the combination of strains used in the present work, showing complementary action on amino acid catabolism, provided an appropriate enzymatic system to produce 2-methylbutanal from isoleucine.

3.5. Sensorial analysis

Six trained panelists scored intensity of aroma in homogenates of cheese samples in water from 0 (absent) to 10 (very strong). Results of sensory analysis are shown in Table 2. Increase of aroma development during cheese ripening was significantly ($P<0.05$) detected after 45 days of ripening. Panelists learned to identify the presence of the compound 2-methylbutanal in the cheese samples using a homogenate of cheese in water containing 2-methylbutanal, as reference. The aroma was defined as intense but not typically cheese-like. The taste threshold of 2-methylbutanal has been reported to be 0.13 μg/g (Sheldon et al., 1971).

Table 2
Aroma evaluation of cheeses manufactured with *L. lactis* IFPL359-GFP (non-producing bacteriocin starter) and with *L. lactis* IFPL3593-GFP (Lacticin 3147-producing starter)

Cheese	Ripening time			
	7 days	15 days	30 days	45 days
IFPL359-GFP	4.3 (1.86)^{a,x}	3.4 (0.80)^{a,x}	3.9 (1.28)^{a,x}	5.9 (1.28)^{a,y}
IFPL3593-GFP	2.7 (0.82)^{a,x}	2.8 (1.17)^{a,x}	4.8 (1.17)^{a,y}	7.7 (1.37)^{b,z}

Scores are mean values (SD) from 0 (absent) to 10 (very strong). Different letters in the same column (a,b) indicate significant differences ($P<0.05$) between cheeses at the same time of ripening. Different letters in the same line (z,y,x) indicate significant differences ($P<0.05$) during ripening.

Aroma conferred by methyl aldehydes has been defined as malty in Cheddar cheese (Morgan, 1976) or spicy in Parmesan cheese (Barbieri et al., 1994). Moreover, methyl aldehyde-producing lactococcal strains have been used to develop new chocolate-like flavours in Gouda cheese (Ayad et al., 2000). It is assumed that a balance of total volatile compounds in cheese during ripening is needed to develop an appropriate cheese flavour. At 45 days of ripening, higher scores ($P<0.05$) for aroma intensity were given to cheeses manufactured with the bacteriocin-producer IFPL3593-GFP than those manufactured with IFPL359-GFP.

In conclusion, the combination of lactococcal strains showing complementary action on isoleucine transamination and α-keto acid decarboxylation resulted in formation of 2-methylbutanal from this amino acid. Moreover, the use of a starter producing Lacticin 3147 enhanced microbial enzymatic reactions in cheese and accelerated formation of the volatile amino acid-derived compound. Therefore, an increase of the aroma of cheese was accomplished.

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