Original article

Lipolysis of semi-hard cheese made with a lacticin 481-producing *Lactococcus lactis* strain and a *Lactobacillus helveticus* strain

Marta Ávila, Javier Calzada, Sonia Garde, Manuel Nuñez*

Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Carretera de La Coruña Km 7, Madrid, 28040 Spain

Abstract – Free fatty acids (FFA) liberated from milk fat by lipases and esterases during cheese ripening affect flavour, by themselves or as precursors for the synthesis of other volatile compounds. The effect of the lacticin 481-producing strain *Lactococcus lactis* subsp. *lactis* INIA 639 (BP) on the release of intracellular esterases from a *Lactobacillus helveticus* strain used as adjunct culture and FFA levels during ripening of semi-hard cheese was investigated. Cheeses were manufactured using either *L. lactis* subsp. *lactis* INIA 639, the bacteriocin non-producing strain *L. lactis* subsp. *lactis* INIA 437, or a combination of both strains, as mesophilic starter cultures. The *Lb. helveticus* strain, sensitive to lacticin 481, was added to all vats. Cheeses made with the BP strain exhibited on day 50 esterase activity values up to 29% higher than those of cheese made without the BP strain. Levels of most individual FFA in cheese were increased by milk inoculation with the BP strain. On day 50, total ($C_{4:0}$-$C_{18:2}$) FFA were 628 to 638 mg·kg^{-1} in cheeses made with the BP strain, and 576 mg·kg^{-1} in cheese made without the BP strain, mostly due to the higher accumulation of FFA from day 25 to day 50 in the former cheeses. Bacteriocin-mediated lysis of the *Lb. helveticus* strain and subsequent release of its esterases appeared to be responsible for accelerated lipolysis in cheese.

lacticin 481 / lipolysis / cheese / *Lactobacillus helveticus*

摘要 – 由产乳链球菌素 481 的乳酸链球菌和瑞士乳杆菌生产的半硬质干酪的脂解作用。摘要 在干酪的成熟过程中由于脂肪酶和酯酶的作用使得乳脂肪分解释放出的游离脂肪酸, 释放出脂肪酸可以自身合成或者作为前体合成其他挥发性化合物, 形成的挥发性化合物影响着干酪的风味。产乳链菌素 481 的乳酸乳球菌乳酸亚种 (*Lactococcus lactis* subsp. *lactis*) INIA 639 (BP) 影响着附属发酵剂瑞士乳杆菌 (*Lactobacillus helveticus*) 胞内酯酶的释放以及在半硬质干酪成熟期间游离脂肪酸的水平。分别以乳酸乳球菌乳酸亚种 INIA 639、不产细菌素的乳酸乳球菌乳酸亚种 INIA 437、或者两者的混合菌株作为嗜温菌发酵剂生产干酪。将对乳链球菌素 481 敏感的瑞士乳杆菌加到所有的发酵罐中。用 BP 菌株生产的干酪发酵到50 d 时呈现了较高的酯酶活性, 与未使用 BP 菌株加工的干酪比较, 前者的酯酶活性比后者高29%。接种 BP 菌株的干酪中多数游离脂肪酸组分浓度增加。当成熟到第 50 d 时, 用 BP 菌株生产干酪总游离脂肪酸 ($C_{4:0}$-$C_{18:2}$) 浓度在 628～638 mg·kg^{-1}, 而未使用 BP 菌株的干酪中游离脂肪酸的浓度为 576 mg·kg^{-1}, 其原因是由于前者在干酪成熟 25～50 d 内游离脂肪酸的积累较高。瑞士乳杆菌的自溶作用释放出的酯酶加速了干酪中的脂解作用。

乳链球菌素481 / 脂解作用 / 干酪 / 瑞士乳杆菌

---

* Corresponding author (通讯作者): nunez@inia.es

Résumé – Lipolyse d'un fromage à pâte pressée fabriqué avec une souche de *Lactococcus lactis* productrice de lacticine 481 et une souche de *Lactobacillus helveticus*. Les acides gras libres (AGL) libérés à partir de la matière grasse du lait par des lipases et estérases pendant l'affinage du fromage influent sur sa saveur, soit directement, soit comme des précurseurs pour la synthèse d'autres composés volatils. L'effet de la souche *Lactococcus lactis* subsp. *lactis* INIA 639 productrice de lacticine 481 (PB) sur la libération des estérases intracellulaires par une souche de *Lactobacillus helveticus* utilisée comme levain additionnel et sur les concentrations des acides gras libres au cours de l'affinage d'un fromage à pâte pressée a été étudié. Des fromages ont été fabriqués avec, comme des levains lactiques mésophiles, soit la souche *L. lactis* subsp. *lactis* INIA 639, soit la souche *L. lactis* subsp. *lactis* INIA 437 ne produisant pas des bactériocines, soit un mélange de ces deux souches. Une souche de *Lb. helveticus* sensible à la lacticine 481 a été ajoutée à toutes les cuves. Les fromages fabriqués avec la souche PB montraient après 50 j une activité estérase de 29 % plus élevée que celle du fromage fabriqué sans la souche PB. Les concentrations de la plupart des acides gras libres dans le fromage augmentaient avec l'addition de la souche PB au lait. Après 50 j, les AGL totaux ($C_{4:0}$-$C_{18:2}$) étaient de 628 à 638 mg·kg^{-1} dans les fromages fabriqués avec la souche PB, et de 576 mg·kg^{-1} dans le fromage fabriqué sans la souche PB, principalement du fait de l'accumulation plus élevée des AGL du jour 25 au jour 50 dans les fromages fabriqués avec la souche PB. La lyse de la souche de *Lb. helveticus* suivie par la libération de ses estérases semblait responsable de la lipolyse accélérée dans le fromage.

lacticine 481 / lipolyse / fromage / *Lactobacillus helveticus*

1. INTRODUCTION

Lactic acid bacteria represent an important source of enzymes such as proteinases, peptidases, esterases and amino acid and free fatty acid (FFA) catabolic enzymes, which make possible the transformation of milk constituents into flavour compounds and aroma precursors of cheese. Esterases and lipases from lactic acid bacteria are responsible for liberating short-chain FFA from milk fat, which directly affect cheese flavour, and for the synthesis of short-chain ethyl esters under certain conditions during ripening [13–16]. Cell lysis becomes essential to favour the access of esterases to their substrates, as these enzymes are intracellular. Certain adjunct cultures, such as some *Lactococcus lactis* and *Lactobacillus reuteri* strains, may have a definite role in lipolysis, with a differential release of some volatile FFA, depending on cheese age [22].

Bacteriocin-producing (BP) cultures have been used satisfactorily to enhance the lysis of sensitive lactic acid bacteria in cheese. Most works were focused on their impact on proteolysis, and few have reported how lipolysis is affected by

bacterial lysis. Collins et al. [4] observed a relationship between autolysis of starter lactic acid bacteria and increased accumulation of FFA during Cheddar cheese ripening, and suggested that it was due to an elevated release of intracellular lipolytic activity in cheese. Adjunct cultures with high enzymatic potential may be added to milk to enrich the enzymatic pool to be released into the cheese matrix. The use of attenuated cells of autolytic *Lb. helveticus* I as adjunct culture increased esterase activity and lipolysis in cheese slurries [7]. A positive effect of the lysis of an autolytic *Lb. helveticus* strain on enhancing the levels of some FFA during Cheddar cheese ripening has also been reported [12]. Nonetheless, BP strains have rarely been combined with adjunct cultures to increase cheese lipolysis, even though a nisin-producing *Lactococcus* strain in combination with *Lactobacillus* adjunct cultures resulted in higher lipolysis during ripening of Cheddar cheese, expressed as fat acidity value [2, 21].

In previous works [10, 11], the effects of milk inoculation with *L. lactis* subsp. *lactis* INIA 639, a lacticin 481-producing strain, and *Lb. helveticus* LH 92, a strain sensitive

to lacticin 481, on the proteolysis, texture, volatile fraction and flavour of a semi-hard cheese were investigated. The objective of the present work was to study the effect of this lacticin 481-producing strain on the esterase activity and the FFA content of semi-hard cheese.

2. MATERIALS AND METHODS

2.1. Lactic cultures

The mesophilic lactic cultures used in the experiments were lacticin 481-producing *L. lactis* subsp. *lactis* INIA 639 (BP), *L. lactis* subsp. *lactis* INIA 437, a bacteriocin non-producing (BNP) culture with technological characteristics similar to those of the BP strain and resistant to lacticin 481, or a combination of both lactococcal strains. Strains of lactococci were from the INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain) culture collection. An appropriate BNP mutant of the lacticin 481 producer with technological characteristics close to those of the parental strain could not be obtained, and *L. lactis* INIA 437 was used as a substitute. A commercial *Lb. helveticus* strain (LH 92, Rhodia Iberia, Madrid, Spain), sensitive to lacticin 481 [10], was used as thermophilic culture.

2.2. Single-culture cheese manufacture

To assess the esterase activity of the individual lactic cultures used for cheese manufacture and the release of FFA during the first 24 h, three single-culture cheeses were made, each from 2 L of commercial pasteurised whole cow's milk, in duplicate experiments, carried out on different days. Milk at 33 °C was inoculated with 5 mL·L^{-1} of *L. lactis* INIA 639, *L. lactis*

INIA 437 or *Lb. helveticus* milk cultures. Addition of rennet (0.25 mL Maxiren, 1:15 000 strength, Gist Brocades, Delft, The Netherlands) took place 30 min after culture inoculation. The curds were cut 40 min later into 6–8 mm cubes and scalded at 37 °C for 5 min. Curds from each vat were pressed in cylindrical moulds at 20 °C for 22 h, to obtain cheeses of approximately 250 g in weight.

2.3. Mixed-culture cheese manufacture

Semi-hard cheeses were manufactured from pasteurised whole cow's milk inoculated with mixed cultures in duplicate experiments, carried out on different days. Each experiment consisted of three vats, each containing 50 L of milk, which was heated at 33 °C. *L. lactis* INIA 437 was added at 5 mL·L^{-1} to vat 1 (BNP cheese) and at 2.5 mL·L^{-1} to vat 2; *L. lactis* INIA 639 was added at 2.5 mL·L^{-1} to vat 2 (BNP + BP cheese) and at 5 mL·L^{-1} to vat 3 (BP cheese); *Lb. helveticus* LH 92 was added at 5 mL·L^{-1} to all vats. Rennet (6 mL Maxiren) was added to milk 30 min after lactic culture inoculation. The curds were cut 40 min later into 6–8 mm cubes and scalded at 37 °C for 15 min. Whey was drained off and curds were distributed into cylindrical moulds. Three cheeses, of approximately 2 kg in weight, were obtained from each vat. The cheeses were pressed overnight at 20 °C and 1.5 kg·cm^{-2} pressure, salted at 12 °C for 24 h in brine (160 g NaCl·L^{-1}), and ripened at 12 °C and 85% RH. On day 7 the cheeses were coated with two layers of polyvinyl acetate containing pimaricine.

2.4. Microbiological and chemical analyses

Lactococci counts were determined in duplicate on M17 agar containing 5 g·L^{-1}

lactose (Biolife, Milano, Italy) using a Spiral plater (Interscience, 78860 Saint-Nom-La-Bretèche, France), after incubation at 30 °C for 48 h aerobically. Bacteriocin-producing lactococci were determined on the surface of double-layer M17 agar plates, with the lower layer inoculated with 1 mL·L^{-1} of a 16-h culture of *L. lactis* subsp. *cremoris* HP as the indicator microorganism: colonies forming a zone of growth inhibition in the lower layer were considered to be *L. lactis* INIA 639. Lactobacilli counts were determined on MRS agar (Biolife) plates incubated anaerobically for 48 h at 44 °C.

Esterase activity released into cheese was measured on duplicate samples of cheese extracts obtained according to Garde et al. [10] using α-naphtylbutyrate (Sigma-Aldrich, Steinheim, Germany) as chromogenic substrate, with the reaction conditions described by Morales et al. [20]. One unit of esterase activity was defined as the amount of enzyme(s) producing 1 pmol of α-naphtol per min per g of cheese under standard assay conditions. Cheese pH was determined according to Garde et al. [10].

For the determination of free fatty acids, cheese pieces were wrapped in aluminium foil, vacuum-packed, and kept frozen at –40 °C. They were thawed overnight at 4 °C prior to analysis. Acetic and propionic acid and FFA from butyric (C$_{4:0}$) to linoleic acid (C$_{18:2}$) were analysed following the method described by de Jong and Badings [5]. FFA were extracted from cheeses using a solid-phase extraction technique, with pentanoic, nonanoic and heptadecanoic acids added as internal standards. Individual FFA were separated, identified and quantified by gas chromatography with flame ionisation detection as described by Fernández-García et al. [8]. Eleven standard solutions of fatty acids were used for the calculation of calibration curves.

2.5. Statistical analysis

Statistical analysis was performed by means of the SPSS Win version 8.0 program (SPSS Inc., Chicago, IL, USA). Analysis of variance was carried out with type of mesophilic starter, cheese age and cheese-making experiment as main effects. Comparison of means at $P < 0.05$ was performed using Tukey's test.

3. RESULTS AND DISCUSSION

3.1. Single-culture cheeses

Esterase activity, counts of lactic acid bacteria, pH values and total (C$_{4:0}$-C$_{18:2}$) FFA concentrations of 1-day-old single-culture cheeses are presented in Table I. Cheeses made with *L. lactis* strains showed lactic acid bacteria counts of 9.6–9.7 log units, and those made with *Lb. helveticus* counts of 9.0 log units. Low extracellular esterase activity was recorded in the three single-culture cheeses, suggesting scarce spontaneous lysis of lactic acid bacteria and release of intracellular enzymes.

There were no significant ($P < 0.05$) differences between total (C$_{4:0}$-C$_{18:2}$) FFA of 1-day-old single-culture cheeses, which ranged from 701 to 726 mg·kg^{-1} (Tab. I). Values reported for total FFA in 1-day-old Cheddar cheeses range from 580 to 1230 mg·kg^{-1} [13, 14], depending on the starter composition, whereas a total FFA concentration of 2222 mg·kg^{-1} was obtained for 3-day-old Emmental cheese [16].

The main volatile acid extracted with FFA was acetic acid, which results from the metabolism of lactose and citrate by lactic acid bacteria. Levels of acetic acid were 1.2 times higher in *Lb. helveticus* cheese than in *L. lactis* cheeses (Fig. 1), despite the lower counts reached by lactobacilli (Tab. I). Palmitic, oleic and stearic acids, the most abundant fatty acids

Table I. Esterase activity[1], lactic acid bacteria counts[1], pH values[1] and total free fatty acids[1] (FFA) in 1-day-old single-culture cheeses made from milk inoculated with 5 mL·L^{-1} of *L. lactis* subsp. *lactis* INIA 437 (BNP), *L. lactis* subsp. *lactis* INIA 639 (BP) or *Lb. helveticus* (LH).

	L. lactis INIA 437	*L. lactis* INIA 639	*Lb. helveticus*
Lactic acid bacteria counts	9.70 ± 0.02[a]	9.64 ± 0.02[a]	8.98 ± 0.02[b]
Cheese pH	4.82 ± 0.01[b]	4.91 ± 0.03[a]	4.77 ± 0.09[c]
Esterase activity	0.04 ± 0.01[b]	0.05 ± 0.02[b]	0.13 ± 0.01[a]
Sum (C$_{4:0}$–C$_{18:2}$) FFA	716.2 ± 29.4[a]	701.4 ± 44.5[a]	726.0 ± 70.9[a]

[1] Mean ± SD (n = 4) of duplicate determinations in two cheese-making experiments. One unit of esterase activity was defined as the amount of enzyme(s) producing 1 pmol of α-naphtol per min per g of cheese under standard assay conditions. Counts of lactic acid bacteria are expressed in log cfu·g^{-1} cheese, and FFA in mg·kg^{-1} cheese.
[a–c] Means within a row with different superscripts differ ($P < 0.05$).

in cow's milk [3], were also the most abundant FFA in single-culture cheeses (Fig. 1). Total levels of short-chain FFA (SCFFA, C$_{4:0}$-C$_{8:0}$) were slightly lower ($P < 0.05$) in single-culture cheese made with *Lb. helveticus*, but long-chain FFA (LCFFA, C$_{16:0}$-C$_{18:2}$) and medium-chain FFA (MCFFA, C$_{10:0}$-C$_{14:0}$) levels were similar in the three cheeses.

3.2. Lactic acid bacteria counts and pH values in mixed-culture cheeses

Lactococci counts were higher in BNP cheese than in cheeses made with the BP strain throughout the ripening period (Tab. II), and differences increased as cheeses aged. In cheese made with both strains of lactococci, counts of the BP strain were 8.79, 8.43 and 6.78 log units on days 7, 25 and 50, respectively (data not shown). Lactobacilli counts were also higher in BNP cheese than in cheeses made with the BP strain throughout the ripening period (Tab. II). There was a decrease of 1.35 log units in lactobacilli counts from day 7 to day 50 in BNP cheese, whereas the respective decreases in BNP + BP cheese and BP cheese were 1.50 and 0.25 log units. Lower counts of lactic acid bacteria have been reported in previous works when a BP strain was used as an adjunct culture in cheese manufacture [2, 9, 21].

Semi-hard cheeses made with different combinations of lactic cultures showed no significant ($P < 0.05$) differences in pH values during the 50-day ripening period (Tab. II).

3.3. Esterase activity in mixed-culture cheeses

Esterase activity remained constant in BNP cheese during ripening, but increased ($P < 0.05$) in BNP + BP and BP cheeses from day 25 to day 50 (Tab. III). Addition of the BP strain to milk resulted in higher ($P < 0.05$) levels of esterase activity with respect to BNP cheese. Thus, esterase activity released in the matrix of the BNP + BP cheese was 17% and 29% higher than that of BNP cheese on days 25 and 50, respectively, and esterase activity in BP cheese 19% and 24% higher, respectively. Release of intracellular esterases, due to the lysis of *Lb. helveticus* cells, occurred in accordance with the enhanced liberation of intracellular aminopeptidases [10]. Addition of freeze-shocked cells of highly autolytic *Lb. helveticus* strains as adjuncts
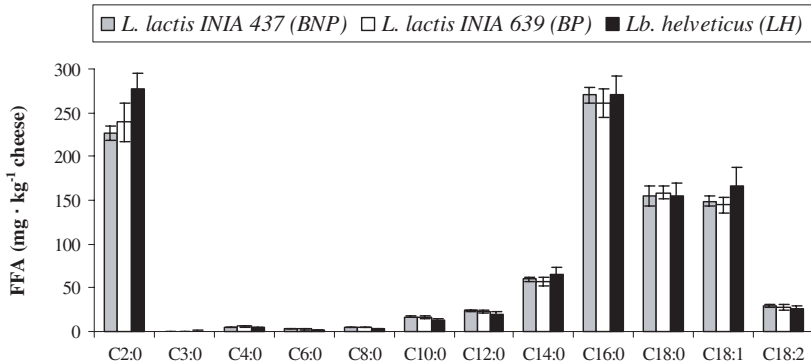
Figure 1. Concentration of free fatty acids (mg·kg^{-1}) in 1-day-old single-culture cheeses made from milk inoculated with 5 mL·L^{-1} of *L. lactis* subsp. *lactis* INIA 437 (BNP), *L. lactis* subsp. *lactis* INIA 639 (BP), or *Lb. helveticus* LH 92 (LH) milk cultures.

Table II. Lactic acid bacteria counts[1] and pH values[1] during ripening of semi-hard mixed-culture cheeses made from milk inoculated with *L. lactis* subsp. *lactis* INIA 437 (BNP), *L. lactis* subsp. *lactis* INIA 639 (BP) and *Lb. helveticus* LH 92 (LH).

	Age (days)	5 mL·L^{-1} BNP + 5 mL·L^{-1} LH	2.5 mL·L^{-1} BNP + 2.5 mL·L^{-1} BP + 5 mL·L^{-1}	5 mL·L^{-1} BP + 5 mL·L^{-1} LH
Lactococci counts	7	9.35 ± 0.16[a]	9.21 ± 0.07[b]	9.13 ± 0.05[b]
	25	9.38 ± 0.03[a]	8.99 ± 0.04[b]	8.64 ± 0.30[c]
	50	9.32 ± 0.01[a]	8.65 ± 0.04[b]	7.44 ± 0.04[c]
Lactobacilli counts	7	8.20 ± 0.19[a]	6.27 ± 0.26[b]	5.78 ± 0.25[c]
	25	7.20 ± 0.09[a]	5.51 ± 0.25[c]	5.89 ± 0.02[b]
	50	6.85 ± 0.13[a]	4.77 ± 0.36[c]	5.53 ± 0.28[b]
Cheese pH	7	4.97 ± 0.08[a]	4.91 ± 0.06[a]	4.97 ± 0.04[a]
	25	4.87 ± 0.04[a]	4.89 ± 0.03[a]	4.90 ± 0.06[a]
	50	4.81 ± 0.05[a]	4.89 ± 0.02[a]	4.86 ± 0.01[a]

[1] Mean ± SD (n = 4) of duplicate determinations in two cheese-making experiments. Counts of lactic acid bacteria are expressed in log cfu·g^{-1}.
[a–c] Means within a row with different superscripts differ ($P < 0.05$).

in cheese slurries made with *Lactococcus* strains also resulted in elevated release of esterase activity [13].

3.4. Free fatty acids in mixed-culture cheeses

Levels of total (C$_{4:0}$-C$_{18:2}$) FFA in mixed-culture cheeses on day 25 (Tab. III) were lower than those in single-culture cheeses on day 1 (Tab. I). This difference could be partly explained by the fact that different milks were used in the two experiments. Also, the actual release of FFA in mixed-culture cheeses during the first 25 days of ripening could have been higher than that shown by the data in Table III, since the liberated FFA which underwent esterification and/or other reactions during that period are not taken into account.

Table III. Esterase activity[1] and concentrations of free fatty acids (FFA)[1] during ripening of semi-hard mixed-culture cheeses made from milk inoculated with *L. lactis* subsp. *lactis* INIA 437 (BNP), *L. lactis* subsp. *lactis* INIA 639 (BP) and *Lb. helveticus* LH 92 (LH).

	Age (days)	5 mL·L^{-1} BNP + 5 mL·L^{-1} LH	2.5 mL·L^{-1} BNP + 2.5 mL·L^{-1} BP + 5 mL·L^{-1} LH	5 mL·L^{-1} BP + 5 mL·L^{-1} LH
Esterase activity	25	0.77 ± 0.02^b	0.90 ± 0.06^a	0.92 ± 0.04^a
	50	0.79 ± 0.04^b	1.02 ± 0.06^a	0.98 ± 0.03^a
Sum C$_{2:0}$-C$_{3:0}$	25	247.8 ± 33.3^a	229.2 ± 17.0^a	190.5 ± 16.4^b
	50	252.5 ± 13.0^a	221.8 ± 19.0^b	181.0 ± 8.7^c
SCFFA (C$_{4:0}$-C$_{8:0}$)	25	14.9 ± 0.6^b	16.9 ± 1.4^a	16.8 ± 0.9^a
	50	21.9 ± 2.3^b	24.8 ± 2.4^a	24.2 ± 1.5^a
MCFFA (C$_{10:0}$-C$_{14:0}$)	25	78.6 ± 4.9^a	83.0 ± 4.8^a	76.2 ± 10.3^a
	50	89.3 ± 5.0^b	96.2 ± 6.7^a	94.2 ± 4.3^a
LCFFA (C$_{16:0}$-C$_{18:2}$)	25	421.5 ± 13.1^b	452.5 ± 21.2^a	426.9 ± 28.5^{ab}
	50	465.2 ± 15.6^b	516.9 ± 11.0^a	509.2 ± 7.1^a
UFFA (C$_{18:1}$-C$_{18:2}$)	25	135.6 ± 18.7^b	149.2 ± 17.3^a	144.5 ± 4.6^{ab}
	50	158.1 ± 20.0^b	185.4 ± 9.7^a	184.4 ± 12.1^a
Sum C$_{4:0}$-C$_{18:2}$	25	515.0 ± 14.8^a	552.4 ± 23.2^a	519.9 ± 39.1^a
	50	576.4 ± 14.3^b	637.9 ± 15.4^a	627.5 ± 4.1^a

[1] Mean ± SD (n = 4) of duplicate determinations in two cheese-making experiments. One unit of esterase activity was defined as the amount of enzyme(s) producing 1 pmol of α-naphtol per min per g of cheese under standard assay conditions. FFA concentrations are expressed in mg·kg^{-1} cheese.

[a–c] Means within a row with different superscripts differ ($P < 0.05$).

Lipolysis, measured as total FFA, increased ($P < 0.05$) in all cheeses from day 25 to day 50 (Tab. III), and so did the levels of all individual FFA. The most notable increases, in the three mixed-culture cheeses, were those of butyric and caproic acids, increasing by an average of 53% and 57%, respectively, from day 25 to day 50 (Fig. 2), most probably due to the high specificity of bacterial lipolytic enzymes towards FFA located in the position *sn*-1,3 of the triglyceride, where SCFFA are predominantly esterified [6]. The enhanced release of esterases in cheeses made with the BP culture resulted in significantly higher concentrations of SCFFA on day 25, a difference which persisted on day 50 (Tab. III).

In our cheeses, SCFFA comprised around 4% of total (C$_{4:0}$-C$_{18:2}$) FFA content, MCFFA 15% and LCFFA 81%, and this pattern hardly varied over ripening or with BP addition. Butyric acid (~2% of total FFA), myristic acid (~10% of total FFA) and palmitic acid (~32% of total FFA) were the main FFA of their respective groups. The high concentrations of free palmitic, oleic and stearic acids (Fig. 2) reflect their abundance in milk fat rather than a preferential release from triglycerides. The degree of lipolysis observed in the present work was not very intense, with total (C$_{4:0}$-C$_{18:2}$) FFA contents on day 50 ranging from 576 to 638 mg·kg^{-1}. This may be due to the limited access of lactic acid bacteria esterases to suitable
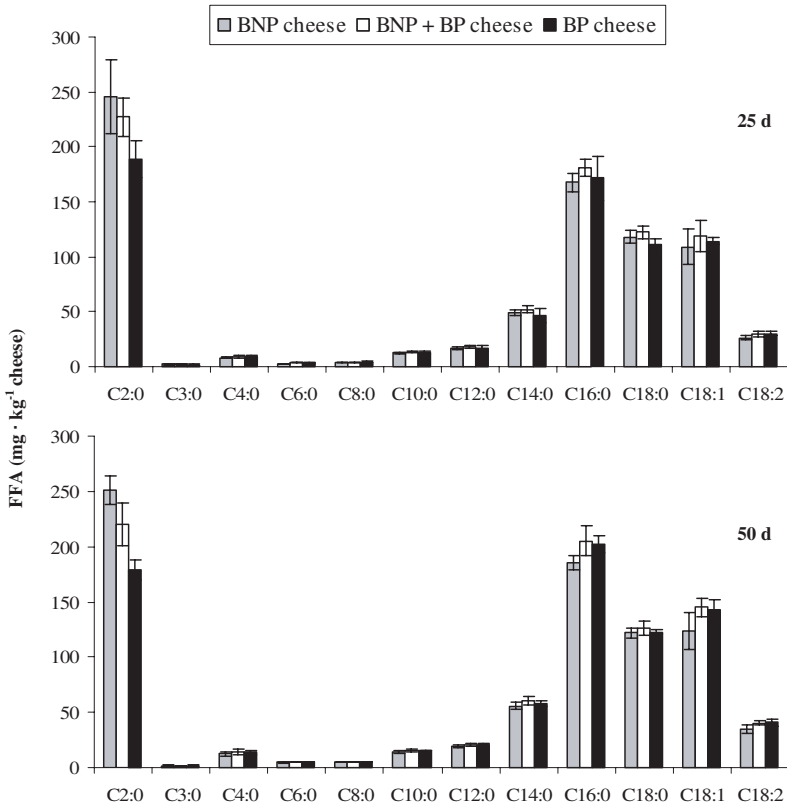
Figure 2. Concentrations of free fatty acids (mg·kg^{-1}) after 25 and 50 days of ripening in semi-hard mixed-culture cheeses made from milk inoculated with *L. lactis* subsp. *lactis* INIA 437 (BNP), *L. lactis* subsp. *lactis* INIA 639 (BP) and *Lb. helveticus* LH 92 (LH) milk cultures (BNP cheese, 5 mL·L^{-1} BNP + 5 mL·L^{-1} LH; BNP + BP cheese, 2.5 mL·L^{-1} BNP + 2.5 mL·L^{-1} BP + 5 mL·L^{-1} LH; BP cheese, 5 mL·L^{-1} BP + 5 mL·L^{-1} LH).

fat substrates during cheese ripening [13]. Similar values have been reported for internal bacterially ripened varieties such as Edam, Swiss and Cheddar [18], although higher concentrations may be attained in Cheddar [14] and, in particular, in Emmental cheese [16], depending on starter composition.

Acetic acid was the most abundant acid in all the cheeses during ripening, while propionic acid was found at very low concentrations (Fig. 2). The concentrations of acetic acid in BNP + BP and BP cheeses

on day 50 were 12% and 29% lower than in BNP cheese (Fig. 2), respectively. The lower counts of lactococci and, especially, of lactobacilli in cheeses made with *Lb. helveticus* and *L. lactis* INIA 639 than in cheese made without BP culture (Tab. II) could be the cause of the decrease in the acetic acid content of cheeses made with BP culture, as it originates mainly from the metabolism of lactic acid bacteria [19].

Addition of *L. lactis* INIA 639 led to higher ($P < 0.05$) concentrations of total ($C_{4:0}$-$C_{18:2}$) FFA on day 50 (Tab. III).

Total FFA content was 11% higher in BNP + BP cheese, and 9% higher in BP cheese than in BNP cheese, which may be attributed to the bacteriocin-mediated lysis of *Lb. helveticus* cells and subsequent release of esterase in cheeses made with BP culture. The use of attenuated cells of highly autolytic *Lb. helveticus* I as adjuncts resulted in the increase in esterase activity and lipolysis in cheese slurries [7], and in a 1.7 times higher fat acidity value in 6-month-old Cheddar cheese compared with control cheese [17]. Also, the combination of a nisin Z-producing *Lactococcus* strain with the autolytic *Lb. bulgaricus* UL12 strain achieved an increase of 1.5 times in the fat acidity value of 6-month-old Cheddar cheese [21], and an increase of 2.5 times if combined with *Lb. casei* L2A [2], when compared with the respective control cheeses.

In the present work SCFFA, MCFFA, LCFFA and unsaturated FFA (UFFA, $C_{18:1}$-$C_{18:2}$) were on average 12%, 7%, 10% and 17% higher, respectively, in cheeses made with than in those made without BP culture, on day 50. Total ($C_{4:0}$-$C_{18:2}$) FFA accumulated at a higher rate from day 25 to day 50 in BNP + BP cheese (16% increase) and BP cheese (21% increase) than in BNP cheese (12% increase).

Levels of all individual FFA were higher ($P < 0.05$) in BNP + BP and BP cheeses than in BNP cheese (Fig. 2) on day 50, except for stearic acid and, in BP cheese, myristic acid. Contrarily, the use of freeze-shocked *Lb. helveticus* CNRZ 32 cells in Gouda cheese-making only achieved a slight increase in butyric acid concentration [1]. More recently, Collins et al. [4] reported an overall significant increase in caprylic, myristic, palmitic and stearic acids over 8 months of ripening in Cheddar cheese manufactured with *L. lactis* AM2, a highly autolytic strain. Also, a positive effect of the highly autolytic *Lb. helveticus* DPC4571 strain and its lysis on enhancing

levels of butyric, capric, myristic, palmitic and oleic acids in 8-month-old Cheddar cheese has been observed [12].

3.5. Impact on sensory characteristics

Flavour characteristics of the cheeses were reported in previous works [10, 11]. Cheeses made from milk inoculated with the BP culture received higher scores for overall taste quality, but there were no significant differences in taste intensity between cheeses. Umami taste was higher and bitter taste lower when BP culture was added, whereas sour, sweet, and salty taste were not affected [10]. No rancid flavour defect, attributable to excessive lipolysis, was found for any of the cheeses.

Cheese odour was not influenced by the BP culture. However, aroma quality and intensity were significantly enhanced by BP culture addition [11]. Some volatile compounds, including ethanol, ethyl acetate, ethyl butanoate and ethyl hexanoate, reached higher levels (more than doubled) in cheeses made with the BP culture, and significant positive correlations were found between the levels of ethyl butanoate and ethyl hexanoate in cheeses and the respective scores for aroma quality and intensity [11]. Both the enhancement of lipolysis and the higher ethanol concentrations in cheeses made from milk inoculated with the BP culture might be responsible for the increased levels of ethyl esters in those cheeses, and these elevated ester concentrations would contribute to their higher aroma quality and intensity scores.

4. CONCLUSIONS

The combination of lacticin 481-producing *L. lactis* subsp. *lactis* INIA 639 with a *Lb. helveticus* strain sensitive to this bacteriocin enhanced the release of

intracellular esterases during ripening of semi-hard cheese. Milk inoculation with the lacticin 481 producer caused a more rapid evolution of lipolysis and resulted in higher concentrations of most individual FFA without affecting the overall pattern of lipolysis in cheese. The procedure presented here was shown to be a simple and non-costly method for the acceleration of cheese lipolysis.

REFERENCES

[1] Bartels H.J., Johnson M.E., Olson N.F., Accelerated ripening of Gouda cheese. 2. Effect of freeze-shocked *Lactobacillus helveticus* on proteolysis and flavour development, Milchwissenschaft 42 (1987) 139–144.

[2] Benech R.O., Kheadr E.E., Lacroix C., Fliss I., Impact of nisin producing culture and liposome-encapsulated nisin on ripening of *Lactobacillus* added-Cheddar cheese, J. Dairy Sci. 86 (2003) 1895–1909.

[3] Christie W.W., The composition and structure of milk lipids, in: Fox P.F. (Ed.), Developments in dairy chemistry. Lipids, Vol. 2, Applied Science Publishers, London, UK, 1983, pp. 1–36.

[4] Collins Y.F., McSweeney P.L.H., Wilkinson M.G., Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening, J. Dairy Res. 70 (2003) 105–113.

[5] de Jong C., Badings H.T., Determination of free fatty acids in milk and cheese. Procedures for extraction, clean up, and capillary gas chromatographic analysis, J. High Res. Chromatogr. 13 (1990) 94–98.

[6] Deeth H.C., Touch V., Methods for detecting lipase activity in milk and milk products, Aust. J. Dairy Tech. 55 (2000) 153–168.

[7] El-Soda M., Madkor S.A., Tong P.S., Evaluation of commercial adjuncts for use in cheese ripening: 3. Properties of heat-shocked adjuncts in buffer and cheese slurry systems, Milchwissenschaft 54 (1999) 262–265.

[8] Fernández-García E., Carbonell M., Calzada J., Nuñez M., Seasonal variation of the free fatty acids contents of Spanish ovine milk cheese protected by a designation of origin: A comparative study, Int. Dairy J. 16 (2006) 252–261.

[9] Garde S., Tomillo J., Gaya P., Medina M., Nuñez M., Proteolysis in Hispánico cheese manufactured using a mesophilic starter, a thermophilic adjunct culture and bacteriocin-producing *Lactococcus lactis* subsp. *lactis* INIA 415 adjunct culture, J. Agric. Food Chem. 50 (2002) 3479–3485.

[10] Garde S., Ávila M., Gaya P., Medina M., Nuñez M., Proteolysis of Hispánico cheese manufactured using lacticin 481-producing *Lactococcus lactis* ssp. *lactis* INIA 639, J. Dairy Sci. 89 (2006) 840–849.

[11] Garde S., Ávila M., Fernández-García E., Medina M., Nuñez M., Volatile compounds and aroma of Hispánico cheese manufactured using lacticin 481-producing *Lactococcus lactis* subsp. *lactis* INIA 639 as adjunct culture, Int. Dairy J. 17 (2007) 717–726.

[12] Hannon J.A., Kilcawley K.N., Wilkinson M.G., Delahunty C.M., Beresford T.P., Flavour precursor development in Cheddar cheese due to lactococcal starters and the presence and lysis of *Lactobacillus helveticus*, Int. Dairy J. 17 (2007) 316–327.

[13] Hickey D.K., Kilcawley K.N., Beresford T.P., Wilkinson M.G., Starter bacteria are the prime agents of lipolysis in Cheddar cheese, J. Agric. Food Chem. 54 (2006) 8229–8235.

[14] Hickey D.K., Kilcawley K.N., Beresford T.P., Sheehan E.M., Wilkinson M.G., Starter strain related effects on the biochemical and sensory properties of Cheddar cheese, J. Dairy Res. 74 (2007) 9–17.

[15] Holland R., Liu S.-Q., Crow V.L., Delabre M.-L., Lubber M., Bennet M., Norris G., Esterases of lactic acid bacteria and cheese flavour: milk fat hydrolysis, alcoholysis and esterification, Int. Dairy J. 15 (2005) 711–718.

[16] Lopez C., Maillard M.-B., Briard-Bion V., Camier B., Hannon J.A., Lipolysis during ripening of Emmental cheese considering organization of fat and preferential localization of bacteria, J. Agric. Food Chem. 54 (2006) 5855–5867.

[17] Madkor S., Tong P.S., El-Soda M., Ripening of Cheddar cheese with added attenuated adjunct cultures of lactobacilli, J. Dairy Sci. 83 (2000) 1684–1691.

[18] McSweeney P.L.H., Biochemistry of cheese ripening: introduction and overview, in: Fox P.F., McSweeney P.L.H., Cogan T.M., Guinee T.P. (Eds.), Cheese: Chemistry, Physics and Microbiology, Vol. 1: General Aspects (3rd edn.), Elsevier Academic Press, London, UK, 2004, pp. 347–360.

[19] McSweeney P.L.H., Sousa M.J., Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review, Lait 80 (2000) 293–324.

[20] Morales P., Medina M., Nuñez M., Cysteine proteinase and tributyrin esterase production by *Micrococcus* sp. INIA 528 under cheese-making conditions, Milchwissenschaft 60 (2005) 143–146.

[21] Sallami L., Kheadr E.E., Fliss I., Vuillemard J.C., Impact of autolytic, proteolytic, and nisin-producing adjunct cultures on biochemical and textural properties of Cheddar cheese, J. Dairy Sci. 87 (2004) 1585–1594.

[22] Tungjaroenchai W., White C.H., Holmes W.E., Drake M.A., Influence of adjunct cultures on volatile free fatty acids in reduced-fat Edam cheeses, J. Dairy Sci. 87 (2004) 3224–3234.