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Inhibitory activity of *Lactobacillus plantarum* TF711 against *Clostridium sporogenes* when used as adjunct culture in cheese manufacture

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Bacteriocins produced by lactic acid bacteria are of great interest to the food-processing industry as natural preservatives. This work aimed to investigate the efficacy of bacteriocin-producing *Lactobacillus plantarum* TF711, isolated from artisanal Tenerife cheese, in controlling *Clostridium sporogenes* during cheese ripening. Cheeses were made from pasteurised milk artificially contaminated with 10^4 spores m/l *C. sporogenes*. Experimental cheeses were manufactured with *Lb. plantarum* TF711 added at 1% as adjunct to commercial starter culture. Cheeses made under the same conditions but without *Lb. plantarum* TF711 served as controls. Evolution of microbiological parameters, pH and NaCl content, as well as bacteriocin production was studied throughout 45 d of ripening. Addition of *Lb. plantarum* TF711 did not bring about any significant change in starter culture counts, NaCl content and pH, compared with control cheese. In contrast, clostridial spore count in experimental cheeses were significantly lower than in control cheeses from 7 d onwards, reaching a maximum reduction of 2·2 log units on day 21. Inhibition of clostridia found in experimental cheeses was mainly attributed to plantaricin activity, which in fact was recovered from these cheeses.

Keywords: Biopreservation, plantaricin TF711, *Clostridium sporogenes*, bacteriocin, cheese.

Lactic acid bacteria (LAB) have a long and safe history of use as preservatives in dairy fermentations where they are commonly employed as starter cultures (O'Sullivan et al. 2002). Preservative ability of LAB is due to production of antimicrobial metabolites, such as organic acids, hydrogen peroxide and bacteriocins, proteinaceous substances usually active against similar or closely related bacteria (Klaenhammer 1988; Leroy & De Vuyst 2004). Bacteriocin-producing LAB are ideally suited to food application as they are natural food isolates with generally recognised as safe status and with a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria (Deegan et al. 2006). Therefore, the use of LAB bacteriocins as part of the hurdle technology has gained great attention in the last two decades since they may help to reduce the use of chemical preservatives and the intensity of heat and other physical treatments, satisfying demands of consumers for more naturally preserved foods (Gálvez et al. 2007).

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LAB bacteriocins can be used either by adding concentrated preparations as food additives, or by adding bacteriocin-producing strains (i) directly in food as starter culture, (ii) as adjunct or co-culture in combination with a starter culture, or (iii) as protective culture in non-fermented foods (Ross et al. 2002). It is generally accepted that the use of bacteriocin-producing cultures in food has a considerable advantage over using purified bacteriocin preparations since the latter application requires extensive and costly purification schemes, toxicology tests, and may suggest a non-natural image of additives (Anastasiou et al. 2007). Finally, successful application of bacteriocinogenic LAB in fermented foods, such as cheeses, depends on the ability of the strain to grow under processing and storage conditions and to produce enough bacteriocin to inhibit target pathogenic and spoilage bacteria without interfering with the starter culture required for fermentation (Gálvez et al. 2007). Some recent studies in cheeses have demonstrated the efficacy of bacteriocin-producing LAB in controlling the growth of non desirable microorganisms (Martínez-Cuesta et al. 2010; Garde et al. 2011; Dal Bello et al. 2012; Vera Pingitore et al. 2012).

During a screening program for bacteriocinogenic LAB from raw Tenerife goats' milk cheese, *Lb. plantarum* TF711 was selected (Hernández et al. 2005). This strain produces plantaricin TF711, a promising bacteriocin due to its technological properties (thermo stability, pH stability and stability against surfactants) and its wide inhibitory activity spectrum, including, among others, *Clostridium sporogenes*. *C. sporogenes* is one of the causative agents of late blowing of semi-hard and hard cheeses, a defect of great economical importance characterised by excessive gas production and accompanying off-flavours (Bergére & Lenoir, 2000). Prevention of late blowing is a difficult issue since *Clostridium* spores are ubiquitous and concentrations as low as 1 spore/ml in cheese milk is enough to induce this defect, if conditions are suitable for spore germination and growth of vegetative cells in cheese (Garde et al. 2013). In addition, because of the cultural, physiological and genetic similarity, *C. sporogenes* is often used as a model to study the growth ability of *C. botulinum* without health risk (Hatheway 1993; Carminati et al. 2001).

In this study, we investigated the ability of *Lb. plantarum* TF711 to inhibit *C. sporogenes* growth when used as adjunct culture in cheese manufacture.

Materials and methods

Microorganisms and preparation of spore suspensions

Clostridium sporogenes ATCC 3584 was obtained from Colección Española de Cultivos Tipo (CECT) and grown in Clostridia Nutrient Medium (CNM, Fluka, Buchs, Switzerland) at 37 °C 72 h under anaerobic conditions (95% H_2 + 5% CO_2; AnaeroGen, Oxoid, Basingstoke, UK). Plantaricin producer *Lb. plantarum* TF711 was used as adjunct culture in cheese-making after confirming its inability to produce biogenic amines by the procedure described by Bover-Cid & Holzapfel (1999). Lactobacilli were routinely grown in de Man, Rogosa and Sharpe (MRS, Scharlau, Barcelona, Spain) at 30 °C for 48 h. All strains were maintained as frozen stocks in their culture medium with 20% glycerol at −80 °C.

Clostridia spore suspensions were prepared by incubation in 50 ml CNM broth at 37 °C under anaerobic conditions for 5 d. After centrifugation (3500 ***g***, 30 min; TJ-6 centrifuge, Beckman-Coulter, California, USA), pellet was resuspended in 5 ml CNM broth containing glycerol (20% (v/v) final concentration), and suspension was stored in aliquots of 1 ml at −20 °C. After heating suspensions at 75 °C for 15 min, spore concentration was determined by plating on CNM agar followed by anaerobic incubation at 37 °C for 72 h. Before inoculation in vat milk, spore suspensions were heated at 75 °C for 15 min (to kill vegetative cells).

Manufacture of cheeses

Cheese-making was done in duplicate (at 2 weeks interval) at laboratory scale and following conventional manufacturing methods. Commercially purchased pasteurised cows' milk with 0·02% $CaCl_2$ was heated to 32 °C and subsequently inoculated with commercial starter containing *Lactococcus lactis* subsp. *lactis, Lac. lactis* subsp. *cremoris* and *Streptococcus thermophilus* (Choozit™ RA21, Danisco, Copenhagen, Denmark) added at the manufacturer's recommended dose (9·47 g/1000 l of milk). Milk was then contaminated with 10^4 spores/ml *Clostridium sporogenes* ATCC 3584 and distributed into two 6 l vats, which were further individually inoculated according to the following scheme: control vat: no adjunct culture added; and experimental vat: inoculated with 1% of an overnight culture of *Lb. plantarum* TF711 in sterilised reconstituted skimmed milk. After agitation, milk was held for 30 min at 32 °C. Commercial rennet (25 mg/l; CHY-MAX™ Powder Extra NB; Chr Hansen, Hørsholm, Denmark) was added and, after agitation, milk was left to settle at 32 °C until coagulation (ca. 30 min). Curd was cut into grain size (ca. 5 mm) while the temperature was increased to 37 °C with agitation. Curd was placed in plastic cylindrical moulds, pressed for 4 h at room temperature and dry salted overnight. Once removed from the moulds, cheeses were vacuum packed in plastic bags and ripened at 12 °C for 45 d. One cheese (ca. 1 kg weight) was obtained for each vat. Cheeses were sampled at days 1, 7, 14, 21, 30 and 45, and were analysed as described below.

Microbiological analysis

Cheese samples (5 g) taken in duplicate were homogenised in 45 ml sterile 2% (w/v) sodium citrate solution. Decimal dilutions in sterile 0·1% peptone water were prepared and the following microorganisms were estimated on duplicate plates: lactococci on M-17 agar (Scharlau) incubated at 30 °C 24 h (double agar layer); lactobacilli on Rogosa agar (RA, Fluka) at 37 °C 24 h in an anaerobic jar (double agar layer); *Clostridium* spores on CNM agar at 37 °C 72 h in an anaerobic jar (surface spread) after treatment of the homogenate or dilutions at 75 °C for 15 min.

Determination of pH and NaCl content

The pH of grated cheese (5 g) homogenised with 45 ml distilled water was determined using a Crison GLP-21 pH-meter (Crison, Barcelona, Spain). NaCl content was determined according to standard of American Public Health Association (Bradley et al. 1993). All determinations were performed in duplicate.

Detection of bacteriocin activity

Bacteriocin activity in cheeses was determined by the agar well diffusion assay, basically as described by Rodríguez et al. (2005). Duplicate cheese samples (5 g) were homogenised with 10 ml sterile 20 mM HCl at 50 °C. After centrifugation (13 000 ***g***, 20 min, 4 °C, Avanti J-E, Beckman-Coulter), supernatants were adjusted at pH 6 with 2 M NaOH, filter sterilised (0·22 μM pore size

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Table 1. Evolution of microbiological parameters (log CFU/g) throughout ripening of cheeses contaminated with spores of *Clostridium sporogenes* with no adjunct culture (control) or with adjunct *Lactobacillus plantarum* TF711 (experimental)

Parameter	Cheese	Days of ripening					
		1	7	14	21	30	45
Lactococci	Control	9·20 ± 0·19	9·20 ± 0·13	9·42 ± 0·12	9·50 ± 0·12	9·52 ± 0·10	9·66 ± 0·08
	Experimental	9·16 ± 0·21	9·25 ± 0·12	9·41 ± 0·14	9·46 ± 0·21	9·58 ± 0·14	9·69 ± 0·04
Lactobacilli	Control	<1	2·36 ± 0·18	3·49 ± 0·10	5·43 ± 0·14	6·17 ± 0·11	6·55 ± 0·14
	Experimental	6·53 ± 0·14*	6·94 ± 0·08*	7·98 ± 0·13*	8·14 ± 0·07*	8·55 ± 0·11*	8·79 ± 0·12*
Clostridia spores	Control	4·08 ± 0·07	5·09 ± 0·11	5·49 ± 0·14	5·75 ± 0·11	6·01 ± 0·12	6·40 ± 0·10
	Experimental	4·05 ± 0·12	4·85 ± 0·07*	3·86 ± 0·11*	3·56 ± 0·07*	4·51 ± 0·13*	4·54 ± 0·12*

Data are mean ± SD of duplicate determinations in two replicate cheese-making trials
*Significantly different ($P < 0·05$) from the respective value in control cheese

Durapore® PVDF, Merck Millipore, Cork, Ireland), and concentrated to 1/10 of their original volume in a vacuum concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany). A volume of 50 µl of each neutralised and concentrated supernatant was placed in duplicate into wells (6 mm diameter) made in plates of RCM soft agar (1% agar) containing 5×10^5 CFU/ml *Clostridium sporogenes* ATCC 3584 as indicator microorganism. After pre-diffusion at 4 °C for 2 h, plates were incubated at 37 °C for 72 h in anaerobic conditions. Bacteriocin activity was determined by measuring the diameter of inhibition zones around wells and expressed in mm.

Statistical analysis

The SPSS Statistics 19 software (International Business Machines Corporation, New York, USA) was used for statistical processing. One way analysis of variance (ANOVA) was applied to microbiological and physicochemical data to determine the presence of significant differences between experimental and control cheeses for each ripening time. Differences were considered significant at $P < 0·05$.

Results

Evolution of microbiological parameters

Table 1 shows the evolution of microbiological parameters throughout ripening of cheeses contaminated with *C. sporogenes* ATCC 3584 without (control) or with adjunct *Lb. plantarum* TF711 (experimental). Lactococci counts were similar for control and experimental cheeses throughout ripening, with values around 9·2 log CFU/g at day 1 that slightly increased with time, reaching at 45 d a mean value around 9·7 log CFU/g. As expected, lactobacilli counts in control cheese were significantly lower than in experimental cheese at all sampling times. The day after manufacture, lactobacilli were below detection limit (<1 log CFU/g) in control cheese whereas 6·5 log CFU/g were found in experimental cheese. Thereafter, lactobacilli counts of both cheeses increased with maturation, reaching at 45 d mean values around 6·6 and 8·8 log CFU/g for control and experimental cheese, respectively. Clostridial spore count were the same in control and experimental cheese the day after manufacture (mean value around 4·1 log spores/g). From here, counts in control cheese increased with time, reaching at the end of ripening a mean value of 6·4 log spores/g. Yet, spore counts in experimental cheese gained almost 1 log unit during the first week, then dropped to 3·6 log spores/g on day 21, and finally rose to 4·5 log spores/g on day 45. Statistical analysis confirmed that spore counts in experimental cheeses were significantly lower than in control cheeses from 7 d onwards, reaching a maximum reduction of 2·2 log units on day 21. For both types of cheeses, clostridial spore count was significantly different ($P < 0·05$) at each ripening time compared with the initial value, except for the 14-d experimental cheese (not shown). Moreover, cheese blowing (characterised by appearance of holes together with unpleasant odour) was observed after 14 d in experimental and control cheeses, although this defect was less pronounced in cheeses with *Lb. plantarum* TF711.

Evolution of pH and NaCl content

Evolution of pH and NaCl content of experimental and control cheeses throughout ripening is shown in Table 2. Use of bacteriocinogenic *Lb. plantarum* TF711 as adjunct did not bring about any significant change in NaCl content or in pH, compared with control cheese. NaCl content of cheeses increased from 1·8 to 2·2% throughout ripening; while pH was around 5·1–5·2 during the first 21 d and slightly increased thereafter to reach a value around 5·4 at 45 d.

Bacteriocin activity

Table 3 shows bacteriocin activity recovered throughout ripening from experimental and control cheeses. No inhibition on indicator *Clostridium* was observed in any of the plates containing samples of control cheese. Contrarily, clear inhibition zones were seen around wells containing experimental cheese preparations at all sampling times, except the day after manufacture. Highest diameter of growth inhibition was observed at 21 d of ripening, coinciding with the lowest clostridial spore count found in experimental cheeses

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Table 2. Evolution of pH and NaCl content throughout ripening of cheeses contaminated with spores of *Clostridium sporogenes* with no adjunct culture (control) or with adjunct *Lactobacillus plantarum* TF711 (experimental)

Parameter	Cheese	Days of ripening					
		1	7	14	21	30	45
pH	Control	5·22 ± 0·03	5·17 ± 0·03	5·09 ± 0·03	5·23 ± 0·03	5·29 ± 0·03	5·36 ± 0·05
	Experimental	5·20 ± 0·06	5·16 ± 0·04	5·10 ± 0·03	5·25 ± 0·03	5·31 ± 0·06	5·37 ± 0·07
NaCl (%)	Control	1·80 ± 0·08	1·95 ± 0·06	1·98 ± 0·05	2·05 ± 0·06	2·10 ± 0·08	2·20 ± 0·08
	Experimental	1·78 ± 0·05	1·90 ± 0·08	1·95 ± 0·06	1·98 ± 0·05	2·05 ± 0·06	2·15 ± 0·06

Data are mean ± SD of duplicate determinations in two replicate cheese-making trials
*Significantly different ($P < 0·05$) from the respective value in control cheese

Table 3. Bacteriocin activity (mm diameter growth inhibition) throughout ripening of cheeses contaminated with spores of *Clostridium sporogenes*, with no adjunct culture (control) or with adjunct *Lactobacillus plantarum* TF711 (experimental)

Cheese	Time (days)	Bacteriocin activity
Control	1	ND
	7	ND
	14	ND
	21	ND
	30	ND
	45	ND
Experimental	1	ND
	7	11 ± 1
	14	17 ± 3
	21	22 ± 1
	30	14 ± 2
	45	15 ± 2

Data are mean ± SD of duplicate determinations in two replicate cheese-making trials
ND, not detected

Discussion

The aim of this study was to investigate the efficacy of bacteriocin-producing *Lb. plantarum* TF711 in the control of *C. sporogenes* in cheeses. For this purpose, cheeses were manufactured using *Lb. plantarum* TF711 as adjunct culture to a commercial starter, and the evolution of microbiological and physicochemical parameters, as well as bacteriocin production was evaluated during maturation and compared with that of control cheeses made without adjunct lactobacilli.

Lactococci counts and pH were similar for control and experimental cheeses throughout ripening confirming that neither growth of the starter culture nor its acidification capability were affected by adjunct *Lb. plantarum* TF711, which is a prerequisite for using bacteriocin-producing LAB as preservatives in cheeses. Although *Lb. plantarum* TF711 was not added, lactobacilli were recovered from control cheeses from 7 d onwards, reaching at the end of maturation values around 6·5 log CFU/g. The presence of lactobacilli in control cheeses was probably due to post-pasteurization contamination of the milk and/or survival in heat-shocked state as has been described (McSweeney et al. 1994; Ortigosa et al. 2006; González & Zárate 2012).

Clostridial spore count was significantly lower in experimental cheese than in control cheese from 7 d onwards. This difference in spore numbers between experimental and control cheese was mainly attributed to bacteriocin activity and not to acid production, as pH values in experimental cheese did not differ significantly from those of control cheese. In addition, we were able to detect bacteriocin activity in experimental cheese at all sampling times, except the day after manufacture, confirming plantaricin production by *Lb. plantarum* TF711 and successful extraction from the cheese matrix. Variable results on bacteriocin detection and stability throughout cheese ripening have been published. Several studies have reported bacteriocin production in situ that was stable during the whole ripening process (Foulquié Moreno et al. 2003; Rilla et al. 2003; Rodríguez et al. 2005; Anastasiou et al. 2007). Other authors have detected bacteriocin at the beginning of cheese ripening but not thereafter (Martínez-Cuesta et al. 2010), whereas others have found a decline of bacteriocin activity during cheese ripening (Benech et al. 2002). Finally, inability to recover bacteriocin from cheeses, although suppression of undesirable microorganisms took place during the whole ripening period, has also been reported (Farías et al. 1999; Bogovič Matijašić et al. 2007; Izquierdo et al. 2009).

Despite anticlostridial activity of *Lb. plantarum* TF711, spore counts in experimental cheese increased the first week, then decreased until day 21, and increased thereafter, reaching at 45 d a value 0·5 log units higher than initially. One possible explanation of such behaviour could be based on changes in the concentration of plantaricin throughout cheese ripening. Thus, the number of spores increased during the first week before bacteriocin produced by *Lb. plantarum* TF711 reached its minimum inhibitory concentration in cheese. Then, plantaricin exerted antimicrobial activity against target clostridia and, therefore, spore counts decreased. Finally, plantaricin fell to non-inhibitory concentration, probably due to depletion and/or loss of stability (Garde et al. 2014), permitting spore counts to increase again towards the end of ripening. This approach to changes in bacteriocin concentration during

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ripening is consistent with the size of inhibition zones found in our experimental cheeses when tested by the agar well diffusion assay (Table 3).

In previous studies with cheeses made from milk inoculated with bacteriocin-producing LAB and clostridial spores, some authors reported a decrease in spore counts of more than 3 log units throughout ripening (Rilla et al. 2003; Martínez-Cuesta et al. 2010). Alternatively, other studies found that spore numbers remained fairly constant (Garde et al. 2011) or even increased around 2·5 log units (Bogovič Matijašić et al. 2007) at the end of ripening compared with the initial value. An increase in the number of spores in cheese indicates spore germination, growth of vegetative cells and subsequent sporulation (Bogovič Matijašić et al. 2007; Garde et al. 2011). Furthermore, growth of *Clostridium* cells produces gas and butyric acid, giving rise to blowing defect. In this study, blowing was observed after 14 d in control cheese, and to a lesser extent in experimental cheese, thus confirming spore germination and subsequent metabolic activity of vegetative cells. In agreement with our results, Bogovič Matijašić et al. (2007) also found signs of blowing as a result of *C. tyrobutyricum* growth in cheeses made from milk contaminated with $2{\cdot}5 \times 10^3$ spores/ml with bacteriocin-producing *Lactobacillus gasseri*.

The finding that *Lb. plantarum* TF711 was not sufficient for complete inhibition of *Clostridium* could possibly be due to the high concentration of spores used for contaminating milk in our experiment (10^4 spores/ml). It is likely that the effect on lower concentrations of spores would be more inhibitory than was found in the present work. Finally, as *C. sporogenes* is often applied as a non pathogenic surrogate for *C. botulinum* in spore inactivation studies (Naim et al. 2008), the results obtained in this work suggest that adjunct *Lb. plantarum* TF711 may also help to improve cheese safety if spores of the pathogenic species were present.

Conclusions

The presence of bacteriocin-producing *Lb. plantarum* TF711 in cheese resulted in inhibition of *C. sporogenes* compared with control cheese, without affecting growth and acidification capability of the starter culture involved in maturation. Despite anticlostridial activity of *Lb. plantarum* TF711, which was mainly attributed to plantaricin activity, some spores were able to germinate, grow, and sporulate again, giving rise to blowing defect in experimental cheeses. Nevertheless, considering that the inoculation level of clostridial spores in our work was much higher than what could be expected in naturally contaminated milk, more successful inhibition would be expected in real cheese production.

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