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Inhibition of *Clostridium tyrobutyricum* by *Streptococcus macedonicus* ACA-DC 198 under conditions mimicking Kasseri cheese production and ripening

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ABSTRACT

Three fermentations in skim milk were used to study the effectiveness of the bacteriocin-producing *Streptococcus macedonicus* ACA-DC 198 strain to inhibit *Clostridium tyrobutyricum* LMG 1285T spore outgrowth under conditions prevailing during Kasseri cheese production and ripening. In fermentation A, *Clostridium* spores were used solely; in fermentation B, *S. macedonicus* ACA-DC 198 and *Clostridium* spores were used; in fermentation C, a commercial starter culture and *Clostridium* spores were used. The temperature program applied was similar to that of Kasseri cheese production and ripening. The presence of macedocin, the bacteriocin produced by *S. macedonicus* ACA-DC 198, was confirmed in fermentation B. The results showed that macedocin was able to inhibit the outgrowth of *Clostridium* spores, since significantly higher inhibition in spore outgrowth was detected in fermentation B than in fermentation C.

1. Introduction

Clostridium tyrobutyricum is a Gram-positive, anaerobic, spore-forming bacterium, that is considered to be the predominant causative agent of the "late blowing" defect in semi-hard and hard cheeses with long ripening times, such as Kasseri, Gruyère, Emmental, Gouda, Parmigiano, and Grana Padano, in which *Clostridium* spore outgrowth is favoured by the relatively high cheese pH of approximately 5.3–5.5 (Steffen et al., 1993; Walstra et al., 1993). The biochemical basis of this defect is the fermentative transformation of lactic acid to acetic and butyric acid, with the latter compound causing rancid flavour, and also the formation of carbon dioxide and hydrogen gas, which are responsible for the distension of the holes that in extreme cases results in cheese bursting. These defects, e.g., distribution and appearance of the holes, splitting and off flavours found in contaminated cheeses, result in loss of commercial value, and a high negative economic impact in cheese production.

C. tyrobutyricum spores present in cheese originate from milk, which is contaminated during the milking process. Silage feed of poor microbiological quality is one of the most important sources of contamination of raw milk with spores that are able to survive the pasteurization process (DasGupta and Hull, 1989). Although *C. tyrobutyricum* is the most frequently isolated spore former from late blown cheeses, *Clostridium beijerinckii*, *Clostridium butyricum* and *Clostridium sporogenes* are also significant secondary causes of gas production in cheese (Klijn et al., 1995; Le Bourhis et al., 2005, 2007). The growth of these bacteria is critically influenced by the ripening time and temperature, lactic acid concentration, salt concentration, pH, moisture and fat content, and the presence of other microorganisms in cheese. The shape, size and texture of the cheese can also play a significant role (Aureli and Franciosa, 1999).

The most common approaches to prevent the late blowing defect include bactofugation or microfiltration of milk, or addition of nitrate or lysozyme (Lodi, 1990; Stadhouders, 1990; Van den Berg et al., 2004). However, since the application of these treatments or the use of additives often results in unacceptable secondary side effects, an alternative is the application of protective lactic acid bacteria (LAB) cultures, which offer great potential in bio-preservation of foods. Their primary usefulness is to provide natural technologies for spoilage control, as healthier and more convenient options (e.g., lower salt, ambient storage) are developed by food processors. Furthermore, it should be stressed that nowadays, biopreservation, in contrast to the chemical preservation of foods, reflects the consumers' demands for clear labelling of safe and

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minimally processed foods of adequate shelf life and convenience, and also the global need for increasing the supply of healthy and safe foods.

Besides organic acids and other low molecular-mass inhibitory compounds such as diacetyl and hydrogen peroxide, many LAB produce ribosomally synthesized peptides or proteins, the bacteriocins, which exert an antimicrobial effect against not only closely related bacteria, but often also against spoilage and pathogenic bacteria (De Vuyst and Vandamme, 1994). In some recent studies the production and efficacy of bacteriocins in cheese have been demonstrated (Anastasiou et al., 2007; Bogovič Matijašić et al., 2007; Foulquié Moreno et al., 2003; Rilla et al., 2003; Rodríguez et al., 2005).

Kasseri is a Greek traditional cheese of protected of origin (PDO, European Commission, 1996) manufactured from ewes' milk or mixtures of ewes' and goats' milk, without the use of starters. It is a semi-hard cheese of the pasta filata type. Traditionally it is produced with milk clotted with the use of rennet. The curd is then cut, scalded up to 45 °C, drained and kept at 15–18 °C to reach a pH value of 5.20–5.40. The acidified curd is sliced and kneaded in hot water (70–80 °C) to give the characteristic homogeneous plastic texture. Then dry salt is added, and the fresh Kasseri cheese is moulded, and ripened for 3 months.

Streptococcus macedonicus ACA-DC 198, isolated from the microbiota of naturally fermented Greek Kasseri cheese (Tsakalidou et al., 1998), produces the lantibiotic macedocin exhibiting antimicrobial activity against, among others, *C. tyrobutyricum* (Georgalaki et al., 2002; Zoumpopoulou et al., 2008). Macedocin is a promising biopreservative due to its broad antimicrobial spectrum (Georgalaki et al., 2002). Moreover, *S. macedonicus* ACA-DC 198 has been successfully used as an adjunct culture in Kasseri cheese production (Anastasiou et al., 2007; De Vuyst and Tsakalidou, 2008). It has been shown that *S. macedonicus* ACA-DC 198 survives under the stress conditions prevailing during Kasseri cheese production, and under these conditions the macedocin produced during the initial stages can be detected until the end of the ripening period.

The aim of the present study was to evaluate the ability of *S. macedonicus* ACA-DC 198 to inhibit *C. tyrobutyricum* spore outgrowth under conditions prevailing during Kasseri cheese production and ripening. For this purpose, fermentations in milk contaminated with *Clostridium* spores were performed using a temperature program similar to the one applied during Kasseri cheese production and ripening.

2. Materials and methods

2.1. Bacterial strains and media

S. macedonicus ACA-DC 198 was isolated from naturally fermented Kasseri cheese and belongs to the ACA-DC culture collection of the Laboratory of Dairy Research (Department of Food Science and Technology, Agricultural University of Athens, Athens, Greece). The strain was subcultured twice (inoculum of 1%, v/v) in skim milk (10%, w/v; Oxoid, Basingstoke, Hampshire, UK), containing yeast extract (0.3%, w/v; Oxoid), at 37 °C for 18 h. The commercial, freeze-dried, mixed thermophilic cheese culture, TCC-3 (Chr. Hansen, Hørsholm, Denmark), containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was used in control fermentations. The starter culture was subcultured twice (inoculum of 0.1%, v/v) in skim milk (10%, w/v), containing yeast extract (0.3%, w/v) at 37 °C for 18 h. *S. macedonicus* ACA-DC 198 and the commercial starter culture were added as 0.1% (v/v) inoculum into the fermentor. *C. tyrobutyricum* LMG 1285T was subcultured in RCM broth (Biokar Diagnostics, Beauvais, France) at 37 °C for 48 h (inoculum of 1%, v/v) under anaerobic conditions (BBL GasPak™ Anaerobic System, Becton Dickinson, NJ, USA).

2.2. Spore preparation

C. tyrobutyricum spores were prepared by incubation of RCM broth cultures at 37 °C under anaerobic conditions for 8 days. After centrifugation (4300 × *g*, 15 min, 20 °C), the pellet was washed twice with sterile distilled water, resuspended in sterile skim milk (10%, w/v), and stored in aliquots of 1.5 mL at −20 °C. The spore concentration in the suspension was determined, after heat treatment at 75 °C for 15 min, by serial dilution in Ringer and plating on RCM plates, followed by anaerobic incubation at 37 °C for 5 days. Before inoculating the spores into the fermentor, the spore suspension was heated at 75 °C for 15 min.

2.3. Fermentations

Fermentations were carried out in a computer-controlled 2.5-L glass fermentor (BioFlo 3000C, New Brunswick Scientific Co., New Brunswick, NJ, USA) with temperature and pH controls. The fermentor contained 1.5 L of skim milk (10%, w/v, Oxoid), supplemented with yeast extract (0.3%, w/v, Oxoid), and it was sterilized at 121 °C for 10 min. The NaCl solution mimicking Kasseri cheese production was sterilized separately and added aseptically to the fermentor. The pH decrease was followed on line (initial pH 6.40), while the temperature was maintained constant within 0.1 °C of the set point. A temperature and NaCl concentration profile mimicking that of Kasseri cheese manufacturing was replicated in the fermentor. Anaerobic conditions were maintained by continuous nitrogen sparging (2 L min^{-1}through the liquor), while slow agitation at 100 rpm was maintained to ensure the homogeneity of the fermentation medium. All fermentations lasted 27 h. The initial temperature of the milk was 37 °C and after inoculation of the fermentation medium, the milk inoculated sample (Mi) was taken.

Subsequently, the temperature was kept constant for 1.5 h, after which it was increased to 45 °C, and the incubation continued for another 20 min. Then, the temperature was decreased to 25 °C, sample B1 (Baski 1, fresh cheese curd) was taken, while the temperature was maintained constant overnight.

After 24 h of fermentation, sample B2 (Baski 2, ripened cheese curd) was taken and then the temperature was increased to 75 °C (Baski scalding). After 5 min at 75 °C, the temperature was decreased to 25 °C and the NaCl solution was added aseptically (final concentration of 3%, v/v). Sample K0 (unripened Kasseri cheese) was taken and subsequently the content of the fermentor was dispensed aseptically in sterile vials. Vials were flushed with nitrogen to maintain anaerobic conditions and they were kept at 20 °C for up to 120 days (conditions of Kasseri cheese ripening). Samples K10, K30, K60, K90, and K120 corresponding to 10, 30, 60, 90, and 120-day Kasseri cheese ripening, respectively, were analysed.

Three sets of fermentations were performed and each set was repeated three times. In fermentation A, *Clostridium* spores (10^3 cfu mL^{-1}) were used solely; in fermentation B, *S. macedonicus* ACA-DC 198 (10^6 cfu mL^{-1}) and *Clostridium* spores (10^3 cfu mL^{-1}) were used; in fermentation C, the commercial starter culture (10^6 cfu mL^{-1}) and *Clostridium* spores (10^3 cfu mL^{-1}) were used. The concentration of the commercial starter culture was previously adjusted, so that the rate of pH decrease in fermentation C was similar to that of fermentation B (data not shown), while during the initial stages of fermentation A (up to K0) the pH was adjusted using 5 N HCl, so that the rate of pH drop was compatible with fermentation B.

2.4. Microbiological analyses

The enumeration of *C. tyrobutyricum* spores in fermentation samples was performed using the Most Probable Number (MPN) method. More specifically, 1 mL aliquots of serial decimal dilutions in quarter-strength Ringer's solution were inoculated in three tubes, each with 9 mL of BBMB medium (Biokar Diagnostics), which contains lactate as the fermentable organic compound. The tubes were sealed with paraffin and incubated at 37 °C for 6 days. Tubes were considered positive if they showed visible gas formation at the end of the incubation period. Two sets of tubes were prepared. One set was heated at 75 °C for 15 min to kill the vegetative cells and to determine the number of spores. The other set was not heated, so that both vegetative cells and spores capable of vegetating without heat pre-treatment, produced gas and were enumerated. *S. macedonicus* ACA-DC 198 and *S. thermophilus* were enumerated through plating on M17 agar (Biokar Diagnostics), at 37 °C for 48 h, while *L. delbrueckii* subsp. *bulgaricus* was plated on MRS agar (pH 5.4; Biokar Diagnostics) and incubated at 37 °C for 3 days, under anaerobic conditions.

2.5. Detection of macedocin

The agar well diffusion assay was used to determine macedocin activity in cell-free culture supernatants of the fermentation samples (Tagg and McGiven, 1971), using *Lactococcus lactis* LMG 6890T as indicator strain. Briefly, 15 mL of M17 agar (Biokar Diagnostics), containing 0.1% (v/v) of a fresh culture of the sensitive strain, was poured into a Petri dish, and wells of 5 mm diameter were made in the solidified medium. The wells were filled with 50 μL of cell-free culture supernatants of the fermentation samples (pH adjusted to 6.5) and the plates were examined for inhibition zones after 12 h of incubation at 30 °C. Activity was expressed in arbitrary units (AU) per mL, corresponding to 50 μL of the highest dilution causing a clear zone of inhibition of the indicator organism.

2.6. HPLC analysis

The lactose as well as the organic acid content of cell-free culture supernatants of the fermentation samples was determined by HPLC (LC 1150 HPLC Pump, GBC Scientific Equipment, Dandenong, Victoria, Australia). The fermentation samples (2 mL) were extracted with 5 mL of distilled water at 40 °C using a Stomacher (Seward, London, UK). The pH was adjusted to 4.5–4.6 and the mixture was incubated at 40 °C for 1 h. After centrifugation at 12,000 × *g* for 30 min at 4 °C, 1 mL of the supernatant was precipitated with $HClO_4$ (final concentration 6.4%) overnight, centrifuged (12,000 × *g* for 1 h, 4 °C) and filtered. A 20-μL sample of the filtrate was injected into an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad, Hercules, CA, USA) connected to a refractive index detector (model LC 1240; GBC Scientific Equipment). Elution was performed at 35 °C with 5 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1}. Data acquisition and processing were performed by the WinChrom™ Chromatography Data Acquisition Software v. 1.32 (GBC Scientific Equipment).

2.7. Statistical analysis

Mean values of particular parameters in fermentations A, B, and C were compared by Tukey's test, with an alpha level of 0.05. The following parameters were statistically evaluated: the concentration of *Clostridium* spores, the concentration of *Clostridium* vegetative cells, as well as the pH values during fermentation and Kasseri cheese ripening. All analyses were performed with Statgraphics Centurion software (version XV).

3. Results and discussion

S. macedonicus ACA-DC 198 is a natural cheese isolate, which produces macedocin, a lantibiotic with high anti-clostridial activity (Georgalaki et al., 2002; De Vuyst and Tsakalidou, 2008). The idea that *S. macedonicus* ACA-DC 198 might be able to inhibit *C. tyrobutyricum* in cheese came from previously established data of *in vitro* inhibition of *C. tyrobutyricum* vegetative cells through a well diffusion assay (Georgalaki et al., 2000). Furthermore, when *S. macedonicus* ACA-DC 198 was used as an adjunct culture in Kasseri cheese production, macedocin was detected in the cheese from the initial stages of cheese making and remained constant throughout ripening (Anastasiou et al., 2007). Before going to cheese manufacture with milk contaminated with *C. tyrobutyricum* spores, this study used the controlled environment of the fermentor to apply the conditions that prevail during Kasseri cheese manufacture and ripening (temperature, pH, salt, and anaerobic conditions) and investigated the inhibition of *C. tyrobutyricum* by *S. macedonicus* ACA-DC 198. The fermentation medium was sterile skim milk.

Three sets of fermentations were performed. In fermentation A, spores of *C. tyrobutyricum* LMG 1285T were solely used; in fermentation B, *S. macedonicus* ACA-DC 198 and *Clostridium* spores were used; in fermentation C, a commercial starter culture together with *Clostridium* spores were used. Through preliminary experiments it was shown that the commercial starters did not produce any antimicrobial compound, apart from lactic acid, towards both spores and vegetative cells of *C. tyrobutyricum* (data not shown). Moreover, neither the commercial starters nor *S. macedonicus* ACA-DC 198 produced any gas, when grown in BBMB medium, which would interfere in the MPN method used to enumerate *C. tyrobutyricum*.

In control fermentation A, inoculated with *C. tyrobutyricum* only, the rate of pH drop was adjusted to be compatible with fermentation B. Thus the initial pH of sample Mi (inoculated milk) was lowered by the addition of 5 N HCl from 6.40 ± 0.04 to 4.82 ± 0.03 in sample B2. Afterwards, the pH remained essentially stable until the end of ripening (K120, pH of 4.46 ± 0.03). No vegetation and outgrowth of *Clostridium* spores were detected, since *C. tyrobutyricum* does not ferment lactose, and consequently, it is not able to grow in milk (Wiedmann et al., 1999) (Fig. 1). The spore counts

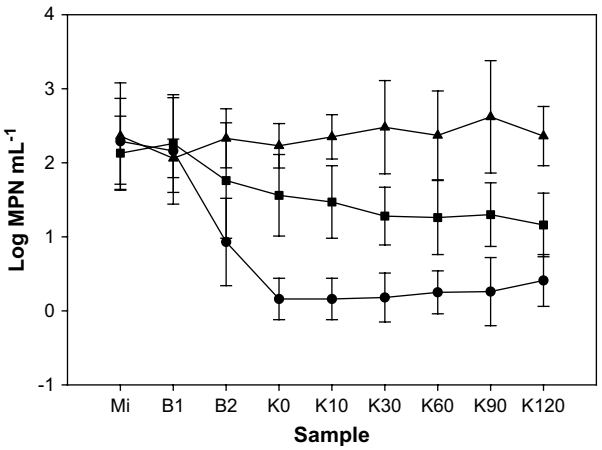

Fig. 1. Inhibition of *Clostridium* spores during milk fermentations mimicking Kasseri cheese production and ripening with: *Clostridium* spores (fermentation A; ▲), *S. macedonicus* ACA-DC 198 and *Clostridium* spores (fermentation B; ●), commercial starter culture and *Clostridium* spores (fermentation C; ■). Data reported are means from three repetitions ± standard deviations. Mi: Milk inoculated; B1: Baski 1, fresh cheese curd; B2: Baski 2, ripened cheese curd; K0: Kasseri at day 0; K10, K30, K60, K90, and K120: Kasseri after 10, 30, 60, 90, and 120 days of ripening, respectively.

ranged from 2.36 to 2.89log MPN mL^{-1} in all samples tested during the fermentation and throughout the ripening period.

In fermentation B, where *S. macedonicus* ACA-DC 198 and *C. tyrobutyricum* LMG 1285T were included, the initial pH value was 6.40 ± 0.03 and the pH decreased to 4.81 ± 0.05 at sample B2 (Fig. 2), reflecting the growth of *S. macedonicus* in milk. Counts of *S. macedonicus* increased during Baski ripening (from Baski 1 to Baski 2) and declined after Baski scalding (Kasseri 0) (Fig. 3). The counts then decreased gradually and by the end of ripening were diminished. Macedocin was produced during the initial stages of fermentation, reaching a maximum activity of 160 AU mL^{-1} in sample B2 and then remaining constant until the end of the ripening period (Figs. 3 and 4). At the beginning of fermentation B, the mean concentration of *Clostridium* spores was 2.29log MPN mL^{-1} (Fig. 1). This value decreased in sample B2 (no significant difference, $P > 0.05$), while the lowest value was detected in sample K0 (0.16log MPN mL^{-1}, significant difference, $P < 0.05$) and then remained at a very low level throughout the ripening period. Statistical analysis showed that in fermentation B, all samples from K0 to K120 showed significant differences ($P < 0.05$) from samples Mi and B1. Moreover, when fermentation B was compared to fermentation A, significant differences were observed in all samples from K0 to K120. In these samples, the reduction in spore concentration ranged from 1.95 to 2.36log units. This reduction in spore concentration detected in fermentation B could result either from the production of lactic acid and the concomitant reduction of pH, since low pH values are inhibitory to *C. tyrobutyricum* growth (Kleter et al., 1984), or from the inhibition of *Clostridium* spores by macedocin as well as from the combined influence of both of them.

In fermentation C, the growth of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* showed a growth profile similar to that of *S. macedonicus*. Counts of these strains increased during Baski ripening, declined after Baski scalding, and then kept decreasing until the end of ripening (Fig. 3). The starting pH of 6.40 ± 0.05 decreased to 4.57 ± 0.07 in sample B2, while the respective pH value in fermentation B was 4.81 ± 0.03 (Fig. 2). Slightly lower pH values were detected in all samples of fermentation C analysed thereafter. When compared with fermentation B, however, the differences were not significant ($P > 0.05$). In fermentation C, the initial concentration of *Clostridium* spores was 2.26log MPN mL^{-1}. This value decreased in sample B2 to 1.76log MPN mL^{-1} and afterwards it showed a slight decrease until the end of the ripening period; however, the differences were not significant ($P > 0.05$) (Fig. 1). The slight inhibition of *Clostridium* spores could be attributed to the reduction of pH of the fermentation medium solely,

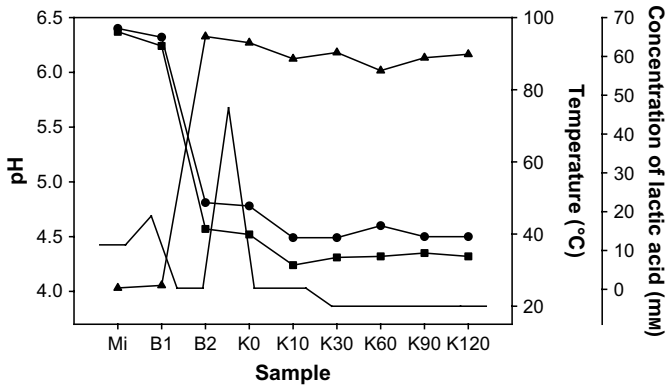

Fig. 2. Evolution of pH (●; fermentations A and B, ■; fermentation C), lactic acid content (▲; representative plot of fermentations B and C), and temperature programme applied (continuous line) during milk fermentations mimicking Kasseri cheese production and ripening.

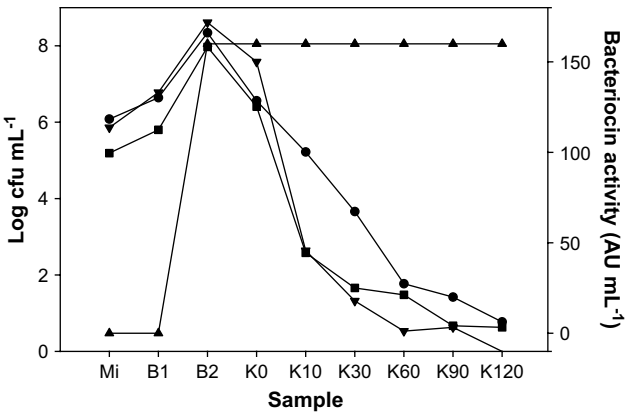

Fig. 3. Growth of *S. macedonicus* ACA-DC 198 (●; fermentation B with *S. macedonicus* ACA-DC 198 and *Clostridium* spores), *S. thermophilus* (▼; fermentation C with commercial starter culture and *Clostridium* spores), *L. delbrueckii* subsp. *bulgaricus* (■; fermentation C), and bacteriocin activity (▲). Mean values from three repetitions. Mi: Milk inoculated; B1: Baski 1, fresh cheese curd; B2: Baski 2, ripened cheese curd; K0: Kasseri at day 0; K10, K30, K60, K90, and K120: Kasseri after 10, 30, 60, 90, and 120 days of ripening, respectively.

since the commercial starter culture did not produce any molecules that inhibit *Clostridium* spores. No significant differences were found between fermentations C and A, while when fermentation C was compared with fermentation B, significant differences in clostridial spore count were detected in the fresh Kasseri sample (K0) and in the 10-day Kasseri (K10) sample, where the spore concentration was reduced to 1.40 and 1.31log units, respectively ($P < 0.05$). These results show that *C. tyrobutyricum* spores were indeed inhibited by macedocin.

The concentration of *C. tyrobutyricum* vegetative cells was determined in all fermentations. For this purpose, non-heated samples were analysed during the MPN determination. However, together with the vegetative cells, spores capable of vegetating without the heat pre-treatment were also determined (Fig. 5). In all fermentations (A, B, and C) the trends for vegetative clostridial cells were the same as the trends described in Fig. 1 for clostridial spores. More specifically, in fermentation A, the concentration of *Clostridium* vegetative cells was constant. In fermentation B, all samples, starting from B2, where macedocin has been produced, until the end of Kasseri cheese ripening showed significant differences with samples Mi and B1 ($P < 0.05$). In fermentation C, a non-significant decrease in *Clostridium* cell concentration was detected in sample K0, compared to the previous time points (Mi, B1 and B2) and thereafter there was only a slight decrease, however not significant. Furthermore, when fermentation B was compared to fermentation A, significant differences ($P < 0.05$) were found in

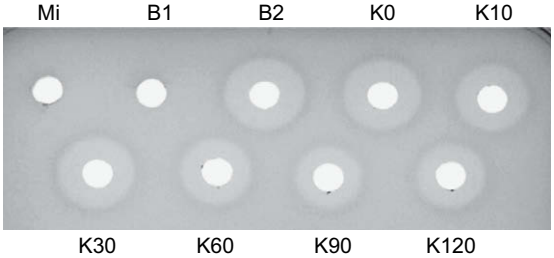

Fig. 4. Macedocin detection in cell-free culture supernatants of samples from fermentation B (with *S. macedonicus* ACA-DC 198 and *Clostridium* spores). Mi: Milk inoculated; B1: Baski 1, fresh cheese curd; B2: Baski 2, ripened cheese curd; K0: Kasseri at day 0; K10, K30, K60, K90, and K120: Kasseri after 10, 30, 60, 90, and 120 days of ripening, respectively.

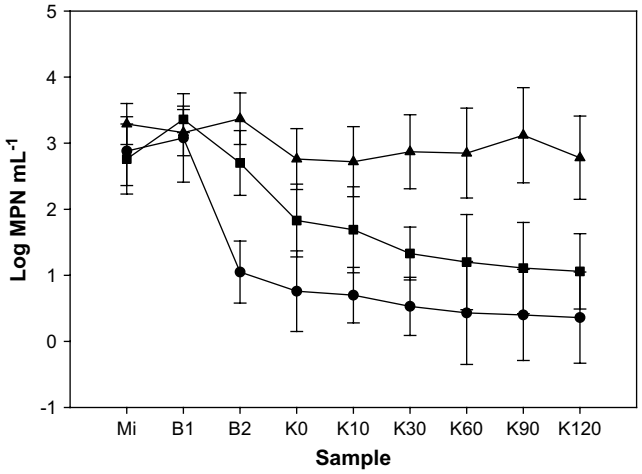

Fig. 5. Inhibition of *Clostridium* vegetative cells and spores during milk fermentations mimicking Kasseri cheese production and ripening with: *Clostridium* spores (fermentation A; ▲), *S. macedonicus* ACA-DC 198 and *Clostridium* spores (fermentation B; ●), commercial starter culture and *Clostridium* spores (fermentation C; ■). Data reported are means from three repetitions ± standard deviations. Mi: Milk inoculated; B1: Baski 1, fresh cheese curd; B2: Baski 2, ripened cheese curd; K0: Kasseri at day 0; K10, K30, K60, K90, and K120: Kasseri after 10, 30, 60, 90, and 120 days of ripening, respectively.

all samples from K0 until K120. Since the rate of pH drop was comparable in the two fermentations (data not shown), the inhibition in fermentation B could be attributed to the effect of macedocin on *Clostridium* cells. No significant differences were detected between fermentations B and C, in contrast to the significant ones obtained for spores (Fig. 1). This could be attributed to the increased sensitivity of vegetative cells to the pH reduction, when compared with spores. It is reasonable to assume that the initial cell inhibition by the low pH could probably make them less susceptible to the macedocin effect.

According to the literature, the inhibition of spore outgrowth and the inhibition of vegetative cells occur by different mechanisms (Liu and Hansen, 1993). Furthermore, it has been reported that sublancin 168, a bacteriocin produced by *Bacillus subtilis* 168 is about 1000-fold more effective in inhibiting spore outgrowth than in inhibiting the same cells in exponential growth (Paik et al., 1998).

Since the growth of *C. tyrobutyricum* can result in the production of butyrate from lactate, it was important to determine lactate production in fermentations B and C. HPLC analysis of fermentation samples was performed to determine the organic acid content and revealed the consumption of lactose and the production of lactic acid due to the growth of *S. macedonicus* or the commercial starter culture. The lactic acid production followed a similar pattern in both fermentations B and C (Fig. 2), while no butyric acid indicative of *C. tyrobutyricum* metabolism was detected. This was also the case for fermentation A, where no vegetation and outgrowth of *Clostridium* spores occurred (data not shown).

In recent challenge studies, cheese was manufactured with milk inoculated with a bacteriocin-producing strain, while the pathogen or the spoilage microorganisms were either added to the milk (Bogovič Matijašić et al., 2007; Nuñez et al., 1997; Rodríguez et al., 2005), or to the fresh cheese (McAuliffe et al., 1999; Rilla et al., 2003). In these cheeses, the production of bacteriocins and their ability to inhibit the target microorganisms have been demonstrated. More specifically, Bogovič Matijašić et al. (2007) prepared a semi-hard cheese from milk artificially contaminated with *C. tyrobutyricum* spores and with the bacteriocin-producing *Lactobacillus gasseri* K7 strain to inhibit *Clostridium*. They observed differences between test and control cheeses concerning the concentration of spores, however these were non-significant. Late blowing occurred in all cheeses, both test and control, but this was less obvious in the cheeses with added *L. gasseri*.

It has been reported that in nature very low concentrations of clostridial spores are able to induce late blowing (1 *C. tyrobutyricum* spore mL^{-1}; Fryer and Halligan, 1976; 5–10 *C. tyrobutyricum* spores L^{-1}; Crawford, 1987) and also that spores cultivated from pure cultures were less successful in provoking late blowing than spores present in milk from natural contamination (Klijn et al., 1995). To increase the probability of inducing late blowing, a high concentration of spores was used in the present study for the fermentation experiments (10^3 spores mL^{-1} of milk). It is likely that the effect of macedocin-producing *S. macedonicus* ACA-DC 198 on lower concentrations of *Clostridium* spores would be more inhibitory than the effect found in the present work.

It should be stressed that the results of the present study are based on a simulation of the Kasseri cheese preparation and ripening. Under real cheese conditions there are several other factors that could negatively affect the activity of the macedocin, such as the use of whole milk containing fat instead of skim milk, the addition of rennet, or even the interaction with the natural Kasseri cheese microflora. However, since the results of this study are promising, it would be interesting to prepare pilot scale Kasseri cheese to evaluate the effectiveness of macedocin-producing *S. macedonicus* ACA-DC 198 in inhibiting *C. tyrobutyricum* spores under conditions of real cheese production and ripening.

4. Conclusions

The macedocin producer strain *S. macedonicus* ACA-DC 198 has been successfully used to inhibit vegetation and outgrowth of *C. tyrobutyricum* spores in milk fermentations, under conditions mimicking Kasseri cheese production and ripening. The reduction achieved was up to 1.40log units, in the 10-day Kasseri ferment sample, when compared with the fermentation in which a commercial starter culture composed of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was used. The inhibition of clostridial spores observed in a fermentation with *S. macedonicus* ACA-DC 198 as starter culture in the presence of clostridial spores could not be attributed solely to low pH and organic acids, since the pH values and concentrations of lactic acid did not differ significantly between fermentations with *S. macedonicus* ACA-DC 198 or the commercial starter culture in the presence of clostridial spores. It can be concluded that *S. macedonicus* ACA-DC 198 could be used as an adjunct culture for controlling *C. tyrobutyricum* during Kasseri cheese production, providing an additional tool for microbiological control, beyond good manufacturing practices in cheese making.

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