Isolation of Anticlostridially Active Lactobacilli from Semi-hard Cheese

Štěpán TŮMA, Kateřina KUČEROVÁ and Milada PLOCKOVÁ

Department of Dairy and Fat Technology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology in Prague, Prague, Czech Republic

Abstract

Tůma Š., Kučerová K., Plocková M. (2008): **Isolation of anticlostridially active lactobacilli from semi-hard cheese**. Czech J. Food Sci., **26**: 324–332.

The group of 7 strains of facultatively heterofermentative lactobacilli (FHL) of the non starter lactic acid bacteria (NSLAB) isolated from semi-hard cheeses using a well agar diffusion assay possessed a different level of inhibition activity against 16 different strains of *Clostridium* sp. belonging to the species *Cl. butyricum*, *Cl. tyrobutyricum*, *Cl. beijerinckii*, and *Cl. sporogenes*. Two selected strains with the production of partially indentified anticlostridially active inhibitory substances, *Lb. paracasei* ST68 (producing hydrogen peroxide) and *Lb. paracasei* 171R2 (producing bacteriocin and hydrogen peroxide), were tested for the inhibition activity against gas producing *Cl. butyricum* 10702 and *Cl. tyrobutyricum* 184 in cheese slurry. The effects of both strains were different in reducing the numbers of gas producing *Cl. butyricum* 10702 by > 3 log cycles and *Cl. tyrobutyricum* 184 by > 1 log cycles in cheese slurry during the tested storage periods of 24 days at 13°C. The *Cl. tyrobutyricum* strains isolated from spoiled cheese were identified by species-specific PCR for *Cl. tyrobutyricum*.

Keywords*:* anticlostridial activity; Edam cheese; non-starter lactobacilli; PCR

Semi-hard cheese is one of the most commonly consumed cheeses in the Czech Republic. The microbiological quality of cheese after its production is one of the main factors for good ripening and final quality of cheese. The occurrence and the role of microorganisms in cheese belong to the basic knowledge about the cheese.

There are several desirable (lactococci, lactobacilli) and undesirable (coliform, *E. coli*, clostridia, moulds, and yeasts) microorganisms occurring in and eventually spoiling the cheese. One of the most undesirable bacteria for cheesemaking is *Clostridium* sp. occurring in raw milk and originating from silage (Christiansen *et al*. 2005). The presence of vegetative cells and especially the clostridial spores cause late blowing effect, due to the butyric acid fermentation in cheese (Klijn *et al*. 1995). The taste and flavour (rancid, distasteful consistence) of cheese are disgusting and not suitable for consumers (Herman *et al*. 1995).

The most frequently isolated species from cheese with the late blowing effect were found to be *Cl. tyrobutyricum*, *Cl. butyricum*, and *Cl. sporogenes* (Herman *et al*. 1995; Cocolin *et al*. 2004). These species were isolated also from silage and raw milk (Cocolin *et al*. 2004). All these clostridia species form butyric acid and gas (carbon dioxide and hydrogen) in various media, but their association

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046137305.

with the late blown defect and the presence of a specific *Clostridium* sp. is very difficult to prove (Klijn *et al*. 1995).

In order to suppress these undesirable microorganisms, the classical techniques (bactofugation, microfiltration of milk, addition of nitrate or lysozyme) (Bogovič Matijašič *et al*. 2007) or protective lactic acid bacteria, especially lactobacilli (Christiansen *et al*. 2005) possessing antagonistic activity against bacteria, can be used.

Lactobacilli constitute the majority of the non starter lactic acid bacteria (NSLAB) population in most cheese varieties during ripening. The facultatively heterofermentative lactobacilli prevail among NSLAB in semi-hard cheese. The dominance of *Lb. paracasei* (Antonsson *et al*. 2002), *Lb. casei*, *Lb. paracasei*, and *Lb. rhamnosus* in semi-hard cheese was reported (Østlie *et al*. 2004).

In several articles lactobacilli strains of different origin, such as *Lb. gasseri* (Bogovič Matijašič *et al*. 2007), *Lb. paracasei* (Rodríguez *et al*. 2000; Christiansen *et al*. 2005; Tůma *et al*. 2005) and *Lb. plantarum* (Rodríguez *et al*. 2000), *Lb. acidophilus* and *Lb. casei* (Jamuna *et al*. 2005) were reported to express anticlostridial activity. However, the reports on the occurrence of anticlostridial NSLAB in semi-hard cheese varieties are rather limited and need to be elucidated (González *et al*. 2007).

The aim of this study was to estimate the numbers of NSLAB during the ripening of semi-hard cheese, to isolate new lactobacilli strains, to characterise them, and to determine their anticlostridial activity in cheese-like conditions.

MATERIALS AND METHODS

Cheese sample. The tested cheeses used for the isolation of lactobacilli strains were manufactured from pasteurised milk (74–76°C, 15–20 s) in three independent processing trials in one Czech cheese factory. A commercial mesophilic DL-starter culture (Chr. Hansen, Denmark) was used according to the standard procedure. The cheese was vacuum packaged in 1.5 kg blocks in plastic bags (WR Grace Australia Ltd, Australia) and stored for 20 weeks at 10°C to 12°C in the storage room.

Microbiological sampling. Cheese samples were aseptically collected and prepared for enumeration according to the standard procedure (EN ISO 8261:2001) after 1, 2, 3, 4, 6, 8, 12, 16, and 20 weeks of ripening. The number of NSLAB was evaluated on Rogosa-SL agar (Hi-Media, India) under anaerobic condition for 5 days at 30°C. The number of *Clostridium* sp. was evaluated on RCM agar (Merck, Germany) under anaerobic incubation for 3 days at 37°C or by MPN method in RCM_L medium with lactate (Ingham *et al*. 1998).

Isolation of lactic acid bacteria from cheese. Single colonies were randomly picked from Rogosa-SL agar. All colonies were initially analysed for colony and cell morphology. The strains were maintained as stock cultures frozen at –80°C in MRS broth containing 150 ml/l glycerol. Working cultures were sub-cultured three times before use in MRS broth at 37°C, 24 h, anaerobically.

Phenotypic and genotypic identification of lactobacilli. All isolated strains used in this work were tested for catalase. Carbohydrate fermentation patterns were determined by using API 50 CHL (Bio Merieux, France). The growths of the selected isolates were examined at 15, 30, 37°C in MRS broth during 48 h (1% inoculum). The effect of salt concentration was examined in MRS containing 2.5, 4.0, 6.5% NaCl at 37°C for 48 hours. Gas production was checked in MRS broth incubated at 37°C for 48 hours. The growth in skim milk during 72 h at 37°C was studied. The selected lactobacilli strains with anticlostridial activity were identified by Rep-PCR (Christiansen *et al*. 2005; Tůma *et al*. 2007) with primers REP1R-Dt (5'-III NCG NCG NCA TCN GGC-3') and REP2R-Dt (5'-NCG NCT TAT CNG GGC CTAC-3') (TAG Copenhagen, Denmark). The profiles obtained were compared with the profiles of type and reference strains of *Lb. paracasei*, *Lb. rhamnosus*, *Lb. zeae*, *Lb. plantarum*, and *Lb. curvatus.*

Clostridium test strains. The strains used in this study were cultivated on RCM agar (Merck, Germany) at 37°C, pH 6.8, anaerobically (AnaeroGen™, Oxoid, Great Britain) for 72 hours. The strains were stored at 4°C until further use and were sub cultured bimonthly (Jo *et al*. 2008).

PCR identification of Cl. tyrobutyricum. The standard strains and strains isolated from cheese were identified by PCR method (Herman *et al*. 1995). DNA was extracted by GenElute™ Bacterial Genomic Kit (Sigma Aldrich, St. Louis, USA) and used for species specific PCR identification of *Cl. tyrobutyricum* (PCR product 233 bp). PCR was performed in a final volume of 25 µl containing 2.5 µl of dNTP mix (2mM), 2.5 µl of 10X PCR buffer, 1 µl of primer CT1F (25µM, 5'-AACTGAAACAGCATGACT-3'), 1 µl of primer CT1R (25µM, 5'-GCTTGACCTT-

TATCTACA-3'), 15 µl of sterile demineralised water, 0.5 µl of Taq polymerace, and 2.5 µl of DNA. PCR was performed in thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, USA) as follows: the initial denaturation 95°C/1 , amplification – 30 cycles (95°C/15 s, 60°C/15 s, 72°C/30 s), final extension 72°C/8 minute. 10 µl of the PCR product was analysed by gel electrophoresis in 1.5% agarose (Sigma Aldrich, St. Louis, USA) with consecutive staining using ethidium bromide and visualisation on UV transilluminator (Vilber Lourmat, Marne-la-Vallée, France). DNA 123 bp marker (Invitrogen) was used as a molecular weight marker.

Anticlostridial activity by overlay method. All lactobacilli isolates were initially screened for anticlostridial activity against standard *Clostridium* sp. strains, using an overlay assay (Magnusson *et al*. 2003). An overnight culture of *Lactobacillus* isolates (3 µl) was inoculated by spotting onto MRS agar plates (20 ml). The plates were incubated at 37°C for 48 h, anaerobically, to allow all colonies to develop. The plates were then overlaid with RCM soft agar (5 ml; 0.75% agar) inoculated with 10^8 cells of the indicator strain *Clostridium* sp. The plates were then incubated (37°C, for 48 h, anaerobically), and subsequently checked for the zones of inhibition around colonies (Zhu *et al*. 2000).

Anticlostridial activity by agar well diffusion assay. The anticlostridial activity of selected lactobacilli was tested by agar well diffusion assay (Rodríguez *et al*. 2000). The cell-free supernatants were obtained from overnight *Lactobacillus* cultures by centrifugation at 4°C, 4000 g, 15 min in order to remove bacterial cells and were stored on ice. The supernatants were adjusted to pH 6 with 1 mol/l NaOH and stored at –20°C until use.

Indicators *Clostridium* sp. isolates were streaked on RCM agar (Difco, Detroit, USA) and incubated anaerobically at 37°C for 72 hours. Individual colonies were inoculated in RCM broth (Difco, Detroit, USA) and incubated anaerobically at 37°C for 48 hours. The suspension of 1% of active culture of *Clostridium* sp. ($OD_{600\ nm}$ 0.7–0.8) and 30 ml RCM agar (55°C) in a Petri dish (ø 9 cm) was prepared and the plates were dried for 2 h at room temperature. Wells of 7 mm were punched with a sterile plug centre cork. The culture of *Lactobacillus* ($OD_{600\ nm}$ 1.6–1.8) or supernatants were added (50 µl) to each well and were maintained at 8°C for 1 h, for facilitating test material diffusion into agar. Plates were incubated anaerobically at 37°C for 3 days and read after 24, 48, 72 h, and the zones of inhibition were measured (Christiansen *et al.* 2005).

Cheese model system. The cheese model system was prepared from Edam cheese. The suspension of Edam cheese (50 g) was mixed with 50 g of citrate buffer (20 g/l) (Lachema, Czech Republic), homogenised (Seward, Thetford, GB), and sterilised at 100°C for 20 minutes. Cheese slurry was inoculated (10 ml/l) with both lactobacilli and clostridia strains and cultivated anaerobically (AnaeroGenTM, Oxoid, GB) at 13°C for 24 days.

MPN method. Serial dilutions of samples were prepared in saline solution. Regenerated medium RCM_L (Merck, Germany) was used for samples preparation. The tubes inoculated with gas producing *Clostridium* strains were heated at 85 ± 1°C, for 10 min, covered by hard-wax cork. The samples were incubated anaerobically (AnaeroGen™, Oxoid, GB) at 37°C for 5 days. The tubes were read after 72 hours. The production of gas was observed (Ingham *et al*. 1998).

Antimicrobial substances assay. The lactobacilli strains were tested for the bacteriocin and hydrogen peroxide production. The strains were cultivated in MRS (Merck, Germany) at 30°C for 16 h, aerobically. The cell-free supernatant was prepared by centrifugation at 4000 g, for 15 min, at 4°C (32R, Hettich, Germany), neutralised to pH 6, and used to determine enzyme and heat sensitivities. The sensitivity to proteinase K EC 3.4.21.64 (Sigma, USA), flavourzyme (Novozymes A/S, Denmark), protamex (Novo Nordisk A/S, Denmark), and α-chymotrypsin EC 3.4.21.1 (Sigma, USA) in buffers recommended by suppliers at the final concentration of 1 mg/ml was tested. To exclude potential inhibition by hydrogen peroxide, the supernatants were treated with catalase EC 1.11.1.6 (Sigma, USA). The enzyme solutions were placed into the supernatant and incubated at 37°C for 30 min (Thuault 1991; Tůma *et al*. 2005). The residual activity was determined by the well agar diffusion assay using clostridial indicator strain.

Heat sensitivity was estimated by heating the supernatants at 25, 37, 55, and 100°C for 30 minutes. The remaining activity was determined by the well agar diffusion assay (Christiansen *et al*. 2005; Tůma *et al*. 2005). Cell-free supernatants were also adjusted to pH 2, 4, 6, 7, and 8 and evaluated for the residual activity (Rodríguez *et al*. 2000).

The cell free supernatant for H_2O_2 production assay was prepared by centrifugation at 6000 g,

at 5°C for 15 minutes. The pellets were washed in a phosphate buffer. The suspension of cells was stored in phosphate buffer at 5°C for 48 h and centrifuged at 6000 g, at 5°C for 15 minutes. The mixture of 5 ml supernatant, 1 ml 0.001% HRP, and 0.1 ml 1% *o*-dianisidin was prepared and cultivated for 10 min at 37°C. The reaction was terminated by the addition of 0.2 ml 4 mol/l HCl. The absorbance was measured at 400 nm (Martín *et al*. 2005). The calibration curve was used to measure optical density at 400 nm (regression equation $A = 0.2826 \times c + 0.024$; $R^2 = 0.9938$; A – absorbance, c – H_2O_2 concentration, µg/l).

RESULTS AND DISCUSSION

NSLAB in cheese

The present study showed that high counts of non-starter lactobacilli were found during the whole ripening time in the tested cheese. In Edam cheese, the total number of non-starter lactobacilli increased from 5.8 log CFU/g after brining to 7.0 log CFU/g after 6 weeks of ripening and remained relatively stable during the next 14 weeks of storage. Similar results, concerning the non-starter lactobacilli, were also found during ripening with the semi-hard cheese Norwegia (Østlie *et al*. 2004) and Danbo cheese (Antonsson *et al*. 2002).

The dominating group of NSLAB isolated from cheese was phenotypically and genotypically characterised and identified as facultatively heterofermentative lactobacilli. NSLAB were subsequently identified by Rep-PCR as *Lb. paracasei* (Christiansen *et al*. 2005; Tůma *et al*. 2007). There were some differences in the occurrence of isolates during the ripening period, but it was difficult to discover any clear tendencies in the prevalence of certain phenotypes (Tůma *et al*. 2007).

Clostridium sp. in cheese

The total counts of *Clostridium* sp. from spoiled cheese were enumerated (Figure 1) by three different techniques. The numbers of clostridia (vegetative cells), found in cheese and evaluated on RCM agar, were from 6.2 to 7.4 log CFU/g. Clostridia (clostridial spores) were enumerated on RCM agar after the heat treatment (85°C, 10 min). The numbers were lower, compared to vegetative cells, between 4.2 to 4.5 log CFU/g. The spores of gas

Figure 1. The Edam cheese spoiled by gas producing *Clostridium* sp. during ripening time

producing clostridia were tested by MPN method. The counts of clostridia spores found in cheese were from 3.3 to 4.1 log CFU/g.

It was observed that the number of clostridial spores, which cause the defect, varies according to the type of milk, cheese, and treatment (bactofugation, nitrate addition) from 5 spores to 6 log CFU/g (Herman *et al*. 1995; Klijn *et al*. 1995; Christiansen *et al*. 2005). The control of spores in cheese milk before the cheese production is time consuming, difficult, and not suitable for cheesemakers. The cheese is produced from one- or two-day-old milk. Thus, there is no time to detect clostridia before the cheese production. The methods for detecting spores of *Cl. tyrobutyricum* based on spore germination and vegetative growth are mostly suitable for the feedback control and confirmation of *Clostridium* sp. in cheese or milk.

Direct detections of vegetative cells and spores based on PCR are available and were described and successfully used (Herman *et al*. 1995). In this work, naturally late blown Edam cheese was analysed and the clostridial strains were isolated and identified by PCR. The late blown cheese used in this study was ripened for 21 days and contained from 3.3 to 4.1 log CFU/g clostridial spores.

Identification of *Clostridium* spp. by PCR

The identification of *Clostridium* spp. at species level on specific media and phenotypic characterisation are not suitable enough. The application of molecular methods such as specific PCR (Herman *et al*. 1995; Cocolin *et al.* 2004) or ARDRA (Jo *et al.* 2008) were well described and successfully used for the identification of dairy-related *Clostri-*

dium spp., especially *Cl. tyrobutyricum*. A variety of new methods have been developed to identify directly the microorganisms in food samples. The advantage of these methods is the exclusion of the time consuming strain isolation. However, many methods show differences between the identification of cultivated and naturally occurring species related to the different food origin. The protocol has to be optimised for the food specific species (Cocolin *et al.* 2004).

PCR (Herman *et al*. 1995) for the identification of *Cl. tyrobutyricum* was used also in this work for the identification of clostridia isolated from Czech semi-hard cheese. Two primers Ct1F and Ct1R were chosen. This primer pair amplifies species specific 233 bp fragment. Examples of PCR-products are shown in Figure 2. The identification at species level was necessary for further testing and possible application of PCR method to detect directly *Cl. tyrobutyricum* in cheese. In all cheese isolates, the typical PCR product (233 bp) was detected, all tested strains were identified as

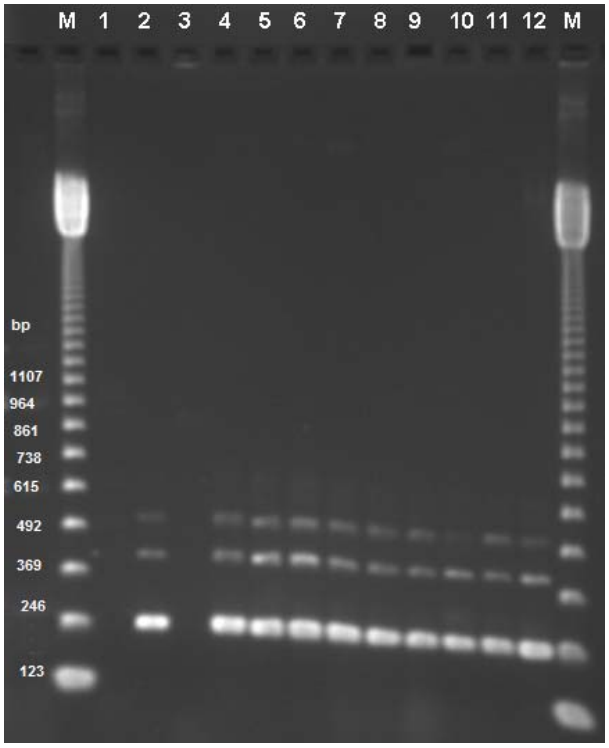

Figure 2. Illustration of PCR identification of *Clostridium* spp. by PCR: Starting from the left side: lane M lane the 123 bp DNA marker; lane 1 *Clostridium butyricum* 10702; lane 2 *Clostridium tyrobutyricum* DMF 6500; lane 3 *Clostridium beijerinckii* 791; lane 4-12 *Clostridium* strains isolated from spoiled Edam cheese

Cl. tyrobutyricum as well as *Cl. tyrobutyricum* DMF6500. The strain *Cl. tyrobutyricum* DMF6005 was used as a positive control and strains *Cl. butyricum* 10702 and *Cl. beijerinckii* 791 as negative control reference strains.

The sensitivity of PCR method used in this study was acceptable for the clear and useful identification of clostridia from the spoiled cheese sample. The sensitivity for clostridia detection in the sample should correlate around 30 *Cl. tyrobutyricum* spores in 100 ml of the sample (Herman *et al*. 1995).

Anticlostridial activity of lactobacilli isolates

All lactobacilli (6) isolates and one reference strain *Lb. paracasei* 171R2 isolated from Danish semi-hard cheese, were primarily screened for their ability to inhibit *Clostridium* spp. strains by overlay method. All strains, showing the inhibition effect, were tested again by well agar diffusion assay (Table 1). All strains inhibited *Cl. sporogenes* 795, *Cl. tyrobutyricum* 189, *Cl. tyrobutyricum* 208, *Cl. tyrobutyricum* 362, *Cl. butyricum* 10702, and *Cl. beijerinckii* M3. However, the lactobacilli showed different inhibition spectrum against *Clostridium* strains, especially the inhibition activity of *Lb. paracasei* strains to various *Cl. tyrobutyricum* strains was observed. The most active isolates *Lb. paracasei* ST68 and *Lb. paracasei* 171R2 were chosen for further testing.

Cheese model system

All clostridia are able to form butyric acid, H_2, and CO_2, however, differences in various media were observed (Cocolin *et al.* 2004). Before the testing of the growth of clostridia in cheese model system, it is useful to test their growth ability and gas production. Testing in cheese slurry in conditions simulating the ripening process of Edam cheese (24 days, at 13°C) is a time consuming procedure. Therefore, the anticlostridial activity of both most active lactobacilli strains was tested preliminary in RCM and milk in conditions (37°C 48 h) optimal for the growth of both lactobacilli and clostridia. The heat treatment of the sample to destroy the vegetative cells, followed by MPN method (most probable number) based on gas production in tubes under anaerobic condition (Cocolin *et al.* 2004) is one of the most suitable methods also for cheesemakers, to confirm

Table 1. The inhibition effect *of Lb. paracasei* against *Clostridium* spp.

Clostridium spp.	*Lb. paracasei* (isolated from cheese)						
	ST68	ST307	ST485	ST486	ST490	ST491	171R2
Clostridium tyrobutyricum 2637	+	–	–	–	–	–	+
Clostridium butyricum 10702	+	+	+	+	+	+	+
Clostridium beijerinckii 791	–	–	–	–	–	–	–
Clostridium sporogenes 795	+	+	+	+	+	+	+
Clostridium tyrobutyricum 39	–	–	–	–	–	–	+
Clostridium beijerinckii M3	+	+	+	+	+	+	+
Clostridium tyrobutyricum K8	–	–	–	–	–	–	+
Clostridium butyricum 112	+	–	–	–	–	–	+
Clostridium tyrobutyricum 184	+	-	+	+	+	+	+
Clostridium tyrobutyricum 189	+	+	+	+	+	+	+
Clostridium tyrobutyricum 194	–	–	–	–	–	–	–
Clostridium tyrobutyricum 208	+	+	+	+	+	+	+
Clostridium tyrobutyricum 215	–	–	–	–	–	–	+
Clostridium tyrobutyricum 218	–	–	–	–	–	–	–
Clostridium tyrobutyricum 220	–	–	–	–	–	–	–
Clostridium tyrobutyricum 362	+	+	+	+	+	+	+

– no inhibition, + inhibition < 10 mm, ++ inhibition > 10 mm

the presence of *Clostridium* sp. in the samples and to enumerate sufficiently all gas producing clostridia species.

Both strains showed anticlostridial activity in RCM during their growth, *Lb. paracasei* 171R2 possessed inhibition activity in milk as well; *Lb. paracasei* ST68 produced antimicrobial substance only at the end of the stationary growth phase in milk.

The testing of anticlostridial activity in cheese slurry was done together with testing in RCM and milk by MPN method. The reference strains *Cl. butyricum* 10702 and *Cl. tyrobutyricum* 184 were used to check the anticlostridial activity in cheese model system, due to the repeatable gas production and sufficient sensitivity. Both lactobacilli strains showed inhibition effects against *Cl. butyricum* 10702 and *Cl. tyrobutyricum* 184. In RCM, *Lb. paracasei* strains inhibited clostridia more than in milk or cheese slurry. The strongest inhibition (> 5 log) was achieved with *Lb. paracasei* ST68 in RCM against *Cl. butyricum* 10702. The inhibition effect of lactobacilli in milk was not significant (≤ 1 log). In cheese slurry, lactobacilli partially inhibited clostridia (Table 2). *Lb. paracasei* ST68 and *Lb. paracasei* 171R2 showed stronger inhibition effects against *Cl. butyricum* 10702 (> 2 log) compared to low inhibition against *Cl. tyrobutyricum* 184 (> 1 log). The lower inhibition effect of lactobacilli in cheese slurry could be due to the lactate production, which supports the growth of clostridia (Herman *et al.* 1995).

The presence of lactobacilli during co-cultivation correlated with pH during cultivation. The pH decreased during co-cultivation more than during the single strain cultivation. In RCM, the pH decreased rapidly due to the production of lactate from glucose, which supports the growth of clostridium and gas production. The milk does not include glucose, only lactose, so clostridia did not reach high densities compared to RCM.

During the cultivation of single *Clostridium* strain, the pH did not decrease, compared to co-cultivation in milk, where pH decreased from 6.5 to 4.5 (data not shown), because lactobacilli were able to ferment lactose and to grow in milk in high cell densities.

Table 2. The inhibition effect *of Lb. paracasei* ST68 and *Lb. paracasei* 171R2 against gas producing *Clostridium butyricum* 10702 and *Clostridium tyrobutyricum* 184 in cheese slurry tested by MPN method (mean value of three independent measurements)

Medium	Incubation		*Cl. butyricum* 10702	*Cl. butyricum* 10702	
				Lb. paracasei ST68	*Lb. paracasei* 171R2
Cheese slurry	24 days/13°C	MPN (ml^{-1})	5.0×10^4	5.0×10^2	5.0×10^2
		pH	5.4	5.5	5.5
		SH	57	61	50
			Cl. tyrobutyricum 184	*Cl. tyrobutyricum* 184	
				Lb. paracasei ST68	*Lb. paracasei* 171R2
		MPN (ml^{-1})	2.0×10^3	3.0×10^2	3.0×10^2
		pH	5.7	5.6	5.65
		SH	48	45	43

Antimicrobial activity

The protective effect of lactobacilli can be caused by depletion of nutrients, low pH, hydrogen peroxide or bacteriocin production (Rodgers *et al.* 2004). The anticlostridial activity and production of antimicrobial substances of selected lactobacilli (7) isolates were tested by well agar diffusion assay. The production of bacteriocin-like substances was confirmed in the neutralised and catalase or proteinase K treated supernatant. Only one isolate, *Lb. paracasei* 171R2, produced bacteriocin-like substance. Two isolates, *Lb. paracasei* ST68 and *Lb. paracasei* 171R2, were selected for further testing because they possessed the strongest and different inhibitory spectrum.

The inhibitory effect of *Lb. paracasei* ST68 was caused by non-proteinaceus compound and hydrogen peroxide. The inhibitory effect of *Lb. paracasei* 171R2 was due to the bacteriocin production and hydrogen peroxide. The activity and production of bacteriocin and non-proteinaceus compound was evaluated, due to the fact of various activities, production, and inhibition activities in different media. Both lactobacilli strains produced antimicrobial substances in MRS and milk. *Lb. paracasei* ST68 produced the inhibitory substance in stationary growth phase, and *Lb. paracasei* 171R2 was able to produce bacteriocin during the whole growth phase in MRS and milk. The bacteriocins can be released and their activity can decrease in time. The fast decrease was already observed not only in MRS, but also in milk (Bogovič Matijašič *et al.* 2007).

Non identified bacteriocin and non-proteinaceus compound were tested for sensitivity to four proteinases, four heat treatments, and five pH values. The bacteriocin of *Lb. paracasei* 171R2 was sensitive to all proteinase enzymes (proteinase K and α-chymotrypsin etc.) as well as bavaricin (Larsen *et al.* 1993), plantaricin 423 (van Reenen *et al.* 1998) and acidocin (Dave & Shah

*T*able 3. Characterisation of protective lactobacilli isolated from cheese

Cheese isolate	Cheese origin	Number of inhibited *Clostridium* strains[a]				Bacteriocin production	H_2O_2 production (μg/l)	Inhibition activity	Identification	
		Cl. tyrobutyricum	*Cl. butyricum*	*Cl. sporogenes*	*Cl. beijerinckii*			cheese slurry	Api-test	Rep-PCR
Lb. paracasei ST68	Edam	6	1	1	0	–	0.42	+	*Lb. pentosus*	*Lb. paracasei*
Lb. paracasei 171R2	Danbo	9	1	1	0	+	0.58	+	*Lb. paracasei*	*Lb. paracasei*

[a]Out of a total of 13 tested; + positive; – negative

1997). The antimicrobial substance produced by *Lb. paracasei* ST68 was not sensitive to any of the enzymes tested.

The hydrogen peroxide production was proved with both strains. The strains *Lb. paracasei* ST68 and *Lb. paracasei* 171R2 did not produce higher amounts of hydrogen peroxide than the other tested lactobacilli isolated from cheese (Plocková *et al.* 1996). *Lb. paracasei* ST68 after 24 h cultivation in MRS, total counts of lactobacilli 3.3×10^9 CFU/g, produced 0.42 μg H_2O_2 in phosphate buffer. *Lb. paracasei* 171R2 produced a higher amount of H_2O_2, 0.58 μg in phosphate buffer, total counts of lactobacilli were 9.9×10^8 CFU/g, cultivation in MRS for 24 h.

Lb. paracasei ST68 and *Lb. paracasei* 171R2 showed specific anticlostridial activity. The anticlostridial activity of lactobacilli strains against *Cl. tyrobutyricum* was strain dependent. The inhibitory spectrum of bacteriocin produced by *Lb. paracasei* 171R2 was as narrow as those of other bacteriocins produced by lactobacilli (Bogovič Matijašič *et al.* 2007).

Evidently, it would be useful to prepare a mixture of *Lb. paracasei* strains with different levels of anticlostridial specifity in order to achieve the broadest anticlostridial activity spectrum. The protective effect need not be caused only by one strain or bacteriocin. The combination of two or more strains with protective effects due to different antimicrobial substances can be used to accelerate the resulting inhibition effect as it was previously suggested (Rodgers *et al.* 2004).

CONCLUSIONS

It was found that non-starter lactobacilli constitute the majority of the NSLAB population occurring naturally in semi-hard cheese type, and that they posses many interesting properties useful to improve the cheese quality in the future. Some of these strains were able to inhibit food spoilage bacteria, due to the production of antimicrobial compounds (bacteriocin, hydrogen peroxide, non-proteinaceus compound). The application of protective lactobacilli as adjunct cultures in semi-hard cheese production is often limited by a narrow activity spectrum and the inactivation due to the interaction with the cheese ingredients. The testing in cheese-like conditions is necessary for a successful application of protective cultures in real cheese.

The lactobacilli isolated from cheese possessed different inhibition effects against *Cl. tyrobutyricum*, *Cl. butyricum*, *Cl. beijerinckii* and *Cl. sporogenes* using a well agar diffusion assay.

At least 7 of the cheese isolates (30) showed a certain level of inhibitory activity. The inhibition effects of selected strains *Lb. paracasei* ST68 (hydrogen peroxide producing) and *Lb. paracasei* 171R2 (bacteriocin and hydrogen peroxide producing) were tested in different cheese-like conditions, and different results were observed in the production and activity of inhibitory substances against gas producing *Clostridium butyricum* 10702 and *Cl. tyrobutyricum* 184 strains isolated from silage. The effects of both strains were similar in reducing the numbers of gas-producing *Cl. butyricum* by > 2 log cycles, and *Cl. tyrobutyricum* 184 > 1 log cycles in cheese slurry, compared to control during the test period.

The anticlostridial active lactobacilli strains were characterised phenotypically and by REP-PCR as *Lb. paracasei*. All *Cl. tyrobutyricum* strains tested were identified by species-specific PCR for their further confirmation in cheese.

Anticlostridial activity seems to be a promising advantage of *Lb. paracasei* strains for their application in cheesemaking. This screening has shown that lactobacilli isolated from Czech Edam cheese inhibited different clostridia strains. The lactobacilli strains should be considered as a part of an adjunct protective culture in semi-hard cheese production.

References

Antonsson M., Ardö Y., Nilsson B.F., Molin G. (2002): Screening and selection of *Lactobacillus* strains for use as adjunct cultures in production of semi-hard cheese. Journal of Dairy Research, **69**: 457–472.

Bogovič Matijašič B., Rajšp M.K., Perko B., Rogelj I. (2007): Inhibition of *Clostridium tyrobutyricum* in cheese by *Lactobacillus gasseri*. International Dairy Journal, **17**: 157–166.

Cocolin L., Innocente N., Biasutti M., Comi G. (2004): The late blowing in cheese: a new molecular approach based on PCR and DGGE to study the microbioal ecology of the alteration process. International Journal of Food Microbiology, **90**: 83–91.

Christiansen P., Petersen M.H., Kask S., Møller P.L., Petersen P., Nielsen E.W., Vogensen F.K., Ardö Y. (2005): Anticlostridial activity of *Lactobacillus* isolated

from semi-hard cheese. International Dairy Journal, **15**: 901–909.

Dave R.I., Shah N.P. (1997): Characteristics of bacteriocin produced by *Lactobacillus acidophilus* LA-1. International Dairy Journal, **7**: 707–715.

EN ISO 8261:2001 (2001): Milk and milk products – General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination. Czech Standard Institute, Prague.

Herman L.M.F., Block J.H.G.E, Waes G.M.A.V.J. (1995): A direct PCR method for *Clostridium tyrobutyricum* spores in up to 100 millilitres of raw milk. Applied and Environmental Microbiology, **61**: 4141–4146.

González L., Sandoval H., Sacristán N., Castro J.M., Fresno J.M., Tornadijo M.E. (2007): Identification of lactic acid bacteria isolated from Genestoso cheese throughout ripening and study of their antimicrobial activity. Food Control, **18**: 716–722.

Ingham S.C., Hassler J.R., Tsai Y., Ingham B.H. (1998): Differentiation of lactate-fermenting, gas-producing *Clostridium* spp. isolated from milk. International Journal of Food Microbiology, **43**: 173–183.

Jamuna M., Babusha S.T., Jeevaratnam K. (2005): Inhibitory efficacy of nisin and bacteriocins from *Lactobacillus* isolates against food spoilage and pathogenic organisms in model and food systems. Food Microbiology, **22**: 449–454.

Jo J.H., Lee D.S., Park D., Park J.M. (2008): Biological hydrogen production by immobilized cells of *Clostridium tyrobutyricum* JM1 isolated from a food waste treatment process. Bioresource Technology, **99**: 6666–6672.

Klijn N., Nieuwenhof F.F.J., Hoolwerf J.D., Waals C.B., Weerkamp A.H. (1995): Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species specific PCR amplification. Applied and Environmental Microbiology, **61**: 2919–2924.

Larsen A.G., Vogensen F.K., Josephsen J. (1993): Antimicrobial activity of lactic acid bacteria isolated from sourdoughs: purification and characterization of bavaricin A, a bacteriocin produced by *Lactobacillus bavaricus* MI401. Journal of Applied Microbiology, **75**: 113–122.

Magnusson J., Ström K., Ross S., Sjörgen J., Schnürer J. (2003): Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters, **219**: 129–135.

Martín R., Olivares M., Marín M.L., Xaus J., Fernández L., Rodríguez J.M. (2005): Characterization of a reuterin-producing *Lactobacillus coryneformis* strain isolater from a goat's milk cheese. International Journal of Food Microbiology, **104**: 267–277.

Østlie H.M., Eliassen L., Florvaag A., Skeie S. (2004): Phenotypic and PCR-based characterization of the microflora in Norvegia cheese during ripening. International Dairy Journal, **94**: 287–299.

Plocková M., Chumchalová J., Pluhařová B. (1996): The pH tolerance, bile resistence and production of antimicrobial compounds by Lactobacilli. Potravinářské Vědy, **14**: 165–174.

Rodgers S., Kailasapathy K., Cox J., Peiris P. (2004): Co-incubation of *Clostridium botulinum* with protective cultures. Food Research International, **37**: 659–666.

Rodríguez E., González B., Gaya P., Nunez M., Medina M. (2000): Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. International Dairy Journal, **10**: 7–15.

Thuault D., Beliard E., Le Guern J., Bourgeois C.M. (1991): Inhibition of *Clostridium tyrobutyricum* by bacteriocin-like substance produced by lactic acid bacteria. Journal of Dairy Science, **74**: 1145–1150.

Tůma Š., Vogensen F.K., Ardö Y. (2005): Anticlostridial activity of *Lactobacillus* strains isolated from semi-hard cheese. Czech Dairy Journal, **93**: 18–21.

Tůma Š., Vogensen F.K., Plocková M., Chumchalová J. (2007): Isolation of antifungally active lactobacilli from Edam cheese. Acta Alimentaria, **36**: 405–414.

Van Reenen C.A., Dicks L.M.T., Chikindas M.L. (1998): Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. Journal of Applied Microbiology, **84**: 1131–1137.

Zhu W.M., Liu W., Wu D.Q. (2000): Isolation and characterization of a new bacteriocin from *Lactobacillus gasseri* KT7. Journal of Applied Microbiology, **88**: 877–886.

Received for publication July 2, 2008
Accepted July 30, 2008

Corresponding author:

Ing. Štěpán Tůma, Ph.D., Vysoká škola chemicko-technologická v Praze, Fakulta potravinářské a biochemické technologie, Ústav technologie mléka a tuků, Technická 5, 166 28 Praha 6, Česká republika
tel.: + 420 220 443 275, fax: + 420 220 443 285, e-mail: stepan.tuma@vscht.cz