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A New Probiotic Cheddar Cheese with High ACE-Inhibitory Activity and γ-Aminobutyric Acid Content Produced with Koumiss-Derived *Lactobacillus casei* Zhang

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Summary

Cheddar cheese has been manufactured with *Lactobacillus casei* Zhang as the dairy starter adjunct. *L. casei* Zhang had previously been isolated from koumiss collected from Xilin Guole in Inner Mongolia and characterized in detail with regard to their probiotic potential. The addition of *L. casei* Zhang to Cheddar cheese had no adverse effects on sensory criteria. The cheese made with 0.1, 1 and 2 % of the probiotic strain *L. casei* Zhang adjuncts contained high levels of the *Lactobacillus* after 6 months of ripening with final counts of $9.6 \cdot 10^7$, $7.7 \cdot 10^7$ and $1.02 \cdot 10^8$ CFU/g, respectively. In the ripe control cheese, without the addition of probiotic strain *L. casei* Zhang, the number of *Lactobacillus* reached $5.7 \cdot 10^7$ CFU/g. Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) analysis was used to distinguish the added *L. casei* Zhang from the natural flora of the cheese and to determine whether *L. casei* Zhang grew in the cheese. ACE-inhibitory activity and γ-aminobutyric acid (GABA) concentrations in the cheese were measured. Compared with control cheese, experimental cheese with 0.1, 1 and 2 % of probiotic strain *L. casei* Zhang revealed some increase in ACE-inhibitory activity and GABA mass fraction. In the present study, the production of both ACE-inhibitory activity and GABA in the probiotic cheese with the *L. casei* Zhang adjunct isolated from koumiss has been found for the first time. The results suggest that cheese with the probiotic strain *L. casei* Zhang showed good potential for application in the management of hypertension.

Key words: ACE-inhibitory activity, *Lactobacillus casei* Zhang, γ-aminobutyric acid, probiotic Cheddar cheese, koumiss

Introduction

Probiotics have been defined as live microbial food supplements that benefit human health. Viable lactic acid bacteria (LAB) of probiotic foods have several scientifically established and/or clinically proved health effects, such as reduction and prevention of diarrhoea of different origin, improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergy, enhancement of immune potency, and antitumorigenic activities (*1*).

In recent years the consumption of cheese has increased rapidly owing to the fact that this dairy product fulfils many of the current dietary needs. It is a ready-to-eat food, and rich in nutritional components. It has been reported that Cheddar cheese may offer certain ad-

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vantages as a carrier of probiotic microorganisms, having a higher pH than the more traditional probiotic foods (*e.g.* yogurts and fermented milk). Furthermore, the matrix of the cheese and its relatively high fat content may offer protection to probiotic bacteria during passage through the gastrointestinal tract (*2–6*).

High blood pressure has been considered as a risk factor for the development of cardiovascular disease (arteriosclerosis, stroke and myocardial infarction) and end--stage renal disease. Angiotensin I-converting enzyme (ACE; dipeptidyl carboxypeptidase I, EC 3.4.15.1) plays an important role in the regulation of blood pressure. ACE inhibitors may exert an antihypertensive effect. Various lactic acid bacteria produce specific peptides that possess an inhibitory activity against the ACE *in vitro* and demonstrate a hypotensive effect in the hypertensive rat model. The γ-aminobutyric acid (GABA) is well known as a neurotransmitter that regulates inhibitory neurotransmission in mammalian central nervous systems. In addition, GABA has been proved to be effective for lowering the blood pressure of mammals. Milk products fermented by lactic acid bacteria enriched with GABA decreased the blood pressure in spontaneously hypertensive rats, and in mildly and moderately hypertensive patients (*7*).

The beneficial health effects of cheese can be enhanced by the ACE-inhibitory peptides or GABA produced during fermentation and storage. More recently, a great interest in the ACE-inhibitory peptides (*8–14*) or GABA (*15–18*) of cheese has been developed. However, there are no reports on the development and characterization of probiotic Cheddar cheese by koumiss-derived *Lactobacillus*, which not only possesses ACE-inhibitory activity but also can produce GABA.

L. casei Zhang had previously been isolated from koumiss collected in Xilin Guole, Inner Mongolia, PR China, and characterized in detail with regard to their probiotic potential. The ability to withstand the strict acid and bile, and remove cholesterol from the growth media *in vitro* has been detected. *L. casei* Zhang showed relatively high resistance to low pH than other isolated lactobacilli from koumiss. Previous studies had also shown that *L. casei* Zhang was able to enhance natural and acquired immunity in healthy mice and antagonism to *Escherichia coli* in mice as well as the ability to adhere to human intestinal epithelial cells (*19–23*). Soy milk, bovine milk and mare's milk could be served as vehicles for delivery of probiotic *L. casei* Zhang (*24,25*). The expression of H^+-ATPase of *L. casei* Zhang in acid conditions was studied and the results show that the expression of H^+-ATPase is associated with the acidity of the environment in *L. casei* (*26*). In addition, to understand further genetic background and functional mechanism of this strain, we have completed and annotated the whole genome sequence recently (GenBank Accession No. CP 001084).

This study investigates the performance of the probiotic strain *L. casei* Zhang added to Cheddar cheese, as well as the ACE-inhibitory activity and GABA content in the cheese. A better perspective towards this end could help the development of the probiotic cheese for the management of hypertension.

Materials and Methods

Origin and properties of microbial strain

The probiotic strain *Lactobacillus casei* Zhang had previously been isolated in our lab from koumiss collected in Xilin Guole in Inner Mongolia, PR China. The high tolerance of acidic pH and high bile salt by *L. casei* Zhang has been demonstrated in *in vitro* studies. It could grow well at pH=3.0 and 3 h after artificial gastric juice digestion, its survival rate was much greater than of other viable lactic acid bacteria (LAB) tested. The presence of bile salt hydrolase activity was also noticed in *L. casei* Zhang, which was able to catalyze the hydrolyzation of sodium taurocholate in the medium and release free bile salt. The strain was able to sustain a bile salt concentration of 1.6 g per 100 mL of the medium (*22*).

Cheese manufacture

Freeze-dried cheese inoculant R-707 (Chr Hansen, Wisby, Denmark), containing *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*, was used as a starter. Cheese was made with 10 L of pasteurized (63 °C, 30 min) whole milk. Raw milk was purchased from Yili Company (Huhhot, PR China) and its composition of the raw milk was (in %, by mass per volume): total solids 12.25, total proteins 3.02, fat 3.93 and lactose 5.3.

The freeze-dried culture containing $2{\cdot}10^{11}$ CFU/g of *L. casei* Zhang was prepared in our laboratory. The heat--treated (90 °C for 30 min) 10 % (mass per volume) reconstituted skimmed milk was inoculated with 1 % (mass per volume) freeze-dried *L. casei* Zhang. After inoculation, the skimmed milk was incubated at 37 °C for 24 h. Then *L. casei* Zhang suspension ($2.43{\cdot}10^8$ CFU/mL) from the skimmed milk was added as adjunct into the pasteurized cow's milk ($5{\cdot}10^2$ CFU/mL) at a volume fraction of 0.1, 1 or 2 %, together with 0.5 % (mass per volume) cheese starter and $CaCl_2$ (25 g per 100 kg) before rennet (12 g/t of milk) coagulation for 35 min at 35 °C. The curd was cut into 0.63 to 1.59 cm^3 blocks and cooked to 39 °C. The cooking took only 20–60 min and the curd was stirred constantly during this step to avoid uneven cooking or overcooking. Whey was removed from the curd by allowing it to drain out of the vat. The curd was allowed to set for a few minutes. By the end of the setting, the acidity of the whey was somewhere between 0.15 and 0.17. Loaves of curd were cut about 15 cm wide. After 10 min, the loaves were turned and stacking began. Every 10 min, when the loaves had to be turned, they were stacked. When the turning process was complete, the loaves were milled into about 1.3-cm pieces. After all the curd was milled, salt was added at a rate of 2.8 % (by mass), and the curd was placed into the mould to drain off the whey and was left to ripen at 12 °C for 180 days at a relative air humidity of 85 to 90 %. The cheese made without probiotic strain *L. casei* Zhang additive was used as a control.

Sensory evaluation of cheese

Cheese samples were graded blindly after 6 months of ripening by a commercial grader from a local cheese manufacturing plant for the characteristics of 'flavour' and 'body', with maximum scores of 45 and 40, respec-

tively. Minimum scores of 38 and 31 for flavour and body, respectively, are required for commercial Cheddar cheese (*2*).

Determination of ACE-inhibitory activity

Water-soluble extracts (pH=4.6) of each of the cheese samples were prepared according to the method of Kuchroo and Fox (*27*). The pH of the water-soluble extracts was adjusted to pH=8.3 with NaOH and then they were centrifuged at 6000×*g* for 10 min. Subsequently, the supernatant was used to determine the ACE-inhibitory activity and GABA content of the samples.

ACE-inhibitory activity was assayed as described by Chen *et al*. (*28*). A mass of 500 mg of hippuryl-L-histidyl--L-leucine (HHL, Sigma-Aldrich China Inc, Shanghai, PR China) was dissolved in 77.6 mL of 100 mmol/L Na-borate buffer (pH=8.3) containing 300 mmol/L NaCl. Rabbit lung ACE (Sigma-Aldrich China Inc, Shanghai, PR China) was dissolved in the same buffer at a concentration of 53.2 mU/mL. A mixture containing 75 μL of ACE solution and 75 μL of filtered sample (150 mg/mL) was incubated at 37 °C for 10 min, then 75 μL of HHL solution were added and incubated for 30 min. The reaction was stopped by the addition of 250 μL of 1 mol/L HCl. A volume of 10 μL of this solution was injected directly into a Luna C18 column (4.6×250 mm, particle size 5 mm; Phenomenex, Torrance, CA, USA) to separate the product and hippuric acid (HA) from HHL. The column was eluted with 50 % methanol in water (by volume) containing 0.1 % trifluoroacetic acid (TFA) at a flow rate of 0.8 mL/min using a pump and the detector was monitored at 228 nm. The inhibition was calculated from the equation:

$$[(A_c - A_s)/(A_c - A_b)] \cdot 100 \qquad /1/$$

where A_c is the absorbance of the buffer (control), A_s is the absorbance of the reaction mixture (sample), and A_b is the absorbance of the blank when stop solution was added before the reaction occurred.

Determination of γ-aminobutyric acid

The determination of GABA was performed by high--performance liquid chromatography (HPLC). A volume of 1 mL of the sample was diluted with 16 % (mass per volume) trichloroacetic acid (TCA) to 10 mL and shaken for 30 s. The extract was centrifuged for 10 min at 8000×*g*. The supernatant was collected and filtered through a 0.45-μm film (Millipore Express®, Billerica, MA, USA). Samples were then analyzed using an Agilent 1100 HPLC with a fluorescence detector (FLD) at excitation λ(Ex)= 340 nm and emission λ(Em)=450 nm (0–25 min). Separation was carried out with a Zorbax Eclipse AAA (4.6× 150 mm, 3.5 μm, Agilent Technologies, Santa Clara, CA, USA). A linear gradient profile of mobile phase, comprising 40 mmol/mL Na_2HPO_4, pH=7.8 (solvent A) and acetonitrile/methanol/water (45:45:10, by volume (solvent B), 0 % B (0–1.5 min), 0–40 % B (1.5–24.0 min), 40–100 % B (24.0–24.5 min), 100 % B (24.5–29.5 min), 100–0 % B (29.5–30.0 min) and 0 % B (30.0–35.0 min) was applied at a flow rate of 2.0 mL/min. The column temperature was kept at 40 °C. Precolumn derivatization with *o*-phthalaldehyde (OPA) was used and a 0.5-μL portion was injected into the HPLC system (Agilent Technologies, Santa Clara, CA, USA).

Chemical analysis

The total solids (gravimetric drying at 98–100 °C), fat (Rose-Gottlieb method), ash (muffle furnace at 550 °C), and total nitrogen (Kjeldahl method) were determined according to the Association of Official Analytical Chemists (AOAC) methods nos. 925.23, 905.02, 945.46 and 991.20, respectively (*29*). The total protein content was calculated from total nitrogen using a 6.38 conversion factor.

Microbial analysis of probiotic cheese

The cheese samples were homogenized and serial dilutions of homogenized cheese were prepared with 0.9 % NaCl solution. After serial dilution with maximum recovery, total viable count of the *Lactobacillus* was determined by a pour plate method using Rogosa selective lactobacillus (RSL) medium (Merck, Damstadt, Germany). The plates were incubated in anaerobic jar at 37 °C for 48 h.

After six months, 20 individual *Lactobacillus* colonies from each cheese were randomly selected from the RSL agar plates for enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) analysis. Genomic DNA from *Lactobacillus* was isolated using Wizard® Genomic DNA Purification Kit (Promega, WI, USA) according to the manufacturer's instructions. PCR was performed using the upstream primer 5′-ATGTAAGCTCCTGGGATTCAC--3′ and the downstream primer 5′-AAGTAAGTGACTG-GGGTGAGCG-3′ (*30*). The cycling program was 95 °C for 7 min, 52 °C for 60 s, 65 °C for 8 min for the first cycle and 90 °C for 30 s, 52 °C for 60 s, 65 °C for 8 min for the next 35 cycles and one cycle at 65 °C for 16 min. All PCR amplicons were electrophoresed on 2 % (mass per volume) agarose gels at a constant voltage of 4 V/cm. PCR patterns were stained with ethidium bromide (0.5 μg/mL) and visualized under UV light at 254 nm.

Assessment of proteolysis in Cheddar cheese

Urea-PAGE electrophoresis of the cheese samples was carried out according to the method of Tarakçi *et al*. (*31*). The free amino nitrogen (FAN) content of the sample was measured by the method of Church *et al*. (*32*). Water-soluble extracts (pH=4.6) of each of the cheese samples were prepared and water soluble nitrogen (WSN) was determined according to the method described by the AOAC method no. 991.20 (*29*). Results were expressed as the ratio of WSN or FAN to total nitrogen (TN) content.

Statistical analysis

All experiments were performed in triplicate. Data were tested for statistical significance by the Statistical Analysis System software (SAS v. 9.00, SAS Institute Inc., NC, USA).

Results and Discussion

Compositional analysis and sensory evaluation

The comparable values observed for control and experimental cheese samples indicate that incorporation of probiotic *L. casei* Zhang as starter culture, and their survival at high numbers, had no direct effect on cheese composition. All cheese samples could be described as commercial grade with respect to sensory criteria. After 6 months of ripening, probiotic cheese samples achieved the scores of 39 and 36 for flavour and body, respectively (Table 1). Lactic acid bacteria adjuncts had previously been reported to improve or decrease cheese flavour. Acceptability of probiotic cheese with *L. casei* 279 was significantly lower ($p<0.05$) than that of the control cheese with bitterness and sour-acid taste as the major defects after ripening for 9 months at 4 °C. There were positive correlations ($p<0.05$) between the scores of bitterness and the level of water-soluble nitrogen (*33*). The presence of *Enterococcus faecium* PR88 strain in Cheddar cheese at levels greater than or equal to 10^8 CFU/g may positively influence Cheddar flavour during 9 months of ripening at 8 °C (*34*). Slight changes in the gross composition and appreciable differences in the flavour profile were observed between control and experimental cheese samples with *Lactobacillus delbrueckii* ssp. *lactis* UO 004. This strain was capable of surviving at high cell numbers of 10^8 to 10^9 CFU/g in cheese after 28 days of ripening without adversely affecting sensory criteria or appearance of the cheese (*35*).

In this study, laboratory-scale cheese samples with different levels of *L. casei* Zhang were found to have the flavour and body comparable to those of control cheese, indicating that the addition of *L. casei* Zhang to Cheddar cheese had no adverse effects on sensory criteria.

Survival of the strain in cheese

Microbiology of the Cheddar cheese samples after 6 months of ripening and the ERIC-PCR profiles of *Lactobacillus* isolates from the ripened cheese are shown in Figs. 1 to 5. Results demonstrate that cheese made with 0.1, 1 or 2 % of probiotic strain *L. casei* Zhang adjuncts (Fig. 1) contained high levels of *Lactobacillus* after 6 months of ripening, with final counts of $9.6 \cdot 10^7$, $7.7 \cdot 10^7$, and $1.02 \cdot 10^8$ CFU/g, respectively. In the ripe control cheese,

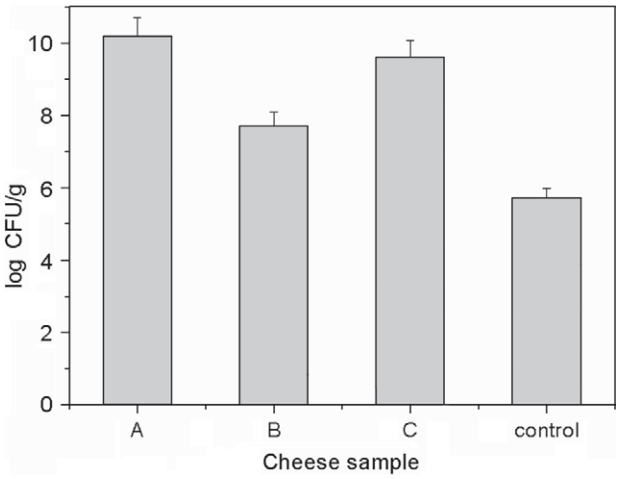

Fig. 1. Survival of lactobacilli of control and probiotic Cheddar cheese samples ripened at 12 °C for 180 days; data are means of triplicate determinations±standard deviation; for abbreviations see Table 1

the number of *Lactobacillus* reached $5.7 \cdot 10^7$ CFU/g. The counts of *Lactobacillus* in all probiotic cheese were significantly higher than that of the control cheese ($p<0.05$). This was confirmed by the comparison with the ERIC-PCR fingerprints generated for *Lactobacillus* isolates from 6-month-ripened cheese. The *Lactobacillus* isolated from the cheese with 0.1, 1 and 2 % of probiotic strain *L. casei* Zhang (Figs. 2 to 5) were mainly identified by ERIC-PCR as *L. casei* Zhang, while the *Lactobacillus* isolated from the control cheese without probiotic strain *L. casei* Zhang were identified by ERIC-PCR as non-starter lactic acid bacteria (NSLAB). Some studies demonstrated that Cheddar cheese can be an effective vehicle for delivery of high numbers of probiotic organisms. Cheddar cheese produced with starter lactococci and *Bifidobacterium longum* 1941, *B. lactis* LAFTI® B94, *L. casei* 279, *L. paracasei* LAFTI® L26, *L. acidophilus* 4962 or *L. acidophilus* LAFTI® L 10 was used to study the survival of the probiotic bacteria during ripening period of 6 months at 4 °C. All probiotic adjuncts survived the manufacturing process of Cheddar cheese at high levels without alteration of the cheese-making process. *L. acidophilus* 4962, *L. casei* 279, *B. longum* 1941, *L. acidophilus* LAFTI® L10, *L. paracasei*

Table 1. Composition and sensory evaluation of control and probiotic Cheddar cheese samples*

Cheese sample	*w*(salt)/%	*w*(total solids)/%	*w*(fat)/%	*w*(protein)/%	Flavour score[a]	Body score[b]
A	1.63±0.01	64.63±0.11	34.94±0.38	25.33±0.37	39	36
B	1.62±0.03	64.71±0.18	34.40±0.36	25.37±0.30	39	36
C	1.60±0.01	65.27±0.10	35.25±0.40	25.51±0.20	39	36
Control	1.60±0.02	64.61±0.17	34.97±0.42	25.37±0.64	38	36

*Composition analyses conducted at 14 days; sensory evaluation was conducted at 6 months; control – starter (freeze-dried cheese inoculant R-707) only was added to the cheese during manufacture, A – 2.0 % probiotic *L. casei* Zhang was added to the starter culture during manufacture of cheese as an adjunct, B – 1.0 % probiotic *L. casei* Zhang was added to the starter culture during manufacture as an adjunct, C – 0.1 % probiotic *L. casei* Zhang was added to the starter culture during manufacture as an adjunct
[a]maximum score=45 and minimum commercial score=38, [b]maximum score=40 and minimum commercial score=31; data are means of triplicate determinations±standard deviation

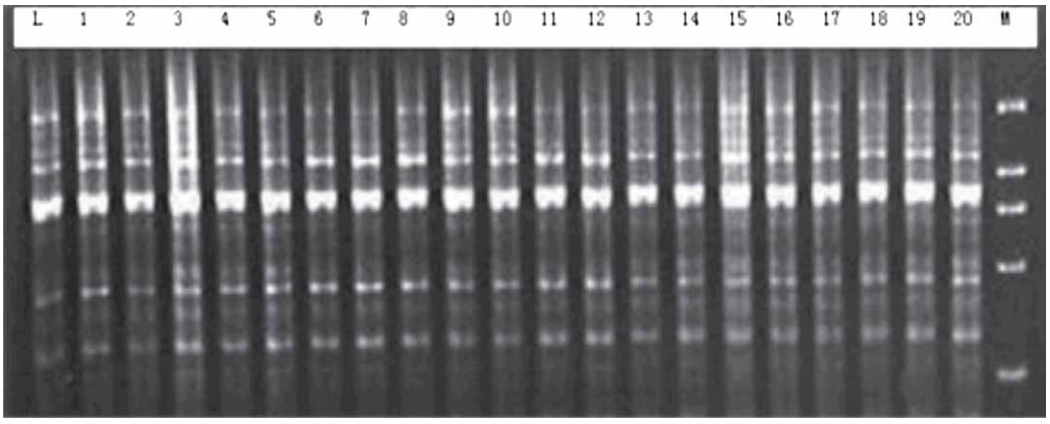

Fig. 2. ERIC-PCR-generated DNA fingerprints for *Lactobacillus* isolates from 6-month-ripened probiotic cheese with 2 % *L. casei* Zhang starter adjunct: lane L shows the ERIC-PCR profile of the *L. casei* Zhang added to the cheese during manufacture, while DL2000 marker is shown in lane M. All other lanes show ERIC-PCR profiles of *Lactobacillus* isolates from 6-month-ripened cheese

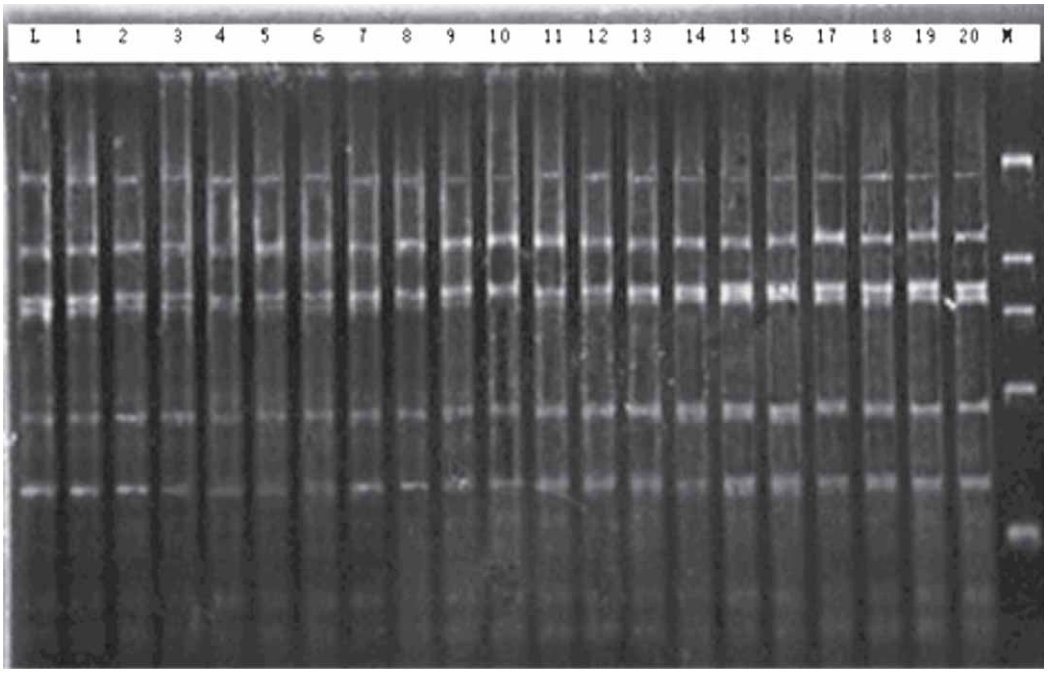

Fig. 3. ERIC-PCR-generated DNA fingerprints for *Lactobacillus* isolates from 6-month-ripened probiotic cheese with 1 % *L. casei* Zhang starter; for legend see Fig. 2

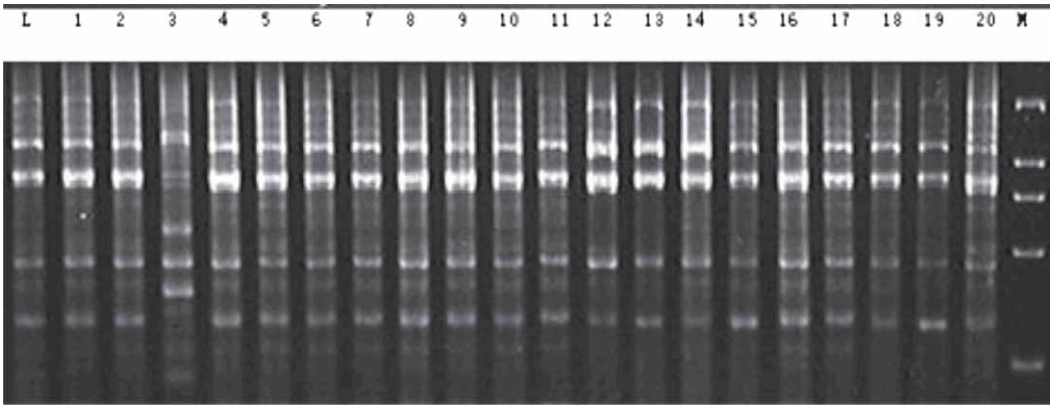

Fig. 4. ERIC-PCR-generated DNA fingerprints for *Lactobacillus* isolates from 6-month-ripened probiotic cheese with 0.1 % *L. casei* Zhang starter; for legend see Fig. 2

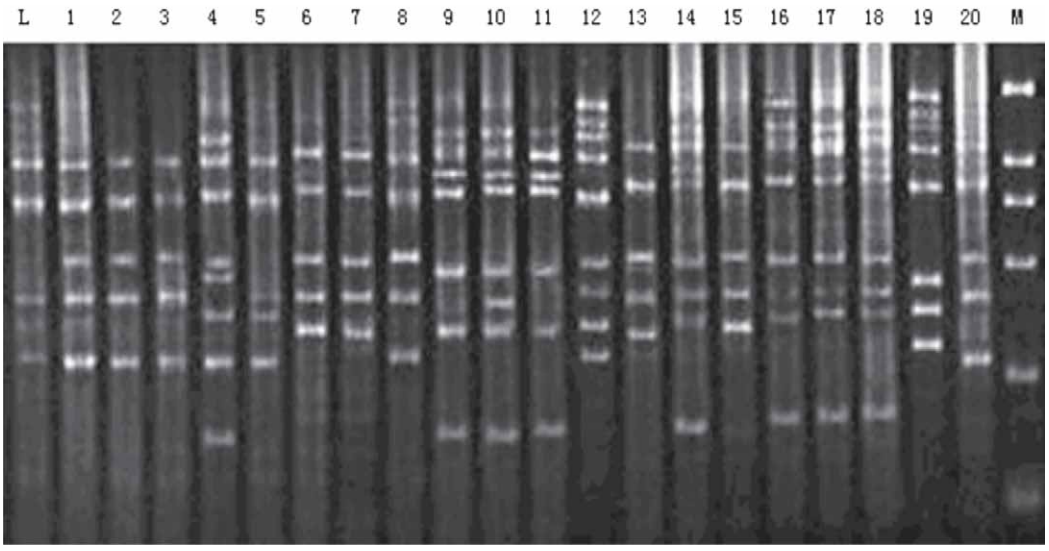

Fig. 5. ERIC-PCR-generated DNA fingerprints for *Lactobacillus* isolates from 6-month-ripened control cheese without the *L. casei* Zhang starter; for legend see Fig. 2

LAFTI® L26 and *B. lactis* LAFTI® B94 can be applied successfully in Cheddar cheese (*36*). Three batches of Cheddar cheese (batch 1, with lactococci as the only starter; batch 2, with lactococci, *L. acidophilus* 4962, *L. casei* 279 and *Bifidobacterium longum* 1941; and batch 3, with lactococci, *L. acidophilus* LAFTI® L10, *L. paracasei* LAFTI® L26 and *B. lactis* LAFTI® B94) were manufactured in triplicate to study the survival and influence of probiotic bacteria on proteolytic patterns. All probiotic adjuncts survived manufacturing process and maintained their viability of >7.5 log CFU/g at the end of ripening (*37*).

Although *Lactobacillus* has been added to Cheddar cheese and has subsequently been found to remain at high levels throughout maturation, no definitive identification method was used in these studies to distinguish the added *Lactobacillus* from the natural flora of the cheese.

ERIC- and repetitive sequence based (REP)-PCR have specific advantages compared to other molecular fingerprinting methods. REP elements and ERIC sequences are dispersed throughout bacterial genomes and PCR studies confirmed that inter-REP and inter-ERIC distances or profiles are typical for given bacterial species and sometimes even for strains within a given species. However, ERIC- and REP-PCR techniques have so far not been extensively used for the differentiation of lactobacillus strains (*30*). In the present study, ERIC-PCR analysis, when used as an identification method, determined that probiotic *L. casei* Zhang strain grew in the cheese.

ACE-inhibitory activity and GABA content in Cheddar cheese

Compared with control cheese, experimental cheese with 0.1, 1 and 2 % of probiotic strain *L. casei* Zhang revealed some increase in ACE-inhibitory activity and GABA content. The basic values of the ACE-inhibitory activity and GABA content of Cheddar cheeses are described in Table 2. The ACE-inhibitory activity in the control cheese was 60.13 %, while in the experimental cheese with 0.1, 1 and 2 % of probiotic strain *L. casei* Zhang the ACE-inhibitory activity was recorded as 91.45, 85.30 and 100 %, respectively.

Table 2. ACE-inhibitory activity and GABA in Cheddar cheese

Cheese sample	ACE-inhibitory activity %	*w*(γ-aminobutyric acid) mg/kg
A	(100±2.56)[a]	(677.35±21.47)[a]
B	(85.30±1.74)[b]	(538.11±20.31)[c]
C	(91.45±3.78)[c]	(525.34±4.11)[c]
Control	(60.13±0.79)[d]	(238.49±22.26)[b]

[a, b, c, d]Means with different superscripts in the same row differ significantly ($p<0.01$); data are means of triplicate determinations±standard deviation; for abbreviations see Table 1

Some ACE-inhibitory peptides have been reported in the cheese. Cheddar cheese samples made with the addition of *L. casei* 279, *L. casei* LAFTI® L26 or *L. acidophilus* LAFTI® L10 had significantly higher ($p<0.05$) ACE-inhibitory activity than those without any probiotic adjunct (*13*). The high ACE-inhibitory activity found in the water-soluble extracts of some investigated hard cheese samples was mainly due to the presence of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) (*12*).

The level of GABA in the control cheese was 238.49 mg/kg, while in the experimental cheese with 0.1, 1 and 2 % of probiotic strain *L. casei* Zhang it was 525.34, 538.11 and 677.35 mg/kg, respectively.

Several GABA-producing lactic acid bacteria have been identified, including *Lactobacillus paracasei* isolated from a Japanese traditional fermented fish, *Lactobacillus brevis* isolated from kimuchi and alcohol distillery lees, and *Lactococcus lactis* from cheese starters (*38*). It has been reported that the mass fractions of GABA in 22 Italian cheese varieties that differ in several technological traits markedly varied from 0.26 to 391 mg/kg. *Lactobacillus*

paracasei PF6, *Lactobacillus delbrueckii* ssp. *bulgaricus* PR1, *Lactococcus lactis* PU1, *Lactobacillus plantarum* C48 and *Lactobacillus brevis* PM17 were the best GABA-producing strains during fermentation of reconstituted skimmed milk (*18*).

In this study, the GABA content could be enhanced by applying *L. casei* Zhang in the cheese. The findings of this study provide a potential to develop health-promoting probiotic *L. casei* Zhang cheese enriched with GABA.

GABA-producing lactic acid bacteria which rapidly produced a large amount of GABA from glutamic acid (Glu) by glutamate decarboxylase (GAD) [EC 4.1.1.15] were also isolated. In the case of *L. brevis*, 4 of the 8 strains tested produced GABA, with the production of 50 mmol/L of GABA from 59 mmol/L of Glu in the glucose yeast extract polypepton medium (*39*). However, the high cost of the culture medium remains a problem for the commercial production. It seems that an economical and simple process of natural GABA production without Glu supplementation could be achieved by the probiotic cheese with the addition of *L. casei* Zhang. It has been reported that GABA can facilitate cell survival by maintaining cellular pH, even under acidic environments, because GAD must consume an H^+ ion for GABA production. Similarly, dairy products with high GABA and GAD activity are capable of sustaining probiotics through the digestive system, by which they made it possible to have probiotic effects, and also they may possess the same acid stability properties that are required to survive in the intestines (*40*). The GABA-enriched milk (1 nmol/mL) was reported to lower the blood pressure in spontaneously hypertensive and normotensive Wistar-Kyoto rats (*41*). Therefore, the amount of GABA in the probiotic cheese made with *L. casei* Zhang adjunct is high enough to have some functional value.

Limited scientific data are available on the ACE-inhibitory activity and GABA concentration in cheese inoculated with *Lactobacillus* isolated from koumiss. The *Lactobacillus* species in koumiss include *L. delbrueckii*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. paracasei*, *L. kefir*, *L. salivarius*, *L. buchneri*, *L. plantarum* (*42*). In the present study, the production of both ACE-inhibitory activity and GABA in the probiotic cheese with *L. casei* Zhang isolated from koumiss have been found for the first time. Cheese already possesses a healthy image and has a long history of safe production. The production of both ACE-inhibitory activity and GABA *in situ* is an appealing approach, as it adds an additional health effect to the products.

Proteolysis in Cheddar cheese

Urea-PAGE patterns of whole cheese samples after 6 months of ripening did not show any differences in the extent of primary proteolysis between the control cheese and those manufactured with the addition of *L. casei* Zhang (Fig. 6). Similarly, others have shown that the addition of lactobacilli had no effect on PAGE electrophoretograms (2).

The ratio of WSN and FAN to TN in whole cheese samples increased progressively throughout the 180 days of ripening period. No significant differences in the concentration of WSN and FAN could be detected in whole cheese samples (Figs. 7 and 8).

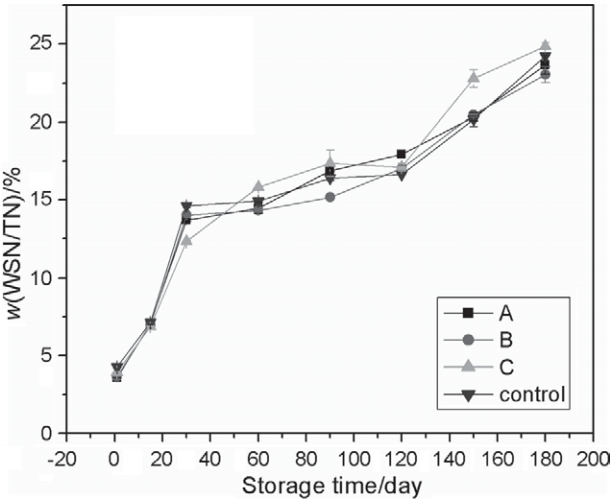

Fig. 7. WSN/TN analysis of Cheddar cheese during 180 days of ripening at 12 °C; TN=total nitrogen, WSN=water soluble nitrogen; data are means of triplicate determinations±standard deviation; for abbreviations see Table 1

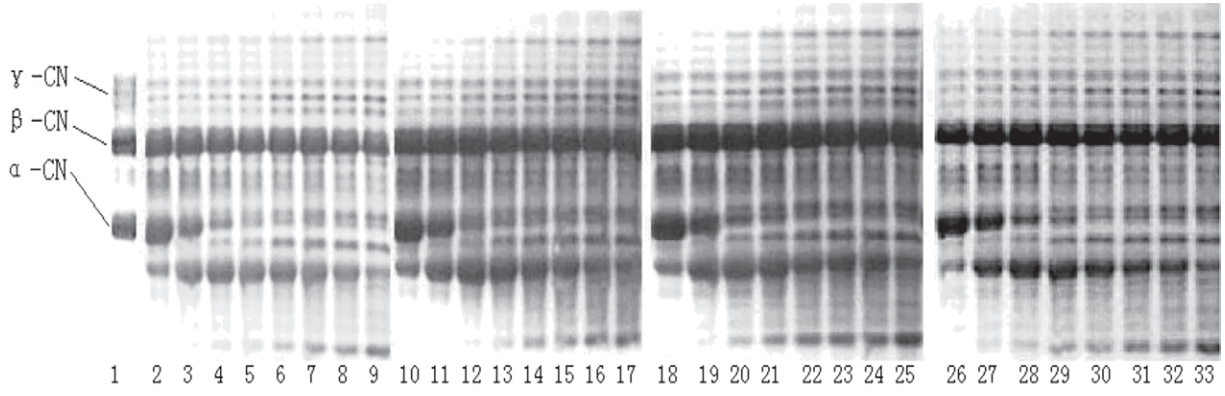

Fig. 6. Urea-PAGE patterns of Cheddar cheese with 2 % *L. casei* Zhang starter (lanes 2–9), 1 % *L. casei* Zhang starter (lanes 10–17), 0.1 % *L. casei* Zhang starter (lanes 18–25), without the *L. casei* Zhang starter (lanes 26–33) for 1, 15, 30, 60, 90, 120, 150 and 180 days, respectively. The standard casein is shown in lane 1

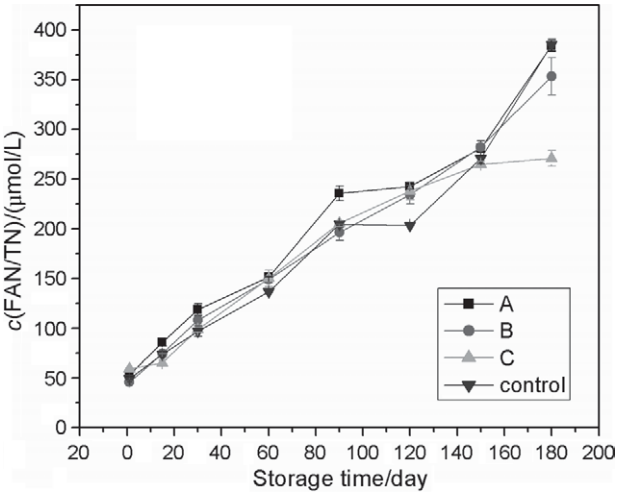

Fig. 8. FAN/TN analysis of Cheddar cheese during 180 days of ripening at 12 °C; TN=total nitrogen, FAN=free amino nitrogen; data are means of triplicate determinations±standard deviation; for abbreviations see Table 1

Conclusions

Cheddar cheese was manufactured with *Lactobacillus casei* (*L. casei*) Zhang as the dairy starter adjunct. The addition of *L. casei* Zhang to Cheddar cheese had no adverse effects on sensory criteria and contained high levels of *Lactobacillus*. ERIC-PCR analysis confirmed that probiotic *L. casei* Zhang strain grew in the cheese. The experimental cheese with 0.1, 1 and 2 % of probiotic strain *L. casei* Zhang revealed some increase in ACE-inhibitory activity and GABA content. The cheese containing the probiotic strain *L. casei* Zhang showed good potential for application in the management of hypertension.

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