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RESEARCH ARTICLE

ACE-inhibitory activity of milk fermented with *Saccharomyces cerevisiae* K7 and *Lactococcus lactis* subsp. *lactis* NBRC 12007

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Abstract: The angiotensin-I converting enzyme (ACE) inhibitory activity of the milk fermented with *Lactococcus lactis* subsp. *lactis* NBRC 12007 and *Saccharomyces cerevisiae* K7 in monoculture and co-culture was evaluated. Bovine full-fat milk was fermented with each strain in monoculture and co-culture at 30 ºC for 72 h, and the *in vitro* ACE inhibitory activity (%) of each milk sample was determined by a fluorogenic assay using H-(2)Abz-Acp(6)-Ala-Phe(4-NO_2)-Leu-OH as the substrate. The ACE inhibitory percentages of the milk samples fermented with *L. lactis* subsp. *lactis* NBRC 12007 and *S. cerevisiae* K7 monocultures and the co-culture was 33, 27 and 25 %, respectively, which varied significantly ($p < 0.05$). Each milk sample was fractionated by semi-preparative HPLC analysis and the ACE inhibitory activity (%) of each fraction was determined using the same substrate, which varied from 20 - 47 %, 16 - 37 % and 16 - 31 % in the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007, *S. cerevisiae* K7 and the co-culture, respectively. The highest ACE-inhibitory activity (47 %) was observed in fraction-2 of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007. The results concluded that the two strains tested were able to hydrolyze milk proteins into ACE-inhibitory peptides in order to produce fermented milk products with ACE-inhibitory activity, both in monoculture and co-culture. Therefore, it can be suggested that these strains can be successfully utilized in the dairy industry in manufacturing fermented milk products with ACE-inhibitory activity as a dietary supplement and/or as an alternative approach for antihypertensive medication.

Keywords: ACE inhibitory activity, *Lactococcus lactis,* milk, *Saccharomyces cerevisiae.*

INTRODUCTION

The angiotensin I-converting enzyme (ACE; kininase II; EC 3.4.15.1) is a carboxy-dipeptidyl-metallopeptidase and a key enzyme associated with the renin angiotensin system, which regulates peripheral blood pressure, where it catalyzes both the production of vasoconstrictor angiotensin-II from angiotensin-I and the inactivation of the vasodilator bradykinin, which ultimately results in hypertension (Gobbetti *et al*., 2000; Kim *et al*., 2004). Hypertension is the main controllable risk factor associated with cardiovascular diseases, and it has been reported that about 25 % of the world population is affected by hypertension while approximately $ 15 billion is spent annually on antihypertensive medication in the United States of America (Vermeirssen *et al*., 2003; FitzGerald *et al*., 2004).

The milk proteins, casein and whey proteins are a good source of bioactive peptides (BAPs) that have a positive impact on body functions and may ultimately influence health. These peptides are inactive within the sequence of precursor proteins, but can be released *in vivo* or *in vitro* by enzymatic digestion or during fermentation with lactic acid bacteria (LAB) (Gobbetti *et al*., 2000; Roy *et al*., 2000; De Noni & Cattaneo, 2010). During microbial fermentation of milk, proteins are hydrolyzed into long oligopeptides by cell wall associated proteinases

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of LAB, transported into the cell and broken down by intracellular peptidases into a range of peptides with different bioactivities (Nielsen *et al*., 2009). These milk-derived BAPs can function as exogenous regulatory substances such as hormones or drugs, which modulate physiological functions through binding interactions to specific receptors on target organs leading to induction of physiological responses (Gobbetti *et al.,* 2000). Among different BAPs, ACE-inhibitory peptides are the most extensively studied group. A number of ACE-inhibitory peptides have been identified from different fermented dairy products such as yoghurt (Chobert *et al*., 2005), cheese (Smacchi & Gobbetti, 1998; Pripp *et al*., 2006), Dahi (Ashar & Chand, 2004) and fermented sour milk products fermented with different LAB species such as lactobacilli (*Lb. helveticus*, *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*), lactococci (*L. lactis* subsp. *cremoris*), *Streptococcus thermophilus* and *Enterococcus faecalis* (Nakamura *et al*., 1995; Gobbetti *et al*., 2000; Roy *et al*., 2000; FitzGerald & Murray, 2006; Nielsen *et al*., 2009). These naturally occurring ACE-inhibitory peptides are reported to be advantageous over the artificially synthesized ACE-inhibitory drugs (vasodilators, diuretics, calcium channel blockers, angiotensin II receptor blockers and ACE-inhibitors such as captopril, enalapril, alecepril, lisinopril). The natural ACE inhibitors are not reported to cause adverse side effects such as hypotension, cough, increased blood calcium levels, fetal abnormalities, reduced renal function, angioedema and skin rashes (Hata *et al*., 1996; FitzGerald *et al*., 2004; Kim *et al*., 2004; Saito, 2008), which are associated with artificially synthesized drugs. Therefore, the concept of functional foods with ACE-inhibitory/antihypertensive activity has attained considerable attention over the past decade as they provide an alternative approach to decrease the requirement of antihypertensive medication.

Several commercial functional dairy products enriched with ACE-inhibitory peptides such as, Evolus® (Valio Ltd. Valio, Finland), Calpis sour milk marketed as Ameal S® (Calpis Food Industry Co., Ltd., Tokyo, Japan) and Casein DP-Peptio® (Kanebo Co., Ltd., Kanebo, Japan) have been introduced as additional or alternative treatments for hypertension. Among these, Calpis and Casein DP-Peptio® are recommended for patients with high blood pressure as "Foods for Specified Health Use (FOSHU)" in Japan (Saito, 2008). The antihypertensive effects of these milk have been tested *in vivo* using hypertensive human subjects and spontaneously hypertensive rat models (Hata *et al*., 1996; Sipola *et al*., 2001; Kim *et al*., 2004). Although hundreds of microorganisms are being used in the food industry, only a few have been tested on milk for their ACE-inhibitory activity. On the other hand, the ability of microbial proteases to produce ACE-inhibitory peptides upon milk fermentation is being currently debated. Therefore, further studies would be helpful to determine the actual contribution of starter culture microorganisms in producing fermented dairy products with ACE-inhibitory activity.

The yeast-lactic fermentation is one of the four types of milk fermentation in addition to the mesophilic, thermophilic and molds-in-lactic fermentation (Walstra *et al*., 2006). Yeasts play an important role in the preparation of certain dairy products including certain cheese types and contribute substantially to the final product due to various interactions between yeasts and LAB, either by contributing to the fermentation by supporting starter cultures, inhibiting undesired microorganisms causing quality defects, or contributing to the final product by means of desirable biochemical changes such as production of aromatic compounds, lipolytic and proteolytic activities (Viljoen, 2001). Kefir is a product of yeast-lactic fermentation made in Russia and southwestern Asia, and is now produced on an industrial scale in various countries. Kumiss is another example for the same type of fermentation and is a well-known milk drink in Russia and western Asia, traditionally made from mares` milk (Walstra *et al*., 2006). Moreover, Calpis sour milk is also a product of yeast-lactic fermentation in which thermophilic *Lb. helveticus* is used with *S. cerevisiae* in co-culture (Nakamura *et al*., 1995; Hata *et al*., 1996). It is obvious that thermophilic *Lactobacillus* and *S. cerevisiae* is an effective combination in producing fermented milk products with antihypertensive properties. The ability of *S. cerevisiae*`s proteolytic enzymes to hydrolyze milk proteins to ACE-inhibitory peptides has been reported earlier (Roy *et al*., 2000), whereas ACE-inhibitory activity was detected in fermented whey supplied with 20 g/L D-glucose and fermented with *S. cerevisiae* in monoculture and in co-culture with *Lb. helveticus* (Vermeirssen *et al*., 2003).

Based on available literature it is evident that considerable attention has been given to identify the ACE-inhibitory activity in milk products fermented with thermophilic *Lactobacillus* strains either in monoculture or in co-culture. However, less attention has been given to identify the ACE-inhibitory activity in fermented milk products in monocultures of *Lactococcus* strains or in co-culture with *S. cerevisiae*, although *Lactococcus* strains are the main mesophilic microorganisms used in dairy industry. Therefore, the objective of the present study was to identify the ACE-inhibitory activity of milk fermented with *S. cerevisiae* K7 and *L. lactis* subsp. *lactis* NBRC 12007, both in monoculture and in co-culture during long fermentation hours.

METHODS AND MATERIALS

Materials

Fluorogenic substrate H-(2)Abz-Acp(6)-Ala-Phe(4-NO_2)-Leu-OH and the angiotensin I-converting enzyme (EC 3.4.15.1) were purchased from Watanabe Chemical Industries, Japan and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Preparation of starter cultures

The strains *S. cerevisiae* Kyokai No. 7 and *L. lactis* subsp. *lactis* NBRC 12007 were obtained from the Production System Informatics Laboratory and Applied Microbiology Laboratory of Saga University, Japan, respectively. Stock cultures of *S. cerevisiae* K7 and *L. lactis* subsp. *lactis* NBRC 12007 were cultured in yeast extract peptone dextrose (YPD) (Difco™, Becton, Dickinson and Company, Sparks, USA) and M17 media (Difco™, Becton, Dickinson and Company, Sparks, USA), respectively. The two strains were inoculated in each specific medium aseptically and incubated overnight at 30 ºC while shaking in sterile conditions.

Production of fermented milk products

Normal bovine full-fat milk was heat treated at 90 ºC for 20 min to destroy contaminant microorganisms and enzymes present in the raw milk, which was then cooled to 30 ºC and supplemented with 1 % (w/v) glucose in order to provide a C-source for the growth of *S. cerevisiae* K7 as it is a non-lactose fermenting yeast. Fifty milliliters of heat treated milk was poured into 50 mL Eppendorf tubes, which were assigned into three treatments: *S. cerevisiae* K7 and *L. lactis* subsp. *lactis* NBRC 12007 in monoculture and in combination as co-culture. For monocultures, each microbial strain was inoculated separately at a concentration of 4.4×10^6 cfu/mL whereas for co-culture, all strains were inoculated at a concentration of 2.2×10^6 cfu/mL per 50 mL of milk. The milk samples were allowed to ferment at 30 ºC for 72 h under micro-aerobic conditions. All operations were carried out under aseptic conditions. At the end of the fermentation, the number of viable lactococci and yeast cells in the fermented milk was determined by plating on M17 and YPD agar, respectively after incubating at 30 ºC for 72 h. The fermented milk samples were stored at -20 ºC until analyses.

Preparation of whey from fermented milk

The fermented milk samples were thawed at 5 ºC and the whey fraction was prepared according to the method previously described by De Noni & Cattaneo (2010).

Semi-preparative high performance liquid chromatography (HPLC) analyses

The fermented milk samples were fractionated into 5 different fractions using semi-preparative HPLC (Tosoh Co., Tokyo, Japan). The HPLC system consisted of pumps (TOSOH CCPS), a column heater (TOSOH SC-8020), degasser (TOSOH SD-8022), UV detector (HITACHI L-4000H) and a chromato-integrator (HITACHI D-2500). Five hundred milliliters of the filtered whey fraction was applied on a Phenomenex Luna 5 µm C18 column (10 × 250 mm; Phenomenex, Torrance, CA, USA) and the peptides were separated at 30 ºC and eluted in a linear gradient mode of acetonitrile (10 – 50 % for 60 min) containing 0.1 % trifluoroacetic acid at a flow rate of 3 mL/min. The elution was monitored at 215 nm.

ACE-inhibitory assay

The ACE-inhibitory activity of each fermented milk sample and each whey fraction obtained from semi-preparative HPLC analysis was determined by the fluorescence method as previously described by Ando *et al*. (2003) with some modifications. Thereafter 175 µL of the buffer solution (pH 7.4) containing 0.22 M NaCl-TrisHCl was placed in a micro-titer well and mixed with 10 µL of H-(2)Abz-Acp(6)-Ala-Phe(4-NO_2)-Leu-OH substrate solution (100 ppm). Ten microliters from each pre-prepared whey samples were dissolved in 20 µL of 0.22 M NaCl-TrisHCl buffer (pH 7.4) and mixed thoroughly. Then 5 µL of this solution (5 µL of buffer solution for blanks) was dissolved in the above mixture containing the fluorogenic substrate and the reaction was initiated by adding 5 µL of ACE enzyme solution (100 mU/mL). The micro-titer plate was immediately placed in a fluorescence microplate reader (FL × 800, Bio Tek Instruments, Inc., Winooski, VT, USA) and the mixture was incubated at 37 ºC for 30 min, where the mixture was excited at 340 nm and the generated fluorescence was measured at 420 nm at 2 min intervals until 30 min. All measurements were carried out in triplicates.

Data were plotted in graphs considering time-kinetic and emission as the independent and dependent variables, respectively. The data obtained for the first 6 min were omitted from further calculations, as a strategy to minimize the associated error due to the variation observed from the rest of the data. Regression equations were derived separately for the control and each whey sample prepared from fermented milk. The decrease in slope over a linear time interval of 15 min was determined separately for the control ($\rho A_{control}$) and

for the whey samples with inhibitors (ρA_{whey}). The ACE inhibition percentage of each sample was calculated according to the following formula:

$$\text{ACE inhibition \%} = [1-(\rho A_{whey} / \rho A_{control})] \times 100$$

Determination of variation in cell density, pH and peptide concentration

Sample preparation

The fermented milk products were prepared according to the same procedure described so far by inoculating the same amount of starter culture microorganisms, *L. lactis* subsp. *lactis* NBRC 12007 and *S. cerevisiae* K7. Variation in cell density, peptide concentration and pH in the fermentation medium of each treatment were determined at 9 sample points obtained at 4, 8, 12, 16, 20, 24, 36, 48, and 72 h of fermentation. Each variable was evaluated in triplicates.

Determination of pH

The pH of the fermented milk samples obtained at each sample point was determined using a digital pH meter (HANNA, pH 211, TOA Electronics Ltd., Tokyo, Japan).

Spectrophotometric determination of cell growth

Cell growth was determined by measuring the cell density in each fermentation medium as the optical density (OD) value according to the method previously described by Exterkate (1984) with some modifications. A volume of 0.5 mL from each sample was transferred into pre-labelled 10 mL eppendorf tubes. The samples were centrifuged (AX-310 Versatile Refrigerated Centrifuge, CS Bio Co., USA) at 3000 × *g* for 20 min at 4 ºC after adjusting the pH to 6.8 with 1 M NaOH solution. The supernatant was pipetted out, diluted with 1 mL of distilled water and mixed thoroughly. Finally, the optical density (OD_{590}) was measured using a UV-1800 spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan) at a wave length of 590 nm. The OD_{590} value at a particular sample point was given as the mean value of three determinations. Milk samples kept for 4, 8, 12, 16, 20, 24, 36, 48, and 72 h under sterile conditions without fermentation were used as blanks to determine the OD_{590} value at each sample point.

Determination of peptide concentration

The peptide concentration of the fermented milk samples obtained at each sample point was determined according to the Bradford micro assay (Bradford, 1976) using a standard curve derived for bovine serum albumin. The fermented milk samples were centrifuged using a Versatile Refrigerated Centrifuge (AX-310, CS Bio Co., USA) at 6000 × *g* for 20 min at 4 ºC. Then 100 µL from each whey fraction was pipetted out into 10 mL eppendorf tubes, mixed with 1 mL of pre-prepared standard coomassie brilliant blue (CBB) solution and allowed to develop the colour complex for 5 min without any disturbance. Five hundred microliters (500 µL) of the solution was mixed with 1.5 mL of distilled water in a 2 mL glass cuvette and the optical density was measured spectrophotometrically (UV-1800, Shimadzu Co., Ltd., Kyoto, Japan) at 595 nm wavelength. Unfermented milk samples kept for 4, 8, 12, 16, 20, 24, 36, 48 and 72 h under similar experimental conditions were used as blanks for each sample point. The experiment was carried out in triplicate.

Statistical analysis

The experiment was conducted as a complete randomized design (CRD). A repeated measures ANOVA (RM-ANOVA) was used to test whether the peptide concentration, pH/acid production and the cell density varied over the experimental period. Each fermentation time was treated as a time point by using the 'repeated' option of Proc Mixed, whereas the starter cultures and fermentation time × starter cultures interaction were used as explanatory variables. Since the analysis revealed the influence of experimental period to be significant on the above variables, data on each experimental period was analyzed separately by using Proc ANOVA in SAS (one-way ANOVA considering the starter cultures as the explanatory variable). The means were separated by the least significant difference test (LSDT). The significant difference between the treatments in terms of percentage of ACE-inhibition was determined by the CATMOD procedure, and the means were separated by orthogonal contrasting using SAS (Version 9.1; SAS Institute, Cary, NC, USA) programme package designed for Windows. All values were reported as mean ± standard error of mean (SEM). All significances were determined at $\alpha = 0.05$.

RESULTS

Ability of the strains to grow in milk

Both strains tested in this study were able to grow in the heat treated milk under the applied conditions both in monoculture and in co-culture, and was reproducible. These milk products were produced from heat treated milk under sterile conditions as a measure to exclude

any possible interference caused by contaminant microorganisms. Figure 1 shows the cell growth in the fermentation medium over the experimental period. The cell density in the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 peaked (OD_{590} = 0.535 ± 0.024) after 8 hours, decreased drastically until 48 hours and then slightly increased to 0.241 ± 0.019 at the end of the fermentation. However, in the milk fermented with the co-culture, it increased gradually until 20 hours of fermentation, peaked (OD_{590} = 0.549 ± 0.039) and decreased steadily afterwards. The growth of *S. cerevisiae* K7 was characterized by four clearly demarcated growth phases, which peaked (OD_{590} = 0.577 ± 0.03) after 24 hours of fermentation.

During the first 12 hours of fermentation, cell density of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 was higher ($p < 0.05$) than that of the other treatments but, was lower ($p < 0.05$) thereafter. Although the initial growth of *S. cerevisiae* K7 and the co-culture was lower, it remained significantly higher from 16 hours until the end of fermentation. The cell density of the milk fermented with monocultures of *L. lactis* subsp. *lactis* NBRC 12007 and *S. cerevisiae* K7, and co-culture at the end of the fermentation was 0.241 ± 0.019, 0.323 ± 0.023 and 0.389 ± 0.05, respectively, which varied significantly. Cell enumerations done at the end of the fermentation process revealed that the live microorganism count was 5×10^6 cfu/mL for *L. lactis* subsp. *lactis* NBRC 12007 in monoculture, 6×10^6 cfu/mL for *S. cerevisiae* K7 in monoculture and, 4×10^6 and 9×10^6 cfu/mL, respectively for lactococci and yeast in the co-culture. It was observed that the live microorganism count for lactococci was low in the co-culture than that in the monoculture. However, a higher number of yeast cells remained viable in the co-culture than that of the monoculture.

Variation in acid production of the starter cultures during milk fermentation

During milk fermentation, lactic acid bacteria convert lactose into lactic acid, which lowers the pH of the product and preserves it from the growth of unwanted microorganisms. The rate of acid production plays a critical role in the manufacture of certain fermented dairy products such as Cheddar cheese. Accumulation of organic acids in the fermentation medium is reflected by the decreasing pH values over the fermentation period. Therefore, pH of the fermentation medium directly reflects the level of acid production by the inoculated starter culture microorganisms. Figure 2 shows the variation in acid production among the treatments over the experimental period. Initial pH of the milk was 6.65 and after 72 hours of fermentation, the lowest pH was observed in the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 (4.41 ± 0.08) while the pH remained at 4.79 in the other two fermented milk products ($p < 0.05$). However, the final pH of the control was 6.38, which was higher than that of the fermented milk samples ($p < 0.05$). According to the repeated measures analysis, acid production of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 and the co-culture was higher than that of *S. cerevisiae* K7 over the experimental period ($p < 0.05$). The fastest acidification to pH 4.6,

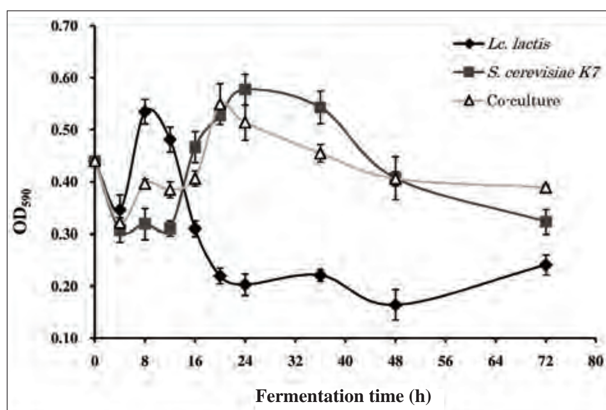

Figure 1: Variation in cell density (OD_{590}) of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007, *S. cerevisiae* K7 and co-culture over the experimental period. Data are expressed as mean ± SEM (n = 3) whereas the significance was determined at α = 0.05.

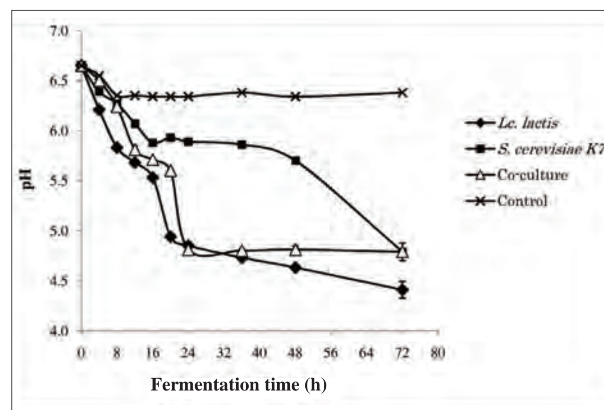

Figure 2: Variation in pH of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007, *S. cerevisiae* K7 and co-culture over the experimental period (72 h) (α = 0.05). Data are expressed as mean ± SEM (n = 3) whereas the significance was determined at α = 0.05.

where the caseins are coagulated at its isoelectric point was observed in the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007. In general, there was a considerable decrease in pH during the first 16 hours in all treatments except the control; however remained almost at the same level in the milk fermented with *S. cerevisiae* K7 over the next 32 hours (16 - 48 h) when the other two treatments showed a drastic reduction (Figure 2). Interestingly, the pH of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 continuously decreased at a slow rate, while it remained at the same level in the co-culture fermented milk.

Variation in peptide concentration during fermentation

The peptide concentration in the fermentation medium reflects the proteolytic acitivity of the stater cultures. Repeated measures analysis showed that the amount of peptides liberated from the hydrolysis of milk proteins during fermentation was higher ($p < 0.05$) in the milk fermented with the co-culture than that of the monocultures (Figure 3). There was no significant difference observed between the two monocultures over the experimental period ($p > 0.05$). However, it was higher in the milk fermented with the co-culture and *S. cerevisiae* K7 at the end of the fermentation ($p < 0.05$). Peptide concentration of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 varied from 0.42 ± 0.01 to 0.77 ± 0.03 mg/mL over the experimental period, peaked after 16 hours and then decreased gradually. It was lower in the milk fermented with *S. cerevisiae* K7 during the first 16 hours, and then remarkably increased to 0.57 ± 0.03 mg/mL corresponding to the higher cell growth followed by another reduction until 48 hours. However, the peptide concentration peaked at 72 hours (0.61 ± 0.03 mg/mL) despite its decreasing cell density. In contrast, the peptide concentration was significantly higher in the milk fermented with the co-culture than that of the monocultures at the initiation, and peaked after 20 hours (0.72 ± 0.03 mg/mL). Moreover, the peptide concentration in the fermentation medium corresponded well with its growth profile.

The HPLC chromatographs obtained at the end of the fermentation (72 hours) suggested that the majority of the originated peptides were short peptides having shorter retention times (Figure 4).

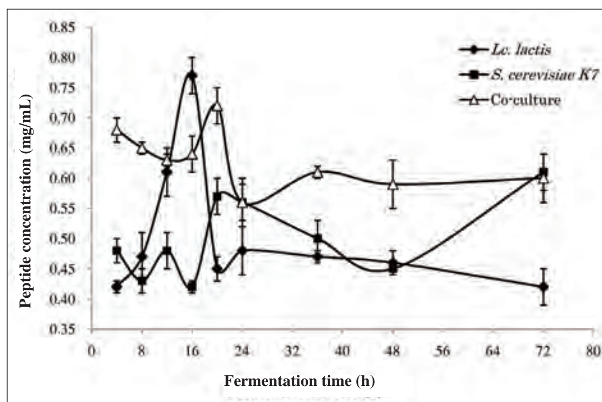

Figure 3: Variation in peptide concentration (mg/mL) in the whey frcations of the milk fermented with monocultures of *L. lactis* subsp. *lactis* NBRC 12007, *S. cerevisiae* K7 and co-culture. Data are expressed as mean ± SEM (n=3) whereas the significance was determined at α = 0.05.

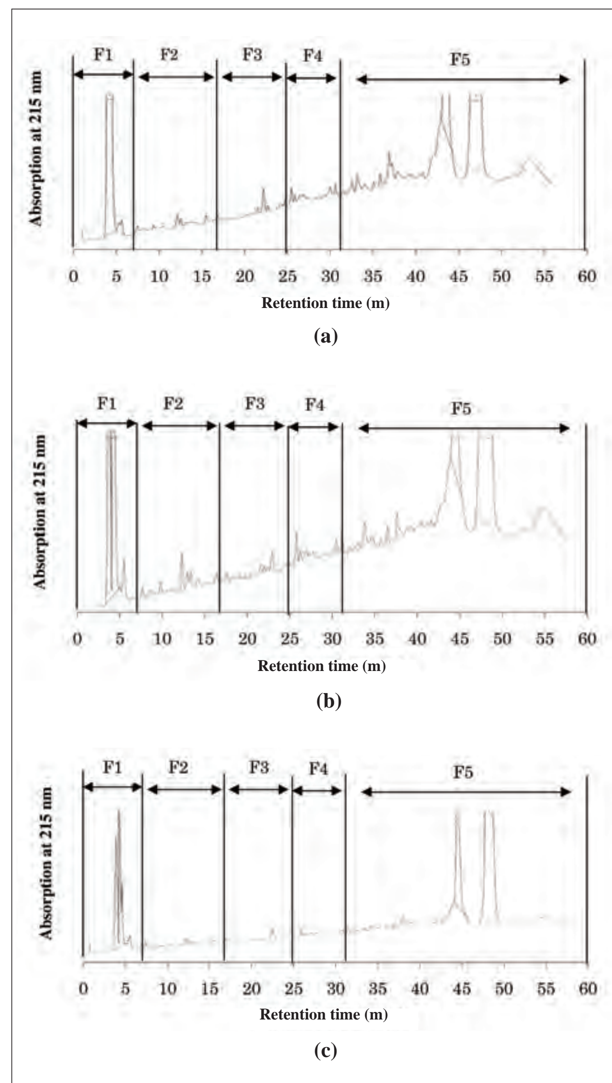

Figure 4: HPLC chromatographs of whey samples of milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 (a); *S. cerevisiae* K7 (b) and the co-culture (c) after 72h of fermentation (F = fractions)

ACE-inhibitory activity

The *in vitro* ACE-inhibitory activity of the fermented milk samples was moderate and varied significantly among the treatments. The average ACE-inhibition percentages were 33, 27 and 25 % in the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007, *S. cerevisiae* K7 and the co-culture, respectively. The ACE-inhibitory activity of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 was greater than that of the other two treatments ($p < 0.05$). Therefore, it can be suggested that *L. lactis* subsp. *lactis* NBRC 12007 is the most efficient strain in producing fermented milk with ACE-inhibitory activity. In addition, the milk fermented with monocultures exhibited higher ACE-inhibitory activities than that of the co-culture ($p < 0.05$). Moreover, the *in vitro* ACE-inhibitory activity (%) was detected in all fractions obtained from HPLC of the three treatments, which varied from 20 to 47 % in *L. lactis* subsp. *lactis* NBRC 12007, 16 to 38 % in *S. cerevisiae* K7 and 16 to 31 % in the co-culture treated milk (Figure 5). Several fractions of milk samples fermented with the monoculture of *L. lactis* subsp. *lactis* NBRC 12007 showed ACE-inhibitory activities of more than 40 % (42.72 ± 0.19 in fraction 1 and 47.1 ± 4.5 in fraction 2). However, the values for the same fractions of the milk fermented with *S. cerevisiae* K7 were more than 35 % but less than 40 % (37.53 ± 0.05 and 36.96 ± 5.85 in the fraction 1 and 2, respectively). The highest ACE-inhibitory percentage, 47 % was found in fraction 2 of the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007, which was significantly higher than that of the other fractions. In general, fractions with higher ACE-inhibitory percentages were always associated with the early-eluting fractions (1 and 2), which were greater than that of the other fractions (fraction 3, 4 and 5) in all treatments ($p < 0.05$). This revealed that the higher ACE-inhibitory activity was attributed to the smaller peptides having short retention times than large peptides having longer retention times. Interestingly, the highest inhibitory activity was observed in fraction 5 for the yeast strain although it was lower than that of the *L. lactis* subsp. *lactis* in other fractions (1, 2, 3 and 4).

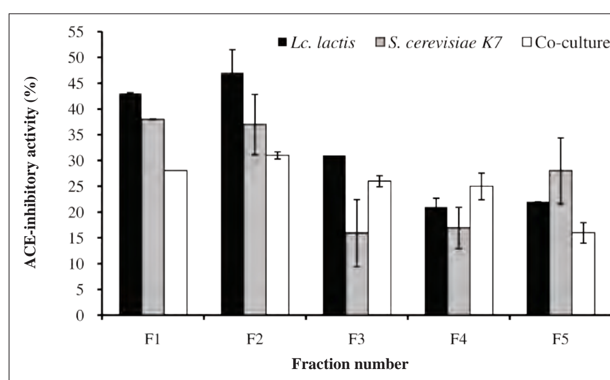

Figure 5: ACE-inhibition percentages (%) of different whey fractions of fermented milk obtained from semi-preparative HPLC after 72h of fermentation with *L. lactis* subsp. *lactis* NBRC 12007, *S. cerevisiae* K7 and co-culture (F=fraction). Data are expressed as mean ± SEM (n = 3) whereas the significance was determined at $\alpha = 0.05$.

DISCUSSION

ACE-inhibitory activity of fermented milk

The scope of this study was to determine the *in vitro* ACE-inhibitory activity of milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 and *S. cerevisiae* K7 in monoculture and in co-culture. *L. lactis* is the major mesophilic microorganism used in the manufacture of a variety of fermented dairy products. It has been reported that the formation of bioactive peptides by LAB in fermented milk is a rare event as they cannot hydrolyze milk proteins into physiologically active substances (Meisel & Bockelmann, 1999). *L. lactis* is not considered as highly proteolytic as *Lb. helveticus* strains. However, the milk fermented with the *Lactococcus* strain used in this study; *L. lactis* subsp. *lactis* NBRC 12007, was able to hydrolyze milk proteins into ACE-inhibitory peptides with an average inhibitory activity of 33 %. This value is higher than that of the Danish commercial fermented milk product A38®, which has previously shown to have an ACE-inhibitory activity (31 %) similar to that of the Japanese Calpis sour milk (Nielson *et al*., 2009). The ACE-inhibitory value obtained for *L. lactis* subsp. *lactis* NBRC 12007 in the current study was considerably higher than that of the values reported by Nielson *et al*. (2009) for *L. lactis* subsp. *lactis* CHCC 3906 and CHCC 3923, and almost similar to that of the *L. lactis* subsp. *cremoris* F3 and W5. Further, it was considerably higher than that was fermented with *Lb. helveticus* strains, MI 1264, MI 637, MI 1169, MI 4080 and MI 1262, as well as *Lb. acidophilus* CHCC 3777 and *S. thermophilus* S2.

In contrast, *S. cerevisiae* is already being utilized in the dairy industry, specially in the production of antihypertensive Calpis sour milk in combination with *Lb. helveticus*. The ability of *S. cerevisiae* to grow in milk and to hydrolyze milk proteins into ACE-inhibitory peptides have been well documented (Roy *et al*., 2000; Vermeirssen *et al*., 2003). *S. cerevisiae* K7 is widely utilized in the manufacture of 'sake', which is well-known for its high proteolytic activity. Several ACE-inhibitory peptides have already been identified

from sake and sake lees (Saito *et al*., 1994), while the *in vivo* hypotensive activity of these peptides have been observed in spontaneously hypertensive rats (SHR) after oral administration. However, there is no evidence of using this strain in the dairy fermentation so far. The results of the present study showed that *S. cerevisiae* K7 is able to grow in milk and hydrolyze milk proteins in order to produce fermented milk with ACE-inhibitory activity both in monoculture and co-culture. It seems that considerable attention has been given to identify the ACE-inhibitory activity and related peptides originating from milk fermented with co-cultures of *S. cerevisiae* and *Lactobacillus* strains. In contrast, milk fermentation with *S. cerevisiae* and *Lactococcus* strains under mesophilic temperatures has received less attention. In this study, milk fermented with the co-culture showed an average ACE-inhibitory activity of 23 %, which is considerably higher than that of the values reported, even for some *Lb. helveticus* strains, *Lb. acidophilus* CHCC 3777 and *S. thermophilus* S2 (Nielson *et al*., 2009).

It has been argued that fermentation is ineffective in producing ACE-inhibitory active peptides from milk proteins. For instance, Pihlanto-Leppälä *et al*. (1998) reported that ACE-inhibitory activity was observed only after digestion of milk proteins with digestive enzymes but not after fermentation with different starter cultures. In addition, Vermeirssen *et al*. (2003) have observed that fermented whey protein samples did not show higher ACE-inhibitory activity after *in vitro* digestion compared to the unfermented whey protein digests. Nevertheless, a study conducted by Hata *et al*. (1996) using elderly hypertensive patients showed that the daily ingestion of Calpis sour milk has decreased systolic and diastolic blood pressure significantly after 4 weeks of treatment compared with patients ingesting chemically acidified milk as a placebo. In addition, *in vivo* hypotensive ability of different fermented dairy products produced with various starter cultures including *Lactococcus* and *Saccharomyces* strains have been tested on SHR and human models (Fitzgerald & Murray, 2006). All these observations suggest that fermentation plays a crucial role in ACE-inhibitory activity in milk. The milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 and *S. cerevisiae* K7 and the co-culture showed significantly higher *in vitro* ACE-inhibitory activities than chemically acidified milk (1.3 %) as reported by Nielson *et al*. (2009). Further, Nakamura *et al*. (1995) have found that some of the peptide sequences obtained after microbial fermentation of milk have not yet been found upon enzymatic digestion. Therefore, it can be predicted that these strains are also able to produce novel peptide sequences; thus the identification of these ACE-inhibitory peptides in each fraction is in progress. On the other hand, some authors have argued that more ACE-inhibitory peptides breakdown than the new peptides formed during fermentation leading to a decrease in the overall ACE-inhibitory activity (Mullally *et al*., 1997). However, ACE-inhibitory activities of milk fermented with each starter culture in the present study was found to be moderate even after 72 hours of fermentation, suggesting that a considerable ACE-inhibitory activity can still be attained even after a long fermentation period. Moreover, the highest ACE-inhibitory activities in all treatments can be attributed to the early eluting fractions of the fermented milk products, which contain short peptides. It is suggested that these small peptides, which originated after a long period of fermentation could be more resistant to gastrointestinal digestion upon oral administration since they have a lower susceptibility to further breakdown into short peptides with the activity of gastric enzymes. Despite *S. cerevisiae* showing the highest inhibitory activity in fraction 5, it was well below the values observed in the fractions having short peptides (fraction 1 and 2). One can argue that the long peptides originated from the hydrolytic activity of this yeast strain may exhibit higher inhibitory activities *in vitro*. However, the observed inhibitory activities can breakdown as they are susceptible to intestinal proteolytic enzymes during gastro-intestinal digestion.

Growth profile of the microorganisms

As *S. cerevisiae* K7 has never been employed in dairy fermentation, its ability to grow in milk and hydrolyze milk proteins into different peptides were determined by evaluating the cell density, acid production and peptide concentration in the fermentation medium, with reference to that of the *L. lactis* subsp. *lactis* NBRC 12007 over the experimental period. Growth profiles of the monocultures and co-culture of the strains showed that the OD_{590} value in the fermentation medium decreased after it peaked. This could be due to the competition among individual microorganisms for nitrogen and energy sources, low availability of the substrates in the medium during the latter part of the fermentation process and autolysis of microbial cells. Accelerated growth of *L. lactis* subsp. *lactis* NBRC 12007 during the initial phase suggests that it was able to utilize the available substrates in the medium at a rapid rate. A higher cell density was observed 48 hours after the milk was fermented with *Lactococcus* strain in monoculture, while it decreased in other treatments. This may be due to its ability to grow in the fermentation medium by utilizing nitrogen substrates released from the autolysis of microbial cells and traces of lactose or some other organic acid available in the medium as the carbon source. The highest cell density at the end of the fermentation process was observed

in the milk fermented with the co-culture. This was confirmed by the viable cell counts obtained at the end of the fermentation in which the co-culture accounted for a sum of 13×10^6 cfu/mL. It seems that the co-culture favours the growth of yeast, since the number of viable yeast cells was higher than that of the monoculture. This may be due to favourable interactions such as mutualism, commensalism and symbiosis between yeast and lactococci. It has been reported that LAB lowers the pH while producing organic acids, which are then utilized by the yeasts as an energy source that improves their growth. Then the growing yeast provides growth factors (amino acids, certain vitamins and other compounds essential for bacterial growth) while elevating the acid production, which would then enhance bacterial growth (Viljoen, 2001). Low viable counts observed for *S. cerevisiae* K7 in monoculture may be due to the absence of these favourable interactions in addition to the inability to utilize available lactose in milk once the added glucose is completely utilized. The less viable lactococci counts observed in the co-culture than that of the monoculture may be due to the antagonistic effect of the yeasts as they secrete antibacterial compounds (Viljoen, 2001).

The growth of *S. cerevisiae* in monoculture was characterized by four clearly demarcated growth phases resembling the typical growth of yeast in fermentation. A lag phase was observed until 12 hours, in which the yeast cells acclimate to the growing conditions. The exponential growth/multiplication phase followed for 24 hours, where the highest cell density was observed. Then a stationary phase lasted upto 36 hours, in which the yeast cells actively converted sugar into alcohol followed by an exponential decline phase observed until the end of the fermentation, where the cell density continuously decreased due to the accumulation of alcohol, which is toxic to the yeast cells.

The unfermented milk samples kept under similar experimental conditions during different time periods corresponding to that of each sample point served as blanks. Therefore, the OD_{590} value measured at each sample point should provide more accurate measurements as it may eliminate any interference caused by other components present in the milk apart from microbial cells.

Acid production

Microbial fermentation plays a crucial role in acid production that helps in coagulating milk and hindering the growth of unwanted microorganisms including those contributing to the spoilage of dairy products. Although *S. cerevisiae* is a non-lactose fermentative yeast, it was able to produce acids during fermentation. It is likely that *S. cerevisiae* K7 has grown in milk during the initial growth phase by utilizing the added glucose (1 % w/v) as the carbon-source. Moreover, it is an alcohol fermentative yeast that produces CO_2 as a by-product, which produces carbonic acid (H_2CO_3) in an aqueous medium. Therefore, the accumulation of H_2CO_3 can be the most possible reason behind the observed pH decrease during the first 12 hours of fermentation with respect to *S. cerevisiae* K7. Absence of readily available glucose in the medium may have halted the growth of yeast for the next 20 hours before it was able to restart the acid production by utilizing the energy and N-sources released from the autolyzed yeast cells. The fastest acidification to pH 4.6 and the continuation of acid production in the milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 suggests that it is the most efficient strain in acid production.

Peptide concentration

In this experiment, milk samples kept under similar experimental conditions corresponding to each sample point were used as blanks. This would help to obtain a more accurate determination of the peptide concentrations at each sample point as it would eliminate any possible interference caused by peptides originated from proteolytic activity of the remaining heat resistant proteases other than the peptides originating from microbial fermentation. The higher peptide concentration observed in the milk fermented with the co-culture may be due to the combined proteolytic actvity of the two strains. It has been reported that the yeast protease B activity of *S. cerevisiae* is optimal at pH 4.8 (Roy *et al.,* 2000). This could be the main reason for the increase in peptide concentration in the milk fermented with *S. cerevisiae* K7 during the latter part of the fermentation once its pH reached 4.8. It seems that the lactococci strain in monoculture was producing more peptides at the beginning of fermentation coupled with its increasing cell density during the same period. However, the same phenomenon was not obserevd in the latter part of fermentation as the peptide concentration was decreasing although there was a slight increase in cell density. Therefore, it can be suggested that ample amounts of N- and C-sources available in the medium (milk proteins and added glucose, respectively) at the beginning of fermentation might also help to produce more peptides. Based on this assumption, it can be argued that the lower peptide concentrations observed for the lactococci in monoculture may be due to the utilization of available peptides for cell growth during the latter part of fermentation.

CONCLUSION

For the first time, this study reports the occurrence of *in vitro* ACE-inhibitory activity of milk fermented with *L. lactis* subsp. *lactis* NBRC 12007 and *S. cerevisiae* K7 in monoculture and co-culture under mesophilic temperature. The ACE- inhibitory activities in fermented milk were moderate after 72 hours of fermentation in which the highest inhibitory activity was found in the monoculture of *L. lactis* subsp. *lactis* NBRC 12007. *S. cerevisiae* K7 can be recommended for the functional food industry as a potential yeast strain, which can hydrolyze milk proteins into physiologically active peptides. Similarly, the combination of *L. lactis* and *S. cerevisiae* can be identified as a potential combination in manufacturing fermented dairy products with ACE-inhibitory activity. This combination will be much beneficial for the products, which usually have a long fermentation process such as cheese.

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