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Research Article

Antioxidant Activity of Peptides from Fermented Milk with Mix Culture of Lactic Acid Bacteria and Yeast

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Abstract: The aim of the present study is to investigate the production of antioxidant peptides during milk fermentation with co-culture of Lactic Acid Bacteria (LAB) and yeast. Five LAB strains, previously screened with higher hydrolysis activity and *Debaryomyces hansenii* H2 which isolated from Tibet kefir were used in the study. The peptides separated from fermented milk were analysed antioxidant activity with DPPH radical scavenging, hydroxyl radical scavenging, chelation of metal ions and reducing power assays. The growth of *Streptococcus. thermophilus*, *Lactobacillus. delbrueckii* ssp. bulgaricus and *Lactococcus. lactis* was enhanced with co-cultures and *L. acidophilus* was inhibited in co-culture with yeast. In co-culture with yeast, a significant decrease of the acidity was observed among all the fermentation and the pH reached higher values than in single LAB cultures. Except for *L.delbrueckii* ssp. bulgaricus, there was no significant difference of protein hydrolysis with other test LAB strains between co-culture and single culture. The co-incubation of LAB with the yeast developed a stronger antioxidant activity in DPPH radical and hydroxyl radical scavenging and no significant ($p>0.05$) difference in chelation of metal ions. The reducing power of *L. delbrueckii* ssp. bulgaricus and *L. helveticus* in co-culture was significant higher than those of single culture.

Keywords: Antioxidant peptides, co-culutre, *Debaryomyces hansenii*, fermented milk, lactic acid bacteria

INTRODUCTION

Reactive Oxygen Species (ROS) and free radicals are highly reactive molecules with one or more unpaired electrons, which can directly attack biological macromolecules, including DNA, RNA, protein and other substances and eventually cause cell injury (Behrend *et al*., 2003). Many degenerative diseases, including cancer, cardiovascular diseases, diabetes and aging, have been proved to be relevant with damaging effects of ROS and free radicals (Opara, 2004; Devasagayam, 2009). Food containing antioxidant substances, which can reduced the reactive activity of ROS and free radicals, may be used to help the human body to reduce oxidative damage.

Proteins of bovine milk, especially the Caseins, are a source of antioxidant peptides obtained either by enzymatic hydrolysis or by fermentation. The antioxidant activity of milk protein hydrolysates and individual peptides released after hydrolysis have been reported by several studies (Hernandez-Ledesma *et al*., 2005; Rival *et al*., 2001a, b). The antioxidant activity has been attributed to certain amino acid sequences and high concentrations of histidine and some hydrophobic amino acids (Suetsuna *et al*., 2000). The antioxidant peptides have been identified in fermented milk with lactic acid bacteria and these peptides had an important role in the oxidative stability of yoghurt (Abubakr *et al*., 2012; Virtanen *et al*., 2007).

Dairy yeast is the major member of the microflora and contribute to characteristics of cheese and fermentation milk (kefir, Kumis, etc.). Dairy yeasts interacted with LAB in many dairy products. They can ferment lactose and assimilate lactate to promote the growth of LAB and produce flavor compounds with proteolytic and lipolytic activities to give cheese characteristic flavor. However, little attention was drawn to antioxidant peptide production with yeast and LAB co-fermentation. *Debaryomyces hansenii* is a common species often found its occurrence in cheese and dairy product. It is reported that the caseinolytic activity of *D. hansenii* was higher than that of lactobacilli. The objective of this study was to investigate the interactions on growth, protein hydrolysis and antioxidant activity between selected LAB strains and dairy yeast *D. hansenii* by analyzing single- and co-cultures of yeasts and bacteria in milk.

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MATERIALS AND METHODS

Microorganism and medium: Yeast strains *D. hansenii* H2 was isolated and identified from Tibet kefir. LAB strains were screened from isolates originated from commercial starters with higher protein hydrolysis activity, including *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. bulgaricus, *Lactococcus lactis*, *Lactobacillus acidophilus* and *Lactobacillus helveticus*. YPD and MRS (de Man Rogosa and Sharpe) medium was used for yeasts and LAB cultivation repectively. YPD medium was composed of 2% (wt/vol) glucose, 0.5% (wt/vol) yeast extract, 1% (wt/vol) peptone. MRS medium was made of 4.8% MRS broth powder (HangZhou Medium Company) and 0.1% Tween80. Both media were sterilized at 121°C for 15 min. Yeasts and bacteria strains were preserved at -20°C YPD and MRS medium, to which 50% (v/v) glycerol had been added.

Preparation of fermented milk: Yeasts and LAB were inoculated in medium with 1% inoculum level from preserved tube as inoculum for milk fermentation. Yeasts was grown for 48 h at 28°C and LAB at 37°Cd. The preculture was inoculated with 1% (v/v) inoculums level each in 10% (w/v) reconstituting skim milk, which was sterilized at 108°C for 10 min in advance. The milk inoculated with yeast and LAB fermented at 30°C for 48 h.

Viable microorganisms: LAB were enumerated (pour plate) on cycloheximide (20 mg/L) MRS agar (1% agar added in MRS broth) and incubating at 37°C for 48 h. Yeast was enumerated (spread plate) on chloramphenicol (100 mg/L) YPD agar (1.5% agar) and incubated at 28°C for 48 h.

pH and total titratable acidity measurement: Fermented milk samples (10 g) were mixed with 90 mL of distilled water thoroughly. The pH value was recorded and the acidity was titrated using 0.1 mol/L NaOH to final pH 8.5. The TTA was expressed in mL 0.1 mol/L NaOH/10 g sample.

Protein hydrolysis: The level of protein hydrolysis was assayed by measuring free amino group of fermented milk using OPA method (Nielsen *et al*., 2001). 2 mL of sample was added to 1 mL of distilled water and mixed thoroughly, then 5 mL of 12% (v/w) Trichloroacetic Acid (TCA) was added and mixed together. After 10 min standing, the mixture was centrifuged at 3000 g for 10 min and the supernatant was collected. 0.4 mL of supernatant was added to 3 mL OPA reagent and reacted at room temperature for 20 min. The absorbance was measured with a spectrophotometer at 340 nm. The results, the OPA index were expressed as the concentration of serine of sample, whin corresponding to standard curve.

Preparation of fermented milk whey and peptides: The fermented milks were adjusted to 3.8 with 1 mol/L HCL. After centrifugation at 3000 g for 20 min, supernatant was collected and the pH was readjusted to 7.0 with 1 mol/L NaOH and recentrifuged at 3000 g for 10 min. The supernatant was collected for peptide concentration assay. Peptide concentration was was measured by the method of Lowry *et al*. (1951) with Bovine Serum Albumin (BSA) as standard (Lowry Protein Assay Kit, Shanghai Sangon Biotech). The supernatant (150 mL) was passed through cation exchange column (Dowex WX2, 2.60×40 cm), washing with water. The peptides were eluted with 200 mL of 2 mol/L aqueous ammonia. The eluent was lyophilized to evaporate ammonia and the residue re-dissolved in distilled water at a final concentration of 0.2 mg/mL for antioxidant activity assay.

Antioxidant activity assay:

DPPH radical scavenging activity: The sample (1 mL,) was mixed 1 mL of 0.1 mmol/L DPPH dissolved in 95% (v/v) ethanol. The mixture was shaken and left for 30 min at room temperature. Absorbance of the resulting solution was measured at 517 nm. Distilled water was used as blank instead of the sample. The scavenging activity was calculated using the following equation (Shimada *et al*., 1992):

$$\text{DPPH radical scavenging activity} = \frac{A_0 - A_S}{A_0} \times 100$$

where,
A_0 = The absorbance at 517 nm of blank
A_s = The absorbance at 517 nm of sample

Hydroxyl radical scavenging activity: 1, 10-phenanthroline (0.75 mmol/L, in phosphate buffer pH 7.4) and FeSO4 (0.75 mmol/L, in phosphate buffer pH 7.4) were mixed thoroughly. Then H_2O_2 (0.01%) and the sample were added. The mixture was incubated at 37°C for 60 min and the absorbance was measured at 536 nm. The scavenging activity was calculated using the following equation (De Avellar *et al*., 2004):

$$\text{Hydroxyl radical scavenging activity} = \frac{A_S - A_1}{A_0 - A_1} \times 100$$

where, A_S is the absorbance of the sample mixture, A_1 is the absorbance of control solution containing 1, 10-phenanthroline, $FeSO_4$ and H_2O_2 and A_0 is the absorbance of blank solution containing 1, 10-phenanthroline and $FeSO_4$.

Chelation of metal ions (Fe^{2+}): An aliquot of sample (0.2 mg/mL) was diluted to 3.7 mL with deionised water. A solution of 2 mmol/L ferrous chloride (0.1 mL) was added. After 3 min, the reaction was inhibited by the addition of 5 mmol/L ferrozine (0.2 mL). The mixture was shaken vigorously and left at room

temperature for 10 min. Absorbance of the resulting solution was measured at 562 nm. A blank without sample was prepared in a similar manner. EDTA (0.1 mg/mL) was used as positive control. The chelating capacity was calculated as follow (Dorman *et al*., 2004):

$$\text{Chelation Activity} = \frac{A_C - A_S}{A_C} \times 100$$

where,
A_S = The absorbance of the sample mixture
A_c = The absorbance of positive control

Reducing power: One millilitre of sample (0.2 mg/mL) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min followed by the addition of 1 mL 10% TCA. An aliquot (2 mL) from the incubation mixture was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride in test tubes. After 10 min the absorbance of resulting solution was measured at 700 nm. An increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid (0.2 mmol/L) was used as positive control (Dorman and Hiltunen, 2004).

Statistical analysis: The results were expressed as the mean±SD. The statistical analysis was performed using SPSS 16.0 (IBM SPSS, New York, USA). The significance of differences between the samples was analyzed with the one-way ANOVA analysis ($p<0.05$).

RESULTS AND DISCUSSION

Viable microorganisms: The viable counts of LAB and yeast in single LAB culture and co-culture with *D. hansenii* H2. were showed in Table 1. After 48 h fermentation at 30°C, the final counts of LAB strains were in the range 8.06-9.55 log cfu/mL in single culture and 7.77-9.58 log cfu/mL in co-culture. The growth of most of test LAB strains, except *L. helveticus*, was significantly different in co-culture than single culture. In particular, the growth of *S. thermophilus*, *L.delbrueckii* ssp. bulgaricus and *Lc. lactis* was enhanced with co-cultures. By contrast, *L. acidophilus* was inhibited in co-culture with yeast *D. hansenii* H2. The viable counts of the three *L. helveticus* were not changed statistically by co-culture with yeast. The viable counts of yeast *D. hansenii* H2 in co-culture with different LAB was variable. Compared to the viable counts reached at 6.64 log cfu/mL in single yeast culture, *D. hansenii* H2 was inhibited by all LAB strains and the final counts was in range of 5.54-6.38 log cfu/mL.

pH and Titratable acidity: The pH and titratable acidity after 48 h fermentation with each LAB strain with and without *D. hansenii* H2 were reported in Table 2. In single culture, four LAB strains lowered the pH from 6.5 to levels ranging between 3.53 to 4.24 and resulted in milk clotting. By contrast, *Lc. Lactis* reduced the pH to 5.15 and did not clot the milk. In co-culture with yeast, a significant decrease of the acidity was observed among all the fermentation and the pH reached higher values than in single LAB cultures. In correspondence with the result of viable counts, *D. hansenii* could lower the acidity to promote LAB growth. This can be explained with attribute to the ability of to ferment or assimilate lactic acid. *D. hansenii* also was able to use other organic acids, such as acetic acid which produced by heterofermentative LAB.

Protein hydrolysis: To evaluate the degree of protein hydrolysis during fermentation, free amino groups and peptides concentration of milk whey were determined by OPA method and Lowry method, respectively. The result showed that the amount of free amino groups depended on the strain used. *L. helveticus* showed the

Table 1: Viable counts of LAB and yeast in single LAB culture and co-culture with *D. hansenii* H2

LAB strains	Initial LAB inoculums (log cfu/mL)	Final LAB counts in sigle culure (log cfu/mL)	Final LAB counts in co-culutre (log cfu/mL)	Initial yeast inoculums (log cfu/mL)	Final yeast counts in co-culure (log cfu/mL)
S. thermophilus	6.29±0.15	8.20±0.14	8.75±0.21a	4.93±0.07	6.38±0.16
L. delbrueckii ssp. bulgaricus	6.68±0.24	8.06±0.23	8.70±0.12a	5.06±0.12	5.86±0.11
L. acidophilus	6.42±0.19	8.44±0.26	7.77±0.14a	5.04±0.13	5.73±0.19
L. helveticus	6.50±0.23	9.55±0.11	9.58±0.51	4.96±0.06	5.88±0.08
Lc. lactis	6.53±0.15	9.07±0.15	9.42±0.08a	5.09±0.05	5.54±0.05

A means the result of LAB in co-culture was significant different than in single culture ($p<0.05$)

Table 2: The pH and Titratable acidity in single LAB culture and co-culture with *D. hansenii* H2

LAB strains	pH in single LAB culture	pH in co-culture	Titratable acidity in single LAB culture (mL0.1M NaOH/10 g sample)	Titratable acidity in co-culture (mL0.1M NaOH/10 g sample)
S. thermophilus	4.24±0.13	4.47±0.08a	9.3±1.3	6.4±0.2a
L. delbrueckii ssp. bulgaricus	3.53±0.13	3.91±0.11a	15.9±1.2	10.3±0.4a
L. acidophilus	4.10±0.05	4.59±0.24a	13.8±0.9	8.1±0.8a
L. helveticus	3.82±0.10	4.45±0.21a	17±1.2	12.4±0.3a
Lc. lactis	5.15±0.07	5.67±0.12a	7.2±0.5	4.4±0.5a

A means the result of LAB in co-culture was significant different than in single culture ($p<0.05$)

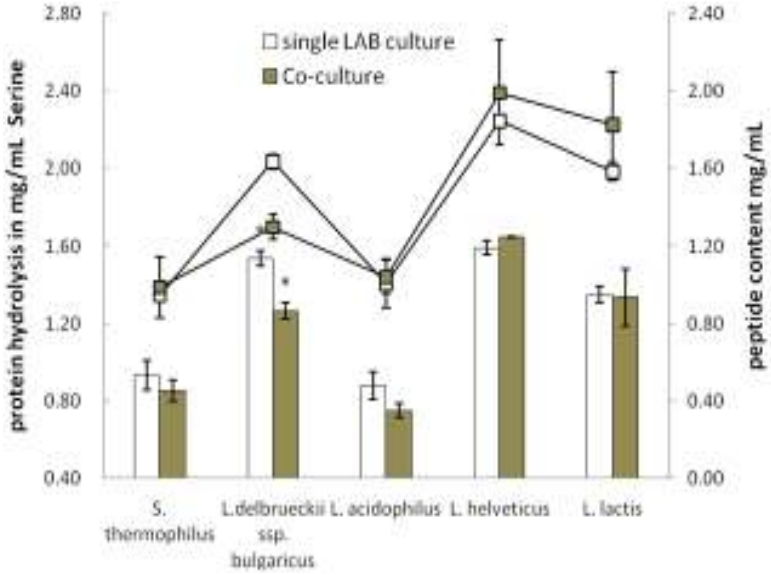

Fig. 1: Protein hydrolysis and peptide content of whey in single LAB culture and co-culture with yeast

Table 3: Antioxidant activity of peptides from fermented milk with single LAB culture and co-culture

Strains	DPPH scavenging activity (%)	Hydroxyl radical scavenging activity (%)	Fe^{2+} ion-chelation capacity (%)	Reducing power (absorbance at 700 nm)
S. t	70.56±4.94	38.36±2.26	42.34±3.60	0.269±0.041
S. t+ *D. h*	79.75±1.95[a]	46.76±1.86[a]	39.33±3.01	0.250±0.062
L. b	85.15±4.31	43.15±0.64	24.85±2.62	0.449±0.104
L. b+ *D. h*	93.75±1.05[a]	49.67±1.00[a]	26.16±2.94	0.567±0.128[a]
L. a	70.43±3.37	30.83±1.02	28.10±3.45	0.138±0.035
L. a+ *D. h*	79.04±1.12[a]	53.73±3.16[a]	26.50±2.10	0.146±0.054
L. h	63.02±2.04	37.48±1.45	23.20±1.44	0.451±0.117
L. h+ *D. h*	74.78±1.66[a]	42.46±2.03[a]	25.96±0.66	0.581±0.101[a]
Lc. l	80.62±2.48	40.53±1.20	37.14±0.75	0.213±0.061
Lc. l+ *D. h*	86.84±1.28[a]	45.48±2.51[a]	40.09±2.48	0.252±0.080

A means peptides within the same antioxidant assay of single and co-culture without a common letter differ significantly ($p<0.05$)

highest proteolytic activity, followed by *L. delbrueckii* ssp. bulgaricus and *Lc. Lactis*. The similar trend was observed in the result of peptides concentration. The *L. helveticus*, *L. delbrueckii* ssp. bulgaricus and *Lc. Lactis* samples had a higher proportion of peptides. In the case of co-culture with yeast, *L. delbrueckii* ssp. bulgaricus showed a lower amount of hydrolysed proteins both in OPA and peptide content assay when co-fermented with the yeast in comparison with the activity of the single culture. There was no significant difference of protein hydrolysis with remaining LAB strains between co-culture and single culture (Fig. 1).

Antioxidant activity of peptides: The antioxidant activity of peptides from single culture and co-cculture fermented milk was evaluated in four assay systems in vitro, including DPPH radical scavenging, Hydroxyl radical scavenging, Fe^{2+} chelation and reducing power (Table 3). The results showed that the DPPH radical-scavenging activity of all the samples was above 60%, ranged from 63.02 to 91.75%. The Hydroxyl radical scavenging activity of all the samples was in the range of 30.83% to 53.73%. Among all the test LAB strains, the milk co-fermented with the yeast showed an increase in both two radical assays than single culture. Milk protein derived peptides had ability to act as electron donors and could react with free radicals to convert them to more stable products. Kudoh *et al*. (2001) isolated antioxidant peptide from *L. delbrueckii* ssp. bulgaricus fermented milk and report that the peptide showed strong DPPH radical scavenging activity. This was in agreement with observations of our study that peptides released by fermentation can act as a good electron donors to scavenge radicals.

The ability to chelate with metal ions often contributes to some antioxidant activity. Transition metals are able to catalyze reaction to release reactive oxygen species, such as hydroxyl radical and superoxide anion (Stohs and Bagchi, 1995). Furthermore, ferrous ions can catalyses the breakdown of lipid peroxides which leads to the formation of off-flavour during the food shelf life. Peptides from fermented milk with test LAB strains exptressed between 23.20-42.34% of chelating ability at 0.2 mg/mL concentration. *S. thermophilus* and *Lc. lactis* showed higher activity of chelation in fermented milk peptides than other test LAB. There was no significant

($p>0.05$) difference higher or lower between single LAB culture and co-culture with yeast. Casein-derived peptides can inhibit lipid oxidation by chelating metals with some amino acid contained carboxyl and amino groups in the side-chain of the acidic and basic amino acids, such as Glu and Asp. The acidic amino acids contained of peptides was reported to contribute mainly to the chelation of transition metal ions (Saiga *et al*., 2003). The result of this study showed that the peptides from LAB fermented milk can chelate metal ions like ferrous ions.

The reducing power of peptide samples were listed in Table 3. Ascorbic acid, used as a positive control, had a reducing power of 0.751±0.052 increased absorbance at 700 nm at the concentration of 0.2 mmol/L. The peptides from *L. delbrueckii* ssp. bulgaricus and *L. helveticus* fermented milk showed higher reducing activities, which were equivalent to 60-67% of reducing power of positive control. In co-culture with yeast, the reducing power of these two strains was significant higher than those of single culture, of which were equivalent to 75-77% of reducing power of positive control. In contrast, the reducing activities of peptides with the remained LAB strains were relatively lower, which was in the range of 0.138-0.272. Meanwhile, the samples fermented with the remained LAB strains were not found a significant difference in reducing activity between co-culture and single culture. The high reducing power of fermented milk peptides could be attribute to the reducing ability amino acid present in the sequence which were exposed by protein hydrolysis during fermentation and could react with free radicals to stabilize and block chain reactions.

CONCLUSION

In the present study, the co-fermentation of different LAB with the yeast *Debaryomyces hansenii* was investigated. The result showed that the yeast can use the fermentation products for its growth to lowered the acidity and enhance the growth of *S. thermophilus*, *L. delbrueckii* ssp. bulgaricus and *Lc. lactis*. Except for *L. delbrueckii* ssp. bulgaricus, there was no significant difference of protein hydrolysis with other test LAB strains between co-culture and single culture. The yeast can increase the antioxidant activity of fermented milk peptides in DPPH radical and hydroxyl radical scavenging. The ability of peptides from LAB yeast co-fermented milk to interact with radical species could lead to the development of novel food ingredients relevant in health promotion and disease prevention.

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