Combination of germination and fermentation to improve antioxidant activity of soymilk

HENDRY NOER FADLILLAH – LILIS NURAIDA – AZIS BOING SITANGGANG – NURHENI SRI PALUPI

Summary
Combination of germination and fermentation is expected to enhance the production of bioactive substances in plant-based foods. The present study aimed to improve antioxidant activity of soymilk by combining soybean germination and lactic fermentation of the soymilk made from germinated soybean. The results showed that *Pediococcus acidilactici* YKP4 and *Lacticaseibacillus rhamnosus* BD2 were the isolates that produced highest antioxidant activity in germinated soymilk. Germination increased the content of reducing sugars with the highest values reached after 72 h, but germination time of soybean did not affect the growth of *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2. Germination increased significantly ferric reducing antioxidant power (FRAP) after 24 h and 2,2-diphenyl-1-picrylhydrazy (DPPH) scavenging activity after 48 h as Trolox equivalents increased from 225.94 ± 1.87 g·kg^{-1} to 247.47 ± 2.10 g·kg^{-1} and from 21.16 ± 1.05 g·kg^{-1} to 28.06 ± 0.66 g·kg^{-1}, respectively. Antioxidant activity of fermented germinated soymilks was higher than of the unfermented counterpart, with the highest activities obtained using *P. acidilactici* YKP4 after 24 h as expressed in DPPH radical-scavenging activity of 152.53 ± 7.57 g·kg^{-1} and FRAP of 339.62 ± 0.91 g·kg^{-1}. Fermentation with *P. acidilactici* YKP4 also produced the highest peptide concentration of 2.38 ± 0.14 mg·ml^{-1}.

Keywords
antioxidant; germination; fermentation; lactic acid bacteria; peptide

Soya-containing food has been known for the excellent nutrition value and, in particular, as a source of proteins and lipids [1]. It is also known to contribute to reducing the risk of several diseases such as coronary heart disease or cancer [2]. Isoflavones are the biologically active compounds in soybean that have received the most attention, because of their multiple functions such as estrogenic effects and antioxidant activity [3]. The nutrient content, their availability and bioactive compounds present in the soybean are affected by processing that the beans undergo [1]. One of the processes that affect the nutrient bioavailability in soybean is germination. It may improve bioavailability of nutrients and hence increase health benefits of legume seeds, by repressing antinutrient factors such as trypsin inhibitors or phytate, which both inhibit nutrient absorption [4]. Degradation of carbohydrates and lipids also takes place during germination [5]. Total soluble sugars were found to increase in germinated soybean, mainly glucose [6].

The other process that affects the nutrient bioavailability in soybean is fermentation. Fermented soya products were reported to exhibit health benefits, such as antioxidant and anti-inflammatory activities [7]. Lactic acid bacteria (LAB) are involved in fermentation of many types of foods. Certain LAB are also known as probiotics. The use of probiotic LAB in fermentation could promote positive effects on health through their probiotic activity and by producing beneficial metabolites or bioactive compounds [8]. Fermented foods are the potential source of probiotics. Some of

Hendry Noer Fadlillah, Department of Food Science and Technology, IPB University, Dramaga Campus, Bogor 16680, Indonesia; Department of Food Technology, Faculty of Life Science, International University Liaison Indonesia (IULI), Intermark South Tangerang 15310, Indonesia.
Lilis Nuraida, Nurheni Sri Palupi, Department of Food Science and Technology, IPB University, Dramaga Campus, Bogor 16680, Indonesia; Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University, Dramaga Campus, Bogor 16680, Indonesia.
Azis Boing Sitanggang, Department of Food Science and Technology, IPB University, Dramaga Campus, Bogor 16680, Indonesia.

Correspondence author:
Lilis Nuraida, e-mail: lnuraida@apps.ipb.ac.id

LAB strains isolated from Indonesian kefir grain, including *Lactobacillus kefiri* and *Lacticaseibacillus rhamnosus*, were reported to be probiotics [9]. Proteolytic activity of LAB degrades proteins into peptides, from which several of them are biologically active, such as antioxidants, lowering cholesterol effects, angiotensin converting enzyme (ACE) inhibitors or immunomodulators [10, 11]. In addition to this, lactic acid fermentation was also reported to improve bioavailability of isoflavones in soya products by converting the glucosidic form into aglycone moieties, the latter being easier absorbed in the body [12, 13].Combination of germination and fermentation could be applied to improve antioxidant properties of soya products and enhance their functional characteristics. The purpose of this research was to evaluate the effect of germination on the growth of LAB and antioxidant activity of soymilk during lactic fermentation.

MATERIALS AND METHODS

Cultures

Pediococcus acidilactici YKP4, *Lactiplantibacillus plantarum* 1W22408, *Limosilactobacillus fermentum* S206, *Lacticaseibacillus rhamnosus* BD2, *Lacticaseibacillus rhamnosus* R2, *Lactobacillus delbrueckii* BD7 and *Lactococcus lactis* ssp. *lactis* BD17 were obtained from Food Microbiology Laboratory, SEAFAST Center, IPB University (Bogor, Indonesia). The strains were previously isolated from tempe, kefir granules or breastmilk [9, 14] and were selected on the basis of the highest proteolytic activity [9, 15].

Selection of lactic acid bacteria

Preparation of starter cultures

LAB cultures were suspended in 10 ml of de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom) and then incubated at 37 °C for 24 h. The cultures were inoculated into soymilk (3 %, v/v) and incubated at 37 °C for 24 h to be used as starter cultures for the fermentations [15].

Soymilk preparation and fermentation

Soybean germination for soymilk was prepared based on Jiang et al. [16] with modifications. Soybeans was cleaned and soaked for 10 h at the ratio of dry soybean-to-water of 1 : 10. The soaking water was renewed every one hour. Germination was carried out for 24 h at room temperature (± 27 °C).

Germinated soybean was washed in tap water. Soymilk was prepared according to Zheng et al. [17] with minor modifications. Germinated soybean was soaked in hot water with a temperature of 85 °C for 20 min at the ratio of germinated soybean-to-water of 1 : 8, homogenized for 15 min and filtered using filter cloth. It was sterilized using retort MC-40 series (ALP, Tokyo, Japan) for 100 °C for 15 min [17]. After sterilization, the soymilk was cooled and inoculated with individual starters (2 %, v/v). Incubation was done at 37 °C for 24 h. The fermented milk was analysed for viable counts of LAB, pH, total soluble solid (*TSS*), antioxidants, proteins and peptides.

Enumeration of lactic acid bacteria

Enumeration of LAB viable counts was done according to Zhao and Shah [18] on MRS agar (Oxoid) using pour plate method and incubated at 37 °C for 24 h. Colonies on plates with 25–250 colonies were used for enumeration and the results were expressed as logarithm of colony forming unit per millilitre.

pH and total soluble solids

Total soluble solids (*TSS*) of soymilk were measured by refractometer (Atago, Tokyo, Japan) and expressed in degrees Brix. The pH value was analysed by using pH meter (pH 700, (Eutech Instruments, Singapore, Singapore).

Reducing sugars

Fermented germinated soymilk was homogenized and then centrifuged at 6 000 ×*g* for 15 min in a centrifuge Z 383 K (Hermle Labortechnik, Wehingen, Germany) [19]. The supernatant was collected and filtered through Minisart syringe cellulose acetate membrane filter (pore size 0.22 μm; Sartorius, Göttingen, Germany). The pH of the filtered supernatant was adjusted to 7.4 by adding 1.0 mol·l^{-1} NaOH. The supernatant was used for reducing sugar, antioxidant, peptide and protein analyses.

Reducing sugars were analysed by using the dinitrosalicylic acid (DNS) method [20]. One millilitre of the supernatant was mixed with 3 ml of DNS solution in the 10 ml test tube. The mixture was heated in the boiling water for 5 min and then cooled in iced water. The concentration was determined by measuring absorbance at a wavelength of 550 nm using UV-Vis spectrophotometer UV-2450 (Shimadzu, Tokyo, Japan). Glucose (concentration range 0–30 mg·ml^{-1}) was used as the standard to calculate the reducing sugars concentration. The standard curve was:

$$y = 1.183x + 0.102 \quad (1)$$

with a coefficient of determination of 0.9841.

Antioxidant activity

Antioxidant activity was determined as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity [21] and ferric reducing antioxidant power (FRAP) [22]. DPPH radical-scavenging activity was analysed by mixing 1.0 ml sample supernatant with 1.0 ml DPPH reagent (0.2 mmol·l^{-1} DPPH solution in 95% methanol, v/v) and incubated in the dark at room temperature (± 27 °C) for 30 min. Absorbance was measured at a wavelength of 517 nm. The antioxidant activity was calculated based on Trolox standard curve (concentration range 0–0.5 mg·ml^{-1}) as follows:

$$y = -1.5759x + 0.7660 \quad (2)$$

with a coefficient of determination of 0.9917. The analytical result was expressed as Trolox equivalents.

FRAP value was analysed by reacting 100 μl sample with 10 μl of FRAP reagent consisting of 10 ml of 300 mmol·l^{-1} acetate buffer (pH 3.6), 1 ml of 10 mmol·l^{-1} 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) solution (10 mmol·l^{-1} TPTZ in 40 mmol·l^{-1} HCl) and 1 ml of 20 mmol·l^{-1} $FeCl_3$ solution). It was incubated at 37 °C for 30 min. Absorbance was measured at a wavelength of 593 nm [22]. Trolox (concentration range 0–0.5 mg·ml^{-1}) was used as the standard, giving a standard curve of

$$y = 1.9431x - 0.2038 \quad (3)$$

and a coefficient of determination of 0.9903. The analytical result was expressed as Trolox equivalents.

Proteins and peptides concentration

Protein concentration was analysed by Lowry's method [23]. The supernatant (0.5 ml) was reacted with 0.7 ml Lowry's reagent consisting of 1 ml of solution A (20 mmol·l^{-1} $CuSO_4{\cdot}5H_2O$ and 30 mmol·l^{-1} Na-citrate) and 50 ml of solution B (0.1 mol·l^{-1} Na_2CO_3 and 0.1 mol·l^{-1} NaOH). The incubation was done for 20 min at room temperature (± 27 °C). The mixture was added with 0.1 ml Folin-Ciocalteu solution (Sigma Aldrich, St. Louis, Missouri, USA). After incubation for 30 min, absorbance was measured at a wavelength of 750 nm. Bovine serum albumin (BSA) was used as a standard (concentration 0–3 mg·ml^{-1}) giving a standard curve of

$$y = 0.0003x + 0.0011 \quad (4)$$

with a coefficient of determination of 0.9858.

Peptides concentration was analysed by the *o*-phthaldialdehyde (OPA) method [24]. Supernatant (50 μl) was added with 2 ml of OPA reagent (consisting of 25 ml of 100 mmol·l^{-1} of sodium tetraborate, 2.5 ml of 20% w/w sodium dodecyl sulfate and 1.1 ml of OPA solution, mixed with 21.4 ml of H_2O), then incubated at room temperature (± 27 °C) for 2 min. absorbance was measured at a wavelength of 340 nm. Tryptone casein (Sigma Aldrich) of concentration 0–3 mg·ml^{-1} was used to obtain the standard curve

$$y = 0.0002x + 0.0485 \quad (5)$$

with a coefficient of determination of 0.9920.

Effect of germination

Soybean germination was done as mentioned above with the germination time up to 72 h. Preparation of fermented soymilk from germinated soybean was done using a similar method as above. In this step, *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2 representing the cultures with the highest proteolytic activity and the highest antioxidant activity were used to ferment soymilk. Fermentation was done at 37 °C for 24 h. Analyses of fermented germinated soybean milk were done for viable counts of LAB, pH, *TSS*, antioxidants, proteins and peptides.

Effect of fermentation time

The cultures with the highest antioxidant activity were used in this step. The soybean was germinated for 24 h and then fermented for 0, 24, 48, and 72 h. Viable counts of LAB, pH, *TSS*, antioxidants, proteins and peptides were analysed. The optimum fermentation time was the one that produced highest antioxidant activity.

Statistical analysis

The mean values and standard deviations were calculated from triplicate trials. The data were analysed by Analysis of Variance, followed by Duncan's Multiple Range tests at significance $p < 0.05$ by SPSS software version 25 (IBM, New York, New York, USA).

RESULTS AND DISCUSSION

Selection of lactic acid bacteria

Growth of lactic acid bacteria

The seven isolates grew in germinated soymilk as demonstrated by the increase in LAB counts and the decrease in pH (Tab. 1). At the start of fermentation, LAB counts were approximately 6 log CFU·ml^{-1} and reached 8.68–9.15 log CFU·ml^{-1} after 24 h. There were no significant differences in LAB counts between the isolates after 24 h fermentation, however, the pH

Tab. 1. Characteristics of soymilk produced from germinated soybeans, after 24 h of fermentation.

	Total LAB [log CFU·ml^{-1}]	pH	Proteins [mg·ml^{-1}]	Peptides [mg·ml^{-1}]
Soymilk after germination at 0 h fermentation	6.17 ± 0.03 ^{a}	7.08 ± 0.01 ^{a}	3.96 ± 0.11 ^{a}	0.73 ± 0.02 ^{ab}
Soymilk fermented by lactic acid bacteria				
Pediococcus acidilactici YKP4	8.85 ± 0.28 ^{b}	5.51 ± 0.04 ^{d}	3.90 ± 0.09 ^{a}	2.38 ± 0.14 ^{e}
Lacticaseibacillus rhamnosus BD2	9.15 ± 0.03 ^{b}	5.20 ± 0.09 ^{ef}	3.91 ± 0.10 ^{a}	0.92 ± 0.09 ^{bcd}
Lactobacillus delbrueckii BD7	8.82 ± 0.21 ^{b}	5.68 ± 0.03 ^{c}	3.93 ± 0.03 ^{a}	1.02 ± 0.10 ^{cd}
Limosilactobacillus fermentum S206	9.11 ± 0.04 ^{b}	5.12 ± 0.06 ^{fg}	3.95 ± 0.02 ^{a}	2.32 ± 0.25 ^{e}
Lactiplantibacillus plantarum 1W22408	8.80 ± 0.27 ^{b}	5.24 ± 0.10 ^{e}	3.91 ± 0.04 ^{a}	1.16 ± 0.13 ^{d}
Lacticaseibacillus rhamnosus R2	8.68 ± 0.24 ^{b}	5.10 ± 0.01 ^{g}	3.93 ± 0.01 ^{a}	0.98 ± 0.03 ^{bcd}
Lactococcus lactis ssp. *lactis* BD17	8.69 ± 0.29 ^{b}	5.98 ± 0.04 ^{b}	3.93 ± 0.03 ^{a}	0.84 ± 0.08 ^{bc}

Different superscripts in the same column indicate significant differences ($p < 0.05$) between samples.
LAB – lactic acid bacteria.

Tab. 2. Antioxidant activity of fermented soymilk produced from germinated soybeans.

	DPPH scavenging activity [g·kg^{-1}]	FRAP [g·kg^{-1}]
Soymilk after germination at 0 h fermentation	44.24 ± 4.77 ^{ab}	225.54 ± 4.68 ^{b}
Soymilk fermented by lactic acid bacteria		
Pediococcus acidilactici YKP4	152.53 ± 7.57 ^{e}	339.62 ± 0.91 ^{g}
Lacticaseibacillus rhamnosus BD2	122.96 ± 9.87 ^{d}	320.50 ± 8.21 ^{f}
Lactobacillus delbrueckii BD7	55.83 ± 8.31 ^{bc}	253.20 ± 1.07 ^{d}
Limosilactobacillus fermentum S206	69.35 ± 11.32 ^{c}	286.22 ± 0.85 ^{e}
Lactiplantibacillus plantarum 1W22408	54.34 ± 12.38 ^{abc}	247.00 ± 1.00 ^{cd}
Lacticaseibacillus rhamnosus R2	53.90 ± 10.94 ^{abc}	281.47 ± 5.69 ^{e}
Lactococcus lactis ssp. *lactis* BD17	46.57 ± 8.03 ^{ab}	242.53 ± 1.03 ^{c}

Different superscripts in the same column indicate significant differences ($p < 0.05$) between samples. Antioxidant activity is expressed as Trolox equivalents.
DPPH – 2,2-diphenyl-1-picrylhydrazyl, FRAP – ferric reducing antioxidant power.

values were different between isolates. The decrease in pH value of germinated soymilk samples after fermentation indicated the LAB activity producing lactic acid and other organic acids. The growth of LAB in fermented soymilk was also reported by other researchers, who reported LAB counts of 8.98–9.62 log CFU·ml^{-1} [15] and 8.54–9.20 log CFU·ml^{-1} [17] after 24 h fermentation in soymilk. The pH value of the fermented soymilk was also reported to decrease with increasing the fermentation time [17].

Peptides concentration

Fermentation increased the peptide concentration and the increases were different between isolates indicating variation in proteolytic activities between isolates (Tab. 1), however the protein concentration was not different before and after fermentation by all isolates. The highest proteolytic activity was indicated by the highest peptide concentration. *P. acidilactici* YKP4 produced the highest peptide concentration followed by *Limosilactobacillus fermentum* S206 (Tab. 1). Formation of peptides during soymilk fermentation was also reported by Rubak et al. [15] with concentration in the range of 2.86–4.68 mg·ml^{-1} after 24 h fermentation. The peptides are resulted from the proteolytic activity of LAB to break down protein into simpler compounds [25].

Antioxidant activity

Fermentation by using *P. acidilactici* YKP4 provided highest antioxidant activity based on DPPH-scavenging activity and FRAP measurements. This strain was followed by *Lb. rhamnosus* BD2 as shown in Tab 2. Fermentation during 24 h by *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2 for 24 h increased DPPH radical-scavenging activity 3.45- and 2.80-fold, respectively, compared to from unfermented soymilk prepared after 24 h germination. *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2 were selected for further study based

on their ability to produce the highest antioxidant activity. The ability of *P. acidilactici* to enhance antioxidant activity was also reported by SHARMA et al. [25]. Application of *P. acidilactici* to ferment soymilk enhanced DPPH radical-scavenging activity 1.8-fold, after 24 h fermentation [25]. Inoculation of *P. acidilactici* was previously found to increase total antioxidant activity of alfalfa silage and reduced lipid oxidation [26]. The ability of *Lb. rhamnosus* BD2 to enhance antioxidant activity in fermented reconstituted skim milk was reported by YUSUF et al. [19]. Fermentation of soymilk by *Lb. rhamnosus* CRL981 was previously found to improve antioxidant activity of soymilk. DPPH radical-scavenging activity reached 29.5 % after 24 h fermentation [27].

Antioxidant activity of fermented soymilk after germination could be derived from its peptides and isoflavones [28]. Hydrolysis of proteins in soymilk during fermentation generates peptides including those with antioxidant activity. *Limosilactobacillus fermentum* S206 produced higher amounts of peptides than *Lb. rhamnosus* BD2, but its antioxidant activity was lower, indicating that *Lb. rhamnosus* BD2 may produce peptides different from those of *Limosilactobacillus fermentum* S206. Bioactivity of peptides depends on several factors, such as the size of the molecule and amino acid sequences. Different peptide size and sequence will provide different bioactivity. Peptides with antioxidant activity were reported to include peptides with aromatic aminoacid residues, sulfhydryl groups

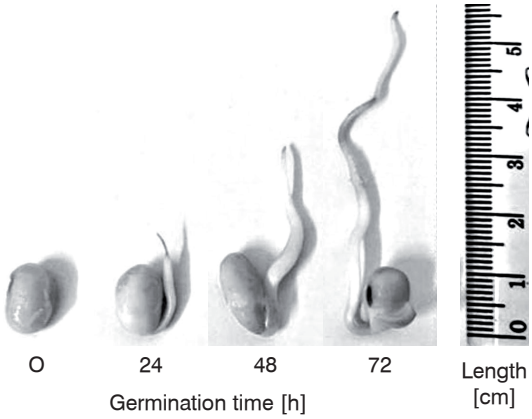

Fig. 1. Physical appearance of a soybean during germination.

(-SH), and alanine or leucine at N or C terminus [13, 19, 29]. Besides the presence of bioactive peptides, antioxidant activity of fermented soymilk may also be influenced by phenolic compounds. Soya products are an excellence source of isoflavones that are converted into active forms by fermentation. Activity of β-glucosidase of LAB may contribute to the increase of soymilk antioxidant activity by converting glucoside isoflavone to their aglycone form. The bioavailability and antioxidant activity of isoflavone aglycones is known to be higher than those of the glucosides [13, 18]. Antioxidant activity of soymilk was previously reported to increase during fermentation. This was related

Tab. 3. Effect of germination on fermentation profile of soymilk.

Germination time [h]	pH	Total soluble solids [°Brix]	Reducing sugars [mg·ml^{-1}]	Total lactic acid bacteria [log CFU·ml^{-1}]
Soymilk before fermentation				
0	7.17 ± 0.08	4.70 ± 0.10^{a}	4.78 ± 0.04^{a}	
24	7.14 ± 0.06	4.90 ± 0.10^{a}	11.62 ± 1.64^{b}	
48	7.09 ± 0.05	5.10 ± 0.10^{a}	22.69 ± 1.45^{c}	
72	7.07 ± 0.01	5.10 ± 0.20^{a}	45.03 ± 2.57^{d}	
Soymilk fermented by *Pediococcus acidilactici* YKP4				
0	5.05 ± 0.20^{a}	2.00 ± 0.10^{a}	4.88 ± 0.15^{a}	8.68 ± 0.27^{a}
24	5.21 ± 0.17^{a}	1.90 ± 0.10^{a}	5.73 ± 0.45^{b}	8.59 ± 0.44^{a}
48	5.01 ± 0.17^{a}	1.60 ± 0.10^{b}	18.77 ± 0.18^{c}	8.41 ± 0.74^{a}
72	5.48 ± 0.07^{a}	1.50 ± 0.00^{b}	27.24 ± 0.47^{d}	8.78 ± 0.46^{a}
Soymilk fermented by *Lacticaseibacillus rhamnosus* BD2				
0	4.95 ± 0.00^{a}	2.50 ± 0.10^{a}	7.61 ± 0.35^{a}	9.63 ± 0.40^{ab}
24	4.87 ± 0.06^{a}	2.00 ± 0.10^{b}	10.12 ± 0.23^{b}	9.28 ± 0.02^{a}
48	5.20 ± 0.09^{b}	2.00 ± 0.00^{b}	16.11 ± 1.76^{c}	9.42 ± 0.04^{ab}
72	5.51 ± 0.09^{c}	1.60 ± 0.10^{c}	20.70 ± 1.51^{d}	9.88 ± 0.32^{b}

Different superscripts in the same column indicate significant differences ($p < 0.05$) between samples.

to biotransformation of isoflavones. The glucoside isoflavones decreased during fermentation, while the aglycones increased [18].

Effects of germination time

Chemical composition and antioxidant activity

The physical appearance during germination of soybean is shown in Fig. 1. Tab. 3 shows that *TSS* did not increase significantly during the germination process, but the level of reducing sugars increased significantly after 24 h, 48 h and 72 h of germination. Bueno et al. [30] indicated that the increase in reducing sugars is the result of amylase activity, which releases free glucose from complex carbohydrates [30]. Germination activates α-amylase resulting in more digestible carbohydrates, including monosaccharides such as glucose and galactose [31]. This result was also in line with results of the previous research that showed an increase in concentration of glucose and galactose in soya after germination, with the highest increase observed in glucose [6].

The germination process increased the DPPH radical-scavenging activity and FRAP of soymilk (Fig. 2). The highest DPPH radical-scavenging activity of soymilk was obtained from soybean germinated for 72 h. However, germination for 48 h provided the best antioxidant activity based on the FRAP measurement. The increase in antioxidant activity in germinated soybean extract was in line with the increase in total phenolic compounds and flavonoids [28] and the total isoflavone aglycones [32]. Total phenolics and flavonoids content of germinated grains increased by 4- and 2.5-fold from non-germinated beans and total isoflavone aglycones increased 2.5-fold after three days of germination [32]. Germination was also reported to release peptides that may also contribute to the increase in antioxidant activity and bioavailability of proteins [30].

Fermentation of germinated soymilk

The pH value of germinated soymilks decreased after fermentation. The decrease was a result of fermentation that metabolized the soluble solid as shown by the significant decrease in *TSS* after fermentation by *P. acidilactici* YKP4 or *Lb. rhamnosus* BD2. During fermentation, LAB metabolize soluble nutrients [33], including reducing sugars as also shown by the lower concentration of reducing sugars compared to the unfermented soymilk (Tab. 3). Fermentation of soymilk obtained from 72 h germination resulted in the highest decrease of reducing sugars in soymilk fermented by *P. acidilactici* YKP4 (from 45.03 mg·ml^{-1} to 27.24 mg·ml^{-1}) and by *Lb. rhamnosus* BD2 (from 45.03 mg·ml^{-1} to 20.70 mg·ml^{-1}). The decrease in reducing sugars in soya yoghurt was previously reported by Kim and Han [20]. Concentration of reducing sugars in soya yoghurt was decreased by 0.1–0.6 % during 24 h to 96 h fermentation [20]. The germination process could give advantage to the growth of LAB by hydrolysis of carbohydrates, which produces simple sugars that may serve as the source of energy for LAB [20, 33].

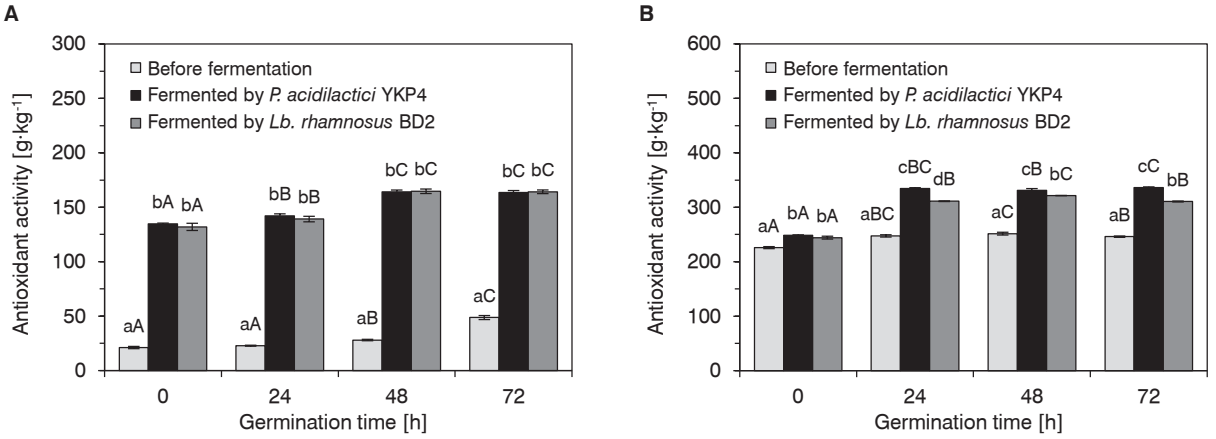

Fig. 2. Antioxidant activity of germinated soymilk before and after fermentation 24 h with different time of germination.

A – DPPH scavenging activity, B – ferric reducing antioxidant power.
Different superscripts indicate significant differences ($p < 0.05$) between samples. Lowercase letters represent differences between treatment within the same germination time. Capital case represent differences between germination time within the same treatment.
Antioxidant activity is expressed as Trolox equivalents. DPPH – 2,2-diphenyl-1-picrylhydrazyl.

The growth of *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2 was not affected by the germination time. The viable counts were not significantly different among the germination times ($p < 0.05$) as shown in Tab. 3. In previous studies, when the viable counts reached 9 log CFU·ml^{-1}, the growth reached the maximum [17]. The present results were also in line with LAB counts determined previously in soymilk fermented by *Lb. rhamnosus* NS4 (7.70 log CFU·ml^{-1}) and NS6 (9.25 log CFU·ml^{-1}) for 24 h [34]. Other study showed that the maximum counts of *P. acidilactici* in soymilk were 10^8 CFU·ml^{-1} and declined subsequently [25].

Fermentation increased antioxidant activity of soymilk with or without previous germination based on DPPH radical-scavenging activity and FRAP assay (Fig. 2). The increase was higher than by germination alone. Fermentation of soymilk after germination resulted in higher DPPH radical-scavenging activity (Fig. 2A). DPPH radical-scavenging increased 6.36- and 6.24-fold after fermentation by *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2 for 24 h, respectively. The increase in the antioxidant activity after fermentation of germinated soybeans was not intensified by the germination time. The highest effect of 24 h fermentation by *P. acidilactici* YKP4 was observed in case of soybeans germinated for 24 h (6.22-fold increase), followed by 48 h (5.85-fold) and 72 h (3.36-fold). Similar effects were observed for fermentation by *Lb. rhamnosus* BD2 for 24 h. The highest increase was observed in soybeans germinated for 24 h (26.08-fold increase), and then 48 h (5.87-fold) and 72 h (3.37-fold). The DPPH radical-scavenging activity indicated that those soymilks contained compounds being able to donate hydrogen to stabilize free radicals.

Similar pattern of fermentation effects was also shown in the antioxidant activity as FRAP (Fig. 2B), although the increase was lower than that of DPPH radical-scavenging activity. The effect of fermentation was more pronounced in soymilk after 24 h germination, therefore germination of 24 h was considered as the most suitable for increasing the antioxidant activity by combination of germination and fermentation. In addition, undesirable flavour of soymilk with more pungent taste of sprouts appeared after 48 h and 72 h germination.

Effects of fermentation time

Growth of lactic acid bacteria

Fig. 3 shows the effect of the fermentation time on the growth of LAB in soymilk after 24 h germination. The LAB counts of both cultures increased sharply after 24 h fermentation, however, the counts of *P. acidilactici* YKP4 were lower. After 24 h, there was no significant increase in viable counts of *Lb. rhamnosus* BD2, while *P. acidilcatici* YKP4 showed considerable increase until the end of fermentation. At the end of fermentation, the viable counts of both cultures were similar. The present results indicated that the growth of *P. acidilactici* was slower than *Lb. rhamnosus* in soymilk after germination.

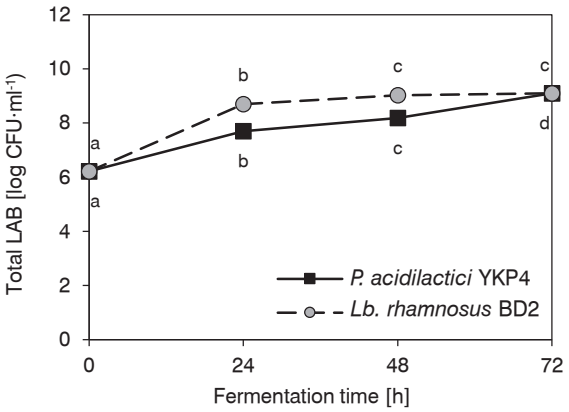

Fig. 3. Effects of the fermentation time on the viable counts of lactic acid bacteria in soymilk.

Different superscripts indicate significant differences ($p < 0.05$) between samples.
LAB – lactic acid bacteria.

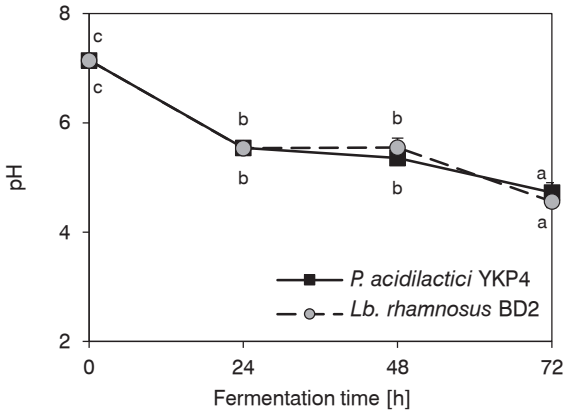

Fig. 4. The pH value during fermentation of soymilk produced from germinated soybean.

Different superscripts indicate significant differences ($p < 0.05$) between samples.

pH and total soluble solids

Fermentation time is very important, since it influences the quality and nutritional composition of fermented products, including the formation of bioactive compounds [35]. The substrates could be

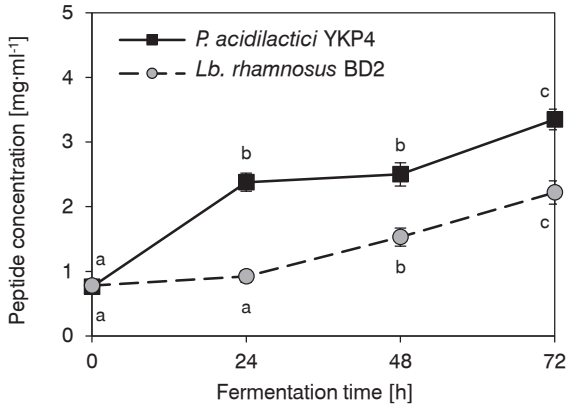

Fig. 5. Peptides concentration in soymilk produced from germinated soybean during fermentation.

Different superscripts indicate significant differences ($p < 0.05$) between samples.

degraded or transformed over the time and this may influence the level of bioactive compounds and their bioactivity [35]. The acidity of soymilks decreased significantly along fermentation time, both in *Lb. rhamnosus* BD2 and *P. acidilactici* YKP4 fermentation (Fig. 4). The initial pH of germinated soymilk was 7.15 ± 0.03 and decreased gradually during fermentation. The pH values after 24 h fermentation by *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2 were pH 5.55 ± 0.01 and pH 5.54 ± 0.01, respectively. These were significantly lower than those of unfermented samples. Although after 24 h fermentation the viable counts of *P. acidilactici* YKP4 were lower than those of *Lb. rhamnosus* BD2 (Fig. 3), there was no difference in the pH values. The pH values continuously decreased until the end of fermentation (72 h), reaching pH 4.73 ± 0.18 and pH 4.56 ± 0.11. The present results are in accordance with previous reports on soymilk fermentation [20, 25]. The decrease in pH is caused by LAB activity. During the growth of LAB, several organic acids are produced, including lactic acid and acetic acid [8]. The starter culture used in the present research was categorized as homofermenter [36, 37] with lactic acid as the major organic acid produced.

TSS values of fermented soymilks were significantly lower than those of unfermented counterparts after 24 h, but further fermentation from 24 h to 72 h did not change *TSS*.

Peptides concentration and antioxidant activity

The peptides concentration increased during fermentation, with the highest values reached in soymilk fermented of *P. acidilactici* YKP4 after germination (Fig. 5). The peptides concentration increased significantly until 72 h of fermentation, both in soymilk fermented by *P. acidilactici* YKP4 and *Lb. rhamnosus* BD2, with the values of 3.35 ± 0.16 mg·ml^{-1} and 2.22 ± 0.18 mg·ml^{-1}, respectively.

The DPPH scavenging activity during fermentation by both starter cultures increased during fermentation until 72 h with *P. acidilactici* YKP4 producing higher values. The results were in line with the production of peptides (Fig. 6A.). Higher peptides concentration in samples fermented by *P. acidilactici* YKP4 resulted in higher DPPH scavenging activity. The results suggest that peptides were continuously produced by the bacterial cells, causing DPPH scavenging activity, even if there was no significant increase in viable counts.

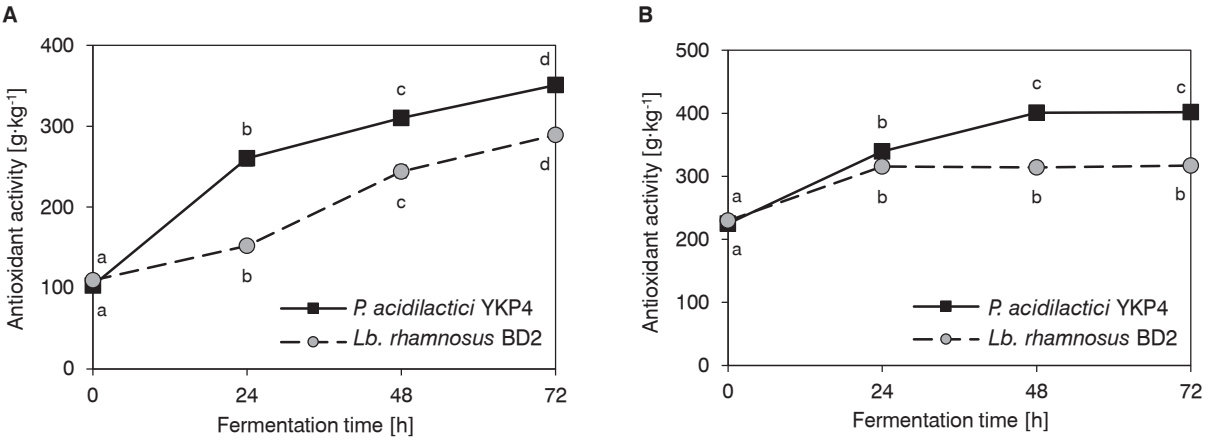

Fig. 6. Effects of the fermentation time on antioxidant activity of soymilk produced from germinated soybean.

A – DPPH scavenging activity, B – ferric reducing antioxidant power.
Different superscripts indicate significant differences ($p < 0.05$) between samples.
Antioxidant activity is expressed as Trolox equivalents. DPPH – 2,2-diphenyl-1-picrylhydrazyl.

The increase in antioxidant activity of fermented soymilk during extended fermentation time was also reported by ZHAO and SHAH [18] and it positively correlated with proteolytic activity of LAB during fermentation.

The significant increase in antioxidant activity expressed in FRAP assay was observed after 24 h fermentation by the two LAB (Fig. 6B.). The FRAP-based values of soymilk after germination fermented by *P. acidilactici* YKP4 subsequently increased until 72 h. In contrast, there was no increase in FRAP values in case of *Lb. rhamnosus* BD2. After 72 h, the FRAP values soymilk fermented by *P. acidilactici* YKP4 was higher than that fermented by *Lb. rhamnosus* BD2. The FRAP values correlated with the pattern of microbial growth. *Lb. rhamnosus* BD2 showed no significant increase in viable counts after 24 h, while *P. acidilactici* YKP4 showed subsequent increase in viable counts.

The different pattern of DPPH scavenging activity and FRAP of *P. acidilactici* YKP4 and *Lb. rhamnous* BD2 could be due to differences in the type of peptides produced. Peptides with radical-scavenging activity have a higher ability to donate hydrogen and transfer electrons. On the other hand, the FRAP value of peptides reflects metal ion chelating or their activity as a reducing agent.

CONCLUSIONS

P. acidilactici YKP4 and *Lb. rhamnosus* BD2 produced highest antioxidant activity among six other LAB isolates used in this study. Germination of soybean increased reducing sugars significantly, but did not affect the growth of the LAB isolates. Germination increased antioxidant activity and the increase was affected by germination time. Antioxidant activity of fermented soymilk after germination increased with increasing fermentation time. *P. acidilactici* YKP4 produced more peptides and higher antioxidant activity than *Lb. rhamnosus* BD2. Lactic fermentation increased antioxidant activity of soymilk produced from germinated soybean. Thus, combination of germination and fermentation evidently provides a significant enhancement of antioxidant activity of soymilk.

Acknowledgements

The author would like to thank the Director General Higher Education, Research, and Technology, the Ministry of Education, Culture, Research, and Technology, Indonesia for providing research funding under the scheme of Doctoral Dissertation Research 2021 for the recipient Lilis Nuraida with the grant number 1/E1/KP.PTNBH/2021 date 8 March 2021 and 001/E5/PG.02.00PT/2022 date 16 March 2022.

REFERENCES

1. Messina, M.: Soy and health update: Evaluation of the clinical and epidemiologic literature. Nutrients, *8*, 2016, pp. 1–42. DOI: 10.3390/nu8120754.
2. Isanga, J. – Zhang, G. N.: Soybean bioactive components and their implications to health – A review. Food Reviews International, *24*, 2008, pp. 252–276. DOI: 10.1080/87559120801926351.
3. Watanabe, S. – Uehara, M.: Health effects and safety of soy and isoflavones. In: Singh, R. B. – Watson, R. R. – Takahashi, T. (Eds.): The role of functional food security in global health. London : Academic Press, 2019, pp. 379–394. ISBN: 9780128131480. DOI: 10.1016/B978-0-12-813148-0.00022-0.
4. Ohanenye, I. C. – Tsopmo, A. – Ejike, C. E. C. C. – Udenigwe, C. C.: Germination as a bioprocess for enhancing the quality and nutritional prospects of legume proteins. Trends in Food Science and Technology, *101*, 2020, pp. 213–222. DOI: 10.1016/j.tifs.2020.05.003.
5. Shi, H. – Nam, P. K. – Ma, Y.: Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination. Journal of Agricultural and Food Chemistry, *58*, 2010, pp. 4970–4976. DOI: 10.1021/jf100335j.
6. Megat, R. M. R. – Azrina, A. – Norhaizan, M. E.: Effect of germination on total dietary fibre and total sugar in selected legumes. International Food Research Journal, *23*, 2016, pp. 257–261. ISSN: 2231-7546. <http://www.ifrj.upm.edu.my/23%20(01)%202016/(38).pdf>.
7. Jang, C. H. – Oh, J. – Lim, J. S. – Kim, H. J. – Kim, J. S.: Fermented soy products: beneficial potential in neurodegenerative diseases. Foods, *10*, 2021, article 636. DOI: 10.3390/foods10030636.
8. Nuraida, L.: A review: Health promoting lactic acid bacteria in traditional Indonesian fermented foods. Food Science and Human Wellness, *4*, 2015, pp. 47–55. DOI: 10.1016/j.fshw.2015.06.001.
9. Yusuf, D. – Nuraida, L. – Dewanti-Hariyadi, R. – Hunaefi, D.: In vitro characterization of lactic acid bacteria from Indonesian kefir grains as probiotics with cholesterol-lowering effect. Journal of Microbiology and Biotechnology, *30*, 2020, pp. 726–732. DOI: 10.4014/jmb.1910.10028.
10. Sitanggang, A. B. – Lesmana, M. – Budijanto, S.: Membrane-based preparative methods and bioactivities mapping of tempe-based peptides. Food Chemistry, *329*, 2020, article 127193. DOI: 10.1016/j.foodchem.2020.127193.
11. Sharma, R. – Garg, P. – Kumar, P. – Bhatia, S. K. – Kulshrestha, S.: Microbial fermentation and its

role in quality improvement of fermented foods. Fermentation, *6*, 2020, article 106. DOI: 10.3390/fermentation6040106.
12. Xu, L. – Du, B. – Xu, B.: A systematic, comparative study on the beneficial health components and antioxidant activities of commercially fermented soy products marketed in China. Food Chemistry, *174*, 2015, pp. 202–213. DOI: 10.1016/j.foodchem.2014.11.014.
13. Fadlillah, H. N. – Nuraida, L. – Sitanggang, A. B. – Palupi, N. S.: Production of antioxidants through lactic acid fermentation: current developments and outlook. The Annals of the University Dunarea de Jos of Galati, Fascicle VI – Food Technology, *45*, 2021, pp. 203–228. DOI: 10.35219/foodtechnology.2021.2.13.
14. Rubak, Y. T. – Nuraida, L. – Iswantini, D. – Prangdimurti, E.: Angiotensin-I-converting enzyme inhibitory peptides in milk fermented by indigenous lactic acid bacteria. Veterinary World, *13*, 2020, pp. 345–353. DOI: 10.14202/vetworld.2020.345-353.
15. Rubak, Y. T. – Nuraida, L. – Iswantini, D. – Prangdimurti, E.: Proteolytic activity of indigenous lactic acid bacteria and Angiotensin-I-Converting Enzyme (ACE) inhibitory activity in fermented soy milk. Pakistan Journal of Nutrition, *19*, 2020, pp. 295–302. DOI: 10.3923/pjn.2020.295.302.
16. Jiang, S. – Cai, W. – Xu, B.: Food quality improvement of soy milk made from short-time germinated soybeans. Foods, *2*, 2013, pp. 198–212. DOI: 10.3390/foods2020198.
17. Zheng, Y. – Fei, Y. – Yang, Y. – Jin, Z. – Yu, B. – Li, L.: A potential flavor culture: *Lactobacillus harbinensis* M1 improves the organoleptic quality of fermented soymilk by high production of 2,3-butanedione and acetoin. Food Microbiology, *91*, 2020, article 103540. DOI: 10.1016/j.fm.2020.103540.
18. Zhao, D. – Shah, N. P.: Changes in antioxidant capacity, isoflavone profile, phenolic and vitamin contents in soymilk during extended fermentation. LWT – Food Science and Technology, *58*, 2014, pp. 454–462. DOI: 10.1016/j.lwt.2014.03.029.
19. Yusuf, D. – Nuraida, L. – Dewanti-Hariyadi, R. – Hunaefi, D.: In vitro antioxidant and α-glucosidase inhibitory activities of *Lactobacillus* spp. isolated from Indonesian kefir grains. Applied Food Biotechnology, *8*, 2020, pp. 39–46. DOI: 10.22037/afb.v8i1.30367.
20. Kim, H. J. – Han, M. J.: The fermentation characteristics of soy yogurt with different content of D-allulose and sucrose fermented by lactic acid bacteria from Kimchi. Food Science and Biotechnology, *28*, 2019, pp. 1155–1161. DOI: 10.1007/s10068-019-00560-5.
21. Chen, P. – Zhang, Q. – Dang, H. – Liu, X. – Tian, F. – Zhao, J. – Chen, Y. – Zhang, H. – Chen, W.: Screening for potential new probiotic based on probiotic properties and α-glucosidase inhibitory activity. Food Control, *35*, 2014, pp. 65–72. DOI: 10.1016/j.foodcont.2013.06.027.
22. Subrota, H. – Shilpa, V. – Brij, S. – Vandna, K. – Surajit, M.: Antioxidative activity and polyphenol content in fermented soy milk supplemented with WPC-70 by probiotic Lactobacilli. International Food Research Journal, *20*, 2013, pp. 2125–2131. ISSN: 2231-7546. <http://www.ifrj.upm.edu.my/20%20(05)%202013/12%20IFRJ%2020%20(05)%202013%20Subrota%20353.pdf>
23. Lowry, O. H. – Roserbrough, N. J. – Farr, A. L. – Randall, R. J.: Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, *193*, 1951, pp. 265–275. DOI: 10.1016/s0021-9258(19)52451-6.
24. Church, F. C. – Swaisgood, H. E. – Porter, D. H. – Catignani, G. L.: Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. Journal of Dairy Science, *66*, 1983, pp. 1219–1227. DOI: 10.3168/jds.S0022-0302(83)81926-2.
25. Sharma, A. – Noda, M. – Sugiyama, M. – Ahmad, A. – Kaur, B.: Production of functional buttermilk and soymilk using *Pediococcus acidilactici* BD16 (*alaD*$^+$). Molecules, *26*, 2021, article 4671. DOI: 10.3390/molecules26154671.
26. Zhang, Y. X. – Ke, W. C. – Bai, J. – Li, F. H. – Xu, D. M. – Ding, Z. T. – Guo, X. S.: The effect of *Pediococcus acidilactici* J17 with high-antioxidant activity on antioxidant, α-tocopherol, β-carotene, fatty acids, and fermentation profiles of alfalfa silage ensiled at two different dry matter contents. Animal Feed Science and Technology, *268*, 2020, article 114614. DOI: 10.1016/j.anifeedsci.2020.114614.
27. Marazza, J. A. – Nazareno, M. A. – Giori, G. S. – Garro, M. S.: Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. Functional Foods, *4*, 2012, pp. 594–601. DOI: 10.1016/j.jff.2012.03.005.
28. Ruiz de la Bastida, A. – Peirotén, Á. – Langa, S. – Rodríguez-Mínguez, E. – Curiel, J. A. – Arqués, J. L. – Landete, J. M.: Fermented soy beverages as vehicle of probiotic lactobacilli strains and source of bioactive isoflavones: A potential double functional effect. Heliyon, *9*, 2023, article E14991. DOI: 10.1016/j.heliyon.2023.e14991.
29. Sarmadi, B. H. – Ismail, A.: Antioxidative peptides from food proteins : A review. Peptides, *31*, 2010, pp. 1949–1956. DOI: 10.1016/j.peptides.2010.06.020.
30. Bueno, D. B. – da Silva Júnior, S. I. – Seriani Chiarotto, A. B. – Cardoso, T. M. – Neto, J. A. – Lopes dos Reis, G. C. – Tavano, O. L.: The germination of soybeans increases the water-soluble components and could generate innovations in soy-based foods. LWT – Food Science and Technology, *117*, 2020, article 108599. DOI: 10.1016/j.lwt.2019.108599.
31. Nkhata, S. G. – Ayua, E. – Kamau, E. H. – Shingiro, J. B.: Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. Food Science and Nutrition, *6*, 2018, pp. 2446–2458. DOI: 10.1002/fsn3.846.
32. Huang, X. – Cai, W. – Xu, B.: Kinetic changes of nutrients and antioxidant capacities of germinated soybean (*Glycine max* L.) and mung bean (*Vigna radiata* L.) with germination time. Food

Chemistry, *143*, 2014, pp. 268–276. DOI: 10.1016/j.foodchem.2013.07.080.

33. Egea, M. B. – Santos, D. C. Dos – Neves, J. F. – Lamas, I. B. – Filho, J. G. D. – Takeuchi, K. P.: Physicochemical characteristics and rheological properties of soymilk fermented with kefir. Biointerface Research in Applied Chemistry, *13*, 2022, article 127. DOI: 10.33263/BRIAC132.127.
34. Hati, S. – Patel, N. – Mandal, S.: Comparative growth behaviour and biofunctionality of lactic acid bacteria during fermentation of soy milk and bovine milk. Probiotics and Antimicrobial Proteins, *10*, 2018, pp. 277–283. DOI: 10.1007/s12602-017-9279-5.
35. Maleke, M. – Doorsamy, W. – Abrahams, A. M. – Adefisoye, M. A. – Masenya, K. – Adebo, O. A.: Influence of fermentation conditions (temperature and time) on the physicochemical properties and bacteria microbiota of *Amasi*. Fermentation, *8*, 2022, article 57. DOI: 10.3390/fermentation8020057.
36. Keles, G. – Demirci, U.: The effect of homofermentative and heterofermentative lactic acid bacteria on conservation characteristics of baled triticale-Hungarian vetch silage and lamb performance. Animal Feed Science and Technology, *164*, 2011, pp. 21–28. DOI: 10.1016/j.anifeedsci.2010.11.017.
37. Coudeyras, S. – Marchandin, H. – Fajon, C. – Forestier, C.: Taxonomic and strain-specific identification of the probiotic strain *Lactobacillus rhamnosus* 35 within the *Lactobacillus casei* group. Applied and Environmental Microbiology, *74*, 2008, pp. 2679–2689. DOI: 10.1128/AEM.02286-07.

Received 13 January 2023; 1st revised 25 July 2023; accepted 22 August 2023; published online 28 October 2023.