

## SELECTION OF *LACTOBACILLUS* STRAINS FOR IMPROVEMENT OF ANTIOXIDANT ACTIVITY OF DIFFERENT SOY, WHEY AND MILK PROTEIN SUBSTRATES

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### Abstract

Due to the health benefits and potential food preservative applications, natural antioxidants were recently attracted great attention of many food manufacturers which tend to produce healthy foods. The special challenge in this field is production of antioxidants on food matrixes that can be suitably used for a wide range of products. In that sense, soy, whey and milk substrates have a great potential as a carriers for delivery of antioxidants into the many food products. The aim of this study was selection of appropriate strain-substrate combination that allows the production of high level of antioxidant activity.

Soy, whey (cow's and goat's) and milk substrates were fermented with *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus reuteri* ATCC 23272 strains at 37 °C during 24 h. The antioxidant activity of the substrates was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant potential (FRAP) assays that evaluate antioxidant activities by different reaction mechanisms.

Based on the results, increased antioxidant activity was reached in all tested strain-substrate combinations. High levels of antioxidant activities, in the range 29.7-72.2%, were reached in all substrates fermented with *Lb. rhamnosus* strain. Among all combinations, goat whey fermented with *Lb. rhamnosus* exhibited the highest increase of DPPH scavenging activity (for 21.1%) as well as FRAP antioxidant activity (for 0.761 mmol Fe<sup>+2</sup> L<sup>-1</sup>) compared to the unfermented substrate.

Thus, the study introduces the *Lb. rhamnosus* as highly effective in production of antioxidants during the

fermentation of goat whey. Furthermore, goat whey fermented with *Lb. rhamnosus*, used in liquid or lyophilized form, represents an excellent carrier for delivery of antioxidants into the different dairy and confectionery products.

**Key words:** Soy, Whey, Milk, Antioxidant activity, DPPH, FRAP, *Lb. rhamnosus*.

### 1. Introduction

The oxidative stability of products is a great concern to the food industry. Oxidation processes in food can result in strong off-flavours and in deterioration of the nutritional quality of products. The oxidative stability is the result of a delicate balance between the anti- and pro-oxidative processes in food products influenced by factors such as: degree of fatty acid unsaturation, content of transition metal ions, and content of antioxidants as tocopherols and carotenoids [1, 2, 3, 4]. Since oxidation only can occur in case of an imbalance between the presence of reactive oxidants and the antioxidant defense mechanism, sensitivity to oxidation can be reduced by the addition of ingredients that enhances the antioxidative capacity of products [5].

Due to the health benefits and potential food preservative applications, natural antioxidants were recently attracted great attention of many food manufacturers which tend to produce healthy foods. The special challenge in this field is production of antioxidants on food matrixes that can be suitably used for a wide range of

food products. In that sense, soy, whey and milk substrates have a great potential as carriers for delivery of antioxidants into the many food products.

Dairy substrates possess antioxidant activity resulting from the presence of such components as bioactive peptides derived from casein, whey proteins, lactoferrin, urate, ascorbate,  $\alpha$ -tocopherol,  $\beta$ -carotene, coenzyme Q10, and various enzymatic systems (superoxide dismutase, catalase, and glutathione peroxidase) [6, 7]. In addition, soy substrates are potent antioxidative ingredients due to the presence of phenolic components and isoflavones. All of these compounds possess metal-chelating capabilities and radical scavenging properties [8]. Antioxidant activity of these substrates could be enhanced by fermentation that tends to enhance targeted property by releasing different by-products [9] or by presence of starter microorganisms that also possess some antioxidant potential [6, 7]. During fermentation, soy and dairy proteins could be hydrolyzed by the extracellular proteinases of the LAB, resulting in an increase of peptides that can contribute to the antioxidant activity of fermented substrates. The amount of peptides formed during fermentation depends on the used strain, specifically on its proteolytic activity, as well as the used substrate [10]. Optimal strain-substrate combination is crucial point in the production of high potent antioxidative formulation that can be easily used in different food products.

The literature shows an extreme diversity in methods that measure a typical nature of antioxidative activity: scavenging of free radicals, reducing power or chelating activity [11]. Among them, the Diphenyl Picryl Hydrazyl (DPPH) method measures the degree in which a product can reduce the free radical 2,2-diphenyl-1-picrylhydrazyl [12], while the Ferric Reducing Antioxidant Power (FRAP) method gives an idea of the ferric reducing antioxidant power of a product [13].

The aim of this study was selection of appropriate strain-substrate combination that allows the production of high level of antioxidant activity. Soy, whey (cow's and goats) and milk substrates were fermented with *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus reuteri* ATCC 23272 and *Lactobacillus rhamnosus* ATCC 7469 strains, at 37 °C during 24 h. The antioxidant activity of the substrates was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant potential (FRAP) assays that evaluate antioxidant activities by different reaction mechanisms.

## 2. Materials and Methods

### 2.1 Culture and media

The strains *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus reuteri* ATCC 23272, used in this study, were supplied by Ameri-

can Type Culture Collection (ATCC, Rockville, USA). Stock cultures were stored at -18 °C in 3 mL vials containing De Man Rogosa Sharpe (MRS) broth (Fluka, USA) and 50% (v/v) glycerol as a cryoprotective agent. To prepare the laboratory cultures, a drop of desired stock culture was transferred to 3 mL of MRS broth and incubated for 18 h under anaerobic conditions at optimal growth temperature (37 °C). The working cultures were pre-cultured twice in MRS broth prior to experimental use.

Sweet cow's whey powder - CW (Lenic Laboratories, Belgrade, Serbia), with the following composition: proteins 12.11% (w/w), lipids 1.00% (w/w), and carbohydrates 69.62% (w/w), was reconstituted to contain 8% (w/v) dry matter. Sweet goat's whey powder - GW (Stanojević Pharm, Novi Sad, Serbia), with the following composition: proteins 21.99% (w/w), lipids 2.79% (w/w), and carbohydrates 64.39% (w/w), was reconstituted to contain 8% (w/v) dry matter. Whey protein concentrate - WPC (DMV International, Nederland) with the following composition: proteins 80.0% (w/w), lipids 6.30% (w/w), and carbohydrates 6.50% (w/w), was reconstituted to contain 5% (w/v) dry matter. Milk protein concentrate - MPC (Megglosat HP ME) with the following composition: proteins 86.0% (w/w), lipids 1.9% (w/w), and carbohydrates 3.2% (w/w), was reconstituted to contain 5% (w/v) dry matter. Soy protein concentrate - SPC (Sojaprotein a.d., Bečej, Serbia) with the following composition: proteins 68.0% (w/w), lipids 1.2% (w/w), and carbohydrates 20.5% (w/w), moisture 8.0% was reconstituted to contain 5% (w/v) dry matter. Soy protein isolate - SPI (Sojaprotein a.d., Bečej, Serbia) with the following composition: proteins 90.0% (w/w), lipids 1.0% (w/w), and carbohydrates 2.2% (w/w), was reconstituted to contain 5% (w/v) dry matter. The reconstituted substrates were pasteurized at 60 °C for 60 min and stored at 4 °C (no longer than one day) prior to its use as a fermentation medium. All chemicals were obtained from Sigma-Aldrich.

### 2.2 Fermentation

Experiments were conducted in 200 mL Erlenmeyer flasks containing 100 mL of reconstituted substrates. Samples were inoculated by adding 2% (v/v) of activated cultures and incubated at 37 °C for 24 h. During the incubation time samples were taken every 6 h for determination of DPPH and FRAP values as measures of antioxidant activity.

### 2.3 Antioxidant activity

#### 2.3.1 DPPH scavenging activity

Antioxidant activity of fermented substrates was determined by its ability to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, which was measured according to the modified method [14]. A stock solution of 0.1 mM DPPH (Sigma-Aldrich, Australia) was

prepared by dissolving in methanol. After the fermentation samples were macerated with methanol and centrifuged at 8000 rpm for 20 min. at 4 °C. Methanol (1.5 mL) and DPPH (1.0 mL) were added to the supernatant (0.5 mL). Control sample was prepared by mixing methanol (1.5 mL) and DPPH (1.5 mL), while methanol was used as blank sample. Mixtures were allowed to stand 30 min. in dark, at room temperature. The antioxidant activity was analyzed by reading the absorbance at 517 nm. DPPH scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(cA - Aa) / cA] \times 100 \quad (1)$$

Where Aa and Ac represent absorbance of sample and control, respectively.

### 2.3.2 FRAP method

The operating FRAP method was conducted following the procedure: 4.5 mL of FRAP solution and 150 µL of extract concentration of 1 mg/mL was poured in the test tube. FRAP solution was prepared just before use. It consists of 25 mL of acetate buffer (300 mmol/L, pH 3.6), 2.5 mL of TPTZ (2,4,6-tripyridyl-s-triazine) and 2.5 mL of 20 mmol/L solution of  $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ . The contents of the test tubes were strongly mixed and after 5 min. absorbance was measured at 593 nm. Blank was FRAP solution. The results were calculated from the standard curve of  $\text{FeSO}_4 \times 7\text{H}_2\text{O}$  (200–1000 µmol/L) and expressed as mmol  $\text{Fe}^{2+}$ /L [15].

### 2.4 Statistical analysis

The experiments were performed in triplicate. All values are expressed as mean  $\pm$  standard deviation. Mean values were analyzed using two-way ANOVA. The Tukey post hoc test was performed for means

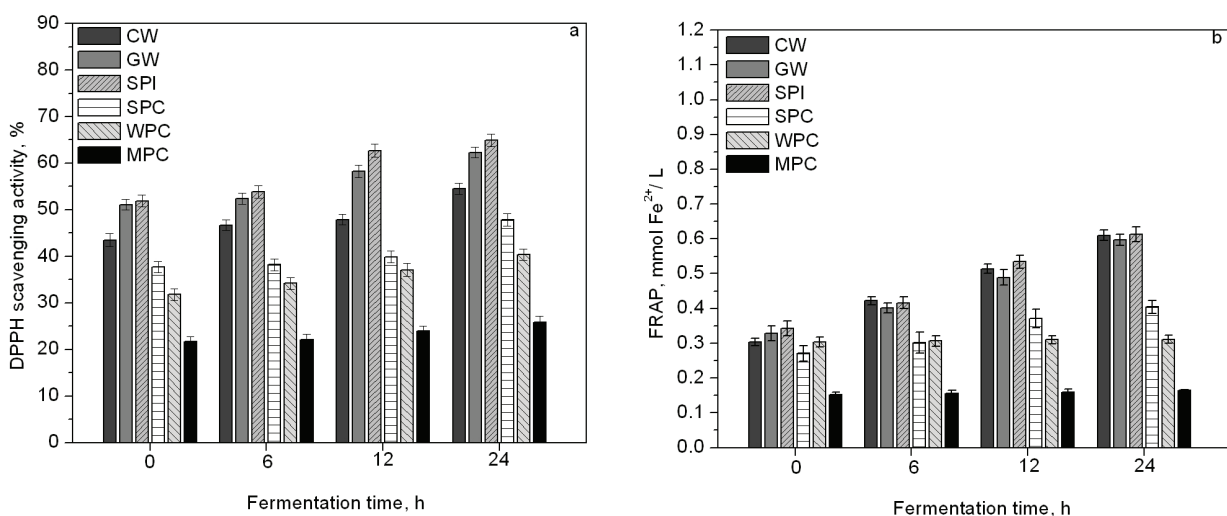
comparison (Origin Pro 8 (1991-2007), Origin Lab Co., Northampton, USA). Differences were considered significant at  $P < 0.05$ .

## 3. Results and Discussion

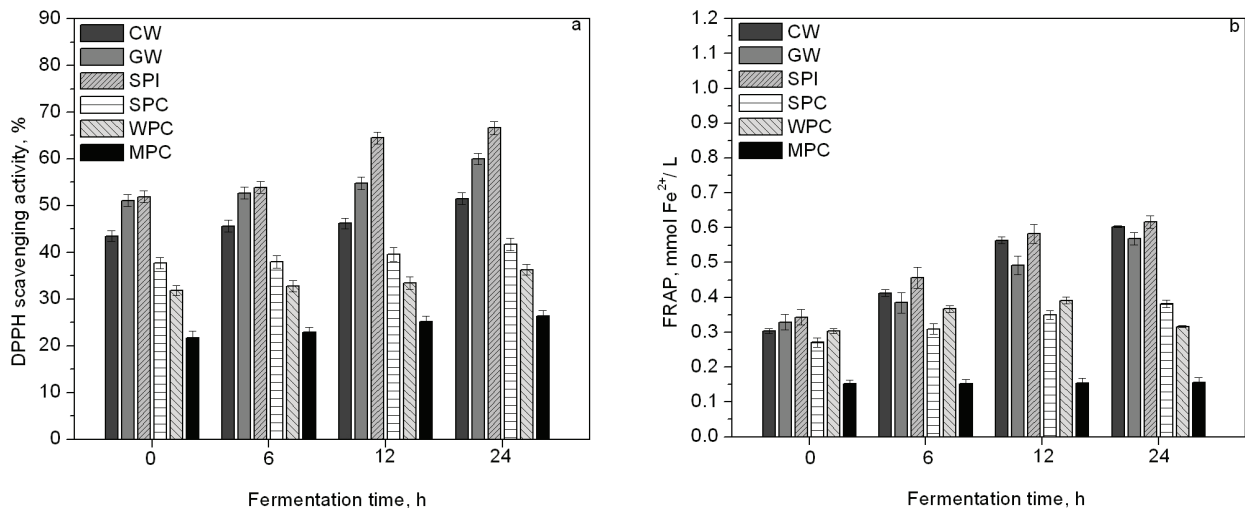
### 3.1 Antioxidant activity

The development of antioxidant activity during 24 h fermentation was studied with DPPH and FRAP assays. Influences of different microorganisms on changes in antioxidant activities of different substrates during 24 h of fermentation are shown in Figures 1, 2 and 3.

As shown in Figure 1, strain *Lb. acidophilus* increases antioxidant activity of all tested substrates during 24 h of fermentation. The maximal DPPH scavenging activity of 64.9% (Figure 1a) was reached after 24 h fermentation of soy protein isolate (SPI). Same trend of antioxidant activity increasing was observed in FRAP assay, where the maximal metal-chelating ability, i.e. FRAP value of 0.6127 mmol  $\text{Fe}^{2+}$ /L (Figure 1b) was registered after 24 h fermentation in sample SPI. Obtained results, suggests that strain *Lb. acidophilus* is capable to produce high amount of antioxidant compounds on soy protein substrate, probably due to its higher proteolytic activity on this protein source. Obtained results are much better than those reported in the literature [10] where the *Lb. acidophilus* is assigned as strain that produces high antioxidant activity of 42.0% in fermented milk, measured by ABTS assay. Based on the fact that in present study the minimal DPPH and FRAP values (25.9%, 0.1640 mmol  $\text{Fe}^{2+}$ /L, respectively) are reached after 24 h in milk protein sample MPC, it could be said that enzymatic system of *Lb. acidophilus* much efficiently hydrolyzes the soy and whey protein sources leading to the production of significantly higher antioxidant activity.



**Figure 1. Changes of the a) DPPH scavenging activity, and b) FRAP antioxidant activity of different substrates fermented with *Lb. acidophilus* ATCC 4356 strain at 37 °C during 24h**

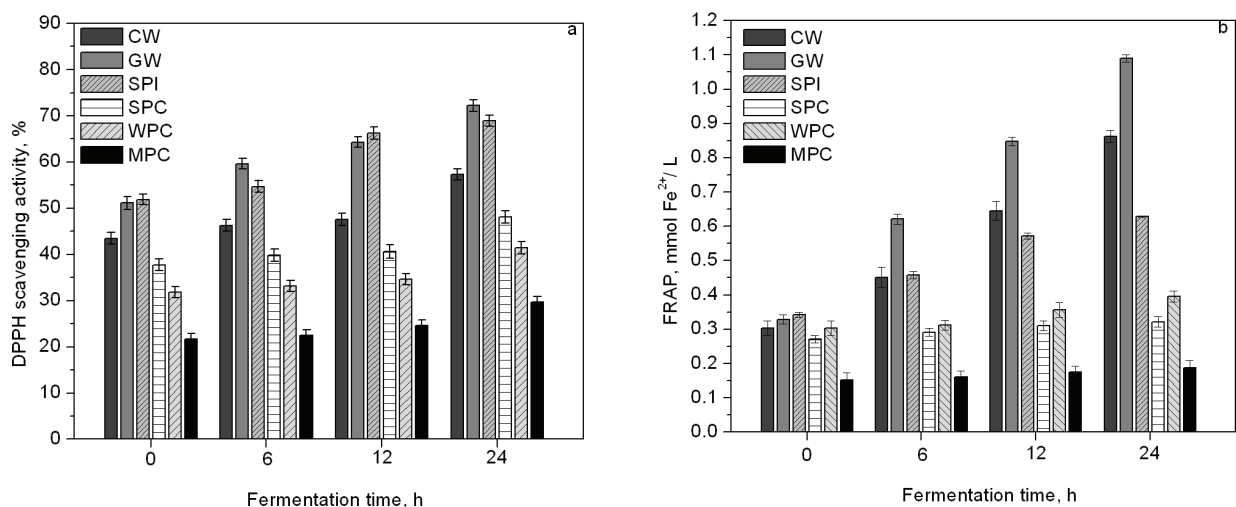


**Figure 2. Changes of the a) DPPH scavenging activity, and b) FRAP antioxidant activity of different substrates fermented with *Lb. reuteri* ATCC 23272 strain at 37 °C during 24h**

Strain *Lb. reuteri* significantly ( $P < 0.05$ ) increases antioxidant activity of all tested substrates during 24 h of fermentation. The maximal DPPH scavenging activity of 66.6% (Figure 2a) was reached after 24 h fermentation of soy protein isolate (SPI). In addition, the maximal value of metal-chelating ability, i.e. FRAP value of 0.6167 mmol Fe<sup>2+</sup>/L (Figure 2b) was reached after 24 h fermentation of SPI sample. Obtained results are lower than those registered for *Lb. acidophilus*, suggesting that *Lb. reuteri* much efficiently hydrolyzes soy proteins than *Lb. acidophilus* strain. Obtained results are contrast to those reported in the literature [10], where the *Lb. reuteri* produces lower antioxidant activity (30.0%) in milk substrate than *Lb. acidophilus* strain, measured by ABTS assay. Based on the literature findings, it was expectable that *Lb. reuteri* reaches lower DPPH and FRAP values than *Lb. acidophilus*, but the fact that *Lb. reuteri* produces higher antioxidant activity in SPI

sample than *Lb. acidophilus* suggests that enzymatic system of *Lb. reuteri* possesses advanced capability to hydrolyze soy protein isolate compared to the *Lb. acidophilus* strain.

As shown in Figure 3, strain *Lb. rhamnosus* increases antioxidant activity of all tested substrates during 24 h of fermentation. The maximal DPPH scavenging activity of 72.2% (Figure 3a) was reached after 24 h fermentation of goat's whey substrate (GW). Same trend of antioxidant activity increasing was observed in FRAP assay, where the maximal metal-chelating ability, i.e. FRAP value of 1.0897 mmol Fe<sup>2+</sup>/L (Figure 3b) was reached after 24 h fermentation of GW sample. Obtained results suggest that strain *Lb. rhamnosus* produces significantly higher antioxidant activity during the hydrolysis of goat's whey compared to the other substrates and microorganisms. It is capable to produce high



**Figure 3. Changes of the a) DPPH scavenging activity, and b) FRAP antioxidant activity of different substrates fermented with *Lb. rhamnosus* ATCC 7469 strain at 37 °C during 24h**

amount of antioxidant compounds probably due to high proteolytic activity [16]. Obtained results are contrast to those reported in the literature [10], where the *Lb. rhamnosus* produces lower antioxidant activity of fermented milk than *Lb. acidophilus* and *Lb. reuteri* strains, measured by ABTS assay. In addition it could be said that *Lb. rhamnosus* more efficiently hydrolyzes the goat's whey protein substrate than *Lb. acidophilus* and *Lb. reuteri* strains, and leads to the production of significantly higher antioxidant activity in goat's whey compared to the other tested substrate.

Obtained DPPH and FRAP values are in agreement to those reported in the literature [17, 18, 19] related to the production of antioxidant activity in studied substrates.

It is interesting to note that the lowest DPPH and FRAP values are obtained in fermentation of milk protein concentrate (MPC), regardless of the applied microorganism. This observation is in contrast to the literature [10] that reports the high antioxidant activity measured by ABTS assay obtained during the fermentation of whole milk by studied microorganisms. It is probably due to the fact that casein is slowly degradable protein [20] which digestion does not allow the production of antioxidant peptides by used microorganisms during 24 h.

Based on the presented results it is evident that the fermentation of goat's whey by *Lb. rhamnosus* allows the production of formulation with high antioxidant activity that could be used as supplement in wide range of food products.

#### 4. Conclusions

- Based on the results, increased antioxidant activity was reached in all tested strain-substrate combinations.

- High levels of antioxidant activities, in the range 29.7 - 72.2%, were reached in all substrates fermented with *Lb. rhamnosus* strain.

- Among all combinations, goat whey fermented with *Lb. rhamnosus* exhibited the highest increase of DPPH scavenging activity (for 21.1%) as well as FRAP antioxidant activity (for 0.761 mmol Fe<sup>2+</sup>/L) compared to the unfermented substrate. Thus, the study introduces the *Lb. rhamnosus* as highly effective in production of antioxidants during the fermentation of goat whey.

- Furthermore, goat whey fermented with *Lb. rhamnosus*, used in liquid or lyophilized form, represents an excellent carrier for delivery of antioxidants into the different dairy and confectionery products.

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#### 5. References

- [1] Barrefors P., Granelli K., Appelqvist L. A., Bjorck L. (1995). *Chemical characterization of raw milk samples with and without oxidative off-flavour*. Journal of Dairy Science, 78, pp. 2691-2699.
- [2] Havemose M. S., Weisbjerg M. R., Bredie W. L. P., Nielsen J. H. (2004). *Influence of feeding different types of roughage on the oxidative stability of milk*. International Dairy Journal, 14, pp. 563-570.
- [3] Kristensen D., Hedegaard R. V., Nielsen J. H., Skibsted L. H. (2004). *Oxidative stability of buttermilk as influenced by the fatty acid composition of cows' milk manipulated by diet*. Journal of Dairy Research, 71, pp. 46-50.
- [4] Lindmark-Mansson H., Akesson B. (2000). *Antioxidative factors in milk*. British Journal of Nutrition, 84, pp. 103-110.
- [5] Halliwell B. (1996). *Antioxidants in human health and disease*. Annual Review of Nutrition, 16, pp. 33-50.
- [6] Chen J., Lindmark-Mansson H., Gorton L., Åkesson B. (2003). *Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods*. International Dairy Journal, 13, pp. 927-935.
- [7] Kullisaar T., Zilmer M., Mikelsaar M., Vihalemm T., Annuk H., Kairane C., Kilk A. (2002). *Two antioxidative lactobacilli strains as promising probiotics*. International Journal of Food Microbiology, 72, pp. 215-224.
- [8] Lopes G. K. B., Schulman H. M., Hermes-Lima M. (1999). *Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions*. Biochimica et Biophysica Acta - General Subjects, 1472, pp. 142-152.
- [9] Yang J. H., Mau J. L., Ko P. T., Huang L. C. (2000). *Antioxidant properties of fermented soybean broth*. Food Chemistry, 71, pp. 249-254.
- [10] Virtanen T., Pihlanto A., Akkanen S., Korhonen H. (2007). *Development of antioxidant activity in milk whey during fermentation with lactic acid bacteria*. Journal of Applied Microbiology, 102, pp. 106-115.
- [11] Antolovich M., Prenzler P. D., Patsalides E., McDonald S., Robards K. (2002). *Methods for testing antioxidant activity*. Analyst, 127, pp. 183-198.
- [12] Brand-Williams W., Cuvelier M. E., Berset C. (1995). *Use of a free radical method to evaluate antioxidant activity*. Food Science and Technology - Lebensmittel-Wissenschaft und Technologie, 28, pp. 25-30.
- [13] Benzie I. F. F., Strain J. J. (1996). *The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay*. Analytical Biochemistry, 239, pp. 70-76.
- [14] Balakrishnan G., Agrawal R. (2014). *Antioxidant activity and fatty acid profile of fermented milk prepared by *Pedococcus pentosaceus**. Journal of Food Science and Technology, 51, pp. 4138-4142.
- [15] Pavlović M. D., Buntić A. V., Šiler-Marinković S. S., Dimitrijević-Branković S. I. (2013). *Ethanol influenced fast microwave-assisted extraction for natural antioxidants obtaining from spent filter coffee*. Separation and Purification Technology, 118, pp. 503-510.

- [16] Cagno R. D., Quintob M., Corsettic A., Minervinia F., Gobbettia. (2006). *Assessing the proteolytic and lipolytic activities of single strains of mesophilic lactobacilli as adjunct cultures using a Caciotta cheese model system*. International Dairy Journal, 16, pp. 119-130.
- [17] Najgebauer-Lejko D., Grega T., Tabaszewska M. (2014). *Yoghurts with addition of selected vegetables: acidity, antioxidant properties and sensory quality*. Acta scientiarum polonorum, Technologia alimentaria, 13, pp. 35-42.
- [18] Yadav D., Vij S., Hati S., Singh B. P., Dhanday M., Dahiya M., Vandna V. (2012). *Evaluation of Total Antioxidant Activity of Soy Yoghurt*. Indian Journal of Dairy Science 65, pp. 220-224.
- [19] Najgebauer-Lejko D., Sady M. (2015). *Estimation of the antioxidant activity of the commercially available fermented milks*. Acta scientiarum polonorum. Technologia alimentaria, 14, pp. 387-396.
- [20] Luhovyy B. L., Akhavan T., Anderson G. H. (2007). *Whey Proteins in the Regulation of Food Intake and Satiety*. Journal of the American College of Nutrition, 26, pp. 704-712.