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Protein characterisation and antioxidant potential of fresh cheese obtained by kombucha inoculum

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Abstract

Protein profile, *in vitro* gastrointestinal digestion and antioxidant potential of two fresh cheese samples obtained by kombucha inoculum and traditional starter culture have been investigated in this study. There is a significant difference in protein profile and bioactive potential between fresh cheese samples. Kombucha fresh cheese contains higher proportion of protein, predominantly the casein fractions (α-casein and ß-casein) compared to fresh cheese obtained by traditional culture. After gastrointestinal digestion (with pepsin and pancreatin), significantly higher amount of soluble protein and higher degree of hydrolyses were measured in kombucha fresh cheese sample than sample obtained by traditional culture. During gastrointestinal digestion, for fresh cheese with traditional starter both fractions of casein are degraded more rapidly by pepsin than in kombucha fresh cheese. The hydrolysates of cheese obtained by kombucha inoculum showed higher phenolic content and higher antiradical scavenging abilities than hydrolysates of fresh cheese produced with traditional starter. These results suggest that kombucha inoculum contributes to improvement of product's bioactive potential.

Key words: fresh cheese; kombucha inoculum; protein profile; antioxidant potential

Introduction

Fresh cheeses represent a large group of nutritionally high-quality cheeses without ripening (cottage, quark, cream cheese, mascarpone) and have a significant role in the diet of the population. These are products with a sour-milk taste or a very mild sour taste, homogeneous and spreadable consistency. Conventional mesophilic starter culture (*Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* with *Leuconostoc mesenteroides* subsp. *cremoris* and *Lc. lactis* subsp. *lactis biovar diacetylactis*) is most commonly used for the production of fresh cheese. Starter culture has the role of decomposition of lactose to lactic acid through a series of enzymatic reactions. The most important task of the produced lactic acid is to stimulate the action and activity of enzymes for coagulation of milk, to help the separation of whey from curd and to inhibit the growth of undesirable and pathogenic bacteria (Schulz-Collins and Senge, 2004).

The development of the technology of functional dairy products, especially different types of cheese is the subject of modern scientific research. Fresh cheese is a good source of bioactive peptides and other essential nutrients. Various metabolites are produced during the complex biochemical transformations of milk components and directly affect the bioactive potential and other specific properties of the product (Milanović et al., 2017). During cheese processing caseins are hydrolyzed into peptides by different proteases and peptidases from milk. Most studies revealed that proteolysis during fermentation by combination of lactic acid bacteria strains can lead to reduction of imunoreactivity of milk proteins (casein and ß-lactoglobulin). The bacteria *Streptococcus thermophilus* and *Lactobacillus helveticus* significantly contribute to the reduction of allergic properties of whey proteins (Bu et al., 2010). Therefore, modern research is aimed at examining the possibility of using different starter cultures in the production of fermented dairy products in order to reduce allergenic power.

Beside milk acidification with lactic acid bacteria strains, a non-conventional starter culture such as kombucha inoculum can also be used in cheese production (Vukić et al., 2021). Kombucha is a fermented drink that is usually prepared from black or green tea. Fermentation is enabled by the presence of a symbiotic culture of bacteria and yeasts (De Fillippis et al., 2018). The quality of kombucha is also affected by the type of substrate (different teas, whey, wine, milk, etc.) and fermentation temperature, as well as the microorganisms responsible for the composition of metabolites formed during fermentation (Leonarski et al., 2021; Coelho et al., 2020; Cardoso et al. 2020; Jafari et al. 2020). Numerous studies have confirmed that polyphenols from tea can also be used as a supplement in the production of fermented dairy products. The main active polyphenolic components present in tea are catechins and phenolic acids, which can react with milk proteins and affect the sensory and functional properties, microbiological quality and oxidative stability of fermented dairy products (Amirdivani and Baba, 2011; Najgebauer-Lejko et al., 2011).

Recent researches have been dealing with possibilities of kombucha application as non-conventional starter for milk fermentation (Vukić et al., 2018; Kanurić et al., 2018; Iličić et al., 2019; Vukić et al., 2021). Metabolic activities of kombucha and their effect on fermentation process, antioxidant potential and protein characteristics of fermented milks have been studied by different authors (Hrnjez et al, 2014; Vukić et al., 2014; Malbaša et al., 2009, 2014).

In the literature, there are no data about protein profile of fresh cheese obtained by kombucha inoculum. Therefore, the aim of this research is based on the effect of non-conventional starter culture (kombucha inoculum) and traditional starter culture on milk acidification kinetics, protein profile and biological potential of fresh cheeses. Changes of milk proteins was estimated through the analyses of soluble proteins during *in vitro* gastrointestinal digestion (using the enzymes pancreatin and pepsin) of fresh cheese samples. The antiradical activity of the hydrolysates was also an objective of this study.

Materials and methods

Materials

For the production of fresh cheese, pasteurized milk was used with milk fat content of 2.8 %, manufactured by *Mlekoprodukt* AD from Zrenjanin. Milk inoculation was performed using different starter cultures: FD-DVS XPL-1 (Chr. Hansen A/S, Denmark) containing *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *lactis biovar diacetylactis, Leuconostoc* and *Streptococcus thermophilus*; and kombucha inoculum produced in laboratory conditions, cultivated on black tea (*Camellia sinesis* - 1.5 g/L) with the addition of saccharose concentration 70 g/L. 10 % (v/v) of the inoculum from the previous fermentation was added to the tea cooled to room temperature. Incubation was performed at 25 °C during 7 days. The pH value of the kombucha inoculum was 4.72. Coagulation enzyme CHY-MAX®Powder Extra NB enzyme manufactured by Chr Hansen A/S from Denmark, (composition: sodium chloride, chymosin and casein peptone). Pepsin (with declared activity minimum 0.7 FIP U/mg) and pancreatin enzyme (with declared activity of 4 × USP specifications of units (0.03 N-benzoyl-L-tyrosine ethyl ester, BTEE (N-benzoyl-L-tyrosine ethyl ester) units/mg), were obtained from AppliChem (Darmstadt, Germany) and Sigma (St. Louis MO, USA), respectively. Reagents and solutions used in the analysis are: solution A (2 % Na_2CO_3; 0.02 % K, Na tartrate in 0.1M NaOH), solution B (0.5 % copper sulphate), solution C: mix A and B in a ratio of 50:1 and Folin-Ciocalteu reagent (dilute 1:2 with distilled water).

Fresh cheese manufacture

Fresh cheese was produced in laboratory conditions at the Faculty of Technology in Novi Sad. Two types of fresh cheese were prepared: with XPL-1 culture (a concentration of 0.02 g/L) and kombucha inoculum (a concentration of 100 mL/L). Starter culture and kombucha inoculum were added

to milk heated to 35 °C. After 30 minutes, a coagulation enzyme CHY-MAX®Powder Extra NB enzyme, EC 3.4.23.4, was added to both cheese samples (with starter culture and with kombucha inoculum) at 35 °C and at a concentration of 0.5 % (1 % solution of enzyme, Chr Hansen, Denmark). Coagulation at 35 °C lasted until a pH of ~4.5-4.6 was reached. After that, the coagulum was cut, pasteurized at 60 °C with gentle stirring for 5 minutes and cooled (to 25 °C). Whey was separated through a cloth. All cheese samples were homogenized using a mixer, packed in cups with lids and stored in the refrigerator at temperature 4 °C. Fresh cheese sample produced with kombucha inoculum (KFC) and fresh cheese produced with XPL-1 starter culture (XFC) were analysed immediately after production.

Chemical analyses of milk and fresh cheese

Chemical analysis of milk composition and fresh cheese samples was performed by employing standard methods: dry matter content after drying at 105 °C (SRPS EN ISO 5534:2007), fat according to Van Gulik (SRPS ISO 3433:2013), total nitrogen according to Kjeldahl (SRPS EN ISO 8968-4:2016) and ash after mineralization at 550 °C (Carić et al., 2000). The content of non-casein nitrogen (NCN) in milk and cheese was determined by treating the sample with a 10 % solution of acetic acid and then with a solution of 1 mol/L sodium acetate, whereby casein precipitation occurs. Non-casein nitrogen is determined in the filtrate by the standard Kjeldahl method. Non-protein nitrogen (NPN) was determined by the addition of 24 % trichloroacetic acid with intensive mixing that causes precipitation of protein fractions in milk and cheese sample, and then the content of non-protein nitrogen is determined in the filtrate by Kjeldahl method. Based on the obtained results, casein, whey protein content and real proteins were calculated (Carić et al., 2000, SRPS EN ISO 8968 - 4: 2016). All chemical analyses and assays were performed in triplicate for all produced samples (n) and values were expressed as average value. pH was measured by an electric pH-meter (pH Spear, Eutech Instruments, Oakton, England) and pH-meter Mettler Toledo, Switzerland.

Protein digestion

Digestion was performed in a glass laboratory beaker using a combination of two enzymes: pepsin and pancreatin. Fresh cheese sample with starter culture XPL-1 was diluted with water in the ratio 1:5, while cheese sample with kombucha was dissolved in the ratio 1:10 and heated to temperature of 37 °C on an electric stirrer. The first aliquot (1-1.5 mL) is then taken and the pH is measured. The aliquot was centrifuged for 5 minutes, the solution was separated and labelled. The rest of the solution on the electric mixer is still maintained at a temperature of 37 °C. HCl was added until a pH of ~2.5-3 was reached and then 0.05 g of pepsin was added. After 60 minutes, the aliquot (1-1.5 mL) was heated for 5 minutes at 100 °C, centrifuged (Eppendorf, Centrifuge 5424) and the solution separated and labelled. The rest of the solution was further maintained on an electric stirrer at 37 °C, NaOH was added until a pH of ~7.5 was reached and then 0.05 g of pancreatin was added. After 120 minutes, the aliquot (1-1.5 mL), was heated for 5 minutes at 100 °C, centrifuged, the solution separated and labelled. The collected supernatants were further analysed.

Determination of protein concentration and the degree of hydrolysis

Fresh cheese suspension and trihloracetic acid (0.44 mol/dm^3) were mixed in 1:1 volume ratio and incubated in the fridge, for 30 min. Thereafter, the mixture was centrifuged (Eppendorf, Centrifuge 5424). Soluble proteins were determined by the method of Lowry et al. (1951). The bovine serum albumin as the standard protein (0.5 mg/mL) was used for calibration. The measurement is based on the spectrophotometric (colorimetric) method, where the concentration of soluble proteins from the calibration line is determined on the basis of the absorption value of the coloured solution. The colour intensity is proportional to the protein content of the sample, and absorbance was measured spectrophotometrically at 660 nm (SP6-550 UV/VIS, spectrophotometer, Pye Unicam, England). The degree of hydrolyses (DH) was calculated as the ratio of 0.22 mol/dm^3 trichloroacetic acid (TCA) soluble proteins to total proteins in the hydrolysate, and expressed as a percentage.

$$DH\ (\%) = (TCA/\text{total proteins}) \times 100$$

Capillary electrophoresis

Protein characterization was performed by electrophoretic separation by automatic capillary chip electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Protein 80 Plus LabChip kit. Data analyses were performed with the Agilent 2100 Expert software. Before applying the samples to the chip, they were prepared according to the method proposed by Tidona et al. (2011) with some modifications. Applied method for preparation of milk samples: in 100 µL of milk was added 400 µL of deionized water, vortex treatment was applied for a few seconds, the sample was mixed with buffer in a ratio of 1: 1.5 (buffer 2 x treatment buffer (0.125 M tris-HCl pH 6.8, 4 % SDS, 2 % glycerol, 5 % 2-mercaptoethanol). After that, we applied a vortex treatment (for a few seconds), heat denaturation (100 °C, 5 min) and centrifugation of the samples (20 min, 14.000 rpm). Applied method for preparation of cheese samples: in 20 mg of cheese sample was added 250 µL of buffer (2 x treatment buffer (0.125 M tris-HCl pH 6.8, 4 % SDS, 2 % glycerol, 5 % 2-mercaptoethanol) and vortex treatment was applied for a few seconds. After heat denaturation (100 °C, 5 min), dissolved samples were diluted with 750 µL buffer and centrifuged (20 min, 14.000 rpm). The results of chip electrophoresis analysis are presented in software in two different ways in the form of quantitative profiles (electropherograms) and in the form of simulated gel images.

Total phenolic content

Total phenolic content in cheese samples and cheese hydrolysates was done by the Folin-Ciocalteu spectrophotometric method (Singleton and Rosi, 1965; Kähkönen et al., 1999). The values for absorbance of the samples were measured at 750 nm (6300 Spectrophotometer, Jenway, UK). Results are expressed as mg of gallic acid equivalents (GAE) per g of cheese sample (mg GAE/g), and in hydrolysates was expressed as mg of gallic acid equivalents (GAE) per mL of sample (mg GAE/mL).

DPPH assay

The ability to neutralize 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH) was measured using a modified method by Brand-Williams et al. (1995). DPPH reagent and diluted cheese sample were added to a glass cuvette (2.9 mL + 0.1 mL) and incubated at room temperature for 60 min. Absorbance was measured at 517 nm using a UV-VIS spectrophotometer (6300 Spectrophotometer, Jenway, UK). Results were reported as μM of Trolox equivalent per g of cheese sample (μM TE/g). Measurements in cheese hydrolysates were performed according to the same principle. Results were reported as μM equivalent of Trolox per mL of cheese hydrolysate (μM TE/mL).

FRAP assay

The ability to reduce Fe^{3+} was determined by the FRAP assay (Benzie and Strain, 1996). Properly diluted cheese samples and FRAP reagent were mixed (0.1 mL + 2.9 mL) and incubated in the dark at 37 °C for 10 min. Absorbance measurements were performed at 593 nm with UV - VIS spectrophotometer (6300 Spectrophotometer, Jenway, UK). Results were expressed as μM of Fe^{2+} equivalents per g of cheese sample (μM Fe^{2+}/g). Reduction capacity of the cheese hydrolysate was determined in the same way, and the results were expressed as μM of Fe^{2+} equivalent per mL of cheese hydrolysate (μM Fe^{2+}/mL).

ABTS assay

The ability to neutralize ABTS free radicals was determined using a modified spectrophotometric method reported by Re et al. (1999). Properly diluted cheese samples and ABTS reagent were mixed (0.1 + 2.9 mL) and incubated at room temperature for 5 h in the dark. Absorbance was measured at 734 nm with UV – VIS spectrophotometer (6300 Spectrophotometer, Jenway, UK). Results were expressed as μM of Trolox equivalents per gram of cheese sample (μM TE/g), and for cheese hydrolysate samples as μM Trolox equivalents per mL of sample (μM TE/mL).

Statistical analysis of results

Statistical analysis was performed in order to determine the influence of kombucha inoculum on the quality of fresh cheese. Each experiment was repeated at least three times. Analysis of variance (ANOVA) was performed using Statistica software program. The difference between the mean values was determined at the level of 5 % statistical significance using Duncan's test.

Results and discussion

Acidification process

Milk fermentation process by kombucha inoculum and traditional starter culture XPL-1 at temperature 35 °C was followed through measuring of pH value. Selected starter

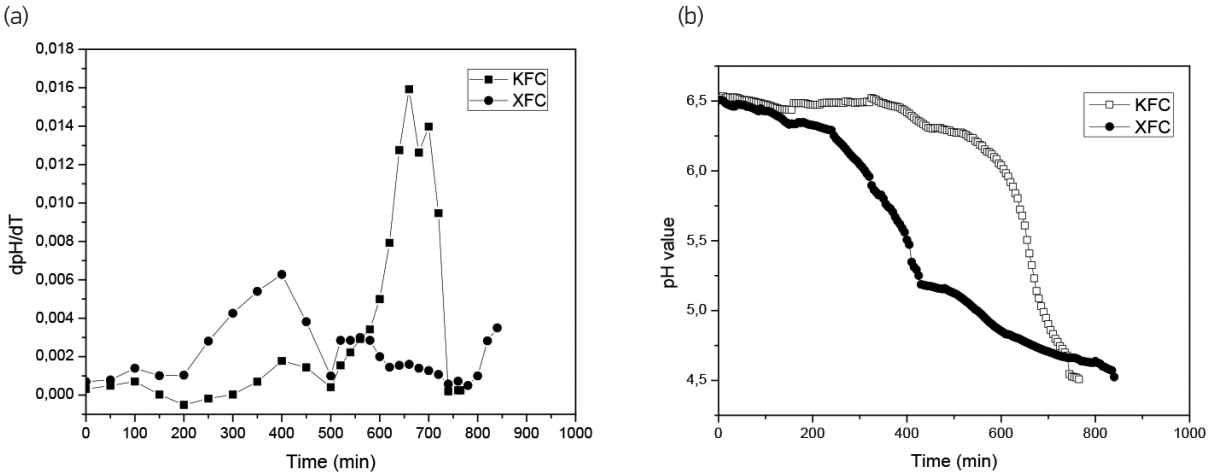


Figure 1. a) Acidification rate of milk by applying of kombucha inoculum and traditional starter culture b) Changes of the pH during milk fermentation by applying of kombucha inoculum and traditional starter culture

cultures had different acidification rate (Fig. 1a). It is evident that the fermentation process was faster in sample made with kombucha inoculum than in the sample produced by traditional starter culture (XPL-1). Acidification time of kombucha fresh cheese was 78 minutes shorter than traditional fresh cheese. Milk with kombucha inoculum reached a maximum acidification rate at 650 min (pH 5.00), while sample with tradition culture XPL-1 had maximum at 400 min (pH 5.25). Different acidification times are the result of different metabolic activity of the applied starter cultures. Decrease of pH during milk fermentation is a result of lactose fermentation. Consequently, the content of L-lactic acid increased in kombucha fermented milk samples (Vukić et al., 2014). Also, Ozyurt (2020) studied the fermentation process of beverages produced by kombucha cultivated on goat, cow and soya milk. The author found that using kombucha inoculum, the fermentation time of cow's milk to reach pH 4.4, is significantly shorter and it amounts to 6 hours. Malbaša et al. (2014) investigated fermentation process of milk with 0.8, 1.6 and 2.8 % fat content at 37, 40 and 43 °C using wild thyme kombucha starter. These authors revealed that the fermentation time of milk at 43 °C ranged from 10 to 14 hours. Also, they concluded that fermentation was significantly shorter at higher fermentation temperature regardless of milk fat content. Evidently, different factors, such as starter culture type, milk composition, fermentation temperature, macromolecules interaction contribute to decrease pH value of gel and different rates of coagulum formation during fresh cheese production.

Chemical composition of fresh cheese samples

The process of milk fermentation using kombucha inoculum and conventional (traditional) starter culture (XPL-1) significantly affects the chemical composition and profile of individual protein fractions in fresh cheese samples. Composition of fresh cheese samples are compared in Table 1. There are significant differences in chemical composition and protein contents between fresh cheese fermented by kombucha and fresh cheese produced by traditional starter culture. Dry matter of fresh cheese produced with XPL-1 starter culture (37.40 %) was significantly lower than in kombucha fresh cheese (49.83 %). These results are in accordance with the Serbian Regulation (2014). In this Standard, fresh cheeses are required to have the total solids content higher than 20 %. Also, our results of fresh cheese produced with XPL-1 showed similar chemical composition with literature data (Miloradović et al., 2018), while dry matter of kombucha fresh cheese was 45 % higher compared to cows´ Quark type cheese. Traditional fresh cheese contained 13.23 % protein and 19.0 % fat, while kombucha fresh cheese contained 21.33 % protein and 26.75 % fat. Casein content was 58 % higher in the sample of cheese with kombucha compared to cheese with traditional starter culture. It is evident that fresh cheese with kombucha contains a significant amount of milk proteins in which casein fractions are predominantly present and whey proteins in a much smaller amount. The content of casein in cheese obtained by kombucha represents 86.31 % of total solids non-fat, while fresh cheese with traditional culture contained 62.77 %.

Table 1. *Composition of milk and fresh cheese samples*

Parameter	Milk	XFC	KFC
Dry matter, %	12.21±0.71	37.40±1.60	49.83±0.35
Moisture, %	87.79±0.71	62.60±1.60	48.61±0.35
Protein, %	3.01±0.02	13.23±0.04	21.33±0.65
Casein, %	2.10±0.02	11.55±0.18	19.92±0.33
Whey protein, %	0.60±0.02	1.68±0.02	1.41±0.02
Fat, %	2.80±0.50	19.0±0.00	26.75±0.25
Ash, %	0.70±0.00	0.88±0.01	1.48±0.06
FDM*, %	22.93	50.80±1.60	53.68±0.30
SNF*, %	9.40	18.40±1.60	23.08±0.30
MNFS*, %	-	77.28±1.60	65.91±0.30

Data represents the mean ±SD of three independent experiments.
XFC - fresh cheese produced with traditional starter culture;
KFC - fresh cheese produced with kombucha inoculum,
** - calculated value;*
SNF - solid-non-fat, MNFS-moisture in non-fat substance,
FDM - fat in the dry matter.

Hydrolysis of fresh cheese protein

Milk proteins are nutritionally very important because they have numerous beneficially effects on the gastrointestinal (GI) and immune systems. Milk proteins are the important source of bioactive peptides. Bioactive peptides can be released by hydrolysis (e.g. pepsin, trypsin and chymotrypsin) during gastrointestinal digestion or by activity of proteases and peptidases released by lactic acid bacteria (LAB) (Santiago-Lopez et al., 2018). The solubilized protein content in supernatant related to protein content in undigested matrix represents the amount of soluble proteins in supernatant of digestion mixtures (Rinaldi et al., 2014). Soluble proteins and changes in hydrolysis degree (DH) at steps of the simulated gastrointestinal digestion are shown in Fig 2. Due to the presence of digestive enzymes the amount of soluble proteins increased during *in vitro* GI digestion for both fresh cheese samples. The content of soluble proteins during protein digestion was significantly higher in the sample of fresh cheese with kombucha than in cheese with traditional starter culture. The low amount of soluble proteins measured in both of samples after first initial phase (before addition of enzymes). The content of soluble proteins in XFC ranges from 20 mg/g in the initial phase to 90 mg/g after digestion with pancreatin. In kombucha fresh cheese, the content of soluble proteins after the third phase (decomposition by pancreatin) is 100 % higher in relation to the solubility of proteins in the XFC sample. The highest increase was observed in kombucha fresh cheese after digestion with pancreatin (180 mg/g sample).

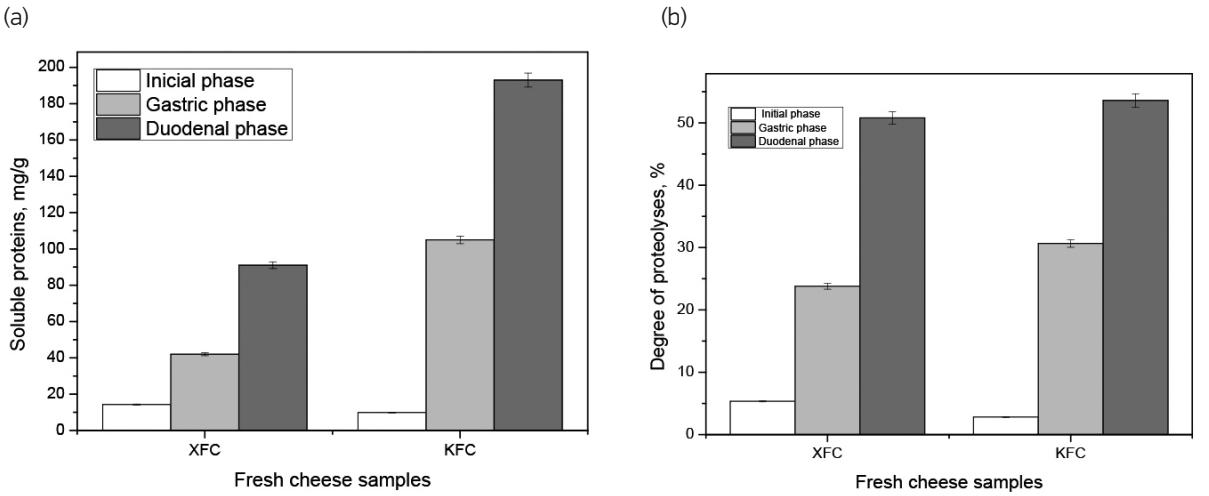


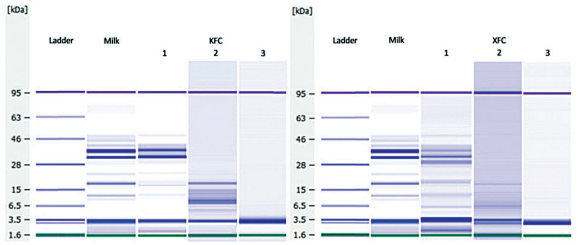
Figure 2. Soluble proteins and degree of proteolysis in fresh cheese sample after gastric and duodenal phases of in vitro digestion

The degree of proteolysis was calculated based on the content of soluble proteins and total proteins in the samples. The initial value of the degree of proteolysis in both cheese samples was less than 5 % and was slightly higher in the XFC sample (Fig 2b). The results of the degree of hydrolysis in cheese samples before digestion are higher compared to the data from the literature (Hrnjez et al., 2014; Tagliazucchi et al., 2017). Hrnjez et al. (2014) investigated the degree of proteolysis in fermented dairy products with starter culture and kombucha during storage. They concluded that at the end of fermentation the highest degree of proteolysis (compared to milk - 1.7 ± 0.02) had a sample with kombucha inoculum K (2.56 ± 0.151 %), a slightly lower value of the sample with probiotic starter culture P (2.35 ± 0.041 %), and the lowest value for the sample with yogurt starter culture Y (2.25 ± 0.034 %). Maximum proteolysis in fresh cheese samples was achieved in gastric phase with pancreatin. DH in gastric phase was higher in the KFC sample (53.60 %) than in XFC sample (50.8 %). Kombucha fresh cheese has 22.35 % less water and 27.27 % higher casein content in dry matter without fat than fresh cheese produced with XPL-1 culture, which contributed to higher protein solubility and a higher degree of proteolysis during the digestion of the kombucha fresh cheese. Our results can be correlated with results of Fang et al. (2016) who found that *in vitro* GI digestion depends on the chemical composition and texture of the sample, as well as on enzyme treatment. These authors concluded that water content negatively affect cheese disintegration suggesting that the disintegration in increased in low moisture and MNFS (moisture in the non-fat substance) cheese. Fang et al. (2016) found that cheese with high casein density are firmer and harder to disintegrate, but the composition has a stronger influence on cheese disintegration than the textural properties. Also, the addition of enzymes enabled 72 % of cheese disintegration to be achieved at the end of duodenal digestion, while it reached 30 % when no enzymes were added.

Protein characterisation during in vitro GI digestion

Lab on chip gel images of milk, undigested samples and digested fresh cheese samples during *in vitro* GI digestion are shown in Fig.3a. The first image shows comparison of the intensity of the bands of milk and fresh cheese samples. It is evident that casein fractions are predominantly present in kombucha fresh cheese, while fractions with molecular weights less than 10 kDa are more present in the fresh cheese obtained with traditional culture. Based on this results, a relative amount of protein fractions in undigested milk and fresh cheese samples was calculated (Fig.3b). The obtained relative amount of α-casein in tested milk was 34.81 %, whereas relative concentration of ß-casein was 24.46 % (Fig. 3a). The α-casein (31.08 %) and ß-casein (47.53 %) were detected in kombucha fresh cheese. During *in vitro* GI digestion, for XFC fresh cheese sample both fractions of casein are degraded more rapidly by pepsin than protein fraction in kombucha fresh cheese (Fig.3a). At the end of simulated duodenal digestion, the bands of caseins were not visible, while the band of whey proteins was still intense, that it has not been totally solubilized and digested. It means that the present whey protein aggregates may be also resistant to duodenal digestion. This is in accordance with other studies that have confirmed resistance of ß-lactoglobulin to digestion with pepsin (Hodgkinson et al., 2018). In digested cheese samples, whey proteins were detected in low relative amount, but it was higher in fresh cheese with XPL-1 culture than in kombucha fresh cheese. Since these proteins are one of the main milk allergens, it can be assumed that cheese with kombucha inoculum is a smaller allergen than traditional fresh cheese and can be used for a special diet for people with protein allergies.

(a)



(b)

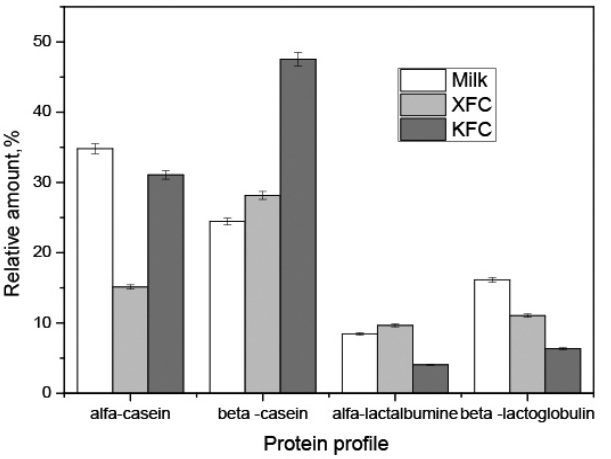


Figure 3. a) Comparison of gel images of undigested (milk and fresh cheese samples) and digested samples b) Relative amount of selected protein fraction in digested fresh cheese samples; (1) first lane undigested fresh cheese, (2) second lane at the beginning of GI digestion, after the addition of pepsin and (3) third lane - after the addition of pancreatin

Antioxidant capacity of fresh cheese samples

Fermentation process, protein profile, content of soluble proteins and degree of proteolysis significantly influence antioxidant potential of fresh cheese samples. The results of antioxidant activity and total phenol content in milk, kombucha inoculum, fresh cheese samples and their hydrolysates are shown in Table 2. Antioxidant activity of milk and kombucha inoculum (0.0287 μM Fe^{2+}/g and 0.0056 μM Fe^{2+}/g, respectively) was lower than in cheese samples. Considering antioxidant capacity (AC), the obtained results showed higher antioxidant capacity (AC) of fresh cheese with traditional culture (XFC) (1.24 μM Fe^{2+}/g) than kombucha fresh cheese (0.93 μM Fe^{2+}/g). The results of DPPH assay showed different ability for milk, kombucha and fresh cheese samples. DPPH value of milk and kombucha inoculum were 0.0014 and 0.007 μM TE/g, respectively. Statistically significant increase ($p<0.05$) in antioxidant activity was found in both fresh cheese samples. Maximum DPPH value (0.95 μM TE/g) was observed in kombucha fresh cheese, while DPPH of cheese sample XFC was 0.93 μM TE/g. Among all samples, the best sample which showed the highest ABTS was fresh cheese produced by a traditional starter culture (XPL-1). The ABTS radical scavenging abilities of kombucha fresh cheese (1.2203 μM TE/g) was significantly lower than in control sample (2.0148 μM TE/g). These results are in accordance with literature data. Hrnjez et al. (2014) investigated biological potential of fermented milks produced by application probiotic (P), yoghurt (Y) and non conventional starter culture kombucha (K). They found that the highest ABTS value had the sample Y (50.6542), slightly lower value the sample P (47.4733) and finally the lowest value the sample K (41.0446). The DPPH value after production was the highest in sample kombucha fermented milk product (17.8832), lower in probiotic sample (13.2299) and in yoghurt (9.4586). The antioxidant activities of fresh cheese hydrolysates is also presented in Table 2. It is evident that FRAP, DPPH and ABTS of radical scavenging abilities of kombucha fresh cheese hydrolysate are higher than in hydrolysates of fresh cheese produced with traditional starter culture (XPL-1), but DPPH and ABTS not statistically significant. The higher antioxidant potential of the hydrolysate prepared from fresh kombucha cheese can be explained by the fact that this cheese sample has a higher protein content

Table 2. The antioxidant activities of milk, kombucha inoculum and fresh cheese samples and their hydrolysates

Samples	FRAP (μM Fe2+/g)	DPPH (μMTE/g)	ABTS (μMTE/g)	TP (mg GAE/g)
Milk	0.0287±0.0011^{a}	0.0014±0.0001^{a}	0.0312±0.0014^{a}	1.2027±0.0069^{c}
Kombucha	0.0056±0.0001^{a}	0.0070±0.0001^{a}	0.0041±0.0000^{a}	0.1252±0.0094^{a}
XFC	1.2416±0.2293^{d}	0.93207±0.2173^{b}	2.0148±0.0206^{c}	0.98±0.03^{b}
KFC	0.9333±0.0532^{c}	0.9524±0.1380^{b}	1.2203±0.6192^{b}	5.81±0.28^{d}
XFCh	0.3329±0.018^{b}	0.1076±0.0191^{d}	0.0339±0.0043^{a}	0.094±0.0014^{a}
KFCh	0.382±0.034^{b}	0.2003±0.0172^{a}	0.0547±0.0003^{a}	0.1061±0.0015^{a}

KFC - fresh cheese produced with kombucha inoculum; XFC - fresh cheese produced with traditional starter culture; KFCh - hydrolysate of fresh cheese with kombucha inoculum; XFCh - hydrolysate of fresh cheese with starter culture XPL-1; Mean values±standard error of three trials in the same column with different small letters superscripts indicate significant difference at $p<0.05$ among samples.

(casein fraction) as well as a higher content of phenols which significantly contribute to the biological potential of the product. The share of casein in fat free dry matter in the kombucha cheese sample is 27.26 % higher compared to fresh cheese produced by traditional culture (XPL-1). Rival et al. (2001) found that the antioxidant potential of casein fractions ranges from ß-casein> αs1-casein> αs2-casein> α lactalbumin> ß-lactglobulin. Since the relative proportion of ß-casein is predominantly present in kombucha cheese, the hydrolysate of kombucha fresh cheese had greater antioxidant potential compared to traditional fresh cheese (XFC).

In addition to the significant protein content, the phenol content in kombucha fresh cheese significantly affected the antioxidant potential of the cheese samples. Total phenolic content of kombucha inoculum was 0.1252 mg GAE/g and in milk sample 1.203 mg GAE/g, respectively. Phenolic contents of the inoculum have influenced the total phenolic content of fresh cheese. Kombucha fresh cheese sample showed a higher total phenol content than fresh cheese produced by traditional starter culture. Total phenols determined in kombucha fresh cheese sample was 5.81 mg GAE/g, while in traditional fresh cheese content of phenol was 0.98 mg GAE/g. Application of kombucha contributed the increase in phenolic components in fresh cheese sample. These results are in accordance with literature data (Ozyurt 202; Cardoso et al., 2020). Ozyurt (2020) concluded that total phenolic contents of black tea – based beverages increased with fermentation time and ranged from 227.78 to 312.26 µg GAE/g. Also, Cardoso et al. (2020) found high concentrations of most phenolic concentration and catechin in kombucha. Black tea kombucha had a total phenolic content of 1.09 mg GAE/mL, which was approximately 55.7 % higher than that of green tea kombucha with 0.70 mg GAE/mL. According to Cardoso et al. (2020), 127 phenolic components have been identified in green and black kombucha tea that contribute to higher antioxidant capacity. Antioxidant activity is associated with the formation of low molecular weight components and the degradation of polyphenols present in tea by enzymes produced by bacteria and yeasts during fermentation (Cardoso et al., 2020; Coelho et al., 2020). Total phenolic components and content of some components in milk and kombucha inoculum which possess strong antioxidant activity are expected to be directly related to fresh cheese antioxidant capacity. The higher antioxidant potential of fresh cheese hydrolysate produced using kombucha compared to the sample of cheese produced by traditional culture can be related to higher content of soluble proteins and the degree of proteolysis of cheese samples during digestion. Given the results of the biological potential of the analysed cheese samples, it can be concluded that the kombucha cheese sample provides better protein bioavailability. Due to the content of phenolic components and antioxidant ability, kombucha has a positive effect on health and it is recommended for people who are exposed to oxidative stress (Cardoso et al., 2020; Coelho et al., 2020).

Conclusions

The application of non-conventional kombucha inoculum in the technology of fermented dairy products has shown a significant impact on fermentation process, protein profile and biological potential of fresh cheese. There is a significant difference in protein profile and biological potential between kombucha fresh cheese sample and fresh cheese obtained by traditional starter culture. Protein digestion in two fresh cheese samples was investigated using i*n vitro* gastrointestinal digestion. The hydrolysates of fresh cheese obtained by kombucha inoculum showed higher digestibility compared to the sample with traditional starter culture in all phases of digestion. Kombucha fresh cheese showed higher antioxidant activity compared to milk and kombucha inoculum. Kombucha inoculum as non-conventional starter culture could be good solution to additionally increase content of phenol and their antioxidant activity in fresh cheese. Based on these results, it can be concluded that kombucha inoculum contributes to fresh cheese enrichment and thereby enhances its potential as functional food.

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Proteinski profil i antioksidativni potencijal svježeg sira dobivenog korištenjem kombucha inokuluma

Sažetak

U ovom radu ispitani su proteinski profil, *in vitro* gastrointestinalna digestija i antioksidativni potencijal dva uzorka svježeg sira dobivenog korištenjem kombucha inokuluma i tradicionalne starter kulture. Utvrđena je značajna razlika u proteinskom profilu i biološkom potencijalu između uzoraka svježeg sira. Kombucha svježi sir sadržava veći udio proteina, pretežno frakcija kazeina (α-kazein i ß-kazein) u odnosu na svježi sir koji je dobiven tradicionalnom starter kulturom. Tijekom gastrointestinalne digestije, frakcije kazeina brže se razgrađuju pepsinom u uzorku svježeg sira s tradicionalnim starter kulturom nego u kombucha svježem siru. Nakon gastrointestinalne digestije (s pepsinom i pankreatinom), u uzorku kombucha svježeg sira utvrđena je znatno veća količina topljivih proteinskih frakcija i viši stupanj hidrolize u odnosu na uzorak dobiven tradicionalnom starter kulturom. Hidrolizati sira dobiveni inokulumom kombuche sadržavali su veće koncentracije fenola i imali veću sposobnost uklanjanja antiradikala od hidrolizata svježeg sira proizvedenog s tradicionalnom starter kulturom. Rezultati pokazuju da primjena inokuluma kombuche doprinosi poboljšanju biološkog potencijala svježeg sira.

Ključne riječi: svježi sir; kombucha inokulum; proteinski profil; antioksidativni potencijal

References

1. Amirdivani, S., Baba, A.S. (2011): Changes in yogurt fermentation characteristics, and antioxidant potential and *in vitro* inhibition of angiotensin-I- converting -enzyme upon the inclusion of peppermint, dill and basil, *Food Science and Technology - LWT,* 44, 1458-1464. http://dx.doi.org/10.1016/j.lwt.2011.01.019
2. 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 (1), 70-76. https://doi.org/10.1006/abio.1996.0292
3. Brand-Williams, W., Cuvelier, M.E., Berset. C. (1995): Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology* 28 (1), 25-30. https://doi.org/10.1016/S0023-6438(95)80008-5
4. Bu, G., Luo, Y., Zhang, Y., Chen, F. (2010): Effect of fermentation by lactic acid bacteria on the antigenicity of bovine whey protein, *Journal of the Science of Food and Agriculture* 90, 2015-2020. http://dx.doi.org/10.1002/jsfa.4046
5. Cardoso, R.R., Neto, R.O., dos Santos D'Almeida, C.T., do Nascimento, T.P., Pressete, C.G., Azevedo, L., Martino, H.S.D., Cameron, L.C., Ferreira, M.S.L., de Barros, F.A.R. (2020): Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. *Food Research International* 128, 108782. https://doi.org/10.1016/j.foodres.2019.108782
6. Carić, M., Milanović, S., Vucelja, D.(2000): *Standardne metode analize mleka i mlečnih proizvoda, Standard method of analysis of milk and dairy products*. Prometej, Novi Sad.
7. Coelho, R.M.D., de Almeida, A.L., do Amaral, R.Q.G, da Mota, R.N., de Sousa, P.H.M. (2020): Kombucha: Review. *International Journal of Gastronomy and Food Science* 22, 100272. https://doi.org/10.1016/j.ijgfs.2020.100272
8. De Fillippis, F., Troise, A.D., Vitaglione, P., Ercolini, D. (2018): Different temperatures select distinctive acetic acid bacteria species and promotes organic acids production during Kombucha tea fermentation. *Food Microbiology* 73, 11-16. http://dx.doi. org/10.1016/j.fm.2018.01.008
9. Fang, X., Rioux, L., Labrie, S., Turgeon, S. (2016): Disintegration and nutrients release from cheese with different textural properties during *in vitro* digestion. *Food Research International* 88, 276-283. https://doi.org/10.1016/J.FOODRES.2016.04.008

10. Hodgkinson, A.J., Wallace, O.A.M., Boggs, I., Broadhurst, M., Prosser, C. G. (2018): Gastric digestion of cow and goat milk: Impact of infant and young child in vitro digestion conditions. *Food Chemistry* 245, 275-281. http://dx.doi.org/10.1016/j.foodchem.2017.10.028
11. Hrnjez, D., Vaštag, Ž., Milanović, S., Vukić, V., Iličić, M., Popović, Lj., Kanurić, K. (2014): The biological activity of fermented dairy products obtained by kombucha and conventional starter cultures during storage. *Journal of Functional Foods* 10, 336-345. https://doi.org/10.1016/j.jff.2014.06.016
12. Iličić, M., Bjekić, M., Milanović, S., Vukić, V., Kanurić, K., Vukić, D. (2019): Comparison characteristics of fresh cheese made with kombucha inoculum and traditional starter. *Acta Periodica Technologica,* Issue 50, 86-92. https://doi.org/10.2298/APT1950086I
13. Jafari, R., Naghavi, N.S., Khosravi - Darani, K., Doudi, M., Shahanipour, K. (2020): Kombucha microbial starter with enhanced production of antioxidant compounds and invertase. *Biocatalysis and Agricultural Biotechnology* 29, 101789. https://doi.org/10.1016/j.bcab.2020.101789
14. Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., Heinonen, M. (1999): Antioxidant activity of plant extracts containing phenolic compounds. *Journal Agriculture and Food Chemistry* 47 (10), 3954-3962. http://dx.doi.org/10.1021/jf990146l.
15. Kanurić, K., Milanović, S., Ikonić, B., Lončar, E., Iličić, M., Vukić, V., Vukić. D. (2018): Kinetics of lactose fermentation in milk with kombucha starter. *Journal of Food and Drug Analysis* 26, I229-I234. http://doi.org/10.1016/j.jfda.2018.02.002
16. Leonarski, E., Cesca, K., Zanella, E., Stambuk,B.U., de Oliveira, D., Poletto, P. (2021): Production of kombucha-like beverage and bacterial cellulose by acerola byproduct as raw material. *LWT - Food Science and Technology* 135, 110075. https:// doi.org/10.1016/j.lwt.2020.110075
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193 (1), 265-275. http://dx.doi.org/10.1016/S0021-9258(19)52451-6
18. Malbaša, R., Milanović, S., Lončar, E., Djurić, M., Carić, M., Iličić,M., Kolarov, Lj. (2009): Milk-based beverages obtained by Kombucha application. *Food Chemistry* 112, 178-184.
19. Malbaša, R., Vitas, J., Lončar, E., Grahovac, J., Milanović, S. (2014): Optimisation of antioxidant activity of kombucha fermented milk products. *Chech Journal of Food Science* 32, 477-484. https://doi.org/10.17221/447/2013-CJFS
20. Milanović, S., Iličić, M., Carić, M. (2017): *Fermentisani mlečni proizvodi*, Fermented dairy products, Tehnološki fakultet, Novi Sad, p. 242. ISBN 978-3-319-42457-6; CIP 637.146; COBISS.SR-ID 313762823
21. Miloradović, Z., Miočinović, J., Kljajević, N., Tomašević, I., Pudja, P. (2018): The influence of milk heat treatment on composition, texture, colour and sensory characteristics of cow's and goats' Quark-type cheeses. *Small Ruminant Research* 169, 154-159. https://doi.org/10.1016/j.smallrumres.2018.09.012
22. Najgebauer-Lejko, D., Sady, M., Grega T., Walczycka, M. (2011): The impact of tea supplementation on microflora, pH and antioxidant capacity of yoghurt, *International Dairy Journal* 21, 568-574. https://doi.org/10.1016/j.idairyj.2011.03.003
23. Ozyurt, V.H. (2020): Changes in the content of total polyphenols and antioxidant activity of different beverages obtained by Kombucha "tea fungus". *International Journal of Agriculture, Environment and Food Sciences* 4 (3), 255-261. https://doi.org/10.31015/jaefs.2020.3.3
24. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999): Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26 (9-10), 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

25. Rinaldi, L., Gauthier, S.F., Britten, M., Turgeon, S.L. (2014): In vitro gastrointestinal digestion of liquid and semi- liquid dairy matrixes. *LWT- Food Science and Technology*, 57, 99-105. https://doi.org/10.1016/j.lwt.2014.01.026
26. Rival, S.G., Fornaroli, S., Boeriu, C.G., Wichers, H.J., (2001): Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *J. Agr. Food Chem.* 49 (1), 287-294.
27. Santiago-López, L., Aguilar-Toalá, J.E., Hernández-Mendoza, A., Vallejo-Cordoba., B., Liceaga., A.M., González-Córdova, A.F. (2018): Invited review: Bioactive compounds produced during cheese ripening and health effects associated with aged cheese consumption. *Journal of Dairy Science* 101 (5). https://doi.org/10.3168/jds.2017-13465
28. Singleton, V.L., Rossi, J.A. (1965): Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16 (3), 144-158.
29. SRPS EN ISO 5534:2007. Sir i topljeni sir - Određivanje sadržaja ukupne suve materije (Referentna metoda). Cheese and processed cheese- Determination of total solids content (Reference method)
30. SRPS EN ISO 8968-4:2016. Mleko i proizvodi od mleka - Određivanje sadržaja azota - Deo 4: Određivanje sadržaja proteinskog i neproteinskog azota i izračunavanje stvarnog sadržaja proteina (referentna metoda). Milk and milk products - Determination of nitrogen content - Part 4: Determination of protein and non-protein nitrogen content and true protein content calculation (Reference method)
31. SRPS ISO 3433:2013. Sir - Određivanje sadržaja masti - Metoda po Van Guliku. Cheese -- Determination of fat content - Van Gulik method.
32. Serbian regulation, 2014. Amendment on Serbian Regulation (2010): Pravilnik o kvalitetu proizvoda od mleka i starter kultura (Sl.glasnik RS).Quality of Dairy Products and Starter Cultures, 33/10. Official Bulletin of the Republic Serbia, Belgrade, Serbia.
33. Schulz-Collins, D., Senge, B. (2004): Acid- and Acid/Rennet-curd Cheeses, Part A: Quark, Cream Cheese and Related Varietes. In: Cheese, Physics and Microbiology, vol.2, pp.301-328.
34. Tagliazucchi, D., Shamsia, S., Helal, A., Conte, A. (2017): Angiotensin-converting enzyme inhibitory peptides from goats' milk released by in vitro gastro-intestinal digestion. *International Dairy Journal* 71, 6-16. https://dx.doi.org/10.1016/j.idairyj.2017.03.001
35. Tidona, F., Sekse, C., Criscione, A., Jacobsen, M., Bordonaro, S., Marletta, D. (2011): Antimicrobial effect of donkeys' milk digested in vitro with human gastrointestinal enzymes. *International Dairy Journal* 21, 158-165. http://dx.doi.org/10.1016/j.idairyj.2010.10.008
36. Vukić, D., Vukić, V., Milanović, S., Iličić, M., Kanurić, K. (2018): Modeling of rheological characteristics of the fermented dairy products obtained by novel and traditional starter cultures. *J Food Sci Technol.* 55, 2180-2188. http://doi.org/10.1007/s13197-018-3135-9
37. Vukić, V., Hrnjez, D., Kanurić, K., Milanović, S.,Iličić, M., Torbica A., Tomić J. (2014): The effect of kombucha starter culture on gelation process, microstructure and rheological properties during milk fermentation. *Journal of Texture Studies* 45, 261-273.
38. Vukić, V., Milanović, S., Iličić., M., Vukić, D., Kanurić, K., Carić, M. (2021): Sveži sir fermentisan kombuhom/Fresh Cheese fermented by kombucha. *Patent* A23C 19/032 (2006.01) (11) 61625 B1.