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THE ANTIOXIDANT ACTIVITY OF KOMBUCHA FERMENTED MILK PRODUCTS WITH STINGING NETTLE AND WINTER SAVORY

This paper investigates the antioxidant activity of fermented milk products obtained by kombucha fermentation. Two starter cultures were used as follows: starter obtained after kombucha fermentation on sweetened stinging nettle extract, and starter obtained after kombucha fermentation on sweetened winter savory extract. The starters were added to milk with 0.8, 1.6 and 2.8% milk fat. Fermentation was carried out at 37, 40 and 43 °C and stopped when the pH reached 4.5. Antioxidant activity to hydroxyl and DPPH radicals was monitored using response surface methodology. Kombucha fermented milk products with stinging nettle (KSN) and with winter savory (KWS) showed the same antioxidant response to hydroxyl and different response to DPPH radicals. The synergetic effect of milk fat and fermentation temperature to antioxidant activity to hydroxyl radicals for both types of kombucha fermented milk products (KSN and KWS) was established. The optimum processing conditions in term of antioxidant activity were: milk fat around 2.8% and process temperature around 41 and 43 °C for KSN and KWS, respectively.

Keywords: kombucha, fermented milk products, antioxidant activity, stinging nettle, winter savory.

Kombucha is a symbiotic association of several yeasts (genera *Schizosaccharomyces*, *Saccharomycodes*, *Saccharomyces*, *Zygosaccharomyces*, *Candida*, *Pichia*, *Kloeckera*, *Brettanomyces* and *Torulopsis*) and acetic acid bacteria (*Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*), *Acetobacter xylinoides*, *Bacterium gluconicum*, *Acetobacter aceti*, *Acetobacter pasteurianus*) [1-3]. The exact microbiological composition depends on the culture origin. Kombucha is also frequently called tea fungus in the literature, although there is actually no fungus involved in the fermentation [4].

Under aerobic conditions, kombucha symbiosis is capable of converting a very simple substrate (usually black or green tea sweetened with sucrose), over a period of 7-10 days, into a slightly carbonated, mildly sour and refreshing beverage. A jelly-like membrane, metabolic product of *Gluconacetobacter xylinus*, floats in the nutrient solution of tea and sugar. At a temperature of 20-30 °C, it multiplies continuously. At first, it spreads over the entire surface of the tea, and then thickens [5]. This beverage is composed of sugars, organic acids, as well as ethanol, 14 amino acids, water soluble vitamins, minerals, antimicrobial active matters and some hydrolytic enzymes [6,7].

It has been reported that the kombucha beverage helps with headaches, gastric illnesses, diabetes, nervousness and aging problems, possesses antibiotic properties, participate in regulation of gastric, intestinal and glandular activities, relief of joint rheumatism, gout and hemorrhoids, has a positive influence on the cholesterol level, arteriosclerosis, toxin excretion and blood cleansing, provides resistance to cancer and increases the immune system performance [1].

Kombucha is most commonly prepared by cultivation of sweetened black tea, but other substrates, such as coca-cola, wine, beer, fruit drinks, milk and herbal teas can be used [8]. It has been also reported the possibility of production of kombucha beverage in a larger scale using different inoculum concentrations [9,10].

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Satureja montana L., commonly known as winter savory, belongs to the *Lamiaceae* family, *Nepetoideae* subfamily and *Mentheae* tribe. *S. montana* L. is native to the Mediterranean and is found throughout Europe, Russia and Turkey. *S. montana* L. is a strong aromatic herb and has been used for centuries as a spice for food and teas. *S. montana* L. has biological properties that are related to the presence of its major essential oils chemical compounds thymol and carvacrol. The whole plant, the essential oil and extracts are used in the traditional medicine for their digestive, carminative, aphrodisiac, bactericidal and fungicidal activities. It has been demonstrated the antimicrobial activity of *S. montana* L. essential oil or extracts against both Gram-positive and Gram-negative bacteria as well as antidiarrhoeal, antioxidant, antiproliferative activities on human erythroleukemic K562 cells, on HeLa (cervix epithelioid carcinoma), HT-29 (colon adenocarcinoma) and MCF-7 (breast adenocarcinoma) cell lines. Anticholinesterase and anti-HIV-1 properties of *S. montana* L. were also demonstrated [11].

Stinging nettle (*Urtica dioica* L.) is a plant with long history as herbal remedy. Stinging nettle is a very nutritious food that is easily digested and is high in minerals (especially iron), vitamin C and pro-vitamin A. Tea made from leaves has traditionally been used. The stinging nettle leaves are a good source of essential amino acids, ascorbic acid, available and unavailable carbohydrates, fatty acids and carotenoids and several mineral elements. Beneficial effects in health have been reported for stinging nettle intake. The stinging nettle extracts inhibit the expression of several cytokines as well as eicosanoid formation in stimulated peripheral blood cells. Besides, the extracts are used as an adjuvant remedy in rheumatoid arthritis with a proven therapeutic efficiency [12].

Fermented milk products represent a very diverse group of nutritive highly valuable products that are obtained by milk fermentation with appropriate starter culture. Many different types of fermented milk products are manufactured throughout the world [13]. The investigations proved that fermented milk products can be produced by application of kombucha starters to cow's milk. The metabolic activities of kombucha cultures (grown-up on black tea, green tea and topinambur) are similar but different from the activity of yoghurt starter. The chemical compositions of the kombucha fermented milk beverages differ significantly from the composition of yoghurt. The differences are large for dry matter and protein content [14].

The interest has increased in naturally-occurring antioxidants that can be used to protect humans from damage caused by oxidative stress. Overproduction of reactive oxygen species (ROS) in humans, by endogenous or external sources, leads to oxidative stress. In healthy cells, there is an equilibrium between the production of highly reactive species and the different defense systems, either enzymatic or non-enzymatic. Oxidative damage due to free radical accumulation, defined as oxidative stress, occurs when equilibrium is disrupted. As a consequence, many diseases and even ageing are promoted [15].

Vitamin C is one of the compounds characteristic for kombucha beverages [6]. As an effective reducing agent, vitamin C shows a powerful scavenging property against free radicals and activated oxygen species. This is a water-soluble compound which acts as non-enzymatic, chain-breaking antioxidant. As an electron donor, vitamin C protects by neutralizing ROS [16]. Vitamin C supplementation has been shown to reduce levels of oxidative stress, thereby reducing potential damage to tissues. While the direct antioxidant protection afforded by vitamin C is limited to water-soluble environments; vitamin C plays an antioxidant role in lipids through its regeneration of fat-soluble vitamin E [17].

Milk and dairy products are the major source of the total fatty acid intake of the human diet. Polyunsaturated fatty acids (PUFAs) have unique structural and functional characteristics. They are distinguished by two main functions. The first function relates to their roles in regulating the architecture, dynamics, phase transition and permeability of membranes, and modulating the behaviour of membrane-bound proteins such as receptors, ATPases, transport proteins and ion channels. The second role of PUFAs as precursors of a wide variety of metabolites (such as prostaglandins, leukotrienes and hydroxy-fatty acids) is regulation of critical biological functions [18]. Epidemiologic studies have shown that consumption of fish oil, rich in ω-3 PUFA, reduces the risk of cardiovascular disease in humans. PUFA exerts its beneficial effect on cardiovascular function through decreasing plasma triacylglycerol concentration and inhibiting free radical production [19].

The antioxidant activities of fermented beverages obtained by kombucha on black or green tea to certain free radicals were reported by some authors [20,21]. Sai Ram *et al.* [22] found that traditional kombucha tea has potent antioxidant and immunopotentiating activities.

In statistically based approaches, response surface methodology (RSM) has been extensively used for optimization of different biotechnological processes. RSM is a collection of mathematical and statistical techniques widely applied in the food industry to determine the effects of several variables and to optimize conditions [23]. In RSM, the experimental responses to the design of experiments are fitted to quadratic function. The number of successful applications of RSM suggests that the second-order relation can reasonably approximate several of the fermentation systems.

This article considers the antioxidant activity of kombucha fermented milk products. The kombucha starter cultures were created by fermentation of native kombucha on sweetened stinging nettle and winter savory extracts. The kombucha starters were cultivated on milk with different milk fat contents at three different temperatures. The present study was designed to describe changes of antioxidant activity to hydroxyl and DPPH radicals, PUFA and vitamin C content employing mathematical relationships in order to enable prediction of these indicators of the kombucha fermented milk products quality, depending on milk fat content and fermentation temperature. Further, the developed prediction models were used to generate optimum processing conditions.

EXPERIMENTAL

Milk

Pasteurized, homogenized cow's milk with 0.8, 1.6 and 2.8% milk fat, from the manufacturer AD IMLEK Beograd, branch Novosadska mlekara, Novi Sad (Serbia), was used for the production of fermented milk products in the laboratory.

Kombucha starter cultures

The local kombucha culture contains at least five yeast strains (*Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bisporus*, *Torulopsis* sp. and *Zygosaccharomyces* sp.), which were determined in previous investigations. Primary kombucha bacterium belongs to the strains of the genus *Acetobacter* [21].

Two different kombucha inoculums were used as starter cultures for milk fermentation:

- Fermentation liquid of kombucha after 7 days long fermentation at 25 °C on stinging nettle extract (2.25 g/L) sweetened with sucrose (7%).

- Fermentation liquid of kombucha after 7 days long fermentation at 25 °C on winter savory extract (2.25 g/L) sweetened with sucrose (7%).

Herbal teas were purchased in the local health food store.

Fermentation

Kombucha starters with winter savory and stinging nettle were cultivated on milk with different contents of milk fat (0.8, 1.6 and 2.8%) at 37, 40 and 43 °C. The amount of inoculum added into milk was 10% (v/v). Fermentation was performed until the pH value of 4.5 was reached. Milk gel is the result of milk fermentation process. During this process, the pH value of milk is lowering and at the isoelectric point of casein (pH value 4.6) the gel is formed by the aggregation of casein micelles in clusters and chains, *i.e.*, in a three-dimensional protein matrix. After the fermentation, the milk gel was cooled to the temperature of 8 °C, homogenized by mixer, and the samples were stored in a refrigerator. It provided a production of eighteen different fermented milk products. Products obtained using kombucha starter with stinging nettle were labeled with mark KSN while products obtained using kombucha starter with winter savory were labeled with mark KWS.

Fermentation was done in triplicate.

Methods of analysis

AA_{DPPH} was determined according to Živković *et al.* [24].

AA_{OH} was determined according to Deeseenthum and Pejovic [25].

Antioxidant activities were represented as differences between the values obtained for kombucha fermented milk products and milk.

pH values were measured by a pH-meter (Iskra, MA 5713, Kranj, Slovenia).

Vitamin C was determined using the HPLC system Agilent 1100 (USA). A C-8 column of dimensions 150 mm×4.6 mm and diameter 5 μm was used. As a mobile phase, ammonium acetate (0.1 mol/L), at pH 5.1, was applied. All chemicals were HPLC purity grade. The flow rate was 0.4 mL/min. The loop volume was 20 μL. Vitamin C was detected by DAD at 254 nm. After vitamin C extraction using acetic solution (8%) of *m*-phosphoric acid (3%), the samples for HPLC analyses were prepared by filtering through hydrophilic membrane filter, whose pore diameter was 0.45 μm.

PUFA content was determined using the GC-MS technique according to Kravić *et al.* [26].

All experiments in this study were carried out in triplicate and the results were averaged. The reproducibility of these measurements was good and the deviations between parallel experiments were in the range of ±5.2%. The results were statistically processed by analysis of variance at the significance level of α = 0.05. The adequacy of the model was evaluated by coefficient of determination (R^2) and model p-value. For the description of the responses Y (AA_{DPPH}, AA_{OH}, PUFA and vitamin C), a second-degree polynomial model was fitted to data (Eq. (1)):

$$Y = b_0 + \sum b_i X_i + \sum b_{ii}^2 X_{ii}^2 + \sum b_{ij} X_i X_j \quad (1)$$

where b_0 is the intercept, b_i represents the linear, b_{ii} the quadratic and b_{ij} the interaction effect of the factors. The factor variables and their values are: X_1, milk fat (0.8, 1.6 and 2.8%) and X_2, fermentation temperature (37, 40 and 43 °C).

Statistical and graphical analyses of the data were performed using Statistica 9.1 software [27]. Plotting responses as a function of two factors drew response surface plots. Plots were generated using the same software.

For the determination of optimal values of processing variables, the method of desired function was applied [28].

Sensory characterization of kombucha fermented milk products was performed by qualified evaluators together with the common consumers, who evaluated each particular element of quality as follows: appearance (0-1), colour (0-1), consistency (0-4), odour (0-2) and taste (0-12). It provides the possibility for total mark in a range from 0-20.

RESULTS AND DISCUSSION

Development of fermentation

In accordance with the plan of experiments, eighteen kombucha fermented products were made. Starters were added to the milk in the amount of 10% (v/v) which provides the yeasts count of approximately 10^5 cells/mL of substrate and acetic acid bacteria count of approximately 1.4×10^5 cells/mL of substrate. The number of microorganisms was slightly higher in comparison to some previous investigations of kombucha fermentation on black and green tea [21]. Development of all fermentation processes was followed by measuring pH values in time. As the end of fermentation, a value typical for yoghurt (pH 4.5) was adopted. The fermentation temperature was the most significant factor for the duration of fermentations. The processes lasted for about 16 to 17 h at 37 °C, 11 to 12 h at 40 °C, and 9 to 10 h at 43 °C on all substrates using both kombucha starters. pH decreased very slowly in the first half of the fermentation, then dropped exponentially, almost till the end of the process, and stagnated at the end. The described dynamics of fermentation was very similar to the processes performed by Malbaša *et al.* [14] who applied some different types of kombucha inoculums.

It is important to say that products obtained using kombucha starters were similar to the yoghurt. They were without separated whey, with uniform colour, and flavour typical for fermented milk products, *i.e.*, for yoghurt. Taste was mild and pleasant with aroma characteristic for used herb extract.

Antioxidant activity, PUFA and vitamin C content of the KSN fermented milk products

AA_{DPPH}, AA_{OH}, PUFA and vitamin C content are the selected responses for the investigation of antioxidant activity of kombucha fermented milk products using RSM methodology. The results of the statistical analyses for AA_{DPPH}, AA_{OH}, PUFA and vitamin C content in KSN fermented milk products are presented in Table 1. The coefficients in Table 1 are related to actual variables.

The ANOVA results for selected responses are reported in Table 2. Relatively high values of coefficient of determination (R^2 > 0.9), obtained for all res-

Table 1. Regression equation coefficients for the response of KSN products

Effects	AA_{DPPH}		AA_{OH}		PUFA		Vitamin C	
	Coefficient	p-Value	Coefficient	p-Value	Coefficient	p-Value	Coefficient	p-Value
Intercept								
b_0	-1209.75	0.0826[b]	136.2680	0.2842	-21.8609	0.7071	-34.2905	0.9618
Linear								
b_1	-87.02	0.0477[a]	-19.5874	0.0463[a]	-11.4216	0.0322[a]	82.0702	0.1167
b_2	66.21	0.0672[b]	-5.9883	0.3357	1.5065	0.6087	1.7987	0.9599
Quadratic								
b_{11}	5.52	0.1405	-0.3375	0.6224	0.3618	0.3294	-16.8750	0.0225[a]
b_{22}	-0.87	0.0589[b]	0.0611	0.4188	-0.0231	0.5336	-0.0315	0.9438
Interaction								
b_{12}	1.57	0.0856[b]	0.5862	0.0238[a]	0.2579	0.0341[a]	-0.5559	0.5670

[a]Effects are statistically significant, p = 0.05; [b]effects are statistically significant, p = 0.10

ponses indicate good fit of experimental data to Eq. (1). The model *F*-value 23.87218, 13.61834, 6.859321 and 49.38151 for AA_{DPPH}, AA_{OH}, PUFA and vitamin C content, respectively, implies that models for selected responses are significant at 95% confidence level (Table 2).

DPPH free radicals are relatively stable chemical species. They are synthetic products, which are suitable and usually used for the investigation of antioxidant activity of this kind of radicals. The coefficient of determination for the response of AA_{DPPH} in KSN fermented milk products indicates high correlation between observed and predicted values, *i.e.*, that less than 4.6% of the variations could not be explained by the model (Table 2). As for significance of the polynomial coefficients, their *p*-values suggest that the most important linear factor is milk fat. Besides, linear and quadratic effects of fermentation temperature, as well as interaction between independent variables are significant at level 0.10. The effects of milk fat and fermentation temperature on AA_{DPPH} are shown in Figure 1a. It can be observed that obtained model predicts highest values of AA_{DPPH} at fermentation temperature of 37-40 °C and milk fat content in the range of 0.8--1.2%.

Hydroxyl radicals are very reactive ones. They belong to group of reactive oxygen species (ROS) and are possible to generate in the human body. This is the reason why humans must consume food products that concern health protection. $AA_{\cdot OH}$ of the kombucha fermented milk products was determined. The coefficient of determination for the response of AA_{OH} in KSN fermented milk products advocated a high correlation between observed and predicted values (Table 2). Only 1.8% of the variations could not be explained by the model. As for significance of the polynomial coefficients, their *p*-values suggest that the most important factors influencing AA_{OH} are linear factor of milk fat and interaction between milk fat and fermentation temperature. According to positive interaction between the two independent variables (Table 1) there is synergetic effect of milk fat and fermentation temperature on AA_{OH}. The effects of independent variables on AA_{OH} in KSN fermented milk products are shown in Figure 1b. From the presented results, it is evident that AA_{OH} is increased at higher milk fat content at all applied fermentation temperatures. Obtained model predicts highest values of AA_{OH} for milk fat content in the range of 2.4--2.8% and fermentation temperature between 42 and 43 °C.

PUFAs are normally occurring constituents of milk. It was interesting to measure the PUFA content in kombucha fermented milk products because of its importance in stabilization of free radicals which directly manipulates the AA. The total PUFA content which is the sum of free fatty acids and fatty acids incorporated in triglycerides is determined. The used milk substrates with 2.8, 1.6 and 0.8% of milk fat contained 0.14, 0.04 and 0.02 g/100 g of PUFAs, respectively. The obtained results of PUFA content in kombucha fermented milk products are expressed as the relative percent difference compared to appropriate milk. The main reason for the presence of PUFAs in kombucha fermented milk products is the fact that they are constituents of milk which was mentioned above. However, the PUFA content was not the same in milk and kombucha fermented milk products obtained from the related milk which indicates that kombucha fermentation process affects the content of PUFAs. The coefficient of determination for the response of PUFA content in KSN fermented milk products shows that 6.6% of the variations could not be explained by the model (Table 2). Among polynomial coefficients, significant effect at the level 0.05 have linear factor of milk fat and interaction between milk fat and fermentation temperature. The effects of independent variables on PUFA content in KSN fermented milk products are shown in Figure 1c. At high values of milk fat content, observed response is enhanced with the increase of fermentation temperature. Obtained model predicts highest values of PUFA content at fermentation temperature of 42-43 °C and milk fat content in the range of 2.4-2.8%.

Vitamin C is one of the most important natural antioxidants. It is common product of kombucha meta-

Table 2. Analysis of variance (ANOVA) for the response of KSN products; DF - degree of freedom, SS - sum of squares, MS - mean squares

Response	Source								
KSN products	Residual			Model			*F*-Value	*p*-Value	R^2
	DF	SS	MS	DF	SS	MS			
AA_{DPPH}	3	41.993	13.9976	6	2004.922	334.1536	23.87218	0.012541	0.954
AA_{OH}	3	2.07644	0.692146	6	56.55526	9.425877	13.61834	0.027934	0.982
PUFA	3	0.529437	0.176479	6	7.263163	1.210527	6.859321	0.071314	0.934
Vitamin C	3	82.270	27.423	6	8125.270	1354.212	49.38151	0.004338	0.946

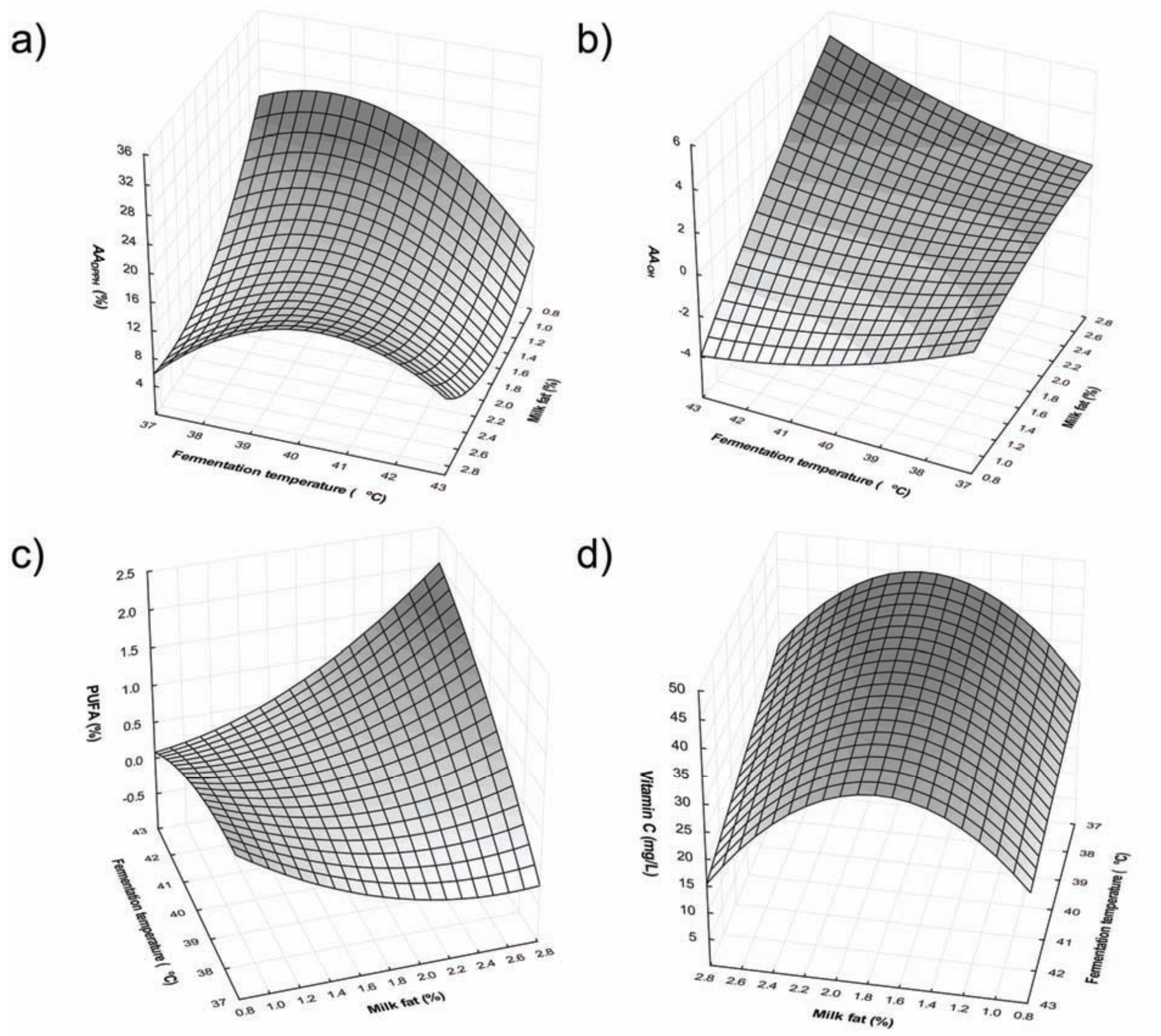

Figure 1. The effects of milk fat and fermentation temperature on AA_{DPPH} (a), $AA_{\cdot OH}$ (b), PUFA (c) and vitamin C (d) in the KSN products.

bolism in traditional kombucha beverages obtained on black and green tea [6,21]. In this investigation, the vitamin C has been chosen for the estimation of the antioxidant activity because it is the metabolite of kombucha fermentation. It was found that kombucha is capable for the biosynthesis of this vitamin during fermentation on milk using starters obtained by its fermentation on sweetened stinging nettle or winter savory. The contribution of kombucha fermentation to antioxidant activity was monitored using vitamin C analysis. For the response of vitamin C in KSN fermented milk products, the R^2 value indicates that less than 5.4% of the variations could not be explained by the model (Table 2). As for significance of the polynomial coefficients, their *p*-values suggest that the most imperative factor influencing vitamin C content is quadratic effect of milk fat content. The effects of both independent variables on content of vitamin C in KSN fermented milk products are given in Figure 1d. Highest values of selected response are predicted at milk fat content of 1.6-2.2% and lowest values of fermentation temperature. These contents are up to three times higher in comparison to traditional kombucha products with black and green tea [21].

Antioxidant activity, PUFA and vitamin C content of the KWS fermented milk products

The results of analyses of the statistics for AA_{DPPH}, AA_{OH}, PUFA and vitamin C content in KWS fermented milk products are showed in Table 3. The coefficients in Table 3 are related to actual variables.

The ANOVA results for selected responses are reported in Table 4. Relatively high values of coefficient of determination ($R^2 > 0.9$), obtained for all responses indicate good fit of experimental data to Eq. (1), which is similar to KSN products. The model *F*-value 68.14656, 35.73508, 9.331435 and 60.09839

Table 3. Regression equation coefficients for the response of KWS products

Effects	AA_{DPPH}		AA_{OH}		PUFA		Vitamin C	
	Coefficient	*p*-Value	Coefficient	*p*-Value	Coefficient	*p*-Value	Coefficient	*p*-Value
Intercept								
b_0	1737.739	0.0266[a]	184.4424	0.0532[b]	170.0604	0.0277[a]	-335.789	0.5347
Linear								
b_1	7.912	0.7658	-18.4581	0.0121[a]	-0.7467	0.7771	-11.162	0.7105
b_2	-86.676	0.0268[a]	-8.5768	0.0632[b]	-8.5328	0.0275[a]	20.310	0.4596
Quadratic								
b_{11}	-8.764	0.0397[a]	0.4132	0.3231	0.1236	0.6542	2.313	0.4734
b_{22}	1.077	0.0271[a]	0.0967	0.0800[b]	0.1072	0.0267[a]	-0.276	0.4250
Interaction								
b_{12}	0.577	0.3786	0.4853	0.0084[a]	0.0083	0.8906	0.014	0.9834

[a]Effects are statistically significant, $p = 0.05$; [b]effects are statistically significant, $p = 0.10$

for AA_{DPPH}, AA_{OH}, PUFA and vitamin C content, respectively, demonstrates that models for selected responses are significant at 95% confidence level too (Table 4).

The coefficient of determination for the response of AA_{DPPH} in KWS fermented milk products was very high (Table 4). It means that less than 4.4% of the variations could not be explained by the model. The *p*-values of the polynomial coefficients exhibit that the most important linear factor is fermentation temperature. Quadratic effects of milk fat and fermentation temperature are also significant at level 0.05. The effects of milk fat and fermentation temperature on AA_{DPPH} are shown in Figure 2a. The created model predicts the highest values of AA_{DPPH} for milk fat content in the range of 1.4-2.2% and fermentation temperature of 43 °C. This prediction is completely different in comparison to KSN fermented milk products.

The coefficient of determination for the response of AA_{OH} in KWS fermented milk products suggested that only 0.7% of the variations could not be explained by the model (Table 4). The p-values indicate that the most important factors influencing AA_{OH} are the same as for the KSN products, i.e. linear factor of milk fat and interaction between milk fat and fermentation temperature. Positive interaction between the two independent variables (Table 3) also hints the synergetic effect of milk fat and fermentation temperature on AA_{OH}. Linear and quadratic effects of fermentation temperature are significant at level 0.10. The effects of fermentation temperature and milk fat on AA_{OH} in KWS fermented milk products are shown in Figure 2b. At lower milk fat contents AA_{OH} decreases at higher values of fermentation temperature. On the other hand, at higher milk fat contents with the increase of fermentation temperatures, AA_{OH} increases. The highest values of AA_{OH} are calculated for milk fat content in the range of 2.4-2.8% and fermentation temperature between 41.5 and 43 °C using the obtained model, which is very similar to AA_{OH} predictions for KSN fermented milk products.

For the response of PUFA content in KWS fermented milk products, the coefficient of determination signifies that 7.4% of the variations could not be explained by the model (Table 4). Linear and quadratic effects of fermentation temperature are significant at the level 0.05. The effects of independent variables on PUFA content in KWS fermented milk products are given in Figure 2c. From the presented results it is unmistakable that the obtained model predicts the highest values of PUFA content in KWS fermented milk products at fermentation temperature of 43 °C

Table 4. Analysis of variance (ANOVA) for the response of KWS products; DF - degree of freedom, SS - sum of squares, MS - mean squares

Response	Source						*F*-Value	*p*-Value	R^2
KSN products	Residual			Model					
	DF	SS	MS	DF	SS	MS			
AA_{DPPH}	3	34.356	11.4522	6	4682.554	780.4257	68.14656	0.002696	0.956
AA_{OH}	3	0.66919	0.223064	6	47.82721	7.971201	35.73508	0.006975	0.993
PUFA	3	0.339533	0.113178	6	6.336667	1.056111	9.331435	0.047171	0.926
Vitamin C	3	43.630	14.5435	6	5244.230	874.0383	60.09839	0.003247	0.912

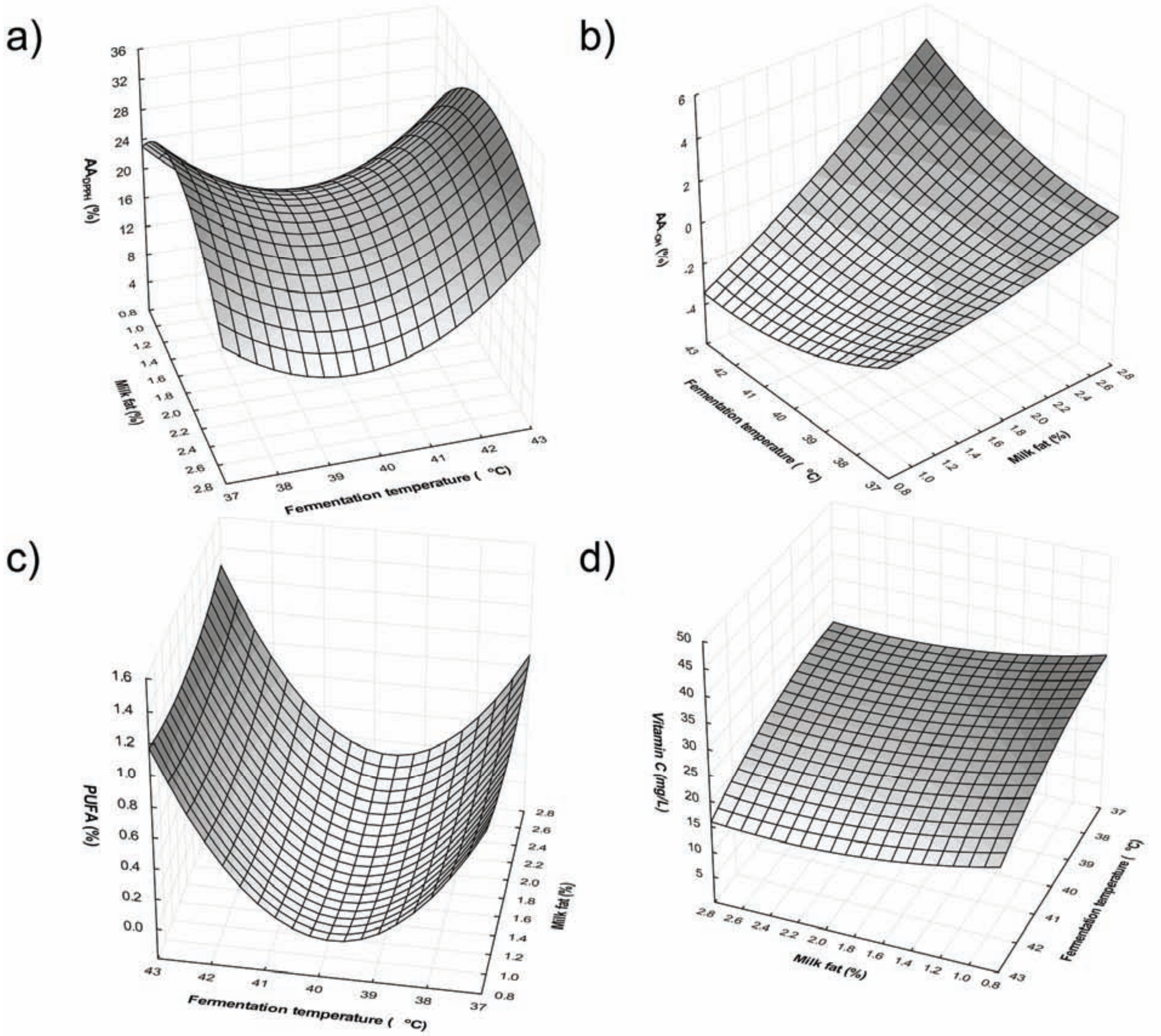

Figure 2. The effects of milk fat and fermentation temperature on AA_{DPPH} (a), $AA_{\cdot OH}$ (b), PUFA (c) and vitamin C (d) in the KWS products.

and milk fat content in the range of 2.4-2.8%. It is almost identical in comparison to KSN fermented milk products.

The effects of milk fat and fermentation temperature on content of vitamin C in KWS fermented milk products are given in Figure 2d. Highest predictions of vitamin C content are at lowest values of milk fat and fermentation temperature. The prediction is opposite to KSN fermented milk products in terms of calculated milk fat. However, it is for about 50% higher value in comparison to traditional kombucha beverage with black tea [21].

Optimization of processing variables

The developed models can be used for simulation and optimization of factor variables. The desirability function is one of the most widely used methods for optimization of multiple response processes in science and engineering. It combines multiple responses into one response called desirability function by choice of value from 0 (one or more characteristics are unacceptable) to 1 (all process characteristics are on target). Each of the estimated responses is transformed into an individual desirability value ranging from 0 to 1. The value of individual desirability increases as the desirability of the corresponding response increases. The overall desirability of the process is computed as geometric mean of the individual desirability functions. The software necessitates assigning goals to the processing variables and the responses. In the first set of optimized conditions all four responses were maximized and in the second set only the responses of AA_{DPPH} and AA_{OH} were included in maximization. The results of developed optimazation are presented in Table 5.

Table 5. Optimized values of processing variables and the predicted responses

Variables and responses	Condition	KSN		KWS	
		Optimum value	Desirability	Optimum value	Desirability
First set					
Milk fat	In range	0.94	0.52	1.74	0.63
Temperature	In range	37		37	
AA_{DPPH}	Maximize	21.27		29.60	
AA_{OH}	Maximize	0.04		-0.18	
PUFA	Maximize	0.75		0.75	
Vitamin C	Maximize	31.97		26.46	
Second set					
Milk fat	In range	2.8	0.61	2.76	0.83
Temperature	In range	40.95		42.91	
AA_{DPPH}	Maximize	15.33		24.95	
AA_{OH}	Maximize	3.24		4.09	
PUFA	In range	1.44		1.21	
Vitamin C	In range	20.34		16.20	

It could be concluded that defined optimum values significantly varies under different sets of optimized conditions. In the case of simultaneous maximization of all selected responses the desirability function has lower values for both used inoculums. In the case of simultaneous maximization of AA_{DPPH} and AA_{OH}, while the other two responses (PUFA and vitamin C) were set in the range of obtained experimental results, desirability function has higher values, 0.61 and 0.83, for KSN and KWS, respectively. During maximization of all responses lower values of AA_{OH} are achieved for selected optimum values of milk fat 0.94 and 1.74% for KSN and KWS, respectively, while the temperature for both applied inoculums was 37 °C. On the other hand, in the case of maximization of only AA_{DPPH} and AA_{OH}, the results suggest that AA_{DPPH} has to some extent lower values while increase in values of AA_{OH} are more significant (Table 5). So it may be concluded that for this set of optimal values (milk fat around 2.8% and temperature around 41 and 43 °C) suggest that antioxidant activity may be result of other kombucha metabolites rather than just vitamin C, as its values are lower for second set of optimization conditions.

Sensory evaluation test

A certain difference between sensory characteristics of the KWS and KSN fermented milk products might be expected. The temperature of fermentation showed the major impact to sensory characteristics. The KWS products obtained at 43 °C had the average mark 18.3 of 20, which was excellent. The other KWS products have been marked significantly lower with average mark 11.2 of 20. The KSN products had the average mark 17.3 of 20 after fermentation at 40 and 43 °C, which was very good. The KSN products obtained at 37 °C have been marked considerably lower with average mark 13.1 of 20. The presented results of sensory evaluation correlate very well to optimized values of process temperature, which were 42.91 °C for the KWS products and 40.95 °C for the KSN products (Table 5).

CONCLUSIONS

These investigations proved that the fermented milk beverages can be successfully produced by application of kombucha starter obtained by cultivation on sweetened stinging nettle and winter savory extracts. The conclusions can be drawn as follows:

- application of different kombucha starters causes a development of different antioxidant activities;
- there is synergetic effect of milk fat and fermentation temperature to AA_{OH} for both types of kombucha fermented milk products;
- responses and predictions for PUFA content and AA_{OH} are very similar for kombucha fermented milk products obtained with both starters;
- response surface methodology proved to be adequate modeling tool for mathematical representation of AA_{DPPH}, AA_{OH}, PUFA and vitamin C content depending on milk fat content and fermentation temperature and also a useful tool for optimizing process conditions;
- it was determined that maximization of the different antioxidant activities requires different content of milk fat and fermentation temperature.

- design expert analysis suggests that antioxidant activity may be result of other kombucha metabolites besides just vitamin C and PUFA.

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NAUČNI RAD

ANTIOKSIDATIVNA AKTIVNOST FERMENTISANIH MLEČNIH PROIZVODA DOBIJENIH POMOĆU KOMBUHE KULTIVISANE NA ČAJU OD KOPRIVE I RTANJSKOM ČAJU

Ovaj rad istražuje antioksidativnu aktivnost fermentisanih mlečnih proizvoda dobijenih pomoću kombuhe. Za fermentaciju mleka su korišćene dve starter kulture: starter dobijen nakon kultivacije kombuhe na zaslađenom ekstraktu koprive i starter dobijen nakon kultivacije kombuhe na zaslađenom ekstraktu rtanjskog čaja. Starteri su dodati mleku sa 0,8, 1,6 i 2,8% mlečne masti. Proces fermentacije mleka je izveden na 37, 40 i 43 °C i zaustavljen kada je dostignuta vrednost pH od 4,5. Antioksidativna aktivnost na hidroksi i DPPH radikale je modelovana korišćenjem metode odzivnih površina. Fermentisani mlečni proizvodi dobijeni pomoću kombuhe kultivisane na čaju od koprive i rtanjskom čaju su pokazali isti trend antioksidativne aktivnosti prema hidroksi, a različit prema DPPH radikalima. Ustanovljen je sinergistički efekat mlečne masti i temperature fermentacije za antioksidativnu aktivnost prema hidroksi radikalima za oba tipa dobijenih fermentisanih mlečnih proizvoda. Optimalni procesni uslovi po pitanju antioksidativne aktivnosti su: mlečna mast oko 2,8% i temperatura fermentacije oko 41 i 43 °C za fermentisane mlečne proizvode dobijene pomoću kombuhe kultivisane na čaju od koprive i rtanjskom čaju, redom.

Ključne reči: kombuha, fermentisani mlečni proizvodi, antioksidativna aktivnost, kopriva, rtanjski čaj.