Optimisation of the Antioxidant Activity of Kombucha Fermented Milk Products

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Abstract

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The antioxidant activity of fermented milk products obtained by using kombucha starter produced by fermentation on sweetened wild thyme extract was investigated. The starter was added to milk containing 0.8, 1.6, and 2.8% milk fat, at fermentation temperature of 37, 40, and 43°C. The fermentation process was terminated when the pH reached 4.5. Antioxidant activities to DPPH and hydroxyl radicals, the contents of MUFAs, PUFAs, vitamin C, and sensory mark, were monitored using the response surface methodology (RSM) and the method of desired function. Kombucha fermented milk products containing wild thyme (WT) showed opposite antioxidant response to DPPH and hydroxyl radicals in terms of milk fat. Optimum processing conditions for WT products in terms of antioxidant activity were: milk fat 2.78% and process temperature 37°C. In order to obtain WT products with a high sensory mark, these conditions are completely different (milk fat 1.10% and process temperature 43°C).

Keywords: tea fungus; fermentation; antioxidants; wild thyme; RSM

Recent studies have shown the possibility of obtaining fermented milk products by using kombucha as a starter culture (Malbaša *et al.* 2009; Iličić *et al.* 2012; Pejić *et al.* 2012; Vitas *et al.* 2013).

The symbiotic association of acetic acid bacteria and yeasts is known as kombucha (Dufresne & Farnworth 2000; Teoh *et al.* 2004; Nguyen *et al.* 2010). Kombucha beverage is the most commonly prepared by the biotransformation of sweetened black or green tea, but other substrates, such as herbal teas, can be also used (Dufresne & Farnworth 2000; Malbaša *et al.* 2009). Kombucha is rich in compounds that are known to be strong antioxidants, such as vitamins C and B_2, as well as polyphenols, primarily catechins (Dufresne & Farnworth 2000; Jayabalan *et al.* 2008; Malbaša *et al.* 2011a).

It is very well known that herbal extracts may provide a variety of health benefits. The extract of wild thyme possesses antioxidant and antihypertensive activities (Mihailovic-Stanojevic *et al.* 2013) and other biological activities (Rehman *et al.* 2009).

Some of the naturally occurring antioxidant constituents of the kombucha fermented milk products with winter savory and stinging nettle are vitamin C (mainly from kombucha) and polyunsaturated fatty acids (PUFAs) (primarily from milk fat) (Vitas *et al.* 2013). The antioxidant properties of PUFAs, as well as of monounsaturated fatty acids (MUFAs) are described in the literature (Berry 1997; Ambrozova *et al.* 2010).

The aim of this article is the optimisation of the antioxidant activity of fermented milk products obtained with kombucha starter prepared on wild thyme substrate considering the fact that no kind of antioxidative analysis of this type of kombucha products has been performed up to our knowledge. Also, the influence of the content of MUFAs on the antioxidant activity of the kombucha fermented milk products has been estimated for the first time besides the influence of PUFAs and vitamin C contents on the antioxidant activity to hydroxyl and DPPH radicals.

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MATERIAL AND METHODS

Milk. Pasteurised, homogenised cow's milks with 0.8, 1.6, and 2.8% milk fat (approximate values of the milk fat content at the local market), from the manufacturer in Novi Sad (Serbia) was used for the production of fermented milk products. The used milk contained, on average, 3.23% of protein, 4.20% of lactose, and 10.77% of dry matter content.

Kombucha starter cultures. The used local kombucha culture contained 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* (Malbaša *et al.* 2009).

The kombucha starter culture, used in this investigation, was the fermentation liquid of kombucha obtained after 7 days of fermentation at 25°C on wild thyme (*Thymus serpyllum*, originated from Fruška gora mountain) extract (2.25 g/l) sweetened with sucrose (7%). Using Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, USA) technique (with UV-DAD detection) the following phenolic acids with antioxidant activity were determined rosmarinic acid (78.02 mg/g, the most abundant); caffeic acid (0.15 mg/g); *p*-hydroxybenzoic acid (0.02 mg/g).

The yeasts count was 4.8×10^5 cells/ml while acetic acid bacteria count was 1.02×10^6 cells/ml.

Herbal tea was purchased in the local health food store.

Fermentation. Kombucha starter with wild thyme was cultivated in milks with different contents of milk fat (0.8, 1.6, and 2.8%) at 37, 40, and 43°C. The inoculum was added to the milk in the amount of 10% (v/v). The fermentation was performed until the pH value of 4.5 was reached. The obtained milk gel was then cooled to the temperature of 8°C, homogenised with a mixer, and the samples were stored in a refrigerator.

The products obtained by using kombucha starter with wild thyme (WT) were labelled as follows: WT2.8-37, WT2.8-40, WT2.8-43, WT1.6-37, WT1.6-40, WT1.6-43, WT0.8-37, WT0.8-40, and WT0.8-43, depending on the milk fat content of the milk used and the process temperature applied.

The fermentation was done in triplicate.

Antioxidant activity to DPPH and hydroxyl radicals (AA_{DPPH} and AA_{OH}). The antioxidant activity to DPPH radicals (AA_{DPPH}) was determined according to Živković *et al.* (2009). DPPH radical standard was produced by Sigma-Aldrich (Steinheim, Germany).

The antioxidant activity to hydroxyl radicals ($AA_{\cdot OH}$) was determined according to Deeseenthum and Pejovic (2010). ·OH radicals were generated by Fenton's reaction. The material formed radicals react with deoxyribose, the malondialdexyde produced giving a pink compound with thiobarbituric acid (both AlfaAesar, Karlsruhe, Germany) .

Determination of pH, MUFAs, PUFAs, vitamin C, and phenolic acids contents. pH-values were measured by a pH-meter (PT-70; Boeco, Hamburg, Germany).

MUFAs and PUFAs contents were determined using the GC-MS technique according to Malbaša *et al.* (2011b). Hewlett-Packard (HP) 5890 Series II GC coupled with HP 5971A MSD was employed. Multi-Standard solution of 37 fatty acids methyl esters (37 component FAME Mix, 47885-U) from Supelco (Bellefonte, USA) was used, as well as Wiley commercial data base of mass spectra. By means of the method used 100% fatty acids were quantified.

Vitamin C was determined using the HPLC technique according to Vitas *et al.* (2013). Agilent 1100 Series HPLC with UV-DAD detector (Agilent Technologies, Palo Alto, USA) was used. Vitamin C standard was produced by J.T. Baker (Deventer, the Netherlands).

Phenolic acids were determined by HPLC according to Bendini *et al.* (2003). The HPLC equipment consisted of an integrated system with a pump HP 1100 (Agilent Technologies, Palo Alto, USA), stationary phase C18 (5 µm particle size, 150 × 4.5 mm *i.d.*, and UV-DAD detector (280 nm). The mobile phase used was a mixture of two components (95% A + 5% B), where A was the mixture of twice-distilled water and acetic acid (98 : 2, v/v) and B was the mixture of methanol and acetonitrile (1 : 1, v/v) at a flow-rate of 0.5 ml/min and elution with linear gradient. The standards of phenolic acids were dissolved in the mobile phase (95% A + 5% B). The compounds were identified in each sample by comparing their retention times and UV-vis spectra with the standards.

The antioxidant activities, MUFAs, PUFAs, and vitamin C contents were represented as differences between the values obtained for kombucha fermented milk products and milk with the corresponding milk fat content.

Chemicals were of analytical, HPLC, and GC purity grade.

Sensory analysis. The sensory analysis of the obtained kombucha fermented milk products was performed by qualified evaluators (4 persons) together

with untrained consumers (3 persons), using a 5-point category scale and the descriptive test (IDF 1984). A scoring range from 1 (lowest) to 5 (highest) was used. The representative characteristics of the products obtained do not have the same influence on the quality. The determined coefficient of significance served for the correction (by multiplying) of the obtained sensory score. The examined parameters of quality with the appropriate coefficients of significance were: appearance – 1, colour – 2, odour – 3, consistency – 4, and flavour – 10. The coefficients of significance were chosen in accordance with the influence of the product characteristics on the quality, and balanced in the such that the total sum makes 20. By adding the individual corrected scores, the single complex indicator which reflects the overall sensory quality is formed. The total sensory mark (SM) is expressed as a percentage of the maximum possible quality. ISO 22935-1:2009 and ISO 22935-2:2009 were applied. Total number of 27 samples were evaluated in three sessions, meaning that 9 samples were evaluated per one session.

All analyses were performed after the production of the samples.

Statistical analysis. All experiments 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%.

In statistically based approaches, the response surface methodology (RSM) has been extensively used for the optimisation of different processes (Šumić *et al.* 2013). In this study, for the description of the responses Y (AA_{DPPH}, $AA_{.OH}$, MUFAs, PUFAs, vitamin C, and sensory mark (SM)), a second-degree polynomial model was fitted to the data:

$$Y = b_0 + \Sigma b_i X_i + \Sigma b_i^2 X_i^2 + \Sigma b_i X_i X_j \quad (1)$$

where: b_0 – intercept; b_i – linear; b_{ii} – quadratic; b_{ij} – interaction effect of the factors. The factor variables and their ranges: X_1 – milk fat (0.80, 1.60, and 2.80%), X_2 – fermentation temperature (37–43°C, interval value 3°C)

The statistical analysis and graphical representation of the data were performed using Statistica 9.1 software (StatSoft, Tulsa, USA). The adequacy of the model was evaluated by the coefficient of determination (R^2) and model P-value ($P = 0.05$).

For the determination of optimal values of the processing variables, the method of desired function was applied (Design-Expert v. 7.1.5; Stat-Ease, Inc., Minneapolis, USA).

RESULTS AND DISCUSSION

Fermentation process. Kombucha starter was added to the milk in the amount which provided the yeasts count of approximately 4.8×10^4 cells/ml of the substrate, and acetic acid bacteria count of approximately 1.02×10^5 cells/ml of the substrate. This was done because at the beginning of the milk fermentation process kombucha starter was added to the milk in the amount of 10% (v/v), which caused the lowering in the numbers of yeasts and bacteria. The number of yeasts was slightly higher, and the number of bacteria was slightly lower in comparison to some previous investigations of kombucha fermentation on winter savory and stinging nettle extract (Vitas *et al.* 2013). The development of all fermentation processes was monitored by measuring pH values in the time course. The value typical for yoghurt and kefir production (pH 4.5) was adopted of the end of fermentation. The fermentation profiles are presented in Figure 1.

The 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 that in the processes performed by Malbaša *et al.* (2009) who applied some different types of kombucha inoculums.

It is obvious (Figure 1) that the fermentations was significantly shorter at higher fermentation temperatures regardless of the milk fat content.

RSM of the WT fermented milk products. The range of values obtained for the antioxidant activity to DPPH and hydroxyl radicals, MUFAs, PUFAs, and vitamin C contents, which were used for the statistical analysis, were as follows: AA_{DPPH} 1.36–36.12%; AA_{OH} –3.11–5.12%; MUFAs –1.18–3.26%; PUFAs 0.52–1.56%; vitamin C 12.36–31.19 mg/l. The fermentation process leads to the degradation of compounds with antioxidant activity, i.e. causes that the products have a lower antioxidant activity than milk. The measured values of the antioxidant activity of milk and milk with the added inoculum did not differ significantly and were in the range from 0.00% to 0.03%.

The results of the statistical analyses for AA_{DPPH}, AA_{OH}, MUFAs, PUFAs, and vitamin C contents, as well as SM of the WT products, are presented in Table 1. The coefficients given in Table 1 are related to actual variables.

The ANOVA results for the selected responses are reported in Table 2. Relatively high values of the

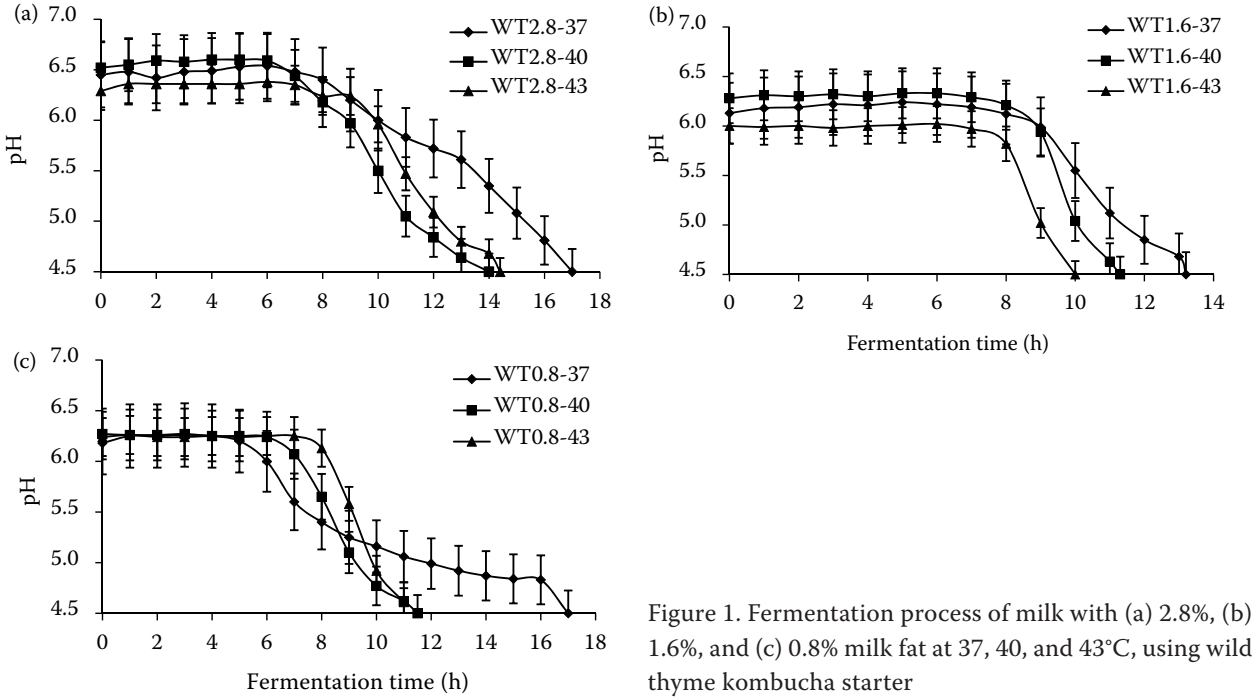

Figure 1. Fermentation process of milk with (a) 2.8%, (b) 1.6%, and (c) 0.8% milk fat at 37, 40, and 43°C, using wild thyme kombucha starter

coefficient of determination ($R^2 > 0.9$), obtained for all responses, indicate a good fit of experimental data with Eq. (1). The model *F*-value 42.28, 8.27, 6.83, 33.81, 64.22, and 1489.64 for AA_{DPPH}, AA_{OH}, MUFAs, PUFAs, and vitamin C contents, as well as SM, respectively, implies that the models for the selected responses are significant at 95% confidence level (Table 2).

Antioxidant activity of WT fermented milk products to DPPH and hydroxyl radicals. Although the antioxidant activity of fermented milk products with the addition of medicinal herbs, such as basil, dill, and peppermint, was established (Amirdivani & Baba 2011), it is not completely acceptable to compare kombucha milk products and their antioxidant activity with the products that belong to the group of yoghurts with medicinal herbs because different starter cultures are responsible for the milk fermentation process.

DPPH and hydroxyl free radicals are two different types in terms of reactivity and origin. DPPH radicals are stable ones but hydroxyl radicals are very reactive. Hydroxyl radicals are generated in the human body, while DPPH radicals are synthetic products, which are suitable for the investigation of antioxidant activity (Malbaša *et al.* 2011a).

Table 1. Regression equation coefficients for the response of WT fermented milk products

Effects	Response											
	AA_{DPPH} (%)		$AA_{\cdot OH}$ (%)		MUFAs (%)		PUFAs (%)		Vitamin C (mg/l)		SM (%)	
	coefficient	*P*	coefficient	*P*	coefficient	*P*	coefficient	*P*	coefficient	*P*	coefficient	*P*
Intercept												
b_0	3005.26	0.01[a]	156.18	0.34	274.91	0.03[a]	57.47	0.12	−141.59	0.74	110.23	0.73
Linear												
b_1	68.45	0.10	−20.40	0.08[b]	3.32	0.45	−4.84	0.05[b]	11.54	0.64	9.08	0.63
b_2	−152.01	0.01[a]	−6.99	0.38	−13.60	0.03[a]	−2.64	0.15	8.33	0.70	−9.11	0.58
Quadratic												
b_{11}	9.09	0.06[b]	−0.15	0.87	−0.26	0.56	0.46	0.06[b]	10.17	0.02[a]	−13.19	< 0.05[a]
b_{12}	1.95	0.01[a]	0.07	0.45	0.17	0.03[a]	0.03	0.16	−0.09	0.73	0.19	0.39
Interaction												
b_{12}	−2.65	0.03[a]	0.59	0.05[a]	−0.09	0.40	0.08	0.10[b]	−1.21	0.10[b]	0.88	0.11

Effects are statistically significant [a]$P = 0.05$, [b]$P = 0.1$

Table 2. Analysis of variance (ANOVA) for the response of WT fermented milk products

Response	Source						*F*-value	*P*	R^2
	residual			model					
	df	SS	MS	*df*	SS	MS			
AA_{DPPH} (%)	3.00	51.69	17.23	6.00	4371.10	728.52	42.28	0.01	0.98
AA_{OH} (%)	3.00	3.54	1.18	6.00	58.49	9.75	8.27	0.06	0.97
MUFAs (%)	3.00	1.60	0.53	6.00	21.86	3.64	6.83	0.07	0.95
PUFAs (%)	3.00	0.14	0.05	6.00	9.34	1.56	33.81	0.01	0.93
Vitamin C (mg/l)	3.00	28.69	9.56	6.00	3684.64	614.11	64.22	<0.01	0.96
SM (%)	3.00	16.37	5.46	6.00	48783.63	8130.60	1489.64	<0.01	0.99

df – degree of freedom; SS – sum of squares; MS – mean squares; SM – sensory mark

As concerns the significance of the polynomial coefficients their *P*-values suggest that the factors influencing AA_{DPPH} in WT products significant at level 0.05, are the linear and quadratic effects of the fermentation temperature as well as the interaction between the milk fat content and fermentation temperature. Quadratic effect of milk fat is significant at level 0.10 (Table 2). The effects of independent variables on AA_{DPPH} are shown in Figure 2a. It can be observed that the obtained model predicts the highest values of AA_{DPPH} at the fermentation temperature of 43°C and milk fat content of 0.80%.

The *P*-values of significance suggest that the most important factor influencing AA_{OH} is the interaction between the milk fat and fermentation temperature. Positive interaction indicates that a synergetic effect exists between the two independent variables on AA_{OH}. The linear factor of milk fat is also significant at level 0.10 (Table 2). The effects of independent variables on AA_{OH} in WT products are shown in Figure 2b. From the presented results is it evident that AA_{OH} increases with the increase of the milk fat content at all fermentation temperatures applied. At lower milk fat contents, AA_{OH} decreases at higher

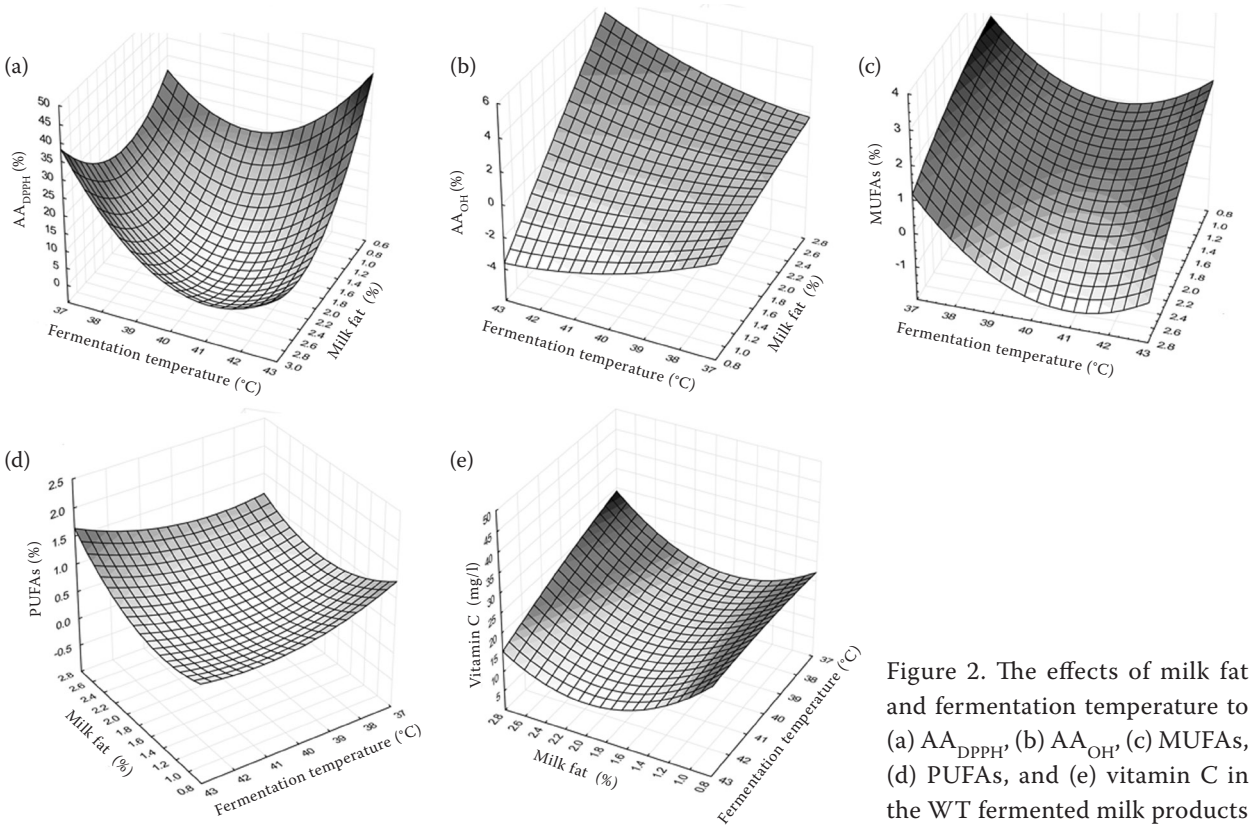

Figure 2. The effects of milk fat and fermentation temperature to (a) AA_{DPPH}, (b) AA_{OH}, (c) MUFAs, (d) PUFAs, and (e) vitamin C in the WT fermented milk products

values of the fermentation temperature but at higher milk fat contents AA_{OH} in WT products also increases with the increase of the fermentation temperature. The obtained model predicts the highest values of AA_{OH} for the milk fat content in the range of 2.4–2.8% and fermentation temperature between 41.5°C and 43°C.

The values predicted for AA_{OH} are very similar and for those AA_{DPPH} are very different in comparison with the reference data related to kombucha fermented milk products with stinging nettle and winter savory (Vitas *et al.* 2013). This indicates that kombucha inoculum affects the antioxidant activity of kombucha fermented milk products.

MUFAs and PUFAs contents of WT fermented milk products. PUFAs and MUFAs are the usual constituents of milk fat. MUFAs may reduce cardiovascular disease risk with their antioxidant, antithrombotic, and antihypertensive properties (Feldman 1999).

Among polynomial coefficients for MUFAs content the significant effects at level 0.05 are linear and quadratic effects of the fermentation temperature (Table 2). The effects of independent variables on MUFAs in WT products are shown in Figure 2c. From the presented results is it evident that the obtained model predicts the highest values of MUFAs at the lowest fermentation temperature applied. The increase of the fermentation temperature up to 41°C affects MUFAs content reduction. On the other hand, the fermentation temperature increase in the range of 41–43°C resulted in MUFAs content progress. Also, the results indicate that MUFAs increase with the decrease of the milk fat content at all fermentation temperatures applied.

The significances at level 0.10 for PUFAs content resides in the linear and quadratic effects of milk fat content (Table 2). The effects of independent variables on PUFAs content in WT products are shown in Figure 2d. The results indicate that PUFAs increased with the increase of the milk fat content at all fermentation temperatures applied, thus the obtained model predicts the highest values of PUFAs at the highest values of the milk fat content and fermentation temperature. The established pattern for PUFAs content is in accordance with the results obtained for kombucha fermented milk products with winter savory and stinging nettle (Vitas *et al.* 2013).

Vitamin C content of WT fermented milk products. The activity of vitamin C present in kombucha fermentation system is modified in the positive way by the chemical environment in the fermented beverage (Malbaša *et al.* 2011a).

Polynomial coefficients for vitamin C content show that the significant effect at level 0.05 is given by quadratic effects of the milk fat content. The interaction between the milk fat and fermentation temperature is significant at level 0.10 (Table 2). The effects of both independent variables on the content of vitamin C in WT products are given in Figure 2e. The highest values of the selected response are predicted at milk fat content of 2.6–2.8% and fermentation temperature in the range of 37–39°C.

Sensory evaluation test of WT fermented milk products. Sensory analysis sugested that the obtained WT products, according to the descriptive test, were similar to the group of fermented milks like yoghurt and kefir. They were without separated whey, with uniform colour and flavour typical of that type of products, i.e. for yoghurt. The taste was mild and pleasant with the aroma characteristic of the wild thyme extract used.

The results of the sensory evaluation of the WT products are presented in Figure 3.

It can be observed that, with the increase of the process temperature, the total sensory mark of all samples also increased. The best total sensory mark (100%) was achieved by sample WT1.6-43. The lowest total sensory mark was obtained with samples WT2.8-37 and WT0.8-37 being 40, i.e. 50%. The average value of total sensory mark, when the milk fat content is taken into account, suggests that the highest mark was achieed wiht the samples produced from milk with 0.8% milk fat (80%), followed by the samples produced from milk with 2.8% milk fat (75%) and those produced from milk with 1.6% milk fat (73.33%).

The most important factors influencing SM are the quadratic effects of the milk fat content. Obtained results indicate that SM increases with the increasing fermentation temperature at all milk fat contents applied. The presented model predicts the highest values of SM for the milk content around 1.8% and the highest values of fermentation temperature applied.

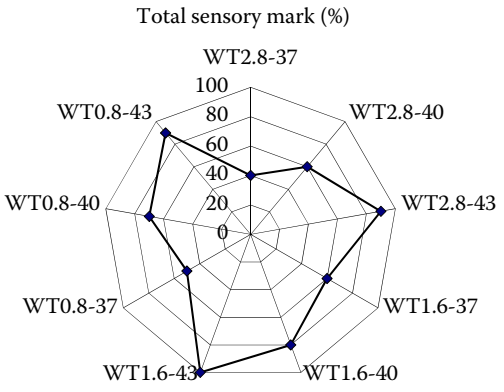

Figure 3. Sensory mark of WT fermented milk products

Table 3. Optimised values of processing variables and the predicted responses of WT fermented milk products

Variables and responses	First set			Second set			Third set		
	condition	optimum value	desirability	condition	optimum value	desirability	condition	optimum value	desirability
Milk fat (%)	is in range	2.78		is in range	2.78		is in range	1.10	
Temperature (°C)	is in range	37.00		is in range	37.00		is in range	43.00	
AA_{DPPH} (%)	maximise	33.31		maximise	33.31		maximise	28.20	
AA_{OH} (%)	maximise	1.72	0.68	maximise	1.72	0.78	maximise	–2.20	0.38
MUFAs (%)	maximise	1.18		is in range	1.18		is in range	1.73	
PUFAs (%)	maximise	1.12		is in range	1.12		is in range	0.68	
Vitamin C (mg/l)	maximise	28.71		maximise	28.71		maximise	16.43	
SM (%)	is in range	40.00		is in range	40.00		maximise	96.36	

Optimisation of processing variables. The desirability function is one of the most widely used methods for optimisation of multiple response processes in science and engineering.

In the first set of optimised conditions AA_{DPPH}, AA_{OH}, MUFAs, PUFAs, and vitamin C contents were maximised. In this case, the conditions were set in order to define the process parameters for the production of WT product of the highest antioxidant activity possible. The results of the developed optimisation are presented in Table 3. According to the obtained results, the highest value of the desirability function (0.68) was achieved at the milk fat content of 2.78% and fermentation temperature of 37°C.

In the second set, only the responses of AA_{DPPH}, AA_{OH}, and vitamin C content were included in maximisation in order to define the process parameters for the production of WT product with the highest antioxidant activity possible. In this way, it is possible to establish the contribution of kombucha fermentation to the antioxidant activity, since vitamin C is one of the major antioxidant metabolites of kombucha fermentation process. The results suggest that this goal is achieved under the same values of the milk fat content and fermentation temperature as were those for the first set of optimised conditions.

In the third set, a higher value of SM was predicted. The goal was to define the conditions for the production of WT product with a high antioxidant activity which is also sensory acceptable for consumers. At the milk fat content of 1.10% and fermentation temperature 43°C, the predicted value of SM was very high (96.36) but predicted values of AA_{DPPH}, AA_{OH}, and vitamin C are lower compared to first and second sets. It can be concluded that in order to obtain WT product with excellent sensory characteristics, there must be a compromise in terms of its antioxidant activity.

CONCLUSION

The conclusions for the obtained kombucha fermented milk products can be drawn as follows:

– Higher values of the milk fat and fermentation temperature lead to higher AA_{OH} and PUFAs contents;
– Response and prediction for MUFAs and AA_{DPPH} are very similar;
– Design expert analysis suggests that the processing conditions have significantly greater influence on the values of AA_{DPPH}, AA_{OH} and vitamin C in comparison to the contents of MUFAs and PUFAs;
– There must be consent to lower values of antioxidant activities in order to obtain a product with excellent sensory characteristics.

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