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FORMULATION OF FERMENTED TEA CONTAINING JERUSALEM ARTICHOKE EXTRACT USING RESPONSE SURFACE METHODOLOGY

SIBEL YIGITARSLAN*

* *Suleyman Demirel University, Faculty of Engineering and Natural Sciences, Chemical Engineering Department, 32040 Isparta, Turkey*

*Corresponding author: yildizsibel@sdu.edu.tr

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Abstract

Currently, the development of functional beverages has gained growing interest in food biotechnology. This study focused on developing a tea enriched through the fermentation of Jerusalem artichoke (JA) extracts, which are known to contain significant prebiotics such as inulin and fructo-oligosaccharides. The fermentation was conducted using a novel probiotic strain, *Lactobacillus LA-5*. The study utilized three factors to optimize the functional properties of the beverage: the amounts of JA extract (ranging from 25% to 75% v/v), the sugar content (5% to 10% w/v), and the fermentation time (spanning 4 to 8 hours). The density, titratable acidity, and viable cell count of the beverage were determined. Response surface methodology and the Box-Behnken design were utilized, with bacterial growth, total phenolic content, and pH serving as the responses. The eleven production conditions identified include 10 grams of sucrose, 35 to 37.5 milliliters of JA extract (v/v, %), and a fermentation time ranging from 6.2 to 6.92 hours. These conditions resulted in the highest optical density values (0.746 to 0.757), total phenolic content (165.1 to 166.7 mg GAE/g), and a pH of 4.09. The product formulated in this study could be of interest for consumers, especially for tea-consuming people.

Keywords: fructooligosaccharide, jerusalem artichoke, inulin, fermentation, probiotic, prebiotic, response surface methodology

Introduction

The fermented food and beverages based on the microbial enzymatic bioconversion of components to biologically active metabolites (*i.e*. exopolysaccharides, phenolic compounds, bioactive peptides, organic acids, etc.) can provide not only numerous health benefits, including antioxidant, anti-inflammatory, antimicrobial, and antidiabetic effects, but also foods having extended shelf life, nutritional value, and

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sensory properties (Icer *et al.*, 2023). Beverage fermentation is an ancient ritual in Turkey; boza and kefir are well-known examples in the market (Bayat and Yıldız, 2023). Although Turkey is the most tea-consuming country in the world (Yildirim and Karaca, 2022), with black tea being the most popular one, there is no fermented tea product in the market. Black tea is known for its health-promoting effects and antimicrobial and antioxidant properties due to the polyphenols in its structure (Sena *et al.*, 2020; Chong *et al.*, 2023). The major polyphenolics of black tea include catechins, theaflavins, thearubigins, amino acids, and alkaloids (Chang *et al.*, 2020). Also, in recent studies, black tea has been declared an excellent carrier for microorganisms (Nikkam *et al.*, 2023).

Plant-based beverages, such as fluids obtained by water extraction of the plant, have also gained interest due to being a good alternative for people with lactose intolerance, milk allergies. Obtaining these kinds of beverages, requires less water and land usage, production of a lower carbon footprint, and ethical concerns on animal welfare (Penha *et al.*, 2021). The application of fermentation on plant-based beverages has been proved to improve sensory properties by increasing flavor compounds and decreasing off-flavor ones, to enhance nutritional composition by increasing vitamin, amino acid, and protein levels and decreasing phytic acid and saponins, and to increase functional profile by increasing digestibility and bioavailability of nutrients (Hidalgo-Fuentes *et al.*, 2024). In a very recent study, increased total phenolic content was obtained within lactic acid bacteria-fermented plant-based beverages (Ozturk *et al.*, 2024).

Lactobacillus acidophilus is a gram-positive, rod-shaped probiotic bacterium. *L. acidophilus* strengthens the immune system by increasing IgG and IgA levels, improves overall gut health by producing acetic and lactic acid. Due to decreasing pH levels, inhibition of the growth of pathogenic microorganisms such as *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus*, and *S. epidermis* occurs. These phenomena inhibit proliferation of colon cancer cells by decreasing generation of reactive oxygen species and carcinogens, and exhibits antiproliferative and antioxidant activities against the HT-29 cell line (Liu *et al.*, 2024). Studies showed that the role of *L. acidophilus* LA-5 promoted antioxidant effects during fermentation processes (Chan *et al.*, 2018; Ozturk *et al.*, 2024). Additionally, *L. acidophilus* produces exopolysaccharides used in food and pharmaceutical industries for enhancing the quality of the product and bioactive peptides (SAGNPN, AAAAG, NVLVG, etc.) that exhibit antioxidant, antiviral, and antifungal activities (da Silva *et al.*, 2023). Because of these health-promoting effects, *L. acidophilus* has been preferred in fermentation processes of the food industry for the production of cheese, yogurt, fermented and/or fortified milk, meat and baking products, and fermented beverages (Shen *et al.*, 2018; Liu *et al.*, 2024). In addition, fruit juices, herbal teas, grains and nuts, and vegetables have been fermented by *L. acidophilus* species in various studies (Buruleanu *et al.*, 2012; Liu *et al.*, 2024; Ozturk *et al.*, 2024). It was reported that fermentation resulted in a reduction in glucose and sucrose levels, lower pH values due to the increase in lactic acid concentration, and the inhibition of the growth of contaminating microorganisms (Buruleanu *et al.*, 2012). These effects contribute to the nutritional value of the final product, enhancing its shelf life, flavor, and taste. It has also been proven that the addition of specific nutrients such as lactose, sucrose, prebiotics, ascorbic acid, and cysteine improves the growth and viability of *L. acidophilus* (Sahin Buyuktortop and Yigitarslan, 2012; Meng *et al.*, 2021).

Prebiotics are food components that beneficially affect the host by selectively stimulating the growth and/or activity of the probiotic bacteria. The disaccharides (lactulose and lactitol), oligosaccharides (fructo-, galacto-, and xylo-oligosaccharides), and polysaccharides (inulin and resistant starch) are examples of prebiotics (Yigitarslan Yildiz, 2006). Both inulin and fructooligosaccharides (FOS) have the generally recognized as safe (GRAS) status, and the most common plants used as sources are chicory, Jerusalem artichoke (JA), asparagus, and onion. The inulin and FOS content of JA (75-98% of dry matter) depends on the growth and harvest conditions and the parameters of the extraction process. Although JA contains not only prebiotic sugars but also phenolic compounds having antioxidant properties (Alyas *et al.*, 2020), the fermentation of JA to obtain functional beverages has been studied scarcely in the literature (Chong *et al.*, 2023; Yigitarslan Yildiz, 2006).

Response surface methodology (RSM) is an approved and the most common statistical method used in processes of food science to optimize ingredients and processing conditions as variables because of its high efficiency (Koocheki *et al.*, 2009). The main advantages of RSM are the requirement of a reduced number of experiments and evaluation of the effects of multiple factors both individually and interactively (Mazlum and Lodos, 2025). RSM provides a polynomial equation for describing these effects on the response as a surface. It has been successfully used in several researches, including food formulations (Koocheki *et al.*, 2009; Mazlum and Lodos, 2025; Goktas *et al*., 2015).

Based on the background mentioned above, this study aimed to formulate a new beverage of lacto-fermented black tea and JA extract with the aid of response surface methodology based on a three-parameter, three-level Box-Behnken experimental design and evaluate some of the physicochemical properties.

Materials and methods

Materials

Freshly harvested JA and Çaykur Black Tea were purchased from a local farmer and a local market, both in Isparta, Turkey, respectively. Citric acid, sodium carbonate, sodium chloride, Folin-Ciocalteu reagent, cysteine, sucrose, and gallic acid were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Man, Rogosa, and Sharp (MRS) broth was purchased from Merck (Darmstadt, Germany). *L. acidophilus* LA-5 was obtained from Chr. Hansen (İstanbul, Turkey).

Preparation of tea

Tea infusions were prepared generally by the addition of 1.5-6 g of tea to 1 L of boiling water (Chong *et al.*, 2023), and after 5 minutes, filtration was applied. It was declared that water-tea weight ratios were not effective on total phenolic content and antioxidant activity of black tea, whereas an increase in temperature from 60 to 100ºC tripled the total phenolic content (Chang *et al.*, 2020). In this study, black tea was prepared by brewing 6 grams of tea powder in 1 L of distilled boiling water (100ºC) for 5 minutes, which was filtered using Whatman filter paper. Then, 0.05 g of cysteine was added into 1 L of tea to increase the viability of *L. acidophilus* (Dave and Shah, 1997) and decrease the fermentation time (Meng *et al.*, 2021). Finally, it was rapidly cooled, on ice, to room temperature.

Preparation of JA extract

According to the results of Yigitarslan Yildiz (2006), February was the most suitable harvest date for the JA to obtain the highest yields (78.4%) of the lowest degree of polymerization (DP=1-2 (15%), 3-4 (65%), and 4-8 (10%)); 10-day storage at 4ºC was required for the conversion of sugars into functional sugar by its own enzyme, and 3-minute ultrasonic extraction produced the best performance; it decreased the amount of sugars of DP 1-2 and increased the amount of fructooligosaccharides having DP 3-6. In addition, increasing the ultrasonic extraction temperature from 20ºC to 60ºC tripled the functional sugar content of the extracts. In that, it was found that 3-min ultrasonication of rectangular prism grated (2×2×40 mm) JA tubers (25 g) in 26 mM citric acid added water (100 ml) conditions achieved a 10% increase in the yield, and the highest functionality was obtained due to the combined effect of acid and ultrasonication of hydrolysis of inulin. In order to obtain the highest functional sugar-containing extracts, ultrasonic extraction (ultrasonic bath; MODEL 1510 MT, frequency of 40 kHz, power of 50 W, tank capacity of 1.8 L) was applied to February-harvested and 10-day-stored grated JA tubers in citric acid-added water solutions at 60ºC for 3 minutes. At the end of extraction, the mixture was filtered using Whatman filter paper, and all of the extracts were mixed.

Activation of L. acidophilus

L. acidophilus LA-5 culture was activated twice in MRS broth at 37ºC for 24 h. The active culture in MRS broth was diluted using physiological saline (0.85% NaCl) (Meng *et al.*, 2021).

Experimental design

The experiments were designed to study the effect of independent variables on the responses by the Box-Behnken design. The independent variables were the amounts of JA extract (x_1) and sugar (sucrose) (x_2) and the fermentation time (x_3). They were coded at three levels according to Equation 1:

$$x_i = \frac{(x_i - x_0)}{\Delta x} \quad (1)$$

where x_i, x_0, and Δx are the coded value of the ith independent variable, the value at the center point, and the step change, respectively. Coded parameters with ranges are given in Table 1. The selected responses were optical density to evaluate the bacterial growth, pH of the medium for estimation of lactic acid production, and the total phenolic content. The behavior of the response is related to the empirical second-order polynomial given in Equation 2:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (2)$$

where Y is the predicted value of the response, x_i, x_j, …, x_k are the input variables, x_i^2, x_j^2, …, x_k^2 are square effects, and x_ix_j, x_jx_k, and x_ix_k are the interaction effects. β_0, β_i, β_{ii}, β_{ij}, and ε imply the intercept, the linear coefficients, the quadratic effect coefficients, the interaction effect coefficients (for i, j = 1, 2, …, k), and a random error, respectively. If the model improvement was needed statistically, some additional terms were also considered. For each response, three center points, and thus a set of a total of 15 experiments, were realized.

Table 1. Experimental range and levels of independent variables.

Independent variable	Factor code	Ranges and levels		
		-1	0	+1
Amount of JA extract (v/v %)	x_1	25	50	75
Amount of sugar (w/v %)	x_2	5	7.5	10
Fermentation time (h)	x_3	4	6	8

Measurements of responses

In each assay, the determined amount of tea was mixed with the extract, and then the respective amount of sucrose was added. After mixing, the temperature of the mixture was set at 37ºC in a shaking water bath. After that, 200 µl of *L. acidophilus* was added. Fermentation was realized at 37ºC with 130 rpm up to the determined time. During the fermentation, the bacterial growth in the medium was investigated spectrophotometrically. At the end of the determined fermentation time, 2 ml of the medium were analyzed in a spectrophotometer (Perkin-Elmer) at 600 nm. Blank solutions were tea mixtures containing respective amounts of JA and sugar. pH values were measured on an electronic pH meter. The concentrations of the total polyphenols in the medium were determined using the Folin-Ciocalteu method (Goktas *et al.*, 2015). In the analysis, 0.4 ml of the sample was mixed with 5.1 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent. Further, 1.5 ml of sodium carbonate solution (20% by weight) was added into the medium immediately, and after mixing, they were kept in the dark for two hours at room temperature. The color resulting from the colorimetric reaction between polyphenols in the sample and the Folin reagent was analyzed by UV-vis spectrophotometer (Perkin-Elmer) at 765 nm. The gallic acid equivalents (GAE) of total phenolics were calculated from the calibration curve (R^2=0.9989) according to Equation 3.

$$\text{Absorbance} = 0.01532 \times \text{concentration} \left(\frac{\mu g}{mL}\right) \qquad (3)$$

and the results were expressed as mg GAE/g dry herb.

Numerical optimization

In order to determine the optimal conditions for the production of vegetable-containing fermented tea, the following criteria were chosen in the numerical optimization section of the Design-Expert program:maximum optical density to approve the maximum amount of probiotic content in the fermented beverage, maximum total phenolic content to maintain the maximum amount of bioactive components present, and to ensure that the beverage was minimally erosive, pH was set at a target value of 4.09, which was the pH of an orange juice (Reddy *et al.*, 2016).

Characterization of the fermented beverage

The density of the fermented beverage was determined by weighing 10 ml of beverage at the end of fermentation (Yigitarslan Yildiz, 2006). Total phenolic content of the beverage was analyzed by the Folin-Ciocalteu method described above. Total soluble matter of beverage was determined by air oven drying (NUVE, Turkey) at 105ºC for 24 h (Gad *et al.*, 2010). Titratable acidity of the beverage was evaluated by titration of the sample with sodium hydroxide solution (Islam *et al.*, 2002). A volume of 10 ml of the beverage was diluted with 20 ml of distilled water and then titrated with 0.1M NaOH solution, using a phenolphthalein indicator. The results were given as the percentage of lactic acid, which was calculated by Equation 4.

$$\%\ \text{lactic acid} = \frac{\text{mL of NaOH} \times 0.1\text{N}}{\text{volume of sample titrated}} \times 0.09 \times 100 \qquad (4)$$

The viable bacterial cell count was also analyzed. Suitable dilutions of beverage with 0.9 g/100 ml NaCl were plated onto the MRS agar plates. They were incubated anaerobically at 37ºC for 24 h, and the results were calculated in log CFU/ml (Abdollahzadeh *et al.*, 2018).

Statistical analysis

All analyses were conducted in triplicate, and mean scores of the results were reported. Design Expert 12.0 (Stat-Ease Inc., USA) software was used to fit the experimental data and to perform regression and graphical analysis. Three-dimensional surface plots of each response were constructed based on its respective fitted and statistically approved polynomial function. Differences in composition and sensory scores were detected using Anova one-way analysis of variance. A significance level of $p < 0.05$ was applied in the study.

Results and discussion

Model development

Independent variables, both in coded and actual values, and responses of each run were represented in Table 2. The pH values and total phenolic content for each reference in the Table 2 are provided for comparison purposes. The pH values of all fermentation media were lower than their corresponding references. The main reason for the pH decrease of the medium is the growth of *L. acidophilus*, lactic acid-producing bacteria, as indicated by optical densities.

According to the results of this study, a direct relation between the bacterial growth and the decrease in pH of the media was obtained in most of the experiments. The highest bacterial growth and the highest pH decrease were observed in assay 12, but some discrepancies were also found, e.g., assay 3. The sugar composition (sucrose, fructooligosaccharides, and inulin content) of the medium seemed to impact the organic acid fermentation rate.

The results of the total phenolic content of the fermented medium showed that *L. acidophilus* fermentation increased the total phenolic components in each run. The juice's phenolic content was in the range of 98.69-183.61 mg GAE/g. The increase in the total phenolic content ranged from 0.6 (assay 9) to 20% (assay 12).

Table 2. Experimental design and responses of the study.

	Independent Variables						Responses				
	Coded value			Actual value							
Assay No	X_1	X_2	X_3	X_1	X_2	X_3	Optical density	pH	TPC	pH_{ref}	TPC_{ref}
1	0	0	0	50	7.5	6	0.7307	4.01	142.25	5.01	122.16
2	1	0	-1	75	7.5	4	0.7328	3.78	107.25	4.82	93.95
3	-1	0	-1	25	7.5	4	0.5602	4.10	156.84	5.20	150.38
4	0	1	-1	50	10	4	0.7053	4.07	132.80	5.01	122.16
5	0	-1	1	50	5	8	0.7295	3.97	138.83	5.01	122.16
6	1	-1	0	75	5	6	0.7675	3.67	111.96	4.82	93.95
7	-1	0	1	25	7.5	8	0.6833	4.25	160.34	5.20	150.38
8	0	-1	-1	50	5	4	0.6135	4.21	131.45	5.01	122.16
9	1	0	1	75	7.5	8	0.8008	3.55	98.69	4.82	93.15
10	0	0	0	50	7.5	6	0.7348	4.02	146.00	5.01	122.16
11	-1	-1	0	25	5	6	0.6169	4.37	173.70	5.20	150.38
12	1	1	0	75	10	6	0.8239	3.53	113.34	4.82	93.95
13	0	1	1	50	10	8	0.7990	3.83	136.67	5.01	122.16
14	-1	1	0	25	10	6	0.7088	4.22	183.61	5.20	150.38
15	0	0	0	50	7.5	6	0.7318	3.89	143.80	5.01	122.16

The independent variables and their respective values were processed by the Design-Expert program to construct the model equations representing the relation between the response and the variables. To determine the fitness of the constructed models to the experimental data, regression analysis and analysis of variance (ANOVA) were used. For all of the responses, the quadratic model was found appropriate at a 95% confidence interval, since all of the statistical criteria were approved in each case. The F-values were 283.32 for the optical density (Table 3), 0.0960 for pH (Table 4), and 39.76 for the total phenolic content (Table 5). There was only a 0.01%, 1.02%, and 0.04% chance that an F-value could occur due to noise for the optical density, pH, and the total phenolic content, respectively. Since the corresponding probability values were less than the F-values, it was concluded that the quadratic model was significant. The lack-of-fit values were insignificant (0.0001 for the optical density, 0.0371 for the pH, and 105.48 for the total phenolic content). It is generally accepted that a coefficient of determination (R^2) value of at least 80% is representative of the well-fitness of the model (Koocheki *et al.*, 2009). All values of the coefficient of determination were above 0.8 (Tables 3, 4, and 5). In general, the closer regression coefficients were preferable (Mazlum and Lodos, 2025). The values of the regression coefficients of the optical density (Table 3) and the total phenolic content (Table 5) were much closer to the adjusted-R^2 values than those of pH (Table 4). Different acid productions having different pH values in dissimilar amounts may have resulted in this discrepancy. Other criteria are the value of adequate precision (signal-to-noise ratio) greater than 4, low standard deviation, and low coefficient of variance. These criteria were also obtained in the quadratic models of each response. As a result, the quadratic model of each response was statistically approved. The good correlation between the predicted and experimental values can be seen in Figure 1.

Thus, the regression models of each response were predicted as follows:

$$\text{Optical density} = 0.7324 + 0.0695x_1 + 0.0387x_2 + 0.0501x_3 - 0.0089x_1x_2 - 0.0138x_1x_3 - 0.0056x_2x_3 - 0.0104x_1^2 + 0.0072x_2^2 - 0.0278x_3^2 \quad (5)$$

$$\text{pH} = 3.97 - 0.3012x_1 - 0.0712x_2 - 0.0700x_3 + 0.0025x_1x_2 - 0.0950x_1x_3 + 0.00x_2x_3 - 0.0629x_1^2 + 0.0371x_2^2 + 0.0096x_3^2 \quad (6)$$

$$\text{Total phenolic content} = 144.02 - 30.41x_1 + 1.31x_2 + 0.7738x_3 - 2.13x_1x_2 - 3.01x_1x_3 - 0.8775x_2x_3 - 1.26x_1^2 + 2.90x_2^2 - 11.98x_3^2 \quad (7)$$

Response surfaces and effects of independent variables

Based on equations 5, 6 and 7, three-dimensional response surface plots were constructed to interpret the interactive effects of the independent variables on each of the responses. The greater the F-value and the smaller the p-value, the greater the effect of the variable on the response (Goktas *et al.*, 2015). According to Table 3 and the more red-colored surfaces present in the three-dimensional plots in Figure 2, it can be stated that all of the independent variables chosen were significantly affected by the optical density.

Table 3. ANOVA for the quadratic model of the optical density.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	0.0753	9	0.084	283.22	<0.0001
X_1	0.0386	1	0.0386	1306.33	<0.0001
X_2	0.0120	1	0.0120	405.34	<0.0001
X_3	0.0201	1	0.0201	679.31	<0.0001
$X_1 X_2$	0.0003	1	0.0003	10.66	0.0223
$X_1 X_3$	0.0008	1	0.0008	25.68	0.0039
$X_2 X_3$	0.0001	1	0.0001	4.21	0.0955
X_1^2	0.0004	1	0.0004	13.39	0.0146
X_2^2	0.0002	1	0.0002	6.47	0.0517
X_3^2	0.0029	1	0.0029	96.57	0.0002
Pure error	9.10^{-6}	2	4.10^{-6}		
Residual	0.001	5	0.0000		
Total	0.0755	14			

R^2=0.9980, Adj.R^2=0.9945, Adeq.Precision=60.2928, Std. dev.=0.0054, C.V.%=0.7594

It is reasonable because *L. acidophilus* is known to metabolize both sucrose and fructooligosaccharides (Sahin Buyuktortop and Yigitarslan, 2012), and time was another factor for growth since it is the requirement of more fermentation.

Table 4. ANOVA for the quadratic model of pH.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	0.8636	9	0.0960	10.08	0.0102
X_1	0.7260	1	0.7260	76.28	0.0003
X_2	0.0406	1	0.0406	4.27	0.0938
X_3	0.0392	1	0.0392	4.12	0.0982
$X_1 X_2$	0.0000	1	0.0000	0.0026	0.9611
$X_1 X_3$	0.0361	1	0.0361	3.79	0.1090
$X_2 X_3$	0.0000	1	0.0000	0.0000	1.0000
X_1^2	0.0146	1	0.0146	1.54	0.2703
X_2^2	0.0051	1	0.0051	0.5335	0.4979
X_3^2	0.0003	1	0.0003	0.0356	0.8577
Pure error	0.0105	2	0.0052		
Residual	0.0476	5	0.0095		
Total	0.9112	14			

R^2=0.9478, Adj.R^2=0.8538, Adeq.Precision=10.9059, Std. dev.=0.0976, C.V.%=2.46

Table 5. ANOVA for the quadratic model of the total phenolic content.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	8057.86	9	895.28	39.76	0.0004
X_1	7396.32	1	7396.32	328.49	<0.0001
X_2	13.73	1	13.73	0.6097	0.4702
X_3	4.79	1	4.79	0.2127	0.6640
$X_1 X_2$	18.19	1	18.19	0.8079	0.4099
$X_1 X_3$	36.36	1	36.36	1.61	0.2597
$X_2 X_3$	3.08	1	3.08	0.1368	0.7266
X_1^2	5.87	1	5.87	0.2607	0.6314
X_2^2	30.98	1	30.98	1.38	0.2936
X_3^2	529.55	1	529.55	23.52	0.0047
Pure error	7.10	2	3.55		
Residual	112.58	5	22.52		
Total	8170.14	14			

R^2=0.9862, Adj.R^2=0.9614, Adeq.Precision=21.0019, Std. dev.=4.75, C.V.%=3.43

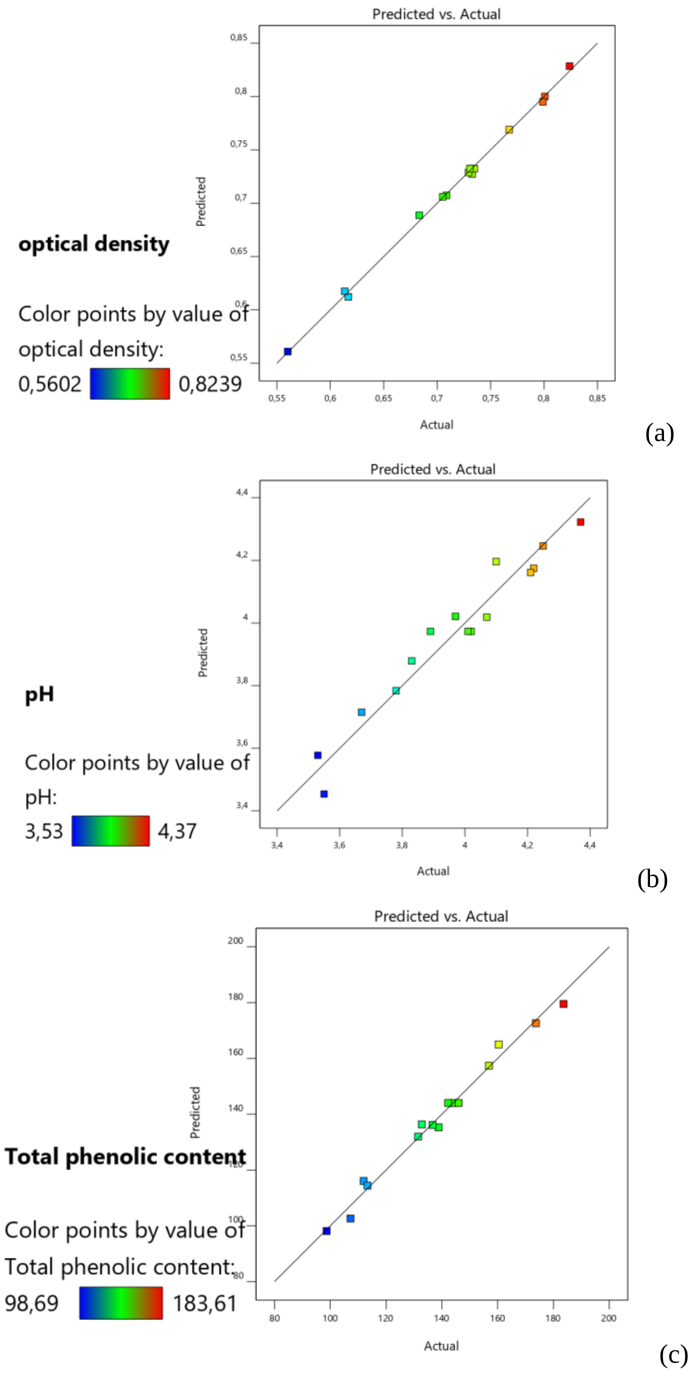


Figure 1. Comparison of the predicted and actual values for (a) the optical density, (b) pH, (c) the total phenolic content.

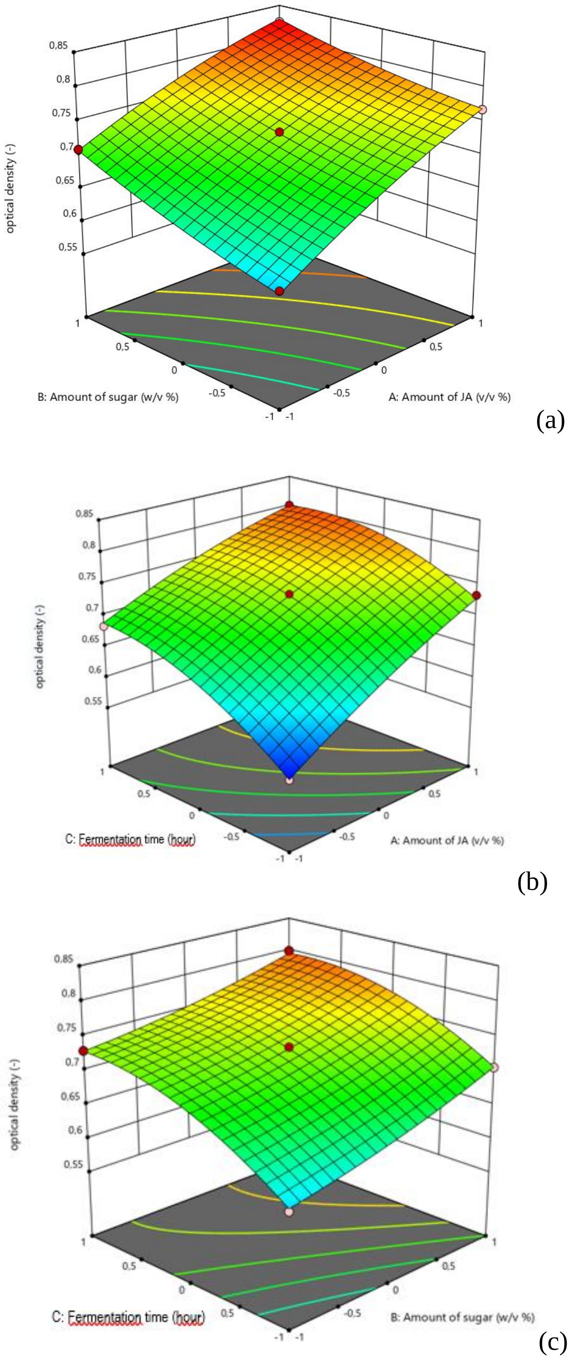


Figure 2. Surface plots of interactive effects of variables on optical density (a)Amount of JA extract (x_1) and amount of sugar (x_2), (b)Amount of JA extract (x_1) and fermentation time (x_3), (c)Amount of sugar (x_2) and fermentation time (x_3).

In contrast to optical density, time was not so effective on the pH of the medium (Figure 3c). The reason for this was that the medium was already acidic (pH 4-5), and although lactic acid, propionic acid, and butyric acid were produced during the fermentation process, the total change in the pH of the medium containing the combination of these acids with different pH values was small. Comparing the colors of the surfaces, due to the less red-colored regions present in Figure 2 than in Figure 1, it can be stated that the parameters studied were less effective on the pH of the fermentation media than on the bacterial growth.

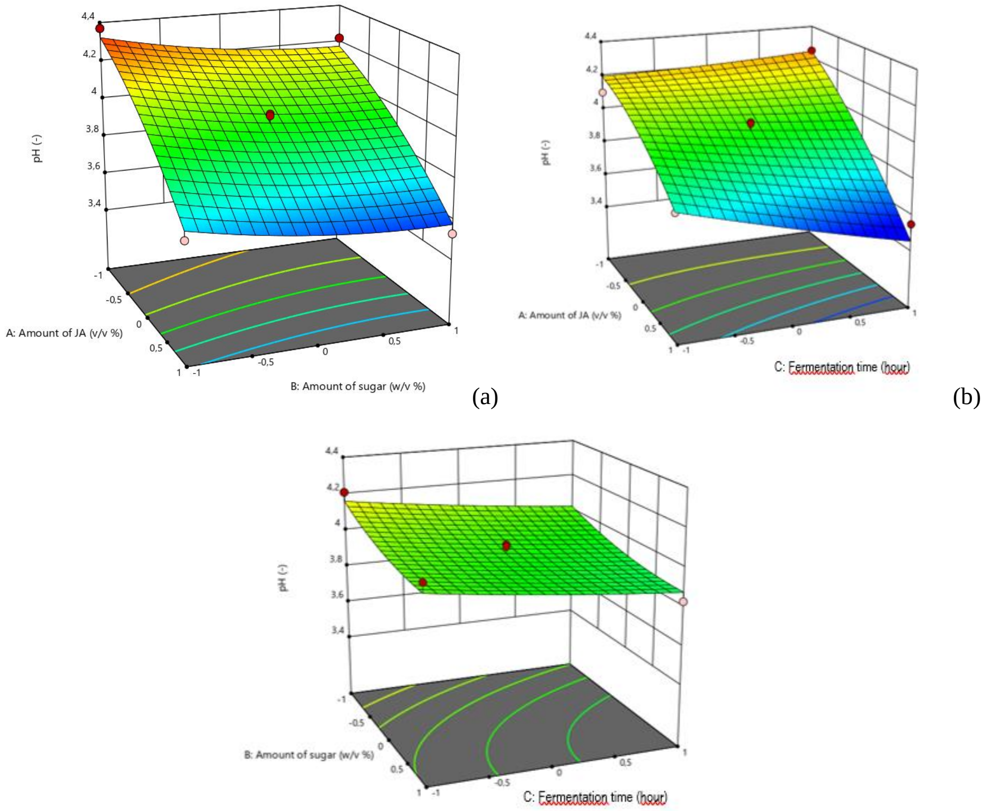
For the total phenolic content, time was found to be more effective on the response than pH (Figure 4c). In particular, the interactive effect of the amount of JA extract and the fermentation time variables showed a significant effect that maximized the total phenolic content of the beverage (Figure 4b). This may be due to the fact that *L. acidophilus* bacteria produces more phenolic compounds when it metabolizes fructooligosaccharides. In addition, according to Figure 4a, it was determined that increasing the amount of sucrose from minimum to maximum value increased the total phenolic compounds from 152.8 mg GAE/g to only 160.34 mg GAE/g (when the amount of JA in coded value was -0.77 v/v), while increasing the amount of JA extract from its minimum to maximum value caused the total phenolic content to increase from 81.19 mg GAE/g to 152.2 mg GAE/g (at all amounts of sugar), approximately twice as much.

The amount of JA was the most effective single parameter, and the fermentation time was the least effective one for all of the responses (Table 3-5). The interactive effect of the amount of JA extract and the amount of sucrose was the dominant variable in all of the responses (Figures 2a, 3a, and 4a), as can be concluded from the highest F-values within interactive effects in ANOVA tables. The concurrent effect of the amount of sucrose and the fermentation time was found to be less effective on both the pH of the media and its phenolic content, whereas it was effective on the bacterial growth.

According to these response surfaces, it can be concluded that prebiotic sugar fermentation resulted in the production of higher amounts of both phenolics and lactic acid (pH of 3.5). For the maximum bacterial growth, the amounts of JA extract and sucrose should be in the range of [+0.5, 1], and the fermentation time should be in the range of [0, 1] (Figure 2). For minimal pH, the amount of JA extract should be in the range of [+0.5, 1], and the amount of sucrose and time should be in the range of [0, 1] (Figure 3). For maximum total phenolic content, the amount of JA extract should be in the range of [-0.5, -1], time should be in the range of [-0.5, +0.5], and the amounts of sugar should be in the range of [-1, 1] (Figure 4).

Numerical solution

The three-dimensional surface plots are useful to see the interactive effects of the independent parameters on the selected response. But, from these plots, it is difficult to determine the specific levels of each independent variable combination for a target response or for a set of criteria. In such cases, the best way is to use numerical optimization. Eleven different production conditions that meet the criteria of maximum optical density and total phenolic content and a pH of 4.09 were obtained by using the numerical optimization section of the program (Table 6).



(c)

Figure 3. Surface plots of interactive effects of variables on pH (a)Amount of JA extract (x_1) and amount of sugar (x_2), (b)Amount of JA extract (x_1) and fermentation time (x_3), (c)Amount of sugar (x_2) and fermentation time (x_3).

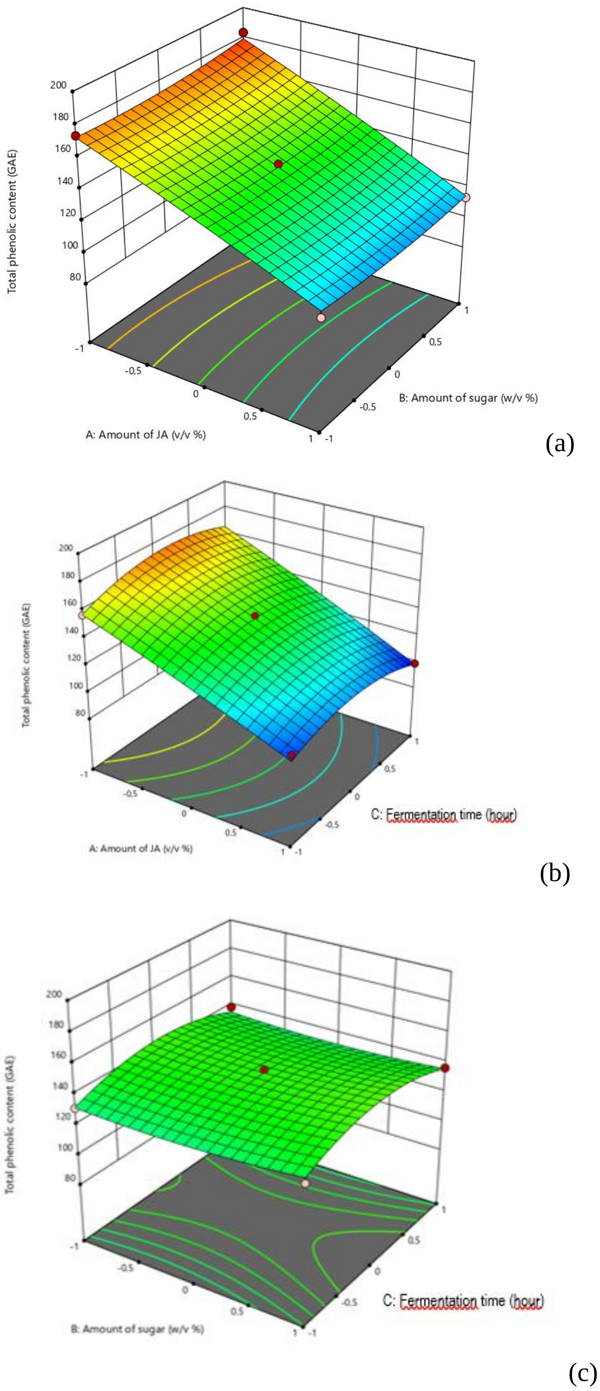


Figure 4. Surface plots of interactive effects of variables on the total phenolic content (a)Amount of JA extract (x_1) and amount of sugar (x_2), (b)Amount of JA extract (x_1) and fermentation time (x_3), (c)Amount of sugar (x_2) and fermentation time (x_3).

To produce the vegetable containing fermented tea having desired characteristics 10g of sucrose and 35-37.5 ml of JA extract (v/v %) must be used in the fermentation media, and fermentation time must be in the range of 6.2-6.92 hours.

Physicochemical properties of the beverage

In order to prove the numerical solution and to evaluate the physicochemical properties of the beverage, the vegetable-containing tea was prepared under the conditions specified at solution 1 given in Table 6. The density of the beverage was 0.9993 g/ml, the pH was 4.09, the optical density was 0.757, and the total phenolic content was 165.814 mg GAE/g, approving the numerical solution with a maximum 0.3 percent deviation in the amount of the total phenolic content. The total soluble matter of the product was determined as 1.23 g/L, and the titratable acidity was 1.87% and 3.48% lactic acid initially and at the end of fermentation, respectively. At 0 hours, the viable counts of *L. acidophilus* were 4.42, 4.40, and 4.40 CFU/ml in black tea, black tea + 10 g sucrose, and the beverage produced under optimum conditions, respectively. At the end of 7 hours, these values were determined as 9.06, 12.36, and 17.14 CFU/ml, respectively. Fermentation of black tea as the sole nutritional source doubled the viable count of *L. acidophilus*. The supplementation of sucrose to black tea resulted in a 36.42% increase in viable count, and further supplementation of prebiotics resulted in a further 38.67% increase in viable count of bacteria.

Table 6. Numerical optimization solutions.

No	X_1	X_2	X_3	OD	pH	TPC
1[a]	-0.593	1.000	0.461	0.757	4.09	165.319
2[a]	-0.593	1.000	0.466	0.757	4.09	165.218
3[a]	-0.593	1.000	0.447	0.757	4.09	165.433
4[a]	-0.594	1.000	0.478	0.758	4.09	165.166
5[a]	-0.593	1.000	0.437	0.756	4.09	165.515
6[a]	-0.594	1.000	0.486	0.758	4.09	165.085
7[a]	-0.592	1.000	0.412	0.756	4.09	165.715
8[b]	-0.591	1.000	0.392	0.755	4.09	165.847
9[b]	-0.595	1.000	0.558	0.759	4.09	164.335
10[c]	-0.590	1.000	0.333	0.753	4.09	166.220
11[d]	-0.583	1.000	0.138	0.746	4.09	166.720

Criteria: Amount of JA-in range, Amount of sugar-in range, Fermentation time-in range, optical density-max., pH-target = 4.09, total phenolic content-max; Desirability: [a] 0.837, [b]0.836, [c]0.835, [d]0.827

In this study, the juices were prepared at fifteen different assay conditions that were determined by the Box-Behnken experimental design. Lower pH values and higher total phenolic content compared to initial values were obtained in each run. The bacterial growth followed by the optical density was concluded as the main reason for these observations due to the fermentation of sucrose and prebiotics and the formation of organic acids. Similar pH values (3.50-4.23) for lacto-fermented herbal teas (thyme, pomegranate peel, rosemary, and echinacea) were also declared in the previous study (Ozturk *et al.*, 2024). In that slight modulation of total phenolic content was observed in 24 hours of fermentation of pomegranate and echinacea infusions, but not rosemary infusions. The maximum amount of total phenolic content was nearly 100 mg GAE/g of dry matter. In this study, higher total phenolic content was obtained due to the supplementation of prebiotics by the addition of JA extract. This is in agreement with previous reports declaring that Lactobacillus species are able to increase the production of polyphenols in many foods and beverages (Wang *et al.*, 2018; Letizia *et al.*, 2023), unlike Bacillus species (Nikkam *et al.*, 2023). In a previous study, Buruleanu *et al.* (2012) investigated cabbage and cucumber juice as substrates for *L. acidophilus* LA-5 and noted that the highest decrease in pH (2.51 units) was observed in cucumber juice, and a 2.27-unit decrease was found in cabbage juice (Buruleanu *et al.*, 2012). The highest acidification rate was observed up to 8 hours, and then the pH decrease slowed down and remained nearly constant between 8 and 24 hours. It was explained by the fastest growth rate of bacteria observed in the first 8 hours of the fermentation. Comparing the required time for fermentation, it can be concluded that supplementation of prebiotics within JA extract resulted in lower fermentation time. According to the results of this study, the change in pH depends not only on the bacterial growth but also on the organic acid type produced, which further depends on the sugar composition. In addition, an extremely dangerous microorganism called *Clostridium botulinum* cannot grow or produce toxins in foods having a pH below 4.6 (classified as highly acidic) (Odlaug and Pflug, 1978). All fermented beverages in this study had low enough pH values to be considered safe; thus, there was no need to add additives. Although the type and the amount of organic acid were not analyzed in detail, the decrease in pH was assumed to be due to lactic acid production since its pH (3.5) was lower than the initial pH values of all beverages. The highest lactic acid production (6.1 mg/ml) compared to propionic (3.3 mg/ml) and butyric acids (3.1 mg/ml) was also declared in previous research on the organic acid production of *L. acidophilus* at the end of fermentation of Orafti P95 (a mixture of fructooligosaccharides) (Sahin Buyuktortop and Yigitarslan, 2012). The supplementation with FOS resulted in the increase by 36.07% and 4.32% of the viable count of *Bacillus coagulans* in green tea and black tea infusions, respectively (Nikkam *et al.*, 2023). A similar increase was also obtained in this study. This is due to the fact that both of the bacteria are probiotic and are capable of metabolizing prebiotics. The growth of Lactobacillus species on vegetable juices, which depends on the type and the amount of vegetable, was also declared in other studies (Buruleanu *et al.*, 2012; Penha *et al.*, 2021).

In the study, the most effective single parameter on the responses was the amount of JA, whereas the fermentation time was the least. Due to the fact that the medium at the beginning of fermentation was already acidic, the time was not so effective at changing the pH of the medium. In addition, *L. acidophilus* is a bacterium capable of fermenting both sucrose and prebiotics; the time required to decrease the pH down to the desired value of 4.09 was enough to ferment the sugar component, independent of its type. All of the variables studied, either singly or in combination, were highly effective on the bacterial growth, but considering the pH and total phenolic content of the beverage, sugar amount and composition were dominant. It was concluded that fermentation of prebiotics resulted in nearly twice the amount of phenolic components, and sugar composition was also found to be effective on the organic acid fermentation rate. Three-dimensional response surfaces supported this conclusion, but since there is no study on this subject, it should be examined in detail by analyzing the amounts of each prebiotic type consumed and organic acids produced in each run. HPLC-MS/MS analysis of phenolics was applied in the research that studied the fermentation of thyme, rosemary, echinacea, and pomegranate extract by lactic acid bacteria, and it was noted that both the types and the amounts of certain phenolics change depending on the plant fermented (Ozturk *et al.*, 2024).

Conclusions

This study demonstrated that tea, the most consumed beverage in Turkey, can be transformed into a functional beverage, and the obtaining parameters were established. The final product of the study is a beverage containing probiotics, prebiotics, and antioxidants. Taking the aforementioned evidence into account, this product presents itself as a potential alternative to traditional tea-based functional beverages aimed at promoting human longevity and well-being. Its advantages include enhanced fermentation products, which lead to improved bioaccessibility and bioavailability. It is anticipated that the beverage produced under the conditions obtained in this study will protect the bodies of individuals with diabetes and liver problems from pathogenic microbial attacks due to its composition. Furthermore, it may protect the body from oxidative effects by scavenging free radicals, which is particularly important in the prevention of hypertension and atherosclerosis. Future studies should focus on a detailed characterization of the product's composition. This should include an examination of the levels of bioactive compounds, such as bioactive peptides, as well as an analysis of metabolomics and proteomics. Additionally, researchers should assess the reduction of antinutritive components, identify the types and quantities of organic acids present, and evaluate the antioxidant capacity. The product's health-enhancing and disease-preventing effects should also be investigated through animal and human clinical trials. Of course, consumer acceptance will also be another factor influencing industrial production.

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