A beetroot-based beverage produced by adding *Lacticaseibacillus paracasei*: an optimization study

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Abstract

The present study aimed to optimize the production conditions of a beetroot-based beverage using *Lacticaseibacillus paracasei*. The experiment was meticulously planned and executed using the Box–Behnken design in Response Surface Methodology (RSM), which provided 17 different combinations. The independent variables in this study included the heat treatment temperature (60–80°C, the heat treatment time (10–30 s), and the incubation temperature (24–36°C). The variables evaluated in this study included pH, total acidity (TA), total phenolic content (TPC), total betalain content (TBC), yellow color (OD420), general acceptance (GA), and total count of lactic acid bacteria (LAB). The samples were determined to have TPC ranging from 67.90 to 437.15 mg GA/L and TBC ranging from 147.52 to 747.21 mg/L. The samples subjected to incubation temperatures of 30°C and 36°C were typically favored by the panelists and exhibited better results in terms of chemical analysis compared to the samples incubated at 24°C. According to RSM, heat treatment at 60°C for 22 min and an incubation temperature of 31°C were the optimum production conditions. Under these optimal conditions, RBJ has maximum TBC, total LAB, and GA scores, indicating nutrient-dense and appealing to consumers.

Keywords: probiotic, functional foods, betalain, fermentation, response surface methodology

Introduction

Consumer demand for products with health benefits has recently increased for various reasons. Notably, functional foods have garnered great interest as they contain components such as antioxidants, bioactive peptides, vitamins, minerals, prebiotics, and probiotics that offer several health benefits (Mantzourani *et al*., 2019). Probiotics-based foods are gaining prominence at the fastest rate among all functional foods, and they represent 70% of the functional food market (Lillo-Pérez *et al*., 2021).

As the demand for functional probiotics increases, more efforts are being made to design, produce, and commercialize various functional food products. Although fermented dairy products have been traditionally considered the best carriers of probiotics, consumption of milk-based products can be limited due to lactose intolerance, allergies, dyslipidemia, and veganism (Perricone *et al*., 2014). Non-dairy foods, particularly vegetables, can produce probiotic beverages as they are rich in phytochemicals and phytonutrients and provide a protective environment for probiotics. Fermented or minimally processed vegetables, such as beets, carrots, radishes, artichokes, cabbage, broccoli, celery, etc., have garnered interest as alternative probiotic carriers and have been previously studied (Bengoa *et al*., 2021; Chaturvedi & Chakraborty, 2022; Jones, 2014;

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Perricone *et al*., 2014; Zohary *et al*., 2012). Owing to its rich content of nutritional components, various health effects, and palatability, beetroot can be a suitable option for producing a probiotic beverage. Contrary to adding to the dairy matrix, adding probiotics to non-dairy foods is more complicated. The primary difficulties encountered in this situation are attributed to the inherent characteristics of these products, including a low pH and a high concentration of organic acids. These features, along with the storage time and conditions required to preserve the viability of probiotics, contribute to the hurdles experienced (Gomes *et al*., 2021). One drawback of non-dairy beverages is that consumers may experience some unpleasant flavors from microorganisms (Tesfaye *et al*., 2019).

Beetroot (*Beta vulgaris* L.) is a flowering plant belonging to the *Amaranthaceae* family. Although it is native to the Mediterranean region, it is cultivated in America, Europe, and India (Chawla *et al*., 2016; Zohary *et al*., 2012). Compared to their fellow subspecies, *B. vulgaris subsp. vulgaris* (*altissima*), known as sugar beet, the beetroot subspecies has approximately two times lower sugar content (Wruss *et al*., 2015). Therefore, beetroot is used in food products (pickles, salad, and juice) rather than sugar production. With its low sugar content, Beetroot juice enhances exercise performance (Jones, 2014).

The Lacticaseibacillus (Lc.) group comprises the closely related *Lc. casei* (Bujna *et al*., 2018; Chaturvedi & Chakraborty, 2022). *Lc. paracasei* (Bengoa *et al*., 2021; Marnpae *et al*., 2022). *Lc. rhamnosus* (Coda *et al*., 2011; Hill *et al*., 2018; Zalán *et al*., 2010), and *Lc. plantarum* (Coda *et al*., 2011; Hill *et al*., 2018) are among the most widely studied and applied probiotic species of *Lactobacilli* in non-dairy foods as most effects of probiotics are strain-specific, *Lc. paracase*i strains, which have high intrinsic heterogeneity, seem to be an optimal group for selecting a novel probiotic strain with unique health-promoting properties (Huys *et al*., 2013). It is crucial to acknowledge that probiotic strains exhibit varying tolerance levels to external stresses, dependent upon their sources. Probiotic strains obtained from food sources typically exhibit greater resistance to variations in temperature and pH during the processing of food products. However, they have a lower rate of survival when passing through the gastrointestinal tract compared to probiotic strains obtained from the gut (Min *et al*., 2019). Additionally, it is important to carefully evaluate the application of probiotic bacteria, as the quantities and types of metabolites produced during fermentation might vary, potentially impacting the quality of the final product. Nevertheless, the ability of probiotic bacteria to survive and remain intact in unfavorable environmental conditions encountered during food processing, storage, and consumption remains a significant obstacle in the advancement of probiotic product development.

Although achieving a high probiotic load while designing a probiotic product is desirable, products should be optimized while considering the effects of different variables. The optimization of production parameters for food items should involve an examination of the effects of different independent variables to obtain the best result. Response surface methodology (RSM) offers the possibility to simultaneously examine multiple variables affecting the productivity of the process, allowing the optimal characterization of the response of a process to changes in parameters using a minimum number of trials. It can be broadly applied to improve existing product designs. The Box–Behnken design (BBD) is one of the most popular designs in RSM, and it offers the possibility to work with 3–21 independent variables. RSM has been successfully used to optimize the production parameters of various fermented products (Bertolini *et al*., 2020; Chaturvedi & Chakraborty, 2022; Mauro & Garcia, 2019).

In previous studies, the development of probiotic beverages with beetroot juice (RBJ) (Kyung *et al*., 2005; Kazimierczak *et al*., 2014; Malik *et al*., 2019), and also with other vegetables (Mantzourin *et al*., 2019; Mantzourin *et al*., 2020; Mesquita *et al*., 2020; Marnpae *et al*., 2022) was investigated by researchers. This research primarily focuses on determining the survival rates of bacteria used in the production of beverages. Currently, there is a lack of research on choosing an appropriate preservation approach in the production of RBJ to ensure the safety of a beverage while minimizing the degradation of healthy bioactive compounds, such as betalains. No study recommends the most appropriate temperature treatment for vegetable juice production, considering the relationship between temperature and betalains, as well as the general acceptance of the people.

To overcome this issue, a comprehensive optimization study was conducted utilizing RSM to provide a manufacturing method for RBJ that ensures both the safety and maximum preservation of its bioactive compounds. In addition, a probiotic starter culture was used to enhance the health benefits of the beverages. 17 distinct samples were produced, and the production method that exhibited the highest betalain and total LAB levels and was preferred by customers was identified.

Materials and Methods

Materials

The red beetroots (*Beta vulgaris* L.) used in beverage production were obtained from the local market on the

same day of the fermentation. The strain *Lc. paracasei* 431 was obtained from the CHR-HANSEN (Hoersholm, Denmark).

Methods

Preparation of cultures before fermentation

Lc. paracasei 431 were stored at –20°C until used. The strains were cultivated in a tube containing 10-mL MRS broth medium using a shaking incubator (IST-3075, Jeio Tech Lab Companion) under the specified conditions: 37°C, 250 rpm, for one night. Following the incubation period, 1 mL from the previously mentioned suspension was inoculated into 10 mL of sterile beetroot juice and cultivated under similar conditions. The most recently generated suspension, which contains approximately 6–7 log CFU/mL total LAB, was utilized in the fermentation experiments.

Preparation of juices and heat treatment

After peeling, washing, and slicing, beetroots were pressed through a juicer (Philips, HR1861), and vegetable juice was used throughout the process. Subsequently, a mixture was prepared consisting of vegetable juice (62.5%), water (37.5%), salt (2%), garlic (1.25%), and a small portion of bay leaf. Our team adjusted the rates of the components in the preliminary studies. The mixture was thereafter covered and exposed to a heat-treatment process in a shaking water bath (Stuart, SD-40, Staffordshire, UK). Temperature measurement was done using a thermometer (Extech Instruments, HD 200, Nashua, USA) kept in a cotton-covered control flask with an equal amount of components utilized. After heat treatment, the juices were allowed to cool to the ambient room temperature. Subsequently, a suspension of 2% *Lc. paracasei* (approximately 6–7 log CFU/mL) was inoculated into the juices, ensuring aseptic conditions were maintained throughout the process.

Fermentation

Experimental setup and optimization

The optimization of production parameters for the probiotic RBJ beverage was conducted using Response Surface Methodology (RSM). A Box-Behnken design (BBD) consisting of three independent variables was implemented using a three-level structure, resulting in 17 runs, including five center points. The independent variables examined in this study included heat treatment temperature (60–80°C), heat treatment time (10–30 min.), and incubation temperature (24–36°C) (Table 1). The fermentation process was conducted in an incubator (Memmert, IN 55, Schwabach, Germany). As independent variables and levels, we prefer the temperature and time range commonly used for conventional fruit and vegetable production (González-Aguilar *et al*., 2004; Atter *et al*., 2015; Alcántara-Zavala *et al*., 2021). Furthermore, we selected the fermentation temperature range of 24–36°C, considering the traditional manufacturing method's ambient temperature and the optimal LAB growing temperature at 30–36°C.

Table 1. Independent variables and levels.

	Levels		
Independent variables	**–1**	**0**	**1**
Heat treatment temperature (°C)	60	70	80
Heat treatment time (min.)	10	20	30
Incubation temperature (°C)	24	30	36

The BBD experimental design incorporated several response parameters for analysis, including pH, total acidity (TA), total phenolic content (TPC), total betalain content (TBC), and yellow color (OD420) for chemical analysis. Additionally, we evaluated general acceptance (GA) through sensory analysis and conducted total LAB counts for microbiological analysis.

The optimization study aimed to determine the optimal conditions for maximizing TBC, total LAB, and GA score to develop a beetroot-based beverage that is both nutritious and attractive to customers.

Chemical analysis after fermentation

pH

The pH values of fermented RBJ samples were determined using a pH meter (Hanna HI 1221, Czeck).

Total acidity

TA levels of the samples were calculated in terms of lactic acid (%) using the method recommended by (Cemeroğlu, 1992).

Total phenolic content

TPC of the samples was determined spectrophotometrically using the method proposed by Cemeroğlu, (1992).

In the procedure, 40 µL of the sample was combined with 200 µL of Folin–Ciocalteu (tenfold dilution) reagent, followed by 2400 µL of distilled water. The resulting mixture was thoroughly mixed using a vortex. The abovementioned mixture was subjected to a period of darkness

lasting 5 min. Subsequently, 600 µL of a 20% saturated Na_2CO_3 solution was combined with 760 µL of distilled water, followed by thoroughly mixing the resultant solution. The samples were subjected to a period of 30 min in darkness, after which the absorbance at a wavelength of 765 nm was quantified utilizing a spectrophotometer (PG Instruments, T80, UK).

To construct a standard gallic acid curve, a range of gallic acid concentrations were prepared and subsequently analyzed using the TPC analysis mentioned previously. After determining the absorbance levels for various concentrations, we generated a graph. The equation employed to depict the graph was $y = 0.001x + 0.0031$, with an R^2 value of 0.999.

Analysis of total betalain content

The TBC in the samples was determined by the sum of the content of betaxanthin and betacyanin, which are detected in a spectrophotometer (PG instruments, T80, UK) by the method recommended by Stintzing *et al*. (2003) which is described below.

The samples were centrifuged at 5000 rpm for 20 min, and the obtained supernatants were filtered through filter paper and diluted with citrate–phosphate buffer (pH 6.5) to ensure that the absorbance did not exceed 1.00. After adjusting the appropriate dilution ratios, absorbance values of the samples at 538 nm (betacyanin) and 476 nm (betaxanthin) were recorded, and the amounts of betaxanthin and betacyanin in the samples were calculated using the following equation.

$$\text{Betalain content [mg/L]} = [(A \times DF \times MW \times 1000)/(\varepsilon \times L)]$$

where A is absorption at 538 nm and 476 nm for betacyanins and betaxanthins, respectively; DF is the dilution factor; L is the path length of the cuvette (1 cm); and MW is molecular weight (550 g/mole for betacyanin 339 g/mole for betaxanthin).

Color determination

The samples were diluted forty times with distilled water, and yellow color absorbance (OD420) values were determined using a spectrophotometer (PG instruments, T80, UK).

The OD420 values correspond to alterations in the yellow pigments found in beets, which are more susceptible to heat compared to the red pigments (Prieto-Santiago *et al*., 2020; Zin, Anucha, *et al*., 2020). Additionally, these values also reflect changes in the formation of browning compounds during heat processing (Marquez *et al*., 2013).

Microbiological analysis

The total counts of LAB in the samples were assessed following the fermentation process. The samples were initially diluted for microbiological analysis using a sterile solution of 0.85% sodium chloride. Subsequently, the final three dilutions (10^{-5}, 10^{-6}, 10^{-7}) were inoculated into MRS plates utilizing the spread plate method with two parallel runs. The colonies that formed in Petri dishes were quantified, and the enumeration of LAB in the samples was performed using the formula derived from the Bacteriological Analytical Manual of the United States Food and Drug Administration (FDA, 2011).

Sensory analysis

The RBJ samples produced according to the experimental design described in Table 1 were stored at 4°C for at least 2 h after fermentation and then tasted by the panelists. The sensory testing was conducted at the Food Product Evaluation Laboratory within the Faculty of Engineering at Sivas Cumhuriyet University, Turkey, with three repetitions. A total of 40 non-trained panelists were selected from the student and staff populations at the faculty. The sensory panel consisted of participants aged 20–55, with 64% female. The samples provided were assessed using the nine-point hedonic scale, which ranges from 1 (indicating extreme dislike) to 9 (indicating extreme liking).

Statistical analysis

The experiments were designed with a Box-Behnken design of an experiment, a type of RSM, using the Minitab 20.0 software (State College, PA). All statistical analyses were performed on the same software. The effects of independent variables on responses were examined using the variance analysis (ANOVA) test. The statistical significance of the effects of each independent variable's linear, quadratic, and binary interactions on the responses was analyzed using the Fisher's test (F test) at a 95% confidence level. In determining the model's suitability, the variation stemming from regression is expected to be significant, and the lack of fit is expected to be insignificant at a 95% confidence level. Moreover, the model's fit was assessed using regression coefficient (R^2) and adjusted regression coefficient (Adj-R^2). All experiments were carried out in two repetitions; statistical analysis was made after the averages were placed in the trial design. A one-sample t-test was performed to investigate the statistically significant difference between validation and optimization results ($P < 0.05$).

Validation

A validation process was conducted to assess and verify the accuracy of the parameters obtained during the optimization process. Firstly, the beetroot juices were produced by adding *Lc. paracasei* under optimal conditions. Subsequently, a revaluation was conducted on the samples, covering an analysis of TBC, total LAB, and GA levels.

Results and Discussions

The fermentation process continued until the RBJ samples' pH levels reached a value lower than 4.00. For samples incubated at 30°C and 36°C, this usually occurred within 48 h, but samples incubated at 24°C needed about 96 h to obtain the same pH levels.

Chemical analyses

Following the fermentation process, the RBJ samples were analyzed to determine their level of pH, TA, TPC, TBC, and OD420.

The pH values of RBJ samples ranged from 3.45 to 3.89 (Table 2). The results presented in Table 3 indicate that the model exhibited statistical significance ($P < 0.05$). Furthermore, both the heat treatment temperature and the incubation temperature were found to significantly impact the pH values of the samples ($P \leq 0.05$). The data indicates that the samples incubated at a temperature of 24°C exhibited pH values that were comparatively higher than those observed at 30°C and 36°C. The pH values of the samples were assessed and found to be within the expected range for a fermented beverage. These results align with previous studies, which reported pH values of 3.80 (Kazimierczak *et al*., 2014) and 3.6 (Yoon *et al*., 2006).

The TA values of the samples were varied between 0.51% and 0.79%. The data is consistent with prior investigations, which reported TA of the samples between 0.60% and 0.74% (Kazimierczak *et al*., 2014), 0.95% (Yoon *et al*., 2006), and 0.78% (Panghal *et al*., 2018).

The TPC of the RBJ samples exhibited a range of values from 67.90 to 437.15 mg GA/L, as shown in Table 2. Based on the data shown in Table 3, it was seen that the incubation temperature had a significant impact on the TPC values of the samples, both at the linear level and the quadratic term ($P < 0.001$). The samples subjected to incubation at 30°C and 36°C exhibited the greatest TPC values. These values agree with prior research conducted on products obtained from red beetroot (Czyzowska *et al*., 2006; Prieto-Santiago *et al*., 2020). The samples of RBJ produced in this investigation appear to contain a significant amount of phenolic compounds. Beets possess a high concentration of betalains, which are a group of betalamic acid derivatives that include betacyanins and betaxanthins, as well as phenolic compounds (Wootton-Beard & Ryan, 2011). Regularly consuming vegetable juices abundant in phenolic compounds makes a substantial contribution to the daily consumption of these bioactive compounds that promote health. The presence of bioactive constituents inhibits the oxidation mechanism in low-density lipoprotein (LDL) and effectively modulates the total amount of cholesterol. Consequently, this leads to a decrease in the possibility of platelet aggregation and adhesion, which is associated with the formation of atherosclerosis (Granato *et al*., 2015).

As seen in Table 2, the TBC of the RBJ samples was within the range of 147.52–747.21 mg/L. Table 3 demonstrates that the incubation temperature was the only variable that exhibited a statistically significant impact on the TBC at both the linear and quadratic levels ($P < 0.05$). The samples incubated at 30°C and 36°C exhibited the highest levels of TBC. The investigation's findings are consistent with the results obtained by the researchers who employed heat treatment to RBJ at 120°C for 10–60 min (Prieto-Santiago *et al*., 2020), and who produced RBJ using probiotic strains *Lc. paracasei* 0916, *Lc. paracasei* 0923, and *Lc. paracasei* 0920 (Czyzowska *et al*., 2006).

The OD420 values of the RBJ samples exhibited a range of 1.26–20.24, as presented in Table 2. The results shown in Table 3 show that the incubation temperature had a significant linear effect on the OD420 values of the samples ($P < 0.001$). The samples that were incubated at temperatures of 30°C and 36°C had the highest OD420 values.

The section titled "Testing the Fit of the Model" presents a comprehensive discussion of TPC, TBC, and OD420 values.

Microbiological analysis

As seen in Table 2, the total LAB count of the samples varied between 8.17 and 9.01 log CFU/ml. According to Table 3, the model established on total LAB count was insignificant ($P > 0.05$). The primary factor that results in a lack of any detectable difference is the optimal bacterial growth conditions provided by the red beetroot juice environment. A probiotic drink should have at least 6 log CFU/mL and 7–8 log CFU/mL to remain within an acceptable range (Ozcan *et al*., 2021). All samples of the RBJ met this criterion.

Table 2. Experimental design created with Box-Behnken and results of responses (chemical, sensory, and microbiological analysis results).

Independent variables				Responses						
				Chemical analyses					Sensory analysis	Microbiological analysis
Sample code	Heat treatment temperature (°C)	Heat treatment time (min.)	Incubation temperature (°C)	pH	Total acidity (LA, %)	TBC (mg/L)	TPC (mg GA/L)	OD420	GA	Total LAB counts (log CFU/mL)
12	60	20	36	3.53±0.02	0.62±0.06	609.66±90.15	422.45±12.55	16.94±5.3	4.41±0.01	8.52±0.10
1	70	10	36	3.45±0.12	0.56±0.11	379.18±46.97	310.95±0.95	19.02±0.18	3.50±0.05	8.60±0.03
4	60	30	30	3.58±0.02	0.73±0.06	483.95±8.32	317.45±3.55	7.52±0.40	3.65±0.15	8.95±0.11
3	80	30	30	3.82±0.14	0.62±0.06	599.23±37.45	342.90±5.00	7.56±0.40	3.90±0.10	8.17±0.48
7	60	20	24	3.58±0.01	0.68±0.00	219.03±3.38	95.40±1.50	1.5±0.18	1.00±0.00	8.81±0.01
6	70	20	30	3.74±0.05	0.68±0.11	605.81±12.20	300.00±5.25	5.2±0.04	4.40±0.00	8.60±0.32
14	80	20	24	3.80±0.23	0.79±0.00	239.59±6.77	145.90±3.00	3.82±0.18	1.00±0.00	9.01±0.07
2	70	10	24	3.89±0.13	0.68±0.11	196.55±1.73	67.90±2.00	1.26±0.40	1.00±0.00	8.81±0.07
13	70	30	24	3.82±0.23	0.62±0.06	147.52±88.52	146.40±3.50	5.9±0.04	1.00±0.00	8.75±0.01
5	80	20	36	3.57±0.03	0.68±0.00	727.49±94.91	314.45±0.45	20.24±1.16	4.00±0.00	8.77±0.19
15	60	10	30	3.67±0.04	0.56±0.00	503.04±51.97	330.20±0.20	6±0.04	4.00±0.00	8.79±0.11
8	70	20	30	3.71±0.07	0.68±0.00	457.44±1.80	300.00±1.00	6.24±0.04	4.40±0.00	8.86±0.21
11	80	10	30	3.67±0.08	0.51±0.06	587.78±48.67	437.15±5.25	6.12±0.20	3.90±0.10	8.33±0.16
9	70	20	30	3.59±0.09	0.68±0.11	747.21±18.80	320.00±5.00	13±0.04	4.60±0.00	8.98±0.12
16	70	20	30	3.56±0.04	0.68±0.11	503.87±1.90	330.40±5.00	6.24±0.04	4.60±0.00	8.37±0.32
17	70	20	30	3.61±0.07	0.79±0.00	563.34±2.80	307.95±3.55	13±0.04	4.00±0.00	8.80±0.16
10	70	30	36	3.67±0.08	0.68±0.11	603.83±8.70	256.45±0.45	14.08±0.04	3.90±0.10	8.77±0.30

TA, Total acidity; TPC, total phenolic content; TBC, total betalain content; OD420, yellow color; GA, general acceptance.

Table 3. ANOVA and regression coefficient of the second-order polynomial model for the response variables.

		Estimated coefficients						F values					
Variables	**DF**	**pH**	**TA**	**TPC**	**TBC**	**OD420**	**GA**	**pH**	**TA**	**TPC**	**TBC**	**OD420**	**GA**
Model	9	3.64	0.6975	311.7	575.5	8.74	4.4	3.9**	1.32	13.07***	6.08*	5.28**	49.46***
Linear	3							7.99*	1.49	21.59***	11.6*	13.94*	85.29***
Heat treatment temperature (°C) (X_1)	1	0.0631	0	9.4	42.3	0.72	−0.0326	5.81**	0	0.5	1.63	0.41	0.12
Heat treatment time (min.) (X_2)	1	0.0275	0.0422	−10.4	21	0.33	0.0063	1.1	3.09	0.61	0.4	0.09	0
Incubation temperature (°C) (X_3)	1	−0.1081	−0.0281	106.1	189.7	7.22	1.4763	17.04**	1.37	63.68***	32.78***	41.31***	255.75***
Square	3							1.18	1.85	14.69***	5.83**	1.15	62.54***
X_1 x X_1	1	−0.0241	−0.0183	47.2	42.6	−0.69	−0.142	0.45	0.31	6.63**	0.87	0.2	1.25
X_2 x X_2	1	0.0646	−0.0745	−1.9	−74.6	−1.25	−0.395	3.2	5.07	0.01	2.67	0.65	9.64**
X_3 x X_3	1	−0.0016	0.0098	−114.3	−169.2	2.58	−1.655	0	0.09	38.91***	13.72**	2.77	169.15***
2-Way Interaction	3							2.55	0.63	2.91	0.81	0.76	0.55
X_1 x X_2	1	0.0575	X_1*X_2	−20.4	7.6	−0.02	0.087	2.41	0.17	1.17	0.03	0	0.45
X1 x X_3	1	−0.0437	X_1*X_3	−39.6	24.3	0.24	−0.103	1.4	0.17	4.44	0.27	0.02	0.62
X_2 x X_3	1	0.0725	X_2*X_3	−33.3	68.4	−2.39	0.1	3.83	1.54	3.13	2.13	2.27	0.59

Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001.
TA, total acidity; TPC, total phenolic content; TBC, total betalain content; OD420, yellow color; GA, general acceptance.

Sensory analysis

The development of innovative products involves a crucial process called sensory analysis. Sensory analysis can facilitate an in-depth knowledge of a product's distinctive features, enhance the researcher's confidence in its quality, and establish the sensory attributes that correspond to consumer preferences.

In this study, the panelists' average GA scores varied from 1 to 4.60 on a 5-point scale. The findings presented in Table 2 indicate that the incubation time exhibited a statistically

significant influence at both the linear and quadratic levels ($P < 0.001$), while the heat treatment time had a statistically significant effect only at the linear level ($P < 0.05$). The samples subjected to fermentation at 24°C exhibited comparatively lower scores than those fermented at 30°C and 36°C.

While the primary reason for consuming probiotic products is their potential health benefits, it is also important for these products to possess acceptable sensory qualities. The flavor components and sweetness of the RBJ developed in this investigation were highly regarded by the panelists. Hence, beetroot exhibits promising prospects for use in the development of innovative products that are likely to be appreciated by consumers. This finding has been documented in prior research. The panelists expressed less appreciation for RBJ, produced with probiotic LAB, than freshly squeezed juice (Panghal *et al*., 2018). However, the same researchers declared that microbial fermentation possesses the potential to serve as a viable technological approach to developing food that promotes health.

Mathematical models built on responses

The modeling of any process should follow two main steps: (a) establishing a mathematical model or empirical relationship and (b) testing the adequacy of the proposed model through model validation. These steps address the inherent variability in the model, namely the methodology for accurately predicting the data by utilizing empirically obtained responses (Puri & Banerjee, 2013). The results of this investigation were interpreted in the same order.

Estimation of model parameters and construction of model equations

In multiple regression analysis, certain independent variables can have an insignificant contribution to the whole model. Including irrelevant terms in models can result in unnecessary complexity for the model. The complexity of the model could hinder comprehension and interpretation. The simplification and enhancement of model comprehensibility are achieved by eliminating extraneous terminology (Krolzig & Hendry, 2001; Vu *et al*., 2015).

Insignificant terms were eliminated from the model by following the rules of hierarchy. After eliminating insignificant factors for each response, ANOVA was conducted again. ANOVA tables are given in detail in the section Appendix A (Tables A1–A6).

The F-values of the ANOVA test were used to determine which model could more satisfactorily explain the responses (Ohale *et al*., 2017). The polynomial model with the highest degree and significant terms demonstrating a good and normal relationship between the parameters was chosen based on the F-value results. The F values and statistically significant levels of the responses were given in Table 3. The linear model demonstrated statistical importance at a significance level of $P < 0.05$, with an F value of 7.99 for pH and an F value of 13.94 for OD420. The F values for the quadratic model were 14.69 ($P < 0.001$) and 5.83 for TPC and TBC ($P < 0.01$), respectively. Further, the F values for the linear model were 721,59 ($P < 0.001$) and 11.6 ($P < 0.05$) for the same variables ($P < 0.001$). For GA of RBJ samples, F values were determined to be 5.82 and 4.26 ($P < 0.001$). Based on the findings, it was concluded that the linear model provides a better representation of pH and OD420 than the quadratic model, and the quadratic model provides a more suitable fit than the linear model for TPC TBC and GA of RBJ samples. The equation in its simplified form was presented in Table 4 after eliminating irrelevant terms. Among all the responses, the proposed TA and total LAB model were found to be insignificant ($P > 0.05$), so equations for these responses were not provided.

Figure 1 presents the correlation between the estimated values of the samples using the derived equations and the observed values. The R^2 values of RBJ samples, which indicate the degree of correlation between the predicted and observed values for pH, TPC, TBC, OD420, and GA, were 0.932, 0.874, 0.792, 0.767, and 0.978, respectively. The obtained higher R^2 values suggested that the equations employed can accurately explain and predict the observed data.

Testing the fit of the model

The statistical analysis did not include testing the adequacy of models that had no significant effect on the responses (TA, total LAB) for the RBJ ($P > 0.05$). Upon removal of irrelevant terms, the coefficient of

Table 4. Mathematical models of the responses obtained after eliminating insignificant terms.

Responses	Equations
pH	$pH = 3.704 + 0.0631 \times X1 + 0.00275 \times X2 - 0.01802 \times X3$
TPC (mg GA/L)	$TPC = -817 - 65.0 \times X1 - 1.04 \times X2 + 208.4 \times X3 + 0.471 \times X12 - 3.178 \times X32$
TBC (mg/L)	$TBC = -4999 + 4.23 \times X1 + 2.10 \times X2 + 316.5 \times X3 - 4.75 \times X32$
OD420	$OD420 = -32.81 - 0.072 \times X1 + 0.033 \times X2 + 1.204 \times X3$
GA	$GA = -46.00 - 0.00326 \times X1 + 0.1616 \times X2 + 3.017 \times X3 - 0.00403 \times X22 - 0.04618 \times X32$

*Each independent variable is encoded with X, and the codes are as follows: X_1: Heat treatment temperature (°C), X_2: Heat treatment time (min.), and X_3: Incubation temperature (°C).

determination (R^2) values for pH, TPC, TBC, and OD420 were found to be 56.86%, 87.36%, 79.24%, and 76.60%, respectively (Table A1, Tables A2–A5). Occasionally, including additional variables in the model leads to a substantial increase in the R^2 coefficient. This issue can be avoided by utilizing adjusted coefficients of determination (R^2_{adj}) (Montgomery, 2017). The R^2_{adj} values of pH, TPC, TBC, and OD420 were determined 46.91%, 81.61%, 72.32%, and 71.30%, respectively (Table A1, Tables A2–A5). As seen in Table 4, the lack of fit value was insignificant only for TBC and OD420 ($P > 0.05$). An ideal model is expected to have a statistically significant regression model and a statistically insignificant lack of fit value. An additional requirement for the adequacy of the model is to get a minimum value of 0.7 for the R^2 or R^2_{adj} derived from the terms that exhibit statistical significance in the model (Iqbal & Khan, 2010). It is important to note that the models have demonstrated effectiveness in predicting TBC and OD420. However, the model's performance in predicting pH and TPC is comparatively less satisfactory. This observation suggests that the models used for TBC and OD420 effectively represent the experimental data.

The effect of independent variables on the TBC of the samples is shown in Figure 2. The results show that an incubation temperature of 30°C–36°C resulted in the highest TBC when the heat treatment temperature was maintained at 70°C (Figure 2A). Moreover, when the heat treatment time was kept constant at 20 min, the higher TBC was obtained at an incubation temperature of 30°C–36°C (Figure 2C). An extended period of fermentation at 24°C proved to increase the activity of enzymes, potentially contributing to the accelerated degradation of betalain. Throughout the pressing of beetroot, several endogenous enzymes, including β-glucosidases, polyphenol oxidases (PPO), and peroxidases, have the potential to be transferred into the vegetable juice. These enzymes may then exhibit their catalytic effects throughout the process of fermentation (Sadowska-Bartosz & Bartosz, 2021). Similar decreases due to the degradation of betaxanthin in fresh red beetroot juice caused by fermentation at 26°C reported by another study (Janiszewska-Turak *et al*., 2022). Therefore, food processing methods with low deterioration should be adopted, as betalains are challenging to stabilize. As shown in Figure 2B, the TBC of the samples increased in response to increased heat treatment temperature and time while the incubation temperature was held constant at 30°C. Indeed, the main factor impacting the stability of betalains throughout the storage and processing of fruits and vegetables is temperature (Herbach *et al*., 2006; Herbach & Stintzing, 2004). In this study, the increase in the TBC values observed despite the increase in heat treatment temperature may be attributed to product degradation, which would have influenced the color of the samples. A study reported that exposing beetroot juices to high temperatures can yield a higher-than-normal TBC (Herbach *et al*., 2006).

A 3D surface graph was used to analyze the effect of the independent variables on the OD420 values of the samples. Figure 3A and 3C demonstrate that the impact of incubation temperature on the OD420 values was more significant compared to the influence of heat treatment time and temperature. The observed decrease in OD420 yellow color values as heat treatment temperatures increase might be related to the sensitivity of betalains, the compounds responsible for imparting yellow color in RBJ samples. As previously indicated, betalains may undergo degradation at temperatures exceeding 50°C. Additional research has demonstrated that subjecting betalain-rich juices to heat treatment leads to a phenomenon of discoloration, resulting in the generation of juices with a lighter color and a more yellow appearance (Herbach & Stintzing, 2004; Herbach *et al*., 2006; Prieto-Santiago *et al*., 2020). Betacyanin, which is responsible for imparting red color, is more tolerant to heat and processing conditions than betaxanthins, which are responsible for imparting yellow color (Prieto-Santiago *et al*., 2020; Zin *et al*., 2020). According to a study on the thermal degradation of yellow pigments, despite their antioxidant capacity, yellow pigments displayed three times lower stability than red pigments (Mikołajczyk-Bator & Pawlak, 2016). Another study reported that antioxidants such as ascorbic acid, ascorbic acid, citric acid, and other preservatives could stabilize betalains to a certain extent. However, antioxidant phenolic compounds do not stabilize pigments, as the degradation of betalains does not

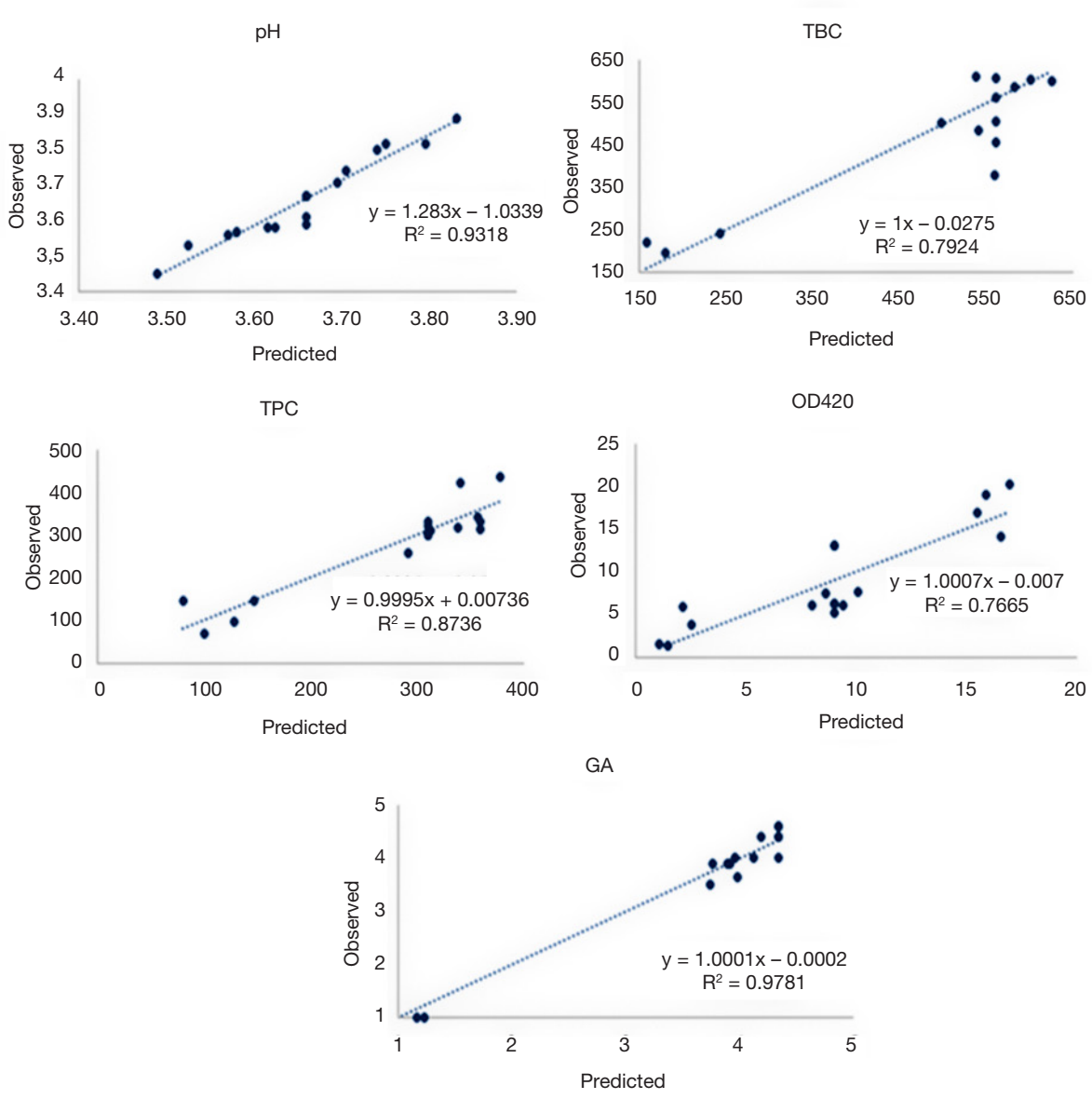

Figure 1. Correlation between the predicted values and the observed values. Abbreviations of the responses: total phenolic content (TPC), total betalain content (TBC), yellow color (OD420), general acceptance (GA).

involve free radical formation (Khan, 2016). In contrast, another investigation showed that a heat treatment temperature of 85°C for RBJ samples enhanced the yellow color. Additionally, it was found that red colored pigments exhibited greater resistance to temperature fluctuations compared to yellow color pigments (Herbach *et al.*, 2004). The same observation was reported in a study concerning the pitaya beverage (Herbach *et al.*, 2004). Dissimilar attitude on yellow color values of the samples that were exposed to heat treatment may be caused by severity of treatment (Prieto-Santiago *et al.*, 2020). Figure 3B shows that heat treatment temperature had a greater effect than heat treatment time, but it should be noted that the OD420 values did not change considerably. The OD420 value is called the browning index too (Marquez *et al.*, 2013). Darkening of color can stem from enzymatic reactions catalyzed by PPO (Ndiaye *et al.*, 2022; Zin, Borda, *et al.*, 2020). The increase in heat treatment temperature can be explained by these browning products.

Figure 4A and 4C show that incubation temperature was also an influential independent variable affecting the GA of RBJ samples. Figure 4B shows that heat treatment time and temperature did not majorly affect the GA of the samples. Data on GA also confirm that incubation temperature is the most influential variable, as in the case of TBC and OD420.

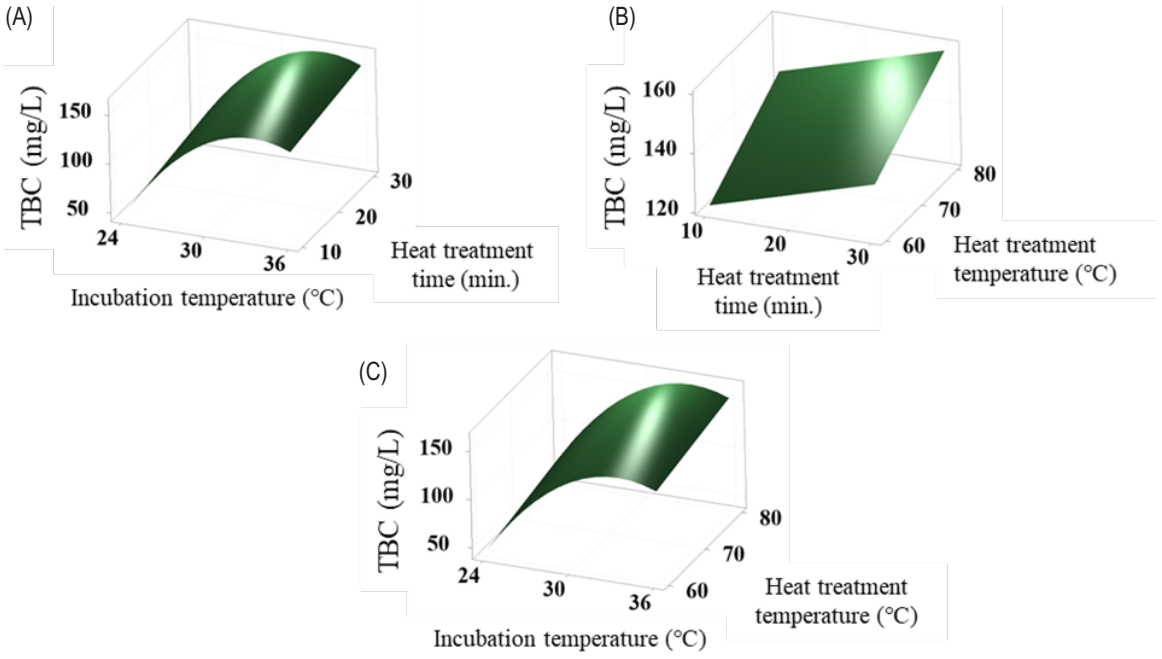

Figure 2. 3D surface response graph illustrating the effect of independent variables on TBC values of RBJ. Abbreviations of the responses: total betalain content (TBC).

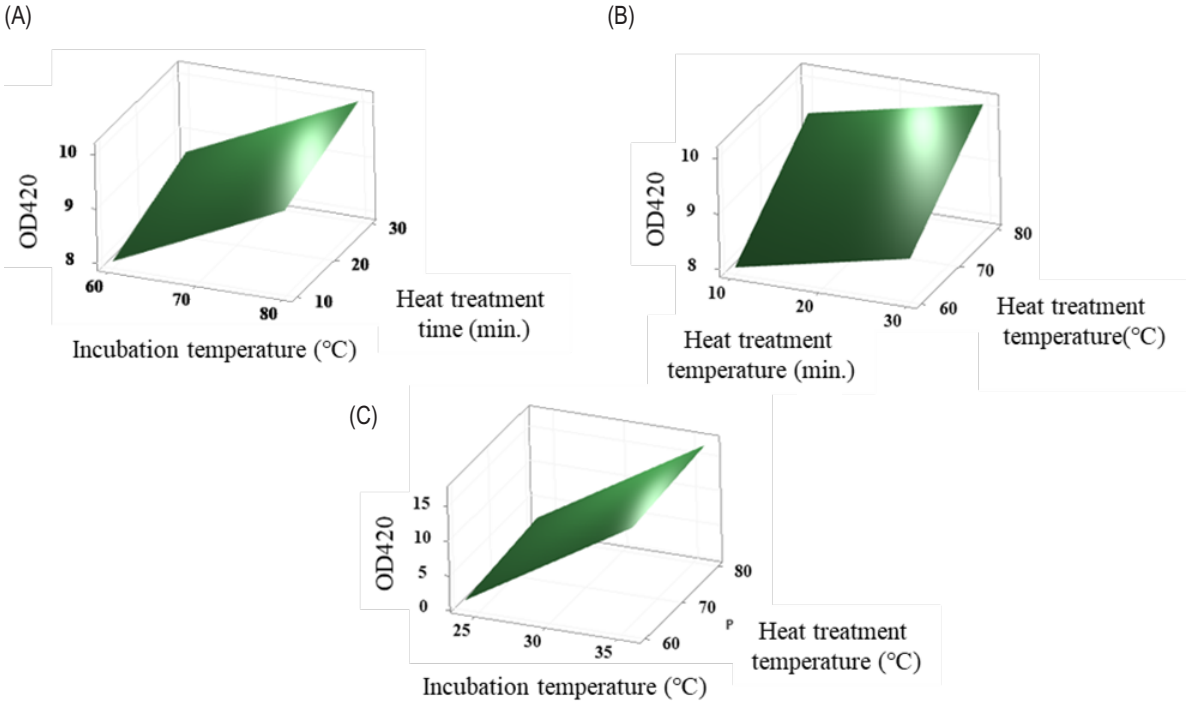

Figure 3. 3D surface response graph illustrating the effect of independent variables on OD420 values of RBJ. Abbreviations of the responses: yellow color (OD420).

Optimization and Validation

The Box–Behnken design in RSM was used to optimize the production of a beverage fermented with probiotic *Lc. paracasei*. The study was targeted to maximize the responses belonging to TBC, GA, and total LAB count. The optimal parameters for the production of RBJ were determined to be a heat treatment of 60°C for 22 min and a fermentation temperature of 31°C. Based on the findings of the optimization analysis, it is anticipated that the production of RBJ under optimal conditions will result in a TBC of

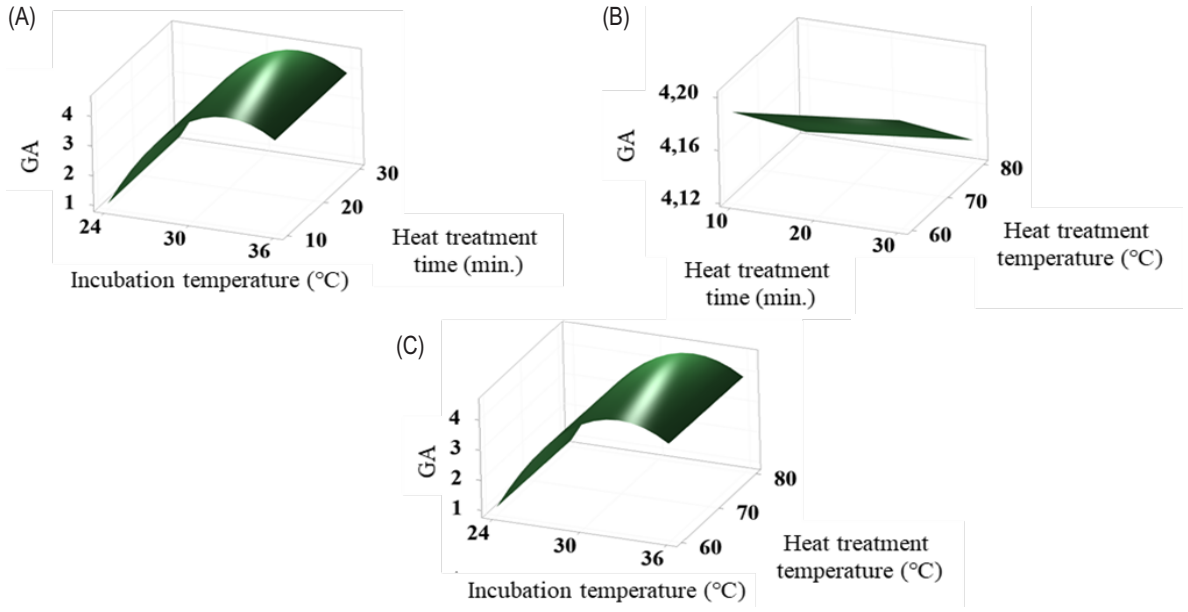

Figure 4. 3D surface response graph illustrating the effect of independent variables on GA values of RBJ. Abbreviations of the responses: general acceptance (GA).

614.769 mg/L, GA score of 4.6, and total LAB count of 8.9 log CFU/mL.

RBJ was produced under optimal conditions for validation, resulting in the following measurements: TBC, GA score, and total LAB count were detected at 512.1 ± 17.5 mg/L, 4.7 ± 0.1, and 8.6 ± 0.1 log CFU/mL, respectively. There was no statistically significant difference between the data obtained through optimization and after optimization ($P > 0.05$).

Conclusions

The study showed the effect of different temperature treatments and fermentation temperatures on RBJ betalains. The optimization study has identified the parameters that minimize the degradation of betalains and maximize the total LAB level, a feature that consumers will highly value. This study makes a valuable contribution to the current state of the literature by successfully developing a nutritional and health-promoting beverage incorporating probiotic microorganisms. Additionally, elucidating the composition of nutrients and major and minor elements present in red beetroot juices produced by various processing methods would contribute to effective marketing strategies for the products.

The study's findings indicated that the incubation temperature significantly impacted the chemical parameters and GA of the samples. The samples incubated at a temperature of 24°C exhibited reduced TPC, TBC, and OD420 compared to the remaining samples. Additionally, the panelists did not prefer these samples. The RBJ samples that completed fermentation at 30°C and 36°C exhibited high levels of TBC and TPC and were also deemed acceptable by the panelists.

Regularly using vegetable juices abundant in phenolic compounds makes a substantial contribution to the daily consumption of bioactive compounds and generates favorable health outcomes.

Additionally, elucidating the composition of nutrients and major and minor elements present in red beetroot juices produced by various processing methods would contribute to effective marketing strategies for the product.

Author Contributions

Conceptualization, H.A.K, and G.D..; methodology, H.A.K and G.D..; software, H.A.K..; validation, H.A.K, G.D., F.S. and.; formal analysis, H.A.K, and G.D.; investigation, H.A.K, and G.D..; resources, H.A.K and F.S; data curation, H.A.K, and F.S. writing—original draft preparation, H.A.K and G.D.; writing, review and editing, H.A.K and F.S.; project administration, H.A.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data used to support this study's findings are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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