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Application of Box Behken Design and Mixture Design to Produce a Probiotic Kefir Fortified with Chia, Oats and Dried Fig: Investigation of Antibacterial Properties with Sterilized Cow, Goat and Camel Milk

Lamia Ayed [1, 2, *], Karima Bekir[3], Houda Ben Abdallah[1, 2], Sami Achour[2] and Tarek Zmantar[1]

[1]*Laboratory of Analysis, Treatment and Valorization of Pollutants of the Environmental and Products, Faculty of Pharmacy, Route Avicenne, 5000 Monastir, University of Monastir, Tunisia*

[2]*Higher Institute of Biotechnology, Monastir (ISBM), 5000 Monastir, Tunisia*

[3]*Unit of Research Analysis and Processes Applied to the Environment UR17ES32 at the Higher Institute of Applied Sciences and Technology, Mahdia 5121, Tunisia*

[*]**Corresponding Author**: Lamia Ayed, Laboratory of Analysis, Treatment and Valorization of Environmental Pollutants and Products, Faculty of Pharmacy Monastir, Route Avicenne, 5000, Monastir-Tunisia, Tel: +21673461000, Fax: +21673461830, E-mail: alym712@yahoo.fr

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Abstract

Kefir with probiotics has a variety of advantages, including the ability to fight against various diseases. This study investigated the effect of prebiotics (Chia, oats and dried fig) on the quality of a beverage fermented by kefir in sterilized milk (cow, goat and camel). Influence of milk and prebiotics type, as well as the probiotic strain was evaluated on the sensory quality of kefir, and antibacterial effects of fermented milk during 24h of storage.Number of total aerobic mesophilic bacteria (TAMB), *Lactobacillus, Lactococcus,* and yeast was determined using Standard Plate Count Agar, MRS (De Man, Rogosa and Sharpe) agar, M17 agar, and Sabouraud dextrose agar with chloramphenicol. The ideal fermentation conditions were as follows: 5% (w/v) of kefir grains in 300 ml each of cow, goat, and camel milk, with a 24- to 48-hour fermentation period by means of rotational Box Behken and Mixture Design. This mixture was tested at 25 °C for 36 hours with 0.777% chia, 26.8% oats, and 72.4% dried fig added as supplements. Prebiotic usage also resulted in a decrease in sensory acceptability (>3.7). More than 108 CFU/mL of *Lactobacillus, Lactococcus,* and yeasts. It has been demonstrated that milk from cows, goats, and camels makes a good substrate for kefir grain fermentation. The experimental factors considerably influenced the analyzed responses, as shown by a second-order polynomial response surface equation and response surface graph. Higher-than-78% determination coefficients (R2) indicated that the created models were well suited to the experimental data.

Keywords: Box Behken Design; Oats; Kefir; Chia; Dried Fig; Antibacterial Properties

Introduction

Turkish word kef, which translates to "a pleasant taste," is where the word kefir comes from. Other names for kefir grains include prophet's millet, Mohomet grains, kefyr, kephir, kefer, kiaphur, knapon, kepi, and kippi [1]. It is a gelatinous granule with an uneven, rough, and convoluted surface that is between 1-6 mm or occasionally up to 15 mm in diameter and resembles a cauliflower floret [2]. Exopolysaccharides and a variety of microbes, primarily bacteria and yeasts, make up the grain [3]. Chemically speaking, kefiran, or exopolysaccharide, is made up of glucose and galactose [4]. Kefir is a fermented acidic beverage with a low alcohol level that is made from kefir grains [5]. Kefir is created by incubating heat-treated milk with kefir grains, a mixture of polysaccharides, proteins, symbiotic lactic acid bacteria (such as *Lactobacillus, Lactococcus, Leuconostoc*, and *Streptococcus*), and yeast (such as *Saccharomyces, Candida, Kluyveromyces, Debaryomyces*, and *Torulaspora*) [6,7]. Prebiotics increase the action of probiotics because they have a better chance of surviving in the gut. To enhance host immunity, they also increase Immunoglobulin A (IgA) levels and regulate cytokine production [8]. Functional beverage fortification with prebiotics, such oats, may encourage the growth of microbes during fermentation in addition to giving humans access to nutrients and bioactive chemicals. Oats are an excellent source of affordable proteins and unsaturated fatty acids. According to the definition of prebiotics, they are "the fermenting component that permits specific adjustments in the activity and/or composition of the gut flora that benefit the host's health and well-being". Kefir can be made from a variety of milk sources, including mare, goat, sheep, soy, coconut, rice, and hazelnut [9]. Regardless of any potential health benefits, a product's sensory acceptability determines whether or not it is included in the daily diet [10]. Although there is not universal agreement on this, high sensory acceptance may be required to predict a product's likelihood of commercialization as well as its life cycle and market success [11]. The pleasurable emotions that food products elicit can boost the satisfaction of eating them, which can also inspire individuals to purchase them and affect their decision to do so. Moreover, contrasting food products with similar characteristics, prices, and packaging may require major consideration of specific emotions. The relationship between the emotions evoked during swallowing and the senses' acceptance or rejection of food products is still the subject of few investigations [11]. Response surface methodology, which combines mathematical and statistical methodologies, is commonly used in experimental models to optimize the formulation system, providing optimum answers and the rheological properties. The Box-Behnken design methodology, one of the response surface methodologies, is often used in the pharmaceutical sector in contrast to central composite design, which necessitates more treatment combinations [12]. According to Gouveia [13], RSM is a useful optimization technique that allows for the simultaneous study of the process, enabling the discovery and quantification of significant interactions between the variables and the prediction of the process' ideal conditions through predictive models.

The aim of the present study was to develop and optimize the production of a fermented kefir drink isolated from traditional kefir grains fortified with chia, oats and dried fig in fresh cow, goat and camel milk, displaying antibacterial activity related to their probiotic potential. The formulation process was optimized using a Box-Behnken and mixture design method. After that, the hedonic, sensory, properties of the products as well as the probiotics' survival were assessed. In this manner, the effectiveness of various milk varieties and three prebiotics were assessed during the kefir production process.

Materials and Methods

Samples

Fresh cow, goat and camel milk were collected from a pool of 3 healthy cows, goat and camel as described by M'hir et al [14]. Before attaching the milking pumps, the teats were washed with water and then dipped in an antiseptic solution (Chlorhexidine Active Mastitis Prevention) with 0.5% chlorhexidine gluconate as the active ingredient. The milk was immediately frozen at 4°C until use. The laboratory for the analysis, treatment, and valorization of environmental pollutants and products (Faculty of Pharmacy of Monastir, Monastir, Tunisia) provided the original grain for the kefir grains utilized in this investigation, which were from a traditional culture and were preserved in UHT milk at 4 °C.

Screening of Process Parameters Using Box Behnken Factorial Experimental Design for Formulation of Fermented Milk Beverage

The Box Behnken factorial design is frequently used in experiments, and it has the advantage of allowing researchers to examine multiple factors as well as how they interact to affect the answer using Minitab 14 [15]. It should be emphasized that only variables that influence response are selectable. According to the Box-Behnken design method, optimization of a number of parameters (including cow milk, goat milk, camel milk, incubation period, temperature, and light intensity) was explored.

The optimal combination that ensures the maximum of Enumeration of total aerobic mesophilic bacteria (TAMB), *Lactobacillus, Lactococcus,* and yeast was then determined by comparing the predicted and experimental values. Six variables were selected at three levels: low level (-1), middle point (0) and high level (+1), as shown in Table 1. These variables were cow milk (mL), goat milk (mL), camel milk (mL), incubation time (hour), temperature (°C), and light intensity (watt). A Box-Behnken experimental design was used to identify the parameter significantly affecting the acceptability of the kefir probiotic (Table 2).

First, fresh cow, goat and camel milk were heated to 20–30 °C and inoculated with kefir grains (20%) followed by fermentation for 24-48 h at 20–30 °C and light intensity 25W-75W. Then, the grains were collected by filtering milk through a sieve, and the kefir samples were stored at 4 °C [14].

The design included 54 sets of tests with various parameter combinations (Table 2). In order to eliminate bias and to lessen the impacts of response variability caused by unimportant factors, the experiment was completed in a random order [16]. The quadratic polynomial model was used to represent the link between the six components and the corresponding responses.

The quadratic polynomial model was chosen to represent the link between the six components and the corresponding responses.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_{11} X_1^2 + \beta_{22} X2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{55} X_5^2 + \beta_{66} X_6^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{16} X_1 X_6 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{26} X_2 X_6 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{36} X_3 X_6 + \beta_{45} X_4 X_5 + \beta_{46} X_4 X_6 + \beta_{56} X_5 X_6 \quad \text{(Equation 1)}$$

Table 1: Variables and experimental Box-Behnken design levels

	Variables		**Experimental range**		
Factors	**Parameters**	**Units**	**Low (-1)**	**Middle (0)**	**High (+1)**
X1	Temperature	°C	20	25	30
X2	Incubation time	hour	24	36	48
X3	Light intensity	watt	25	50	75
X4	Cow milk	mL	100	200	300
X5	Goat milk	mL	100	200	300
X6	Camel milk	mL	100	200	300

Table 2: Box Behnken design modelling for experimental and predicted *Lactobacillus, Lactococcus* and Yeast of fermented kefir

	Parameters						**Experimental response**			**Predicted response**		
Run order	**X1**	**X2**	**X3**	**X4**	**X5**	**X6**	***Lactobacillus*** **(log FU/mL)**	***Lactococcus*** **(log FU/mL)**	**Yeast (log CFU/mL)**	***Lactobacillus*** **(log FU/mL)**	***Lactococcus*** **(log FU/mL)**	**Yeast (log CFU/mL)**
1	20	24	50	100	200	200	6.6	7.59	5.23	6.80396	7.79396	5.35813
2	30	24	50	100	200	200	6.8	7.79	5.43	6.80604	7.90688	5.43604

3	20	48	50	100	200	200	7.2	8.41	5.89	7.22896	8.53063	6.01479
4	30	48	50	100	200	200	7.1	8.41	5.98	7.18104	8.62354	6.13271
5	20	24	50	300	200	200	7.12	8.45	5.99	7.00771	8.28771	5.83354
6	30	24	50	300	200	200	7.02	8.23	5.78	7.02229	805813	5.65896
7	20	48	50	300	200	200	7.22	8.55	5.88	7.18271	8.48438	5.87021
8	30	48	50	300	200	200	7.32	8.49	5.86	7.14729	8.23479	5.73563
9	25	24	25	200	100	200	6.88	7.58	5.22	6.66167	7.39125	5.16542
10	25	48	25	200	100	200	6.99	7.88	5.98	6.83417	7.61792	5.60708
11	25	24	75	200	100	200	7.45	6.33	4.77	7.34	6.54792	4.89708
12	25	48	75	200	100	200	7.56	6.43	4.88	7.5325	6.65958	4.97375
13	25	24	25	200	300	200	6.24	6	4.55	6.135	5.42542	4.16125
14	25	48	25	200	300	200	6.25	6.1	4.65	6.4925	6.22708	4.81792
15	25	24	75	200	300	200	7.33	8.12	5.57	7.35333	8.03708	5.64792
16	25	48	75	200	300	200	7.38	8.19	5.59	7.73083	8.72375	5.93958
17	25	36	25	100	200	100	6.59	7.52	5.61	6.7625	7.82229	5.73813
18	25	36	75	100	200	100	7.29	8.24	6.01	7.08083	7.96521	5.65604
19	25	36	25	300	200	100	6.87	7.75	5.99	6.7225	7.67479	5.87229
20	25	36	75	300	200	100	7.55	8.4	6.23	7.64583	8.30771	6.13021
21	25	36	25	100	200	300	6.97	7.87	5.74	6.60917	7.63354	5.77604
22	25	36	75	100	200	300	7.19	8.25	6.19	7.6025	8.65396	6.37146
23	25	36	25	300	200	300	6.27	7.45	5.09	6.21417	7.39604	5.38021
24	25	36	75	300	200	300	7.72	8.88	6.38	7.8125	8.90646	6.31563
25	20	36	50	100	100	200	7.29	8.78	6.18	7.36729	8.79396	6.23729
26	30	36	50	100	100	200	7.08	8.54	6.09	7.08688	8.25438	6.02271
27	20	36	50	300	100	200	7.19	8.64	6.21	7.38354	8.65521	6.25521
28	30	36	50	300	100	200	6.89	7.21	5.44	7.11563	7.77313	5.78813
29	20	36	50	100	300	200	7.14	8.35	6.11	6.88313	7.83813	5.75813
30	30	36	50	100	300	200	7.28	8.65	6.21	7.11771	8.58354	6.16854
31	20	36	50	300	300	200	7	8.19	6	7.02438	8.42438	6.07104
32	30	36	50	300	300	200	7.38	8.79	6.29	7.27146	8.82729	6.22896
33	25	24	50	200	100	100	6.79	7.09	5.28	6.825	7.12208	5.34792
34	25	48	50	200	100	100	6.87	7.12	5.31	7.2125	7.46875	5.67458
35	25	24	50	200	300	100	6.58	7	5.19	7.07333	7.82125	5.83375
36	25	48	50	200	300	100	7.77	8.88	6.41	7.64583	8.74292	6.37542
37	25	24	50	200	100	300	7.55	8.65	6.4	7.54167	8.44208	6.13958
38	25	48	50	200	100	300	7.88	8.91	6.68	7.51917	8.43375	6.33125
39	25	24	50	200	300	300	6.99	7.27	5.47	6.78	7.26625	5.40042
40	25	48	50	200	300	300	7.11	8.21	6.17	6.9425	7.83292	5.80708

41	20	36	25	200	200	100	7.21	8.45	6.38	7.11333	8.40896	6.32313
42	30	36	25	200	200	100	7.24	8.56	6.39	7.05667	8.29063	6.23979
43	20	36	75	200	200	100	7.87	8.99	6.51	7.57667	8.64438	6.31354
44	30	36	75	200	200	100	7.92	9.1	6.62	7.835	8.83104	6.42521
45	20	36	25	200	200	300	6.55	7.68	5.88	6.9	8.27771	6.13854
46	30	36	25	200	200	300	6.05	7.28	5.71	6.60833	7.95438	5.97021
47	20	36	75	200	200	300	8.12	9.45	6.72	8.03833	9.39063	6.80646
48	30	36	75	200	200	300	8.23	9.66	6.84	8.06167	9.37229	6.83313
49	25	36	50	200	200	200	7.19	8.39	6.29	7.35	8.53167	6.48667
50	25	36	50	200	200	200	7.17	8.37	6.25	7.35	8.53167	6.48667
51	25	36	50	200	200	200	7.28	8.45	6.31	7.35	8.53167	6.48667
52	25	36	50	200	200	200	7.23	8.43	6.3	7,35	8.53167	6.48667
53	25	36	50	200	200	200	7.45	8.68	6.87	7.35	8.53167	6.48667
54	25	36	50	200	200	200	7.78	8.87	6.9	7.35	8.53167	6.48667

Response Surface Methodology (RSM) Using Mixture Design of Experiments

Following the BBD screening of the significant factors, the RSM using the mixture design was carried out to learn more about the significant effects, and the interactions between the prebiotics (Chia (0-100%), Oats (0-100%) and dried fig (0-100%)) on the fermented beverage, and establish the ideal value for each variable that would affect the sensory analysis (Table 3). Following a screening study, response surface methodology (RSM) is used to investigate the area of interest of the factors found in the earlier investigation [16,17]. The formulation of chemicals, fertilizers, insecticides, and other items, as well as food experiments, frequently uses the mixture design. Regression analysis can be used to assess the link between formulation and performance with fewer experiments [18].

Table 3: Mixture design matrix with the experimental analysis

Assay	Chia	Oats	Dried fig	Experimental Overall acceptability	Predicted Overall acceptability
1	1	0	0	3.8	3.50333
2	0	0	1	3.5	3.58764
3	0	1	0	2.92	2.66037
4	0.33	0.33	0.33	3.8	3.50333
5	0.5	0.5	0	2.9	2.92532
6	0.5	0	0.5	3.3	3.67259
7	0	0.5	0.5	3.6	3.63987
8	0.16	0.66	0.16	2.5	3.16593
9	0.66	0.16	0.16	3.68	3.34774
10	0.16	0.16	0.66	4.1	3.72411

Characterization of Kefir Products

Enumeration of total aerobic mesophilic bacteria (TAMB), *Lactobacillus, Lactococcus,* and yeast was determined using Standard

Plate Count Agar, MRS (De Man, Rogosa and Sharpe) agar, M17 agar, and Sabouraud dextrose agar with chloramphenicol, were performed by incubation of kefir sample at 37 °C for 72 h. White and opaque colonies grown on petri dishes were counted after incubation. Results were expressed as log of colony-forming units per mL of fermented beverage (log CFU/mL).

Sensory Evaluation

An escalating hedonic scale from 1 (very disliked) to 5 (extremely loved) was used to ask a group of 50 participants, who were regular consumers of homemade milk kefir. 30 men and 20 women, ages 30-49, to rate the overall acceptability (OA) (extremely liked). Probiotic samples in clear vials totaling 20 mL were displayed. Based on the scores obtained by panellists, each attribute was indicated as mean ± standard deviation. As a solution for optimization, the average score for each sample formulation was calculated [14]. The study complies with all regulations and confirmation that informed consent was obtained.

Antimicrobial Activity

The capability of the strains to inhibit a group of foodborne pathogens was determined using an agar spot test. Overnight test cultures were spotted (2 µL) on the surface of modified MRS agar (without ammonium citrate and sodium acetate) and incubated anaerobically for 24 h at 30°C. Cells were then inactivated with chloroform for 30 min. *Pseudomonas aeruginosa* (ATCC 27853),*Aeromonas hydrophila* (ATCC 7966T), *Escherichia coli* (ATCC 35218), *Listeria monocytogenes* (ATCC 1915), *Salmonella typhimurium* (ATCC 1408), *Candida albicans* (ATCC 90028), *Staphylococcus aureus* (ATCC 25923), *Vibrio parahaemolyticus* (ATCC 17802) and *Vibrio alginolyticus* (ATCC 177449) were used as indicators. A 100-µL volume of an overnight culture of each indicator was mixed with 10 mL of brain heart infusion (Difco) soft agar (0.7%), and poured onto MRS agar plates. Inhibition but no clear-cut halo or a halo 1 mm was scored as positive (+); and an inhibition zone between 2 and 5 mm surrounding the colony was recorded as (++).

Results and Discussion

Box Behnken Factorial Design Modelling for Product a Probiotic Kefir

Modulization of *Lactobacillus, Lactococcus* and Yeast

Statistical optimization of various parameters related to *Lactobacillus, Lactococcus* and Yeast using Box-Behnken factorial design was applied elsewhere [15].

Table 2 displays the results of the Box-Behnken factorial design (experimental and anticipated values). The coefficients of a second order polynomial equation used in the quadratic model that links the operations of the surface response technique to the independent variables are as follows:

$$\mathbf{Y}_{Lactobacillus} = 7.35 - 0.00833\ X_1 + 0.13750\ X_2 + 0.47917\ X_3 + 0.04250\ X_4 - 0.08208\ X_5 + 0.00333\ X_6 + 0.10264\ X_1^2 - 0.16528\ X_2^2 - 0.11819\ X_3^2 - 0.23986\ X_4^2 - 0.05653\ X_5^2 + 0.06431\ X_6^2 - 0.01250\ X_1X_2 + 0.07875\ X_1X_3 + 0.00312\ X_1X_4 + 0.12875\ X_1X_5 - 0.05875\ X_1X_6 + 0.00500\ X_2X_3 - 0.06250\ X_2X_4 + 0.04625\ X_2X_5 - 0.10250\ X_2X_6 + 0.15125\ X_3X_4 + 0.13500\ X_3X_5 + 0.16875\ X_3X_6 + 0.03125\ X_4X_5 - 0.08875\ X_4X_6 - 0.25250\ X_5X_6 \quad (Eq2).$$

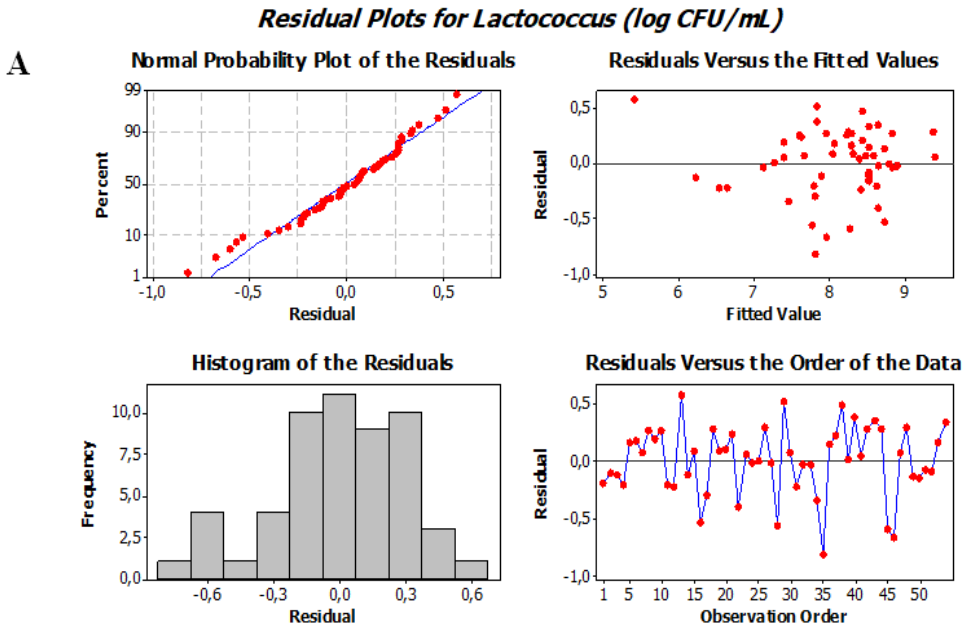
R-Sq = 78,5% ; R-Sq(adj) = 71,2%

$$\mathbf{Y}_{Lactococcus} = 8.53167 - 0.03417\ X_1 + 0.22833\ X_2 + 0.41333\ X_3 + 0.02625\ X_4 + 0.02458\ X_5 + 0.10250\ X_6 + 0.41708\ X_1^2 - 0.52458\ X_2^2 - 0.55750\ X_3^2 - 0.18417\ X_4^2 - 0.37083\ X_5^2 + 0.25500\ X_6^2 - 0.00500\ X_1X_2 + 0.07625\ X_1X_3 - 0.08562\ X_1X_4 + 0.32125\ X_1X_5 - 0.05125\ X_1X_6 - 0.02875\ X_2X_3 - 0.13500\ X_2X_4 + 0.14375\ X_2X_5 - 0.08875\ X_2X_6 + 0.12250\ X_3X_4 + 0.86375\ X_3X_5 + 0.21938\ X_3X_6 + 0.18125\ X_4X_5 - 0.02250\ X_4X_6 - 0.46875\ X_5X_6 \quad (Eq3).$$ R-Sq = 85.9% ; R-Sq(adj) = 67,3%.

$\mathbf{Y}_{\text{Yeast}} = 6.48667 - 0.01417\ X_1 + 0.18333\ X_2 + 0.21333\ X_3 + 0.01958\ X_4 - 0.00958\ X_5 + 0.05583\ X_6 + 0.16153\ X_1^2 - 0.57847\ X_2^2 - 0.48972\ X_3^2 - 0.31472\ X_4^2 - 0.26722\ X_5^2 + 0.22278\ X_6^2 + 0.01000\ X_1X_2 + 0.04875\ X_1X_3 - 0.06312\ X_1X_4 + 0.15625\ X_1X_5 - 0.02125\ X_1X_6 - 0.09125\ X_2X_3 - 0.15500\ X_2X_4 + 0.05375\ X_2X_5 - 0.03375\ X_2X_6 + 0.08500\ X_3X_4 + 0.43875\ X_3X_5 + 0.16938\ X_3X_6 + 0.07375\ X_4X_5 - 0.13250\ X_4X_6 - 0.30625\ X_5X_6$ (Eq4). R-Sq = 84.0% ; R-Sq(adj) = 19,9%

For *Lactobacillus, Lactococcus,* and Yeast, the presented model's R^2 and adjusted R^2 values were (78.5%, 85.9%, and 84.0%) and (71.2%, 67.3%, and 19.9%, respectively. This further highlights the model's high relevance. The importance of the independent variable and its interactions with other components, as well as the accuracy of the model, were assessed in this case using analysis of variance (ANOVA). Tables 4,5 and 6 display the results of the ANOVA, and the p-values they include highlight the significance of the model. The anticipated p-values for the quantity of *Lactobacillus, Lactococcus,* and Yeast, respectively, must be 0.001, 0.0001, and 0.001, 0.0001 for a model to be deemed significant. On the other hand, the p-value for lack of fit was discovered to be 0.46 for Lactobacillus, 0.034 for Lactococcus, and 0.494 for yeast, suggesting a negligible lack of fit model, confirming that the model demonstrates strong fitting to the relevant response. When the p-value is less than 0.01, a model is deemed significant; when it is more than 0.1, it is deemed insignificant [18]. The results of the ANOVA analysis emphasize the importance of first order, second order, and interaction components even more.

Plotting studentized residuals against the expected probability of the experiment led to the creation of Figure 1. The acceptable normal residual plots for *Lactococcus, Lactobacillus*, and yeast are shown in Figures 1A, 1B, and 1C. The histograms demonstrate that the normalized residual typically follows a normal distribution with a mean of 0 and a standard deviation of 1. The mistakes were regularly distributed and minor; the studentized residuals vs run plot shows points randomly distributed between 0.5 and + 0.5. The real (experimental) and predicted (Fitted) values are near to one another in all graphs, which is better than gravimetric analysis. As a result, it had an odd structure and no evident pattern. This indicates that the model is acceptable and that there is no cause to believe that any of the runs violated the constant variance assumptions.



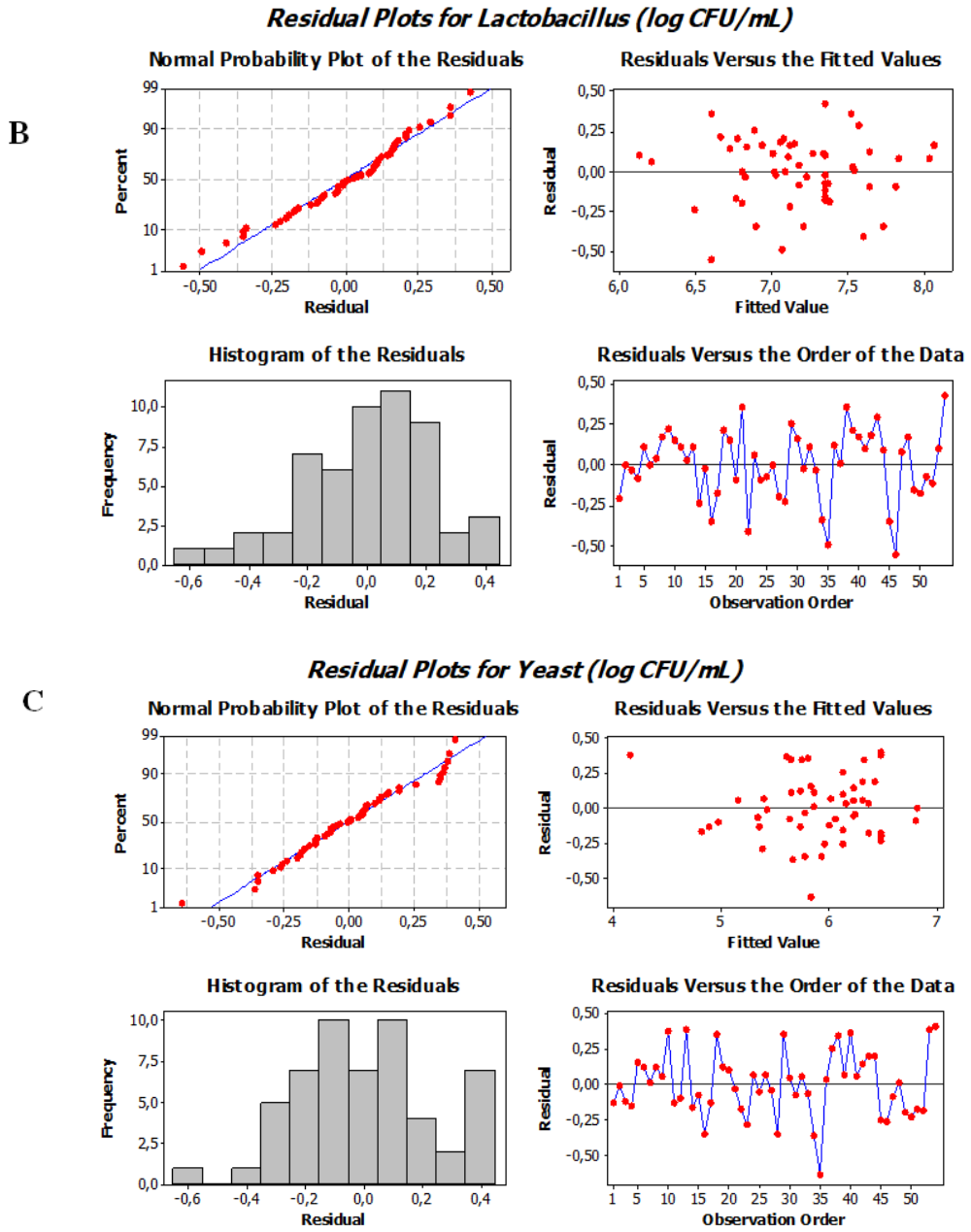


Figure 1: Residual plots for Lactococcus (A), Lactobacillus (B), and Yeast (C) of beverage fermented by kefir of Box behnken design

Table 4: Estimated regression coefficients for *Lactobacillus, Lactococcus* and Yeast (CFU/mL)

Term	P *Lactobacillus*	P *Lactococcus*	P Yeast
Constant	0	0	0
X1	0.895	0.7	0.832
X2	0.037	0.015	0.01
X3	0	0	0.003
X4	0.503	0.767	0.77
X5	0.201	0.781	0.886
X6	0.958	0.253	0.407
X1*X1	0.293	0.004	0.123

X2*X2	0.096	0.001	0
X3*X3	0.227	0	0
X4*X4	0.019	0.181	0.005
X5*X5	0.559	0.01	0.014
X6*X6	0.507	0.068	0.037
X1*X2	0.909	0.974	0.931
X1*X3	0.474	0.62	0.675
X1*X4	0.968	0.433	0.444
X1*X5	0.246	0.044	0.185
X1*X6	0.592	0.739	0.855
X2*X3	0.964	0.851	0.434
X2*X4	0.569	0.382	0.189
X2*X5	0.551	0.192	0.514
X2*X6	0.353	0.564	0.771
X3*X4	0.175	0.427	0.466
X3*X5	0.224	0	0.001
X3*X6	0.037	0.051	0.047
X4*X5	0.775	0,244	0.526
X4*X6	0.42	0,883	0.259
X5*X6	0.028	0.005	0.013

Table 5: Analysis of Variance for *Lactobacillus*

Source	Degrees of freedom	Sum of adjusted squares	Adjusted mean of square	F-ratio	P-value
Regression	27	8.9437	0.33125	3.52	0.001
Linear	6	6.1712	1.02853	10.94	<0.0001
Square	6	1.0463	0.17438	1.85	0.127
Interaction	15	1.7263	0.11509	1.22	0.315
Residual Error	26	2.4441	0.094		
Lack-of-Fit	21	2.1719	0.10343	1.9	0.46
Pure Error	5	0.2722	0.05444		
Total	53	11.3878			

Table 6: Analysis of Variance for *Lactococcus*

Source	Degrees of freedom	Sum of adjusted squares	Adjusted mean of square	F-ratio	P-value
Regression	27	29.1709	1.0804	5.85	<0.0001
Linear	6	5.6627	0.94379	5.11	0.001
Square	6	13.0683	2.17804	11.79	<0.0001
Interaction	15	10.4399	0.696	3.77	0.001
Residual Error	26	4.8012	0.18466		
Lack-of-Fit	21	4.6015	0.21912	5.49	0.034
Pure Error	5	0.1997	0.03994		
Total	53	33.9721			

Interaction Effects of Operational Factors on the Probiotic Kefir Fortified

A contour map (Figure 2-4) was created to verify the expected levels of *Lactobacillus, Lactococcus,* and yeast (7.833, 9.786 and 6.513, respectively). US law stipulates that beverages made with kefir grains must have cell survival rates of > 4.040 log CFU/ml of total lactic acid bacteria and > 2.737 log CFU/ml of yeast [19]. The mixed design's ternary contour map illustrates how changes in the variables affect the answers. To explore the interaction effects of the researched variables on the responses, pair-wise combinations of two-dimensional contour plots of expected answers were also added to the data as an extra visual interpretation.

The higher number of *Lactobacillus, Lactococcoccus* and Yeast yield were obtained when Incubation time and Light proportions were (0.038; 0.999), (0.102; 0.999) and (-0.029;0.975) respectively.

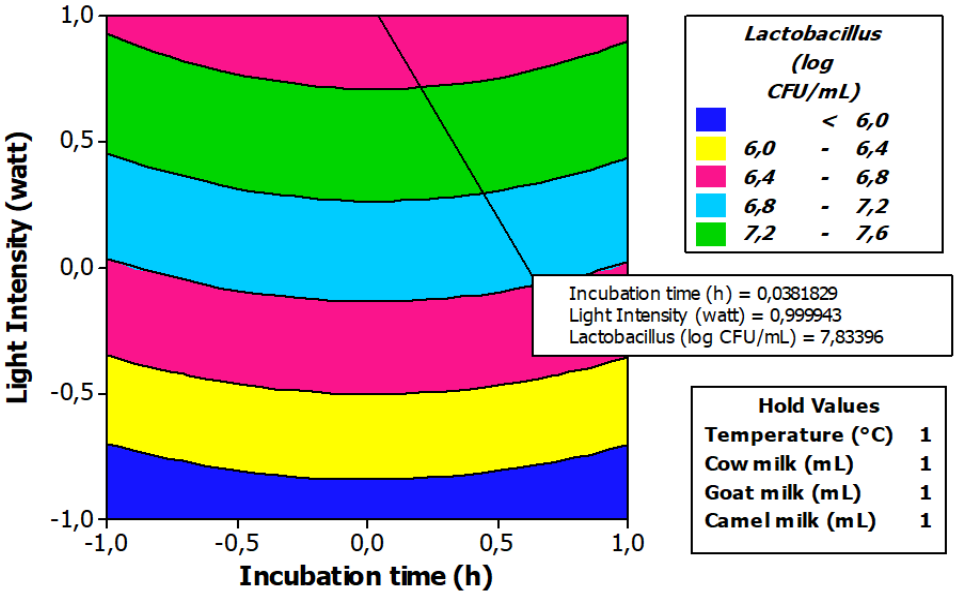


Figure 2: Contour plot showing interaction between Incubation time and Light intensity on numeration of *Lactobacillus*

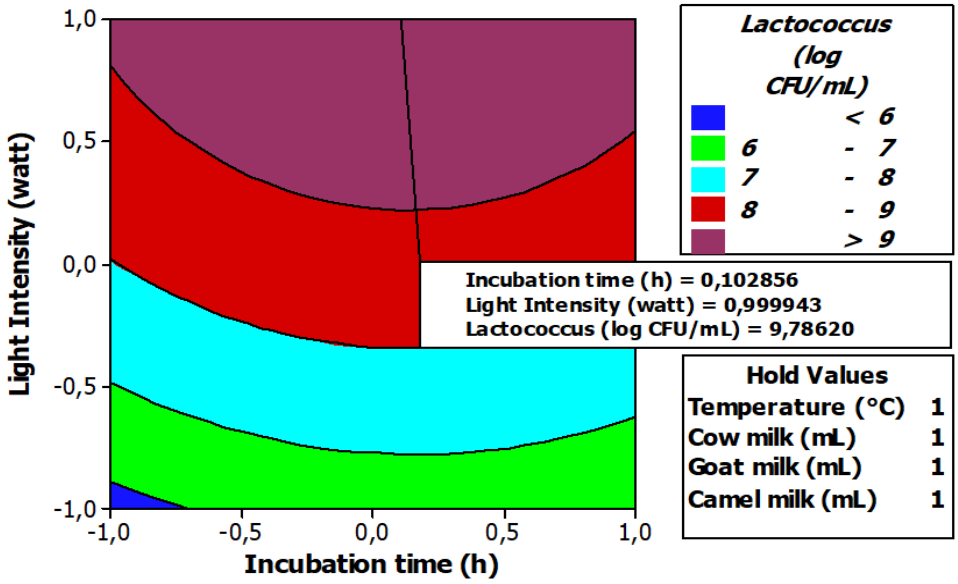


Figure 3: Contour plot showing interaction between Incubation time and Light intensity on numeration of *Lactococcus*

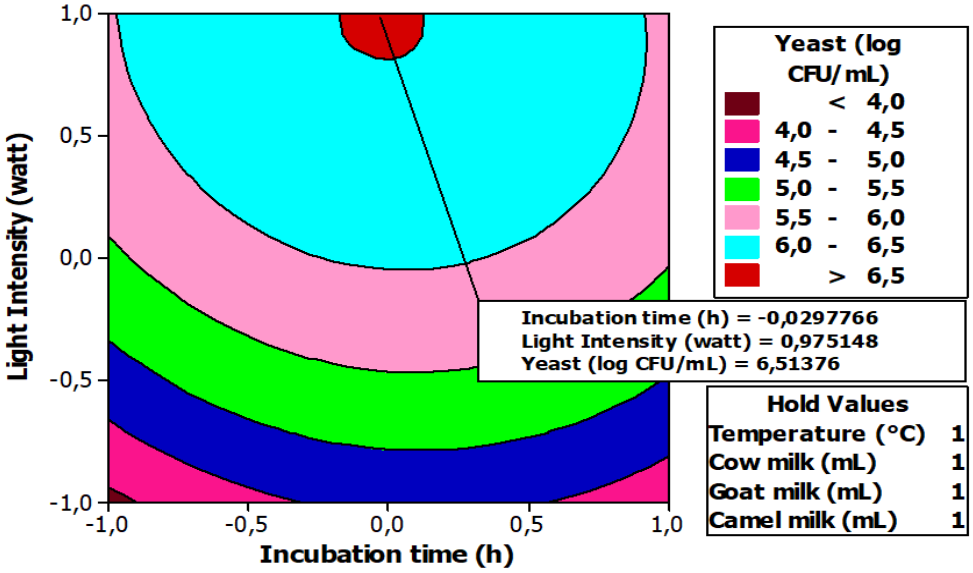


Figure 4: Contour plot showing interaction between Incubation time and Light intensity on numeration of Yeast

Response Surface Methodology Design Modelling for Product Fortified Probiotic Kefir and Sensory Attributes

The experimental and anticipated values of the Chia, Oats, and dried Fig content in the Overall Acceptability (OA) of fortified kefir utilizing mixture starter are shown in Table 3. The regression models for OA were created by fitting linear regression. The orthogonality of the design allowed for the independent estimation of each effect in Eq 5. Response Surface Methodology (RSM) is typically used in screening studies to investigate the components that were found in the study's focus region [20]. Regression analysis can be used by the mixture design to estimate the link between formulation and performance with fewer experiments (Zhang et al., 2015).

YOverall acceptability = 3.1731 Chia + 2.6603 Oats + 3.5876 Dried fig + 0.0343 Chia*Oats + 1.1688 Chia* Dried fig + 2.0634 Oats* Dried fig; (Eq5) R2= 52.90%.

The study's R2 score was 0.529, meaning that 52.90% of the total variations were assigned to independent variables and that the model for Overall Acceptability was unable to account for 47.10% of the total variations. Ayed et al. [20] claim that the developed model, where a high value of R2 was attained, is capable of providing a good estimation for the response within the process conditions range.

In order to secure customers' preferences and raise the products' perceived worth, artisanal producers should offer more enticing and suitable packaging in order to gain a larger market share over time. Future market trends can be accurately predicted by conducting additional research on consumer attitudes, tastes, and perceptions of traditional fermented foods and the employment of microbial cultures in our food systems that target the millennial generation. Food producers may find this knowledge useful in creating novel goods, maintaining culinary customs, and safeguarding food diversity. A mixed contour plot (Figure 5A) and surface plot (Figure 5B) were created to help verify the experimental results that were obtained (OA: 4.1). Chia, Oats, and Dried Fig proportions of 0.77%, 26.80%, and 72.41% produced the highest OA (3.74) yields. Figure 5B shows the three-dimensional graph mixing surface plots. The three factors' separate and combined effects, as well as their subsequent impact on the response, were also described in the mixture surface plot [21].

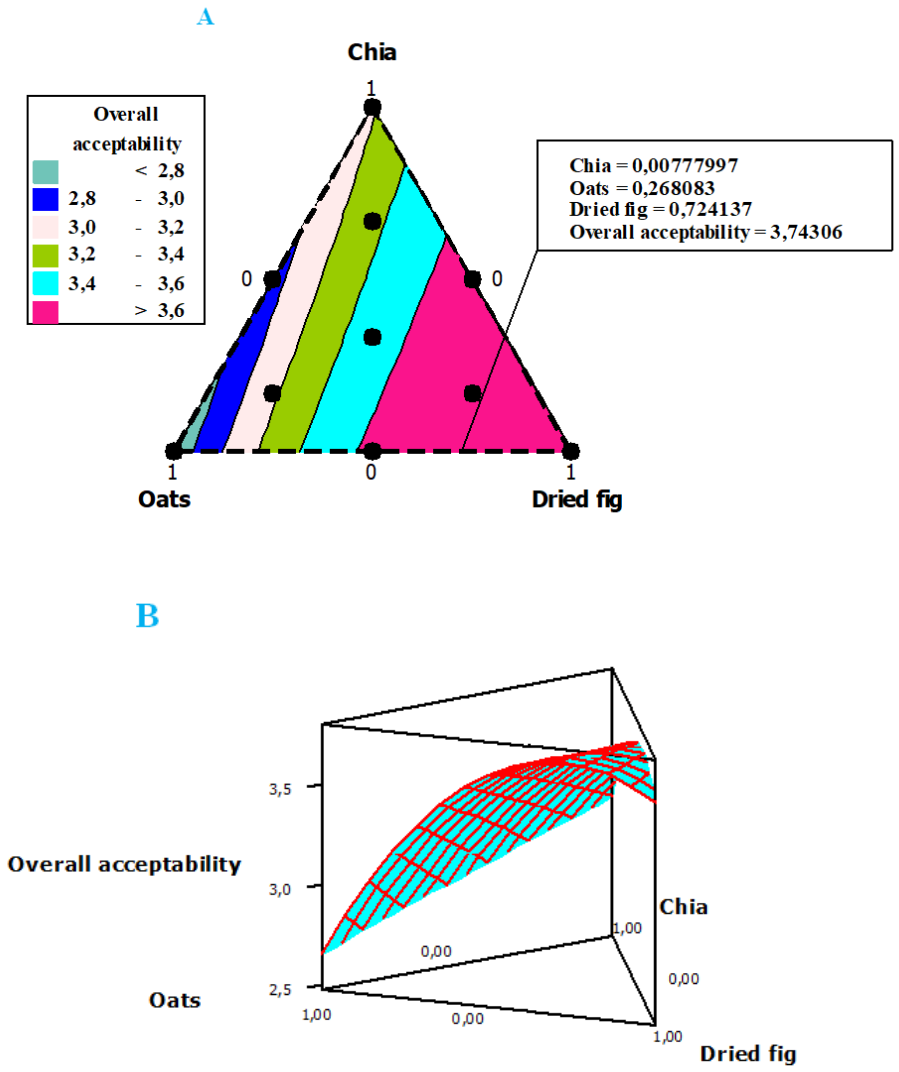


Figure 5: Response Surface Methodology: Contour and surface plots for overall acceptability of fortified fermented probiotic by Chia, Oats and Dried fig.

Antimicrobial Properties

Pathogens were inhibited by most strains in the agar spot test (Table 7). The antibacterial The majority of strains in the agar spot test suppressed pathogens (Table 8). The addition of fermented fresh cow, goat, and camel milk did not result in the establishment of an inhibitory zone for Pseudomonas aeruginosa, Staphylococcus aureus, or Candida albicans in the antibacterial activity test. The generation of organic acids from fermentation may be the cause of the antibacterial activity [22]. Moraes et al. [23] speculate that the antibacterial action of kefir grains may be attributed to unidentified bioactive substances, such as antimicrobial peptides (bacteriocins) or polysaccharides (exopolysaccharides like kefirana), in addition to organic acids. Although complete kefir supernatants or many isolated strains from kefir grains have been reported to have antimicrobial activity, antibiotic or germicidal effects from bioactive substances obtained from specific strains have not yet been proven. So, exploring novel sources of naturally occurring chemicals with antibacterial capabilities is essential given the rise of germs that are resistant to antibiotics. Dana et al. [24] showed that the EPS produced by *Lactobacillus kefiranofaciens* DN1 might be used in the food business to assure food safety or developed into an alternative treatment for foodborne illnesses. To fully understand the mechanism of this EPS's antibacterial effect against pathogenic bacteria, more research is necessary. In particular, transmission electron microscopy could be used to look at how the structural changes in the bacterial cell wall affect gene expression in microbes that have been exposed to EPS.

Table 7: Analysis of Variance for Yeast

Source	Degrees of freedom	Sum of adjusted squares	Adjusted mean of square	F-ratio	P-value
Regression	27	14.3493	0.53146	5.04	<0.0001
Linear	6	1.99	0.33166	3.15	0.019
Square	6	8.7716	1.46194	13.87	<0.0001
Interaction	15	3.5877	0.23918	2.27	0.032
Residual Error	26	2.7402	0.10539		
Lack-of-Fit	21	2.2616	0.1077	1.13	0.494
Pure Error	5	0.4785	0.09571		
Total	53	17.0895			

Table 8: Antibacterial activity of the drink optimized

BeverageBacteria	kefir +Camel	kefir +Goat	kefir +Cow	Optimized fortified fermented probiotic
Pseudomonas aeruginosa	0	0	0	0
Aeromonas hydrophila	11	12	10	14
Escherichia coli	12	0	0	21
Listeria monocytogenes	9	0	0	11
Salmonella typhimurium	12	10	0	16
Candida albicans	0	0	0	0
Staphylococcus aureus	0	0	0	0
Vibrio parahaemolyticus	13	8	0	16
Vibrio alginolyticus	10	9	0	19

Conclusion

In this study, a Box-Behnken and mixture design (5% (w/v) of kefir grains in cow, goat, and camel milk (300ml), with 0.777% chia, 26.8% oats, and 72.4% dried fig, light intensity 25-75watt, and fermentation period for 24-48 h) allowed for the standardization of the fermentation process. Cell survival for *Lactobacillus, Lactococcus,* and yeasts at this time was 10^8 UFC/mL. The inclusion of dried fig, chia, and oats increased the fermented beverage's sensory acceptability while preserving the survival of desired microorganisms. Additionally, this product showed a decrease in the cell density of *Aeromonas hydrophila* (ATCC 7966T), *Escherichia coli* (ATCC 35218), *Listeria monocytogenes* (ATCC 1915), *Salmonella typhimurium* (ATCC 1408), *Vibrio parahaemolyticus* (ATCC 17802) and *Vibrio alginolyticus* (ATCC 177449).

Declaration of Competing Interest

None

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