Development of Browntop Millet Based Probiotic Fermented Beverage Using Response Surface Methodology

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Abstract — This study explores the development of a Browntop Millet (BTM)-based probiotic fermented drink, leveraging BTM's high nutritional value and resilience. To enhance nutritional value and digestibility, the BTM flour was fermented using Lactobacillus helveticus and a commercial lactic starter culture. The optimization process involved varying levels of BTM flour (8-12%), skim milk powder (0-4%), and fermentation time (6-12 hours). The final optimized formulation included 9% BTM flour, 3% skim milk powder, and 9 hours of fermentation. The resulting beverage had a pH of 4.4, 0.79% lactic acid, 10.5° Bx total soluble solids, and a viscosity of 7.80 mPa.s, with a probiotic count of 8.25 log CFU/mL, confirming its potential as a functional food with sensory appeal and health benefits.

Keywords: Browntop Millet, Probiotic, Beverages, Fermentation, SMP, Lactic acid Bacteria, Lactobacillus helveticus MTCC 5463

I. Introduction

Functional foods are foods that offer health benefits beyond basic nutrition, potentially reducing the risk of diseases and improving overall well-being. These foods contain bioactive compounds that positively impact health, such as enhancing heart health, boosting the immune system, and lowering the risk of chronic diseases [1]. The global market for functional foods has seen significant growth due to increasing consumer awareness of health and wellness, rising prevalence of chronic diseases, and advancements in food technology. In 2021, the market was valued at approximately $177 billion and is projected to reach around $268 billion by 2027, with a compound annual growth rate (CAGR) of 7.9% from 2022 to 2027 [2].

Millets, a group of small-seeded grasses grown as cereal crops, are particularly valued for their nutritional richness and resilience in arid and semi-arid regions of Asia and Africa. Rich in dietary fiber, protein, B-vitamins, and essential minerals like iron, magnesium, and phosphorus, millets also have the advantage of being naturally gluten-free, making them suitable for individuals with celiac disease or gluten intolerance [3]. Among millets, browntop millet (BTM) stands out for its high energy content, significant amounts of protein, and its wealth of essential nutrients and phytochemicals, including calcium, iron, and flavonoids. BTM thrives in challenging environments, providing nutritional and economic security, especially in marginal farming areas [4]. The fermentation of millets, including BTM, further enhances their nutritional value by breaking down anti-nutritional factors like phytic acid, improving digestibility, and supporting gut health through the presence of beneficial bacteria. This process, common in traditional cuisines across Asia and Africa, not only increases the bioavailability of vitamins and minerals but also acts as a natural preservative, extending the shelf life of millet-based products while enhancing their flavor and texture. However, the fermentation process can be labor-intensive and requires careful monitoring to ensure consistent quality and safety [5]. Lactic acid bacteria (LAB), commonly used in fermentation, are known for their probiotic benefits, including balancing intestinal microbiota, enhancing gut barrier function, and supporting the immune system. Well-studied LAB strains like *Lactobacillus acidophilus* and *Bifidobacterium bifidum* play a crucial role in maintaining gut health and supporting overall well-being, making them important components in the development of functional foods [6, 7].

II. Material and Methods

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III. Materials

The materials used in the development of the Browntop Millet (BTM) based probiotic fermented beverage were carefully sourced from various suppliers. BTM grains were procured from Me2 Millet Pvt. Ltd. in Anand, Gujarat, while skimmed milk powder (SMP) and sugar were obtained from the local market in Anand, Gujarat. Additionally, xanthan gum, a food-grade stabilizer, was sourced from Suvidhinath Laboratories in Vadodara, Gujarat.

IV. Methods

The methods for development of BTM based probiotic fermented beverage were discussed in following sections.

A. Production of BTM flour

1) Grinding

Grinding of raw BTM grains was done under hygienic conditions using a hammer mill available at Food Processing Technology Laboratory, Department of FPT, College of FPT & BE, AAU, Anand, Gujarat. This process thoroughly pulverizes the grains, producing a fine, uniform flour suitable for further processing and analysis.

2) Sieving

After milling, the BTM flour was passed through a 100-mesh sieve to reduce the particle size. This sieving process ensures a finer and more uniform brown millet flour. The high quality, uniform flour improves the texture, mixing properties and overall quality of the flour for use in making fermented beverages.

- Influence of temperature on the sedimentation index of BTM beverages

  The stable drink was found using the sedimentation index in preliminary tests to help choose the temperature. This process was used to calculate the beverage's sedimentation index. Mixture of 100 mL RO and 10 g BTM flour was made. Water that has been left at a temperature above 55 °C for ten minutes after being added to a water bath at a different temperature. When the product was heat treated, it was kept for 48 h at refrigerator temperature. Sedimentation index: After being moved to clear PET bottles, samples (100 mL) were kept at room temperature for 48 h in order to evaluate stability. It was measured how high the upper clear liquid phase (H_t) and the total height of the liquid head (H_0). Additionally, the calculation of IS (percent) was $100 \times H_t/H_0$ [8].
- Development of BTM based probiotic fermented beverage

  Dry ingredients, e.g. BRM flour, SMP, sugar, and xanthan gum were weighed according to their addition rate (either constant or as recommended by RSM). The starter culture was added according to the supplier's instructions.

  First of all, the R.O. water was heated to 55°C and then the dry ingredients were added with constant stirring until the temperature reached 80°C. It was kept at this temperature for 10 min and then cooled to 40°C. The drink was inoculated with starter culture as previously described and then mixed thoroughly to evenly distribute the starter culture. After mixing, the drink was packaged in PET bottles and incubated at 37°C for different incubation times. After incubation, the drink was transferred to the refrigerator and used for sensory, physicochemical and microbiological evaluation.
- Statistical design to optimize BTM flour content, SMP and fermentation time for development of BTM based probiotic fermented drinks

Run	Factor 1	Factor 2	Factor 3
	A: Rate of flour (%)	B: Rate of SMP (%)	C: Fermentation time (h)
1	9.0	1.0	7.0
2	10.0	0.0	8.0
3	11.0	1.0	7.0
4	10.0	2.0	8.0
5	10.0	2.0	8.0
6	10.0	2.0	8.0
7	12.0	2.0	8.0
8	9.0	1.0	9.0
9	9.0	3.0	7.0
10	10.0	2.0	10.0
11	10.0	2.0	8.0
12	10.0	2.0	6.0
13	8.0	2.0	8.0
14	9.0	3.0	9.0
15	10.0	4.0	8.0
16	11.0	3.0	9.0
17	11.0	3.0	7.0
18	11.0	1.0	9.0
19	10.0	2.0	8.0
20	10.0	2.0	8.0

Table 1: Experimental run for BTM based probiotic fermented beverage proposed by CCD of RSM (Run No. 01-20)

V. Result and Discussion

A. Optimization of Process Temperature for Beverage

Treatments	Temperature (℃)	Consistency	Average IS (%)
T1	70℃	Acceptable	26.09 ± 2.17
T2	75℃	Acceptable	27.76 ± 1.00
T3	80℃	Acceptable	38.39 ± 1.76
T4	85℃	Not Acceptable	45.06 ± 2.53
T5	90℃	Not Acceptable	59.29 ± 2.64
T6	95℃	Not Acceptable	± 3.19

Table 2: Effect of temperature on sedimentation index of BTM based beverage

Table 2 shows that treatment T6 gives the highest IS (68.16 ± 3.19%) while T1 gives the lowest IS (26.09 ± 2.17%). T4, T5 and T6 treatments resulted in higher IS values, but due to gel formation the consistency became unsuitable for a drink. Therefore, among all treatments, T3, i.e. 80 °C temperature for 10 min, selected as a further process. T3 treatments have an IS (38.39 ± 1.76%) that still shows water phase separation. To overcome water phase separation, different amounts of xanthan gum stabilizer were evaluated. The results of xanthan gum content in the sedimentation index of the beverage are shown in Table 3. The aim of this approach is to increase the viscosity and stability of the drink.

B. Optimization of Xanthan Gum for Beverage

Table 3 shows that treatment T1 with 0.2% xanthan gum at 80°C achieves an IS of 90%, while treatments T2 to T5 with xanthan gum levels ranging from 0.25% to 0.4% consistently achieve an IS of 100 % to reach. Since all treatments after T1 achieve the desired IS of 100%, treatment T2 with 0.25% xanthan gum is selected as the standard for further investigation. This choice balances achieving optimal IS with minimizing xanthan gum addition, ensuring desired consistency and cost effectiveness for the functional beverage formulation.

Treat-ments	Temperature (℃)	Holding time (min)	Rate of xanthan gum	Average IS (%)
T1	80	10	0.20%	90
T2			0.25%	100
T3			0.30%	100
T4			0.35%	100
T5			0.40%	100

Table 3: Effect of xanthan gum on sedimentation index of BTM based beverage

Based on the preliminary trials and review of literature the rate of sugar addition was decided at the constant rate @ 5 % to the beverage. The commercial starter culture powder was added as per the instruction of the culture supplier while the *Lactobacillus helveticus* was inoculated after activation in sterilized skim milk @ 1 % to the beverage.

C. Influence of independent variables on the developmental properties of BTM-based probiotic fermented beverages

The responses obtained after conducting 20 runs, as tabulated in Table 4 were analyzed individually using the quadratic regression model in the design expert software. The ANOVA of the models were used to check the significance of the model and effect of individual factors on the responses. The fitted quadratic regression model for individual responses and effects on them are discussed as under.

1) Changes in overall acceptability score

Overall acceptability is an indicator of the sensory quality of the product as a whole and includes taste, consistency, color and appearance. The average overall acceptance scores for each product are summarized in Table 4. The developed probiotic beverage samples received overall acceptability scores ranging from 7 to 8 on the 9-point hedonic scale, as shown in Table 4. The highest overall acceptance value (8) was observed for run 14, which used 9% BTM flour, 3% SMP and a fermentation time of 9 hours. The lowest overall acceptance value (7) was observed for run 2, which used 10% BTM flour, 0% SMP and a fermentation time of 8 hours.

As shown in Table 5, the quadratic model for overall acceptance had an F-value of 21.84 and a *P*-value of <0.0001, indicating that the model was highly significant. The lack of fit was not significant compared to the pure error, indicating that the model was a good fit. The multiple regression equation created to predict overall adoption, which is influenced by various factors, is as follows:

$$\text{Overall acceptability} = +79 - 0.1063*A + 0.2187*B + 0.1187*C - 0.0125*AB + 0.0125*AC + 0.1125*BC - 0.0182*A^2 - 0.0432*B^2 - 0.0557C^2$$

Where, A, B and C are coded variables for rate of BTM flour, rate of SMP and fermentation time (h).

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A: Rate of BTM flour (%)	B: Rate of SMP (%)	C: Fermentation time (h)	Overall acceptability (9- point hedonic scale)	Probiotic viable count (log CFU/mL)
1	9.00	1.00	7.00	7.30	6.95
2	10.00	0.00	8.00	7.00	6.80
3	11.00	1.00	7.00	7.10	7.10
4	10.00	2.00	8.00	7.70	7.66
5	10.00	2.00	8.00	7.70	7.64
6	10.00	2.00	8.00	7.60	7.62
7	12.00	2.00	8.00	7.30	7.94
8	9.00	1.00	9.00	7.30	7.65
9	9.00	3.00	7.00	7.50	7.73
10	10.00	2.00	10.00	7.60	8.40
11	10.00	2.00	8.00	7.60	7.72
12	10.00	2.00	6.00	7.20	7.17
13	8.00	2.00	8.00	7.80	7.50
14	9.00	3.00	9.00	8.00	8.25
15	10.00	4.00	8.00	7.90	7.85
16	11.00	3.00	9.00	7.80	8.19
17	11.00	3.00	7.00	7.30	7.23
18	11.00	1.00	9.00	7.20	7.95
19	10.00	2.00	8.00	7.50	7.65
20	10.00	2.00	8.00	7.50	7.40

Table 4: Experimental design matrix and responses characteristics of BTM based probiotic fermented beverage (Run No. 01-20)

Where, A = Rate of BTM Flour (%), B = Rate of SMP (%), C = Fermentation Time (h), OAA = Overall acceptability, PC= Probiotic viable count (log CFU/mL)

As shown in Table 6, the adjusted R^2 and predicted R^2 values for overall acceptance were 0.90 and 0.79, respectively. The coefficient of determination (R^2) was 0.95, indicating that the model is significant with a coefficient of variation of 1.12%. The adequate precision value (APV) for overall acceptability was 17.71, well above the desired minimum value of 4.00, indicating that the model is reliable for predicting responses within the design space.

Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Model	1.38	9	0.1533	21.84	<0.0001	Significant
A-Rate of flour	0.1806	1	0.1806	25.74	0.0005	
B-Rate of SMP	0.7656	1	0.7656	109.11	<0.0001	
C-Fermentation time	0.2256	1	0.2256	32.15	0.0002	

AB	0.0012	1	0.0012	0.1781	0.6819	
AC	0.0013	1	0.0013	0.1781	0.6819	
BC	0.1013	1	0.1013	14.43	0.0035	
A^2	0.0083	1	0.0083	1.18	0.3020	
B^2	0.0469	1	0.0469	6.68	0.0272	
C^2	0.0780	1	0.0780	11.11	0.0076	
Residual	0.0702	10	0.0070			
Lack of Fit	0.0302	5	0.0060	0.7543	0.6177	Not significant

Table 5: ANOVA of fitted quadratic regression model for overall acceptance of BTM based probiotic fermented beverage

Std. Dev	0.084	R^2	0.9516
Mean	7.49	Adjusted R^2	0.9080
C.V. %	1.12	Predicted R^2	0.7938
		Adeq Precision	17.72

Table 6: Fit statistics data of overall acceptability of BTM based probiotic fermented beverage

2) Effect on probiotic count of developed BTM based probiotic fermented beverage

The microbial quality of the developed probiotic beverage samples, as measured by probiotic count, ranged from 6.95 log CFU/mL to 8.4 log CFU/mL as listed in Table 4. The lowest probiotic count (6.95 log CFU/mL) was observed for run 1, which used 9% BTM flour, 1% SMP and a fermentation time of 7 h. Conversely, the highest probiotic count (8.4 log CFU/mL) was recorded for run 10, which used 10% BTM flour, 2% SMP and a fermentation time of 10 h.

As shown in Table 7, the quadratic model for probiotic count had an F value of 22.11 and a *P* value of 0.0001, indicating that the model was highly significant. The lack of fit was not significant compared to the pure error, indicating that the model was a good fit. The multiple regression equation generated to predict the number of probiotics influenced by various factors is as follows:

$$\text{Probiotic count} = +7.62 + 0.0481*A + 0.2406*B + 0.3431*C - 0.1263*AB + 0.0737*AC - 0.0088*BC + 0.0281*A^2 - 0.0707*B^2 + 0.0443*C^2$$

Where, A, B and C are coded variables for rate of BTM-flour, rate of SMP and fermentation time (h).

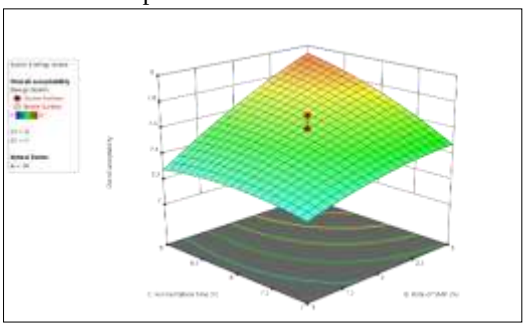
As shown in Table 8, the values of adjusted R^2 and predicted R^2 for the probiotic count were 0.90 and 0.73, respectively, while the coefficient of determination (R^2) was 0.95, indicating that the model with a coefficient of variation of 1, 67% is significant. The adequate precision value (APV) for the probiotic count was 18.04, which was well above the minimum desirable value for APV (4.00), indicating that the model can be used to predict response within the design space.

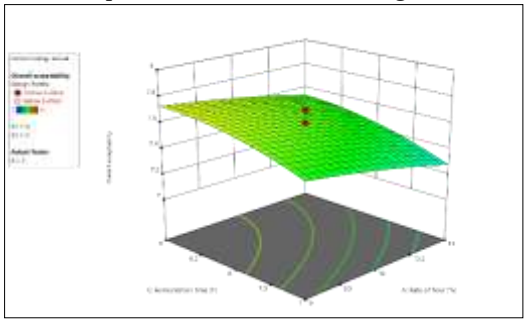
Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.11	9	0.3451	22.11	<0.0001	Significant
A-Rate of flour	0.0529	1	0.0529	3.39	0.0955	
B-Rate of SMP	0.8556	1	0.8556	54.81	<0.0001	
C-Fermentation time	1.78	1	1.78	114.17	<0.0001	
AB	0.0925	1	0.0925	5.92	0.0352	
AC	0.0684	1	0.0684	4.38	0.0627	
BC	0.0061	1	0.0061	0.3876	0.5475	
A^2	0.0175	1	0.0175	1.12	0.3149	
B^2	0.1317	1	0.1317	8.44	0.0157	
C^2	0.0457	1	0.0457	2.92	0.1180	
Residual	0.1561	10	0.0156			
Lack of Fit	0.0950	5	0.0190	1.55	0.3205	Not significant

Table 7: ANOVA of fitted quadratic regression model for probiotic count

Std. Dev	0.1275	R^2	0.9526
Mean	7.62	Adjusted R^2	0.9099
C.V. %	1.67	Predicted R^2	0.7314
		Adeq Precision	18.0485

Table 8: Fit statistics data of probiotic count of BTM based probiotic fermented beverage





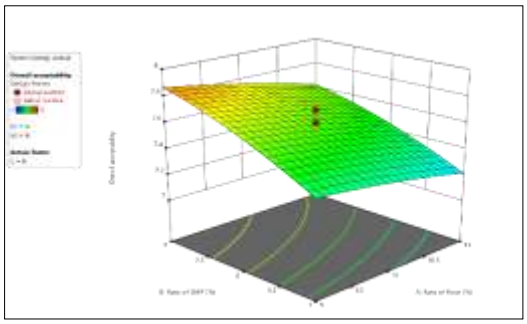


Fig. 1: Interaction of process variables on overall acceptability of BTM based probiotic fermented beverage

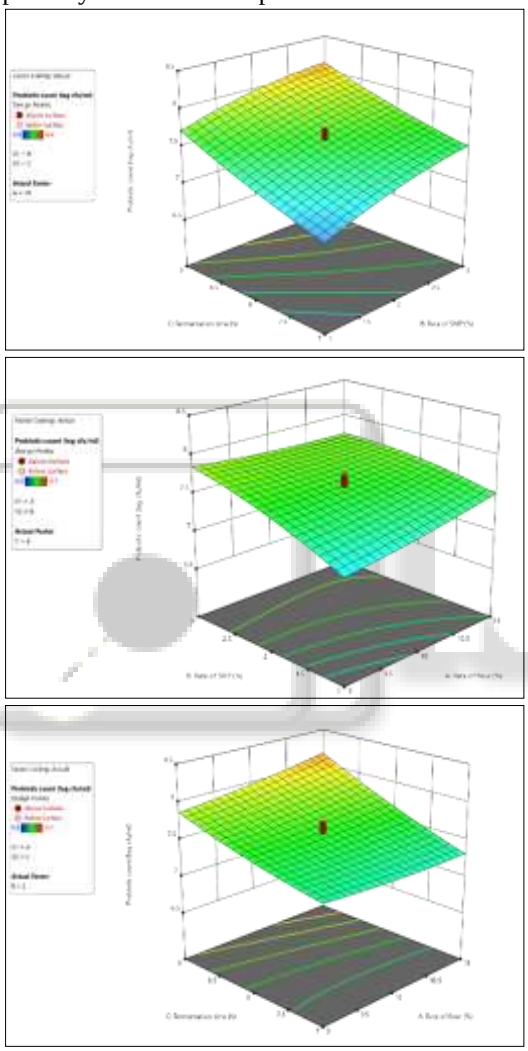


Fig. 2: Interaction of process variables on probiotic count of BTM based probiotic fermented beverage

Optimization of the process for developing BTM-based probiotic fermented beverage using the quadratic regression model of response surface methodology

A process optimization for the development of BTM-based probiotic fermented beverages was carried out to identify the best factors, namely: rate of BTM flour (A), rate of SMP (B) and fermentation time (C), which were the most suitable in terms of overall acceptability and probiotic viable count product would lead. The data obtained for the responses were analyzed in the Design Expert software. The objectives/criteria set for optimization are listed in Table 9.

Parameter	Units	Constraints	Lower Limit	Upper Limit
Independent Variables/Factors				
BTM flour	%	is in range	8.00	12.00
SMP	%	is in range	0.00	4.00
Fermentation time	h	is in range	6.00	10.00
Dependent Variables/Responses				
Sensory quality	9- Point hedonic scale	Maximize	7.00	8.00
Probiotic count	log CFU/mL	Maximize	6.95	8.40

Table 9: Selected criteria for process optimization of BTM-based probiotic fermented beverage

Considering their limitations and parameters, the RSM proposed an optimized solution as shown in Table 10, with 9% BTM flour, 3% SMP and the 9 h fermentation time of the optimized probiotic drink as the model is 90.4 percent desirable.

Parameter	Units	Optimized Value
BTM flour	%	9.00
SMP	%	3.00
Fermentation time	H	9.00
Overall acceptability	9- Point Hedonic Scale	8.02
Probiotic count	log CFU/mL	8.19
Desirability	0.935	

Table 10: Optimized solution for BTM based probiotic fermented beverage

Investigation of the Physical-Chemical, Microbial and Proximate Properties of Optimized BTM-Based Probiotic Fermented Beverage

Parameter	Results
pH	4.4 ± 0.02
Acidity (%lactic acid)	0.79 ± 0.01
TSS (°Bx)	10.5 ± 0.5
Viscosity (mPa.s)	7.80 ± 0.01

Table 11: Physicochemical properties of optimized BTM based probiotic fermented beverage

The analysis results indicate a drink with distinctive properties. A pH of 4.4 indicates that the drink is slightly acidic. The acid content is relatively low at 0.79% lactic acid and contributes to a mild acidity. Total soluble solids (TSS) at 10.5°Bx reflects moderate sugar content, indicating balanced sweetness. The viscosity measured at 7.80 mPa.s indicates a thin consistency, which is typical for most juices or light drinks. Overall, the drink has a harmonious balance of slight acidity and sweetness, with a thin, easy-drinking texture.

Microbial Count	Log CFU/mL
Probiotic count	8.25 ± 0.06
Yeast & Mold	Absent
Coliform	Absent

Table 12: Microbial analysis of optimized BTM based probiotic fermented beverage

Microbial evaluation of the sample shows that the probiotic count is also significant at 8.25 log CFU/mL,

suggesting a robust population of beneficial microorganisms. The yeast and mold were absent in the samples which is positive and indicates that there is no spoilage or contamination from these fungi. Likewise, the absence of coliform bacteria, which are indicators of fecal contamination and potential pathogens, indicates good hygienic conditions during the preparation of product. Overall, high viable probiotics and the absence of harmful microbes indicate a potentially functional and safe product.

Parameters	Results (%)
Moisture	84.68 ± 0.04
Protein	2.05 ± 0.06
Fat	0.61 ± 0.03
Ash	0.21 ± 0.02
Fibre	0.89 ± 0.02
Carbohydrates	12.45 ± 0.05

Table 13: Proximate composition of optimized BTM based probiotic fermented beverage

Table 13 shows the nutritional composition of a BTM-based probiotic fermented beverage per 100 mL. It contains 2.05 ± 0.06 g of protein, 0.61 ± 0.03 g of fat, 0.21 ± 0.02 g of ash, 0.89 ± 0.02 g of fiber and 12.45 ± 0.03 g carbohydrates. The product has high moisture content at 84.68 ± 0.04 g, indicating high water content typical of such drinks. This overall composition highlights that the beverage is a balanced food with a good mix of macronutrients and essential minerals and is therefore well suited to a varied diet.

VI. Conclusion

The Quadratic Model Showed A Well Fit for the Overall Acceptability and Probiotic Count Of Btm Based Probiotic Fermented Beverage, With R^2 Values Of 0.9516 And 0.9526, Respectively. The Anova Table For Overall Acceptability Presents The Statistical Analysis Of A Model Evaluating The Effects Of Various Factors On An Outcome. The Model Is Significant, As Indicated By The F-Value Of 21.84 And A *P*-Value Of <0.0001, Suggesting That The Factors Being Studied Have A Substantial Impact On The Response Variable. Among The Factors, The Rate Of Skimmed Milk Powder (Smp) Shows The Most Significant Effect With An F-Value Of 109.11 and A *P*-Value Of <0.0001. The Fermentation Time and the Rate of Flour Also Show Significant Effects with *P*-Values of 0.0002 and 0.0005, Respectively. The Interaction Effects Ab And Ac, However, Are Not Significant, With *P*-Values Of 0.6819. The Quadratic Terms B^2 and C^2 Are Significant, Suggesting Non-Linear Effects. The Lack Of Fit Is Not Significant (*P*=0.6177), Indicating That The Model Adequately Fits The Data. Overall, The Model Provides A Robust Understanding Of The Factors Influencing The Outcome.

The Anova Table For Probiotic Count Summarizes The Effects Of Different Factors On A Response Variable, With The Overall Model Being Significant (F-Value = 22.11, $P < 0.0001$), Indicating That The Factors Under Investigation Significantly Influence The Outcome. Among The Individual Factors, The Rate Of Skimmed Milk Powder (Smp) And Fermentation Time Are Highly Significant, With *P*-Values Of <0.0001, Demonstrating A Strong Impact On The Response Variable. The Rate Of Flour Has A Marginally Significant Effect ($P = 0.0955$), Suggesting Its Influence May Be Less Pronounced. Interaction Between Rate Of Flour And Smp (Ab) Is Significant ($P = 0.0352$), Indicating A Combined Effect On The Response, While The Interaction Between Rate Of Flour And Fermentation Time (Ac) Is Borderline Significant ($P = 0.0627$). The Quadratic Term B^2 Is Also Significant ($P = 0.0157$), Suggesting Non-Linear Effects, While Other Interactions And Quadratic Terms Are Not Significant. The Lack Of Fit Is Not Significant ($P = 0.3205$), Indicating A Good Model Fit. Overall, The Analysis Highlights Key Factors And Interactions Affecting The Outcome.

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