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Research Article

Traditional Cereal Food as Container of Probiotic Bacteria "*Lb. rhamnosus* GG": Optimization by Response Surface Methodology

Asma Gharbi Yahyaoui,[1,2] Taroub Bouzaiene,[1] Fathia Aouidi,[1,3] Abdelkarim Aydi,[4] and Moktar Hamdi[1]

[1]*Laboratory of Microbial Ecology and Technology LETMi-05ES08, National Institute of Applied Sciences and Technology, University of Carthage, BP 676, 1080 Tunis, Tunisia*
[2]*Higher Institute of Technological Studies of Bizerte, Campus University Menzel Abderrahman, BP 65, 7035 Bizerte, Tunisia*
[3]*Higher Institute of Biotechnology of Beja, University of Jendouba, Avenue Habib Bourguiba, BP 382, 9000 Béja, Tunisia*
[4]*Chemical and Materials Engineering Department, College of Engineering, Northern Border University, P.O. Box 1321, Arar, Saudi Arabia*

Correspondence should be addressed to Asma Gharbi Yahyaoui; gharbi.yahyaoui.asma@hotmail.fr

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This research paper aims at optimizing three parameters involved in solid state fermentation (SSF) using *Lactobacillus rhamnosus* GG (LGG) to improve a traditional cereal food "Bsissa" in order to elaborate a new probiotic fermented breakfast cereal. A Box-Behnken experimental design was used and the optimal fermentation conditions were liquid to solid ratio: 1.2 (vw^{-1}), fermentation time: 12 h, and sucrose concentration: 10.48 g (100 g DM)$^{-1}$. Under these conditions, the viable LGG cells, the free amino nitrogen content, and the total acidity were obtained to be 9.1 $\log_{10}$(cfu g^{-1}), 12.95 (mg g^{-1}), and 6.46 (μmol g^{-1}), respectively. After three weeks of refrigerated storage, the viability of LGG in the fermented Bsissa was 8.23 $\log_{10}$(cfu g^{-1}). This study shows a new possibility to make an acceptable nonfermented dairy product based mainly on cereals, leguminous plants, spices, and aromatic herbs, which are suitable substrates able to support the high probiotic viability.

1. Introduction

Nowadays, the demand of probiotic functional foods is growing rapidly due to increased awareness of consumers [1]. Probiotic foods are categorized as functional foods containing a single or mixed culture of microorganisms with various prophylactic properties and efficient at a level of 10^6 (cfu g^{-1}) [2]. These foods affect beneficially the consumer's health by improving their intestinal microbial balance and composition of intestinal flora. Moreover, they reduce blood pressure levels and the risk of colon cancer and increase the resistance against invasion of pathogens [3–6].

Lactobacillus rhamnosus GG is one of the most monitored probiotic strains which was clinically studied and was found to enhance human natural resistance and healthy digestive system and inhibits the adhesion of some pathogenic bacteria [7]. These cells showed a high tolerance to the acidic conditions and responded to sudden changes in their environmental osmotic conditions by accumulating sucrose in order to protect both the membranes and internal organs. Thus, this strain could be successfully used in the processing of foods containing sugars and also in preservation processes. Technologically, the LGG adheres well to fibers, creating a possibility for cereal-based probiotic products [8]. *Lb. rhamnosus* GG is used in food industry not only as probiotics but also as a protective culture in fermented and nonfermented dairy products, beverages, ready-to-eat foods, dry sausages, and salads.

Dairy products provide a suitable environment for probiotics. They support the growth and the viability of these bacteria [9]. However, with an increase in the consumer vegetarianism and lactose intolerance, there is a demand for the vegetarian probiotic products. The development of new nondairy probiotic food products is challenging; that is why, other foods such as cereal-based products, meat products, ice creams, fruit drinks, vegetable drinks, and many others have been examined for their potential as probiotic carriers. Cereal-based probiotic products show a beneficent effect for the consumer's health. They have health-benefiting microbes and potentially prebiotic fibers [9]. Moreover, cereal-based products belong to a highly nutritive culinary recipe category. They provide nutriments, such as minerals, polyphenols, dietary proteins, carbohydrates (glucose, glycerol, stachyose, xylose, fructose, maltose, sucrose, and arabinose), vitamins, and nonnutrients, such as dietary fiber and oligosaccharides. The traditional foods made from cereal grains usually lack flavor and aroma [10]. The production of volatile compounds during cereal fermentation, such as organic acids (butyric, succinic, formic, valeric, caproic, lactic, acetic, etc.), alcohols (ethanol, *n*-propanol, isobutanol, isoamyl alcohol, etc.), aldehydes and ketones (acetaldehyde, formaldehyde, isovaleraldehyde, *n*-valeraldehyde, 2-methyl butanol, etc.), and carbonyls compounds (furfural, methional, glyoxal, etc.), contributes to a complex blend of flavors in fermented cereals and makes them more appetizing [11]. In addition, the fermentation of cereals leads to a general improvement in the shelf life of cereal products [12]. Acids formed during the fermentation process lower the pH to value 4 or less, thus inhibiting the growth of numerous spoilage organisms [13, 14].

Lactic acid fermentation of cereals is a long-established processing method used to realize various beverages (boza, bouza, and Kenya busaa), gruels (togwa), porridge (ogi, yosa), and mixtures (tarhana, balao balao) in Asia, Africa, and some other countries [12]. Bsissa, zumita, Utshu, Bazin, and Asida are the most common cereal-based breakfast in the *Maghreb* Union Countries. These water based porridges and beverages of semolina or grilled barley and wheat are known for their nutritional and satiating effect. The Bsissa is an aromatized cereal powder composed basically of cereals, leguminous plants, aromatized dried herbs, and spices. It is realized through four steps: cleaning the vegetable raw material, roasting cereals and leguminous seeds, grinding the mix into fine flour, sieving well, and storing away on a jar. The recipe of Bsissa changes according to each region standard of living as well as the urban or rural status of the people. There are several forms of uses for Bsissa. It could be consumed as a liquid meal or a solid one. It could be mixed with water, oil, or milk until it becomes a paste. Other components could be added such as sugar, dried fruit, honey, crushed almonds, crushed pomegranate seeds, and dates in order to improve the flavor of the meal.

Response Surface Methodology (RSM), originally described by Box and Wilson, enables evaluation of the effects of several process parameters and their interactions on the response variables. RSM are useful tools: statistical and mathematical techniques that has been successfully used for developing, improving, and optimizing biochemical and biotechnological process related to food systems, such as fermentation of tempeh from hardened chickpeas [15], ash gourd beverage [16], apple juice and whey based novel beverage fermented using kefir grains [17].

This study is evaluating, up to our knowledge for the first time, the potential of Bsissa as substrates for the development of a probiotic product by the use of *Lactobacillus rhamnosus* GG. Response surface methodology based on Box-Behnken design was applied in order to optimize fermentation conditions of solid state fermentation of Bsissa. Cells growth evaluation, total acidity, and free amino nitrogen production were investigated during the fermentation process of Bsissa.

2. Materials and Methods

2.1. Preparation of Bsissa Powder. The Bsissa powder is a mixture of wheat grains, chickpea, carob, fenugreek seeds, and marjoram dried leaves. All materials were purchased from a local supermarket in Tunis. After sorting and cleaning, marjoram leaves were crumbled and carob pods were cut into pieces. Wheat grains, chickpeas, and fenugreek seeds were roasted in the oven at 250°C, respectively, for 6 minutes, 10 minutes, and 2 minutes. After mixing and cooling, the 5 ingredients were ground. Then, it was passed through a fine sieve and kept in a well-sealed jar until use.

2.2. Microorganism Inoculum Preparation. A strain of *Lactobacillus rhamnosus* GG (ATCC 53103) belonged to the collection of the Laboratory of Ecology and Microbial Technology (LETMI) and was stored frozen at Man Rogosa and Sharpe broth (MRS) containing glycerol (10% vv^{-1}) at −80°C. The lactic acid bacteria were activated by transferring into MRS broth and cultured three times for 18 h at 37°C before use. For the preparation of the inoculum, the lactic strain was cultured in MRS broth for 12–14 h at 37°C. Then, the bacterial cells were pelleted by centrifugation at 500 g for 10 minutes at 4°C, washed twice with phosphate buffered saline (PBS), pH = 7, and resuspended in physiological water. An inoculum with a population of probiotic cells of 10^7 cfu g^{-1} was used for the inoculation of Bsissa samples.

2.3. Experimental Box-Behnken Design. The Box-Behnken design experiments were applied in order to study the main effects, the interaction effects, and the quadratic effects of sucrose concentration g (100 g DM)$^{-1}$, fermentation time (h), and liquid to solid ratio (vw^{-1}) on the growth of *Lactobacillus rhamnosus* (cfu g^{-1}), free amino nitrogen content (mg^{-1}), and total acidity (μmol eq. lactic acid g^{-1}), following lactic fermentation of Bsissa.

The three independent variables were studied at three different levels: low, medium, and high, coded as −1, 0, and +1, respectively (Table 1). A total of 17 experiments were carried out (Table 2). Twelve experiments were augmented with five replications in the center points of the design to estimate the pure error and to check the absence of bias between several sets of experiments.

Table 1: Levels and code of independent variables used for Box-Behnken experimental design.

Independent variable	Coded symbol	Variable levels		
		Low	Center	High
		−1	0	1
Sucrose concentration g (100 g DM)$^{-1}$	X_1	2.1	6.29	10.48
Fermentation time (h)	X_2	4	11	18
Liquid to solid ratio (vw^{-1})	X_3	1.2	1.5	1.8

To find the optimum set of fermentation conditions, a second-degree model was used according to

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}{X_1}^2 + b_{22}{X_2}^2 + b_{33}{X_3}^2 + b_{12}X_{12} + b_{13}X_{13} + b_{23}X_{23}, \quad (1)$$

where $X_i = (x_i - x_0)/\Delta x_i$ is the coded value: x_i is the actual value of the independent variable, x_o is the actual value at the center point, and Δx_i is the step change value. Y is the predicted response, a_o is the constant, a_1, a_2, and a_3 are linear coefficients, a_{12}, a_{13}, and a_{23} are cross product coefficients, and a_{11}, a_{22}, and a_{33} are quadratic coefficients.

2.4. Lactic Acid Fermentation of Bsissa. A mass of 50 g of autoclaved Bsissa was mixed with a volume of sterilized water and a mass of sucrose according to the Box-Behnken design (Table 2). The paste was aseptically homogenized and inoculated with 1% (vw^{-1}) of *Lactobacillus rhamnosus* inoculum. The solid state fermentation was carried out at 30°C for a fixed time, according to Box-Behnken design (Table 2).

2.5. Analytical Methods

2.5.1. Viable Cells Enumeration. Bsissa samples were aseptically diluted (1 : 10) in sterile peptone water (0.1%). Sample dilutions (0.1 mL) were plated into MRS agar and were incubated at 37°C for 48 h under aerobic conditions. Total viable cells were estimated by colony forming units (cfu) and were expressed as $\log_{10}$ per gram.

2.5.2. pH and Titrable Acidity Determination. A mass of 10 g of samples were homogenized with 90 mL of distilled water for 20 min in a Laboratory Incubator Shaker. The pH value was measured at room temperature after standardizing the combined glass electrode with pH 4.01 and 7.01 standard buffer solution. The total titrable acidity (TTA) was determined by titrating each fermented sample with 0.1 N NaOH, using phenolphthalein as an indicator, until pH of 8.5 was attained. Each mL of 0.1 N NaOH is equivalent to 9.008 mg of lactic acid. The total titrable acidity was expressed in μmolg^{-1}

$$\text{Total titrable acidity } \left(\mu\text{mol eq. lactic acid g}^{-1}\right) = \frac{V_{\text{NaOH}} \times 9.008}{\text{MW}} \times 10, \quad (2)$$

where V_{NaOH} is the volume of NaOH (mL) and MW is the molar mass of lactic acid (g mol^{-1}).

2.5.3. Determination of Free Amino Nitrogen Content. Free amino nitrogen content in the fermented Bsissa was determined according to the official AOAC [18] method 945.18-B.

2.6. Statistical Analysis. Data from the BBD were analyzed using NEMROD-W (Version 9901, LPRAI Company) for the regression analysis and the graphical optimization. Student's t-test permitted us to check the statistical significance of the regression coefficients. Fisher's test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model. The models of each response for the full factorial design were expressed in terms of code variables and without taking into account the statistically insignificant terms.

3. Results

3.1. Efficiency of the Bsissa Fermentation. During the fermentation of Bsissa under a liquid to solid ratio of 1.2 and sucrose concentration of 10.48 g (100 g DM)$^{-1}$, the cell population increased rapidly from 7.87 $\log_{10}$(cfu g^{-1}) to 9.33 $\log_{10}$(cfu g^{-1}) after 18 h of fermentation and then remained constant until 48 h of fermentation (Figure 1(a)).

Kinetics of pH changes during the fermentation period showed a significant ($p < 0.05$) reduction in the pH from 5.5 to 4.9 after 18 h of fermentation. The pH showed a gradual reduction from 4.9 at 18 h to 4.63 at 48 h of fermentation (stationary phase). Hence, the total acidity increased significantly ($p < 0.05$) from 4.5 μmol g^{-1} up to 9.57 μmol g^{-1} at the end of the fermentation (48 h) (Figure 1(b)).

3.2. Experimental Results of the BBD. The matrix design of the coded independent variables and experiment are shown in Table 2. Each test was performed in duplicate and the central point was repeated five times (runs 13, 14, 15, 16, and 17). Results demonstrate that sucrose concentration, fermentation time, liquid to solid ratio, and their interactions have an effect on LGG growth and total acidity. In particular, the highest results of LGG growth (9.12 $\log_{10}$(cfu g^{-1})) and total acidity (8.2 μmol eq. lactic acid g^{-1}) were obtained working with the highest fermentation time (18 h), an intermediate sucrose concentration (6.29%), and a lowest liquid to solid ratio (1.2). On the other hand, the maximum free amino nitrogen (13.7 mg g^{-1}) was reached at the highest fermentation time (18 h), an intermediate liquid to solid ratio (1.5), and the lowest sucrose concentration (2.1%). For this reason, a simultaneous optimization of the three responses was considered to be necessary to select the best overall conditions for the process.

Table 2: Box-Behnken experimental design with experimental values of viable *Lb. rhamnosus* GG cells, free amino nitrogen content, and total acidity.

Number Exp	Actual and coded level of variables			Experimental values			Predicted values		
	X_1 g (100 gDM)$^{-1}$	X_2 (h)	X_3 (vw^{-1})	Y_1 $\log_{10}$(cfu g^{-1})	Y_2 (mg g^{-1})	Y_3 (μmol eq. lactic acid g^{-1})	Y_1 $\log_{10}$(cfu g^{-1})	Y_2 (mg g^{-1})	Y_3 (μmol eq. lactic acid g^{-1})
1	2.1 (−1)	4 (−1)	1.5 (0)	8.390	12.440	5.700	8.391	12.438	5.650
2	10.48 (1)	4 (−1)	1.5 (0)	8.270	12.660	4.700	8.266	12.660	4.663
3	2.1 (−1)	18 (1)	1.5 (0)	9.030	13.700	8.100	9.034	13.700	8.138
4	10.48 (1)	18 (1)	1.5 (0)	8.890	11.260	8.100	8.889	11.263	8.150
5	2.1 (−1)	11 (0)	1.2 (−1)	8.870	14.010	6.950	8.869	14.011	6.944
6	10.48 (1)	11 (0)	1.2 (−1)	9.040	13.220	6.100	9.044	13.219	6.081
7	2.1 (−1)	11 (0)	1.8 (1)	8.990	13.270	6.100	8.986	13.271	6.119
8	10.48 (1)	11 (0)	1.8 (1)	8.540	11.850	6.000	8.541	11.849	6.006
9	6.29 (0)	4 (−1)	1.2 (−1)	8.270	13.070	5.400	8.270	13.071	5.456
10	6.29 (0)	18 (1)	1.2 (−1)	9.120	13.060	8.200	9.118	13.059	8.169
11	6.29 (0)	4 (−1)	1.8 (1)	8.290	12.070	4.700	8.293	12.071	4.731
12	6.29 (0)	18 (1)	1.8 (1)	8.710	11.950	8.050	8.710	11.949	7.994
13	6.29 (0)	11 (0)	1.5 (0)	9.000	12.880	7.370	8.986	12.830	6.920
14	6.29 (0)	11 (0)	1.5 (0)	8.970	12.710	7.100	8.986	12.830	6.920
15	6.29 (0)	11 (0)	1.5 (0)	9.120	12.960	6.500	8.986	12.830	6.920
16	6.29 (0)	11 (0)	1.5 (0)	8.960	12.680	6.700	8.986	12.830	6.920
17	6.29 (0)	11 (0)	1.5 (0)	8.880	12.920	6.930	8.986	12.830	6.920

X_1: sucrose concentration g (100 g DM)$^{-1}$; X_2: fermentation time (h); X_3: liquid to solid ratio (vw^{-1}); Y_1: viable *Lb. rhamnosus* GG ($\log_{10}$(cfu g^{-1})); Y_2: free amino nitrogen content (mg g^{-1}); Y_3: total acidity (μmol eq. lactic acid g^{-1}, Y_3).

*When $t = 0$ h, LGG = 7.8 ± 0.075 $\log_{10}$(cfu g^{-1}) in all experimental samples; FAN = 13.11 ± 0.09 mgg^{-1} and total acidity = 4.7 ± 0.18 μmol g^{-1}.

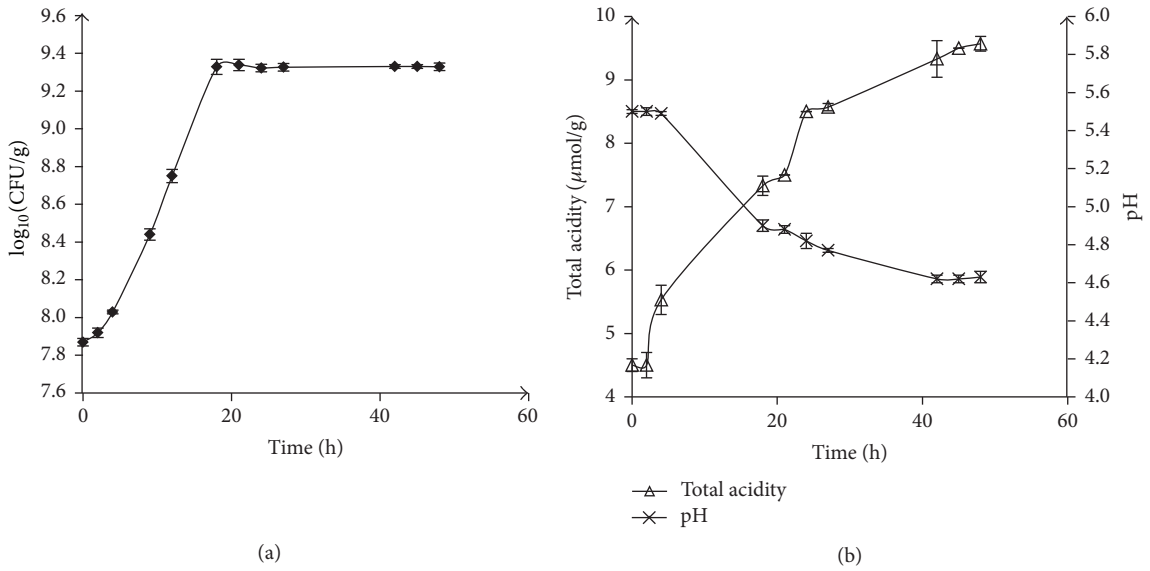

Figure 1: Evolution of *Lb. rhamnosus* (a) and pH and total acidity (b) during fermentation of Bsissa.

3.3. Statistics

3.3.1. Application Response Surface Methodology to Viable Lb. rhamnosus GG (Y_1). The second-order model describing viable *Lb. rhamnosus* GG as a simultaneous function of sucrose concentration (X_1), fermentation time (X_2), and liquid to solid ratio (X_3) is described by (3) after neglecting the statistically insignificant terms ($p < 0.05$):

$$Y_1 = 8.986 - 0.068X_1 + 0.316X_2 - 0.096X_3 - 0.302{X_2}^2 - 0.087{X_3}^2 - 0.155X_{13} - 0.107X_{23}\,(\pm 0.066), \quad (3)$$

where Y_1 is the viable *Lb. rhamnosus* GG response, X_{13} is the interaction effect between sucrose concentration (X_1) and liquid to solid ratio (X_3), and X_{23} is the interaction effect between fermentation time (X_2) and liquid to solid ratio (X_3).

The regression coefficients obtained to predict polynomial model for LGG growth are summarized in Table 3. The significance of all regression coefficient was checked by means of Student's test (variation of data about mean value) and p values (probability) (Table 3). We can see that ${X_1}^2$ (sucrose concentration × sucrose concentration) and X_{12} (sucrose concentration × fermentation time) were found to be statistically insignificant ($p > 0.05$). The goodness of fit of the model was examined by F-test and the determination coefficient R^2. The analysis of variance (Table 4) showed that this elaborated regression model was highly significant ($p < 0.1\%$) as is evident from the Fisher F-test and has a very low probability value [(p model $> F$) $< 0.1\%$]. The value of 1 (>0.05) for lack of fit implies that it is not significant compared to the pure error and that the model equation was adequate for predicting viable *Lb. rhamnosus* GG ($\log_{10}$cfu g^{-1}, Y_1). The fitness of the model was further confirmed by a satisfactory value of determination coefficient (R^2), which was calculated to be 0.980 (Y_1). Furthermore, the predicted responses of the final quadratic model, along with the corresponding values observed, are given in Table 2. The agreement between the predicted results of the model and the experimental data is very strong as shown by a high value of correlation coefficient, ${R^2}_{\text{pred}}$ (0.968 (Y_1)). The residuals were examined to check the adequacy of the model. The residuals were plotted against the predicted value. No unusual behavior was notified (horizontal band) [19], confirming the adequacy of the regression model. The effect of interaction of the three components on growth of LGG was tested by contour plots for all possible combinations of factors, keeping one factor constant at a time. The interactions between the variables can be inferred from the shapes of the contour plots [20]. Circular contour plots indicate that the interactions between the variables are negligible. In contrast, elliptical ones indicate the evidence of the interactions (Figure 2).

Figure 2(B1) showed that there was a significant decrease in LGG growth from 8.97 $\log_{10}$(cfu g^{-1}) to 8.66 $\log_{10}$ (cfu g^{-1}), when liquid to solid ratio was between 1.5 (vw^{-1}) and 1.8 (vw^{-1}) and sucrose concentration was between 6.29% and 10.48%, which was corresponding to the extremely significant interaction of X_1X_3, shown in Table 4. However, the greater LGG values were shown for a liquid to solid ratio of [1.2; 1.65] and for fermentation time between 11 h and 13 h (Figure 2(C1)). This interaction is, indeed, confirmed by the interaction X_2X_3 shown in Table 4.

3.3.2. Application Response Surface Methodology to Free Amino Nitrogen Content (Y_2). The second-order model describing free amino nitrogen (FAN) content as a simultaneous function of sucrose concentration (X_1), fermentation time (X_2), and liquid to solid ratio (X_3) is described by

Table 3: Coefficients by regression analysis and their significance values obtained by ANOVA.

Nomenclature of the coefficients	Viable *Lb. rhamnosus* GG ($\log_{10}$ cfu g^{-1}, Y_1)				Free amino nitrogen content (mg g^{-1}, Y_2)				Total acidity (μmol eq. lactic acid g^{-1}, Y_3)			
	Coefficient	Ecart-type	*t*.exp.	Signif. %	Coefficient	Ecart-type	*t*.exp.	Signif. %	Coefficient	Ecart-type	*t*.exp.	Signif. %
b_0	8.986	0.029	304.93	***	12.830	0.043	299.04	***	6.920	0.117	59.30	***
b_1	−0.068	0.023	−2.90	*	−0.554	0.034	−16.33	***	−0.244	0.092	−2.64	*
b_2	0.316	0.023	13.57	***	−0.034	0.034	−1.00	35.5%	1.494	0.092	16.19	***
b_3	−0.096	0.023	−4.13	**	−0.528	0.034	−15.55	***	−0.225	0.092	−2.44	*
b_{11}	−0.039	0.032	−1.22	26.1%	0.118	0.047	2.51	*	−0.285	0.127	−2.24	5.8%
b_{22}	−0.302	0.032	−9.40	***	−0.432	0.047	−9.25	***	0.015	0.127	0.12	90.5%
b_{33}	−0.087	0.032	−2.70	*	0.140	0.047	2.99	*	−0.348	0.127	−2.73	*
b_{12}	−0.005	0.033	−0.15	87.8%	−0.665	0.048	−13.86	***	0.250	0.130	1.92	9.5%
b_{13}	−0.155	0.033	−4.70	**	−0.158	0.048	−3.28	*	0.188	0.130	1.44	19.2%
b_{23}	−0.107	0.033	−3.26	*	−0.028	0.048	−0.57	58.9%	0.138	0.130	1.05	32.8%

Test Student (*t*.exp.); *: $p < 5\%$; **: $p < 1\%$; ***: $p < 0.1\%$.

Table 4: Analysis of variance and regression analysis.

Source	Sum of squares	DF	Mean squares	F-value	Signif. %
Viable *Lb. rhamnosus* GG ($\log_{10}$ cfu g^{-1}, Y_1)					
Model	1.4966	9	0.1663	38.2972	***
Residual	0.0304	7	0.0043		
Lack of fit	0.0001	3	0.0000	0.0033	100.0%
Pure error	0.0303	4	0.0076		
Total	1 .5270	16			
Free amino nitrogen content (mg g^{-1}, Y_2)					
Model	7.4485	9	0.8276	89.9226	***
Residual	0.0644	7	0.0092		
Lack of fit	0.0000	3	0.0000	0.0005	100.0%
Pure error	0.0644	4	0.0161		
Total	7.5129	16			
Total acidity (μmol eq. lactic acid g^{-1}, Y_3)					
Model	20.0954	9	2.2328	32.7892	***
Residual	0.4767	7	0.0681		
Lack of fit	0.0169	3	0.0056	0.0489	98.4%
Pure error	0.4598	4	0.1149		
Total	20.5721	16			

DF: degrees of freedom; F: variance ratio; ***: P (probability) <0.1%.

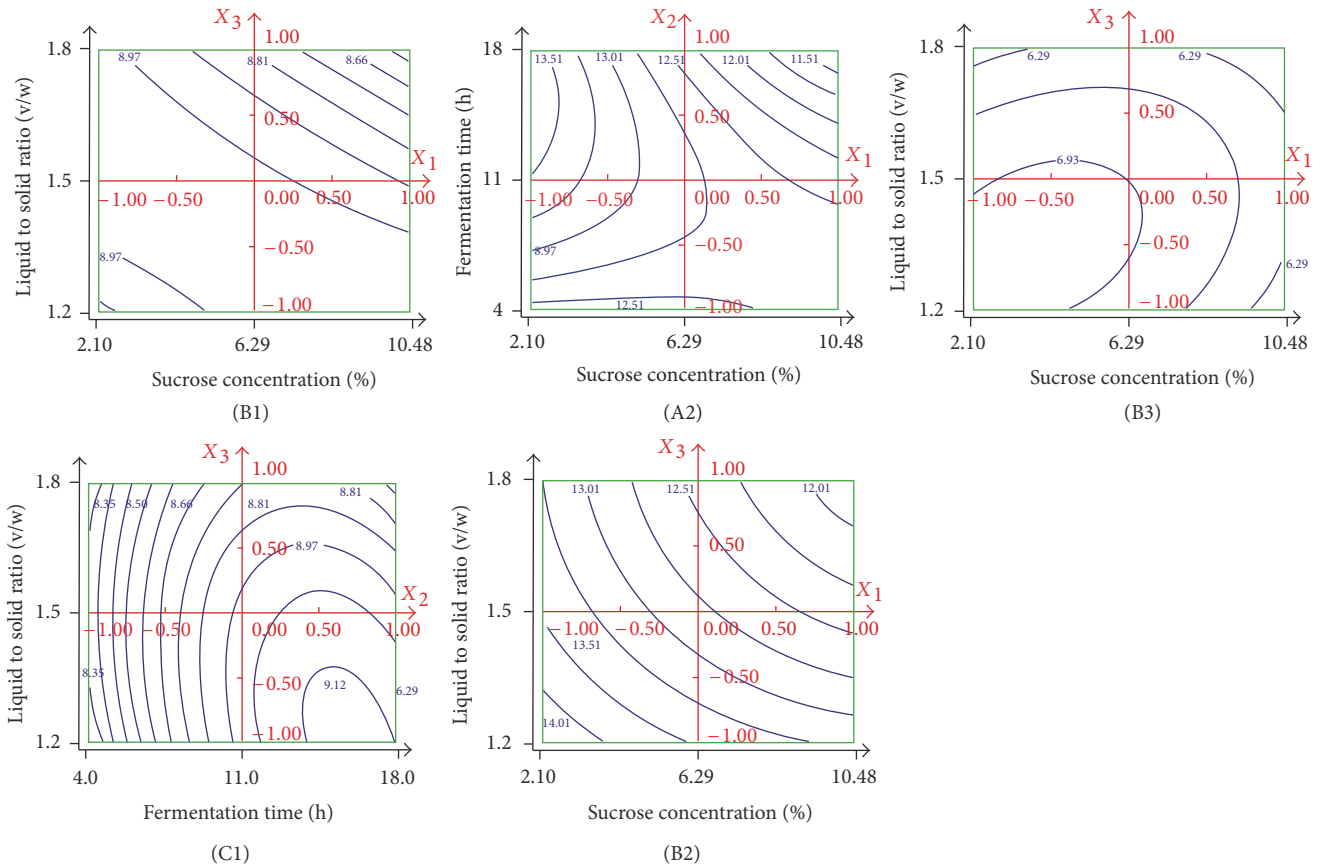

Figure 2: Response surface and contour plots that represent the effect of fermentation time and sucrose concentration (A); liquid to solid ratio and sucrose concentration (B); liquid to solid ratio and fermentation time (C) and their interaction on (1) viable *Lb. rhamnosus* GG cells when X_1, X_2, and X_3 are 6.29 g (100 g DM)$^{-1}$, 11 h, and 1.5(vw^{-1}); (2) free amino nitrogen content when X_1, X_2, and X_3 are 6.29 g (100 g DM)$^{-1}$, 11 h, and 1.5(vw^{-1}); (3) total acidity when X_1, X_2, and X_3 are 6.29 g (100 g DM)$^{-1}$, 11 h, and 1.5(vw^{-1}). X_1: sucrose concentration g (100 g DM)$^{-1}$; X_2: fermentation time (h); X_3: liquid to solid ratio (vw^{-1}).

$$Y_2 = 12.83 - 0.554X_1 - 0.528X_3 + 0.118{X_1}^2 - 0.432{X_2}^2 + 0.14{X_3}^2 - 0.665X_{12} - 0.158X_{13}\,(\pm 0.096), \quad (4)$$

where Y_2 is the free amino nitrogen content, X_{12} is the interaction between sucrose concentration (X_1) and fermentation time (X_2), and X_{13} is the interaction between sucrose concentration (X_1) and liquid to solid ratio (X_3).

Figure 2(A2) represents the FAN response surface versus the actual level of fermentation time and sucrose concentration. The results of the Fisher test for analysis of variance (Table 3) revealed that the regression is statically significant at 100% of confidence level. Moreover, the value of the determination coefficient ($R^2 = 0.991$) points out the goodness of the regression, which can be used to explain 99.1% of the total variation in the response.

We can see from Table 3 that X_2 (fermentation time) and X_{23} (fermentation time × liquid to solid ratio) had no significant effect on free amino nitrogen content ($p > 0.05$). The linear term of the fermentation time seems to have no significant effect on the response, contrary to the quadratic term. This last increase is when the fermentation time is between 10 h and 12 h. Under 10 h and below 12 h of fermentation, FAN keeps decreasing.

3.3.3. Application Response Surface Methodology to Total Acidity (Y_3). The application of the response surface methodology to total acidity yielded the following regression equation:

$$Y_3 = 6.92 - 0.244X_1 + 1.494X_2 - 0.225X_3 - 0.348{X_3}^2\,(\pm 0.261), \quad (5)$$

where Y_3 is total acidity response and X_1, X_2, and X_3 are the coded value of sucrose concentration, fermentation time, and liquid to solid ratio, respectively.

The coefficients of ${X_1}^2$ (sucrose concentration × sucrose concentration), ${X_2}^2$ (fermentation time × fermentation time), X_{12} (sucrose concentration × fermentation time), X_{13} (sucrose concentration × liquid to solid ratio), and X_{23} (fermentation time × liquid to solid ratio) were found to be statistically insignificant ($p > 0.05$). Besides, the analysis of variance reveals that this regression is statically significant at 98.4% confidence level. Besides $R^2 = 97.7\%$ points out that only 2.3% of the total variation in the response is not explained by the model. The linear regression coefficient of fermentation time (X_2) puts in evidence the positive effect of this parameter on the response, making the total acidity clearly growing with the fermentation time. The response surface to predict the total acidity within the region under investigation is shown in Figure 2(B3). The quadratic term of fermentation time is insignificant. The maximum total acidity (8.25 μmol g^{-1}) was obtained in 2.1% of sucrose concentration, 18 h of fermentation time, and 1.5 of liquid to solid ratio.

In this study, total acidity increases with fermentation time, contrary to sucrose concentration and liquid to solid ratio.

3.4. Optimization of the Fermentation Conditions. Optimization of the Bsissa fermentation by LGG was carried out via a multiple response method called desirability (D) function to optimize different combinations of process parameters such as sucrose concentration, fermentation time, and liquid to solid ratio. The goal of optimization was to choose the best fermentation conditions that give us the higher biomass ($\log_{10}$(LGG) > 9 ± 0.1) and free amino nitrogen (>13 ± 0.1) with a total acidity bigger than 6 μmol g^{-1} and lower than 7 μmol g^{-1}. Figures 3(a), 3(b), and 3(c). show that desirability is higher when fermentation time is closer to 12 h (11-12 h), liquid to solid ratio closer to 1.2 (1.2–1.6), and sucrose concentration closer to 10.48% (8.4%–10.48%).

Among all the optimum points, the best desirability value 88.4% was found in sucrose concentration of 10.48%, fermentation time of 12 h, and liquid to solid ratio of 1.2 (vw^{-1}).

Three test experiments were also carried out according to the optimum fermentation conditions. The result showed that the value of LGG growth, FAA content, and total acidity was closer to the values predicted earlier, with a low relative error ($p < 0.05$), declaring the effectiveness of the RSM method to optimize the fermentation conditions of Bsissa.

3.5. Viability of LGG in Fermented Bsissa during Conservation. The Bsissa was fermented under the optimized conditions and then stored at 4°C. The viability of LGG in fermented Bsissa and the value of the pH and the total acidity over the refrigerated storage were monitored at 3-day intervals for 3 weeks. Figure 4 shows that the number of LGG decreased significantly from 2.5×10^9 cfu g^{-1} (day 1) to 1.7×10^8 at day 21. Total viable count, coliforms, yeasts, and molds were not detected (<1 $\log_{10}$(cfu g^{-1})) during storage up to 21 days. Figure 5 showed the evolution of pH and total acidity during refrigerated storage of Bsissa. After 3 days of storage, the pH of the fermented Bsissa decreased from 4.3 to 4.1 and continues to decrease significantly ($p < 0.05$) to 3.4 during the 21 days of refrigerated storage (average of 0.043 ΔpH day^{-1}). The total acidity of the fermented Bsissa increased throughout 21 days of storage from 6.6 μmol/g to 10.23 μmol/g. Single factor ANOVA analysis showed that the pH and the total acidity changes were significant ($p < 0.05$).

4. Discussion

Since Bsissa has been traditionally consumed as a non-fermented beverage, carrying preliminary experiments was essential to test the fermentability of the cereal-mix by *Lactobacillus rhamnosus* GG. After 18 h of fermentation, the viable cell counts were well above the suggested minimum limit of 7.7 $\log_{10}$(cfu g^{-1}) for a probiotic product to confer a therapeutic effect based on a 100 g daily dose [21]. The LGG strain used in the present study showed good growth in Bsissa. This aromatized cereal medium was appropriate for growth of LGG because it provides all the required nutrients. This could be attributed to the presence of considerable amounts of glucose in carob [22] and wheat [23]. Kocková and Valík [24] showed that *Lb. rhamnosus* GG was able to grow in cereal, pseudocereal, and leguminous substrates

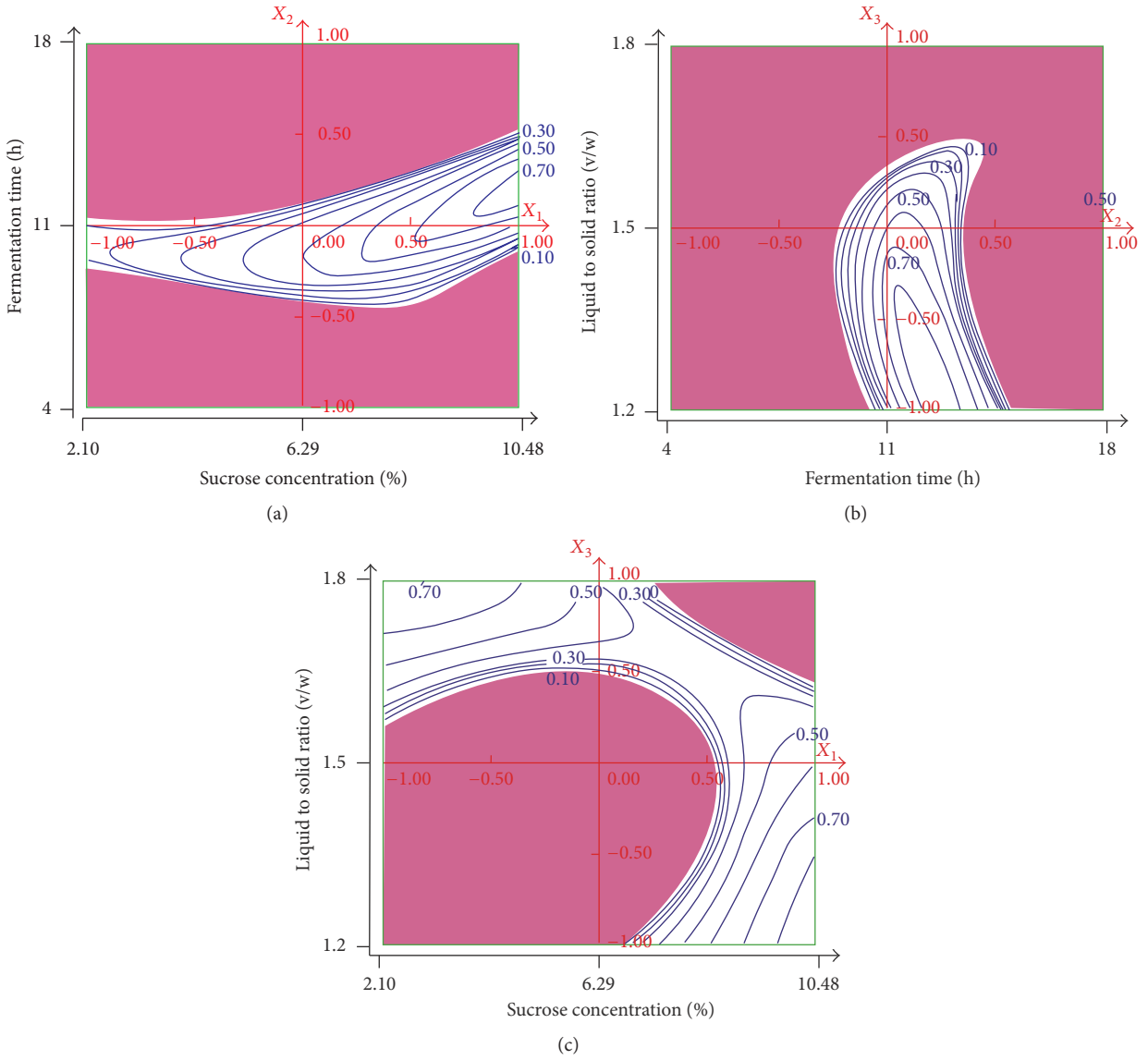

FIGURE 3: Desirability fitted 2D surface that represent the effect of fermentation time and sucrose concentration (a); liquid to solid ratio and fermentation time (b); liquid to solid ratio and sucrose concentration (c), when X_1, X_2, and X_3 are 10.48 g (100 g DM)$^{-1}$, 12 h, and 1.2(vw^{-1}). X_1: sucrose concentration g (100 g DM)$^{-1}$; X_2: fermentation time (h); X_3: liquid to solid ratio (vw^{-1}).

(buckwheat, dark buckwheat, barley, oat, soya, and chickpea, in combination with oat) with only a small content of residual water (from 48.8% to 79.8%), unlike in porridges or beverages (95%). The growth of LGG was lower in cereal and pseudocereal in comparison to porridges and beverages due probably to the lower water content.

Application of probiotic cultures in cereal products, such as Bsissa, represents a great challenge. The formulation of the new probiotic Bsissa requires several factors to be considered such as final viable cells in the product, total acidity, and viability of probiotic during conservation. In this study, the main parameters of the fermentation, sucrose concentration g (100 g DM)$^{-1}$, fermentation time (h), and liquid to solid ratio (vw^{-1}), were optimized by using a Box-Behnken design at constant temperature 30°C.

Results showed that the sucrose concentration poorly affects the LGG growth, contrary to fermentation time, which had the higher significant effect on cell growth. No interaction was revealed between the two variables, which correspond properly to the insignificant interaction (87.8%) (Table 4). However, the interaction between sucrose concentration (from 6.29 to 10.48%) and liquid to solid ratio (from 1.5 to 1.8) leads to a decrease in LGG from 8.97 $\log_{10}$(cfu g^{-1}) to 8.66 $\log_{10}$(cfu g^{-1}). Liquid to solid ratio under 1.5 had no effect on LGG growth. The greater LGG values were shown for a liquid to solid ratio of [1.2; 1.65] and for a fermentation time between 11 h and 13 h. After 13 h of fermentation, LGG shows a slight decrease, especially when the liquid to solid ratio is higher than 1.35. LGG growth shows a greater result when fermentation time and liquid to solid ratio have

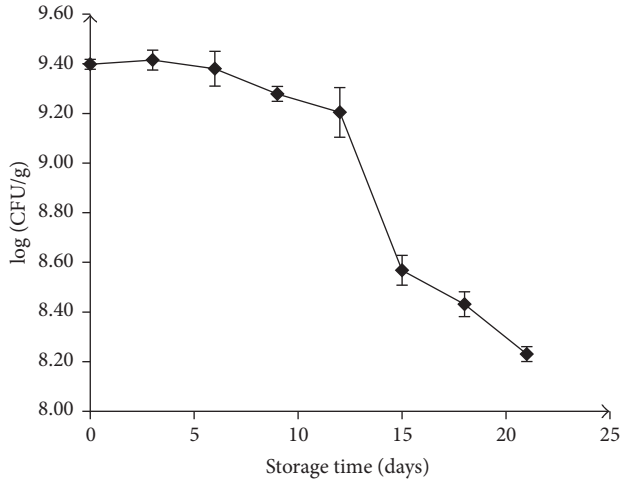

Figure 4: Effect of storage time at 4°C on the viable LGG of fermented Bsissa.

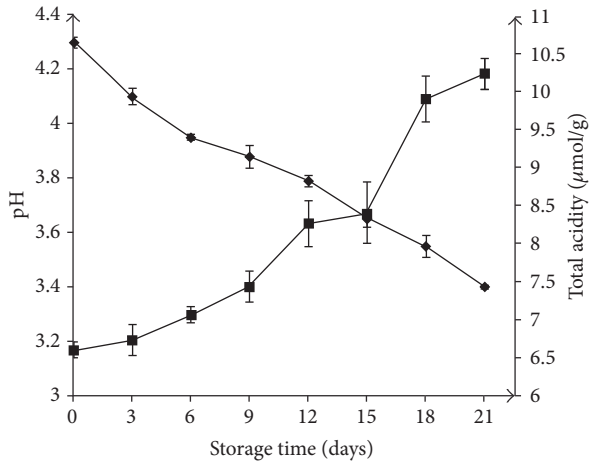

Figure 5: Effect of storage time on the pH (◆) and the total acidity (■) of fermented Bsissa.

an opposite code sign, corresponding to fermentation time of [11; 15] and liquid to solid ratio of [1.2; 1.5]. This interaction is, indeed, confirmed by the interaction X_2X_3 shown in Table 4.

Thus, maximum LGG was found when liquid to solid ratio was in the center point of the experimental design. Similar results were reported by Chen [25], who found that negative effect on the bacterial growth was shown when liquid to solid ratio was too low or too high. In fact, a poor liquid to solid ratio gave a little medium to satisfy microorganism growth; however, high amount created free medium and decreased the porosity of the liquid film on the surface and oxygen transfer needed for the growth of microorganisms.

In our study, when sucrose concentration is 6.28 g $(100\,\text{g DM})^{-1}$ and liquid to solid ratio is 1.5 (vw^{-1}), LGG increased from 7 $\log_{10}(\text{cfu g}^{-1})$ to 8.99 $\log_{10}(\text{cfu g}^{-1})$ after 11 h of fermentation at 30°C.

The cell numbers increased by 1.99 $\log_{10}(\text{cfu g}^{-1})$ at the center point of the experiment. Comparable results were reported by Kocková et al. [26] and Kocková and Valík [24] during the fermentation of cereals (rye, barley, oat, and millet), pseudocereals (amaranth, buckwheat, and dark buckwheat), and legumes (soya and chickpea) substrates. The cell numbers increase greater than 2 $\log_{10}(\text{cfu g}^{-1})$ (7.40–8.80 $\log_{10}(\text{cfu g}^{-1})$) after 10 h of fermentation at 37°C. Other studies reported that higher fermentation time was needed to obtain a similar result. Maize porridge with malted barley addition was fermented by LGG at 37°C and needed 24 h to increase probiotic cells by only 1.2 $\log_{10}(\text{cfu g}^{-1})$ [27]. Similar results were found during the lactic fermentation of the cashew apple juice (16 h) [28] and the mare's milk (24 h) [29]. It has been suggested that the fermentation time and the final probiotic biomass is a function of the probiotic strain and the food matrix. The LGG growth in this study reached 9 $\log_{10}(\text{cfu g}^{-1})$ after 11 h of fermentation. The same amount of lactic bacteria was obtained in Togwa after 20 h of fermentation [30] and in York cabbage after 24 h [31]. In other studies, final lactic acid growth in food matrix was found to be 8 $\log_{10}(\text{cfu g}^{-1})$ such as cereal-based probiotic beverages (oats, barley, and malt substrates) [32]. The effect of sucrose concentration on lactic bacteria was controversial. In fact, sucrose did not support the good growth of the *Lb. Plantarum* [33]. However, it supported the growth of the *Lb. casei* in probiotic roselle juice [34]. Chan-Blanco et al. [35] and Nancib et al. [36] found that *L. casei subsp. rhamnosus* is unable to consume the sucrose in an appropriate period of time. It uses firstly glucose as a carbon source, followed by fructose [37].

FAN increased when the fermentation time is between 10 h and 12 h. Under 10 h and below 12 h of fermentation, FAN keeps decreasing. Similar results were described by [38]. They found that FAN content increased during the lag phase and at the end of fermentation of malt media by *Lb. acidophilus*. In fact, the increase in FAN concentration during the lag phase is probably due to the hydrolysis of intracellular proteins. However, the increase in FAN concentration towards the end of incubation signaled the initiation of the disintegration of intracellular proteins, which was probably the very first step of cell autolysis. At the end of fermentation FAN concentration decreased linearly, even throughout the stationary phase to provide maintenance energy for the cells [10, 38].

Total acidity increases with fermentation time, contrary to sucrose concentration and liquid to solid ratio. Reference [39] studied the effect of sucrose concentration on lactic acid production. The productivity of lactic acid by *Lactobacillus casei subspecies rhamnosus* decreases with the high sugar concentration probably owing to substrate inhibition, the osmotic pressure, the low water activity, and the accumulation of toxic byproducts (e.g., high concentration of LA) especially after a long fermentation time [39]. However, in probiotic roselle juice fermented by *Lactobacillus plantarum* and *Lactobacillus casei*, the addition of sucrose (7 and 14%) at the beginning of fermentation increased the amount of titrable acidity by at least two times (more than 1.1% lactic acid) after 48 h of fermentation to reach a pH inferior to 3.5 [34]. This result was also confirmed by Costa et al. [40] during pineapple juice (86% sucrose) fermentation. It

has been suggested that lactic acid production reduced the pH. At low pH value sucrose hydrolysis is enhanced. Then, when sucrose concentration increases, the acidity of the final product increases too.

This study proves that decreasing liquid to solid ratio increased total acidity. Reference [41] found that maximum glutamic acid production by *Brevibacterium* was at maximum experimental moisture content (85–90%) due to homogeneous distribution of the nutrients. However, when the liquid to solid ratio is over 90%, the support may form clumps and affect negatively the bacterial growth [25].

The Bsissa was fermented under the optimized conditions calculated by the experimental design (Section 3.4). After 21 days of refrigerated storage, this fermented cereal food showed a high viability of LGG. In fact, the counts of *Lb. rhamnosus* remained viable above the minimum effective dose for therapeutic cultures of 10^6 cfu g^{-1} [42]. This result indicates that LGG is capable of surviving under highly acidic conditions during storage at 4°C. High survival rates of LGG in fermented products during storage under refrigerated conditions have been reported in earlier studies. Klu et al. [43] and Randazzo et al. [44] reported high survival of LGG (>6 logs) in peanut butter after 48 weeks of storage. The final pH after 21 days of storage in the Bsissa was 3.34, which is similar to the results (pH between 3.4 and 4.4) reported by Helland et al. [27] during the fermentation of a cereal based product.

In this study, the viability of a probiotic strain *Lactobacillus rhamnosus* GG was tested on a traditional nonfermented Tunisian aromatized cereal: "Bsissa." A Box-Behnken design with the RSM was successfully applied to the optimization of the fermentation conditions of the Bsissa. Quadratic models were elaborated to describe the relationship between the LGG growth (Y_1), the free amino nitrogen production (Y_2), and the total acidity (Y_3) and the fermentation time, the solid to liquid ratio, and the sucrose concentration. Among all the optimum points, the best desirability value (88.4%) was found in sucrose concentration of 10.48%, fermentation time of 12 h and liquid to solid ratio of 1.2 (vw^{-1}). Under these conditions, LGG, free amino nitrogen content, and total acidity were, respectively, 9.1 $\log_{10}$(cfu g^{-1}), 12.95 mg g^{-1}, and 6.46 μmol g^{-1}. The Bsissa was fermented under these optimal conditions and the viability of LGG was high (>6 $\log_{10}$(cfu g^{-1})) during 21 days of storage. This study shows a new possibility to make an acceptable fermented product based mainly on cereals, leguminous plants, spices, and aromatics, which are suitable substrates that can support the high probiotic viability during cold storage for 21 days.

The amount of free amino nitrogen was investigated in the nonfermented and the fermented Bsissa under the optimized condition. These values were 13.11 mg/g and 13.42 mg/g, respectively. Thus, the fermentation improved the nutritive value of the Bsissa by increasing the amount of free amino nitrogen. In addition, it was an efficient way to extend the shelf life of the Bsissa by the combination of the heat treatment and the fermentation. In conclusion, the fermentation contributed to improve the nutritive value of the final product and to formulate a new probiotic cereal meal with original aroma, different from the traditional mix. This kind of study can facilitate the development of new, fermented, nondairy, nutritionally well-balanced food products.

Competing Interests

No conflict of interests is declared.

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