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Research Article

Optimization of Parameters Using Response Surface Methodology to Develop a Novel Kefir-Like Functional Beverage from Cheese Whey Enriched with Myrtle Juice

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Whey, liquid wastewater from cheese production, is one of the sources of dietary protein and lactose that are still largely unused for human consumption. It is only in recent years that it has aroused the interest of industries and sought as a valuable raw material and thus represents an opportunity for the manufacture of new products. The manufacture of fermented whey drink requires the mixing of whey with fruit juice or an aromatic plant to improve its organoleptic properties and acceptability. Myrtle, an aromatic medicinal plant, known for its health benefits is not well exploited for making dairy products. This is the first report on the development of kefir-myrtle beverage. Three factors were optimized (whey permeates (%), myrtle's juice (%), and kefir grains as inoculum (%)) using a central composite design with response surface methodology. The analyses showed that the number of lactic acid bacteria (LAB) and yeast cells varied from 5.4 to 9.2 $\log_{10}$ CFU/mL and from 4.3 to 6.2 $\log_{10}$ CFU/mL, respectively. A decrease in pH and an increase in the total polyphenol content and antioxidant activity were observed. The analysis of variance indicated the goodness of fit of the model with R^2 from 0.827 to 0.966. The absolute average deviation values of each model were low and ranged from 1.61% to 4.23%. The optimized fermented kefir whey beverage accomplished an overall acceptability of 5.41 (1 to 9 preference scale) and a high number of LAB cells (8.53 $\log_{10}$ CFU/mL). The viability of LAB and yeast cell was maintained at 7.61 and 6.19 $\log_{10}$ CFU/mL, respectively, after 14 days of storage.

1. Introduction

The dairy industry in Tunisia is one of the important food industries in the country. This sector comprises 25 enterprises that processed daily about 3.8 million liters, and 13% of the total volume of produced milk is destined for cheese production [1]. In the past, whey was considered a byproduct, but now it is considered a coproduct. Its valorization is both an economic and ecological issue since it has a high chemical oxygen demand (COD) [2]. Indeed, when whey is discharged into rivers, it generates eutrophication problem and toxicity modifying the physicochemical properties of aquatic ecosystems [3].

The relatively new interest in this byproduct results mainly from its composition rich in proteins, lactose, and water-soluble vitamins and minerals. Furthermore, it represents a well-balanced source of essential amino acids [4].

There are different processes to value whey. Among the most used technologies are drying, evaporation, reverse osmosis, nanofiltration, and ultrafiltration. By its biochemical composition, whey can be also considered an excellent culture medium for microorganisms.

Since lactose is the main component of whey solids, various biotechnological processes have been developed to use whey as a substrate to produce important industrial products having functional properties. For example, it can be

converted into prebiotic such as galactooligosaccharides (GOS), lactulose, lactobionic acid, and tagatose [5]. In addition, lactic acid fermentation of whey makes it rich in GOS [6], which exerts a stimulating effect on the growth of probiotic bacteria [5]. Bioactive peptides manifesting antihypertensive, antioxidant, immunomodulatory, and antimicrobial activities can also be released during fermentation [7].

On the other hand, lactic acid fermentation was known as an important tool to increase the bioavailability of polyphenols in food. To date, kefir grains were largely exploited for dairy and nondairy beverages fermentation. Numerous studies described the health benefits of kefir including antihypertensive, antidiabetic, anti-inflammatory, anticancer, antioxidative, and antihypercholesterolemic properties reviewed by Azizi et al. [8]. These therapeutic's aspects made kefir a suitable proposal for commercial intention.

However, to improve whey fermented beverages' flavor and increase their consumption among young people, they should be mixed with fruits juice [9–12]. In fact, fruit and vegetables are rich in nutrients and phytochemicals such as vitamins, minerals, and phenolic compounds [13]. Due to their various micronutrients, fruits and vegetables are often considered as "functional foods" helping to prevent various diseases such as cancer, obesity, and diabetes [14].

Myrtus communis L. is an aromatic and medicinal plant belonging to the family of Myrtaceae. It is widespread in the Mediterranean regions, such as North Africa and Southern Europe, and also found in South America, Australia, and in some areas of the Himalaya [15]. Myrtle berries have a long history of application in the pharmaceutical and food industries. They contain many biologically active compounds such as phenolic compounds, flavonoids, and anthocyanins [16], which are thought to be responsible for their antioxidant proprieties. They also have various positive effects on human health, and they are used as antiseptic, analgesic, cardiotonic, diuretic, anti-inflammatory, stomachic, nephroprotective, antidote, hemostatic, brain tonic, and antidiabetic properties [16].

Experimental designs can be used as a method for formulating food products. They allow selecting the factors that influence the response, modeling the variations in the system response according to the fluctuations of the factors, and validating experimentally the model described by a mathematical equation. However, a suitable range for each factor is also an important consideration for the accuracy of the final model [17–19].

The aim of this study is to optimize the formula of a functional whey beverage. Optimization was done by a central composite design (CCD) to determine the optimum ratio of whey permeate, myrtle juice, and kefir inoculum on pH, lactic acid bacteria (LAB), and yeast viability, % radical scavenging activity, polyphenol content, and overall acceptability. From this design, the surface plots of considered responses with respective second-order polynomial models were obtained. Finally, the analysis of variance (ANOVA) was employed to judge the adequacy of the model, and the optimized formulation was validated experimentally.

2. Materials and Methods

2.1. Kefir Grains and Raw Material. Kefir grains (KG) used in this study were collected from Tunisian households and preserved by the laboratory of microbial ecology and microbial technology (LETMi, INSAT) [11, 20]. The grains were cultured in sterile cow milk and renewed daily to maintain their viability. Kefir grain is polysaccharides and protein matrixes consisting of a symbiotic community were LAB (10^8 CFU/g) and yeasts (10^5 CFU/g). The predominant LAB in the used grains are related to *Leuconostoc* spp., *Lactobacillus* spp., and *Lactococcus* spp. *Saccharomyces* spp. and *Zygosaccharomyces* spp. are the dominant yeasts [21]. Kefir grains produce 0.6% lactic acid titratable acidity.

Cheese whey was collected from an artisanal cheese maker, and whey permeate was provided by dairy industry. The composition of liquid cheese whey was lactose 5.01% (w/v), proteins 1.22% (w/v), fat 0.34% (w/v), and ash 0.8% (w/v). Whey permeate contains lactose 85% (w/v), proteins 3% (w/v), and ash 7% (w/v).

Lactose, total proteins, the total fat content, and the ash were determined using the HPLC method [22], the Kjeldahl method [23], and a solvent extraction [24] and by heating the samples at 550°C [25], respectively. The pH of the beverages was measured using a pH meter (Mettler-Toledo EL20).

Myrtle fruits (*Myrtus communis*) were collected from the area of Nefza (north-west of Tunisia, latitude 36° 58′ 31″N, longitude 9° 04′ 51″ E, altitude 500 m, far 147 km from Tunis, the capital), purchased from the local market in January 2019. The berries were washed and crushed with a mixer by adding distilled water (8°Brix). The mixture was filtered to obtain a juice that was pasteurized at 70°C for 15 min and used immediately.

2.2. Beverage Formulation Using a Response Surface Methodology (RSM). The solution of cheese whey and whey permeate were sterilized for 20 min at 120°C and then mixed with myrtle juice according to the plan of RSM. Obtained beverages (400 mL) were placed in bottles and inoculated with kefir grains (Figure 1).

Central composite design (CCD) was used to develop a novel kefir-like functional beverage from cheese whey enriched by myrtle juice (MJ). The RSM is a combination of experiment design, statistics, empirical modeling, and mathematical optimization techniques. The number of experiments to be carried out was determined rationally, which avoids redundancies of information. In addition, the implementation of an optimization procedure allows the study of the interactions between the different factors. It is possible that a factor that apparently has no effect on the phenomenon being studied and influences this phenomenon indirectly through an interaction. The construction of the response surfaces is carried out following the adjustment of the model using mathematical functions such as polynomials [26].

The central composite design used for the developing of whey kefir-like beverage consists of nineteen experiments

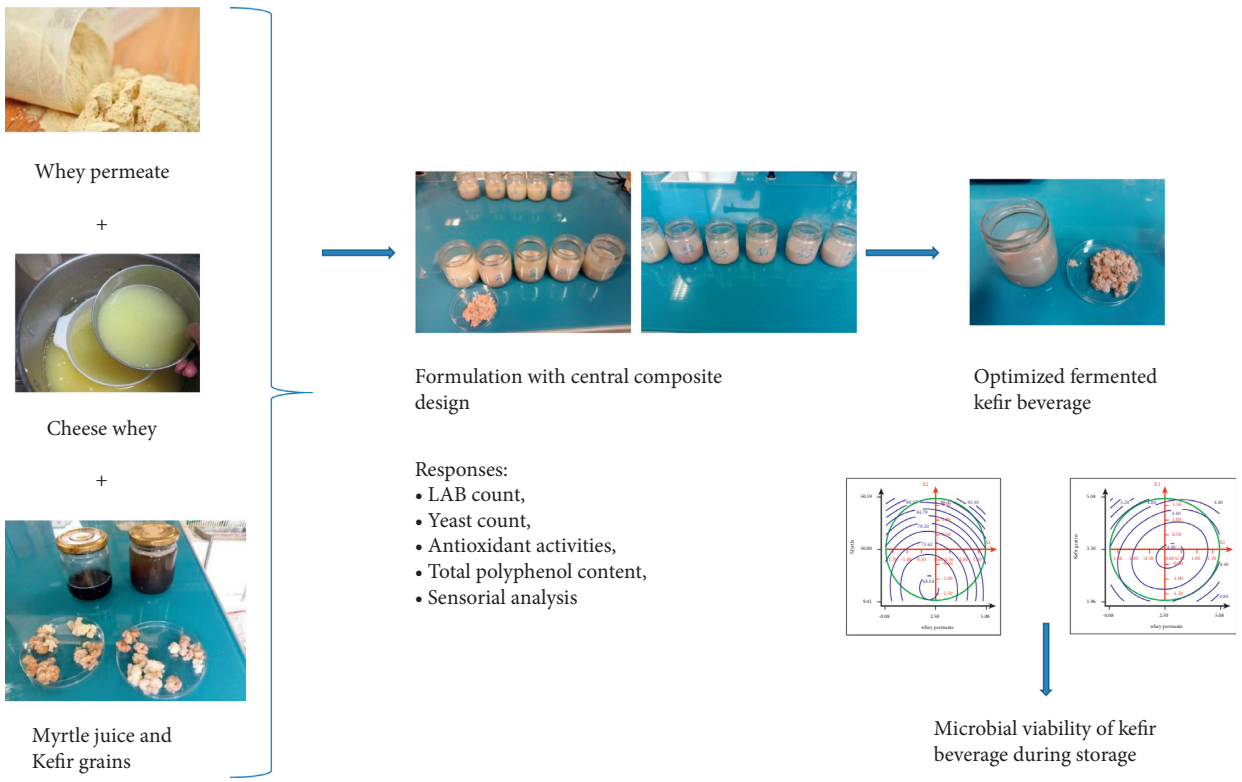

Figure 1: Whey valorization by developing a kefir-like functional beverage from cheese whey, whey permeate, and myrtle juice.

with five replicates at the central points. The independent variables (whey permeate X1, myrtle juice X2, and kefir grains inoculum X3) in the design were assigned into five levels, coded −1.68, −1, 0, 1, and 1.68 as described in Table 1. The responses were pH, lactic acid bacteria viability, yeast viability, total polyphenol content (TPC), antioxidant capacity (DPPH), and overall acceptability (OA). The results obtained from the CCD were used to fit a second-order polynomial equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b22X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3, \quad (1)$$

where Y is the predicted response; X_1, X_2, and X_3 are the independent variables; b_0 the model constant; b_1, b_2, and b_3 are the linear effect of variables; b_{11}, b_{22}, and b_{33} are the squared effect of variables; and b_{12}, b_{13}, and b_{23} are the interaction effect of variables. These coefficients were calculated using software NEMROD-W (version 99901, LPRAI Company).

2.3. Lactic and Yeast Counts. Lactic acid bacteria and yeasts were quantified using conventional culture techniques [27]. LAB were quantified on MRS agar (with cycloheximide 150 μg/mL) and yeasts on PDA supplemented with chloramphenicol (100 mg/L); they were incubated at 37°C and 30°C for 48 h, respectively. The results were expressed as CFU/mL. The numbers of LAB and yeasts were converted to log CFU/mL.

2.4. Polyphenol Content and Free Radical Scavenging Capacity (DPPH Assay). The TPC was determined following the method of Folin-Ciocalteau [28], modified by Karaaslan et al. [29]. The mixture of each beverage (0.03 mL) and distilled water (2.730 mL) was added to the Folin–Ciocalteau reagent (0.15 mL). After adding sodium carbonate (20%), the mixture was left at room temperature for 30 min. The absorbance was measured at 750 nm (Jenway 63200 UV/Vis). The TPC was expressed as mg of gallic acid equivalent/mL of the sample. The correlation coefficient was $R^2 = 0.991$.

The free radical scavenging activity of the samples was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) method according to the method proposed by Balakrishnan and Agrawal with some modifications [30]. Each sample (700 μL) was added to 700 μL DPPH methanolic solution (0.035 mol/L). The mixture was shaken and allowed to stand at room temperature for 30 min. Antioxidant capacity was measured by recording the absorbance at 517 nm using a spectrophotometer (Jenway 63200 UV/Vis). Methanol was used as the blank. All the determinations were performed in triplicate. A mixture of DPPH solution and methanol (instead of the sample) was used as the negative control for this assay. Percentage of DPPH radical scavenging activity was the result of antioxidant activity. The scavenging activity was calculated by the following equation:

$$\text{DPPH scavenging activity}\,(\%) = \frac{(A\,(\text{control}) - A\,(\text{sample}))}{A\,(\text{control})} \times 100. \quad (2)$$

Table 1: Experimental range and levels of the three factors used in the central composite design for beverage formulation.

Level	Independent variables		
	X_1 (whey permeate fortification, % w/v)	X_2 (fruit juice, % w/v)	X_3 (kefir grains, % w/v)
+1.68	5	50	5
+1	4	42	4.4
0	2.5	30	3.5
−1	1	18	2.6
−1.68	0	10	2

2.5. Sensory Evaluation. A total of 40 persons have contributed to a panel test; they were from the food technology department and the dairy industry (students and staff, ranging 24–56 ages). The evaluation was based on the hedonic sensory acceptance of beverage samples using a 9-point hedonic scale. The drinking water was given to tasters to rinse their mouth between each sample. Beverages were evaluated for color, odor, sweetness, acidity, taste, and overall acceptability [31].

2.6. Statistical Analysis. All experiments were carried out in triplicate. All data are reported as the mean ± standard deviation. Statistics were performed with the analysis of variance (ANOVA) procedure (STATIGRAPHICS 202 Centurion XVI software, Statpoint Technologies, Warrenton, USA). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

Whey-based milk drinks can sometimes have unpleasant flavors. The improvement of their organoleptic characteristics can be obtained by the addition of fruit concentrates and/or by fermentation. To obtain a novel kefir-like functional beverage, the whey was fortified by WP, and myrtle juice was then fermented by kefir grains. The effects of whey permeate; myrtle juice and kefir grains' levels on the nutritional and organoleptic characteristics of the obtained beverages were investigated using a CCD. These independent variables (WP (X_1), fruit juice (X_2), and percentage of inoculum (X_3) levels) were prescribed into five levels (−1.68, −1, 0, +1, and +1.68). Tables 2 and 3 present the experimental values of total polyphenol content (mg EGA/mL), radical scavenging activity, LAB and yeasts' viability, pH, and sensory evaluation for each run of the CCD. Results from the 19 runs were fitted to a second-order polynomial equation, and the removal of nonsignificant terms was assigned. When the values of R^2 and R^2adj. are close to 1, the results indicate the adequacy of the fitted models. Yaakob et al. [32] mentioned that R^2 should be at least 80% and Cruz et al. [33] reported that the values of R^2adj. should be over 70%. However, the calculation of R^2 and absolute average deviation values (AAD) together should be better to determine the accuracy of the model. R^2 must be close to 1.0, and the AAD between the predicted and observed data must be as small as possible [34]. AAD values of six predicted models were calculated; they were 4.23% for TPC, 1.61% for antioxidant activities, 2.75% for LAB number, 1.96% for yeasts counts, 2% for pH, and 4.13% for overall acceptability. Therefore, the model illustrates the global variability; it is predictive (Supplementary Materials (available here)).

3.1. Effects of the Variables on Total Phenolic Content and Antioxidant Activity. Myrtle is considered a preventive element against diseases related to oxidative stress. Indeed, it is rich in many chemical compounds including phenolic compounds and essential oils [35]. Many groups of phenolics were identified such as phenolic acids, hydrolyzable tannins (gallotannins), flavonoids, and anthocyanins [36]. Messaoud and Boussaid [37] reported that their antioxidant activities are due to their phenolic compounds. The TPC values of obtained beverages varied from 55.25 ± 0.68 to 90.45 ± 0.07 mg EGA/mL, and the DPPH radical scavenging from 65.25 ± 0.22 to 91.6 ± 0.29% (Table 2). R^2 and adj R^2 for TPC and DPPH radical scavenging activity are close to 1. The AAD values were 4.23% and 1.61%, respectively.

An increase in myrtle juice or whey permeate or kefir inoculum level improves the TPC and DPPH radical scavenging activity ($p < 0.001$; Table 4). It must be mentioned that a p value lower than 0.001 showed that the model is highly significant in the response. The coefficients for linear and quadratic models are also highly significant ($p < 0.01$; Table 4). The highest level of TPC and DPPH radical scavenging activity was obtained with 5.08% WP, 50.59 fruit juice (% w/v), and 5.04% inoculums (Figures 2 and 3).

With regard to interaction, myrtle juice and kefir inoculum's level affect positively the TPC and the antioxidant activity. These results could be due to the metabolic activities of microorganisms in kefir grains. In fact, microbial enzymes break down polyphenol compounds and form aglycones [38]. These latter can be also liberated from their corresponding glycosides, contributing to enhancing the bioavailability of polyphenol [39] and increasing their quantitative amount [40, 41]. Sabokbar et al. [42] showed that the addition of kefir to apple juice enhanced both the total phenolic content and antioxidant activities. During the last years, several works have been interested in the action of lactic acid bacteria and kefir microflora on phenolic compounds [43–46]; they cited many enzymes, which are involved in the hydrolysis mechanisms and which are inducible as a specific stress response.

The improvement of the antioxidant activity after fermentation (Table 5) is in line with other studies [11, 47–51]. The increase of DPPH radical scavenging of fermented beverages indicated that fermentation may produce metabolites with higher and better antioxidant activity. The release of bioaccessible phenolic compounds can be one of

Table 2: Matrix of the central composite design for three variables and the measured responses (DPPH scavenging activity and total phenolic content).

Run	Independent variables			Response variables	
	X_1 (whey permeate, % w/v)	X_2 (myrte juice, % w/v)	X_3 (kefir grains, % w/v)	DPPH scavenging activity (%)	Total phenolic content (mg EGA/mL)
1	1.0	18	2.6	68.40 ± 0.61	57.58 ± 0.28
2	4.0	18	2.6	72.60 ± 0.26	59.75 ± 0.42
3	1.0	42	2.6	71.25 ± 0.36	64.80 ± 0.49
4	4.0	42	2.6	75.60 ± 0.26	63.60 ± 1.04
5	1.0	18	4.4	70.80 ± 0.06	69.97 ± 0.08
6	4.0	18	4.4	73.60 ± 0.30	70.68 ± 0.29
7	1.0	42	4.4	91.36 ± 0.13	85.00 ± 0.04
8	4.0	42	4.4	95.95 ± 0.38	89.45 ± 0.39
9	−0.0	30	3.5	80.45 ± 0.03	82.20 ± 0.91
10	5.0	30	3.5	85.62 ± 0.01	81.69 ± 0.14
11	2.5	10	3.5	69.80 ± 0.04	55.25 ± 0.68
12	2.5	50	3.5	91.60 ± 0.29	90.45 ± 0.07
13	2.5	30	2.0	65.25 ± 0.22	69.30 ± 0.26
14	2.5	30	5.0	89.85 ± 0.20	81.20 ± 0.42
15	2.5	30	3.5	71.50 ± 0.44	63.10 ± 0.13
16	2.5	30	3.5	72.05 ± 0.15	62.50 ± 0.14
17	2.5	30	3.5	71.95 ± 0.43	63.01 ± 0.026
18	2.5	30	3.5	72.42 ± 0.02	62.90 ± 0.35
19	2.5	30	3.5	71.15 ± 0.38	63.05 ± 0.39

Table 3: Matrix of the central composite design for three variables and the measured responses (LAB viability, yeast viability, pH, and overall acceptability).

Run	Independent variables			Response variables		pH	Overall acceptability
	X_1 (whey permeate, % w/v)	X_2 (myrte juice, % w/v)	X_3 (kefir grains, % w/v)	LAB viability ($\log_{10}$ CFU/mL)	Yeast viability ($\log_{10}$ CFU/mL)		
1	1.0	18	2.6	6.28 ± 0.06	4.15 ± 0.01	5.02 ± 0.01	3.90 ± 0.10
2	4.0	18	2.6	6.80 ± 0.06	4.61 ± 0.06	4.88 ± 0.02	4.90 ± 0.20
3	1.0	42	2.6	6.02 ± 0.03	4.85 ± 0.02	4.78 ± 0.06	4.90 ± 0.10
4	4.0	42	2.6	7.39 ± 0.04	5.68 ± 0.09	4.69 ± 0.04	4.40 ± 0.10
5	1.0	18	4.4	6.98 ± 0.04	5.50 ± 0.07	4.45 ± 0.05	4.10 ± 0.20
6	4.0	18	4.4	7.09 ± 0.02	5.17 ± 0.04	4.41 ± 0.04	4.60 ± 0.10
7	1.0	42	4.4	7.10 ± 0.02	5.11 ± 0.03	4.36 ± 0.03	5.60 ± 0.10
8	4.0	42	4.4	9.23 ± 0.03	6.21 ± 0.07	4.21 ± 0.03	5.10 ± 0.20
9	−0.0	30	3.5	5.47 ± 0.09	4.79 ± 0.01	4.54 ± 0.04	4.90 ± 0.10
10	5.0	30	3.5	8.25 ± 0.04	6.02 ± 0.01	4.39 ± 0.03	4.10 ± 0.00
11	2.5	10	3.5	7.90 ± 0.02	5.19 ± 0.03	4.41 ± 0.05	4.70 ± 0.10
12	2.5	50	3.5	8.30 ± 0.03	5.90 ± 0.06	4.19 ± 0.04	5.60 ± 0.26
13	2.5	30	2.0	6.83 ± 0.06	4.39 ± 0.03	4.65 ± 0.01	4.60 ± 0.10
14	2.5	30	5.0	8.67 ± 0.04	5.29 ± 0.04	3.94 ± 0.05	4.50 ± 0.00
15	2.5	30	3.5	7.60 ± 0.07	5.10 ± 0.02	4.35 ± 0.03	4.40 ± 0.36
16	2.5	30	3.5	7.60 ± 0.03	5.31 ± 0.03	4.31 ± 0.02	3.40 ± 0.10
17	2.5	30	3.5	7.41 ± 0.04	5.25 ± 0.03	4.29 ± 0.08	4.10 ± 0.10
18	2.5	30	3.5	7.70 ± 0.03	5.19 ± 0.06	4.30 ± 0.02	3.95 ± 0.05
19	2.5	30	3.5	7.67 ± 0.02	5.26 ± 0.04	4.29 ± 0.02	4.05 ± 0.35

Overall acceptability score (9-point hedonic scale).

the reasons, and their antioxidant capacity is always associated with their health-promoting properties. Hernández-Ledesma et al. [52] reported the antioxidant activity for some peptide chains in whey, which had higher radical scavenging activity than butylated hydroxyanisole (BHA). The antioxidant activity of kefir has also been shown, and it is due to some potential compounds like glutathione, organic acids, and kefiran [8, 51, 53].

The multiple coded equations in terms of coded factors generated for these responses are shown as follows:

Table 4: Analysis terms for the quadratic model representing the investigated responses (TPC, DPPH (%), LAB and yeasts' number, pH, and overall acceptability).

Model terms	Coefficient estimate	*t*.exp	*p* value	Coefficient estimate	*t*.exp.	*p* value
Total phenolic content (mg/mL)				*DPPH scavenging activity (%)*		
b_0	63.199	584.42	***	71.950	325.27	***
b_1	0.386	5.89	**	1.804	13.46	***
b_2	7.620	116.32	***	6.255	46.68	***
b_3	6.545	99.91	***	6.241	46.57	***
b_{11}	5.148	78.57	***	3.220	24.02	***
b_{22}	1.933	29.50	***	2.394	17.86	***
b_{33}	2.781	42.45	***	1.280	9.55	**
b_{12}	0.046	0.54	NS	0.243	1.39	NS
b_{13}	0.524	6.12	**	−0.145	−0.83	NS
b_{23}	2.841	33.20	***	4.632	26.46	***
R^2	0.843			0.966		
Adj.R^2	0.686			0.931		
LAB (log_{10} CFU/mL)				*Yeasts (log_{10} CFU/mL)*		
b^0	7.614	151.07	***	5.226	145.45	***
b^1	0.645	21.12	***	0.302	13.88	***
b^2	0.239	7.83	**	0.265	12.16	***
b^3	0.513	16.80	***	0.309	14.18	***
b^{11}	−0.358	11.71	***	0.042	1.93	NS
b^{22}	0.081	2.64	NS	0.092	4.21	*
b^{33}	−0.043	−1.41	NS	−0.158	−7.24	**
b^{12}	0.359	8.99	**	0.225	7.91	**
b^{13}	0.044	1.10	NS	−0.065	−2.29	NS
b^{23}	0.241	6.05	**	−0.140	−4.92	**
R^2	0.909			0.942		
Adj.R^2	0.818			0.885		
pH				*Overall acceptability*		
b_0	4.299	386.48	***	3.984	26.50	***
b_1	−0.049	−7.31	**	−0.062	−0.68	NS
b_2	−0.080	−11.85	***	0.294	3.23	*
b_3	−0.229	−34.06	***	0.083	0.91	NS
b_{11}	0.108	15.95	***	0.164	1.80	NS
b_{22}	0.049	7.30	**	0.394	4.33	**
b_{33}	0.047	7.04	**	0.182	2.00	NS
b_{12}	−0.008	−0.85	NS	−0.312	−2.63	*
b_{13}	0.005	0.57	NS	−0.062	−0.53	NS
b_{23}	0.017	1.99	NS	0.187	1.58	NS
R^2	0.836			0.827		
Adj.R^2	0.672			0.654		

NS: nonsignificant; * $p < 0.05$, * * $p < 0.01$, and * * * $p < 0.001$.

$$\begin{aligned} \text{TPC} &= 63.199 + 0.386X_1 + 7.620X_2 + 6.545X_3 + 5.148X_1^2 + 1.933X_2^2 + 2.781X_3^2 + 0.524X_{13} + 2.841X_{23}, \\ \text{DPPH\%} &= 71.95 + 1.804X_1 + 6.255X_2 + 6.241X_3 + 3.22X_1^2 + 2.394X_2^2 + 1.28X_3^2 + 4.632X_2X_3. \end{aligned} \quad (3)$$

3.2. Effects of the Variables on pH and LAB and Yeast Cell Viability of Kefir Microorganisms. The effects of independent variables on LAB and yeast cell viability are shown in Table 3. Their number depends on whey permeate, fruit juice, and kefir inoculum level. The linear effects were significantly positive ($p < 0.001$; Table 4). The interaction effects of whey permeate-fruit juice and fruit-kefir grains were also significant ($p < 0.01$; Table 4). An increasing inoculum level allows enhancing LAB and yeasts' number. The same result was observed by Sabokbar and Khodaiyn [9] when they fermented a mixture of pomegranate juice and whey by kefir grains. They reported that the cell density of LAB increased by 1.3 Ulog when they increase the inoculum from 5% to 8%. However, for yeasts' number, nonsignificant difference was observed. M'hir et al. [11] obtained the same result when they prepared a fermented beverage from whey, whey permeate, and date syrup and when they prepared a kefir beverage made with carob, oat flour, and whey permeate [54]. This increase of LAB due to the increase of the level of juice can be explained by the probiotic effect of myrtle (Figure 4). In fact, in the studies of Mangia et al. [55] and Öztürk et al. [56], they showed that myrtle juice exerts a positive effect on lactobacilli.

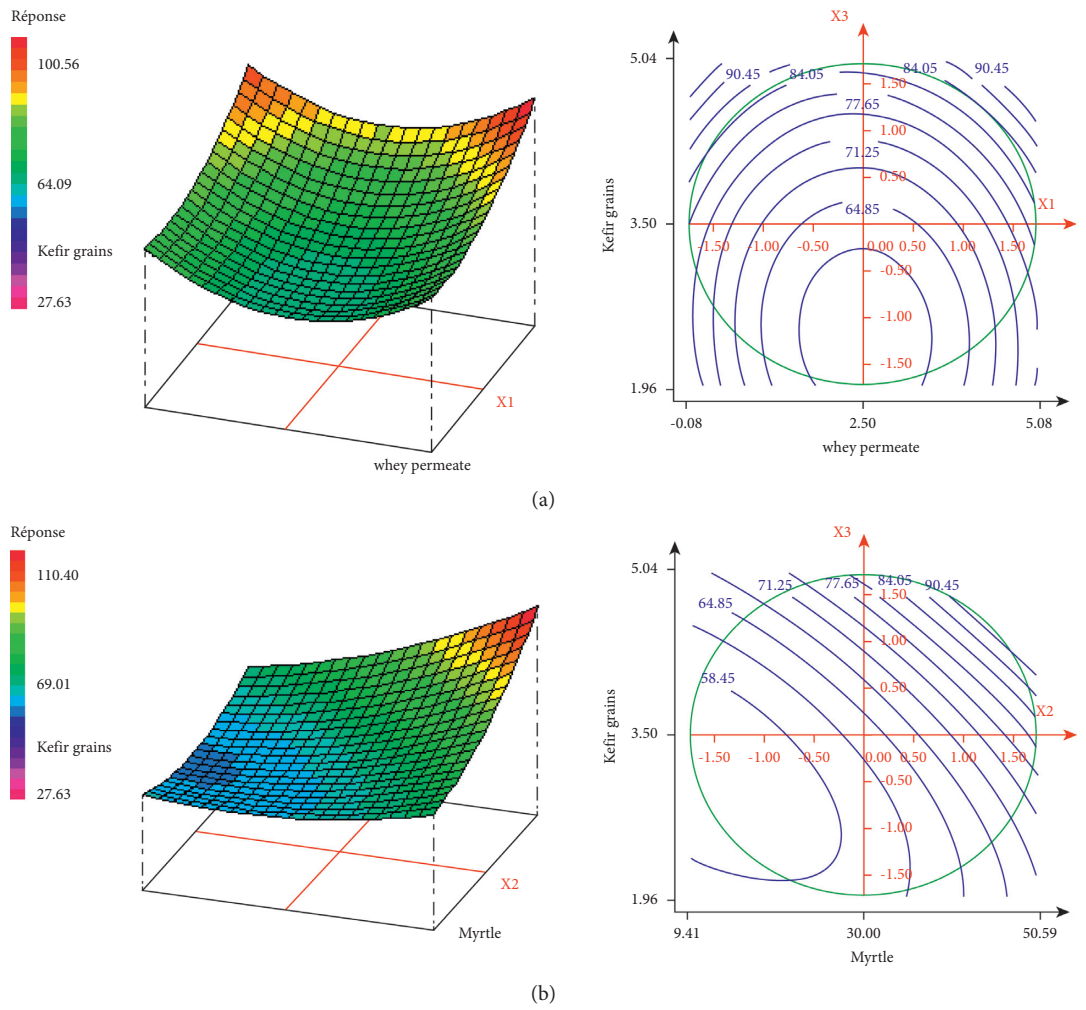

FIGURE 2: Response surface and contour plots representing the effect of WP and KG (a) and the effect of MJ and KG (b) on total polyphenol content (X_1: WP (% w/v); X_2: MJ (% w/v); and X_3: KG (% w/v)).

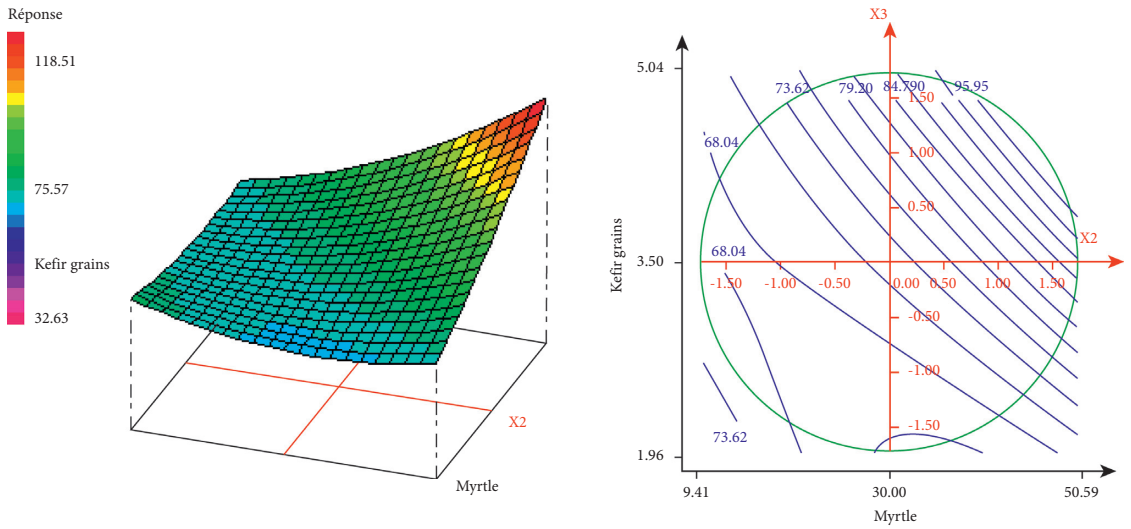

FIGURE 3: Response surface and contour plots representing the effect of MJ and KG on DPPH radical scavenging activity (%; X_2: MJ (% w/v) and X_3: KG (% w/v)).

Table 5: Levels of total phenolic content and antioxidant activities before and after fermentation for each beverage.

Exp	Total phenolic content (mg GAE/mL)		Antioxidant activities (%)	
	Unfermented	Fermented	Unfermented	Fermented
1	$48.75^{b} \pm 0.70$	$57.58^{b} \pm 0.69$	$60.88^{a} \pm 0.51$	$68.40^{b} \pm 0.67$
2	$49.88^{b} \pm 0.17$	$59.75^{c} \pm 0.56$	$63.15^{b} \pm 0.91$	$72.60^{fg} \pm 0.31$
3	$58.46^{de} \pm 0.70$	$64.80^{e} \pm 0.56$	$63.88^{b} \pm 0.45$	$71.25^{de} \pm 0.75$
4	$57.20^{d} \pm 0.96$	$63.60^{de} \pm 0.47$	$65.77^{c} \pm 0.74$	$75.60^{h} \pm 0.43$
5	$52.75^{c} \pm 0.63$	$69.97^{fg} \pm 0.49$	$60.76^{a} \pm 0.62$	$70.80^{cd} \pm 0.28$
6	$59.00^{ef} \pm 0.44$	$70.68^{g} \pm 0.39$	$65.20^{c} \pm 0.42$	$73.60^{g} \pm 0.38$
7	$57.25^{d} \pm 0.89$	$85.00^{i} \pm 0.14$	$69.45^{e} \pm 0.77$	$91.36^{l} \pm 0.70$
8	$58.91^{ef} \pm 0.96$	$89.45^{j} \pm 0.62$	$71.49^{f} \pm 0.41$	$95.95^{m} \pm 0.76$
9	$60^{fg} \pm 0.45$	$82.20^{h} \pm 0.26$	$67.77^{d} \pm 0.35$	$80.45^{i} \pm 0.65$
10	$63.89^{h} \pm 0.28$	$81.69^{h} \pm 0.70$	$71.67^{f} \pm 0.88$	$85.62^{j} \pm 0.65$
11	$45.11^{a} \pm 0.19$	$55.25^{a} \pm 0.34$	$60.78^{a} \pm 0.75$	$69.80^{c} \pm 0.24$
12	$60.68^{g} \pm 0.51$	$90.45^{j} \pm 0.79$	$75.94^{g} \pm 0.53$	$91.60^{l} \pm 0.51$
1 3	$58.30^{de} \pm 0.91$	$69.30^{f} \pm 0.77$	$60.76^{a} \pm 0.36$	$65.25^{a} \pm 0.32$
14	$58.91^{ef} \pm 0.96$	$81.20^{h} \pm 0.26$	$68.25^{de} \pm 0.91$	$89.85^{k} \pm 0.51$
15	$52.10^{c} \pm 0.28$	$63.10^{d} \pm 0.67$	$65.61^{c} \pm 0.57$	$71.50^{def} \pm 0.33$
16	$52.73^{c} \pm 0.40$	$62.50^{d} \pm 0.14$	$65.61^{c} \pm 0.12$	$72.05^{ef} \pm 0.84$
17	$51.77^{c} \pm 0.33$	$63.01^{d} \pm 0.09$	$65.96^{c} \pm 0.15$	$71.95^{def} \pm 0.84$
18	$52.77^{c} \pm 0.45$	$62.90^{d} \pm 0.84$	$65.23^{c} \pm 0.30$	$72.42^{ef} \pm 0.79$
19	$52.05^{c} \pm 0.44$	$63.05^{d} \pm 0.93$	$66.12^{c} \pm 0.12$	$71.15^{de} \pm 0.30$

Values are expressed as mean ± standard deviation (three determinations). Data in the same row with different superscript letters are significantly different at $p < 0.05$.

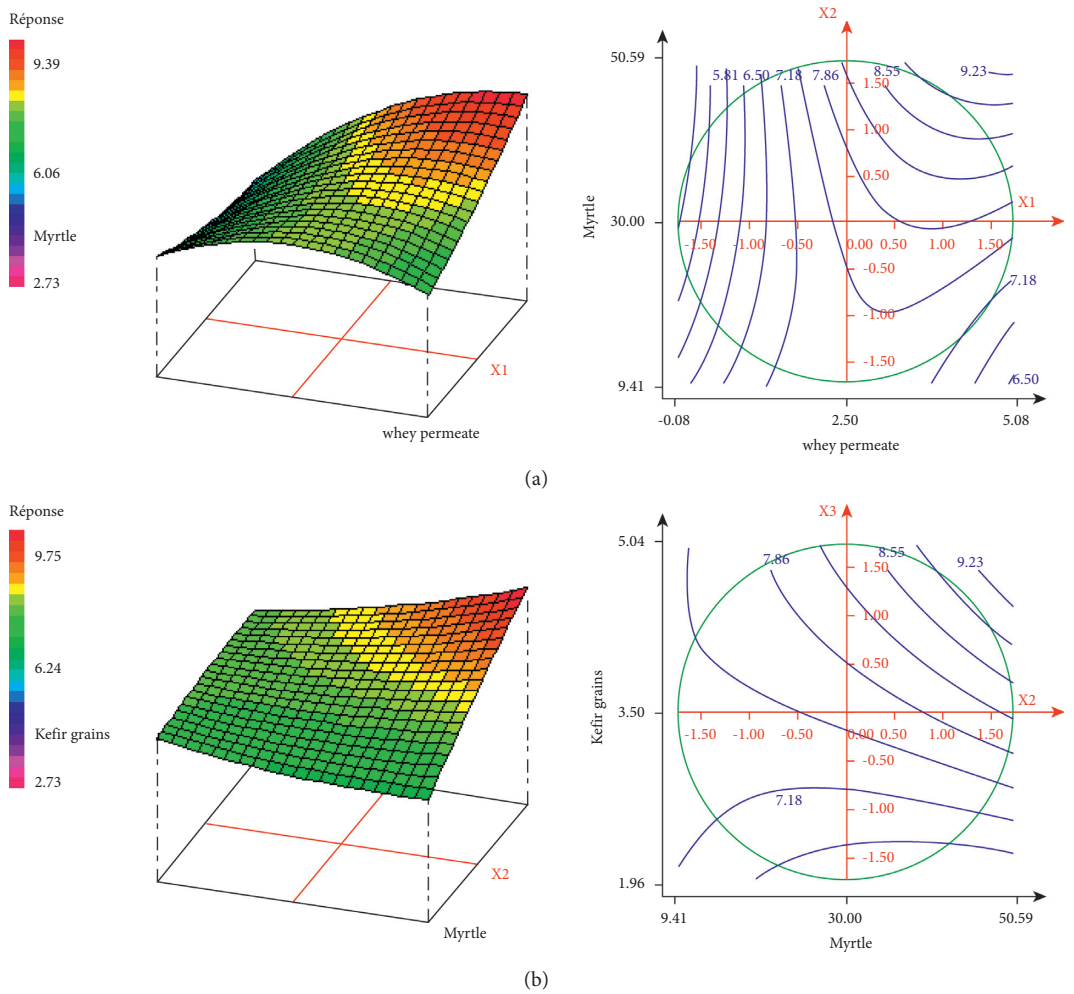

Figure 4: Response surface and contour plots representing the effect of WP and MJ (a) and the effect of MJ and KG (b) on LAB count (X_1: WP (% w/v); X_2: MJ (% w/v); X_3: KG (% w/v)).

The positive interaction between myrtle juice and kefir inoculum on cell density was significant ($p < 0.01$; Table 4). Filannino et al. [45] showed that phenolic compounds can exert a positive effect on LAB growth.

As a consequence of kefir microflora growth, the pH decline during fermentation (pH ranging between 5.02 and 3.94; Table 3) due to lactic acid increase, which can inhibit spoilage and pathogenic bacteria development in the beverage [9]. The pH decrease was significantly related to whey permeate ($p < 0.01$), juice ($p < 0.001$), kefir grains ($p < 0.001$), and their quadratic effects ($p < 0.01$). However, the pH was not significantly influenced by their interaction (Table 4).

The number of viable cells of yeast and LAB of the obtained beverage were in accordance with codex Standard FAO/WHO [57], suggesting at least 10^4 and 10^7 CFU/mL of bacteria and yeast counts, respectively.

The multiple coded equations in terms of coded factors generated for these responses are shown as follows:

$$\begin{aligned}
\text{LAB} &= 7.614 + 0.645X_1 + 0.239X_2 + 0.513X_3 - 0.358X_1^2 + 0.359X_{12} + 0.241X_{23},\\
\text{Yeasts} &= 5.226 + 0.302X_1 + 0.265X_2 + 0.309X_3 + 0.092X_2^2 - 0.158X_3^2 + 0.225X_{12} - 0.14X_{23},\\
\text{pH} &= 4.229 - 0.049X_1 - 0.08X_2 - 0.229X_3 + 0.108X_1^2 + 0.049X_2^2 + 0.047X_3^2.
\end{aligned} \tag{4}$$

3.3. Effects of the Variables on Sensory Evaluation. Sensory analysis is a real lever for the development of new food products. Indeed, the characterization of their organoleptic properties is essential to guarantee their acceptability by consumers.

Only myrtle juice linear and quadratic terms (b_2 and b_{22}) have relevant effects on the overall acceptability (Table 4). As noticed in Figure 5, a significant interaction was detected between whey permeate (X_1) and myrtle juice (X_2). The OA scores ranged from 3.9 ± 0.1 to 5.6 ± 0.26 on a 9-point hedonic scale (Table 3). The most appreciated samples were the beverages from experiment 7 (1% WP, 42% myrtle juice, and 4.4% kefir grains) and 12 (2.5% WP, 50% myrtle juice, and 3.5% kefir grains). The acceptability ratings were highest in the samples containing a high amount of fruit juice. It can be explained by the fact that Myrtle's juice masked the unsavory taste of cheese whey. In the study of Öztürk et al. [56], black and white myrtles improved the taste scores of probiotic goat milk ice cream samples by masking the low pH resulting from fermentation. Koksoy and Kilic [58] showed that acid odor was masked with the fruit aromas resulting in more acceptable drinks for consumption.

The level of juice and whey used in many studies was different; it largely depends on the fruit matrix. For example, Islam et al. [12] suggested that the optimum formula was with 25% whey and 75% pineapple juice. However, Pereira et al. [59] added only 10% of mango fruit to liquid whey protein and concentrated permeates.

Phenolic compounds contained in myrtle juice have also an impact on the obtained beverages' sensory attributes. Indeed, they have an important effect on color, perceived taste, and flavor. Pinto and Vilela [60] reported that different color palettes may influence our taste and flavor perception. The polynomial model for OA is presented by the following equation:

$$\text{OA} = 3.984 + 0.294X_2 + 0.394X_2^2 - 0.312X_1X_2. \tag{5}$$

3.4. Validation. Validation tests were performed in order to determine the LAB and yeasts' cell count, the pH, the TPC, the DPPH antioxidant activity, and OA under optimized condition (2.83% (w/v) at whey permeate, 48.45% (w/v) myrtle juice, and 3.71% (w/v) inoculum). The validation results are demonstrated in Table 6 in which the number of LAB and yeasts, the antioxidant activity, TPC, and the OA were mentioned. TPC showed a lower value than the predicted ones. Taking into account the standard deviations of measured and calculated responses, the results obtained indicated that the experimental values were in good agreement with the predicted values. This suggested that the fitted model is satisfactory and accurate.

3.5. Microbial Viability of Kefir Culture and the Change in Physicochemical Parameters during Storage. The results for the effects of storage temperature at 4°C on the counting of LAB and yeasts and on physicochemical parameters of the developed beverage are shown in Table 7. A reduction in the load of LAB was observed from the first day of storage. LAB were more sensitive to storage than yeasts. Indeed, the loss in the viability of LAB cells was more important than this of yeasts. A very little and nonsignificant decrease of yeasts counts was recorded after 14 days of storage at 4°C. However, the viability is maintained during the two weeks of storage at a high level for all tested microflora as recommended by FAO/WHO 2006 [57]. The number of LAB and yeast viable cells was 7.61 ± 0.14 and 6.19 ± 0.24, respectively, after 2 weeks of storage at 4°C. These results were in agreement with the reports of previous investigators [59, 61]. The microorganisms' viability largely depended both on medium composition and on storage temperature. The pH influence on cell viability has been widely mentioned [62]. Existing exopolysaccharides, as kefiran, might help improve the survival of microorganisms in an acidic or frozen medium by giving a protective envelop that may preserve them from stressful conditions. The resistance of LAB in acidic conditions is due to the action of the proton pump, the change in their cell membrane composition, and other mechanisms [63]. Nualkaekul and Charalampopoulos [64] suggested that *Lactobacillus* probably uses the energy

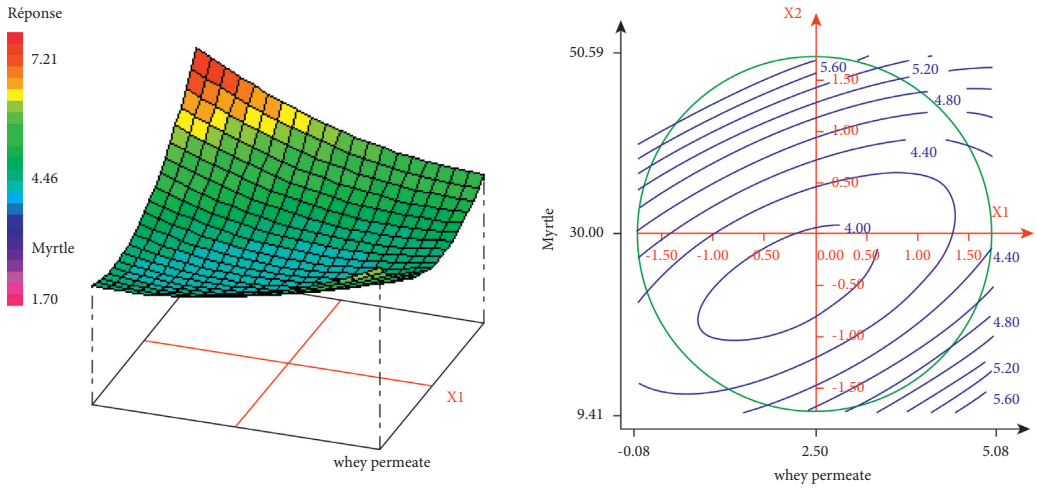

Figure 5: Response surface and contour plots representing the effect of WP and MJ on overall acceptability (X_1: WP (% w/v) and X_2: MJ (% w/v)).

Table 6: Predicted and experimental values of responses under optimum conditions.

Response variables	Experimental	Predicted
TPC (mg GAE/mL)	81.56 ± 0.78	82.77
DPPH (%)	91.61 ± 0.61	91.26
LAB viability ($\log_{10}$ CFU/mL)	8.53 ± 0.30	8.64
Yeast viability ($\log_{10}$ CFU/mL)	6.21 ± 0.27	6.01
pH	4.18 ± 0.02	4.24
OA score	5.41 ± 0.25	5.37

Mean values ± standard deviation (duplicate determination); OA score on 1–9 scale (with 100% d (i): the percentage of calculated desirability).

Table 7: Microbial viability and the change in physicochemical parameters during refrigerated storage.

	LAB ($\log_{10}$ CFU/mL)	Yeast ($\log_{10}$ CFU/mL)	Total polyphenol content (mg EAG/mL)	Scavenging activity (%, DPPH)	Overall acceptability (1–9 scale)	pH
0j	$8.53^{a} \pm 0.60$	$6.21^{a} \pm 0.04$	$81.56^{d} \pm 0.50$	$91.61^{d} \pm 0.63$	$5.41^{ab} \pm 0.12$	$4.18^{d} \pm 0.02$
1j	$7.85^{a} \pm 0.18$	$6.55^{a} \pm 0.09$	$76.13^{c} \pm 0.67$	$88.44^{c} \pm 0.71$	$5.53^{b} \pm 0.17$	$3.99^{c} \pm 0.02$
7j	$7.89^{a} \pm 0.14$	$6.23^{a} \pm 0.16$	$73.86^{b} \pm 0.67$	$86.73^{b} \pm 0.42$	$5.44^{ab} \pm 0.07$	$3.82^{b} \pm 0.02$
14j	$7.61^{a} \pm 0.14$	$6.19^{a} \pm 0.24$	$69.12^{a} \pm 0.15$	$84.57^{a} \pm 0.21$	$5.13^{a} \pm 0.06$	$3.69^{a} \pm 0.01$

Mean ± SD. Data in the same row with different superscript letters are significantly different at $p < 0.05$.

generated through their metabolic activity in order to maintain their viability. During the storage time, the pH value decreased to 3.69 with the progress of the storage period. This acidification through storage could be caused by residual microbial activity. The same result was observed by Islam et al. [12].

The storage has a significant impact on polyphenol content and antioxidant activity. A significant decrease of TPC and of DPPH free radical scavenging activity (%) was observed during storage. The loss of phenolic compounds could be explained by their oxidation.

On day 1 of the postproduction, the sensory properties of the fermented beverage have been slightly improved and then decreased under refrigerated storage. The decrease in product quality was linked to the increase in the contents of organic acids. Indeed, a mild increase in acidity was observed from the second day of storage.

4. Conclusions

The supplementation of cheese whey allowed developing kefir-like beverage having functional proprieties such as high polyphenol content and good antioxidant capacity. The RSM was successfully employed to optimize the LAB and yeast cell counts, the TPC, the antioxidant activity, and the OA with the incorporation of different concentrations of WP, myrtle juice, and kefir grains. The beverage obtained by mixing (2.83% whey permeate and 3.71% kefir grains) gave acceptable sensorial properties. The enumeration of LAB and yeast cells showed that the obtained beverage fulfilled the criterion of probiotic beverage in an acceptable manner even after 14 days of storage at 4°C. Sensory attributes may be ameliorated with further investigations like using myrtle syrup and/or adding another carbon source.

Data Availability

The data used to support the findings of this study are included within the article (Tables 2–7).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Analyses of variance of central composite design are provided in Supplementary Materials. (*Supplementary Materials*)

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