Analysis of Volatile Compounds in Probiotic Yogurt During Storage Through Solid-Phase Microextraction Gas Chromatography

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ABSTRACT

Two different yogurts, control and probiotic with *Bifidobacterium* BB-12 were produced and analyzed for their contents of total solids, proteins, pH, counts of probiotic bacteria, and volatile composition during refrigerated storage for 28 days. The response surface methodology (RSM) was used to optimize the extraction of volatile compounds from the probiotic yogurt containing through HS-SPME combined with gas chromatography–mass spectrometry (GC–MS). Post-acidification and decrease in protein content were noted in both yogurts during storage. The results showed that the extraction temperature and the addition of salt were statistically the most influential factors for the extraction of higher amounts of volatile compounds. The volatile compounds detected in the probiotic yogurt were 2-butanone, 2,3-butanedione, 2,3-pentanodione, acetone and hexanoic acid. During the 28 days of storage, the only differences noted were between the amounts of 2,3-butanedione, 2,3-pentanodione and hexanoic acid.

Keywords: Probiotic yogurt, volatile compounds, Bifidobacterium BB-12, solid-phase microextraction, GC-MS, response surface methodology

1. INTRODUCTION

Yogurt is a very popular fermented milk product, widely consumed all over the world. The production of high-quality yogurt requires control of several factors such as the chemical composition of milk base, type of milk, processing conditions and types of starter culture used to produce aroma compounds during incubation period for the manufacture of yogurt [1]. One possible method of enhancing those properties further is by creating yogurt that contains probiotics. Probiotics are live microorganisms which when administered in adequate amounts confer health benefits [2] by improving microbial balance in the host's gut flora and defenses against pathogenic microorganisms. The species which are most frequently used as probiotics belong to the genera Lactobacillus and *Bifidobacterium* [3]. *Bifidobacterium* BB-12® is a probiotic microorganism that is widely consumed in the form of probiotic yogurt. Probiotic yogurt containing this microorganism is reported to have beneficial

effects on metabolism preventing gastrointestinal illness [4]. However, it is crucial that the viable counts of probiotic bacteria not decreased below to 6 log CFU/ml throughout the product's shelf life. Thus, they are in sufficient numbers in order to exert the desired therapeutic effects [3].

One of the basic parameters through which starter cultures for yogurts are characterized is their ability to produce volatile compounds. The aroma and flavor of yogurt and dairy products occur basically because of the production of non-volatile and volatile acids and carbonyl compounds [5]. Carbonyl compounds and free fatty acids in yogurt are influenced by the type of starter culture, type and quality of raw milk, incubation, cooling and storage [6]. Even though *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are lactic acid bacteria used for yogurt production, variations in the strains affect the synthesis of carbonyl compounds [5].

Volatile compounds are generally present in trace amounts and require analysis through gas chromatography (GC) coupled to mass spectrometry (MS), with a prior step involving the extraction and pre-concentration of the volatile fraction. This analysis has been a challenge to many researches. Chen [7] reported that different techniques have been applied for the extraction and concentration of the volatile flavor compounds in yogurt and other cultured dairy products. However, many different methods are time-consuming, expensive and likely to introduce artifact resulting from sample preparation and solvent interaction steps. The solid-phase microextraction (SPME) method has become the method of choice for aroma analysis, allowing solvent-free, rapid sampling with low cost and ease of operation [8]. In addition, it is sensitive, selective and also compatible with low detection limits [7]. Considering that SPME is a technique based on physicochemical processes of equilibrium between the matrix and the headspace, and between the headspace and the material coating the fiber, the success of its use depends on factors such as the chemical nature of the compounds to be extracted, the temperature used during extraction and the extraction time to the headspace [8]. However, due their advantages, SPME has been widely used in the extraction volatile and semi-volatile compounds from biological, environmental, food and drink samples [7]. By using headspace (HS) SPME, it is possible to reduce matrix effects and any other interferences present in the liquid sample. On other hand, equilibrium is reached faster through HS-SPME than through direct immersion (DI) SPME as there is no liquid to stop diffusion of the analytes onto the coating [9].

In relation to dairy products, the SPME technique has been used to determine the shelf life of yogurt and of fresh cheese [10], to provide a quantitative analysis of thermally derived off-flavour compounds of milk [11], and to assess the impact of processing and/or storage on the stability of the flavor of whey powders [12]. Therefore, the aim of this work was to optimize the extraction of volatile compounds of probiotic yogurt by using the response surface methodology (RSM) based on HS-SPME combined with gas chromatography–mass spectrometric (GC–MS) in order to extract, identify and quantitatively monitor the concentration of selected volatile compounds of the probiotic yogurt during refrigerated storage for 28 days.

2. MATERIAL AND METHODS

2.1 Material

Commercial pasteurized milk (3 g fat/100 ml), thermophilic culture (YCX-11®, Chr. Hansen, Honsholm, Denmark) containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, and probiotic culture composed of *Bifidobacterium* BB-12 (BB-12®, Chr. Hansen, Honsholm,

Denmark) were used for sample preparation. MRS agar (Merck, Darmstadt, Germany), lithium chloride (Vetec, Rio de Janeiro, Brazil), sodium propionate (Vetec, Rio de Janeiro, Brazil) and AnaeroGen® (Oxoid, Hampshire, UK) were used for the microbiological analysis. Acetone (2-propanone), diacetyl (2,3-butanedione), 2,3-pentanodione, 2-butanone and hexanoic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the reagents were either of analytical grade or chromatographic.

2.2 Manufacture of yogurts

Two yogurts, one denoted as control and the other as probiotic, were manufactured according to the procedures of Almeida et al. [13], with modifications. Aliquots of the milk (1 l) were heated to 42 ± 1 ºC and inoculated with thermophilic culture, while in the probiotic yogurt *Bifidobacterium* BB-12 was also added. The cultures were used in the following concentrations, 0.0032 g/100 ml and 0.0200 g/100 ml, respectively. Both yogurts were incubated at 42 ± 1 ºC until pH 4.6 was reached. After fermentation, the yogurts were cooled to 4 ± 1 ºC, gently stirred, put into plastic pots sealed with aluminum and then stored in refrigeration (4 ± 1 ºC) until analyses were done. All analyses were performed on days 1, 14, and 28 of storage.

2.3 Microbiological analysis

The viability of *Bifidobacterium* BB-12 in the probiotic yogurt was evaluated. For the enumeration of probiotic culture, the MRS Agar modified with addition of 0.2 g/100 ml of lithium chloride and 0.3 g/100 ml of sodium propionate (LP-MRS) were used as proposed by Vinderola and Reinheimer [14]. The plates were incubated in anaerobic jars containing AnaeroGen® at 37 ± 1 ºC for 72 h. After this incubation period, the count of viable probiotic cells was carried out, expressed as log of colony-forming units per milliliter (log CFU/ml). The analyses were carried out in triplicate.

2.4 Physicochemical analysis

The yogurts (control and probiotic) were investigated for total solids by drying to constant weight at 85 ºC and for protein content through the Kjeldahl method (N x 6.38) [15]. The pH values were determined with a pH meter (Quimis, model Q-400A, Brazil) through the potentiometric method. All the analyses were carried out in triplicate.

2.5 Analysis of volatile compounds by gas chromatography-mass spectrometry

2.5.1 Optimization of headspace solid phase microextraction (HS-SPME) parameters

The volatile compounds of the samples were extracted through the headspace method. A randomized 23 central composite design (CCD) along with response surface methodology (RSM) was used to study extraction temperature (40 to 60 ºC), extraction time (30 to 50 min) and the effects of ionic strength through addition NaCl (0 to 6 g) on the amount of volatile compounds adsorbed by SPME fiber from the probiotic yogurt. The experimental design was composed of seventeen combinations of the independent variables; eight factorial points (levels -1 and 1), six axial points (level -1.682 and 1.682) and three repetitions in the central point, as shown in Table 1. Due to systematic errors, all the experiments were carried out at random in order to minimize the effect of unexplained variability on the responses obtained. The response evaluated during all the experiments was the total sum of the peak areas, obtained in the GC-MS analysis. SPME was performed with a commercially available fiber housed in its manual holder (Supelco, Bellefonte, PA, USA). All extractions were carried out

using a DVB/CAR/PDMS (divinylbenzene/ carboxen/ polydimethylsiloxane) fiber, 50/30 µm film thickness (Supelco, Bellefonte, PA, USA). Prior to use, the fiber was conditioned at 270 ºC for 1 hr. Twenty gram sample amount was put into 40 mL glass vials with a valve cap (Supelco, Bellefonte, PA, USA). During the extraction, the samples were stirred continuously with a magnetic stir bar on a stir plate spinning at 750 rpm. The fiber was carefully put in the same place for each exposure for the headspace to obtain maximal repeatability. After sampling, the SPME fiber was introduced into the GC-MS injector and kept in the splitless mode and maintained at 270 ºC for 10 min for thermal desorption of the analytes. Each sample was analyzed in triplicate, using a fresh vial and aliquot for each replicate.

Table 1: Central composite design (CCD) with the independent variables and their levels used for the experimental designa.

Tests	Levels		
	Extraction temperature (ºC)	Extraction time (min)	Salt concentration (g NaCl)
1	-1 (40)	-1 (30)	-1 (0)
2	1 (60)	-1 (30)	-1 (0)
3	-1 (40)	1 (50)	-1 (0)
4	1 (60)	1 (50)	-1 (0)
5	-1 (40)	-1 (30)	1 (6)
6	1 (60)	-1 (30)	1 (6)
7	-1 (40)	1 (50)	1 (6)
8	1 (60)	1 (50)	1 (6)
9	-1.68[b] (38.32)	0 (40)	0 (3)
10	1.68 [b] (61.68)	0 (40)	0 (3)
11	0 (50)	-1.68 [b] (28.32)	0 (3)
12	0 (50)	1.68 [b] (51.68)	0 (3)
13	0 (50)	0 (40)	-1.68 [b] (1.68)
14	0 (50)	0 (40)	1.68 [b] (7.68)
15	0 (50)	0 (40)	0 (3)
16	0 (50)	0 (40)	0 (3)
17	0 (50)	0 (40)	0 (3)

[a] *Factors coded (in bracket) and reals levels used in the full experimental design for extraction of volatile compounds.*

[b] *α = ± 1.68 for three independent variables.*

2.5.2 GC-MS analysis

A Shimadzu GC-2010 gas chromatography coupled to a mass spectrometer was used to analyze the components in the headspace of the samples. Helium (99.999 %) was used as carrier gas. The capillary column used was Rtx-5MS (30 m x 0.25 mm i.d. x 0.25 µm df) (Restec, USA). Column temperature was held at 40 ºC for 1 min and increased to 120 ºC at a rate of 4 ºC/min, and finally to 280 ºC at a rate of 15 ºC/min. The temperature of the injector was 270 ºC and the time of desorption of the fiber into the injection port was 10 min. The temperature of the detector was 250 ºC. Electron impact mass spectra were recorded at a voltage of 70 eV over the 40-400 m/z mass range.

2.5.3 Component identification

Volatile compounds predominant were identified by comparing their experimental spectra with those of NIST'98 [16], and by comparison of their retention times with authentic standards.

2.5.4 Quantitative analysis

Acetone (2-propanone), diacetyl (2,3-butanedione), 2,3-pentanodione, 2-butanone and hexanoic acid were quantified. Each quantified peak was required to have a minimum signal-to-noise ratio (S/N) of 5. Quantitative results were obtained by using the method of standard addition. Standard solutions were added to multiple aliquots of a sample of yogurt. The sample without standard solutions was also analyzed. The samples were extracted and analyzed through HS-SPME/GC-MS, as previously described. The compounds were quantified based on a calibration curve that was generated by plotting the detected response versus the amount spiked from each standard. Each sample measurement was repeated three times.

2.6 Statistical analysis

The regression coefficients for linear quadratic and interaction terms were determined by using multiple linear regression (MLR). A Student's t-test was used to verify the statistical significance of the regression coefficients derived from the model. From manufacture of yogurts, three experimental trials were carried out in independent days and three replicates were analyzed each time. The analysis of variance (ANOVA) was applied to validate the model and to determine significant differences between the samples of the yogurts in all the parameters investigated. The regression coefficients were then used to generate response surfaces. All the calculations and graphics of the experimental design were performed by using the STATISTICA 13.3 software (TIBCO Software Inc., Palo Alto, CA). A difference was considered statistically significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Microbiological analysis

In relation to the cell viability of *Bifidobacterium* BB-12, the yogurt was considered probiotic as there was no decrease in viable cell count between the 1st and the 28th day of refrigerated storage (Table 2). Tripathi and Giri [3] stated that the recommended count of viable probiotic cells for a probiotic food should be equal to or greater than 6 log CFU/ml during storage and the best way to administer probiotics is by regular ingestion, which confers the presence of these microorganisms in high numbers in the intestine, either maintaining or improving intestinal microbial balance. Similar results on the survival of *Bifidobacterium* were found by Saarela et al. [17], who evaluated the cell stability of *B. animalis* subsp. lactis in skim milk and in fruit juices and observed that the cells were stable in milk for only two weeks, whereas the same stability was not noted in the juices. Cunha et al. [18] evaluated the stability of bifidobacteria in fermented lactic beverage added with whey and also noted the stability of probiotic bacteria during storage of their products.

3.2 Physicochemical analysis

Mean values for total solids, protein and pH of both types of yogurt are shown in Table 2. When compared to the samples on the same days of storage no differences ($P < 0.05$) were noted in total solids content, indicating that there were no changes due to processing. These results were lower than those obtained by Cunha et al. [18] with fermented milk made with no addition of whey.

In both yogurts, the values for protein decreased during the storage period ($P < 0.05$). Similar protein values were obtained by Thamer and Penna [19] in probiotic milk added with

whey. According to Donkor et al. [20] both the probiotic bacteria and the bacteria used in yogurt production need peptides and amino acids for their growth. The primary enzymes of lactic bacteria, which are responsible for proteolysis of milk proteins, offer an increase of amino acid and nitrogen necessary for the fermentative bacteria, causing a decrease in protein content.

The pH values were similar to those found in probiotic yogurt containing bifidobacteria by Kempka et al. [21]. Lankaputhra and Shah [22] reported that the pH range between 4.0 and 5.0 is ideal for maintaining the viability of probiotics. During storage, post-acidification of the yogurts was observed; however, their pH still remained within the recommended ranges. Kailasapathy [23] stated that, when at refrigeration temperatures between 0 and 5 ºC, the maintenance of β-galactosidase activity is responsible for post-acidification of fermented milk and also that refrigeration temperature and storage time of fermented milk would account for the variation in pH.

Table 2: Viable *Bifidobacterium* BB-12 counts, total solids, protein and pH of yogurts, on day 1, 14 and 28 of storage at 5 ± 1 ºC.

Yogurts	Days	Viable counts (log CFU/ml)	TS[d] (g/100g)	Protein[e] (g/100g)	pH
Control	1	-	11.28 ^{A,a} ± 0.02	2.76 ^{A,a} ± 0.33	4.75 ^{A,a} ± 0.01
	14	-	11.23 ^{A,b} ± 0.04	2.72 ^{A,a} ± 0.01	4.74 ^{A,a} ± 0.00
	28	-	11.33 ^{A,a} ± 0.05	2.58 ^{A,b} ± 0.00	4.62 ^{A,b} ± 0.00
Probiotic	1	7.9	11.26 ^{A,a} ± 0.02	2.73 ^{A,a} ± 0.00	4.62 ^{B,a} ± 0.00
	14	7.8	11.14 ^{A,b} ± 0.05	2.65 ^{B,b} ± 0.01	4.61 ^{B,b} ± 0.00
	28	7.8	11.20 ^{B,b} ± 0.01	2.67 ^{B,b} ± 0.03	4.39 ^{B,c} ± 0.01

$^{A-B}$ Within a column, different superscript uppercase letters denote significant differences ($P < 0.05$) amongst control and probiotic yogurts for the same periods of storage.
$^{a-c}$ Within a column, different superscript lowercase letters denote significant differences ($P < 0.05$) among the different periods of storage for each studied yogurt.
[d] TS= Total Solids.
[e] Proteins = Total nitrogen x 6.38.

3.3 Analysis of volatile compounds by gas chromatography-mass spectrometry

3.3.1 Optimization of HS-SPME parameters

Table 3 shows the effects observed on the studied factors in the response of the volatile compounds extracted from the probiotic yogurt besides those caused by the interactions among such factors. The t-test for the model was significant ($P < 0.05$) for the quadratic coefficient of extraction temperature and addition of salt (NaCl) and for interaction between extraction time and addition of salt, thus indicating that only these variables can adequately explain the variation noted in the extraction of volatile compounds within the levels studied in this work.

The model built for the volatile compounds of the probiotic yogurt is represented by Equation (1), and the answer (A) is the total chromatographic peak area. A response surface was plotted to facilitate the visualization of the significant factors derived from the statistical analysis (Figure 1).

$$A = -458.006 + 20.295\,T - 0.204\,T^2 + 6.6485\,s - 1.690\,s^2 + 0.139\,t\,s \quad (1)$$

where T (°C) is the extraction temperature, s (g NaCl) is the amount of salt added and t (min) the extraction time.

The optimum region of volatile compounds extraction from the probiotic yogurt was obtained at 50 ºC with 5 g of NaCl. A similar temperature was used by Contarini and Povolo [24] in the extraction of volatile compounds from milk. The use of high temperatures during headspace extraction may selectively concentrate certain volatiles on the displacement of others.

Table 3: Results of the variance profile of volatile compounds of probiotic yogurt through SPME and GC-MS.

	Sum of squares	DF[c]	Mean square	*F* value	*P* value
Linear					
Temperature (ºC) (L)[a]	0.355	1	0.355	0.001763	0.967680
Time (min) (L)	28.862	1	28.862	0.143297	0.716244
Salt (g) (L)	50.893	1	50.893	0.252677	0.630622
Quadratic					
Temperature (ºC) (Q)	1596.636	1	1596.636	7.927125	0.025940[d]
Time (min) (Q)[b]	251.653	1	251.653	1.249429	0.300551
Salt (g) (Q)	1086.836	1	1086.836	5.396025	0.053164[d]
Interaction					
1L/2L	676.523	1	676.523	3.358863	0.109508
1L/3L	124.624	1	124.624	0.618747	0.457312
2L/3L	1139.306	1	1139.306	5.656529	0.049001[d]
Model fit	771.163	5	154.233	0.482930	0.778744
Pure error	638.737	2	319.369		
Total SQ	8227.464	16			

[a]*L= linear effect; bQ= quadratic effect; cDF= degrees of freedom. d Values significantly differents (P < 0.05).*

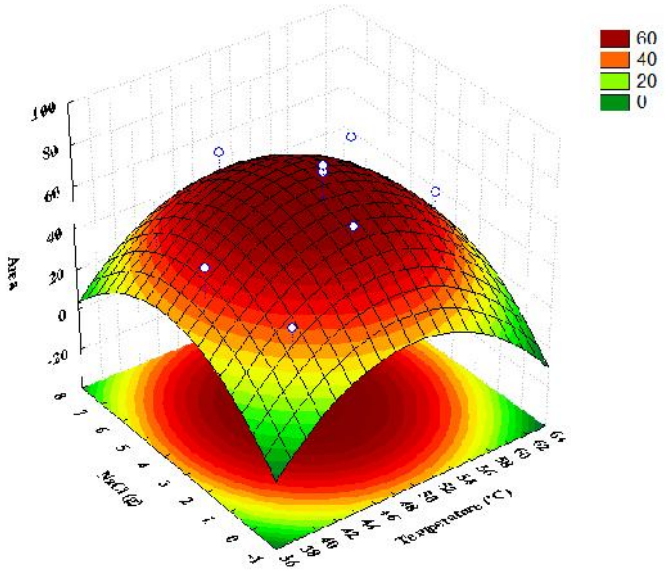

Fig. 1. Response surface obtained by central composite design using coded variables where the response was total chromatographic peak area. Extraction time set at 45 min.

As reported by Yang and Peppard [25], the addition of salt increased the sensitivity of the extraction of volatile compounds by SPME due to the “salting out” effect.

It is important to assess the fitted model to ensure that it provides sufficient approximation to the results obtained in the experimental conditions. The normality of the data, which was checked by using a normal probability plot of the residuals and the difference between the observed and predicted values from the regression, showed that the experimental points were normally distributed around the line, indicating that the normality assumption was satisfied. A determination coefficient value (R^2) of 0.83 was obtained for this model, which indicates a good fit between the observed and the predicted response values. The plots of the residuals versus the predicted values (Figure 2) showed that the residuals were scattered randomly around zero. Thus, the variance analysis results were valid as the model assumptions were satisfied.

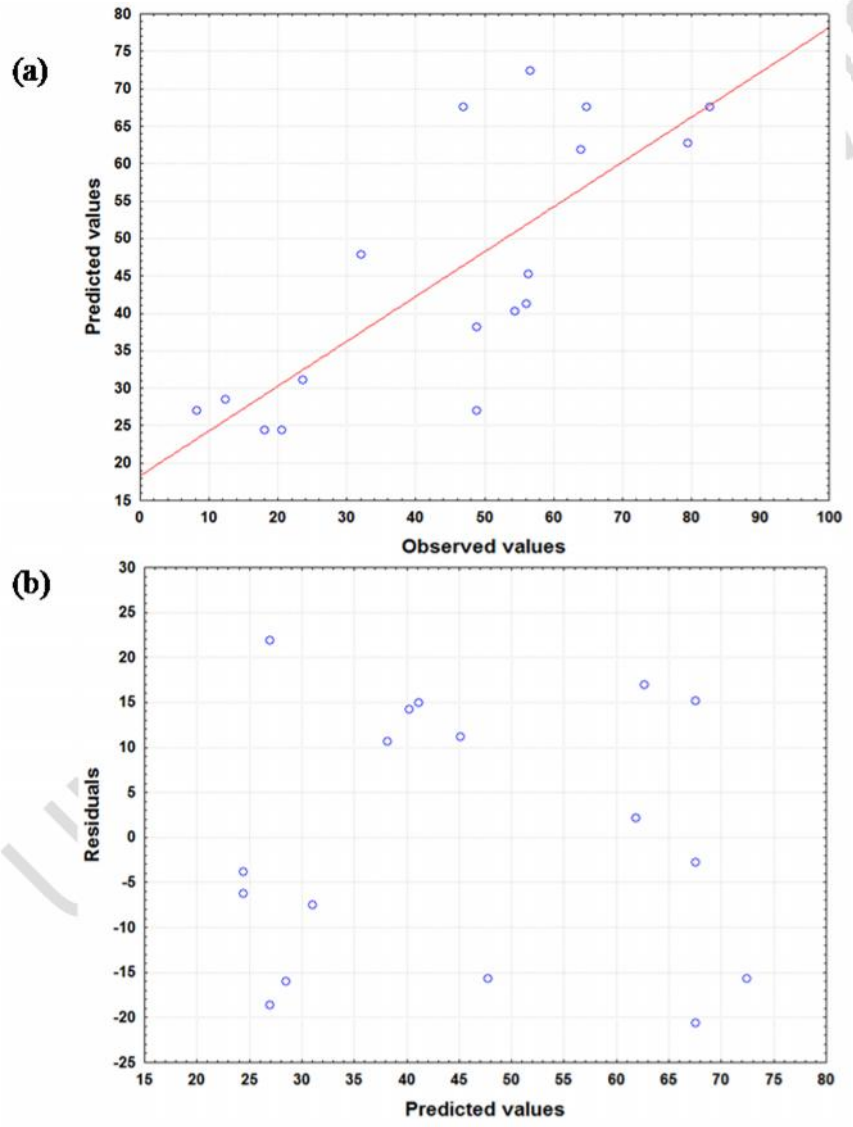

Fig. 2. (a) Plot of the predicted versus observed values. (b) Plot of residuals versus observed for total area of volatile compounds in probiotic yogurt.

3.3.2 Component identification and quantitative analysis of volatile compounds through GC-MS

The volatile compounds detected in the probiotic yogurt were 2-butanone, 2,3-butanedione, 2,3-pentanodione, acetone and hexanoic acid. These compounds were previously described by Imhof et al. [26] and Ott et al. [6] as impacting on the flavor of yogurt. However, different strains of probiotic bacteria can produce different aroma profiles. Cruz et al. [27] and Cruz et al. [28] evaluated the effect of the addition of glucose oxidase in stirred probiotic yogurt added of *B. longum*, and observed the production of aroma compounds diacetyl and acetaldehyde.

In the present work, the volatile composition of the probiotic yogurt was stable during the 28 days of storage. Condurso et al. [10] and Chen [7] reported that volatile compounds are formed due to numerous biochemical changes which occur during the fermentation process and storage of yogurt. Zourari et al. [29] stated that diketones, 2,3-butanedione and 2,3-pentanodione in yogurts come only from pyruvate, since thermophilic starter cultures are not able to metabolize citrate. According to Tsau et al. [30] and Monnet and Corrieu [31] species of *S. thermophilus* possess an α-acetolactate synthase and an acetohydroxy acid synthase, which produce α-acetolactate and 2-hydroxyacetolactate, respectively, from pyruvate. As reported by Monnet and Corrieu [31], both these α-aceto acids are generally metabolized into more neutral compounds to maintain pH homeostasis. These acids can be converted either into 2,3-butanedione and 2,3-pentanodione by spontaneous decarboxylation or into branched-chain amino acids in milk, such as valine, leucine or isoleucine, by means of enzymatic mechanisms. Tsau et al. [30] reported that methyl ketones such as 2-butanone and acetone (2-propanone) derive from β-oxidation of saturated free fatty acids and from decarboxylation of β-ketoacids and, therefore, they depend on the lipolytic activity of yogurt strains.

Probiotic yogurts showed the same volatile compounds profile, and the quantification was carried out in the probiotic yogurt sample during the storage period. The volatile compounds contents are shown in Table 4. During the 28 days of storage, only the differences between the amounts of 2,3-butanedione, 2,3-pentanodione and hexanoic acid ($P < 0.05$) were observed. On the first day of storage, the compound 2-butanone was detected in larger quantities, while on the last day (28) 2,3-butanedione was the major compound. This result is consistent with a research by Xu et al. [32], who quantified the volatile compounds in fermented milk prepared with probiotics and noted predominance of 2,3-butanedione. However, Vazquez-Landaverde et al. [11] noted 2,3-butanedione as the component in second largest quantity present in milk samples. The concentration of 2,3-pentanodione increased during the 28 days of storage ($P < 0.05$). Similar results were obtained by Gallardo-Escamilla et al. [33], with 0.07 mg of 2,3-pentanodione per kilogram of yogurt.

Table 4: Concentration (mg/kg) of the volatile compounds from probiotic yogurt during storage at 5 ± 1 ºC.

Compounds	Days of storage		
	1	14	28
2-butanone	2.93 [a] ± 0.88	0.75 [b] ± 0.15	3.11 [a] ± 0.21
2,3-butanodione	2.72 [b] ± 0.30	2.94 [b] ± 0.34	4.92 [a] ± 0.17
2,3-pentanodione	0.05 [c] ± 0.02	0.09 [b] ± 0.01	0.13 [a] ± 0.02
Acetone	2.40 [a] ± 0.25	1.89 [b] ± 0.16	2.63 [a] ± 0.23
Hexanoic acid	0.85 [c] ± 0.14	1.48 [b] ± 0.15	1.92 [a] ± 0.22

[a-c] *Different letters in the same row indicate significant differences between means ($P < 0.05$).*
[d] *Mean ± standard deviation ($n=3$).*

The acetone content detected in the probiotic yogurt (2.40 mg/kg) remained stable during storage and was higher than that obtained by Serra et al. [34] in yogurts. Kneifel et al. [35] analyzed samples of yogurt containing *Bifidobacterium* spp. and detected significant amounts of 2-butanone, 2,3-butanedione and acetone, which are consistent with some of the compounds detected in this present work.

The concentration of hexanoic acid increased over the period of refrigerated storage ($P < 0.05$). Different results were obtained by Condurso et al. [10], who analyzed yogurt samples after 30 days of refrigerated storage and noted hexanoic acid amounts of 4.9 mg/kg and 2.1 mg/kg for 2,3-butanedione. Finally, it was verified that the profile of volatile compounds hardly changes during refrigerated storage.

4. CONCLUSION

It was observed post-acidification and decrease in protein content in probiotic yogurt during storage. The results showed that the extraction temperature and the addition of salt were statistically the most influential factors for the extraction of higher amounts of volatile compounds. Thus, the optimum region of volatile compounds extraction from the probiotic yogurt was obtained at 50 ºC with 5 g of NaCl. The volatile compounds detected in the probiotic yogurt were 2-butanone, 2,3-butanedione, 2,3-pentanodione, acetone and hexanoic acid. During the 28 days of storage, the only differences noted were between the amounts of 2,3-butanedione, 2,3-pentanodione and hexanoic acid.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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