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Natamycin and nisin to improve shelf life and minimize benzene generation in lemon soft drinks

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Abstract

Benzoic acids preservatives may be converted to benzene in soft drinks. The use of alternative antimicrobial compounds, specifically nisin and natamycin, would reduce benzene formation. Initially, doses of nisin and natamycin were tested against Lactobacillus plantarum lactic bacteria and Zygosaccharomyces bailii yeast. Using 167 UI/mL of nisin and 0.017% w/v of natamycin no microbial growth was reached on lemon-flavored soft drinks. The benzene formation was analyzed in a standard formulation (380 mg/L of sodium benzoate and 180 mg/L of potassium sorbate) and nisin and natamycn formulation (167 UI/mL of nisin and 0.017% w/v of natamycin). The soft drinks were stored without UV exposure at 20 °C and with UV sunlight exposure at 30 °C, for 120 days at PET bottles. The initial benzene content was 1.29 µg/kg for standard and 0.09 µg/kg for nisin and natamycin formulation; these values increased to 11.00 µg/kg and 0.18 µg/kg, respectively. The sensory attributes did not differ from each formulation, either initially or after 120 days of storage. The lower benzene concentrations obtained using nisin and natamycin suggest that these compounds are attractive alternatives to benzoic acids, keeping the sensory quality and microbial stability during shelf life.

Keywords: natamycin; nisin; soft drinks; benzene; preservatives.

Practical application: Natamycin and nisin was used to produce a stable soft drink with sensory quality and less benzene.

1 Introduction

The manufacturing of carbonated soft drinks is a very large industry (Fabietti et al., 2001). Soft drinks are susceptible to microbial spoilage because their high carbon-to-nitrogen (C/N) ratio and low pH (3.5) allows the growth of acetic and lactic acid bacteria, molds, and yeasts (Belletti et al., 2007). Benzoic and sorbic acids have been used as preservatives in soft drinks (Arisseto et al., 2013), but by associated of benzoic acid to ascorbic acids can generate a benzene accumulation in soft drinks (Nyman et al., 2010). At low pH, highly reactive hydroxyl radicals can form by an ascorbic acid-assisted pathway, catalyzed by iron (Fe^{3+}) and/or copper (Cu^{2+}) (Nyman et al., 2010). The hydroxyl radical is thought to react with benzoic acid to generate an unstable benzoic acid radical, which subsequently loses CO_2 to form benzene (Nyman et al., 2010; International Council of Beverages Associations, 2006). Elevated benzene content of soft drinks was firstly detected by the industry in 1990-1991, especially with high heating and light exposure (International Council of Beverages Associations, 2006).

Natamycin and nisin, which are generally recognized as being safe (Kallinteri et al., 2013; Li et al., 2012) can be used of alternative to place of sodium benzoate and reduce the benzene formation (International Council of Beverages Associations, 2006; Nyman et al., 2010). The use of nisin is approved as an antimicrobial in food (Aouadhi et al., 2014). Secreted by *Lactococcus lactis* subsp. *lactis*, nisin is a hydrophobic, cationic polypeptide (Aouadhi et al., 2014) with more antimicrobial activity against Gram-negative bacteria (Kallinteri et al., 2013). Nisin can be used in litchi juices (Li et al., 2012), cheeses (Aly et al., 2012), beer and other beverages (Delves-Broughton, 2005).

Natamycin is a macrolide polyene antifungal, produced by the aerobic fermentation of *Streptomyces natalensis* (Tsiraki & Savvaidis, 2014). Natamycin is a natural fungicide commonly employed in the food industry, especially for dairy products (Kallinteri et al., 2013). Because natamycin is very poorly absorbed, there is an adequate margin of safety in its current applications, and there is no concern for the induction of antimicrobial resistance (European Food Safety Authority, 2009).

This study evaluated the ability of antimicrobials natamycin and nisin to increase the shelf life, inhibit microbial growth and decrease benzene formation, compared to the preservative sodium benzoate in lemon-flavored soft drinks.

2 Material and methods

2.1 Microorganisms and preservatives

The yeast strain used as indicator strain in this study was *Zygosaccharomyces bailii* BCV 08, from the yeast collection of Laboratory of Mycology from Federal University of Rio Grande

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do Sul (UFRGS). The yeast was maintained in Potato Dextrose Agar (Acumedia, Lansing, MI, USA) and at 4 °C. The bacteria strain used was *Lactobacillus plantarum* ATCC 8014 and was maintained in Orange Serum Agar (Acumedia) at 4 °C.

The natamycin and nisin were commercial products, Natamax® and Nisaplin® respectively, obtained from Dupont™ Danisco® (Reigate, UK). Each 1 g of Nisaplin® contains approximately 2.5% (w/w) nisin (10^6 UI/g of Nisaplin®); Natamax® had a content of 50% (w/w) of natamycin. Sodium benzoate and potassium sorbate were purchased from Emerald Kalama Chemical, LLC (Kalama, USA).

2.2 Antimicrobial effects

The effects of natamycin and nisin singly and combined were evaluated. For natamycin, three doses were tested: i) 0.017% (w/v), ii) 0.025% (w/v) and iii) 0.050% (w/v). Nisin was used in the following doses: i) 167 IU/mL, ii) 250 IU/mL and iii) 500 IU/mL. So, to reach a nisin activity concentration of 500 IU/mL, 0.5 mg of Nisaplin® was dissolved in 1 mL. The doses of the natural preservatives were defined based on Brazilian food laws, the European Food Safety Authority (European Food Safety Authority, 2009) and information obtained from the suppliers of the commercial products.

The tests with antimicrobial agents were conducted on flat-bottomed 96-well microtiter plates (Steels et al., 2000). Starter cultures of *Z. bailii* (24 hours, 28 °C, 120 rev/min) or *L. plantarum* were accurately diluted in YPD or MRS broth, respectively, to obtain an optical density (600 nm) of 0.004 to 0.008, with approximately 10^5 CFU/mL, and then diluted 100X prior to inoculation. Aliquots of 20 µL of inoculum were transferred to each well on a flat-bottomed 96-well microtiter plate, with 100 µL of YPD or MRS broth and 80 µL of preservative solution. The plates were capped, sealed and incubated for 5 days at 28 °C (yeast culture) or 37 °C (bacteria culture) and, after the optical density was measured at 600 nm (SpectraMax MI5, Molecular Devices, CA, USA). The positive control was performed in wells without preservative addition, and the negative control was performed without the microbial inoculum.

The effect of each preservative was evaluated by the decrease of the optical density at 600 nm value compared with the positive control, calculating the microbial inhibition index (%). Also, the antimicrobial effect of nisin and natamycin were compared with traditional weak acids preservatives in soft drinks, using 180 mg/L of potassium sorbate and 380 mg/L sodium benzoate.

2.3 Lemon soft drinks production and shelf life

Lemon-flavored soft drinks were prepared aseptically in 600-mL PET bottles based on Brazilian legislation standard norms. Table 1 lists the specifications of the different formulations used in the study. The simple syrup was prepared with cane sugar solution in mineral carbonated water to obtain a final sugar value of 65 °Brix.

The bottles were stored using two different conditions: (i) 20 ± 2 °C without light exposition (best condition) and (ii) 30 ± 2 °C with light exposition (critical condition). The storage conditions were performed according to typical shelf life used by retailers on Brazilian market. Therefore, as the benzene formation is accelerated by UV exposition (Nyman et al., 2010) and this effect could be assessed also.

Table 1. Specifications of lemon soft drinks used in the tests. The values with same letters in the line are not significantly different according to Tukey's test (95%).

Parameter	Standard formulation	Formulation with nisin and natamycin
Sugars (°Brix)	9.35 ± 0.1[a]	9.5 ± 0.1[a]
Titratable acidity (g of citric acid/L)	0.14 ± 0.05[a]	0.12 ± 0.06[a]
pH	2.97 ± 0.04[a]	3.03 ± 0.05[a]
Turbidity (NTU)	3.7 ± 0.25[a]	3.45 ± 0.05[a]
Density (g/cm^3)	1.034 ± 0.005[a]	1.035 ± 0.005[a]
Carbonic gas pressure (v/v)	3.8 ± 0.02[a]	3.75 ± 0.04[a]
Sodium benzoate (mg/L)	380	-
Potassium sorbate (mg/L)	180	-
Nisin (UI/mL)	-	167
Natamycin (% w/v)	-	0.017

The dosages of natamycin and nisin in the soft drink formulations were defined by primary experiments with indicator microorganisms. All results were compared with the control standard formulation of lemon soft drinks and the shelf life test was conducted under controlled temperature conditions during 120 days. The samples were collected four times during this period: (I) *SF0* - shelf life start, (II) *SF1* - after 45 days, (III) *SF2* - after 90 days, and (IV) *SF3* - after 120 days, at shelf life ending. The composition, microbial stability and sensory quality of soft drinks were evaluated in each sample collected.

2.4 Analytical procedures

The membrane filtration method was used to count the viable cells in samples. 100 mL of lemon soft drink were mixed with 100 mL of 0.02% peptone water (Oxoid) and then filtered aseptically though membranes with pore sizes of 0.41 µm and 0.80 µm (Merck Millipore, Billerica, MA, USA) for bacteria and yeast/molds counter, respectively, as proposed by Akond et al. (2009). The membranes were disposed in Petri dishes containing Plate Count Agar (Acumedia) supplemented with 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) to enumerate the active total bacteria and at 35 °C for 48 hours. Orange Serum Agar (Acumedia) was used to enumerate the lactic bacteria (25 °C for 120 hours) and Potato Dextrose Agar (Acumedia) and 28 °C for 96 hours for yeast and molds couting. The results are expressed in logarithms of the number of colonies (log CFU/mL). To confirm the acid lactic bacteria, each colony growth was tested for catalase production (Mohd Adnan & Tan, 2007).

The soft drinks were maintained for 30 minutes in an ultrasonic bath (Hanna Struments, São Paulo, Brazil) to eliminate de carbonic gas. The pH was measured using a pH meter (Hanna Instruments) equipped with a glass electrode; the total acidity (g/L of citric acid), sugars (°Brix, refractometer; Instruterm, São Paulo, Brazil) and turbidity (NTU; Hanna Instruments) measurements were performed in triplicate following AOAC official methods (Association of Official Analytical Chemists, 2002).

Benzene was measured according to Arisseto et al. (2013) using headspace-solid phase microextraction and gas chromatography/mass spectrometry (HS-SPME-GC/MS). For the SPME, a 75-μm fiber of CAR/PDMS was used at 40 °C. The GC/MS system consisted of a HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a mass spectrometer (MSD 5973; Agilent) and capillary column HP-INNOWAX of 60 m X 0.25 mm X 0.25 μm (Agilent). Helium was used as the carrier gas at a flow rate of 0.7 mL/min. The injector temperature was at 230 °C and oven temperature program was started at 30 °C (for 0.1 min), then increased at 2 °C/min to 40 °C (5 min), and 12 °C/min to 200 °C. The quadrupole and ionization source were maintained at 150 °C and 230 °C, respectively.

2.5 Sensory analysis

The multiple comparison test was used, placing the natamycin and nisin formulations side by side of standard formulation (lemon soft drink with 180 mg/L of potassium sorbate and 380 mg/L sodium benzoate), used as control sample. A five-point scale was used, starting at moderately better than the standard (5) to moderately worse than the standard (1), as proposed by Lawless & Heymann (2010). The test was realized by an expert panel (n=15), trained to identify the sensory attributes of soft drinks. The assessors evaluated four different sensory attributes important to sensory quality of soft drinks: sweet flavor, acid flavor, bitterness and chemical-type flavor notes. Sensory analyzes were carried out in a climatized (22 °C) individual cabins, and evaluated under white light, thereby ensuring comfort and privacy for the assessors.

The samples (50 mL of lemon soft drinks at 8 °C) were coded and presented randomly to the assessors. Soft drinks samples were used in pre-testing panel-test sessions to let the assessors familiarize with the products under investigation, the scale used and the terminology related. Those sessions were also used to standardize panel's attributes definitions and assessors perception.

2.6 Statistical analysis

The results were subjected to one-way ANOVA test for significant differences between the different time and conditions of soft drinks storage, also in sensory tests analysis. When significance was reached, Tukey's (HSD) post-hoc test (with a confidence interval of 95%) was performed. These analyses were performed using the Statistica 7.0 software package (Statsoft, Tulsa, OK, USA) and all the experiments were performed in triplicate.

3 Results and discussion

3.1 Antimicrobial effect of nisin and natamycin

The natamycin and nisin effect against *Z. bailii* and *L. plantarum* cultures was examined. In soft drinks, for selective conditions and composition (low pH and high sugar content), the growth of lactic acid bacteria and yeasts and molds is favored (Ndagijimana et al., 2004; Akond et al., 2009; Fitzgerald et al., 2004; Ilaslan et al., 2014). Nevertheless, even at minor dosage of natamycin and nisin, was possible verify a great inhibitory index. The Figure 1 shows the inhibitory index (%) of the preservatives against spoilage microorganisms.

Initially, the maximal recommended doses of both preservatives were used (0.1% w/v of natamycin and 500 UI/mL of nisin). Then, half reduction of these doses, the inhibition index was maintained (Figure 1) and reduced again. The utilization of lower doses is advantageous to decrease the formulation costs and the negative sensorial characteristics produced in soft drinks. As a result, the minimal doses used (0.017% w/v of natamycin and 167 UI/mL of nisin), were sufficient to controlling the growth of standard microorganisms.

The natamycin is considered to be a fungicide with a dose-dependent effect (Kallinteri et al., 2013); as a result, for all the tested doses, the yeasts growth was completely inhibited (Figure 1). Natamycin acting directly on the cell wall (te Welscher et al., 2008) and is commonly used in cheeses (Ollé Resa et al., 2014) and in beverages as soft drinks (Nyman et al., 2010).

Regarding the bacteria control, the maximum inhibition average of *L. plantarum*, achieved with 0.1% (w/v) natamycin, was approximately 25% (Figure 1). *L. plantarum* was completely inhibited (100%) only with nisin employment even with the low level tested. The total inhibition (100%) of yeasts was only observed for the application of natamycin, at all tested doses. This effect is due to the action spectrum of the preservatives tested (Nyman et al., 2010). On the other hand, the data suggested that a total inhibition (100%) of microorganisms was possible by the simultaneous application of natamycin and nisin.

Nisin has a related effect with food composition and its utilization is recommended to improve the quality and stability of innumerable foods (Aly et al., 2012). For example, in Port Salut cheese, a film of natamycin (0.027% w/w) inhibits the growth of *Saccharomyces cerevisiae*, and a film of nisin (272 UI/g) and natamycin (0.027% w/w) controls the growth of *Listeria innocua*, allowing the consumer to receive a safer

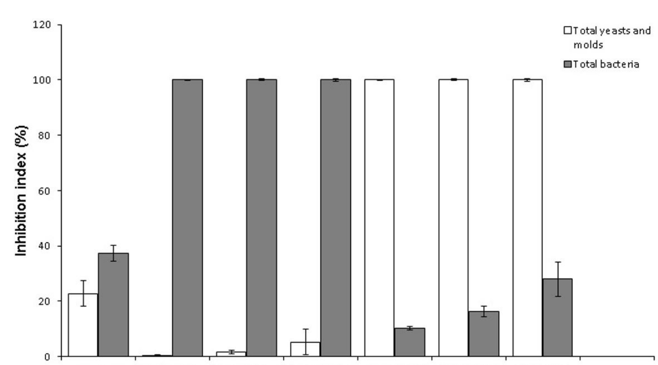

Figure 1. Inhibition index (%) of microorganisms growth test on microplates using various nisin doses (167 UI/mL: Nis167; 250 UI/mL: Nis250 and 500 UI/mL: Nis500) and natamycin doses (0.017%: Nat0.017; 0.025%: Nat0.025 and 0.050%: Nat0.050) and Standard (180 mg/L of potassium sorbate and 380 mg/L sodium benzoate). The standard error bars were calculated using triplicate testing.

product (Ollé Resa et al., 2014). To control the growth of lactic bacteria in ale beer, a minimum of 37.5 mg/mL of nisin is required (Delves-Broughton, 2005).

3.2 Microbiological changes during shelf life

Soft drinks produced were considered stable to microbial contaminations of lactic bacteria, molds and yeasts, even after 120 days and for both tested conditions. For the shelf life, were applied the minimal doses of natural preservatives (167 UI/mL of nisin and 0.017% of natamycin). The Figure 2 shows the effect of each preservative against bacteria and yeasts after 120 days.

The microbial population in lemon soft drinks after shelf life was significantly reduced comparing of 20 °C and 30 °C of storage temperature in both formulations (Figure 2). Using sodium benzoate and potassium sorbate, at the end of the shelf life, 2 log CFU/100 mL of yeasts and molds, 2 log CFU/100 mL of lactic bacteria and 5 log CFU/100 mL of active bacteria were observed. Using the formulation with nisin and natamycin the microbial growth was preserved below 1 log CFU/100 mL, for all conditions tested.

When using nisin and natamycin, the microbial stability was better than the formulation with sodium benzoate and potassium sorbate, indicating that its use may extend the shelf life of soft drinks. Microbial growth was shown even using sodium benzoate (380 mg/L) and potassium sorbate (180 mg/L) (Figure 2). Maximal levels permitted by Brazilian laws (500 mg/L of sodium benzoate and 200 mg/L of potassium sorbate) must be applied combined and can inhibits yeast, molds and bacteria growth (World Health Organization, 2000).

The antimicrobial action of weak-acid preservatives is pH-dependent, and it has been established that they are much more active in acidic environments (Martorell et al., 2007). Yeasts show tolerance to low pH and are associated to spoilage of soft drinks (Martorell et al., 2007). For this reason, the yeast growth would be achieved even using benzoic and sorbic acid. This pH effect can be avoided using alternative preservatives such as nisin and natamycin. Nisin is very stable at pH range of 3.0 to 3.5 (Delves-Broughton, 2005). Natamycin also is stable at low pH values, but its activity can be reduced by low pH value of 3.0 (Tsiraki & Savvaidis, 2014). The lemon-flavored soft drinks used have a pH value about 3.0, which can maintain the stability of nisin and natamycin.

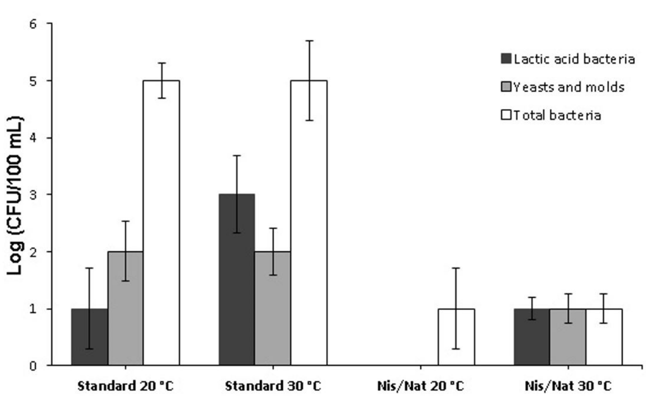

Figure 2. Growth of microorganisms from formulations of lemon soft drinks after 120 days using standard formulation (potassium sorbate and sodium benzoate) and formulation containing nisin and natamycin. The standard error bars were calculated based on triplicate testing.

High levels of yeasts could lead to alterations and decrease the quality of soft drinks (Belletti, et al., 2007). Moreover, the high sugars and salts concentration in soft drinks favor osmophilic yeast development, such as *Zygosaccharomyces* sp., *Rhodototula* sp. and *Pichia* sp. (Pribylova et al., 2003). The common yeasts alteration is characterized by substantial carbonic gas production, which causes PET packaging deformation, sediment production and a fermentative aroma bouquet (Loureiro & Querol, 1999). The formation of carbonic gas is not a health risk but causes visual damage to the package and increases economic losses for the industry (Ndagijimana et al., 2004).

Concerning the condition at 30 °C and after 120 days, was reached a total level of 3 log CFU/100 mL for lactic bacteria, 5 log CFU/100 mL for active bacteria and 2 log CFU/100 mL for yeasts. For the natamycin and nisin soft drink, the microbial growth was not detected at 20 °C, but at 30 °C, 1 log CFU/100 mL was observed for the bacteria and yeast counts. The temperature may have increased yeast and bacterial growth in both lemon soft drink formulations. On other hand, the antimicrobial activity of natamycin and nisin is very stable even to increasing temperature (Ollé Resa et al., 2014; Delves-Broughton, 2005).

3.3 Sensory and composition evaluation

The total acidity, pH, sugars value and turbidity were evaluated as these parameters represent important factors for control the soft drink quality. All parameters were initially within acceptable legal and quality limits for lemon-flavored soft drinks. Significant differences were not observed for sugars values when comparing the beginning and the ending of shelf life for both formulations and conditions tested (Table 2).

An increase of acidity and pH value decrease, from 45 days of storage, was observed (Figure 3A and 3B). The pH increases slightly between 45 and 90 days, and was reduced after 120 days of storage (Table 2). This effect was significant (95%) when compared the acidity and pH level from the initial to the end of storage time (120 days), for all formulations and conditions tested. The enhancement of acidity and pH decline were also verified in standard formulation, confirming that the use of nisin and natamycin not modify these lemon soft drinks parameters.

An increase on acidity may have been due to chemical equilibrium modifications inside the PET bottles (Nyman et al., 2010). During the storage, precipitation and dissolving of salts (Dias et al., 2011) and even the incorporation of oxygen during bottling and storage (Arisseto et al., 2013) are common, modifying their compositions and pH value. PET bottle permit gas exchanges and allows light to pass through (Katoch et al., 2010), which would cause alterations on pH and acidity levels (Nyman et al., 2010).

The sensory characteristics were measured using the multiple comparison test. The formulation with nisin and natamycin was considered equal to formulation with sodium benzoate

Table 2. Quality parameters and benzene accumulation in lemon soft drinks formulations during shelf life at different conditions. The values with same letters in the line are not significantly different according to Tukey's test (95%).

Parameter	Standard formulation				Nisin and natamycin formulation			
	0 days		120 days		0 days		120 days	
	20 °C	30 °C	20 °C	30 °C	20 °C	30 °C	20 °C	30 °C
Sugars (°Brix)	9.6 ± 0.15[a]	9.6 ± 0.1[a]	9.8 ± 0.12[a]	9.8 ± 0.15[a]	9.5 ± 0.1[a]	9.5 ± 0.1[a]	9.9 ± 0.25[a]	9.6 ± 0.15[a]
Total acidity (g/L)	0.12 ± 0.01[b]	0.12 ± 0.01[b]	0.21 ± 0.01[a]	0.17 ± 0.01[b]	0.12 ± 0.01[b]	0.12 ± 0.01[b]	0.20 ± 0.01[ab]	0.18 ± 0.01[b]
pH	3.36 ± 0.005 [a]	3.14 ± 0.015[b]	3.29 ± 0.025[d]	3.06 ± 0.01[d]	3.07 ± 0.01[d]	3.06 ± 0.01[d]	3.02 ± 0.02[e]	2.93 ± 0.005[f]
Turbidity (NTU)	3.70 ± 0.15[ab]	3.70 ± 0.12[ab]	3.40 ± 0.05[a]	3.75 ± 0.05[a]	3.45 ± 0.05[bc]	3.45 ± 0.15[bc]	3.29 ± 0.01[c]	3.70 ± 0.01[a]
Benzene (µg/kg)	1.29 ± 0.1[c]	1.29 ± 0.1[c]	4.0 ± 0.01[b]	11.0 ± 0.25[a]	0.09 ± 0.01[e]	0.09 ± 0.01[e]	0.18 ± 0.05[e]	0.7 ± 0.12[d]

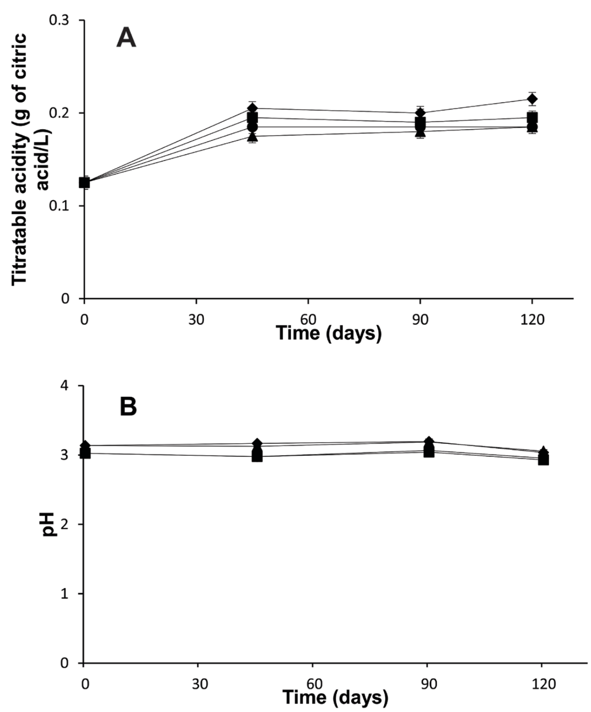

Figure 3. Evaluation of lemon soft drinks compositions at beginning of shelf life (SF0), 45 days (SF1), 90 days (SF2) and 120 days (SF3). A: total acidity; B: pH. Squares: 20 °C, formulation with nisin and natamycin; diamonds: 20 °C, formulation with sodium benzoate and potassium sorbate; circles: 30 °C, formulation with nisin and natamycin; triangles: 30 °C, formulation with sodium benzoate and potassium sorbate. The standard error bars were calculated based on triplicate testing.

and potassium sorbate. These results were identical for all the sensory attributes investigated (bitterness, sweetness, acidity and chemical-type notes). These findings demonstrate that nisin and natamycin did not alter significantly the lemon soft drink flavor quality, including at the shelf life ending.

3.4 Benzene formation

The use of benzoic acid as a preservative in soft drinks directly leads to the formation of benzene (Nyman et al., 2010). Thus, the use of natural preservatives may be a strategy to minimize benzene generation (Belletti et al., 2007). Comparing the formulation with nisin and natamycin and the standard formulation, the benzene formation was significantly reduced when using these alternative preservatives. For the standard formulation, the storage time and temperature had a direct effect on the benzene formation (Table 2).

From the start of the shelf life (SF0), after 120 days and at 20 °C, an increase of approximately 210% in the benzene level was reached (Table 2); however, at 30 °C this enhancement was 750%, and the quantity of benzene detected was 4 µg/kg and 11 µg/kg, respectively. Higher temperatures can lead to an increase in benzene formation in soft drinks (Arisseto et al., 2013), which is a compound with great toxicity for humans (Türkoğlu, 2007), and its level is regulated (International Council of Beverages Associations, 2006).

The initial benzene concentration in the standard formulation was 1.29 µg/kg (Table 2). Using natamycin and nisin, at the beginning of the shelf life, 0.09 µg/kg of benzene was detected, and, at the end, values of 0.18 µg/kg at 20 °C and 0.7 µg/kg at 30 °C were detected.

4 Conclusions

Natamycin and nisin can be used to preserve the quality of lemon-flavored soft drinks. At doses of 0.017% (w/v) of natamycin and 167 UI/mL of nisin, it was possible to inhibit the growth of *L. plantarum* and *Z. bailii* cultures, and even after 120 days, using these same doses, the lemon soft drinks remained stable. Besides the lemon soft drink produced offered good quality with a composition that adhere legal and technological specifications. Using natamycin and nisin, the soft drink produced results in lower benzene formation while maintaining the sensory and microbiological quality. Therefore, the natamycin and nisin concomitant association provides an alternative to the use benzoic and sorbic acid.

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