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Original article

Control of lactic acid bacteria in fermented beverages using lysozyme and nisin: test of traditional beverage boza as a model food system

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Summary The objective of this study was to increase quality and limited shelf-life of boza (3–15 days), a traditional Balkan origin fermented beverage using lysozyme (LYS) and/or nisin (NIS). For this purpose, the effectiveness of NIS, LYS and LYS:NIS combinations was first tested in a broth medium at 4 °C for 3 weeks on *Lactobacillus plantarum*, one of the frequently isolated lactic acid bacteria in boza. Stability of LYS and NIS in boza, their effects on LAB counts, and chemical and sensory properties of boza were then evaluated during cold storage at 4 °C. Results of LAB counts as well as pH, D- and L-lactic acid, and titratable acidity measurements showed that LAB in boza containing NIS (250 μg g^{-1}) or LYS:NIS (500:250 μg g^{-1}) could be controlled without reducing LAB counts below 6 log CFU mL^{-1} during 2 weeks shelf-life. In contrast, LYS (500 μg g^{-1}) alone could not control LAB in boza to delay its acidic spoilage. Positive effects of NIS and LYS:NIS application on quality of boza were also proved with sensory analysis by panelists and e-nose measurements. This work showed that use of natural GRAS agents in preservation of fermented beverages containing probiotic LAB is possible without affecting their characteristic aroma and flavour.

Keywords Boza, biopreservation, lysozyme, nisin, shelf-life, lactic acid bacteria.

Introduction

Boza is a traditional fermented beverage that is a colloidal suspension of hydrocolloids with a sweet and slightly sour taste. It is consumed in a large geographical area including majority of countries in Balkan Peninsula, in some parts of Caucasus and in Turkey where it is served together with cinnamon and unsalted roasted chickpeas during winter months (Gotcheva *et al*., 2001; Yeğin & Üren, 2008). It is produced from a slurry containing mixture of different pulses and cereals such as bulgur, maize, chickpea, millet, wheat or rice by fermentation of a microbial flora dominated heavily by lactic acid bacteria (LAB). Depending on its cereal composition, and fermentation and storage conditions, total dry matter, protein, total sugar and ash content of boza could vary between 5.57% and 29.82%, 0.27% and 2.75%, 10.62% and 22.59%, and 0.02% and 0.17%, respectively (Altay *et al*., 2013). However, the characteristic pleasant sour taste of boza is originated from its lactic acid content that vary between 0.3% and 0.6% (w/v) (Arici & Daglioglu, 2002; Akpinar-Bayizit *et al*., 2010; Petrova & Petrov, 2017). Some yeasts are also involved in boza fermentation (Arici & Daglioglu, 2002; Akpinar-Bayizit *et al*., 2010), but the conditions during fermentation allow formation of only 0.5–2% alcohol by volume in this beverage (Petrova & Petrov, 2017).

The results of clinical studies proved the positive effect of probiotics and prebiotics on human gastrointestinal system and numerous diseases such as allergic diseases, obesity, insulin resistance syndrome, type 2 diabetes, hypertension, coronary heart disease, non-alcoholic fatty liver disease, and cancer and its side effects (Markowiak and Śliżewska, 2017; Salmerón, 2017). Thus, the global interest in fermented functional beverages rich in prebiotics and probiotics has been increasing continuously (Altay *et al*., 2013; Marsh *et al*., 2014). The health benefits of boza are attributed to its (i) probiotic LAB, (ii) prebiotics originated from exopolysaccharides (EPS) produced by LAB (Heperkan *et al*., 2014) and (iii) cereal dietary fibre (Prado *et al*., 2008; Todorov *et al*., 2008; Vasudha & Mishra,

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2013; Heperkan *et al*., 2014; Dogan & Ozpinar, 2017; Petrova & Petrov, 2017; Salmerón, 2017). Kancabaş & Karakaya (2013) also reported that boza is also a good source for bioactive peptides with antihypertensive activity. However, content and profile of probiotic, prebiotic and bioactive peptide of boza in different countries could be variable due to differences in mixture of cereals and microbiota used in fermentation. For example, it was reported that the Bulgarian boza is formed 70% by LAB, whereas microbiota of Turkish boza is formed 98% by LAB (Petrova & Petrov, 2017). Different LAB isolated from Turkish boza include *Lactobacillus plantarum* (Kivanc *et al*., 2011), *L. fermentum* (Hancioğlu & Karapinar, 1997; Kivanc *et al*., 2011), *L. sanfrancisco*, *L. coryniformis*, *L. confusus, Leuconostoc paramesenteroides, Leu. mesenteroides* subsp. *mesenteroides, Leu. mesenteroides* subsp. *dextranicum, Leu. oenos* (Hancioğlu & Karapinar, 1997), *L. brevis* (Kivanc *et al*., 2011; Dogan & Ozpinar, 2017), *L. paracasei* subsp. *paracasei, L. acidophilus, L. paraplantarum, L. graminis, Leu. citreum, Lactococcus lactis* subsp. *lactis, Enterococcus faecium* and *Pediococcus species* (Kivanc *et al*., 2011). However, Heperkan *et al*. (2014) reported that *L. plantarum*, *Lc. lactis*, *L. brevis* and *L. fermentum* are the most frequently cited species in boza worldwide.

Shelf-life of boza varies from 3 to 15 days, depending on the amount of fermentable carbohydrates, profile of microbiota and storage conditions (Gotcheva *et al*., 2001; Tangüler, 2014). Shelf-life highly correlates to the amount of organic acids (mainly lactic acid) produced in the beverage by fermentation (Blandino *et al*., 2003; Altay *et al*., 2013). The pH of different boza could change between 3.43 and 3.86 (Altay *et al*., 2013). However, boza having a pH below 3.5 might be over-fermented by the LAB, and it could be too sour for consumption of consumers (Blandino *et al*., 2003; Altay *et al*., 2013). The temperature abuse during transportation and storage causes undesirable changes in composition (fermentable sugars, lactic acid and other organic acids and alcohol) and sensory properties of boza due to uncontrolled growth of microbiota (Akpinar-Bayizit *et al*., 2010). Thus, the use of bio-based preservation methods to control LAB in boza without reducing its probiotic potential, and without affecting its characteristic aroma and flavour attract a great industrial interest.

In this study, natural and Generally Recognised as Safe (GRAS) biopreservatives lysozyme (LYS) and nisin (NIS) were used alone or in combination to control LAB in Turkish boza and to delay its acidic spoilage without causing a considerable destruction in LAB. NIS, an antimicrobial peptide that is produced by certain strains of *Lactococcus lactis* spp. *lactis,* and LYS, an antimicrobial enzyme obtained from hen egg white, have been used extensively in preservation of dairy products (Delves-Broughton *et al*., 1996; Teerakarn *et al*., 2002). Lysozyme is also successfully employed in wines to control malolactic fermentation by LAB (Liburdi *et al*., 2014). The antimicrobial effect of LYS originates from its ability to split bonds between the N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer in bacterial cell wall. On the other hand, NIS owes its antimicrobial activity to its cationic nature that enables its interaction with the anionic phospholipids at the bacterial surfaces and helps forming pores at the bacterial membrane (Sudagidan & Yemenicioğlu, 2012). Both NIS and LYS are effective mainly on Gram-positive bacteria (Appendini & Hotchkiss, 2002) with a particular synergistic effect on LAB (Chung & Hancock, 2000). Thus, this study aimed not only the use of NIS and LYS alone, but also the exploitation of the synergy between LYS and NIS to control LAB in a fermented beverage. The preservation of boza with natural antimicrobials without a significant destruction on LAB opens a new perspective to standardise/increase shelf-life of this probiotic beverage and increase its worldwide consumption as a functional food.

Materials and methods

Materials

Hen egg white lysozyme (L6876 with a minimum activity of 40 000 unit mg^{-1} protein), nisin from *Lactococcus lactis* (N5764), *Micrococcus lysodeiktikus* as a substrate of lysozyme in enzyme activity determination and Tween 20 were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA). D-/L-Lactic acid kit was obtained from NYZTech (Cat. No. AK00141, Lisbon, Portugal). MRS broth, peptone water and agar used in the enumeration of LAB were obtained from Merck (Darmstadt, Germany). The strain of *Lactobacillus plantarum* (NRRL-B4496) obtained from ARS Culture Collection (NRRL) was kindly provided by Dr. Burcu Öztürk, from the Department of Food Engineering, Izmir Institute of Technology, İzmir. Four batches of freshly prepared boza (obtained from mixture of corn, wheat and millet) were purchased from Şemikler Bozacısı in Karşıyaka, İzmir (Turkey) at different time periods.

Effects of NIS and/or LYS on *L. plantarum* in broth medium

Variable amounts (250 or 500 μg mL^{-1}) of NIS and/or LYS were added into 45 mL of MRS broth adjusted to pH 4.0 with 0.1 N HCL and it was inoculated with 5 mL of *L. plantarum* culture (10^7 CFU mL^{-1}) that was used as model LAB to estimate the potential antimicrobial effects of NIS and

LYS. The inoculated broth was then cold stored at 4 °C for 21 days and the survivors of *L. plantarum* were enumerated periodically at 0th, 3rd, 7th, 14th and 21st days. For this purpose, broth from each group was serially diluted with 0.1% sterile peptone water and enumerated using pour plate method in MRS agar after 2-day incubation at 30 °C. The enumeration was performed in duplicate at each dilution.

Preparation of NIS and/or LYS containing boza for cold storage

The experiments conducted during cold storage of boza were carried out using four different batches of boza samples. Batch#1 was used in determination of LYS and NIS stability in cold stored boza; Batch#2 was used in determination of LYS and/or NIS effect on LAB, pH, titratable acidity, and D- and L- lactic acid content in boza during cold storage. Batch#3 and Batch#4 were used in sensory analysis and e-nose analysis, respectively. Each batch was separated into four different groups by weighting into sterile bottles under aseptic conditions. These treatment groups were as follows: (i) Control group; (ii) NIS (250 μg g^{-1}) containing group; (iii) LYS (500 μg g^{-1}) containing group and (iv) LYS:NIS (500:250 μg g^{-1}) containing group. The applied NIS and LYS concentrations of boza were the maximum allowed concentrations in food products according to US Food and Drug Administration (FDA) (FDA, 1988) and European Union Regulation (European Commission (EC) Regulation No. 2066/2001 (EC, 2016), respectively. After addition of NIS and/or LYS, samples stirred extensively with a sterile glass rod were cold stored for 28 days at 4 °C. Samples taken periodically under aseptic conditions were used in different tests given below. Two bottles were prepared for each treatment group.

Stability of LYS in cold stored boza

The stability of LYS was determined by monitoring change in its activity in cold stored boza samples at 0th, 3rd, 7th, 14th, 21st and 28th days. The measurements were conducted at pH 3.5 and pH 6.5 to determine activity changes at a minimal pH observed in boza and at pH close to enzyme's optimum, respectively. Briefly, 0.5 mL of LYS containing boza was transferred into an Eppendorf tube and it was diluted 1:3 with 0.05 M citrate-phosphate or 0.05 M Na-phosphate buffers at pH 3.5 and 6.5, respectively. The samples were then centrifuged at 15 000 ***g*** at 4 °C for 15 min. After that, 0.1 mL of the supernatants was then brought to 30 °C and mixed with 2.4 mL of *Micrococcus lysodeikticus* (0.26 mg mL^{-1}) solution prepared in 0.05 M citrate-phosphate buffer at pH 3.5 or 0.05 M Na-phosphate buffer pH 6.5 at 30 °C. The measurement of residual activity of LYS was conducted at 660 nm by a spectrophotometer (Shimadzu Model 2450, Japan) equipped with a constant temperature cell holder working at 30 °C. The absorbance measurements were recorded for 2 min and activity calculated from the slope of the initial linear portion was given in Units per g of boza (1 Unit corresponds to 0.001 changes in absorbance per minute).

Stability of NIS in cold stored boza

The stability of NIS in boza was determined by monitoring change in its concentration in cold stored samples at 0th, 3rd, 7th, 14th, 21st and 28th days. The NIS concentration was determined by the classical agar diffusion method (Teerakarn *et al.*, 2002) using *L. plantarum* NRRL-B4496 as test microorganism. Serial dilutions of NIS (500 IU mL^{-1}) prepared in sterile 0.05 M citrate-phosphate buffer at pH 4.0 were used to prepare the standard calibration curve. Briefly, the bacterial culture was inoculated into MRS broth and incubated for 24 h at 30 °C. Then, culture of freshly grown cells was adjusted to 0.5 Mac Farland unit with 0.1% of peptone water, and the diluted culture was seeded into MRS test agar which was prepared by adding 0.75% of agar and 20 mL L^{-1} of 50% of Tween 20 into MRS broth. In all, 20 mL of the inoculated agar was then poured into Petri dishes and solidified for almost 3 h at room temperature. Three wells were then opened on the surface of each agar using a sterile 6 mm-diameter cork-borer, and 50 μL of NIS containing boza sample (or NIS solution for preparation of standard curve) was added into the wells. The boza samples were prepared by 1:3 dilution of 0.5 mL of sample with sterile 0.05 M citrate-phosphate buffer at pH 4.0, and clarification of mixture by 15 min centrifugation at 4 °C and at 15 000 ***g***. The Petri dishes were incubated in an anaerobic jar with Aerocult C (Merck, Darmstadt, Germany) at 37 °C for 16–18 h to grow the test bacteria and observe formation of visible clear zones formed around wells by NIS. The diameter of each well and clear zone were measured from three different points using a digital caliper (Mitutoyo IP67, Japan). The experiment was conducted in duplicate with three replications. The standard curve was obtained by plotting logarithm of NIS concentration *vs.* diameter of the clear zones. The concentration of NIS in boza was expressed as IU per g of boza.

Effects of NIS and/or LYS on LAB in cold stored boza

The change in LAB counts of control, NIS (250 μg g^{-1}), LYS (500 μg g^{-1}) and LYS:NIS (500:250 μg g^{-1}) containing boza were monitored at 0th, 3rd, 7th, 14th, 21st and 28th days. For this purpose, 10 mL of boza sample from each group was serially diluted

with 0.1% sterile peptone water. The LAB was enumerated using double layer pour plate method in MRS agar incubated for 2 days at 30 °C. The enumeration was performed in duplicate for each sample and LAB counts were expressed as log CFU mL^{-1} of boza.

Monitoring of D- and L- lactic acid concentrations in cold stored boza

The concentration of D-lactic acid and L-lactic acid in boza was determined spectrophotometrically at 340 nm for 0th, 14th, 21st and 28th days of cold storage using D-/L-Lactic acid kit (NZYTech) according to the manufacturer's instructions. The measurements were performed in duplicate and results were expressed as g per L of boza.

Monitoring of pH and titratable acidity in cold stored boza

10 mL of boza was diluted 10-fold with deionised water, mixed by vortex for 5 s and measured with a pH meter (WTW, Inolab, Multilevel-3, Germany) (AOAC, 2006). The measurements were performed twice.

Titratable acidity of boza samples was determined by the titration method described by Cemeroğlu (2007). The samples were diluted 10-fold with deionised water and titrated with 0.1 N NaOH until endpoint identified by phenolphthalein indicator. The acidity was expressed as per cent in lactic acid equivalents. The measurements were performed twice.

Sensory analyses of cold stored boza

Sensory analyses of cold stored boza samples were conducted at 0th, 7th and 14th days of cold storage as described by Akpinar-Bayizit *et al.* (2010). The analysis was performed by ten semi-trained panelists who rated six attributes (colour, odour, texture, taste, mouthfeel and overall acceptability) for each sample on a five-point hedonic scale where one and five corresponded to dislike extremely and like extremely, respectively. Panelists were chosen among the graduate students of Food Engineering Department at İzmir Institute of Technology who were willing to taste boza. Since the panelists did not consume boza frequently, a briefly 30-min training session was conducted to the panelists to remind desired attributes of boza using fresh boza at optimal quality and to inform them about possible negative changes in the sensory attributes of boza during storage. Panelists assessed the samples in individual booths at sensory analysis laboratory. Samples were served at 10 ± 1 °C in glasses coded with three-digit numbers. The sensory evaluation was performed twice in different sessions.

E-nose analysis of cold stored boza

The effects of NIS and/or LYS on aroma profiles of cold stored boza were determined at 0th, 7th and 14th days using an e-nose (zNose™ 7100 vapor analysis system, EST, Newbury Park, CA, USA) containing a 1-m DB-5 column and a surface acoustic wave (SAW) detector with a parts per billion sensitivity. The analysis method was modified from Kadiroğlu *et al.* (2011). Briefly, 10 g of boza sample was put into a 20 mL septa-sealed screw cap vial and kept at 40 °C for 40 min in an incubator to equilibrate the headspace volatile components. The vapour of the sample was introduced into e-nose device through an injection 5-cm-long needle. The operating conditions of e-nose were programmed as follows: injection time of 10 s, inlet temperature of 200 °C, valve temperature of 165 °C, SAW detector temperature of 20 °C, column ramp temperature from 40 to 180 °C at 6 °C s^{-1}, helium flow rate of 4 cm^3 min^{-1} and data acquisition time of 10 s. Data were collected in every 0.02 s using Microsense software (Newbury Park, CA, USA). Two vials for each treatment group were prepared and e-nose measurement for each vial was run in triplicate.

The data of e-nose analysis were analysed using partial component analysis (PCA) that is a multivariate method. This analysis was performed using SIMCA 13.0.3 software (Umetrics, Malmö, Sweden). PCA is unsupervised technique that reduces the dimensionality of the data matrix to convert a set of large uncorrelated variable into a few new linearly correlated variables called as principal components (Uncu & Ozen, 2016). The first principal component (PC1) covers maximum variation of data and it is orthogonal to the second principal component (PC2) which is also orthogonal to the PC3 and covers as much of the remaining variation in the data as possible compared to PC3 and so on (Kara, 2009). The scatter plot obtained from the results of PCA shows that how the different observations distributed to differentiate from each other by forming a cluster.

Statistical analysis

The statistical comparisons of the mean values for microbiological, analytical and sensory analyses were performed using analysis of variance (ANOVA) with Fisher's multiple comparison test (Minitab, State College, PA). Differences at $P < 0.05$ were considered statistically significant.

Results and discussion

Effects of NIS and/or LYS on *Lactobacillus plantarum* in broth media

This work was aimed to prevent the acidic spoilage of boza by LAB within its expected shelf-life (15 days)

without causing a substantial inhibition in these potentially probiotic bacteria. *Lactobacillus plantarum* is one of the most frequently isolated lactic acid bacteria in boza not only in Turkey (Kivanc *et al*., 2011), but also worldwide (Heperkan *et al*., 2014). Thus, different concentrations of NIS and/or LYS were first tested on this bacterium in MRS broth at pH 4.0, a pH close to that of freshly prepared boza. The results presented at Table 1 showed that LYS alone was not effective on *L. plantarum* during 21-day incubation. The samples with LYS:NIS combinations at 1:1 (250:250 μg mL^{-1} or 500:500 μg mL^{-1}) ratio showed significantly ($P < 0.05$) lower *L. plantarum* counts than control at 0th day. In contrast, LAB counts of all other samples were quite similar with that of control at 0th day. A significant reduction in respect of initial *L. plantarum* counts of each sample occurred within 3 days in presence of NIS alone at 250 μg mL^{-1}, and LYS:NIS combination at 250:500 μg mL^{-1}. It took 7 days for LYS:NIS combination at 500:500 μg mL^{-1} to cause a significant reduction ($P < 0.05$) in respect of initial *L. plantarum* count of samples while this took 14 days for samples containing NIS alone at 500 μg mL^{-1} and LYS:NIS at 250:250 μg mL^{-1}. At the end of 14 days, a period almost equivalent to the expected shelf-life of boza, NIS alone at 250 μg mL^{-1}, NIS alone at 500 μg mL^{-1} and LYS:NIS combination at 250:250 gave similar bacterial counts ($P > 0.05$) that were 1.6– to 1.8 decimal (D) lower than that of control. On the other hand, combination of LYS:NIS at 500:500, 250:500 and 500:250 μg mL^{-1} gave the highest inhibition levels within 14 days with 3.4, 3.1 and 2.4 D lower *L. plantarum* counts than control, respectively. Further incubation of broths containing NIS alone at 250 or 500 μg mL^{-1}, and LYS:NIS combinations at 500:500 or 250:500 μg mL^{-1} for 21 days did not considerably change decimal differences between *L. plantarum* counts of these samples and control. However, decimal differences between broths containing LYS:NIS at 250:250 or 500:250 μg mL^{-1} and control reached to 2.4 and 2.9 D at the end of 21 days, respectively. Thus, at the studied storage conditions, the ranking of the effectiveness on *L. plantarum* was as follows: LYS:NIS at 500:500, LYS:NIS at 250:500, LYS:NIS at 500:250, LYS:NIS at 250:250, NIS at 500 and NIS at 250 μg mL^{-1}. These results clearly showed the concentration-dependent inhibitory activity of NIS on *L. plantarum* during cold storage. Moreover, it is also clear that NIS was more effective on *L. plantarum* when it was combined with LYS. In the literature, the occasional synergy of LYS and NIS combinations against some LAB strains such as *L. sake* and *L. curvatus* had been reported by Chung & Hancock (2000). In the current work, the synergy between LYS and NIS was observed when test results at some specific concentrations were evaluated carefully. For example, it is important to report that LYS:NIS combination at 250:250 μg mL^{-1} caused significantly lower (minimum 0.7 D) *L. plantarum* counts than LYS alone at 500 μg mL^{-1} or NIS alone at 500 μg mL^{-1} at the end of 21 days.

Stability of LYS and NIS in boza

The residual activities of LYS (at 500 μg g^{-1}) in cold stored boza with or without the presence of NIS (at 250 μg g^{-1}) were determined at different pH values. Activity measurements at pH 3.5 were determined to understand the levels of LYS activity ($LYS_{3.5}$) at a minimal pH observed for boza (Fig. 1a). According to Smolelis & Hartsell (1952), LYS showed its optimal antimicrobial activity at pH 6.6. Thus, activities at pH 6.5 ($LYS_{6.5}$) were measured to determine changes in enzyme activity close to its optimal pH without acidic stress on enzyme (Fig. 1b). The $LYS_{3.5}$ activities in boza changed between 2000 and 4000 U g^{-1} during 28-day cold storage. The activity at acidic conditions showed some fluctuations in presence of NIS, but it

Table 1 Effects of different concentrations of NIS and/or LYS on *Lactobacillus plantarum* in broth media at 4 °C

Concentrations (μg mL^{-1})		*L. plantarum counts* (log CFU mL^{-1})				
LYS	NIS	Day 0	Day 3	Day 7	Day 14	Day 21
–	–	$6.62 \pm 0.15^{b,A}$	$6.99 \pm 0.20^{a,A}$	$6.88 \pm 0.10^{ab,A}$	$7.10 \pm 0.36^{a,A}$	$6.94 \pm 0.12^{ab,A}$
250	–	$6.66 \pm 0.12^{c,A}$	$6.74 \pm 0.13^{bc,B}$	$6.94 \pm 0.02^{a,A}$	$6.88 \pm 0.10^{ab,A}$	$6.99 \pm 0.12^{a,A}$
500	–	$6.68 \pm 0.10^{a,A}$	$6.74 \pm 0.12^{a,B}$	$6.80 \pm 0.12^{a,A}$	$6.82 \pm 0.10^{a,A}$	$6.80 \pm 0.05^{a,A}$
–	250	$6.62 \pm 0.02^{a,A}$	$5.52 \pm 0.02^{c,E}$	$5.74 \pm 0.02^{b,CD}$	$5.53 \pm 0.06^{c,B}$	$5.34 \pm 0.04^{d,B}$
–	500	$6.47 \pm 0.13^{a,AB}$	$6.37 \pm 0.06^{a,C}$	$6.14 \pm 0.41^{a,B}$	$5.29 \pm 0.05^{b,B}$	$5.22 \pm 0.43^{b,B}$
250	250	$5.95 \pm 0.48^{ab,C}$	$6.25 \pm 0.08^{a,C}$	$5.83 \pm 0.31^{ab,C}$	$5.30 \pm 0.38^{b,B}$	$4.53 \pm 0.89^{c,C}$
500	500	$6.26 \pm 0.36^{a,BC}$	$5.92 \pm 0.03^{ab,D}$	$5.50 \pm 0.16^{b,D}$	$3.73 \pm 0.51^{c,D}$	$3.51 \pm 0.05^{c,D}$
250	500	$6.39 \pm 0.08^{a,AB}$	$5.79 \pm 0.12^{b,D}$	$4.39 \pm 0.14^{c,E}$	$4.05 \pm 0.38^{cd,D}$	$3.76 \pm 0.41^{d,D}$
500	250	$6.59 \pm 0.13^{a,A}$	$5.52 \pm 0.09^{b,E}$	$5.65 \pm 0.11^{b,CD}$	$4.71 \pm 0.13^{c,C}$	$4.00 \pm 0.31^{d,CD}$

Different lower-case and capital letters indicated statistically significant differences at rows and columns ($P < 0.05$), respectively.

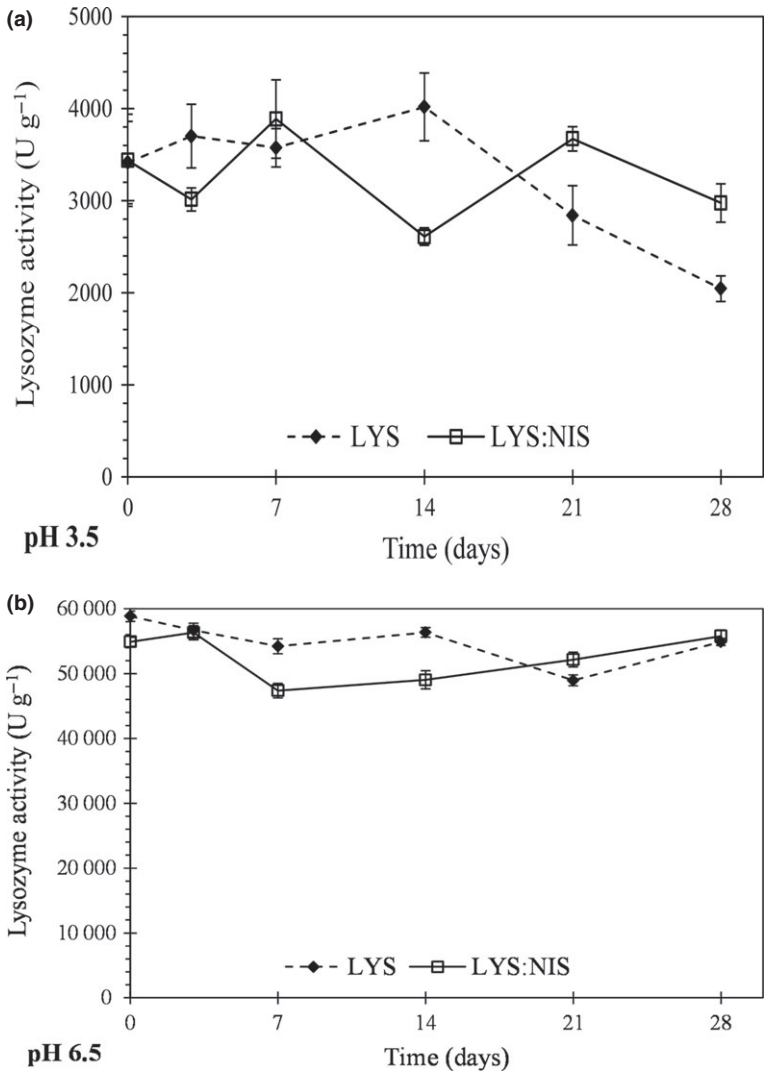

Figure 1 Stability of LYS in boza (Batch# 1) cold storage at 4 °C (activity determination at pH 3.5 (a) and pH 6.5 (b)).

did not drop below 2500 U g^{-1} during cold storage. The $LYS_{3.5}$ activity in boza without NIS remained quite stable within 14 days. However, $LYS_{3.5}$ showed a considerable loss (almost 50%) in activity at 21st and 28th days without the presence of NIS. In contrast, the $LYS_{6.5}$ activities of boza showed significantly lower fluctuation than $LYS_{3.5}$ activities, and they varied between 47 000 and 59 000 U g^{-1} during 28 days of cold storage. Thus, it is clear that the LYS activity levels determined at pH 3.5 for boza accounted for only 3–8% of its activity determined at pH 6.5. It is also important to note that minimum 80% of $LYS_{6.5}$ activities were maintained with or without the presence of NIS in boza during 28 days of cold storage. These results clearly showed that the factors that caused instability and activity loss of LYS in boza were reversible and appeared due to acidic conditions.

The stability of NIS (at 250 μg g^{-1}) in boza was also determined during 28 days of cold storage (Fig. 2). The results clearly showed that almost 57% of initial antimicrobial activity for NIS was destabilised within 3 days. However, the destabilisation of NIS in boza slowed down after 3 days. It should be reported that the NIS maintained almost 30% and 20% of its initial antimicrobial potential after 14 and 28 days of cold storage, respectively.

Effects of NIS and/or LYS on LAB in boza

The effect of NIS at 250 μg g^{-1}, LYS at 500 μg g^{-1} and LYS:NIS combination at 500:250 μg g^{-1} on LAB of cold stored boza is presented in Table 2. The initial LAB counts of control, NIS, LYS or LYS:NIS containing boza samples did not show a statistically significant difference ($P > 0.05$). Thus, it is clear that NIS

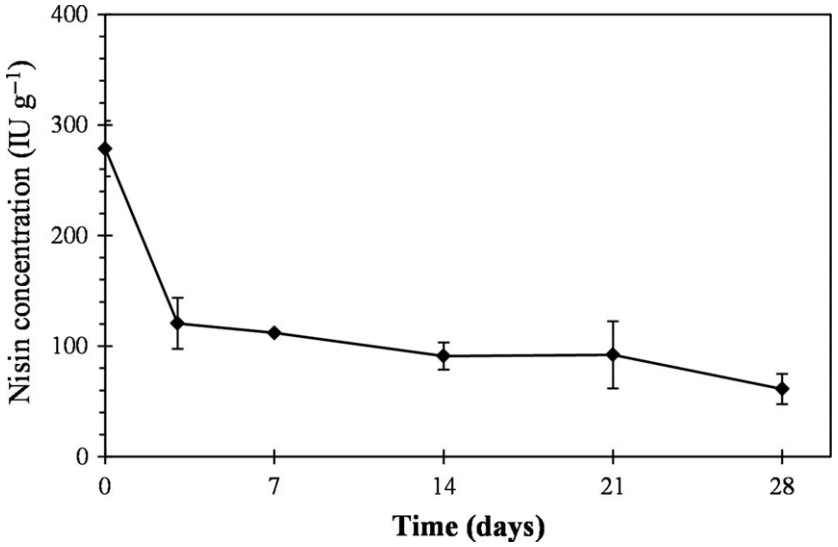

Figure 2 Stability of NIS in boza (Batch# 1) cold storage at 4 °C.

Table 2 Effects of different concentrations of NIS and/or LYS on lactic acid bacterial counts in boza (Batch# 2) at 4 °C

Concentrations (μg g^{-1})		LAB counts (log CFU mL^{-1})					
LYS	**NIS**	**Day 0**	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
–	–	$6.84 \pm 0.23^{a,A}$	$6.72 \pm 0.24^{a,A}$	$6.71 \pm 0.12^{a,A}$	$6.71 \pm 0.18^{a,A}$	$6.66 \pm 0.18^{a,A}$	$6.80 \pm 0.07^{a,A}$
500	–	$6.53 \pm 0.36^{a,B}$	$6.80 \pm 0.19^{a,A}$	$6.68 \pm 0.36^{a,A}$	$6.74 \pm 0.25^{a,A}$	$6.65 \pm 0.10^{a,A}$	$6.78 \pm 0.08^{a,A}$
–	250	$6.80 \pm 0.14^{a,AB}$	$6.35 \pm 0.22^{b,B}$	$6.30 \pm 0.21^{bc,B}$	$6.26 \pm 0.19^{bc,B}$	$6.02 \pm 0.08^{d,B}$	$6.13 \pm 0.08^{cd,B}$
500	250	$6.70 \pm 0.04^{a,AB}$	$6.39 \pm 0.32^{b,B}$	$6.21 \pm 0.27^{bc,B}$	$6.00 \pm 0.29^{cd,B}$	$5.78 \pm 0.15^{d,C}$	$5.83 \pm 0.09^{d,C}$

Different lower-case and capital letters indicated statistically significant differences at rows and columns ($P < 0.05$), respectively.

and/or LYS did not show a potent LAB inhibition at the applied concentrations. It is also important to report that the initial LAB count of control boza sample (6.9 log CFU mL^{-1}) was within the range of LAB counts determined for this fermented beverage by different researchers (between 5.9 and 7.9 log CFU mL^{-1}) (Gotcheva *et al*., 2000; Morea, 2008; Osimani *et al*.,2015). The cold storage of control and LYS containing boza samples for 28 days did not cause a significant change ($P > 0.05$) in their LAB counts. This finding was expected since LYS alone did not also show a considerable antimicrobial activity on *L. plantarum* even in the broth media. In contrast, boza samples containing NIS alone and LYS:NIS combination showed significantly lower ($P < 0.05$) LAB counts than control and LYS containing boza samples starting from the 3rd day of cold storage. Samples containing NIS and LYS:NIS did not show a significant difference ($P > 0.05$) in their LAB counts within the first 14 days of cold storage. However, boza samples containing LYS:NIS showed significantly lower ($P < 0.05$) LAB counts than NIS containing boza samples at 21st and 28th days. The overall reduction in the initial LAB counts of boza samples at 14th and 28th days of cold storage reached to 0.54 and 0.67 decimal (D) for NIS, and 0.7 and 0.87 D for LYS:NIS containing boza samples, respectively. The LAB counts of NIS containing boza samples were ≥6.0 log CFU mL^{-1} during 28 days of cold storage. However, LAB counts of LYS:NIS containing samples after 14th day of cold storage drop below 6.0 log CFU mL^{-1} that is essential for a food to be accepted as a probiotic (Espitia *et al*., 2016). These results suggested that the NIS is the main antimicrobial compound effective on LAB. The LYS showed antimicrobial effect on LAB at the later stages of cold storage only when NIS presented in the boza. This finding once more suggested the effectiveness of LYS and NIS combination against LAB.

Effects of NIS and/or LYS on pH, titratable acidity and lactic acid concentration of boza

The pH and titratable acidity of samples containing NIS at 250 μg g^{-1}, LYS at 500 μg g^{-1} and LYS:NIS combination at 500:250 μg g^{-1} are presented in Fig. 3a and b, respectively. The drop of pH, but the increase in titratable acidity in control samples and samples containing LYS alone were observed very

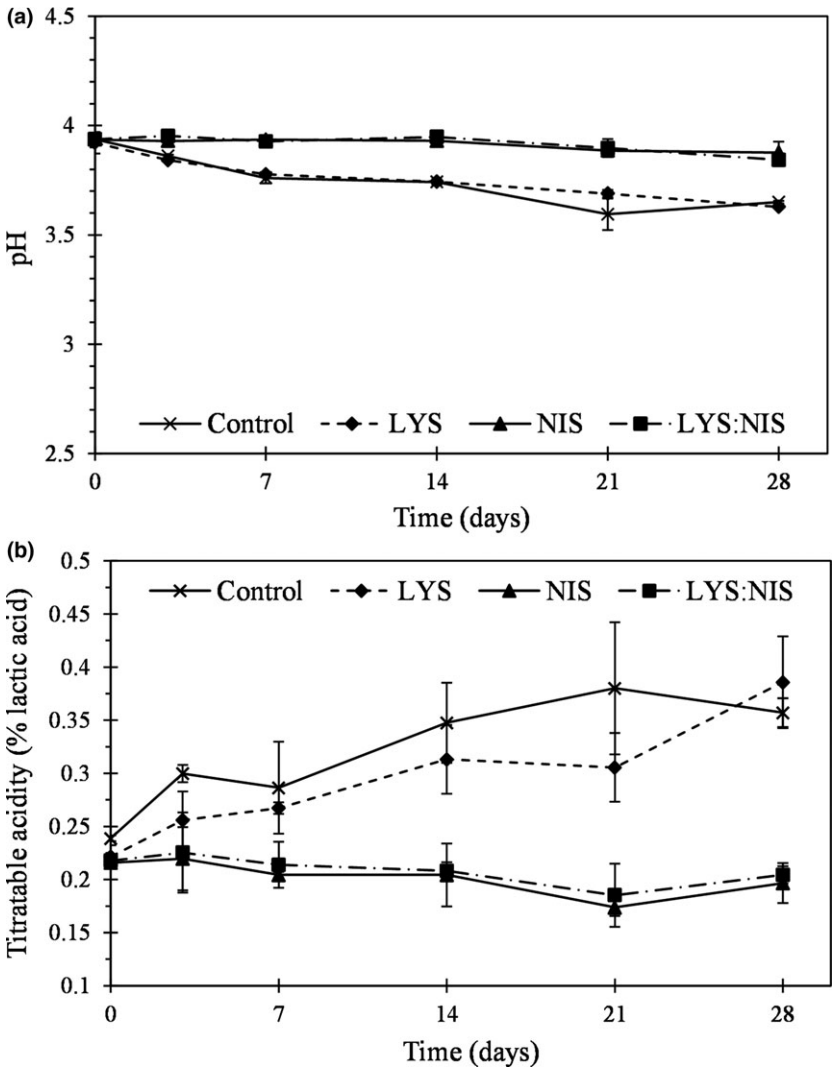

Figure 3 Changes in pH (a) and titratable acidity (b) of boza (Batch# 2) samples during cold storage at 4 °C.

Table 3 D-/L-Lactic acid concentrations of boza (Batch# 2) samples during cold storage

Concentrations (μg g^{-1})		D-Lactic acid concentrations (g L^{-1})			
LYS	**NIS**	**Day 0**	**Day 14**	**Day 21**	**Day 28**
–	–	$0.84 \pm 0.02^{d,A}$	$1.10 \pm 0.04^{c,B}$	$1.61 \pm 0.01^{a,A}$	$1.35 \pm 0.01^{b,B}$
500	–	$0.89 \pm 0.04^{c,A}$	$1.29 \pm 0.04^{b,A}$	$1.20 \pm 0.03^{b,B}$	$1.49 \pm 0.01^{a,A}$
–	250	$0.92 \pm 0.02^{b,A}$	$0.91 \pm 0.00^{b,C}$	$0.99 \pm 0.00^{a,C}$	$1.01 \pm 0.00^{a,D}$
500	250	$0.83 \pm 0.04^{c,A}$	$1.16 \pm 0.01^{a,B}$	$1.03 \pm 0.01^{b,C}$	$1.09 \pm 0.01^{ab,C}$
		L-Lactic acid concentrations (g L^{-1})			
–	–	$0.01 \pm 0.01^{b,B}$	$0.08 \pm 0.02^{a,A}$	$0.04 \pm 0.00^{ab,A}$	$0.05 \pm 0.03^{ab,B}$
500	–	$0.00 \pm 0.00^{b,B}$	$0.14 \pm 0.08^{a,A}$	$0.03 \pm 0.00^{ab,A}$	$0.05 \pm 0.01^{ab,B}$
–	250	$0.05 \pm 0.00^{bc,A}$	$0.10 \pm 0.02^{ab,A}$	$0.02 \pm 0.01^{c,A}$	$0.12 \pm 0.01^{a,A}$
500	250	$0.00 \pm 0.00^{c,B}$	$0.06 \pm 0.01^{a,A}$	$0.03 \pm 0.00^{b,A}$	$0.05 \pm 0.00^{ab,B}$

Different lower-case and capital letters indicated statistically significant differences at rows and columns ($P < 0.05$), respectively.

clearly during cold storage. The pH and titratable acidity of control and LYS containing boza samples did not differ significantly from each other during cold storage, but they differentiated significantly ($P < 0.05$) from boza samples containing NIS and LYS:NIS combination after 7th day of cold storage. These data supported the ineffectiveness of LYS alone on LAB to control their total acid production capacity. In contrast, the almost unchanged pH and titratable acidity of boza samples containing NIS and LYS:NIS combination clearly showed the successful control of acid production capacity of LAB in the presence of NIS. The measurements of D- and L-lactic acid in boza (Table 3) showed that LAB in boza lacked to form sufficient amounts of L-lactic acid ($\leq$0.14 g L^{-1}). This result is expected since D-lactic acid was already reported as the main lactic acid isomer produced by LAB during boza fermentation (Gotcheva *et al.*, 2000). In the current study, the initial D-lactic acid contents in different boza samples were similar, and they changed between 0.8 and 1.0 g L^{-1} ($P > 0.05$). D-lactic acid in different NIS containing (NIS or LYS:NIS) boza samples showed almost no change during 28 days of cold storage. Thus, it is clear that NIS alone showed the most effective inhibition of D-lactic acid production capacity of LAB in boza. All other samples showed a moderate increase in their D-lactic acid content at the 14th day of cold storage. Samples containing LYS:NIS combination showed a decline in their D-lactic acid content at 21st day of cold storage, and no more D-lactic acid formation was determined in these samples at 28th day. In contrast, controls and samples containing LYS alone continued to form D-lactic acid at 21st and 28th days of cold storage, respectively. It is important to note that the D-lactic acid content of control samples increased much more rapidly than LYS containing samples, and it was almost doubled at the end of 21st day. These results suggested that the LYS alone also delayed D-lactic acid formation in boza, but this finding did not show sufficient parallelism with respect to pH and titratable acidity measurements in boza. On the other hand, higher effectiveness of NIS alone than LYS:NIS combination to reduce D-lactic acid formation showed the different responses of D-lactic acid formation mechanism in LAB in presence of different antimicrobials. Further studies are needed to determine the detailed organic acid profile of boza in presence of different preservatives. However, this work clearly showed the possibility of using NIS alone and LYS:NIS combinations to control acidic spoilage of boza by LAB.

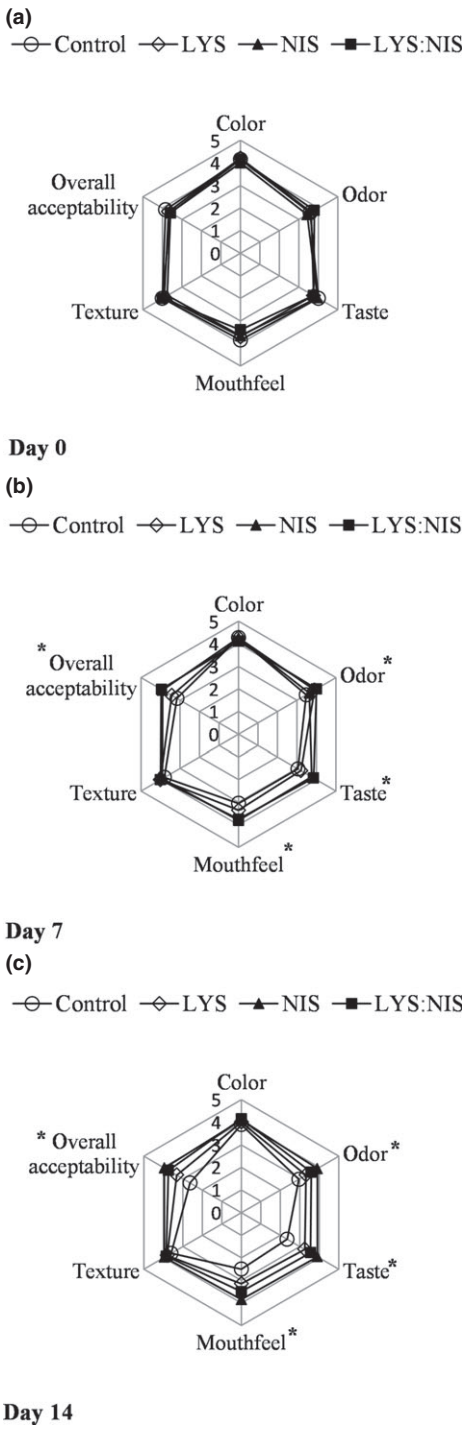

Figure 4 Sensory attributes of different boza (Batch# 3) samples during cold storage at 4 °C (at 0th (a), 7th (b) and 14th (c) days); * indicates statistically differed attributes ($P < 0.05$)).

Sensory analysis

The average scores of sensory attributes during cold storage of boza samples containing NIS at 250 μg g^{-1}, LYS at 500 μg g^{-1} and LYS:NIS combination at 500:250 μg g^{-1} are given in Fig. 4a–c. The samples were evaluated for 14 days since their declared shelf-

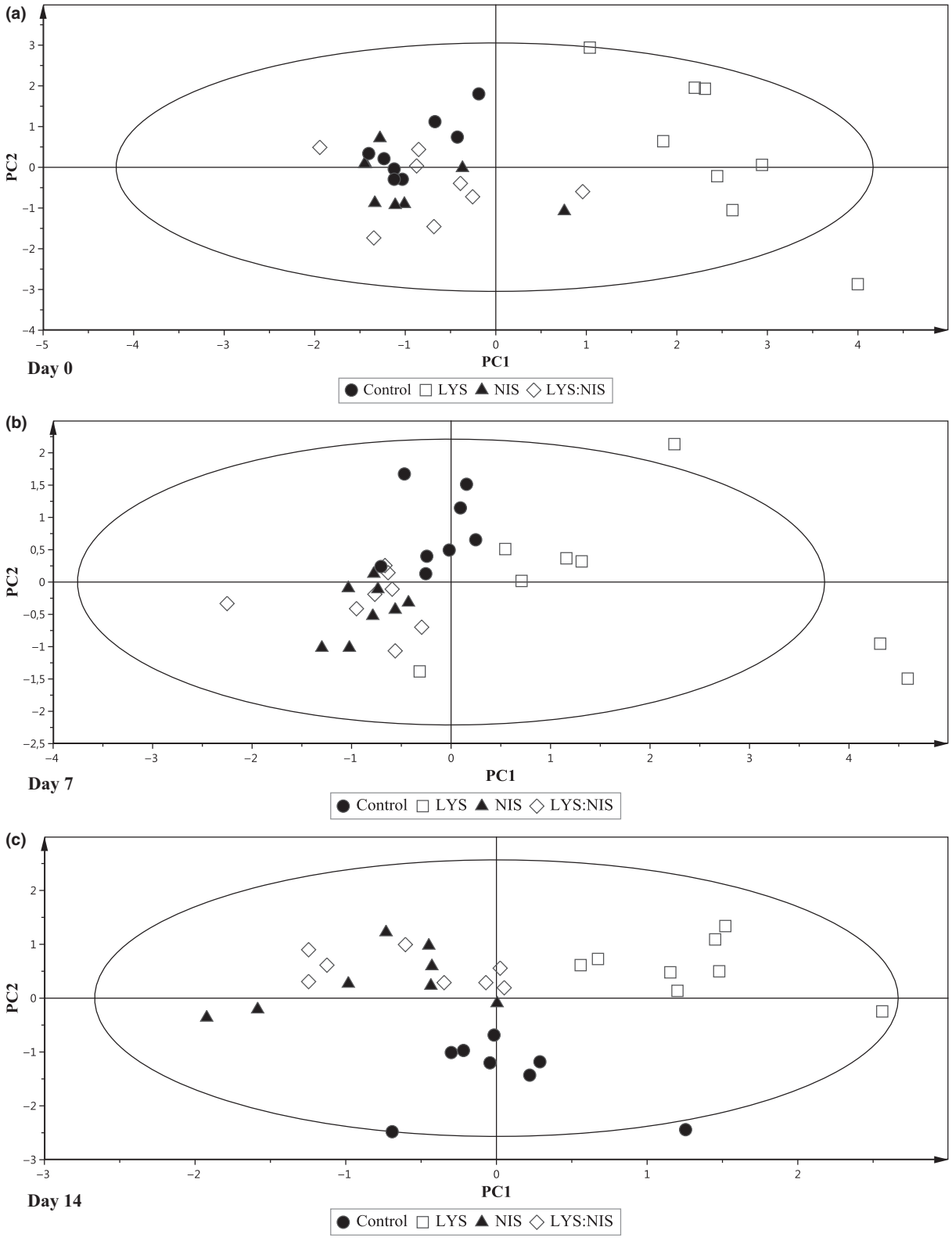

Figure 5 PCA score plots of different boza (Batch# 4) samples during cold storage at 4 °C (at 0th (a), 7th (b) and 14th (c) days).

life was expired within 15 days. It should be reported that the panelists did not detect any significant differences among colour and texture of different boza samples during 14 days of cold storage ($P > 0.05$). At the end of 7 and 14 days, NIS and LYS:NIS containing samples got the highest taste, mouthfeel and overall acceptability scores that are all significantly higher than those of the control ($P < 0.05$). In contrast, taste, mouthfeel and overall acceptability scores of LYS containing samples were not significantly different than those of controls at the end of 7 days ($P > 0.05$). The mouthfeel of LYS containing samples and controls did not also differ significantly after 14 days. However, LYS containing samples had significantly higher scores for taste and overall acceptability than controls at the end of 14 days ($P < 0.05$). Moreover, no significant differences were determined among mouthfeel and overall acceptability scores of LYS, NIS and LYS:NIS containing samples ($P > 0.05$) at 7th and 14th days of cold storage. However, taste and odor scores of LYS: NIS and NIS containing samples were significantly higher than those of LYS containing samples at 7th and 14th days, respectively ($P < 0.05$).

E-nose analysis

E-nose that mimics human olfactory system (Wilson & Baietto, 2009) was used to detect the changes in food aroma originated form food spoilage, adulteration and loss of quality parameters (Casalinuovo *et al*., 2006; Hai & Wang, 2006; Marina *et al*., 2010; Kim *et al*., 2015). In addition, combination of e-nose with chemometric methods showed a great potential to distinguish the aroma fingerprint of food products such as extra virgin olive oils obtained from olives of different cultivars, geographical origins and harvest years (Kadiroğlu *et al*., 2011). Wasnin *et al*. (2014) employed e-nose to detect aromatic changes in durian fruit pulp during fermentation. Rajamäki *et al*. (2006) used e-nose to detect aromatic changes in refrigerated modified atmosphere packaged broiler chicken carcasses. On the other hand, Santos *et al*. (2010) employed the device to detect early signs of deterioration in red wines.

In the current study, the e-nose analysis was conducted to differentiate the aroma fingerprints of boza samples treated with different antimicrobials on 0th, 7th and 14th days of cold storage (Fig. 5a–c). The PCA results showed that the total variation (R^2) and prediction ability (Q^2) of e-nose data obtained at 0, 7 and 14th days were 73.8% and 21.4%, 92.4% and 52.1%, and 89.3% and 55.7%, respectively. The results obtained at 0^{th} day of cold storage showed that LYS containing group separated from control, NIS and LYS:NIS containing groups (Fig. 5a). The control group started to separate from NIS and LYS:NIS containing groups at 7th day of storage (Fig. 5b). On the other hand, control and LYS containing groups clustered and got separated from NIS and LYS:NIS containing groups at the 14th day (Fig. 5c). In contrast, data for NIS and LYS:NIS containing groups did not show an apparent discrimination at 7th and 14th days. These clustering profiles showed parallelism with sensory analysis that distinguished better taste of NIS and LYS:NIS containing samples than those of control and LYS containing samples.

Conclusion

The results of this study clearly showed that the use of natural antimicrobial agents such as LYS and NIS is possible to improve quality attributes and shelf-life of boza without causing a considerable destruction of its LAB. Using NIS alone in boza yielded the most positive effect at the test conditions, but further combinations are needed to obtain a more beneficial effect from combinational application of NIS with LYS. In contrast, due to the highly acidic nature of boza, LYS activity alone is clearly insufficient to control LAB in this fermented beverage. The control of acidic spoilage in fermented probiotic beverages without causing microbial destruction is a great challenge. In fact, this is a major global problem in commercialisation of traditional fermented beverages. The results of this work are quite promising to obtain shelf-stable traditional probiotic beverages. However, further studies are needed at pilot scale with boza having different cereal composition and LAB profile. Moreover, further studies are also needed with alternative more popular LAB fermented beverages such as ayran, a yogurt drink and acidophilus milk. Such easily scalable and economically feasible bio-based methods could help making a great contribution to local economies and increase global consumers benefited from fermented functional foods.

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