

Survival of *Listeriamonocytogenes* during the production and ripening of Boza

Sadik BUYUKYORUK¹, Devrim BEYAZ¹, Ergun Omer GOKSOY¹, Filiz KOK¹,
Pelin KOCAK¹, Nevin TUZCU², Ozlem Sahan YAPICIER³

DOI: 10.25177/JFST.4.5.RA.441

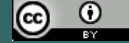
Research

Received Date: 10th Dec 2018

Accepted Date: 15th May 2019

Published Date: 20th Jun 2019

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¹Adnan Menderes University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Isikli/Aydın/Turkey;

²Cumhuriyet University, Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences Sivas/ Turkey;

³Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology, Burdur/ Turkey

CORRESPONDENCE AUTHOR

Sadik BUYUKYORUK,
Tel.: +902562470700;
E-mail: sbuyukyoruk@adu.edu.tr

CITATION

Sadik Buyukyoruk, Survival of *Listeriamonocytogenes* during the production and ripening of Boza (2019) SDRP Journal of Food Science & Technology 4(5)

ABSTRACT

This study intended to determine the survival of *Listeria monocytogenes* during the production and fermentation of the traditional Turkish wheat-based beverage, Boza. *L. monocytogenes* was inoculated at a level of 5.70 log cfu/ml and survival at 30°C was studied during Boza fermentation. The study also determined the pH changes during that period. The results indicated that *L. monocytogenes* count tended to decrease rapidly together with a sharp decrease in the pH of boza after 48th hour storage. Additionally, *L. monocytogenes* was not detected after 72 hour of boza production. It was concluded that this result was attributed by the low pH formation during boza fermentation as well as the antilisterial activity of the *Enterococcus faecalis* and *Enterococcus faecium* strains that were isolated from the boza as the dominant species.

Keywords: Boza; *Enterococcus* spp; *Listeria monocytogenes*; ribotyping; survival.

INTRODUCTION

Boza is a beverage prepared by adding drinking water to one or more types of purified grits or flour from different cereal varieties (e.g. proso millet, rice, wheat, corn). In the traditional method, this procedure is followed by cooking and adding sugar. Finally, alcohol and lactic acid fermentations are applied to end the preparation. It has been reported that proso millet is the most favorable ingredient for the ideal boza (Anonymous, TS 9778, 1992; Arici and Daglioglu, 2002). This fermentative beverage is mostly consumed in Balkan countries; being produced by the fermentation of lactic acid bacteria (LAB) (*Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*) and yeasts (Hancioglu and Karapinar, 1997; Zorba *et al.*, 2003). Table 1 presents the microorganisms isolated from boza that have been reported by different studies. The beverage has a sour or sweet taste depending on the production method. It has a yellow color and a high viscosity. It has a unique acidic-alcohol odor, a low pH as well as a low alcohol content (Anonymous, TS 9778, 1992; Arici and Daglioglu, 2002; Hancioglu and Karapinar, 1997; Zorba *et al.*, 2003). Table 2 presents the composition of this beverage reported in the results of various studies.

L. monocytogenes is a gram-positive, non-spore forming, rod-shaped bacterium. The most incriminated food products include ready-to-eat food, meat, dairy, and aquaculture products (Meloni *et al.*, 2009). *Listeriosis* can have a variety of symptoms including a mild fever, meningitis, septicemia, newborn infections and abortus (Salamina *et al.*, 1996; Lokerse *et al.*, 2016). It emerges with as high as 30% mortality rate in pregnant women, newborn infants, elderly persons, and individuals with suppressed immune system (McLaughlin, 1997).

Food products are formulated, produced and processed in order to minimize the growth of pathogenic microorganisms and target spoilage organisms. Nevertheless, the final product might contain pathogenic or spoilage organisms, or these organisms could grow in the formulation. They may also be already present in the product due to the contamination caused by the

problems in the process or hygiene practices. For this reason, preventing the growth of undesirable organisms during the intended shelf life is necessary. There are few studies in the literature on the survival of pathogenic microorganisms in Boza. Also, there are no studies examining the survival of *L. monocytogenes* in the pH environment changing during the Boza fermentation. This study aims to determine the survival of *L. monocytogenes* during the production of boza and evaluate the microflora of the ripened boza as well as its inhibitory effect on the microflora on *L. monocytogenes*.

Table 1. Microorganisms isolated from boza as reported by studies.

Hancioglu and Karapinar (2002)	LAB (<i>Lactobacillus</i> and <i>Leuconostoc</i> spp.), Yeast (<i>Saccharomyces</i> spp.)
Botes <i>et al.</i> (2007)	LAB (<i>Lactobacillus</i> spp.), Yeast (<i>Candida</i> spp. and <i>Issatchenkia</i> spp.)
Gotcheva <i>et al.</i> (2000)	LAB (<i>Lactobacillus</i> and <i>Leuconostoc</i> spp.) Yeast (<i>Candida</i> spp. and <i>Geotrichum</i> spp.)
Kivanc <i>et al.</i> (2011)	LAB (<i>Lactococcus</i> spp., <i>Leuconostoc</i> spp., <i>Lactobacillus</i> spp., <i>Enterococcus</i> spp., <i>Pediococcus</i> spp.)

LAB: Lactic Acid Bacteria

Table 2. Composition of boza and the chemical values.

Dry Matter (%)	24.85	Zorba <i>et al.</i> (2003)
Dry Matter (%)	15.3–31.1	Yegin and Uren (2008)
Protein, (%)	1.06	Zorba <i>et al.</i> (2003)
Protein, (%)	0.50–0.99	Yegin and Uren (2008)
Carbohydrates, (%)	12.3	Zorba <i>et al.</i> (2003)
pH	3.16–4.02	Yegin and Uren (2008)
Alcohol(ethanol), (%)	0.13	Kose and Yucel (2003)

MATERIALS AND METHODS

Boza production

In this study, boza was produced under laboratory conditions in accordance with the traditional formulation. Firstly, a mixture of corn, wheat, and rice (at a ratio of 2:1:1, respectively), free from impurities and brans, was prepared. Sugar and starter culture was not added. This was continuously mixed for an hour with

the addition of water (water/mixture ratio 1:1) until it became homogenous. Then, hot water (~ 65 °C) was added at 4:1 ratio. This new mixture was cooked and mixed for 45 minutes. Afterwards, it was cooled until reaching room temperature, diluted with water (2.5:1 ratio), and added sugar (20%), mixing it continuously (Hancioglu and Karapinar, 1997).

L. monocytogenes strains ATCC 13932 (4b strain) and ATCC 19111 (1/2a strain) at 5.70 log cfu/mL, (designated hour 0) were inoculated individually to the prepared beverage. Then, *L. monocytogenes* count was immediately calculated at zero hour. *L. monocytogenes* enumeration was conducted according to TS EN ISO 11290-2 (Anonymous, TS EN ISO 11290-2, 2001). This procedure was carried out at regular time intervals.

Microbiological Analyses

Boza sample (10 mL), was homogenized in 90 mL dilution medium that containing 0.1% peptone and 0.85% NaCl. For the isolation of *Lactococcus*, *Enterococcus*, and *Lactobacillus* strains, appropriate dilution rates were inoculated on M17 agar [Oxoid, added with 10% membrane filtered lactose solution (Oxoid, w/v)], Kanamycin esculin azide agar base (Oxoid, added with kanamycin supplement), and MRS agar (Oxoid), respectively. Diluted samples were incubated at 30°C for 2 days under aerobic conditions on M17 agar, at 40°C for 2 days under aerobic conditions on Kanamycin esculin agar, and at 37°C for 2 days under anaerobic conditions on MRS agar. Three colonies from each medium were randomly taken, and purified in M17 broth [Oxoid, added with 10% membrane filtered lactose solution (Oxoid, w/v)], for *Lactococci* and *Enterococci* spp, and in MRS broth (Oxoid) for *Lactobacilli* (Tormoet *al.*, 2015). This procedure was repeated twice. Gram staining and catalase tests were applied to the purified colonies, and Gram positive and catalase negative colonies were then stored in tubes containing 20% glycerol at -80°C.

Experimental design

For the determination of phenotypic aspects, especially for differentiation of *Enterococci* and *Lactococci*, biochemical tests were performed. These tests were

carried out at 10°C, 15°C, 40°C and 45°C in an optimal salt concentration of 2.5%, and at 30°C in 2%, 4% and 6.5% salt concentrations using M17 and MRS broth media (Tormoet *al.*, 2015). The pH of the boza was determined by using a pH meter (Inolab 7110, pH & oxygen meter).

Automated EcoRI ribotyping

Based on the information above, the automated *EcoRI* ribotyping identified 48 isolated suspected lactic acid bacteria. The standard method of the automated RiboPrinter® Microbial Characterization System was conducted for *EcoRI* ribotyping in accordance with the manufacturer's instructions. To obtain the pure culture samples, MRS plates were incubated for 24–48 h at 30°C using appropriate colony samples. The microbial samples were analyzed following manufacturer's instructions of the producer. The ribotype profiles used DuPont identification database DUP2003 to compare of the isolates with each other. When the corresponding pattern matched one of the patterns of the DuPont Identification Library with a similarity ≥ 0.85 , that isolate was accepted as an identification. RiboPrinter™ (DuPont Qualicon) was used for dividing the isolates automatically into ribogroups based on the similarity of the respective ribotype patterns (Kivanc *et al.*, 2011).

Determination of anti-Listerial activity

The isolated *Enterococci* strains were resuscitated at 30°C for 24 h in M17 broth. Further the strains were then grown at 30°C for 20 h under anaerobic conditions. For the determination of the anti listerial activity of isolates (whole culture broth), the study used an agar spot-on-a-lawn method (Todorov and Dicks, 2005) with the replacement of *L. monocytogenes* ATCC 19118 as the indicator strain. Wells of uniform diameter (6 mm) were cork bored in the agar and sealed with 15 mL of tempered soft agar. The cultures obtained from overnight cultures were applied to 4 different processes. (I): centrifuged at $2500 \times g$ for 5 min. (II): neutralized by addition of 5 N NaOH to exclude the effects of organic acids. (III): treated with catalase (in 10 mM phosphate buffer, pH 7.0 to a final concentration of 1 mg/mL) for 30 min at 25 °C to exclude inhibitory effects of hydrogen peroxide and

controls included phosphate buffer with and without catalase. (IV): added Proteinase K enzyme in 0.5 µg/mL final concentration and incubated for 4 h at 30 °C together with and without samples which including enzyme. Each of the solutions was sterilized with Millipore membrane filter (0.22 mm pore diameter) before loading to wells. Then, 20 µl samples were inoculated onto the surface of the BHI agar plates that had been inoculated with approximately 5 log cfu/ml of *L. monocytogenes*. The plates were incubated under aerobic conditions at 35°C for 15 h, and the diameters of clear zones of inhibition were calculated using the Computer Scanning System (Multi-ScanBase v. 14.02). The results were expressed in mm. PCR-identified *Enterococcus faecium* and *Enterococcus faecalis* strains obtained from the previous studies were used as positive control (Buyukyoruk et al., 2014).

RESULTS

After 48 hours of sharp decline in pH (from 6.55 to 3.52), the number of microorganisms also decreased significantly (from 5.60 log cfu/mL to 3.00 and/or 3.62 log cfu/mL) through ripening period. Although the pH value decreased to 3.70, inoculated *L. monocytogenes* strains were dominant in boza samples after 24 hours of storage at 4°C and reached up to 7.00 log cfu/ml. However, *L. monocytogenes* 1/2a and *L. monocytogenes* 4b strains could not be detected in microbiological tests after 72 hour Table 3 and Table 4.

Table 3. Hourly changes in *Listeria monocytogenes**:

Hours	<i>L. monocytogenes</i> ATCC 13932 ^χ	<i>L. monocytogenes</i> ATCC 19111 ^χ
0 (zero)	5.63 ± 0.035	5.61 ± 0.030
1	5.25 ± 0.050	5.39 ± 0.080
2	4.95 ± 0.040	5.67 ± 0.015
4	5.62 ± 0.030	6.34 ± 0.020
8	6.50 ± 0.015	7.3 ± 0.015
12	6.61 ± 0.020	7.2 ± 0.025
24	7.00 ± 0.020	6.57 ± 0.025
48	3.00 ± 0.020	3.62 ± 0.035
72	Not detected, <1	Not detected, <1

*The average of five consecutive repetitions
χ: logcfu/mL± standard variation

Table 4. Hourly changes in pH though of the ripened Boza*

Hours	pH
0 (zero)	6.55
1	7.02
2	6.57
4	6.55
8	6.47
12	3.85
24	3.70
48	3.52
72	3.48

* : The average of five consecutive repetitions

The ribotyping results revealed 23 *Enterococcus faecium* and 21 *Enterococcus faecalis* strains. (Fig 1 and Fig 2). Out of these isolates, 14 *Enterococcus faecium* and 8 *Enterococcus faecalis* strains had antilisterial activity. *Enterococci* showed an antilisterial activity in the form of a turbid zone round about the well and bacteriostatic activity against *L. monocytogenes*. Five of the strains were similar with *Corynebacterium macginleyi* (between 55-58% similarity), *Xanthomonas campestris* (58% similarity) and *Corynebacterium simulans* (54% similarity) from the Dupont Identification Library.

	Sample Number	Label	DuPont ID Label	RiboPrint™ Pattern
1	604-45-S-1	40	Enterococcus faecalis	
2	604-45-S-2	41	Enterococcus faecium	
3	604-45-S-3	42	Enterococcus faecalis	
4	604-45-S-4	47	Enterococcus faecalis	
5	604-45-S-5	48	Enterococcus faecium	
6	604-45-S-6	94	Enterococcus faecium	
7	604-45-S-7	138	Enterococcus faecium	
8	604-45-S-8	140	Enterococcus faecalis	

Figure 1. Dupont-ribotyping outputs of *E. faecalis* and *E. Faecium*

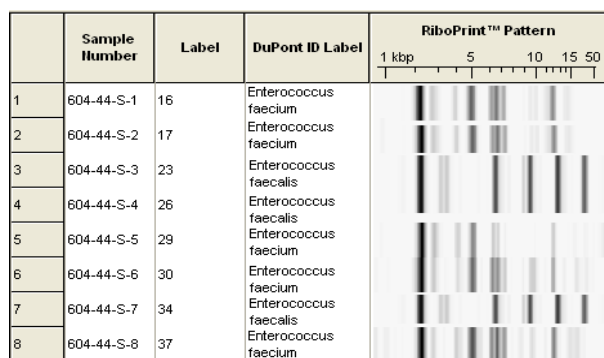


Figure 2. Dupont-ribotyping outputs of *E. faecalis* and *E. faecium*

DISCUSSION

Based on the literature research, no study was found regarding to *L. monocytogenes* in boza depending on pH changing and the food infection case due to boza consumption in Turkey. However *L. monocytogenes* is an important pathogen due to some characteristics of bacteria. The organism is the causative agent of listeriosis, a severe disease with high hospitalization and case fatality rates. *L. monocytogenes* can survive and grow over a wide range of environmental conditions such as refrigeration temperatures, low pH and high salt concentration (Gandhi and Chikindas, 2007). Since *this bacteria* is one of the most dangerous microorganism indicated above, *L. monocytogenes*, should be considered as a remarkable source of public health problems, even its initial contamination level is low (Sarimehmetoğlu and Kaymaz, 1994; Yıldırım and Sarimehmetoğlu, 2006; Şireli *et al.*, 2008; Dikici *et al.*, 2013; Zoz *et al.*, 2017; Changcheng Liet *et al.*, 2017).

In this study, 1/2a and 4b serotypes were selected as reference *L. monocytogenes* strains, in which the previous one was foodborne and the latter was clinical strain. Although 13 serotypes have been described for *L. monocytogenes* (Shi *et al.*, 2015), among these serovars, the 4b serotype is regarded to be responsible for the majority of human listeriosis cases, and serovar 1/2a is the most prevalent serovar in foods (Jamali and Thong, 2014).

Boza harbours a diverse population of lactic acid bacteria. Diversity in bacterial and yeast populations

from the different samples may be due to differences in raw materials, production processes and storage conditions. Examining the studies on the flora of boza (Table 1), only one study has reported the isolation of *Enterococcus* (Kivanc *et al.*, 2011). The present study concluded that *Enterococcus* was the dominant flora in boza.

Hancioglu and Aktug-Gonul (1999), examined the survival and growth of *E. coli* O157:H7, *Salmonella* Typhimurium, and *Staphylococcus aureus* in boza, and found that *E. coli* O157:H7 was not inhibited and survived at pH 3.7 during a 32 hour fermentation. The growth of *S. typhimurium* and *S. aureus* were reported to be inhibited after 12 hour fermentation (pH <4.5). Guven K and Benlikaya N (2005), have reported that the acidic pH environment in boza had an important effect on the inhibition of *Bacillus cereus*. In another study, the antimicrobial effect of boza on various microorganisms was shown to be (in descending order) as total bacteria >*E. coli*>*L. monocytogenes*>*S. pneumoniae*>*B. cereus* (Pehlivanoglu *et al.*, 2015).

Helland *et al.*, (2004), reported that boza samples had a probiotic effect as they contained more than 10⁶ cfu/mL viable microorganisms, which was sufficient for functional food in their study. Both lactic acid bacteria and yeasts were responsible for the spontaneous fermentation of many types of foods including boza (Arici and Daglioglu 2002). Cetinkaya and Soyutemiz (2004), have suggested that pathogens and spoilage microorganisms in fermented foods may be inhibited during the growth of lactic acid bacteria. In the present study, the inactivation of *L. monocytogenes* strains after a certain period of time was associated with the decrease in pH and the antilisterial activity of *Enterococcus faecium* and *Enterococcus faecalis* strains.

In this study, *L. monocytogenes* did not survive after the production and ripening procedures of boza. However, good manufacturing practices should be considered in the production of ready-to-eat foods since this microorganism is abundant in nature and potentially dangerous for public health.

Funding

This study was supported by Adnan Menderes University Scientific Research and Projects as Project No. VTF-11006.

REFERENCES

- [1] Anonymous, 1992. TS 9778, Boza.
- [2] Anonymous, 2001. TS EN ISO 11290-2, Microbiology of food and animal feeding stuffs- Horizontal method for the detection and enumeration of *Listeria monocytogenes*- Part 2: Enumeration method.
- [3] Arici M, Daglioglu O, 2002. Boza: A lactic acid fermented cereal beverage as a traditional Turkish food. *Food Rev Int* 18: 39-48. [View Article](#)
- [4] Botes A, Todorov SD, von Mollendorff JW, Botha A, Dicks LMT, 2007. Identification of lactic acid bacteria and yeast from boza. *Process Biochem* 42: 267-270. [View Article](#)
- [5] Buyukyoruk, S, Naim DA, Emre YG, Devrim B, Pelin K, 2014. Species distribution, molecular characteristics and vancomycin resistance gene profiles of *Enterococcus* spp. isolates from home-made western cheeses in Turkey. *Int J Dairy Technol* 67 (1): 103-9. [View Article](#)
- [6] Cetinkaya F, Soyutemiz GE, 2004. A study on survival of *Listeria monocytogenes* during manufacture and ripening of kashar cheese. *Turk J Vet Anim Sci* 28: 927-932.
- [7] Changcheng Li C, Huang L, Hwan CA, 2017. Effect of temperature and salt on thermal inactivation of *Listeria monocytogenes* in salmon roe. *Food Control* 73: 406-410. [View Article](#)
- [8] Dikici A, Ilhak OI, Calicioglu M, 2013. Effects of essential oil compounds on survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in çiğ köfte. *Turk J Vet Anim Sci* 37: 177-182.
- [9] Gandhi M., Chikindas ML, 2007. *Listeria*: A foodborne pathogen that knows how to survive. *Int J Food Microbiol* 113: 1-15. PMID:17010463 [View Article](#) [PubMed/NCBI](#)
- [10] Gotcheva V, Pandiella SS, Angelov A, Roshkova ZG, Weeb C, 2000. Microflora identification of the Bulgarian-cereal based fermented beverage Boza. *Process Biochem* 36: 127-130. 00192-8 [View Article](#)
- [11] Güven K, Benlikaya N, 2005. Acid pH produced by lactic acid bacteria prevent the growth of *Bacillus cereus* in boza, a traditional fermented Turkish beverage. *J Food Safety* 25: 98-108. [View Article](#)
- [12] Hancioglu O, Karapinar M, 1997. Microflora of boza, a traditional fermented Turkish beverage. *Int J Food Microbiol* 35: 271-4. 01230-5 [View Article](#)
- [13] Hancioglu O, Aktug-Gonul S, 1999. *Biyoteknoloji Dergisi* XI. Kukem-Biyoteknoloji Kongresi Ozel Sayisi, 23: 93-4.
- [14] Helland MH, Wicklund T, Narvhus JA, 2004. Growth and metabolism of selected strains of probiotic bacteria, in maize porridge with added malted barley. *Int J Food Microbiol* 91: 305-313. PMID:14984778 [View Article](#) [PubMed/NCBI](#)
- [15] Jamali H, Thong KL, 2014. Genotypic characterization and antimicrobial resistance of *Listeria monocytogenes* from ready-to-eat foods. *Food Control* 44: 1-6. [View Article](#)
- [16] Kivanc M, Yilmaz M, Cakir E, 2011. Isolation and identification of lactic acid bacteria from boza, and their microbial activity against several reporter strains. *Turk J Biol* 35: 313-324.
- [17] Kose E, Yucel U, 2003. Chemical composition of Boza. *J Food Technol* 1: 191-3.
- [18] Lokerse RFA, Maslowska-Corker KA, van de Wardt LC, Wijtzes T, 2016. Growth capacity of *Listeria monocytogenes* in ingredients of ready-to-eat salads. *Food Control* 60: 338-345. [View Article](#)
- [19] McLauchlin J, 1997. The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Rev Med Microbiol* 8: 1-14. [View Article](#)
- [20] Meloni D, Galluzzo P, Mureddu A, Piras F, Griffiths M, Mazzette R, 2009. *Listeria monocytogenes* in RTE foods marketed in Italy: Prevalence and automated EcoRI ribotyping of the isolates. *Int J Food Microbiol* 129: 166-173. PMID:19100643 [View Article](#) [PubMed/NCBI](#)
- [21] Salamina G, Dalle Donne E, Niccolini A, Poda G, Cesaroni D, Bucci M, Fini R, Maldini M, Schuchat A, Swaminathan B, Bibb W, Rocourt J, Binkin N, Salmaso S, 1996. A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiol Infect* 117: 429-436. PMID:8972666 PMID:PMC2271639 [View Article](#) [PubMed/NCBI](#)
- [22] Sarimehmetoğlu B, Kaymaz Ş, 1994. Türk salamura beyaz peynirinde yapım ve olgunlaşma aşamalarının *Listeria monocytogenes* üzerine etkisi. *Ankara Üniv Vet Fak Derg* 41 (2): 234-242. [View Article](#)
- [23] Shi W, Qingping W, Jumei Z, Moutong C, Zean Y, 2015. Prevalence, antibiotic resistance and genetic diversity of *Listeria monocytogenes* isolated from retail ready-to-eat foods in China. *Food Control* 47: 340-7. [View Article](#)

- [24] Şireli UT, Göncüoğlu M, Pehlivanlar S, 2008. Growth of *Listeria monocytogenes* in çiğ köfte (raw meat ball). *Ankara Üniv Vet Fak Derg* 55: 83-7.
- [25] Todorov SD, Dicks LMT, 2005. *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against gram-negative bacteria. *Enzyme Microb Tech* 36: 318-326. [View Article](#)
- [26] Tormo H, Lekhal DAH, Roques C, 2015. Phenotypic and genotypic characterization of lactic acid bacteria isolated from raw goat milk and effect of farming practices on the dominant species of lactic acid bacteria. *Int J Food Microbiol* 210: 9-15. PMID:26082325 [View Article](#) [PubMed/NCBI](#)
- [27] Yegin S, Uren A, 2008. Biogenic amine content of boza: A traditional cereal-based, fermented Turkish beverage. *Food Chem* 111: 983-7.
- [28] Yıldırım Y, Belgin Sarımeahmetoğlu B, 2006. Beyaz peynir yapımında bazı probiyotik bakterilerin kullanılmasının *Listeria monocytogenes* üzerine etkisi. *Erciyes Üniv Vet Fak Derg* 3(1): 1-7.
- [29] Zorba M, Hancıoğlu O, Genç M, Karapınar M, Ova G, 2003. The use of starter cultures in the fermentation of boza, a traditional Turkish beverage. *Process Biochem* 38: 1405-11. 00033-5 [View Article](#)
- [30] Zoz F, Grandvalet C, Lang E, Iaconelli C, Gervais P, Firmesse O, Guyot S, Beney L, 2017. *Listeria monocytogenes* ability to survive desiccation: Influence of serotype, origin, virulence, and genotype. *Int J Food Microbiol* 248: 82-9. PMID:28288399 [View Article](#) [PubMed/NCBI](#)