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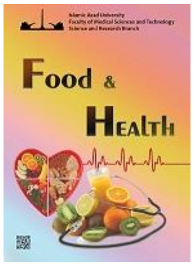

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An evaluation of stress impacts on survival of probiotic bacteria in yogurt

Noushin Mohajeri [1], Peyman Mahasti Shotorbani [2*], Afshin Akhondzadeh Basti [3], Zhaleh Khoshkhoo [1], Ali Khanjari3 [3]

[1] Department of Food Science and Technology, Tehran North Branch, Islamic Azad University, Tehran, Iran
[2] Department of Food Quality Control and Hygiene, Science and Research Branch, Islamic Azad University, Tehran, Iran
[3] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

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ABSTRACT

The aim of this study was to investigate the impacts of NaCl, bile salts, and their combinations on the viability of *Lactobacillus casei* in probiotic yogurt. For this purpose, the antibacterial activity of NaCl and bile salts was investigated via the microdilution technique by determining the minimum inhibitory concentration (MIC) against *L. casei*. Further, the stress effects of 50% MIC on NaCl and bile salts on the *L. casei* were examined by comparing the stress treatments with the control in terms of the *L. casei* population, pH, acidity, and syneresis percentage in probiotic yogurt during storage in the refrigerator for 28 days. According to the results, the *L. casei* population and pH decreased in all the treatments during the storage time, such that the intensity of the decrease in the control treatment was lesser than in other stress treatments ($p<0.05$). The acidity and percentage of syneresis during the storage time increased for all the treatments, with the increase being less in control than in the other stress treatments ($p<0.05$). The control scored the highest in the sensory evaluation ($p<0.05$). In conclusion, applying stresses below the MIC had a negative effect on the survival of *L. casei* in the probiotic yogurt until the end of 28 days and the outcome was not as expected.

1. Introduction

Today, particular attention is being given to functional foods, which have nutritional value as well as positive effects on human health. People are excited about eating products containing probiotics. However, products containing probiotics are amongst the ones in which there have been health allegations. These allegations are even advertised in the media during the last few years (1). Various studies on probiotics for humans' advantages have focused on the innovative formulation, and some even provided valuable information on probiotics linked to health and well-being (2). Probiotics are microorganisms that improve the gut microbial balance. These often include the *Lactobacillus* and *Bifidobacterium* species, as they have a historically prolonged and reliable value. Also, identified as generally recognized as safe (GRAS), all are the predominant inhabitants in the human intestines (3, 4). *Lactobacillus casei* is a gram-positive, mesophilic, microaerophilic, catalase-negative, and spore-free bacterium and a facultative hetero-formative bacterium with high acid production capacity (5). *L. casei* is a probiotic strain linked with anti-hypertensive antioxidant, anti-carcinogenic, anti-hypocholesterolemic and characteristics (6, 7). Fermented milk, such as yogurts, has the potential to act as a medium for producing value-adding materials because of its favorable sensory attributes, nutritional properties, high-grade harmony, and ample content of essential nutrients; besides, yogurt consumption enhances gut macrobiotic activity, mitigates immune responses, and increases gastrointestinal functionality by adjusting lactose intolerance. Technologists and manufacturers have reviewed distant fortifications by combining probiotics and nutraceutical compounds (4, 8). Today, probiotic yogurt is the most popular and widely consumed probiotic product in the world. The survival of probiotic bacteria in yogurt and similar products is an important challenge during storage in probiotic products. The

*Corresponding author: Department of Food Quality Control and Hygiene, Science and Research Branch, Islamic Azad University, Tehran, Iran.

E-mail address: p-mahasti@srbiau.ac.ir (Peyman Mahasti Shotorbani).

minimum acceptable concentration of probiotic strains for beneficial and therapeutic effects should be at least 10^6-10^7 CFU g $^{-1}$ or mL in the final product (9). The main problem in production was maintaining the survival rate of probiotic strains during storage of product with due attention to high acidity, oxygen stress, and nutrient deficiencies. The main reasons for reducing the viability of probiotic strains in the stomach were low pH and bile salts in the intestines (10). Many studies have been carried out on the viability of probiotics under the stomach's acidic conditions, bile salts of the small intestine (11), and survival rate of probiotics were studied in the cold storage of foods (12). Various methods such as microencapsulation, the addition of prebiotics and essential oils (13), and different procedures were used to increase the survival rate of probiotic strains during storage of functional products applied stresses were less than the minimum inhibitory concentration (MIC). They produced resistance-inducing genes (14). In many cases, the viability of probiotic bacteria is not sufficient, and it is necessary to evaluate the viability of probiotic bacteria in yogurt by applying different procedures to increase the resistance of bacterial cells against stresses. This study aimed to evaluate the effects of stresses less than the MIC of NaCl (NC) and bile salts (BS) or a combination of them on the viability of probiotic *L. casei* and later we monitored through the storage time, physicochemical and sensory properties of yogurt containing stressed probiotic during storage time.

2. Materials and methods

2.1. Materials

Cow's milk (3% fat) was supplied from Pegah, Tehran Plant, and Starter culture of yogurt (*Streptococcus thermophilus* and *Lactobacillus bulgaricus* sub spp. *delbrukii*) was purchased from Hanson Company, Denmark.

2.2. Preparation of inoculums

Lactobacillus casei (ATCC39392) was supplied from the Microbial Collection of Pasteur Institute of Iran. It remained in laboratory samples in a glycerol stock at -70°C and transferred in de Man, Rogosa & Sharpe (MRS) broth (Merck, Germany) at 37°C without shaking. Working cultures were prepared from stock cultures in two successive transfers (1% inoculum) in MRS broth at 37°C for 18 h. *L. casei* cells were inoculated from working cultures to MRS broth. After 18 h incubation at 37ºC, optical density (OD) (absorbance) of 0.1 at 600 nm, using a Spectronic 20 spectrophotometer (Varian, USA) was applied for determining the population of *L. casei.* Cell concentration was 1.5×10^8 CFU mL^{-1} for inoculation. The enumeration of *L. casei* was performed according to the serial dilution method and cultivating on (MRS) agar (Merck, Germany) after incubation for 24 h at 37ºC.

2.3. Determination MIC of NaCl, and bile salt

A 96-well plate with a volume of 300 µL was used in this experiment. Sequential concentrations of essential oil of bile salt (0%, 0.05%, 0.02%, 0.03%, 0.06%, 0.07%, 0.08%, 0.1%, 0.2% and 0.3%) and NaCl (0%, 1%, 2%, 3%, 4% and 5%) were used in De Man, Rogosa and Sharpe agar (MRS) broth (Merck, Germany). Media contained 5% DMSO and were transferred to 96-well plates. Then, 250 µL of different concentrations of NaCl, and bile salts along with 20 µL of *L. casei* suspension (5×10^6 CFU mL^{-1}) were added to all well. The contents of each well were mixed with a shaker for 2 min. The microplates were closed by Parafilm and then incubated for 24 h at 37°C in an anaerobic jar (Merck, Germany). At the end of incubation time, turbidity or non-turbidity was evaluated in the wells.

2.4. Adaptation and challenging conditions

For putting 50% MIC stress on bacteria, 2 g NC was added to 100 mL Mueller Hinton broth medium (Merck, Germany) and also 0.15 g BS was added to 100 mL Mueller Hinton broth medium. Treatments were kept at room temperature for 120 min and then were centrifuged in falcon tubes. After centrifugation, upper layers were separated and inoculated to yogurt (10^8 per mL). Enumeration of *L. casei* was conducted by serial dilution method (Most Probable Number) at 0, 7, 14, 21, and 28 days of storage to evaluate survival rate.

2.5. Physicochemical properties of yogurt samples

The experiments for physicochemical properties were pH, acidity, and syneresis. pH and titratable acidity were concluded based on the method described by Yangilar and Yildiz (15). Moreover, the percentage of syneresis was averaged according to the method by Wacher-Rodarte *et al.* (16). 5 ML of the yogurt sample were centrifuged at 2.208 g for 20 min at 4°C, and the volume of isolated whey was calculated after 1 min. Lastly, we displayed the Syneresis rate (%) as the separated whey volume per 100 g of yogurt (16).

2.6. Sensory evaluation

Sensorial tests were executed based on a 5-point Hedonic scale. The lowest score was intensely disliked, and the highest score was 5 as remarkably like samples (17). Sensory properties were measured as follows: flavor, texture, and overall acceptability. Sensory evaluations were carried out during 28 days of storage. Ten trained panelists performed judgments.

2.7. Statistical analysis

All experiments were performed in completely randomized design as triplicates and the result was reported as mean ± SD. The comparisons of data mean were performed by Tukey test. Two-way ANOVA was used for the determination of significance or non-significance of data ($p<0.05$).

3. Results and discussion

3.1. MIC results

The MIC values of NC, and BS against *Lactobacillus casei* were 4%, and 0.3% (v/v), respectively.

3.2. Survival of Lactobacillus casei

The effect of MIC of salts in return to the survival of *L. casei* was quite high; the inhibitory effect of concentrations less than MIC was also seen on pathogenic bacteria. Hence, the usage of concentrations of NaCl, and bile salts less than MIC may also eliminate pathogens without harm to probiotics (18). The viability of probiotic strains during production, food storage, and passage through the gastrointestinal tract is a major challenge in fermented dairy products. Researchers reported that enumeration of probiotic strains for beneficial and therapeutic effects should be at least 10^6 to 10^7 CFU g^{-1} or mL in products (9). The results showed that the interaction effects of treatment and storage time on the *L. casei* population were significant ($p<0.05$) (Fig.1).

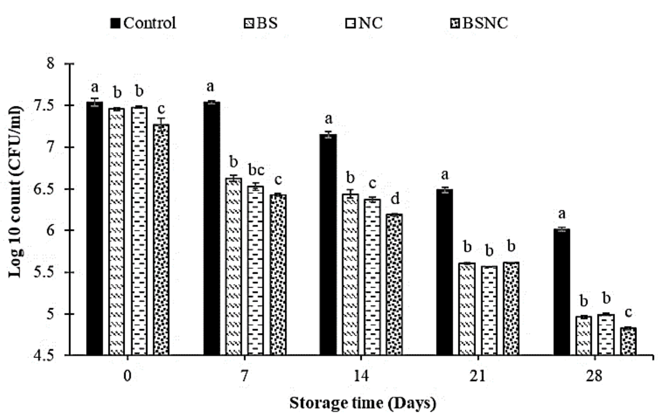

Fig 1. Effect of NaCl (NC), bile salts (BS), and their combinations on the survival of *L. casei* ATCC-39392 (log CFU mL^{-1}). Deviation bars designate the standard error of the method (n=3).

The viability of *L. casei* decreased in all treatments except for control at 7 days of storage time ($p<0.05$). The increased survival rate of *Lactobacillus acidophilus* LA5, *Lactobacillus fermentum*, and *Bifidobacterium* Bb-12 in yogurt at 7 days of storage was reported by Azizkhani and Parsaeimehr (9). The viability of *L. casei* of all samples significantly decreased during 28 days of storage ($p<0.05$). The bacterial population decline was attributed to the accumulation of organic acid during growth and fermentation. The main reasons for reducing pH are converting lactose to lactic acid, type of starter culture, duration of storage, and fermentation temperature (19). Several studies reported a decline in probiotic strains' survival rate during storage time (9, 20). Indeed, decreased pH of products during storage was due to activation of the beta-galactosidase enzyme at 0-5°C, as well as post-acidification. pH values decreased to 4.2. The viability of probiotic bacteria is affected by increased hydrogen ions compared to lactate ions (21). One of the main criteria for selecting probiotic bacteria is resistance to NaCl and bile salts (22). Following 28 days of storage time, the highest survival rate of *L. casei* has detected for the control (6.01±0.02 log CFU mL^{-1}) treatment, which was significantly different from others ($p<0.05$). According to the obtained results, treatments under stress with 0.15% BS were to be effective in the food at end of the 21st day. However, the survival rate of *L. casei* in BS stress treatment was significantly decreased from the 21st to the 28th day of storage time. It was less than the acceptable limit ($p<0.05$). Probiotic bacteria have different mechanisms of protection against stress, one of which is the bile hydrolysis system. The resistance of some strains to bile salts is associated with bile salt hydrolysis activity. Therefore, the hydrolysis of the bile salts will reduce their toxicity and side effects (23). According to Taranto *et al*. (24), *Lactobacillus delbrucium* subsp. *Bulgaricus* treated with different concentrations of thiorodoxylate (one of the bile salts) showed different levels of activity of the hydrolysis system (24). The researchers reported that in some bacterial cells, the bile hydrolysis system's activity was significantly stronger than others, which resulted in cells showing greater resistance to higher concentrations and longer exposure times to these bile salts (24). When probiotic bacteria are exposed to bile salts, cellular homeostasis disorders occur. Destruction of lipid membranes and cell membrane proteins leads to bacteria's death (23). The survival rate of *L. casei* with considering salt stress was within acceptable limit until the end of 21 days of storage, and these findings are following other research that studied the viability of probiotic strain more than 2% of concentration (25). Other researchers reported that probiotic strains' viability decreased in samples with high concentrations of salt (4%) (26).

3.3. pH

The suitable pH for commercial yogurt is 4.5. pH enhanced shelf life of the yogurt maintained mild taste and optimum appearance. Undesirable pH (less than 4), which had been resulted by *Lactobacillus bulgaricus*, produced large amounts of lactic acid, acetaldehyde, and by-products from proteolytic activity (27). The data for pH (Fig. 2) showed that the effect of time and type of samples were significant ($p<0.05$). The pH of all the yogurt samples decreased significantly during the storage period ($p<0.05$), and higher pH was attributed to samples under stress. At the end of 28 days of storage, the highest and lowest pH values for BSNC and the control treatments were 4.23±0.05 and 3.93±0.02, respectively ($p<0.05$). The main reason for the lower pH of BS, and NC salts compared to the control sample was due to the interactions between starters and employed probiotics. Vinderola *et al* (28) showed that probiotics play an important role in decreasing the survival rate of starters. Therefore, it can be concluded that the control sample with higher survival rate compared to other treatments, suppressed the starter's growth rate so pH stayed at a higher level. However, treatments with less survival rate could not stop starter bacteria reproduction, thus pH decreased and acidity increased significantly. Salt also affected pH and acidity values by influencing the growth of microorganisms, which may cause lactic acid production during storage time (25). Some studies reported that probiotic yogurt's pH decreased during storage time (9, 27).

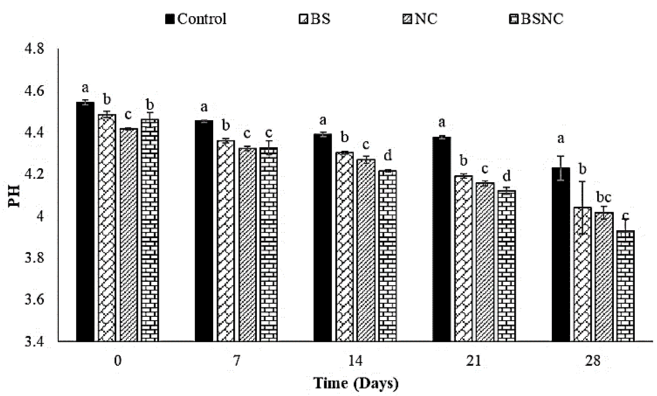

Fig 2. Effect of NaCl (NC), bile salts (BS), and their combinations on changes in pH of probiotic yogurt. Deviation bars designate the standard error of the method (n=3).

3.4. Titratable acidity (TA%)

The acidification degree of probiotic yogurt is a crucial process control model that directly impacts the gel intensity and the commercially available fermentation period (8). The titratable acidity (Fig. 3) results showed that interaction between group and time was significant ($p<0.05$). The acidity of all samples significantly increased during storage time ($p<0.05$). The acidity of the control sample was less than those of stress treatments ($p<0.05$).

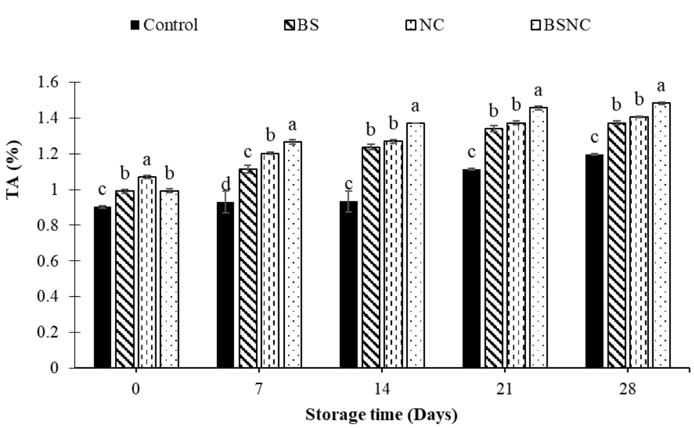

Fig 3. Effect of NaCl (NC), bile salts (BS), and their combinations on acidity changes of probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

After 28 days of the storage time, the highest and lowest acidity values were related to treatments under the stress of BSNC (1.48±0.006) and control (1.19±0.008), respectively. No significant difference was observed between NC and BC treatment during 28 days of storage ($p>0.05$). The production of organic acids by lactic acid bacteria was the main reason for increased acidity during storage time (29). The consumption of lactose by lactic acid bacteria led to lactic acid production, and then the acidity of samples increased. The production of lactic acid in yogurt is the main factor in producing a unique flavor. Due to casein instability, conversion of the colloidal calcium phosphate complex to soluble calcium phosphate, calcium excretion, and casein coagulation take place at pH 4-4.6 (30).

3.5. Syneresis

Significant factors such as heterogeneity, high acidity, storage, breakage of protein strand, and structural rearrangement that will induce yogurt's whey to leakage are recognized as substantial defects (15).

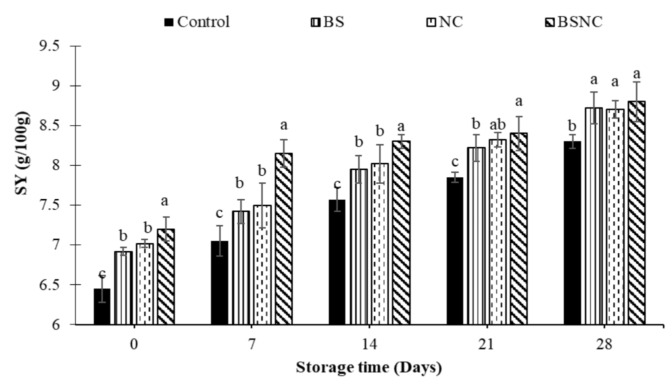

Fig.4. Effect of NaCl (NC), bile salts (BS), and their combinations on syneresis changes in probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

Results showed that exchange within-group and time was notable ($p<0.05$). As shown in Fig. 4, the amount of syneresis significantly rose in all samples during storage time ($p<0.05$). The increase in intensity for control treatment was less than that for stress treatments ($p<0.05$). Among the stressful treatments, the treatments under stress BSNC had the highest percentage of syneresis, respectively, during 21 days of storage ($p<0.05$), but at the end of the storage period no significant difference was observed between stress treatments ($p>0.05$). The main reason for increased syneresis in probiotic yogurt during storage might be the activation of microorganisms of starter culture and their effects on long-chain biopolymers, which could be an important factor in reducing the softness and enhancing the syneresis of the yogurt during storage (21). At the same time, Akgun (31) found that the syneresis rate in probiotic yogurt samples increased during storage at 4°C. The consistency of yogurt increased by stabilizers, an increase in the amount of milk casein concentration, and a reduction of acidification rate (32).

3.6. Sensory properties

In the overall acceptability of food products by consumers, sensory characteristics represent a vital role. Researches affirm that flavor is the first criterion for food acceptance, followed by health considerations as the second rule (8). Table 1 showed the results of sensory characteristics (flavor, texture, and general acceptance) during 28 days of storage time. The interaction between group and time on sensory characteristics was significant ($p<0.05$). The sensory scores of examples decreased during storage time. A more severe drop in sensory score was observed for stress treatments ($p<0.05$). Flavor scores for all treatments showed there were no significant differences among all samples on the first day of storage ($p>0.05$). The highest and lowest scores were attributed to control (4±0) and BSNC (3.11±0.33) at 28 days of storage time, respectively. The reduction of flavor scores in all samples during storage might be related to increasing acidity and reduced starter bacteria activity, which induced flavor components (20). The texture feature results showed no significant difference within samples on the first day of storage ($p>0.05$). The percentage of syneresis causes a change in the firmness of the sample. The firmness of yogurt decreased with increasing of syneresis. Indeed, the percentage of syneresis was inversely correlated to yogurt's firmness (33). The highest and lowest scores were attributed to control (4.33±0.50) and NC (3.22±0.44) at 28 days of storage time, respectively. Overall acceptance of samples indicated no significant difference among all samples on the first day of storage ($p>0.05$).

Table 1. Sensory properties of probiotic yogurt samples with NaCl (NC), bile salts (BS), and their combinations.

Sensory properties	Yogurt samples	Storage time (Days)				
		0	7	14	21	28
Flavor	Control	5.00±0.00^{a}	5.00±0.00^{a}	5.00±0.00^{a}	4.67±0.50^{a}	4.00±0.00^{a}
	BS	5.00±0.00^{a}	4.00±0.00^{b}	4.00±0.00^{b}	4.00±0.00^{b}	3.33±0.50^{b}
	NC	5.00±0.00^{a}	4.00±0.00^{b}	4.00±0.00^{b}	3.78±0.44^{b}	3.33±0.50^{b}
	NCBS	4.67±0.50^{a}	4.00±0.00^{b}	3.78±0.44^{b}	3.22±0.44^{c}	3.11±0.33^{b}
Texture	Control	5.00±0.00^{a}	5.00±0.00^{a}	5.00±0.00^{a}	4.67±0.23^{a}	4.33±0.50^{a}
	BS	5.00±0.00^{a}	4.00±0.00^{b}	4.33±0.50^{bc}	4.33±0.50^{bc}	3.44±0.53^{b}
	NC	5.00±0.00^{a}	4.33±0.50^{b}	4.33±0.50^{bc}	4.33±0.50^{bc}	3.22±0.44^{b}
	NCBS	4.67±0.50^{a}	4.33±0.50^{b}	4.00±0.00^{c}	3.22±0.44^{d}	3.44±0.44^{b}
Overall acceptability	Control	5.00±0.00^{a}	5.00±0.00^{a}	4.89±0.33^{a}	4.67±0.50^{a}	4.00±0.00^{a}
	BS	5.00±0.00^{a}	4.00±0.00^{b}	4.00±0.00^{b}	4.00±00.70^{b}	3.22±0.44^{b}
	NC	5.00±0.00^{a}	4.00±0.70^{b}	4.00±0.00^{b}	4.00±0.00^{b}	3.22±0.44^{b}
	NCBS	4.67±0.50^{a}	4.33±0.50^{b}	4.00±0.00^{b}	3.56±0.53^{c}	3.22±0.44^{b}

The highest score was attributed to control (4±0) at 28 days of storage time. Overall, about all sensory scores, at the end of the storage period no significant difference was observed between stress treatments ($p>0.05$). Treatments that contained more probiotic counts had better flavor and texture than treatments with less probiotic counts, so the overall acceptance scores of yogurts with more probiotic counts were higher than the yogurt with less probiotic counts (under stress). Proteolytic strains of *Lactobacillus* could produce taste through carbohydrate metabolism, proteolysis, and low lipolysis processes. Enzymes of *Lactobacillus* hydrolyzed casein and produced large and medium bioactive peptides. Proteolytic enzymes may subsequently degrade these peptides from starter bacteria, non-acidic lactic acid probiotic bacteria to small peptides and free amino acids, which are the major contributors to taste in dairy products (32, 34).

4. Conclusion

According to the results, the pH and population of *L. casei* decreased for all the treatments during the storage time, while their acidity and percentage of syneresis increased. The enumeration of *L. casei* was in the range of the recommended amount (10^6-10^7 CFU mL^{-1}) for probiotic yogurt without any stress. Overall, applying stresses below the MIC had no effect on the survival of *L. casei* in the probiotic yogurt until the end of 28 days and the outcome was not as expected.

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