JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

OBSERVATION OF THE SUITABILITY OF SINGLE STRAINS OF STREPTOCOCCUS THERMOPHILUS AND LACTOBACILLUS DELBRUECKII SUBSP. BULGARICUS ISOLATED FROM LOCAL DAIRY SOURCES IN TURKEY AS YOGURT STARTER COMBINATIONS

Irem Uzunsoy[1], Sebnem Ozturkoglu Budak*[2], Tuba Sanli[2], Birce Taban[2], Aykut Aytac[3], Nuray Yazihan[4,5], Ahmet Levent Bas[6], Barbaros Ozer[2]*

Address(es):
[1]Zonguldak Bülent Ecevit University, Çaycuma Vocational School of Food and Agriculture, Department of Food Processing, 67900, Çaycuma, Zonguldak, Turkey.
[2]Ankara University, Faculty of Agriculture, Department of Dairy Technology, 06110, Dışkapı, Ankara, Turkey.
[3]Hacettepe University, Faculty of Engineering, Department of Food Engineering, 06800, Beytepe, Ankara, Turkey.
[4]Ankara University, Faculty of Medicine, Department of Pathophysiology, 06100, Sıhhiye, Ankara, Turkey.
[5]Ankara University, Institute of Health Sciences, Department of Food, Metabolism and Clinical Nutrition, 06100, Sıhhiye, Ankara, Turkey.
[6]Selçuk University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, 42130, Konya.

*Corresponding author: iremuzunsoy@beun.edu.tr

https://doi.org/10.55251/jmbfs.9241

ARTICLE INFO

Received 23. 7. 2022
Revised 3. 5. 2023
Accepted 10. 5. 2023
Published 1. 8. 2023

Regular article

OPEN ACCESS

ABSTRACT

The present study investigated the suitability of combinations one *Streptococcus thermophilus* and five *Lactobacillus delbrueckii* subsp. *bulgaricus* isolates for industrial yogurt productions. The isolates were selected after a detailed pre-screening procedure based on acidification performances, production of volatile aroma compounds, texture developing properties and sensory evaluations of yogurts made from single cultures. The fermentation profiles of the combinations were investigated by monitoring the small oscillatory rheological parameters and pH reductions during fermentation. The variations in some physical, chemical and organoleptic characteristics of the yogurt samples were also monitored during cold storage at 4 ºC for 21 days. Results indicated that all the combinations reduced the milk acidity to pH 4.6 within 4-5 hour at 43 ºC. Gelation profiles of the samples were close to each other (close tan δ values) with different elastic (G') and loss (G") moduli, indicating that similar chemical bondings took place in gel formation with different levels. All the samples showed a shear-thinning non-Newtonian flow behavior. Total number of starter bacteria was higher than 6 log cfu/mL in each sample after 21 days of storage. The post-acidification rates were also limited (between 4.21-4.37 after 21 days of cold storage). Untrained sensory evaluation panel found no marginal differences between the samples regarding appearance, body and texture, and aroma and flavor of the samples. To conclude, the combinations examined in this study were found to be suitable for commercial yogurt productions.

Keywords: Yogurt, starter combinations, gelation, rheology, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*

INTRODUCTION

The main element that gives traditional dairy products their usual characteristics is their natural flora. Today, unique characteristics of many traditional dairy products (*e.g.* artisanal cheeses, kefir etc.) rely on the metabolic activities of the natural flora of raw milk. However, this is not the case for yogurt because yogurt milk must be subjected to high heat treatment to obtain a proper gel matrix prior to being inoculated with yogurt culture. In rare practices, yogurt is inoculated with yogurt of previous productions (**Ozer, 2006**). Considering the volume of yogurt production on a global scale, this practice is not applicable. Therefore, the use of defined cultures in large scale yogurt production is inevitable. The first use of starter culture in yogurt production was realized in 1919. Until early 1960s, home-made or local yogurt productions were dominant over industrial productions (**Aryana and Olson, 2017**). With the introduction of the use of commercial defined starter cultures in yoghurt production, a significant success has been achieved in product standardization. Estimating the end product quality and process time has brought important commercial advantages for yogurt producers. Today, both industrial applications and international regulations mandate the use of combination of *Streptococcus thermophilus* (*Str. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) as the starter culture in the production of yogurt. The balance between the strains of these two starter bacteria is of critical importance for obtaining a well-balanced textural and, aroma and flavor characteristics (**Tamime and Robinson, 2007**).

Yogurt starter bacteria synthesize one or more metabolites that each other needs for development and encourage their growth mutually (**Angelov *et al.*, 2009; Smid and Lacroix, 2013; de Souza Oliveira *et al.*, 2012**). Since *Str. thermophilus* does not have an advanced proteolytic capacity, it cannot synthesize essential amino acids and free peptides required for its growth (**Sieuwertz *et al.*, 2008**). In contrast, *Lb. bulgaricus* has the ability to produce free amino acids (valine, glycine, histidine *etc.*) from caseins, which are used by *Str. thermophilus* as growth factors (**Ginovart *et al.*, 2002; El-Zahar *et al.*, 2003**). The growth of *Lb. bulgaricus* is stimulated in the presence of formic acid, predominantly released by *Str. thermophilus* (**Zourari *et al.*, 1992; Settachaimongkon *et al.*, 2014**). In addition, the CO_2 released during the urea metabolism of *Str. thermophilus*, is an effective factor enhancing the growth of *Lb. bulgaricus* (**Routray and Mishra, 2011**). The growth rate of each bacteria and accordingly rate of fermentation are promoted by this proto-cooperation, when it is compared with single culture performances (**Yamauchi *et al.*, 2019**).

One of the basic principles of food processing is to ensure that traditional foods are passed through the industrial process with the concept of food safety, while preserving their basic characteristics which are the reason for consumers' preference. This approach aims to preserve the original/traditional quality of the product as much as possible. The desired properties in the final product are reached beyond any doubt with the selection of suitable starter culture strains used in the production of yogurt at the industrial level. The formation of the characteristic taste/aroma and textural properties of yogurt is a result of the metabolic activities of the bacteria used and is highly strain-dependent. Since all fermentation metabolism is regulated by starter bacteria, correct understanding of the metabolic activities of yogurt bacteria and the proto-cooperation between them is very important in terms of modifying the characteristic properties of yogurt to meet consumers' demands (**Ozer, 2006**). In our previous study, single isolates of *Str. thermophilus* and *Lb. bulgaricus* obtained from local yogurt samples obtained from different areas of Turkey were screened for their suitability to industrial yogurt productions (**Uzunsoy *et al.*, 2021**). Based on the fermentation profiles (gelation characteristics, pH reduction profiles, aroma development capacities) of single isolates, candidate combinations of yogurt bacteria for industrial yogurt productions were selected. This study aimed to evaluate bacterial strain combinations selected in **Uzunsoy *et al.* (2021)** for their suitability for industrial yogurt productions based on gelation profiles, physical characteristics, lipolytic capacities and sensory properties. Since the proteolytic activities of individual strains used in the formation of starter combinations in the present study were low

(**Uzunsoy, 2018**), we did not consider the proteolysis as evaluations parameter in the present case.

MATERIAL AND METHODS

Bacterial strains and chemicals

Bacterial isolates [one *Str. thermophilus* (ST) and five *Lb. bulgaricus* (LB), Table 1] used in this study were obtained from culture collection created by the project titled "Development of industrial yogurt starter combinations from single strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from local dairy products in Turkey" (Project No: 112D052, financially supported by The Scientific and Technological Research Council of Turkey-TUBITAK). The details of the technological performances of single isolates and geographical locations where the isolates obtained are given in **Uzunsoy *et al.* (2021)**. All chemicals were provided from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, MO, USA) unless otherwise stated. Cow's milk was supplied from Ankara University Dairy Farm and yogurt production was performed at Pilot Dairy Plant of Ankara University, Ankara, Turkey.

Table 1 Combinations of isolates used in the study

Bacteria	Strain	Codes in the present study
Str. thermophilus	KLDS SM	ST5
Lb. bulgaricus	MGB27-2	LB2
	IMAU11365	LB3
	SKB1083	LB5
	MN-BM-F01	LB6
	JCM1002	LB7

Preparation of combined cultures and yogurt production

For pre-activation of stock cultures, freeze-dried isolates of *Lb. bulgaricus* and *Str. thermophilus* were inoculated into 5 mL of MRS and M17 broths, respectively. *Lb. bulgaricus* strains were incubated at 43°C for 72 h under anaerobic conditions and *Str. thermophilus* strains were incubated at 37°C for 24 h aerobically, and were kept at 4°C for 24 h after incubation. These pre-activated strains of *Str. thermophilus* and *Lb. bulgaricus* (*ca*. 9 log cfu/mL) were mixed in a 1:1 ratio to prepare culture combinations as widely attributed in the literature (**Tamime and Robinson, 2007**). Yogurt samples were produced by inoculating *ca*. 9 log cfu/mL of combined cultures into 500 mL of fresh cow's milk [3.2% (w/v) milk fat and 3.0% (w/v) protein] which was heat-treated at 90 °C for 10 min. Samples were incubated at 43°C until pH 4.6 was attained, and then were stored at 4 °C for 21 days.

Determination of acidification profiles of combined strains

The pH values of milk samples inoculated with each combination of yogurt bacteria were monitored during fermentation with hourly intervals at 43 ºC until pH 4.6 was attained. pH measurements were achieved by means of a combined electrode pH-meter (Hanna Instruments, Rhode Island, USA).

Quantification of free fatty acids (FFAs) of yogurt samples

Lipid extraction. Lipid extraction from yogurt samples was carried out *as per* **de Jong and Badings (1990)**. For this purpose, 3 g of yogurt sample was mixed with 8 g of anhydrous sodium sulfate. This mixture is transferred to a capped flask, and 0.3 mL of 2.5 M H_2SO_4, 1 mL of internal standard solution [valeric acid ($C_{5:0}$), heptanoic acid ($C_{7:0}$), heptadecanoic acid ($C_{17:0}$), each at a concentration of 0.5 g/L] and 15 mL ether/heptane (1:1) were added to each sample mixture. The samples were mixed by vortex for 1 min and centrifuged at 490 x *g* for 2 min. The upper clear phase was transferred to capped bottles containing 1 g of anhydrous sodium sulfate, and collected for solid-phase extraction.

Solid-Phase Extraction. The aminopropyl columns (AccuBond II SPE, Agilent Technologies Ltd., CA, USA) were preconditioned with 10 mL of heptane before use. The collected supernatant from the previous step was passed through the column under vacuum and the triglycerides were eluted. Then, neutral triglycerides were removed by passing 20 mL of hexane:2-propanol (3:2, v/v) mixture. The fatty acids attached to the column filling material were extracted with 2.5 mL of ether solution containing 2% formic acid and the entire free fatty acid extract was taken into 1 mL amber vials (Agilent Technologies Ltd., CA, USA), capped with polytetrafluoroethylene (PTFE) septa (Agilent Technologies Ltd., CA, USA). FFAs were determined by injecting 0.5 µL of this solution directly on the gas chromatography [(GC 6890 series, Agilent Technology, CA, USA) equipped with a flame ionization detector (FID) and TR-FFAP capillary column (30 m x 0.25 mm x 0.25 µm CP7420, Agilent Technologies Ltd, CA, USA)]. Nitrogen (purity: ≥99.999%) was used as carrier gas at a flow rate of 2 mL/min. The oven temperature was kept at 260°C and the injector temperature was set at 250°C. 0.5 µL aliquots were injected on column with a split ratio of 1:40. The temperature program was initially kept at 90 °C for 1 min, then increased by 7 °C *per* min to 240 °C and kept constant at this temperature for 15 min.

For calculation of FFAs concentrations, a standard mixture was prepared at five concentrations (100-500 mg/kg). 0.05 g of each fatty acid was weighed and made up to 50 mL with 6% formic acid prepared in ether. The standard mixture prepared as 1000 mg/kg master stock was injected into the device under the same conditions and the standard curve was drawn. The concentrations of FFAs were calculated using the equations (1) and (2) and expressed as mg/kg:

$$C_i = \frac{A_i}{A_{st}} \times C_{st} \times RF \times DF \quad (1)$$

$$RF = \frac{\text{Standard peak area}}{\text{Compound peak area}} \times \frac{\text{Concentration of compound}}{\text{Concentration of internal standard}} \quad (2)$$

where $\mathbf{C_i}$ is the concentration of the compound (mg/kg), A_i is the peak area of the compound, A_{st} is the peak area of the internal standard, C_{st} is the concentration of internal standard (mg/kg), RF is the response factor and DF is the dilution factor. Butyric acid ($C_{4:0}$), caproic acid ($C_{6:0}$) and caprylic acid ($C_{8:0}$) were evaluated on the basis of valeric acid; capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$) and myristic acid ($C_{14:0}$) on the basis of heptanoic acid; palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) on the basis of heptadecanoic acid.

Rheological characterization of yogurts and fermenting milks

Small deformation dynamic rheological analysis

Small deformation analysis of fermenting milk and yogurt samples was conducted on a dynamic rheometer (Kinexus Pro+, Malvern Instruments Ltd., Worcestershire, UK). Both gelation profiles of fermenting milks during fermentation and small oscillatory rheological profiles of yogurt samples were determined *as per* **Ozer *et al.* (1998)**. To determine the transition of fermenting milks from sol to gel phase, milk samples inoculated with starter bacteria were left to gel between two parallel plates (ϕ 20 mm) at 43 °C and time-dependent changes in elastic modulus (G', storage modulus) and viscous modulus (G", loss modulus) were monitored. tan δ (G"/G') values of the samples were calculated at G'=1 Pa. The frequency and deformation rate were applied as 1 Hz and 3%, respectively, and the temperature was controlled at 43 ºC throughout the measurement by a water bath connected to the rheometer. The measurements were ceased when the pH of fermenting milk was attained 4.6.

Frequency sweep tests were used to analyze the small oscillatory rheological properties of the yogurt samples at 1 and 21 days of cold storage. Measurements were carried out using a parallel plate geometry with 20 mm diameter at 4 ºC. The frequency range applied was 0.01 to 10 Hz. tan δ values of yogurt samples were calculated at 1 Hz. Apparent viscosities *versus* shear rate (0.01 to 10 s^{-1}) was also measured to determine the flow behavior of the samples. All rheological measurements were carried out within the linear viscoelastic region defined by preliminary tests and the convenience to non-Newtonian flow models was determined by Power Law model. Measurements were done in triplicate.

Large deformation texture analysis

The resistance of yogurt samples to large deformation forces was measured with the back extrusion test using a Texture Analyzer (TX.2TA model, Stable Micro Systems, Godalming, UK). The device was operated with a cell load of 5 kg and the samples were subjected to the penetration test under a 30 mm cylindrical probe. The penetration depth and the penetration rate were 15 mm and 1 mm/s, respectively. Measurements were carried out at 4 °C and firmness, cohesiveness, consistency and index of viscosity values were recorded. Measurements were done in triplicate.

Sensory analysis

The yogurt samples were organoleptically evaluated at the end of cold storage (at day 21). Sensory evaluations were made using the 5-point hedonic scale (**Isleten and Karagul-Yuceer, 2006**), with volunteer adult tasters of both genders (n=50) who consume yogurt regularly. Before the test, participants were provided with information about the yogurt samples offered to them and then were given oral instructions on how to conduct the test. An evaluation sheet with a balanced 5-point hedonistic verbal scale (1-dislike a lot, 2-dislike a little, 3-neither like nor dislike, 4- like a little, 5- like a lot) was given to each consumer. Odorless and tasteless cracker and water were also given to cleanse the palate between samples.

Enumeration of starter cultures

For the enumeration of *Str. thermophilus* and *Lb. bulgaricus* colonies M17 agar (spread pale technique) and acidified-MRS agar (pH 5.4, pour plate technique) medium were used, respectively. Serial dilutions were prepared in Ringer's solution. For *Str. thermophilus*, incubation was carried out under aerobic conditions at 37 °C for 24 h, and for *Lb. bulgaricus*, petri plates were incubated at

43 °C for 72 h under microaerophilic conditions created by an anaerobic medium kit (Anaerocult®A, Merck Millipore, Darmstadt, Germany). Colony numbers were calculated by equation 3 (**Anonymous, 2005**).

$$N = \frac{C}{\left[V \times (n_1 + 0.1n_2) \times d\right]} \quad (3)$$

where ***N*** is the number of microorganisms in 1 g of yogurt sample, ***C*** is the total number of colonies in all counted petri dishes, ***V*** is the volume of diluted sample transferred into counted petri dishes (mL), n_1 is the number of petri dishes counted from the first dilution, n_2 is the number of petri dishes counted from the second dilution and ***d*** is the dilution rate of the higher concentration of the two consecutive dilutions counted.

Statistical analysis

Statistical analyses were performed by one-way ANOVA using SPSS version 17.0 (Cary, NC, USA). Differences between the groups were determined by least-significant difference (LSD) test ($p>0.05$) for sensory evaluation. Texture analysis results were reported as mean ± SD. Experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Acidification performances of combined cultures

One of the most important factors in selection of starter cultures for yogurt production is the acidification performances of the bacteria (Liu *et al.*, 2016). Slow acidification rate during fermentation can cause defects that directly affect the quality of yogurt, such as serum separation and insufficient aroma/flavor development (Tamime and Robinson, 2007). In addition, the prolongation of the fermentation period also leads to economic losses. In general, strains with fast acidification abilities during fermentation and limited post-acidification capability are preferred in yogurt production. The pH-time profiles of the yogurt samples are presented in Figure 1.

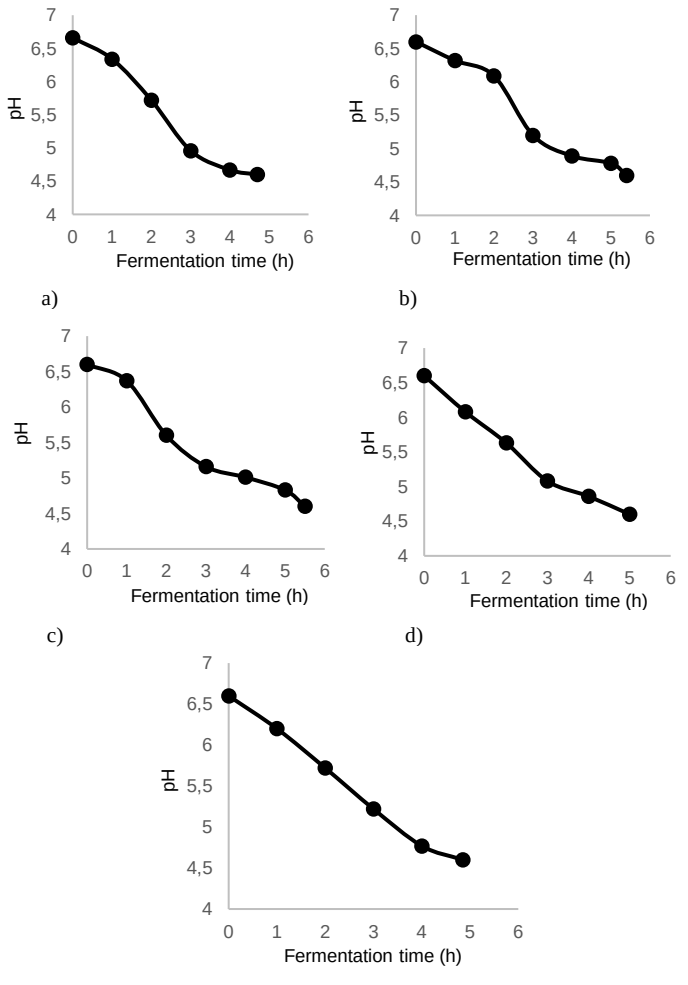

Figure 1 pH profiles of the starter combinations during fermentation (fermentation end point: pH 4.6). (a) ST5/LB2, (b) ST5/LB3, (c) ST5/LB5, (d) ST5/LB6, (e) ST5/LB7. For sample codes please refer to Table 1. Standard error bars are smaller than symbol dimension

In industrial scale yogurt production, the whole fermentation process is expected to complete within 4-6 hours. Overall, all combinations reached to pH 4.6 around 5 h with slight differences in the pH-time profiles, ST5/LB2 observed as the most prominent combination. Acidification velocity of yogurt milk is one of the major parameters determining coagulation process during fermentation (**de Brabandere and de Baerdemaeker, 1999**). During yogurt fermentation, the pH-driven changes in casein micelle stability occur in three distinct phases. In the first phase, the pH of milk drops to *ca*. 6 where very small amount of colloidal calcium phosphate is dissolved, indicating small changes in casein micelle stability. In the present case, all combinations reached to pH *ca*. 6 within less than two hours. In the second phase, pH of fermenting milk reaches to 5.1-5.2 where colloidal calcium phosphate is completely dissolved (**de Brabandere and de Baerdemaeker, 1999**). All the combinations reached to this pH range within 3 hours. In the final stage, the pH reaches to 4.6 where all the caseins are solubilized and yogurt gel matrix is formed. Total fermentation times of the yogurt samples varied between 282 and 330 min. These figures may well be optimized to *ca*. 240 min (as mostly desired by industrial applications) by adjusting the cocci to bacilli balance in the commercial combinations. In the present case, the ratio between *Str. thermophilus* and *Lb. bulgaricus* isolates was set to 1:1.

Gelation profiles of fermenting milks

The rheological changes during gelation of fermenting milks are presented in Figure 2. In general, gelation profiles of all combinations were similar. Although the time intervals varied, an adaptation period was observed before gelation started (at G'=1 Pa) in all combinations. This was in line with the fact that colloidal calcium phosphate is dissociated very slowly at higher pHs (i.e. ~6.0). This period also coincides with the lag phase of bacterial growth (**Tamime and Robinson, 2007**). The combination of ST5/LB3 had the shortest transition time from sol to gel phase (25 min) and the combination of ST5/LB7 had the longest (157,5 min). The latter combination yielded a fairly weak gel during fermentation compared to other samples. Yogurt gel is defined as a typical viscoelastic acid casein gel, and the viscous (G") and elastic (G') characteristics change simultaneously during gelation (**Sodini *et al.*, 2004**). Overall, all samples showed a gelation profile which is characteristic to yogurt type viscoelastic gels, being elastic modulus (G') higher than viscous modulus (G"). On other hand, the sample produced from ST5/LB3 combination had remarkably higher G' values than G" values. None of the samples reached to the physically metastable position (plateau region) which is expected for a yogurt-type gels and the increase in curd firmness continued during fermentation. In general, a negative relationship was noted between the rate of pH decrease and the rate of increase of G'. The tan δ values (G"/G') of the samples were found to be close to each other (between 0.191 and 0.275). This evidenced that similar chemical weak (mainly hydrophobic and electrostatic) and strong (covalent thiol-disulfide) bonds were present in all of the samples with varying degrees as was noted differences in the G' and G" values (Fig 2). During yogurt-type protein matrix formation, G´ values increase as a result of the additional bonds between proteins and new arrangements in the protein network structure (**Nguyen *et al.*, 2014**). As the isoelectric point of the β-casein (pI 5.1) is approached during fermentation, the electrostatic repulsions around the caseins decrease and hence interactions of caseins increase. This eventually leads to increases in elastic modulus of the matrix.

Small oscillatory rheological properties of yogurts

Since yogurt gel shows weak viscoelastic properties, it cannot protect its structural integrity under the influence of high shear forces (**Ozer and Robinson, 1998**). Therefore, in order to determine the physical properties of the product correctly and to minimize experimental errors and artefacts in dynamic rheological measurements, it is important to carry out all measurements within a linear viscoelastic range (**Benezech and Maingonnat, 1994; Lee and Lucey, 2010**). Overall, strong gels keep their structural integrity within the linear viscoelastic region over a greater strain range compared with weak gels (**Steffe, 1996**). In this study, a series of preliminary experiments were carried out to determine the linear viscoelastic region and a shear stress range between 0.01 to 10 Pa at 1 Hz was set as measuring conditions. All small deformation oscillatory measurements were carried out within this linear viscoelastic region (not shown). No deformation was observed in the gel structures within the shear stress range tested.

Figure 3 shows the frequency sweep profiles of the samples. All samples showed a distinct frequency-dependency and no structural deformation occurred in the samples at any frequency point.

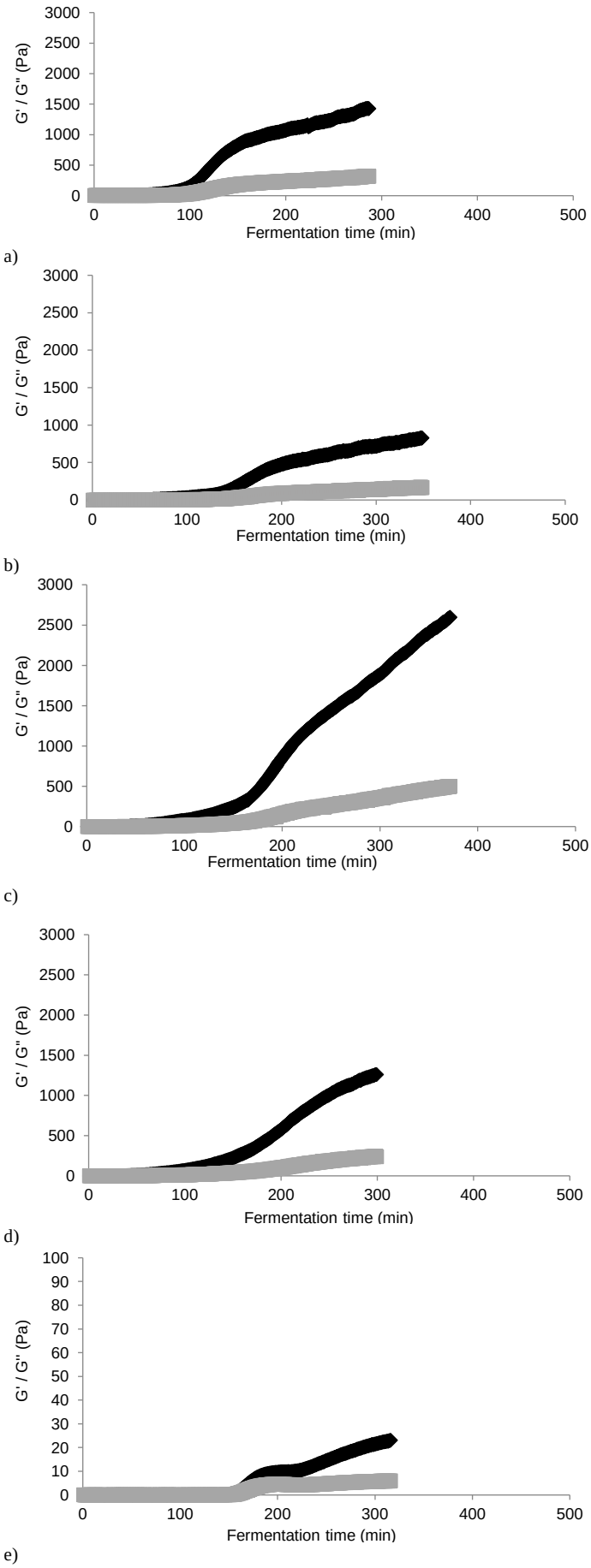

Figure 2 Gelation profiles of yogurt samples as measured by small oscillatory rheology. Black and grey lines represent storage (G') and loss (G") modulus, respectively. (a) ST5/LB2, (b) ST5/LB3, (c) ST5/LB5, (d) ST5/LB6, (e) ST5/LB7. Standard error bars are smaller than symbol dimension

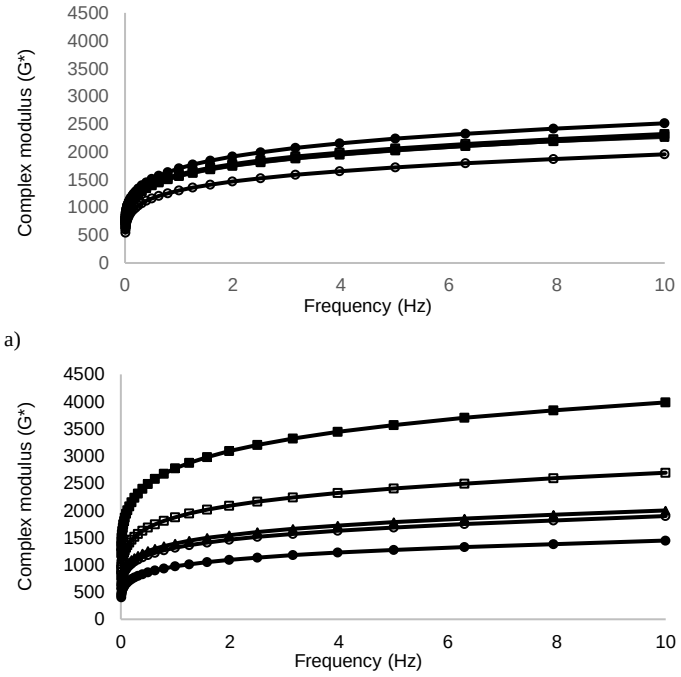

Figure 3 Frequency sweep profiles of yogurt samples at day 1 (a) and day 21 (b). (●) ST5/LB2, (▲) ST5/LB3, (■) ST5/LB5, (○) ST5/LB6, (□) ST5/LB7. Frequency range applied was 0.01 to 10 Hz. For sample codes please refer to Table 1. Standard error bars are smaller than symbol dimensions. G*=|G'+*i*G"|

Complex moduli (G*=|G'+*i*G"|) of the samples which represent overall viscoelasticity of the gels were closer to each other at day 1 (Fig 3a). However, at day 21, the differences between the samples became more remarkable (Fig 3b). Over the range of frequency, G' values of the samples were greater than G" values, indicating a solid-like behavior (not shown). The high frequency dependent feature indicates that all gels have a heterogeneous structure, which is expected for yogurt (**Ozer and Robinson, 1998**). The increase in the network moduli of the gels with the increase in frequency indicates the loosening of the protein bonds during the measurement period. Frequency-dependent moduli increase without any deformation indicates that permanent protein bonds with limited relaxation (expressed as G') are dominant. While the combination of ST5/LB2 stood out as the sample with the most stable gel structure at day 1, this sample showed a time-dependent weakening at the end of storage period (Fig 3b). On contrary, the samples ST5/LB5 and -to a lesser extent- ST5/LB7 showed rheological improvement during cold storage. Differences in frequency sweep profiles of yogurt may be due to the interference of technological parameters applied as well as the specific characteristics of the starter culture strain (*i.e.* acidification kinetics, exopolysaccharide production) (**Rohm and Kovac, 1994**). The time-dependent variation in rheological characteristics of yogurt samples may stem from dynamic reorganization of protein bonds during the cold storage process. This restructuring process is closely related to the glycolytic and proteolytic activities of bacteria under cold storage conditions. In the present case, the isolates used in yogurt production had low or moderate proteolytic activities as were determined during the screening of single isolates (**Uzunsoy, 2018**).

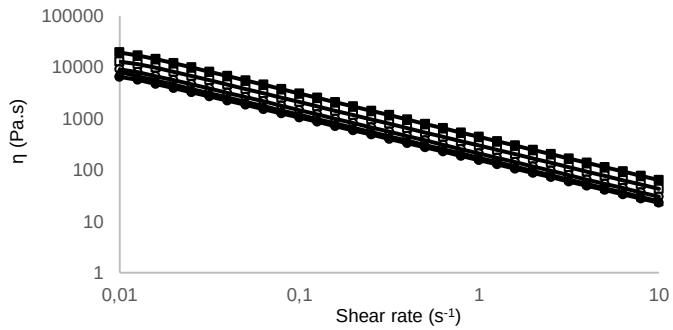

Figure 4 Apparent viscosities of 21-day old yogurt samples as a function of shear rate. (●) ST5/LB2, (▲) ST5/LB3, (■) ST5/LB5, (○) ST5/LB6, (□) ST5/LB7. For sample codes please refer to Table 1

Therefore, it is fair to assume that the rheological changes were due to restructuring of yogurt network as a function of acidification during cold storage. All samples showed a shear-thinning behavior as a function of shear rate (Fig 4). This is a

characteristic behavior for yogurt type-gels as the weak chemical bonds contributing to yogurt network are disrupted by increasing shear (**Abu-Jdayil *et al.*, 2000; Bond and Moraru, 2014**).

The compatibility of the dynamic rheological data of the experimental yogurts to Power Law model was evaluated (Table 2). The fitness of the data set to Power Law model was satisfactory as the correlation coefficients were over 99% in all samples. The highest shear viscosity (Pa.s) value was obtained in ST5/LB7 combination. The close flow behavior indices of the samples indicated that the interaction forces (weak and strong protein interaction forces) forming the yogurt gel structure were similar in all samples. This was further supported by tan δ values of the samples. The tan δ values of the samples were between 0.254 and 0.282, indicating that the interaction forces contributing to the yogurt gel matrix formation were the same but the degree of contributions was slightly different (**Ozer and Robinson, 1998**).

Table 2 Compatibility of the yogurt samples to Power Law Model

Combinations	Power Law Model			
	Shear viscosity (Pa.s)	Correlation coefficient	*n* (flow behavior index)	tan δ
ST5/LB2	4.334	0.999	0.165	0.282
ST5/LB3	5.236	0.998	0.153	0.281
ST5/LB5	5.824	0.998	0.160	0.261
ST5/LB6	4.757	0.998	0.170	0.280
ST5/LB7	10.55	0.999	0.161	0.254

For sample codes please refer to Table 1

The results of large deformation texture analysis of yogurts are presented in Table 3. Firmness is the most important parameter for the evaluation of the physical quality especially in set-type yogurts (**Ozer *et al.*, 1998**). No remarkable differences were noted between the samples regarding firmness values at day 1. The firmness of the samples increased during cold storage, being slightly more pronounced in the combination of ST5/LB2. The similar trends were observed for consistency and cohesiveness values as well. Cohesiveness gives information about the resistance of the inner bond to deformation forces in a gel matrix (**Chandra and Shamasundar, 2015**). The firmness, consistency, cohesiveness and index of viscosity results of the yogurts are consistent with (**Haktan, 2022**) and different from some studies (**Miocinovic, 2016; Bierzuńska *et al.*, 2019**), showing that these textural parameters of yogurt differ according to the starter culture (**Rohm and Kovac, 1994; Hess *et al.*, 1997; Rawson and Marshall, 1997**).

Table 3 Back extrusion test results of 1-day and 21-day old yogurt samples

Combinations	Firmness (g)		Consistency (g/s)		Cohesiveness (g)		Index of viscosity	
	1	21	1	21	1	21	1	21
ST5/LB2	154.5±0.62	202.3±9.16	1958.2±4.23	2268.0±11.09	74.5±6.05	111.3±10.72	155.9±10.02	222.3±18.27
ST5/LB3	135.2±3.76	175.7±1.52	1566.9±73.78	2083.8±32.92	70.5±4.86	88.7±1.10	155.1±12.57	186.5±1.25
ST5/LB5	152.7±1.37	188.6±0.85	1792.1±4.49	2180.8±28.62	80.6±8.95	89.7±0.13	164.7±2.01	181.4±7.94
ST5/LB6	144.6±7.77	164.4±11.51	1708.7±119.75	1911.9±31.39	72.1±3.11	111.1±3.73	167.7±6.21	145.5±66.78
ST5/LB7	140.3±10.19	165.4±13.79	1636.9±55.58	1891.4±64.21	61.6±4.15	105.2±5.29	140.7±9.46	94.0±22.36

For sample codes please refer to Table 1

As seen in Table 3, the cohesiveness values increased in all yogurts, being less pronounced in ST5/LB5 combination. This indicated that the structural integrity of the yogurt gel matrices was improved during cold storage. There are a number of factors affecting the textural characteristics of yogurt matrix, including starter culture, acidification kinetics, heat treatment, physical interventions, protein ratio, homogenization, exopolysaccharide production, post-incubation pH and cooling rate (**Rohm and Kovac, 1994; Hess *et al.*, 1997; Rawson and Marshall, 1997**). Since all of these parameters were kept constant throughout the experiment, except for the combination of bacteria and acidification kinetics, it seems highly likely that the textural differences between samples were due to the change in fermentation kinetics. Although the pH of the incubation end point was the same, the proto-cooperation between *Str. thermophilus* and *Lb. bulgaricus* could significantly affect acidification kinetics during fermentation and cold storage, as observed in the differences of incubation times to reach pH 4.6.

Lipolytic activities of combined cultures

Figure 5 represents the variation in free fatty acids (FFAs) values of yogurt samples during storage. In general, the concentrations of total, medium- and long-chain FFAs in all combinations decreased over the storage period (Fig 5a). Long-chain FFAs were dominant in all samples (Fig 5d). The combination of ST5/LB7 yielded the highest FFAs both at day 1 and day 21. The decrease in FFAs over the storage period may be the result of oxidation or ester formation by metabolism of lactic acid bacteria (**Menendez *et al.*, 2000**) and was also reported for yogurt in **Regula (2007)** and **Guler and Gulsoy-Balci (2011)**. Interestingly, linolenic acid ($C_{18:3}$), which is one of the primary polyunsaturated fatty acids in dairy products (**Seckin *et al.*, 2005; Florence *et al.*, 2009**) was not detected in ST5/LB2 combination. This fatty acid was present in ST5/LB5 and ST5/LB6 samples at low concentrations at day 1 and levelled off after 21 days of storage. Palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic acids ($C_{18:2}$) were the most abundant fatty acids in all combinations. The FFA composition of yogurt shows similarity to milk. While triacylglycerol lipase of *Str. thermophilus* is effective on tributyrin and triolein, its effect on milk fat is partially limited. *Lb. bulgaricus* has an intracellular esterase activity that acts on *orto*- and *para*-nitrofenyl. In general, it is known that the changes in the concentrations of FFAs in yogurt vary according to the starter bacteria strain and milk type (**Florence *et al.*, 2012; Sumarmono *et al.*, 2015**). Although the yogurt bacteria have limited lipolytic activities, the FFAs formed by starter bacteria may well contribute to the aroma and flavor of yogurt (**Vagenas and Roussis, 2012**).

Changes in the colony counts of yogurt bacteria and pH of yogurts during storage

The changes in colony numbers of the *Str. thermophilus* and *Lb. bulgaricus* are presented in Figure 6. Overall, there is no clear difference between the samples regarding the counts of *Str. thermophilus* at both storage days (Fig 6a) and a limited decrease was observed in the *Str. thermophilus* colony counts during storage period. On contrary, the number of *Lb. bulgaricus* was lower than *Str. thermophilus* at both storage days. It is the fact that the symbiotic relationship between *Str. thermophilus* and *Lb. bulgaricus* tends to favor the former one. One should bear in mind that breaking up the long chains of *Str. thermophilus* comprising 5-20 cells likely change the ratio in favor of *Str. thermophilus* as *Lb. bulgaricus* is in short chains of rods (2-3 cells) (**Ozer and Robinson, 1999**).

The change in pH values of the samples during the 21-day storage period is given in Figure 7. At the beginning of the storage, pH values of all samples varied between 4.50 and 4.69 reached 4.21-4.37 after 21 days of storage. One of the desired characteristics of yogurt bacteria for commercial productions is to reduce the pH of milk to 4.6 within 4-5 hours during fermentation, but to develop acidity more slowly under cold storage conditions. From this point of view, all combinations were found to be suitable for commercialization. The resistance of single isolates used in the present study to freeze-drying conditions was also high enough as was reported in **Uzunsoy (2018)**.

Sensory properties of yogurt samples

Sensory scores of 21-day old yogurt samples are presented in Figure 8. As the detailed descriptive sensory profiling of the samples was discussed in **Uzunsoy *et al.* (2021)**, here only the evaluations of untrained consumers are presented. Regarding appearance and body and texture characteristics, the combination of ST5/LB5 received the highest scores. Overall, no clear differences were noted among the samples but aroma and flavor scores of all combinations were lower than other sensory characteristics assessed. The descriptive sensory analysis of the culture combinations revealed that fruity and insufficient yogurt aroma were dominant in the end products (**Uzunsoy *et al.*, 2021**). Since the proteolytic activities of the individual strains used in the present study were low (Uzunsoy, 2018), the interference of proteolysis on the aroma and flavor development is

expected to be negligible. This could be related with the imbalance between strains in the combinations as 1 to 1 ratio between *Lb. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus* was used in the present case. Our current study is focusing on the determination of optimized balance between yogurt starter strains for improving the aroma and flavor of the end products. No visual wheying-off on top of the yogurt pots was evident in the samples. None of the panelists criticized the samples as inconsumable.

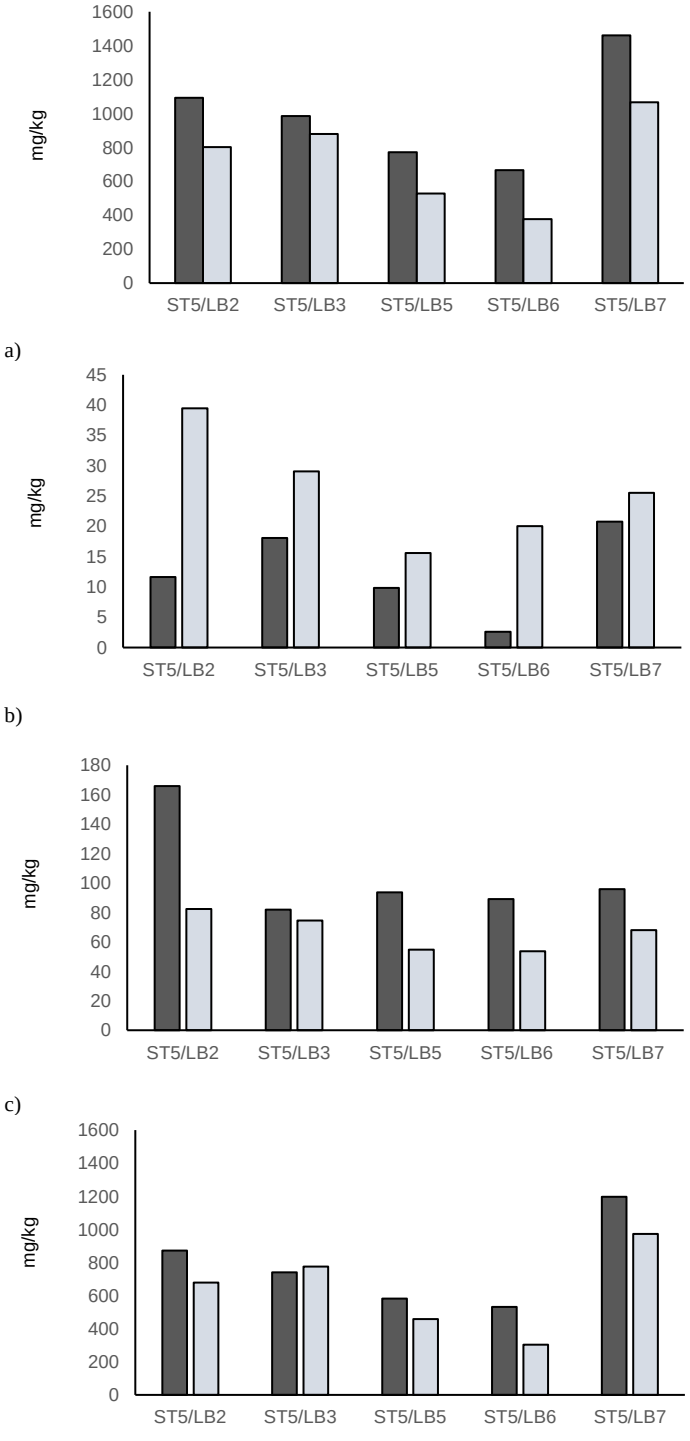

Figure 5 Variation in (a) total, (b) short-chain, (c) medium chain and (d) long chain free fatty acids concentrations of yogurt samples during storage. Black and grey bars represent the 1st and 21st days of storage, respectively. For sample codes please refer to Table 1

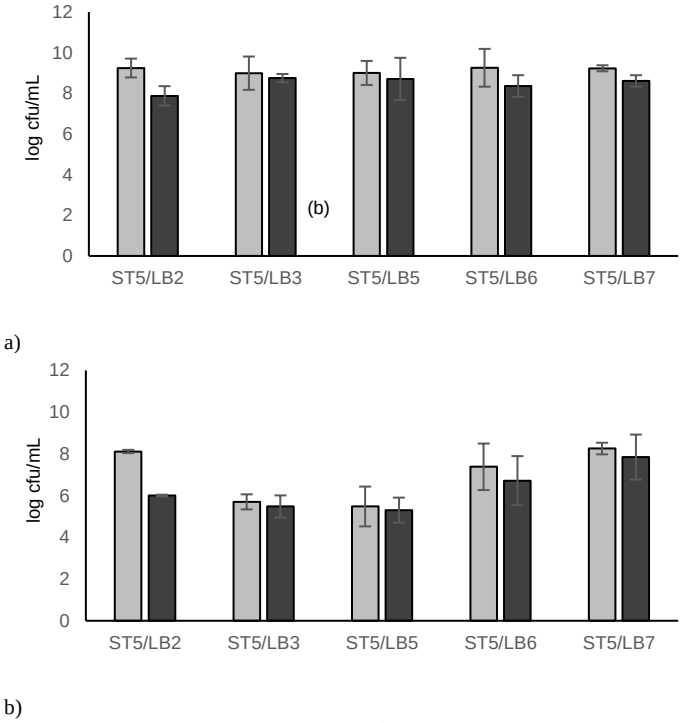

Figure 6 Changes in colony numbers of (a) *Str. thermophilus* and (b) *Lb. delbrueckii* subsp. *bulgaricus* during storage period (n=2). Grey and black bars represent 1-day old and 21-day old yogurt samples, respectively

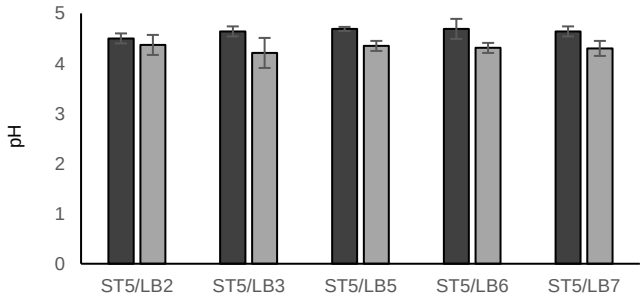

Figure 7 Changes in pH of yogurt samples during cold storage (n=2). Black and grey bars represent 1-day old and 21-day old samples, respectively

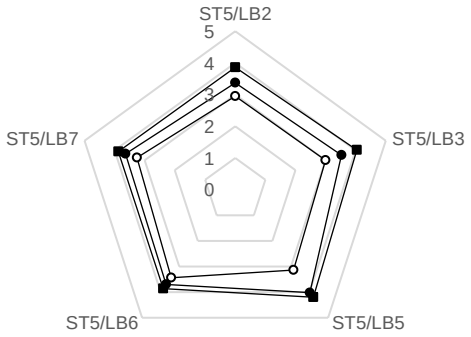

Figure 8 Sensory profiles of 21-day old yogurt samples. (■) Appearance, (●) Body & Texture, (○) Aroma & Flavor (n=50)

CONCLUSION

The isolates used in the study were selected from 48 *Str. thermophilus* and 15 *Lb. bulgaricus* isolates previously taking into account their technological compatibilities and growth characteristics (**Uzunsoy, 2018**). Among the prescreened isolates, 5 *Str. thermophilus* and 7 *Lb. bulgaricus* isolates were further evaluated for their technological performances and organoleptical properties in **Uzunsoy *et al.* (2021)**. In the present study, the most promising combinations made up of one *Str. thermophilus* isolate with five *Lb. bulgaricus* strains were evaluated for gelation profiles, fermentation abilities, post-acidification properties, large and

small deformation rheological characteristics and consumers test for organoleptical properties. Results showed that all the combinations reduced the pH of milk to 4.6 within 5 hours with similar pH-time profiles to commercial yogurt cultures. All the samples showed a shear-thinning rheological behaviour which is also characteristic for yogurt type gels. No marginal differences were noted between the textural characteristics of combinations. Consumer preferences of the samples regarding sensory properties were also close to each other. Aroma and flavor scores of the samples were lower than other sensory parameters evaluated. Therefore, further studies should focus on establishing the most suitable balance between cocci and bacilli in the combinations to improve aroma and flavor of the end products.

Acknowledgment: This study was financially supported jointly by The Scientific and Technical Research Council of Turkey (Project No: 112D052) and FARMAPARK R&D Biotechnology, Machinery, Chemistry, Food LC (Konya, Turkey). The authors declare that they do not have any conflict of interest. Dr. Irem Uzunsoy undertook yogurt productions, gelation analysis and fermentation processes. Dr. Sebnem Ozturkoglu Budak carried out fatty acids analysis. Dr. Tuba Sanli run the sensory analysis. Prof. Birce Taban was responsible for the microbiological analysis. Prof. Aykut Aytac, Prof. Nuray Yazihan, Prof. A. Levent Bas and Prof. Barbaros Ozer were responsible for the conceptualization, management of the project, running textural analysis, statistical evaluations and writing the paper.

REFERENCES

Abu-Jdayil, B., Shaker, R. R. & Jumah, R. Y. (2000). Rheological behavior of concentrated yogurt (Labneh). *International Journal of Food Properties*. 3: 207-216. https://doi.org/10.1080/10942910009524628

Angelov, M., Kostov, G., Simova, E., Beshkova, D. & Koprinkova-Hristova, P. (2009). Proto-cooperation factors in yogurt starter cultures. Revue de Génie Industriel. 2009; 3: 4-12.

Anonymous. (2005). Calculation of the number of colonies. In Halkman, K. (ed) Merck Food Microbiology Practices. Turkey: Basak Publishing.

Aryana, K. J. & Olson, D. W. (2017). A 100-year review: Yogurt and other cultured dairy products. *Journal of Dairy Science*, 100(12), 9987-10013. https://doi.org/10.3168/jds.2017-12981

Benezech, T. & Maingonnat, J. F. (1994). Characterization of the rheological properties of yoghurt-a review. *Journal of Food Engineering*. 21: 447-472. https://doi.org/10.1016/0260-8774(94)90066-3

Bierzuńska, P., Cais-Sokolińska, D. & Yiğit, A. (2019). Storage stability of texture and sensory properties of yogurt with the addition of polymerized whey proteins. *Foods*, 8, 548-556. https://doi.org/10.3390/foods8110548

Bond, D. D. & Moraru, C. I. (2014). Use of micellar casein concentrate for Greek-style yogurt manufacturing: Effects on processing and product properties. *Journal of Dairy Science*. 97: 1259-1269. https://doi.org/10.3168/jds.2013-7488

Chandra, M. V. & Shamasundar, B. A. (2015). Texture profile analysis and functional properties of gelatin from the skin of three species of fresh water fish. *International Journal of Food Properties*. 2015; 18(3): 572-584. https://doi.org/10.1080/10942912.2013.845787

de Brabandere, A. G. & de Baerdemaeker, J. G. (1999). Effects of process conditions on the pH development during yogurt fermentation. *Journal of Food Engineering*. 41: 221-227. https://doi.org/10.1016/S0260-8774(99)00096-5

de Jong, C. & Badings, H. T. (1990). Determination of free fatty acids in milk and cheese. Procedures for extraction, clean-up and capillary gas chromatographic analysis. *Journal of High Resolution Chromatography*. 13: 94-98. https://doi.org/10.1002/JHRC.1240130204

de Souza Oliveira, P. R., Torres, B. R., Perego, P., de Oliveira, M. N. & Converti, A. (2012). Co-metabolic models of *Streptococcus thermophilus* in co-culture with *Lactobacillus bulgaricus* or *Lactobacillus acidophilus*. *Biochemical Engineering Journal*. 62: 62-69. https://doi.org/10.1016/j.bej.2012.01.004

El-Zahar, K., Chobert, J., Sitohy, M., Dalgalarrondo, M. & Haertlé, T. (2003). Proteolytic degradation of ewe milk proteins during fermentation of yoghurts and storage. *Nahrung*. 3: 199-206. https://doi.org/10.1002/food.200390046

Florence, A. C. R., Silva, R. C., Espírito Santo, A. P., Gioielli, L. A., Tamime, A. Y. & Oliveira, M. N. (2009). Increased CLA content in organic milk fermented by bifidobacteria or yoghurt cultures. *Dairy Science and Technology*. 89: 541-553. https://doi.org/10.1051/dst/2009030

Florence, A. C. R., Béal, C., Silva, R. C., Bogsan, C. S. B., Pilleggi, A. L. O. S., Gioielli, L. A. & Oliveira, M. N. (2012). Fatty acid profile, trans-octadecanoic, α-linolenic and conjugated linoleic acid contents differing in certified organic and conventional probiotic fermented milks. *Food Chemistry*. 135: 2207-2214. https://doi.org/10.1016/j.foodchem.2012.07.026

Ginovart, M., López, D., Valls, J. & Silbert, M. (2002). Simulation modelling of bacterial growth in yoghurt. *International Journal of Food Microbiology*. 73: 415-425. https://doi.org/10.1016/s0168-1605(01)00668-7

Guler, Z. & Gursoy-Balci, A. C. (2011). Evaluation of volatile compounds and free fatty acids in set types yogurts made of ewes', goats' milk and their mixture using two different commercial starter cultures during refrigerated storage. *Food Chemistry*. 127: 1065-1071. https://doi.org/10.1016/j.foodchem.2011.01.090

Haktan, K. (2022). Physicochemical, aromatical, rheological and sensory properties of yoghurts produced from different mixture rates of buffalo and cow milks, M.Sc. thesis, Tekirdağ: Namık Kemal University.

Hess, S. J., Roberts, R. F. & Ziegler, G. R. (1997). Rheological properties of nonfat yogurt stabilized using *Lactobacillus delbrueckii* ssp. *bulgaricus* producing exopolysaccharide or using commercial stabilizer systems. *Journal of Dairy Science*. 80: 252-263. https://doi.org/10.3168/jds.S0022-0302(97)75933-2

Isleten, M. & Karagul-Yuceer, Y. (2006). Effects of dried dairy ingredients on physical and sensory properties of nonfat yogurt. *Journal of Dairy Science*. 89: 2865 2872. https://doi.org/10.3168/jds.s0022-0302(06)72559-0

Lee, W. J. & Lucey, J. A. (2010). Formation and physical properties of yogurt. *Asian Australian Journal of Animal Science*. 23(9): 1127-1136. https://doi.org/10.5713/ajas.2010.r.05

Liu, W., Yu, J., Sun, Z., Song, Y., Wang, X., Wang, H., Wuren, T., Zha, M., Menghe, B. & Heping, Z. (2016). Relationships between functional genes in *Lactobacillus delbrueckii* ssp. *bulgaricus* isolates and phenotypic characteristics associated with fermentation time and flavor production in yogurt elucidated using multilocus sequence typing. *Journal of Dairy Science*. 99(1): 89-103. https://doi.org/10.3168/jds.2015-10209

Menendez, S., Centeno, J. A., Godínez, R. & Rodríguez-Otero, J. R. (2000). Effects of *Lactobacillus* strains on the ripening and organoleptic characteristics of Arzúa-Ulloa cheese. *International Journal of Food Microbiology*. 59: 37-46. https://doi.org/10.1016/s0168-1605(00)00286-5

Miocinovic, J., Miloradovic, Z., Josipovic, M., Nedeljkovic, A., Radovanovic, M. & Pudja, P. (2016). Rheological and textural properties of goat and cow milk set type yoghurts. *International Dairy Journal*, 58, 43-45. https://doi.org/10.1016/j.idairyj.2015.11.006

Nguyen, H. T. H., Ong, L., Kentish, S. E. & Gras, S. L. (2014). The effect of fermentation temperature on the microstructure, physicochemical and rheological properties of probiotic buffalo yoghurt. *Food and Bioprocess Technology*. 7: 2538-2548. https://doi.org/10.1007/s11947-014-1278-x

Ozer, B. (2006). *Yoğurt Bilimi ve Teknolojisi*. Izmir: Sidas Yayinevi.

Ozer, B. H., Robinson, R. K., Grandison, A. S. & Bell, A. E. (1998). Gelation properties of milks concentrated by different techniques. *International Dairy Journal*. 8: 793-799. https://doi.org/10.1016/S0958-6946(98)00115-0

Ozer, B. & Robinson, R. K. (1999). The behaviour of starter cultures in concentrated yoghurt (labneh) produced by different techniques. *Lebensmittel-Wiss. u.-Technologie*. 32: 391-395. https://doi.org/10.1006/fstl.1999.0566

Rawson, H. L. & Marshall, V. M. (1997). Effect of 'ropy' strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* on rheology of stirred yogurt. *International Journal of Food Science and Technology*. 32(3): 213-220. https://doi.org/10.1046/j.1365-2621.1997.00395

Regula, A. (2007). Free fatty acid profiles of fermented beverages made from ewe's milk. *Lait*. 87: 71-77. https://doi.org/10.1051/lait:2006024

Rohm, H. & Kovac, A. (1994). Effects of starter cultures on linear viscoelastic and physical properties of yogurt gels. Journal of Texture Studies. 25(3): 311-329. https://doi.org/10.1111/j.1745-4603.1994.tb00763

Routray, W. & Mishra, H. N. (2011). Scientific and Technical Aspects of Yogurt Aroma and Taste: A review. *Comprehensive Reviews in Food Science and Food Safety*. 10: 208-220. https://doi.org/10.1111/j.1541-4337.2011.00151

Seckin, A. K., Gursoy, O., Kinik, O. & Akbulut, N. (2005). Conjugated linoleic acid (CLA) concentration, fatty acid composition and cholesterol content of some Turkish dairy products. *LWT Food Science and Technology*. 3: 909-915. https://doi.org/10.1016/j.lwt.2004.09.011

Settachaimongkon, S., Nout, M. J. R, Antunes Fernandes, E. C., Hettinga, K. A., Vervoort, J. M., van Hooijdonk, T. C. M., Zweitering, H. M., Smid, E. J. & van Valenberg, H. J. F. (2014) Influence of different proteolytic strains of *Streptococcus thermophilus* in co-culture with *Lactobacillus delbrueckii* subsp. *bulgaricus* on the metabolite profile of set-yoghurt. *International Journal of Food Microbiology*. 177: 29-36. https://doi.org/10.1016/j.ijfoodmicro

Sieuwertz, S., de Bok, F. A. M., Hugenholtz, J. & van HylckamaVlieg, J. E. T. (2008). Unraveling microbial interactions in foods: from classical to genomics approaches. *Applied and Environmental Microbiology*. 74(16): 4997-5007. https://doi.org/10.1128/AEM.00113-08

Smid, E. J. & Lacroix, C. (2013). Microbe–microbe interactions in mixed culture food fermentations. *Current Opinion in Biotechnology*. 24: 148-154. https://doi.org/10.1016/j.copbio.2012.11.007

Sodini, I., Remeuf, F., Haddad, S. & Corrieu, G. (2004). The relative effect of milk base, starter, and process on yogurt texture: a review. *Critical Reviews in Food Science and Nutrition*. 44: 113137. https://doi.org/10.1080/10408690490424793

Steffe, J. F. (1996). Oscillatory testing. pp. 313–324. In Steffe J. (ed) *Rheological methods in food process engineering*, 2nd ed. USA: Freeman Press.

Sumarmono, J., Sulistyowati, M. & Soenarto, X. (2015). Fatty acids profiles of fresh milk, yogurt and concentrated yogurt from peranakan etawah goat milk. *Procedia Food Science*. 3: 216-222. https://doi.org/10.1016/j.profoo.2015.01.024

Tamime, A. Y & Robinson, R. K. (2007). *Yogurt Science and Technology*, 3rd ed. Oxford: Woodhead Publishing.

Uzunsoy, I. (2018). Determination of suitability of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains isolated from traditional

yogurt samples for industrial yogurt productions and developing starter combinations, Ph.D. thesis. Ankara: Ankara University.
Uzunsoy, I., Ozturkoglu Budak, S., Sanli, T., Taban, B., Aytac, A., Yazihan, N. & Ozer, B. (2021). Assessment of growth and technological performances of yogurt bacteria isolated from local Turkish yogurts. *Food Science and Engineering*. 2(2): 52-64. https://doi.org/10.37256/fse.222021758
Vagenas, G. & Roussis, I. G. (2012). Fat-derived volatiles of various products of cows', ewes', and goats' milk. *International Journal of Food Properties*. 15: 665-682. https://doi.org/10.1080/10942912.2010.498542
Yamauchi, R., Maguin, E., Horiuchi, H., Hosokawa, M. & Sasaki, Y. (2019). The critical role of urease in yogurt fermentation with various combinations of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Dairy Science*. 102(2): 1033-1043. https://doi.org/10.3168/jds.2018-15192
Zourari, A., Accolas, J. P & Desmazeud, M. J. (1992). Metabolism and biochemical characteristics of yogurt bacteria. A review. *Lait*. 72: 1-34.