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Research Article

Effects of the Incorporation of Hydrogen and Nitrogen into Milk on the Reducing and Acidification Capacities of Yoghurt Bacteria

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The use of gases in different food applications has increased in the last decades. The incorporation of hydrogen into yogurt was reported to improve the organoleptic properties of some dairy products. A study is needed to understand the effect of this application on the behavior of yogurt bacteria during the fermentation stage. The effects of H_2- and N_2-incorporation into milk on the acidification and reducing capacities of *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST) were envisaged. A data acquisition multiparameter interface equipped with pH and redox electrodes was used for continuously measuring and recording values. H_2 increased the acidification capacity of ST (+10%) and the reducing capacity of LB (+13.7%), whereas N_2 increased the acidification capacity of ST (+13.3%) and decreased the reducing capacity of LB (-21.47%) and ST (-18.88%). H_2 increased the coagulation time of LB (+12.96%) and ST (+6.15%). Results could reveal the promotive role of hydrogen on the metabolic activity of yogurt bacteria in single cultures and these bacteria grow better in the presence of hydrogen in milk. These results will allow yogurt manufacturers to better understand the behavior of yogurt bacterial strains when the preparation of gas-incorporated yogurt is envisaged and better control its quality attribute notes.

1. Introduction

The role of the starter in the yoghurt preparation is to provide acidity, viscosity, and desirable aroma and flavor to the product. These bacteria can decrease the pH value of the milk from 6.3-6.5 to 4.6 by the fermentation process of lactose into mainly lactic acid as an end metabolite. When the pH value reaches the isoelectric point ($t_{coagulation}$), i.e. 4.6, the characteristic taste and specific aroma of yoghurt are achieved. The yoghurt strains are known as thermophilic lactic acid bacteria, with fermentative activity in facultative anaerobic conditions with a preference for anaerobic conditions [1, 2]. Many studies revealed the effect of the incorporation of different gases into milk on yoghurt quality attributes and survival properties of starters [3–5]. Ebel et al. [3] reported different techniques to protect probiotic bacteria from oxygen toxicity in fermented milk. These include the use of oxygen-consuming strains, the use of oxygen scavengers such as ascorbate and L-cysteine, and the use of specific packaging material or microencapsulation.

The use of gases to achieve these desirable goals has been increasingly explored. The advantages of the use of different gases in dairy products were described in the review of Adhikari et al. [6]. The authors reported many gases such as air, carbon dioxide, and nitrogen, which were applied in different dairy products such as ice cream, whipped cream, and butter for obtaining different improvements in their texture and mouthfeel as well as shelf-life extension.

Recent research revealed the advantageous uses of hydrogen gas as a therapeutic antioxidant in the health field [7–9]. The hydrogen gas (H_2) was reported to possess many beneficial roles in the physiology and pathology of the cell. Ohsawa et al. [10] reported in their research the selective antioxidant role of hydrogen in decreasing the reactive

oxygen species (ROS) levels, especially the most cytotoxic and the strongest oxidant species i.e. the hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^{-}$), permitting protection of the cell components against oxidative stresses/damages. The work of Ohsawa et al. [10] has opened the door for more research related to the potential uses of hydrogen as a therapeutic antioxidant [11]. Furthermore, the H_2 saturated-solutions such as hydrogen-rich water (HRW) were widely studied for their antioxidative, anti-inflammatory, and DNA-protection properties in the case of heavy metal contaminations [12, 13], inhibition of the formation of biogenic amines [14] and the accumulation of heavy metals [15], and preservation of quality attribute notes of butter [16].

Few reports envisaged the importance of hydrogen and nitrogen incorporation in food products. Recent reviews were published about the applications of different gases in the food industries such as dairy and beverage [6, 8, 17–19]. In the last two decades, the use of hydrogen in food industries has been recently paid attention by researchers and food processors to its specific advantageous properties [14, 20]. Furthermore, the use of hydrogen-incorporated gaseous mixtures was found to protect antioxidants and freshness notes of different food products such as orange juice [18, 21], strawberries [22], dried apricot [23], dried apple [24], fat-free fermented milk [3], nonfat yogurt ([4]), acid skimmed milk gels [25], polyunsaturated fatty acids-enriched dairy beverage [26], butter [14–16], white cheese [27], and fish [28].

The use of gases in dairy products was gradually advised by different reports [6, 17–20, 29]. Regarding dairy products, some studies revealed the advantageous incorporation of hydrogen-incorporated gaseous mixtures, i.e. N_2-H_2, into the yoghurt preparations such as the improvement of textural and rheological properties of yoghurt [5, 25]. However, the modification of the fermentation medium by the addition of any external materials such as food additives, plant extracts, and gases can affect the acidification and reducing capacities of yoghurt bacteria leading to modifying the quality attribute notes of the product [17, 30, 31]. Thus, we hypothesize that the incorporation of gases such as hydrogen and nitrogen may similarly lead to modifying the acidification and reducing capacities of lactic acid bacteria (LAB) during fermentation. In this work, we aimed to explore the effect of the incorporation of H_2 and N_2 into milk on the behavior of yoghurt bacteria by studying their acidification and reducing capacities in single and mixed cultures. This is the first study revealing the impact of gas incorporation effect on the acidification and reducing properties of yoghurt starters, which will help the dairy industry to better control the quality of the product.

2. Materials and Methods

2.1. Media and Culture Conditions. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains were obtained from Ankara University, Food Engineering Department, Laboratory of Microbiology. The preparation of preculture and culture was performed as described previously by Alwazeer, Bulut, et al. [17]. Briefly, 10 μL of a preculture was added into a 100 mL MRS broth bottle and left for further incubation at 45°C for 13 h.

2.2. Incorporation of Gases into Milk Samples. The skim milk powder (Uğuray, Turkey) was added to an appropriate volume of distilled water to obtain 12% (w/w) total solids milk. The milk was heated at 90°C for 5 min followed by a cooling step up to 43°C. The gases (N_2, H_2) (99.9%, Elite Gaz Teknolojileri, Ankara, Turkey) were separately bubbled in Schott glass bottles containing 100 mL milk samples. The gases were supplied at a 1 L/min rate for about 10 min before the inoculation step of bacterial inoculum through a 0.45 μm filter (Chromafil Xtra PTFE-45/25, Macherey-Nagel, Germany). One milliliter of each LB and ST culture was separately centrifuged (Neofuge 23 R-Q/Teuc 40-2011, China) at 5000 g for 20 min at 4°C. The pellet was washed twice with sterile peptone water followed by a similar centrifugation step. The bacterial pellet was then collected by a small volume of pasteurized milk for making bacterial preparations. The previously gas-bubbled milk samples, as well as the non-bubbled sample (control), were then inoculated with these bacterial preparations (LB, ST, and mixed LB + ST) followed by an incubation step at 43°C for 12 h. During this stage, the time taken to reach a pH value of 4.6 ($t_{coagulation}$) and its corresponding $E_{h7coagulation}$ value were recorded.

2.3. Acidification and Reducing Capacity Measurements. The pH and E_h values of milk samples were continuously recorded for 12 h by a data acquisition multiparameter interface (Consort multiparameter analyzer C3040, Belgium). An SP60X (Consort, Belgium) oxidoreduction electrode and an SP10R (Consort, Belgium) pH electrode were used. The calculation of E_{h7}, acidification capacity, and reducing capacity was performed as follows:

$$E_{h7} = E_h - 38(7 - pH_m), \tag{1}$$

where E_h is the electrode potential value referred to as the normal hydrogen electrode and pH_m is the measured pH value of the milk sample.

$$\text{Acidification capacity} = \frac{pH_{initial}\text{ of milk} - 4.6}{t_{coagulation}},$$
$$\text{Reducing capacity} = \frac{\left(E_{h7}\text{ initial of milk} - E_{h7coagulation}\right)}{t_{coagulation}}. \tag{2}$$

2.4. Statistical Analysis. The analysis of variance (ANOVA) was performed using SPSS 22 software at a 95% confidence interval. The significance level of the differences between the applications was calculated using Duncan's test. The Pearson correlation test was applied to determine the relationship between the applications. All assays were performed in three replications.

3. Results and Discussion

3.1. Acidification Capacity. For evaluating the effect of gas incorporation on the acidification and reducing activities of yoghurt bacteria, the E_{h7} and pH values were continuously recorded during 12 h of fermentation (Figure 1). The results shown in Table 1 showed that the acidification rate values change according to both the strain and the gas type. Both N_2- and H_2-incorporated milk samples showed no change in the acidification rate of LB and mixed LB + ST. However, the H_2-incorporation showed an increase in the acidification capacity of ST by +10.0%, while N_2-incorporation led to an increase by +13.3% compared to the control ($p < 0.05$) (Table 1). Shekar and Bhat [32] found an increase in acid production rate in buffalo milk inoculated separately by several lactic acid bacteria including both *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* when the initial oxygen content of milk decreased. Beshkova et al. [1] reported that the mixed culture of *L. delbrueckii* subsp. *bulgaricus* 2-11 and *S. thermophilus* 15HA produced more intensive lactic acid in 10 and 20% oxygen conditions compared with the anaerobic ones and in mixed cultures than in the pure culture under the same conditions.

In control and H_2-incorporated milk samples, both LB and mixed LB + ST exhibited a higher acidification rate than ST ($p < 0.05$) (Table 1). However, in N_2-incorporated milk samples, the mixed LB + ST showed a higher acidification rate than the two single strains with a higher value attributed to LB compared to ST ($p < 0.05$).

For all types of gas and control, the mixed LB + ST exhibited the highest value of acidification rate compared to single cultures (Table 1). In control samples, LB, ST, and mixed LB + ST exhibited, respectively, 0.0049, 0.0030, and 0.0054 pH unit/min (Table 1). Horiuchi and Sasaki [33] reported that the acid production by *L. delbrueckii* subsp. *bulgaricus* 2038 and *S. thermophilus* 1131 in reduced dissolved oxygen samples (prepared by injecting nitrogen gas inside inoculated milk samples during an undefined time) were promoted in mixed cultures (called LB81), while there was no influence in single cultures. The authors concluded that the dissolved oxygen does not interfere with the growth of single cultures while it does with mixed cultures. The difference in the methods and strains between our study and the previous one could explain these observations.

The synergistic effect between LB and ST appears obviously in mixed LB + ST samples. This symbiosis phenomenon, or so-called protocooperation, was widely revealed for these strains. An increase in the acidification rate in mixed cultures of yoghurt bacteria compared to single cultures was widely reported in the literature [34–36]. This phenomenon was related to the enhancement of the lactic acid production rate during mixed culture fermentation [37, 38]. Furthermore, it was reported that *S. thermophilus* produces a growth stimulator factor, i.e. formic acid, for *L. delbrueckii* subsp. *bulgaricus* under anaerobic or almost anaerobic conditions [39]. It was reported that when milk samples were incorporated with thyme and grape seed extracts, the highest values of acidification capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST) were 0.0065 pH unit/min and 0.0068 pH unit/min, respectively [17]. This shows that any modification of the composition of the fermentation medium can lead to a change in the behavior of the starter affecting its growth kinetics and then modify the properties of the product.

3.2. Reducing Capacity. Figure 1 shows the change in the E_{h7} value of milk samples in different conditions (Control, N_2, and H_2) inoculated with different strains (LB, ST, and mixed LB + ST) during incubation at 43°C for 720 min. The results shown in Table 1 reveal that the reducing capacity changes according to the type of gas and the bacterial strain.

While the H_2-incorporation of milk samples increased the reducing capacity of LB by +13.69% with up to 0.94 mV/min, N_2-incorporation samples decreased it by -21.47% with down to 0.65 mV/min compared to control samples (0.83 mV/min) ($p < 0.05$) (Table 1). Regarding ST, while the H_2-incorporation of milk samples did not exhibit any change in reducing capacity, the N_2-incorporation samples decreased it by -18.88% down to 0.56 mV/min compared to control samples (0.69 mV/min) ($p < 0.05$) (Table 1). Concerning the mixed LB + ST, the incorporation of both gases did not exhibit any change in reducing capacity compared to the control ($p > 0.05$) (Table 1).

These results reveal the promotive role of H_2 in the reducing capacity of LB in single cultures compared with an inhibitor effect of N_2. This difference in the effect of both gases on the reducing capacity disappeared in mixed bacterial cultures.

This enhancing effect of H_2 in the case of LB could not be related to only the elimination of oxygen from milk samples because both H_2- and N_2-incorporated samples lacked oxygen due to the gas bubbling step. It seems that the presence of hydrogen in milk samples may have led to neutralize many oxidative species produced in LB cells such as $^{\bullet}OH$, O_2^-, and H_2O_2 during the sugar metabolism of LAB [40]. The inhibitory effects of H_2O_2 against LAB were reported to be potentiated in natural environments such as milk (Condon 1987). Although both *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were reported to produce H_2O_2 [41], and the amounts of H_2O_2 produced by *L. delbrueckii* subsp. *bulgaricus* are higher than that of *S. thermophilus* [1]. This can explain the promotive effect of H_2-incorporation on the reducing capacity of LB compared with ST.

The reducing activity of LAB can be related to the production of some metabolites with reducing properties like reduction enzymes, NADH, $FADH_2$, and thiol group included-amino acids and peptides [19]. Free amino acids such as His, Tyr, Thr, and Lys, as well as peptides, liberated from milk proteins by *L. delbrueckii* subsp. *bulgaricus* were reported to exhibit antioxidant properties, which explains the higher reducing capacity of this strain in our study [19, 42]. Although the absence of superoxide dismutase activity, strains of *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *B. longum* showed the capacity to chelate metal ions, scavenge reactive oxygen species, or possess reducing activity [43]. Lin and Yen [44] reported that all 11 studied strains had reactive oxygen species-scavenging ability with the highest hydroxyl radical scavenging activity

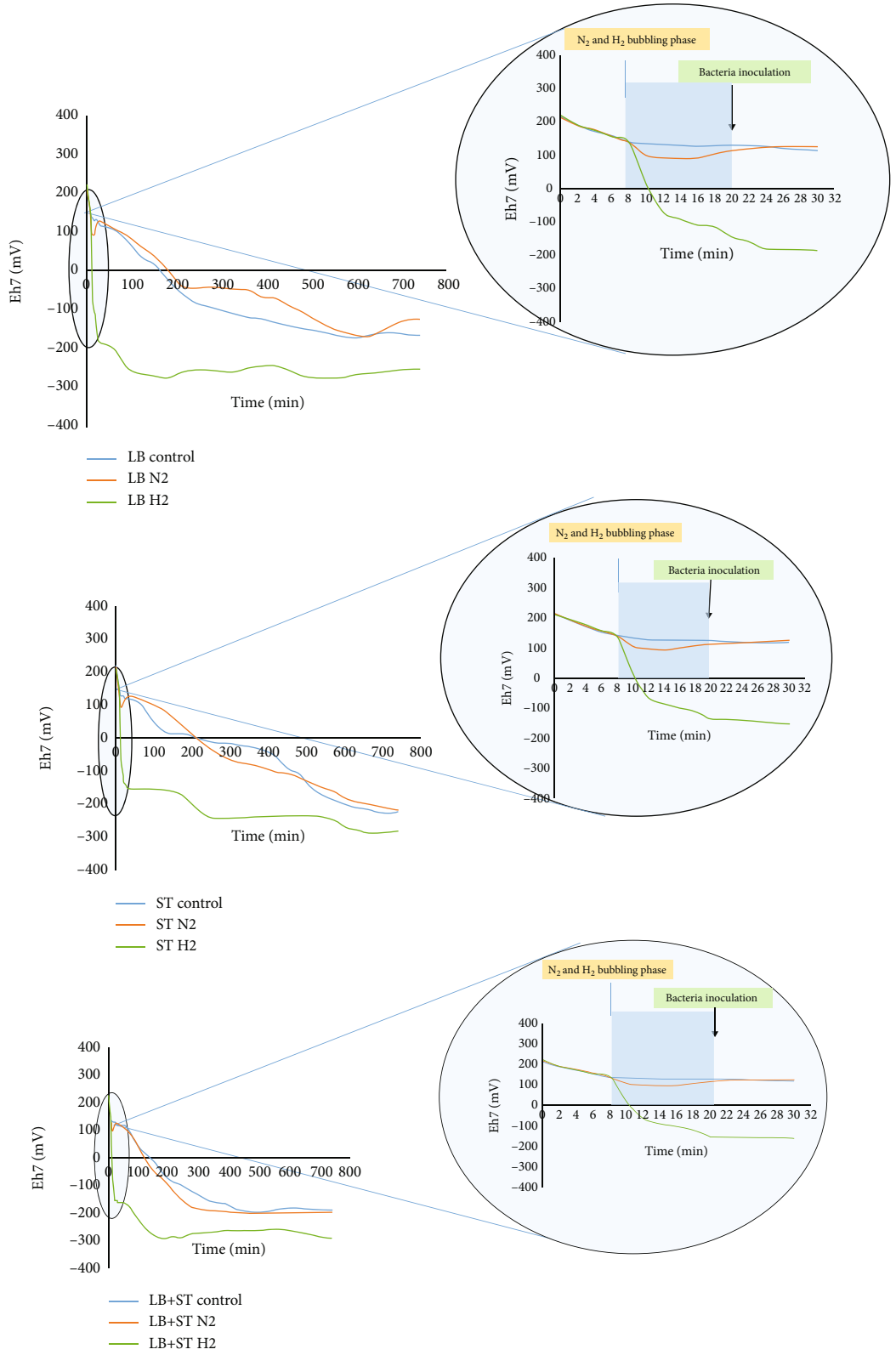

Figure 1: The evolution of E_{h7} (mV) value of different conditions (Control, N_2, and H_2) of milk samples inoculated with *L. delbrueckii* subsp. *bulgaricus* (LB), *S. thermophilus* (ST), and mixed LB + ST during incubation at 43°C for 720 min.

Table 1: Acidification and reducing capacities of *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB), *Streptococcus thermophilus* (ST), and mixed culture of mixed LB + ST inoculated in control, N_2- or H_2-incorporated milk samples during an incubation period of 12 h at 43°C.

Strains	Acidification capacity (pH unit/min)∗		
	Control	N_2	H_2
Lactobacillus delbrueckii subsp. *bulgaricus*	0.0049 ± 0.0005^{aA} (0.294 ± 0.03)	0.0044 ± 0.0002^{aB} (0.264 ± 0.012)	0.0045 ± 0.0001^{aA} (0.27 ± 0.006)
Streptococcus thermophilus	0.0030 ± 0.0000^{bB} (0.18 ± 0.00)	0.0034 ± 0.0001^{aC} (0.204 ± 0.006)	0.0033 ± 0.0005^{aB} (0.198 ± 0.03)
Lactobacillus delbrueckii subsp. *bulgaricus* + *Streptococcus thermophilus*	0.0054 ± 0.0003^{aA} (0.324 ± 0.018)	0.0055 ± 0.0002^{aA} (0.33 ± 0.012)	0.0050 ± 0.0001^{aA} (0.3 ± 0.006)
	Reducing capacity (mV/min)∗		
Lactobacillus delbrueckii subsp. *bulgaricus*	0.83 ± 0.08^{bB} (49.8 ± 4.8)	0.65 ± 0.01^{cB} (39.0 ± 0.6)	0.94 ± 0.03^{aA} (56.4 ± 1.8)
Streptococcus thermophilus	0.69 ± 0.00^{aB} (41.4 ± 0.0)	0.56 ± 0.06^{bC} (33.6 ± 3.6)	0.68 ± 0.05^{aB} (40.8 ± 3)
Lactobacillus delbrueckii subsp. *bulgaricus* + *Streptococcus thermophilus*	1.27 ± 0.23^{aA} (76.2 ± 13.8)	0.97 ± 0.08^{Aa} (58.2 ± 4.8)	1.03 ± 0.31^{aA} (61.8 ± 18.6)

Different small letters on the same row indicate a significant difference between gas conditions ($p < 0.05$). Different capital letters on the same column indicate a significant difference between strains ($p < 0.05$). ∗ Values in brackets represent pH unit/h and mV/h.

attributed to *L. delbrueckii* subsp. *bulgaricus*. On the other hand, Stecchini et al.[45] reported that some nonenzymatic antioxidants such as glutathione and thioredoxin may play a role in the cellular alleviation of oxidative reactions by reducing certain reactive oxygen intermediates such as ROS.

From the previous reports, we can assume that in the presence of H_2, known as a potent reducing agent, the antioxidative metabolic activity of LB and the production of amino acids (e.g. Tyr, Trp, Met, Lys, Cys, and His) and peptides (e.g. His-containing peptides) with reducing property may be accelerated. To prove this hypothesis, a further study on the level of oxidative reaction products especially the H_2O_2 in the absence and presence of H_2 in milk samples inoculated with these bacterial strains should be conducted. Additionally, the analysis of metabolites, amino acids, and peptide profiles produced during fermentation in all culture types with the screening of their reducing/oxidizing activity should be an interesting subject for future study.

On the other hand, the depletion of dissolved oxygen, and the neutralization of some oxidative metabolites such as hydrogen peroxide and free radicals, may explain the high values of the reducing capacity of H_2-incorporated samples in LB cultures. McSweeney et al. [46] reported that the dissolved oxygen content, lactate: pyruvate ratio, ascorbate and thiol levels, and metal ions are the main biological factors influencing the E_h value of dairy products such as cheese [46]. The decline in the E_h value of milk during fermentation has been explained by the consumption of dissolved oxygen and the reducing activity of each *Lactococcus lactis* [47], *Lc. Lactis*, and *Leu. mesenteroides* [48]. Jeanson *et al. [46]* used a mutant that could not consume dissolved oxygen for proving this hypothesis and revealed that this mutant can reduce the culture medium (lowering E_h value), which shows the presence of a reducing activity system in *Lc. lactis*. Moreover, NADH-oxidase of *Lc. lactis* that transforms O_2 into water has been shown to play important role in this mechanism. The increase in the acidification and reducing activities of some LAB species has been related to their enzymatic activities [49]. The values of reducing capacity found in for control sample are similar to that found by the latter authors; who found a maximum reducing capacity value of 0.77 mV/min for *S. thermophilus*. In the latter study, the calculation of acidification and reducing activity values were performed over all the times of fermentation; while in the present study these parameters were calculated when the pH value of the medium reached 4.6.

For *S. thermophilus*, the decrease of its reducing capacity in N_2-incorporated samples could be explained by the low production of formic acid in anaerobic conditions and low level of H_2O_2 [1, 33].

It was reported that when milk samples were enriched with different plant extracts, the highest values of the reducing capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST) were -0.98 mV/min (thyme), -1.92 mV/min (grape seed), and -0.75 mV/min (green tea) samples for LB, ST, and mixed culture of LB + ST, respectively [17].

Regarding the effect of strain type on the reducing capacity in control conditions, we observe that the mixed bacterial cultures, i.e. LB + ST, exhibited the highest levels followed by LB then ST, with, respectively, 1.27, 0.83, and 0.69 mV/min (Table 1). The synergetic effect, i.e. protocooperation, between LB and ST could explain this phenomenon.

On the other hand, previous studies have reported the protective effects of the H_2-incorporated atmosphere in the RAD drying method on the antioxidant activity (DPPH, ABTS, and flavonoids) of apples and apricots [23, 24]. In our laboratory, yoghurt samples produced from H_2-incorporated milk exhibited high antioxidant activity (unpublished

Table 2: Coagulation time and E_{h7} values at the coagulation point, i.e. pH 4.6, of *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB), *Streptococcus thermophilus* (ST), and mixed culture of mixed LB + ST inoculated in control, N_2 or H_2-incorporated milk samples during an incubation period of 12 h at 43 C.

Strains	$t_{coagulation}$ (min) Control	N_2	H_2
Lactobacillus delbrueckii subsp. *bulgaricus*	453 ± 50^{bB}	492 ± 18^{abB}	512 ± 16^{aB}
Streptococcus thermophilus	650 ± 10^{bA}	675 ± 12^{abA}	690 ± 36^{aA}
Lactobacillus delbrueckii subsp. *bulgaricus* + *Streptococcus thermophilus*	407 ± 38^{aB}	423 ± 30^{aC}	463 ± 99^{aB}
	$E_{h7\,coagulation}$ (mV)		
Lactobacillus delbrueckii subsp. *bulgaricus*	-145 ± 1^{bB}	-114 ± 9^{cC}	-274 ± 2^{aAB}
Streptococcus thermophilus	-254 ± 1^{bAB}	-235 ± 36^{bA}	-295 ± 13^{aA}
Lactobacillus delbrueckii subsp. *bulgaricus* + *Streptococcus thermophilus*	-306 ± 14^{aA}	-196 ± 6^{aB}	-265 ± 27^{aB}

$*t_{coagulation}$: Acidification time i.e. the time taken to reach a pH value of 4.6; $E_{h7\,coagulation}$: the E_{h7} value obtained when pH value of the sample reaches 4.6. Different small letters on the same row indicate a significant difference between gas conditions ($p < 0.05$). Different capital letters on the same column indicate a significant difference between strains ($p < 0.05$).

data). These previous reports indicate the advantageous role of hydrogen in the protection of the antioxidant property of food products.

The presence of a promotive role of hydrogen on the acid and the reducing metabolic activity of yogurt bacteria in single cultures can be assumed. The reducing property of hydrogen that can neutralize many types of oxidative species in the cell such as the reactive oxygen species and other free radicals could explain this promotive role of hydrogen on the metabolic activity of yogurt bacteria [9, 10]. Regarding the previous studies reporting the protective effects of the hydrogen-included atmosphere on the different biological activities, we can assume an advantageous role of hydrogen in the total quality attribute notes of products.

In this concept, it is important to indicate that this stimulation role of hydrogen gas has been obtained at levels of gas as low as 4% (v/v). In preliminary assays, we found that N_2-H_2 (96/4%, v/v) mixtures introduced similar effects on the acidification and reducing capacities of the same strains i.e. LB and ST (nonpublished data). Thus, when industrial applications of hydrogen are envisaged, nonexplosible nitrogen and hydrogen mixtures will be applicable especially when safety measures are considered. In another study, bubbling milk with nitrogen and, more particularly, with a nitrogen-hydrogen (96/4%, v/v) mixture has been shown to increase the survival of a probiotic strain i.e. *Bifidobacterium bifidum* during the storage stage without affecting the survival of yogurt strains i.e. *S. thermophilus* and *L. bulgaricus* [3].

3.3. Coagulation Time and $E_{h7coagulation}$. The coagulation time ($t_{coagulation}$, min) in control conditions was as follows: ST > LB = mixed LB + ST with a value of 650, 453, and 407 min, respectively ($p < 0.05$) (Table 2). Results show that the N_2- and H_2-incorporation in milk samples increased significantly the coagulation time of samples in the case of single strains with the highest effect attributed to H_2-incorporated samples (Table 2). The incorporation of hydrogen in milk provided the highest coagulation time in single cultures compared to control, with an increase of +12.9% and+6.15% for LB and ST, respectively. Although the H_2 incorporation increased the coagulation time of LB + ST by +13.7%, this increase was not statistically significant ($p > 0.05$). Our findings agree with those of Beshkova et al. [1] who reported that *Streptococcus thermophilus* 15HA exhibited a shorter time to coagulate milk at a partially aerated medium (20%-oxygen concentration) compared with anaerobic conditions (similar to our N_2- and H_2-incorporated samples) with 180 and 330 min, respectively. The authors explained their results by two possible mechanisms: (1) the protocooperation in the mixed culture (the stimulating effect of *S. thermophilus* 15HA on the growth of *L. delbrueckii* subsp. *bulgaricus* 2-11) and (2) the increase of its proteolytic activity. Furthermore, it has been reported that the symbiotic relationship between *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* led to a decrease in the fermentation time [33].

Our results are also in agreement with those obtained by Horiuchi and Sasaki [33] who found that the fermentation time (the time required for the original acidity of 0.2% to reach 0.7% level) of yoghurt bacteria, i.e. mixed LB + ST, was shorter in normal air conditions (control) than reduced dissolved oxygen milk samples prepared by N_2 bubbling. Furthermore, our results showed that the mixed LB + ST exhibited shorter coagulation times compared to single cultures in all control and gas-incorporated samples (Table 2). Beshkova et al. [1] reported that *Streptococcus thermophilus* 15HA grew better in a mixed culture with *L. delbrueckii* subsp. *bulgaricus* 2-11 in 10 and 20% oxygen conditions than the pure culture under the same conditions, or the anaerobic culture. They found also that the growth of *L. delbrueckii* subsp. *bulgaricus* 2-11 was better in a mixed culture at the given oxygen concentrations regardless of the inhibition effect of oxygen on its growth.

The increase of the coagulation time in the case of H_2 incorporation although the increase in the acidification capacity (in the case of ST) indicates the better survival ability of yoghurt bacteria in the presence of H_2. This protective effect of H_2 could be related to its multiphysiologic beneficial effects e.g. antioxidant activity, antiradicals, and antioxidative stress [9].

Regarding the $E_{h7coagulation}$ parameter, the H_2 incorporation led to an increase in its value by +89.0% and +16.3% for

Table 3: Correlation analysis.

	Sample	Strain	Acidification capacity	Reducing capacity	$t_{coagulation}$	$E_{h7\ coagulation}$
Sample	1	0.000	-0.066	-0.079	0.188	-0.225
Strain		1	0.283	0.471**	-0.199	-0.406**
Acidification capacity			1	0.776**	-0.963**	0.132
Reducing capacity				1	-0.741**	-0.480**
$t_{coagulation}$					1	-0.213
$E_{h7\ coagulation}$						1

** Correlation is significant at the 0.01 level (2-tailed).

LB and ST (Table 2). While the incorporation of N_2 led to a decrease in this parameter only for LB by -21.4% compared to the control. In mixed cultures, there was no influence of gas incorporation on this parameter (Table 2) ($p > 0.05$). These results could be explained by the promotive effect of H_2 on LB compared to ST as discussed above.

On the other hand, the correlation analysis revealed the presence of a strong negative correlation between $t_{coagulation}$ and each acidification capacity ($r = -0.963$, $p < 0.01$) and reducing capacity ($r = -0.741$, $p < 0.01$) (Table 3). Furthermore, a positive correlation ($r = 0.776$, $p < 0.01$) between acidification capacity and reducing capacity was shown (Table 3).

On the other hand, it is important to indicate that the stimulation role of H_2 has been obtained at levels of gas as low as 4% (v/v). In preliminary assays, N_2-H_2 (96/4%, v/v) mixtures introduced similar effects on the acidification and reducing capacities of the same strains i.e. LB and ST (unpublished data).

In another study, it has been proved that bubbling milk with N_2 and, more particularly, with N_2-H_2 (96/4%, v/v) mixture increases the survival of a probiotic strain i.e. *Bifidobacterium bifidum* during the storage stage without affecting the survival of yoghurt strains i.e. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* [3]. When the previous observation is combined with our findings, the application of H_2 in yoghurt and other dairy products seems to be advantageous. Thus, when industrial applications of H_2 are envisaged, nonexplosible mixtures of H_2 and N_2 must be used and safety issues have to be considered.

4. Conclusion

The use of gases in dairy products is increasing gradually due to the recent findings showing the improving effects of some gases on the quality of the product. H_2 increased the acidification capacity of the ST strain, whereas, it prompted the reducing capacity in the LB strain. H_2 showed a promotive role in the acid and the reduction metabolic activities of yoghurt bacteria in single cultures. The therapeutic effects of hydrogen gas revealed in many health reports in human and animal models may be now expanded to cover the health state of yoghurt bacteria that can grow better in the presence of H_2. These results should aid yoghurt manufacturers in better managing the yoghurt bacterial strains when gas incorporation is envisaged.

Data Availability

Derived data supporting the findings of this study are available from the corresponding author on request.

Conflicts of Interest

The authors have declared no conflicts of interest for this article.

Authors' Contributions

Menekşe Bulut is responsible for the conceptualization, validation, methodology, supervision, review, and editing. Duried Alwazeer is responsible for the conceptualization, investigation, methodology, and writing of the original draft. Yusuf Tunçtürk is responsible for the investigation and methodology.

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