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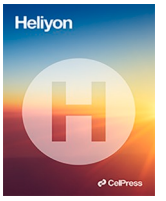

Research article

Development of a functional Greek sheep yogurt incorporating a probiotic *Lacticaseibacillus rhamnosus* wild-type strain as adjunct starter culture

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ABSTRACT

Greek yogurt is a fermented dairy product of high nutritional value that can be used as a matrix for the delivery of probiotics. The aim of this study was to develop a new probiotic Greek sheep yogurt with upgraded quality and functional characteristics. To do this, yogurt was manufactured by fermenting pasteurized milk with the commercial starter culture (*Streptococcus thermophilus* (ST), *Lactobacillus bulgaricus* (LB)) together with a probiotic *Lacticaseibacillus rhamnosus* (LR) wild-type strain (probiotic yogurt; PY). As a control, yogurt manufactured with only the starter culture (ST, LB) was used (conventional yogurt; CY) The survival of all three lactic acid bacteria (LAB) species (ST, LB, and LR) was monitored throughout the products' shelf life (storage at 4 °C for 25 days), and also following exposure to a static *in vitro* digestion model (SIVDM). The population dynamics of total aerobic plate count (APC), *Enterobacteriaceae*, yeasts and molds grown in both yogurts were also determined. The total antioxidant activity (AA) of yogurts was comparatively determined using in parallel two different assays, whereas the Folin-Ciocalteu assay was used to determine their total phenolic content (TPC). At each sampling day, yogurts were also evaluated for their pH, titratable acidity (TA) and main sensory characteristics. The population of probiotic LR remained stable during the shelf life (and above 10^8 CFU/g). Yogurt starters (ST, LB) were not detected following SIVDM, whereas LR (in PY) presented a reduction of about only one log. The AA and TPC of PY were found significantly higher than that of CY ($P < 0.05$). At the end of storage (25th day), neither pH nor TA differed significantly between the two yogurt types, while no fungal growth was observed in the PY. Consumer sensory analysis did not reveal important differences between the two yogurt types during their shelf life. To sum up, the novel yogurt was able to deliver to consumers a high number of probiotic cells (>10^8 CFU/g), presented increased antioxidant power, had an expanded shelf life, and maintained its good sensory attributes.

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1. Introduction

In the last decades, there has been an observed increase in non-communicable chronic diseases, such as obesity, diabetes mellitus, and cardiovascular disorders [1]. The modern lifestyle and more specifically the frequent intake of antibiotics, bad eating habits, physical inactivity, and stress, cause a gastrointestinal dysbiosis, which in turn often contributes to the onset and progression of these diseases [2,3]. However, it appears from several studies that probiotics may have a key role in intestinal homeostasis promoting intestinal health [4,5]. These are live microorganisms that, when administered in appropriate amounts, have a positive effect on the health of the host [6]. The fact that good gut health has proven to be a key factor in the proper functioning of the body, together with the ever-increasing percentage of consumers who have received this knowledge and are interested in healthy food choices, capable of preventing and/or curing the above non-communicable diseases, has led to the consolidation of the probiotic functional food market [7,8].

The viability and stability of probiotics in foods and till their arrival to the gut remains however a technological challenge for industrial producers [9,10]. As it is one of the critical points for probiotics to exert their beneficial effects, their survival during food processing and preservation and following exposure to the different harsh conditions of the digestion process, such as low pH, bile salts, and digestive enzymes' activities, is of utmost importance [11]. Achieving an appropriate level ($>10^6$) of viable cells, able to adhere to the intestinal mucosa to colonize and proliferate, has been the subject of research and the food industry has intensified the efforts to add new probiotic strains to food and find new technological methods [12].

Traditional Greek yogurt is a fermented dairy product of high nutritional value, which seems like a suitable matrix for the integration of probiotic cells and their transport through its consumption in the human body [13]. This is because most consumers already have, and rightfully so, a strong positive opinion of the health profile of this product. This is manufactured by fermenting milk, either of bovine or ewe origin, with the lactic acid bacteria (LAB) species *Streptococcus salivarius* subsp. *thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB). This is indeed in accordance with the Codex Alimentarius Commission International Standard (IS) for fermented milks, which defines yogurt as milk fermented with symbiotic starter cultures of ST and LB, which shall be in a viable state, active and still present in the product through the end of shelf life ($\geq 10^7$ CFU/g) [14]. In addition to this IS and according to the Greek Food Law concerning yogurt, other microorganisms are also allowed to be used in the production of yogurt (e.g., probiotic, technological strains) in addition to the strains of the starter culture, which can be listed on the package, provided that their population will be at least 10^6 CFU/g of product on the date of consumption [15].

The health benefits of probiotics are believed to be maximized if these possess the ability to survive and persist, at least transiently, in the human gastrointestinal tract (GIT) [16,17]. Nowadays, various *in vitro* digestion models are available to predictively evaluate the bioaccessibility and bioactivity of food nutrients [18]. These models have also been used to predict the gastrointestinal survival of probiotic microorganisms, as a preliminary step of studies, before further clinical investigations [19,20]. Among these models, static *in vitro* digestion models (SIVDMs) attempt to simulate the *in vivo* digestion conditions and the fact they are easy to perform, economically affordable, and not time-consuming, makes them useful tools to investigate microorganisms' survivability upon their exposure to simulated GIT conditions [21,22].

Probiotic viability in yogurt during shelf life and subsequent survival after passage through the GIT is thus crucial in conferring the health benefits of the product. Besides nutritional value, the enhancement of yogurt's functional and sensory characteristics contributes to the consumer's acceptance. The present study aimed therefore to develop a new probiotic Greek sheep yogurt with upgraded quality and functional characteristics by incorporating at the beginning of fermentation a probiotic LAB wild-type strain of *Lacticaseibacillus rhamnosus* (LR). To the best of our knowledge, no such product is on the market so far. Its successful production is hopefully expected to further boost sales of traditional Greek sheep yogurt, protecting, and strengthening the health of consumers. For this, some of the main microbiological, physicochemical, functional, and sensory properties of the new yogurt (probiotic yogurt; PY) were determined and compared to those of the yogurt manufactured without the addition of probiotic cells (conventional yogurt; CY). More specifically, the populations of the three LAB species (those of the starter culture and the probiotic) were monitored by selective agar plating throughout the products' shelf life (storage at 4 °C for 25 days) and following passage through a SIVDM. The populations of total aerobic plate count (APC), *Enterobacteriaceae*, yeasts and molds grown in yogurts were also determined throughout the products' shelf life, together with the pH and titratable acidity (TA). Both yogurt types (CY, PY) were also comparatively tested for their antioxidant activity (AA) and total phenolic content (TPC), and periodically evaluated for their main sensory characteristics (appearance, odor, taste, texture, and overall acceptance).

2. Materials and methods

2.1. Yogurts production

The production of both yogurt types took place once and in parallel using milk of the same batch (to exclude any biological variation) at the facilities of the cooperating company (Mystakelli Traditional Dairy Products, Mantamados, Lesvos, Greece). In brief, yogurts were manufactured the traditional way by fermenting pasteurized sheep milk with either only the commercial starter culture (ST and LB; YF-L812 Chr Hansen, Hoersholm, Denmark) (CY), or together with the potential probiotic strain 708 of LR, isolated in our lab from raw sheep milk (PY). This latter strain had been previously found to present an increased resistance to low pH (exposure to pH 2.5 for 2 h at 37 °C), strong adherence to collagen, absence of hemolytic activity, and important antihypertensive angiotensin-converting enzyme inhibitory (ACE-I) activity (preliminary screening *in vitro* experiments; data not presented). The probiotic strain was added in milk together with the commercial starter culture at the beginning of fermentation at a concentration of ca. 10^7 CFU/mL.

At both cases (CY, PY), fermentation took place inside the product's plastic containers for 4.5 h at 45 °C. Following fermentation, the yogurts were kept for 24 h at 4 °C and then transported under refrigeration to the premises of the Laboratory of Food Microbiology and Hygiene (Myrina, Lemnos, Greece) where they were preserved at 4 °C for 25 days.

2.2. Enumeration of microorganisms

Five samplings were carried out at different times during the products' shelf life (5th, 11th, 16th, 20th, and 25th day at 4 °C) to study the population dynamics of a) APC, b) ST, c) LB, d) LR, e) *Enterobacteriaceae*, and f) yeasts and molds in the two yogurt types (CY, PY). In each sampling, 10 g of yogurt were taken to which 90 mL of Maximum Recovery Dilutant (MRD; Lab M, Heywood, Lancashire, UK) were added. The mixture was homogenized in a stomacher (BagMixer® 400; Interscience, Saint Nom la Bretêche, France) for 2 min and then used to make serial decimal dilutions (up to 10^{-8}). The counting of each microorganism was done in the following way: a) Tryptic Glucose Yeast Agar (TGYA; Biolife Italiana, Milano, Italy), pouring method, incubation at 30 °C for 72 h, was used to count APC, b) M17 Agar with 1 % (w/w) lactose (Biolab, Budapest Hungary), pouring method, incubation at 37 °C for 48 h, was used to count ST, c) and d) MRS Agar (pH 5.4) (Oxoid, Thermo Fisher Specialty Diagnostics Ltd., Hampshire, UK), pouring method, incubation at 37 °C for 48 h, was used to count LB and LR (the two species, even though they were in the same plate, could be counted separately as they presented obvious morphological differences in their colonies), e) Violet Red Bile Glucose Agar (VRBGA; Biolife Italiana), pouring method, incubation at 37 °C for 24 h, was used to count *Enterobacteriaceae*, and f) Dichloran Rose Bengal Chloramphenicol Agar (DRBCA; Lab M), spreading method, incubation at 25 °C for 120 h, was used to count yeasts and molds.

2.3. Static in vitro digestion model (SIVDM)

The *in vitro* digestion assay was performed according to the method described by Argyri et al. with some adaptations [23]. More specifically, 1 g of each yogurt (CY, PY) was mixed with 1 mL of sterile distilled water in 6 well plates (Abdos Labtech Private Limited, Uttarakhand, India) and the pH of each mixture was adjusted to 2.0 using 1 M hydrochloric acid (HCl) solution. Then, 0.1 mL of human pepsin solution were added to each well and the plates were placed in a shaking incubator for 2 h at 37 °C. After this first incubation step, 1 mL of piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer reagent was added to each well to elevate the mixture pH and a second incubation step was carried out for 30 min at 37 °C. Afterward, a mixture of pancreatin-bile acids (0.5 mL) was added to each well and the pH was adjusted to 7.0 using 1 M sodium hydroxide (NaOH) solution. A third incubation step was followed for 2 h at 37 °C and at the end the digested yogurt samples were collected for the enumeration of the viable and culturable cells of the three LAB species (ST, LB, and LR). All chemicals used in this assay were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Merck Chemicals (Darmstadt, Germany).

2.4. pH and TA measurements

At each sampling day, the pH and TA of the two yogurt types (CY, PY) were measured. Concerning the pH, this was measured in the stomacher bag following the homogenization of the samples using a digital benchtop pH meter equipped with a glass electrode (Consort C931, Turnhout, Belgium). The TA was determined following a previously described protocol [24]. In brief, 5 g of yogurt were homogenized with 45 mL of distilled water in a beaker. The mixture was then titrated with 0.1 N NaOH using 0.5 mL of phenolphthalein indicator until the color of the solution changed from colorless to pale pink. The volume of NaOH that was consumed was used to calculate the mg of lactic acid (LA; $CH_3CH(OH)COOH$) per 100 g of yogurt. Data were finally expressed in weight-by-weight percentages (% w/w LA).

2.5. AA and TPC measurements

2.5.1. Preparation of yogurt water extracts

Yogurt water extracts were prepared according to the method described by Shori and Baba with slight modifications [25]. Specifically, 10 g of each yogurt type (CY, PY) and 2.5 mL of distilled water were mixed in plastic centrifuge tubes and homogenized. Thereafter, the pH of each mixture was adjusted to 4.0 using 1 M HCl. Samples were then incubated in a shaking incubator for 10 min at 45 °C and followed by centrifugation at 5000 g for 10 min at 4 °C (Frontier 5000 Series Multi Pro Centrifuge, FC5718R, OHAUS Europe GmbH, Nänikon, Switzerland). After the centrifugation, the supernatants were collected, their pH was adjusted to 7.0 using 1 M NaOH and recentrifuged at 5000 g for 10 min at 4 °C. Supernatants were again collected, filtered using 0.45 μm syringe filters (Whatman®, Cytiva, Buckinghamshire, UK) and analyzed according to the Folin-Ciocalteu and Oxygen Radical Absorbance Capacity (ORAC) assays, whereas before Ferric Reducing Antioxidant Power (FRAP) analysis, their pH was readjusted to 4.0. For these latter analyses, all chemicals were purchased from Sigma-Aldrich.

2.5.2. Total AA by FRAP assay

Total AA of the yogurt water extracts was determined by the FRAP assay, as previously described [26]. The method is based on the conversion of the ferric tripyri-dyltriazine complex (TPTZ-Fe^{3+}) to its ferrous form (TPTZ-Fe^{2+}) at low pH in the presence of antioxidants. In particular, 80 μL of FRAP reagent and 20 μL of each sample yogurt water extract were placed in 96-well polystyrene (PS) microtiter plates and the absorbance was measured at 595 nm using the SPARK® multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The total AA was determined using a standard ferrous sulfate ($FeSO_4$) curve and the results were expressed in

mmol of Fe^{2+} per mL of sample extract.

2.5.3. Total AA by ORAC assay

The ORAC assay was performed according to the method proposed by Huang et al. with minor modifications [27]. This is based in the property of fluorescein to lose its fluorescence during its oxidation by peroxy radicals [2,2′-Azo-bis-amidinopropane (ABAP)] in a neutral pH at 37 °C. In the presence of antioxidants, there is an inhibition of free radicals, which is reflected in protection against the

Table 1
Logarithmic populations (log_{10} CFU/g) of the different microbial groups (*S. thermophilus*, *L. bulgaricus*, APC, *Enterobacteriaceae*, yeasts and molds, and *L. rhamnosus*) in the two yogurt types (CY, PY) during their shelf life (storage at 4 °C for 25 days). Data are mean values ± standard deviations. For each microorganism separately, mean values sharing at least one common letter (a-d) do not differ statistically significantly ($P > 0.05$).

Microorganism	Storage day	Yogurt	Log_{10}(CFU/g)
S. thermophilus (ST)	5	CY	$9.25^{a} \pm 0.08$
		PY	$9.30^{a} \pm 0.06$
	11	CY	$8.57^{b} \pm 0.22$
		PY	$9.22^{a} \pm 0.06$
	16	CY	$9.40^{a} \pm 0.16$
		PY	$9.35^{a} \pm 0.21$
	20	CY	$9.31^{a} \pm 0.22$
		PY	$9.26^{a} \pm 0.04$
	25	CY	$9.39^{a} \pm 0.06$
		PY	$9.48^{a} \pm 0.03$
L. bulgaricus (LB)	5	CY	$5.15^{b} \pm 0.21$
		PY	$6.44^{d} \pm 0.16$
	11	CY	$4.74^{a} \pm 0.09$
		PY	<2.00
	16	CY	$4.80^{ab} \pm 0.08$
		PY	<2.00
	20	CY	$4.69^{a} \pm 0.14$
		PY	< DL[a]
	25	CY	$5.74^{c} \pm 0.12$
		PY	< DL[a]
APC	5	CY	$6.26^{a} \pm 0.23$
		PY	$8.13^{b} \pm 0.07$
	11	CY	$6.46^{a} \pm 0.02$
		PY	$8.27^{b} \pm 0.14$
	16	CY	$6.40^{a} \pm 0.05$
		PY	$8.33^{b} \pm 0.18$
	20	CY	$6.26^{a} \pm 0.21$
		PY	$8.04^{b} \pm 0.14$
	25	CY	$5.84^{c} \pm 0.08$
		PY	$9.19^{d} \pm 0.17$
Enterobacteriaceae	5	CY	$2.63^{c} \pm 0.22$
		PY	$2.84^{cd} \pm 0.11$
	11	CY	$1.45^{a} \pm 0.21$
		PY	$1.75^{ab} \pm 0.21$
	16	CY	< DL[a]
		PY	$3.07^{d} \pm 0.07$
	20	CY	$1.77^{ab} \pm 0.10$
		PY	$2.92^{cd} \pm 0.05$
	25	CY	< DL[a]
		PY	$2.03^{b} \pm 0.14$
Yeasts and Molds	5	CY	< DL[b]
		PY	< DL[b]
	11	CY	$2.15^{a} \pm 0.21$
		PY	< DL[b]
	16	CY	$2.15^{a} \pm 0.21$
		PY	< DL[b]
	20	CY	$3.48^{b} \pm 0.14$
		PY	< DL[b]
	25	CY	$3.35^{b} \pm 0.11$
		PY	< DL[b]
L. rhamnosus (LR)	5	PY	$8.13^{ab} \pm 0.09$
	11		$8.07^{a} \pm 0.06$
	16		$8.34^{c} \pm 0.05$
	20		$8.26^{bc} \pm 0.04$
	25		$9.13^{d} \pm 0.12$

[a] Detection limit of the pouring plating method (10 CFU/g; 1 log_{10} CFU/g).
[b] Detection limit of the spreading plating method (10^2CFU/g; 2 log_{10} CFU/g).

change of probe fluorescence. A calibration curve using Trolox™ as standard was used and the results were expressed in μmol of Trolox™ equivalents (TRE) per g of sample.

2.5.4. TPC by Folin-Ciocalteu assay

The TPC of the yogurt water extracts was determined using the Folin–Ciocalteu assay. The method is based on the measurement of the reductive capacity of the Folin–Ciocalteau reagent, in an alkaline environment, in the presence of phenolic compounds [28]. Specifically, 20 μL of Folin reagent, 20 μL of 7.5 % (w/v) sodium carbonate (Na_2CO_3) solution and 50 μL of each sample yogurt water extract were placed in 96-well PS microtiter plates and the absorbance was measured at 765 nm using the SPARK® multimode microplate reader. A calibration curve using gallic acid (GA) as standard was manufactured and the results were expressed in milligrams of GA equivalents (GAE) per mL of sample extract.

2.6. Consumer sensory study

At 5th, 11th, 16th, and 20th day of storage, consumer sensory evaluation (affective testing) of the two yogurt types (CY, PY) was carried out. After the 20th day, some of the conventional yogurts showed visible mold growth on their surface, and thus the sensory evaluation was stopped. Each product was evaluated for five different sensory parameters (appearance, odor, taste, texture, and overall acceptance) by a panel of 15 untrained assessors who were all however familiar with yogurt consumption. Informed consent was obtained from all assessors for taking part in the sensory evaluation. Each assessor was asked to evaluate each sensory parameter for each yogurt type without knowing its identity, using a standardized rating scale ranging from 1 to 5 (1 = I do not like at all, 2 = I do not like, 3 = I neither like nor dislike, 4 = I like, 5 = I like very much). For any value equal or lower than 2 in any of the sensory attributes evaluated, the product was organoleptically rejected, while assessors were also aware of this rejection threshold. To carry out consumer sensory analysis, approximately 25 g of yogurt samples were portioned in uniform paper cups with lid labelled with 3-digit random codes just before the arrival of participants. At each sampling day two replicate samples for each yogurt type and assessor were periodically withdrawn from storage (4 °C), left at room temperature for 30 min and sensory evaluation was then carried out in artificial light and ambient temperature, while water and unsalted crackers were provided to the assessors between samples for palate cleansing.

2.7. Statistics

Each plate count, pH, TA, AA and TPC measurement was repeated three times using independent yogurt samples. Plate counts (CFU/g) were transformed to logarithms before means and standard deviations were computed. Analyses of variance (ANOVA) were applied on all the plate count data ($\log_{10}$ CFU/g) followed by Tukey's multiple range *post-hoc* honestly significant difference (HSD) tests for the discrimination of the means for each microorganism between the two yogurt types (CY, PY) and different storage days (5th, 11th, 16th, 20th, and 25th). The same type of analyses was also applied to the pH and TA data (% w/w LA) to check for any significant differences in pH and TA between the two yogurt types (CY, PY) and different storage days. With respect to the sensory evaluation data, Wilcoxon matched pairs non-parametric tests were applied on each sensory parameter (appearance, odor, taste, texture, and overall acceptance) to check for any significant differences in panelists' responses between the two yogurt types (CY, PY) at each day of sampling. In addition to those tests, Student's t tests were also executed to determine significant differences between the mean values of each sensory parameter throughout the yogurts' shelf life. All the previous statistical analyses were done using the STATISTICA® software, version 12.0 (StatSoft Inc., Tulsa, OK, USA), while all differences are reported at a significance level of 0.05. The statistical analyses for the total AA and TPC were on the other hand performed using the SPSS® package, version 16.1 (SPSS Inc., Chicago, IL, USA). The total AA and TPC of the two yogurt types were expressed as mean ± standard deviation (SD). Student's t tests were used to compare these means and statistical significance was again considered at $P = 0.05$.

3. Results

3.1. Microbial dynamics during yogurt shelf life

The logarithmic populations ($\log_{10}$ CFU/g) of the different microbial groups in the two yogurt types (CY, PY) during their shelf life are presented in Table 1. Concerning the starter culture, the population of ST was predominant in both yogurts and remained stable (ca. 10^9 CFU/g) till the end of the storage period (25th day). LB presented an initial population of 10^5 CFU/g in the CY at the 5th day which was slightly increased to 5.74 $\log_{10}$ CFU/g at the end of storage (25th day). On the contrary, the initial population of LB (6.44 $\log_{10}$ CFU/g; 5th day) declined significantly in PY and this was not detected from the 2nd sampling (11th day) and afterward ($<10^2$ CFU/g). The populations of *Enterobacteriaceae* were similar in both yogurts at the 1st sampling (5th day; 2.63 and 2.84 $\log_{10}$ CFU/g in CY and PY, respectively) and these declined in CY at the 25th day to a below the detection limit level (<10 CFU/g), whereas a population of 2.03 $\log_{10}$ CFU/g of *Enterobacteriaceae* existed in PY at the end of storage (25th day). Yeasts and molds were not detected at either yogurt type at the 1st sampling (5th day; $<10^2$ CFU/g). These, however, appeared in CY from the 2nd sampling (11th day) and afterward and were increased to 3.35 $\log_{10}$ CFU/g at the end of storage (25th day). Yeasts and molds were not at all detected in PY till the end of storage. Concerning the population of the probiotic LR in PY, this was above 10^8 CFU/g from the 1st sampling (5th day) and was increased to 9.13 $\log_{10}$ CFU/g at the end of storage (25th day).

3.2. pH and TA of yogurts

The alterations of pH and TA (% w/w LA) in the two yogurt types (CY, PY) during their shelf life are depicted in Fig. 1A and B, respectively. Regarding the pH, the PY was significantly more acidic than CY during the first four samplings (5th, 11th, 16th, and 20th day of storage) ($P < 0.05$). However, the pH did not significantly differ between the two yogurt types at the end of storage (25th day). Regarding the TA, this did not differ significantly between the two yogurt types, except on 11th and 16th storage days where the content of PY in LA was significantly higher than that of CY. For both yogurt types, the TA increased significantly from the 5th to the 25th day of sampling ($P < 0.05$).

3.3. Survival of LAB species following in vitro digestion

The reductions of the populations of the three LAB species (ST, LB, and LR) following the *in vitro* digestion are depicted in Table 2. In both yogurt types (CY, PY), the two species of the starter culture (ST and LB) failed to survive under the harsh conditions of the SIVDM, with their populations following digestion being below the detection limit of the plate counting method (10 CFU/g). On the contrary, the probiotic LR presented a good survival ability since this was reduced by only 1.2 log following the passage through the SIVDM.

3.4. AA and TPC of yogurts

The total AA and TPC of the two yogurt types (CY, PY) are shown in Table 3. PY presented a statistically significant higher AA and TPC compared to CY based on all the three different assays used for the analyses ($P < 0.05$).

3.5. Consumer sensory evaluation

The results of the evaluation of the five sensory parameters (appearance, odor, taste, texture, and overall acceptance) of the two yogurt types (CY, PY) at the four days of sampling (5th, 11th, 16th, and 20th) are depicted in Fig. 2 in the forms of spider graphs. In addition, Fig. S1 presents these data in the forms of box and whisker plots (also showing standard errors and standard deviations for each mean organoleptic value). Overall, no significant differences existed between the two yogurt types at their sensory attributes during the whole storage period. The only statistically significant difference appeared on the 11th day of storage for the parameter "appearance" where the PY received a higher score compared to the CY ($P < 0.05$). When comparing independently the performance of each sensory parameter throughout the yogurts' shelf life (i.e., from the 5th to the 20th d), a significant downgrading in the taste criterion was evident for PY, while the overall acceptance of both yogurts also worsened from the 5th to the 20th d of sampling (Fig. S2). Despite that, all yogurt samples received sensory scores whose mean values were always above 3.0 (I neither like nor dislike) throughout their shelf life, revealing their satisfactory acceptance from the panelists.

4. Discussion

The development of novel functional products with clinically proven health benefits, such as probably those incorporating probiotics, is highly desirable by the food industry. Although it is not necessary; viability of probiotic bacteria is considered important in order these to protect health. However, the viability of probiotics in fermented dairy products, such as yogurt, during their shelf life is of concern due to the low pH (which is usually lower than 4.5), presence of dissolved oxygen and high amount of organic acids (mainly LA) [9,29,30]. Thus, it is known that there are a number of properties of foods and surroundings that may influence the survival of a given probiotic microorganism during the shelf life; these include storage temperature, pH, oxygen availability and redox potential, type and amount of organic acids, presence, type and amount of micronutrients and other metabolizable substances (e.g., amino acids, peptides, prebiotics), as well as the presence and metabolic activities of other microorganisms [31–34]. This probiotic survival is also

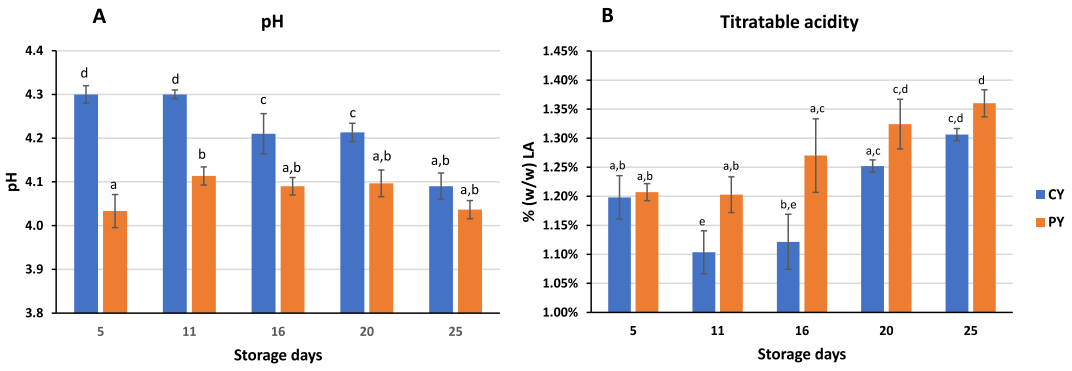

Fig. 1. Alterations of pH (A) and TA (B) in the two yogurt types (CY, PY) during their shelf life (storage at 4 °C for 25 days). Data are mean values ± standard deviations. For each graph separately, mean values sharing at least one common letter do not differ statistically significantly ($P > 0.05$).

Table 2
Logarithmic populations (log_{10} CFU/g) of the three LAB species (ST, LB, and LR) before and following *in vitro* digestion of the two yogurt types (CY, PY). Data are mean values ± standard deviations.

Yogurt type	Microorganism	Log initial[a]	Log final[b]
CY	*S. thermophilus*	9.1 ± 0.4	<1
	L. bulgaricus	5.2 ± 0.4	<1
PY	*S. thermophilus*	8.9 ± 0.3	<1
	L. bulgaricus	<2	<1
	L. rhamnosus	7.4 ± 0.5	6.2 ± 0.3

[a] Populations that existed in yogurts on the 9th day of storage and just before the *in vitro* digestion.
[b] Populations that existed in yogurts following the *in vitro* digestion protocol.

Table 3
AA and TPC of the two yogurt types (CY, PY). Data are mean values ± standard deviations. Different letters in the same column indicate significant differences ($P < 0.05$) between the two yogurt types (CY, PY).

Yogurt type	AA (FRAP assay) (mmol Fe^{2+}/mL)	AA (ORAC assay) (μmol TRE/g)	TPC (Folin-Ciocalteu assay) (mg GAE/mL)
CY	0.05^{a} ± 0.00	3.34^{a} ± 1.07	21.48^{a} ± 2.39
PY	0.06^{b} ± 0.01	7.62^{b} ± 1.13	25.97^{b} ± 2.64

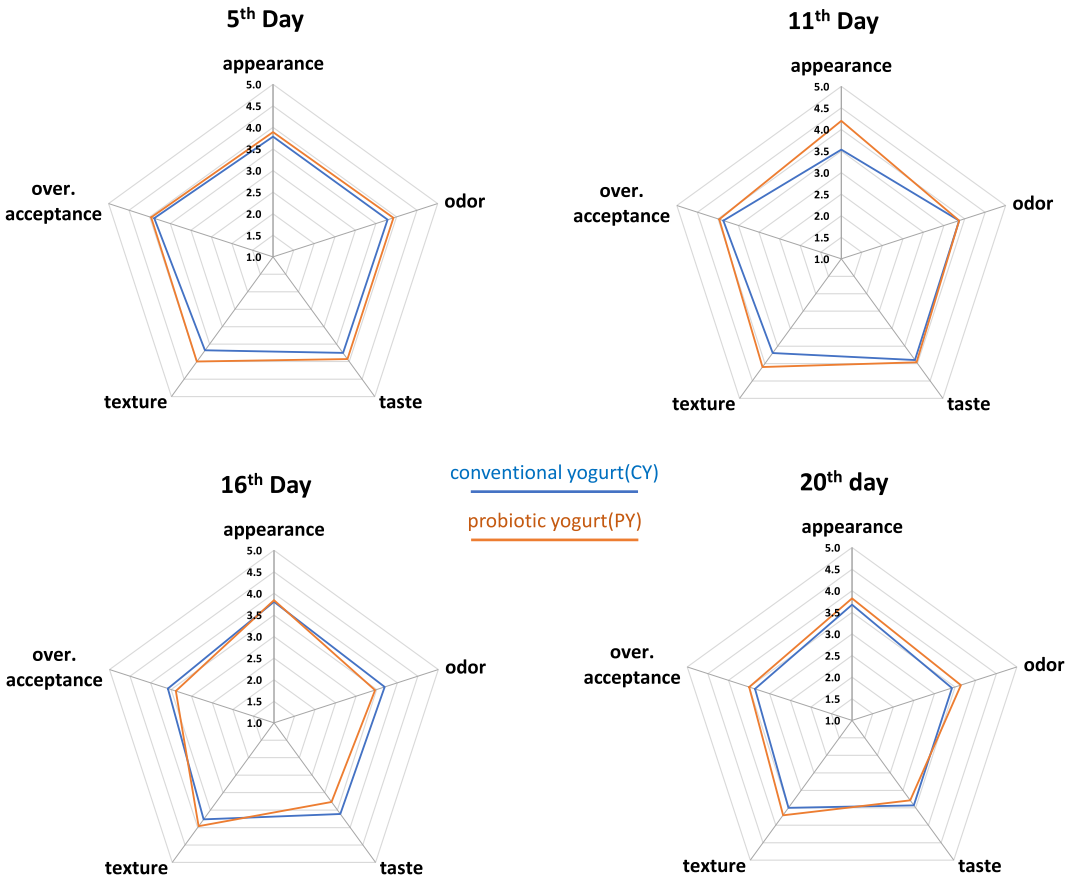

Fig. 2. Results of the evaluation of the five sensory parameters (appearance, odor, taste, texture, and overall acceptance) of the two yogurt types (CY, PY) at the four days of sampling (5th, 11th, 16th, and 20th).

strain-dependent, can be influenced by the initial inoculum level and may be improved by various means such as stress adaptation, two-step fermentation (i.e., the addition of starter culture and probiotics at different time points), the addition of cryoprotectants, prebiotics, cells immobilization, microencapsulation, and the use of oxygen scavengers [9,34,35].

Although the International Scientific Association of Probiotics and Prebiotics (ISAPP) does not assign a specific amount to their

definition of "probiotic", it is generally recommended that the minimum daily intake of viable probiotic cells should be in the range of 10^{6-7} CFU per g of food product, while the vast majority of existing clinical trials indicate that probiotic doses of 10–20 billion CFU per day should be sufficient for maintaining immune and digestive health [28,36,37]. However, the dose-response relation of probiotics in human interventions may be affected by the disease required to be treated, that is individual's purpose for taking probiotics, as well as the recipient's general physiological state of health, and as thus for some specific conditions, higher doses of probiotics may have a greater clinical benefit. It should still be mentioned that the use of the word probiotic on product labels is restricted in Greece, and all other European countries, since this is considered as a health claim and requires approval by the European Food Safety Authority (EFSA) [29]. However, till now, the EFSA has rejected all the health claims concerning probiotics that have been submitted by the manufacturers, researchers, and collection owners, mainly due to insufficient scientific evidence. Surely, the research on probiotic foods should be continued with the exploration of new strains-food combinations.

Sheep milk is a product rich in bioactive substances that have health-promoting functions, and not surprisingly in recent years there is an increasing interest in its consumption [38]. The yogurt that is produced from this milk is also highly desired by consumers, and in Greece and some other countries as well, this is a very popular product. The further improvement of its functional properties is thus an interesting field of research. However, to the best of our knowledge, studies that have attempted to add probiotic microorganisms to that specific product are still rare [39,40]. In addition, no other study has been published on probiotic sheep yogurt that has determined any of its functional properties, such as AA and TPC, or the survival of probiotic bacteria contained in such product following *in vitro* digestion. In this study, the new probiotic sheep yogurt (PY) was found to contain a viable and culturable population of LR that was above 10^8 CFU/g throughout the shelf life period (storage at 4 °C for 25 days). It should be noted that given that the yogurts were produced by the cooperating company (Mystakelli Traditional Dairy Products), which is situated on a different island (Lesvos, Greece) than where the premises of our laboratories are located (Lemnos, Greece), it was not possible to analyze them just after their production. This is because, these had to be transported by boat and under refrigeration from one island to another (sea journey duration of about four and a half hours), while there were no daily scheduled routes. Interestingly, the probiotic population was reduced by about only one log following *in vitro* digestion. Several previous studies have examined the survival of probiotic bacteria, mainly *Lactobacillus* and *Bifidobacterium* species in cow milk yogurts throughout their refrigerated storage, and in some cases following exposure to simulated GIT conditions [41–54].

Besides probiotic viability, the effects of the incorporation of probiotic microorganisms in yogurts, either in free or encapsulated form, on the microbiological, physicochemical, and sensory characteristics of the products have been studied in several of those previous studies. However, in most of the previous studies, commercial, and well-known (studied) probiotic strains of intestinal origin were employed that were obtained from international culture collections (such as and *Bifidobacterium animalis* BB12, *Lactobacillus acidophilus* LA5, and *L. rhamnosus* GG). In our research, we on the other hand chose to use as a probiotic a wild-type strain that was isolated from the same milk that was used to produce the type of yogurt we manufactured. This strain was selected following an *in vitro* screening of a large collection of more than one hundred isolates from that milk for some crucial probiotic properties (data not presented). This screening was done in cooperation with the team of Prof. Tsakalidou from the Laboratory of Dairy Research of the Agricultural University of Athens (Athens, Greece). Such an approach was preferred since this not only sustains the particular local character of the specific product, but in addition, the screening of wild-type isolates from local raw materials and traditional indigenous products may sometimes reveal strains with some novel and interesting probiotic properties [55,56].

Although the two species of the classical yogurt starter culture (ST, LB) are usually vulnerable to the GIT conditions, such as the increased presence of bile salts, these may still present some interesting probiotic properties with most known the improvement of lactose digestion in lactose-intolerant individuals, the release of bioactive antihypertensive and/or antioxidant peptides, and the stimulation of the gut immune system [57,58]. Both these properties do not require the presence of viable cells in the gut. However, there have been conflicting studies concerning the recovery of viable ST and LB from fecal samples after dairy yogurt ingestion. Thus, while some studies failed to show recovery [59,60], some other studies managed to recover viable cells suggesting that these bacteria can sometimes survive transit through the GIT [58,61]. Surely, differences in survival abilities between the different strains of the starter cultures may explain these conflicting results [62,63]. In our study, viable yogurt starters were not detected following SIVDM. One potential limitation of our study that should however be mentioned is that the bacterial viability (either during storage of yogurts or following *in vitro* digestion) was determined following the sole application of classical microbiological procedures based on cell culturing. However, it is known that many bacteria may enter a viable-but-non-culturable (VBNC) state following their exposure to adverse environmental conditions, such as low pH and/or low temperature [64]. Such VBNC bacteria are undetectable by routine selective agar plating but could be still detected and enumerated using other advanced techniques such as real-time viability PCR, flow cytometry or fluorescent *in situ* hybridization [65,66].

It is also interesting to note that while ST was here found to present a very good survival throughout the shelf life of both yogurt types (with counts always above 10^9 CFU/g), the initial population of LB (10^6 CFU/g; before fermentation; data not presented) declined significantly in PY and was not detected from the 11th d and afterward ($<10^2$ CFU/g). It thus seems that the *in-situ* interaction with the added probiotic (LR) negatively affects the viability of LB in yogurt. It should still be noted that an *in vitro* agar well-diffusion antimicrobial assay did not reveal any inhibition of the cell-free supernatant of LR against either strain of the starter culture (data not presented). In addition, microbial survival and growth can surely be influenced by endogenous yogurt parameters including pH and TA. Considering pH, the PY was significantly ($P < 0.05$) more acidic than the CY during almost the whole storage period except the last day of sampling (25th day). On the other hand, the TA differed significantly ($P < 0.05$) between the two yogurt types (CY, PY) only on the 11th and 16th days of storage, where the PY was found to present a higher TA than the CY. This last observation seems to agree with the results of some previous studies which also have shown a higher TA of probiotic yogurts than conventional ones [49,67]. The lack of fungal growth (populations of yeasts and molds $<10^2$ CFU/g) in the probiotic yogurts till the end of the storage period (25th day) is

also another interesting observation since this growth is ultimately associated with the spoilage of those products and the end of their shelf life. It thus seems that the addition of the probiotic increases the marketable shelf life of yogurts. This is quite important for the industry given that traditional Greek sheep yogurt has a limited shelf life that does not usually exceed three weeks. An extension of shelf life duration could facilitate the export efforts of this live product.

Besides their role in intestinal homeostasis, in the last years, probiotics have also gain much of attention as functional antioxidant ingredients, due to their promising protective role against oxidative stress-related diseases [68,69]. In food technology, antioxidants, of either natural or synthetic origin, are added to many foodstuffs, not only to preserve them, but also due to their many health benefits such as anti-aging and anti-inflammatory actions [70]. Concerning yogurts and other dairy products, several studies have examined their antioxidant content by employing various *in vitro* methods such as FRAP, DPPH, ABTS and ORAC assays [71–73]. The antioxidative effect of yogurts is partially assigned to the LAB starters, which during milk fermentation and the resulting proteolysis of milk proteins, can generate a wide range of bioactive peptides [74,75]. Additionally, the higher antioxidant capacity of ewe's against cow's and goat's milk, and dairy products made there off, that has been reported, could be probably attributed to their relatively higher content in protein and casein percentages [76–78]. Interestingly, we here found that the incorporation of the probiotic LR, as an adjunct starter culture before milk fermentation, increased both the total AA and TPC of the produced yogurt.

Important and well-known antioxidants that play a role in disease prevention are phenolic compounds [79]. These are secondary metabolites that are mostly found in the plant cell walls, so yogurt is not considered a source of them [80]. According to some other studies, phenolic compounds that seem to be present in yogurt are actually non-phenolic compounds (such as sugars, amino acids and small peptides) that may interfere with total phenolic compounds' measurement [72]. Phenolic compounds can also be formed by ruminants' gut bacterial flora from plant-derived phenolic compounds and in this way be later found in milk [81]. Our results on the TPC of yogurts (21.48 $\pm$ 2.39 and 25.97 $\pm$ 2.64 mg GAE/mL for CY and PY, respectively) agree with previous reports that indicated a small content of total phenolic compounds in yogurt samples [25,71,72]. However, it was here found a statistically significant difference in TPC between the two yogurt types, with PY presenting a higher TPC. This could be probably explained by the probiotic's specific metabolism in PY, which may lead to an increase in proteolysis, resulting in a rise of amino acids and small peptides and so in a plasmatic elevated TPC [82,83]. In agreement with the higher TPC, the PY was also here found to present increased AA compared to CY.

Besides sufficient viability throughout processing and storage, for a microbial strain to be incorporated into any food as a probiotic, this should not impair its sensory attributes. The consumer sensory analysis we executed did not reveal any important differences between the two yogurt types (CY, PY) for each day of sampling in the five sensory parameters evaluated (appearance, odor, taste, texture, and overall acceptance). In addition, each sensory parameter received an evaluation score whose mean value was always above 3.0, indicating the satisfactory acceptance of the two products from the panelists (consumers) throughout their shelf life. It is also worth noting that it does not appear that any relationship between sensory acceptance and the TA of yogurts exists. For instance, the differences in TA between the two yogurt types (CY, PY) that were observed on the 11th and 16th day of storage did not seem to influence the taste or odor of these yogurts as those were perceived by the panelists. The incorporation of probiotic strains in yogurts has previously shown to alter sometimes their sensory profile, either for better or for worse, depending on the intrinsic biochemical (metabolic) features of the added strain, as well as its interactions with the starter cultures [53,54].

5. Conclusions

A novel functional Greek sheep yogurt was successfully manufactured by incorporating at the beginning of fermentation, as adjunct starter culture, a probiotic *L. rhamnosus* wild-type strain, previously isolated from raw sheep milk. The new product was shown able to deliver to consumers a high number of probiotic cells ($>10^8$ CFU/g) till the end of its storage (at 4 °C for 25 days), presented increased antioxidant power, expanded shelf life, and maintained its good sensory attributes throughout storage. The added probiotic strain was also found to present increased survival to simulated GIT conditions, something that may increase the probability for health benefits. Future clinical studies are to be executed to evaluate *in vivo* the probiotic potential of the new yogurt. Overall, the development of such a functional product is expected to boost the sales of traditional Greek sheep yogurt, facilitate export efforts, also protecting, and strengthening the health of consumers.

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Data availability Statement

The data presented in this study are contained within the article or Supplementary Materials.

CRediT authorship contribution statement

Ioanna Gkitsaki: Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Panagiota Potsaki:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal

analysis, Data curation. **Ioanna Dimou:** Investigation. **Zoi Laskari:** Methodology, Investigation. **Antonios Koutelidakis:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Efstathios Giaouris:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Efstathios Giaouris reports financial support was provided by North Aegean Region. Zoi Laskari reports a relationship with Mystakelli Traditional Dairy Products that includes: employment. Efstathios Giaouris is currently serving as associate editor in Heliyon Food Science and Nutrition. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] L.J. Wilkins, M. Monga, A.W. Miller, Defining dysbiosis for a cluster of chronic diseases, Sci. Rep. 9 (1) (2019) 12918, https://doi.org/10.1038/s41598-019-49452-y.
[2] E.Z. Gomaa, Human gut microbiota/microbiome in health and diseases: a review, Antonie Leeuwenhoek 113 (12) (2020) 2019–2040, https://doi.org/10.1007/s10482-020-01474-7.
[3] P. Illiano, R. Brambilla, C. Parolini, The mutual interplay of gut microbiota, diet and human disease, FEBS J. 287 (5) (2020) 833–855, https://doi.org/10.1111/febs.15217.
[4] K. Dixit, D. Chaudhari, D. Dhotre, Y. Shouche, S. Saroj, Restoration of dysbiotic human gut microbiome for homeostasis, Life Sci. 278 (2021) 119622, https://doi.org/10.1016/j.lfs.2021.119622.
[5] A. Gagliardi, V. Totino, F. Cacciotti, V. Iebba, B. Neroni, G. Bonfiglio, M. Trancassini, C. Passariello, F. Pantanella, S. Schippa, Rebuilding the gut microbiota ecosystem, Int. J. Environ. Res. Public Health. 15 (8) (2018) 1679, https://doi.org/10.3390/ijerph15081679.
[6] S.K. Kim, R.B. Guevarra, Y.T. Kim, J. Kwon, H. Kim, J.H. Cho, H.B. Kim, J.H. Lee, Role of probiotics in human gut microbiome-associated diseases, J. Microbiol. Biotechnol. 29 (9) (2019) 1335–1340, https://doi.org/10.4014/jmb.1906.06064.
[7] P. Markowiak, K. Śliżewska, Effects of probiotics, prebiotics, and synbiotics on human health, Nutrients 9 (9) (2017) 1021, https://doi.org/10.3390/nu9091021.
[8] E.M.M. Quigley, Prebiotics and probiotics in digestive health, Clin. Gastroenterol. Hepatol. 17 (2) (2019) 333–344, https://doi.org/10.1016/j.cgh.2018.09.028.
[9] J. Gao, X. Li, G. Zhang, F.A. Sadiq, J. Simal-Gandara, J. Xiao, Y. Sang, Probiotics in the dairy industry-Advances and opportunities, Compr. Rev. Food Sci. Food Saf. 20 (4) (2021) 3937–3982, https://doi.org/10.1111/1541-4337.12755.
[10] T. Mattila-Sandholm, P. Myllärinen, R. Crittenden, G. Mogensen, R. Fondén, M. Saarela, Technological challenges for future probiotic food, Int. Dairy J. 12 (2–3) (2002) 173–182, https://doi.org/10.1016/S0958-6946(01)00099-1.
[11] L. Cassani, A. Gomez-Zavaglia, J. Simal-Gandara, Technological strategies ensuring the safe arrival of beneficial microorganisms to the gut: from food processing and storage to their passage through the gastrointestinal tract, Food Res. Int. 129 (2020) 108852, https://doi.org/10.1016/j.foodres.2019.108852.
[12] M.K. Tripathi, S.K. Giri, Probiotic functional foods: survival of probiotics during processing and storage, J. Funct.Foods 9 (2014) 225–241, https://doi.org/10.1016/j.jff.2014.04.030.
[13] A. Lourens-Hattingh, B.C. Viljoen, Yogurt as probiotic carrier food, Int. Dairy J. 11 (1–2) (2001) 1–17, https://doi.org/10.1016/S0958-6946(01)00036-X.
[14] CODEX STAN 243-2003, Codex standard for fermented milks, in: Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO), 2003. Available at:. (Accessed 19 December 2023).
[15] Article 82: Yogurt, Chapter IX: products of animal origin other than those of Chapter X, in: Food and Beverage Code, Greek State General Laboratory, 2016. Available at: https://www.aade.gr/sites/default/files/2020-07/82-iss2.pdf. (Accessed 19 December 2023).
[16] I. González-Rodríguez, L. Ruiz, M. Gueimonde, A. Margolles, B. Sánchez, Factors involved in the colonization and survival of bifidobacteria in the gastrointestinal tract, FEMS Microbiol. Lett. 340 (1) (2013) 1–10, https://doi.org/10.1111/1574-6968.12056.
[17] R. Nagpal, A. Kumar, M. Kumar, P.V. Behare, S. Jain, H. Yadav, Probiotics, their health benefits and applications for developing healthier foods: a review, FEMS Microbiol. Lett. 334 (1) (2012) 1–15, https://doi.org/10.1111/j.1574-6968.2012.02593.x.
[18] R. Lucas-González, M. Viuda-Martos, J.A. Pérez-Alvarez, J. Fernández-López, *In vitro* digestion models suitable for foods: opportunities for new fields of application and challenges, Food Res. Int. 107 (2018) 423–436, https://doi.org/10.1016/j.foodres.2018.02.055.
[19] T. Faye, A. Tamburello, G.E. Vegarud, S. Skeie, Survival of lactic acid bacteria from fermented milks in an *in vitro* digestion model exploiting sequential incubation in human gastric and duodenum juice, J. Dairy Sci. 95 (2) (2012) 558–566, https://doi.org/10.3168/jds.2011-4705.
[20] A. Lo Curto, I. Pitino, G. Mandalari, J.R. Dainty, R.M. Faulks, M.S. John Wickham, Survival of probiotic lactobacilli in the upper gastrointestinal tract using an *in vitro* gastric model of digestion, Food Microbiol. 28 (7) (2011) 1359–1366, https://doi.org/10.1016/j.fm.2011.06.007.
[21] T. Bohn, F. Carriere, L. Day, A. Deglaire, L. Egger, D. Freitas, M. Golding, S. Le Feunteun, A. Macierzanka, O. Menard, B. Miralles, A. Moscovici, R. Portmann, I. Recio, D. Rémond, V. Santé-Lhoutelier, T.J. Wooster, U. Lesmes, A.R. Mackie, D. Dupont, Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static *in vitro* digestion models? Crit. Rev. Food Sci. Nutr. 58 (13) (2018) 2239–2261, https://doi.org/10.1080/10408398.2017.1315362.

[22] R. Colombo, L. Ferron, I. Frosi, A. Papetti, Advances in static *in vitro* digestion models after the COST action Infogest consensus protocol, Food Funct. 12 (17) (2021) 7619–7636, https://doi.org/10.1039/d1fo01089a.

[23] K. Argyri, A. Birba, D.D. Miller, M. Komaitis, M. Kapsokefalou, Predicting relative concentrations of bioavailable iron in foods using *in vitro* digestion: new developments, Food Chem. 113 (2) (2009) 602–607, https://doi.org/10.1016/j.foodchem.2008.07.089.

[24] S.Y. Yang, K.S. Yoon, Effect of probiotic lactic acid bacteria (LAB) on the quality and safety of Greek yogurt, Foods 11 (23) (2022) 3799, https://doi.org/10.3390/foods11233799.

[25] A.B. Shori, A.S. Baba, Antioxidant activity and inhibition of key enzymes linked to type-2 diabetes and hypertension by *Azadirachta indica*-yogurt, J. Saudi Chem. Soc. 17 (3) (2013) 295–301, https://doi.org/10.1016/j.jscs.2011.04.006.

[26] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1) (1996) 70–76, https://doi.org/10.1006/abio.1996.0292.

[27] D. Huang, B. Ou, M. Hampsch-Woodill, J.A. Flanagan, R.L. Prior, High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format, J. Agric. Food Chem. 50 (16) (2002) 4437–4444, https://doi.org/10.1021/jf0201529.

[28] A. Kandyliari, P. Potsaki, P. Bousdouni, C. Kaloteraki, M. Christofilea, K. Almpounioti, A. Moutsou, C.K. Fasoulis, L.V. Pol-ychronis, V.K. Gkalpinos, A.G. Tzakos, A.E. Koutelidakis, Development of dairy products fortified with plant extracts: antioxidant and phenolic content characterization, Antioxidants 12 (2) (2023) 500, https://doi.org/10.3390/antiox12020500.

[29] R. Nyanzi, P.J. Jooste, E.M. Buys, Invited review: probiotic yogurt quality criteria, regulatory framework, clinical evidence, and analytical aspects, J. Dairy Sci. 104 (1) (2021) 1–19, https://doi.org/10.3168/jds.2020-19116.

[30] A. Talwalkar, K. Kailasapathy, A review of oxygen toxicity in probiotic yogurts: influence on the survival of probiotic bacteria and protective techniques, Compr. Rev. Food Sci. Food Saf. 3 (3) (2004) 117–124, https://doi.org/10.1111/j.1541-4337.2004.tb00061.x.

[31] J. Flach, M.B. van der Waal, M. van den Nieuwboer, E. Claassen, O.F.A. Larsen, The underexposed role of food matrices in probiotic products: Reviewing the relationship between carrier matrices and product parameters, Crit. Rev. Food Sci. Nutr. 58 (15) (2018) 2570–2584, https://doi.org/10.1080/10408398.2017.1334624.

[32] N.M. Meybodi, A.M. Mortazavian, M. Arab, A. Nematollahi, Probiotic viability in yoghurt: a review of influential factors, Int. Dairy J. 109 (2020) 104793, https://doi.org/10.1016/j.idairyj.2020.104793.

[33] E.W. Ng, M. Yeung, P.S. Tong, Effects of yogurt starter cultures on the survival of *Lactobacillus acidophilus*, Int. J. Food Microbiol. 145 (1) (2011) 169–175, https://doi.org/10.1016/j.ijfoodmicro.2010.12.006.

[34] N.P. Shah, Probiotic bacteria: selective enumeration and survival in dairy foods, J. Dairy Sci. 83 (4) (2000) 894–907, https://doi.org/10.3168/jds.S0022-0302(00)74953-8.

[35] G. Mitropoulou, V. Nedovic, A. Goyal, Y. Kourkoutas, Immobilization technologies in probiotic food production, J. Nutr. Metab. 2013 (2013) 716861, https://doi.org/10.1155/2013/716861.

[36] A.C. Ouwehand, A review of dose-responses of probiotics in human studies, Benef. Microbes 8 (2) (2017) 143–151, https://doi.org/10.3920/BM2016.0140.

[37] M.E. Sanders, M.L. Marco, Food formats for effective delivery of probiotics, Annu. Rev. Food Sci. Technol. 1 (2010) 65–85, https://doi.org/10.1146/annurev.food.080708.100743.

[38] Z. Flis, E. Molik, Importance of bioactive substances in sheep's milk in human health, Int. J. Mol. Sci. 22 (9) (2021) 4364, https://doi.org/10.3390/ijms22094364.

[39] M. Sidira, V. Santarmaki, M. Kiourtzidis, A.A. Argyri, O.S. Papadopoulou, N. Chorianopoulos, C. Tassou, S. Kaloutsas, A. Galanis, Y. Kourkoutas, Evaluation of immobilized *Lactobacillus plantarum* 2035 on whey protein as adjunct probiotic culture in yoghurt production, LWT - Food Sci. Technol. 75 (2017) 137–146, https://doi.org/10.1016/j.lwt.2016.08.026.

[40] Š. Zamberlin, D. Samaržija, The effect of non-standard heat treatment of sheep's milk on physicochemical properties, sensory characteristics, and the bacterial viability of classical and probiotic yogurt, Food Chem. 225 (2017) 62–68, https://doi.org/10.1016/j.foodchem.2017.01.001.

[41] G.S. Rutella, D. Tagliazucchi, L. Solieri, Survival and bioactivities of selected probiotic lactobacilli in yogurt fermentation and cold storage: new insights for developing a bi-functional dairy food, Food Microbiol. 60 (2016) 54–61, https://doi.org/10.1016/j.fm.2016.06.017.

[42] M. Sidira, G. Saxami, D. Dimitrellou, V. Santarmaki, A. Galanis, Y. Kourkoutas, Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool, J. Dairy Sci. 96 (5) (2013) 3369–3377, https://doi.org/10.3168/jds.2012-6343.

[43] A. Terpou, A. Bekatorou, M. Kanellaki, A.A. Koutinas, P. Nigam, Enhanced probiotic viability and aromatic profile of yogurts produced using wheat bran (*Triticum aestivum*) as cell immobilization carrier, Process Biochem 55 (2017) 1–10, https://doi.org/10.1016/j.procbio.2017.01.013.

[44] T. Zhou, R. Huo, L.Y. Kwok, C. Li, Y. Ma, Z. Mi, Y. Chen, Effects of applying *Lactobacillus helveticus* H9 as adjunct starter culture in yogurt fermentation and storage, J. Dairy Sci. 102 (1) (2019) 223–235, https://doi.org/10.3168/jds.2018-14602.Erratumin:J.DairySci, 2019, 102(2):1885.

[45] B. Aktas, M. Budinich, L. Hoza, S.A. Rankin, J.R. Broadbent, J.L. Steele, Shelf-life studies of putative probiotic *Lacticaseibacillus casei* strains in milk and model yogurt, Food Sci. Technol. Int. (2022) 10820132221112260, https://doi.org/10.1177/10820132221112260.

[46] M. Afzaal, A.U. Khan, F. Saeed, A. Ahmed, M.H. Ahmad, A.A. Maan, T. Tufail, F.M. Anjum, S. Hussain, Functional exploration of free and encapsulated probiotic bacteria in yogurt and simulated gastrointestinal conditions, Food Sci. Nutr. 7 (12) (2019) 3931–3940, https://doi.org/10.1002/fsn3.1254.

[47] C.P. Champagne, Y. Raymond, N. Guertin, C.J. Martoni, M.L. Jones, I. Mainville, Y. Arcand, Impact of a yogurt matrix and cell microencapsulation on the survival of *Lactobacillus reuteri* in three *in vitro* gastric digestion procedures, Benef. Microbes 6 (5) (2015) 753–763, https://doi.org/10.3920/BM2014.0162.

[48] A.G. Cruz, W.F. Castro, J.A. Faria, P.C. Lollo, J. Amaya-Farfán, M.Q. Freitas, D. Rodrigues, C.A. Oliveira, H.T. Godoy, Probiotic yogurts manufactured with increased glucose oxidase levels: postacidification, proteolytic patterns, survival of probiotic microorganisms, production of organic acid and aroma compounds, J. Dairy Sci. 95 (5) (2012) 2261–2269, https://doi.org/10.3168/jds.2011-4582.

[49] A. Dantas, S. Verruck, M.H.M. Canella, E. Hernandez, E.S. Prudencio, Encapsulated *Bifidobacterium* BB-12 addition in a concentrated lactose-free yogurt: its survival during storage and effects on the product's properties, Food Res. Int. 150 (Pt A) (2021) 110742, https://doi.org/10.1016/j.foodres.2021.110742.

[50] S.S. Dias, D. de Souza Vergílio, A.M. Pereira, S.J. Klososki, V.A. Marcolino, R.M.S. da Cruz, G.N. Costa, C.E. Barão, T.C. Pimentel, Probiotic Greek yogurt: effect of the addition of prebiotic fat substitutes on the physicochemical characteristics, probiotic survival, and sensory acceptance, J. Dairy Res. 88 (1) (2021) 98–104, https://doi.org/10.1017/S0022029921000121.

[51] N. Innocente, M. Biasutti, F. Rita, R. Brichese, G. Comi, L. Iacumin, Effect of indigenous *Lactobacillus rhamnosus* isolated from bovine milk on microbiological characteristics and aromatic profile of traditional yogurt, LWT - Food Sci. Technol. 66 (2016) 158–164, https://doi.org/10.1016/j.lwt.2015.10.031.

[52] M. Kaur Sidhu, F. Lyu, T.P. Sharkie, S. Ajlouni, C.S. Ranadheera, Probiotic yogurt fortified with chickpea flour: physicochemical properties and probiotic survival during storage and simulated gastrointestinal transit, Foods 9 (9) (2020) 1144, https://doi.org/10.3390/foods9091144.

[53] E. Mani-López, E. Palou, A. López-Malo, Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria, J. Dairy Sci. 97 (5) (2014) 2578–2590, https://doi.org/10.3168/jds.2013-7551.

[54] M.C.E. Ribeiro, K.S. Chaves, C. Gebara, F.N.S. Infante, C.R.F. Grosso, M.L. Gigante, Effect of microencapsulation of *Lactobacillus acidophilus* LA-5 on physicochemical, sensory and microbiological characteristics of stirred probiotic yoghurt, Food Res. Int. 66 (2014) 424–431, https://doi.org/10.1016/j.foodres.2014.10.019.

[55] J.J. Ahire, C. Jakkamsetty, M.S. Kashikar, S.G. Lakshmi, R.S. Madempudi, *In vitro* evaluation of probiotic properties of *Lactobacillus plantarum* UBLP40 isolated from traditional indigenous fermented food. Probiotics Antimicrob, Proteins 13 (5) (2021) 1413–1424, https://doi.org/10.1007/s12602-021-09775-7.

[56] G. Zoumpopoulou, K. Papadimitriou, V. Alexandraki, E. Mavrogonatou, K. Alexopoulou, R. Anastasiou, M. Georgalaki, D. Kletsas, E. Tsakalidou, E. Giaouris, The microbiota of Kalathaki and Melichloro Greek artisanal cheeses comprises functional lactic acid bacteria, LWT - Food Sci. Technol. 130 (2020) 109570, https://doi.org/10.1016/j.lwt.2020.109570.

[57] G.S. Rutella, D. Tagliazucchi, L. Solieri, Survival and bioactivities of selected probiotic lactobacilli in yogurt fermentation and cold storage: new insights for developing a bi-functional dairy food, Food Microbiol. 60 (2016) 54–61, https://doi.org/10.1016/j.fm.2016.06.017.

[58] M. Elli, M.L. Callegari, S. Ferrari, E. Bessi, D. Cattivelli, S. Soldi, L. Morelli, N. Goupil Feuillerat, J.M. Antoine, Survival of yogurt bacteria in the human gut, Appl. Environ. Microbiol. 72 (7) (2006) 5113–5117, https://doi.org/10.1128/AEM.02950-05.
[59] R. del Campo, D. Bravo, R. Cantón, P. Ruiz-Garbajosa, R. García-Albiach, A. Montesi-Libois, F.J. Yuste, V. Abraira, F. Ba-quero, Scarce evidence of yogurt lactic acid bacteria in human feces after daily yogurt consumption by healthy volunteers, Appl. Environ. Microbiol. 71 (1) (2005) 547–549, https://doi.org/10.1128/AEM.71.1.547-549.2005.
[60] M.C. Pedrosa, B.B. Golner, B.R. Goldin, S. Barakat, G.E. Dallal, R.M. Russell, Survival of yogurt-containing organisms and *Lactobacillus gasseri* (ADH) and their effect on bacterial enzyme activity in the gastrointestinal tract of healthy and hypo-chlorhydric elderly subjects, Am. J. Clin. Nutr. 61 (2) (1995) 353–359, https://doi.org/10.1093/ajcn/61.2.353.
[61] D.D. Mater, L. Bretigny, O. Firmesse, M.J. Flores, A. Mogenet, J.L. Bresson, G. Corthier, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt, FEMS Microbiol. Lett. 250 (2) (2005) 185–187, https://doi.org/10.1016/j.femsle.2005.07.006.
[62] L. Morelli, Yogurt, living cultures, and gut health, Am. J. Clin. Nutr. 99 (5 Suppl) (2014), https://doi.org/10.3945/ajcn.113.073072, 1248S-50S.
[63] C. Vázquez, J.I. Botella-Carretero, R. García-Albiach, M.J. Pozuelo, M. Rodríguez-Baños, F. Baquero, M.A. Baltadjieva, R. del Campo, Screening in a *Lactobacillus delbrueckii* subsp. *bulgaricus* collection to select a strain able to survive to the human intestinal tract, Nutr. Hosp. 28 (4) (2013) 1227–1235, https://doi.org/10.3305/nh.2013.28.4.6540.
[64] K. Dong, H. Pan, D. Yang, L. Rao, L. Zhao, Y. Wang, X. Liao, Induction, detection, formation, and resuscitation of viable but non-culturable state microorganisms, Compr. Rev. Food Sci. Food Saf. 19 (1) (2020) 149–183, https://doi.org/10.1111/1541-4337.12513.
[65] M.C. Scariot, G.L. Venturelli, E.S. Prudêncio, A.C.M. Arisi, Quantification of *Lactobacillus paracasei* viable cells in probiotic yoghurt by propidium monoazide combined with quantitative PCR, Int. J. Food Microbiol. 264 (2018) 1–7, https://doi.org/10.1016/j.ijfoodmicro.2017.10.021.
[66] C. Davis, Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria, J. Microbiol. Methods 103 (2014) 9–17, https://doi.org/10.1016/j.mimet.2014.04.012.
[67] R. Soni, N.K. Jain, V. Shah, J. Soni, D. Suthar, P. Gohel, Development of probiotic yogurt: effect of strain combination on nutritional, rheological, organoleptic and probiotic properties, J. Food Sci. Technol. 57 (6) (2020) 2038–2050, https://doi.org/10.1007/s13197-020-04238-3.
[68] A. Hoffmann, P. Kleniewska, R. Pawliczak, Antioxidative activity of probiotics, Arch. Med. Sci. 17 (3) (2021) 792–804, https://doi.org/10.5114/aoms.2019.89894.
[69] Y. Wang, Y. Wu, Y. Wang, H. Xu, X. Mei, D. Yu, Y. Wang, W. Li, Antioxidant properties of probiotic bacteria, Nutrients 9 (5) (2017) 521, https://doi.org/10.3390/nu9050521.
[70] C. Zehiroglu, S.B. Ozturk Sarikaya, The importance of antioxidants and place in today's scientific and technological studies, J. Food Sci. Technol. 56 (11) (2019) 4757–4774, https://doi.org/10.1007/s13197-019-03952-x.
[71] J. Kim, M. Kim, I. Choi, Physicochemical characteristics, antioxidant properties and consumer acceptance of Greek yogurt fortified with apple pomace syrup, Foods 12 (9) (2023) 1856, https://doi.org/10.3390/foods12091856.
[72] A.B. Shori, Storage quality and antioxidant properties of yogurt fortified with polyphenol extract from nutmeg, black pepper, and white pepper, Electron. J. Biotechnol. 57 (2022) 24–30, https://doi.org/10.1016/j.ejbt.2022.03.003.
[73] M. Stobiecka, J. Król, A. Brodziak, Antioxidant activity of milk and dairy products, Animals (Basel) 12 (3) (2022) 245, https://doi.org/10.3390/ani12030245.
[74] M.A. Ali, M.M. Kamal, M.H. Rahman, M.N. Siddiqui, M.A. Haque, K.K. Saha, M.A. Rahman, Functional dairy products as a source of bioactive peptides and probiotics: current trends and future prospectives, J. Food Sci. Technol. 59 (4) (2022) 1263–1279, https://doi.org/10.1007/s13197-021-05091-8.
[75] H.S. Aloğlu, Z. Oner, Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt, J. Dairy Sci. 94 (11) (2011) 5305–5314, https://doi.org/10.3168/jds.2011-4285.
[76] G. Niero, S. Currò, A. Costa, M. Penasa, M. Cassandro, C. Boselli, G. Giangolini, M. De Marchi, Short communication: Phenotypic characterization of total antioxidant activity of buffalo, goat, and sheep milk, J. Dairy Sci. 101 (6) (2018) 4864–4868, https://doi.org/10.3168/jds.2017-13792.
[77] E. Papadaki, I.G. Roussis, Assessment of antioxidant and scavenging activities of various yogurts using different sample preparation procedures, Appl. Sci. 12 (2022) 9283, https://doi.org/10.3390/app12189283.
[78] L. Yilmaz-Ersan, T. Ozcan, A. Akpinar-Bayizit, S. Sahin, Comparison of antioxidant capacity of cow and Ewe milk kefirs, J. Dairy Sci. 101 (5) (2018) 3788–3798, https://doi.org/10.3168/jds.2017-13871.
[79] C. Rice-Evans, N. Miller, G. Paganga, Antioxidant properties of phenolic compounds, Trends Plant Sci. 2 (4) (1997) 152–159, https://doi.org/10.1016/S1360-1385(97)01018-2.
[80] A.K. Rashwan, A.I. Osman, W. Chen, Natural nutraceuticals for enhancing yogurt properties: a review, Environ. Chem. Lett. 21 (2023) 1907–1931, https://doi.org/10.1007/s10311-023-01588-0.
[81] S.Y. Tsen, J. Siew, E.K.L. Lau, F.A.B. Roslee, H.M. Chan, W.M. Loke, Cow's milk as a dietary source of equol and phenolic antioxidants: differential distribution in the milk aqueous and lipid fractions, Dairy Sci. Technol. 94 (2014) 625–632, https://doi.org/10.1007/s13594-014-0183-4.
[82] R. Sharma, B. Diwan, B.P. Singh, S. Kulshrestha, Probiotic fermentation of polyphenols: potential sources of novel functional foods, Food Prod. Process. and Nutr. 4 (2022) 21, https://doi.org/10.1186/s43014-022-00101-4.
[83] S. Ahmed, A. Noor, M. Tariq, A. Zaidi, Functional improvement of synbiotic yogurt enriched with *Lacticaseibacillus rhamnosus* and aloe vera gel using the response surface method, Food Prod. Process. and Nutr. 5 (2023) 38, https://doi.org/10.1186/s43014-023-00153-0.