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Research Article
Functional Yogurt Fortified with Phenolic Compounds Extracted from Strawberry Press Residues and Fermented with Probiotic Lactic Acid Bacteria

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Abstract

Background and Objective: The objective of the present study was to characterize polyphenol-enriched extracts from industrial plant by-products (strawberry press residues) and to investigate the effect of the addition of these extracts on the growth and activity of yogurt starter culture. **Materials and Methods:** Polyphenol-enriched extracts from strawberry press residues (SPE) were obtained by adsorption technology. The total polyphenol and total monomeric anthocyanin content of the extracts and the yogurt samples were determined. Anthocyanin identification and quantification was performed using UHPLC-DAD. The total antioxidant capacity was evaluated using DPPH-(2,2 diphenyl 1 picryl hydrazyl hydrate) radical and FRAP-(ferric reducing antioxidant power) assays. **Results:** The SPE was characterized by high total polyphenol content (46400.0±23.93 mgGAE.100 g^{-1} dry extract), high total monomeric anthocyanin content (5901.25±0.011) and high antioxidant activity (DPPH = 2427±5.00 μmol TE g^{-1} and FRAP = 1664±1.77 μmol TE g^{-1}). Four main anthocyanin groups were identified in the extracts: cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside and pelargonidin 3-malonoyl glucoside. The growth and acidification activity of the probiotic lactic acid bacteria were not affected by the enrichment of milk with polyphenol extracts. **Conclusion:** The results reported in the present study indicated that polyphenol-enriched extracts from industrial plant by-products (strawberry press residues) could be considered relevant sources of bioactive compounds. They also proved to be an interesting choice for improving the functional characteristics of probiotic yogurts.

Kay words: Antioxidants, functional dairy products, HPLC-DAD, polyphenols, probiotic bacteria, strawberry press residues, yogurt

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Yogurt is a popular fermented dairy product which has gained consumers' positive perception as a functional dairy product with health promoting ingredients[1]. The health benefits of yogurt are correlated with the presence of living microorganisms like lactic acid bacteria, which originate from the starter cultures[2].

Food polyphenols have been widely studied and found to possess a number of important bioactivities such as the prevention of cardiovascular diseases and many types of cancer and age-related illnesses[3]. There is an increasing awareness that the health benefits of dietary polyphenols may be due to their antioxidative activities[4]. Therefore, yogurt with added polyphenol-enriched extracts from natural sources may be a convenient food format to satisfy consumer interest in original yogurt nutrients, the beneficial effects of starter cultures and the health benefits of added polyphenol extracts[5].

Recently, research interest has been focused on the use of agricultural products and by-products as a potential source of phenolic compounds[6]. By-products obtained during fruit and vegetable processing usually contain a significant amount of phenolic compounds. Some studies have already been done on the utilization of vegetable by-products as potential sources of polyphenols with antioxidant activity[7]. The natural antioxidants available in these sources protect the human body against free radicals and oxidative stress[8].

Over the last few years, the identification and development of polyphenol-enriched extracts from different plants has become a major area of health and medicine-related research[9]. In this respect, it is important to quantify the phenolic content of the extracts and to evaluate its contribution to the antioxidant activity. Among natural antioxidants, phenolic-rich extracts like those derived from different fruit by-products have been reported as good alternatives since they are readily available as industrial wastes[10]. They could thus be of great benefit from both an economic and an environmental perspective as sources of low cost natural antioxidants[11].

The combination of phenolic compounds, especially when extracted from natural sources and probiotic lactic acid bacteria may represent an innovative approach for the development of functional fermented milks. Therefore, the present study aimed at producing and characterizing functional yogurt fortified with polyphenols extracted from strawberry press residues (SPE) and fermented with probiotic lactic acid bacteria.

MATERIALS AND METHODS

Materials

Plant materials: The strawberry by-products were supplied by Santulita Ltd. (Sofia, Bulgaria). The press residues were stored frozen at -18°C until used to produce the polyphenol extracts.

Bacterial strains: The starter cultures of lactic acid bacteria used in the present study consisted of *Lactobacillus delbrueckii* sub sp. *bulgaricus* S19 (*L. bulgaricus*) and *Streptococcus thermophilus* S13 (*S. thermophilus*). They belong to the laboratory collection of the Department of Microbiology at the UFT, Plovdiv.

Polyphenol extract from strawberry press residues: Polyphenol-enriched extracts from strawberries were obtained by adsorption technology. To remove the sugars, salts and amino acids, the extracts were purified using a column (465×30 mm) filled with Amberlite XAD 16 HP. The purified extracts were lyophilized for 48 h.

Yogurt samples: The control and experimental batches of yogurt were prepared in laboratory conditions (Department of Milk and Dairy Products Technology at the UFT, Plovdiv, Bulgaria) from a single vat of milk according to the following procedure: cow's milk (M = 3.7%) was heated to 95°C for 15 min, cooled to t = 45±1°C and divided into two lots: one experimental lot (Batch S1) fortified with polyphenols to 0.4 mg of polyphenols 100 g^{-1} using SPE and an unfortified control batch (Batch K1). The experimental and control batches of milk were inoculated with 2% Bulgarian yogurt starter culture in *L. bulgaricus*: *S. thermophilus* ratio of 1:5. All samples were packaged in containers and incubated at t = 44±1°C until they reached pH = 4.5±0.1. At the end of the incubation, the yogurts were cooled down to approximately 4°C and then stored at the same temperature for 15 days.

Methods

Microbiological analysis: Viable cell counts of *S. thermophilus* S13 and *L. bulgaricus* S19 were determined every 60 min during the fermentation process by cultivations on synthetic culture media M17 and MRS (Merck, Darmstadt, Germany). The methodology described in Standard EN ISO 7889[12] was followed. The samples were prepared according to ISO[13]. *Lactobacillus bulgaricus* was counted on MRS (Merck, Darmstadt, Germany) spread plates in which the pH had been adjusted to pH 5.4. After incubation at 41°C for 48 h, *L. bulgaricus* colonies were observed as small star-shaped

Table 1: Contents of total polyphenols (TPP) and total monomeric anthocyanins (TMA), antioxidant capacity (DPPH and FRAP) value and total anthocyanins/phenolics ratio (TMA/TPP) of an extract from strawberry press residues

Characteristics	Mean ±SD (n=6)
TPP[a] (mg GAE 100 g^{-1})	46400.0±23.93
TMA[b] (mg GAE 100 g^{-1})	5901.25±0.011
TMA/TPP (%)	12.00
DPPH[c] (μmol TE g^{-1})	2427.00±5.00
FRAP[c] (μmol TE g)	1664.00±1.77

[a]Results are expressed as mg gallic acid equivalents per 100 g dry extract, [b]Results are expressed as mg cyanidin 3-glucoside equivalents per 100 g dry extract. [c]Results are expressed as μmol Trolox equivalents per 1 g dry extract

white colonies. *Streptococcus thermophilus* was counted on M17-lactose (Merck, Darmstadt, Germany) spread plates after incubation at 37°C for 48 h.

Physicochemical analyses

pH and lactic acid: The pH values of the samples were determined with an MS 2011 pH meter (Microsyst, Plovdiv, Bulgaria) equipped with a Sensoret pH electrode (Garden Grove, CA, USA). The lactic acid content was determined by the titration method[14]. The residual lactose content was calculated on the basis of the results for initial lactose content in milk and lactic acid formation during the fermentation process.

Total polyphenols, total monomeric anthocyanins and antioxidant capacity tests: All measurements were performed with a Helios Omega UV-vis spectrophotometer equipped with VISION*lite* software (all from Thermo Fisher Scientific, Madison, WI, USA). Before the analyses, 200 mg of lyophilized extracts were dissolved in 40 ml aqueous methanol (80%). After overnight extraction at 10°C, the mixture was filtered through a paper filter. The extraction was performed in triplicate.

The total polyphenol (TPP) content was determined by the method of Singleton and Rossi[15]. The results were expressed mg gallic acid equivalents (GAE) per 100 g of dry extract. The total monomeric anthocyanin (TMA) content was determined by the pH-differential method[16]. The results were expressed as mg cyanidin 3-glucoside equivalents (CGE) per 100 g of dry extract.

The total antioxidant capacities were determined by the DPPH (2,2 diphenyl 1 picryl hydrazyl hydrate) and FRAP (ferric reducing antioxidant power) assays. The results of both tests were expressed as μmol Trolox equivalents (TE) per g of dry extract.

UHPLC-DAD and LC-MS analysis: The separation of strawberry anthocyanins was performed using a Nexera UPLC system (Shimadzu, Kyoto, Japan) equipped with two model LC-30AP high pressure pumps, a model DGU-20A5R degasser, a model SIL-30AC autosampling unit (cooled at 10°C), a model CTO-20AC column oven (set at 40°C) and a model SPD-M20A diode array detector. The column used was an Acquity HSS T3 (Waters, Ireland) (150×2.1 mm, 1.8 μm particle size) equipped with a security guard column. The mobile phase consisted of 5% (v/v) formic acid in water (eluent A) and 5% (v/v) formic acid in acetonitrile (eluent B). The monitoring was performed at 520 nm at a flow rate of 0.4 mL min^{-1}. The injection volume was 5 μL and the samples (strawberry extracts) were membrane-filtered (0.45 μm) prior to the analyses.

The identification was confirmed by mass spectrometric analysis. Therefore, the same UPLC method as described above was applied using an Acquity UPLC system (Waters Milford, MA, USA), consisting of a binary pump (BSM), an autosampler (SM) cooled at 20°C, a column oven (CM) set at 40°C, a diode array detector (PDA) and Acquity TQD triple-quadrupole mass spectrometer with an electrospray interface (ESI), operating in positive mode. An Acquity UPLC HSS T3 column with 1.8 μm particle size (150×2.1 mm) and guard column (5×2.1 mm) was used for separation. The mobile phase consisted of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The injection volume was 5 μL. The monitoring was performed at 520 nm at a flow rate of 0.4 mL min^{-1}. The mass spectrometer was tuned using a delphinidin-3-O-glucoside solution.

Sensory analysis: The evaluation of the sensory quality of the yogurt samples was performed by 10 panelists according to a five-point hedonic scale from 1 = dislike a lot to 5 = like a lot. The color, appearance, body, texture, aroma and taste of the yogurt samples were evaluated. The tests were repeated three times.

Statistical analysis: The results reported in the present study are expressed as the mean values of at least three analytical determinations. The coefficient of variation expressed as percentage ratios between the standard deviations and the mean values was found to be 5% in all cases. The means were compared using one-way ANOVA performed with Microsoft Excel and Tukey's test at a 95% confidence level.

RESULTS AND DISCUSSION

Phytochemical characterizations of the polyphenol extract from strawberry press residues: The application of polyphenol-enriched extract as a functional additive to

fermented milk requires its preliminary phytochemical characterization. The data on total polyphenols, total monomeric anthocyanin content and the antioxidant capacity of the polyphenol extract from strawberry press residues are presented in Table 1.

High contents of total polyphenols (46400.0±23.93 mg GAE.100 g^{-1} dry extract) and total monomeric anthocyanins (5901.25±0.011 mg CGE.100 g^{-1} dry extract) in the SPE were established. These data indicate that the strawberry press residues are a valuable source of substances that may impart added value to a large number of products. Moreover, the phenolic compounds in extracts from plant by-products are associated with positive health effects on the human organism[17]. A lot of researchers[18,19] have reported a positive correlation between the total phenolic content and the free-radical scavenging activity of fruit polyphenol extracts. In the present study, the higher polyphenol levels in the SPE resulted in a higher antioxidant capacity as quantified by DPPH and FRAP assays (Table 1). According to data presented by Yoncheva *et al.*[20], the total anthocyanins/total polyphenols ratio in strawberry fruits was about 4%, while in the present study, the TMA/TPP ratio in the SPE was three times higher (12%). Probably, most of the anthocyanins in the strawberries are contained in those fruit parts that remain in the press residues after juice extraction. Therefore, their ratio in the total polyphenols is higher in the strawberry press residues in comparison with the strawberry fruits.

Anthocyanins are the glycoside forms of anthocyanidins which are responsible for the red color of fruits and vegetables and have a characteristic absorption wavelength of approximately 500-520 nm in their HPLC-DAD[21]. The HPLC-DAD profiles of the anthocyanins in the SPE are presented in Fig. 1. The anthocyanins showed very intense peaks in the positive ionization mode of LC-MS because of the acidic flavylium cations, their natural and most stable forms[22]. The main anthocyanins in strawberries are glycosides of pelargonidin (λ_{max} at 502 nm) and cyanidin (λ_{max} at 516 nm)[23].

The results on the identification and quantification of the anthocyanins in the SPE are shown in Table 2. Four main groups of anthocyanins were identified on the basis of their HPLC DAD and LC-MS data: cyanidin 3-glucoside, pelargonidin 3-glucoside (the major anthocyanin in strawberries), pelargonidin 3-rutinoside and pelargonidin 3-(malonoyl)glucoside. The presence of these anthocyanins in strawberry is consistent with a previous study[24].

Effect of polyphenols on the growth and activity of yogurt starter culture: The fermentation of lactose to lactic acid by

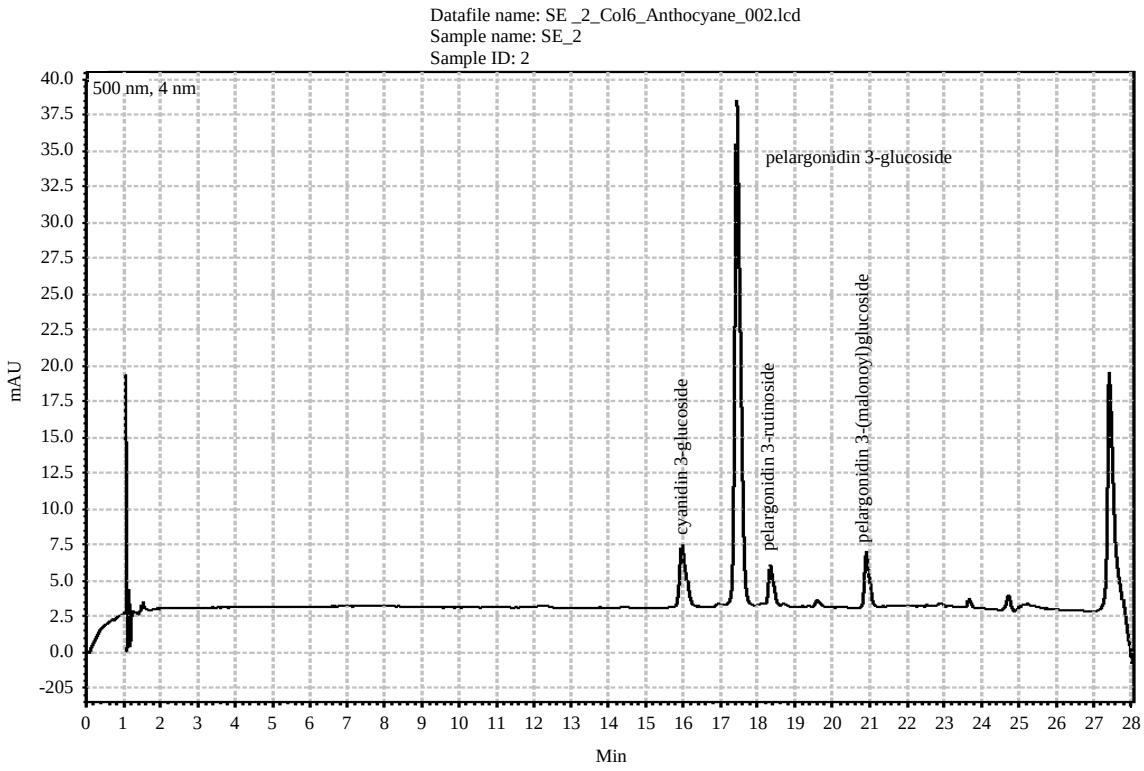

Fig. 1: HPLC-DAD separation (λ = 520 nm) of anthocyanins from a strawberry extract

Table 2: Identification of anthocyanins in an extract from strawberry press residues

No.	t_{RUV} (min)	t_{RMS} (min)	UV_{max} (nm)	$(M)^+$ (m/z)	MS^2 (m/z)	MS^3 (m/z)	Peak assignment	Contents (mg g^{-1})
1	-	12.31	516	449	287	-	cyanidin 3-glucoside	32.98
2	-	15.07	502	433	271	-	pelargonidin 3-glucoside	232.76
3	-	16.54	502	579	433/271	-	pelargonidin 3-rutinoside	18.70
4	-	19.86	502	519	433/271	-	pelargonidin 3-(malonoyl)glucoside	24.88
Total								309.32

lactic acid bacteria is the main process in yogurt production. Lactic acid production is important for the formation of the sensory characteristics of yogurt. As a result of this process, the pH values of milk decrease and the concentration of lactic acid increases, respectively[25]. The fermentation process is closely related to the growth and activity of lactic acid microflora. The results on the changes in the starter lactic acid bacteria count, the pH values, lactic acid and residual lactose concentrations during fermentation of control and polyphenol-enriched yogurts are presented in Fig. 2 and 3. During incubation of milk samples, a significant decrease ($p<0.05$) in the pH values from 6.67 ± 0.05 to 4.83 ± 0.07 and an increase in the lactic acid concentration from 1.65 ± 0.03 g L^{-1} to 6.05 ± 0.05 g L^{-1} was observed. The residual lactose content of the yogurt samples at the end of the coagulation process was in the range of 37.00 ± 0.45 g L^{-1}. The results obtained (Fig. 2 and 3) indicated that the addition of SPE to milk did not influence significantly the lactic acid production process and milk coagulation time, respectively.

During milk incubation, rapid growth of lactic acid bacteria in all experimental samples was observed. The *L. bulgaricus* and *S. thermophilus* counts increased from $6.2\pm0.1.10^5$ CFU g^{-1} to $1.4\pm0.3.10^7$ CFU g^{-1} and from 3.7 ± 2.10^6 CFU g^{-1} to $1.8\pm0.9.10^8$ CFU g^{-1}, respectively. During the first two hours of incubation a higher increase in the *S. thermophilus* count in comparison with the *L. bulgaricus* count was found. This could be explained with the two specific stages of the symbiotic growth of *S. thermophilus* and *L. bulgaricus*. At the first incubation stage (up to the 2nd hour), more rapid growth of *S. thermophilus* was established. After the 2nd hour until the end of incubation, the *L. bulgaricus* count increased more rapidly. No significant differences ($p<0.05$) in the *S. thermophilus* and *L. bulgaricus* counts in the control and polyphenol-enriched yogurts were observed. These data indicated that the enrichment of milk with strawberry polyphenols did not affect significantly the growth and acidifying activity of the starter culture. Our results were in agreement with the findings of Chouchouli *et al.*[26], who reported that the fortification of yogurts at 5-10 mg gallic acid equivalents/100 g did not affect the yogurt pH and Lactobacilli counts.

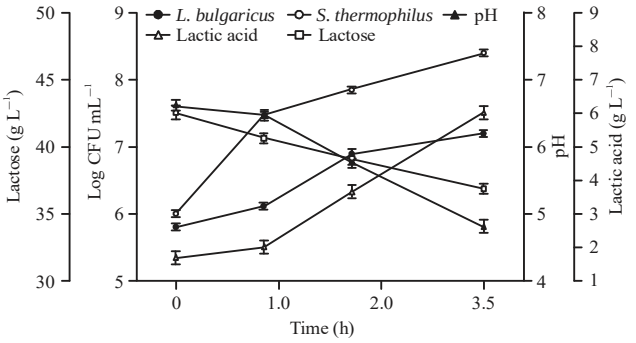

Fig. 2: Growth and acidification activity of the starter culture during the fermentation process of control yogurt

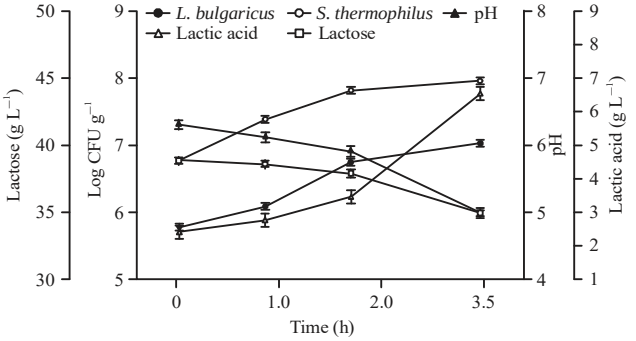

Fig. 3: Growth and acidification activity of the starter culture during the fermentation process of polyphenol-enriched yogurt

Quality characteristics of polyphenol-enriched yogurt: The main physicochemical and microbiological characteristics of control and polyphenol-enriched yogurt samples are presented in Table 3.

It could be seen that the pH values, lactic acid and residual lactose contents in the control yogurt and polyphenol-enriched yogurt did not differ significantly ($p<0.05$). Singh *et al.*[27] also reported that the addition of a strawberry polyphenol extract did not affect the composition and physicochemical parameters of stirred dahi. The viable cell counts of probiotic lactic acid bacteria *L bulgaricus* S19 and *S. thermophilus* S13 in the experimental samples were above 10^8 CFU g^{-1}. An important prerequisite for the functional characteristics of yogurt is that it should contain a high count of viable probiotic bacteria[28].

Table 3: Physicochemical and microbiological characteristics of yogurts enriched with polyphenol extracts

Parameters	Samples	
	Control yogurt	Polyphenol-enriched yogurt
pH	4.34±0.04	4.44±0.02
Lactic acid (g L^{-1})	8.37±0.02	8.46±0.04
Lactose (g L^{-1})	34.65±0.2	33.17±0.3
Total count of lactic acid bacteria (CFU g^{-1})	$3.6.10^8$	$2.7.10^8$
Lb. delbrueckii subsp. bulgaricus (CFU g^{-1})	$7.2.10^7$	$1.1.10^7$
Str. thermophilus (CFU g^{-1})	$2.9.10^8$	$2.6.10^8$
TPP (mg GAE.100 mL^{-1})	-	45.6±1.5
DPPH (µmol TE.100 mL^{-1})	32.0±3.5	120.0±2.5
FRAP (µmol TE.100 mL^{-1})	41.0±4	105.0±2.5

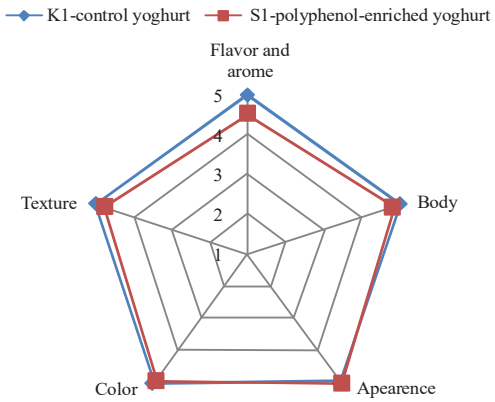

Fig. 4: Sensory evaluation of control and polyphenol-enriched yogurts

The results obtained in the DPPH and FRAP tests showed that the antioxidant capacity of yogurt fortified with SPE was two or three times as high as that of the control yogurt. These data indicated that the SPE addition contributed significantly to the enhancement of the antioxidant capacity of yogurts and of their functional properties, respectively. Similar results were reported by Skrede *et al.*[18]. The authors monitored the antioxidant activity of commercially prepared fermented milk supplemented with bilberry (10°Brix juice) and black currant (13°Brix juice) extracts during storage (1-3 weeks). The antiradical power (ARP, µmol TE g^{-1}) and oxygen radical absorbance capacity (ORAC, µmol TE g^{-1}) were increased due to the addition of fruits.

The results from the sensory analysis of yogurt samples fortified with SPE and of the control yogurt are presented in Fig. 4.

Control yogurt samples with probiotic strains of *L. bulgaricus* and *S. thermophilus* lactic acid bacteria received the highest score of 25 points for the sensory characteristics tested. They were characterized with typical flavor and aroma, homogeneous cream-like texture and white color with a creamy tinge which is characteristic of cow's milk. The scores given to the flavor and aroma characteristics of the polyphenol-enriched yogurt were a little lower compared to the control samples. This could have been due to the specific SPE flavor, which affected the yogurt aroma. Nevertheless, the results obtained (Fig. 4) showed that the sensory evaluation score of the polyphenol-enriched yogurt was similar to that of the control yogurt.

CONCLUSION

The results reported in the present study showed that the fortification of milk with polyphenols extracted from strawberry press residues had no negative effect on the growth and acidification activity of the probiotic bacteria. The polyphenol-enriched yogurt had similar physicochemical and organoleptic characteristics to natural yogurt. The fortified yogurts contained more polyphenols and exhibited higher antiradical and antioxidant activities than the controls. It is concluded that, at the supplementation levels tested, the production of functional yogurts with SPE is feasible.

SIGNIFICANCE STATEMENT

This study has explored the production and characterization of functional yogurt fortified with polyphenols extracted from strawberry press residues and fermented with probiotic lactic acid bacteria. Among natural antioxidants, phenolic-rich extracts like those derived from strawberry press residues have been reported as good alternatives, since they are readily available as industrial wastes. They could thus be of great benefit from an economic and environmental perspective as sources of low cost natural antioxidants. This study will help the researcher to uncover the critical areas of the combination of phenolic compounds, especially when they are extracted from natural sources and

probiotic lactic acid bacteria. Thus a new theory on an innovative approach to the development of functional fermented milks may be formulated.

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