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ORIGINAL PAPER

Growth and activity of Bulgarian yogurt starter culture in iron-fortified milk

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Abstract Bulgarian yogurts were manufactured and fortified with 8, 15 and 27 mg of iron kg^{-1} of yogurt. The growth and acidifying activity of the starter culture bacteria *Streptococcus thermophilus* 13a and *Lactobacillus delbrueckii* subsp. *bulgaricus* 2-11 were monitored during milk fermentation and over 15 days of yogurt storage at 4 °C. Fortifying milk with iron did not affect significantly the growth of the starter culture during manufacture and storage of yogurt. Counts of yogurt bacteria at the end of fermentation of iron-fortified milks were between 2.1×10^{10} and 4.6×10^{10} CFU ml^{-1}, which were not significantly different from numbers in unfortified yogurts. In all batches of yogurt, the viable cell counts of *S. thermophilus* 13a were approximately three times higher than those of *L. delbrueckii* subsp. *bulgaricus* 2-11. Greater decrease in viable cell count over 15 days of storage was observed for *S. thermophilus* 13a compared to *L. delbrueckii* subsp. *bulgaricus* 2-11. Intensive accumulation of lactic acid was observed during incubation of milk and all batches reached pH 4.5 ± 0.1 after 3.0 h. At the end of fermentation process, lactic acid concentrations in iron-fortified yogurts were between 6.9 ± 0.4 and 7.3 ± 0.5 g l^{-1}. The acidifying activity of starter culture bacteria in the control and iron-fortified milks was similar. There was no increase in oxidized, metallic and bitter off-flavors in iron-fortified yogurts compared to the control. Iron-fortified yogurts did not differ significantly in their sensorial, chemical and microbiological characteristics with unfortified yogurt, suggesting that yogurt is a suitable vehicle for iron fortification and that the ferrous lactate is an appropriate iron source for yogurt fortification.

Keywords Yogurt · Iron-fortified · Yogurt starter culture · Growth · Activity

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Introduction

Iron deficiency is one of the major nutritional problems in the world, primarily affecting women of childbearing age, infants, and children [7, 36]. It is usually the result of insufficient dietary intake of iron, poor utilization of iron from ingested food, or a combination of the two [11]. One way of increasing the iron intake is by the fortification of foods regularly consumed by the groups at risk [12, 15, 29–31]. Dairy products are an excellent source of calcium and phosphorus [38], but they contain very little iron [16, 22]. Fortification of milk with iron would help meet this nutritional need for people from the risk groups who typically consume more dairy products. There are reports for the manufacture of iron-fortified milk [28, 32, 33], iron-fortified yogurts [13, 25] and iron-fortified cheeses [27, 34, 35, 41, 42]. Yogurt could be a suitable vehicle for iron fortification because of its widespread consumer acceptance in many countries, primarily by women, children and teenagers. The right selection of an appropriate source of iron is important for the quality of iron-fortified yogurt produced. Different sources of iron have been used for iron fortification in dairy industry: microencapsulated iron [23, 24, 28] sodium iron EDTA [NaFe(III)EDTA] [8]; protein-chelated iron [13]. Currently there are no reports about the usage of

ferrous lactate for yogurt fortification. It has a priority over the sources mentioned before, because it is cheaper and easy for preparation and application. However, before any such fortification is undertaken in yogurt, the effects of the iron fortification source and the level on the starter culture physiology during the manufacture and shelf-life of yogurt, chemical composition and sensory characteristics must be ascertained.

The aim of the present study was to examine the growth and acidifying activity of starter culture bacteria *Streptococcus thermophilus* 13a and *Lactobacillus delbrueckii* subsp. *bulgaricus* 2-11 during the manufacture and storage of iron-fortified Bulgarian yogurt.

Materials and methods

Isolation of lactic acid bacteria strains

The *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains were isolated from homemade cow and sheep milk yogurts, manufactured in certain high-mountain regions (in the Rhodope Mountains, Bulgaria), known as traditional producers of the original Bulgarian yogurt. The fermented milks have been used in households as starter cultures for 8–10 months. In the continuous use of these milks for starter cultures, the lactic acid microflora is subjected to natural selection, and in this way durable proto-cooperative relationships are established between the two species, which are able continually to manifest their properties in natural combinations. Identification of strains was by PCR-generated DNA banding patterns obtained with species-specific primers 5′-CCGAGCTCAACAGAGTT TGATCCTGGCTCAG-3′ and 5′-GGTCGACCGTTAAT ACGACTCACTATAGGGATACCTTGTTACGACTT-3′. The methods of Torriani et al. [37], and Ward and Timmins [39] were used. The monocultures *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 were maintained by weekly transfers in test tubes with sterilized cow skim milk (115 °C, 15 min) and stored at 4 °C. They are included in the lactic acid bacteria bank of the Laboratory of Applied Microbiology at the Institute of Microbiology of the Bulgarian Academy of Sciences.

Preparation of the yogurt starter culture

The *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* strains were used to form associations which were tested as yogurt starter cultures. The starter culture *S. thermophilus* 13a + *L. delbrueckii* subsp. *bulgaricus* 2-11 was selected following studies on the morphology of the cultures, the ratio between the two strains, the starter culture activity, and the organoleptic properties of the manufactured yogurt [1, 2]. It was proved that the thermophilic streptococci and lactobacilli can successfully maintain stable co-existence and the desired ratio (3:1 = *S*:*L*) during a 3-year-study of the starter culture.

The mixed culture, composed by mixing pure cultures, was obtained in the following way: whole cow milk, sterilized and cooled to 45 °C, was inoculated with 1% (v/v) of *S. thermophilus* 13a + 1% (v/v) of *L. delbrueckii* subsp. *bulgaricus* 2-11, then incubated at 45 °C. The mixed culture with the desired ratio (3:1 = *S*:*L*) was subjected to daily transfers for 3 months and weekly transfers for 1 month.

Yogurt manufacture

The control and experimental batches of yogurt were manufactured in laboratory conditions (Department of Milk and Dairy Products Technology at the University of Food Technologies, Plovdiv, Bulgaria) from a single vat of milk according to the following procedure: non-fat cow milk ($M = 0.1\%$) was divided into four lots—three experimental lots (Batches L, M and H) that were fortified with iron to ether 8, 15 and 27 mg of iron kg^{-1} with ferrous lactate hydrate solution (Fluka, Sigma-Aldrich, Switzerland) and an unfortified control batch. The experimental and control batches of milk were then heated to 95 °C for 15 min, cooled to $t = 45 \pm 1$ °C and inoculated with 2% Bulgarian yogurt starter culture. The mixes were packaged in containers and incubated at $t = 44 \pm 1$ °C until they reached pH 4.5 ± 0.1; the containers were then transferred to a cold room (5 ± 1 °C) and stored for 15 days.

Microbial analysis

Viable cell counts of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 were determined every 30 min during the fermentation process and after every day of storage by cultivations on synthetic culture media M17 and MRS (Merck, Darmstadt, Germany). The methodology described in IDF Standard 149 A:1997 [18] was followed. The samples were prepared according to IDF Standard 122 C:1996 [17]. *Lactobacillus delbrueckii* subsp. *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. delbrueckii* subsp. *bulgaricus* colonies were observed as small star-shaped, white colonies. *Streptococcus thermophilus* was counted on M17-lactose (Merck, Darmstadt, Germany) spread plates after incubation at 37 °C for 48 h.

Chemical analysis

Iron concentrations of yogurts were determined by ICP-OES method with the help of ICP-OES Spectrometer

Modula (Spectro Analytical Instruments, Spectro, Germany), $\lambda = 238.204$ nm. The pH was determined with a pH meter MS 2011 (Microsyst, Plovdiv, Bulgaria), equipped with a pH electrode Sensoret (Garden Grove, CA, USA). Lactic acid and lactose contents were determined by enzymatic methods as described by Boehringer Mannheim [3]. Yogurts were analyzed for dry matter [21], fat content [20] and total protein (calculated as total nitrogen × 6.38). Nitrogen determination was performed in duplicate by the Kjeldahl method using Kjeltec Auto 1030 Analyzer (Tecator Sweden) combined with the Digestion System 20.

Sensory analysis

The presence of oxidized, metallic, or bitter off-flavors in yogurts that had been fortified with 8, 15, and 27 mg of iron kg^{-1} of yogurt was evaluated by a trained panel from the Department of Milk and Dairy Products Technology at University of Food Technologies (Plovdiv, Bulgaria). The taste panel evaluated the yogurts after 1, 7, and 15 days storage at 4 °C. Each judge was given four samples at a time and asked to evaluate each for the presence of bitter, oxidized and metallic off-flavors on a nine-point rating scale (1 = not perceptible to 9 = extremely strong) [13].

Statistical analysis

The experimental results were analyzed mathematically and statistically by factor dispersion analysis [4]. The influence of the various factor levels was evaluated by Scheffe's S-method for multiple comparisons. The results obtained were statistically processed with specialized software (Sigma Plot 2001, Microsoft Excel 2000). The level of confidence used was $\alpha = 0.05$. The results given in the diagrams and tables are mean values from four parallel observations.

Results

The growth and acidifying activity of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 during fermentation of the control (Fig. 1a) and iron-fortified (Fig. 1b, c, d) milks were followed. Iron fortification had no effect on the incubation time required for the yogurt mixes to reach pH 4.5 ± 0.1. The starter culture bacteria intensively accumulated lactic acid during incubation and all batches reached pH 4.5 ± 0.1 after 3.0 h. Intensive growth of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 during

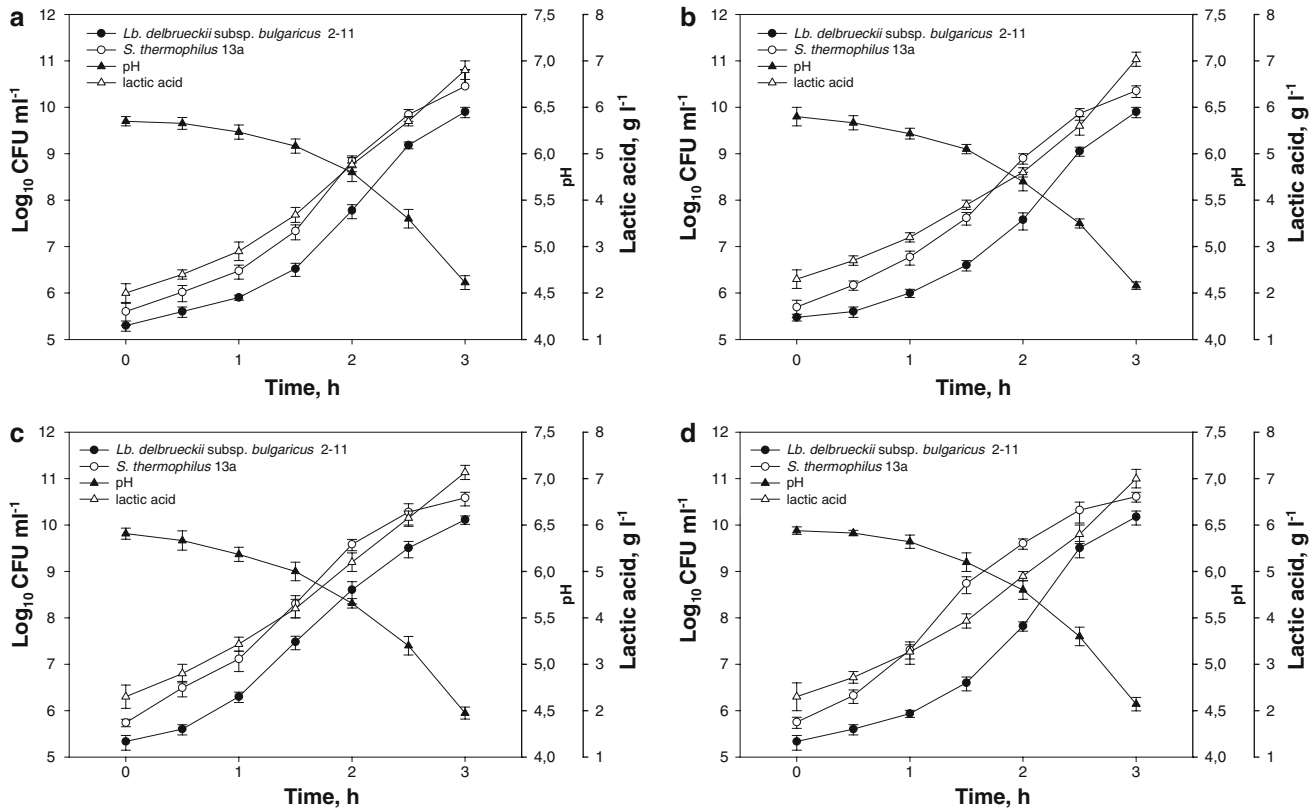

Fig. 1 Growth and acidifying activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2-11 and *Streptococcus thermophilus* 13a during milk fermentation: **a** Control; **b** Batch L (8 mg iron kg^{-1} of milk); **c** Batch M (15 mg iron kg^{-1} of milk); **d** Batch H (27 mg iron kg^{-1} of milk)

milk coagulation was observed. For the control batch, the count of *L. delbrueckii* subsp. *bulgaricus* 2-11 increased from 2.8×10^5 CFU ml^{-1} at the beginning to 9.4×10^9 CFU ml^{-1} at the 3rd hour of incubation, the count of *S. thermophilus* 13a increased from 6.2×10^5 to 2.1×10^{10} CFU ml^{-1}, respectively (Fig. 1a). The growth of the starter culture bacteria in iron-fortified batches was similar (Fig. 1b, c, d). Relatively high viable cell counts of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 (at the 3rd hour of incubation) were found for Variant M—3.4×10^{10} and 1.2×10^{10} CFU ml^{-1}, respectively. In all batches of yogurt the viable cell counts of *S. thermophilus* 13a were approximately three times higher than those of *L. delbrueckii* subsp. *bulgaricus* 2-11. The acidifying activity of starter culture bacteria in the control and iron-fortified milks was similar. During the incubation of the control sample, lactic acid was accumulated and its concentration increased from 2.2 ± 0.5 to 6.8 ± 0.2 g l^{-1}; pH decreased from 6.11 ± 0.07 to 4.61 ± 0.06 (Fig. 1a). At the end of the fermentation process, the lactic acid accumulated in iron-fortified yogurts was between 6.9 ± 0.4 and 7.3 ± 0.5 g l^{-1}; pH values varied from 4.52 ± 0.06 to 4.58 ± 0.04 (Fig. 1b, c, d). No significant ($P < 0.05$) effect both of the iron fortification of the milk and of the concentration of ferrous lactate on the growth and acidifying activity of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 during milk fermentation was observed.

Viability and lactic acid production of starter culture bacteria during storage of the control (Fig. 2a) and iron-fortified (Fig. 2b, c, d) yogurts were also similar. During the 15-day storage of the control yogurt the viable cell counts of *L. delbrueckii* subsp. *bulgaricus* 2-11 and *S. thermophilus* 13a decreased from 9.6×10^{10} to 3.7×10^9 CFU ml^{-1} and from 2.1×10^{11} to 1.6×10^9 CFU ml^{-1}, respectively. Greater decrease in viable cell counts and, respectively, lower viability during storage was observed for *S. thermophilus* 13a compared to *L. delbrueckii* subsp. *bulgaricus* 2-11. The changes in viable cell counts of starter culture bacteria during the storage of iron-fortified yogurts were similar (Fig. 2b, c, d). The concentration of viable lactic acid bacteria in iron-fortified yogurts at the 15th day of storage remained a little higher compared to the control yogurt (Fig. 2). Relatively high counts of *L. delbrueckii* subsp. *bulgaricus* 2-11 and *S. thermophilus* 13a at the end of the storage period were found for Variant M—4.2×10^9 and 2.1×10^9 CFU ml^{-1}, respectively. The results obtained

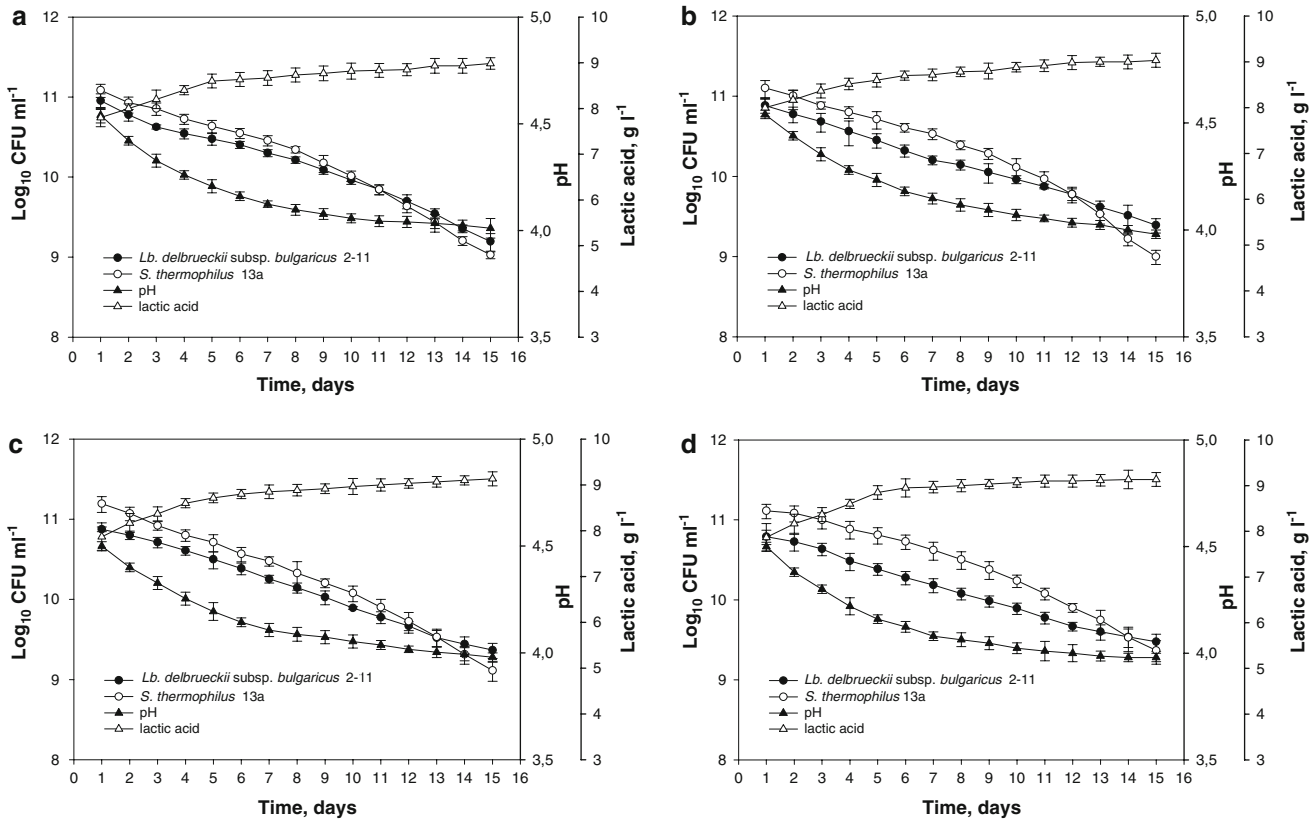

Fig. 2 Viability and acidifying activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2-11 and *Streptococcus thermophilus* 13a during storage of yogurt at $t = 5 \pm 1$ °C: **a** Control; **b** Batch L (8 mg iron kg^{-1} of yogurt); **c** Batch M (15 mg iron kg^{-1} of yogurt); **d** Batch H (27 mg iron kg^{-1} of yogurt)

(Fig. 2) showed that the accumulation of lactic acid was significantly delayed during the refrigerated storage of yogurts. The course of the acid production process during the storage of control and iron-fortified yogurts was similar. The main increase of lactic acid concentration and the decrease of pH values were observed up to 5th day of the storage. Further lactic acid production during storage was very small. For all studied yogurt samples the lactic acid concentrations at the end of the storage were in the range of 9.2 ± 0.2 g l^{-1}, and the pH values were in the range of 4.02 ± 0.06.

The results obtained from the chemical analysis showed similarities in the composition of control and experimental batches of yogurt (Table 1). The dry matter, total protein, fat and lactose contents of control and iron-fortified yogurts did not differ significantly ($P < 0.05$). It was proved that the desired levels of iron fortification in experimental yogurt batches were reached.

The results from the sensorial analysis (Table 2) showed that there was no increase in oxidized, metallic, or bitter off-flavors in yogurts that had been fortified with 8, 15, and 27 mg of iron kg^{-1} of yogurt. The trained panel did not detect any significant differences in overall quality between fortified and unfortified yogurts.

Table 2 Sensorial evaluation of control and experimental batches of yogurt

Yogurt	Off-flavors		
	Metallic	Bitter	Oxidized
Control	1.5 ± 0.2a	1.4 ± 0.7c	1.2 ± 0.4b
Batch L	1.7 ± 0.2a	1.5 ± 0.5c	1.5 ± 0.6b
Batch M	1.6 ± 0.2a	1.7 ± 0.6c	1.4 ± 0.5b
Batch H	1.5 ± 0.3a	1.8 ± 0.5c	1.6 ± 0.5b

Same column bearing a common letter did not differ significantly ($P < 0.05$)

Discussion

The results obtained showed that the iron fortification of milk with the addition of ferrous lactate in concentrations of 8, 15 and 27 mg of iron kg^{-1} of milk does not affect significantly the growth and viability of *L. delbrueckii* subsp. *bulgaricus* 2-11 and *S. thermophilus* 13a during milk fermentation and storage of yogurt. The lower viability of *S. thermophilus* 13a during storage was probably due to the lower pH values, which inhibited to a greater extent *S. thermophilus* than *L. delbrueckii* subsp. *bulgaricus*. Our results about the viability of starter culture bacteria during the storage of yogurt batches are in agreement with the findings of Hekmat and McMahon [13]. The authors did not found significant differences in the count of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* between yogurts fortified by adding $FeCl_3$, whey protein-chelated and casein-chelated iron, and unfortified yogurt stored for 30 days. Kim et al. [25] found similarity in the growth of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* during the manufacture and storage of iron-fortified and unfortified drink yogurt. The similarity in the growth of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in iron-fortified and unfortified yogurts could be explained by the characteristics of their physiology. Lactic acid bacteria are reported to be an exception among living organisms in that they show no iron requirements [5, 6, 40]. Elli et al. [10] reported that only marginal differences in the growth of *Lactobacillus* spp. were observed in iron-depleted media supplemented with five free bases. The authors found that *Lactobacillus* spp. require iron under particular environmental conditions with limited or specific nucleotide sources. Such requirements could not be expected in milk, which is a medium, very rich in nutritional sources. On the other hand, there are reports that some *Lactobacillus* spp. have the ability to bind ferric hydroxide at their cell surface, rendering it unavailable to pathogenic microorganisms [26].

The similarity in the course of lactic acid production during the manufacture and storage of iron-fortified and unfortified yogurts reported in the present study is in agreement with the findings of Hekmat and McMahon [13] and Kim et al. [25]. The shorter milk coagulation time (3 h) in our study is probably due to the higher acidifying activity of the yogurt starter culture used.

Iron-fortified Bulgarian yogurts were successfully manufactured at the target iron concentrations (Table 1). Iron-fortified yogurts did not differ significantly in their

Table 1 Mean values for iron content (Fe), dry matter (DM), total protein (TP), fat and lactose content of control and experimental batches of yogurt

Yogurt	Chemical composition parameters				
	Fe (mg kg^{-1})	DM (%)	TP (%)	Fat (%)	Lactose (%)
Control	0.70 ± 0.12a	9.6 ± 0.2d	3.6 ± 0.1c	0.10 ± 0.02a	3.5 ± 0.3c
Batch L	8.04 ± 0.10c	9.5 ± 0.1d	3.7 ± 0.2c	0.10 ± 0.03a	3.6 ± 0.5c
Batch M	15.00 ± 0.11b	9.7 ± 0.2d	3.6 ± 0.2c	0.10 ± 0.04a	3.6 ± 0.5c
Batch H	27.06 ± 0.10d	9.7 ± 0.3d	3.5 ± 0.3c	0.10 ± 0.03a	3.5 ± 0.4c

Same column bearing a common letter did not differ significantly ($P < 0.05$)

sensorial, chemical and microbiological characteristics from the unfortified yogurt (Tables 1, 2). One cup (120 g) of iron-fortified yogurt from the batches L, M and H would provide more than 10% of the recommended daily allowance of iron for infants and children (7–10 mg), teenagers (11–15 mg) and pregnant women (37 mg) [14, 19]. Drago and Valencia [9] have established that milk fermentation causes an increase in iron availability. This fact suggests that yogurt is a suitable vehicle for iron fortification. Currently yogurt has gained widespread consumer acceptance in many countries in the world, so the iron fortification of yogurt can be used as a long-term strategy for reducing the prevalence of iron deficiency in the risk groups.

Conclusion

Iron-fortified Bulgarian yogurts were manufactured at iron concentrations to supply daily iron requirements of infants, children, teenagers and pregnant women. The iron fortification of the milk and the concentration of ferrous lactate had no significant ($P < 0.05$) effect on the growth and lactic acid production of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 during milk fermentation. The viability and acidifying activity of *S. thermophilus* 13a and *L. delbrueckii* subsp. *bulgaricus* 2-11 during storage of control and iron-fortified yogurts were also similar.

Iron-fortified yogurts did not differ significantly in their sensorial, chemical and microbiological characteristics from the unfortified yogurt. The obtained results showed that ferrous lactate is an appropriate iron source for yogurt fortification, which is cheap and easy for preparation and application.

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