Food Sci Biotechnol (2019) 28(6):1837–1844
https://doi.org/10.1007/s10068-019-00625-5

In vitro bioaccessibility of added folic acid in commercially available baby foods formulated with milk and milk products

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Received: 4 January 2019 / Revised: 10 April 2019 / Accepted: 30 April 2019 / Published online: 9 May 2019

Abstract Milk contains a certain amount of folate binding proteins. The binding capacity varies in acidic conditions and affects the bioavailability of folic acid. Folic acid is commonly added into baby foods to ensure adequate intake of infants. The aim of this study was to determine the bioaccessibility of added folic acid in baby foods formulated with milk and milk products under different gastric pH values by an in vitro digestive system. The bioaccessibility of folic acid ranged between 56–71 and 35–49% in infant formula samples, between 59–78 and 31–67% in cereal-based baby foods, and between 42–67 and 38–57% in follow-on baby milk at gastric pH 1.5 and pH 4, respectively. Our results demonstrate that the bioaccesibility of folic acid that is added to baby food is affected by gastric pH. Therefore, it was observed that the bioaccesibility of folic acid was lower in the higher gastric pH.

Keywords Folic acid · Bioaccessibility · Folate binding protein · Milk · Baby foods

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Introduction

As a water soluble vitamin, folic acid, also known as folate is not found naturally in foods. It is also called as pteroylglutamic acid, because it contains only one glutamic acid in the structure (Ball, 2004). Folic acid is commonly used in food supplements and as a food fortification ingredient because it is the most oxidized, stable, and bioavailable of all the folates in foods (Elliot, 2008). Folate exists in foods predominantly as polyglutamyl forms of tetrahydrofolate (THF), 5-methyl-THF, and 10-formyl-THF (Gregory et al., 1984). Folate is found in the form of 5-methyl-THF in plasma and converted to THF, which is the biochemically active form, in the liver. In addition, synthetic folic acid is converted to THF by the dihydrofolate reductase (DHFR) enzyme. THF plays many roles in cell replication and methylation reactions. Also, it plays an important role in the remethylation of homocysteine (Hcy) to methionine (Ball, 2004). An increased Hcy level in plasma may cause cardiovascular diseases (Eichholzer et al., 2001), different types of cancer (He and Shui, 2014), and neural tube defects (Eichholzer et al., 2006). According to some clinical studies, folic acid supplementation reduces the level of homocysteine in children (Papandreou et al., 2010).

The amount of natural folate in milk is very low and predominantly consists of 5-methyl-THF form of folate (Konings et al., 2001). According to data from the United States Department of Agriculture (USDA) National Nutrient Database, the total amount of folate in cow's milk is in the range of 5–10 μg/100 g, while in milk powder and skimmed milk, it is in the range of 15–60 μg/100 g (USDA, 2018). As seen, milk contains low amounts of folate. Thus, folic acid is commonly added into baby foods to ensure adequate intake of infants. According to the EU

Law, the maximum limit for folic acid in baby foods, if added, is 50 μg/100 kcal, and the daily reference intake is 150 μg of dietary folate equivalents (EC, 2006).

Processed and untreated milk contains a certain amount of folate binding proteins (FBP), and it can be bound to folates at a rate of 1:1 (Salter et al., 1981). FBP isolated from cow's milk has 222 amino acids and contains eight disulphide bridges. The molecular weight is about 30,000–35,000 Da (Svendsen et al., 1979). Studies indicated that FBP prevents the uptake of folate by intestinal bacteria, protects folate from degradation (Ford, 1974; Tani and Iwai, 1984) at body temperature, and assists in the transport of folate to the intestinal mucosa (Salter and Blakeborough, 1988). In addition, FBP plays an important role in increasing the bioavailability of folates (Swiatlo et al., 1990). FBP has about 100-fold higher affinity to folic acid than the 5-methyl-THF (Nygren-Babol et al., 2005). Some in vitro studies show that FBP is resistant to pepsin and intestinal proteases (Arkbåge et al., 2003; Verwei et al., 2004). Under acidic gastric conditions (pH < 4.5), folic acid is liberated from FBP, but it is recombined with FBP in the small intestine (pH 6–7). Limited in vitro human studies show that adding FBP to milk and yogurt decreases folate bioaccessibility (Arkbåge et al., 2003; Verwei et al., 2005). With no addition of FBP in milk, the folic acid and 5-methyl-THF bioaccessibility decreased, and folic acid bioaccessibility was lower than that of 5-methyl-THF (Jong et al., 2005).

Bioavailability of nutrients is usually studied by use of in vitro systems because conducting in vivo studies is difficult in terms of time, cost, and ethical problems (Lee et al., 2016; Minekus et al., 2014; Sopade and Gidley, 2009). The digestion, structural changes, and absorption of nutrients can be examined in simulated mouth, stomach, and small intestine model systems (Menard et al., 2014).

There are very few studies of the bioavailability and bioaccessibility of folic acid found in milk and milk products. To date, in vivo and in vitro studies of the bioavailability and bioaccessibility of added folic acid have been directed to only adult gastric pH conditions. The aim of this study was to examine the bioaccessibility of added folic acid in baby foods formulated with milk and milk products under conditions of different gastric pH values simulated by an in vitro gastrointestinal digestive model system.

Materials and methods

Materials

Ascorbic acid, acetonitrile (ACN), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), acetic acid (ACS reagent, $\geq$ 99.7%), 2-mercaptoethanol, alpha-amylase (from *Aspergillus oryzae* powder, 1.5 U/mg) and pancreatin (from porcine pancreas 8 × USP specifications) were obtained from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Protease (subtilisin A from *Bacillus licheniformis*, 350 U/mL) was obtained from Megazyme (UK). Folic acid (96–102%, pure) was procured from Acros Organics (Springfield Township, NJ, USA). Strong anion-exchange (SAX) column (Bond Elut SAX, 500 mg, 3 mL) was obtained from Agilent Technologies (Santa Clara, CA, USA) and used for purification of folic acid. In this study, all other chemicals were used in high purity.

Sampling

Three groups of folic-acid-fortified baby foods were included in this study. The sample included three different brands of follow-on milk, three different brands of milk-based infant formula, and eight different brands of cereal-based baby food formulated with milk powder were procured from different markets in Istanbul, Turkey. The contents of the baby foods are given in Table 1.

Preparation of solutions and standards

Phosphate buffer solution consisted of potassium dihydrogen phosphate (0.1 M) at pH 7.5, and L-ascorbic acid (1%). The elution solution containing sodium acetate (0.1 M) consisted of 0.82 g of sodium acetate, 10 g of sodium chloride, and 1 g of ascorbic acid weighed into a 100 mL volumetric flask. The mixture was dissolved in deionized water. Then, the pH was adjusted to 4.5 with 1 M NaOH, and the volume was completed with deionized water. The SAX column conditioning buffer solution (0.01 M) was prepared by diluting the buffer solution with deionized water and the pH was again set to 7.2.

The standard stock solution of folic acid was prepared in 0.1 M phosphate buffer solution. Each standard is freshly

Table 1 Folic acid extraction with different amounts of pancreatin

Pancreatin (g)	Folic acid (μg/100 g)[1]	Recovery (%)
0.25	122 ± 2.9[a]	53
0.5	161 ± 4.5[b]	71
1	232 ± 5.7[c]	101
1.5	228 ± 4.5[c]	99

Values refer to the mean ± SD ($n = 3$) for quantification of folic acid in the reference material (infant formula/adult nutrition formula)

[1]The different letters in the same column indicate that there are statistical differences between the applications (ANOVA $p < 0.05$, Tukey's test)

prepared on daily basis. Working standard solutions were prepared from the stock solution.

In vitro gastrointestinal procedure and bioaccessibility of folic acid

In this study, the bioaccessibility of folic acid in test samples was studied by simulating the human digestive system. This model includes the mouth, stomach, and small intestinal medium. This procedure was a modified version of the one described previously by Lee et al. (2016).

Digestive enzymes and other solutions (organic, inorganic)

Saliva, gastric juice, duodenal juice, and bile juice were prepared using enzymes, organic, and inorganic chemicals.

Saliva

1.7 mL of NaCl (175.3 g/L), 8 mL of urea (25 g/L), 15 g of uric acid, 280 mg α-amylase, and 25 mg mucin were dissolved in a 500 mL volumetric flask with deionized water. The final volume was then completed with deionized water, and the pH was around 6.8 ± 0.2. We adjusted pH value with acids or bases when it was not at the desired value.

Gastric juice: 6.5 mL of HCl (37 g/L), 18 mL of $CaCl_2{\cdot}H_2O$ (22 g g/L), 1 g of bovine serum albumin, 2.5 g of pepsin, and 3 g mucin were dissolved in a 500 mL volumetric flask with deionized water. The final volume was then completed with deionized water, and the pH was around 1.5 ± 0.02. We adjusted pH value with acids or bases when it was not at the appropriate value.

Duodenal juice: 6.3 mL of KCl (89.6 g g/L), 9 mL of $CaCl_2{\cdot}2H_2O$ (22.2 g g/L), 2 g of bovine serum albumin, 1 g of pancreatin, and 1.5 g of lipase were dissolved in a 500 mL volumetric flask with deionized water. The final volume was completed with deionized water, and pH was around 8.0 ± 0.2. We adjusted pH value with acids or bases when it was not around the desired value.

Bile juice: 68.3 mL of $NaHCO_3$ (84.7 g g/L), 10 mL of $CaCl_2{\cdot}2H_2O$ (22.2 g g/L), 1.8 g of bovine serum albumin, and 30 g of bile were dissolved in a 500 mL volumetric flask with deionized water. The final volume was completed with deionized water, and the pH was around 7.0 ± 0.2. As in the above juices, we adjusted pH value with acids or bases when it was not at the appropriate value.

In vitro digestion procedure

In the mouth phase, 5 g of test sample were mixed with 5 mL of saliva solution in a 100 mL beaker and then homogenized for 20 s with a vortex. This mixture was incubated for 5 min at 37 °C in a shaking water bath. In the gastric phase, 12 mL of gastric juice were added to the fluid obtained from the mouth phase. This was mixed for 20 s with a vortex, and the mixture was incubated again for 2 h at 37 °C in a shaking water bath. Next, 10 mL of duodenal juice and 5 mL of bile juice were added to the fluid obtained after the gastric phase. This mixture was incubated again for 2 h at 37 °C in a shaking water bath. Once the digestion process was completed, the final volume was diluted with deionized water to 50 mL or 100 mL. The samples were then centrifuged for 10 min at 17,226×*g*, filtered through a 0.22 µm CA filter, and stored in a freezer at − 80 °C degrees until they were analyzed. Just before analyzing, purification of folic acid was carried out as described below.

Extraction of folic acid

Folic acid extraction methods have been described by Rychlika et al. (2007) and used with some modifications. Since natural folates were not analyzed in our study, rat plasma and chicken pancreas were not used as deconjugation enzymes. In this study, two different methods were used for extraction of folic acid. In the first method, protease and alpha amylase were used as a di-enzyme treatment. In the second method, only pancreatin was used. The first method proceeded as follows: 10 mL of milk sample, 2 g of infant formula, and 2 g of cereal-based baby food were separately weighed in a 100 mL Erlenmeyer flask. Then, 40 mL of the phosphate buffer (0.1 M) solution was added into each flask, and the mixture was stirred for 10 min in a magnetic stirrer. To prevent oxidation, approximately 25 µL of 2-mercaptoethanol was added. One hundred milligrams of alpha amylase enzyme was added to the prepared mixture and incubated for 2 h at 37 °C in a shaking water bath. Then, the enzyme was denatured in a water bath at 100 °C for 10 min. After cooling to room temperature, 0.2 mL of protease was added and the samples were incubated again for 2 h at 37 °C in a shaking water bath. To provide enzyme denaturation, the extract was placed again in a water bath at 100 °C for 10 min. After cooling to room temperature, the final volume was then brought up to 50 mL with phosphate buffer solution. The samples were transferred into 50 mL Falcon tubes, centrifuged for 10 min at 8613×*g*, and filtered through a 0.45 µm cellulose-acetate (CA) filter (ISOLAB Laborgerate GmbH, Wertheim, Germany). Then, the supernatant was purified with a SAX column.

Optimization of pancreatin treatment

Unlike the first method, as a mixture of enzymes, pancreatin alone was used in the second method; and enzymatic incubation was performed in a single step. For method optimization in this study, the certified reference material (Standard Reference Material 1849: Infant Formula) was used. Different amounts of pancreatin (0.25, 0.5, 1, and 2 g) were added to the samples for digestion.

Purification of the sample with a SAX column

The SAX column was conditioned with 5 mL of methanol and 5 mL of conditioning solution, respectively. Then, 5 mL of filtered supernatant solution was loaded onto the SAX column. The vacuum pump was set to elute the sample at a flow rate of 1 mL/min. Column washing was carried out using 10 mL of 0.01 M phosphate buffer solution. After this step, 2 mL of elution solution was slowly added to the column to collect the folic acid, and the vacuum pump was set to elute the sample at a flow rate of 0.3 mL/min. The eluate was collected in a test tube containing 15 µL of 2-mercaptoethanol, filtered through a 0.45 µm CA filter, and stored at − 20 °C until analysis.

Chromatographic conditions

The quantification of folic acid in baby foods was determined by a reversed-phase high-performance liquid chromatography (HPLC) method. HPLC conditions described by Rychlika et al. (2007) were used with some modifications. The HPLC system was a Shimadzu LC 20AT pump with a Shimadzu RF-10AXL UV–Vis (Shimadzu Corporation, Kyoto, Japan). The mobile phase was formed with 8% ACN in 0.33 M buffer solution. Then, the pH of the mobile phase was adjusted to 2.4 with ortho-phosphoric acid. Afterwards, it was filtered under vacuum using a 0.20 µm CA filter paper. Folic acid was detected with a UV–Vis detector, and the wavelength was set at 290 nm. Zorbax SB-C8 4.6 × 250 mm 5 µm column (Agilent, USA) was used for separation of folic acid. The column oven temperature was maintained at 30 °C, and the flow rate was set to 1 mL/min. The injection volume was set at 20 µL, and separation was completed in 40 min.

Quantification and quality control

Quantification of folic acid was performed by measurement of the peak area. The peak area was plotted against the concentration. In the study, the certified reference material (Standard Reference Material 1849: Infant Formula), was used to check the accuracy and the performance of the method. At the same time, we also participated in a proficiency test for analyzing breakfast cereal test material, which was organized by FAPAS (Food Analysis Performance Assessment Scheme in UK). All analyses were performed in triplicate, and the average value was used.

Statistical analysis

All analyses were performed in triplicate, and the average value was given with standard deviation. Significant differences between the applications were statistically evaluated by one-way analysis of variance (ANOVA $p < 0.05$, Tukey's test).

Results and discussion

Method optimization

Tri-enzyme extraction (Rychlika et al., 2007) has been suggested for folate and folic acid analyses. An incubation with rat plasma and chicken pancreatin has been suggested for use after the amylase and protease extraction, and for deconjugation of folate glutamate chains. Since natural folates were not analyzed in this study, only di-enzyme (alpha-amylase with protease) extraction was applied in the folic acid analysis. Through this extraction method, the amount of folic acid in the reference material was determined as 231 ± 3.8 µg/100 g (assigned value 230 ± 5.5 µg/100 g), and recovery was 101%. In our study, pancreatin was used as an alternative to di-enzyme extraction. As pancreatin includes amylolytic, lipolytic, and proteolytic enzymes (Minekus et al., 2014), it can hydrolyze polysaccharides, lipids, and proteins. Therefore, it can degrade polysaccharide to dextrins or smaller glucose molecules, lipids to glycerol and fatty acids, and proteins to smaller peptides. As shown in Table 1, using different amounts of pancreatin, the reference material (infant formula) was incubated (at pH 7) for 2 h at 37 °C. Consequently, the amount of folic acid was determined to be 232 ± 5.7 and 228 ± 4.5 µg/100 g with a recovery of 101% and 99% by using 1 g and 1.5 g of pancreatin, respectively. There were no statistical differences between the two extractions. For the quality of the research, we participated in the FAPAS test. Our result was found in the acceptable range ($-2 \leqslant$ Z score $\leqslant +2$). During the study, for folic acid analysis, instead of di-enzyme extraction, the second method (the extraction with pancreatin) was performed.

Folic acid content in baby foods

As seen in Table 2, the amount of folic acid was between 83.3 and 130.0 µg/100 g in infant formulas, between 15.3

Table 2 Product types with their main contents and total amount of folic acid in test samples before and after in vitro digestion

Product type	Main product content	Folic acid (μg/100 g)		
		Initial value[1]	pH 1.5[1]	pH 4[1]
Infant formula 1 (0–6 months)	Milk powder	83.3 ± 3.2[a]	48.3 ± 2.5[b]	29.0 ± 2.6[c]
Infant formula 2 (from the 2nd month)	Milk powder	130.3 ± 4[a]	92.7 ± 3.5[b]	51.7 ± 4.5[c]
Infant formula 3 (from the 6th month)	Milk powder	90.3 ± 4.5[a]	50.7 ± 2.5[b]	44.0 ± 3.5[c]
Follow-on milk 1	Milk, UHT	30.3 ± 2.5[a]	12.7 ± 1.5[b]	11.7 ± 2.1[b]
Follow-on milk 2	Milk, UHT	15.3 ± 1.5[a]	10.3 ± 1.2[b]	8.7 ± 1.2[b]
Follow-on milk 3	Milk, UHT	22.0 ± 1.0[a]	11.3 ± 1.5[b]	10.0 ± 2.0[b]
Cereal-based baby food 1 (6–36 months)	Cereal + milk powder	130.0 ± 5.5[a]	100.0 ± 3.6[b]	87.7 ± 4.2[c]
Cereal-based baby food 2 (from the 2nd month)	Cereal + milk powder	61.3 ± 5.0[a]	38.3 ± 2.1[b]	20.7 ± 2.1[c]
Cereal-based baby food 3 (6–36 months)	Cereal + milk powder	105.3 ± 5.0[a]	82.0 ± 1.7[b]	69.0 ± 3.0[c]
Cereal-based baby food 4 (6–36 months)	Cereal + milk powder	138.7 ± 5.5[a]	83.3 ± 2.5[b]	50.0 ± 4.0[c]
Cereal-based baby food 5 (6–36 months)	Cereal + milk powder	137.3 ± 4.2[a]	91.0 ± 3.5[b]	80.7 ± 3.1[c]
Cereal-based baby food 6 (6–36 months)	Cereal + milk powder	124.3 ± 5.5[a]	80.3 ± 2.5[b]	65.0 ± 2.6[c]
Cereal-based baby food 7 (6–36 months)	Cereal + milk powder	95.0 ± 3.6[a]	69.0 ± 3.6[b]	53.7 ± 3.2[c]
Cereal-based baby food 8 (6–36 months)	Cereal + milk powder	73.7 ± 5.9[a]	43.7 ± 3.5[b]	22.7 ± 2.5[c]

Values refer to the mean ± SD (n = 3) for quantification of folic acid in the test samples and in vitro digestion

[1]The different letters in the same rows indicate that there are statistical differences between the applications (ANOVA $p < 0.05$, Tukey's test)

and 30.3 μg/100 g in follow-on milks, and between 61.3 and 138.7 μg/100 g in cereal based baby foods. In a study conducted by Campos-Giménez et al. (2018), the folic acid amount in infant formulas was between 10 and 43 μg/100 g. The amount of folic acid in those infant formulas indicated in that study was lower than the infant formulas included in our study. According to the USDA, the amount of folic acid in cereal-based baby foods ranges between 212 and 132 μg/100 g, while our study's results ranged between 61 and 138 μg/100 g (USDA, 2018). Although the folic acid amount in the cereal-based baby foods used in Turkey was lower, it ranged within the limits of the amounts required by the EU Law. In the USDA, the amount of folic acid in infant formulas (82–128 μg/100 g) was very close to our results (83–130 μg/100 g) (USDA, 2018). In addition, Brandon et al. (2014) reported that the amount of folic acid in infant formulas ranged from 43 to 275%, which is higher than the reported amounts. In our study, the folic acid ranged from 50 to 100%, which is higher than the reported amount on the product labels. Milk and milk products include 5-methyl-THF, a natural form of folates. Campos-Giménez et al. (2018) suggest that infant formulas contain 5-methyl-THF between 0.9 and 2 μg/100 g, while this ranges between 15 and 60 μg/100 g in another study. In addition, cow's milk contains 5-methyl-THF between 5–7 μg/100 g. As milk and milk products contain a low amount of 5-methyl-THF, folic acid can be added into baby foods and various foods as food supplements in accordance with the permitted limits.

In vitro study

The bioaccessibility of folic acid in infant formulas, follow-on milks, and cereal-based baby foods were analyzed in an in vitro gastrointestinal tract, including the mouth, gastric (pH 1.5 and pH 4), and small intestine. The results are summarized in Table 2.

The bioaccessibility of folic acid ranged between 56–71 and 35–49% in infant formula samples (Fig. 1), and between 59–78 and 31–67% in cereal-based baby foods (Fig. 2) at gastric pH 1.5 and pH 4, respectively. Although the bioaccessibility of folic acid significantly decreased in

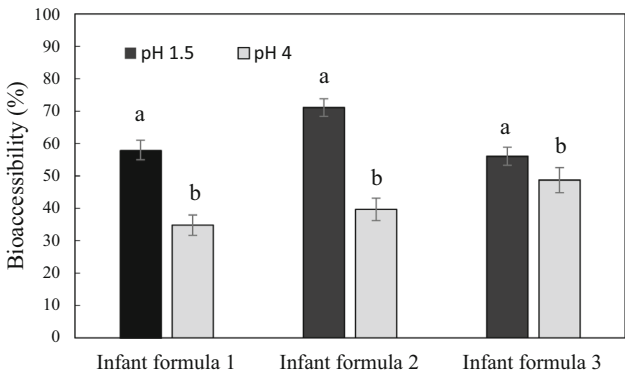

Fig. 1 Bioaccesibility of folic acid in infant formula(s). The bioaccesibility was determined dividing the value after digestion by the value obtained before digestion. The different letters atop columns for each sample indicate that there are statistical differences between the applications (ANOVA $p < 0.05$, Tukey's test)

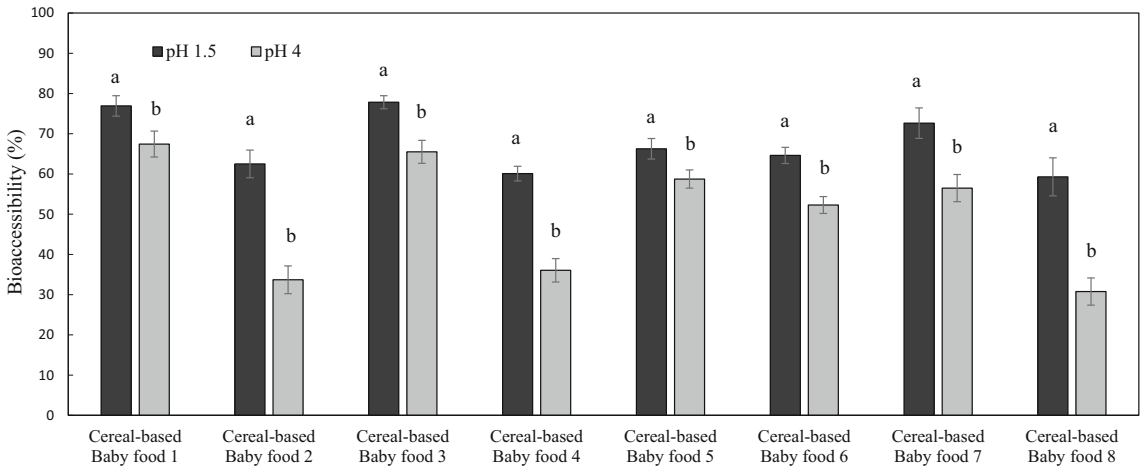

Fig. 2 Bioaccesibility of folic acid in cereal-based baby food formulated with milk powder(s). The bioaccesibility was determined dividing the value after digestion by the value obtained before digestion. The different letters atop columns for each sample indicate that there are statistical differences between the applications (ANOVA $p < 0.05$, Tukey's test)

all samples (infant formulas and cereal-based baby foods) in both gastric pH values, the highest decrease was observed at the gastric pH 4. As seen in Fig. 3, the bioaccessibility of folic acid in follow-on baby milk ranged between 42–67 and 38–57% at gastric pH 1.5 and pH 4, respectively. Comparing the decreases in both pH values, there is no significant difference between pH 1.5 and pH 4 regarding follow-on milk products.

This study examined the bioaccessibility of folic acid in infant formulas, cereal-based baby foods, and follow-on milks in an in vitro gastrointestinal system. In infant formulas, the bioaccessibility decreased to 62% on average at a gastric pH 1.5, while it decreased to 41% at a gastric pH 4. In cereal-based baby foods, the bioaccessibility decreased to 68% on average at a gastric pH 1.5, while it decreased to 41% at a gastric pH 4. In follow-on milks, the bioaccessibility decreased to 54% on average at a gastric pH 1.5, while it decreased to 47% at a gastric pH 4, and no significant decrease was found between these two decreases. Overall, when all samples were evaluated, the bioaccessibility decreased to 63% on average at a gastric pH 1.5, while it decreased to 47% at a gastric pH 4. Verwei et al. (2003) reported that when folic acid was added into ultra-high temperature (UHT) pasteurized milk, its bioaccessibility ranged between 58 and 61%, but when it was added instead of the natural form of 5-methyl-THF, its bioaccessibility was reported as 71%. In the same study, the bioaccessibility of folic acid decreased to 44–51% after the addition of FBP. As observed, the bioaccessibility of folic acid decreased by the FBP effect. The decrease in the in vitro bioaccessibility of folic acid in follow-on milk products included in our study was similar to those results.

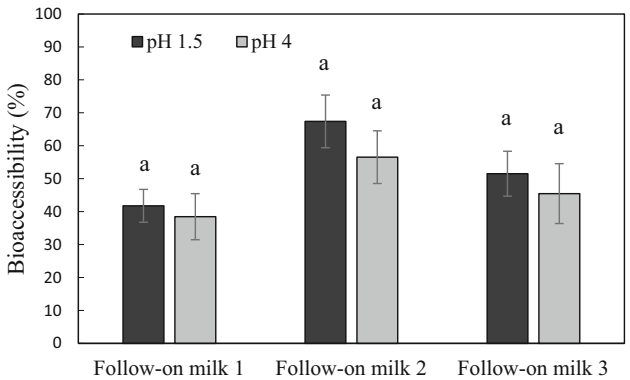

Fig. 3 Bioaccesibility of folic acid in milk products for babies. The bioaccesibility was determined dividing the value after digestion by the value obtained before digestion. The different letters atop columns for each sample indicate that there are statistical differences between the applications (ANOVA $p < 0.05$, Tukey's test)

It was reported that the amount of FBP ranged between 2280 and 4450 mg/kg in cow's milk powder (Swiatlo et al., 1990), and it was 168 ± 20 mg/kg in pasteurized cow's milk and 14 mg/kg or less in UHT milk (Wigertz et al., 1997). FBP can be bound to folates at a rate of 1:1 (Salter et al., 1981). In this study, infant formulas and cereal-based baby foods contain 10–15% milk powder. Moreover, it was reported that the binding capacity of FBP to folic acid is 100-fold higher than 5-methyl-THF (Nygren-Babol et al., 2005). Therefore, it is expected that folic acid will bind to FBPs. The bioaccessibility of folate in spinach ranged between 78 and 80% (Verwei, 2004). The same study indicated that the bioaccessibility of folic acid in milk products was lower than that in products which do not contain milk. As the bioaccessibility of folic acid was low in our study, the FBP in these products was strongly bound to folic acid. Before the gastric passage, folate and folic acid were bound to FBP at similar rates (76–79%), but during the gastric passage, folic acid remained bound to

FBP at a similar rate of 80–81%, while the 5-methyl-THF bound rate decreased from 79 to 5% (Verwei et al., 2004). This previous study indicated that both folic acid and 5-methyl-THF may still bind to FBP after gastric passage and FBP can affect the rate of absorption of both folate compounds from the small intestine. In particular, the digestive system is not fully developed in infants between 0 and 6 months of age, and the digestive enzymes, the concentration of these enzymes, and the gastric acidity of this age group of infants are very different than in adults (Bourlieu et al., 2014). Therefore, in vitro studies should take into account this situation. It was reported that the gastric pH of infants and adults ranged from 3.8 to 4.7 and from 1.5 to 1.8, respectively (Nguyen et al., 2015). FBP begins to be separated from folic acid when the gastric is less than 4.5 (Tani et al., 1983). Results from the present study suggest that at a gastric pH of 1.5, more FBP separates from folic acid and more free folic acid passes into the small intestine than it does at a higher gastric pH.

Pepsin is a protease that is responsible for the hydrolysis of proteins in the stomach. Since the gastric pH of infants is higher than that in adults, pepsin enzyme is less secreted, and the hydrolysis of proteins in the milk is expected to be less in infants than in adults (DiPalma et al., 1991). In addition, as FBP is a whey protein, it is resistant to hydrolysis and it cannot be fully hydrolyzed in infants. Therefore, our results indicate that FBP is less affected by gastric enzymes or acidity at pH 4. Hence, it is more stable when it passes through the small intestine.

In a study conducted on rats, under acidic conditions ($pH < 4.5$), folic acid was released from FBP in the stomach, and it is re-bound in the small intestine (pH 6–7) (Tani et al., 1983). The FBP in the stomach was not affected by the pepsin, and it was reactivated in the small intestine. In general, our results suggested that the bioaccessibility of folic acid was 63% on average at a gastric pH of 1.5, while it was 47% at a gastric acid pH of 4. As seen in our study, the bioaccessibility of folic acid was lower at gastric pH of 4. These data indicated that the gastric pH affects folic acid bioaccessibility. The low bioaccessibility of folic acid at gastric pH 4 than at 1.5 may be explained by the fact that FBP has a higher affinity to folic acid at higher gastric pH values. Therefore, under lower acidic gastric conditions, less folic acid is liberated from FBP.

Since yoghurt is a fermented product, its FBP amount is very low. It was stated that in the case of adding folic acid and folate to yoghurt, the bioaccessibility was at the same level for both (82%). However, the addition of FBP to yoghurt, the bioaccessibility of folic acid decreased to 34%, while the bioaccessibility of folate decreased to 57%. This study conducted with yoghurt, supports the idea that the presence of FBP has an effect on folic acid bioaccessibility (Arkbåge et al., 2003). Examining the literature about the products in our study, we observed a high amount of FBP particularly in infant formula and in cereal-based baby foods. The follow-on milks included in our study were formulated using UHT milk which is a treated milk at ultra high temperature. Despite the heat treatment, FBP remained stable in UHT milk (Wigertz et al., 1997). In another study, it was reported that FBP was affected by the heat treatment (Colman et al., 1981). In our study, the amount of folic acid in follow-on milks was low compared to other products. However, even a small amount of FBP in the milk may affect the bioaccessibility of folic acid.

Information on the bioavailability of folate or folic acid in fortified or non-fortified food products is required to determine whether the daily amount of folate in these products meets daily food requirements. Since clinical studies on infants are allowed to a limited extent, in vitro studies inform us whether we can take supplemental folic acid or other nutrients by consuming our daily foods. The bioaccessibility of folic acid in baby foods is lower at higher gastric pH. These results indicate that the bioaccessibility of folic acid is more likely to be lower for in vivo. Considering in vivo and in vitro studies, daily requirements of nutrients can be reviewed when planning a new formulation.

Acknowledgements We thank the İstanbul Sabahattin Zaim University for their support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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