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Bioaccessibility of folate in faba bean, oat, rye and wheat matrices

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ABSTRACT

Cereals and legumes are rich in folate. However, due to the instability of folate, processing and digestion can induce significant folate loss. In this paper, folate bioaccessibility of faba bean, oat, rye and wheat flours and pastes was studied using a static *in vitro* digestion model. Folate bioaccessibility depended on food matrices, varying from 42% to 67% in flours and from 40% to 123% in pastes. Digestion was associated with the interconversion of formyl folates, as well as the increase of oxidised vitamers and decrease of reduced vitamers. Especially in faba bean, 5-methyltetrahydrofolate showed surprisingly good stability both in digestion and heat treatment, resulting in high bioaccessibility. The physiological concentration of ascorbic acid did not stabilise folate in digestion; however, a higher level helped to maintain reduced vitamers. Heat treatment (10-min paste making) could improve folate bioaccessibility by liberating folate from the food matrices and by altering folate vitamer distribution.

1. Introduction

Folate describes a group of water-soluble vitamers that share a similar structure with pteroyl-L-glutamic acid (folic acid). It acts as a one-carbon donator, playing important roles in the methylation cycle and amino acid and nucleotide metabolism. In addition to preventing megaloblastic anaemia and neural tube defects, folate has also been associated with the development of cardiovascular diseases (Wiebe et al., 2018) and neurodevelopmental disorders (Lintas, 2019). Especially in countries where mandatory fortification is not practiced, such as EU countries, it is important to study natural folate sources.

Cereals and legumes are rich in folate (Saini et al., 2016). In Finland, cereal products account for 28% of the total dietary intake of folate for men and 23% for women (Valsta et al., 2018). At the same time, legumes have become popular in recent years. It is generally reported that 5-methyltetrahydrofolate (5-CH_3-H_4folate) and 5-formyltetrahydrofolate (5-HCO-H_4folate) are the main folate vitamers in cereals and legumes, and legumes usually contain more folate than cereals (Edelmann et al., 2013; Jha et al., 2015).

From a nutritional point of view, and due to the instability of folate, it is essential to study its fate during digestion, as food with high folate contents may have low bioavailable folate and vice versa (Seyoum & Selhub, 1998). Folate bioavailability varies considerably among different foods, as well as among different human studies, ranging from 10% to 98% (Saini et al., 2016). *In vivo* bioavailability has been being the gold standard to assess the availability of nutrients and bioactive compounds in food. However, since *in vivo* studies are expensive and time-consuming, bioaccessibility studies have been used to predict bioavailability and for sample screening for bioavailability study.

Folate bioaccessibility, usually demonstrated by *in vitro* digestion models, is defined as the proportion of folate present in the digesta before absorption in the small intestine (Etcheverry et al., 2012). Unlike bioavailability, bioaccessibility does not take the absorption of folate into account. At present, studies on the effect of food components on folate bioaccessibility often focus on added folic acid, whereas knowledge of bioaccessibility of endogenous folate in staple foods is also needed. In addition, only a few studies have investigated folate vitamers that undergo interconversions during digestion. Öhrvik et al. (2010) reported that around 80% of folate in breads was bioaccessible using a dynamic *in vitro* digestion model (TIM, TNO Gastro-Intestinal Model). Similarly, using the TIM system, Mo et al. (2013) reported 82% folate bioaccessibility for tofu and around 100% for tempe. A recent comprehensive folate bioaccessibility study was carried out by Ringling and Rychlik (2017) using a static *in vitro* model. They studied the folate bioaccessibility of three food matrices, where wheat germ had the lowest bioaccessibility, with around 30%. In addition, they assumed that tetrahydrofolate (H_4folate) had low bioavailability, and the bioavailability of 5-CH_3-H_4folate varied according to the food matrix. Their

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results supported the idea that stability affects folate bioaccessibility.

Recently, a group of researchers introduced a standardised static *in vitro* digestion method (Minekus et al., 2014). Use of this standardised approach would increase the reliability of the bioaccessibility results and decrease the variations among the results of different research groups. Using this method, Bationo et al. (2020) reported folate bioaccessibilities varying from 23% to 81% in seven cereal-based fermented foods from West Africa. However, the folate quantification method they used, as well as the complexity of the samples, made it difficult to explain the discrepancy of folate bioaccessibility among the samples. In addition, with this method, Hiolle et al. (2020) revealed that the release of folic acid in the gastric phase was faster from biscuit and sponge cake than it was from pudding and custard, indicating that food structures generated by different processing methods can affect the bioaccessibility of folate. Food density, texture and microstructure have been considered to influence the digestion of several nutrients, such as protein (Zahir et al., 2020) and starch (Blazek & Gilbert, 2010). However, studies on folate have been less common.

In this research, folate bioaccessibility of faba bean, oat, wheat and rye flours, as well as pastes made of them was studied, using the standardised static *in vitro* digestion method. Wheat was selected as a point of comparison, whereas oat and rye are commonly consumed in many parts of Europe. Interest towards legumes is increasing worldwide. Faba bean is an important source of plant-based protein and can be cultivated in boreal climate. The aims of this study were as follows: 1) to determine folate bioaccessibility in different food matrices and study the influence of *in vitro* digestion on individual folate vitamers; and 2) to investigate the effects of paste-making processing on folate stability and bioaccessibility of the studied food matrices. It was hypothesised that folate bioaccessibility differs among faba bean and cereals, and that heat treatment could improve folate bioaccessibility.

2. Materials and methods

2.1. Enzymes and calibrants

The enzymes and bile extract used were obtained from Sigma-Aldrich (St Louis, MO, USA), including: α-amylase from *Aspergillus oryzae* (A9857), protease (P8811), pepsin (P7125), bile from bovine and ovine (B8381), chymotrypsin (C4129) and trypsin (T0303). For the calibrants used for quantification, (6S)-Tetrahydrofolate (H_4folate, sodium salt), (6S)-5-methyltetrahydrofolate (5-CH_3-H_4folate, calcium salt), (6R,S)-5,10-methenyltetrahydrofolate hydrochloride (5,10-CH^+-H_4folate) and (6S)-5-formyltetrahydrofolate (5-HCO-H_4folate, sodium salt) were purchased from Eprova AG (Schaffhausen, Switzerland). 10-formylfolic acid (10-HCO-PGA) and folic acid (PGA) were purchased from Schirck's Laboratories (Jona, Switzerland). 10-formyldihydrofolate (10-HCO-H_2folate) was synthesized from 5,10-CH^+-H_4folate according to our previous publication (Kariluoto et al., 2004). In addition, the standards were dissolved, and their concentrations were confirmed by a spectroscopic method according to Kariluoto et al. (2004).

2.2. Sample preparation

Wholegrain flours (in 1-kg packages) were obtained from local markets, including rye flour (Helsinki Mills Ltd, Järvenpää, Finland), oat flour (Helsinki Mills Ltd), whole wheat flour (Myllyn Paras, Hyvinkää, Finland) and faba bean flour (Vihreä Härkä, Kalanti, Finland). The flours were stored at − 20 °C until further use. Two different paste samples (labelled A and B) were prepared from flours, and the ratio of flour to water was 1:7 (w/v). Paste A samples were prepared as follows: 2.5 g of flour was mixed with 17.5 mL of Milli-Q water in a 50-mL centrifuge tube. The tubes were flushed with nitrogen gas, closed, and placed in a boiling water bath exactly for 10 min. During the incubation, tubes were occasionally shaken using a vortex to avoid forming a clot. After the 10-minute incubation, the temperature of paste A samples was from 91 to 96 °C, measured by an electronic thermometer with a probe (Testo Ltd, Lenzkirch, Germany). To mimic a domestic cooking method, paste B samples were prepared in a beaker. In brief, 5 g of flour was mixed with 35 mL of Milli-Q water in a 100-mL beaker. Following this, the mixture was placed on a hot plate. On average after 2 min, the flour/water solution began to boil and was kept boiling for 10 min with constant stirring. The temperature was measured immediately after the boiling and paste B samples reached the final temperatures from 98 to 100 °C. Pastes A and B were both analysed for folate bioaccessibility right after they had cooled down to room temperature. In addition, the moisture contents of pastes and flours were analysed using the AACC 44-15A method (AACC, 2000) to report the data on a dry weight basis.

2.3. In vitro digestion

Simulated digestion of the flours and pastes was carried out in triplicate under subdued light using the static *in vitro* model described by Minekus et al. (2014) with modifications. Human salivary α-amylase and porcine pancreatic α-amylase were replaced by α-amylase from *Aspergillus oryzae*.

The activities of individual enzymes and the concentration of bile salt were determined according to Minekus et al. (2014). Briefly, the approach included three different phases, which were as follows: the oral phase (simulated salivary fluid, SSF), gastric phase (simulated gastric fluid, SGF) and intestinal phase (simulated intestinal fluid, SIF). First, 5 g of sample was mixed with 4 mL of SSF (pH 7) containing α-amylase in a 50-mL centrifuge tube. Following this, $CaCl_2$ was added, and the tube was filled with Milli-Q water to the volume of 10 mL (W/V = 5/5). The mixture was incubated at 37 °C for 2 min. Second, 8 mL of SGF (pH 3) with pepsin and $CaCl_2$ was added. The pH of the solution was adjusted to 3 before the volume was brought to 20 mL by Milli-Q water. The gastric digesta was incubated at 37 °C with constant shaking for 2 h. Finally, 10 mL of SIF (pH 7) with bile extract and 6 mL of SIF with α-amylase, as well as $CaCl_2$, chymotrypsin and trypsin were added. After the pH was adjusted to 7, the volume was brought to exact 40 mL by Milli-Q water, and the final mixture was incubated at 37 °C under constant shaking for 2 h. The digesta was obtained after centrifugation (10 000 rpm, 10 min) and stored at − 20 °C until folate analysis. A blank control (where the sample was replaced by 5 mL of Milli-Q water) was carried out in each batch of *in vitro* digestion.

As ascorbic acid is secreted in the human stomach and can affect folate stability (Ringling & Rychlik, 2017), the effect of ascorbic acid in the gastric phase on the stability of folate and its bioaccessibility were studied using faba bean, oat and rye flours. *In vitro* digestion with ascorbic acid was carried out in the same way as previously described, but ascorbic acid was included in the gastric phase. Two different concentration levels were applied, which were as follows: 0.1 μmol/mL (pharmacological concentration) and 100 μmol/mL (excessive concentration).

2.4. Extraction and purification of folate

The extraction was carried out via tri-enzyme treatment with α-amylase, hog kidney conjugase and protease under yellow light, as described previously (Edelmann et al., 2012). In brief, samples (1 g of flour, 2 g of paste) were extracted in triplicate with 15 mL of CHES/HEPES buffer (pH 7.85) containing 2% sodium ascorbate and 10 mM 2-mercaptoethanol in a boiling water bath for 10 min. Then, the pH was adjusted to 4.9, and α-amylase (20 mg) and hog kidney conjugase were added. The extract was subsequently incubated for 3 h at 37 °C, after which time, the pH was adjusted to 7 and protease (4 mg) was added. The extract was incubated for 1 h at 37 °C, and the enzymes were inactivated in a boiling water bath for 5 min. The supernatant was collected after centrifugation (12 000 rpm, 10 min) and filtrated through a 0.45 μm syringe filter. The extraction of digesta was carried out in duplicate in a similar way except for the exclusion of α-amylase

and protease. In short, 10 mL of digesta was mixed with 10 mL of extraction buffer and boiled for 10 min. The pH was then adjusted to 4.9, and the extract was incubated only with hog kidney conjugase for 3 h at 37 °C. The steps of enzyme inactivation and supernatant collection that followed were as previously described. Duplicate blank controls were carried out in each batch of extraction. The purification of folate extracts was carried out by affinity chromatography as described previously (Edelmann et al., 2012). Affinity agarose gel (Affi-Gel 10, Bio-Rad Laboratories, Richmond, CA, USA) coupled with folate-binding protein (Scripps Laboratories, San Diego, CA, USA) was used. Folates were eluted by 0.02 M trifluoracetic acid/0.01 M dithiothreitol into a 5 mL volumetric flask with 10 mg of ascorbic acid and 5 μL of 2-mercaptoethanol. The eluent was filtered through a 0.2-μm syringe filter, flushed with nitrogen, and stored at − 20 °C for no more than 7 days.

2.5. Quantification of folate

The determination of folate vitamers was conducted using a reversed-phase ultra-high performance liquid chromatography (UHPLC) method developed and validated by our laboratory (Edelmann et al., 2012). Vitamers were separated on the HSS T3 column (1.8 μm, 2.1 × 150 mm; Waters, Milford, MA, USA) at 30 °C. During the run, samples were stored in a dark autosampler at 4 °C, and the injection volume was 30 μL. The mobile phases were 30 mM potassium phosphate buffer (Eluent A, pH 2.2) and acetonitrile (Eluent B). Gradient elution (flow rate: 0.4 mL/min) conditions were as follows: 5% B at 0–2.16 min, 5–6.9% B at 2.16–4.71 min, 6.9–15.4% B at 4.71–7.47 min, 15.4% B at 7.47–7.87 min, and finally, to initial conditions from 15.4% to 5% B at 7.87–8.3 min, with reconditioning of the column to 5% B at 8.3–11 min.

Seven monoglutamate folate vitamers were determined and quantified using fluorescence (FL) and a photodiode array (PDA) detectors as follows: H_4folate (tetrahydrofolate) and 5-CH_3-H_4folate (5-methyltetrahydrofolate) were analysed using FL with an excitation wavelength of 290 nm and emission of 356 nm; 10-HCO-PGA (10-formylfolic acid) was analysed using FL, with excitation of 360 nm and emission of 460 nm; 10-HCO-H_2folate (10-formyldihydrofolate), PGA (folic acid) and 5-HCO-H_4folate (5-formyltetrahydrofolate) were analysed using PDA with 290 nm; and 5,10-CH^+-H_4folate (5,10-methenyltetrahydrofolate) was analysed using PDA with 360 nm. The details of the preparation and spectrophotometric purity determination of standard calibrants and their mixture run on UHPLC have been illustrated by Edelmann et al. (2012). The identification of folate vitamers was achieved by comparing the retention times and the ultraviolet (UV) spectra of the sample peaks to those of standard peaks. Quantification was based on calibration curves with peak areas plotted against concentrations. Especially in rye, 5-HCO-H_4folate and PGA peaks were often masked by unknown impurities, hindering the accurate quantification of these vitamers.

2.6. Calculation and statistical analysis

The R Studio platform was used to generate bar plots and analyse the differences among groups. Folate content is expressed as mean ± standard deviation (μg/100 g, $n = 3$) on dry matter (DM) basis. Total folate was expressed as the sum of folate vitamers (without conversion to folic acid equivalent). Two sample *t*-tests were applied to study the differences between folate contents before and after the *in vitro* digestion. In addition, one-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) *post hoc* test were selected for multi-group comparisons relating to folate contents. The formula used to calculate folate bioaccessibility was:

$$FB(\%) = 100 \times \frac{TFD(\mu g/100gDM)}{TF(\mu g/100gDM)}$$

where FB means folate bioaccessibility; TFD means total folate in flour or paste digesta; TF means total folate in flour or paste; DM means dry matter.

Theoretical folate contents in paste samples were calculated using the following equation:

$$TFP(\mu g/100gDM) = \frac{FWF(g) \times FF(\mu g/100gDM) \times \mathrm{DF}(\%)}{FP(g) \times DP(\%)}$$

where TFP means theoretical folate content in paste; FWF means fresh weight of flour; FF means folate contents in flour; DF means dry matter of flour; FP means fresh weight of paste; DP means dry matter of paste; DM means dry matter.

3. Results

3.1. Folate content and bioaccessibility in flours

Faba bean flour had the highest total folate content, with 142.0 ± 5.3 μg/100 g DM (Table 1), followed by rye (49.5 ± 3.0 μg/100 g DM), wheat (46.2 ± 1.0 μg/100 g DM) and oat flours (41.6 ± 2.3 μg/100 g DM). In flour digesta, significantly ($p < 0.05$) lower total folate contents were measured, which brought the folate bioaccessibility values of faba bean, oat, rye and wheat to 63%, 67%, 47% and 42%, respectively (Table 1).

Fig. 1 demonstrates the differences in folate vitamer contents and distributions between flour and digesta samples. 5-HCO-H_4folate was one of the main vitamers for all the flour samples, and *in vitro* digestion decreased its content in all matrices, although the result was not statistically significant in the case of oat. However, it remained one of the main vitamers in faba bean (27%), oat (27%) and rye (55%) flour digesta. The changes of 5,10-CH^+-H_4folate levels were like those of 5-HCO-H_4folate, but the contribution of 5,10-CH^+-H_4folate to the total folate contents was smaller in the digesta than it was in flour. 5-CH_3-H_4folate had a marked contribution to the total folate contents in oat (35%), rye (21%) and wheat (19%) flours, but it was not found in the flour digesta. Nevertheless, in faba bean, the contents of 5-CH_3-H_4folate accounted for around 16% of the total folate contents in both flour (24.6

Table 1
Total Folate Contents and Bioaccessibilities of Flours and Pastes.

Material		Total folate content (μg/100 g DM) Theoretical	Before digestion	After digestion	Folate bioaccessibility (%)
Faba bean	Flour	–	142.0 ± 5.3	96.1 ± 12.1 *	63 ± 10
	Paste A	142.0	134.0 ± 21.7	165.3 ± 18.0	123 ± 13
	Paste B	125.6	193.5 ± 35.6	231.7 ± 10.2	120 ± 5
Oat	Flour	–	41.6 ± 2.3	28.7 ± 4.0 **	67 ± 6
	Paste A	41.6	30.1 ± 3.6	23.5 ± 2.0	78 ± 7
	Paste B	32.9	37.1 ± 9.0	37.5 ± 21.1	101 ± 57
Rye	Flour	–	49.5 ± 3.0	22.6 ± 4.6 **	47 ± 8
	Paste A	49.5	34.6 ± 3.2	31.0 ± 5.1	90 ± 15
	Paste B	41.3	58.2 ± 4.6	31.2 ± 2.6 **	54 ± 5
Wheat	Flour	–	46.2 ± 1.0	20.7 ± 2.3 **	42 ± 4
	Paste A	46.2	37.1 ± 3.9	34.5 ± 6.0	93 ± 16
	Paste B	40.9	27.9 ± 8.7	11.1 ± 3.2 *	40 ± 12

Note: Values are expressed as mean ± standard deviation. For total folate contents before digestion, the standard deviations represent variation among three analytical replicates; for total folate contents after digestion and folate bioaccessibility, the standard deviations represent variation among triplicate digestions In addition, statistically significant differences between the folate contents before and after digestion are marked with * ($p < 0.05$) or ** ($p < 0.01$).

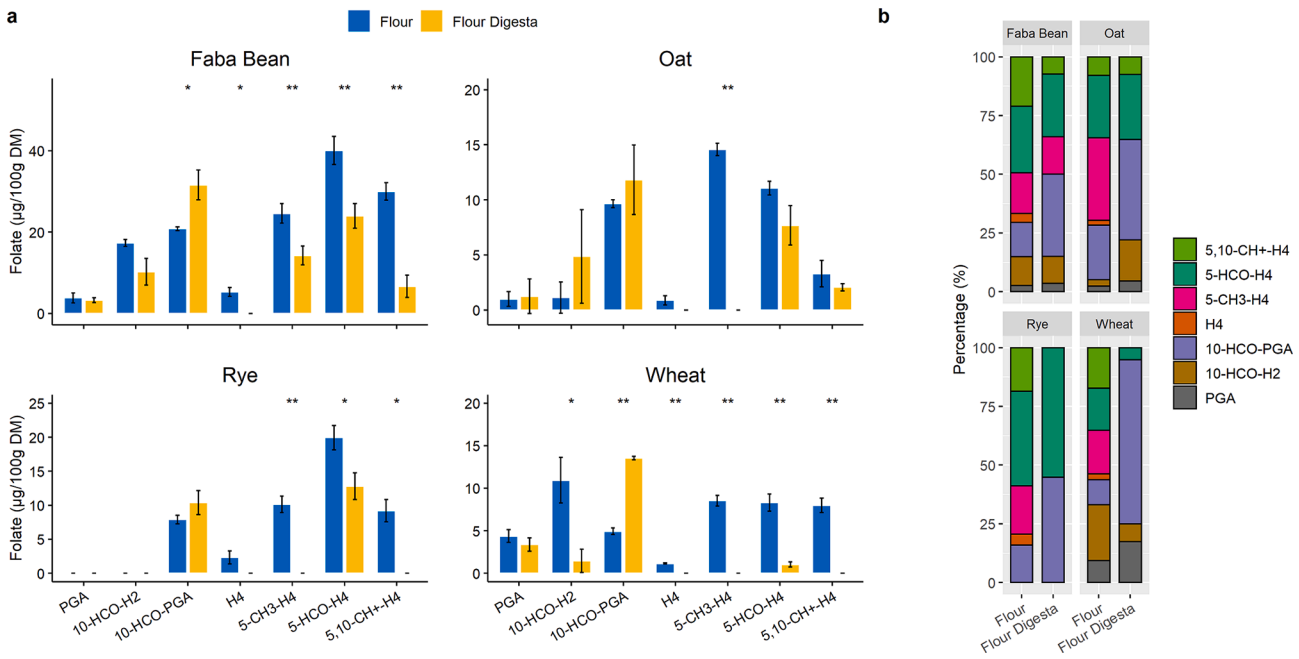

Fig. 1. a) Folate vitamer contents and b) folate vitamer distributions in the flour samples before and after digestion. The error bars of flour and flour digesta represent the standard deviation among triplicate analysis and among triplicate digestions, respectively. In addition, * or ** indicates a significant difference in vitamer contents before and after digestion at a level of $p < 0.05$ or $p < 0.01$, respectively.

± 1.9) and flour digesta (14.3 ± 2.8 μg/100 g DM), although its content decreased by 42% ($p < 0.05$) during digestion. By contrast, an increase of 10-HCO-PGA content was observed in all the flour digesta samples, especially in wheat (by 225%), increasing its contribution in all these samples. At the same time, a significant ($p < 0.05$) change of 10-HCO-H_2folate content was only seen in wheat, with an 85% decrease from flour to digesta. In addition, H_4folate had a small contribution to the total folate content but only in flour matrices.

3.2. Effect of ascorbic acid on folate in flour digesta

Compared with the regular *in vitro* digestion, the addition of ascorbic acid at the physiological level (0.1 μmol/mL) had only a limited effect on total folate contents in all flour digesta samples (Table 2). As for the individual vitamers, significant ($p < 0.05$) changes were only observed in oat. 5-CH_3-H_4folate was not detected in the regular oat digesta, but it was present in small amounts (1.0 ± 0.1 μg/100 g DM) in the oat digesta with added ascorbic acid. The content of 10-HCO-PGA was higher in regular oat digesta than it was in the digesta with added ascorbic acid.

Digesta samples with the addition of 100 μmol/mL ascorbic acid had higher total folate contents than those with either 0.1 μmol/mL or without ascorbic acid. Especially for oat and rye, the total folate contents in digesta were close to those in flours when excessive concentration of ascorbic acid was applied. Most of the individual vitamers in the original flour samples (before digestion) were detected in the respective digesta with the addition of 100 μmol/mL of ascorbic acid; the exception was 10-HCO-H_2folate in oat, which was found in oat flour but not in the digesta. H_4folate, 5-CH_3-H_4folate and 5,10-CH^+-H_4folate contents were notably higher in digesta with 100 μmol/mL ascorbic acid addition than in digesta without ascorbic acid or with the physiological level of ascorbic acid addition.

3.3. Folate content and bioaccessibility in pastes

3.3.1. Heat treatment in closed tubes (Paste A)

The highest total folate content in the Paste A samples was found in faba bean paste, at 134.0 ± 21.7 μg/100 g DM, while in wheat, rye and oat pastes, the total folate contents were around 35 μg/100 g DM (Table 1). No significant difference in total folate level was observed between the paste and paste digesta for any matrices. Faba bean paste digesta contained 165.3 ± 18.0 μg/100 g DM of total folate, resulting in folate bioaccessibility of 123%. Meanwhile, folate bioaccessibility levels from the wheat, rye and oat pastes were 93%, 90% and 78%, respectively (Table 1).

5-HCO-H_4folate was one of the most abundant vitamers both in all Paste A and paste digesta samples (Fig. 2). In addition, it was stable in cereal (oat, rye and wheat) pastes, in terms of both its content and contribution to the total folate. However, in faba bean paste, the content of 5-HCO-H_4folate decreased markedly (52%) during digestion, and its proportion to the total folate fell from 29% in the paste to 14% in the paste digesta. In contrast, 5-CH_3-H_4folate was stable in faba bean samples, whereas it could not be detected in cereal paste digesta, even though it had been the main vitamer in oat paste (37% contribution).

Considerably higher contents of 5,10-CH^+-H_4folate were measured in all the paste digesta samples than in pastes, accounting for higher contribution to total folate levels in paste digesta. 10-HCO-PGA was stable in cereal samples, while a significant increase of its content (58%) was seen in faba bean paste digesta, making it the second dominant vitamer (23%). Moreover, considerably higher amounts of PGA were measured in almost all the paste digesta compared with pastes. In rye samples, the PGA peak was unfortunately masked.

3.3.2. Heat treatment in open beakers (Paste B)

As displayed in Table 1, Paste B from faba bean contained the highest amount of folate, with 193.5 ± 35.6 μg/100 g DM, followed by rye (58.2 ± 4.6 μg/100 g DM), oat (37.1 ± 9.0 μg/100 g DM) and wheat (27.9 ± 8.7 μg/100 g DM) pastes. In faba bean paste digesta, the total folate level was higher than in the paste, bringing the folate bioaccessibility value to 120%. Folate bioaccessibility in oat paste was 101%, whereas significantly lower ($p < 0.05$) total folate contents were measured in rye and wheat paste digesta than in the respective pastes, resulting in folate bioaccessibility of 54% in rye and 40% in wheat.

Fig. 3 shows the individual vitamer contents and vitamer distributions in paste B and its digesta for each material. 5-HCO-H_4folate was the major (30%–65%) folate vitamer in all the paste samples; however, significantly lower contents ($p < 0.05$) were observed in faba bean (by

Table 2
Folate Contents in Flour and Flour Digesta with and without Ascorbic Acid Addition.

Material	Component	Folate content (μg/100 g DM) Digesta without ascorbic acid (regular)	Digesta with 0.1 μmol/mL ascorbic acid	Digesta with 100 μmol/mL ascorbic acid	Before digestion
Faba bean	PGA	5.5 ± 1.2c	5.1 ± 0.4c	2.7 ± 1.2b	1.9 ± 1.2 a
	10-HCO-H_2	14.6 ± 2.6c	12.9 ± 2.4c	18.1 ± 4.0b	14.5 ± 4.4 a
	10-HCO-PGA	29.3 ± 3.0b	28.2 ± 5.2b	22.2 ± 5.9b	17.5 ± 1.3 a
	H_4	–	–	1.3 ± 0.5b	4.9 ± 1.0 a
	5-CH_3-H_4	13.6 ± 2.7c	16.3 ± 2.3 bc	28.4 ± 7.3 a	23.0 ± 2.6 ab
	5-HCO-H_4	22.0 ± 2.6 bc	16.5 ± 1.9c	25.0 ± 4.5b	36.4 ± 2.1 a
	5,10-CH^+-H_4	5.9 ± 3.3c	5.6 ± 0.8c	14.4 ± 3.0b	28.2 ± 2.3 a
	Total	86.1 ± 13.5 bc	84.4 ± 7.0c	112.1 ± 24.4b	126.3 ± 8.3 a
Oat	PGA	2.6 ± 0.9 a	4.1 ± 0.5 a	4.0 ± 0.9 a	2.6 ± 0.7 a
	10-HCO-H_2	–	–	–	4.1 ± 1.3 a
	10-HCO-PGA	10.5 ± 2.3 a	7.2 ± 2.7 bc	9.7 ± 0.8 abc	7.8 ± 0.3 abc
	H_4	–	–	1.0 ± 0.1 a	0.8 ± 0.4 a
	5-CH_3-H_4	–	1.0 ± 0.1c	15.0 ± 1.1b	12.6 ± 0.5 a
	5-HCO-H_4	9.8 ± 2.3 a	8.7 ± 1.5 a	9.8 ± 1.0 a	9.9 ± 0.2 a
	5,10-CH^+-H_4	1.4 ± 0.5b	2.4 ± 0.5b	3.7 ± 0.8 a	2.9 ± 1.1 ab
	Total	23.6 ± 3.3b	24.1 ± 1.9b	42.8 ± 2.8 a	40.8 ± 2.4 a
Rye	PGA	–	–	–	–
	10-HCO-H_2	–	–	2.2 ± 0.8 a	1.9 ± 1.2 a
	10-HCO-PGA	9.1 ± 1.7b	8.3 ± 1.9 ab	6.5 ± 0.8 a	6.2 ± 0.6 ab
	H_4	–	–	1.0 ± 0.1b	1.9 ± 0.6 a
	5-CH_3-H_4	–	–	9.8 ± 0.7 a	8.7 ± 1.5 a
	5-HCO-H_4	14.2 ± 3.3 a	16.5 ± 3.4 a	18.7 ± 2.3 a	18.2 ± 1.3 a
	5,10-CH^+-H_4	0.2 ± 0.4c	1.3 ± 0.5c	6.5 ± 1.2b	8.2 ± 1.0 a
	Total	23.4 ± 5.0b	26.4 ± 4.0b	44.1 ± 2.1 a	44.9 ± 4.1 a

Note: Results are expressed as mean ± standard deviation. For folate contents before digestion, the standard deviations represent variation among three analytical replicates; for folate contents of digesta, the standard deviations represent variation among triplicate digestions. – not detected. Values within the same row with different letters differ significantly ($p < 0.05$).

31%) and rye (by 58%) paste digesta. Meanwhile, digestion significantly decreased the levels of 5-CH_3-H_4folate in cereal pastes, while in faba bean, this vitamer was stable, contributing to 16% and 12% of the total folate in paste and paste digesta. In addition, only in faba bean digesta, significantly higher amounts of 10-HCO-PGA (2-fold increase) and 10-HCO-H_2folate (increased by 48%) were detected compared with paste. In contrast, 10-HCO-H_2folate was one of the main vitamers (32%) in wheat paste, but it was not detected in wheat paste digesta.

3.4. Folate stability in heat treatments

According to Table 1, the effects of the heat treatments on total folate contents differed in the studied materials. Folate contents in all Paste A samples were somewhat lower than the folate contents in the respective original flours (before digestion), and a decrease by 30% was shown for rye paste. In Paste B samples, analysed total folate contents were generally in line with folate contents in flours for faba bean, oat and rye, whereas in Paste B from wheat, the folate content was considerably lower than that in wheat flour. Nevertheless, for all Paste B samples, except wheat paste, the analysed folate contents were higher than the theoretical total folate contents, where folate and moisture contents in flour and the loss of water during heat treatment were considered. Finally, paste B usually had higher levels of analysed total folate than paste A, with an exception for wheat, where lower folate level in paste B than in paste A was observed.

Fig. 4 provides a direct view of the effect of heat treatments on individual vitamers. Lower 5-CH_3-H_4folate contents were observed in oat, rye and wheat after heat treatments, while in faba bean samples, the contents remained similar. In addition, lower levels of 5,10-CH^+-H_4folate were found in all the matrices after the heat treatments. In contrast, higher levels of 5-HCO-H_4folate were detected in Paste B from faba bean and rye than in flour or Paste A. In addition, the content of 10-HCO-H_2folate in Paste B from faba bean increased substantially, by almost 200% compared with faba bean flour. As for 10-HCO-PGA, its content decreased in Pastes A and B from oat compared with that of oat flour, whereas Paste A from wheat had a higher content of 10-HCO-PGA compared with either Paste B or wheat flour. Finally, the contents of PGA in Paste B from faba bean and oat were higher than in flour or Paste A, while H_4folate was only present in flour samples.

4. Discussion

4.1. Folate bioaccessibility in flours and pastes

Folate bioaccessibility varied from 40% to 120% in all the studied food matrices and from 42% to 67% in flours. Little is known about the folate bioaccessibility of raw materials; however, Ringling and Rychlik (2017) reported around 30% folate bioaccessibility in wheat germ. Recent data on folate bioaccessibility or bioavailability in legumes is also scarce. Gregory (1989) summarised a generally high folate bioavailability in beans; however, the precision of the assays was low (0 – 181%). Mo et al. (2013) reported folate bioaccessibility of 81% and around 100% in soybean-based tofu and tempe, respectively.

Paste samples generally had better folate bioaccessibility than flour samples did. One plausible explanation for this is that folate was more easily liberated from the matrices after boiling, especially from faba bean. In contrast, the secondary structure formed during boiling could also have stabilised the folate. The main reaction during paste making is starch gelatinisation. A gelatinised structure could perhaps protect folate during acidic gastric digestion and release it in the intestinal phase, leading to higher folate bioaccessibility for paste samples. Bationo et al. (2020) studied the folate bioaccessibility of four different African foods made from pearl millet with various structures. They reported bioaccessibility values ranging from 24% to 81%, with the highest in batter fritters, suggesting that a dense structure could protect folate from degradation during digestion. In a recent study, different foods were produced with various structures but from the same ingredients (Hiolle et al., 2020). For some reason, the release of added folic acid in the gastric phase was faster from biscuit and sponge cake than it was from pudding and custard, although there were no differences in folic acid release among the studied foods at the end of digestion. Folic acid is the most stable folate vitamer, whereas for labile endogenous folate, a rapid release from food structure in the gastric phase could be detrimental.

Among cereals, oat exhibited better folate bioaccessibility than rye and wheat in flour and Paste B, but it was somewhat lower in Paste A. The composition of oat flour is quite different from that of rye or wheat flour, especially due to β-glucan. β-Glucan forms viscous solutions in

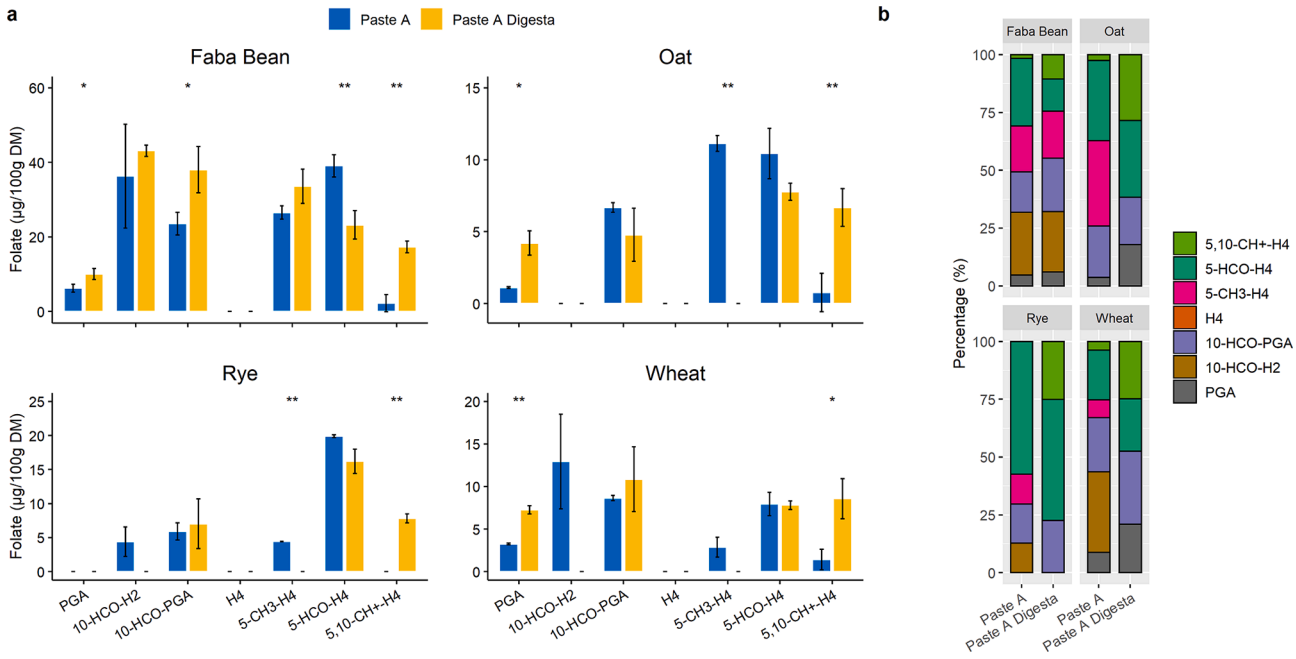

Fig. 2. a) Folate vitamer contents and b) folate vitamer distributions in Paste A samples before and after digestion. The error bars of Paste A and Paste A digesta represent the standard deviation among triplicate analysis and among triplicate digestions, respectively. In addition, * or ** indicates a significant difference in vitamer contents before and after digestion at a level of $p < 0.05$ or $p < 0.01$, respectively.

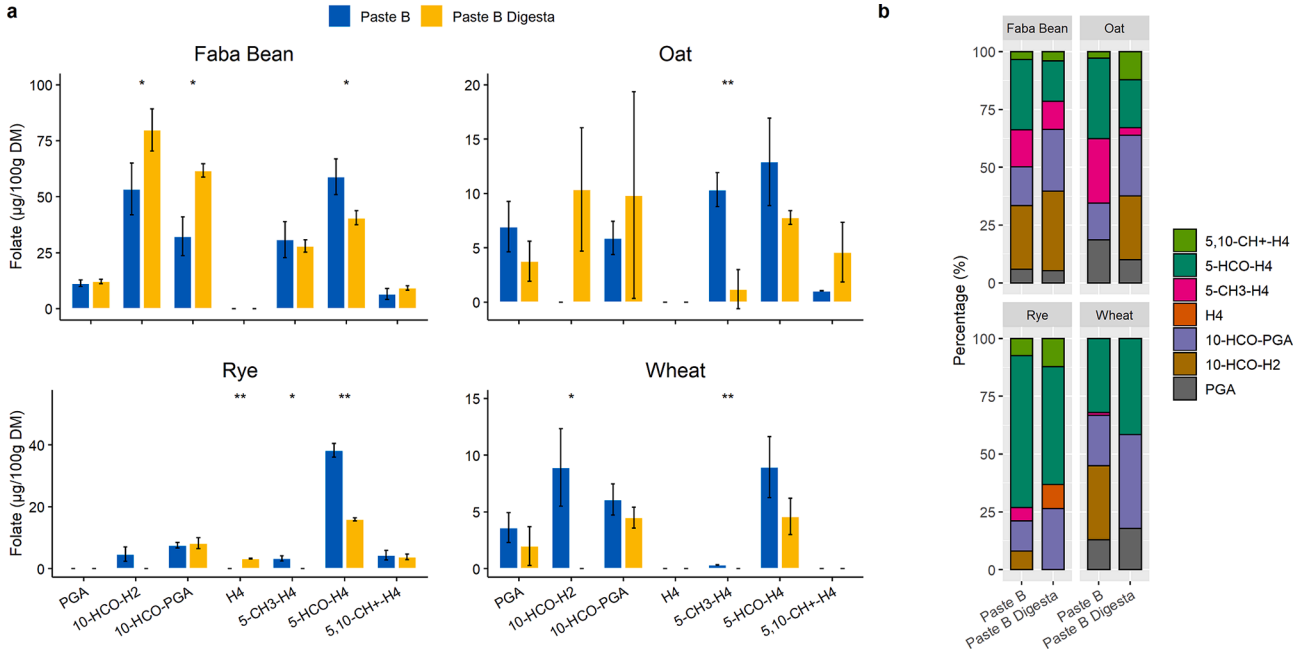

Fig. 3. a) Folate vitamer contents and b) folate vitamer distributions in Paste B samples before and after digestion. The error bars of Paste B and Paste B digesta represent the standard deviation among triplicate analysis and among triplicate digestions, respectively. In addition, * or ** indicates a significant difference in vitamer contents before and after digestion at a level of $p < 0.05$ or $p < 0.01$, respectively.

digestion and has been reported to be stable under gastric conditions of low pH (Kumar et al., 2013). β-Glucan may be able to interact with folate in the viscous structure, protecting it. However, the structure should be loose enough for folate to be liberated in the intestinal phase. In addition to changes in carbohydrates, heat treatment affects protein conformation, potentially releasing folate.

Faba bean exhibited high folate bioaccessibility, with values exceeding 100% in pastes. Theoretically, bioaccessibility should always be below or equal to 100%. *In vitro* digestion can be considered a folate extraction process with higher extraction volume, more enzymes and longer extraction time compared with the normal tri-enzyme extraction used in folate determination. Hence, the high folate bioaccessibility of faba bean pastes means that folate was efficiently liberated during the *in vitro* digestion. However, this indicates that the folate content in faba bean flour was underestimated, and consequently, the true bioaccessibility may be lower. Hefni et al. (2015) reported the total folate levels in faba bean flour ranging from 92 to 140 μg/100 g DM, which is in line with the results from this study (126–142 μg/100 g DM). The

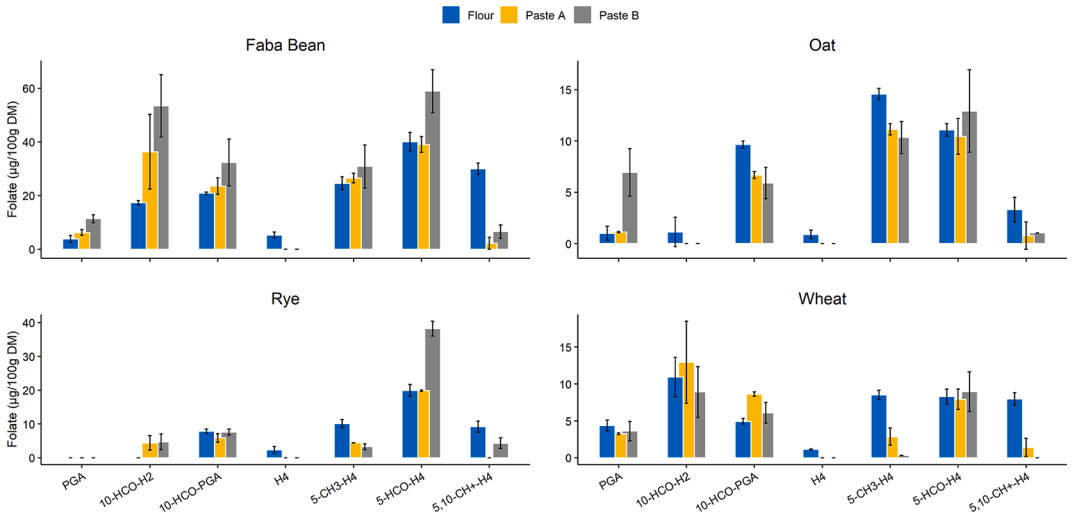

Fig. 4. Folate vitamer contents in a) faba bean, b) oat, c) rye and d) wheat flour, Paste A and Paste B samples. The error bars represent variation among three analytical replicates.

insufficient extraction of folate in faba bean flour seems to be a common problem, and a careful assessment should be made regarding the dietary value of folate in faba bean. In addition, an alternative, or more effective method for folate extraction in legumes should be developed. Nevertheless, differences between folate bioaccessibility in faba bean and cereals could be attributed to different seed structures. Folate in legume seeds is mostly located in cotyledons, which represents most of the total seed mass (Coffigniez et al., 2019), whereas in cereals, folate is concentrated in the bran and germ (Edelmann et al., 2013). Folate in legumes may be more easily released from the starch and protein-rich cotyledons than from the bran or germ structure during the *in vitro* digestion. Faba bean is protein-rich, and high protein concentration could result in a high buffer capacity (Mennah-Govela et al., 2019), creating a relatively stable pH environment that could stabilise folate. The high buffer capacity of faba bean samples was also noticed during the experiments, as more NaOH or HCl was needed during the pH adjustment for faba bean than for cereals.

Information on the individual vitamers suggested that folate in faba bean was more stable than it was in cereals. For example, 5-CH_3-H_4folate was stable—or alternatively, better liberated—in faba bean samples during the *in vitro* digestion, especially in pastes. In all the cereal digesta samples, the content of this vitamer decreased significantly. The relatively good stability of folate in faba bean could be explained by the high antioxidant capacity, possibly mainly from phenolic compounds (Lafarga et al., 2019), which could protect folate from oxidation during *in vitro* digestion. However, similar or even higher capacities have also been reported for cereals (Luo et al., 2015).

4.2. Folate vitamer interconversion and degradation induced by in vitro digestion

Two major trends in the changes of folate vitamers due to *in vitro* digestion could be identified for all matrices; these were interconversions of formyl folates and decrease of reduced vitamers (mainly H_4folate and 5-CH_3-H_4folate). Generally, the interconversion among formyl folates was characterised by the decrease of reduced and intermediate vitamers (5–HCO–H_4folate and 5,10-CH^+-H_4folate) and the increase of oxidised vitamers (10–HCO–H_2folate and 10-HCO-PGA). One exception was 5,10-CH^+-H_4folate in Paste A, where an increased level of this vitamer was observed in paste digesta. Formation of oxidised vitamers during heat treatment could partly explain the generally higher folate bioaccessibility in pastes compared with flours, as these vitamers are more stable, and thus, likely to survive digestion. It is noteworthy that the standard deviations of these vitamers were high. One explanation for this is that variation tends to increase with smaller concentrations. Nevertheless, the fluctuation could also indicate that these vitamers were unstable during the digestion and analysis, and minor changes may have caused interconversion among them. The stability of formyl folates has been thoroughly reviewed by other scholars (Jägerstad & Jastrebova, 2013), who summarised that the reactions are mainly pH rather than temperature driven. 5-HCO-H_4folate is stable in a neutral environment and will convert to 5,10-CH^+-H_4folate under an acidic pH. Therefore, these changes could have been caused by pH changes during the digestion. In addition, one study indicated that iron compounds can catalyse the oxidation of 10-HCO-H_4folate to 10-HCO-H_2folate (Baggott et al., 1998), and 10–HCO–H_4folate could be obtained from conversion of 5,10-CH^+-H_4folate under neutral pH (Jägerstad & Jastrebova, 2013). In our analytical system, 10-HCO-H_4folate could not be determined as such, but it was converted mainly to 10-HCO-PGA. Since the flours we studied are whole grain flours with relatively high mineral contents, iron could have promoted interconversions among formyl folates during *in vitro* digestion. In addition, it is worth mentioning that digestion also resulted in an increase in another oxidised folate, PGA. However, in Paste B, where the intensive heat treatment was applied, this vitamer may have suffered from further degradation, and its content was smaller after digestion.

Among the reduced folate vitamers, H_4folate was almost completely lost during digestion due to its inherent instability. This finding agrees well with the literature showing that H_4folate is unstable even at 37 °C, especially under acidic conditions (De Brouwer et al., 2007). Similarly, 5-CH_3-H_4folate degraded during digestion in all cereal samples. Even under mild conditions, 5-CH_3-H_4folate is readily oxidised to 5-CH_3-5,6-H_2folate, which can be reduced back to 5-CH_3-H_4folate. However, in acidic media, 5-CH_3-5,6-H_2folate may degrade further (Lucock et al., 1995). Since ascorbic acid/ascorbate and other antioxidants are commonly used in folate analysis, it is difficult to estimate the proportion of 5–CH_3–5,6–H_2folate naturally present in foods. Surprisingly, 5-CH_3-H_4folate showed great stability (or better liberation) in the digestion of faba bean pastes, and in flour digesta, a considerable concentration of this vitamer was still left. The reason for this unexpectedly good stability remained unclear and warrants further study; however, it may somehow be related to the antioxidant capacity of faba bean.

Especially in rye sample chromatograms, extra peaks often interfered with 5-HCO-H_4folate and PGA. Under more severe conditions, the oxidative degradation of 5-CH_3-H_4folate produces p-aminobenzoylglutamate (pABG), as well as 4α-hydroxy-5-

methyltetrahydrofolate (MeFox). MeFox has been reported to be abundant in cereal grains (Shahid et al., 2020) and to disturb the quantification of 5-HCO-H_4folate, as well as PGA, because of similar retention (Fazili & Pfeiffer, 2013). However, the coupling of stable isotope dilution assay and mass spectrum technology has been used to address these issues by other groups (Ringling & Rychlik, 2017; Shahid et al., 2020).

Since ascorbic acid is secreted into the stomach (Sobala et al., 1991), we explored the effect of inclusion of ascorbic acid on the folate bioaccessibility of faba bean, oat and rye flours. When the physiological amount of ascorbic acid was added, no significant changes in folate content were oberserved inthe studied samples, and thus, bioaccessibility. However, when an excessive amount (1000 times the physiological amount) of ascorbic acid was added, more folate vitamers, especially 5-CH_3-H_4folate, were retained. This could indicate that the 5-CH_3-5,6-H_2folate naturally present in the studied flours was efficiently reduced to 5-CH_3-H_4folate, as well as that 5-CH_3-H_4folate was stabilised by ascorbic acid. Ringling and Rychlik (2017) included ascorbic acid in their *in vitro* model and found that the influence of ascorbic acid depended on the food matrix. They observed a 94% loss of 5–CH_3–H_4folate in wheat germ even with added ascorbic acid and only a small difference in folate bioaccessibility. This observation is consistent with the results of cereal samples from our study. Another interesting phenomenon was the absence of 10-HCO-H_2folate in the oat flour digesta with 100 μmol/mL ascorbic acid as this vitamer was present in other matrices. 10-HCO-H_2folate could have been converted to 5,10-CH + -H_4folate under acidic environment (Jägerstad & Jastrebova, 2013), resulting in an increase of 5,10-CH + -H_4folate. In addition, 10-HCO-H_2folate can be oxidised to 10-HCO-PGA. The relatively good stability of 10-HCO-H_2folate in faba bean and rye samples could be due to their rich formyl folate pool (Table 2) or the endogenous antioxidants. As the addition of ascorbic acid in a physiological concentration had little effect on the results, we decided to exclude ascorbic acid in our model. Nevertheless, the results stress the importance of folate stability in the context of folate bioaccessibility, and foods with high antioxidant capacity could result in high folate bioaccessibility.

4.3. Changes in folate content and vitamer distribution induced by heat treatments

The evaluation of the effect of heat treatment on folate content and the comparison between the treatments was complicated by the greater than 100% calculated retention in most of the Paste B samples. As expected, the analysed total folate contents in Paste A samples were lower compared with the theoretical values, confirming that the processing led to folate losses (or perhaps that folate could not be liberated from the studied flour matrices). However, in Paste B samples, where a more severe heat treatment was applied, the analysed values were higher than the corresponding theoretical values except for wheat. Especially in faba bean and rye, folate was more easily liberated from Paste B than it was from the corresponding flour, presumably due to the structural changes discussed above. In addition, the heat treatment might improve the liberation of antioxidants as well, which could protect folate in these matrices (Ng & Tan, 2017). In contrast, processing of Paste B from wheat may have been excessive, to the point where the liberation of folate could no longer compensate for the loss. This was further confirmed by the bioaccessibility data for Paste B from wheat, where an even lower folate level was observed for the digesta.

The effect of heat treatments on folate in different foods has been studied by several research groups. A 50-min cooking time did not significantly affect the folate content in pearl millet paste (Bationo et al., 2019). Delchier et al. (2012) reported that folate lost in green beans during boiling could be found in processing media and suggested that leaching was also responsible for the folate loss during these treatments. In addition, an article reported no loss of folate in faba bean after industrial blanching (Hefni & Witthöft, 2014). In rye and wheat baking, approximately 25% folate losses have been reported (Kariluoto et al., 2004).

The information about individual folate vitamers could help us understand the effect of heat treatments in more detail. In most samples, the contents of oxidised vitamers (PGA, 10–HCO–H_2folate and 10-HCO-PGA) increased, while the contents of reduced vitamers (H_4folate, 5-CH_3-H_4folate and 5,10-CH^+-H_4folate) decreased. It is interesting to note that 5–CH_3-H_4folate in faba bean seemed to be highly stable compared with that in the cereal samples during the thermal processing. 5,10-CH^+-H_4folate, which is the intermediate form of 5–HCO–H_4folate, was reported to convert back to 5-HCO-H_4folate under heating at neutral pH (De Brouwer et al., 2007). This could explain the increase of 5-HCO-H_4folate and decrease of 5,10-CH^+-H_4folate observed in paste samples. The increase of 5-HCO-H_4folate contents in Paste B from faba bean and rye could also be due to the liberation from the food matrices during the paste making.

Motta et al. (2017) reported that boiled or steamed quinoa contained more 5-CH_3-H_4folate than raw quinoa did, while no losses were found in buckwheat; in amaranth, the folate content decreased. Similarly, seemingly contradictory results where heat treatments have led to no folate loss or even an increase in folate content have been reported for broccoli (Stea et al., 2007), green beans (Delchier et al., 2012) and lentils (Zhang et al., 2019). This again indicates that food matrices influence folate liberation and stability.

5. Conclusion

This study provided new information about folate bioaccessibility in legume and cereal matrices. Faba bean is a promising source of dietary folate due to its high folate content and better folate bioaccessibility than the levels observed in cereals. In addition, 5–methyltetrahydrofolate in faba bean showed exceptionally good stability in both digestion and heat treatment, which warrants further study. However, other characteristics, such as sensory quality, might hinder the popularity of faba bean among consumers. The physiological concentration of ascorbic acid was not able to stabilise folate during digestion; however, the better retention of reduced vitamers at a higher level of ascorbic acid addition emphasises the great importance of folate stability on bioaccessibility. Our results indicated that the structure formed by the paste-making process can protect folate from oxidation during *in vitro* digestion, enhance folate extractability, and thus, result in better folate bioaccessibility from paste than from the respective flour.

The prediction of folate bioaccessibility is complex. In addition to inherent vitamer distribution in the raw material, processing may improve bioaccessibility by changing folate vitamer distribution towards more stable vitamers, destroying folate-binding structures or forming secondary structures protecting folate. Therefore, further studies about the effect of food macrocomponent structure on folate bioaccessibility are needed. The role of antioxidants should also be elucidated. Finally, from a nutritional point of view, in addition to the determination of folate contents, bioaccessibility and bioavailability of common foods should gain more attention in the future.

CRediT authorship contribution statement

Fengyuan Liu: Conceptualization, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Susanna Kariluoto:** Conceptualization, Writing - review & editing, Supervision. **Minnamari Edelmann:** Writing - review & editing, Supervision. **Vieno Piironen:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129259.

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