Influence of storage conditions and packaging of fortified wheat flour on microbial load and stability of folate and vitamin B12

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ABSTRACT

Flour fortification with folic acid (FA) is implemented in many countries, and the fortification of flour with vitamin B12 has been planned. However, vitamins losses can occur during storage. In this study, fortified wheat flour was packaged either in paper bags or multilayer aluminum/PET bags, and stored in controlled conditions of temperature (25 °C or 40 °C) and relative humidity (65% or 85% RH) for 6 months. FA content, cyanocobalamin content, and microbial quality were regularly assessed. In flours packed in multilayer bags (non-permeable to oxygen and humidity), no significant FA and cyanocobalamin losses were observed, irrespective of temperature and RH. In flours packed in permeable paper bags, the microbial quality deteriorated in flours stored at 85% RH, FA loss reached 22–53% after 6 months at 85% RH, whereas cyanocobalamin loss reached 49–63% after 6 months at 65% RH. This shows that, depending on environmental conditions, packaging choice is of critical importance.

1. Introduction

Micronutrient deficiencies, and the resulting negative health consequences of such deficiencies, affect over an estimated 2 billion people worldwide (Bailey & Black, 2015). Folate is the generic name for vitamin B9, which includes a number of naturally occurring compounds (natural folates) derived from tetrahydrofolate. The term folic acid specifically refers to the fully oxidized monoglutamate form of the vitamin that is used in supplements and fortified foods, and rarely occurs in nature. Vitamin B12 (cobalamin) is synthesized only by certain microorganisms, and the main source of the vitamin is food of animal origin (FAO/WHO, 2002). Deficiency of folate and cobalamin are global health concerns. Although data are scarce, it is estimated that over 20% of women of reproductive age living in low- and middle income countries are folate deficient (Rogers et al., 2018), and the systematic review carried out by Sukumar et al. (2016) showed that the worldwide prevalence of vitamin B12 insufficiency during pregnancy was of 19–29%, with higher rates for the Indian subcontinent and the Eastern Mediterranean.

Folate and vitamin B12 are both necessary for the synthesis of DNA, RNA, and are necessary cofactors for the conversion of homocysteine to methionine, which is required for the synthesis of neurotransmitters and phospholipids. Folate and vitamin B12 deficiencies lead to elevated plasma homocysteine concentrations, which are associated with a greater risk of adverse pregnancy outcomes (still-births, preeclampsia, very low birth weight, preterm delivery, and neural tube defects) (Bergen et al., 2012; Mujawar, Patil, & Daver, 2011; Rogers et al., 2018). In addition, folate and vitamin B12 status is related to megaloblastic anemia, and also with cognitive impairment in the elderly (Morris, Jacques, Rosenberg, & Selhub, 2007). As folate and vitamin B12 have been proven to be crucial for normal embryogenesis, more attention is being paid to the folate and vitamin B12 status in young women during the periconceptional period and pregnancy. Moreover, concern about inadequate vitamin B12 intake is increasing, as vegans, vegetarians, elderly with impaired absorption, and people in countries with low intake of animal products – either for religious reasons or

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because of low income – are at risk of vitamin B12 deficiency (Allen, 2009; Pawlak, Lester, & Babatunde, 2014).

Food fortification allows reaching large segments of at-risk populations without requiring major changes in existing consumption patterns (Osendarp et al., 2018). Mandatory fortification of wheat flour with folic acid was introduced in North America in the late 1990s and in many other countries in the 2000s, which led to reductions in the prevalence of neural tube defects (NTDs) and improvements in folate status (Ray et al., 2002; Sayed, Bourne, Pattinson, Nixon, & Henderson, 2008). However, as the risk of NTDs attributable to vitamin B-12 deficiency increased during the same period (Ray et al., 2007), the question of the fortification of flour with vitamin B12 has been raised (Allen, Rosenberg, Oakley, & Omenn, 2010).

The stability of the micronutrient in the food carrier is considered one of the key factors toward the success (or failure) of a fortification program, as fortified flours may undergo physico-chemical changes during storage and processing. Factors that can typically affect micronutrient stability include uncontrolled conditions during storage and transport, long storage times, composition of the micronutrient premix, and interaction between components of the premix (Allen, De Benoist, Dary, & Hurrell, 2006). Studies carried out in the 1970s on the folic acid retention in fortified cereal-based products during storage and baking suggested that there was little loss (Berry, Bailey, Mulinare, Bower, & Dary, 2010), but the packaging and storage conditions were not always fully described. Recently, Phillips, Pike, Eggett, and Dunn (2017) did not report any significant loss of added folic acid in corn masa flour for up to six month of storage, but the flour was well-packaged and stored at 22 °C and 65% relative humidity. However, the influence of packaging on the stability of fortified flours stored in 'tropical conditions' (*i.e.* high temperature and high relative humidity) has not been investigated yet. Indeed, in many low and middle-income countries, fortified food products are likely to be exposed to severe and non-controlled environmental conditions, such as high temperature (> 40 °C during the dry season in Sahelian countries) or high humidity (> 85% RH in tropical countries), which may lead to vitamin losses during storage. Besides, to the best of our knowledge, the stability of vitamin B12 in fortified wheat flours has not been studied yet.

The aim of our work was to simultaneously evaluate the influence of various factors on the stability of vitamins B9 and B12, in wheat flour fortified with multi-micronutrient (zinc, iron, and vitamins A, B9, B12). The fast degradation of vitamin A and the changes in oxidative status have already been reported (Hemery et al., 2018). In the present work we assessed the combined impact of several factors on the sanitary quality – water activity and microbial quality – and the retention of vitamin B9 and vitamin B12 in wheat flours: i) storage length, ii) type of packaging (oxygen- and moisture-permeable or not), iii) storage temperature, and iv) relative humidity within the storage environment.

2. Materials and methods

2.1. Flour fortification and experimental set-up

The white flour (T55 wheat flour) was milled by Grands Moulins de Dakar (Senegal), then shipped to Montpellier (France), and fortified as reported by Hemery et al. (2018), using a Turbula® T10 Shaker-Mixer (Glen Mills Inc., Clifton, NJ, USA), in order to meet the WHO fortification standards for an average consumption range of 150–300 g of wheat flour per day: 1.3 µg/g folic acid and 0.01 µg/g cyanocobalamin (WHO, 2009). For each fortification batch, 3.21 g of micronutrient premix were added to 6 kg of flour.

Micronutrient premixes supplied by a GAIN premix certified producer were used. As described by Hemery et al. (2018), the premix 1 contained ~20.5 µg/g spray dried cyanocobalamin and ~2.6 mg/g anhydrous folic acid, but also dry vitamin A palmitate, zinc oxide, and ferrous sulphate monohydrate, whereas premix 2 displayed a comparable composition, with the exception that it did not contain ferrous
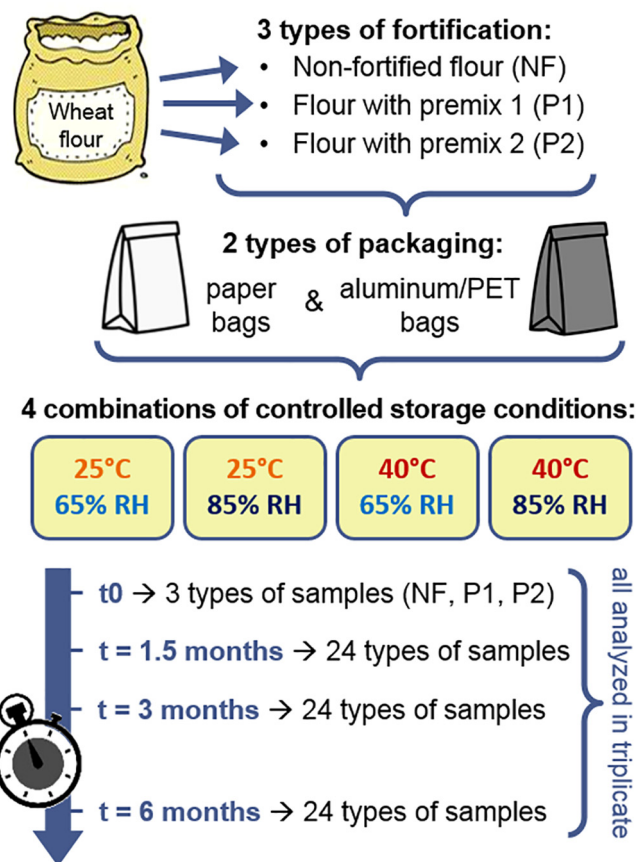

Fig. 1. Study design: fortified and non-fortified wheat flours were packaged and stored under different conditions for up to 6 months. Three replicates were performed for each sampling time and condition.

sulfate, or any other added iron source. The micronutrient levels of premixes were checked before fortification, using the hereafter described methods.

After fortification, three products were obtained: non-fortified flour (NF), 'flour fortified with premix 1' (P1), and 'flour fortified with premix 2' (P2). Those products were sampled in 100 g bags, using two different packaging types: either multi-laminar PET/aluminum/polyethylene bags (12 µm/8 µm/80 µm, non-permeable to oxygen and humidity), or paper bags (permeable to oxygen and humidity) as used in retail markets for the selling of loose flour. As shown in Fig. 1, sealed flour bags were stored in incubators at controlled temperature (at either 25 °C or 40 °C), as described by Hemery et al. (2018).

Relative humidity (RH) conditions were maintained constant by using saturated saline solutions: $NaNO_2$ was used to maintain RH at 62 ± 5%, and KCl to maintain RH at 85 ± 5%. To recreate several climatic conditions comparable to Mediterranean or tropical conditions (during dry and rainy season), 4 different combinations of temperature and RH were used: 25 °C/65% RH, 25 °C/85% RH, 40 °C/65% RH, and 40 °C/85% RH. The RH and temperature were continuously recorded during the 6-month storage, by using data loggers equipped with RH and temperature sensors (EasyLog EL-USB-2, Lascar Electronics, Salisbury, UK). After 1.5 months, 3 months and 6 months of storage, the biochemical composition and microbial quality of NF, P1 and P2 flours (packed in 2 different types of bags, and stored in 4 different conditions) was characterized. At each time, 3 new bags were taken from each treatment, and then discarded after analyses.

2.2. Measurement of water activity

Water activity (a_w) was also characterized after 1.5, 3 and 6 months

of storage. Water activity was measured in triplicates, using an AquaLab CX2 instrument (Decagon Devices Inc., Pullman, USA) at room temperature (18–21 °C).

2.3. Quantification of folate and folic acid

For non-fortified flours, only the total folate content was measured, which corresponds to the "natural folate" content. For fortified flours P1 and P2, both the total folate and the folic acid (FA) contents were measured, and then the "natural folate" content was calculated as follow: natural folate = total folate – FA. All folate values are averages of 3 replicates samples (1 replicate per bag, 3 bags from each treatment).

The total folate content of flours was determined using the microbiological assay described by Kariluoto and Piironen (2009), modified as described by Saubade, Hemery, Rochette, Guyot, and Humblot (2018), using 96-well microtiter plates, with Lactobacillus rhamnosus ATCC 7469 as the growth indicator organism, folic acid (F7876, Sigma-Aldrich, St Louis, MI, USA) as calibrant, and Folic Acid Casei Medium (BD Difco, Sparks, MD, USA) as assay medium. The performance of the method was confirmed by analyzing a certified reference material (BCR 121 Wholemeal flour, IRMM, Geel, Belgium). A blank sample and the reference sample were analyzed in each set of samples.

To measure the folic acid content of fortified samples, the extraction was modified to extract only the added synthetic form of vitamin B9, which was already soluble without the need of an enzymatic extraction. In centrifuge tubes, 15 mL of extraction buffer (50 mM Ches/50 mM Hepes buffer, 10 mM 2-mercaptoethanol, 2% (w/v) sodium ascorbate, pH 7.85) were added to 0.5 g of flour, the tubes were flushed with nitrogen, thoroughly vortexed, and incubated for 10 min at 37 °C in a shaking water bath, in the dark. At the end of incubation, pH was adjusted to 6.1, volume was brought to exactly 50 mL with 0.5% sodium ascorbate (pH 6.1), and the tubes were centrifuged at 20,000 g for 30 min at 4 °C. Supernatants were then diluted by 40 and 80, and folic acid concentration was determined using the microbiological assay described by Kariluoto and Piironen (2009). An enzyme immunoassay kit designed for the determination of folic acid in food (FOL-E01, Immunolab GmbH, Kassel, Germany) was also used on a small set of samples, to confirm the folic acid values obtained with the microbiological method.

2.4. Quantification of vitamin B12 (cyanocobalamin)

Cyanocobalamin was quantified in fortified flours (P1 and P2) using an ELISA method designed for the quantitative determination of vitamin B12 in food products, based on methods described by Reichert and Rubach (1990) and Selva Kumar and Thakur (2011) (test kit ref. CO1702010, Libios, Bully, France). Three grams of flours were dissolved in 10 mL of sample diluent (provided in the kit), and vortexed for 10 s. The pH was then adjusted to 6.5, and potential turbid matter was precipitated by adding 0.5 mL Carrez I reagent (150 g/L potassium *hexa*-cyanoferrate(II)-3-hydrate) and 0.5 mL Carrez II reagent (300 g/L zinc sulfate-7-hydrate). The extract was centrifuged at 13,000*g* for 15 min at 4 °C, and 50 μL of supernatant was pipeted in duplicate into the appropriate wells of the microtiter plate, and the immunoassay was performed as recommended by the test kit supplier. Values are averages of 3 replicate samples.

2.5. Microbiological analyses

For microbial counting, 1 g of flour was diluted in 9 mL of sterile solution of NaCl (0.9%), then decimal dilutions were performed in the same way. Appropriate dilutions (1 mL) were plated in duplicate, using direct plating methods. Counts of aerobic mesophilic bacteria were done by the pour plate method, on plate count agar (PCA medium, BD Difco, Sparks, MD, USA), followed by incubation at 30 ± 1 °C for 72 h

(ISO 4833. , 2013). Enumeration of yeasts and molds was done by the pour plate method, using Sabouraud agar with chloramphenicol (Sabouraud Dextrose Agar, BD Difco, Sparks, MD, USA), followed by incubation at 25 ± 1 °C for 3–5 days (ISO 7954, 1987). Microbial counts were calculated as colony forming units (CFU)/g flour. As no international standards on the microbial quality of flours were available (except for pathogenic bacteria), the total aerobic mesophilic bacteria count (TAMBC) and the total molds and yeasts count (TMYC) were chosen as indicators of food safety, and the thresholds given by the Circular 5788 of the French National Association of Millers (ANMF, 2012) were used. Thresholds for total aerobic mesophilic bacteria count (TAMBC): acceptable if below 20,000 CFU/g, marginal if between 20,000 and 200,000 CFU/g, excessive if above 200,000 CFU/g. Thresholds for total molds and yeasts count (TMYC): acceptable if below 1000 CFU/g, marginal if between 1000 and 10,000 CFU/g, excessive if above 10,000 CFU/g.

2.6. Statistical analyses

In order to identify differences in composition among the various samples during the storage, one-way analyses of variance (ANOVA) followed by Newman-Keuls tests with a 95% confidence interval were carried out, and differences were declared as statistically significant for p-values < 0.05. Linear correlation tests (Pearson's correlation method) were also carried out, with a 95% confidence interval, and the correlation coefficients (R) were considered as statistically significant for p-values < 0.05.

In order to assess the association between the evolution of quality parameters (retention of vitamins, water activity, microbial load) and the 5 factors studied (storage duration, RH, temperature, type of packaging, presence of iron in the premix), general linear models (GLM) were used, taking into account the main effects and the interactions between factors. The normality of the residues was checked. Based on the type III sums of squares, the fraction of the variance of the 6 dependent variables (folic acid retention, natural folate content, vitamin B12 retention, water activity, aerobic mesophilic bacteria count, and yeasts/molds count) that is explained by each of the 5 factors – and their interactions – was calculated. Statistical significance was declared for p-values < 0.05.

Principal component analysis (PCA) was used to evaluate which of the variables of interest were most closely associated with the vitamin contents of flour samples. Correlation matrices and correlation circles were constructed to quantify and represent the inter-correlation among the variables (Variables reported here: storage time, water activity, B9 and B12 vitamins contents, and microbial load; Variables reported by Hemery et al. (2018): vitamin A content, water content, fat acidity, and peroxide value).

Statgraphics Centurion 17.2.01 software (Rockville, MD, USA) was used to carry out all the statistical analyses.

3. Results

3.1. Characterization of the initial samples

The initial non-fortified white flour displayed a moisture content of 13.7% and a water activity of 0.64. The folate contents of the non-fortified flour (0.20 ± 0.01 μg/g) was comparable to the standard values found in the French food composition table (ANSES, 2016) and in the West African food composition table (Stadlmayr et al., 2012), which were of 0.18 and 0.24 μg/g, respectively.

The micronutrient levels of premixes were checked before fortification. The analyzed data were not significantly different (p-value > 0.05) from the values indicated in the certificate of analysis provided by the premix supplier, except for the cyanocobalamin content of the Premix 1 that was of 27.6 ± 2.4 μg/g, instead of 20.5 μg/g as indicated in the certificate (p-value = 0.02).
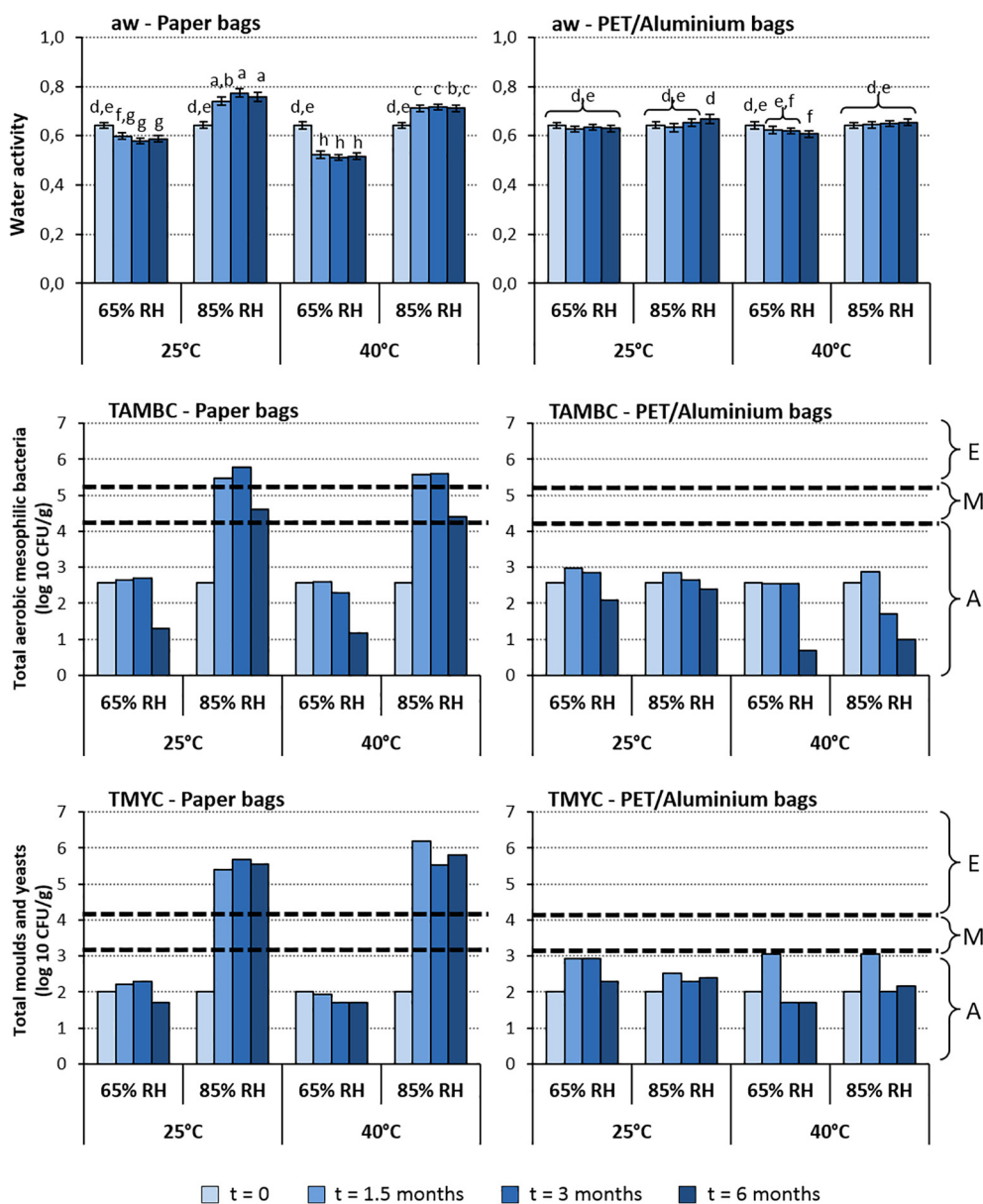
Fig. 2. Influence of packaging, storage conditions and storage length (from 0 to 6 months) on the water activity and microbial counts in flours (RH: Relative humidity, CFU = colony forming units). The flour samples represented here correspond to averages of the values for NF, P1 and P2 samples. Thresholds given by the ANMF Circular 5788, 2012. For total aerobic mesophilic bacteria count (TAMBC): A = acceptable (< 20,000 CFU/g), M = marginal (20,000–200,000 CFU/g), E = excessive (> 200,000 CFU/g). For total molds and yeasts count (TMYC): A = acceptable (< 1000 CFU/g), M = marginal (1000–10,000 CFU/g), E = excessive (> 10,000 CFU/g). For a_w: different letters correspond to significant differences of the mean between samples (p ≤ 0.05).

After fortification, both fortified flours displayed folic acid and cyanocobalamin levels close to the target values, which are 1.30 µg folic acid/g fresh weight (FW) and 0.010 µg cyanocobalamin/g FW for a wheat flour consumption of 150–300 g/day, according to WHO (2009). The folic acid content of fortified flours was of 1.44 ± 0.08 and 1.43 ± 0.05 µg/g FW for flours P1 and P2, respectively, and their cyanocobalamin content was of 0.015 ± 0.001 and 0.013 ± 0.001 µg/g FW for flours P1 and P2, respectively.

3.2. Evolution of the water activity during storage

During the 6-month storage, no significant differences were detected between the water activity of fortified products and that of the non-fortified flour.

Fig. 2 shows that, except for those stored for 6 months at 40 °C/65% RH, the a_w of samples packed in PET/aluminum bags did not significantly change over time (0.64 ± 0.02).

In contrast, the flour samples packed in paper bags exhibited noticeable a_w variations, related to the RH within the storage environment: the flour stored at 65% RH underwent a significant drying, with a_w values of 0.59 ± 0.02 and 0.52 ± 0.02 for samples stored at 25 °C

and 40 °C, respectively. On the other hand, the flour samples stored at 85% RH displayed a significant rise in a_w, with values of 0.75 ± 0.03 and 0.71 ± 0.02 for flour samples stored at 25 °C and 40 °C, respectively. Fig. 2 shows that a_w changed rapidly during the first 1.5 months of storage, but did not significantly change afterwards. A general linear model (GLM) was built, and provided a good explanation of a_w variations ($R^2 > 86\%$) (Table 1). Relative humidity was the main factor influencing a_w, explaining 47% of the variance. The packaging type also highly modulated the influence of RH on a_w, as shown by the significance of the interaction between RH and packaging.

3.3. Folate and folic acid content

Table 2 presents the effect of storage duration and storage conditions on folic acid in fortified flours P1 and P2, and on the natural folate (i.e. the other folate vitamers that were not brought by fortification) in both non-fortified and fortified samples.

For samples packed in PET/aluminum bags, the folic acid content of the fortified flours samples did not significantly change over time during the 6-month storage. When samples were packed in PET/aluminum bags, the natural folate content of flours didn't significantly

Table 1

Factors influencing retention of vitamin B12, retention of folic acid and natural folate, water activity, and microbial load in flour samples: fraction of the variance (%) explained by each factor, and associated probabilities.

| | Fortified flours (P1 and P2) | | | | Non-fortified flours & fortified flours (NF, P1, P2) | | | | | | | |
| | Vitamin B12 retention | | Folic acid retention | | Natural folate | | Water activity (a_w) | | Aerobic bacteria | | Molds and yeasts | |
	% variance	p-value	% variance	p-value	% variance	p-value	% variance	p-value	% variance	p-value	% variance	p-value
Main effects												
Time	6.9	< 0.001	14.7	< 0.001	0.5	ns	0.2	0.044	3.4	< 0.001	5.8	< 0.001
Temperature	0.7	ns	3.8	< 0.001	6.2	< 0.001	3.8	< 0.001	0.8	0.001	0.1	ns
Relative humidity	**21.4**	< 0.001	**6.0**	< 0.001	**15.1**	< 0.001	**46.8**	< 0.001	**24.3**	< 0.001	**36.4**	< 0.001
Packaging	**21.6**	< 0.001	**31.7**	< 0.001	**10.9**	< 0.001	0.2	ns	**24.3**	< 0.001	**13.1**	< 0.001
Premix	0.2	ns	0.2	ns	0.2	ns	0.1	ns	0.0	ns	0.9	< 0.001
Interactions between factors												
Time * Temperature	0.1	ns	0.6	ns	0.7	0.011	0.5	0.001	0.3	0.006	0.7	< 0.001
Time * Humidity	2.5	< 0.001	0.8	0.027	2.4	< 0.001	5.2	< 0.001	2.8	< 0.001	4.1	< 0.001
Time * Packaging	2.6	< 0.001	4.0	< 0.001	1.6	< 0.001	0.0	ns	3.2	< 0.001	1.6	< 0.001
Time * Premix	0.0	ns	0.0	ns	0.0	ns	0.0	ns	0.0	ns	0.9	< 0.001
Temperature * Humidity	0.0	ns	2.8	0.001	**8.1**	< 0.001	0.4	0.047	0.1	ns	1.5	< 0.001
Temperature * Packaging	0.1	ns	3.0	0.001	**9.2**	< 0.001	2.7	< 0.001	0.8	0.001	0.1	ns
Temperature * Premix	0.0	ns	0.0	ns	0.0	ns	0.0	ns	0.0	ns	0.0	ns
Humidity * Packaging	**13.6**	< 0.001	**5.4**	< 0.001	**16.7**	< 0.001	**26.9**	< 0.001	**30.3**	< 0.001	**25.7**	< 0.001
Humidity * Premix	0.1	ns	0.1	ns	0.1	ns	0.0	ns	0.0	ns	0.0	ns
Packaging * Premix	0.2	ns	0.1	ns	0.0	ns	0.0	ns	0.0	ns	0.0	ns
Residual variance	26.6		28.2		13.1		9.7		9.3			
Coefficient of determination of the general linear models (R^2)	**73.4**		**71.8**		**86.9**		**90.3**		**90.7**			

ns: not statistically significant effect. Statistical significance was assumed at $p < 0.05$. Variance calculations based on the type III sums of squares of the general linear models.

Table 2

Influence of packaging and storage conditions on added folic acid and native folates in wheat flour samples over 6 months storage.

Type of packaging	T (°C)	RH (%)	Type of premix	Folic acid – from the added premix (ng/g DM)				Natural folate – from the wheat flour (ng/g DM)			
				t = 0	t = 1.5 months	t = 3 months	t = 6 months	t = 0	t = 1.5 months	t = 3 months	t = 6 months
Paper bags	25	65	NF	–	–	–	–	200 ± 12[i,j]	190 ± 12[i,j]	166 ± 3[i,j]	130 ± 1[k,m]
			P1	1673 ± 91[a]	1513 ± 145[a,b]	1388 ± 74[a,b]	1335 ± 69[b]	196 ± 7[i,j]	180 ± 8[i,j]	161 ± 7[j,k]	127 ± 7[k,m]
			P2	1653 ± 60[a]	1538 ± 128[a,b]	1370 ± 138[a,b]	1406 ± 55[a,b]	204 ± 14[i,j]	188 ± 14[i,j]	165 ± 7[j,k]	128 ± 8[k,m]
		85	NF	–	–	–	–	200 ± 12[i,j]	191 ± 7[i,j]	199 ± 5[i,j]	232 ± 17[i]
			P1	1673 ± 91[a]	1466 ± 189[a,b]	1329 ± 61[b]	1217 ± 90[b,c]	196 ± 7[i,j]	200 ± 12[i,j]	191 ± 11[i,j]	237 ± 16[i]
			P2	1653 ± 60[a]	1371 ± 99[a,b]	1290 ± 70[b]	1288 ± 62[b]	204 ± 14[i,j]	197 ± 8[i,j]	199 ± 5[i,j]	234 ± 26[i]
	40	65	NF	–	–	–	–	200 ± 12[i,j]	185 ± 10[i,j]	158 ± 9[j,k]	94 ± 5[m]
			P1	1673 ± 91[a]	1472 ± 99[a,b]	1407 ± 116[a,b]	1343 ± 84[b]	196 ± 7[i,j]	183 ± 15[i,j]	152 ± 6[j,k]	92 ± 5[m]
			P2	1653 ± 60[a]	1421 ± 159[a,b]	1338 ± 118[a,b]	1294 ± 64[b]	204 ± 14[i,j]	191 ± 11[i,j]	155 ± 8[j,k]	91 ± 11[m]
		85	NF	–	–	–	–	200 ± 12[i,j]	383 ± 30[h]	514 ± 43[f]	541 ± 71[e,f]
			P1	1673 ± 91[a]	1204 ± 213[b,c]	852 ± 168[d]	785 ± 184[d]	196 ± 7[i,j]	443 ± 37[g]	579 ± 95[e]	574 ± 38[e]
			P2	1653 ± 60[a]	1366 ± 186[a,b]	996 ± 81[c,d]	869 ± 159[d]	204 ± 14[i,j]	399 ± 28[h]	542 ± 39[e,f]	570 ± 59[e]
Alum/PET bags	25	65	NF	–	–	–	–	200 ± 12[i,j]	210 ± 5[i,j]	183 ± 3[i,j]	149 ± 6[k]
			P1	1673 ± 91[a]	1624 ± 191[a]	1532 ± 86[a,b]	1476 ± 177[a,b]	196 ± 7[i,j]	189 ± 19[i,j]	190 ± 5[i,j]	147 ± 6[k]
			P2	1653 ± 60[a]	1630 ± 129[a]	1553 ± 194[a,b]	1508 ± 59[a,b]	204 ± 14[i,j]	195 ± 10[i,j]	186 ± 4[i,j]	142 ± 11[k,m]
		85	NF	–	–	–	–	200 ± 12[i,j]	184 ± 13[i,j]	196 ± 12[i,j]	152 ± 7[j,k]
			P1	1673 ± 91[a]	1650 ± 87[a]	1536 ± 128[a,b]	1548 ± 151[a,b]	196 ± 7[i,j]	194 ± 29[i,j]	200 ± 6[i,j]	143 ± 12[k,m]
			P2	1653 ± 60[a]	1604 ± 102[a,b]	1603 ± 101[a,b]	1525 ± 176[a,b]	204 ± 14[i,j]	193 ± 11[i,j]	193 ± 8[i,j]	147 ± 7[k]
	40	65	NF	–	–	–	–	200 ± 12[i,j]	180 ± 10[i,j]	176 ± 5[i,j]	147 ± 23[k,m]
			P1	1673 ± 91[a]	1701 ± 82[a]	1588 ± 122[a,b]	1514 ± 83[a,b]	196 ± 7[i,j]	197 ± 7[i,j]	176 ± 7[i,j]	136 ± 13[k,m]
			P2	1653 ± 60[a]	1604 ± 153[a,b]	1595 ± 140[a,b]	1444 ± 202[a,b]	204 ± 14[i,j]	184 ± 6[i,j]	176 ± 11[i,j]	147 ± 26[k,m]
		85	NF	–	–	–	–	200 ± 12[i,j]	186 ± 4[i,j]	160 ± 3[j,k]	126 ± 10[k,m]
			P1	1673 ± 91[a]	1577 ± 168[a,b]	1607 ± 128[a,b]	1466 ± 195[a,b]	196 ± 7[i,j]	176 ± 14[i,j]	161 ± 7[j,k]	122 ± 10[k,m]
			P2	1653 ± 60[a]	1586 ± 199[a,b]	1501 ± 180[a,b]	1487 ± 46[a,b]	204 ± 14[i,j]	193 ± 9[i,j]	158 ± 7[j,k]	118 ± 17[k,m]

T: Temperature; RH: Relative humidity; DM: dry matter content of the flour; NF: non-fortified flour; P1: flour + premix 1 (with iron) P2: flour + premix 2 (without iron).

Values given as mean ± standard deviation of three repetitions. Different letters correspond to significant differences of the mean between samples (p ≤ 0.05).

change during the first 3 months, but a significant decrease was observed thereafter between the third and the sixth month of storage (Table 2).

For samples packed in paper bags, significant changes in folic acid content occurred. Fig. 4 shows that for flour samples packed in paper bags, a significant negative correlation ($R = -0.64$) was observed between storage length and folic acid retention. Folic acid losses occurred mainly during the first three months of storage, with 17–19% of the initial folic acid lost after 3 months when flour samples were stored at 65% RH in paper bags, irrespective of storage temperature (Fig. 3). When flour samples were stored at 85% RH in paper bags, the folic acid loss after 3 months ranged from 21 to 22% (when stored at 25 °C) to 40–49% (when stored at 40 °C).

For samples stored in paper bags, a significant positive correlation ($R = 0.61$) was observed between a_w and the natural folate content of samples (Fig. 4). The natural folate content decreased at 65% RH and increased at 85% RH. The changes observed for samples stored at 65% RH in paper bags were similar to that observed for samples packed in PET/aluminum bags, with a significant decrease between the third and the sixth month of storage, and the loss of natural folate after 6 months ranged from 24 to 56%. In flour samples stored at 85% RH in paper bags, the natural folate forms increased, especially in samples stored at 40 °C, with a two-fold increase after 1.5 months of storage, and 2.5 to 3-fold increases after 3 and 6 months of storage.

The statistical analysis (Table 1) showed that the retention of folic acid in fortified flours was mostly influenced by the type of packaging and the storage time, that explained 32% and 15% of total variance, respectively. Storage temperature and relative humidity also had a significant – but much lesser – influence (4–6% of the variance). Table 1 also shows that the natural folate content in flours was mostly influenced by RH and by the type of packaging, that explained 15% and 11% of total variance, respectively. The significance of the interactions between factors confirmed that the packaging type also highly modulated the influence of RH (explaining 17% of total variance), and that the temperature within the storage facilities also significantly modulated the influence of RH and packaging type.

3.4. Vitamin B12 retention

The retention of cyanocobalamin did not significantly change over time for flour samples packed in PET/aluminum bags, whereas it exhibited significant changes when the samples were packed in paper bags, depending on the relative humidity of the storage environment (Fig. 3). For these later samples, a significant correlation ($R = 0.76$) was observed between a_w and vitamin B12 retention (Fig. 4). Indeed, 30% to 48% of the initial cyanocobalamin was lost after 1.5 months when flour samples were stored at 65% RH in paper bags, irrespective of storage temperature, and the loss reached 49% to 63% after 6 months. When samples were stored at 85% RH, no significant cyanocobalamin loss was observed for samples stored at 25 °C compared with initial samples, whereas the cyanocobalamin loss reached 21–23% for samples stored at 40 °C (Fig. 3). The factors that mostly influenced the retention of vitamin B12 in fortified flours were the relative humidity and the type of packaging, which explained 21% and 22% of total variance, respectively (Table 1). The significant interaction between packaging and RH (explaining 14% of total variance) showed that the packaging type highly modulated the influence of RH on cyanocobalamin retention.

3.5. Evolution of the microbial load during storage

The microbial quality of flour samples was evaluated during the 6-month storage. Fig. 2 presents the evolution of the total aerobic mesophilic bacteria count (TAMBC) and the total molds and yeasts count (TMYC). The microbial load of samples packed in PET/aluminum bags did not change over time and stayed acceptable, for either TAMBC or TMYC. Samples packed in paper bags stayed acceptable during the 6-months storage when stored at 65% RH, but for those stored at 85% RH the microbial load increased and exceeded the acceptable limits after 1.5 months of storage – for both TAMBC and TMYC – and stayed
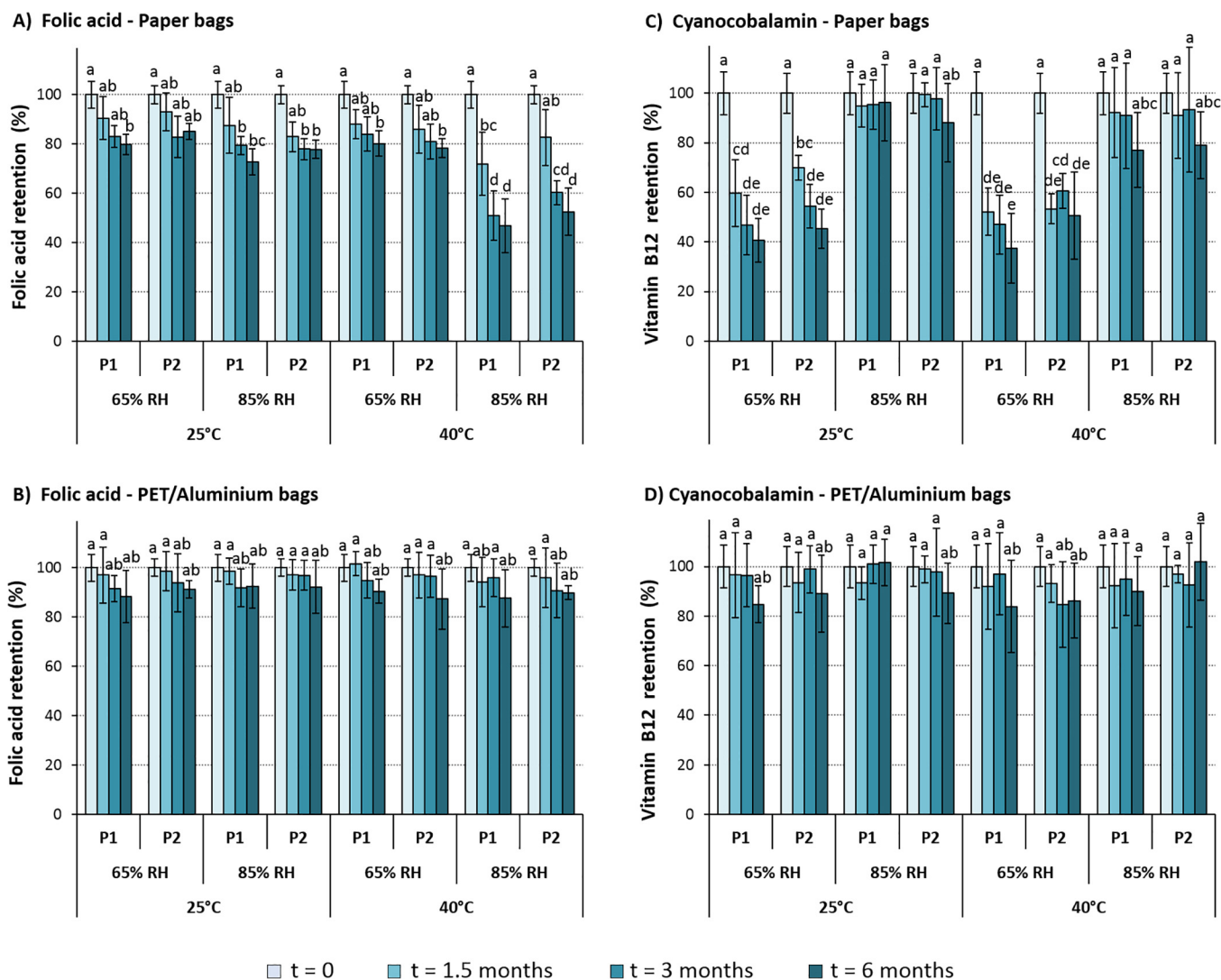
Fig. 3. Evolution of folic acid retention and cyanocobalamin retention (%) in fortified flours during the 6-month storage, for samples stored at various temperature and relative humidity (RH) conditions. P1: flour + premix 1 (with ferrous sulphate), P2: flour + premix 2 (without ferrous sulphate). Different letters correspond to significant differences of the mean between samples (p ≤ 0.05).

elevated until the end of the 6-month storage, irrespective of storage temperature. For flour samples packed in paper bags, Fig. 4 shows that significant positive correlations were observed between a_w and TAMBC ($R = 0.87$), and a_w and TMYC ($R = 0.91$). Table 1 shows that the GLM provided a good explanation of the variations of microbial load ($R^2 > 90\%$). Relative humidity and type of packaging were the main factors influencing both TAMBC and TMYC, with relative humidity being more preponderant for the growth of molds and yeasts than for the growth of aerobic bacteria. The significance of the interaction between RH and packaging also confirmed that the packaging type highly modulated the influence of RH.

3.6. Influence of premix composition

Fig. 3 and Table 1 clearly show that the composition of the added premix (with or without ferrous sulphate) did not have any significant influence on the retention of folate and vitamin B12 in fortified flours, irrespective of storage temperature and packaging.

4. Discussion

This work showed that the factor that mostly influenced the

retention of folic acid and vitamin B12 in fortified flours was the quality of packaging material. Substantial losses of folic acid and vitamin B12 can occur during the storage of fortified flours when stored in packaging material with low barrier properties (*i.e.* permeable to oxygen and water transfers) such as paper bags. However, when fortified flour was stored in a multilayer packaging with good barrier properties, no significant loss of folic acid and vitamin B12 was observed during the 6-month storage, irrespective of temperature and RH in the storage environment. For the fortified flour stored in paper bags, folic acid loss occurred mainly during the first 3 months of storage, with 17–19% loss when stored at 65% RH, and up to 40–49% when stored at 85% RH. Relative humidity highly impacted the retention of vitamin B12 in flours packed in paper bags, with no significant cyanocobalamin loss in fortified flours when stored at 85% RH, but 30–48% loss after 1.5 months and 49–63% after 6 months when stored at 65% RH.

Irrespective of the packaging material, the loss of natural folate after 6 months ranged from 24 to 56% when exposure to high relative humidity was avoided. Swindler (2013) also reported 24% loss of native folate in unfortified wheat flour after six months of storage at room temperature in sealed cans, but no significant loss of added folic acid in enriched wheat flour stored over the same time in the same conditions. Phillips et al. (2017) also showed that folic acid was stable in well-
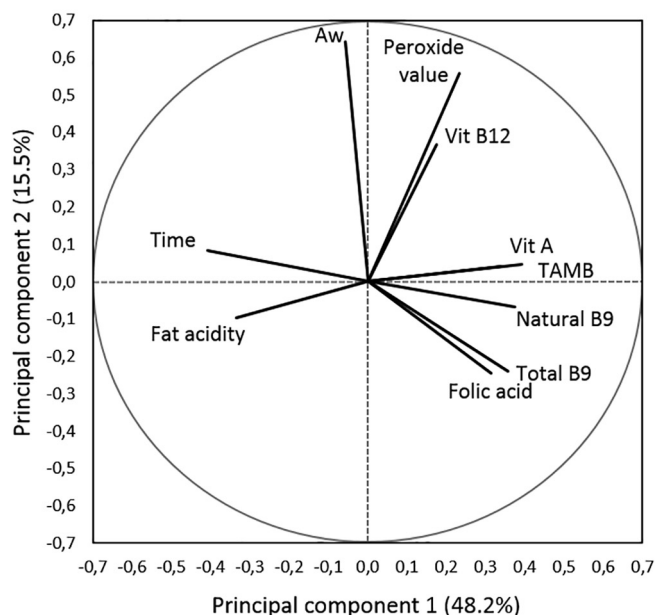
A) Paper bags
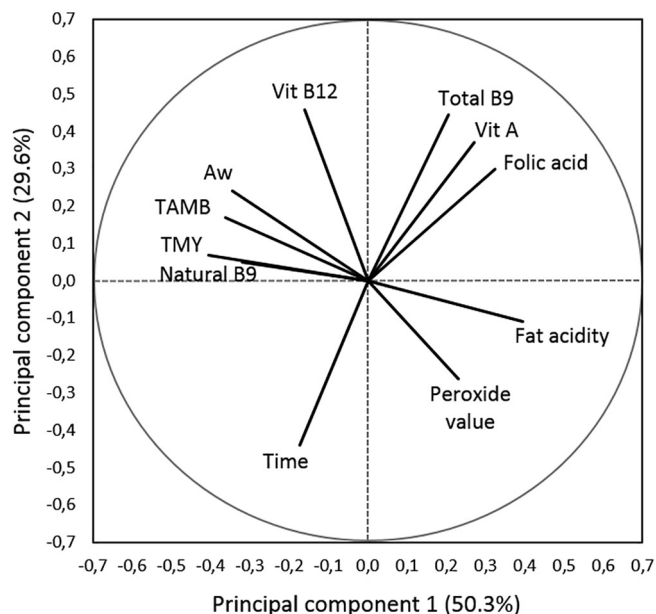
B) PET/Aluminium bags

Fig. 4. Correlation circles of the principal component analysis, for samples packed A) in paper bags, and B) in PET/Aluminum bags·H2O: water content, Aw: water activity, TAMB: total aerobic mesophilic bacteria, TMY: total molds and yeasts, Vit A: vitamin A content, Vit B12: cyanocobalamin content. Data for variables Vit A, H₂O, Fat acidity and Peroxide value were previously reported by Hemery et al. (2018).

packaged fortified flours: they measured the folic acid content of fortified corn masa flour stored at 22 °C and 65% RH for up to six months in kraft plastic-lined bags [*i.e.* multilayer packaging made up of brown paper and plastic], and did not observe any significant differences across the six-month shelf life. In the present study, the folic acid losses observed for samples packed in paper bags, that were stored 3 months or more, was most likely due to oxidative degradation. Indeed, in the presence of air or oxygen and in the absence of antioxidants, folic acid can undergo autoxidation and a split in its C^9–N^{10} bond, giving biologically inactive products (Gazzali et al., 2016; Strandler, Patring,

Jägerstad, & Jastrebova, 2015). Before the current study, vitamin A content was previously analyzed in the same fortified flour samples (Hemery et al., 2018), and is presented in Fig. 4 for comparison purpose. As shown on Fig. 4, for flour samples packed in paper bags, a significant correlation ($R = 0.75$) was observed between the folic acid retention and the vitamin A retention, suggesting that both vitamins are sensitive to the same factors. Moreover, Chapman, Steele, Eggett, Johnston, and Dunn (2010) observed a degradation of the added folic acid during fermentation of freshly ground masa, and speculated that microbial degradation was the cause. Chapman et al. (2010) also showed that folate in masa flour was not affected by iron fortification, as observed in our study.

Vitamin B12 is a unique metalorganic compound and the most chemically complex of all the vitamins (Randaccio, Geremia, Demitri, & Wuerges, 2010), and few studies have been published on the factors influencing the stability of vitamin B12 in foods. This study is the first one to focus on the stability of vitamin B12 in form of added cyanocobalamin in fortified flours. It showed that the drying of the flour (*i.e.* the decrease in a_w when samples were stored at 65% RH in paper bags) led to the fast degradation of cyanocobalamin in fortified flours. This suggests that cyanocobalamin stability might be sensitive to a_w variations and the hydration of the molecule. Ohmori, Kataoka, and Koyama (2007) studied the influence of storage conditions on the stability of cyanocobalamin in powders, granules, and sugar-coated tablets, and showed that cyanocobalamin was less stable in products stored at 57–75% RH/40 °C than in products stored at 6% RH/40 °C, but the authors did not study the stability of vitamin B12 at very high relative humidity values (85%). As the flours samples analyzed here were the same as those analyzed in our precedent study (Hemery et al., 2018), we carried out analyses of correlations between different characteristics of the flour samples and results are presented in Fig. 4 for comparison purpose. As shown on Fig. 4 for flour samples packed in paper bags, significant negative correlations were observed between cyanocobalamin retention and peroxide value ($R = -0.60$), and between cyanocobalamin retention and fat acidity ($R = -0.64$). This suggests that cyanocobalamin might also be sensitive to the presence of oxidizing agents. Berry Ottaway (1993) reported that cyanocobalamin in solution is decomposed by both oxidizing and reducing agents. Johns et al. (2015) showed that the generation of reactive oxygen species such as hydroxyl radicals can result in the oxidation of vitamin B12 and its decomposition. In the present study, only four temperature/RH combinations were tested, which was not enough to precisely model the impact of temperature and RH, and to understand the influence of a_w and oxidative status on the stability of cyanocobalamin in fortified flours. In order to help the stakeholders that are involved in fortification programs to more accurately estimate the vitamin B12 content of fortified flours that are produced and stored in various countries, more research is needed to model the influence of various RH and temperatures conditions, the oxidative status of the flour, and the storage duration on the stability of cyanocobalamin.

The microbial load of flour packed in PET/aluminum bags did not change over time and stayed acceptable. For flour packed in paper bags and stored at 85% RH, the exposure to high relative humidity increased the water activity of the products (average $a_w > 0.7$ after homogenization of samples) and resulted in the development of microorganisms, with microbial load exceeding the acceptable limits after 1.5 months of storage, for aerobic mesophilic bacteria, and for molds and yeasts. Akhtar, Anjum, Rehman, Sheikh, and Farzana (2008) also reported significantly different mold growth depending on the storage conditions of fortified wheat flour. In this study, an increase in the content of natural folate forms was observed in flour samples packed in paper bags stored at 85% RH, and a significant positive correlations was observed between the natural folate content of these flours and the level of microbial counts. This may possibly be explained by the ability of yeasts and certain bacteria to naturally synthesize folate (Moslehi-Jenabian, Lindegaard, & Jespersen, 2010; Saubade, Hemery, Guyot, &

Humblot, 2017). However, this effect on natural folate was accompanied by an increased microbial load above the acceptable limits, which made the products not suitable for human consumption. This microbial load may also have led to the production of microbial lipases, which would explain the lipolysis and free fatty acids production observed by Hemery et al. (2018) in the same fortified wheat flours.

These results – and those previously presented for the same fortified flour samples (Hemery et al., 2018) – show that, if the packaging is permeable to oxygen and water vapor, it may not be possible to simultaneously optimize the retentions of vitamins A, B9 and B12. A good way to better preserve the vitamins in fortified wheat flours would be to use a packaging with good barrier properties to limit oxygen and water transmissions, and to store the products under mild temperature conditions (25 °C) and for no more than 3 months, as those conditions would allow the retention of 50% of vitamin A, 90% of folic acid, and 90% of cyanocobalamin. The use of coated or nanoencapsulated vitamin forms could also allow maximizing vitamin retention during longer storage periods or during storage in more severe conditions (Katouzian & Jafari, 2016). However, additional vitamin loss may also occur during further processing and cooking of the fortified flour, and more research is needed to estimate these losses that could lead to increase the level of fortification accordingly.

In order to encourage the stakeholders involved in fortification programs to consider the choice of appropriate packaging before the implementation of any fortification program, a number of suggestions can be considered:

- More understanding of the turn-around time of the products (once fortified to consumption) is needed, to better identify where packaging might or not be an issue. Where the turn-around time is not clearly known, packaging needs to be considered in a very serious way; including how fortified flour is stored in the household level.
- In trainings on food fortification that are implemented in many countries, packaging issues should be treated as importantly as the knowledge of the level of the fortificant.
- Existing fortification laws should include an explicit chapter or reference to the packaging of fortified products, as currently, the existing fortification laws or national technical guidelines do not specifically refer to packaging.
- An initiative with the major packaging industry could be forged, to look into packaging for fortified flour and beyond, in order to favor the development of low-cost, high-quality packaging adapted to tropical storage conditions.

5. Conclusion

This work showed that the quality of enriched flour packaging, particularly its effectiveness in protecting it from moisture and oxygen, was crucial in limiting losses of added folic acid and cyanocobalamin, and to maintain their levels within the expected ranges. Flour is not an inert product and many reactions can occur during storage, and impact the stability of vitamins. Therefore, the choice of a suitable packaging, which is not permeable to both oxygen and moisture, is of critical importance, and must be taken into account when planning a fortification program in countries with a tropical environment. The cost of vitamins is high and the decision to enrich a food must be based on several factors, such as the target populations, the potential impacts expected for the nutrition and health of these populations, the quality of the product to be fortified and its consequences on vitamin stability, the cost of ensuring the physical and economic availability of the fortified food and its consumption by the target populations. It is therefore essential to ensure that the fortified foods will contain the appropriate content of fortifying agents at the time of consumption, and we recommend that the duration between the fortification of flour and its use by consumers should be shortened as much as possible, and that proper

and efficient packaging should be considered before the implementation of any fortification program. At the same time, more research is needed to better understand the impact of food quality on the retention of added vitamins, and innovation for developing low-cost, high-quality packaging should be stimulated.

CRediT authorship contribution statement

Youna M. Hemery: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Laura Fontan:** Methodology, Validation, Formal analysis, Investigation. **Arnaud Laillou:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Vincent Jallier:** Conceptualization, Supervision. **Regina Moench-Pfanner:** Writing - review & editing. **Sylvie Avallone:** Methodology. **Jacques Berger:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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. The authors state that there was no conflict of interest during this study, and that they were not directly or indirectly affiliated to any profit-making units that may result in a conflict of interest. Arnaud Laillou is a UNICEF staff member, but the opinions and statements in this article are those of the authors and may not reflect the official UNICEF policies.

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References

Akhtar, S., Anjum, F. M., Rehman, S.-U., Sheikh, M. A., & Farzana, K. (2008). Effect of fortification on physico-chemical and microbiological stability of whole wheat flour. *Food Chemistry, 110*(1), 113–119. https://doi.org/10.1016/j.foodchem.2008.01.065.

Allen, L. H., Rosenberg, I. H., Oakley, G. P., & Omenn, G. S. (2010). Considering the case for vitamin B12 fortification of flour. *Food and Nutrition Bulletin, 31*(1, Suppl.), S36–S46.

Allen, Lindsay H. (2009). How common is vitamin B-12 deficiency? *The American Journal of Clinical Nutrition, 89*(2), 693S–696S. https://doi.org/10.3945/ajcn.2008.26947A.

Allen Lindsay H., De Benoist B., Dary O., Hurrell R., & WHO. (2006). Guidelines on food fortification with micronutrients. Retrieved from http://www.who.int/iris/handle/10665/43412.

ANMF. (2012). Cahier des charges microbiologique des farines, circulaire N°5788 de l'Association Nationale de la Meunerie Française.

ANSES. (2016). French food composition table. Table de Composition Nutritionnelle Des Aliments Ciqual, https://pro.anses.fr/tablec[qual/.

Bailey, R. L., Jr., West, K. P., & Black, R. E. (2015). The Epidemiology of global micronutrient deficiencies. *Annals of Nutrition and Metabolism, 66*(Suppl. 2), 22–33. https://doi.org/10.1159/000371618.

Bergen, N. E., Jaddoe, V. W. V., Timmermans, S., Hofman, A., Lindemans, J., Russcher, H., & Steegers, E. A. P. (2012). Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. *BJOG: An International Journal of Obstetrics & Gynaecology, 119*(6), 739–751. https://

doi.org/10.1111/j.1471-0528.2012.03321.x.

Berry, R. J., Bailey, L., Mulinare, J., Bower, C., & Dary, O. (2010). Fortification of flour with folic acid. *Food and Nutrition Bulletin, 31*(1_suppl1), S22–S35. https://doi.org/10.1177/15648265100311S103.

Chapman, J. S., Steele, F. M., Eggett, D. L., Johnston, N. P., & Dunn, M. L. (2010). Stability of native folate and added folic acid in micronutrient-fortified corn masa and tortillas. *Cereal Chemistry, 87*(5), 434–438. https://doi.org/10.1094/CCHEM-12-09-0164.

FAO/WHO (2002). *Human vitamin and mineral requirements. Report of a joint FAO/WHO expert consultation, Bangkok, Thailand.* Rome: FAO and WHO.

Gazzali, A. M., Lobry, M., Colombeau, L., Acherar, S., Azaïs, H., Mordon, S., ... Frochot, C. (2016). Stability of folic acid under several parameters. *European Journal of Pharmaceutical Sciences, 93*, 419–430. https://doi.org/10.1016/j.ejps.2016.08.045.

Hemery, Y. M., Laillou, A., Fontan, L., Jallier, V., Moench-Pfanner, R., Berger, J., & Avallone, S. (2018). Storage conditions and packaging greatly affects the stability of fortified wheat flour: Influence on vitamin A, iron, zinc, and oxidation. *Food Chemistry, 240*, 43–50. https://doi.org/10.1016/j.foodchem.2017.07.084.

ISO 4833 (2013). *Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30 degrees C by the pour plate technique.* International Organization for Standardization ISO 4833-1:2013.

ISO 7954 (1987). *Microbiology – General guidance for enumeration of yeasts and moulds – Colony count technique at 25 degrees C.* International Organization for Standardization ISO 7954:1987.

Johns, P. W., Das, A., Kuil, E. M., Jacobs, W. A., Schimpf, K. J., & Schmitz, D. J. (2015). Cocoa polyphenols accelerate vitamin B12 degradation in heated chocolate milk. *International Journal of Food Science & Technology, 50*(2), 421–430. https://doi.org/10.1111/ijfs.12632.

Kariluoto, S., & Piironen, V. (2009). Total folate. In P. R. Shewry & J. L. Ward (Eds.), HEALTHGRAIN Methods, Analysis of Bioactive Components in Small Grain Cereals (pp. 59–68). St Paul, Minnesota, USA: AACC International. Retrieved from ://WOS:000169828700020.

Katouzian, I., & Jafari, S. M. (2016). Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins. *Trends in Food Science & Technology, 53*, 34–48. https://doi.org/10.1016/j.tifs.2016.05.002.

Morris, M. S., Jacques, P. F., Rosenberg, I. H., & Selhub, J. (2007). Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *The American Journal of Clinical Nutrition, 85*(1), 193–200. https://doi.org/10.1093/ajcn/85.1.193.

Moslehi-Jenabian, S., Lindegaard, L., & Jespersen, L. (2010). Beneficial effects of probiotic and food borne yeasts on human health. *Nutrients, 2*(4), 449–473. https://doi.org/10.3390/nu2040449.

Mujawar, S. A., Patil, V. W., & Daver, R. G. (2011). Study of serum homocysteine, folic acid and vitamin B12 in patients with preeclampsia. *Indian Journal of Clinical Biochemistry, 26*(3), 257–260. https://doi.org/10.1007/s12291-011-0109-3.

Ohmori, S., Kataoka, M., & Koyama, H. (2007). Stability of cyanocobalamin in sugar-coated tablets. *International Journal of Pharmaceutics, 337*(1), 161–168. https://doi.org/10.1016/j.ijpharm.2006.12.044.

Osendarp, S. J., Martinez, H., Garrett, G. S., Neufeld, L. M., De-Regil, L. M., Vossenaar, M., & Darnton-Hill, I. (2018). Large-scale food fortification and biofortification in low- and middle-income countries: A review of programs, trends, challenges, and evidence gaps. *Food and Nutrition Bulletin, 39*(2), 315–331. https://doi.org/10.1177/0379572118774229.

Ottaway, P. B. (1993). Stability of vitamins in food. In P. B. Ottaway (Ed.). *The Technology of Vitamins in Food* (pp. 90–113). Boston, MA: Springer US https://doi.org/10.1007/978-1-4615-2131-0_5.

Pawlak, R., Lester, S. E., & Babatunde, T. (2014). The prevalence of cobalamin deficiency among vegetarians assessed by serum vitamin B12: A review of literature. *European Journal of Clinical Nutrition, 68*(5), 541–548. https://doi.org/10.1038/ejcn.2014.46.

Phillips, R., Pike, O. A., Eggett, D. L., & Dunn, M. L. (2017). Folate stability in folic acid enriched corn masa flour, tortillas, and tortilla chips over the expected shelf life. *Cereal Chemistry, 94*(6), 917–921. https://doi.org/10.1094/CCHEM-02-17-0037-R.

Randaccio, L., Geremia, S., Demitri, N., & Wuerges, J. (2010). Vitamin B12: Unique metalorganic compounds and the most complex vitamins. *Molecules, 15*(5), 3228–3259. https://doi.org/10.3390/molecules15053228.

Ray, J. G., Meier, C., Vermeulen, M. J., Boss, S., Wyatt, P. R., & Cole, D. E. (2002). Association of neural tube defects and folic acid food fortification in Canada. *The Lancet, 360*(9350), 2047–2048. https://doi.org/10.1016/S0140-6736(02)11994-5.

Ray, J. G., Wyatt, P. R., Thompson, M. D., Vermeulen, M. J., Meier, C., Wong, P., ... Cole, D. E. C. (2007). Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. *Epidemiology, 18*(3), 362–366. https://doi.org/10.1097/01.ede.0000257063.77411.e9.

Reichert, N., & Rubach, K. (1990). Estimation of vitamin B12 in vitamin supplemented foodstuffs by means of a competitive protein-binding assay (CPBA) and an ELISA (enzyme-linked immunosorbent assay). *Deutsche Lebensmittel-Rundschau, 86*(10), 307–310.

Rogers, L. M., Cordero, A. M., Pfeiffer, C. M., Hausman, D. B., Tsang, B. L., De-Regil, L. M., ... Bailey, L. B. (2018). Global folate status in women of reproductive age: A systematic review with emphasis on methodological issues. *Annals of the New York Academy of Sciences, 1431*(1), 35–57. https://doi.org/10.1111/nyas.13963.

Saubade, F., Hemery, Y. M., Guyot, J., & Humblot, C. (2017). Lactic acid fermentation as a tool for increasing the folate content of foods. *Critical Reviews in Food Science and Nutrition, 57*(18), 3894–3910. https://doi.org/10.1080/10408398.2016.1192986.

Saubade, F., Hemery, Y. M., Rochette, I., Guyot, J.-P., & Humblot, C. (2018). Influence of fermentation and other processing steps on the folate content of a traditional African cereal-based fermented food. *International Journal of Food Microbiology, 266*, 79–86. https://doi.org/10.1016/j.ijfoodmicro.2017.11.015.

Sayed, A.-R., Bourne, D., Pattinson, R., Nixon, J., & Henderson, B. (2008). Decline in the prevalence of neural tube defects following folic acid fortification and its cost-benefit in South Africa. *Birth Defects Research Part A: Clinical and Molecular Teratology, 82*(4), 211–216. https://doi.org/10.1002/bdra.20442.

Selva Kumar, L. S., & Thakur, M. S. (2011). Competitive immunoassay for analysis of vitamin B12. *Analytical Biochemistry, 418*(2), 238–246. https://doi.org/10.1016/j.ab.2011.07.011.

Stadlmayr, B., Charrondiere, U. R., Enujiugha, V., Bayili, R. G., Fagbohoun, E. G., Samb, B., ... Burlingame, B. (2012). *West African food composition table.* Rome, Italy: FAO.

Strandler, H. S., Patring, J., Jägerstad, M., & Jastrebova, J. (2015). Challenges in the determination of unsubstituted food folates: Impact of stabilities and conversions on analytical results. *Journal of Agricultural and Food Chemistry, 63*(9), 2367–2377. https://doi.org/10.1021/jf504987n.

Sukumar, N., Rafnsson, S. B., Kandala, N.-B., Bhopal, R., Yajnik, C. S., & Saravanan, P. (2016). Prevalence of vitamin B-12 insufficiency during pregnancy and its effect on offspring birth weight: A systematic review and meta-analysis. *The American Journal of Clinical Nutrition, 103*(5), 1232–1251. https://doi.org/10.3945/ajcn.115.123083.

Swindler, J. (2013). *Effect of enrichment-bleaching and low oxygen atmosphere storage on all-purpose wheat flour quality (Master's thesis).* Provo, UT: Brigham Young University Retrieved from https://scholarsarchive.byu.edu/etd/4064.

WHO (2009). *Recommendations on wheat and maize flour fortification. Meeting Report: Interim Consensus Statement, WHO, FAO, UNICEF, GAIN, MI, & FFI.* Geneva: World Health Organization.