Bread cofortified with folic acid and vitamin B-12 improves the folate and vitamin B-12 status of healthy older people: a randomized controlled trial

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ABSTRACT
Background: Mandatory fortification of flour with folic acid has reduced the number of neural tube defects in North America. Concerns that high intakes of folic acid might mask vitamin B-12 deficiency in older persons have delayed the introduction of fortification in many European countries. Cofortification of flour with folic acid and vitamin B-12 could simultaneously improve folate and vitamin B-12 status.

Objective: The objective was to estimate the effect of the consumption of bread fortified with modest amounts of folic acid and vitamin B-12 on folate and vitamin B-12 status in healthy older persons living in the Netherlands, where folic acid fortification is not taking place.

Design: Men and women aged 50–75 y were randomly assigned in this 12-wk double-blind, placebo-controlled trial to consume bread fortified with 138 \( \mu g \) folic acid and 9.6 \( \mu g \) vitamin B-12 daily (n = 72) or unfortified bread (n = 70).

Results: The consumption of fortified bread increased serum folate concentrations by 45% (mean: 6.3 nmol/L; 95% CI: 4.5, 8.1 nmol/L) and serum vitamin B-12 concentrations by 49% (mean: 102 pmol/L; 95% CI: 82, 122 pmol/L) relative to the placebo group. Fortified bread increased erythrocyte folate concentrations by 22% and holo-transcobalamin concentrations by 35%; it decreased homocysteine concentrations by 13% and methylmalonic acid concentrations by 10%. Consumption of fortified bread decreased the proportion of individuals with marginal serum vitamin B-12 concentrations (<133 pmol/L) from 8% at enrollment to 0% after 12 wk.

Conclusion: Bread fortified with modest amounts of folic acid and vitamin B-12 will improve folate and vitamin B-12 status and a considerable proportion of vitamin B-12 deficiency in older people. This trial was registered at clinicaltrials.gov as NCT00353353. Am J Clin Nutr 2008;88:348–55.

INTRODUCTION
Periconceptional intake of folic acid by women of childbearing age reduces the risk of having an infant with a neural tube defect (1–5). Several European countries are considering folic acid fortification to ensure sufficient intake of folic acid for women of childbearing age (6, 7), because many women who plan their pregnancies do not take folic acid supplements in the advised period (8) and many other pregnancies are unplanned (9).

Excessive intake of folic acid may cure (“mask”) vitamin B-12–related anemia and thereby delay the diagnosis of a vitamin B-12 deficiency (10, 11). About one-fifth of older persons have marginal vitamin B-12 status (12–14) and thus have a high risk of developing a vitamin B-12 deficiency. Therefore, folic acid fortification could potentially harm a large proportion of the population. Cofortification of grain products with modest amounts of both vitamin B-12 and folic acid (15, 16) could simultaneously increase folate status and minimize hazards for vitamin B-12–deficient persons, but the effects of cofortification on folate and vitamin B-12 status in the general population have not been previously examined. Previous interventions examined the effects of very high doses of vitamin B-12 supplements (17–20), were performed only in elderly individuals with marginal B-vitamin status (17–22), or used only a limited assessment of vitamin B-12 status (23).

We examined to what extent consumption of bread fortified with modest amounts of folic acid and vitamin B-12 can improve the folate and vitamin B-12 status of older people living in the Netherlands, where fortification is not taking place. The intervention aimed to increase the intake of folic acid by \( \geq 100 \mu g/d \), comparable with the current fortification level in the United States (24), and to increase the intake of vitamin B-12 by \( \geq 6 \mu g/d \), which is \( \approx 2 \) times the reference nutrient intake in the United States (25). We chose to fortify bread with 6 \( \mu g \) vitamin B-12/d because a recent study reported that a dietary intake of at least that dose optimized all vitamin B-12–related markers in 98 postmenopausal women (26).

SUBJECTS AND METHODS
Participants
We recruited participants from a database of persons who had previously expressed interest in participation in trials from the

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Division of Human Nutrition of Wageningen University and through advertisements in local newspapers. Participants had to be between 50 and 75 y of age and had to consume ≥3 slices of bread/d (Figure 1). Exclusion criteria were use of B vitamin supplements in the 3 mo before the study, treatment with vitamin B-12 injections in the past 5 y, or illness or use of medication that interfered with folate or vitamin B-12 metabolism (eg, pernicious anemia or use of antacids). We invited 154 persons who met these criteria to provide a fasting blood sample at the study center at Wageningen University. We excluded 11 applicants with a serum vitamin B-12 concentration <18 pmol/L, serum folate concentration <6.8 nmol/L, or both (Access Immunoassay, Beckman Coulter, Fullerton, CA; cutoff values are laboratory reference limits for deficiency). It was considered unethical in the Netherlands to withhold vitamins from individuals with vitamin concentrations below these cutoffs. We stratified the remaining 143 participants for serum vitamin B-12, serum folate, and age. An independent research assistant randomly assigned

182 persons aged 50-75 y who habitually consumed ≥3 slices of bread per day volunteered for the study

Excluded: n
- use of B-vitamin supplements for 3 months prior to the study (n=3)
- illness or use of medication that interfered with folate or vitamin B-12 metabolism (n=17)
- practical reasons (eg, absent during the planned trial period) (n=8)

154 subjects were eligible and provided a blood sample

Excluded: n=11
- serum vitamin B-12 < 118 pmol/L (n=5)
- serum folate < 6.8 nmol/L (n=5)
- both folate and vitamin B-12 too low (n=1)

143 subjects randomly assigned

n=73 Fortified bread
n=70 Placebo bread

2 dropped out after 7 weeks for medical reasons not related to the trial, 1 participant still provided a blood sample, the other was lost to follow-up

n=72 Analyzed
n=70 Analyzed

FIGURE 1. Recruitment and flow of participants during the study.
participants within each stratum to 1 of the 2 intervention groups using the random number generator from Excel (Microsoft Office Excel 2003; Microsoft Inc, Redmond, WA). The 27 participants, who shared a household with another participant, were allocated to the same treatment to avoid a mix-up of treatments. All participants gave written informed consent. Both trial staff and participants remained blinded to the treatment until all data had been gathered and double-checked. The Medical Ethical Committee of Wageningen University approved the study. Recruitment started in June 2006, and the trial ran from September through December 2006.

Design
The fortified bread group ate bread fortified with folic acid and vitamin B-12, and the placebo group ate unfortified bread. The participants provided a fasting blood sample in the week before the treatment started (baseline). They then consumed the bread for 12 wk and provided a fasting blood sample on the last day of treatment (week 12). We asked the participants to substitute the bread that they usually ate with the bread provided by us, to consume ≥3 slices of this bread per day, and to record the number of slices eaten in a food diary. Furthermore, the participants returned all unused slices of bread at the end of each week, at which time they collected a fresh batch of bread for the next week. We also asked participants to consume their habitual diet and avoid the use of vitamin supplements.

Sample size
We calculated that 55 participants would be required in each group to detect an absolute difference in serum vitamin B-12 response of 50 pmol/L between the groups (18, 22, 23) with a power of 90% and a = 0.025 (one-sided testing), assuming an SD of the response of 80 pmol/L (17, 18, 22). We added another 20 persons per group, because bread intake varies between persons, which increases variability. The trial had >99% power to detect a 6-nmol/L difference in serum folate response (27), assuming an SD of 5 nmol/L (27).

Bread
We wanted to increase the intake of folic acid by 100 µg/d and the intake of vitamin B-12 by 6 µg/d. Individuals in this age group in the Netherlands typically consume 3–5 slices of bread daily (28); thus, our aim was to add 33 µg folic acid and 2 µg vitamin B-12 per slice of bread. We used whole-wheat bread because this type of bread is consumed most widely in the Netherlands (29). One loaf of bread weighed ≈800 g and contained 24 slices.

Pilot studies of bread fortification
We first tested procedures to make the fortified bread. We prepared a solution containing 800 mg folic acid (folic acid from Merck Eprova, Schaffhausen, Switzerland) and 10 g baking powder (mixture of sodium carbonate and ammonium carbonate) per liter of water (baking powder improved solubility of folic acid) and a solution containing 480 mg vitamin B-12 (cyanocobalamin from DSM Nutritional Products, Basel, Switzerland) per liter of water. We mixed 5 mL folic acid solution with 0.5 mL vitamin B-12 solution. A commercial baker added this mixture to the water used to make the dough and baked 5 loaves on each of 3 separate days using a standard recipe. Two samples of 100 g of each bread were freeze-dried, ground, and stored at −20 °C. Samples were weighted, and internal standard ([13C5]folic acid) was added. Samples were extracted in an autoclave at 119 °C for 15 min in buffer (pH 7.8), the extract was filtered and adjusted to pH 4.5, and folic acid was analyzed with a reversed-phase HPLC method with mass spectrometry/mass spectrometry detection (SOP VIT/071; TNO Quality of Life, Zeist, Netherlands). To analyze vitamin B-12, samples were extracted in an autoclave (119 °C for 15 min) with the use of 0.1 mol acetate buffer (pH 4.6) with 50 mg/L potassium cyanide. Vitamin B-12 was analyzed in the filtrated extract with a competitive binding radiodissay with [15C]cyanocobalamin (SOP VIT/065; TNO Quality of Life). The analyzed amount of folic acid was 760 mg/L in the folic acid solution and 408 µg/loaf of bread: recovery of folic acid during bread making was therefore 54%. We performed a second pilot study in a research bakery (DSM Food Innovation Centre, Delft, Netherlands); the recovery of folic acid during those experiments was 73%. The average recovery was therefore 64%, consistent with values previously reported (30, 31). The analyzed amounts of vitamin B-12 were 482 mg/L in the vitamin B-12 solution and 37 µg/loaf of bread; thus, the recovery of vitamin B-12 was 77%. The CV of vitamin concentrations within and between loaves of bread was <5% for both folic acid and vitamin B-12.

Trial
One of the authors (RMW) prepared the vitamin and placebo solutions. The vitamin solutions were prepared with 1288 mg folic acid and 640 µg vitamin B-12 to correct for the incomplete recovery. Because the vitamin solution was red, the placebo solution was colored with tartrazine E102 and carmoisine E122; an assistant coded all solutions, stored them at −20 °C, and delivered them to the baker. The baker baked and sliced the bread for our trial weekly; the fortified and placebo breads were baked on separate days. We analyzed samples of each batch of bread and found that the recovery of folic acid was 64% and of vitamin B-12 was 81%, which was comparable with our pilot experiments. The fortified bread contained 30 µg folic acid per slice and 2 µg vitamin B-12 per slice. The CV of vitamin concentrations between loaves of bread was <7%.

We (RMW and IAB) detected no differences between the fortified and nonfortified breads in taste, smell, or appearance. Participants collected fresh bread once a week on the day of production; they stored bread in the freezer or at room temperature.

Blood analysis
Blood samples were collected into coagulation tubes, stored in the dark for 30 to 120 min at room temperature, and centrifuged at 3000 × g for 10 min at 4 °C. Serum was pipetted off and stored at −80 °C. Serum folate and vitamin B-12 were analyzed with a chemiluminescence immunoassay (Access Immunoassay, Beckman Coulter; CV for folate: 5%; CV for vitamin B-12: 7%) and serum holotranscobalamin with a Microparticle Enzyme Immunoassay (AxSYM-HoloTC, Axis-Shield Diagnostics, Dundee, United Kingdom; CV: 9%). To obtain plasma, blood samples were collected into EDTA-containing tubes. These tubes were placed on ice immediately after venipuncture and centrifuged within 30 min at 3000 × g for 10 min at 4 °C. Plasma
was pipetted off and stored at $-80^\circ$C. The plasma methylmalonic acid concentration was analyzed by HPLC with mass spectrometry/mass spectrometry detection (32) (CV 4%), and plasma total homocysteine concentration was measured by HPLC with fluorescence detection (33) (CV 7%). We collected whole blood into an EDTA-containing tube, hemolyzed the blood, and analyzed folate with a chemiluminescence immunoassay (Access Immunoassay; CV: 8%).

Approximately 50 persons provided additional blood samples for construction of a quality control pool. We coded these pools as simulated participants and randomly submitted ≥20 samples of this pool to the laboratory in between samples: we calculated the CV for each assay from these measurements.

### Data analysis

The main outcomes of the trial were the differences in changes from baseline to week 12 in serum vitamin B-12 and serum folate.
between the fortified bread and placebo groups. Secondary outcomes were the differences in the changes in methylmalonic acid, holotranscobalamin, homocysteine, and erythrocyte folate between the fortified bread and placebo groups.

In addition, we assessed whether the response in markers of vitamin B-12 status depended on baseline vitamin B-12 status. The serum vitamin B-12 assay (Beckman Coulter) defined 133 pmol/L as the cutoff value for marginal or indeterminate vitamin B-12 status. We assessed the changes in each biomarker within the various quintile categories of the biomarkers. We also defined our own cutoffs for vitamin B-12 concentrations below this cutoff at baseline and at the end of the intervention. Furthermore, we divided the participants in quintile categories based on baseline serum vitamin B-12, baseline holotranscobalamin, and baseline methylmalonic acid. We assessed the changes in each biomarker within the various quintile categories of the biomarkers.

To test differences between groups, we used unpaired Student's t tests or ANOVA with a post hoc Bonferroni test for normally distributed variables and Mann-Whitney U tests for variables that were not normally distributed. All statistical analyses were carried out on an intention-to-treat basis with SPSS 12.0.1 (SPSS Inc, Chicago, IL).

RESULTS

The characteristics of the participants assessed during the recruitment were similar for both the fortified and the placebo groups (Table 1). Serum folate and vitamin B-12 concentrations were within the normal range for this age group in the Netherlands (34), but lower than concentrations typically found in the United States, probably because of the use of fortified breakfast cereals, spreads, and supplements in the United States.

Compliance was assessed from the food diaries; 139 of the participants consumed ≥3 slices of bread during ≥90% of the trial period. The fortified bread group, on average, consumed 4.6 slices of bread per day, and the placebo group consumed 4.3 slices per day (Table 2). The mean (±SD) additional intake of folic acid from fortified bread was 138 ± 47 μg/d and of vitamin B-12 was 9.6 ± 3.2 μg/d. Fortified bread increased serum folate concentrations by 45%, or 6.3 nmol/L (95% CI: 4.5, 8.1 nmol/L), and serum vitamin B-12 concentrations by 49%, or 102 pmol/L (95% CI: 82, 122 pmol/L), relative to unfortified bread. Fortified bread increased erythrocyte folate concentrations by 22% and holotranscobalamin concentrations by 35%. Fortified bread decreased homocysteine concentrations by 13% and methylmalonic acid concentrations by 10% relative to unfortified bread.

Exclusion of the 27 participants that shared a household with another participant did not substantially alter the findings of this study (data not shown).

The proportion of participants with marginal or indeterminate vitamin B-12 status (serum vitamin B-12 < 133 pmol/L) decreased from 8% at baseline to 0% at the end of the intervention in the fortified bread group, and the proportion increased from 4% at baseline to 9% after the intervention in the placebo group (Figure 2). In addition, the proportion of participants with marginal vitamin B-12 status according to the other markers of vitamin B-12 status decreased substantially in response to the consumption of fortified bread.

The responses of vitamin B-12 and holotranscobalamin were similar within the different quintile categories of baseline vitamin B-12 and holotranscobalamin status, but the participants in the highest quintile category of baseline methylmalonic acid status had a statistically significantly larger response to the fortified bread than did the participants in the first 2 quintile categories (Figure 3).

DISCUSSION

In this population of healthy older people, bread fortified with modest amounts of folic acid and vitamin B-12 eliminated the presence of low serum vitamin B-12 concentrations. It improved...
serum folate concentrations by 45% and serum concentrations of vitamin B-12 by 49% compared with unfortified bread. Consequently, cofortified bread appears promising as a means of simultaneously improving the folate and vitamin B-12 status of older people.

The sensitivity and specificity of serum vitamin B-12 concentrations in diagnosing deficiency is reportedly low (35) and methylmalonic acid and holotranscobalamin concentrations have been suggested as better indicators (35–37). Because there is no consensus about the best biochemical test to diagnose vitamin B-12 deficiency, we assessed the effect of vitamin B-12 fortification on all available biochemical markers of vitamin B-12 status. Moreover, because there is no agreement on the absolute values for definition of vitamin B-12 deficiency (38), we used 2 different cutoffs for serum vitamin B-12 and showed that consumption of fortified bread markedly improved vitamin B-12 status irrespective of which biomarker or cutoff was used to define vitamin B-12 deficiency.

We fortified bread with folic acid at a level comparable with that applied in flour fortification in the United States (24). The average actual additional intake of folic acid due to fortification in the United States was estimated to be $\approx 200\, \mu g/d$ (39, 40) due to overage. Neural tube defects decreased by 25% since the start of the fortification program in the United States (41) and by 50% in Canada (42). Folic acid fortification at the level used in the current study might prevent $\approx 20\%$ of neural tube defect–affected pregnancies in the United Kingdom (5). In addition to the established effect of folic acid on birth defects, folic acid has been suggested to reduce the risk of cardiovascular disease, certain types of cancer, and the rate of cognitive decline in elderly people (43–45). Vitamin B-12 fortification may reduce both the rate of cognitive decline (46) and the risk of neural tube defects (47).

We chose to fortify bread with $6\, \mu g$ vitamin B-12/d rather than with the current Recommended Dietary Allowance (RDA) of $\approx 3\, \mu g/d$. Actual intake of vitamin B-12 was somewhat higher because participants ate more than the minimum 3 slices per day. We chose the amount of 6 $\mu g$ for several reasons. First, the current RDA was established based on the absence of anemia and neurologic symptoms, whereas it is unknown whether it will also prevent other adverse health consequences (26). Second, a recent study reported that a dietary intake of $\geq 6\, \mu g/d$ optimized all vitamin B-12–related markers in 98 postmenopausal women (26). Last, studies with vitamin B-12–deficient individuals showed that $2.5–10\, \mu g/d$ will improve serum vitamin B-12 concentrations by 20–60 pmol/L (18, 21, 22) and will decrease
methylmalonic acid concentrations by $\approx50\%$ of the effect observed at high doses (18).

A limitation of our study was that it was too short to assess the effect of fortification on other aspects of health status, such as cardiovascular disease (48) and cognitive impairment (46, 49). Indeed, assessment of the effects of cofortification on other markers of health would have required a much larger number of participants and would have required that the trial be continued for a much longer time. Furthermore, because we lacked a control on the causes of low vitamin B-12 status, we were unable to specifically evaluate the ability of cofortified bread to improve the vitamin B-12 status of older persons with atrophic gastritis—the most common cause of low vitamin B-12 status in older people (50).

Delays in the introduction of folic acid fortification in many countries have resulted because of concerns about the masking of vitamin B-12 deficiency, but also for other reasons. Specifically, although high folate intakes are associated with a decreased risk of cancer in epidemiologic studies, animal studies suggest that high doses of folic acid could promote the growth of existing tumors (51). Studies conducted after the introduction of folic acid fortification in the United States in 1998 reported a possible increase in colorectal cancer rates (52) and a higher prevalence of anemia and cognitive impairment in elderly persons with a high serum folate and a low vitamin B-12 status compared with elderly persons with normal status (53). Furthermore, folic acid may interfere with antifolate chemotherapy and with anticonvulsant therapy. However, the evidence underpinning possible negative effects of folic acid fortification is far from conclusive (5). Countries need to consider all possible benefits and harms before they decide to start fortification (5, 54, 55).

Will the low doses of vitamin B-12 administered in our trial be beneficial for individuals with marginal or deficient status? Individuals with pernicious anemia, ie, lack of intrinsic factor, can only passively absorb $\approx1\%$ of crystalline vitamin B-12 (50); such individuals are unlikely to benefit from fortification and will still require parenteral vitamin B-12 therapy by intramuscular injections. However, the main cause of low vitamin B-12 status in older persons is the reduced ability to extract vitamin B-12 from food protein, because of age-related gastric atrophy (36, 46, 56, 57). Absorption of crystalline vitamin B-12 should be unimpaired in such cases; hence, most older persons with a low vitamin B-12 status will probably be responsive to fortified food. On the other hand, a study by Eussen et al (18) suggests that daily oral doses of 650 to 1000 $\mu$g/d were needed to maximally decrease methylmalonic acid concentrations in individuals with a marginal status. This finding appears consistent with the persistence of elevated methylmalonic acid concentrations in 11% of the participants in our trial, despite normalization of other biomarkers of vitamin B-12 status. However, it is important to emphasize that our cutoff for an elevated methylmalonic acid concentration was 0.23 nmol/L, which is lower than the 0.32 nmol/L cutoff in the study by Eussen et al (18).

In conclusion, bread fortified with modest amounts of folic acid and vitamin B-12 improve folate and vitamin B-12 status and may prevent a considerable proportion of vitamin B-12 deficiency in older people. Several large-scale trials of folic acid and vitamin B-12 are currently assessing the effects of long-term dietary supplementation with folic acid and vitamin B-12 on vascular and nonvascular outcomes (48). However, further large-scale trials of vitamin B-12 supplementation are needed to assess the effects on nonvascular outcomes of vitamin B-12 supplementation in elderly populations in the absence of anemia or cognitive impairment.

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The authors’ responsibilities were as follows—RMW and IAB: executed the study; and RMW: wrote the manuscript with extensive input from all authors. All authors participated in the conception and design of the study. PV is an employee of Unilever. Unilever markets food products, some of which are enriched with B vitamins. None of the other authors declared a conflict of interest.

REFERENCES


FOOD FORTIFICATION AND FOLATE AND VITAMIN B-12 STATUS


