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Determination of the bioavailability of food folates in a controlled intervention study.

Mary P Hannon-Fletcher[1*], Nicola C Armstrong [1], John M Scott [3], Kristina Pentieva[1], Ian Bradbury[1], Mary Ward[1], JJ Strain[1], Adele A Dunn[2], Ann M Molloy [3], Maeve A Scullion[1], Helene McNulty[1]

[1]Northern Ireland Centre for Diet and Health, and [2]School of Hotel, Leisure and Tourism, University of Ulster, Coleraine, Northern Ireland BT52 1SA; [3] Department of Biochemistry, Trinity College, Dublin 2, Ireland

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*Author to whom correspondence should be addressed, telephone (44) 2870323247, fax (44) 2870324965; e-mail mp.hannon@ulster.ac.uk

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ABSTRACT

Background: The concept of Dietary Folate Equivalents (DFE) in the US recognizes the differences in bioavailability between natural food folates and the synthetic vitamin, folic acid. However, many published reports on folate bioavailability are problematic as a result of a number of confounding factors.

Objective: The aim was to compare the bioavailability of food folates with folic acid under controlled conditions. To broadly represent the extent to which natural folates are conjugated in foods, we used two natural sources of folate, spinach and yeast, in which folates are present as 50% and100% polyglutamyl folate, respectively.

Design: 96 male subjects were randomized on the basis of their screening plasma homocysteine (tHcy) to one of four treatment groups for an intervention period of 30-days. Each subject received (daily under supervision) either a folate depleted "carrier" meal or a drink plus: a) placebo tablet; b) 200µg folic acid in a tablet; c) 200µg natural folate provided as spinach; or d) 200µg natural folate provided as yeast.

Results: Among those who completed the intervention, responses (increase in serum folate, lowering of tHcy) compared to placebo (n=18) were significant in the folic acid group (n=18), but not in the yeast folate (n=19) or the spinach folate (n=18) groups. Both natural sources of folate were significantly less bioavailable than folic acid. Overall estimations of folate bioavailability were found to be between 30% (spinach) and 59% (yeast) relative to folic acid.

Conclusion: Relative bioavailability estimates were consistent with those from the metabolic study which was used as a basis to derive the US DFE value.

KEY WORDS: Food folate; bioavailability; homocysteine; folic acid.

INTRODUCTION

Folate is attracting major interest in recent years as having an established role in the prevention of neural tube defects (NTD, 1,2) and possible preventive roles against cardiovascular disease (3), certain cancers (4) and neuro-psychiatric conditions (5). For the prevention of NTD, official bodies worldwide recommend women to take an additional 400-μg/d folate before conception and in early pregnancy. However, the achievement of such recommendations is problematic. Although folic acid supplements are very effective in optimizing folate status in women who receive them (6), they do not offer an effective strategy for the primary prevention of NTD because of poor compliance (7). Therefore, in recent years, mandatory fortification of grain products with folic acid has been introduced in the United States (8) and elsewhere (9). Despite impressive decreases in the incidence of NTD since the introduction of these new policies (10,11), fortification remains controversial. It is untargeted and therefore, delivering the required nutrient levels to the at risk group, inevitably results in a proportion of the general population being exposed to high levels. Of greatest concern is the potential for high intakes of folic acid to mask the anemia of vitamin B_{12} deficiency in elderly people, thereby allowing the concomitant irreversible nerve degeneration to go undetected (12). The third approach to optimize folate status, which does not have such health concerns, is by increased consumption of foods naturally rich in folate. However, the effectiveness of this approach has been found to be somewhat limited (13,14,15), a finding generally attributed to the poor bioavailability of natural food folates compared with the synthetic vitamin, folic acid.

Depending primarily on the methodological approach used, previous human studies have estimated the bioavailability of food folates relative to folic acid to range anywhere between 10% and 98% (16-21). The uncertainty regarding folate bioavailability (15) is of particular

concern for countries without folic acid fortification (including some which do not even permit it on a voluntary basis)(22), and therefore, a high dependency on natural food folates as a means to optimize status. In addition, although mandatory folic acid fortification in the US means that there is relatively less reliance placed on natural folate sources, US dietary recommendations are now based on the greater bioavailability of folic acid added to food compared with natural food folates, with the recent introduction of Dietary Folate Equivalents (DFE)(23). The estimated DFE conversion factor of 1.7 is largely based on one metabolic study in non-pregnant women which estimated the bioavailability of food folates to be no more than 50% that of folic acid (19), and other evidence showing that folic acid added to food had about 85% the bioavailability of free folic acid (24).

The aim of this study was to compare the bioavailability of food folates with folic acid under controlled conditions. The approach was to administer natural sources of folate under supervision at a dose (of pre-determined folate content) within the physiological range, but sufficiently concentrated so as to elicit serum folate and plasma homocysteine (tHcy) responses, for comparison with an equivalent dose of folic acid. In order to broadly represent the extent to which natural folates are conjugated in foods, we used two folate-rich sources, spinach and yeast, in which folates are present as 50% and 100% polyglutamyl folate, respectively.

SUBJECTS AND METHODS

Subject recruitment and screening

The Research Ethical Committee of the University of Ulster granted ethical approval, and subjects gave written informed consent at the time of recruitment to the study. Healthy men, aged 18-45 years were recruited between December 2000 and September 2001 from the staff and student population at the University of Ulster, the Causeway Health and Social Services Trust, Coleraine; and the FG Wilson engineering firm, Belfast. All potential subjects were interviewed, using a short medical questionnaire about their general health, medication and supplement use, to identify those meeting the following inclusion criteria: no history of gastrointestinal, vascular, hepatic, renal disease or hematological disorders, not taking B vitamin supplements nor consuming folic acid-fortified foods, not taking drugs known to interfere with folate metabolism. In addition, a blood sample was collected in order to screen volunteers for identification of the 677C→T (thermolabile) variant of the methylenetetrahydrofolate reductase (MTHFR) gene, and to determine plasma homocysteine (tHcy) concentrations. Individuals who were found to be homozygous for the 677C→T mutation (i.e. TT genotype) were excluded from the study.

Intervention

Suitable subjects were randomized on the basis of their screening tHcy levels to one of four treatment groups. The subjects received either a folate depleted "carrier" meal or a drink plus: a) placebo tablet; b) 200µg folic acid in a tablet; c) 200µg folate provided as spinach; or d) 200µg folate provided as yeast.

Pre-intervention treatment

For a 4-week run-in period prior to commencement of the folate intervention described below, all subjects (irrespective of the treatment group) were administered daily with oral supplements of vitamin B_6 (1.6mg/d) and vitamin B_{12} (1.5µg/d), doses equivalent to UK Reference Nutrient Intake values (25). In order to monitor compliance, subjects were provided with these supplements on a weekly basis in a 7-day pill organizer box (Carepac, Farringdon, UK), and asked to return the box at each visit; any missed doses were recorded. This treatment was continued for the duration of the entire study, i.e. until completion of the folate intervention.

Folate intervention

The folate intervention was conducted as a placebo controlled, blind study, which was carried out over a 6-week period, during which treatments were administered 5 days per week (i.e. in total, a 30 day folate intervention). In order to ensure compliance, subjects were supervised while taking the treatments on a daily basis. Each of the four treatments was administered in one of two ways, either as a meal (with other food present) or as a drink (with no other food present).

<u>Administration of treatments as a meal</u>

Each morning a "carrier meal" was freshly prepared by the catering staff at the School of Hotel, Leisure and Tourism, University of Ulster, Portrush, Northern Ireland, under the supervision of two colleagues (MHF and one other author, MAS) from the Northern Ireland Center for Diet and Health (NICHE), University of Ulster, Coleraine. A total of 4 carrier meals were devised and rotated on a weekly menu cycle. Ingredients selected for use in the carrier meals were of low folate content, according to the British Food Composition Tables

(26). The ingredients (for full list see **Appendix**) were thrice boiled in order to reduce the water-soluble micronutrient content (i.e. placed in cold water and taken to boiling temperature for a minimum of one minute, the water was removed and replaced with fresh cold water; this was carried out three times before the food was finally cooked). For each of the carrier meals, a duplicate meal was retained and stored for later analysis of total folate content, this was repeated on two separate occasions during the folate intervention.

Volunteers attended our catering center daily between 12 noon and 2pm to receive their intervention treatments under supervision. The carrier meal was provided as a lunch to all volunteers irrespective of their treatment group allocation. Each subject received either the carrier meal alone (placebo treatment) or the carrier meal enriched to provide 200µg of natural folate provided from one of two natural folate sources, either lyophilized spinach (7.8g; Kanegrade, Stevenage UK) or lyophilized yeast (4.1g; Allinson, Castleford UK), with poly- to monoglutamate ratios of approximately 50:50 and 100:0, respectively. The natural folate source was added to the carrier meal after the meal was fully cooked, and immediately before starting serving, the meal was then maintained under heat lights while being served. In addition, after consuming half of the meal, all subjects received a pill, either placebo or 200µg synthetic folic acid. Subjects drank only water and were not permitted to use additional sauces or seasoning with the meal. Volunteers were instructed to follow their usual dietary pattern for all other meals and snacks consumed during the intervention period.

<u>Administration of treatments as a drink</u>

Volunteers received either a placebo drink or a drink that provided 200µg of natural folate in a disposable plastic cup at their place of work mid morning (10-11am) under the supervision of two colleagues (MHF and NCA). The drinks were prepared freshly before each

administration as follows: 7.8g lyophilized spinach or 4.1g lyophilized yeast (the equivalent of 200µg total folate) were added to 20 ml water. The drinks were mixed vigorously; 50ml of sugar free lemonade was added and again mixed. The placebo drink consisted of 20ml of water and 50ml sugar free lemonade, with no other ingredient. All drinks were prepared at the same time each morning and consumed within 2 hours. Volunteers also received a pill, either placebo or 200µg synthetic folic acid, which was taken after consuming the first half of the drink. Any residue remaining in the cup was rinsed with a small volume of lemonade, which the subject was required to drink in order to ensure the ingestion of the entire treatment dose. Apart from this drink, subjects were instructed to follow their usual dietary pattern for the duration of the study.

Laboratory methods

Blood sampling and analysis

Blood samples were collected following an overnight fast (min 12 hours) at screening, pre-intervention with folate and post-intervention with folate. For each time point (other than screening), two blood samples were collected 2-4 days apart (shown to be the optimal time interval between repeated blood sampling for measurement of tHcy) (27). A total of 22ml of blood was collected from each subject into EDTA-coated pre-evacuated blood tubes for full blood count, whole blood folate, plasma pyridoxal- 5'phosphate (PLP), and tHcy analysis, or into a Vacuette Serum Separator tube (Greiner Labortechnik, Germany) for analysis of serum folate and serum B12. Samples for PLP and tHcy analysis were wrapped in foil and placed on ice immediately after collection. Sample preparation and fractionation were performed within 0.5 to 2.5 h of the time of sampling as described in detail elsewhere (28) and fractions were stored at -70^{O}C for batch analysis at the end of the study and at -20^{O}C for extraction of DNA.

Full blood counts were carried out on whole blood with an automated Coulter Counter (Causeway Health and Social Services Trust Laboratories, Coleraine, Northern Ireland). Plasma tHcy was measured by immunoassay (29). Red blood cell folate (30), serum folate (30), and serum vitamin B-12 (31) were measured by microbiological assay. Plasma PLP was measured by reversed-phase HPLC with fluorescence detection (32). For all assays, samples were analyzed blind, in duplicate and within 6 months of sampling. Quality control was provided by repeated analysis of stored batches of pooled plasma (for tHcy and PLP), serum (for folate and vitamin B-12), and red blood cell lysates (for folate), covering a wide range of values in each case.

From screening samples, DNA was extracted from frozen whole blood by incubating with proteinase K (Gibco Life Technologies, Paisley, UK) as described in detail by Kawasaki (33), or using the QIAamp DNA Blood Mini Kit (UK QIAGEN Ltd., Crawley, West Sussex). The MTHFR 677C→T mutation (i.e. TT genotype) was identified by polymerase chain reaction (PCR) amplification followed by *Hin*F1 restriction digestion (Gibco Life Technologies, Paisley, UK), as previously described (34).

Natural folate analysis

The total folate content of the yeast, spinach and "carrier meals" was measured by microbiological assay with *Lactobacillus casei* NCIB 10463 (30) following thermal extraction and trienzyme (α-amylase, protease and conjugase) treatment according to the procedure of Tamura (1998) (35). The calibration of the assay was performed using folic acid (Sigma Chemical Co, Poole Dorset) as a standard. Under the conditions of the assay in our laboratory (pH 6.7 of the assay medium) *L. casei* shows equivalent responses to the main folate derivatives found in foods. Folate assays were performed both at the start and at the end of the

intervention period (in each case, triplicate measurements on two separate occasions two days apart). The coefficient of interassay variation in folate content of quality control samples was 5.5% (n=48). The folate polyglutamate content in yeast and spinach was determined as the mean difference in total folate content of samples treated with and without folate conjugase.

Dietary assessment

Dietary intake was recorded during the intervention period by a self-administered 4-day food diary (2-week days and 2-weekend days). Food intake data were analyzed for energy and nutrient intakes using the dietary analysis program WISP (WISP for Windows version 1.28, Tinuviel Software, Warrington, UK).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS version 11; SPSS Inc., Chicago, IL, USA) was used to compare the effects of intervention among the treatment groups using ANCOVA. The pre-treatment value was used as a covariate; pre and post treatment values were log-transformed. Treatment comparisons were made using Tukey's test multiple comparisons procedure, values <0.05 were considered significant.

In order to represent the response to intervention of food folates relative to that of folic acid, estimations of relative bioavailability (%) were calculated as follows:

$$RB = \frac{\bar{x}_t - \bar{x}_p}{\bar{x}_f - \bar{x}_p} \text{ x } 100$$

where RB is the relative bioavailability, $\bar{x}_t$ is the treatment group (yeast or spinach) mean response, $\bar{x}_p$ that in the placebo group and $\bar{x}_f$ that in the folic acid group. 95% confidence intervals were calculated by bootstrapping and truncated at zero (36).

RESULTS

Baseline data

Of 127 subjects initially recruited and screened, 96 satisfied the inclusion criteria and proceeded to intervention (24 to each of four treatment groups), of which 74 subjects completed the study **Figure 1**. Subjects either withdrew from the study voluntarily or were withdrawn if their attendance (compliance) was less than 95% (in practice this meant failure to attend on more than one occasion over the 30 d intervention). Subjects who successfully completed the intervention had an attendance rate of 100%. The baseline characteristics of this cohort expressed as median and 25^{th}-75^{th} quartiles are presented in **Table 1**. No subject was found to have deficient status of folate (serum or red cell folate) or related B vitamins (plasma PLP or serum B12) prior to the intervention period.

Following randomization of subjects by tHcy concentrations at screening, there were no significant differences between groups in tHcy, serum folate or red cell folate, either before or after the 4-week run-in period with physiological doses of vitamins B6 and B12 (independent t- test; results not shown). However, as expected, significant increases (before vs. after 4-week run in period, paired t-test) in both PLP (73.4nmol/L vs. 108nmol/L; $p< 0.001$) and serum B12 (297pmol/L vs. 325pmol/L; $p< 0.05$) were observed. Although treatment with vitamins B6 and B12 continued for a further 6-weeks (i.e. throughout folate intervention) no further increase in either parameter was observed (i.e. week 4 vs. week 10).

Natural folate analysis

Analysis of the carrier meals (two separate measurements for each of four meals, each assayed in triplicate) for total folate content showed a mean folate value of 44.89 ± 16.5 µg/meal (**Appendix**).

The total folate content of yeast and spinach, analyzed both at the start and at the end of intervention for each folate source (in each case, triplicate measurements on two separate occasions two days apart) showed that the quantity of natural folate sources required to provide 200 μg folate corresponded to mean weights of 4.1 g (4.16g ± 0.44 at the start; 3.78g ± 0.46 at the end of intervention) of lyophilized yeast, and 7.8 g (7.77g ± 0.94; 7.54g ± 0.86) of lyophilized spinach. The ratio of poly- to mono-glutamate folate in spinach and yeast was found to be 50:50 and 100:0, respectively, whether this was measured at the start or at the end of intervention.

Intervention

In order to determine the relative effects of intervention with the various treatments, the response (post-intervention value minus the pre-intervention value) of each treatment was compared among the four treatment groups (**Table 2).** The folic acid response (both tHcy and serum folate) was significantly different from placebo, spinach and yeast, no other significant differences were observed. The overall bioavailability of these representative natural folate sources relative to folic acid (and adjusted for placebo effect) was estimated to be 30% (tHcy response 23%; serum folate response 36%) for spinach, and 59% (56%; 62%) for yeast. Thus the average bioavailability of these representative food folate sources was estimated to be 45%.

Analysis of food intake data for total energy and total folate are presented in **Table 3**, and showed no significant differences among the four treatment groups.

Percentage responses to intervention (the post-intervention value minus the pre-intervention value expressed as a percentage of the pre-intervention value) for both serum folate and tHcy are shown in **Figure 2.** The percentage response of both parameters to folic acid was significantly different from the response to placebo, spinach and yeast, no other significant differences were observed.

DISCUSSION

In devising the DFE conversion factor in the United States, experts drew heavily on one metabolic study in women conducted several years ago which estimated folate bioavailability from a mixed diet to be no more than 50% relative to that of folic acid (19). Reported estimates of food folate bioavailability since then are highly variable. Representative natural folate sources in the current study were found to be significantly less bioavailable than folic acid, with estimated relative bioavailability consistent with the report of Sauberlich *et al.* based on a mixed diet (19).

The bioavailability of folates from various foods is considered to be dependent on the content of monoglutamyl and polyglutamyl folates, and the presence of components that can inhibit both intestinal folate deconjugation and specific transport processes of folate (17). Dietary folates (excluding fortified foods) are comprised of about one third monoglutamate (derived mainly from bread and meat) and two-thirds polyglutamate (derived mainly from vegetables) (37). In the current study, we used two folate-rich sources, spinach and yeast, not because we wished to specifically study these foods as sources of folate *per se*, but rather to broadly represent the extent to which natural folates are conjugated in foods. Thus while yeast is not an important dietary source of folate, its inclusion in our study enabled us to compare a folate source which was entirely in the polyglutamate form with another which had a much lower content (50%) of polyglutamyl folate. Although we showed no significant difference in the responses between these two food folate sources, the trend seen was consistently towards higher bioavailability (whether based on serum folate or tHcy responses) of folate from yeast compared to spinach. Given that folate in yeast is all in the polyglutamate form, and is even reported to contain potent inhibitors of certain conjugases (38), our results provide no evidence to support the view that the extent of glutamation is a limiting factor in the

bioavailability of folates from natural sources. Such observations are in good agreement with previous findings (39) from studies using exogenous deuterium-labeled monoglutamyl and polyglutamyl folates added to various foods, which showed equivalent bioavailability for the two folate forms. Results from the current study and the aforementioned study (39), are consistent with earlier observations (40) indicting that the activity of human jejunal brush border conjugase exceeds that needed for hydrolysis of polyglutamyl folates within the range of dietary intake and, therefore, was not rate limiting in the absorption process.

Apart from the activity of the conjugase enzyme, factors considered to influence the bioavailability of ingested folates include the presence or absence of other components in the diet or in the intestinal milieu that may inhibit or enhance absorption (41). Pfeiffer et al. (24) for example reported a small reduction in absorption of [^{13}C5] folic acid when administered after a light breakfast meal compared to its administration without food. Although in the current study all four treatments were administered in one of two ways, either in a drink with no other food present or as part of a meal, a sub-analysis of the overall results comparing the responses according to the route of administration was not possible because of insufficient subject numbers completing the meal arm (across the four treatment groups). Further studies are clearly required to address this issue.

Reported estimates of relative bioavailability of food folates show great variation, ranging from 10% to 98% (16-21), depending on the methodological approach and response index used. The interpretation of bioavailability studies in free-living subjects involving the provision of folate rich foods may be particularly problematic as a result of a number of confounding effects. The current study, in which all treatments were administered daily under supervision and were of predetermined folate content, allowed a number of potential

confounding effects to be overcome, including poor subject compliance and displacement of the usual dietary folate intake with intervention foods (13). In addition, all of the administered natural folate (provided as spinach or yeast) came from the same batch and was not subjected to cooking or further processing prior to ingestion, thereby eliminating the confounding effect of folate losses during cooking, which may be considerable in the case of green vegetables (42). Although stable-isotopic studies overcome these potential confounders, their applicability is limited somewhat in that in order to improve the precision of short-term studies, pre-saturation of tissues with folate is recommended, thereby creating a non-physiological condition (24). The bioavailability of natural folate sources estimated in the current study is much lower than that from a previous long-term intervention study (21) which estimated the bioavailability of food folates relative to folic acid to be between 60 and 98% (depending on the endpoint used). The strength of the latter study (21) lies in its attempt to assess folate bioavailability from a mixed diet rather than from individual foods. The unexpected findings, however, may be the result of one or more of the following confounding factors. First, the response of folic acid may have been underestimated as a result of administering 500µg folic acid every-other-day, on the assumption that it would be equivalent to 250µg daily. Higher intakes of folic acid (i.e. doses in excess of 266µg) have been shown to exceed the metabolic capacity of the intestinal mucosa, resulting in the appearance of unreduced folic acid in the circulation (43), the uptake of which may not be equivalent to the reduced vitamin. A second limitation of the study is that the natural food folate and folic acid groups did not receive comparable doses of the vitamin (350µg of natural folate daily versus 500µg of synthetic folic acid every-other-day), although this clearly was not intended in the study design. There was some attempt to correct for the different doses at the analysis stage. Corrected values, however, may not necessarily represent relative food folate bioavailability

to the same degree as a study, such as the current one, in which equivalent doses were administered daily throughout the intervention period.

We used two response indices to assess folate bioavailability, serum folate and a functional biomarker of folate status, plasma tHcy, previously shown by us to be a reliable index which responds to low dose folic acid in a dose dependent manner (44). Our estimations of bioavailability of natural folate (relative to folic acid) are similar whether they are based on tHcy or serum folate responses (yeast folate 56% vs 62%; spinach folate 23% vs 36%, respectively). Thus, our results show good internal robustness in the estimation of folate bioavailability from natural sources. However, the use of tHcy responses in the determination of relative folate bioavailability required the inclusion in our study design of a 4-week pre-treatment period with physiological doses of vitamin B_{12} and $B_{6.}$ This was necessary in order to ensure that any homocysteine-lowering owing to the presence of these vitamins in foods was corrected prior to the folate intervention. Red cell folate responses were not used in the estimation of folate bioavailability because we considered that the duration of the intervention (30 d) was insufficient to observe a complete turnover of the red cell folate population (i.e. 120 days), and therefore fully reflect the effect of the red cell folate response.

In conclusion, the bioavailability of natural folate is now important given that the alternatives only offer partial solutions for addressing sub-optimal folate status in the general population, either because of limited compliance (in the case of folic acid supplementation) or safety concerns (in the case of fortification). By comparing the bioavailability of representative natural folate sources with folic acid, we estimate the relative bioavailability of natural folate to be approximately 45%. In addition to losses of natural folates due to their incomplete bioavailability shown here, in practice losses prior to ingestion may also decrease the amount

of available folate from natural sources, particularly in the case of green vegetables (42). Finally, the estimations of relative bioavailability in the current study are consistent with those estimated in the metabolic study by Sauberlich et al. (19) that was the cornerstone of the recently derived US DFE value.

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Contribution of authors:

Mary P Hannon-Fletcher: lead role in writing of the manuscript, data collection and analysis.

Nicola C Armstrong: assisted in manuscript preparation, lead role in data collection and analysis.

John M Scott: study design, assisted in manuscript preparation.

Kristina Pentieva: laboratory analysis and manuscript preparation.

Ian Bradbury: statistical analysis and statistical aspects of manuscript preparation.

Mary Ward: data analysis, assisted in manuscript preparation.

JJ Strain: study design, assisted in manuscript preparation.

Adele A Dunn: supervisor of food delivery, assisted in dietary aspects of manuscript writing.

Ann M Molloy: laboratory analysis, assisted in manuscript preparation.

Maeve A Scullion: assisted in food delivery, laboratory aspects and manuscript preparation.

Helene McNulty: study coordinator, study design, writing of manuscript.

Conflict of Interest:

None of the authors listed: Mary P Hannon-Fletcher, Nicola C Armstrong, John M Scott, Kristina Pentieva, Ian Bradbury, Mary Ward, JJ Strain, Adele A Dunn, Ann M Molloy, Maeve A Scullion and Helene McNulty, have any financial or personal interests in any company or organization sponsoring research, including advisory board affiliations which require to be disclosed.

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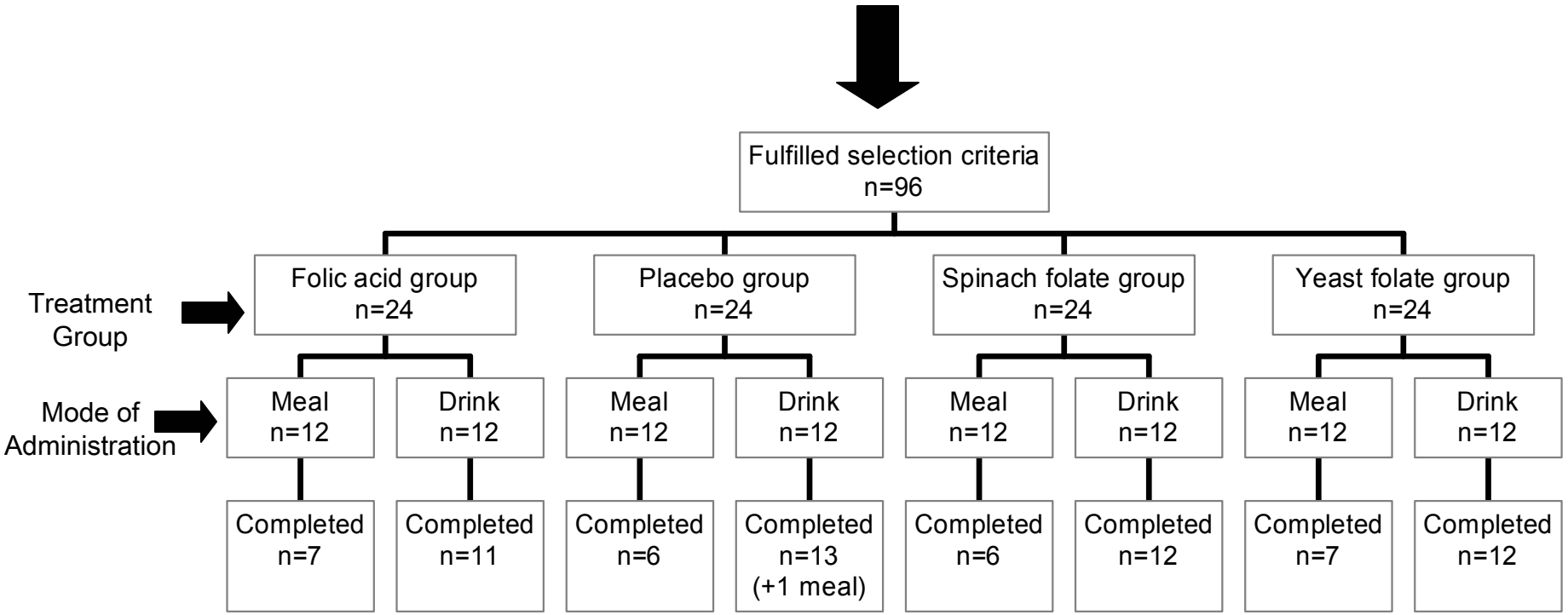
127 Subjects Screened
Fulfilled selection criteria n=96
Treatment Group
Folic acid group n=24
Placebo group n=24
Spinach folate group n=24
Yeast folate group n=24
Mode of Administration
Meal n=12
Drink n=12
Meal n=12
Drink n=12
Meal n=12
Drink n=12
Meal n=12
Drink n=12
Completed n=7
Completed n=11
Completed n=6
Completed n=13 (+1 meal)
Completed n=6
Completed n=12
Completed n=7
Completed n=12

Table 1: General characteristics, dietary status and laboratory nutrient status of subjects at screening*.

Parameter	Reference range	Median	25th –75th quartile
Age (years)	18-45 years	31.0	23.3 – 38.5
Body Mass Index (kg/m^2)	18.5-24.9[1]	26.3	23.5 – 28.8
Energy intake (MJ/d)	-	8.0	7.0 – 11.0
Folate intake (µg/d)	200[2]	183	140 - 235
B12 intake (µg/d)	1.5[2]	2.9	2.0 - 4.4
B6 intake (mg/d)	1.4[2]	1.9	1.8 - 2.4
Riboflavin intake (mg/d)	1.3[2]	1.3	1.0 - 1.5
Plasma Homocysteine (µmol/L)	5-15[3]	10.5	8.9 - 12.3
Serum Folate (nmol/L)	6.1-45[4]	16.67	12.3 – 22.1
Red Cell Folate (ng/mL)	150-1000[4]	385	303 - 485
Serum B12 (pg/mL)	150-1000[4]	415	291 - 554
Plasma PLP[5] (nmol/L)	>30[5]	67.7	48- 89

*median and 25th – 75th quartile.

n=74.

[1]Normal range of body mass index (BMI) for healthy males.

[2]Reference nutrient intake in the United Kingdom for males between the ages of 19-50y (26).

[3]Reference range for normal homocysteine concentration (44).

[4]Laboratory reference ranges for normal serum and red cell folate concentrations; Vitamin Research Laboratory, Trinity College Dublin.

[5]PLP: Pyridoxal 5'- phosphate, vitamin B6 (45).

Table 2: Plasma total homocysteine (tHcy) and serum folate responses to a 30-day intervention with 200μg natural folate/folic acid.

	Pre-intervention	Post-intervention	Response[1]	Relative Bioavailability[2]
Plasma homocysteine (μmol/L)				
Placebo (n=18)	11.6 ±3.7	11.8 ± 3.3	0.2 ± 1.2[b]	
Folic Acid (n= 18)	11.5 ± 3.0	10.1 ± 1.9	-1.4± 2.1[a]	
Spinach Folate (n=18)	12.1 ± 2.9	11.7 ± 2.5	-0.4 ± 1.1[b]	**23%**
Yeast Folate (n=19)	11.9 ± 3.2	11.2 ± 2.7	-0.7± 0.9[b]	**56 %**
Serum folate (nmol/L)				
Placebo (n=18)	15.9 ± 9.4	15.4 ± 8.4	-0.4 ± 4.2[b]	
Folic Acid (n= 18)	17.2 ± 11.9	21.6 ± 13.1	4.4 ± 4.8[a]	
Spinach Folate (n=18)	13.5 ± 5.1	15.2 ± 6.5	1.8 ± 4.0[b]	**36%**
Yeast Folate (n=19)	15.0 ± 7.2	17.6 ± 5.69	2.6 ± 3.5[b]	**62%**

Values are mean ± standard deviation and represent double (2-4 days apart), fasting samples. Responses were compared using Analysis of Covariance (ANCOVA) on log-transformed data (response = log $^{post}/_{pre}$). Means not sharing a common superscript letter are significantly different ($p < 0.05$) based on Tukey's test for multiple comparisons.

[1] Response refers to the post-intervention value minus pre-intervention value.

[2]Relative bioavailability refers to the response of yeast or spinach relative to the response of folic acid and corrected for the placebo response, for calculation see text. (95% CI calculated

by bootstrapping and truncated at zero%: spinach: tHcy, 0-80%; serum folate, 0-90%); yeast: tHcy, 20-170%; serum folate, 20-170%),

Table 3: Dietary total folate and total energy intake[1] in all treatment groups.

Treatment Group	Folic Acid	Placebo	Spinach	Yeast
Total Dietary Folate (µg/d) [2]	202 ± 84	186 ± 70	184 ± 70	211 ± 86
Total Energy (MJ/d)	9.11± 2.19	8.29 ± 2.17	8.70 ± 2.47	9.72 ± 2.37

Values are mean ± standard deviation.

No significant differences were observed for total folate ($p = 0.31$) or total energy ($p = 0.49$) intakes among the treatment groups, one-way ANOVA.

[1]Dietary intakes were measured mid-intervention.

[2]Folate intake values do not include the contribution from the folate treatments administered daily (200µg/d folic acid/ folate).

Appendix 1. General cooking instructions and ingredients used to prepare "carrier" meals (serve 4).

General Instructions for all meals to be prepared:	**Chicken Duxelle (Total folate: 63.2 ± 22.6µg/meal)**	**Pasta Bake (Total folate: 38.1± 15.4µg/meal)**
All vegetables, meat, pasta and rice used were thrice boiled. Dry ingredients such as herbs, spices, stock cubes (chicken & beef) salt & pepper and other ingredients used for flavourings such as Tabasco, Worchester sauce, gravy browning (black jack), honey, brown sugar, mustard (dry) and fresh garlic were not required to be pre-boiled. Bisto, soy sauce and canned tomatoes were not used.	Onions- 100g Mushrooms- 400g Chicken- 600 g Margarine- 25 g Eggs- 2 Milk-90ml Breadcrumbs-100g Nutmeg-10g Dry sherry-150ml Water- 360ml Demiglaze-70.35g Turnips-740g Carrotts-438g Salt-3g Pepper-1.5g	Pasta-200g Mushrooms-132g Smoked bacon (without fat)-147g Peppers (red)-124g Margarine-50g Flour (plain white)-50g Milk-720ml Salt-1.5g Cheese-371g Gloves-2 Bay leaf-1

Following the thrice-boiling ingredients used in the sauce were pan fried in oil (groundnut or olive) and garlic. Spices & herbs and flavourings were added to the pan and cooked together with the meat and vegetables for a few minutes and finally the stock (wine/sherry etc.) was added.	**Thai Green Curry (Total folate: 38.6 ± 5.3µg/meal)** Chicken- 655g Olive oil- 35g Chicken stock- 250ml (or half chicken stock cube) Garlic -6g Root ginger-4 g Coconut milk-115ml Green curry paste-5g Coriander leaves-10.5g Lime juice -9.5g Salt- 3g Rice-200g Black pepper-1g	**Chicken and Gammon Pie (Total folate: 39.5 ± 15.2µg/meal)** Chicken breast-375g Smoked gammon-350g Mushrooms-100g Margarine-50g Flour-50g Cream-200ml 1 chicken stock cube Water-750ml Carrots-480g Parsnips-300g Pastry (frozen)

Folate content of the carrier meals is expressed as mean ± standard deviation.

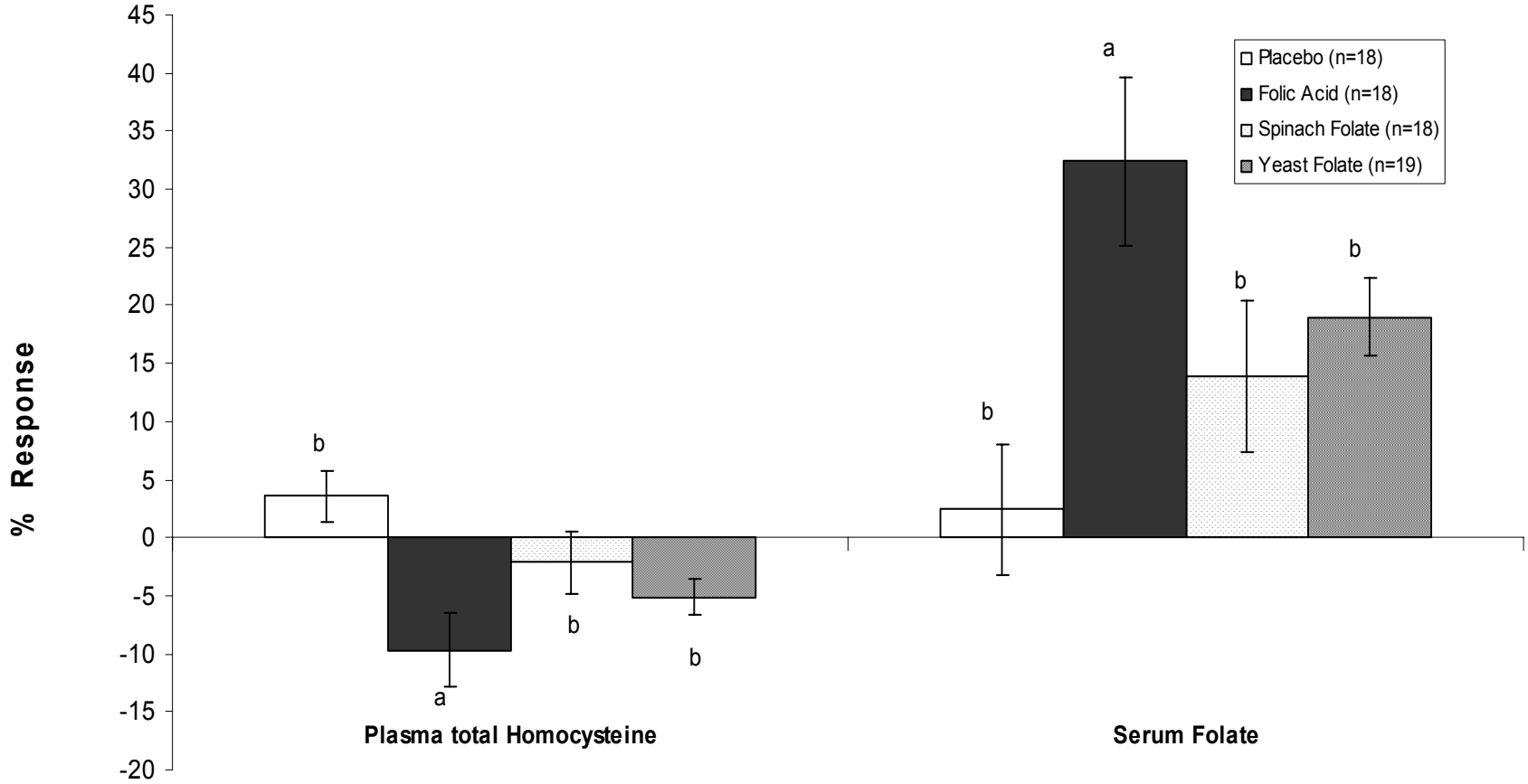
% Response
45
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
Placebo (n=18)
Folic Acid (n=18)
Spinach Folate (n=18)
Yeast Folate (n=19)
b
a
b
b
b
a
b
b
Plasma total Homocysteine
Serum Folate

Figure 1: Subject group allocation and rates of completion.

[1]one subject, who had been assigned to receive the placebo treatment as a meal, reported on day one that he would be unable to attend the catering centre at the specified time each day. This subject agreed to be reassigned to receive the treatment as a drink, to be administered daily (mid-morning) at his place of work.

Figure 2: Comparison of percentage response of plasma homocysteine and serum folate to a 30-day intervention with 200μg folate as either synthetic folic acid or natural folate source[1].

Values are mean ± SEM and represent double (2-4 days apart) fasting samples. Percentage responses (homocysteine and serum folate) among the four groups were compared using log-transformed data for normalization purposes. Means not sharing a common letter are significantly different ($p < 0.05$) based on Tukey's test for multiple comparisons.

[1]Natural folate sources: spinach folate, 50% polyglutamyl folate; yeast folate, 100% polyglutamyl folate.