THE SHORT-TERM BIOAVAILABILITY OF MICROENCAPSULATED FOLIC ACID AND L-5-METHYL-TETRAHYDROFOLATE

by

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ABSTRACT

Background: Folic acid (FA) is added to grain products in Canada to reduce the incidence of neural tube defects. There have been concerns that FA fortification may have adverse effects on the population. L-5-methyltetrahydrofolate (L-5-methyl-THF) is an alternative form of folate and may be safer than FA. However, L-5-methyl-THF has poor stability relative to FA limiting its use as a food fortificant. Microencapsulation of L-5-methyl-THF in a polyglycerol monostearate coating could improve its stability in food matrices.

Objective: To determine if microencapsulated FA and L-5-methyl-THF, either in capsule form or in skim milk powder, increases plasma folate concentrations over 8 hours, to the same extent, compared to free-form FA and L-5-methyl-THF.

Methods: In a repeated-measures crossover design, participants (n=15) were randomly administered seven treatments as single doses. The doses were based on the molar equivalent of 400 μg of FA. Treatments included: placebo, FA, microencapsulated FA, L-5-methyl-THF, microencapsulated L-5-methyl-THF, microencapsulated FA in milk powder, and microencapsulated L-5-methyl-THF in milk powder. Blood was collected at baseline and at frequent intervals over the 8-hour test period. Plasma folate was quantified at each time point and corrected to baseline values. Area under the curve (AUC) was calculated for each treatment and differences between treatments were evaluated using repeated-measures ANOVA.

Results: The AUC values (± SEM) for all six folate treatments were significantly greater than the placebo ($P<0.05$). AUC for L-5-methyl-THF was greater than FA (347±35 vs. 181±12 8h.nmol/L; $P<0.001$). The AUC for microencapsulated FA was not different than FA (222±12 vs. 181±14 8h.nmol/L; $P=0.12$). However, the AUC was less for microencapsulated L-5-methyl-THF than L-5-methyl-THF (147±17 vs 347±35 8h.nmol/L; $P<0.001$). The AUC for microencapsulated FA in milk powder was less than microencapsulated FA (120±11 vs. 222±12 8h.nmol/L; $P<0.001$) but microencapsulated L-5-methyl-THF in milk powder was not different than microencapsulated L-5-methyl-THF (178±14 vs 147±17 8h.nmol/L; $P=0.284$).

Conclusions: L-5-methyl-THF may have greater short-term bioavailability than FA. Microencapsulation had no effect on FA but decreased the bioavailability of L-5-methyl-THF. Adding microencapsulated FA to a skim milk powder decreased the bioavailability however there was no effect on microencapsulated L-5-methyl-THF.

PREFACE

This study was approved by the University of British Columbia Clinical Research Ethics Board. Certificate number: H10-00290.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
CRC	Colorectal cancer
DHFR	Dihydrofolate reductase
DRI	Dietary reference intake
EDTA	Ethylenediaminetetraacetic acid
FA	Folic acid
GRAS	Generally recognized as safe
L-5-methyl-THF	L-5-methyltetrahydrofolate
MFA	Microencapsulated folic acid
MMA	Methylmalonic acid
MRC	Medical research council
MTHFR	Methyltenetrahydrofolate reductase
NTD	Neural tube defect
PGMS	Polyglycerol monostearate
RBC	Red blood cell
THF	Tetrahydrofolate
tHcy	Total homocysteine
UL	Tolerable upper intake level

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1 INTRODUCTION

Folate is a B vitamin and refers to a group of compounds required for DNA synthesis and the transfer of methyl groups [1]. Folic acid is a synthetic oxidized form of folate that is used in supplements and as a fortificant because of its high stability compared to naturally occurring folates [2]. In 1998, the Canadian government mandated the addition of folic acid to grain products to reduce the incidence of neural tube defects. While folic acid has significantly reduced the rates of neural tube defects in Canada, there exist concerns that folic acid may have adverse effects in certain populations [3]. For example, excessive intakes of folic acid may mask the hematologic signs of vitamin B_{12} deficiency which may delay diagnosis and allow the progression of neurologic damage [4]. Furthermore, folic acid fortification in North America has coincided with a temporal rise in colon cancer rates [5].

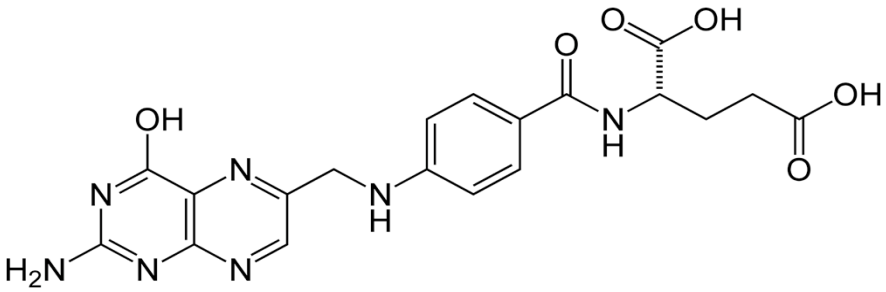
An alternative form of folate, L-5-methyltetrahydrofolate (L-5-methyl-THF), has become available commerically. L-5-methyl-THF is a fully reduced, naturally occurring form of folate [2]. It is less likely than folic acid to mask a vitamin B_{12} deficiency; therefore, it may be a safer fortificant [6]. While L-5-methyl-THF shows equal bioavailability compared with folic acid when given as a supplement, it is not as stable as folic acid in foods limiting its use as a fortificant [7]. Microencapsulating L-5-methyl-THF in a polyglycerol monostearate coating may improve its stability in food matrices [8]. However, it remains to be determined whether microencapsulated L-5-methyl-THF and FA have comparable bioavailability.

2 LITERATURE REVIEW

2.1 Chemical Structure

Folate is the generic name that encompasses the structurally related forms of the water-soluble vitamin B_9 [9]. Folic acid (pteroyl monoglutamate) is the synthetic form of folate used in supplements and fortified foods (**Figure 2.1**). Folic acid has the chemical formula $C_{19}H_{19}N_7O_6$ and a molecular weight of 441.4 g/mol [2]. The pteridine ring of folic acid is fully oxidized and resistant to chemical oxidation; therefore, it has a higher stability compared to naturally occurring, reduced forms of folate [10].

Figure 2.1. Chemical structure of folic acid [10]



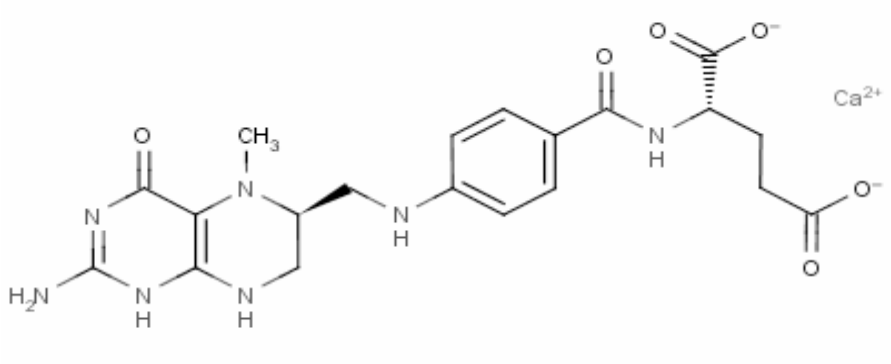
Naturally occurring folates exist in the polyglutamyl form containing a variable number of glutamate residues. There are numerous reduced forms of the vitamin that involve one-carbon substitutions of the pteridine ring (**Table 2.1**). However, the predominant tetrahydrofolate exists in the fully-reduced form L-5-methyltetrahydrofolate (L-5-methyl-THF) [10]. L-5-methyl-THF has the chemical formula $C_{20}H_{25}N_7O_6$ and a molecular weight of 464.5 g/mol [1].

Table 2.1. One carbon substitutions and oxidation states of tetrahydrofolates [2]

Form of Folate	Oxidation State	One-carbon Substitution
5-formyl-THF	Formate	-CHO
10-formyl-THF	Formate	-CHO
5-formino-THF	Formate	-CH=NH
5,10-methenyl-THF	Formate	=CH-
5,10-methylene-THF	Formaldehyde	$=CH_2$
5-methyl-THF	Ethanol	$-CH_3$

L-5-methyl-THF can also be artificially synthesized and is available in the pure crystalline form as a calcium salt marketed under the brand name Metafolin® by Merck Eprova^{AG} (**Figure 2.2**). Both the natural and synthetic forms of L-5-methyl-THF are biologically active and are directly bioavailable within the body [2]. L-5-methyl-THF has received the regulatory status of GRAS (generally recognized as safe); both forms can be legally used as a dietary ingredient in supplements and food products in Canada and a number of countries around the world [11].

Figure 2.2 Chemical Structure of L-5-methyl-THF [2]



2.2 Absorption, Distribution, Metabolism

2.2.1 Absorption

Prior to intestinal absorption, naturally occurring polyglutamate folates are hydrolyzed to the monoglutamyl form by the enzyme glutamate carboxypeptidase II (GCPII; EC: 3.4.17.21) also known as folate conjugase [12]. Folic acid and synthetic L-5-methyl-THF exist in the monoglutamate form and do not need to undergo this process. Absorption of both natural folates and folic acid occurs primarily in the brush-border membrane of the proximal small intestine [13]. Transport across the membrane is facilitated by the proton coupled folate transporter (PCFT). PCFT is a saturable transporter that has a similar affinity for both reduced and oxidized forms of folates [13].

During transport, folic acid is metabolized to the biologically active form of folate, L-5-methyl-THF [1].This occurs in a two-step reaction in which folic acid is first reduced to dihydrofolate (DHF) and then tetrahydrofolate (THF) by the enzyme DHF reductase (DHFR; EC 1.5.1.3). THF is subsequently metabolized to L-5-methyl-THF via serine hydroxymethltransferase (SHMT; EC: 2.1.2.1) and 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). At high intakes (>200 μg/d), transporters become saturated and unmetabolized folic acid passes directly into portal circulation [14].

2.2.2 Distribution

Folate circulates, primarily as L-5-methyl-THF, bound to plasma proteins such as albumin or in its free-form. L-5-methyl-THF is transported into peripheral tissues via the reduced folate carrier-1 (RFC1) [13]. RFC1 is specific for reduced folates and has a poor affinity for folic acid [9]. A different folate transporter, folate binding protein, is found in a small range of tissues including the placenta, proximal tubules of the kidney and the choroid plexus. Folate binding protein has a similar affinity for both oxidized and reduced forms of folate and is primarily responsible for reabsorption of folate in the kidneys [13]. PCFT, the intestinal transmembrane transporter, is also responsible for the transport of folate into some tissues, such as the liver.

Folate monoglutamates that are transported into cells must undergo metabolism to the polyglutamate form via the enzyme folylopolyglutamate synthetase (FPGS; EC: 6.3.2.16). L-5-methyl-THF is a poor substrate for FPGS and must be converted to THF by the vitamin B_{12} -dependant enzyme methionine synthase (EC: 2.1.1.13) before polyglutamylation can proceed. Conversion to the polyglutamate form of a chain length greater than three facilitates the accumulation and retention of folate by tissues [2].

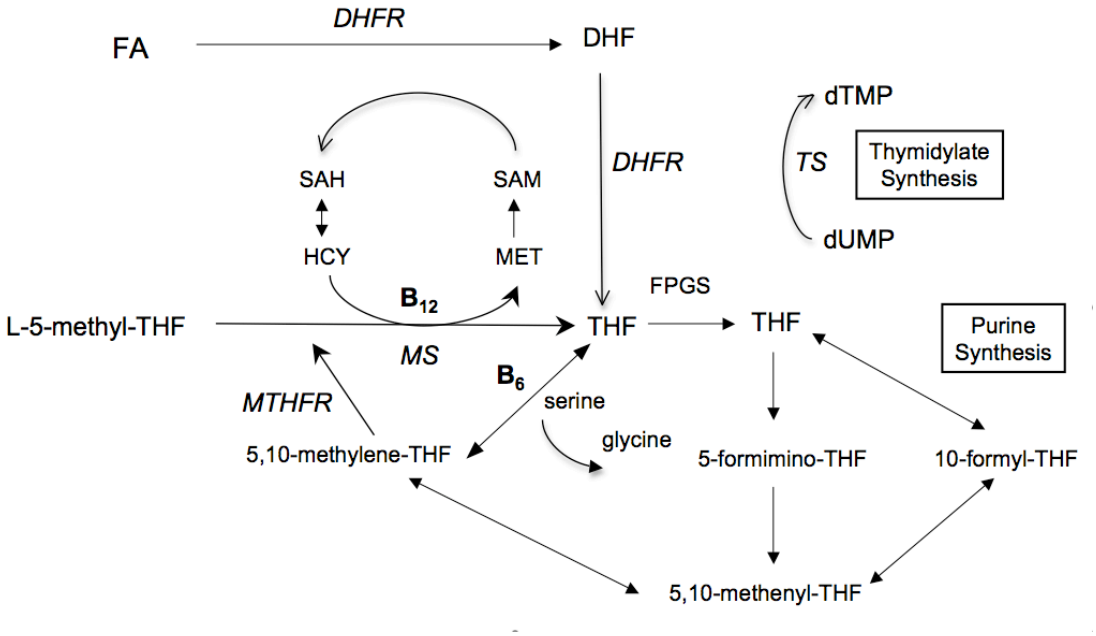
2.2.3 Metabolism

Within the cytosol of cells, folate functions as a coenzyme and is required in three interrelated metabolic pathways (**Figure 2.3**). These pathways are involved in the inter-

conversion between serine and glycine, the production of thymidylate and purines, and the remethylation of homocysteine to methionine. These cycles all take place in the cytosol; mammalian cells also have a large store of mitochondrial folate. Methyl groups that are used for folate metabolism come from formate or serine [1]. THF is converted to 5,10-methylene-THF by pyridoxal phosphate (PLP) and SHMT. This reaction requires vitamin B6 as a cofactor in the conversion of serine to glycine by accepting a hydroxymethyl group from 5,10-methylene-THF.

Folate is essential for the synthesis of thymidylate, a nucleotide. During this reaction, 5,10-methylene-THF methylates 2'-deoxyuridylate 5'-monophosphate (dUMP) to form 2'thymidylate 5'monophosphate (dTMP). Thymidylate synthase (TS; EC: 2.1.1.45) is the rate-limiting enzyme that catalyzes this reaction. In methionine synthesis, 5,10-methylene-THF is converted to 5-methyl-THF by MTHFR. The methyl group is transferred to homocysteine in a vitamin B_{12} dependent reaction. Methionine is then converted to *S*-adenosylmethionine (SAM) by methionine adenosyl transferase (EC: 2.1.5.6). SAM is a key methyl donor for DNA, RNA, proteins and phospholipids. Following methyl donation SAM is converted to *S*-adenosylhomocysteine (SAH) and following liberation of adenosine forms homocysteine [2].

Figure 2.3 One-Carbon Metabolism



FA folic acid; DHF dihydrofolate; DHFR dihydrofolate reducatase; THF tetrahydrofolate; L-5-methyl-THF L-5-methyltetrahydrofolate; MET methionine; SAM *S*-adenosylmethionine; SAH *S*-adenosylhomocysteine; MS methionine synthase; FPGS folate polyglutamate synthetase; MTHFR methylenetetrahydrofolate reductase; TS thymidylate synthase; dUMP

2.3 Consequences of Sub-optimal Folate Status

Folate plays an integral role in numerous metabolic pathways and as such, a folate deficiency poses severe health consequences. A folate deficiency is defined as red blood cell (RBC) folate < 305 nmol/L. This cut-off was established by the Institute of Medicine (IOM) based on reports showing that the risk of megaloblastic anemia increased markedly below this level [15]. Fortunately folate deficiency is uncommon worldwide. Sub-optimal folate status (RBC folate 305-905 nmol/L) is more prevalent in the population and has been associated with adverse health outcomes such as neural tube defects (NTDs) [16], cardiovascular disease [17] and certain cancers [18].

2.3.1 Megaloblastic Anemia

Megaloblastic anemia is characterized by the presence of large, immature red blood cells in the bone marrow. The dysfunctional red blood cells are known as megaloblasts and interfere with the body's ability to deliver oxygen to peripheral tissues [2]. Hematological markers associated with megaloblastic anemia include decreased RBC count, increased mean corpuscular volume (MCV) and hyper-segmented neutrophils [9]. The condition may be caused by severe folate and/or vitamin B_{12} deficiency and leads to a decrease in the production of the nucleic acid thymidylate inhibiting DNA synthesis during red blood cell formation [1]. Treatment with folic acid will correct the anemia if it is caused by a folate deficiency. Megaloblastic anemia resulting from a dietary deficiency of folate is rare in North America given the mandatory folic acid fortification policy [19].

2.3.2 Cardiovascular Disease

A lack of folate interferes with the remethylation of homocysteine to methionine, resulting in elevated plasma total homocysteine concentrations (tHcy) [2]. Interest in homocysteine first came about through studies of individuals with a rare genetic disorder of homocysteine metabolism known as homocystinuria [20]. This condition was characterized by high circulating tHcy with large amounts being excreted into the urine. If untreated, individuals were at increased risk for premature cardiovascular disease. Lowering tHcy reduced cardiovascular risk in this population [20]. This finding raised the notion that mildly elevated blood levels of homocysteine, a common occurrence in the general population, may be involved in cardiovascular disease. Indeed numerous observational studies have shown that an elevated tHcy is associated with increased risk of coronary heart disease, cerebrovascular disease, and peripheral vascular disease [21, 22].

Folic acid, with or without other B-vitamins, has been shown to lower circulating tHcy concentrations [23]. Unfortunately, in secondary prevention randomized studies, B-vitamin supplements, including folic acid, have lowered tHcy but have not reduced clinical events [24-26]. Eight randomized controlled trials that compared folic acid and B-vitamin supplement use with either placebo or usual care trials were included in a recent meta-analysis. No significant differences existed between the intervention and control groups. The overall relative-risk ratios (95% CI) for supplemented patients versus controls were 1.01 (0.97-1.05) for cardiovascular events; 1.01 (0.94-1.07) for coronary heart disease; 0.94 (0.85-1.04) for stroke; and 1.00 (0.95-1.05) for all-cause mortality [17]. Based on the results

of this meta-analysis, there is not sufficient evidence to support recommending B-vitamin supplementation in the secondary prevention of vascular disease.

2.3.3 Cancer

It had been postulated that low folate status may be a risk factor for certain cancers. Potential mechanisms include changes in DNA methylation, misincorporation of uracil into DNA, and strand breakage [27]. Evidence from epidemiological studies has previously shown that a high folate intake is inversely associated with risk of colorectal adenomas [28]. For example, a nested case-control study in the Women's Health Study found that women in the highest quartile of serum folate (>31.3 nmol/L) compared to the lowest (<12.2 nmol/L) had half the risk of developing colorectal cancer.

However, a large randomized controlled trial published by Cole et al. challenged the idea that folate supplementation is an effective strategy in decreasing the risk of developing colorectal cancer. The study found that supplementation with 1 mg/d of folic acid did not reduce the risk in participants that had a recent history of adenomas. It has been hypothesized that high folate intake prior to the existence of adenoma may prevent tumor development, but high folate intake may have carcinogenic effects in the case of an existing adenoma [29]. This hypothesis is discussed further in the section 2.7.3.

2.3.4 Neural Tube Defects

Sub-optimal maternal folate status during pregnancy increases the risk of preterm delivery, low birth weight and growth retardation [30]. However, the most serious risk posed by sub-optimal folate status during pregnancy is a neural tube defect (NTD) [31]. NTDs encompass a range of neurodevelopmental conditions that result from failure of the neural tube to close within the first 28 days post-conception [32]. The two most common types of NTDs are anencephaly and spina bifida. In the former, the brain fails to form properly and the neonate dies at birth or soon after. The latter is caused by the failure of the lower neural tube to close properly and may lead to varying degrees of paralysis [31]. The rate of NTDs in Canada prior to mandatory folic acid fortification was 0.77 per 1,000 births but there was considerable geographical variation across the country. A relationship between periconceptional folic acid intake and NTDs was first postulated in the 1960s. Twenty-five years later, definitive evidence emerged that supplementing women with folic acid during the periconceptional period could significantly reduce the risk of NTDs in offspring. In the UK, the Medical Research Council (MRC) Vitamin Study Research Group conducted a randomized controlled study in 1817 women planning a pregnancy that had a prior history of an NTD-affected pregnancy. A daily folic acid supplement (4 mg), during the periconceptional period reduced the recurrence of NTDs by 72% [33].

A similar study was conducted in Hungary, Czeizel in 1993 in women with no prior history of NTDs. In those women taking a multivitamin supplement containing 800 µg/day of folic acid there were no NTD cases. In comparison, there were six cases of NTDs in women who

received the placebo treatment [34]. Further evidence comes from a recent population-based health campaign involving 247,831 women in three provinces of China. In the province where the incidence of NTDs was highest (5-6 per 1000 births), risk of an NTD-affected pregnancy was reduced by 79% in women who took 400 μg of folic acid per day during the periconceptional period. In the other two provinces, where the incidence of NTDs was lower (1 per 1000 births), NTD-affected pregnancy was reduced by 41% [35]. Based on these findings, a number of health authorities around the world, including Canada, issued recommendations for women planning a pregnancy or at risk of becoming pregnant. Health Canada recommends that all women of childbearing age consume a daily multivitamin supplement containing 400 μg of folic acid [40].

2.4 Strategies to Improve Folate Intake

Three strategies to improve folate intake are dietary approaches, supplementation and fortification. Folate is found naturally in a variety of foods including leafy vegetables, citrus fruits, beans, dairy and whole grains. However, mean intake of natural folate in Canada is well below the 400 μg shown to be effective in NTD prevention [19]. Natural folates are highly susceptible to thermal and chemical degradation as well as leaching from foods [36]. While it is possible for women to meet their folate requirements by consuming foods naturally high in the vitamin, it would require very careful attention to diet. It is difficult to increase red blood cell folate concentrations to a level associated with reduced risk for NTDs. Research shows that RBC folate concentrations >906 nmol/L result in optimal risk reduction (0.8 per 1000 births) against NTDs [16].

Supplementation with folic acid is the most effective strategy to improve folate status [37]. However, folic acid supplements will only be effective in reducing NTD rates if they are consumed regularly for a sufficiently long period prior to conception [38]. This poses a challenge as many pregnancies in Canada are unplanned and the neural tube closes early in pregnancy before many women know they are pregnant [16]. Another consideration is that daily supplementation may not be entirely effective as a result of compliance issues. Compliance with current folate recommendations in Canada has been minimal; it is thought that primary prevention of NTDs is only possible through food fortification because of poor adherence to prenatal supplements [39].

Fortification of the food supply with folic acid provides a practical solution to the challenges posed by unplanned pregnancy and compliance issues [3]. In 1998, the Canadian government mandated the addition of folic acid to the grain supply in recognition of the importance of folic acid in preventing NTDs [40]. Fortification levels were established for three targeted grain products including white flour (0.15 mg per 100g), enriched pasta (0.2 mg per 100g) and cornmeal (0.15 mg per 100g). The goal was to increase folic acid intake amongst women of child-bearing age without exceeding the UL of 1000 mg/day [41].

2.5 Folate Status of Canadians Post-Fortification

The folate status of the Canadian population had not been assessed on a nationally representative sample in over 30 years. Recently, the Canadian Health Measures Survey (2007-2009) was conducted to fill this gap. Colapinto et al. [19] used data collected from

this survey to assess the impact of folic acid fortification on the folate status of the population. The authors assessed red blood cell (RBC) folate concentrations from the general population (n = 5248) including women of childbearing age (15-45 years, n = 1162). Findings from this survey showed that less than 1% of the Canadian population had a folate deficiency (RBC < 305 nmol/L) and 22% of women of childbearing age had a folate status less than the optimal concentration (< 906 nmol) for maximal protection against neural tube defects. High folate concentrations (RBC >1360 nmol/L) were identified in 40% of the population. The cut-off for high RBC folate (>1360 nmol/L) reflects the 97th percentile from the National Health and Nutrition Examination Survey (1999-2004) [42]. In summary, findings from the Canadian Health Measures Survey demonstrate that on a population level, folate deficiency is non-existent. However, of concern is that high folate concentrations are detectible amongst a large proportion of the population. This finding highlights the importance of ongoing monitoring of the folate status of Canadians and the associated health outcomes.

2.6 Impact of Fortification on NTD Risk

NTD rates have decreased significantly since the introduction of mandatory fortification in Canada. A study conducted in seven Canadian provinces showed that food fortification with folic acid was associated with a temporal decline in the rates of NTDs by 46% [3]. The greatest risk-reduction was found in provinces with the highest baseline incidence rates. For example, in Newfoundland the difference in rate was 3.8 per 1000 births as compared to British Columbia where there was a difference in rate of 0.21 per 1000 births. A

retrospective population-based study in Ontario women had similar findings, reporting a 51% NTD reduction rate after fortification [43]. A similar study conducted in Nova Scotia reported a relative risk of 0.46 (95% CI: 0.32-0.66) comparing NTD rates pre and post fortification [44].

Canadian data is consistent with findings from other countries that have implemented mandatory folic acid fortification including the United States and Chile. In the Unites States, it was reported by Honein et al. that total NTD prevalence decreased 19% from 37.8 to 30.5 per 100,000 live births [45]. The incidence of NTD rates in Chile post-fortification fell by 40% (RR:0.60, 95% CI: 0.46-0.77) [46].

2.7 Potential Adverse Effects of Excessive Folic Acid

While folic acid fortification has decreased the prevalence of NTDs, there are concerns about the potential adverse effects of high folic acid intakes. Mandatory fortification of the food supply results in exposure of the entire population to higher intakes of folic acid [14]. Furthermore, fortification does not provide an equal distribution of folic acid across population sub-group as non-target groups often receive more folic acid than women of childbearing age. For example, data from the 2004 Canadian Community Health Survey indicate that Canadian males 14-18 y are consuming 254 µg/d folic acid from fortified food, whereas women 19-30 y are consuming 142 µg/d [47]. At normal physiological concentrations, folic acid is converted to L-5-methyl-THF in the intestinal enterocyte. However, at folic acid intakes >200 ug/d, the enzymes responsible for this conversion

become saturated resulting in detectable levels of unmetabolized folic acid in circulation [14]. Unmetabolized folic acid in plasma and tissues may lead to hematological masking and possibly exacerbation of vitamin B_{12} deficiency [48], increased risk of drug-nutrient interactions [49], and potentially increased colorectal cancer risk [5].

2.7.1 Masking of a Vitamin B12 Deficiency

Vitamin B_{12} is found only in animal products; thus, vegans and other individuals with very low animal product intakes are at increased risk of developing vitamin B_{12} deficiency. However, the elderly is the population segment most at increased risk for vitamin B_{12} deficiency as a result of decreased gastric hydrochloric acid (HCL) secretion. Sufficient gastric HCL is required to free vitamin B_{12} from its food matrix thereby allowing it to bind intrinsic factor (IF). IF facilitates the absorption of vitamin B_{12} in the terminal ileum [50]. A chronic vitamin B_{12} deficiency can lead to the development of megaloblastic anemia and serious neurological problems [1]. As discussed in section 2.3.1, megaloblastic anemia is a condition that can result from either folate and/or vitamin B_{12} deficiency. However, neurological problems are a unique consequence of vitamin B_{12} deficiency and do not generally occur with folate deficiency [4].

In vitamin B_{12} deficiency, the activity of methionine synthase is reduced and cystolic folate remains trapped as L-5-methyl-THF and cannot be converted to the THF form. L-5-methyl-THF is a poor substrate for FPGS that is required for the addition of glutamate residues necessary for the retention of folate within the cell [2]. This leads to a functional folate

deficiency resulting in deranged DNA synthesis in erythropoetic cells and megaloblastic anemia. With high levels of circulating unmetabolized folic acid, as might occur in individuals exposed to fortification, folic acid can enter the cell and be reduced to THF, bypassing the requirement for MS [4]. As a result, folate can enter the active folate pool, DNA synthesis is then corrected, and the anemia disappears. Without the presence of megaloblastic anemia, vitamin B_{12} deficiency is harder to detect. Folic acid does not correct the neurological problems and they can worsen leading to permanent damage to the nervous system [1]. The exact amount of folic acid required to "mask" a B_{12} deficiency is not known. The Institute of Medicine has set an upper limit of folic acid intakes at 1000 µg/day because of concerns of "masking of vitamin B12 deficiency" above this intake [15]. Of importance is that the masking of vitamin B_{12} deficiency is thought to occur only with folic acid and not other forms of folate such as L-5-methyl-THF [6, 51].

A study conducted by Mills et al. in the United States post-fortification was designed to examine whether there is an increase in the proportion of elderly people (n = 1573) with low serum vitamin B_{12} concentrations (<258 pmol/L) who do not have anemia. The results showed that within a group of individuals with low vitamin B_{12}, the proportion without anemia did not increase significantly from the pre-fortification period (39.2%) to the post-fortification period (37.6%) [52]. Results from this study suggests that despite the increase in folic acid exposures, there is not evidence to support folic acid fortification of the food supply is associated with increased masking of vitamin B_{12} deficiency.

2.7.2 Exacerbation of Vitamin B_{12} Deficiency

Concerns exist as to the potential for high folic acid intakes to mask and potentially exacerbate vitamin B_{12} deficiency. There is evidence that elderly individuals with low serum vitamin B_{12} and high serum folate concentrations may have exacerbated metabolic abnormalities associated with vitamin B_{12} deficiency including elevated plasma tHcy and methylmalonic acid, as well as low hemoglobin [53]. A limitation of this study was that the number of individuals with high serum folate and low vitamin B_{12} was small (n= 22) and the findings could be confounded by differences between those groups not controlled for in their multivariate adjustments. Mills et al. examined the relationship between high serum folate and biochemical indices of vitamin B_{12} in a population of young individuals without chronic disease and whose folic acid exposure was well quantified. The authors showed that individuals with high folate serum concentrations (>30 nmol/L) and low serum B_{12} (<148 pmol/L) did not have high rates of metabolic abnormalities [48]. However, additional research is needed to further elucidate the effects of high folate concentrations on the biochemical indexes associated with vitamin B_{12} deficiency in various population groups.

2.7.3 Colorectal Cancer

There has been a temporal rise in the rates of colorectal cancer (CRC) in both the United States and Canada that coincides with introduction of folic acid fortification in 1996 and 1998 respectively. A study published by Mason et al. showed that CRC rates have increased by four to six additional cases per 100,000 individuals compared to the pre-1996/1997 rates

[5]. Preceding this, both countries had experienced a downward trend in CRC incidence rates [29]. Cancer cells have higher rates of proliferation as compared to healthy, non-cancerous cells and previous studies have suggested that folate or folic acid supplementation may potentially exacerbate existing neoplasms by functioning as a cofactor in DNA synthesis [29]. This association has only been found with folic acid and not naturally occurring folates [54]. This topic is highly controversial amongst experts in the field and has received considerable attention from the media. However, it should be noted that the link between folic acid fortification and increased rates of colorectal cancer is based solely on a temporal association that could be attributed to an aging population, higher screening rates or another environmental factor.

2.8 Synthetic L-5-methyl-THF

As introduced in section 2.1, L-5-methyl-THF is the naturally occurring, predominant form of folate found in circulation**.** L-5-methyl-THF is available synthetically in the form of a calcium salt from Merck Eprova^{AG}. As discussed below, it appears to have similar bioavailability as compared to folic acid based on results from numerous randomized clinical trials [55-58]. The benefits of L-5-methyl-THF over folic acid is that this form does not need to be enzymatically reduced to become biologically active, and more importantly it cannot mask a vitamin B_{12} deficiency [2, 6]. The latter is an important consideration from a public health perspective considering the Canadian demographic consists of a rapidly aging population [50].

2.9 Relative Bioavailability of Folic Acid versus L-5-methyl-THF

Numerous studies have been conducted in various population groups that have compared the bioavailability of equimolar doses of folic acid and L-5-methyl-THF. It should be noted, that in the context of the short-term studies discussed below, bioavailability is an exclusive measure of intestinal absorption of folate and does not account for physiological retention or excretion. Bioavailability is quantified by calculating the area under the plasma concentration-time curve (AUC), after correcting for time-matched baseline values. Acute single-dose studies provide an understanding of the absorption and short-term bioavailability of these compounds. Long-term studies provide a better understanding of the retention and utilization of folic acid and L-5-methyl-THF [7].

2.9.1 Short-term Studies

A study conducted by Pentieva et al. [58] applied a cross-over design to compare single oral doses of folic acid (500μg) and equimolar L-5-methyl-THF in 21 healthy male subjects. After correcting for baseline values, the area-under-the-curve (AUC) for plasma folate concentrations showed no difference between folic acid and L-5-methyl-THF. Prinz-Langenohl et al. went on to perform a similar study using a four-period cross-over design in 21 female subjects. Single doses of folic acid (400 μg) and L-5-methyl-THF (416 μg), either with or without a folic acid preload (1mg/d for 10 days), were administered in a randomized, double-blind fashion. The preload model is used in bioavailability studies to mimic fortification and provide prolonged exposure of a nutrient preceding a study period to

facilitate physiological adaptation. The results showed that AUC values did not differ significantly between folic acid and L-5-methyl-THF regardless of the preload treatment. The mean AUC (CV) values for L-5-methyl-THF and folic acid without and with the preload was 210 (17.1%) vs. 191 (28.8%) and 278 (14.4%) vs. 277 1h x nmol/L (18.3%). Findings from this study suggest that a preload treatment is not necessary as it does not appear to impact the acute bioavailability of single 400 μg doses of folic acid and L-5-methyl-THF. Results from these two studies, both of which had a similar study design, subject population and treatment dose, show that the short-term bioavailability of L-5-methyl-THF is comparable to folic acid.

2.9.2 Long-term Studies

Venn et al. [59] [55] investigated the effects of low dose folic acid and L-5-methyl-THF on RBC folate, plasma folate, and plasma tHcy over a 24-week period in 167 subjects. Subjects were randomized to folic acid (100 μg), L-5-methyl-THF (113 μg), or placebo. At 24 wk, after adjustment for baseline values, mean (95% CI) tHcy was 14.6% (9.3, 19.5%) and 9.3% (3.7, 14.6%) lower, mean plasma folate was 34% (14, 56%) and 52% (30, 78%) higher, and mean RBC folate was 23% (12, 35%) and 31% (19, 44%) higher in the L-5-methyl-THF and folic acid groups, respectively, than in the placebo group. L-5-methyl-THF was more effective than was folic acid in lowering tHcy ($P < 0.05$). At 24 wk, the increases in plasma folate and RBC folate concentrations did not differ significantly between the two supplemented groups.

The authors subsequently chose to report on the kinetics of blood folate in a subset of this population that included only women of childbearing age. The mean (95% CI) estimated linear increase in plasma folate concentration was 0.3 (0.2, 0.5), and 0.4 (0.2, 0.6) nmol/(L.wk in the L-5-methyl-THF and folic acid groups, respectively. The mean (95% CI) estimated linear increase in RBC folate was 7.4 (4.5, 10.3), and 8.3 (4.4, 12.3) nmol/(L.wk) in the L-5-methyl-THF and folic acid groups, respectively. There were no significant differences in the slopes between the L-5-methyl-THF group and the folic acid group suggesting the kinetics of blood folate increase are similar at least at a low-dose.

Based on the previous findings, Lamers et al. [56] conducted a similar study but using higher doses to compare the effects of L-5-methyl-THF and folic acid on increasing RBC folate concentrations over a 24-week period. The study was a randomized double-blind, placebo-controlled trial in 144 women of childbearing age. Subjects were administered equimolar doses of either folic acid (400 μg) or L-5-methyl-THF (416 μg). The rationale for the doses used in this study was based on the optimal amount of folic acid shown to be effective in reducing NTD risk [33]. Results from this study show that RBC folate concentrations were significantly greater for L-5-methyl-THF group than equimolar folic acid ($P < 0.001$).

Houghton et al. [57] went on to examine the effect of supplementation with L-5-methyl-THF on blood folate concentrations throughout pregnancy and lactation. The authors conducted a 16-week randomized placebo-controlled trial in 72 pregnant women to compare the effectiveness of 400 μg FA and an equimolar dose of L-5-methyl-THF in raising RBC

folate concentrations throughout lactation. Results from this study showed the RBC folate concentrations were higher for the L-5-methyl-THF group compared to the FA group (2178; 95% CI: 1854-2559 nmol/L) vs. (1967; 95% CI: 1628, 2377 nmol/L; $P < 0.05$).

Long-term studies indicate that L-5-methyl-THF is retained and utilized to a similar extent as folic acid in a variety of populations and doses. Therefore, it can be assumed that once absorbed, folate will be retained in tissues and utilized to carry out its metabolic functions. This justifies the use of short-term studies as indicators of long-term bioavailability.

2.10 L-5-methyl-THF as a Food Fortificant

While L-5-methyl-THF is stable and highly bioavailable in supplemental form, its poor stability in food matrices has limited its use as a food fortificant [7]. In contrast, folic acid is fully oxidized and thus is very stable in food matrices [2]. Naturally occurring, reduced forms of folate including L-5-methyl-THF are highly labile and susceptible to degradation. Thermal oxidation and leakage from baking and boiling account for the greatest forms of degradation and these processes can result in folate losses >90% [36]. There is some evidence that L-5-methyl-THF is more stable in complex food matrices than in simple aqueous solutions. For example, folate-binding protein found in milk powder has been shown to impart stabilizing effects on L-5-methyl-THF [60]. This finding has raised interest in whether milk powders are a suitable vehicle for food fortification. In addition, microencapuslation of L-5-methyl-THF may have beneficial effects in improving stability in food matrices.

2.11 Microencapsulation Technologies

Biologically active components of food, including nutrients such as folate, are required to remain structurally intact and metabolically active in order to be absorbed and carry out their desired function within the body. This means that nutrients must be stable enough to withstand the effects of food processing and storage. Microencapsulation technologies provide solutions to protect a core nutrient from environmental conditions and ensure the delivery and release of bioactive nutrient [61]. This process increases the stability of a core nutrient from environmental factors including UV radiation, light, oxygen, humidity and temperature. Previous studies have been shown to efficiently microencapsulate various nutrients including β-galactosidase and iron using the coating material polyglycerol monostearate (PGMS) [8, 62]. PGMS is a lipid composition that forms a micell structure encasing the core nutrient. Lipids are common microencapsulating agents because they effectively protect against environmental factors and have been shown to impart minimal flavor when incorporated into different food products and supplements. There are currently no studies that assess the stability effects of microencapsulating L-5-methyl-THF to facilitate its use as a food fortificant.

3 HYPOTHESIS & OBJECTIVES

Null Hypothesis: Microencapsulated folic acid and L-5-methyl-THF, in capsule form and incorporated into a skim milk powder, will not increase plasma folate response, as calculated by AUC over 8 hours, to the same extent as free-form folic acid and L-5-methyl-THF.

Objectives

Primary Objective:

To determine if microencapsulated folic acid and L-5-methyl-THF, in capsule form and incorporated into a skim milk powder, will increase the plasma folate response, as calculated by AUC over 8 hours, to the same extent as free-form folic acid and L-5-methyl-THF.

Secondary Objectives:

1. To determine if incorporating microencapsulated folic acid and L-5-methyl-THF into skim milk powder will increase the plasma folate response, as calculated by AUC over 8 hours, to the same extent as microencapsulated folic acid and L-5-methyl-THF in capsule form.

2. To determine if free-form L-5-methyl-THF will increase the plasma folate response, as calculated by AUC over 8 hours, to the same extent as free-form folic acid.

4 METHODS AND MATERIALS

4.1 Participants

Canada has folic acid fortification and as such it would be difficult to conduct human folate intervention trials in this country without a lengthy and expensive folate depletion period. There would also be ethical concerns involved with removing folic acid from the diet of subjects, especially women of childbearing age. For these reasons, the study was conducted in Dunedin, New Zealand in collaboration with colleagues at the University of Otago. New Zealand's food supply is not fortified with folic acid. Ethical approval was obtained from both the University of Otago Human Ethics Committee in New Zealand and by the Clinical Research Ethics Board at the University of British Columbia.

Eligible participants for the study were healthy, English speaking men and women between the ages of 18 and 65 years. Individuals were excluded if they were pregnant, planning a pregnancy, taking folic acid supplements, had anemia or chronic disease (i.e. cardiovascular disease, diabetes), or had an allergy or dislike of milk products.

4.2 Recruitment & Screening

Recruitment was conducted over a one-week period immediately preceding the study via posters and email advertisements. An information letter (**Appendix A)** and consent form (**Appendix B**) were provided to individuals who expressed interest in participating. Prior to

enrollment subjects were asked to attend a screening visit to assess their eligibility. A brief questionnaire was administered to collect information on medical history and lifestyle behaviors. The subjects' height and weight were measured and used to calculate their body mass index (BMI). A complete blood count (CBC) was used to screen for anemia. To obtain the blood sample a venipuncture was performed by the study nurse and the sample (5 mL) was collected into a vacutainer containing EDTA. The sample was placed on ice and transported immediately to Southern Community Laboratories where a CBC was performed using an automated hematology analyzer machine. Anemia was defined as hemoglobin < 120 g/L in women and <140 g/L in men. Based on the results of the CBC none of the participants were determined to be anemic.

4.3 Study Design

The study was a repeated-measures cross-over design. Subjects (n=15) were randomly administered seven treatments as a single-dose at weekly intervals. The treatment dosages administered were 400 µg of folic acid or equimolar amounts of L-5-methyl-THF (416 µg). The treatments were: placebo; 400 µg folic acid; 400 µg microencapsulated folic acid; 416 µg L-5-methyl-THF; 416 µg microencapsulated L-5-methyl-THF; 400 µg microencapsulated folic acid in milk powder and 416 µg microencapsulated L-5-methyl-THF in milk powder.

4.4 Supplements

Folic acid and synthetic L-5-methyl-THF (Metafolin ®) were obtained from Merck Eprova (Schaffhausen, Switzerland). Five of the treatments were given as hard gelatin capsules containing cellulose as filler. For the other two treatments, the folates were incorporated into a skim milk powder by blending. The powder was dispensed in 50 g vacuum-sealed sachets with instructions to be reconstituted with 250 mL of water at the time of ingestion. Supplements were coded so that neither the investigators nor the participants were aware of the contents.

4.4.1 Analysis of Supplements

The folic acid or L-5-methyl-THF content of the supplements or milk was quantified using HPLC with fluorescence detection in the laboratory of Dr. David Kitts. **Table 5.1** summarizes the planned and actual folate content measured in each of the seven supplements and milk powders. Based on the results of these analyses, some differences were found between the planned dosages and actual amounts quantified in the supplements. Adjustments were made to allow comparison between treatments. A correction factor was calculated to adjust the values to the molar equivalent of 400 μg of folic acid. For example, the planned amount of folic acid in the microencapsulated folic acid in pill form was 400 μg (907 μmol) and the actual measured amount was 283 μg (657 μmol). Thus the plasma folate values are multiplied by a correction factor of 1.38 (907/657).

Table 4.1. Planned and measured amounts of folic acid (FA) or L-5-methyl-THF in supplements or milk powder.

Treatment	Folate (µg)	
	Planned	**Measured***
Placebo	0	0
Folic Acid	400	414
L-5-methyl-THF	416	412
Microencapsulated Folic acid	400	283
Microencapsulated L-5-methyl-THF	416	405
Microencapsulated Folic acid in Milk Powder	400	400
Microencapsulated L-5-methyl-THF in Milk Powder	416	450

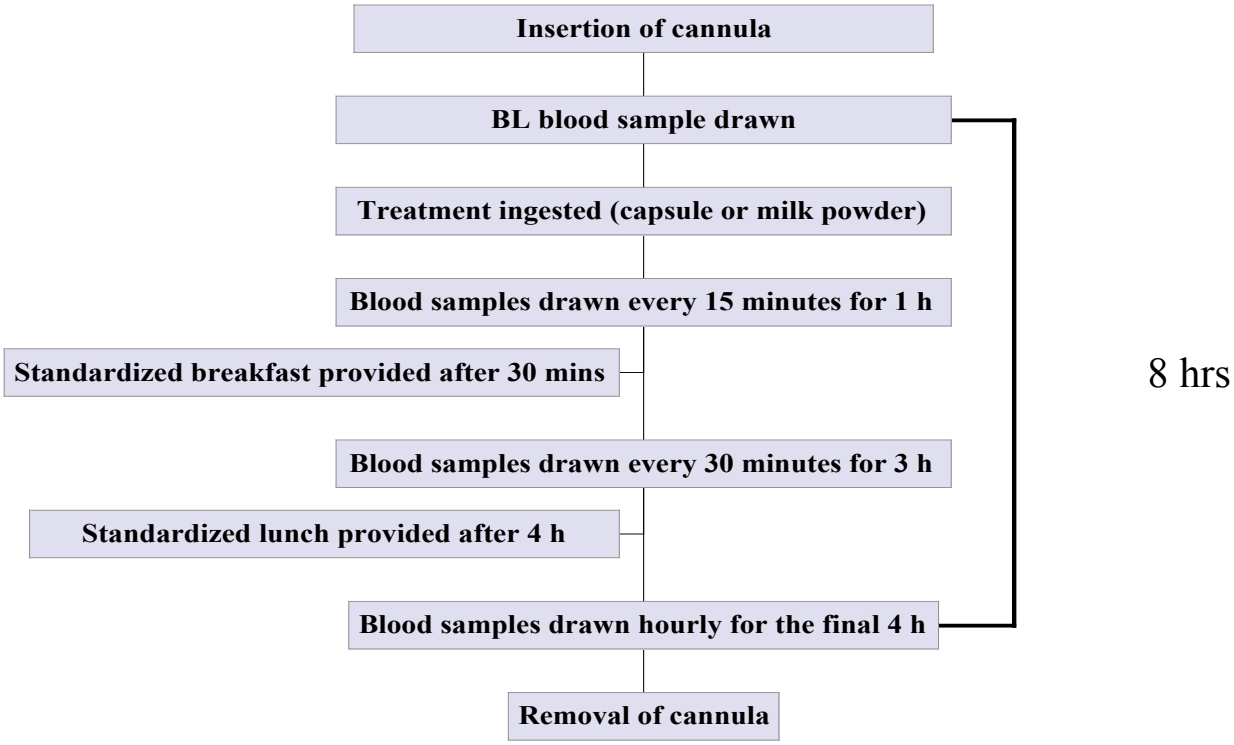
* n = 6 replicates. Folic acid was measured using HPLC with photodiode array detection. L-5-methyl-THF was measured using HPLC with florescence detection.

4.5 Protocol

Subjects attended seven test days staggered at weekly intervals. The study was conducted in the Nutrition Clinic at the University of Otago. Participants arrived in a fasting state and had a venous catheter with a multiple-blood-sampling system was inserted into a dorsal hand vein (Bionector-S, Vygon, Ecouen, France). The purpose of the catheter was to eliminate the need for multiple venipuncture. After a baseline blood sample was drawn, subjects consumed one of the capsules or milk treatments based on a predetermined randomized allocation. Due to safety concern with the catheters, subjects were asked to remain in the clinic and stay as sedentary for the duration of the test period.

A total of fifteen blood samples were collected from each participant per day (8 hours). Samples (5 mL) were drawn at baseline (BL) and then after ingestion of the treatment every 15 minutes for an hour, every 30 mins for the following 3 hours, and then hourly for the final four hours. Care was taken to ensure that the red blood cells were not hemolyzed during ejection from the catheter. The main issues encountered related to blood sampling resulted from dehydration, clotting and poor circulation as a result of subjects being sedentary. When a blood sample could not be obtained, a heating bag was applied to the catheterized arm and oral fluids were given. If the problem persisted the catheter was re-inserted or a venipuncture was performed. All blood samples (5ml) were collected into EDTA-treated tubes, transported on ice and processed within one hour of collection. The samples were centrifuged at 3000 rpm for 15 minutes and subsequently the plasma was removed, aliquoted and stored in a -80°C freezer.

Figure 4.1 Blood Sampling Protocol



4.6 Standardized Meals

During each test day participants consumed a standardized low-folate diet. Breakfast and lunch were provided after the baseline and 4-hour blood collections, respectively. Approximately thirty minutes was allotted for subjects to complete each meal. In addition to meals, participants were also provided with a light afternoon snack following the 6-hour blood collection. Coffee, tea and water were unrestricted and offered throughout the day to maintain hydration. Participants were instructed not to consume any other foods or beverages during the test period.

4.7 Laboratory Analysis

Plasma folate concentrations were quantified using the microbiological assay. The microbiological assay was developed by O'Broin et al. and uses the test organism chloramphenicol-resistant *Lactobacillus casei* (*L. Casei*). The use of a chloramphenicol-resistant organism reduces time by eliminating the need for aspectic techniques. *L.Casei* requires folate for growth and does not distinguish between forms of folate [63]. This method is highly sensitive, relatively inexpensive and can efficiently handle large sample numbers [64].

All samples from a participant (15 samples per treatment x 7 treatments = 105 samples/per participant) were analyzed on the same day to minimize day-to-day assay variation. Samples were analyzed in duplicate and repeated if there was greater than 20% variation between the duplicate. When a sample value exceeded the maximum value of the standard curve, the sample was diluted by an additional 50% in a 20% Na-ascorbate buffer solution and repeated.

Accuracy and inter-assay variability were monitored using external quality controls. This included a whole blood standard (National Institute for Biological Standards and Control, Hertfordshire, United Kingdom) with a certified value of 29.5 nmol/L [mean 27.3 (SD 4.6); CV:17%] and a pooled-plasma sample with a known folate concentration of 48.5 nmol/L. The inter-assay CV was determined to be 8.8%.

4.8 Statistical Analyses

Based on the results from a bioavailability study of L-5-methyl-THF in healthy men, it was estimated that a sample size of n= 15 would be required to detect a 25% difference in any two treatments with 80% power [65]. The pre-planned comparisons between treatments were as follows: placebo and each of the 6 folate treatments; free-form L-5-methyl-THF and free-form folic acid; microencapsulated L-5-methyl-THF and free-form L-5-methyl-THF; microencapsulated folic acid and free-form folic acid; microencapsulated L-5-methyl-THF and microencapsulated L-5-methyl-THF in milk powder; microencapsulated folic acid and microencapsulated folic acid in milk powder.

Plasma folate concentrations were measured at each time-point and adjusted to baseline values. Area under the curve (AUC) was calculated for each treatment using Prism software (Version 4.0. Graphpad Software Inc, San Diego, CA). Prism computes AUC using the trapezoidal method. A number of the plasma folate concentrations fell below baseline, particularly with the placebo. Prism ignores values that fall below baseline; thus, to avoid negative values, 5 nmol/L was added to each plasma folate concentration. As discussed above (section 5.5.1), correction factors were multiplied through to reflect the actual amounts of folate quantified in the supplements. Differences in AUC by treatment were evaluated for each of the pre-planned comparisons by repeated-measures ANOVA using SAS (SAS Institute Inc., Cary, NC, USA). Level of significance was established at $P < 0.05$.

5 RESULTS

All 15 participants completed the trial. The study population consisted of 12 females and 3 males. The mean ± SD age and BMI of participants was 23 ± 3.4 years and 25.6 ± 3.2 kg/m^2 respectively. The AUC 8h x nmol/L (± SEM) for each treatment is presented in **Table 5.1**. The AUCs values for all six folate treatments were significantly greater than the placebo ($P < 0.05$) (**Figure 5.1**). In comparing the bioavailability of free-form folates, the AUC for L-5-methyl-THF was greater than folic acid (347 ± 35 vs. 181+12 8h x nmol/L; $P < 0.001$) (**Figure 5.3**). Microencapsulation using a PGMS coating had differential effects on the bioavailability of folic acid and L-5-methyl-THF. The AUC for plasma folate over 8 hours for microencapsulated folic acid was not significantly different than free-form folic acid (222 ± 12 vs. 181 ± 14 8h x nmol/L; $P = 0.12$) (**Figure 5.4**). However, the AUC for microencapsulated L-5-methyl-THF was less than free-form L-5-methyl-THF (147 ± 17 vs. 347 ± 35 8h x nmol/L; $P < 0.001$) (**Figure 5.5**).

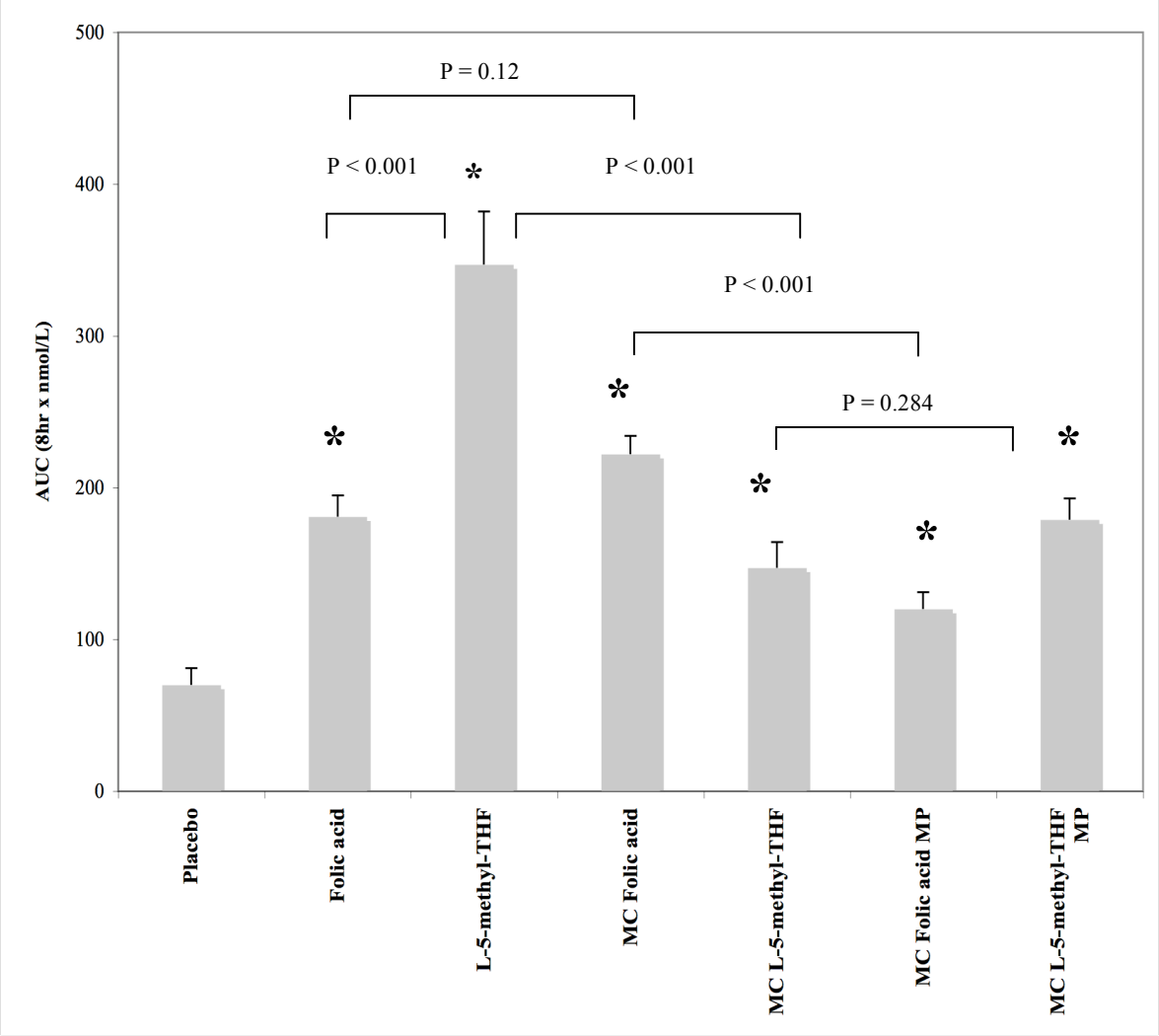
The skim milk powder decreased the bioavailability of microencapsulated folic acid but showed no effect on microencapsulated L-5-methyl-THF. The AUC for microencapsulated folic acid in milk powder was less than microencapsulated folic acid in capsule form (120 ± 11 vs. 222 ± 12 8h x nmol/L; $P < 0.001$) (**Figure 5.4**). Microencapsulated L-5-methyl-THF in milk powder was not significantly different than microencapsulated L-5-methyl-THF in capsule form (178 ± 14 vs. 147 ± 17 8h x nmol/L; $P = 0.284$) (**Figure 5.5**).

Table 5.1. Area under the curve (AUC) for plasma folate over 8-h test periods in healthy men and women after taking supplements or skim milk powder administered in random order at weekly intervals.*

Treatment	Plasma Folate AUC (8 hours x nmol/L)		
	Mean	**SEM**	**95% CI**
Placebo	70	11	47, 93
Folic Acid	181	14	152, 210
L-5-methyl-THF	347	35	271, 423
Microencapsulated Folic acid	222	12	195, 248
Microencapsulated L-5-methyl-THF	147	17	110, 184
Microencapsulated Folic acid Milk Powder	120	11	96, 143
Microencapsulated L-5-methyl-THF Milk Powder	179	14	149, 210

* n = 15; AUCs are adjusted to the molar equivalent of 400 µg of folic acid

Figure 5.1 AUC values (corrected) over 8-h test periods in healthy men and women after taking supplements or skim milk powder administered in random order at weekly intervals (n = 15); Values are adjusted to the molar equivalent of 400 µg of folic acid



MC = Microencapsulated; MP = Milk Powder
* = Significantly different compared to placebo (P < 0.05)

Figure 5.2 Plasma folate (corrected for baseline) over 8-h test periods in healthy men and women after taking supplements or skim milk powder administered in random order at weekly intervals (n = 15); Values are adjusted to the molar equivalent of 400 µg of folic acid

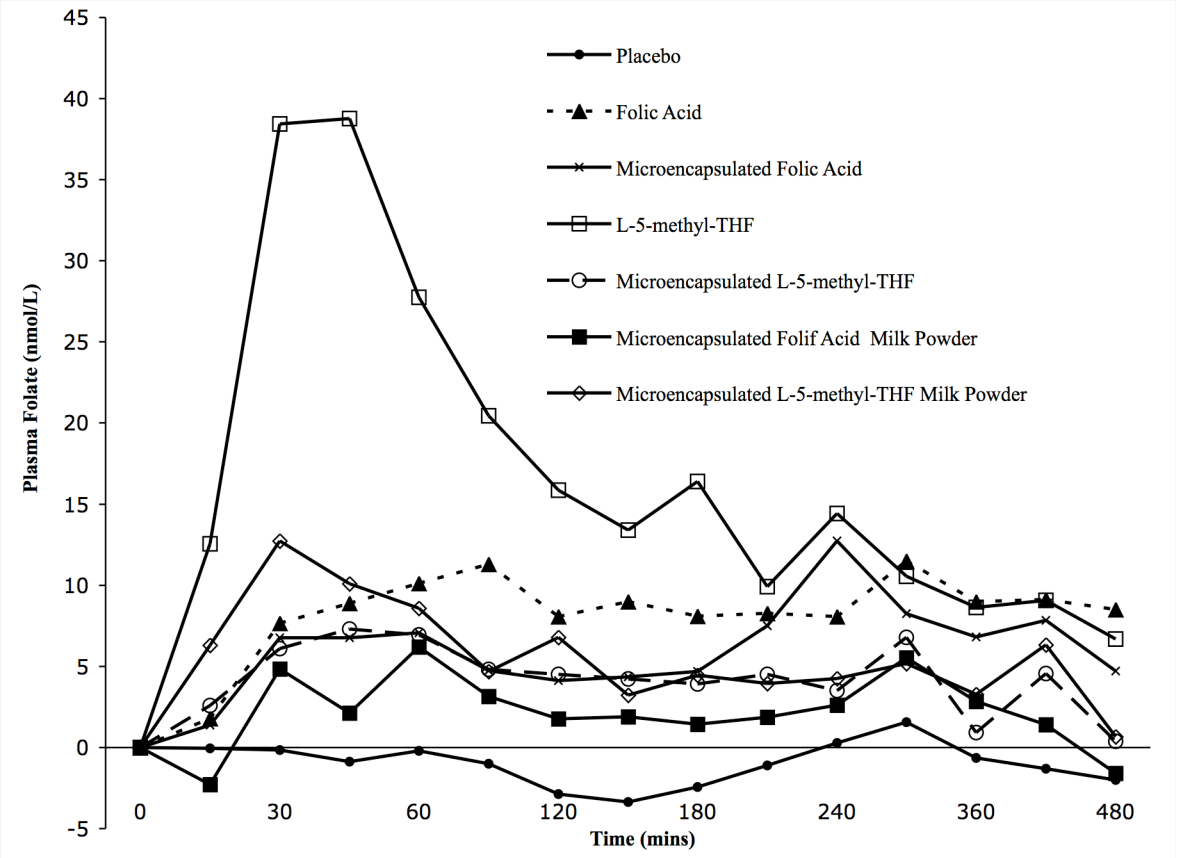


Figure 5.3 Plasma folate (corrected for baseline) over 8-h test periods in healthy men and women after taking supplements containing Folic Acid or L-5-methyl-THF administered in random order at weekly intervals (n = 15); Values adjusted to the molar equivalent of 400 µg of folic acid

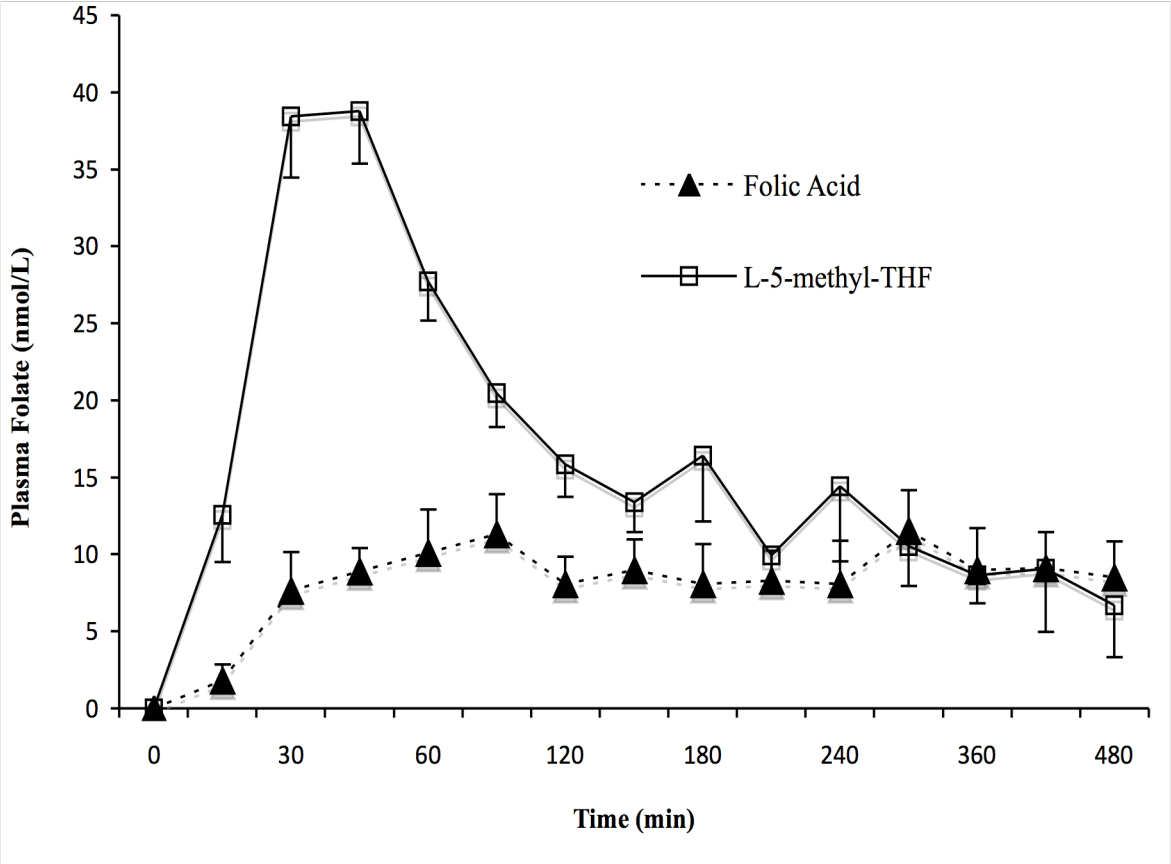


Figure 5.4 Plasma folate (corrected for baseline) over 8-h test periods in healthy men and women after taking supplements or milk powders containing Folic Acid or Microencapsulated Folic Acid administered in random order at weekly intervals (n = 15); Values adjusted to the molar equivalent of 400 µg of folic acid

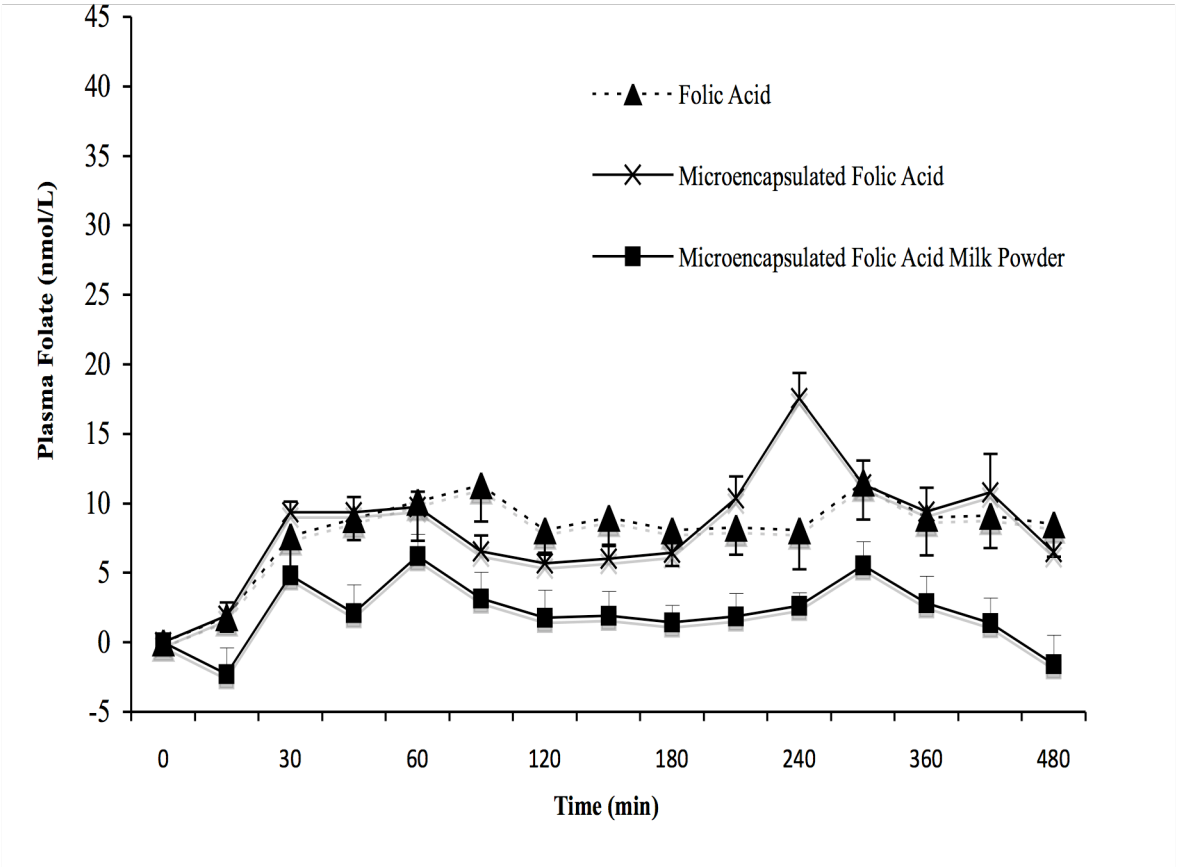
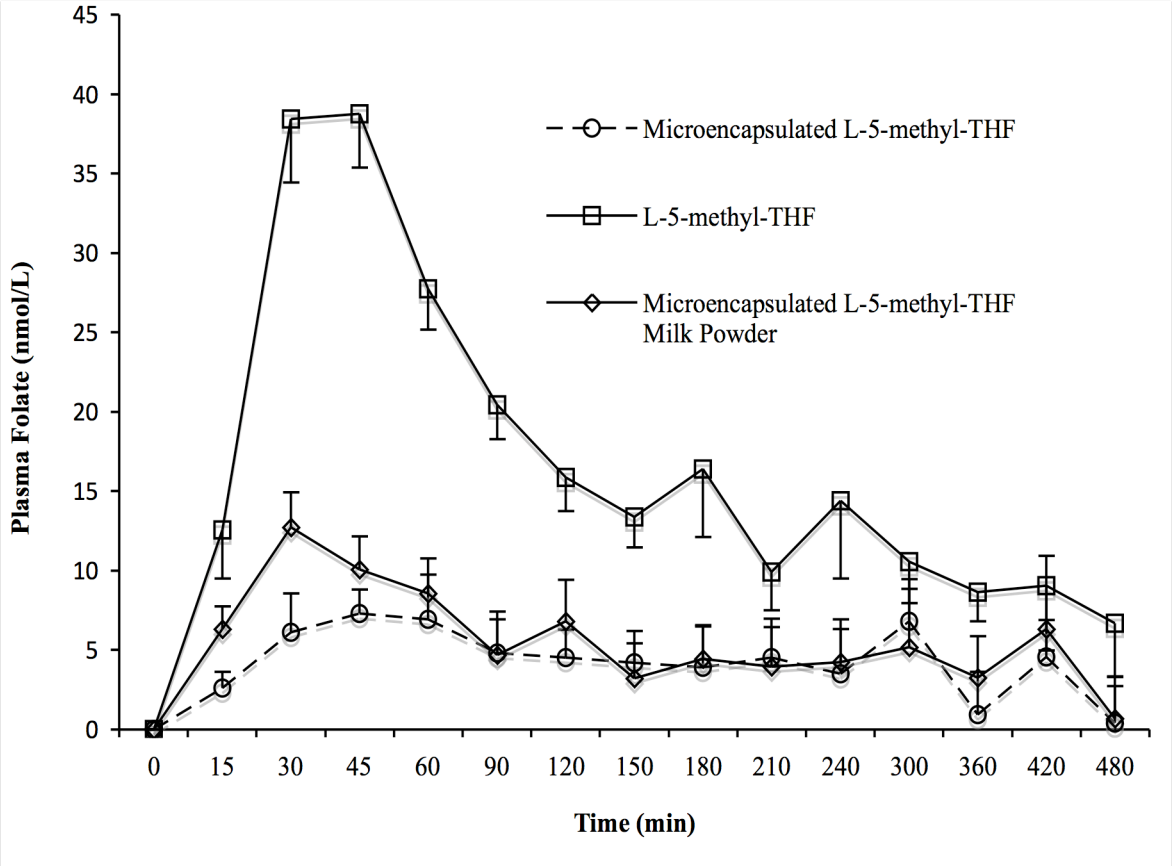


Figure 5.5 Plasma folate (corrected for baseline) over 8-h test periods in healthy men and women after taking supplements or milk powders containing L-5-methyl-THF or Microencapsulated L-5-methyl-THF administered in random order at weekly intervals (n = 15); Values adjusted to the molar equivalent of 400 µg of folic acid



6 DISCUSSION

6.1 Summary of Findings

Previous studies have shown the long-term bioavailability of L-5-methyl-THF to be equal or slightly greater as compared to folic acid [55-58]. Despite the apparent superior bioavailability and potential health advantages of L-5-methyl-THF over folic acid, it has not previously been considered as a food fortificant because of its poor stability. To overcome this limitation, microencapsulation technologies were considered as a process solution to protect L-5-methyl-THF from degradation in food matrices. Microencapsulation techniques have not previously been examined for use with folates. This study compared the bioavailability of microencapsulated L-5-methyl-THF and microencapsulated folic acid to their relative free-forms. Furthermore, microencapsulated L-5-methyl-THF and microencapsulated folic acid were both incorporated into a skim milk powder to assess the effects of a food matrix on bioavailability as compared to capsule form.

Based on the results of the study, microencapsulation using a PGMS coating had varied effects on the bioavailability of folic acid and L-5-methyl-THF. Microencapsulation had no effect on folic acid, however, it decreased the bioavailability of L-5-methyl-THF. Similar to microencapsulation, the milk matrix had differential effects on folic acid compared to L-5-methyl-THF. The skim milk powder decreased the bioavailability of microencapsulated folic acid but showed no effect on microencapsulated L-5-methyl-THF.

6.2 Relative Short-term Bioavailability of Free-form L-5-methyl-THF and Folic Acid

In comparing the short-term bioavailability of free-form folates, the AUC for L-5-methyl-THF was nearly 2 times greater than folic acid (347 ± 35 vs. 181+12 8h x nmol/L; $P < 0.001$) (**Table 5.1**). This result is inconsistent with the findings from two previous studies (Pentieva *et al.*; Prinz-Langernohl *et al*.) that reported the short-term bioavailability of supplemental L-5-methyl-THF and folic acid to be equivalent [58, 66]. Both studies used a comparable design, population (healthy participants) and treatment dose (500 μg and 400 μg amounts of folic acid and equimolar L-5-methyl-THF). It is not clear why there is a marked difference between our results and those of others.

A potential explanation may relate to the timing and frequency of blood sampling. Previous studies collected blood samples every thirty-minutes during the first hour, whereas in our study blood samples were collected every fifteen-minutes. As illustrated in **Figure 5.3**, there was a sharp rise in the mean plasma folate concentration in response to L-5-methyl-THF, which occurred within the first hour. At such high plasma folate concentrations, it is plausible that cellular uptake of L-5-methyl-THF into tissues may become temporarily saturated [67]. Furthermore, urinary and biliary excretion may also be less efficient at elevated levels, resulting in delayed clearance of L-5-methyl-THF from the plasma [68]. If we had not sampled so frequently in the first hour, the sharp peak in plasma folate concentration may have been missed and thus our findings would have been more similar to others [58, 66].

Alternatively, the observed discrepancy in acute bioavailability may be explained by the difference in bioefficacy between L-5-methyl-THF and folic acid. Bioefficacy is defined as the efficiency by which a nutrient is absorbed and converted to its bioactive form [69]. L-5-methyl-THF could be considered more bioeffective than folic acid because it can be used directly in metabolic reactions whereas folic acid must first undergo enzymatic reduction by the enzyme DHFR (section 2.2). DHFR becomes saturated at high concentrations (>200 ug) limiting the rate of absorption at supra-physiological doses [14]. As discussed in section 2.9.2, long-term studies based on RBC folate or tHcy have found the bioavailability of L-5-methyl-THF to be equal or slightly greater than folic acid [56, 58]. This suggests that the potential effect of bioefficacy on short-term bioavailability is not relevant in the long-term.

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6.3 Microencapsulation

The effects of microencapsulation differed between folic acid and L-5-methyl-THF. Microencapsulation using a polyglycerol monostearate coating had no effect on the bioavailability of folic acid. The AUC for microencapsulated folic acid was not significantly different than free-form folic acid (222 ± 12 vs. 181 ± 14 8h x nmol/L; $P = 0.12$). This finding was expected because free-form folic acid is already very stable [2] and thus it is unlikely there would be a marked improvement in bioavailability. Interestingly, microencapsulation reduced the bioavailability of L-5-methyl-THF. The AUC for microencapsulated L-5-methyl-THF was significantly less than free-form L-5-methyl-THF (147 ± 17 vs 347 ± 35 nmol/L; $P < 0.001$). The reason as to why microencpasulation had negative effects on the bioavailability of L-5-methyl-THF and not folic acid is unclear.

There are several variables associated with the microencapsulation process, which may explain why it decreased the bioavailability of L-5-methyl-THF including: the coating material, ratio of coating-to-core-nutrient and the particle size.

6.3.1 Coating Material

In the selection of microencapsulating ingredients, there is a need to balance between a coating material that provides adequate protection while also allowing for the controlled release of a bioactive nutrient at the target absorption site. Polyglycerol monostearate (PGMS), a stearate based coating, was selected as the preferred material. Stearates have previously been shown to efficiently microencapsulate β-galactosidase and iron [8, 62] and protect against degradative factors such as temperature, oxygen, and moisture. However, a limitation of stearate is that it is hydrophobic and requires the action of gastric lipases to breakdown the lipid coating [70]. Based on the results of this study, it is plausible that the stearate coating was too stable and could not be sufficiently broken down by the gastric lipases preventing release of L-5-methyl-THF in the small intestine.

6.3.2 Coating Ratio

Another factor that may have affected the release of microencapsulated L-5-methyl-THF is the coating-to-core ratio. The coating ratio used in the supplements was 75% coating material to 25% core nutrient (3:1). A key consideration is whether the stearate coating may have “buried” the L-5-methyl-THF to an extent that bioavailability was affected. The 3:1

coating ratio used in our supplements may have been too high, negatively impacting bioavailability. In hindsight, it would have been informative to include an additional treatment with a lower coating ratio (ie: 2:1). This treatment would have served as a comparator to determine whether the 3:1 coating ratio was too high and inhibited the release of L-5-methyl-THF from the stearate coating.

6.3.3 Particle Size

Furthermore, it has also been found with stearate-based coatings that the extent and the rate of the release are inversely related to the particle size. The selection of a suitable microparticle size can represent a further control or promotion of the release [71]. Unfortunately, we did not measure particle size in this study. The microencapsulating process results in a larger particle size than the free-form vitamin, which in turn would decrease the rate of release [71]. However, the coating ratio used in microencapsulation is a factor that can be adjusted. A lower coating ratio would decrease the particle size and potentially increase the acute bioavailability.

6.4 Food Matrix Effects

There is limited data available on folate bioavailability from various food matrixes [72]. The need for data on bioavailability from specific foods is particularly important for countries without mandatory fortification programs. Voluntary fortification of target foods may be an effective strategy to improve the folate status of women of childbearing age without

exposing the rest of the population to high levels of folic acid. Folate bioavailability from various foods is dependent on a multitude of factors including the food matrix, environmental conditions (pH and temperature), cooking methods, storage conditions as well as dietary components that impact folate stability [36]. Previous studies have demonstrated that fluid milk and milk powders provide a suitable matrix for folic acid fortification. [73]. In vitro studies using a simulated gastrointestinal model demonstrated that folic acid is readily released from the milk matrix and highly bioavailable [74]. Furthermore, a recent study showed folic acid to be more bioavailable in skim milk as compared to whole milk based on the postprandial plasma folate response [75]. Based on the evidence skim milk powder was selected as the food vehicle to examine the effects of a food matrix on the bioavailability of microencapsulated L-5-methyl-THF and folic acid.

Our results indicate that the effects of the food matrix were varied between the two treatments. Adding microencapsulated L-5-methyl-THF to skim milk powder had no effect on bioavailability (**Figure 5.3**). Microencapsulated L-5-methyl-THF in milk powder was not significantly different than microencapsulated L-5-methyl-THF in capsule form (178 ± 14 vs. 147 ± 17 nmol/L; $P = 0.284$). However, the AUC of microencapsulated folic acid decreased when it was incorporated into skim milk powder. (120 ± 11 vs 222 ± 12 nmol/L; $P < 0.001$). The explanation as to why the milk matrix had differential effects on microencapsulated L-5-methyl and THF is not well understood. Since microencapsulated folic acid and L-5-methyl-THF has not previously been added to skim milk powder, we are limited in our ability to compare and interpret our data in relation to other studies.

There were a several challenges encountered relating to the milk powders that should be taken into consideration when interpreting these results. First, it was very difficult to get the milk powders into a homogenous solution and they had a tendency to settle out and form clumps. Milks typically consist of approximately 10% solids and as such our protocol specified that 375mL of water be added to 37.5g of milk powder. The prepared milks were reported by participants to have a thick consistency with a "chalk like" flavor, which were generally unpalatable. The research team encountered ongoing difficulty in ensuring the participants consumed the milk beverages within a reasonably short time frame.

Another issue encountered was that the leftover powder was often found congealed to the bottom or sides of the milk glass. In these cases the powder would have to be scraped down and reconstituted with additional water. Based on these challenges, it is highly likely that on multiple occasions not all 37.5 g of milk powder was consumed or it was delivered over a prolonged time frame. Both of these factors may have imparted small but potentially significant effects on bioavailability, either by decreasing the magnitude or delaying the plasma response. There were no notable differences in the physical and sensory properties between the two milk powders; thus it should be acknowledged that this explanation does not reconcile the differences of the milk matrix on the bioavailability of microencapsulated L-5-methyl-THF and folic acid.

7 STRENGTHS AND LIMITATIONS

This study had numerous strengths. Treatments were randomly administered in a standardized, controlled environment and staggered at weekly intervals to prevent carry-over effects. To provide identical physiological and dietary conditions, standardized low-folate meals and snacks were offered at the same time each day. The planned treatment dosage (400 μg folic acid and 416 μg L-5-methyl-THF) was chosen to reflect the current recommendations for folate intake for women of childbearing age. Women who can become pregnant are recommended to obtain 400 μg/day of folic acid from supplements or fortified foods [15]. Furthermore, based on previous studies [58], it was assumed that 400 μg would be a sufficient oral dose to obtain a measurable plasma response. As expected, the AUC values all six folate treatments were significantly greater than the placebo (**Figure 5.1**).

However, we do acknowledge a number of limitations. The primary limitation of this study is that all the administered folate treatments did not contain the planned dosages. As discussed in the section 5.5.1, the planned dosage for every treatment was set based on the molar equivalent of 400 μg of folic acid. Five of the six treatments contained 90-100% of the planned dosage. However, the amount quantified in the microencapsulated folic acid treatment was only 283 μg (70.1% of the planned dosage). To enable us to conduct statistical analyses and draw comparisons, a correction factor was calculated and applied to the AUC values. The AUCs were adjusted based on the quantified folate content to the molar equivalent of 400 μg of folic acid. This strategy was based on the premise that a linear dose-response relationship exists for L-5-methyl-THF and folic acid. A major flaw to this

rationale is that the existence of a linear dose-response relationship is only speculative and has not been definitively established. Therefore, the use of correction factors decreases the validity of our results.

An additional limitation was that the time-period used to measure the plasma folate response was too short. The plasma concentration-time graph (**Figure 5.2**) illustrates that 8 hours was not sufficient to allow the plasma concentration values return to baseline. Extrapolating our data, it appears a 10-12 hour window would be required to fully capture this response. This result was unexpected because previous short-term bioavailability studies have shown that 8 hours is a sufficient time period [58, 65]. It is conceivable that microencapsulation may have delayed intestinal absorption explaining why it took longer for the concentration values to peak and subsequently return to baseline levels.

8 FUTURE DIRECTIONS

To my knowledge this was the first study to examine of the effects of microencapsulation using a PGMS coating on the bioavailability of folic acid and L-5-methyl-THF. As discussed in section 6.3.1, the results suggest that PGMS is not a suitable coating material for microencapsulation of L-5-methyl-THF. The lipid emulsion is likely too stable and resistant to breakdown by gastric lipases. A recommendation for a future study direction would be to experiment with hydrophilic coating materials that will break down more readily promoting the release the core nutrient. An alternative direction would be to keep the PGMS coating and instead examine various coating ratios to enhance the effectiveness of the microencapsulation process. Our results suggest that a 3:1 coating ratio may be too high and that a lower coating ratio may be more effective. Testing of different microencapsulating materials or coating ratios would again need to be carried out both *in vitro* as well as in *vivo* using randomized-human clinical trials.

9 CONCLUSIONS

L-5-methyl-THF appears to have a greater acute bioavailability than folic acid. Microencapsulation of the folates with a polyglycerol monosterate coating had varied effects on folic acid and L-5-methyl-THF, showing no effect on folic acid but a decrease in the bioavailability of L-5-methyl-THF. Adding microencapsulated folic acid to milk powder decreased the bioavailability but had no effect on microencapsulated L-5-methyl-THF.

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APPENDICES

Appendix A: Letter of Information

ABSORPTION OF DIFFERENT FORMS OF FOLATE

INFORMATION SHEET FOR PARTICIPANTS

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you of any kind and we thank you for considering our request.

What is the Aim of the Project?

Folate is a B-vitamin that has been shown to reduce the risk of certain types of birth defects when taken early in pregnancy. To reduce birth defects, folic acid, a type of folate, is added to bread or flour in some countries such as Australia and the United States. Other countries such as New Zealand have been reluctant to add folic acid to bread due to potential concerns about long-term consumption of folic acid on the health of the population. Folic acid is only one type of folate but is the form usually added to bread or flour because it is very stable (i.e. doesn't breakdown during the baking process). Another form of folate, L-5-methyltetrahydrofolate (Metafolin^{TM}), has recently become available. Researchers at the University of Otago have previously shown that Metafolin^{TM} works as well as folic acid at increasing blood levels of folate when given as a pill. However Metafolin^{TM} is not as stable as folic acid when added to food. We have developed a method of protecting Metafolin^{TM} in food by covering it with a protective covering, a process called microencapsulation. The purpose of this study is to determine whether microencapsulated Metafolin^{TM} either in pill form or added to a food (milk) increases blood folate levels over the short term as well as folic acid.

What Type of Participants are being sought?

We are seeking males and females aged between 18 and 65 years of age. They should be in good health. People who are in one or more categories listed below will not be able to participate in the project.

- People who use vitamin supplements
- People with a chronic disease e.g. heart disease, diabetes
- People with allergies to milk

What will Participants be Asked to Do?

Should you agree to take part in this project, this study will involve nine visits to the clinic at the Department of Human Nutrition. At the first appointment you will be asked to sign a consent form and we will weigh you and measure your height. We will also take a blood sample to measure your iron status. At each of the 8 clinic visits you will have a catheter inserted into a vein in your arm. This catheter will be worn for the next 8 hours and will be used for blood sampling, removing the need for repeated needles. A blood sample (5 ml) will be collected from the catheter. You will then be asked to consume one of the following in a random order each week:

1. Free-form folic acid in a capsule
2. Free-form Metafolin^{TM} in a capsule
3. Microencapsulated folic acid in a capsule
4. Microencapsulated Metafolin^{TM} in a capsule
5. Microencapsulated folic acid in milk
6. Microencapsulated Metafolin^{TM} in milk
7. Placebo in a capsule

You will then be offered a standardised breakfast. Blood samples will then be collected from the catheter every ¼ hour for the first hour, every ½ hour for the next three hours, and then every hour for four hours. You will be able to do work (e.g. work at your laptop) between blood draws. We will offer you a standardised lunch between the blood draws at 4 and 5 hours. Blood will be used to measure folate and DNA methylation. Please be aware that you may decide not to take part in the project without any disadvantage to yourself of any kind.

What Benefits or Risks come from Participating in the Study?

The risks associated with the study are very small. Giving blood can be associated with short-term pain and bruising may occur. Blood samples will be drawn by an experienced registered nurse to minimise participant discomfort. You will be given $70 for each clinic visit plus $40 for completing all 8 treatments for a total of $600 for the entire study.

Can Participants Change their Mind and Withdraw from the Project?

You may withdraw from participation in the project at any time and without any disadvantage to yourself of any kind.

What Data or Information will be Collected and What Use will be Made of it?

We will be collecting personal information regarding your sex, age, weight, height and general health. The purpose of collecting this information is so that we are able to describe the overall

characteristics of the population. Only Dr Rachel Brown and Sarah Harvey will have access to personal information and even then only ID numbers will identify individuals.
The data collected will be securely stored in such a way that only those mentioned above will be able to gain access to it. At the end of the project, any personal information will be destroyed immediately except that, as required by the University's research policy, any raw data on which the results of the project depend will be retained in secure storage for five years, after which it will be destroyed.

The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve your anonymity. Any data will in no way be linked to any specific participant.
You are most welcome to request a copy of the results of the project should you wish.

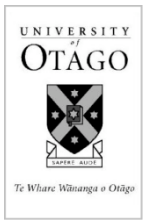
No records which identify you by name, initials or date of birth will be allowed to leave the Investigators' offices. Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of University of Otago Research Ethics Board for the purpose of monitoring the research.

What if Participants have any Questions?
Your participation in this study is appreciated. If you have any questions about our project, either now or in the future, please feel free to contact either:-

Sarah Harvey	or	Dr Rachel Brown
Department of Human Nutrition		Department of Human Nutrition

This study has been approved by the University of Otago Human Ethics Committee

Appendix B: Consent Form



ABSORPTION OF DIFFERENT FORMS OF FOLATE

CONSENT FORM FOR PARTICIPANTS

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.
I know that:-

1. My participation in the project is entirely voluntary;

2. I am free to withdraw from the project at any time without any disadvantage;

3. Personal identifying information will be destroyed at the conclusion of the project but any raw data on which the results of the project depend will be retained in secure storage for five years, after which they will be destroyed.

Please indicate the following:

- I consent to any remaining samples being disposed of using standard disposal methods at the end of the study

YES / NO

- I wish to have any remaining samples disposed with appropriate karakia at the end of the study

YES / NO

- I wish to have any remaining samples returned to me at the end of the study

YES / NO

4. I may experience slight pain during the blood test;

5. I have not had any previous adverse effects from drinking milk;

6. I understand that reasonable precautions have been taken to protect data transmitted by email but that the security of the information cannot be guaranteed;

The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve my anonymity.

I agree to take part in this project.

Full Name (please print)……

Signature of participant.....

Date .....

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