

# Folates and Dairy Products: A Critical Update

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In recent years, folates have come into focus due to their protective role against child birth defects, for example, neural tube defects. In addition, folates may have a protective role to play against coronary heart disease and certain forms of cancer. During the last few years most countries have established increased recommended intakes of folates, for example, between 300–400 $\mu\text{g}$  per day for adults. This review of folates in milk and dairy products compares some recent data based on high pressure liquid chromatography (HPLC) analyses and radioprotein-binding assays, with previous data based on microbiological assays. All three methods show similar ranges for folates in cow's milk, 5–10 $\mu\text{g}$  per 100g, the variation being due to seasonal variations. Data on folates in fermented milk (buttermilk and yogurt) are also similar for these methods. Different starter cultures, however, might explain some of the variations in folate content and folate forms. Most cheese varieties contain between 10 $\mu\text{g}$  and 40 $\mu\text{g}$  folate per kg, with slightly higher values for whey cheese. Ripened soft cheeses may contain up to 100 $\mu\text{g}$  folate per 100g. Most previous and recent studies using HPLC indicate that 5-methyl-tetrahydrofolate (5-methyl-THF) is the major folate form in milk, but more studies are needed concerning folate forms in other, especially fermented dairy products. Relatively new data on actual concentrations in different dairy products show folate-binding proteins (FBP) to occur in unprocessed milk, but also in pasteurised milk, spray-dried skim milk powder and whey. In contrast, UHT milk, fermented milk and most cheeses only contain low levels or trace amounts.

## **Key teaching points:**

- The last decade has recognised health benefits of folates regarding their prevention of neural tube defects in babies and occlusive vascular diseases caused by elevated plasma homocysteine, their link to mental fitness and possibly some forms of cancer in adults.
- When publishing the dietary reference intakes (DRI) in 1998, the US Food and Nutrition Board included the concept of these possible health-protective effects of folates by increasing recommendations for adults to 400 $\mu\text{g}/\text{day}$  from the previous 200 $\mu\text{g}/\text{day}$ . The fact that the average daily intake of folate among Western populations is generally lower than these recently set recommendations, emphasizes the need for a critical evaluation of the dietary sources of folates.
- The low concentrations of folates occurring in foods, along with a great number of unstable chemical forms of this vitamin, make analysis of dietary folates very demanding. A continuously up-dating and re-evaluation of folate figures currently present in food tables and data bases and mainly based on microbigeal assay, are absolutely necessary. Recently developed, more specific and carefully validated methodology, for example, HPLC and protein-binding methods, should be used.
- Milk and especially fermented dairy products like yogurt, buttermilk and different varieties of cheeses are already recognised as good dietary sources of folates. More quality-assured methodology in up-dating folate concentrations in the whole set of dairy products, especially those based on fermentation, would probably strengthen their significance further as folate sources.
- Raw and pasteurised milk contain folate binding proteins (FBP). Thermal treatments above pasteurisation generally denature FBP. To what extent this denaturation of FBP will affect the retention of folates during processing and storage of dairy products need further investigation. Likewise the role of intact FBP for the bioavailability of dairy folates should be paid more attention.
- In order to judge which foods are good or bad sources of folates, we need to have validated figures on the absorption and bioavailability of food folates. Still reliable data concerning to what extent dietary folates actually are absorbed are incomplete, mainly owing to lack of suitable methodology for studies on humans. However, such work is in progress.

## INTRODUCTION

Folates represent an important B vitamin, participating in one-carbon transfer reactions required in many metabolic pathways, especially purine and pyrimidine biosynthesis (DNA and RNA) and amino acid interconversions. Recent studies on folates focus on their protective role against neural tube defects occurring during early pregnancy [1,2]. In addition, a growing body of evidence suggests an association between folate intake and a reduced risk of occlusive vascular disease. Low plasma folate concentrations have been shown to correlate with elevated levels of plasma homocysteine, an independent risk for coronary heart disease [3,4]. Furthermore there is growing evidence that a low folate status increases the risk of cancer, particularly colon cancer [5,6]. As a consequence, several experts and authorities advocate an increase in the consumption of folates, especially for women of fertile age. A level of 400  $\mu\text{g}$  folate per day has been suggested, which was actually the RDI value used until 1989 [7], when it was reduced to 200  $\mu\text{g}$ . A recommended increase in the daily intake of folates emphasizes the need for a critical evaluation of the dietary sources of folates. Considering the fact that food data on folates rely on microbiological determination of broad specificity and insufficient methodological quality control, it is obvious that dietary folates need to be evaluated using more specific methods. Another important issue concerns the bioavailability of dietary folates, which has been estimated to range between 40% to 70%, based on relatively few studies [for a review see 8].

On average, milk and dairy products provide 10% to 15% of the daily folate intake in many Western countries, especially among the younger population. Expressed in terms of nutrient density (calculated as the ratio between nutrient content per joule and recommended intake per joule), figures close to or exceeding a value of "one" indicate that a particular food is a good source. Milk, especially low-fat milk products, shows nutrient density values for folates of approximately one for adults and two for children aged one to three years [9].

Below follows a brief presentation of folates in terms of chemistry, chemical stability, analysis, bioavailability, physiology, deficiency and dietary requirements. Thereafter, milk and dairy products are reviewed with regard to their folate content. Data obtained from microbiological assays are compared with recent data based on HPLC analyses. Major forms of folates and concentration ranges due to seasonal variations are presented together with data on folate retention during processing and storage. Special emphasis is placed on the

significance of milk and dairy products as folate sources, taking into consideration new data on folate-binding proteins.

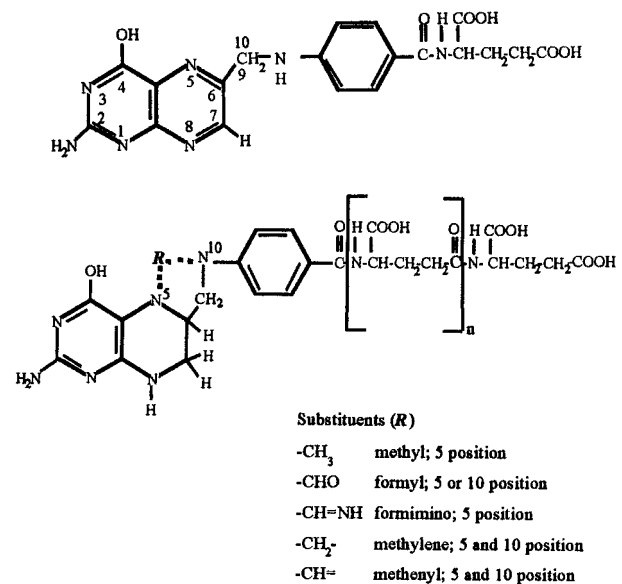
## FOLATES

### Chemical Structure

The generic term "folate" refers to the class of compounds having a chemical structure and nutritional activity similar to that of folic acid (pteroyl-L-glutamic acid, Fig. 1a). In nature, the vitamin exists primarily as reduced, one-carbon-substituted forms of pteroylglutamates, differing in substituent and number of glutamyl residues attached to the pteroyl group. Five different one-carbon units (methyl, formyl, formimino, methylene and methenyl) are known (Fig. 1b). Most of the naturally occurring dietary folates have a side chain of five to seven glutamate residues connected by  $\gamma$ -peptide linkages [10].

### Folate Stability

There are considerable differences in stability between various reduced folates (THF). In most cases, folic acid exhibits substantially greater stability than the reduced folates. The order of stability of these latter forms is 5-formyl-THF > 5-methyl-THF > 10-formyl-THF > THF. Moreover, the stability



**Fig. 1a.** Structure of folic acid (pteroyl-L-glutamic acid). **1b.** Structure of native food folates, e.g. reduced, one-carbon-substituted forms of polyglutamates.

Abbreviations: ATCC = American Type Culture Collection, DNA = deoxyribonucleic acids, ELISA = enzyme-linked immunosorbent assay, FBP = folate binding protein(s), HPLC = high performance liquid chromatography, MA = microbiological assay, RPBA = radio protein binding assay, RDI = recommended dietary intake, RNA = ribonucleic acids, THF = tetrahydrofolate, UHT = ultra high temperature.

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is pH-dependent, with the reduced folates being most stable at pH >8 and pH <2 and least stable between pH 4–6 [10]. The chemical reactivity of some important folate compounds makes the vitamin one of the most vulnerable to losses during food processing. Considerable losses have been reported following a number of different processes [10]. Oxidative degradation enhanced by oxygen, light and heat is responsible for a major part of these losses. Oxidation results in a splitting of the molecule into biologically inactive forms, of which *p*-aminobenzoylglutamate is one major form. If present in sufficient amounts, antioxidants, e.g., ascorbic acid and thiols, protect folates from being oxidized. The rate of reaction for folate breakdown in the presence of oxygen depends on the type of folate derivative and the nature of the food matrix, in particular in respect to pH, buffer composition, catalytic trace elements and antioxidants. In addition, folates are easily lost by leakage into water during processing.

### Analytical Methods

Techniques potentially suitable for the measurement of folate in foods include microbiological assays, HPLC, and competitive-binding radioassay procedures [for a review, see 11]. The measurement of folates is complicated by the need to account for all forms of the vitamin, which could easily include several dozen compounds if each form of the folate nucleus exists in all possible combinations with various polyglutamate chain lengths. Microbiological assays serve as the traditional method of folate analysis and are based on the nutritional requirements of microorganisms. *Lactobacillus rhamnosus* (*Lactobacillus casei*, ATCC 7469) is used to analyse total folate in food and responds to most native folates, although the response decreases as the number of glutamyl residues linked to the pteroyl group increases. In order to measure all the polyglutamated forms, these must be enzymatically deconjugated prior to analysis. Although the method is quite unspecific and crude, most data on folate levels compiled in food tables and used for labelling purposes are still based on this method.

Competitive-binding radioassays involve competition between folate in the sample or standard and radio-labelled folate for the binding to a folate-binding protein, typically from milk. In spite of the speed and convenience of these assays, their application to food analysis is limited due to a varying affinity for different forms of folate. In particular, formyl forms of THF show very low affinities. Likewise, the affinity towards polyglutamyl folates needs further investigations. In addition, the binding is strongly pH-dependent [12]. Only a very limited number of studies have reported folate concentrations in milk, but in no other dairy products, using this method [13–15].

Several HPLC methods have been developed for the measurement of folates in foods and other biological material. These methods have been applied to separate and detect the individual forms of folates, especially 5-methyl-THF,

5-formyl-THF and THF, mostly involving fluorescence detection [for ref, see 11]. So far, no HPLC method has been approved as suitable for food analysis in general, although intercalibration work is in progress [16,17]. Extraction, enzymatic pre-treatment and sample clean-up must be optimised to permit application to various types of food. Only a few studies have been published on folates in milk and dairy products based on HPLC analyses [18–24].

### Bioavailability

Monoglutamate folates are absorbed by an active energy-dependent, carrier-mediated process at physiological concentrations and by passive absorption at higher concentrations [25]. Absorption takes place mainly in the jejunum and is markedly influenced by pH with a maximum absorption at pH 6.3. The polyglutamic folates must be cleaved to their monoglutamate forms by a pteroylpolyglutamate hydrolase, referred to as folate deconjugase, before uptake can take place in the intestinal epithelial cells (primarily in the jejunum). Reisenauer *et al.* [26] reported the existence of two separate folate deconjugase activities in human jejunal mucosa: one soluble and intracellular, the other membrane-bound and concentrated in the brush border. Human brush border deconjugase is a zinc-dependent exopeptidase with optimum activity at pH 6.5, resulting in the stepwise hydrolysis of polyglutamyl folates. This enzyme has been shown to play the principal role in the digestion of dietary folate. The bioavailability of naturally occurring folate in food is incomplete, varying between 40% and 70% [27–29]. Most of these data rely on only two methods of determination. In man, following pre-saturation of tissues with repeated doses of synthetic folic acid, various single food items have been ingested and the urinary excretion of folates monitored to assess the bioavailability. The other method in use is a rat bioassay [28,30,31]. The bioavailability of naturally occurring folates in most foods has not been fully determined under conditions of actual consumption, including the consequences of interactions between various food constituents. The mean bioavailability of polyglutamyl folates is estimated to typically 70% relative to the monoglutamyl species, which indicates the rate-limiting nature of intestinal deconjugation [10]. Among the factors responsible for incomplete bioavailability is the possible degradation of labile tetrahydrofolate forms in the acidic gastric environment. Recently, Wigertz [32] showed substantial losses (10% to 60%) of 5-methyl-THF using an *in vitro* method for studying the retention of native milk folates during the digestion. In the presence of 0.01% sodium ascorbate, however, a retention close to 90% was obtained.

Using a rat bioassay model, Swiatlo *et al.* [33] have reported that the bioavailability of synthetic folic acid is higher in the presence of cow's milk. The experimental diets were based on 20% milk solids and 0ng, 200ng, 400ng and 600ng added synthetic folic acid per gram of diet. Thus, native milk folate

was only around 10ng/g diet. The results of this study showed that incorporation of human or bovine milk into diets significantly ( $p < 0.01$ ) enhanced the bioavailability of added folic acid. In contrast, goat milk reduced the bioavailability of added folic acid by 44%. The cause for these results is not clear. Hypothetically, differences in folate binding protein content of the diets or coprophagy and colon folate biosynthesis in the rat might have interfered. Similar results were recently found in a preliminary balance study on nine healthy ileostomists [32]. Ileostomists consuming daily either one litre of milk or fermented milk together with a standardised diet excreted less folate into their ileostomy bags than after replacement of the milk or fermented milk by a beverage based on carbonised sugarised water. Another interesting aspect of the bioavailability of milk folates is the possibility that folate-binding proteins may have a positive impact. Although claimed that this protein resists degradation during the gastrointestinal passage in suckling pups, there are no systematic studies performed on humans. However, the ileostomy study compared the excretion of dairy folates from milk and fermented milk and did not find any difference in excreted amounts of 5-methyl-THF [32]. In contrast to milk, fermented milk did not contain any folate binding protein. However, this study is the first one using human ileostomists and needs to be repeated under more specific and controlled conditions, where milk or dairy products are the sole folate source and not, as in this study, a part of the folate intake.

### Physiology, Deficiency and Requirements

The liver is the major storage site in the human body where folates are stored as polyglutamates. Folate is essential for one-carbon transfer reactions required in many metabolic pathways, including purine and pyrimidine biosynthesis (DNA and RNA) and amino acid interconversions. A negative folate balance is followed by a depletion of folate stores and, ultimately, anaemia. Folate deficiency is first manifested in erythrocytes and bone marrow cells, as these cells have a relatively high turnover rate. These cells become megaloblastic (enlarged), due to lack of the DNA and RNA necessary for normal cell division and protein/enzyme synthesis. Folate deficiency is developed in the presence of malnutrition, due to low intake of folate-containing foods, or as a result of severe alcoholism. A more important risk factor is malabsorption, especially for diseases affecting either intestinal pH or the jejunal mucosa, e.g., celiac disease. Secondary folate deficiency (also giving rise to megaloblastic anaemia) may be due to vitamin B<sub>12</sub> deficiency.

Another important cause of folate deficiency is an increased requirement. Pregnancy is known to double the need of dietary folates; recommendations in most countries are therefore set to 400  $\mu\text{g}$  dietary folates per day, whereas some countries, e.g., the UK and the USA, instead recommend additional 400  $\mu\text{g}$  folic acid per day as supplements [36]. Low maternal folate

status has been associated with premature birth, low birth-weight and increased risk of neural tube defects in the offspring. Of all cases of neural tube defects, one half have spina bifida alone, and another half have anencephaly with or without spina bifida. Neural tube defects result when the neural tube fails to close during the early stages of pregnancy. Normally, the tube is closed by the end of the sixth week after the last menstrual period, i.e., before most women are even aware of their pregnancy. It is, however, unclear which precise mechanisms are involved in the development of these anomalies, although several studies in recent years show a reduced risk of neural tube defects following folate supplementation [1].

Previous recommendations concerning intakes of essential nutrients aimed to cover the needs of the consumer in order to prevent deficiency diseases. Recent knowledge has raised the question of whether recommendations should also consider the potential of a nutrient to reduce the risk of chronic disease among the middle-aged and elderly [7,35,36]. In addition to data regarding folate and neural tube defects, a growing body of evidence suggests an inverse association between folate intake and coronary heart disease. This association is based on the fact that plasma homocysteine levels increase in response to inadequate folate intake. Elevated plasma homocysteine levels are recognised as an independent risk factor for coronary heart disease. It is assumed that homocysteine is responsible for the development of some forms of occlusive vascular disease. Homocysteinaemia might also develop due to genetic enzyme defects [37,38].

A low folate status has also been associated with elevated risk of cancer. A large number of epidemiological studies have provided evidence of a positive association between high levels of folate intake and reduction in cancer risk. The data are strongest concerning risk of colorectal cancer, which represents the second leading cause of death due to malignancies [5,6].

## FOLATES IN DAIRY PRODUCTS

Tables 1,2 and 3 summarise current data of folates in typical dairy products. Table 1 compares data from different national food tables usually based on microbiological determination if not otherwise stated. In general, the total folate values in Table 1 vary with a factor of 2, sometimes even three- to fivefold, suggesting weak methodological control besides variation of food samples. Table 2 compares folate concentrations in dairy products based on different analytical methods: the microbiological assay (MA), the radio protein binding assay (RBPA) and HPLC. Wide variations within each dairy product are demonstrated, but in general the different methods show values of the same magnitude, though the RBPA do not quantify formylated folate forms and HPLC data are usually only based on the sum of a few reduced folate monoglutamyl forms. Table

**Table 1.** Folate Content in Common Dairy Products, ( $\mu\text{g}/100\text{g}$ ): Comparison of Food Tables from Different Countries

Dairy products	Sweden	Finland	Britain	Germany	Denmark	USA	France	N. Zealand
	folate( $\mu\text{g}/100\text{g}$ )							
Milk, whole	6	6	6	7	9	5	7	5
Milk 3% fat	7	–	–	–	–	5	5	5
Milk 1.5% fat	7	4	–	–	4	–	5	–
Whipping cream	4	7	7	4	6	4	4	6
Skimmed milk powder	–	51	–	21	21	–	–	–
Whey cheese 8% to 30% fat	5–12	–	–	–	–	–	–	–
Quark	12	22	–	16	–	–	–	45
Cottage cheese 5% fat	12	–	27	–	–	12	16	17
Yogurt plain 3% fat	15	8	18	13	17	7/12	3	12
Brie 28% fat	65	–	58	65	90	65	150	–
Camembert 23% fat	62	59	102	44*	62	62	96	51
Feta cheese	18	62	23	–	62	–	–	23
Cheese blue 30% fat	36	–	50	40	36	36	94	24
Cheddar 33% fat	–	–	33	19	20	18	20	16
Edam cheese 28% fat	–	35	40	–	–	16	18	20

\* analyzed by HPLC.

– not reported.

**Table 2.** Folate and Folate Binding Protein in Dairy Products

Dairy product	folate ( $\mu\text{g}/100\text{g}$ )			FBP (nmol/100g)
	MA <sup>1</sup>	RPBA <sup>2</sup>	HPLC <sup>3</sup>	ELISA <sup>4</sup>
Milk*	5–10	5–10	5–10	16–21
Milk (UHT)	5–8	4–8	4–8	<2
Skimmed milk powder	50	–	60	196
Whey	2	–	6	10
Whey cheese	5–12	–	35–50	3–4
Fermented milk	2–19	5–10	5–13	<1
Cottage cheese	12–27	–	3–21	54
Camembert	10–100	–	44	–
Brie	10–100	–	–	–
Hard cheese	10–40	–	12–18	1

FBP folate binding protein.

UHT ultra high temperature processing.

\* pasteurized.

– not reported.

<sup>1</sup> microbiological assay; total folates, data from food tables [40,67–74].<sup>2</sup> radioprotein binding assay; formylated folate forms not included [13–15].<sup>3</sup> high performance liquid chromatography; only 5-methyl-THF [21–23].<sup>4</sup> enzyme-linked immunosorbent assay [23].

2 also presents data on the amounts of folate binding proteins (FBP) in some dairy products. Table 3 presents compiled data on the occurrence of the major folate forms in dairy products based on HPLC analysis. Below, these data are discussed in more detail.

## MILK

### Folate Content

Food composition tables and review papers based on microbiological assay report total folate values for cow's milk in the range of 5–7  $\mu\text{g}/100\text{g}$  (Table 1; [39,40]). Commercial radio-protein-binding assays based on folate-binding proteins from

bovine milk produce ranges between 4  $\mu\text{g}$  and 8  $\mu\text{g}$  per 100g (Table 2; [13–15]). Using HPLC, 5-methyl-THF concentrations after deconjugation of various milks range between 4.1  $\mu\text{g}$  and 4.4  $\mu\text{g}/100\text{g}$ . Thus, using three independent methods of which HPLC is the most specific, similar amounts of folate in cow's milk are found (Table 2 and 3).

Most studies [17,20,24,32,41] with one exception [20] indicate 5-methyl-THF as the major form (>90%) of folate in milk. The degree of polymerisation of folates is of importance for the bioavailability of milk folates. Using a microbiological assay, Karlin [42] found that the total 5-methyl-THF in cow's milk consisted of 60% monoglutamate folates and 40% of polyglutamate folates. According to Selhub [43], cow's milk contained monoglutamyl and polyglutamyl folates in the ratio

**Table 3.** Concentration of Individual Folate Forms in Dairy Products Analyzed by HPLC

Dairy product	folate $\mu\text{g}/100\text{g FW}$			Reference
	THF	5-CH <sub>3</sub> -THF	5-CHO-THF	
Milk 1.5% fat*	<1	4	—	24
Milk 1.5% fat*	—	7 <sup>#</sup>	—	22,23,32
Milk 3% fat*	—	7 <sup>#</sup>	—	22,23,32
Milk 3.8% fat*	<1	3	15	20
Milk UHT	—	7 <sup>#</sup>	—	22,23,32
Skimmed milk powder	—	60	—	22,23,32
Whey	—	6	—	22,23,32
Whey cream cheese	—	51	—	22,23,32
Cottage cheese (Keso <sup>®</sup> )	—	22	—	22,23,32
Buttermilk 2.5% fat	<1	4	5	24
Buttermilk (Filmjöl <sup>®</sup> )	—	10 <sup>#</sup>	—	22,23,32
Yogurt plain	2	1	3	24
Yogurt plain 3% fat	—	5 <sup>#</sup>	—	22,23,32
Yogurt 3.5% fat	<1	1	13	20
Camembert	14	17	15	20
Hard cheese Edam type	1	2	4/msk	24
Hard cheese (Herrgård <sup>®</sup> )	—	12 <sup>#</sup>	—	22,23,32
Hard cheese (Grevé <sup>®</sup> )	—	18 <sup>#</sup>	—	22,23,32
Hard cheese (Västerbotten <sup>®</sup> )	—	13 <sup>#</sup>	—	22,23,32
Hard cheese (Emmentaler)	<1	<1	6	20
Milk powder CRM 421 <sup>†</sup>	—	25	—	17

HPLC high-performance liquid chromatography.

FW fresh weight.

THF tetrahydrofolate.

5-CH<sub>3</sub>-THF 5-methyltetrahydrofolate.

5-CHO-THF 5-formyltetrahydrofolate.

\* pasteurized.

<sup>#</sup> recovery corrected values.

— below detection limit.

— not reported.

msk masked by impurities.

<sup>†</sup> vitamin enriched milk powder, reference material from European Commission [17].

3:1. HPLC analyses before and after deconjugase treatment indicated that the 5-methyl-THF of cows' milk constituted between 40% to 70% of polyglutamic folates [43].

### Seasonal Variation

Several studies over time indicate higher folate values for cow's milk during summer (May–September) than winter, ranging between 4  $\mu\text{g}$  and 10  $\mu\text{g}$  folate per 100g on an annual basis [23,40–42]. However, Hoppner and Lampi [44] found no significant variation of folate content in skim milk obtained from local stores in Canada. A seasonal change in milk folates seems logical considering that folate is an unstable vitamin with highest concentrations occurring in fresh green plants fed to the cows during the summer compared to longer stored winterfodder.

### Processing and Storage

Pasteurisation has only minor effects on the folate content of milk, causing losses of less than 10% [22,40,45]. According

to a study by Andersson & Öste [14], folate levels in pasteurised milk were not reduced during storage beyond the expiration date. The milk, packaged in commercial paperboard cartons, was stored open in a refrigerator to simulate household conditions and was exposed to daylight at room temperature for two 30-minute periods per day [14].

Losses of folates are, however, observed during UHT treatment of milk [22,40] and the storage of aseptically packed UHT milk [45]. The amount of oxygen that is solubilized in the milk before processing, and the level of ascorbic acid, play a role in the extent of folate loss. Andersson & Öste [13] reported a 43% reduction of microbiologically available folates during indirect UHT treatment, with an oxygen level of 5.4 ppm in the milk. When the oxygen level was reduced to 0.6 ppm, only 4% of the folates were lost. The amount of oxygen available, either in the headspace of the package or from the atmosphere by diffusion through the package material, has a marked influence on the retention of folates during the storage of UHT milk [13]. Milk in packages with an oxygen-permeable seal lost all folates within three weeks of storage at room temperature. Ascorbic acid may exhibit a protective effect towards folates, and it has

been shown that the addition of ascorbic acid to UHT milk prolongs the storage stability of folates [13]. Recent advances in milk processing have resulted in reduced oxygen levels (<1 ppm) in UHT milk. A storage study performed on UHT milk containing 0.3 ppm dissolved oxygen stored at 5°C for four months showed no loss of 5-methyl-THF [22].

According to food tables and recent HPLC studies, spray-dried milk powder contains on average around 30–60 µg total folates per 100g. Data from spray-drying of milk indicate rather modest losses of folates according to Wigertz *et al.* [22,23]. The stability of folates in dry milk was also investigated in a study by Kneifel *et al.*, [46] who found that folates, together with vitamin C, were the most labile among the vitamins during storage.

## FERMENTED MILK

### Folate Content

Several review articles on the nutritive value of cultured dairy products, e.g., buttermilk and yogurt, have reported that the folate content of such milk products vary widely, ranging from 4 µg to 19 µg/100g [39,40,47–50]. Food composition tables based on microbiological assays report total folate values of between 5 µg and 18 µg per 100g for various fermented milk products (Table 1). A few studies based on HPLC analyses support the data obtained with microbiological assays (Table 2). In addition, one study using RBPA found plain yogurt to contain 5.4 µg folate per 100g [23]. A recent study based on HPLC found buttermilk and yogurt to contain 9.7 µg and 4.7 µg 5-methyl-THF/100g, respectively [44]. The plain yogurt in their study consisted of a culture of *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, which could continuously alter the composition and concentration of folate. However, the significantly lower levels of 5-methyl-THF found in plain yogurt compared with buttermilk (inoculated with *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* ssp. *cremoris*) seemed to be in accordance with observations by Rao & Shahani [51]. They found that the total folate levels in skimmed milk fermented by *L. bulgaricus* decreased from 9.8 µg to 1.6 µg/100g within 36 hours of incubation, while *S. thermophilus* and *L. acidophilus* increased the total folate levels substantially. In the presence of both *L. bulgaricus* and *S. thermophilus* (see plain yogurt) the former might have consumed the folates produced by the latter.

Table 3 shows data on different folate forms reported in fermented milk. Müller [20] found yogurt and buttermilk to contain approximately 13.7 µg and 7.5 µg/100g respectively, with approximately 80% to 90% of the total folate appearing in the 5-formyl-THF form. Vahteristo *et al.* [24] reported THF, 5-methyl-THF and 5-formyl-THF in buttermilk and plain yogurt; 5-formyl-THF was the major form. Wigertz *et al.* [23]

found 5-methyl-THF to be the major folate form in buttermilk and plain yogurt, while other forms of folate, such as THF, only corresponded to approximately 10% of the total folate concentration. However, due to instability and high variability, this form of folate was not reported. In addition, Wigertz *et al.* [23] found no 5-formyl-THF, but quantification was limited due to low fluorescence sensitivity for this form. The difference in folate forms found in these three HPLC studies might be due to the use of different cultures during fermentation or to differences in the methodology of analysing native folates.

### Storage

Reddy *et al.* [52] found that storage of yogurt at 5°C for 8 to 16 days resulted in considerable reduction of the folate content. In contrast, Rao & Shahani [51] claimed that there was no appreciable change in the folate level when fermented milk was stored at 5°C for five days. Similar findings were reported by Wigertz *et al.* [23], who found no significant loss of folate in any of the fermented milk products at the end of their shelf-life (2 weeks in refrigerator).

## CHEESE AND WHEY

### Folate Content

Unripened soft cheeses, for example, plain cottage cheese, contain between 12 µg and 27 µg total folates per 100g based on microbiological assays (Table 1) and HPLC analyses (Table 2). Ripened soft cheeses, for example, Brie and Camembert, have been reported to contain between 50 µg and 100 µg total folate/100g, probably due to the synthesis of folates by microorganisms during ripening (Table 1). Most hard cheeses, among them, Edam, Gouda, Emmental and Cheddar, have been reported to contain 20 to 40 µg of total folates/100g based on microbiological assays (Table 1). HPLC analyses have shown lower values of 7 µg total folate/100g mainly of 5-formyl-THF [20] and between 2 µg and 18 µg/100g, mainly as 5-methyl-THF (Table 2 and 3). Liquid whey contains 2 µg microbiologically active folate per 100g according to food tables, and approximately 6 µg 5-methyl-THF/100g according to HPLC analyses (Table 2 and 3). Whey cream spread and whey cheese contain 12 µg and 5 µg folate per 100g respectively, according to food tables based on microbiological assay (Table 1). Corresponding figures using HPLC were 30 µg and 50 µg total 5-methyl-THF/100g, respectively (Table 2 and 3). The higher figures in whey cheese might be due to its high concentrations of whey proteins rich in folate binding proteins (FBP).

### Processing and Storage

The manufacture of one kg of cheese requires about 10 litres of milk. During curdling, the water-soluble material, whey

proteins and water-soluble vitamin, is separated from the semi-solids of casein, fat and salts. The 5-methyl-THF concentration in whey and cheese obtained by HPLC indicates that approximately 50% of the milk folates are recovered in the whey. Since FBP concentration is also reduced by approximately a half, this means that the 5-methyl-THF in the whey fraction could still be bound to FBP. Generally, curdling due to rennet occurs at a pH of approximately 5.5 to 6; a complete dissociation between FBP and bound folates occurs only below 3.5 [53]. The folate levels and/or chemical forms in whey products are very dependent on the harshness of the processing conditions [53]. Variations of folate levels in cheese might be due to different cheese starters which either produce or consume folates.

Studies on the effects of storage and ripening on the folates of hard cheese are few. Reif *et al.* [54] observed that the starter culture of a cottage cheese actively synthesised folates during the setting period, resulting in a significant increase in folate concentrations in the final product. Wigertz *et al.* [23] reported no significant change in the concentration of 5-methyl-THF over a two week period, whereas a significant decrease (about 30%) was seen in hard cheese stored for six months. Again, one must bear in mind that biosynthesis or utilisation of B vitamins by lactic-acid-producing bacteria depend greatly on the strain of the organisms used and the manufacturing procedure. Taking into account the different stages of cheese making—drainage of whey, the coagulation of milk proteins, the lowering of pH and the ripening procedure through the action of different enzymes or species of microorganisms—low levels of milk folates in cheese seem likely. Furthermore, the higher concentration of 5-methyl-THF observed in cottage cheese compared to hard cheese could also be explained by the addition of pasteurised cream to the final product and the considerable amount of whey left in the product after processing.

## MILK FOLATE-BINDING PROTEIN (FBP)

### Background

The existence of a “milk folate binder” was originally suggested by Ghitis and associates [55] and was later supported by Metz *et al.* [56]. Ford and colleagues [57] partially purified a folate-binding factor from bovine milk with a molecular mass of 28 to 30 kDa. The binding factor clearly had a preference for oxidised folates, but also bound 5-methyl-THF and other reduced folates. The binding of folates was found to be pH-dependent. Below pH 3.5, the folate-binder complex completely dissociated, but binding activity was regained when the solution was neutralised [for reviews of this literature, see 57–60].

The physiological role of these folate-binding proteins is unclear. Ford *et al.* [57] suggested that in human milk, the

folate-binding proteins may initially act in the mammary gland as a trapping agent to accumulate folate from blood plasma into the milk. However, after ingestion by the infant, it could assist in the absorption of folate by preventing uptake by intestinal bacteria. It might also directly promote the transport of folate across the intestinal mucosa. Bound folate and free folate are absorbed in different ways in the gastro-intestinal tract. While free monoglutamic folate is absorbed in the jejunum, the protein-bound folate is mainly absorbed in the ileum and at a much slower rate than free folate [61]. A slower rate of transport, coupled with protection from intestinal bacteria, may improve the bioavailability of folate when bound to proteins in milk. In fact, breastfed babies have been reported to have a better folate status than bottlefed babies. While breastfed babies sustain their folate status on an intake of 55 µg folate per day, bottlefed babies need 78 µg/day. It has been suggested that the discrepancy is due to the occurrence of folate-binding proteins in human milk which are not present in the heat-processed milk formula [62,63].

### FBP Concentrations in Milk and Dairy Products

Using an ELISA method originally developed by Høier-Madsen *et al.* [64], the first values of FBP concentrations in processed and unprocessed cow's milk were recently presented by Wigertz *et al.* [23]. The concentration of FBP in unprocessed milk was found to be  $21.1 \pm 0.7$  nmol/100g, whereas a significantly ( $p < 0.05$ ) reduced concentration of  $16.8 \pm 2.0$  nmol/l (still in the high range) was observed in pasteurised milk. Similar FBP values were found in pasteurised milk in another recent study by Wigertz *et al.* [23]. These findings are in good agreement with earlier findings by Ford *et al.* [57], who found a 10% decrease in folate-binding properties after low-temperature, long-term pasteurisation. In a study from Gregory [65], partial denaturation and similar folate-binding capacities for unprocessed and commercially pasteurised skimmed milk were observed. In contrast, in a study by Areekul *et al.* [66], where the folate-binding capacity was monitored using  $^3\text{H}$ -labelled folic acid, more than a 90% reduction in folate-binding capacity in pasteurised milk (75°C for 15s) was observed. Similar results to those of Areekul *et al.* [66] have been reported by Kohashi *et al.* [67], who compared unprocessed milk and milk pasteurised at 63°C for 30 min, at 75°C for 15s and at 120°C for 1s. A possible explanation of these conflicting results might be that the conditions used for pasteurisation are very close to those at which denaturation of FBP takes place. Thus, small fluctuations in the processing conditions may have a relatively large impact on the denaturation state of FBP.

UHT milk and plain yogurt were found to contain only very low levels of FBP concentrations, 0.5–1.5 nmol/l and <1 nmol/100g, respectively (Table 1). The low levels of FBP found in UHT milk could be explained by the heat treatment process of 140°C for 5 seconds. The yogurt was also subjected to heat treatment (90°C for 10 minutes) prior to inoculation. However,

even if the heating step in yogurt production had been omitted, folate in fermented milk would most likely have occurred in the free form, since low pH such as that found in yogurt is known to cause dissociation between FBP and the folate [68].

In spite of the high temperatures employed in the spray-drying process, most of the FBP was found to be retained in skimmed milk powder. During cheese manufacturing, FBP was recovered both in the whey fraction and curd, around half of it in each fraction. Cottage cheese and hard cheeses contained 54nmol and approximately 1.5nmol FBP/100g, respectively. Low FBP concentrations (3–4nmol/100g) were recorded for whey cream spread and whey cheese. These variations in FBP concentration may be explained by differences in processing conditions, for example, heating and pH. Furthermore, the microbiological activity of the cheese starters could destroy FBP through proteolysis. Since the ELISA method has been applied very recently on acidic and/or fat-rich milk samples like whey, curds and cheese products, one cannot exclude methodological interferences. Thus, further studies are necessary to confirm the data on FBP concentrations in cheese and whey products (Table 2).

## CONCLUSION

This survey of folates in milk and dairy products compares some recent data based on HPLC analyses and competitive-binding radioassays with previous data based on microbiological assays. All three methods show similar ranges for folate concentrations in cow's milk, 5–10 $\mu$ g per 100g, taking into consideration seasonal variations. In addition, data on folates in fermented milk products (buttermilk and yogurt) are comparable by these methods. Different starter cultures, however, might explain some of the variations in folate content and folate forms determined. An overall trend suggests that fermented milk contains slightly higher amounts of folates, sometimes double, depending on the starter culture used. Most cheese varieties contain between 10 $\mu$ g and 40 $\mu$ g folate/100g, with slightly higher values for whey cheese. Ripened soft may contain up to 100 $\mu$ g folate/100g. Most studies indicate 5-methyl-THF to be the major form in milk, but further studies are required to determine dominant folate forms in other dairy products.

Considering the bioavailability of dairy folates, HPLC studies indicate that approximately half of the folates in milk and dairy products occur as polyglutamic folates which require intestinal hydrolysis before they can be absorbed. In respect to the suggested role of folate-binding proteins in facilitating the absorption of folates from milk, new data on actual concentrations in different dairy products are now available. These data clearly show folate-binding proteins to occur in unprocessed milk, but also in pasteurised milk, spray-dried skim milk powder and whey. In contrast, UHT milk, fermented milk and most cheeses only contain low levels or trace amounts of FBP. The role of FBP, if any, requires further elucidation. One question

that needs to be answered is whether FBP can resist gastrointestinal proteolysis, thereby acting like an "intrinsic factor" for folates. For example, if unsaturated FBP binds dietary folates, pasteurised milk, with its excess of FBP, could be used as a means of enhancing the bioavailability of not only milk folates but also dietary folates in general.

Thus, FBP might have several applications for the dairy industry: i) stabilise dairy folates and eventually also other dietary folates during processing and storage; ii) improve the bioavailability of dairy folates and other dietary folates consumed simultaneously; iii) be used as a vehicle for developing folate-rich food products; iv) be a useful compound in extracting and clean-up of dietary folates prior to analyses.

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