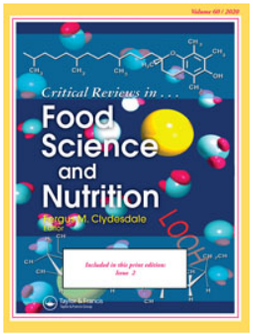

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REVIEW



# Bacterial folate biosynthesis and colorectal cancer risk: more than just a gut feeling

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**ABSTRACT**

Folate is a B-vitamin with an important role in health and disease. The optimal folate status with regard to human health remains controversial. A low intake of natural folate as well as excessive intake of synthetic folic acid, were previously linked to an increased risk of colorectal cancer or with aberrant molecular pathways related to carcinogenesis in some studies. Importantly, most studies conducted so far, solely focused on dietary intake or circulating levels of folate in relation to cancer risk. Notably, diet or dietary supplements are not the only sources of folate. Several bacteria in the gastrointestinal tract can synthesize B-vitamins, including folate, in quantities that resemble dietary intake. The impact of bacterial folate biosynthesis concerning human health and disease remains unexplored. This review highlights current insights into folate biosynthesis by intestinal bacteria and its implications for processes relevant to cancer development, such as epigenetic DNA modifications and DNA synthesis. Moreover, we will reflect on the emerging question whether food-grade or intestinal bacteria can be considered a potential target to ensure sufficient levels of folate in the gastrointestinal tract and, hence the relevance of bacterial folate biosynthesis for disease prevention or treatment.

**KEYWORDS**

Folate; biosynthesis; colon; intestinal bacteria; colorectal cancer; DNA methylation; one-carbon metabolism

## Introduction

Folate represents a group of water-soluble vitamins named tetrahydrofolates (THFs), which together with other B-vitamins, play an essential role in human metabolism (Scott 1999). Mammalian cells are not able to produce folate and are, therefore, dependent on other sources. Natural forms of folate, mostly present in the form of polyglutamate derivates, are commonly found in green leafy vegetables, fruits, liver, bread, potatoes and dairy products (Konings et al. 2001). Synthetic folic acid (pteroylmonoglutamic acid) is commonly used in dietary supplements or fortified food products, given its fully oxidized and stable composition. An inadequate status or intake of folate has been implicated in the etiology of congenital defects, neurodegenerative conditions, cardiovascular diseases, and some cancers (Chen et al. 2014; De-Regil et al. 2015; Galeone et al. 2015; Porter et al. 2016; Smith and Refsum 2016; Wang et al. 2012). Implementation of folic acid fortification programs for the prevention of neural tube defects (Czeizel et al. 1994; Wolff et al. 2009) have been shown to increase folic acid intake (Hoey et al. 2007; Quinlivan and Gregory 2003), and hence circulating levels of folate in the general population (Ganji and Kafai 2006; Hoey et al. 2007; Pfeiffer et al. 2005). An improved folate status can benefit public health, not only through prevention of neural tube defects (Honein et al. 2001), but also in terms of decreased risk of anemia (Ganji and Kafai 2009) and potentially stroke (Hsu et al. 2018; Yang et al. 2006).

There have, however, also been substantial concerns about potential detrimental effects of prolonged and excessive intake of folate, particular in the form of high-dose folic acid supplements (Yeung et al. 2008). One issue which has been heavily debated, but remains unsolved, is the question whether folate is associated with cancer risk (Kim 2018; Mason and Tang 2017). Colorectal cancer is among the most common cancers, and has been extensively studied in relation to folate intake or status (Kim 2008; WCRF/AICR 2017). Remarkably, epidemiological as well as animal studies provided crucial evidence suggesting that folate might play a dual role in colorectal carcinogenesis (Ulrich and Potter 2007). It has been suggested that either a deficiency or excessive intake of folate or folic acid may increase colorectal cancer risk, especially if precancerous lesions exist

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(Mason and Tang 2017). The proposed relationship between folate and colorectal cancer risk can be visualized in a so-called *U*-shaped curve (Ulrich 2007). Despite extensive research, the exact boundaries for this *U*-shaped curve and the underlying mechanisms linking folate to colorectal cancer risk are not fully understood (Neuhouser et al. 2011). Moreover, it is not clear whether the *U*-shaped curve applies to natural as well as synthetic forms of folate and the need for further studies extending this field of research is recognized (Blumberg et al. 2018; Miller and Ulrich 2013).

It should be noted that most epidemiological studies conducted so far focused on dietary intake or circulating levels of folate in relation to colorectal cancer risk (Chuang et al. 2013; Kennedy et al. 2011; Kim et al. 2010; Moazzen et al. 2017). Notably, diet is not the only source of folate. Several intestinal bacteria in the colon, but also in the small intestine, are capable of biosynthesis of natural forms of folate as well as vitamin $B_{12}$ and other B-vitamins (Camilo et al. 1996; Magnusdottir et al. 2015; Rossi et al. 2011). It has been shown that specific transporters in the colon actively absorb folate (Said 2013) and as such contribute to folate levels in peripheral tissues and the circulation (Aufreiter et al. 2009; Lakoff et al. 2014; Pompei et al. 2007b). These findings indicate that intestinal bacteria contribute to folate metabolism and that colonic contents represent a substantial and natural source of folate. Remarkably, the role of bacterial folate biosynthesis with regard to colorectal cancer risk or other aspects of human health remains unexplored, highlighting the need for further studies. In this critical review, current knowledge on bacterial folate biosynthesis, folate absorption in the colon and further metabolism in the host will be briefly summarized. Next, we will discuss colonic folate sources in relation to potential mechanisms underlying the development of colorectal cancer. Finally, we will critically reflect on the relevance of bacterial folate biosynthesis for disease prevention, and the emerging question whether food-grade or intestinal bacteria can be considered a potential target to ensure an optimal folate status.

## Bacteria as folate producers

The folate biosynthesis pathway in bacteria is well-defined (de Crécy-Lagard et al. 2007; Magnusdottir et al. 2015) and has been previously reviewed in detail (LeBlanc et al. 2011; Rossi et al. 2011). Various lactic acid bacteria naturally occurring in the human or animal gastrointestinal tract or used as starters in fermented foods are recognized for their folate-producing capacities (LeBlanc et al. 2011; Rossi et al. 2016). With regard to food-grade lactic acid bacteria, specifically the species *Lactococcus lactis* and *Streptococcus thermophilus* are well-known folate producers (LeBlanc et al. 2011; Sybesma et al. 2003b; Tomar et al. 2009). Of the genus *Lactobacillus*, the strain *Lb. plantarum WCFS1* has been studied frequently in relation to its ability to produce folate (Laiño et al. 2014; Li et al. 2016; Wegkamp et al. 2010). *Lb. plantarum WCFS1* is generally considered an interesting candidate for research on folate biosynthesis, given its ability to survive in the gastrointestinal tract of rodents and human (Marco et al. 2009; Vesa et al. 2000), suggested probiotic properties (de Vries et al., 2006), and the presence of a functional folate gene cluster (Kleerebezem et al. 2003; Rossi et al. 2011).

Also, the genus *Bifidobacterium* comprises various species that can reside in the human or animal gastrointestinal tract and that are recognized for their potential health-promoting effects (Pokusaeva et al. 2011; Rivière et al. 2016). The folate biosynthesis capacity in *Bifidobacterium* spp. seems strain specific and largely depends on origin or residence of the bacteria (D'Aimmo et al. 2014; Milani et al. 2014; Pompei et al. 2007a; Sugahara et al. 2015). Strains of *Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium catenulatum* were commonly recognized as strong folate producers (D'Aimmo et al. 2012; Pompei et al. 2007a; Sugahara et al. 2015). Evidence for folate production by other species of the genus *Bifidobacterium* is less consistent (D'Aimmo et al. 2012; Lin and Young 2000; Sugahara et al. 2015).

Besides the lactic acid bacteria and *Bifidobacterium* species, also other bacterial as well as yeast species are capable of folate production (Greppi et al. 2017; Hugenholtz et al. 2002). There is considerable interest in the application of lactic acid bacteria to increase levels of folate and other B-vitamins in fermented food products (e.g. dairy) (Crittenden et al. 2003; Divya et al. 2012; Laiño et al. 2014; Santos et al. 2008; Sybesma et al. 2003b). Metabolic engineering of bacteria aiming at elevated folate production through modulation of the bacterial genome provides interesting leads for this active field of research. Engineered strains of *L. lactis* (Sybesma et al. 2003a; Wegkamp et al. 2007), *Lactobacillus gasseri* (Wegkamp et al. 2004), *Lactobacillus reuteri* (Santos et al. 2008) and *Lb. plantarum WCFS1* (Wegkamp et al. 2009) have been developed and showed increased folate production of up to ~100-fold compared to wildtype strains during cultivation. Safety of the engineered strains remains an important issue that needs to be carefully considered especially in the context of biofortification or probiotic applications (Sybesma et al. 2006). An *in vivo* safety study showed that mice receiving engineered *L. lactis* strains during 30 days did not show aberrant general health, growth rates, hematological parameters, or other physiological parameters as compared to mice exposed to the wildtype strain (LeBlanc et al. 2010b). Next, the same strain showed the ability to increase liver, kidney and serum folate levels and to revert a partial megaloblastic anemia in rats on a folate deficient diet (LeBlanc et al. 2010a). To the best of our knowledge, other functional outcomes and specifically folate-mediated processes in the host have not been further studied in relation to (engineered) bacterial folate biosynthesis so far.

Predictions of folate biosynthesis capacity of a selected set of 256 human gut bacterial genomes imply that folate biosynthesis capacity is notably present in the phyla Bacteroidetes, Fusobacteria and Proteobacteria (Magnusdottir et al. 2015). Since most studies on folate biosynthesis focus on bifidobacteria and lactic acid bacteria, this observation stresses that there is still a huge gap in our knowledge about bacteria involved in intestinal folate biosynthesis.

## Absorption and metabolism of bacterially synthesized folate in the colon

The primary site of absorption of folate derived from dietary sources is the small intestine. Dietary THF polyglutamates are hydrolyzed to monoglutamate forms (Darcy-Vrillon et al. 1988) in the intestinal brush border prior to absorption in the proximal small intestine (duodenum and jejunum) (Said and Mohammed 2006; Visentin et al. 2014). Absorption at the apical site of the membrane is thought to be predominantly mediated through the proton-coupled folate transporter (PCFT, or SLC46A1) (Qiu et al. 2006) in a pH-dependent manner (Visentin et al. 2014). It has been suggested that release from the basolateral membrane of the enterocyte occurs via multidrug resistance-associated proteins (MRP3) (Visentin et al. 2014). Similar to natural forms of folate, synthetic folic can be easily absorbed in the proximal small intestine through action of the PCFT (Visentin et al. 2014). After absorption, folic acid is first converted to dihydrofolate (DHF) by DHF reductase (DHFR) (Bailey and Ayling 2009) and subsequently to THF, which can participate in metabolism once in the polyglutamated form (Bailey et al. 2015).

Apart from dietary folate or folic acid being primarily absorbed in the small intestine, absorption of biosynthesized folate can occur in the colon (Visentin et al. 2014) (Figure 1). Early evidence showing the potential of bacterially synthesized folate to be incorporated in tissues of the host came from animal studies using the tritiated folate precursor *p*-amino benzoic acid ($^3$H *p*ABA) (Asrar and O'Connor 2005; Rong et al. 1991). The $^3$H folates were detected in liver and kidney of rats and piglets after cecal administration of $^3$H *p*ABA, indicating that bacterially synthesized folate is absorbed across the colon and can be incorporated in peripheral tissues (Asrar and O'Connor 2005; Rong et al. 1991). Subsequent human studies were able to confirm these findings and provided additional evidence of folate absorption through the human colon (Aufreiter et al. 2009) (Lakoff et al. 2014). An elegant study by Lakoff et al. using caplets containing [$^{13}C_5$]5-formyl-THF and specifically designed to disintegrate in the colon, showed an average absorption rate of $0.33 \pm 0.09$ nmol/h, which is estimated to be ~100 times slower as compared to folate absorption in the small intestine (Lakoff et al. 2014; Wright et al. 2003). It is, however, important to realize that transit time in the colon is substantially longer as compared to the small intestine (Fallingborg et al. 2007), which has implications for the total amount of folate that can be absorbed through the colon (Lakoff et al. 2014). Based on the work of Lakoff et al., it is estimated that ~322–396 µg of folate per day can be absorbed in the colon, which resembles the average daily dietary requirement of

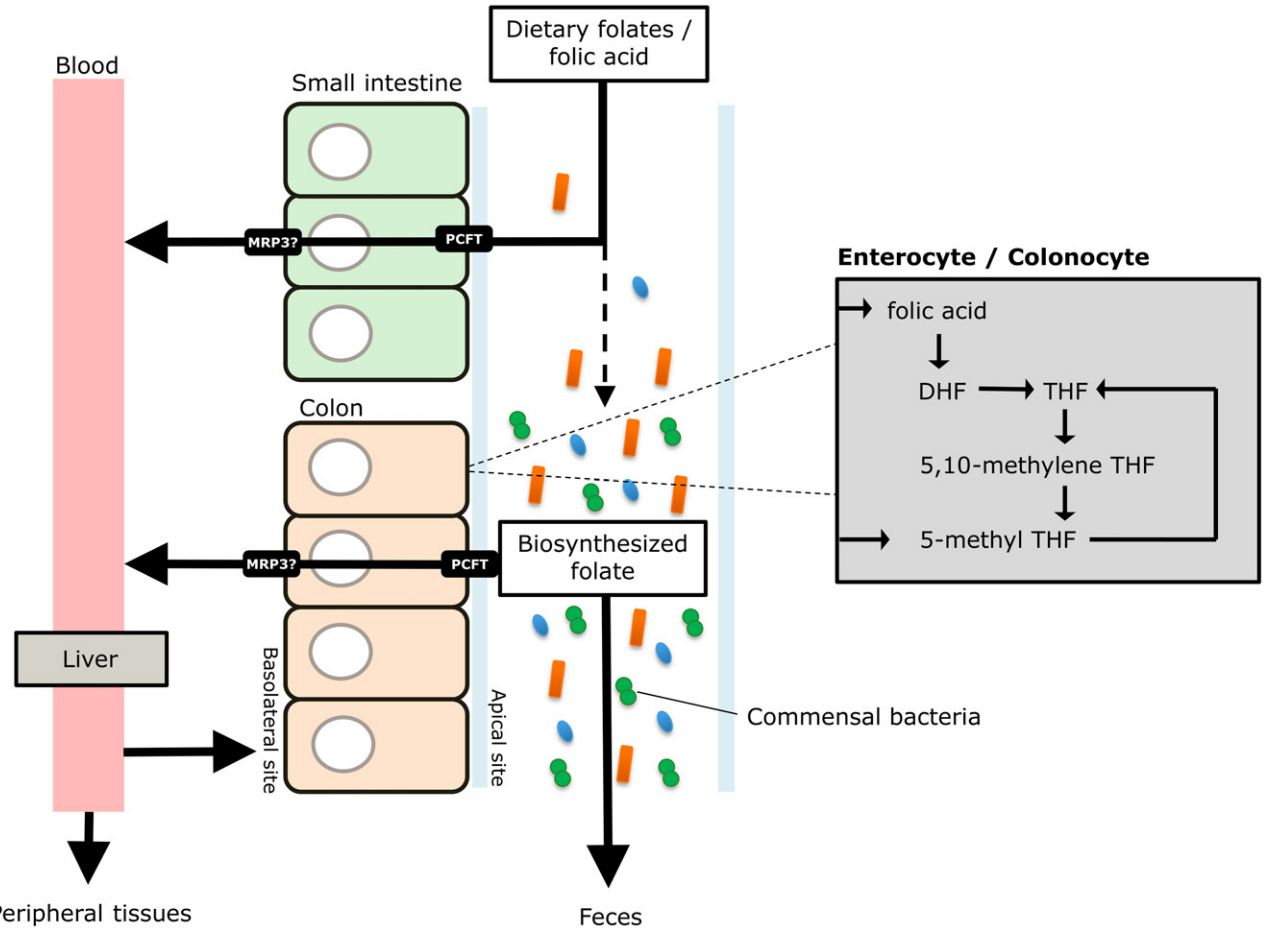


Figure 1. Schematic and simplified overview of absorption and metabolism of folate from dietary sources or bacterial biosynthesis. Abbreviations: DHF dihydrofolate, MRP3 multidrug resistance-associated protein 3, PCFT proton-coupled folate transporter, THF tetrahydrofolate.

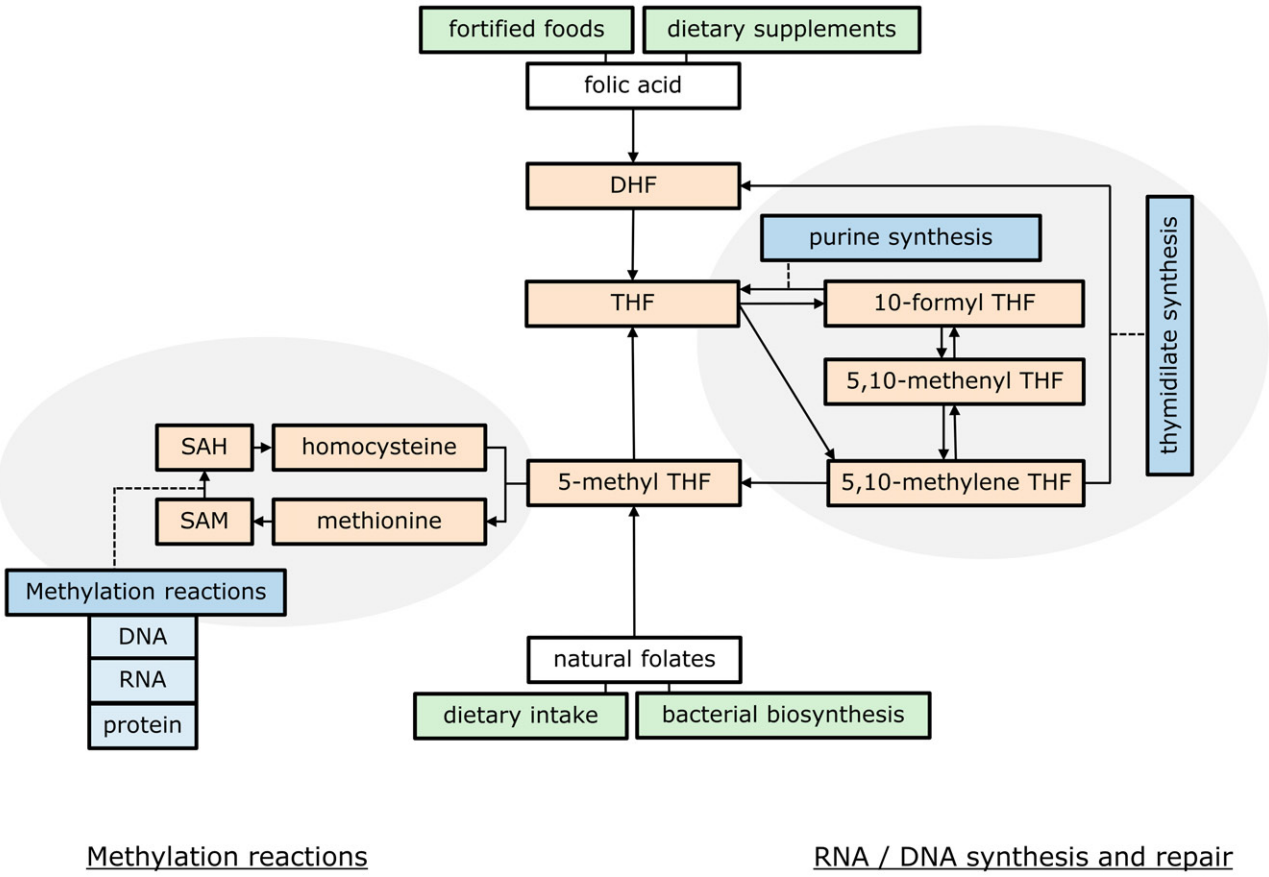


**Figure 2.** Overview of basic concepts and main processes involved in folate-mediated one-carbon metabolism. Abbreviations: DHF dihydrofolate, SAM S-adenosylmethionine, SAH S-adenosylhomocysteine, THF tetrahydrofolate.

250–320 µg of dietary folate equivalents (DFE) for adults (EFSA-report 2014; Institute of Medicine 1998).

Although it has been clearly demonstrated that *in situ* produced bacterial folate (1) can be absorbed in the colon (Dudeja et al. 1997; Kumar et al. 1997), (2) is detected in the circulation (Aufreiter et al. 2009; Lakoff et al. 2014), and (3) is incorporated in extra-intestinal tissues (Asrar and O'Connor 2005; Rong et al. 1991), the exact mechanisms of metabolism and absorption in the colon are not yet fully elucidated. The PCFT is expressed in the colon, although at lower levels as compared to the small intestine (Qiu et al. 2006). Moreover, the pH in the colonic lumen, ranging from pH 7.05–7.46 (right to left colon) (McDougall et al. 1993) exceeds the optimal pH for the PCFT (pH 5.5) (Qiu et al. 2006) suggesting that other mechanisms for absorption may be involved as well (Visentin et al. 2014).

Fecal excretion of folate implies that not all biosynthesized folate is absorbed in the colon (Kim et al. 2004; Rong et al. 1991). The exact amount of folate produced and released by the intestinal bacteria is difficult to assess, since folate is distributed across different tissues after intestinal absorption, which hinders complete assessment of the recovery of folate. Moreover, absorption of folate may depend on various factors (McNulty and Pentieva 2010), including variations in genes responsible for folate uptake and metabolism (DeVos et al. 2008; Qiu et al. 2006; Zhao et al. 2017) and the bioavailability of the specific forms of folate (Melse-Boonstra et al. 2002; Winkels et al. 2007). Also, the complex ecosystem in the colon complicates quantification of bacterial folate biosynthesis, since other microorganisms use folate as well (Pompei et al. 2007b). Moreover, it remains speculative whether folate is actively excreted of released in the intestinal lumen as a result of cell lysis. It has been reported that up to one third of bacteria in the feces is not viable anymore, which included some bifidobacterial species (Ben-Amor et al. 2005).

## Metabolism in the host: folate-mediated one-carbon metabolism

Bacterially biosynthesized folate available in the colonic lumen can participate in host metabolism after absorption. From a biological point of view, folate sources in the colon are of particular relevance to colonic health given the proximity of the intestinal bacteria and continuous exposure of colonocytes to locally produced folate. Moreover, colonic epithelial tissue is characterized by rapid cellular turnover (Creamer et al. 1961) and heavily depend on DNA synthesis and genomic stability mediated through folate or other B-vitamins (Kim 2004).

As a carrier and donor of one-carbon units, folate is involved in a series of biochemical reactions commonly referred to as one-carbon metabolism. One-carbon metabolism is characterized by strict regulatory mechanisms, including allosteric interactions (Nijhout et al. 2008). The main output of one-carbon metabolism includes *de novo* purine

**Table 1:** Overview of studies focusing on colonic tissue folate levels in relation to disease biomarkers or mechanisms

| Study | Population | Tissue source | Method[a] | Biomarker or mechanism | Main findings |
|---|---|---|---|---|---|
| Cravo, 1998 | Individuals with ulcerative colitis (n = 23) | Mucosa obtained through rectosigmoid biopsies | Microbiological assay | Microsatellite instability (MSI) | Patients with MSI in rectal mucosa had lower tissue levels of folate (55 ± 37 ng/mg) compared to patients without MSI (105 ± 65 ng/mg) (p = 0.10). |
| Kim, 2001b | Individuals with polyps (n = 20) participating in a randomized trial (5mg folic acid/day or placebo) | Normal-appearing mucosa obtained through rectosigmoid biopsies | Microbiological assay | Genomic DNA methylation and *p53* DNA strand breaks | Folate supplementation resulted in increased colonic tissue folate levels, increased extent of genomic DNA methylation and fewer *p53* strand breaks. After 1 year of intervention, also changes in the placebo group were observed. |
| Protiva, 2011 | Healthy, high-risk individuals undergoing folate depletion (n = 10) or supplementation (1 mg/day, n = 10) during 8 weeks | Normal-appearing mucosa obtained through rectosigmoid biopsies | Microbiological assay | Genome-wide gene expression, genomic DNA methylation, promoter methylation, and *p53* DNA strand breaks | No changes in genomic or promoter-specific DNA methylation were observed after depletion or supplementation. The extent of *p53* strand breaks increased with depletion. Pathways related to inflammation and immune response were upregulated after supplementation, and downregulated after depletion. |
| Liu, 2012 | Individuals with colorectal cancer (n = 67) | Normal-appearing mucosa obtained through endoscopic biopsies | LC-MS/MS | Folate species distribution in tissue versus blood of the same individuals | A lower proportion of 5-methylTHF and a higher proportion of formyl-THF, THF and folic acid were found in colonic tissue as compared to blood. |
| McGlynn, 2013 | Individuals with polyps (n = 40) | Colonocytes (isolated epithelial cells from colonic biopsies) | Microbiological assay | Uracil misincorporation and global DNA methylation | Lower folate levels were found in polyps compared to adjacent or normal mucosa. Extent of uracil misincorporation and global DNA hypomethylation was highest in polyps compared to other sites. |
| Odin, 2013 | Individuals with colorectal cancer (n = 77) | Tumor and adjacent normal tissue excised from surgical specimens | LC-MS/MS | Folate species distribution in tissue | Higher levels of methylene-THF and THF were found in tumor tissue compared to matched normal mucosa. |
| Hanks, 2013 | Individuals without colorectal polyps and cancer (n = 336) | Mucosa obtained through rectal biopsies | Microbiological assay | Genomic- and gene-specific DNA methylation | Colonic tissue folate levels were borderline associated with genomic DNA methylation and methylation of *MGMT*, but not other genes in colonic mucosa. |
| O'Reilly, 2016 | Individuals with polyps (n = 20) participating in a randomized trial (600 μg folic acid/day or placebo) | Colonocytes (isolated epithelial cells from colonic biopsies) | Microbiological assay | Uracil misincorporation and global DNA methylation | Supplementation resulted in increased tissue folate levels and less DNA hypomethylation as well as a decrease in uracil misincorporation. |

[a]Method for assessment of folate levels in colonic tissue.

Abbreviations: 95% CI: 95% confidence interval, MGMT: O(6)-methylguanine-DNA methyltransferase gene, MSI: microsatellite instability, LC-MS/MS: liquid chromatography tandem mass spectrometry.

(adenine and guanine) and thymidylate biosynthesis, which are essential for DNA and RNA synthesis (Ducker and Rabinowitz 2017) (Figure 2). In case of folate deficiencies, production of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP) by thymidylate synthase (TS) can be hampered, resulting in accumulation of dUMP and eventually uracil misincorporation (Duthie 2011). DNA repair enzymes are able to remove the uracil groups from the DNA, but this procedure may result in single-strand breaks and genomic instability (Blount et al. 1997; Reidy 1988).

Besides production of purines and thymidylate, folate-mediated one-carbon metabolism is involved in methylation reactions. The 5-methyl THF-mediated conversion of homocysteine to methionine and subsequently S-adenosylmethionine (SAM), determines the availability of methyl groups that can be transferred to proteins, RNA and DNA. Enzymatic transfer of the methyl group to cytosine residues (CpG sites) in the DNA is facilitated by DNA methyltransferases (DNTMs) and is referred to as DNA methylation.

DNA methylation is considered an epigenetic phenomenon with a well-established role during cancer onset and progression (Feinberg 2018; Kanwal and Gupta 2012). Gene-specific DNA methylation of CpG sites within promoter and enhancer regions is considered crucial for control of transcriptional activity (Jaenisch and Bird 2003; Kulis et al. 2013). Excessive methylation (hypermethylation) of promoter regions is thought to repress binding of the transcription machinery and is therefore mostly associated with silencing of the respective genes (Ferreira and Esteller, 2018; Esteller 2008). An extensive list of tumor-suppressor genes which are inactivated through excessive DNA methylation or other epigenetic mechanisms has been identified, which emphasizes the role of aberrant DNA methylation during carcinogenesis (Llinàs-Arias and Esteller 2017).

At the same time, an overall loss of DNA methylation marks, referred to as global or genomic hypomethylation, is frequently observed in cancer cells (Feinberg and Vogelstein 1983; Weber et al. 2005). DNA hypomethylation is associated with genomic instability and as such can contribute to cancer development or progression (Eden et al. 2003; Gaudet et al. 2003). This traditional concept of hypermethylation in CpG-dense regions of gene promoters and loss of methylation at other genomic positions may be seen as a gain-and-loss function (Esteller 2008), although the exact mechanisms are not fully understood. Moreover, it becomes increasingly clear that the picture is more complex and research in the active field of cancer epigenetics moved forward by focusing on complementary hypotheses regarding other genomic regulatory elements such as enhancers (Bae et al. 2016), DNA hydroxymethylation and the role of TET enzymes (Haffner et al. 2011), and potential interactions with other epigenetic modifications (Feinberg 2018; Murtha and Esteller 2016). Nevertheless, plasticity and profound disease-related profiles make DNA methylation a likely candidate explaining the link between folate and colorectal cancer risk (Costa-Pinheiro et al. 2015; Herceg et al. 2018; Jia et al. 2016). This hypothesis is further substantiated by previous evidence showing that intake or status of folate is associated with DNA methylation of critical genes during early development and in adult life (Joubert et al. 2016; Kok et al. 2015; O'Reilly et al. 2016; Pauwels et al. 2017; Steegers-Theunissen et al. 2009; van den Donk et al. 2007; Waterland and Jirtle 2003).

## Colonic folate sources: implications for colorectal cancer risk?

Despite great promise from pioneering studies (Kim et al. 2001a; McGlynn et al. 2013; Meenan et al. 1997; Strozzi and Mogna 2008), little attention has been paid to the role of colonic folate sources in relation to DNA methylation and other molecular parameters. Some studies actually determined folate levels in cells or tissues of interest, i.e., isolated colonocytes or colonic mucosa and analyzed these in relation to (1) dietary folate intake, (2) circulating levels of folate, (3) health outcomes, or (4) potential disease mechanisms. Although these studies provided important answers regarding the role of colonic folate sources, there are also numerous questions that remain to be answered, which will be illustrated here. First, colonic folate levels have been consistently shown to increase with higher dietary or supplemental intake of folate or folic acid (Kim et al. 2001b; O'Reilly et al. 2016; Powers et al. 2007; Protiva et al. 2011). Observed correlations, however, were relatively modest and variation in dietary intake is rather limited as compared to variation in colonic folate levels (Flood et al. 2011). These findings point towards other factors, such as bioavailability of different folate species, genetic variants in folate-metabolizing enzymes or composition of the intestinal microbial communities and hence folate biosynthesis capacity. Second, the correlation between colonic folate levels and systemic markers of folate (serum and red blood cell content) has been extensively studied, but there are still some controversies regarding the use of serum or red blood folate as biomarker of colonic folate levels (Kim et al. 2001a; Meenan et al. 1996; Williams et al. 2013). Third, colonic folate concentrations have been associated with risk of (advanced) adenomas in 813 asymptomatic women undergoing screening by colonoscopy (Flood et al. 2011). This finding highlights the clinical relevance of colonic folate sources and provides leads for further studies focusing on health and disease outcomes.

Also potential cancer-related mechanisms have been studied in relation to colonic folate levels (Table 1). Tissue folate levels were borderline associated with genomic DNA methylation and inversely associated with methylation of the tumor suppressor gene *MGMT*, but not other candidate genes, in the colon of individuals without colorectal neoplasia (Hanks et al. 2013). Kim et al. demonstrated that 6 months of folic acid supplementation resulted in higher colonic folate levels and fewer strand breaks in the tumor suppressor gene *p53* as well as an increased extent of global DNA methylation as compared to a placebo group (Kim et al. 2001b). In another study focusing on folate depletion as well as supplementation, which has been reflected in

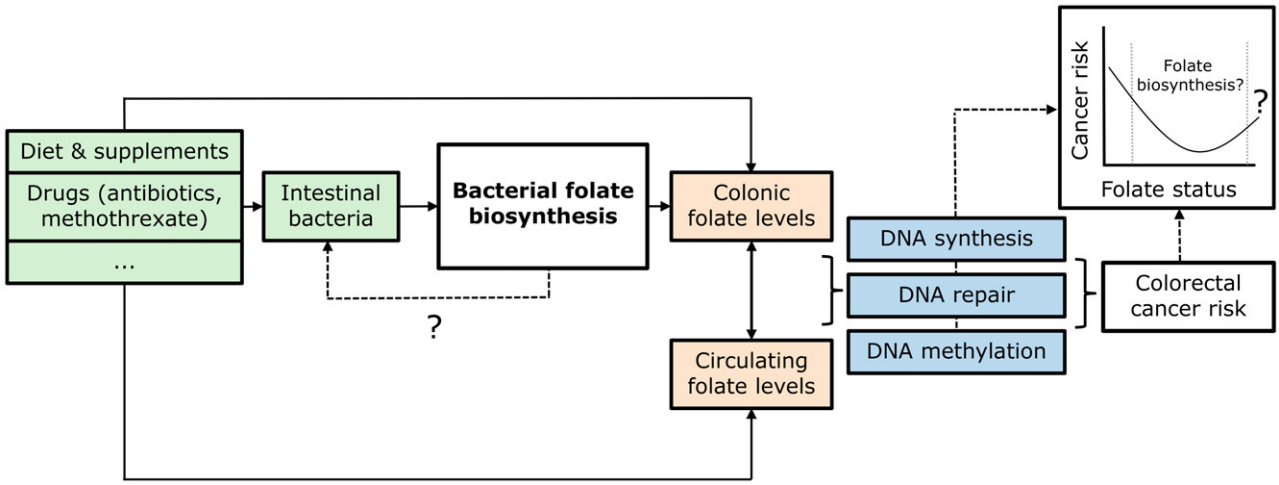


**Figure 3.** Proposed model showing how bacterial folate biosynthesis may be linked to colorectal cancer risk. Capacity of bacterial folate biosynthesis depends on the composition of the intestinal microbiota, which in its turn depends on host and environmental factors. Bacterial folate biosynthesis is thought to contribute to levels of folate in the colon as well as other organs and the circulation. The supply of natural folate through bacterial folate biosynthesis contributes to folate-mediated one-carbon metabolism and as such may have implications for DNA synthesis, repair and methylation. As an endogenous source of folate, bacterial biosynthesis is hypothesized to impact the suggested *U*-shaped relationship between folate levels and colorectal cancer risk.

colonic folate levels, it was shown that *p53* DNA strand breaks increased with depletion, whereas global or gene-specific DNA methylation did not change during the intervention of eight weeks (Protiva et al. 2011). Pronounced gene expression changes pointed towards upregulated expression of pathways related to inflammation and immune response upon folic acid supplementation, whereas these pathways were downregulated during depletion (Protiva et al. 2011). In a study among 40 individuals with adenomatous polyps, folate levels in the polyp were lower as compared to adjacent sites and normal colonic mucosa (10–15 cm distal to the polyp), which may be due to an increased demand for folate during cellular growth (McGlynn et al. 2013). Correspondingly, the extent of global DNA hypomethylation and uracil misincorporation was highest in the polyps as compared to the other colonic sites (McGlynn et al. 2013). Follow-up work from this group demonstrated that an intervention with folic acid during 6 months resulted in increased colonocyte folate levels and less DNA hypomethylation and uracil misincorporation in individuals with adenomatous polyps (O'Reilly et al. 2016).

## Conclusion and perspectives

Altogether, emerging evidence suggests that intestinal bacteria contribute to folate metabolism and that the colon represents a substantial local folate source. The importance of colonic folate depots is evident from a clinical and mechanistic perspective. Although previous studies started to unravel potential disease mechanisms linked to colonic folate depots, the bacterial folate biosynthesis capacity needs to be further explored in relation to colorectal cancer risk as well as other aspects of human health (Figure 3).

One important aspect that deserves further attention is the suggested *U*-shaped relationship between folate status and colorectal cancer risk. Despite extensive research, the optimum folate status and boundaries for this curve have not been defined. Current dietary reference intakes (DRI) and biomarker reference values for folate deficiency are predominantly based on hematological conditions (e.g. megaloblastic anemia) (EFSA-report 2014; Institute of Medicine 1998; WHO 2012). To what extent these values also apply to (colorectal) cancer risk, and how folate levels in the colon are reflected in these values need to be established. Moreover, it is unclear whether the *U*-shaped curve applies to natural as well as synthetic forms of folate. So far, evidence suggesting that excessive intake of folate may increase colorectal cancer risk in individuals with precursor lesions merely comes from studies with high-dose folic acid exposures (Cole et al. 2007; van den Donk et al. 2007). An explanation for this observation could either be that (1) excessive intake of folate is not likely to result from dietary intake only; or (2) that synthetic folic acid may exert different effects, including suggested effects on immune function (Sawaengsri et al. 2016; Troen et al. 2006), as compared to natural forms of folate. It should also be noted that folate is closely related to other B-vitamins and linked metabolites, including vitamin $B_{12}$, $B_2$, $B_6$ as well as homocysteine, choline and methionine, and imbalances in these key-role players may result in aberrant regulatory mechanisms in one-carbon metabolism (Molloy 2010). An interesting subsequent question is whether promotion of bacterial folate biosynthesis can assure a sufficient folate status, and at the same time circumvent potential detrimental effects of excessive folic acid intake and imbalances in one-carbon metabolism. These and other emerging questions that remain to be answered and that provide important leads for future studies are summarized in Box 1.

The concept that food-grade or intestinal bacteria can be considered a potential target to ensure continuous exposure to natural folate in the colon is fascinating from a food science as well as cancer biology perspective. Promotion of folate biosynthesis, either in fermented foods or in the gastrointestinal tract of the host, may contribute to an increased folate status. It has even been suggested that enhancement of the natural folate content of various food

**Box 1 Emerging issues that need to be addressed**

Leads for future studies that need to address whether:

- a distinction should be made between natural and synthetic forms of folate when considering the suggested *U*-shaped relationship between folate status and colorectal cancer risk.
- the suggested *U*-shaped relationship between folate status and colorectal cancer risk applies for both levels of folate in the circulation or in colonic tissue.
- bacterial folate biosynthesis impacts the *U*-shaped relationship between folate status and colorectal cancer risk, and whether there is a critical lower and upper value for biosynthesized folates.
- bacterial folate biosynthesis impacts one-carbon metabolism of the host.
- promotion of bacterial folate biosynthesis, either in the gastrointestinal tract or via biofortification, may be considered a future alternative to folic acid fortification programs.
- genes encoding folate production pathways are present in the genomes of a wide variety of intestinal microbes that have not been studied with respect to intestinal folate biosynthesis.
- the host is exposed to bacterial folate through bacterial cell lysis or active excretion and whether specific microorganisms interact with regards to their folate biosynthesis and use.

products, for instance through fermentation fortification by lactic acid bacteria (Saubade et al. 2017) may be considered an alternative to folic acid fortification programs (LeBlanc et al. 2011; LeBlanc et al. 2010a). Also administration of folate-producing bacteria may directly promote folate biosynthesis in the gastrointestinal tract of the host. So far, *in vivo* human and animal studies showed that biosynthesis of folate was induced by oral administration of *Bifidobacterium* resulting in increased levels of folate in feces (Strozzi and Mogna 2008) as well as blood and peripheral tissues (Pompei et al. 2007b). As indicated earlier, also administration of metabolically engineered lactic acid bacteria showed potential to improve folate status in the host (LeBlanc et al. 2010a), although safety and acceptability of this procedure need to be further determined. Also dietary strategies may promote abundance of folate-producing bacteria in the colon and as such favor biosynthesis of folate. For example, dietary oligosaccharides increased the amount of folate in colonic contents, but did not alter indicators of folate status in blood, liver or kidney, in piglets (Aufreiter et al. 2011). Similarly, intake of dietary fiber or human milk has been effective in raising levels of folate in the circulation, colonic tissue or feces in rodents, which may be due to an increased abundance of bifidobacteria (Krause et al. 1996; Sepehr et al. 2003; Thoma et al. 2003). As a proof of principle, Chan et al. demonstrated that switching from a Western-style low-fiber, high-fat diet to a fiber-rich diet which is low in fat for 2 weeks resulted in increased folate levels in colonic evacuates in African Americans (Chan et al. 2018). Impact on intestinal microbiota composition and bacterial folate biosynthesis capacity has been suggested, next to effects on transit time, as a potential explanation for these findings (Chan et al. 2018).

Obviously, the observation that bacterial folate biosynthesis contributes to folate levels in tissues and the circulation is not only of interest in relation to colorectal cancer etiology, but also provides tremendous opportunities for other research fields. Of particular interest is the use of anti-folate drugs. Well-known anti-folate drugs include certain antibiotics and anti-malaria agents (Estrada et al., 2016; Verhoef et al., 2017). Also methotrexate used for the reduction of symptoms of rheumatoid arthritis and treatment of cancer, as well as other chemotherapeutics (e.g. 5-fluorouracil), target folate-mediated one-carbon metabolism (Chon et al., 2017; Yu et al., 2018). Toxicity as well as efficiency of these drugs have been linked to folate status of the patients (den Boer et al., 2014; den Hoed et al., 2015; Verhoef et al., 2017; Yan et al., 2016). The folate biosynthesis capacity may as such have important implications for treatment outcomes, since folate levels in the circulation as well as the target tissue (e.g. colon) may depend on this capacity. Hence, levels of folate, and biosynthesis capacity or composition of the intestinal microbiota, may be considered predictive factors for treatment outcomes and consideration of these factors may fuel development of personalized treatment strategies. Based on the fact that genomes from a wide diversity of intestinal microbes contain genes encoding folate biosynthesis pathways, it can also be speculated that disturbances of bacterial composition, for example by use of antibiotics, may have important consequences for the folate status and subsequently health of the host at different stages throughout the life course, which is an important issue for further research (Kok et al. 2018).

In conclusion, folate biosynthesis can contribute to colonic as well as circulating folate depots and hence may have implications for colorectal cancer risk, but also other aspects of human health. Our understanding of the relevance of colonic folate sources in relation to colorectal cancer risk as well as other disease outcomes should be fueled by well-controlled experiments focusing on stimulation or inhibition of bacterial folate biosynthesis capacity that allow careful assessment of plausible mechanisms, with an initial focus on DNA methylation, DNA synthesis and DNA repair. Future epidemiological studies should not only focus on dietary intake or circulating levels of folate, but should also consider colonic folate sources in relation to disease outcomes. Although collection of colonic tissue may not be feasible and ethical in large cohorts, alternatives such as folate-production capacity of bacteria in feces can provide meaningful information. Pioneering research focusing on strategies to modulate the bacterial *in situ* folate biosynthesis capacity may provide further answers to the question whether modulation of the intestinal bacteria can be considered a target to increase levels of natural folate, although the safety, efficiency and effects on health-related outcomes need to be carefully assessed first.

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