

Assessing a Fermented Whey Beverage Biofortified with Folate as a Potential Folate Source for Humans

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Folate, a vital water-soluble vitamin (B₉), requires specific attention as its recommended daily intake frequently is not reached in countries without mandatory fortification. In this regard, biofortification with microorganisms like *Bifidobacterium* and *Streptococcus* offers a compelling approach for enhancing food with natural folates. A randomized, nonblinded, and monocentric human pilot study is conducted to assess the bioavailability of a folate-biofortified fermented whey beverage, comprising 3 intervention days and a controlled replenishment phase before and during the assay. Folate plasma concentration (5-CH₃-H₄ folate) is determined using a stable isotope dilution assay and LC-MS/MS detection. Biokinetic parameters ($c_{\max}$ and $t_{\max}$) are determined, and areas under the curve (AUC) normalized to the basal folate plasma concentration are calculated. An average bioavailability of 17.1% in relation to the 5-CH₃-H₄ folate supplement, ranging from 0% to 39.8%, is obtained. These results reiterate the significance of additional research into folate bioavailability in general and dairy products. Further investigations are warranted into folate-binding proteins (FBP) and other potential limiting factors within the food and individual factors. In summary, biofortification via fermentation emerges as a promising avenue for enhancing the natural folate content in dairy and other food products.

growth and renewal and acts as a coenzyme in the metabolism of one-carbon groups and DNA synthesis. The various folate forms differ chemically regarding the oxidation levels, substituents like methyl- or formyl-groups, the lengths of their glutamate residue, and their biochemical activity.^[1] Since humans cannot produce folates in vivo, they must be obtained from external sources through diet or nutritional supplements.^[2] Meeting the recommended daily intake (RDI) of folates is especially crucial for pregnant women (RDI of 600 µg) and women of childbearing age (RDI of 400 µg), as a deficiency increases the risk of neural tube defects (NTDs) and other fetal malformations. Additionally, inadequate folate levels have been linked to higher risks of cardiovascular diseases, colorectal cancer, and Alzheimer's disease.^[3]

Despite the consequences, many people worldwide are unaware of the folate deficiency situation, even in developed countries.^[4] As a result, hundreds of

thousands of babies (prevalence 2.5 cases per 1000 live births) are born with NTDs each year, which is highly related to folate deficiency of the mother during conception and the early phase of pregnancy.^[5,6] Folate deficiency is part of a global problem. Still,

1. Introduction

Folates, also known as vitamin B₉, are an essential micronutrient. This group of different water-soluble vitamins is vital for cell

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it is even more prominent in low- and middle-income countries, which experts summarize under the term “hidden hunger.” It describes the deficiency of essential micronutrients, like vitamins and minerals, even though they may be consuming enough calories to meet their energy needs and is predominantly driven by poverty.^[7,8] The effectiveness of global strategies to combat folate deficiencies has been well-documented, resulting in a significant reduction in the prevalence of neural tube defects (NTDs) over the past few decades.^[5,6] However, despite the proven efficacy of synthetic vitamins in addressing folate deficiencies, these interventions remain inaccessible to a considerable portion of vulnerable populations. Barriers to accessibility extend beyond poverty, encompassing factors such as low levels of women’s education, unplanned pregnancies, limited access to antenatal care services, and inadequate availability of supplements and fortified foods among vulnerable groups.^[4,6]

Regarding folates, fortification programs of wheat and maize flours and rice have been established, and awareness campaigns have been launched.^[9–12] However, fortified foods and supplementation often fail to reach impoverished populations and those residing in remote rural areas due to challenges like low literacy rates, limited access to healthcare facilities, and lower socioeconomic status.^[11–16] This leads to significantly higher rates of folate deficiency among women of reproductive age in low- and middle-income countries compared to high-income countries.^[15] By contrast, certain consumers, particularly in developed nations, prioritize a nutritious, well-rounded diet and over-rely on supplements.^[17–19] Knowing the adverse effects of folic acid excess (over 1 mg day^{−1}), like masking of vitamin B₁₂ deficiency, accumulation of nonmetabolized folic acid, and raised concerns about cancer risks,^[20] numerous researchers are seeking alternative sources of natural folates that are more stable, safer, and efficacious.^[21] One approach could be the search for “new” or yet unused natural folate sources, like fruits and leaves.^[22] Another option is biofortification, which means food’s “natural” intrinsic folate enhancement with the help of specific folate-producing microorganisms such as bacteria or yeasts.

While milk naturally contains a low concentration of folate, approximately 5–10 µg 100 mL^{−1},^[23] it only contributes to 10–15% of the daily folate intake in countries with high dairy consumption (without breast milk).^[24] Notably, natural folate production within dairy products can be achieved through fermentation with lactic acid bacteria (LAB) and other microorganisms. Milk and whey act as optimal media for fermentation because of their nutrient composition, which supports LAB growth and intrinsic folate production.^[21] Numerous studies have documented the ability of strains like *Bifidobacterium longum* (*B. longum*), *Streptococcus thermophilus* (*S. thermophilus*), and *Lactiplantibacillus plantarum* (*L. plantarum*), and other strains of the *Lactobacillaceae* family to produce intra- and extracellular folates during milk fermentation, while the variety of synthesized folate forms depends on each strain and species of bacteria.^[21,25,26] Liquid milk, reconstituted skim milk powder, and yogurt are ideal culture media for achieving high folate levels, especially 5-CH₃-H₄folate, through fermentation.^[26–29] The production of folates by selected bacterial strains within suitable food matrices, such as whey, can enhance both the stability and bioactivity of these folates.^[26,30] Dairy products contain proteins capable of binding with folates, enhancing the stability of the folates they produce.^[21] Consequently, these

products present a promising option for fortification with natural folate.^[24]

Whey is often mistakenly regarded as a waste product when, in fact, it should be considered as a by- or co-product. It holds significant potential for creating value-added products, including whey powders, proteins, edible films and coatings, lactic acid, various biochemicals, bioplastics, biofuels, and similar bioproducts derived from residual materials in the dairy industry.^[31] However, the substantial quantities of waste food, in general, can still pose environmental challenges if not managed sustainably and disposed into the environment.^[30] Another utilization involves the development of functional food and beverages, which aligns with our biofortification approach. In addition to employing whey as a culture medium for fermentation, we also utilize grape pomace and seeds. The extraction of high-potency substances from these industrial side streams is also in harmony with the United Nations’ Sustainable Development Goals (SDGs), with particular emphasis on SDG 12, centered on sustainable consumption and production principles. Natural resources should be utilized sustainably and efficiently, while waste should be prevented or recycled.^[32]

Unfortunately, the high folate content from 200 to 400 µg 100 g^{−1} in the fermented whey beverage does not necessarily correlate with a sufficient supply for human individuals, as the bioavailability of folates can vary significantly. In this context, bioavailability refers to the percentage of ingested folates absorbed by the body, which is available for metabolic processes or stored in the organism.^[27–30,33] The overall bioavailability of folates from different food sources is 50% (relative to a folic acid supplement), depending on several influencing factors that may inhibit folate absorption and metabolism.^[34] The distribution of poly- or monoglutamates and folate vitamers, the composition of the food matrix, dietary fiber, low pH levels, and the instability of labile folate vitamers in the food and during digestion might hinder folate bioavailability. On the other hand, specific stabilizing food components like folate-binding proteins or ascorbate and zinc can enhance folate absorption.^[35] Internal and individual factors influencing bioavailability include methyl-tetrahydrofolate reductase gene (MTHFR) polymorphisms, sex, and ethnicity of the individual. Due to MTHFR polymorphism, the folate biomarker response can be influenced, which impacts the conversion of folates to active forms of folate and impairs bioavailability.^[36–38] Understanding the absorption and bioavailability of nutrients from foods in interaction with dietary components can help individuals to better reflect on the adequate intake of nutrients. In addition, these findings may help to design foods, meals, and diets that support the delivery of bioavailable nutrients to specific target populations. Human studies can help understand the bioavailability of folates from individual food sources and support the data generation needed for comprehensive dietary recommendations.

This human pilot study aimed to determine the bioavailability of folates produced in a fermented whey beverage during a short-term intervention. Therefore, the plasma folate concentration was analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and stable isotope dilution assay (SIDA). The evaluation was conducted using the area under the curve (AUC) method and was determined in comparison to the bioavailability of a 5-CH₃-H₄folate supplement. To the best of our

knowledge, this is the first study that assesses the bioavailability of folates produced by microorganisms in a fermented whey-based beverage.

2. Experimental Section

2.1. Folate and Additional Analysis

2.1.1. Chemicals

The HPLC- and LC-MS grade solvents acetonitrile (ACN), methanol (MeOH), and water were obtained from VWR (Ismaning, Germany). Ascorbic acid, formic acid (>95%), and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, sodium acetate trihydrate, and sodium hydroxide were purchased from Merck in Darmstadt, Germany. Disodium hydrogen phosphate (anhydrous) and sodium chloride were obtained from Alfa Aesar and Baker J.T. (Thermo Fisher) in Karlsruhe, Germany. Rat serum and chicken pancreas containing γ -glutamyl hydrolase (EC 3.4.19.9) were purchased from Biozol in Eching, Germany, and Difco in Sparks, MD, USA. Dithiothreitol (DTT) was obtained from AppliChem in Darmstadt, Germany. The unlabeled reference compounds ((6S)-H₄folate, (6R,S)-5-CH₃-H₄folate, (6R,S)-5-CHO-H₄folate, PteGlu, and 10-CHO-folate) were purchased from Schircks Laboratories in Jona, Switzerland. The isotopological internal standards (Pte[¹³C₅]Glu, (6S)-H₄Pte[¹³C₅]Glu, (6S)-5-CH₃-H₄Pte[¹³C₅]Glu-Ca, (6S)-5-CHO-H₄Pte[¹³C₅]Glu-Ca, and 10-CHO-Pte[¹³C₅]Glu) were obtained from Merck & Cie KmG in Schaffhausen, Switzerland. The Strata SAX cartridges (quaternary amine, 100 mg 1 mL⁻¹ and 500 mg 3 mL⁻¹) for solid-phase extraction were acquired from Phenomenex in Aschaffenburg, Germany.

2.1.2. Buffers and Solutions

The methods described by Striegel et al.^[64] for the folate quantification in blood plasma and Striegel et al.^[39] for the folate analysis in food were followed for the preparation of buffers, stock solutions, enzymes, and extraction processes with slight modifications.

For the extraction, a buffer was prepared by dissolving 2 g L⁻¹ of ascorbic acid and MES (200 mmol L⁻¹) in a solution containing 0.1 g of DTT and adjusted to pH 5 using 5 M NaOH. A phosphate buffer (100 mmol L⁻¹) was prepared for dissolving folate standards and as part of the equilibration buffer for SAX cartridges. This buffer was obtained by adjusting a 100 mmol L⁻¹ disodium hydrogen phosphate aqueous solution to pH 7.0 using a potassium dihydrogen phosphate (100 mmol L⁻¹) aqueous solution. The equilibration buffer was prepared by combining 0.2 g L⁻¹ of DTT and 10 mmol L⁻¹ phosphate buffer with deionized water. The two different elution buffers consisted of 5% (for monoglutamate analysis) respectively, 10% aqueous sodium chloride (for polyglutamate analysis), 100 mmol L⁻¹ aqueous sodium acetate, 0.1 g of DTT, and 1% ascorbic acid.

For the enzymatic treatment, lyophilized chicken pancreas (30 mg) was added to a 30 mL aqueous phosphate buffer solution (100 mmol L⁻¹) containing 1% ascorbic acid, adjusted to pH 7. Both the chicken pancreas enzyme and the enzyme γ -glutamyl

hydrolase (EC 3.4.19.9) resulting from rat serum were treated with activated carbon for 30 min and filtered using a 0.45 μ m molecular filter.

For the stock solutions of the reference compounds, 10 mg of PteGlu and 2 mg each of H₄folate, 5-CH₃-H₄folate, 5-CHO-H₄folate, and 10-CHO-PteGlu were dissolved in 3 and 10 mL (for PteGlu) phosphate buffer, respectively, and brought up to 10 and 100 mL (for PteGlu) using the extraction buffer. The exact concentrations of the freshly prepared unlabeled analytes were determined on each extraction day using HPLC-DAD with PteGlu as the internal standard (IS) for 5-CH₃-H₄folate, 5-CHO-H₄folate, and 10-CHO-PteGlu, and 5-CH₃-H₄folate as the internal standard for the determination of H₄folate. For LC-MS/MS analysis, the stock solutions were further diluted at 1:20 and 1:10 (for PteGlu). The labeled standards ([¹³C₅]-PteGlu, [¹³C₅]-H₄folate, [¹³C₅]-5-CH₃-H₄folate, [¹³C₅]-5-CHO-H₄folate, and [¹³C₅]-10-CHO-PteGlu) were dissolved once in concentrations of 60–70 μ g mL⁻¹ in extraction buffer and further diluted to a final concentration of 8–11 μ g mL⁻¹. The labeled reference solutions were stored in the dark at –20 °C.

2.1.3. Plasma Sampling and Analysis

The sampling and analysis of the blood plasma were done according to Striegel et al.^[64] with slight modifications. After sample collection, venous blood was centrifuged at 4 °C for 10 min, and the plasma was transferred to an Eppendorf tube containing 20 mg of ascorbic acid. The plasma samples were stored in the dark at –20 °C until further processing.

To quantify the plasma folate content, 60 μ L plasma was mixed with 1 mL of extraction buffer, and the corresponding amount of internal standard [¹³C₅]-5-CH₃-H₄folate was added. After 30 min of equilibration at room temperature with stirring, 1 mL ACN was added, and the mixture was centrifuged for 20 min at 4 °C. The supernatant was used for solid-phase extraction (SPE) with strong anion exchange cartridges (SAX, 1 mL, 100 mg). The eluate was membrane filtered and stored in the dark at –20 °C until measurement on the LC-MS/MS system.

The method described by Striegel et al.^[64] for determining plasma folate content was optimized as part of a preliminary study. The optimization led to a precipitation step with ACN to remove proteins from the matrix and the introduction of a blank sample consisting of the extraction buffer and the internal standards. This blank was processed the same way as the other blood samples to account for any potential contamination of the samples, i.e., with unlabeled standard, and subtracted from the results.

All vitamers were analyzed using the LC-MS/MS method, but only 5-CH₃-H₄folate was evaluated as this vitamer was the metabolized folate form in blood plasma. The assessment of relative bioavailability was conducted with reference to the 5-CH₃-H₄folate supplement, assumed to have a complete absorption rate of 100%.

2.1.4. Folate Analysis of Food

The whey drink and the standardized food for the human study were subjected to a triple analysis, following the protocol outlined

by Striegel et al.^[39] and Obermaier et al.^[22] Other than for the plasma samples, the study evaluated the five most important folate vitamers to cover the total folate content: PteGlu, H₄folate, 5-CH₃-H₄folate, 5-CHO-H₄folate, and 10-CHO-PteGlu.

To quantify the samples, 20 mg of freeze-dried samples were utilized. After a 15-min equilibration with a 10 mL buffer, the specific internal standards were added in quantities equivalent to the expected concentration of the unlabeled analytes in the sample. Following another 15-min equilibration time and a subsequent 10-min boiling step, a mixture of 900 µL chicken pancreas solution and 400 µL rat serum was used for deconjugation. The samples were then incubated overnight at 37 °C, followed by a 10-min boiling step and adding 10 mL acetonitrile to the cooled samples. The resulting supernatant from each sample was purified with a solid-phase extraction (SPE) using strong anion-exchange cartridges (SAX, 3 mL, 500 mg). After membrane filtration, the purified samples were subjected to measurement using liquid chromatography-tandem mass spectrometry (LC-MS/MS). All presented outcomes were normalized based on dry biomass.

2.1.5. Instrumental Conditions

The instrumental conditions for the analysis were based on Striegel et al.^[39] and Obermaier et al.^[22]

To check the purity of the unlabeled reference solutions, a Shimadzu HPLC/DAD system (Shimadzu, Kyoto, Japan) was used with a reversed-phase column (C18 EC, 250 × 3 mm, 5 µm, 100 Å, precolumn: C18, 8 × 3 mm, Machery-Nagel, Düren, Germany). A sample volume of 10 µL was injected, and the column oven temperature was set to 25 °C. The mobile phases comprised A) 0.1% acetic acid and B) methanol. The analysis was performed at a flow rate of 0.4 mL min⁻¹. The gradient started at 10% B, followed by a 7-min equilibration time. Subsequently, B concentration was linearly increased to 50% within the next 14 min. The gradient then linearly reached 100% B in 2 min and was held at 100% B for 1 min. Finally, the mobile phase returned to the starting condition (10% B) in 2 min and was equilibrated for 9 min before the next run.

For the LC-MS/MS measurement, a Shimadzu Nexera X2 UH-PLC system (Shimadzu, Kyoto, Japan) with a Raptor ARC-18 column (2.7 µm, 100 × 2.1 mm, Restek, Bad Homburg, Germany) and a Raptor ARC-18 precolumn (2.7 µm, 5 × 2.1 mm, Restek, Bad Homburg, Germany) as the stationary phase was utilized. The injection volume was 10 µL, and the separation was performed at 30 °C.

The mobile phases consisted of (A) 0.1% formic acid and (B) ACN with 0.1% formic acid. The analysis was conducted at a flow rate of 0.4 mL min⁻¹. The gradient elution started at 3% B, followed by a linear increase to 10% B within the next 2.5 min, which was then held for 2.5 min. Subsequently, the gradient linearly increased to 15% B over 5 min and to 50% B within 1 min, where it was maintained for 1 min. Finally, the gradient returned to the starting condition (3% B) for 4 min.

The triple quadrupole mass spectrometer (LCMS-8050, Shimadzu, Kyoto, Japan) operated in the positive ESI mode for all analytes. Before the study, the ion source parameters were optimized by injecting each labeled and unlabeled standard solution (1 mg L⁻¹). The heat block, dilution line, and interface tempera-

ture were maintained at 400, 250, and 300 °C, respectively. The drying, heating, and nebulizing gas flow were set to 10, 10, and 3 L min⁻¹, respectively. Collision-induced dissociation gas was applied at 270 kPa, and the interface voltage was set to 4 kV. The column effluent was only directed to the mass spectrometer from 2.1 to 7.0 min. Multiple reaction monitoring (MRM) was used for data acquisition. Detailed conditions can be found in the [Supporting Information](#).

Table 1 displayed the MRM scanning parameters for 5-CH₃-H₄folate and its corresponding internal standard, [¹³C₅]-5-CH₃-H₄folate. The MRM parameters used for quantifying the other four vitamers could be found in the previous work from Obermaier et al.^[22] **Figure 1** showed a chromatogram of 5-CH₃-H₄folate, and the matching IS in a blood plasma sample.

The measurements were analyzed and evaluated using LabSolutions software 5.118 (Shimadzu, Kyoto, Japan).

2.1.6. Additional Analysis of the Fermented Whey Drink

All analyses were performed in technical triplicates.

Microbiological Testing: Two samples from two different batches were sent to the Food Microbiology laboratory of the Faculty of Pharmaceutical Sciences, University of Sao Paulo, and tested for counts of molds and yeasts, *Enterobacteriaceae*, *Escherichia coli*, and the presence of *Salmonella* spp.^[40]

Chemical Composition: The moisture was determined by drying in a vacuum oven at 70 °C for 24 h, and the ash content was determined by the muffle incineration method at 550 °C.^[41] Protein analysis was performed by Micro Kjeldahl, using a value of 6.38 as a conversion factor.^[42] The fat content was determined using the Gerber method, according to the Association of Official Analytical Chemists (AOAC) method.^[43] The carbohydrate content was calculated by difference to reach 100%.^[44] The results of these analyses were displayed in the [Table S2, Supporting Information](#).

Polyglutamate Analysis: The semiquantitative polyglutamate analysis was conducted according to Schillert et al.^[45] and was described in the [Supporting Information](#). The analysis of the 5-CH₃-H₄PteGlu₂₋₇ content was performed using a semiquantitative approach, with [¹³C₅]-5-CH₃-H₄folate as an internal standard.

Analysis of Sulfate and Phosphate: The quantification of sulfate and phosphate was done by ion chromatography (IC), according to Toelstede and Hofmann.^[46] The detailed approach can be found in the [Supporting Information](#).

Lactate Quantification: The lactate content was determined by ¹H qNMR measurements, according to Frank et al.^[47] The procedure was described in the [Supporting Information](#).

2.1.7. Statistics and Biokinetic Calculations

The analysis was conducted in technical triplicates and duplicate injections at the LC-MS/MS instrument. The plasma folate results were calculated as nmol L⁻¹, including the relative standard deviation (RSD, %). The results of the analyzed food were expressed in µg 100 g⁻¹, based on the molecular mass of PteGlu. The error bars in the subsequent figures represent the variation. Statistical tests for significant differences were not performed

Table 1. MRM scanning parameters for 5-CH₃-H₄folate and [¹³C₅]-5-CH₃-H₄folate.

Compound	Precursor [<i>m/z</i>]	Product [<i>m/z</i>]	Dwell time [ms]	Q1 Pre bias [V] ^{a)}	CE [V] ^{b)}	Q3 Pre bias [V] ^{c)}
5-CH ₃ -H ₄ folate	460.10	313.30	50	−26.0	−19.0	−16.0
		180.30	50	−24.0	−20.0	−30.0
[¹³ C ₅]-5-CH ₃ -H ₄ folate	465.30	313.30	50	−24.0	−20.0	−16.0
		180.30	50	−24.0	−35.0	−20.0

MRM, multiple reaction monitoring; *m/z*, mass-to-charge-ratio; ms, milliseconds. ^{a)} Voltage used to promote ionization of precursor ion, displayed in Volt [V]; ^{b)} Collision energy; ^{c)} Voltage used to promote ionization of product ion.

since the sample data were only indicative and not representative in sample number and size.

For the evaluation of the biokinetics, the following parameters were calculated: *c*_{max} (maximum plasma concentration), *t*_{max} (time of *c*_{max}), and the area under the curve (AUC), which was determined by the linear trapezoidal rule via the integral of the concentration–time curve. To determine the plasma concentration curve, all calculated levels of 5-CH₃-H₄folate were visualized, and the mentioned parameters were determined. The relative bioavailability was calculated in relation to the 5-CH₃-H₄folate supplement, assuming complete absorption with 100%.

Due to varying initial plasma folate values, a normalization to the start or predose value (*c*₀) of the individual study day and subject was performed to get a baseline correction. Only the positive AUC values were considered to prevent negative AUC values, which could occur in certain instances when the plasma concentration of 5-CH₃-H₄folate falls below the baseline. This means all negative values were excluded from the final normalized AUCs. This method of AUC calculation was also conducted and successfully tested by Mönch et al.^[48] In the following paragraphs, whenever AUC values were mentioned, they specifically pertain

to AUC values that have been standardized with respect to the initial values, as explained before.

2.2. Human Study

2.2.1. Ethical Permission

The study was approved by the ethics committee of the Faculty of Medicine of the Technical University of Munich (#75/22 S). The study was registered in the German Clinical Trial Register (DRKS00028996), and participants provided written informed consent before being included in the study.

2.2.2. Study Participants

The inclusion criteria for this study were: healthy volunteers, aged between 18 and 30 years, both males and females, Caucasians, BMI < 30 kg m^{−2}, nonsmokers, and written informed consent. The study protocol defined the following exclusion criteria: current participation in intervention studies, antibiotics,

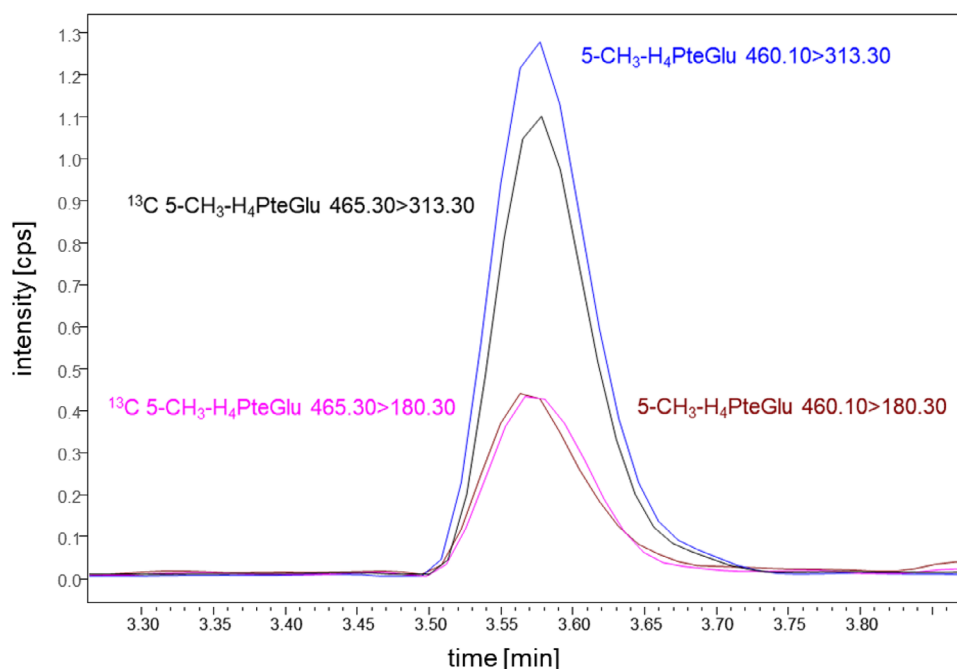


Figure 1. Chromatogram of 5-CH₃-H₄folate and the corresponding IS in blood plasma sample.

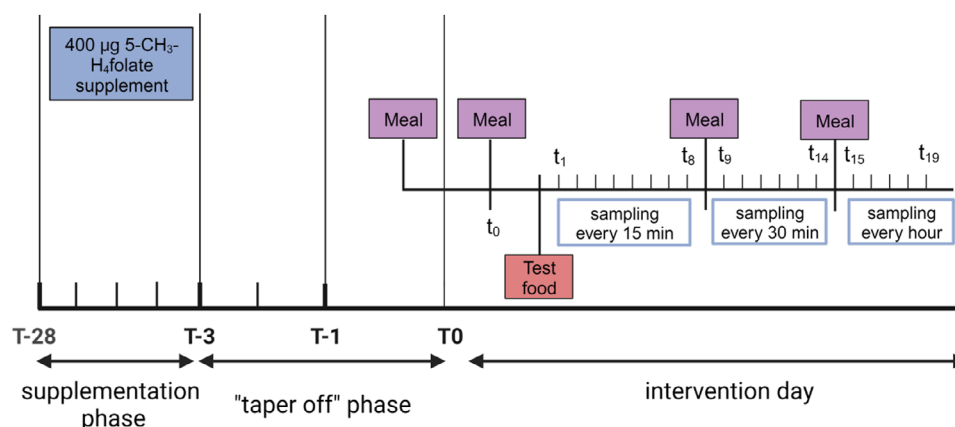


Figure 2. Timeline of the human study with supplementation and washout-phase, blood sampling points and time points of the standardized meals (created with BioRender.com).

medication like methotrexate, and other medications that interfere with the folate metabolism, active smoking, alcohol abuse, vitamin B₁₂ deficiency, elevated homocysteine levels, anemia (based on complete blood count including the Hb, erythrocyte volume and staining coefficient), chronic illness, blood donation in the past 3 months, allergy or intolerance to dairy products, and vegans. The study protocol specified that the following criteria would lead to an exclusion or a drop-out during the study: antibiotic use during the experimental period, acute illnesses during the test phase, withdrawal of consent to participate, and violation of experimental conditions. In this pilot study, six subjects (three males/three females) were planned to be enrolled, of whom only two males and one female finished the study due to unexpected events that complied with the drop-out/exclusion criteria.

2.2.3. Study Design

The study was a randomized, nonblinded, monocentric intervention study with 3 intervention days and a controlled replenishment (taper-off or wash-out) phase before and between the study days.

Before the start of the study, a comprehensive screening of 20 participants was conducted, including questionnaires on inclusion and exclusion criteria, blood tests with the assessment of folate, vitamin B₁₂ status, and hemoglobin status. A genetic analysis was also performed to identify polymorphisms of the MTHFR gene. Only participants with a heterozygous point mutation at position A1286 (c.665C > C, c.1286A > C) were selected to allow for better data comparison. This gene mutation was known to reduce enzyme activity by 60%.^[49]

Height, weight, and other anthropometric data like fat-free mass, total body water, and intracellular water were determined using noninvasive bioimpedance analysis (BIA) for the six selected participants. For female participants, pregnancy was excluded prior to the study through a conventional pregnancy test (urine test). An overview of the individual subject parameters could be found in Table S3, Supporting Information.

Four weeks before the first study day, participants received a daily dietary supplement as tablets containing 400 µg of metafolin

(5-CH₃-H₄folate) to replenish liver and erythrocyte folate stores. During the intervals between the study days, participants continued to take 400 µg of the commercial folate supplement daily to maintain folate levels. This supplement was discontinued 2 days before each intervention (wash-out phase) to deplete and stabilize plasma folate levels. This study design was illustrated in Figure 2. Notably, there were two different sections of "folate-free" days. On the one hand, the 2-day taper-off phase (displayed in Figure 2), which was done prior to every of the 3 study days, and, on the other hand, a single folate-free test day as an intervention study day. Before the folate-free test days, the 2 folate-free wash-out days were done as well.

Participants consumed standardized low-folate meals the evening before each test day, 2 h before the start, and on the test day itself (after 2 and 5 h). They recorded their dietary intake during these meals. On the 3 test days, venous blood samples (4.9 mL each, totaling 98 mL per test day) were collected from a venous indwelling cannula before consuming the food (0 min) and at 19 subsequent time points (15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270, 300, 360, 420, 480, 540, and 600 min). The suitability of a commercial whey powder (reconstituted with milk) was tested for use on the folate-free control day. Analyzed food items with a folate content of less than 5 µg 100 g⁻¹ folate was verified for consumption as standardized meals before and during the study.

2.2.4. Test Foods

The commercially available whey powder (see point 1, reconstituted with milk) was checked for its use on the folate-free day. It showed a folate content of less than 2 µg 100 g⁻¹ and no increase in the plasma folate concentration in a prestudy with one study subject and, therefore, it was chosen as test food for the folate-free intervention day. Also, the other food items (see point 4) were considered suitable for use as standardized meals before and during the study because no increase in the folate concentration was observed after consumption in the pretrial.

- 1) *Fermented whey-based beverage powder*: obtained from USP (São Paulo, Brazil).

Table 2. Folate content and vitamer distribution of fermented whey powder in [$\mu\text{g } 100 \text{ g}^{-1}$], calculated as PteGlu, standard deviation is calculated from technical triplicates.

Vitamer	Folate content [$\mu\text{g } 100 \text{ g}^{-1}$ as PteGlu]
PteGlu	6.41
H ₄ folate	30.66
5-CH ₃ -H ₄ folate	99.44
5-CHO-H ₄ folate	88.59
10-CHO-PteGlu	22.60
Total folate content	247.70 $\pm$ 5.37

On each study day, each participant was given 82 g of the freeze-dried whey powder reconstituted in 200 mL milk. This amount of whey powder contained 80 μg 5-CH₃-H₄ folate (the content of other folates was listed in Table 2).

- 2) *Supplement (commercial)*: pure encapsulation Folate 400, containing 400 μg 5-CH₃-H₄ folate (metafolin). (The folate content of the capsules of $400 \pm 2.43 \mu\text{g}$ 5-CH₃-H₄ folate was investigated with the method mentioned in Chapter 2.1.4 without using enzymes.)
- 3) *"Folate-free" whey powder*: whey powder 'Raab Vitalfood Bio Süßmolke Natur.

Each participant consumed 82 g of the "folate-free" whey powder from Raab Vitalfood mixed with 200 mL milk on the folate-free test day.

- 4) *Standardized food for human trial*: cherry jam, butter, honey, rice and corn wafers, rice (cooked), and Emmental and Gouda cheeses. (The analyzed folate contents of the different food items were indicated in Table S1, Supporting Information).

2.2.5. Preparation of the Fermented Whey Drink

The commercial starter and probiotic Christian Hansen strains *S. thermophilus* (TH-4) and *B. longum* subsp. *infantis* (BB-02) were used for fermentation. The strains and conditions of fermentation were selected based on previous results for folate production by the USP research group (data not shown here). Some studies had also demonstrated the significant folate-producing potential of *S. thermophilus* and *B. longum* leading to elevated folate values, e.g., up to 200 $\mu\text{g } 100 \text{ g}^{-1}$ in yoghurt fermentation.^[30,50,51] First, they were (metabolically) activated separately in their cultivation medium (HJ broth and MRS-cis) in order to cultivate them and incubated at 37 °C overnight. After, they were centrifuged $10\,000 \times g \times 5 \text{ min}$, washed three times, resuspended in sterile saline solution (0.85%), and inoculated in 100 mL of a mixture of whey and milk (50% reconstituted and pasteurized powder milk [Molico, Nestlé, Brasil] and 50% of pasteurized whey) overnight. This culture was used as a starter inoculum (10%, v/v) for the bioreactor fermentation, added to a 1 L of 50% reconstituted and pasteurized powder milk (Molico, Nestlé, Brasil) and 50% of pasteurized whey supplemented with 10% of grape by-product water extract. A hot-water extraction obtained the water-extract^[52] from

grape by product (peel and seeds of a mixture of Merlot [Bento Gonçalves, RS] and Alicante Bouschet [Petrolina, PE] types of grapes), supplied by the Brazilian Agricultural Research Corporation (EMBRAPA), Food Agroindustry Department, Guaratiba, Rio de Janeiro, Brazil. Fermentation was conducted in a bioreactor BioFlo 115 (New Brunswick, Eppendorf, Hamburg, Germany) with 1.5 L capacity, using lactic acid food-grade solution as acid and a 5 M solution of potassium hydroxide food-grade (Merck, Germany) as the base. Samples were withdrawn at 0 h and after 24 h fermentation for enumeration of cells and folate quantification. After the pH declined to 5.5 (after 2–3 h), it was automatically maintained at 5.5 for 24 h at 37 °C, using 40 mL of potassium hydroxide per liter of beverage. The final population of TH-4 and BB-02 were 9.2 ± 0.01 and $7.8 \pm \log \text{CFU mL}^{-1}$, respectively. The drink was freeze-dried to generate a whey powder and shipped to the Technical University of Munich (TUM).

3. Results

3.1. Folate Results of the Fermented Whey Powder

Table 2 shows the folate content and vitamer distribution of the fermented whey powder.

Due to the limited amount of fermented whey, we could only use 82 g per participant on the intervention day. This quantity provided 203.1 $\mu\text{g } 100 \text{ g}^{-1}$ total folates, including 81.5 $\mu\text{g } 100 \text{ g}^{-1}$ 5-CH₃-H₄ folate for each volunteer. However, this fell short of our target of 400 μg 5-CH₃-H₄ folate, which we used on the reference day with the commercial supplement. This will be further discussed later in Chapter 4.

3.2. Outcome of the Human Short-Term Study

Throughout the whole study, we did not observe any changes in the participants' responses to these meals, which means that the low-folate meals before and during the study did not affect the study's results.

The plasma folate concentrations were determined on 3 independent study days for the three remaining participants, whose names were pseudonymized by a random three-letter code (ZEY, SSV, and BOI). Figure 3 shows the postabsorptive plasma concentration of the folate-free day, the test day with the fermented whey beverage, and the reference day with the folate supplement for the subject ZEY. Table 3 presents the biokinetic parameters of subject ZEY for the 3 study days. The other two participants, BOI and SSV, showed very similar results; the corresponding information for these subjects can be found in Table S4 and S5, Supporting Information, Tables 4,5.

The plasma concentration curve of the folate-free days showed no maximum for all three individuals with average concentrations of 28.1 nmol L⁻¹ (subject ZEY), 35.9 nmol L⁻¹ (subject BOI), and 44.7 nmol L⁻¹ (subject SSV). These values demonstrate the high variations between the three individuals. These deviations do not only apply within different participants but also for the 3 different study days of the same participant. Especially, the varying start values c_0 of 31.8 nmol L⁻¹ (subject ZEY), 41.7 nmol L⁻¹ (subject BOI), and 52.3 nmol L⁻¹ (subject SSV) are the decisive

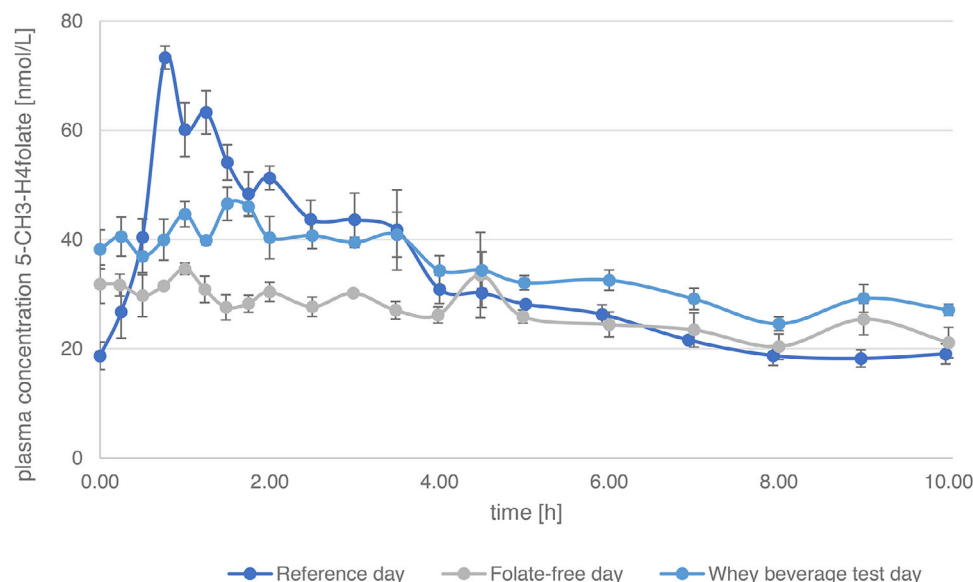


Figure 3. Postresorptive plasma concentration of the folate-free day, test day with the fermented whey beverage, and the reference day with the folate supplement (subject ZEY). Standard deviation [nmol L⁻¹], calculated from a technical triplicate.

Table 3. Summary of biokinetic parameters observed on the folate-free test day, the whey drink test day, and the folate supplement test day for subject ZEY.

Study day	Total study time [h]	c_0 [nmol/L]	c_{min} [nmol/L]	c_{max1} [nmol/L]	c_{max2} [nmol/L]	t_{max1} [h]	t_{max2} [h]	Normalized AUC [nmol L ⁻¹ h ⁻¹]
Folate-free day	10.00	31.84	20.39	34.64	–	1.00	–	1.49
Whey beverage test day	10.00	38.22	24.58	46.55	–	1.50	–	22.52
Reference day	9.97	18.73	18.24	73.32	63.3	0.77	1.25	256.96

variable for the measured folate concentrations on the folate-free test day. Therefore, we decided to normalize the AUC values to the basal value (initial starting value) to cope with the high inter-individual- and interday variability. The normalization led to AUC values for the folate-free day from 0.03 nmol h⁻¹ (SSV), 1.5 nmol L⁻¹ h⁻¹ (ZEY), and 3.1 nmol L⁻¹ h⁻¹ (BOI), showing

that there was almost no absorption of folates during the 10-h study day.

The reference day with the consumption of a 400 µg 5-CH₃-H₄folate supplement showed two maximum for all three subjects. ZEY reached the first and highest maximum of 73.3 nmol L⁻¹ (c_{max1}) after 0.8 h and a c_{max2} of 63.3 nmol L⁻¹

Table 4. Summary of biokinetic parameters observed on the folate-free test day, the whey drink test day, and the folate supplement test day for subject BOI.

Study day	Total study time [h]	c_0 [nmol L ⁻¹]	c_{min} [nmol L ⁻¹]	c_{max1} [nmol L ⁻¹]	c_{max2} [nmol L ⁻¹]	t_{max1} [h]	t_{max2} [h]	Normalized AUC [nmol L ⁻¹ h ⁻¹]
Folate-free day	9.88	41.68	25.66	45.03	–	0.28	–	3.10
Whey beverage test day	10.00	57.33	40.49	61.20	–	0.77	–	2.28
Reference day	10.05	20.82	20.82	76.80	69.45	0.78	1.53	374.42

Table 5. Summary of biokinetic parameters observed on the folate-free test day, the whey drink test day, and the folate supplement test day for subject SSV.

Study day	Total study time [h]	c_0 [nmol L ⁻¹]	c_{min} [nmol L ⁻¹]	c_{max1} [nmol L ⁻¹]	c_{max2} [nmol L ⁻¹]	t_{max1} [h]	t_{max2} [h]	Normalized AUC [nmol L ⁻¹ h ⁻¹]
Folate-free day	10.03	52.34	36.19	52.54	–	0.25	–	0.03
Whey beverage test day	9.97	51.40	40.26	55.49	–	0.73	–	11.25
Reference day	10.13	20.48	20.48	66.31	111.00	0.50	1.75	476.27

after 1.3 h (see Figure 3). The plasma folate concentration of participant BOI (Figure S1, Supporting Information) increased at almost the same time as ZEY after 0.8 h to a similar $c_{\max 1}$ of 76.8 nmol L^{-1} . The second maxima after 1.5 h showed a plasma folate concentration of 69.5 nmol L^{-1} . The postabsorption plasma curve of subject SSV exhibited slight variations but followed a similar trend, with two observable maxima. Unlike BOI and ZEY, the first increase to 66.3 nmol L^{-1} after 0.5 h is the smaller one, and the highest folate concentration of $111.0 \text{ nmol L}^{-1}$ was measured after 1.8 h (see Figure S2, Supporting Information). So, SSV showed the overall highest folate level with a 1-h-delayed absorption compared to the other two participants. The baseline values on the reference day exhibited less variation, with ZEY at 18.7 nmol L^{-1} , while BOI and SSV were at 20.8 and 20.5 nmol L^{-1} , respectively (unlike subject ZEY, the initial concentrations consistently measured the lowest values throughout the entire study day). To facilitate comparison, we calculated the normalized AUC values once more. In ascending order, ZEY had an AUC of $257.0 \text{ nmol L}^{-1} \text{ h}^{-1}$, while BOI followed with $374.4 \text{ nmol L}^{-1} \text{ h}^{-1}$, and SSV exhibited the highest value with an AUC of $476.3 \text{ nmol L}^{-1} \text{ h}^{-1}$.

In order to determine the bioavailability of the fermented whey beverage, we proceeded in the same way on the test day as we did on the other 2 study days. Unsuspectably, no “real” maximum could be observed in the postresorptive plasma curves of all three participants. The normalized AUC values of $22.5 \text{ nmol L}^{-1} \text{ h}^{-1}$ (ZEY), $2.3 \text{ nmol L}^{-1} \text{ h}^{-1}$ (BOI), and $11.3 \text{ nmol L}^{-1} \text{ h}^{-1}$ (SSV) underline this observation as they are more in line with the AUCs of the folate-free days than with the values obtained on the reference day. These findings demonstrate a similar pattern and suggest that the folate absorption from the fermented whey beverage differed from that of the folates from the supplement. However, it should be noted that only approximately 20% of the desired 5- $\text{CH}_3\text{-H}_4$ folate-concentration of $400 \mu\text{g}$ was administered with the whey beverage due to the low folate content of the test material.

Although the postabsorption plasma curves of the 3 test days did not show any decisive maxima, we calculated the bioavailability with the help of the normalized AUC values as we observed higher AUC values compared to the folate-free day, except for subject BOI. To calculate the relative bioavailability, the AUC values of the folate-free control day were subtracted from the calculated AUC values of the reference and the test day. Taking the absolute dose of folates into account, the relative bioavailability of the whey beverage compared to the supplement was 39.8% for subject ZEY and 11.4% for subject SSV. The evaluation of the folate plasma curve of participant BOI showed a slightly higher AUC for the folate-free day with $3.1 \text{ nmol L}^{-1} \text{ h}^{-1}$ than for the beverage test day ($2.3 \text{ nmol L}^{-1} \text{ h}^{-1}$). Therefore, subtracting the AUC from the folate-free would lead to negative values. These calculated negative values were analytically not different from 0 as the values range in the analytical standard deviation of the measurements. In conclusion, the folate intake with the fermented whey beverage on the test day was lower than or equal to the folate-free day, and no bioavailability was observed for participant BOI.

Therefore, taking the arithmetic mean of the calculated bioavailability of all three participants, the average bioavailability was 17.1%.

The other vitamers (PteGlu, H_4 folate, 5-CHO- H_4 folate, and 10-CHO-PteGlu) were also analyzed in all plasma samples, but

the results were not considered in this study due to their low amounts under the estimated LoDs and LoQs.

3.3. Results of the Additional Analysis

We performed supplementary analyses to gain deeper insights into the safety and composition of the fermented whey beverage and to rule out further possibilities for the surprisingly low bioavailability.

The pH value of the fermented whey drink was 5.8, while the osmotic potential was determined at 709 mosm.

3.3.1. Microbiological Testing

Counts of molds and yeasts, *Enterobacteriaceae* and *E. coli*, were below 10 CFU g^{-1} , and *Salmonella* spp. was not detected in the samples, indicating that they were in accordance with the Brazilian microbiological criteria for dairy-fermented food products.^[40]

3.3.2. Anion Analysis

The average phosphate content was $457 \pm 86.91 \text{ mg } 100 \text{ g}^{-1}$, comparable to hard cheese like Gouda or Edam.^[53] The sulfate content was $101 \pm 21.70 \text{ mg } 100 \text{ g}^{-1}$, similar to sulfate-rich “healing water” or mineral water with high mineralization.^[54] All samples were negative for chloride and nitrate.

The lactate content was $174 \pm 49.82 \text{ mg } 100 \text{ g}^{-1}$, which may be considered low. For comparison, buttermilk contains $983 \text{ mg } 100 \text{ mL}^{-1}$, and kefir around $926\text{--}1000 \text{ mg } 100 \text{ mL}^{-1}$.^[55]

3.3.3. Polyglutamate Analysis

Table 6 shows the results of the semiquantitative polyglutamate analysis, indicating a content of approx. $99 \mu\text{g } 100 \text{ g}^{-1}$ 5- $\text{CH}_3\text{-H}_4$ PteGlu. This value is closed to the summed content of mono- and polyglutamates from the semiquantitative experiment with $97 \mu\text{g } 100 \text{ g}^{-1}$ ($\pm 2\%$). The analysis indicates that 70% of the measured 5- $\text{CH}_3\text{-H}_4$ PteGlu was monoglutamates. The second most common polyglutamate was heptaglutamate, which was around 9%. The remaining 20% accounts for 5- $\text{CH}_3\text{-H}_4$ PteGlu_{2,4}. The abundance of hexaglutamate was below 1% and pentaglutamate was not detectable.

4. Discussion

First, previous internal analyses at our institute showed that folate biofortification and fermentation were successful. As studies from Albuquerque et al. already demonstrated, folate production is not only strain-dependent but can also be stimulated by adding fruit by-products or other substrates.^[30,56] Our results (not presented in this paper) stated that the folate content of the fermented beverage was lower when only whey or fruit by-products were used. The fermentation with whey and fruit by-products resulted in the greatest folate production through *S. thermophilus*

Table 6. Results of the semiquantitative polyglutamate analysis (rounded to the nearest integer), calculated in $\mu\text{g } 100 \text{ g}^{-1}$ as 5-CH₃-H₄PteGlu.

	5-CH ₃ -H ₄ PteGlu	5-CH ₃ -H ₄ PteGlu ₂	5-CH ₃ -H ₄ PteGlu ₃	5-CH ₃ -H ₄ PteGlu ₄	5-CH ₃ -H ₄ PteGlu ₅	5-CH ₃ -H ₄ PteGlu ₆	5-CH ₃ -H ₄ PteGlu ₇	Sum [$\mu\text{g } 100 \text{ g}^{-1}$ as 5-CH ₃ -H ₄ PteGlu]
Folate content [$\mu\text{g } 100 \text{ g}^{-1}$]	67	6	7	7	0	1	9	97
Percentage of total folate [%]	70	6	7	7	0	1	9	100

(TH-4) and *B. longum* subsp. *infantis* (BB-02). Controlling and maintaining the pH at 5.5 also enhanced the levels obtained in the fermentation (data not shown), as demonstrated by Meucci et al. in their studies on the pH influence on folate production by LAB and probiotic strains.^[57] Folate production by microorganisms is an interesting approach to enhance the vitamin content in food, and the bioavailability of folates produced during fermentation could be shown in studies with rodents.^[26,58]

The results of the present study, even though the authors are aware of the limitations regarding limited statistical power due to the small participant sample size, demonstrate that the general design of the human trial was adequate. The folate absorption on the reference day showed a clear increase in the folate level, and the plasma curve on the folate-free day was flat, showing no folate absorption. Therefore, it can be asserted that the participants adhered to the supplementation phase instructions before and between the study days, and the duration of the supplementation phase proved adequate for replenishing the participants' folate storage pools. The use of 5-CH₃-H₄PteGlu as a reference rather than folic acid was selected according to the report of Striegel et al.^[64] and the results of a study by Wright et al. (2010). Even though the latter authors conducted a long-term intervention study, it is stated that the natural folate form is a better choice for use in a human trial due to the discrepancies in erythrocyte folate concentrations shown for folic acid. To avoid the impairment of future results and investigate the relative bioavailability of folate from food sources, 5-CH₃-H₄PteGlu should be used as a referencing supplement, which we followed in our study.^[59]

Furthermore, the selection of the standardized low-folate meals was appropriate, as no observable increase in folate concentrations occurred following their consumption on the study days. By adhering to fixed mealtime intervals, we ensured consistent dietary conditions for every treatment, as previous research has shown that folate plasma concentrations typically increase when individuals are fasting.^[48,60,61] This approach helped to maintain a stable folate balance and prevented fluctuations by fasting and disruptions in the enterohepatic circulation. A study day duration of 10 h proved suitable. It can be reduced to 7 or 8 h, considering primary folate absorption occurs within the initial 4–5 h. Given our focus on a single type of polymorphism, the impact of these factors should be relatively minor, strengthening the study's power.

On the reference day, when the folate supplement was administered, the first maximum in plasma concentration occurred approximately 0.50–0.78 h later. This pattern is comparable to the findings of Striegel et al.,^[64] who also used 5-CH₃-H₄folate as a reference, although administered with a water-pectin-sugar mixture, thus explaining the faster absorption in our study. Furthermore, the second peak concentrations align with the study's results. Striegel et al. postulated that the ingested 5-CH₃-H₄folate is transported into the bile and is reabsorbed for distribution to

various tissues and the liver. Our findings provide further evidence supporting this hypothesis. Another hypothesis points to the direction of the role of gut microbiota in folate production, as several studies have shown that microbial folate can be absorbed and utilized by the host, particularly in the colon. Certain bacteria present in the gut, such as *Bacteroides*, *Enterococcus*, and *Lactobacillus* species, possess the enzymatic machinery necessary for folate biosynthesis through various biochemical pathways. This microbial-derived folate can supplement dietary folate intake, potentially influencing host folate levels. The understanding of the contribution of gut microbial folate production to host folate status has not yet been thoroughly investigated. Further research into the mechanisms regulating microbial folate synthesis, absorption, and utilization will provide insights into optimizing gut health and supporting folate production in the human gut.^[62,63] Additionally, our study confirms that plasma concentrations rapidly increased on the day of folate supplementation, followed by a gradual decline.

Conversely, on the day of the beverage test, plasma concentration was higher by the end of the study day. This could be an artifact since the initial values on this particular day were the highest among all three subjects. However, when considering the curves and maxima, it also suggests that the absorption of natural folates from the fermented whey beverage requires more time. Similar trends were observed in the absorption of folates from strawberries.^[64] This delay in absorption is likely attributed to the breakdown of polyglutamates and the encapsulation of folates within the matrix, which appear to be limiting factors. We determined the polyglutamate composition in the fermented whey beverage to gain further insights into the potential effects of the deconjugation process of folate polyglutamates in the intestine. Our analysis revealed that the majority of the 5-CH₃-H₄PteGlu, approximately 70%, already exists in the form of monoglutamate. It is known that *S. thermophilus* species produce intracellular and extracellular folate, especially in mono, di-, and triglutamate forms,^[21] which could be partly confirmed in this analysis. This suggests that the inhibition of conjugase activity in the intestine, caused by specific ingredients, can essentially be ruled out as a significant contributing factor to the observed lack of bioactivity. Furthermore, it is worth noting that the general bioavailability of polyglutamates is commonly estimated to be around 70%.^[65,66] However, the AUC values and the calculated bioavailability of only 17.1% on average lead to the conclusion that the bioavailability was negatively influenced.

The examination of the osmotic potential and pH of the whey beverage revealed interesting results. The pH of the whey beverage, at 5.8, did not deviate significantly from the pH optimum of the conjugase to explain enzyme activity inhibition.^[67] However, the osmotic potential of liquids, which indicates how rapidly they can be absorbed and processed by the body, did yield meaningful insights into the low plasma folate concentrations observed

in the conducted human study. The measured osmolality of 709 mosmol L⁻¹ for the whey beverage is deemed high. High osmolalities are also typical in soft drinks, juices, or energy drinks, generally attributed to their high sugar content. Our product's carbohydrate content of 5.5% is not excessively high and cannot account for the high osmolality. Regardless, the optimal osmotic pressure of beverages for fluid absorption in the human body is approximately 300 mosmol L⁻¹. Hyperosmolality in drinks, characterized by significantly higher osmolality as observed in the whey beverage, can influence the osmotic pressure in the body and disrupt normal intestinal function. When consuming a beverage with high osmolality, the body must retain more water in the intestine to balance this concentration difference. This can lead to digestive disorders, diarrhea, or an unfavorable distribution of fluids in the body. Additionally, high osmolality can impair the absorption of nutrients and electrolytes, as the intestine works less efficiently when overloaded.^[68–70] This circumstance could explain the reduced bioavailability of the whey beverage observed here.

Given that the elevated osmolality cannot be ascribed to the sugar content, the subsequent stage involved the quantification of prevalent anions. However, they did not demonstrate lifted levels of phosphate, sulfate, or lactic acid compared to other food products. Thus, these factors cannot elucidate the observed high osmolality values. Moreover, there is no indication that the determined concentrations of anions impeded folate absorption or could have impacted the efficiency of intestinal conjugase. Although carbohydrate content was not high in the beverage, the ash content (2%) was a little elevated compared to other fermented drinks (0.8–1.4%) due to the high mineral content in whey, which may have influenced the osmotic potential and therefore the absorption of folates.

Another explanation could be the known matrix effects and the existing folate-binding proteins in milk. It is known that 5-CH₃-H₄folate found in untreated milk is typically bound to FBP.^[71–73] Several studies have investigated the effect of folate-binding proteins (FBP) on bioavailability, which could explain the bioavailability demonstrated in this study. On the one hand, Mason and Selhub found that folates attached to FBP are still accessible in the intestinal microbiota; consequently, the uptake does not necessitate the prior separation of the vitamin-binding protein complex.^[73] On the other hand, Witthöft et al. proved that adding folate-binding proteins (FBP) notably reduced folate absorption from dairy products. These protein bonds can be partially denatured after undergoing ultra-high temperature (UHT) treatment, potentially allowing folates to exist freely in UHT milk.^[74] In pasteurized milk, as used in this study, the heating process only partially denatures FBP, resulting in folate remaining bound to FBP.^[75] Nevertheless, fortified dairy products are particularly suitable without active FBP.^[74]

Due to the conflicting results of several studies, the general bioavailability of milk and milk products is complex to assume.^[48,60,71] Mönch et al. performed human studies on the bioavailability of Camembert cheese. These studies revealed a notably higher bioavailability of over 64% for a low-fat Camembert compared to a different brand with only 8.8% bioavailability. They explain these variations with varying folate distribution between the soft dough and firm rind and differences in the proportions of individual folate vitamers.^[60] Again, these findings emphasize

the significance of the food matrix, even within the same food category. Considering the effect of FBP and other matrix effects, it might be valuable to explore this option by examining the participants' stools and tracer studies of labeled metabolites, which could help further investigate the metabolic pathway.

Besides the matrix effects and the inherent challenge of deglutamylation, the study has additional limitations. The pilot had a small number of three participants who completed the entire protocol, which allowed us to draw conclusions about the low folate concentration in the test product. Although the test design seems feasible, a higher dose would be needed in a follow-up trial. Furthermore, the overall 5-CH₃-H₄PteGlu content in the whey beverage was lower than our target, measuring 83 µg compared to 400 µg in the supplement. However, it is worth highlighting that the 5-CHO-H₄folate content in the administered quantity of whey powder was 73 µg, which can also exhibit bioavailability.^[36] Furthermore, folic acid is completely converted to 5-CH₃-H₄PteGlu and, thereby, becomes bioactive. Thus, the total folate content would reach 201 µg folate in the 82 g of whey powder, further diminishing the calculated bioavailability of folates from the fermented whey drink. Consequently, the bioavailability for the participant ZEY would decrease to 16.4%, and for the participant SSV, it would decline to 4.7%. The bioavailability or the undetectable folate uptake for participant BOI logically remains unchanged at 0%. By referencing the total folate content, the bioavailability of the whey drink would be reduced to an average of only 7.0%. Another obstacle in evaluating the study data was the high variations in interday and interindividual baseline values. Therefore, a normalization of the AUCs to the baseline of the corresponding study day and subject was used, which is reasonable in this case. Furthermore, the analysis would have been simplified if the folates in the fermented whey beverage had been isotopically labeled. This would have facilitated data evaluation since the baseline value over the whole study day would consistently be 0. Additionally, isotopically labeled tracer studies would not be impacted by excess unlabeled folates in the organisms present after the replenishment phase.

5. Conclusion

This research sheds light on a promising alternative for enhancing folate intake. Given the substantial impact of folates on human health, this becomes especially critical in regions where mandatory folate fortification is unknown or impossible. Increasing awareness regarding valuable natural sources of folates and their absorption potential is imperative for maintaining optimal folate levels and overall well-being. By leveraging innovative approaches and interdisciplinary collaborations, we can work towards improving folate accessibility and addressing nutritional deficiencies, particularly in vulnerable populations.

For the first time, the bioavailability of folates derived from microorganisms within a fermented whey-based drink was assessed in a human trial. The outcomes of this study indicate an overall bioavailability of 17.1% for the folates found in the fermented whey beverage, demonstrating considerable interindividual variation among the subjects.

While the study presents valuable insights into the potential of fermented whey beverages as folate sources, several limitations warrant consideration, which have been discussed. The small

sample size and variations in baseline values underscore the need for larger-scale studies with more diverse populations to validate the findings as we did not achieve statistical power, making biokinetic interpretation rather challenging.

The deconjugation process and encapsulation within the matrix may be influenced by the presence of FBP, which might be a limiting factor in folate absorption. Additional factors such as elevated salt content, pH levels, or polyglutamate distribution may offer potential explanations. However, this highlights again the complex dynamics of folate bioavailability and a more comprehensive investigation of these factors. The observed bioavailability underscores the need for further refinement in production methods and formulation strategies.

These findings again emphasize the importance of further research on folate bioavailability in general and those derived from dairy products, as bioavailability remains a concern due to various factors influencing folate absorption and metabolism. Biofortifying folates through fermentation is a promising approach for enhancing natural folate content. Besides its health benefits, fermentation is a more cost-effective long-term method on larger scales.^[76]

The following studies could assess the bioavailability of 5-CHO-H₄ folate and 5-CH₃-H₄ folate administered in capsules or a whey drink. Additionally, a follow-up study could test the bioavailability when adding 400 µg 5-CH₃-H₄ folate to the existing whey beverage to get more insight into the tested food. However, it is important to investigate the dose-dependency of 5-CH₃-H₄ folate more thoroughly by testing reduced levels of this vitamer, e.g., 80 µg (as in the self-study), but with a higher number of participants. This could clarify the impact on our analysis's detectability and statistical power.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: L.O., M.R.; Data curation: L.O., A.C.C.C.; Formal analysis: L.O., T.S., M.R.; Methodology: L.O., T.S., M.R.; Project administration: L.O., M.R.; Supervision: M.R.; Validation: L.O., T.S.; Writing – original draft: L.O.; Writing – review & editing: A.C.C.C., T.S., M.R., S.M.I.S., B.D.G.M.F.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Keywords

bioavailability, biofortification, folates, human study, stable isotope dilution assay

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