

# Kinetics of iron absorption from ferrous fumarate with and without galacto-oligosaccharides determined from stable isotope appearance curves in women

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## ABSTRACT

**Background:** Prebiotic galacto-oligosaccharides (GOS) are novel enhancers of iron absorption from ferrous fumarate (FeFum). However, the mechanism(s) of this effect, and whether it occurs in the proximal or distal gut, are uncertain.

**Objectives:** We studied: 1) *in vitro*, the effect of GOS on iron solubility and dialyzability from FeFum; 2) in volunteers, the absorption kinetics of FeFum given with and without GOS using stable isotope appearance curves (SIAC).

**Methods:** We measured iron solubility at various pH and dialyzability from FeFum with and without GOS. In crossover design, iron-depleted women [ $n = 11$ ; median serum ferritin (SF) 15.2; IQR: 12.6–21.2  $\mu\text{g/L}$ ] received 2 14-mg iron doses as labeled (<sup>57</sup>Fe, <sup>58</sup>Fe) FeFum 14 d apart with and without 15 g GOS in randomly assigned order. Multiple blood samples were collected over a time period of 24 h and 14 d later to determine SIAC and fractional iron absorption (FIA), respectively. SIAC data were fitted using nonlinear mixed effects modeling to a 1-compartment model with first-order absorption, and AUC and time of peak serum isotope concentration ( $t_{\text{max}}$ ) were calculated.

**Results:** Iron dialyzability was 75% higher with GOS ( $P < 0.001$ ) and iron solubility was more than doubled at pH 4 and 6 with GOS [both  $P < 0.001$ ]. Mean  $\pm$  SD AUC (5830.9  $\pm$  4717.3  $\mu\text{g/min}$  with GOS, 4454.0  $\pm$  3260.7  $\mu\text{g/min}$  for control), and median (IQR) FIA (20.3% (8.6%–38.7%) with GOS, and 15.6% (10.6%–24.8%) for control) were not different with compared to without GOS ( $P = 0.064$ ;  $P = 0.080$ ). Mean  $\pm$  SD  $t_{\text{max}}$  was not altered with GOS (3.08  $\pm$  0.47 h with GOS; 2.80  $\pm$  0.50 h for control;  $P = 0.096$ ). Iron bioavailability significantly increased with decreasing SF and this effect was significantly enhanced by GOS ( $P = 0.037$ , interaction of GOS with SF).

**Conclusions:** GOS increases iron solubility from FeFum at physiological pH characteristic of the proximal duodenum. The absorption kinetics *in vivo* are consistent with effects on iron absorption in the proximal, rather than distal, parts of the gut. There was no overall effect of GOS on FIA *in vivo*, but the interaction of GOS and SF on FIA might benefit iron-deficient women, an effect potentially

mediated by the higher solubility shown *in vitro*. This study was registered at clinicaltrials.gov as NCT03996421. *Am J Clin Nutr* 2022;115:949–957.

**Keywords:** prebiotic, galacto-oligosaccharides, GOS, iron deficiency, absorption, stable isotope, women, ferrous fumarate, kinetics

## Introduction

Anemia remains a global health problem that affects more than one-third of nonpregnant women, with about half of cases thought to be due to iron deficiency (1, 2). Oral iron supplementation is used as a preventive measure for anemia and is also a first-line treatment of iron deficiency and mild-to-moderate iron deficiency anemia (1–5). Iron absorption from oral iron supplements is often low (6, 7) and adverse gastrointestinal side effects are commonly observed with oral supplement ingestion, reducing compliance (8, 9). Prebiotic galacto-oligosaccharides (GOS) are promising

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Supplemental Figure 1 is available from the “Supplementary data” link in the online posting of the article at <https://academic.oup.com/ajcn/>.

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Abbreviations used: AGP,  $\alpha$ -1-acid glycoprotein; AAS, atomic absorbance spectrometer; CRP, C-reactive protein; DMT-1, divalent metal transporter; FeFum, ferrous fumarate; FePP, ferric pyrophosphate; FeSO<sub>4</sub>, ferrous sulfate; FIA, fractional iron absorption; GOS, galacto-oligosaccharides; Hb, hemoglobin; PF, plasma ferritin; SF, serum ferritin; SIAC, stable isotope appearance curve; sTfR, soluble transferrin receptor;  $t_{\text{max}}$ , time of peak serum isotope concentration.

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enhancers of iron absorption (10, 11) and may be beneficial for the distal gut because they are bifidogenic (12, 13). Ferrous fumarate (FeFum) is commonly used for the treatment of iron deficiency (6) and is fully soluble only at pH 2 (4). Previous studies in iron depleted women [serum ferritin (SF) <30  $\mu\text{g/L}$ ] looking at the effect of GOS on oral iron absorption from FeFum have shown enhancing effects from single doses of 7 g (11) and 15 g GOS, by up to 61% (14).

Putative mechanisms for how GOS acutely enhances iron absorption from FeFum include the following: 1) increasing gastric residence time, allowing greater iron dissolution (15); 2) increasing iron solubility via chelation or via iron reduction by the terminal reducing sugar on GOS molecules (16–18); and/or 3) a bifidogenic effect that rapidly increases production of short chain fatty acids in the proximal colon, decreasing gut luminal pH and increasing iron dissolution (19). It has been proposed that prebiotics can enhance the colonic absorption of minerals such as calcium and magnesium (20) via this latter mechanism, but the exact mechanisms involved remain uncertain (21).

Stable isotope appearance curves (SIACs) can detect the appearance of small amounts of absorbed labeled iron in serum with high precision and be used to distinguish between circulating body iron and absorbed iron, even from low iron doses (22). SIACs are useful tools to investigate the rate, quantity, and pattern of iron absorption from iron compounds (23) and have been validated as a measure of iron absorption (24).

The mechanism of absorption of iron combined with GOS was assessed by determining the effect of GOS on in vitro solubility and dialyzability of FeFum at various pH levels. The absorption profile of iron from FeFum with and without GOS using SIACs was determined in iron-depleted young women to clarify whether the absorption-enhancing mechanism of GOS occurs in the proximal or distal gut. We hypothesized that, compared to without GOS, GOS would: 1) improve iron solubility and dialyzability in vitro; 2) slow down iron absorption from FeFum in vivo, resulting in delayed time of peak serum isotope concentration ( $t_{\text{max}}$ ); 3) increase iron absorption from the distal gut, resulting in an extended or even second absorption peak and/or a less pronounced decrease after the peak in the SIAC; and 4) as a result of items 1–3, GOS would result in a greater AUC of the SIAC and higher fractional iron absorption (FIA) as measured by erythrocyte incorporation 14 d after FeFum ingestion.

## Subjects and Methods

### Determination of iron solubility in vitro

Iron solubility of FeFum with GOS, lactose/sucrose, or on its own as control was tested at pH 2, 4, and 6. The treatment solutions, each 50 mL, consisted of ultrapure water only, or 4 g GOS dissolved in ultrapure water, or 1.1 g lactose and 0.23 g sucrose dissolved in ultrapure water. The amounts of sucrose and lactose were based on the amounts of sucrose and lactose present in the GOS powder. The pH was adjusted to the desired value (2, 4, or 6) with 1 M hydrochloric acid, and the solutions were placed into a shaking water bath at 37°C. For the measurement, portions of 7.5-mg FeFum, corresponding to 2.5 mg iron, were added to the prewarmed treatment solutions and samples were shaken at 150 rpm and 37°C (the effects

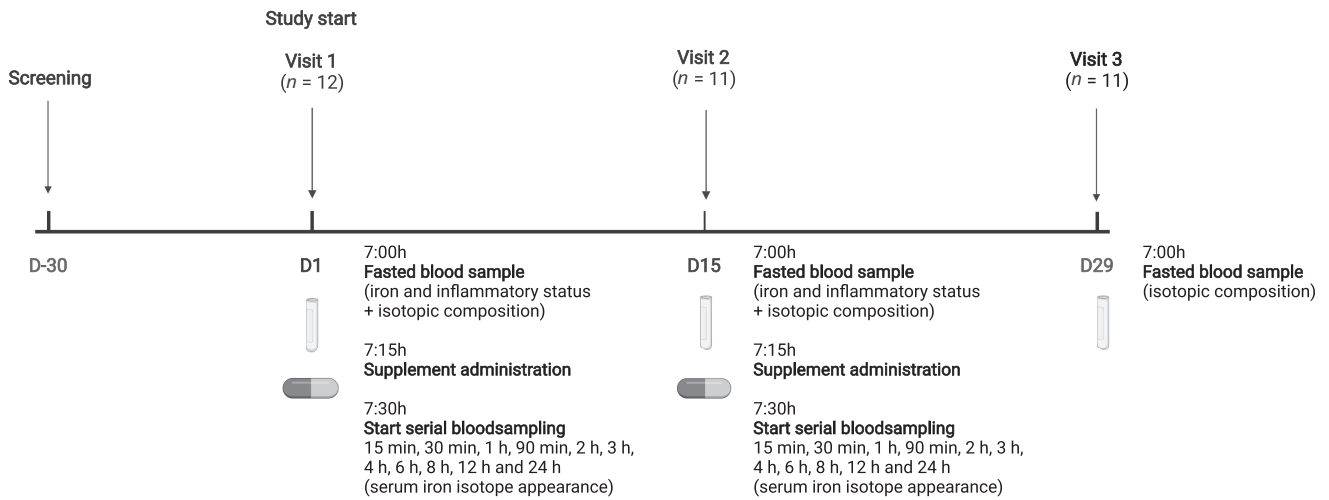
of heating and addition of FeFum on pH were negligible). Shaking was stopped for 1 min at time points 15, 30, 45, and 60 min to let the nonsolubilized contents settle and 1-mL aliquots were collected from the supernatants. After adequate dilution with 0.1-M hydrochloric acid, the iron concentration in the collected aliquots was measured with a flame atomic absorbance spectrometer (AAS) (AA240FS; Agilent Technologies). Each condition was tested in triplicate.

### Determination of iron dialyzability in vitro

Iron dialyzability was tested in a 1.1-g GOS/1-mg iron (in the form of FeFum) ratio, which corresponded to the ratio used in the human study. For determining the iron dialyzability, we adapted the methods described by Miller et al. (25) and Hurrell et al. (26). Supplement combinations were analyzed in triplicate. A total of 21.7 g GOS powder, or 1.3 g sucrose and 6.2 g lactose, were dissolved in 200 mL ultrapure water. The mixtures were brought to pH 2 with 1 M hydrochloric acid and then split into 4 replicates of 40-g weight in Erlenmeyer flasks. To each replicate 2-mg iron as FeFum powder was added. Then, 1.3 mL pepsin solution [200 mg pepsin/mL (porcine; Sigma-Aldrich) in HCl (0.1 M)] was added to all replicates. All samples were then incubated in a shaking water bath (1 h at 37°C and 150 rpm). One replicate of each treatment was used to determine the amount of base ( $\text{NaHCO}_3$ ) needed to raise the pH to 7.5 during the second phase of the digestion: 5 mL of pancreatin solution [4 mg pancreatin (porcine, Sigma-Aldrich) and 25 mg bile extract (porcine; Sigma-Aldrich) per mL in  $\text{NaHCO}_3$  0.1 M] were added to these replicates, and the mixtures were titrated to pH 7.5 with KOH 0.5 M. Dialysis membranes (30-cm long, 6-8000 Da porosity, preboiled in water; SpectraPor 1, 2.4 mm O; Spectrum Laboratories) were filled with 25 mL of an aqueous solution containing an amount of  $\text{NaHCO}_3$  needed to raise the pH of the samples to 7.5, based on the previous titration with KOH, and placed into the 3 remaining sample replicates. All samples were incubated for 30 min at 37°C, after which 5 mL of pancreatin solution was added to each sample, and then the incubation was continued for 1 h more. The dialysis membranes were removed from the samples, and the dialysates were transferred into acid-washed preweighed polyethylene bottles that contained 700  $\mu\text{L}$  65%  $\text{HNO}_3$ . The samples were stored at 4°C overnight and then diluted, and their iron concentration was measured by graphite-furnace AAS (AA240FS; Agilent Technologies) on the subsequent day. The percentage dialyzability was expressed as the quotient of dialyzable iron from the dialysate divided by the dialyzable iron from the reference, which consisted of the same iron compound in water, treated in the same way as the samples.

### Subjects

Iron-depleted (SF 15–30  $\mu\text{g/L}$ ) healthy women were recruited among students and staff of the ETH Zurich and University of Zurich. Inclusion criteria were the following: 1) female aged between 18 and 45 y; 2) plasma ferritin (PF) 15–30  $\mu\text{g/L}$ ; 3) BMI (in  $\text{kg/m}^2$ ) 18.5–24.9; and 4) body weight <70 kg. Exclusion criteria were the following: 1) anemia [hemoglobin (Hb) <120 g/L]; 2) inflammation [C-reactive protein (CRP) >5 mg/L]; 3) chronic digestive, renal, and/or metabolic disease; 4) chronic medications (except for oral contraceptives); 5) use of vitamin, mineral, and pre- and/or probiotic supplements in



**FIGURE 1** Study design and participant flowchart. Adapted from “Experimental Timeline (Horizontal)”, by BioRender.com (2021); (retrieved from <https://app.biorender.com/biorender-templates>).

the previous 2 wk; 6) blood transfusion, blood donation, or significant blood loss over the past 4 mo; 7) difficulties with blood sampling; 8) antibiotic treatment in the previous 4 wk; 9) known hypersensitivity or intolerance to iron supplements, GOS, or lactose; 10) pregnancy (tested in plasma at screening) and/or lactation; 11) smoking; and 12) alcohol consumption >2 units alcohol/d.

### Stable-isotope tracers

$^{57}\text{Fe}$  and  $^{58}\text{Fe}$ -enriched FeFum were prepared from  $^{57}\text{Fe}$  and  $^{58}\text{Fe}$ -enriched elemental iron (95.56% and 99.89% isotopic enrichment; Chemgas), respectively, by Dr. Paul Lohmann, GmbH. Of the 14-mg iron doses administered, 6 mg iron was the respective tracer. Iron compounds were analyzed for iron isotopic composition and tracer iron concentration by reverse isotope dilution mass spectrometry.

### Composition of GOS and control formulations

We used a high-purity GOS powder (Vivinal® GOS Powder) containing high levels of GOS (69%) and low levels of monosaccharides (5%). The amount of GOS powder provided per administration (21.7 g) contained 15 g GOS. GOS content of the powder was measured by high-performance anion-exchange chromatography with pulsed amperometric detection. Before consumption, the GOS powder was dissolved in 300 mL ultrapure water. Ultrapure water (300 mL) containing the same amount of sucrose (1.3 g) and lactose (6.2 g) as the GOS formulation served as control.

### Study Design

We designed the study as a controlled, single-center, prospective crossover trial in which each subject received both test conditions (the outline of the study is shown in **Figure 1**). The study was conducted between September and November 2019 at the Laboratory of Human Nutrition, ETH Zurich. The study was

approved by the ethics Committee of the Canton Zurich (2019-01036) and all subjects provided written informed consent. The trial was registered at [clinicaltrials.gov](https://clinicaltrials.gov) as NCT03996421.

During the screening, about 1 mo before the start of the study, 61 women were assessed for eligibility. The study procedure was explained in detail and written informed consent was obtained. A venipuncture blood sample (4 mL) was collected for the determination of Hb, PF, and CRP, and for excluding pregnancy. Weight was measured to the nearest 0.1 kg and height to the nearest 0.5 cm. An interview was conducted to assess eligibility based on inclusion and exclusion criteria and to obtain demographic characteristics. Finally, 12 eligible women were invited to participate.

The experimental phase lasted for 29 d. Each subject received in total 2 supplemental iron doses of 14 mg iron (as FeFum), containing a 6-mg isotopic tracer ( $^{57}\text{Fe}$  or  $^{58}\text{Fe}$ ; see Stable-isotope tracers). The iron doses were administered in parallel to the GOS ( $^{58}\text{Fe}$ ) or control ( $^{57}\text{Fe}$ ) formulations. The 2 tests ( $^{58}\text{Fe}/\text{GOS}$  or  $^{57}\text{Fe}/\text{control}$ ) were randomly allocated to days 1 and 15 of the study. The participants, but not the investigators, were blinded to the test conditions. The randomization list was created in Excel using simple randomization. On these days, the assigned freshly prepared supplemental formulation and iron were consumed between 07:00 and 08:00 in the morning after an overnight fast (**Figure 1**). The participants consumed the complete test drink and iron in the presence of the investigators. After consuming the test drink, participants were not allowed to eat or drink for 3 h.

Before the intake of the test drink on days 1 and 15, a venous catheter (Vasofix® Braunüle,® B. Braun) was placed for blood sampling. A first blood sample was collected after the overnight fast but before the consumption of the test drink to determine iron and inflammatory status and isotopic composition. At 15 min, 30 min, 1 h, 90 min, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, and 24 h after intake of the test drink, blood samples were collected to determine the appearance of the isotopic tracer in serum. After the 8 h sampling, the catheter was removed, and the subjects were allowed to temporarily leave the study site. The 12 h and 24 h blood samples were obtained by venipuncture. On both days (days 1 and 15), subjects were provided with 3 standardized vegetarian meals and

were allowed to drink water and consume standardized snacks provided by the study team ad libitum after breakfast until 20:00. Breakfast was served 3 h after the supplement administration and the next 2 meals 3 and 8 h after breakfast, respectively. The participants were instructed not to eat from 20:00 until after the 24-h blood sample and were allowed to drink water until midnight. Fourteen days after the second iron administration (day 29), a final fasting blood sample was collected for determination of the isotopic composition for calculation of the FIA of the supplemental iron dose administered on day 15 (Figure 1).

### Laboratory analysis

Venous blood samples were drawn into EDTA coated (4 mL) and serum (4 mL) tubes (BD Vacutainer®). Serum tubes were centrifuged for 10 min at  $1000 \times g$  at 20 degC. We measured Hb in whole blood by using a Sysmex XE\_5000 analyzer (Sysmex Corporation). We furthermore determined SF, soluble transferrin receptor (sTfR),  $\alpha$ -1-acid glycoprotein (AGP), and CRP in serum by using a multiplex immunoassay (27). Anemia was defined as Hb <120 g/L (28), iron deficiency was defined as SF <15  $\mu$ g/L (28) and/or elevated sTfR >8.3  $\mu$ g/mL. CRP and AGP values >5 mg/L and >1 g/L, respectively, were defined as indicating inflammation. Serum samples and whole-blood samples collected for isotopic analyses were mineralized in duplicates by using HNO<sub>3</sub> and microwave digestion followed by separation of the iron from the blood matrix by anion-exchange chromatography and a subsequent precipitation of the isolated iron as ferric hydroxide. All isotopic analyses were performed by multicollector inductively coupled plasma mass spectrometry (Neptune, Thermo Finnigan) (29).

We calculated the FIA of the <sup>57</sup>Fe and <sup>58</sup>Fe isotopic tracers in the fasted whole-blood sample collected 14 d after administration (days 15 and 29) of each of the 2 supplemental iron doses on the basis of the shift in iron isotope ratios and the estimated amount of iron circulating in the body. The shift was calculated based on the isotopic ratio measured in the prior blood sample, e.g., the shift between the sample on days 15 and 29. As iron isotopic composition is highly constant in nature, we used the natural isotopic distribution as the baseline for the first supplement combination (based on data from 60 unlabeled blood samples from female subjects from previous studies in our lab). We calculated serum isotope concentrations according to the principle of isotope dilution (30). Circulating iron was calculated on the basis of the blood volume (31) and measured Hb concentrations (mean Hb value of baseline measurement and endpoint measurement) for each participant. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels are not monoisotopic (30). The total AUC for the appearance of iron isotopes in the serum was calculated by using the trapezoidal rule.

### Data and statistical analysis

The study was powered to detect a 40% within-subject difference in FIA, based on a SD of 0.170 from log-transformed FIA from a previous study by our laboratory (14), a type I error rate of 5%, and 80% power; this calculation yielded a sample size of 10 women. Anticipating a drop-rate of 15%, 12 participants were enrolled.

The primary outcomes were FIA and AUC of the SIAC. Secondary outcomes were  $t_{\max}$  of the SIAC, iron, and inflammatory status of the participants and correlations between iron absorption and markers of iron status and inflammation and BMI. Data was analyzed per protocol. This means that in the final analysis we only included participants who successfully completed the study and had no inflammation on either 2 d of supplement administration (day 1 and day 15). We analyzed biochemical and anthropometric data using Excel (2016; Microsoft) and IBM SPSS statistics (version 24). R software (version 3.6.1) was used to perform a pharmacokinetic analysis with nonlinear mixed effects modeling. FIA and serum isotope concentrations were calculated as described above. The time at maximal plasma concentration ( $t_{\max}$ ) was extrapolated from the data fitted to a 1-compartment model with first-order absorption by the method of residuals. Data were examined for normality by use of the Shapiro-Wilk test (the null hypothesis was rejected if  $P < 0.05$ ). Normally distributed data were expressed as means  $\pm$  SDs and nonnormally distributed data as medians and IQRs. FIA was always reported as medians and IQRs for consistency with our previous studies (10, 11), even though the data were normally distributed in this study. FIA, AUC, and estimated  $t_{\max}$  were compared between the 2 conditions by use of a paired samples t-test. Mean relative iron dialyzability and mean soluble iron were compared by independent samples t-test. Correlations were assessed by using Spearman's rank correlation coefficient ( $\rho$ ). We considered differences at  $P$  values < 0.05 as statistically significant.

For the mixed effects modeling, we used the *nlme*-package (version 3.1-141). Data were fitted to an 11-compartment model with first-order absorption. A parameter was included in the model, expressing the bioavailability divided by the distribution volume. Since we assumed no intraindividual change of the distribution volume from one iron administration to the other, this parameter allowed us to estimate the individual relative change of the bioavailability of iron from FeFum when administration with GOS was compared with administration without GOS. We introduced random effects at the participant level and nested random effects at the administration level within each participant. In an initial model, random effects for all parameters were defined. From this initial model, we eliminated stepwise random effects on parameters with minor random effects and compared the models using the Akaike information criterion. The final model had random effects for the elimination rate constant and relative bioavailability on the participant level and for relative bioavailability on the administration level within each participant. Next, we performed a covariate analysis with this final model. The pharmacokinetic rate constants and the relative change of bioavailability of FeFum administration with GOS compared with without GOS were tested against the ratios (difference in logarithmic scale) of the participants' anatomical characteristics such as height and weight as well as concentrations of CRP and SF relative to the corresponding mean value.

## Results

### Iron dialyzability and solubility

In vitro iron dialyzability and solubility of iron from FeFum with and without GOS are shown in Table 1. Iron dialyzability was higher for FeFum administration with GOS than without



**TABLE 1** In vitro iron dialyzability and solubility of ferrous fumarate with and without GOS ( $n = 3$ )

	FeFum + control <sup>1</sup>	FeFum + GOS	<i>P</i> value <sup>2</sup>
Dialyzability, <sup>3</sup> %	91 ± 7	159 ± 3	<0.001
Mean soluble iron, <sup>4</sup> pH 2, %	84.36 ± 1.50	88.08 ± 3.28	0.149
Mean soluble iron, pH 4, %	41.46 ± 2.26	92.15 ± 1.43	<0.001
Mean soluble iron, pH 6, %	25.07 ± 1.91	68.27 ± 1.96	<0.001

<sup>1</sup>Values presented as means ± SDs unless otherwise indicated. Control consisted of sucrose and lactose, based on the amounts of sucrose and lactose present in the GOS powder. FeFum, ferrous fumarate; GOS, galacto-oligosaccharides.

<sup>2</sup>Compared with independent samples *t*-test.

<sup>3</sup>Relative iron dialyzability (%) (FeFum added as solution = 100% dialyzability).

<sup>4</sup>Mean soluble iron (%) respective to total iron after in vitro digestion for 60 min at respective pH.

GOS ( $P < 0.001$ ). Iron solubility was higher at pH 4 and pH 6 for FeFum with GOS than without GOS (both  $P < 0.001$ ). At pH 2, solubility was >80% in both cases ( $P = 0.149$ ).

### Stable isotope study

Of the 12 women included in the study, 1 dropped out of the study because of complications with blood sampling and 11 women completed the study (compare **Supplementary Figure 1**). None of the women had elevated CRP (>5 mg/L) and/or AGP (>1 g/L) values at visit 1 or 2; thus all data points were included in the final analysis. There were no missing data. Anthropometric as well as iron and inflammatory status of the participants are presented in **Table 2**. Based on an SF <15 µg/L, 5 women (45%) were considered iron deficient during the study.

Mean stable isotope concentrations in serum of the 11 volunteers during 24 h after an oral iron dose of FeFum given with or without 15 g GOS are shown in **Figure 2**. FIA and AUC of the SIAC from FeFum given with or without 15 g GOS are shown in **Figure 3** and **Table 3**. FIA and total absolute AUC of the SIAC from FeFum given with 15 g GOS were not significantly different to those without GOS ( $P = 0.064$  and  $P = 0.080$ , respectively). The estimated  $t_{\max}$  values are shown in **Table 3** and were not significantly different for FeFum intake with or without GOS ( $P = 0.096$ ).

FIA values from FeFum given with and without GOS were both correlated with their corresponding AUC (FeFum only:  $\rho = 0.927$ ,  $P < 0.001$ ; FeFum + GOS:  $\rho = 0.945$ ,  $P < 0.001$ ). SF was the only predictor of iron absorption (FeFum only:  $\rho =$

-0.782,  $P = 0.004$ ; FeFum + GOS;  $\rho = -0.864$ ,  $P = 0.001$ ); Hb, BMI, CRP, AGP, and sTfR were not significant predictors (all  $P > 0.05$ ). In the covariate analysis, iron bioavailability significantly increased with decreasing SF and this effect was significantly enhanced by GOS ( $P = 0.037$ ; significant interaction between SF and GOS), which is also shown by the correlation between iron availability ratios (with or without GOS) and SF ratio (individual/mean) in **Figure 4**. No other significant correlations were found.

### Discussion

Our main findings are the following: 1) in vitro, iron dialyzability from FeFum was 75% greater and iron solubility values at pH 4 and pH 6 were doubled with GOS; 2) in vivo, AUC values of the SIAC and FIA were not different for FeFum with GOS compared with FeFum without GOS; 3) mean  $t_{\max}$  of the SIAC was similar and there was no delayed peak in isotope appearance with compared with without GOS; and 4) iron bioavailability increased with decreasing SF and this effect was enhanced by GOS.

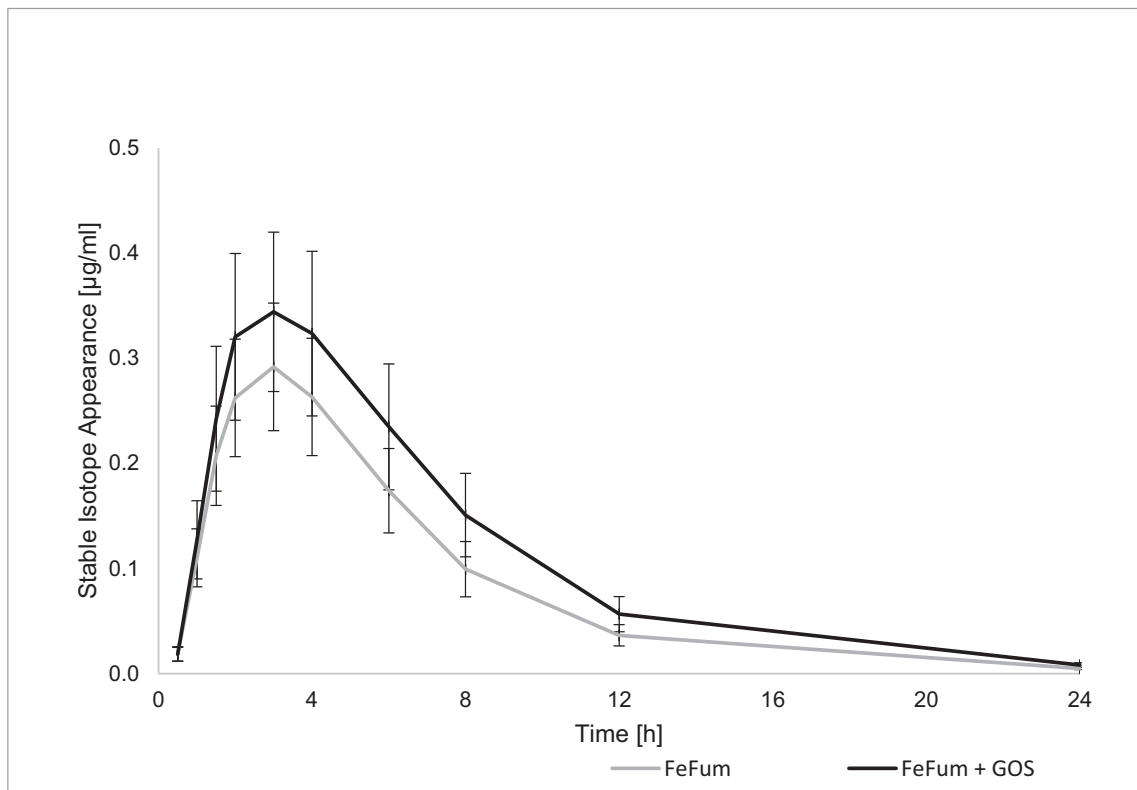
To be available for intestinal absorption through the divalent metal transporter (DMT-1), iron must be solubilized in the upper gastrointestinal tract (32). The in vitro solubility method simulates this first phase of iron absorption and is widely used as a predictor of in vivo iron absorption (33). FeFum is poorly soluble in water but readily soluble at pH 2 (6, 34). Notably, in our studies, the addition of GOS more than doubled iron solubility at pH 4 and pH 6. This finding suggests that once the acidic chyme enters the more alkaline environment of the duodenum, GOS might help keep the iron in solution in its ferrous state, favoring intestinal iron absorption. These data are consistent with the results of our dialyzability studies, in which GOS improved iron dialyzability, suggesting increased iron availability for absorption in humans (35, 36).

One putative mechanism for how GOS may enhance iron absorption from FeFum is by increasing gastric residence time and thus allowing greater iron dissolution at low gastric pH. For example, lipids that increase gastric residence time can substantially increase iron absorption from poorly soluble iron complexes (37). We would expect that a prolongation of gastric residence time would affect the shape of the plasma concentration time curve. However, this effect was not observed in our study. We did not find a significant difference in  $t_{\max}$ , and the shapes of the SIAC curves were similar. These findings suggest that

**TABLE 2** Anthropometric measurements, iron status, and inflammation markers of the participating women at baseline<sup>1</sup>

Parameter	Values ( $n = 11$ )
Age, y	24.5 ± 2.7
Height, cm	167.5 ± 7.5
Weight, kg	58.2 ± 6.1
BMI	20.7 ± 1.4
Hemoglobin, g/L	135 ± 7
Ferritin, µg/L	15.2 (12.6–21.2)
Soluble transferrin receptor, mg/L	4.7 ± 0.6
C-reactive protein, mg/L	0.3 (0.1–1.0)
α-1-acid-glycoprotein, g/L	0.4 ± 0.1

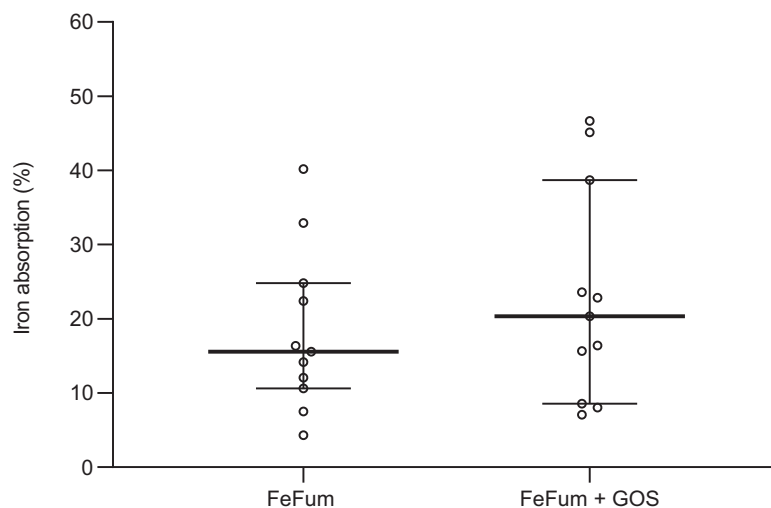
<sup>1</sup>Values are means ± SD or medians (IQRs). Hemoglobin was measured in whole blood. Ferritin, soluble transferrin receptor, C-reactive protein and α-1-acid-glycoprotein were measured in serum.



**FIGURE 2** Circulating mean iron isotope concentration in serum after a 14-mg iron dose (in the form of FeFum) of which 6 mg was  $^{57}\text{Fe}$  (given without GOS) or  $^{58}\text{Fe}$  (given with 15 g GOS) in iron-depleted volunteers ( $n = 11$ ) followed for 24 h. Error bars show the standard error. FeFum, ferrous fumarate; GOS, galacto-oligosaccharides.

increased residence time in the stomach does not play a role in the enhancement of iron absorption associated with GOS but also that it is likely to have an effect in the proximal gut and is not due to higher absorption from the colon. Prebiotics enhance the absorption of calcium and magnesium in humans (20, 38), an effect thought to be mediated by a decrease in

colonic pH due to bifidogenesis and increased production of short-chain fatty acids, which increase mineral dissolution and uptake. In animals, lower fecal pH is associated with higher colonic iron absorption (39, 40). In a previous study in infants, daily consumption of GOS for 3 wk increased iron absorption, which was associated with lower fecal pH and higher numbers



**FIGURE 3** Fractional iron absorption from FeFum with or without 15 g GOS, in iron-depleted women ( $n = 11$ ). FIA was compared by paired samples  $t$ -test ( $P = 0.064$ ). Figure shows medians with IQR. FeFum, ferrous fumarate; FIA, fractional iron absorption; GOS, galacto-oligosaccharides.

**TABLE 3** FIA, AUC, and estimated  $t_{\max}$  in young, iron-depleted women ( $n = 11$ )<sup>1</sup>

	FeFum + control <sup>2</sup>	FeFum + GOS	$P^3$
FIA, %	15.6 (10.6–24.8)	20.3 (8.6–38.7)	0.064
AUC, $\mu\text{g}/\text{min}$	4454.0 $\pm$ 3260.7	5830.9 $\pm$ 4717.3	0.080
$t_{\max}$ , h	2.80 $\pm$ 0.50	3.08 $\pm$ 0.47	0.096

<sup>1</sup>Values are means  $\pm$  SDs or medians (IQRs). FeFum, corresponding to 14 mg elemental iron, was given at each combination, of which 6 mg was isotopically labelled. FeFum, ferrous fumarate; FIA, fractional iron absorption; GOS, galacto-oligosaccharides;  $t_{\max}$ , estimated timepoint of peak serum iron concentration.

<sup>2</sup>Control consisted of sucrose and lactose, based on the amounts of sucrose and lactose present in the GOS powder.

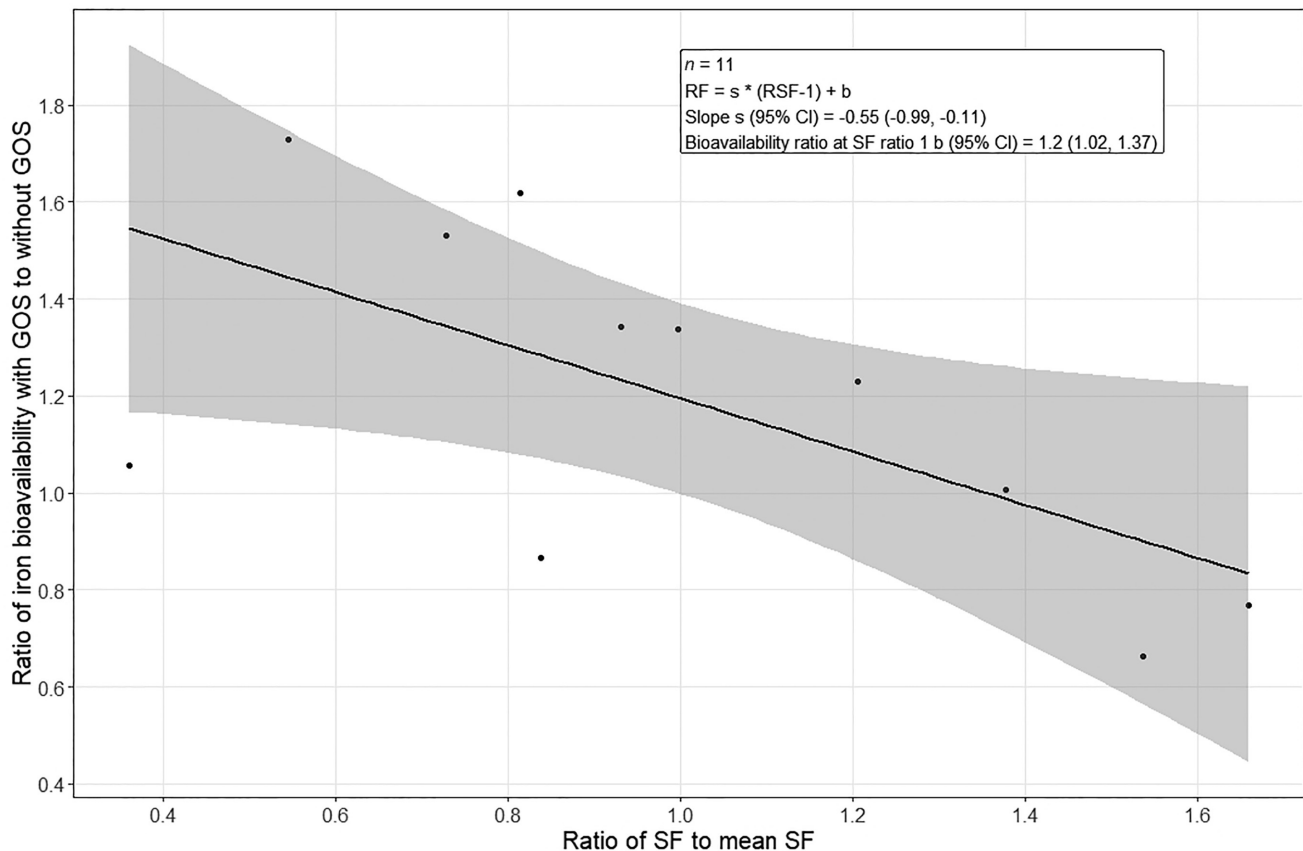
<sup>3</sup>FIA, AUC, and  $t_{\max}$  were compared by paired samples  $t$ -test.

of fecal *Lactobacillus/Pediococcus/Leuconostoc* spp. (41). In a previous study in women, daily consumption of GOS for 4 wk resulted in a similar pattern of decrease in fecal pH and an increase in *Lactobacillus/Pediococcus/Leuconostoc* spp., but this was not associated with increased iron absorption (10). Humans can absorb colonic iron, but colonic iron absorption is thought to be minimal compared with duodenal absorption (42). In humans, radioiron studies have suggested that oral iron is absorbed in 2 periods: the larger fraction ( $\leq 80\%$ ) is absorbed in the first 2–4 h

after dosing, and the remaining iron is absorbed more slowly over the next 22–48 h (43, 44). It is possible that we did not observe this late period of iron absorption because we gave a smaller iron dose and/or we collected our final SIAC blood sample 24 h after dosing, and not later.

In a previous stable iron isotope absorption study in iron-depleted nonanemic Swiss women (10), a single dose of 15 g GOS significantly increased FIA from FeFum given with water (+61%) or with a meal (+28%). However, 4 wk of daily GOS consumption did not increase FIA from FeFum given with a meal if GOS was not simultaneously consumed (10), suggesting a gut conditioning effect from GOS was not responsible for the increase in FIA. Absorption studies in anemic Kenyan infants showed differing results (41, 45). A single dose of 7.5 mg GOS given with FeFum did not significantly increase FIA (45), whereas the same dose of GOS fed daily for 3 wk as well as with the test meal at the end of this period significantly increased iron absorption by 62% from FeFum, suggesting a conditioning effect may have played a role for the increase in FIA (41). Therefore, the mechanism of the enhancing effect of prebiotics on iron absorption may vary by age and/or by baseline anemia or infection status.

Body iron stores are among the main determinants of iron absorption and SF was a significant predictor of FIA in our study (46). Notably, in the pharmacokinetic analysis, we found



**FIGURE 4** Correlation (linear regression) between ratios of serum ferritin to mean serum ferritin and iron bioavailability with GOS to iron bioavailability without GOS ( $n = 11$ ). The slope for the bioavailability ratio (with/without GOS) compared with the SF ratio (individual/mean) and the calculated bioavailability ratio at an SF ratio of 1 are shown; both were significantly different from 0 and 1, respectively (based on the 95% CI), pointing to a significant positive effect of GOS on iron bioavailability at low SF. GOS, galacto-oligosaccharides; RF, ratio of iron bioavailability; RSF ratio of serum ferritin; SF, serum ferritin.

a significant interaction between the effects of GOS and SF on iron bioavailability. GOS enhanced the positive effect of low SF on iron bioavailability. DMT-1, the apical iron transporter on enterocytes, is upregulated in subjects with depleted body iron stores (46, 47). Thus, higher iron solubility from FeFum when consumed with GOS, as suggested by our *in vitro* experiments, may allow for a relatively greater increase in iron absorption in more iron-deficient subjects. Since iron-deficient women may benefit most from an increase in iron bioavailability, the addition of GOS to iron supplements may be a promising approach.

The strengths of our study include the following: 1) we combined *in vitro* and *in vivo* approaches to investigate mechanisms of the GOS effect on iron absorption; 2) we used a prospective crossover design with 2 different stable iron isotopes, allowing within-subject comparisons; 3) we used 2 high-precision methods, SIAC and erythrocyte iron incorporation, to measure and model the kinetics of iron in the plasma; 4) our study participants were iron-depleted young women, a target group for iron supplements. Our study also has limitations. Our small sample size likely increased the probability of type II error in our comparisons. In our sample size calculation, we estimated that with 10 participants we could resolve a 40% within-subject difference in FIA. This may explain why a 31% difference in FIA in the present study was not statistically significant, as similar differences in FIA in our previous studies with GOS were significant, but with 3 times the number of subjects (10, 11). We enrolled a smaller sample size for this mechanistic study because, compared with the previous studies, the design of the present study involved multiple blood samples over the day and was therefore more time intensive and invasive for our participants. Furthermore, the testing of numerous secondary study outcomes has increased the risk of making a type I error.

In summary, our *in vitro* data suggest that GOS increases iron solubility from FeFum at physiological pH characteristic of the proximal duodenum. In volunteers, our data are consistent with effects in the proximal rather than distal gastrointestinal tract on iron absorption. Our *in vitro* findings suggest GOS may maintain iron dissolved from FeFum in the alkaline duodenum, favoring higher intestinal iron absorption in women with relatively low iron stores. Future studies should investigate the kinetics of iron absorption from FeFum with and without GOS when consumed with meals rather than with water only and examine the mechanism by which GOS increases iron solubility from FeFum at higher pH. Also, it would be valuable to study whether mechanisms and the absorption enhancing effect of GOS in more severely iron-deficient anemic women vary from those described here.

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The authors' contributions were as follows—MZ, IHA, FH: designed the study; FH, LS, IHA, CZ: conducted the study; FH, DB, CZ: analyzed the data and performed the statistical analyses; FH, IHA, DB, SDK, MZ: participated in the data interpretation; FH: wrote the first draft of the manuscript; all authors edited the manuscript; and all authors: read and approved the final

version of the manuscript. MBZ's spouse is an employee of Antistress AG—Burgerstein Vitamins who provided technical and financial support for this study through Innosuisse (CTI 27166 2 PFLS-LS). All other authors report no conflicts of interest.

## Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

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