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Published in:
Free Radical Biology and Medicine

DOI:
10.1016/j.freeradbiomed.2022.08.008

Publication date:
2022

Free Radical Biology and Medicine 190 (2022) 169–178

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

The effect of riboflavin supplementation on the systemic redox status in healthy volunteers: A post-hoc analysis of the RIBOGUT trial

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ARTICLE INFO

Keywords:
Riboflavin
Oxidative stress
Redox
Thiols
Gut microbiota
F. prausnitzii

ABSTRACT

Background: Riboflavin is a redox-active vitamin that plays a pivotal role in human energy metabolism. Riboflavin may have beneficial health effects by increasing extracellular antioxidant capacity, thereby alleviating oxidative stress. Reduced levels of free thiols in blood reflect systemic oxidative stress, since they are readily oxidized by reactive species. In this study, we aimed to study the potential of riboflavin supplementation to improve the systemic redox status in healthy volunteers.

Methods: This study was a post-hoc analysis of the RIBOGUT study, a randomized, double-blind, placebo-controlled human intervention trial that investigated the effect of riboflavin supplements on the gut microbiota composition of healthy individuals. Serum free thiols were quantified before and after intervention and adjusted to serum albumin levels. Changes in albumin-adjusted free thiols were analyzed, as well as potential associations with routine laboratory parameters and faecal bacterial quantification by fluorescence in-situ hybridization (FISH).

Results: Participants were randomized to either placebo ($n = 34$), riboflavin 50 mg daily ($n = 32$), or riboflavin 100 mg daily ($n = 33$). At baseline, no significant differences in albumin-adjusted serum free thiols were observed. After intervention with either placebo or riboflavin, albumin-adjusted serum free thiols did not significantly change ($P > 0.05$), however, observed changes were inversely associated with changes in C-reactive protein (CRP) levels ($r = -0.22$, $P < 0.05$). At baseline, albumin-adjusted serum free thiols were positively associated with faecal relative abundances of *Faecalibacterium prausnitzii* ($P < 0.01$).

Conclusion: Riboflavin did not change the systemic redox status in healthy individuals as reflected by serum free thiols, but observed changes in albumin-adjusted free thiol levels were negatively associated with changes in CRP levels. Strikingly, albumin-adjusted free thiols were independently associated with relative abundances of faecal *F. prausnitzii*, which may suggest a potential host redox-microbiota interaction.

1. Introduction

Riboflavin (vitamin B_2) is a water-soluble vitamin that plays a pivotal role in human energy metabolism. It acts as a redox-active cofactor in coenzymes such as flavin-adenine-dinucleotide (FAD) and flavin mononucleotide (FMN), functioning as mediators of various oxidative enzymes, e.g. pyruvate and α-ketoglutaric acid dehydrogenases [1]. Riboflavin is also known for its antioxidant properties, e.g. preventing lipid peroxidation, attenuating ischemia/reperfusion injury, and stimulating activity of antioxidant enzymes (e.g., superoxide dismutase [SOD] and glutathione peroxidase [GPx]) [2]. Recent findings indicate that riboflavin may also modulate

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https://doi.org/10.1016/j.freeradbiomed.2022.08.008
Received 21 June 2022; Received in revised form 27 July 2022; Accepted 4 August 2022
Available online 13 August 2022

the gut microbiota, attenuate inflammation and alter the gut redox potential [3–5].

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage [6]. Although ROS are necessary for cellular physiology, pathological overproduction results in oxidative stress leading to cellular damage [7]. Oxidative stress can be reflected by free thiols, which are organosulfur compounds carrying a free sulfhydryl (R–SH) moiety, which occur both in cells and in extracellular fluids. Free thiols are the main biological targets of reactive species, possess potent antioxidant buffering capacity, and govern a myriad of (protein) functions, enabling both short-term and longer-term biological adaptations [8]. Systemic oxidative stress is reflected by decreased levels of free thiols, as they are readily oxidized by ROS [9,10]. Apart from ROS, however, sulfhydryl moieties may also undergo oxidative modification by other types of reactive species, such as hydrogen sulfide (H_2S) and nitric oxide (NO)-derived metabolites. Recently, an integrative conceptual framework was established that aims to describe the interactions among different types of reactive species, including ROS, reactive nitrogen species (RNS), reactive sulfur species (RSS), and reactive carbonyl species (RCS), as well as their interactions with downstream biological targets [8,11,12]. A prominent role in the RSI has been attributed to RSS, and cysteine-based *redox switches* (consisting of sulfhydryl moieties) have gained a central place in the RSI since they function as the main transducing components of redox regulation [8,13]. By doing so, they modulate a variety of downstream intracellular targets to adapt in relation to changes in metabolic demand. More specifically, this may comprise both structural and functional modulation of proteins, e.g. enzymes, membrane transporters, structural proteins, and transcription factors. Extracellular free thiols comprise a reliable reflection of the overall extracellular redox state, as they capture the balance between total oxidant burden and antioxidant capacity [7]. Furthermore, assessment of free thiols in serum/plasma is an easy, minimally invasive, and reproducible method to evaluate the degree of systemic oxidative stress [11]. Extracellular free thiol status has been investigated in relation to several (cardiovascular) risk factors, and is known to be disturbed in a number of oxidative stress-mediated diseases, e.g. inflammatory bowel diseases (IBD), diabetes mellitus, and cardiovascular diseases [14–16].

In a previous prospective clinical intervention study, we investigated the effects of riboflavin supplementation in patients with IBD, specifically Crohn's disease (CD). In that study, an overall increase in extracellular free thiol levels was observed, indicative of a successful reduction of systemic oxidative stress [17]. Since accumulating evidence indicates that flavins may indeed increase extracellular reducing capacity, thereby alleviating oxidative stress, these compounds deserve further consideration for therapeutic approaches. Therefore, we aimed to study the effect of riboflavin supplementation on the systemic redox status in healthy individuals, as reflected by systemic free thiol levels. In addition, we aimed to study how serum free thiol levels relate to the abundance of selected gut microbial species as well as to standard inflammatory parameters under healthy conditions. We hypothesized that riboflavin supplementation in healthy volunteers would result in antioxidant effects, as reflected by an increase in systemic free thiol levels, and, consequently, an improved whole-body redox status. As free thiols are amenable to therapeutic modulation, it is important to investigate potential nutritional and/or therapeutic agents that may increase human extracellular reducing capacity.

2. Materials and methods

2.1. Study population and study design

This study is a post-hoc analysis of the RIBOGUT trial (NCT02929459), a randomized, double-blind, placebo-controlled human intervention trial in which the effect of riboflavin supplementation on the abundance of *Faecalibacterium prausnitzii* and other members of the gut microbiota was assessed in faeces of healthy volunteers. Furthermore, potential effects on short-chain fatty acid (SCFA) production, gut hormone production, and changes in glucose homeostasis were also evaluated [18]. The RIBOGUT trial was performed at the University Medical Center Groningen (UMCG), Groningen, the Netherlands, and participants were recruited from November 2016 to October 2018. Enrolled participants were randomly allocated to either placebo, riboflavin 50 mg daily, or riboflavin 100 mg daily, for a period of two weeks. Subjects were allowed to participate if they fulfilled the following main inclusion criteria: age 20–60 years, body-mass index (BMI) of $\geq$18.5 and $\leq$ 24.9 kg/m^2, not smoking, no health conditions as judged by an independent medical expert based on medical history and laboratory test results, willingness to maintain normal dietary habits, physical activity, and to refrain from consuming alcoholic beverages 24 h prior to test days, and having a stable body weight in the last six months. Participants were excluded when one (or more) of the following main criteria were met: abnormal laboratory test results of any clinical significance as judged by an independent medical expert, a history of gastrointestinal disorders of any kind, antibiotic use or signs of systemic infection in the last six months, regular use of dietary supplements (e.g. riboflavin, fish oil) one month prior to screening, and use of commercially available probiotic-, prebiotic-, or other supplements that may affect the gut microbiota. The study has been approved by the Institutional Review Board (IRB) of the UMCG (full name in Dutch: "Medisch Ethische Toetsingscommissie", METc, IRB no. 2015/510) and was performed in accordance with the principles of the Declaration of Helsinki (2013).

2.2. Demographics and laboratory measurements

Standard demographic characteristics, including age, sex, and BMI, were registered. In addition, standard laboratory examinations were performed, including hemoglobin, high-sensitive C-reactive protein (hs-CRP), erythrocyte sedimentation rate (ESR), leukocyte and platelet counts, creatinine and estimated glomerular filtration rate (eGFR), albumin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and bilirubin levels. Hemoglobin, ESR, leukocyte and platelet counts were routinely measured on a modular analyzer (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Serum creatinine was measured enzymatically (Roche Modular, Roche Diagnostics, Mannheim, Germany). Serum AST and ALT levels were measured using the standardized kinetic method with pyridoxal phosphate activation (Roche Modular P, Roche Diagnostics). Total bilirubin was measured using a colorimetric assay (2,4-dichloroaniline reaction; Merck MEGA, Darmstadt, Germany). Hs-CRP levels were determined using turbidimetry (Dade Behring Diagnostics, Marburg, Germany). Serum albumin levels were measured using photometry (Roche Modular, Roche Diagnostics, Mannheim, Germany). Estimated glomerular filtration rates (eGFR) were calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [19]. In addition, faecal riboflavin concentrations were determined using liquid chromatography.

2.3. Riboflavin and placebo capsules

Participants received daily supplementation with either placebo or riboflavin in two possible dosages (50 and 100 mg, Riboflavin Universal, E101, vitamin B_2, CAS no. 83-88-5) for a period of 2 weeks. Capsules were produced and kindly supplied by DSM Nutritional Products Ltd, Basel, Switzerland. Riboflavin is authorized as a food additive by the European Union (EU) in accordance with Annex II to Regulation (EC) No 1333/2008 and 1129/2011. Riboflavin is an orange-yellow free-flowing powder that melts at about 280 °C with decomposition. The full chemical name is 7,8-dimethyl-10-(1-D-ribityl)-isoalloxazine; 7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxy-pentyl)isoalloxazine. Riboflavin capsules consisted of hard gel (hydroxypropyl methylcellulose (HPMC),

bovine and/or porcine), 1 mg Silica colloidal anhydrous and 106 mg pregelatinized starch. Placebo capsules consisted of hard gelatin, 0,5% silica and 250 mg pregelatinized starch. Quality control was performed externally by high-performance liquid chromatography with a fluorescence detection (HPLC-FD) method (Eurofins CLF, Friedrichsdorf, Germany). Prior to this method, riboflavin is released from the sample with acid hydrolysis followed by an enzymatic treatment overnight. For purification, an automatic solid phase extraction with C18-SPE cartridges is performed by a Gilson laboratory robot. Subsequently, riboflavin is separated with isocratic HPLC upon which it is detected with fluorescence. This method is based on norm methods (DIN EN 14152 (2014-08)) from the European Standard for the determination of riboflavin (Technical Committee CEN/TC 275 "Food analysis – Horizontal methods", Deutsches Institut für Normung, Germany). Information on the reagents, apparatus, procedure, and calculations performed can be found in this standard, and specific validation data are provided in Clause 8 and Annex B of the standard. The capsules were prepared and packaged by Aenova Holding GmbH, Gronau, Germany, and stored in a dark and cool environment, and delivered to study participants in closed, white containers without any description.

2.4. Faecal bacterial characterization by fluorescence in-situ hybridization (FISH)

Targeted quantification of faecal microbial species, including *Faecalibacterium prausnitzii*, *Enterobacteriaceae*, *Roseburia* and *Clostridium coccoides-Eubacterium rectale* species, was performed as previously described, with minor modifications [20]. After thawing, 4.5 mL filtered phosphate-buffered saline (PBS) was added to 0.5 g of faecal sample, followed by centrifugation at 700×*g* for 2–3 min. Subsequently, the supernatant was 4-fold diluted with fresh 4% paraformaldehyde solution and stored overnight at 4 °C. Serial dilutions were prepared for manual counting of total bacteria and the bacterial species of interest. Each dilution was distributed over gelatin-coated glass slides and then dried at room temperature. The following bacterial probes were added (**Supplementary Table S1**): Eub338 (Rhodamine) for total bacteria, Fprau645 (FITC) for *F. prausnitzii*, Ec1535 (CY3) for *Enterobacteriaceae*, Rint623 (FITC) for *Roseburia*, Erec482 (FITC) for *Clostridium coccoides-Eubacterium rectale* species [21–24]. Slides were hybridized overnight at 50 °C. FISH was performed using an automated device (Biotrack analyzer, Biotrack B.V., Leeuwarden, the Netherlands).

2.5. Faecal quantification of short-chain fatty acids (SCFAs)

Faecal concentrations of SCFAs were measured using gas chromatography-mass spectrometry (GC-MS) analysis. The applied protocol was adapted from Moreau et al. [25] with few modifications. First an eight-point calibration curve was freshly prepared on ice from 0.5 M stored stock solutions of sodium butyrate, -acetate, and -propionate in Milli-Q (stored in aliquots at −80 °C) to reach concentrations of 0.10–20 mM in phosphate-buffered saline (PBS). After sample thawing, homogenization and centrifugation, 200–500 μL of supernatant was diluted to 1,000 μL with PBS. Subsequently, 100 μL internal standard solution (0.5 mg/mL 2-ethylbutyrate in Milli-Q, CAS no. 105-54-4) and 20 μL 20% (w/v) SSA solution (CAS no. 5965-83-3) were added to the samples as well as to 1,000 μL of calibration samples. In addition, two drops of 37% HCl (CAS no. 7647-01-0) were added together with 3–5 2.3 mm Zirconia/Silica beads (BioSpec Products, Bartlesville, USA). Samples were bead-beated at 6,000×*g* for 15 s in three runs at 4 °C using a Precellys 24 tissue homogenizer (Bertin Instruments, Bretonneux, France). Samples were spun down at 16,100×*g* for 20 min at 4 °C, whereafter the supernatant was moved to a glass tube. Calibration samples were not bead-beated and centrifuged after HCl addition. Subsequently, 2 mL of diethylether (CAS no. 60-29-7) was added to the samples, followed by vortexing for 10 min at room temperature and spinning down at 3,000 ×*g* for 10 at 4 °C. From the transparent upper layer, a 500 μL aliquot was transferred to a glass GC-vial. To this vial, 50 μL of MBTSTFA +1% TBDMCS (CAS no. 77377-52-7) was added and left to derivatize overnight at room temperature. Next, 3 μL together with 2 μL of air was injected into the GC-MS (7890A GC Systema and 597 5C inert XI EI/CI MSD with an EI inert 350 source, Agilent Technologies, Santa Clara, USA). Analysis was performed in a split mode with an inlet split ratio of 50:1. Samples were analyzed in the SIM acquisition mode: 2-ethylbutyrate at *m/z* 175, butyrate at *m/z* 145, acetate at *m/z* 117, and propionate at *m/z* 131. The temperatures of the injector, source and quadrupoles were 280 °C, 230 °C, and 150 °C, respectively. A Zebron capillary GC column of 30 m × 0.25 mm, 0.25 μm film thickness was used (ZB-1, Phenomenex, Torrance, USA). The program of the GC oven was as follows: 40 °C for 0 min, to 70 °C at 5 °C per minute, held at 70 °C for 3.5 min, increased to 160 °C at 20 °C per minute, further increased to 280 °C at 35 °C per minute, and finally held at 280 °C for 3 min with a total run time of 20.43 min. Helium was used as carrier gas while the flow was set at 1.0 mL per minute. Data processing was performed using the MassHunter Workstation Software (MassHunter, Agilent Technologies).

2.6. Measurement of serum free thiol levels

Blood samples were collected before and after the intervention period according to a standardized protocol. Serum-containing tubes (with a gel separator for serum separation) were stored in the dark for 20 min at room temperature and centrifuged thereafter. Serum samples were subsequently put on dry ice after processing and stored at −80 °C until further analysis. Free thiol levels were determined as previously described, with minor modifications [26,27]. Samples were thawed and diluted 4-fold using 0.1 mol/L Tris buffer (pH 8.2) (CAS no. 77-86-1). Background absorption of samples was measured at 412 nm using the Varioskan microplate reader (Thermo-Scientific, Breda, the Netherlands), together with a reference measurement at 630 nm. Subsequently, 20 μL 1.9 mmol/L 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's Reagent, CAS no. 69-78-3, Sigma-Aldrich Corporation) was added to the samples, followed by 20 min of incubation time. Then, background absorption was measured a second time, after which concentrations of free thiols were determined by parallel measurement of an L-cysteine (CAS no. 52-90-4, Fluka Biochemika, Switzerland) calibration curve (concentration range: 15.6–1,000 μM) in 0.1 M Tris/10 mM EDTA (pH 8.2). Intra- and interday coefficients of variation (CV) of measurement values were all <10%. Finally, serum concentrations of free thiols were normalized to plate-specific internal controls using the mean of internal controls as normalization factor. Since human serum albumin harbors the largest amount of free thiols in the extracellular compartment and therefore largely determines the amount of potentially detectable free thiols, adjustment to albumin levels was performed in order to indirectly account for this phenomenon as well as for fluid status [8,9].

2.7. Statistical analysis

Characteristics of the study population were presented as means ± standard deviations (SD), medians [interquartile range, IQR] in case of skewed variables, or proportions *n* with corresponding percentages (%). Normality was assessed by visual inspection of normal probability (Q-Q) and kernel density plots. Differences between groups for continuously distributed variables were tested using independent sample *t*-tests and one-way analysis of variance (ANOVA) in case of normally distributed variables, or Mann-Whitney *U*-tests and Kruskal-Wallis tests in case of skewed variables, while for nominal variables chi-square tests were performed, as appropriate. Univariable and multivariable linear regression analyses were performed to identify parameters that independently associated with albumin-adjusted serum free thiol levels. In linear regression analyses, standardized beta (β) coefficients and corresponding *P*-values were reported, indicating strength and direction and

significance of observed associations, respectively. Standardized β-coefficients represented the difference in albumin-adjusted serum free thiol levels per 1-SD increment or decrement for continuous variables or the difference in albumin-adjusted serum free thiol levels compared to the implied reference group for categorical variables. Robust regression using least trimmed squares (LTS) was additionally fitted in case of the presence of relevant outliers. Two-tailed *P*-values ≤ 0.05 were considered statistically significant. Data analysis was performed using SPSS Statistics software package (v.25.0) (SPSS Inc., Chicago, IL, USA) and the Python programming language (v.3.8.5, Python Software Foundation, https://www.python.org), using the *pandas* (v.1.2.3) and *numpy* (v.1.20.0) libraries. Data visualization was performed using the *seaborn* (v.0.11.1) and *matplotlib* (v.3.4.1) packages in Python.

3. Results

3.1. Baseline characteristics of the study population

In this post-hoc analysis of the RIBOGUT trial, 99 participants were enrolled, who were randomly allocated to either the placebo group ($n = 34$), an intervention group receiving daily capsules containing 50 mg riboflavin ($n = 32$), and a second intervention group receiving 100 mg riboflavin daily ($n = 33$) for two weeks. Baseline demographic characteristics, laboratory values, and relative abundances of faecal bacteria are presented in Table 1. There were no notable differences in these characteristics between the three study groups, except for a minor difference in baseline relative abundance of *Enterobacteriaceae* between the placebo group and the intervention group receiving 50 mg of riboflavin (0.003 [0.001; 0.007] % vs. 0.007 [0.003; 0.024] %, post-hoc Dunn's multiple comparisons test: $P = 0.03$). In all study groups, levels of albumin-adjusted serum free thiols were normally distributed (Fig. 1). Importantly, no significant differences in serum free thiol levels, nor in albumin-adjusted free thiol levels were observed between the three groups ($P = 0.440$ and $P = 0.100$, respectively). No adverse events were observed in the study.

3.2. Riboflavin supplementation does not affect systemic redox status in healthy volunteers

The effect of two weeks riboflavin supplementation on the systemic redox status was evaluated by measuring levels of free thiols in serum (Fig. 2, Supplementary Table S2). In the total study cohort, as well as in all separate study groups, both unadjusted and albumin-adjusted serum free thiol levels did not significantly change after the intervention period (all $P > 0.05$). In line with these findings, other laboratory parameters also did not significantly change after the intervention period (Table 2). As expected, faecal riboflavin concentrations significantly increased in the intervention groups in a dose-dependent manner ($\Delta_{T2\text{-}T0}$ for placebo: −245 [−568; 280] ng/g vs. riboflavin 50 mg: +2,250 [735; 3,870] ng/g vs. +3,870 [1,231; 76,340] ng/g; $P < 0.001$), confirming participant's adherence to the allocated intervention. Notably, individual changes in albumin-adjusted serum free thiol levels were significantly inversely associated with changes in blood C-reactive protein (CRP) levels (Pearson's $r = -0.220$, $P < 0.05$) (Fig. 3). Remaining associations between individual changes in study parameters can be found in Supplementary Fig. S1.

3.3. Baseline characteristics stratified by systemic redox status

Next, baseline characteristics of the study population were re-

Table 1
Baseline (T0) characteristics of the study population, separated by interventions groups (placebo: $n = 34$; riboflavin 50 mg dosage: $n = 32$; riboflavin 100 mg dosage: $n = 33$).

Variable	Placebo	Riboflavin 50 mg	Riboflavin 100 mg	*P*-value[b]
	$n = 34$	$n = 32$	$n = 33$	
Age (years)	27.1 ± 9.4	32.5 ± 12.3	32.2 ± 11.4	0.087
Sex (female), *n* (%)	25 (73.5)	22 (68.8)	26 (78.8)	0.655
BMI (kg/m^2)	22.5 ± 1.2	21.7 ± 1.8	21.5 ± 2.2	0.116
FT at T0 (μM)	563.7 ± 105.3	588.1 ± 103.4	593.6 ± 94.6	0.440
AA-FT at T0 (μM/g)	12.0 ± 1.9	12.6 ± 2.5	13.2 ± 2.1	0.100
Laboratory parameters				
Hemoglobin (mmol/l)	8.6 ± 0.8	8.5 ± 0.7	8.6 ± 0.5	0.515
CRP (mg/l)[a]	0.5 [0.3; 1.0]	0.5 [0.3; 1.0]	0.7 [0.3; 1.2]	0.730
ESR (mm/h)[a]	4 [2; 14]	5 [3; 11]	3 [2; 7]	0.172
Leukocytes (x10 [9]/l)[a]	6.4 [5.5; 7.2]	5.7 [4.8; 6.8]	6.7 [5.0; 7.8]	0.150
Platelets (x10 [9]/l)[a]	258 [238; 292]	247 [221; 262]	258 [201; 302]	0.226
eGFR (ml/min/1.73 m [2])[a]	113 [108; 116]	112 [107; 120]	113 [107; 119]	0.879
Creatinine (μmol/l)[a]	74.4 [68.0; 80.0]	74.0 [62.2; 82.1]	72.0 [63.7; 80.2]	0.791
AST (U/l)	22 ± 7	21 ± 6	21 ± 6	0.714
ALT (U/l)[a]	20 [15; 34]	20 [17; 35]	20 [14; 33]	0.837
Albumin (g/l)	47.3 ± 3.3	46.5 ± 3.2	45.6 ± 2.3	0.098
Bilirubin (μmol/l)[a]	8.3 [5.5; 12.1]	8.6 [6.6; 12.1]	9.3 [6.5; 10.5]	0.999
Riboflavin (faeces, ng/g)[a]	2280 [1905; 2765]	1875 [1150; 2563]	1915 [1393; 2688]	0.379
FISH				
F. prausnitzii (%)[a]	2.79 [0.91; 7.43]	2.90 [1.03; 9.35]	3.07 [0.48; 10.2]	0.867
Enterobacteriaceae (%)[a]	0.003 [0.001; 0.007]	0.007 [0.003; 0.024]	0.006 [0.003; 0.012]	**0.036**
Clostridium (%)[a]	7.35 [4.42; 14.1]	10.8 [6.00; 15.4]	9.44 [5.27; 16.0]	0.335
Roseburia (%)[a]	3.92 [1.05; 8.30]	4.18 [1.31; 10.4]	4.27 [0.92; 8.22]	0.770
Total bacterial count (counts/g of faeces)	$3.63x10^{10}$ [$1.92x10^{10}$;$6.45x10^{10}$]	$2.69x10^{10}$ [$1.34x10^{10}$;$6.96x10^{10}$]	$3.55x10^{10}$ [$2.11x10^{10}$;$1.00x10^{11}$]	0.415
SCFAs				
Butyrate (mM)	15.3 [9.2; 20.9]	11.4 [7.1; 18.6]	10.7 [5.9; 16.1]	0.209
Acetate (mM)	51.0 [33.2; 68.1]	37.8 [24.2; 61.1]	47.0 [30.5; 61.1]	0.427
Propionate (mM)	15.8 [10.0; 20.4]	10.9 [7.4; 22.3]	11.3 [8.7; 14.9]	0.289

Data are presented as numbers (proportions *n* (%)) or means ± standard deviations (SD).
[a] Skewed variables are presented as median [interquartile ranges (IQR)].
[b] *P*-values were calculated using one-way analysis of variance (ANOVA) tests (continuous variables), Kruskal-Wallis tests (skewed distributions) or chi-square tests (nominal variables). Significances are indicated in **bold**. Abbreviations: BMI, body-mass index; AA-FT, albumin-adjusted free thiols; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; eGFR, estimated glomerular filtration rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SCFAs, short-chain fatty acids. T0: baseline, pre-placebo/intervention; T2: post-placebo/intervention, 2 weeks afterwards.

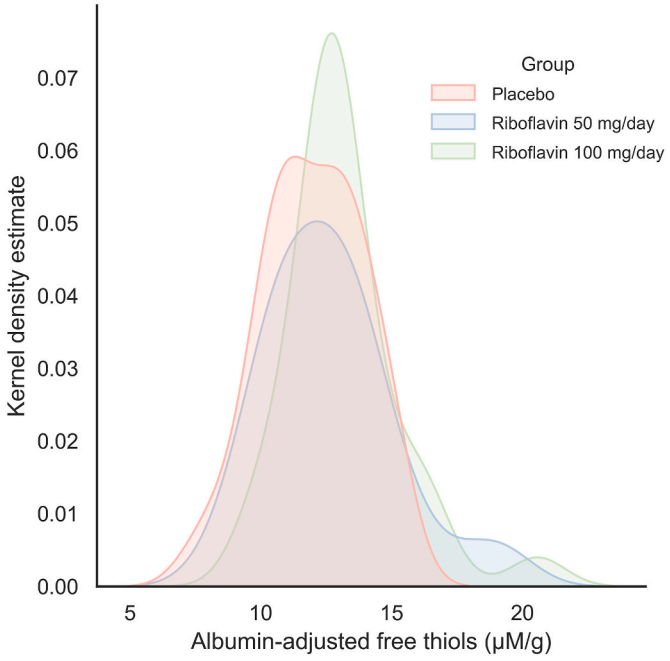

Fig. 1. Kernel density estimation of the distributions of albumin-adjusted serum free thiol levels among the three study groups demonstrate a rather normal distribution: placebo ($n = 34$, red shaded), riboflavin with 50 mg dosage ($n = 32$, blue shaded), and riboflavin with 100 mg dosage ($n = 33$, green shaded). Density estimates were performed using a Gaussian kernel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

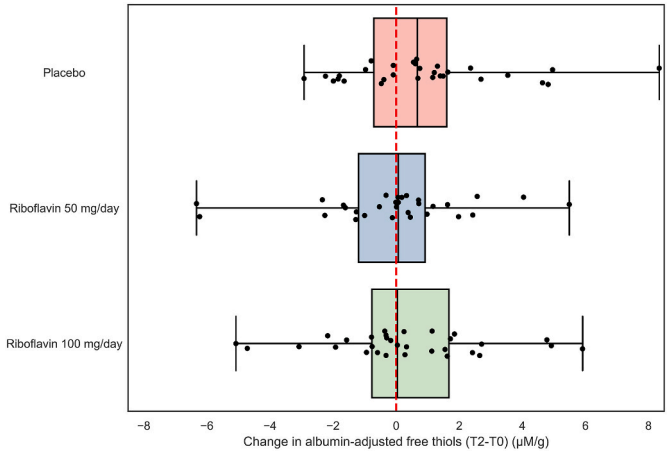

Fig. 2. Riboflavin supplementation does not alter albumin-adjusted serum free thiol levels in healthy volunteers. None of the study groups demonstrate a significant change in albumin-adjusted free thiols after the intervention period. Boxplots represent the distributions of changes (Δ) in albumin-adjusted serum free thiol levels after the 2-week intervention period. Boxplot whiskers represent 1.5 times the interquartile range (IQR). The red dashed vertical line indicates zero (0) change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

evaluated, but then stratified by the average levels of albumin-adjusted (and unadjusted) free thiols at baseline (Table 3, Supplementary Table S3). More female participants were present in the group having below-average albumin-adjusted free thiols (<12.6 μM/g) at baseline ($P = 0.10$, but $P < 0.05$ in below-average unadjusted free thiols). Participants with below-average albumin-adjusted free thiols had relatively lower faecal riboflavin concentrations, although this was not statistically significant (1,920 [1,335; 2,495] ng/g vs. 2,290 [1,598; 2,785] ng/g, $P = 0.08$). In addition, these participants had relatively lower levels of faecal *F. prausnitzii* counts, which was borderline non-significant (1.57 [0.55; 5.18] % vs. 4.59 [0.94; 11.3], $P = 0.06$).

3.4. Albumin-adjusted serum free thiol levels associate with the relative abundance of faecal F. prausnitzii

Subsequently, relationships between albumin-adjusted serum free thiol levels and observed study parameters at baseline were analyzed by treating albumin-adjusted serum free thiols as continuous outcome variable (Table 4). Univariable linear regression analyses demonstrated that albumin-adjusted serum free thiol levels were independently and positively associated with the relative abundance of faecal *F. prausnitzii* ($P = 0.002$) (Fig. 4). Similarly, unadjusted serum free thiol levels were also significantly positively associated with the relative abundance of faecal *F. prausnitzii* ($P = 0.005$, Supplementary Table S4). Multivariable linear regression analyses were not performed as no other relevant correlates with albumin-adjusted serum free thiols could be identified.

4. Discussion

In this post-hoc analysis of the RIBOGUT trial, which was originally aimed to study the effects of two-week-long daily riboflavin supplementation on the composition of the gut microbiota, we analyzed whether riboflavin supplementation could improve the systemic redox status in healthy individuals, as reflected by albumin-adjusted serum free thiol levels. In contrast to our hypothesis, we did not observe significant changes in albumin-adjusted free thiol levels upon riboflavin supplementation, nor any differential effects compared to the placebo group. At baseline, before riboflavin supplementation, albumin-adjusted serum free thiol levels were positively associated with faecal relative abundances of *F. prausnitzii*.

Riboflavin is a precursor for flavin cofactors and participates in numerous intracellular redox reactions, the majority of which are involved in cellular intermediary metabolism [28]. Although riboflavin has known antioxidant properties, this evidence is primarily derived from *in vitro* and animal studies, whereas it is yet unclear whether riboflavin might also act as successful antioxidant treatment in humans [2]. Only a few clinical studies have so far specifically focused on its antioxidant potential. An example includes the assessment of riboflavin as a potential homocysteine-lowering agent in the context of cardiovascular disease, since homocysteine is an established risk factor for cardiovascular disease and its re-methylation back to methionine is partially dependent on riboflavin [29–33]. However, these studies showed rather conflicting results and pointed to the existence of genotype-specific effects. Other human studies demonstrated associations of riboflavin intake with lipid peroxidation [34–36]. For instance, a study that evaluated riboflavin supplementation (on top of standard chloroquine-treatment) in individuals with uncomplicated malaria infection found significantly reduced serum levels of lipid hydroperoxide (LHP), a biomarker of lipid peroxidation [34]. In a case-control study involving children, riboflavin intake was found to be significantly inversely associated with serum levels of malondialdehyde (MDA), which is an established lipid peroxidation biomarker [35]. In our recent study in patients with CD, we observed an overall improvement of the systemic redox status as reflected by increased albumin-adjusted serum free thiol levels [17]. This observation, however, was especially pronounced in patients with high faecal calprotectin (FC) levels, indicative of active disease. In contrast, patients with low FC levels (and lower levels of oxidative stress at baseline) did not show a significant increase in albumin-adjusted free thiols. Interestingly, microbial riboflavin biosynthesis pathways have been demonstrated to be decreased during CD exacerbations [37]. This may imply that a difference in microbial riboflavin-producing capacity may determine a disease-specific response to riboflavin (antioxidant) supplementation. Therefore, the

Table 2
Changes (Δ) in study parameters before (T0) and after the 2-week intervention period (T2) with comparisons between the study groups (placebo; riboflavin 50 mg dosage; riboflavin 100 mg dosage).

Variable	Placebo	Riboflavin 50 mg	Riboflavin 100 mg	*P*-value[a]
	n = 34	*n* = 32	*n* = 33	
AA-FT (μM/g)	0.9 ± 2.5	−0.1 ± 2.4	0.3 ± 2.5	0.307
FT (μM)	9.2 ± 83.0	−20.0 ± 89.2	7.7 ± 97.7	0.343
Hemoglobin (mmol/l)	−0.4 ± 0.5	−0.2 ± 0.5	−0.3 ± 0.3	0.203
CRP (mg/l)[a]	0.0 [-0.2; 0.5]	0.0 [-0.4; 0.2]	−0.1 [-0.7; 0.1]	0.245
ESR (mm/h)[a]	0.5 [-2.3; 2.0]	0.0 [-2.0; 1.3]	−1.0 [-1.0; 1.0]	0.590
Leukocytes ($x10^9$/l)[a]	−0.4 [-1.1; 0.5]	−0.7 [-1.3; 0.2]	−0.5 [-1.4; 0.5]	0.776
Platelets ($x10^9$/l)[a]	1.5 [-18.3; 20.0]	8.0 [-10.0; 15.0]	−2.0 [-27.0; 9.0]	0.295
eGFR (ml/min/1.73m^2)[a]	−1.2 [-5.3; 2.6]	0.4 [-1.7; 3.9]	1.4 [-3.3; 3.8]	0.273
Creatinine (μmol/l)[a]	0.7 [-4.0; 5.8]	−1.0 [-3.7; 1.5]	−2.0 [-4.5; 4.0]	0.361
AST (U/l)	−0.8 ± 7.1	0.2 ± 4.1	−1.3 ± 7.5	0.839
ALT (U/l)[a]	−0.5 [-3.3; 3.0]	−1.0 [-4.0; 1.3]	0.0 [-4.0; 2.0]	0.853
Albumin (g/l)	−2.3 ± 3.6	−1.4 ± 3.3	−0.7 ± 1.9	0.123
Bilirubin (μmol/l)[a]	0.9 [-2.4; 3.5]	1.1 [-2.2; 3.6]	1.0 [-0.4; 4.3]	0.629
Riboflavin (faeces, ng/g)[a]	−245 [-568; 280]	2,250 [735; 3,671]	3,870 [1231; 76,340]	**<0.001**
FISH				
F. prausnitzii (%)[a]	0.81 [-2.66; 3.69]	−0.13 [-4.26; 5.46]	0.05 [-2.32; 5.63]	0.800
Enterobacteriaceae (%)[a]	0.001 [-0.003; 0.003]	−0.001 [-0.014; 0.0003]	−0.0003 [-0.004; 0.002]	0.195
Clostridium (%)[a]	0.82 [-3.34; 7.01]	−0.71 [-6.21; 6.94]	0.37 [-3.12; 5.49]	0.905
Roseburia (%)[a]	−0.25 [-2.71; 1.94]	0.03 [-4.84; 2.78]	−0.27 [-2.36; 3.75]	0.877
Total bacterial count (counts/g of faeces)	$5.16x10^8$ [$-1.62x10^{10}$;$1.8x10^{10}$]	$-3.24x10^9$ [$-1.62x10^{10}$;$6.12x10^9$]	$1.48x10^9$ [$-1.26x10^{10}$;$6.97x10^9$]	0.562
SCFAs				
Butyrate (mM)	−1.0 [-7.6; 4.3]	2.0 [-6.2; 8.5]	2.9 [-1.1; 6.4]	0.172
Acetate (mM)	−3.5 [18.5; 15.3]	6.9 [-5.1; 17.5]	0.1 [-12.3; 13.9]	0.330
Propionate (mM)	−0.6 [-7.4; 6.5]	1.1 [-2.7; 5.3]	−0.3 [-3.2; 4.3]	0.793

Data are presented as mean difference (T2-T0) ± standard deviation (SD) of this difference or median difference with corresponding [interquartile ranges] in case of skewed data.

[a] *P*-values for between-group differences in parameter changes. *P*-values were two-tailed and calculated using one-way analysis of variance (ANOVA) or Kruskal-Wallis tests, as appropriate. Significances are indicated in **bold**. Abbreviations: AA-FT, albumin-adjusted free thiols; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; AST, aspartate transaminase; ALT, alanine transaminase; FISH, fluorescent *in-situ* hybridization; SCFAs, short-chain fatty acids.

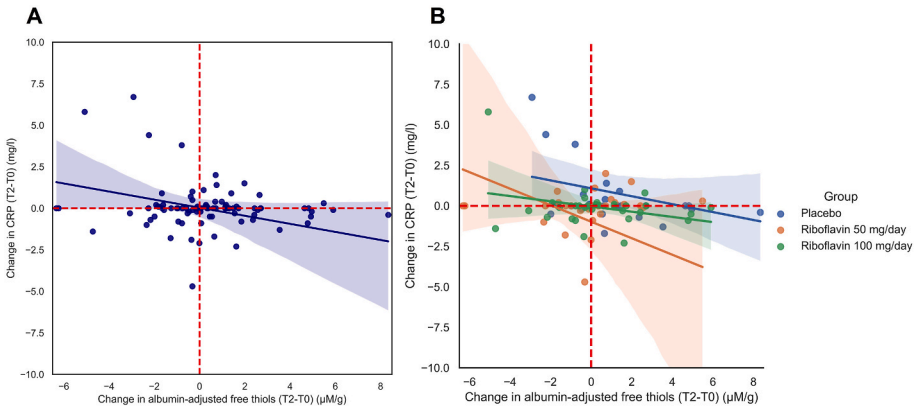

Fig. 3. (A-B) Individual changes in albumin-adjusted serum free thiol levels during the 2-week intervention period are significantly inversely associated with individual changes in serum CRP levels. (**A**) The fitted regression line (dark blue) is accompanied by a 95% confidence interval (CI) (blue shade). The red dashed lines represent zero changes in the corresponding axis parameters. (**B**) Stratification by allocated treatment group does not show significantly differential associations between individual changes in albumin-adjusted serum free thiol levels and serum CRP levels. Lines indicate fitted regression lines, shades represent 95% confidence intervals. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contribution of riboflavin-producing bacteria may be a more important source of endogenous riboflavin than has previously been recognized [38–40].

In view of the above-mentioned findings on *in vivo* antioxidant effects of riboflavin supplementation, a possible explanation for the absence of a distinct antioxidant effect of riboflavin supplementation in the current study may be found in the selection of study participants. Following in- and exclusion criteria, participants were healthy, without having any significant health conditions or unhealthy lifestyle habits. As such, no participants were included who are typically at risk for riboflavin deficiency or malnourishment with subsequent vitamin deficiencies, e.g. pregnant women (especially those who consume little dairy or meat), infants or young children, or elderly people. In addition, riboflavin deficiency is very rare in Westernized populations, while it occurs more frequently in developing countries, which is attributable to a low consumption of dairy products and meat [40]. Another possible explanation for the absence of a clear antioxidant effect constitutes the short intervention period (two weeks) as well as the risk of insufficient study power to detect smaller differences in albumin-adjusted serum free thiol levels before and after intervention. Although faecal riboflavin levels increased in both intervention groups, we further speculate that intestinal riboflavin availability could still have been partially inadequate to exert an evident antioxidant effect. In addition to faecal riboflavin levels, plasma riboflavin levels also increased in the intervention groups, as has been reported in the RIBOGUT study [18]. This indicates that the bioavailability of riboflavin in the systemic circulation was sufficiently elevated upon supplementation, which may exclude a potentially defective absorption in the intestine. Intestinal absorption of riboflavin predominantly occurs in the jejunal region via carrier-mediated active transport, and this has been reported to become saturated up to 30 mg riboflavin in a given meal [40,41]. In order to improve colonic delivery of vitamins, a promising future therapeutic strategy may consist of the application of colon-targeted delivery systems (CTDSs) to locally administer vitamins to the colon, in order to beneficially modulate the

Table 3
Baseline characteristics of the study population, divided by and compared between below- and above-average albumin-adjusted serum free thiol levels.

Variable	Total cohort	Below-average AA-FT (<12.6 μM/g)	Above-average AA-FT (>12.6 μM/g)	*P*-value
	n = 99	*n* = 46	*n* = 53	
AA-FT (μM/g)	12.6 ± 2.2	11.0 ± 1.2	14.2 ± 1.7	**<0.001**
Age (years)[a]	26.0 [23.0; 33.0]	24.0 [22.0; 32.0]	27.0 [24.0–33.0]	0.154
Female, *n* (%)	73 (73.7)	40 (87.0)	33 (62.3)	0.102
BMI (kg/m^2)[a]	21.6 [20.5; 23.2]	21.5 [20.4; 22.9]	22.2 [20.9; 23.8]	0.147
Laboratory parameters				
Hemoglobin (mmol/l)	8.6 ± 0.7	8.4 ± 0.7	8.7 ± 0.7	0.077
CRP (mg/l)[a]	0.5 [0.3; 1.0]	0.6 [0.3; 1.0]	0.5 [0.3; 1.0]	0.880
ESR (mm/h)[a]	4 [2; 9]	5 [3; 11]	4 [2; 9]	0.243
Leukocytes (x10^9/l)[a]	6.2 [5.1; 7.2]	6.3 [5.0; 7.1]	6.1 [5.3; 7.6]	0.757
Platelets (x10^9/l)[a]	254 [225; 280]	253 [231; 279]	254 [214; 288]	0.496
eGFR (ml/min/1.73m^2)[a]	113 [107; 118]	112 [107; 117]	113 [107; 120]	0.525
Creatinine (μmol/l)[a]	74.0 [66.4; 80.7]	74.3 [66.8; 81.8]	73.0 [63.1; 80.3]	0.589
AST (U/l)	22 ± 6	21 ± 7	22 ± 6	0.495
ALT (U/l)[a]	20 [15; 33]	21 [14; 36]	19 [16; 33]	0.751
Albumin (g/l)	46.5 ± 3.0	46.7 ± 3.5	46.3 ± 2.5	0.650
Bilirubin (μmol/l)[a]	8.6 [6.5; 11.5]	8.4 [6.2; 11.6]	8.8 [6.5; 11.4]	0.669
Riboflavin (faeces)[a]	2,110 [1,520; 2,650]	1,920 [1,335; 2,495]	2,290 [1,598; 2,785]	0.084
FISH				
F. prausnitzii (%)[a]	2.92 [0.73; 9.01]	1.57 [0.55; 5.18]	4.59 [0.94; 11.3]	0.058
Enterobacteriaceae (%)[a]	0.005 [0.002; 0.012]	0.005 [0.002; 0.013]	0.005 [0.002; 0.011]	0.759
Clostridium (%)[a]	9.35 [5.45; 15.2]	8.25 [4.92; 14.5]	9.32 [5.58; 16.0]	0.541
Roseburia (%)[a]	4.08 [1.16; 8.85]	3.45 [1.01; 7.95]	3.89 [1.16; 8.08]	0.628
Total bacterial count (counts/g of faeces)	3.47x10^{10} [1.61x10^{10};7.15x10^{10}]	3.61x10^{10} [1.74x10^{10};7.57x10^{10}]	3.58x10^{10} [1.51x10^{10};7.39x10^{10}]	0.984
SCFAs				
Butyrate (mM)	12.8 [7.1; 17.6]	12.8 [8.1; 18.6]	13.1 [6.7; 16.8]	0.468
Acetate (mM)	46.9 [31.2; 61.3]	46.7 [32.4; 68.6]	47.2 [24.3; 61.1]	0.335
Propionate (mM)	12.5 [8.2; 20.0]	13.4 [9.4; 19.5]	12.5 [7.9; 21.2]	0.575

Data are presented as mean ± standard deviation (SD) or proportions (*n*, %).

[a] Skewed data are presented as median [interquartile range]. *P*-values were two-tailed and calculated using independent sample *t*-tests or Mann-Whitney *U*-tests, as appropriate. Significances are indicated in **bold**. Abbreviations: AA-FT, albumin-adjusted free thiols; BMI, body mass index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; eGFR, estimated glomerular filtration rate; AST, aspartate transaminase; ALT, alanine transaminase; FISH, fluorescent *in-situ* hybridization; SCFAs, short-chain fatty acids.

gut microbiota and gut redox potential [3,4,42]. Further, it may be considered unlikely that supplementation with a single antioxidant vitamin would confer marked health benefits, particularly in healthy individuals. Instead, a combined approach, consisting of selected candidate antioxidant vitamins, may become a more promising alternative for consideration in clinical trials, as has recently been illustrated by combining riboflavin with vitamin C [43,44]. Finally, results from this study highlight the notion that exogenous administration of antioxidants should be reserved only for individuals who are affected by inflammatory, oxidative stress-mediated diseases, that are accompanied by pathological overproduction of reactive species, and, thus, reduced levels of serum free thiols [8,14]. Improvement of the systemic redox status by thiol antioxidant administration may be completely absent in healthy individuals, as they would be expected to have physiological levels of reactive species that are effectively scavenged by a robust antioxidant buffering capacity. In fact, antioxidant therapy in rather healthy individuals should be carefully considered to avoid interference with physiological redox signaling processes [11,45]. For example, single-electron oxidation of thiols, forming thiyl radicals, or disturbance of membrane transport signaling processes that are dependent on disulfide bonds, may occur as adverse effects of antioxidant treatment [46,47].

In this post-hoc analysis, a positive association between serum free thiols (when unadjusted for albumin) and albumin levels was observed, which is in line with previous studies on free thiols [14,16,48–50]. Albumin is the most abundant circulating protein and constitutes the predominant source of protein-embedded free thiols, mainly based on its single free cysteine residue (Cys^{34}), but also on its transporting capacity of low-molecular-weight (LMW) free thiols [8,9,51]. As albumin largely determines the amount of potentially detectable free thiols, an adjustment to circulating albumin levels is often performed as an indirect way of accounting for this, theoretically resulting in a more precise reflection of the total extracellular thiol pool [9]. Therefore, in this study, we performed our analyses using albumin-adjusted free thiol levels [14,49,52]. Importantly, in our analyses, individual changes in albumin-adjusted serum free thiol levels before and after riboflavin supplementation were inversely correlated with changes in serum CRP levels. This observation aligns with previous findings from large prospective cohort studies in which (protein/albumin-adjusted) serum free thiols were analyzed in relation to the occurrence of cardiovascular events, to non-alcoholic fatty liver disease (NAFLD), and to renal transplant recipients [16,49,53]. Likewise, in patient cohorts that showed considerably larger variation in systemic inflammation and oxidative stress, serum CRP levels showed consistent negative associations with albumin-adjusted free thiol levels, e.g. in patients with inflammatory bowel disease (IBD) and acute kidney injury (AKI) [14,50]. Altogether, these observations underscore the intimate link between inflammation and oxidative stress.

At baseline, we observed an independent positive association between albumin-adjusted serum free thiols and faecal relative abundances of *F. prausnitzii*, which is one of the most dominant commensal anaerobic bacteria in the human gut. It is known for its anti-inflammatory properties and short-chain fatty acid (SCFA)-producing capacity, especially butyrate, which serves as energy source for the human intestinal epithelium [54–56]. From *in vitro* studies, it has been revealed that *F. prausnitzii* employs a specialized form of anaerobic respiration, acting as redox mediator by using riboflavin and thiols for extracellular electron transfer (EET) through shuttling electrons to oxygen [57,58]. In this way, *F. prausnitzii* can reduce its oxygenated micro-environment, thereby preventing oxidative stress, and facilitating its own growth at the oxic-anoxic interface of the human gut [54]. In a previous pilot intervention study consisting of 11 healthy volunteers, riboflavin supplementation (100 mg daily) for two weeks resulted in an increase in the faecal abundance of *F. prausnitzii* as well as the

Table 4
Univariable linear regression analyses of albumin-adjusted serum free thiols with population characteristics, laboratory parameters, faecal bacterial abundances and SCFA levels.

AA-FT	Univariable analysis	
	B coefficient[a]	*P*-value
Age (years)	0.085	0.418
Female gender	−0.099	0.345
BMI (kg/m^2)	0.079	0.449
Laboratory parameters		
Hemoglobin (mmol/l)	0.120	0.251
CRP (mg/l)	−0.041	0.698
ESR (mm/h)	−0.125	0.231
Leukocytes (x10^9/l)	−0.038	0.716
Platelets (x10^9/l)	−0.095	0.363
eGFR (ml/min/1.73m^2)	−0.086	0.410
Creatinine (μmol/l)	0.061	0.559
AST (U/l)	0.014	0.893
ALT (U/l)	−0.050	0.635
Albumin (g/l)	−0.160	0.124
Bilirubin (μmol/l)	0.063	0.545
Riboflavin (faeces)	−0.095	0.398
FISH		
F. prausnitzii (%)	0.322	**0.002**
Enterobacteriaceae (%)	0.112	0.319
Clostridium (%)	−0.007	0.951
Roseburia (%)	0.030	0.779
Total bacterial count (counts/g of faeces)	0.023	0.828
SCFAs		
Butyrate (mM)	−0.083	0.439
Acetate (mM)	−0.117	0.271
Propionate (mM)	−0.159	0.134

[a] Standardized beta (β) coefficient. Significances are indicated in **bold**. Abbreviations: AA-FT, albumin-adjusted free thiols; BMI, body mass index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; eGFR, estimated glomerular filtration rate; AST, aspartate transaminase; ALT, alanine transaminase; FISH, fluorescent *in-situ* hybridization; SCFAs, short-chain fatty acids.

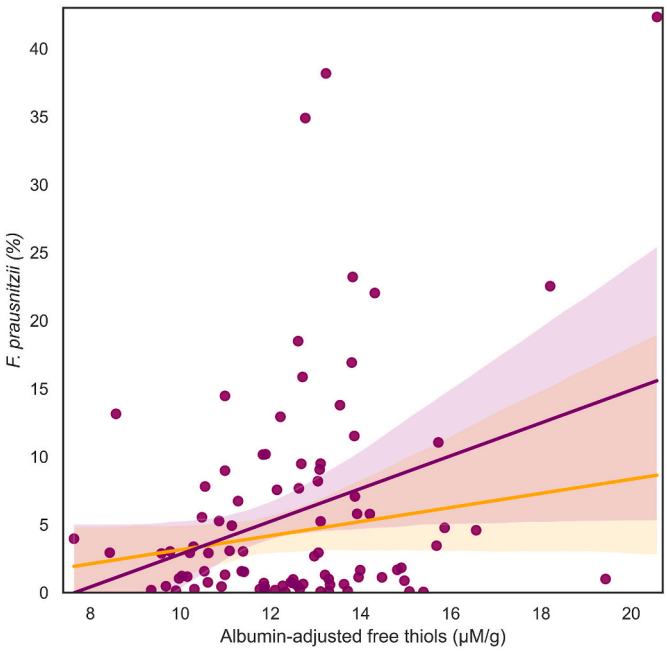

Fig. 4. Baseline faecal abundances of *Faecalibacterium prausnitzii*, as determined by FISH, are significantly positively associated with albumin-adjusted serum free thiol levels. Fitted regression lines are accompanied by 95% confidence intervals (CI). The purple line represents the best fitting line from linear regression using ordinary least squares (OLS), whereas the orange line was fitted using a robust regression method by minimizing least trimmed squares (LTS), the latter being not unduly affected by the outliers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

production of butyrate, which was, however, not confirmed in the RIBOGUT trial [4]. Nevertheless, oral supplementation with riboflavin and thiol-containing compounds may still promote intestinal health by modulating growth and metabolism of commensal anaerobic gut bacteria [59]. Based on current insights, however, this therapeutic strategy could be best targeted to individuals with established oxidative stress, inflammation, and decreased levels of free thiols, e.g. patients with IBD. Hence, the observed association between *F. prausnitzii* and albumin-adjusted free thiols may be indicative of a favorable host redox-microbiota interaction, as a thiol-rich environment could be beneficial for growth of *F. prausnitzii* or the presence of *F. prausnitzii* may generate an environment that promotes a thiol-rich host circulation.

Relevant strengths of this post-hoc analysis include the extensive characterization of the study cohort in a prospective, randomized-controlled trial setting, which enabled us to prospectively evaluate the effect of riboflavin supplementation on albumin-adjusted serum free thiol levels and to establish associations with other available health parameters, e.g. inflammatory parameters and selected gut microbial species. Simultaneously, we were able to study the effects of two different dosages of riboflavin (50 mg and 100 mg, respectively), which, however, did not reveal any trend with regard to observed changes in albumin-adjusted free thiol levels. At the same time, several limitations of this study warrant recognition. For instance, the study population consisted of healthy volunteers who were recruited using strict inclusion- and exclusion criteria, which may therefore limit generalizability of the results to people from the general population. In addition, this selection of study participants resulted in small variations of recorded health parameters, which reduced the likelihood of demonstrating any obvious changes in study parameters. Another limitation constitutes the short follow-up period of this study, which refrained us from studying effects of long-term supplementation on the systemic redox status.

Finally, the present study focused on one single redox parameter – serum free thiols – considered representative of the *in vivo* systemic redox status. However, it is unlikely that one single biomarker would be fully representative, since it could be skewed by the dynamic nature of oxidative stress as a pathophysiological entity. Instead, a combination of integrative components of the redox signaling network would be highly preferred, since this approach would combine read-outs of different types of reactive species as well as multiple redox-regulated metabolic pathways. Such "redox metabolomics" approaches are currently in their infancy, mainly because of several (mainly technical and methodological) constraints [60]. Recent reports are emerging that advocate the use of these approaches, and they provide suggestions for criteria that potential integrative redox biomarkers should fulfill in order to reliably assess the human redox system in clinical and translational settings [11,12,61]. A recently suggested example of an attempt to disentangle the human redox architecture consists of an omics approach targeted at the critical elements of the RSI, consisting of 1) nutritional precursors/substrates (e.g. organic compounds like arginine or methionine, inorganic compounds like O_2 or H_2S, and cofactors such as vitamins), 2) the transducing components, consisting of cysteine-based redox switches e.g. the circulating pool of free thiols, and 3) the stable end products of the RSI, encompassing S-, N- and O-derived metabolites [8]. Another example constitutes the establishment of a mass spectrometry-based analysis of the *thiol redox metabolome*, consisting of the parallel measurement of 12 analytes, including total and free thiols, their disulfides and sulfide, which could be performed in several biological matrices [62]. This could also potentially become a novel diagnostic/prognostic platform for patient stratification and monitoring the effects of therapeutic interventions in redox-mediated diseases. Provided that such measurements would be systematically performed alongside well-documented clinical phenotypes, routine determination of blood parameters, and perhaps complemented by other key factors

central to metabolic and/or mitochondrial function, it should become possible to increase the granularity of redox architecture and indicate the key nodes of interactions among the different types of redox compounds [61].

In conclusion, this post-hoc analysis of the RIBOGUT trial, a prospective, randomized-controlled, double-blinded intervention trial, did not demonstrate a significant effect of riboflavin supplementation on the systemic redox status, as reflected by serum free thiols, in healthy volunteers. Despite small and non-significant alterations in study parameters were observed, changes in albumin-adjusted serum free thiol levels were inversely associated with changes in CRP levels, confirming that inflammation and oxidative stress are two highly associated processes. Interestingly, however, our data showed an independent association between albumin-adjusted serum free thiol levels and faecal relative abundances of *F. prausnitzii*, which aligns with findings from *in vitro* studies and may be suggestive of a potential host redox-microbiota interaction. Future studies are warranted to further elucidate potential interactions between the host redox system and the gut microbiota, as well as the modulating capacity of several other nutritional components that may confer health benefits.

Funding

The RIBOGUT study was financially supported by DSM Nutritional Products Ltd. The research position of ARB was supported by a JSM MD-PhD trajectory grant (no. 17-57) from the Junior Scientific Masterclass of the University of Groningen, the Netherlands. All other authors received no financial support for the research, authorship and/or publication of this specific article.

Authors' contributions

HJMH, GD, HvG and ARB were involved in conceptualization and study design. HJMH, HvG and MS were responsible for funding acquisition and resources. HJMH, MS and JZHvM obtained ethical approval. HJMH, MS, JZHvM, MLCB and HvG gathered the data. ARB performed data curation, data analysis and data visualization. ARB wrote the first draft of the manuscript. All authors contributed to results interpretation and manuscript revision, read and approved the final version of the manuscript.

Declaration of competing interest

GD received an unrestricted research grant from Takeda, and receiver speaker fees from Pfizer and Janssen Pharmaceuticals. All other authors have no conflicts of interest to declare.

Data availability

Data will be made available upon reasonable request to the corresponding author(s).

Acknowledgements

The authors would like to thank all participants of the RIBOGUT study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.08.008.

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