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Enhanced bound antioxidant capacity of oat bran treated with green tea infusion modulates antioxidant release and short chain fatty acid formation during colonic fermentation†

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Bound antioxidants play a significant role in human health due to their slow and continuous release throughout the colon. Oat bran (OB) is a key source of bound antioxidants; however, its antioxidant capacity is limited. This capacity could be enhanced through treatment with green tea infusion (GTI) under optimum conditions, resulting in GTI treated OB. This study investigated the release of antioxidants from GTI treated OB during both *in vitro* digestion and colonic fermentation using *in vitro* batch and SHIME model systems. Antioxidant capacity was determined in both soluble and insoluble fractions using the QUENCHER approach. The released antioxidant capacity during *in vitro* batch fermentation was associated with catechin metabolites. The effects on gut microbiota metabolites, specifically short chain fatty acids (SCFA), were examined. Additionally, the interaction between OB and green tea extract (GTE), referred to as OB + GTE, during co-digestion and fermentation was tested. Compared to GTE alone and OB + GTE, GTI treated OB showed superior antioxidant activity (2–6-fold higher) in both insoluble and soluble fractions during short- and long-term exposure. The antioxidant release during fermentation of GTI treated OB was strongly correlated with the concentration of pyrogallol (r = 0.693) and 3,4-dihydroxyphenylacetic acid (r = 0.625). Half of its antioxidant capacity remained in the insoluble fraction. Both GTI treated OB and OB + GTE stimulated SCFA production, with concentrations of 25.25 ± 2.53 mM and 30.62 ± 5.03 mM in the distal colon, respectively. These findings suggest that the enhanced bound antioxidant capacity of OB has beneficial health effects on colon health by increasing antioxidant capacity and stimulating SCFA production.

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1. Introduction

Oxidative stress, caused by an imbalance between reactive oxygen species (ROS) and the antioxidant defense system in the human body, has been linked to several chronic diseases.[1] The gastrointestinal tract is continuously exposed to foreign substances and microbial pathogens, leading to the generation of ROS and the stimulation of oxidative stress. This plays a critical role in the early stages of intestinal injury and inflammation, contributing to various intestinal diseases, including inflammatory bowel diseases (IBD), enteric infections, ischemic intestinal injury, and colorectal cancer.[2] Oxidative stress throughout the gastrointestinal tract can also alter the composition of the gut microbiota, subsequently influencing the production of gut metabolites and affecting host metabolism through multiple pathways. Maintaining gut homeostasis is essential for preserving overall host health.[3]

Dietary antioxidants play a crucial role in maintaining the oxidative balance in the human body, particularly in the gastrointestinal tract. These antioxidants exist in foods in two primary forms: free or bound to macromolecules (*e.g.*, dietary fiber and structural proteins).[4] While bound antioxidants were often underestimated in the past, recent studies, particularly on bound phenolic acids in cereal grains, have highlighted their beneficial health effects. Unlike free phenolic acids, a substantial fraction of which are rapidly absorbed and metabolized in the small intestine, bound phenolic acids entirely reach the colon, where they are metabolized by the gut microbiota and released slowly and continuously.[5–7] Similarly, free (poly)phenols, which are marginally absorbed in the small intestine, are also metabolized by the local microbiota and

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absorbed, but this induces a rapid and transient spike in plasma antioxidant levels (depleted within 6 hours).[8] On the other hand, (poly)phenols bound to cell wall components, such as dietary fibers, are gradually liberated by the gut microbiota, which allows them to eliminate the free radicals that are persistently formed in the gastrointestinal tract, create a reducing environment, and influence the gut microbiota profile as well as the sustained plasma antioxidant level over time.[5] This prolonged release underpins their pivotal role in microbiota–disease interactions, including intestinal inflammation and chronic diseases.[9]

Cereal grains are significant sources of bound antioxidants in the daily diet, with 83%, 82%, and 67% of their phenolic compounds present in bound forms in wheat, oats, and rye, respectively.[10,11] Phenolic acids including ferulic, cinnamic, and caffeic acids are typically bound to cell wall components such as cellulose, hemicellulose, arabinoxylans, lignin, and structural proteins in the bran fraction of cereal grains.[12] Oat bran (OB) is particularly valuable due to its composition of dietary fiber (10–40%), protein (15–18%) and bound phenolic compounds (359.5–592.4 μg g^{-1}).[13,14]

Studies have shown that OB can reduce oxidative stress and inflammation, as evidenced by increased levels of superoxide dismutase and glutathione, along with decreased concentrations of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), compared to mice fed a high fat diet.[15] Furthermore, OB dietary fiber-bound (poly)phenols have been reported to upregulate the expression of Nuclear factor erythroid 2-related factor 2 (Nrf2), improve antioxidant capacity, and reduce gut inflammation more effectively than OB dietary fiber.[3] The effects of OB on the gut microbiota are also associated with short chain fatty acid (SCFA) production, which has been shown to increase in DSS-induced colitis and IBD model mice following OB feeding.[16,17]

Although OB holds significant potential for human health due to its phenolic acids bound to dietary fiber, its antioxidant capacity is considered limited compared to free antioxidant sources commonly consumed in the daily diet, such as fruits and beverages.[18] In our previous study, the bound antioxidant capacity of OB was significantly increased to a high level (more than 200 mmol Trolox equivalent (TE) per kg) through treatment with green tea infusion (GTI) under optimum conditions (53.3 °C, pH 7.4, and 1 h).[19] Understanding the release behavior of OB with enhanced bound antioxidant capacity, referred to as GTI treated OB hereafter, and its role in modulating the gut microbiota metabolism is crucial for elucidating its impact on colon health.

This study aimed to investigate the release of antioxidants from GTI treated OB and its effects on microbiota metabolites (SCFA) during colonic fermentation using *in vitro* batch fermentation and Simulator Human Intestinal Microbiome Ecosystem (SHIME) models. Additionally, the interaction between untreated OB and free green tea extract (GTE) (poly) phenols, referred to as OB + GTE, during their co-digestion and colonic fermentation was examined to determine potential synergistic effects.

2. Experimental

2.1 Chemicals

All chemicals, standard compounds, and solvents used were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise stated. 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone was purchased from Toronto Research Chemicals (Toronto, ON, Canada). OB and green tea leaves were purchased from a local market in Turkey.

2.2 Preparation of GTI treated OB samples

The insoluble fraction of OB was prepared by implementing a washing cycle to remove soluble components.[17] This procedure consists of a 7-step washing process, in which 5 mL of solvent is added to 1 gram of ground bran sample in the following order: 1 cycle with hexane, 3 cycles with ethanol, and 3 cycles with deionized water, each followed by mixing on a magnetic stirrer (CMAG HS, IKA, Germany) for 5 minutes. At the end of each cycle, the samples were centrifuged at 6080*g* for 2 minutes (Universal 320, Hettich, Germany) and the supernatant was separated from the mixture. To ensure the removal of free soluble antioxidants from the insoluble fraction of OB, antioxidant capacity measurement was performed in the supernatant of the final washing water. The obtained insoluble fraction of OB was lyophilized. To prepare OB with enhanced bound antioxidant capacity (GTI treated OB), GTI was first prepared after brewing a total of 6 g of green tea leaves in 100 mL of boiling water at 80 °C for 15 minutes and subsequently filtering through coarse filter paper. Ten grams of insoluble OB fraction was reacted with 100 mL of GTI under optimum conditions (53.3 °C and pH 7.4) over 1 h, as determined in our previous study.[19] After the reaction, the sample was centrifuged (6080*g* for 2 minutes) to separate the remaining unbound GTI from the pellet. Free antioxidants in the medium but not bound to the OB structure were washed away by rinsing the sample twice with ethanol and water, respectively. The antioxidant capacity of the final washing water was measured to ensure that no free antioxidants remained. GTI treated OB was freeze-dried (Alpha 2–4 LSCplus, Christ, Germany) before use. GTI prepared according to the same procedure was also freeze-dried to obtain GTE to represent free soluble green tea (poly)phenols. The samples were stored at −20 °C.

2.3. *In vitro* digestion procedure

Digestion fluids simulating saliva, gastric juice and duodenal juice were used to mimic the gastrointestinal tract conditions. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated duodenal fluid (SDF) were prepared according to the procedure described by Minekus *et al.* (2014).[20] 1.25 g of OB, 1.25 g of GTI treated OB, 55 mg of GTE, or a mixture of 1195 mg of OB and 55 mg of GTE (to obtain OB + GTE with the same antioxidant capacity as GTI treated OB) was mixed with 1.25 mL of deionized water. Pepsin (25 000 U mL^{-1}, final activity in SGF, 2000 U mL^{-1}) and pancreatin (800 U mL^{-1}, final trypsin activity in SDF, 100 U mL^{-1}) were the enzymes

used during gastric and intestinal digestion, respectively. For each sample, six replicates were conducted. After the intestinal phase, 3 tubes were stored for *in vitro* batch fermentation, while the other 3 tubes were used to measure the antioxidant capacity in the soluble and insoluble fractions of the intestinal phase. To that end, the samples were centrifuged (4 °C, 20 000*g*, and 10 minutes) and the supernatants, representing the soluble fractions, were filtered through a 0.22 μm regenerated cellulose (RC) filter; the remaining pellets were freeze-dried. All the samples were stored at −20 °C.

2.4. *In vitro* colonic fermentation

2.4.1. Fecal inoculum preparation. Fresh fecal materials were collected from 3 different healthy volunteers, 25–35 years old, with body mass indices between 18.5 and 25. All donors had no history of gastrointestinal disorders or antibiotic treatment for at least 6 months before this study. The feces were transferred to an anaerobic container in the morning and used immediately. 40 g of fresh feces with 200 mL of phosphate buffer (sterilized) were transferred to a stomacher bag and homogenized at a speed of 300 for 10 min in a bag mixer (Stomacher 400 circulator, Seward, UK). After a homogeneous suspension was prepared, the fecal suspension was centrifuged at 500*g* for 2 min (Megafuge 8, Thermo Fisher Scientific), and the supernatant was collected for use in *in vitro* batch fermentation. Informed consent was obtained from all volunteers prior to fecal sample collection.

2.4.2. *In vitro* batch fermentation. Basal colon medium was prepared by adding K_2HPO_4 (5.22 g), KH_2PO_4 (16.32 g), $NaHCO_3$ (2.0 g), yeast extract (2.0 g), peptone (2.0 g), mucin (1.0 g), L-cysteine HCl (0.5 g), and Tween-80 (2 mL) to 1 L of deionized water. The basal colon medium, penicillin bottles, aluminum caps, and rubber caps were autoclaved before use. 43 mL of basal colon medium and 20 mL of digested samples were added to penicillin bottles. The bottles were flushed with nitrogen for 30 min in a fume hood. 7 mL of fecal supernatant from each donor was added to the bottles and they were placed in an incubator at 37 °C at 300 rpm. 1 mL of sample was collected at 0 h, 6 h, 24 h, 30 h, and 48 h. The supernatants were filtered through an RC filter after centrifugation at 2000*g* for 5 min. The remaining pellets after 48 h of fermentation were lyophilized. All the samples were stored at −20 °C prior to the analysis. According to the guidelines of the Medical Ethical Advisory Committee of Wageningen University (METC-WU), this research did not need ethics approval.

2.5. SHIME® model

A SHIME® system (ProDigest, Belgium) was employed to mimic colonic fermentation. The reactor comprises a series of double jacketed vessels connected in sequence that mimic the human gastrointestinal tract. The vessels were maintained under anaerobic conditions by daily nitrogen flushing of the head space (10 min). Fig. 1 shows the schematic representation of the experimental plan and configuration of SHIME vessels. Two separate vessels were used to represent the proximal colon (PC) and distal colon (DC) and the pH of each vessel was kept constant using automated pH controllers. The experimental setup was started using a fecal inoculum in each of the two vessels representing the PC and DC. In this study, the fecal inoculum, which had already been stabilized during a previous SHIME experiment, was used and a stabilization period of 7 days prior to the application of the treatment was employed.[21] During this stabilization period, bacteria were fed three times per day with 200 mL (10.92 g L^{-1}) of the basal diet (SHIME® growth medium, PD-NM001B, ProDigest), enabling the microbial community to adapt to the environmental conditions of the different colon vessels. After the stabilization period, the treatment period was initiated. PC 1 was fed 200 mL of basal diet (Feed 1) three times a day as the control. PC 2, PC 3, and PC 4 were fed once daily with Feed 2 (3.5 g of OB in 200 mL basal diet), Feed 3 (3.5 g of OB + GTE in 200 mL basal diet), and Feed 4 (3.5 g of GTI treated OB in 200 mL basal diet) and twice daily with basal medium (Feed 1) over 10 days. Samples were taken from each vessel every day at the same time. Immediately after sampling, aliquots were centrifuged (10 min and 2000*g*), and the pellets and supernatants were separated and frozen at −20 °C until further use.

2.6. Determination of antioxidant capacity

The antioxidant capacities of the samples (OB, OB + GTE, and GTI treated OB) as well as the supernatants and pellets obtained from *in vitro* digestion, *in vitro* batch fermentation, and the SHIME model system were determined using the QUENCHER approach. The QUENCHER method, based on a direct surface interaction, was applied using a DPPH radical solution as described before.[22] Antioxidant capacity was expressed as μmol TE through a calibration curve prepared using Trolox in the range of 0.4–2.4 mmol L^{-1}.

2.7. Determination of total phenolic content

The total phenolic contents in the supernatants obtained from *in vitro* batch fermentation were determined by the Folin–Ciocalteu method as described elsewhere.[23] The phenolic content was determined by means of a calibration curve prepared with gallic acid in the range of 0.05–0.35 mmol L^{-1} and expressed as μmol gallic acid equivalent (GAE).

2.8. Determination of catechin derivatives and metabolites

To analyse the catechin derivatives and their metabolites, the supernatants obtained from *in vitro* batch fermentation were transferred to amber vials after filtration. Analysis was carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan), according to the method described elsewhere.[24] (−)-Epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC), (+)-catechin, gallic acid (GA), quinic acid (QA), (+)-procyanidin B2 (PB2), benzene-1,2,3-triol (pyrogallol), 3′,4′-dihydroxybenzoic acid (3,4-DHBA), 3-(3′,4′-dihydroxyphenyl)propionic acid (3,4-DHPA), 3′,4′-dihyroxyphenyl acetic acid (3,4-DHAA), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (valerolactone), hippuric acid (HA), and 4′-

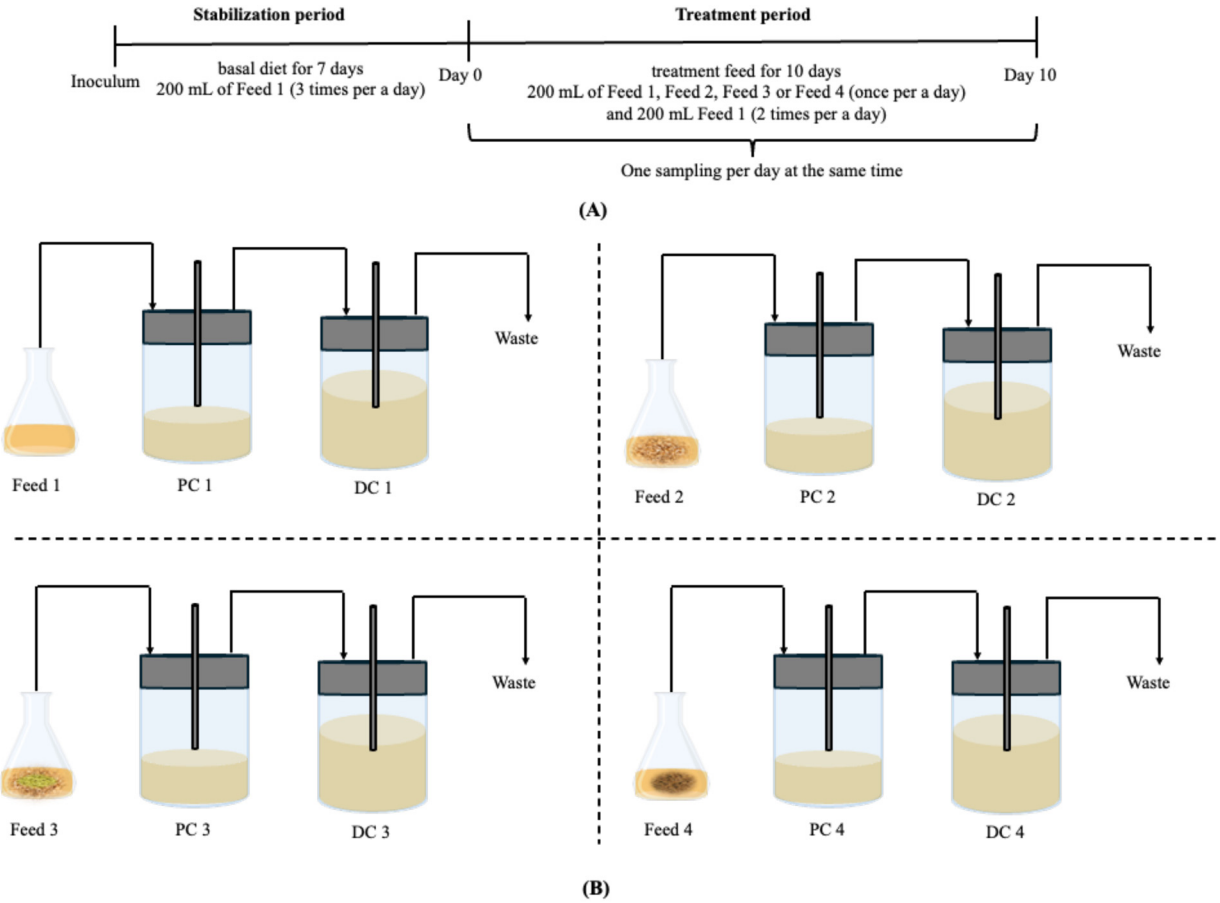

Fig. 1 Schematic representation of a Simulator of the Human Intestinal Microbial Ecosystem (SHIME): (A) experimental plan and (B) configuration. Feed 1: basal diet, Feed 2: basal diet supplemented with oat bran (OB), Feed 3: basal diet supplemented with a combination of OB and green tea extract (OB + GTE), and Feed 4: basal diet supplemented with green tea infusion (GTI) treated OB.

hydroxy-3′-methoxycinnamic acid (ferulic acid) were identified by single reaction monitoring transitions. The most abundant fragment ion was selected for quantitation, and the second and third fragments were selected for structural confirmation (Table S1†). The concentration of each compound was calculated by means of a calibration curve built with the standard compound in the range of 2.5–5000 μg L^{-1}. Data were processed using LabSolutions 5.6 software (Shimadzu Corporation, Japan).

2.9. Determination of SCFA

The supernatants taken from *in vitro* batch fermentation and the SHIME model system were thawed and mixed with an internal standard (0.45 mg mL^{-1} 2-ethylbutyric acid in 0.3 mol L^{-1} HCl and 0.9 mol L^{-1} oxalic acid) at a ratio of 2 : 1 (v/v) for SCFA analysis according to the protocol suggested by Huang *et al.* (2021) using a Shimadzu GC-2014 (Kyoto, Japan) equipped with a flame-ionization detector, a capillary fatty acid-free Stabilwax-DA column (1 μm × 0.32 mm × 30 m) (Restek, Bellefonte, PA, USA) and a split injector.[25] Standard solutions of SCFA (acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, and isovaleric acid) in the range of 0.01–0.45 mg mL^{-1} were prepared and used for identification and quantification. The results were processed using Chromeleon 7.2.10 (Thermo Fisher Scientific, San Jose, CA).

2.10. Determination of nitrogen content

Nitrogen content was estimated by the Dumas combustion method using an analyzer (EA 1112 NC, Thermo Fisher Scientific Inc., Waltham, USA) following the manufacturer's protocol.

2.11. Statistical analysis

Statistical analysis to determine the effects of treatment over time during the SHIME experiment was conducted using linear mixed models in the lme4 package in R (4.4.2). The model included variables and random effects (replicates). The significance of the results was assessed using the Kenward–Roger approach. To evaluate significant differences between groups, a pairwise comparison was performed. Tukey's HSD test was used for multiple comparisons. $p < 0.05$ was considered statistically significant. The relationship between antioxidant capacity and catechin metabolites during fermentation of GTE, OB + GTE, and GTI treated OB was analyzed by princi-

pal component analysis (PCA) using XLSTAT. The data were normalized to the maximum values for each compound prior to analysis. The relationship between catechin metabolites and antioxidant capacity changes during *in vitro* batch fermentation was evaluated using linear regression analysis in Microsoft Excel, and correlation coefficients (r) were reported.

3. Results

3.1. Antioxidant capacity changes during *in vitro* digestion

Table 1 shows the changes in the antioxidant capacity of the soluble and insoluble fractions after *in vitro* digestion and batch fermentation of OB, GTE, OB + GTE, and GTI treated OB. The initial antioxidant capacities of GTI treated OB, GTE, and OB + GTE were the same and were 23-fold higher than that of OB (11.05 ± 0.03 μmol TE). At the end of digestion, the total antioxidant capacities of OB alone and GTI treated OB significantly ($p < 0.05$) increased to levels of 63.42 ± 1.96 μmol TE and 513.93 ± 8.58 μmol TE, respectively, while a statistically significant ($p < 0.05$) reduction in the total antioxidant capacity of GTE was observed after digestion. In the case of OB + GTE, the total antioxidant capacity remained stable throughout the digestion. Additionally, no significant difference (263.05 ± 7.42 μmol TE) was observed in the total antioxidant capacity of GTI treated OB digested *in vitro* without digestive enzymes, confirming that enzymes, but not the digestion conditions, contributed to the increased antioxidant capacity at the end of digestion of GTI treated OB. The percentages of antioxidant capacity in the soluble fractions relative to their total antioxidant capacity were calculated to be 63.66% ± 1.03, 60.36% ± 0.29, 28.07% ± 0.78, and 15.18% ± 1.18 for OB, GTE, OB + GTE, and GTI treated OB, respectively.

3.2. Antioxidant capacity changes during *in vitro* batch fermentation

Digested samples were subjected to *in vitro* batch fermentation using fecal matter from three different donors. The antioxidant capacity release during fermentation was monitored over 48 h, as shown in Fig. 2. In the control samples, which included only the basal colon medium and donor feces, antioxidant release was found to be very low and remained stable over 48 h ($p > 0.05$). In the presence of OB, the antioxidant capacity significantly increased in the first 6 h ($p < 0.05$) and then remained stable (approximately 100 μmol TE) until the end of fermentation. A rapid increase in antioxidant capacity was observed for GTE in the first 6 h, reaching a level of 176.51 ± 3.54 μmol TE. This level was maintained until 24 h but it significantly decreased after that time point ($p < 0.05$). Although the presence of OB together with GTE reduced the rate of antioxidant release from GTE during the first 6 h, there was no statistically significant difference ($p > 0.05$) between GTE and OB + GTE samples after 6 h. In the presence of GTI treated OB, the released antioxidant capacity continued to significantly increase (312.40 ± 48.09 μmol TE) over 48 h, showing a 2-fold higher antioxidant capacity compared to OB + GTE.

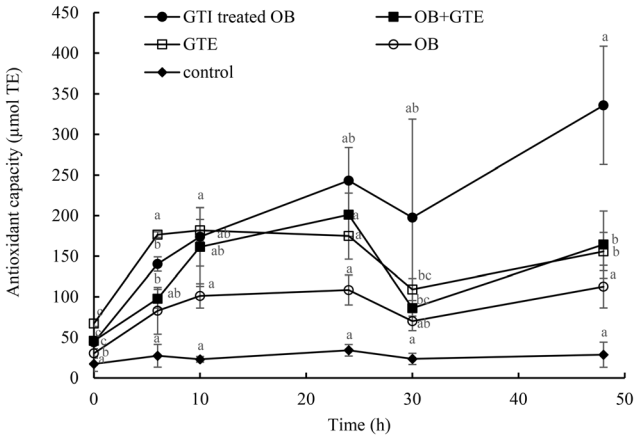

Fig. 2 Antioxidant capacity in the soluble fraction during the *in vitro* batch fermentation of the control (without samples), oat bran (OB), green tea extract (GTE), a combination of OB and GTE (OB + GTE), and green tea infusion (GTI) treated OB over 48 h.

The antioxidant capacity of the insoluble fractions was also determined at the end of 48 h of batch fermentation (Table 1). The antioxidant capacity of OB in the insoluble fraction slightly increased ($p < 0.05$) after 48 h of fermentation, while

Table 1 Antioxidant capacities (ACs) of the insoluble and soluble fractions at the end of *in vitro* digestion and *in vitro* batch fermentation of oat bran (OB), green tea extract (GTE), a combination of OB and GTE (OB + GTE), and green tea infusion (GTI) treated OB

		After *in vitro* digestion			After 48 h of batch fermentation		
	Initial Total AC[a] (μmol TE)	Insoluble AC (μmol TE)	Soluble AC (μmol TE)	Total AC[a] (μmol TE)	Insoluble AC (μmol TE)	Soluble AC (μmol TE)	Total AC[a] (μmol TE)
OB	$11.05 \pm 0.03^{B,c}$	$23.04 \pm 0.82^{D,bc}$	$40.37 \pm 1.58^{C,bc}$	$63.42 \pm 1.96^{D,b}$	$33.4 \pm 3.7^{C,bc}$	$112.61 \pm 12.05^{B,a}$	$146.01 \pm 29.76^{C,a}$
GTE	$258.52 \pm 7.67^{A,a}$	$89.93 \pm 3.76^{C,d}$	$136.97 \pm 6.81^{A,c}$	$226.9 \pm 10.52^{C,b}$	$4.94 \pm 1.16^{D,e}$	$155.86 \pm 23.54^{B,c}$	$160.80 \pm 23.13^{C,c}$
OB + GTE	$254.57 \pm 2.21^{A,a}$	$188.1 \pm 5.07^{B,b}$	$73.38 \pm 2.31^{B,c}$	$261.48 \pm 5.89^{B,a}$	$101.35 \pm 0.89^{B,c}$	$164.33 \pm 41.43^{B,b}$	$265.68 \pm 41.92^{B,a}$
GTI treated OB	$254.30 \pm 1.42^{A,c}$	$435.95 \pm 13.01^{A,b}$	$77.98 \pm 4.92^{B,d}$	$513.93 \pm 8.58^{A,ab}$	$237.45 \pm 31.11^{A,c}$	$312.40 \pm 48.09^{A,c}$	$549.85 \pm 56.05^{A,a}$

Values are presented as mean ± SD. Values with different uppercase superscript letters within the same column and those with lowercase superscript letters within the same row are significantly different ($p < 0.05$). [a] Initial total antioxidant capacity (AC) refers to the AC of lyophilized samples determined by a QUENCHER approach, while the total AC after *in vitro* digestion and 48 h of batch fermentation refers to the sum of ACs in their soluble and insoluble fractions after digestion and fermentation.

there was a significant reduction in the antioxidant capacity of the insoluble fraction of GTE. In the presence of OB together with GTE (OB + GTE), the total antioxidant capacity during *in vitro* batch fermentation did not change significantly ($p > 0.05$), while 54% of the antioxidant capacity bound to the insoluble fraction was released into the soluble fraction at the end of 48 h, reaching a level of 164.33 ± 41.43 μmol TE. There was a slight but not statistically significant ($p > 0.05$) increase in the total antioxidant capacity of GTI treated OB after 48 h, while 55% of antioxidant capacity in the insoluble fraction became soluble at the end of fermentation.

3.3. Relationship between released antioxidant capacity, total phenolic content, and catechin metabolites

Catechin derivatives and certain metabolites including GA, QA, PB2, pyrogallol, 3,4-DHBA, 3,4-DHPA, 3,4-DHAA, valerolactone, HA, FA, and total phenolic content were monitored during 48 h of fermentation. At the beginning of batch fermentation, catechin derivatives (EGCG, EGC, ECG, EC, and catechin) were only detected in the digested GTE, while GTI treated OB included ECG and EC (Table S2†). During batch fermentation, EGCG, ECG, EC, and catechin concentrations decreased in the GTE samples, while EGC and GA concentrations increased in the first 6 h and decreased thereafter. In GTI treated OB samples, EGC, EC, and GA concentrations showed an increasing trend until 10 h of fermentation and tended to decrease thereafter. Pyrogallol, whose concentration was 268.18 ± 39.03 μM after 48 h, was the most abundant catechin metabolite during GTI treated OB fermentation, which was higher than that of GTE. 3,4-DHAA, 3,4-DHPA, and valerolactone showed an increasing trend during 48 h of fermentation of GTI treated OB. In the OB + GTE samples, EGC, GA, 3,4-DHPA, 3,4-DHAA, and valerolactone were found to slightly increase, while there were no significant changes in other metabolites.

The relationship between antioxidant capacity, total phenolic content, and catechin metabolites in GTE, OB + GTE, and GTI treated OB was evaluated by PCA (Fig. 3). The first two principal factors, which explained 47.94% of the data variability, revealed a clear distinction between samples collected at the initial state of batch fermentation (0–10 h) and those after longer periods (24–48 h). This difference could be attributed to variations in the concentrations of compounds including EGCG, EGC, EC, catechin, QA, and GA in GTE samples. Notably, the high antioxidant capacity observed at the end of 48 h fermentation of GTI treated OB by microbiota from the third donor was also correlated with the concentrations of pyrogallol, 3,4-DHAA and total phenolic content. Additionally, specific compounds, 3,4-DHPA and valerolactone, were associated with the samples collected at 24 h, 30 h, and 48 h of fermentation of GTE and GTI treated OB. The correlation coefficients between antioxidant capacity and catechin derivatives and metabolites during colonic fermentation were also determined. There was a strong correlation ($r = 0.718$) between antioxidant capacity and total phenolic content, as well as between antioxidant capacity and pyrogallol ($r = 0.693$), and antioxidant capacity and 3,4-DHAA ($r = 0.625$). However, there was a very weak correlation ($r < 0.300$) between antioxidant capacity and other catechin derivatives and metabolites (EGCG, EGC, ECG, EC, catechin, GA, PB2, 3,4-DHBA, 3,4-DHPA, QA, HA, and valerolactone).

3.4. SCFA changes during *in vitro* batch fermentation

SCFA are the primary end products of dietary fiber fermentation in the colon. Total SCFA, acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid and isovaleric acid concentrations were monitored during *in vitro* batch fermentation over 48 h (Fig. S1†). Acetic acid, propionic acid, and butyric acid were the major SCFA, with acetic acid being the most abundant and showing the same trend as total SCFA changes (Fig. S1†). Compared to control samples, all samples (GTI treated OB, OB + GTE, OB, and GTE) showed significantly increased total SCFA concentration within 24 h of fermentation, while in control samples there was no statistically significant change ($p > 0.05$). GTE increased the SCFA concentration to a level of 43.99 ± 3.29 mmol L^{-1} at the end of 24 h. Compared to GTE alone, the presence of OB in OB + GTE samples stimulated SCFA production, resulting in 1.5-fold higher SCFA concentration. However, there was no statistically significant difference ($p > 0.05$) between OB alone and OB + GTE. The highest concentration of total SCFA (117.13 ± 41.71 mmol L^{-1}) was observed after 24 h of fermentation of GTI treated OB and it subsequently tended to decrease.

3.5. Antioxidant capacity changes during long term exposure (SHIME model)

OB, OB + GTE, and GTI treated OB were fed to the SHIME model over 10 days to evaluate changes in the antioxidant environment in the two compartments of the colon (PC and DC) with the long-term exposure. Changes in the antioxidant capacity in the soluble and insoluble fractions in both the PC and the DC are illustrated in Fig. 4. The antioxidant capacity changes in both compartments throughout the 10 days of fermentation of OB were not found to be statistically significant ($p > 0.05$), while GTI treated OB and OB + GTE caused a significant increase in antioxidant capacity ($p < 0.05$). The antioxidant capacity in the soluble fractions of the PC following feeding with OB + GTE and GTI treated OB was higher (Fig. 4A) than in the DC (Fig. 4B). For GTI treated OB, the antioxidant capacity in the soluble fractions showed an increasing trend after 4 days, reaching a 1.5-fold higher level by day 7 compared to the first day of treatment in both the PC and the DC. After day 7, the antioxidant capacity decreased slightly but significantly ($p < 0.05$) (Fig. 4A and B). In the insoluble fractions, the antioxidant capacity of GTI treated OB significantly ($p < 0.05$) increased, while no significant changes were observed in the insoluble fractions of OB + GTE during the 10-day fermentation period (Fig. 4C and D). Notably, the antioxidant capacity of the insoluble fraction in the DC reached 69.16 ± 7.50 mmol TE per kg at the end of the 10-day treatment with GTI treated OB, representing a 5.3-fold increase compared to the first day of treatment (Fig. 4D). For OB + GTE samples,

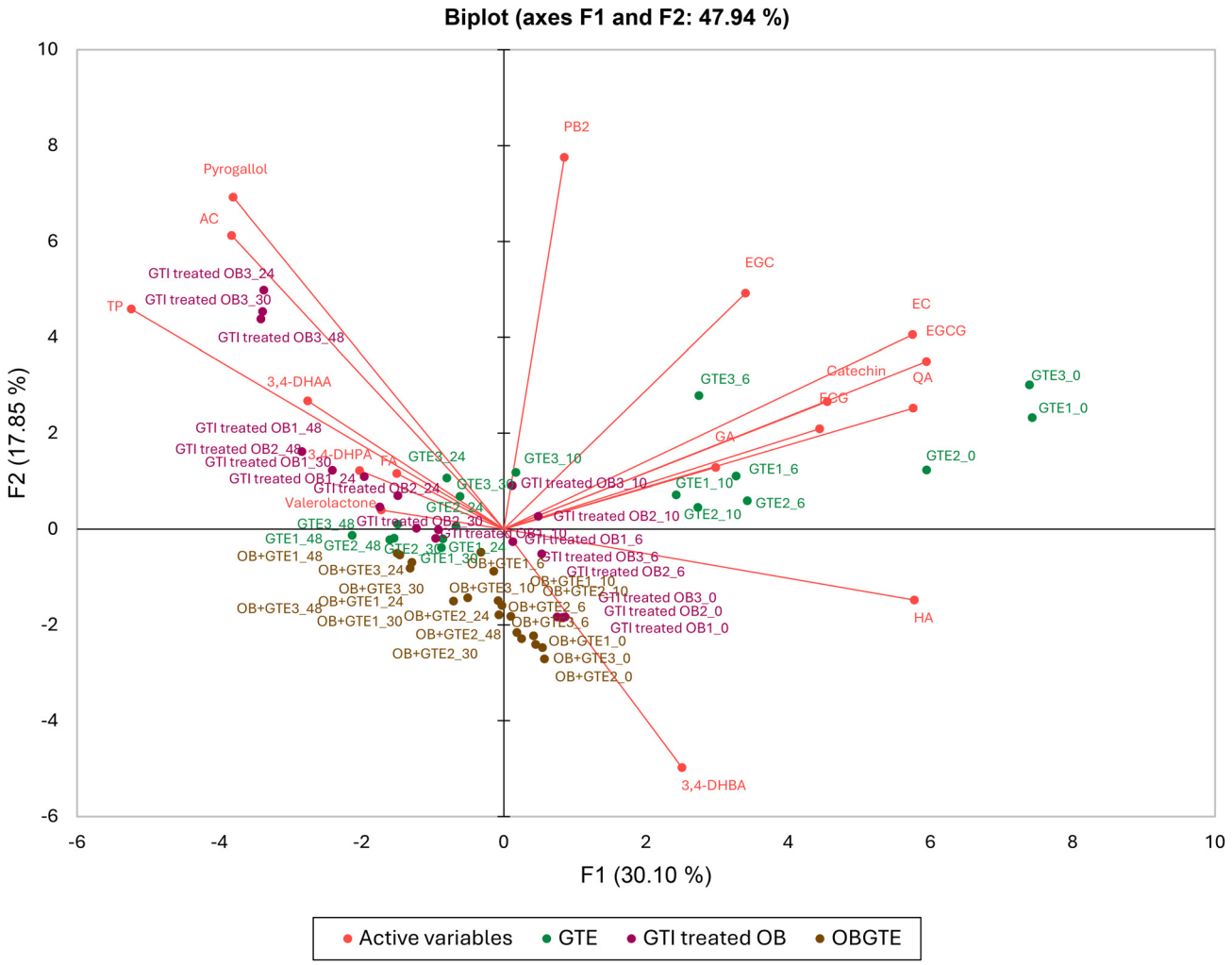

Fig. 3 Principal component analysis (PCA) biplot of the antioxidant capacity (AC), total phenolic content (TPC), and catechin metabolites data during *in vitro* batch fermentation of green tea extract (GTE), a combination of oat bran (OB) and GTE (OB + GTE), and green tea infusion (GTI) treated OB over 48 h. Data were normalized to maximum values for all compounds prior to analysis.

the antioxidant capacities in both compartments and fractions were 2–3 times lower than those of GTI treated OB, despite the initial antioxidant capacities of the feedings being the same (203.55 ± 0.15 μmol TE per kg). The antioxidant capacities of the soluble fractions in OB + GTE samples in both the PC and the DC decreased after the first day of treatment but slightly increased or remained stable throughout the 10 days of colonic fermentation.

3.6. SCFA changes during long term exposure (SHIME model)

The concentrations of acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, and isovaleric acid in the PC and DC were also monitored over a treatment period of 10 days in the SHIME model. For each compound and the total SCFA, the net production compared to their initial concentration before the treatment period is shown in Table S3† and Fig. 5. Accordingly, OB did not cause a statistically significant increase ($p > 0.05$) in total SCFA production in the PC, but the total SCFA concentration was found to be higher in the DC compared to that in the PC (Fig. 5). GTI treated OB and OB + GTE caused a significant increase in SCFA concentration over time, with the total SCFA production of 20.85 ± 0.91 mmol L^{-1} and 35.07 ± 0.70 mmol L^{-1} in the PC at the end of day 10 (Fig. 5A), respectively. OB + GTE caused slightly higher ($p < 0.05$) SCFA production during 10 days of treatment compared to GTI treated OB in the PC and until day 5 in the DC, but it was not significantly different afterwards. Acetic acid showed a similar trend to total SCFA production as it was the dominant SCFA. GTI treated OB significantly ($p < 0.05$) induced butyric acid production in the PC after 7-day treatment compared to the other samples (Table S3†). While OB caused much more valeric acid production in the PC, the presence of GTE (in both GTI treated OB and OB + GTE) reduced valeric acid production.

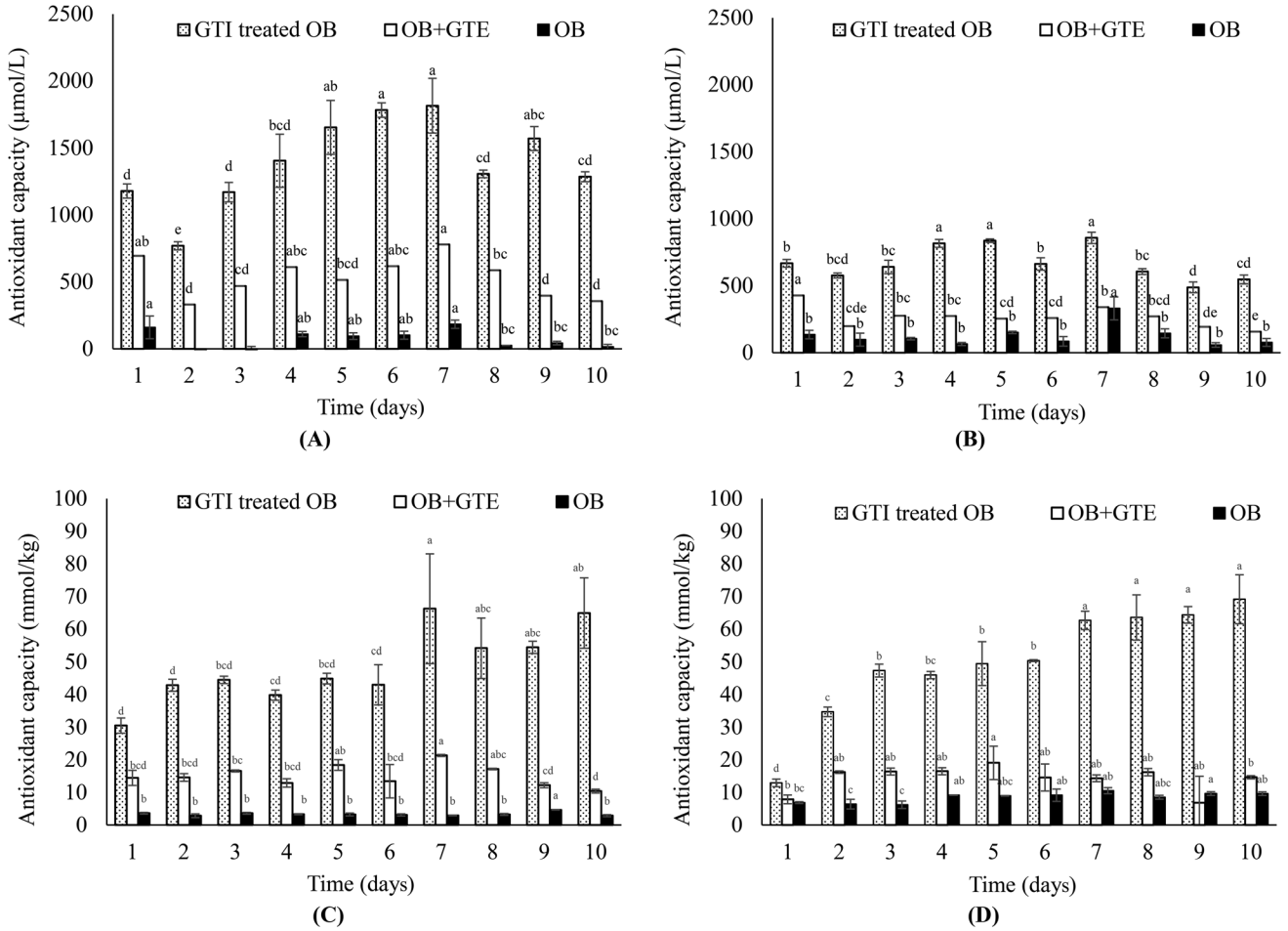

Fig. 4 Antioxidant capacity changes in (A) the soluble fraction in the proximal colon (PC), (B) the soluble fraction in the distal colon (DC), (C) the insoluble fraction in the PC, and (D) the insoluble fraction in the DC during 10 days of exposure to a basal diet supplemented with oat bran (OB), a combination of OB and green tea extract (OB + GTE), and green tea infusion (GTI) treated OB in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME).

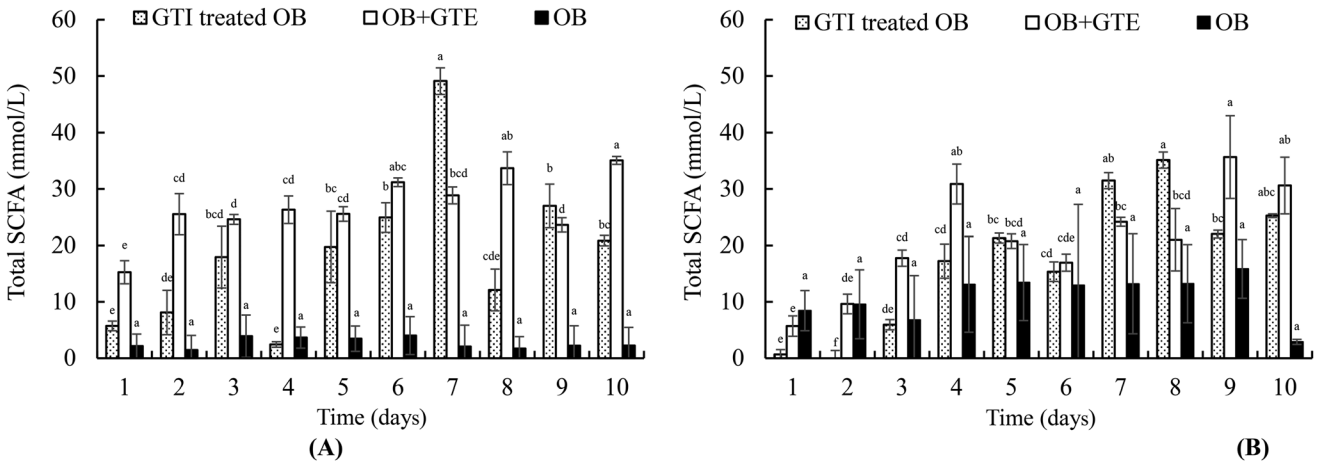

Fig. 5 Total short chain fatty acid (SCFA) concentration net production, normalized to the initial SCFA levels before the treatment period, in (A) the proximal colon and (B) the distal colon during 10 days of exposure to a basal diet supplemented with oat bran (OB), a combination of OB and green tea extract (OB + GTE), and green tea infusion (GTI) treated OB in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME).

4. Discussion

In our previous study, the bound antioxidant capacity of OB increased significantly due to its treatment with GTI under optimum conditions (53.3 °C, pH 7.4, and 1 h).[19] Neutral or alkaline pH was thought to stimulate the oxidation and promote the formation of semiquinone and quinone free radicals of catechins in the reaction environment,[26] which readily react with nucleophilic groups (amino and thiol) in the protein structure bound to the dietary fiber in OB. This reaction led to polymerization on the surface, resulting in a more than 20-fold increase in the bound antioxidant capacity of OB.[19] It is expected that the increased bound antioxidant capacity would promote the antioxidant environment through slow and continuous release throughout the gastrointestinal tract. However, the potential prooxidant effects of antioxidant compounds, depending on their concentration and structure, should also be considered. Therefore, in the present study, the antioxidant release from GTI treated OB with high bound antioxidant capacity was evaluated during both upper digestion and colonic fermentation. The resulting antioxidant environment was also compared to that of OB alone, GTE alone and their combination (OB + GTE), with the latter two adjusted to match the antioxidant capacity of the GTI treated OB. The total antioxidant capacity of OB + GTE was very close to the sum of the individual antioxidant capacities of OB and GTE. However, an increase in antioxidant capacity in the insoluble fraction along with a reduction in the soluble fraction was observed, leading to a decrease in the bioaccessibility of GTE (poly)phenols and triggering their transfer to the colon. Although there were no significant differences between the antioxidant capacities of the soluble fractions of GTI treated OB and OB + GTE, the antioxidant capacity of the insoluble fraction of GTI treated OB was more than twice that of OB + GTE. The increase in the antioxidant capacity in the insoluble fraction of GTI treated OB could be explained by the greater accessibility of the buried proteins and polymerized phenolic compounds during digestion. Exposure to enzymatic digestion could result in the formation of new peptide fractions with antioxidant capacity in the insoluble fractions.[27] The nitrogen content was found to be 2-fold lower in the soluble fraction of GTI treated OB after intestinal digestion compared to OB + GTE, whereas they were identical before digestion (Table S4†). This suggests that the peptide fractions might remain in the insoluble fraction in the presence of polymerized and bound (poly)phenols on OB, which is also consistent with the 2-fold higher antioxidant capacity observed in the insoluble fraction of GTI treated OB. Certain peptide fractions of OB isolate with higher antioxidant capacity than others have been identified before.[28] The increased radical and hydroxyl scavenging potential of OB protein isolates throughout enzymatic digestion due to the appearance of the new peptide fractions has also been reported.[29] Moreover, the possible quinones or semiquinones in GTI treated OB, which had already formed during the reaction between GTE and OB under slightly alkaline conditions, could be regenerated by the free soluble (poly)phenols released during digestion, leading to an increase in the antioxidant capacity of the insoluble fraction. This type of synergistic interaction between insoluble bound phenolic acids in wheat bran and soluble flavonoids such as catechins has been reported before.[30,31]

During the simulated batch fermentation of the samples, the antioxidant capacity in the soluble fraction initially increased, then decreased, and subsequently increased again, with the rate of change varying depending on the sample type. This trend reflects the complex dynamics of the release of antioxidant compounds from the matrix being fermented and the simultaneous conversion of more complex (poly)phenols into simpler ones, which often show a different antioxidant potential compared to the parent compounds. GTI treated OB and OB + GTE preserved their antioxidant capacity during *in vitro* batch fermentation, while 30% of total antioxidant capacity in digested GTE alone was lost at the end of 48 h. Approximately 60% of total antioxidant capacity in GTI treated OB and OB + GTE was released into the soluble fraction at the end of fermentation, while there was more than two-fold higher antioxidant capacity both in the soluble and insoluble fractions of GTI treated OB compared to OB + GTE. The most advantageous aspect of GTI treated OB was that the released antioxidant capacity continued to increase over 48 h, while in the fermentation of GTE or OB + GTE, it rapidly increased in the first 6 h but then remained stable or decreased. The high and continuous antioxidant capacity in GTI treated OB can be explained by the slow release of (poly)phenols and the sustained generation of the catechin metabolites during colonic fermentation. It was already reported that 80% of free catechins in green tea were degraded under simulated human digestive conditions[32] and they are metabolized by the gut microbiota through a series of complex reactions after reaching the colon and absorbed into the circulatory system.[33] The concentration of catechin derivatives in GTE was supposed to follow the order EGCG > EGC > ECG > EC. However, we observed that the EGCG level was lower than those of EGC and EC (Table S2†), as the stability of EGCG under intestinal conditions prior to colonic fermentation was lower than that of other catechin derivatives.[32] In OB + GTE samples, none of the catechin derivatives (EGCG, EGC, ECG, EC, and catechin) were detected after digestion, consistent with their initial concentrations used for *in vitro* batch fermentation (Table S2†). This could be attributed to the presence of trace metals in OB, which might promote the oxidation of catechins under intestinal conditions.[34] Additionally, OB might physically entrap free soluble catechins within the insoluble fraction, further contributing to their absence in the digested samples.

It was observed that free catechin derivatives in GTE alone were quickly metabolized, while the polymerized bound (poly)phenols in GTI treated OB could be released slowly and continuously. The higher antioxidant capacity in the soluble fraction of GTI treated OB may be explained by the high concentrations of pyrogallol and 3,4-DHAA and their superior antioxidant capacity compared to other catechin metabolites. In a previous study, it was revealed that certain microbial metab-

olites including 3,4-DHPA, 3,4-DHAA and valerolactone have a very high antioxidant capacity compared to other metabolites.[35] In our study, we also revealed that pyrogallol (2.85 ± 0.2 μM TE) has the same antioxidant capacity as 3,4-DHAA (2.69 ± 0.02 μM TE), but higher capacity than valerolactone (0.70 ± 0.02 μM TE) and 3,4-DHPA (0.18 ± 0.06 μM TE). It should also be kept in mind that the metabolism of tea (poly) phenols is complex, involving other metabolites such as 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, monohydroxyphenyl-γ-valerolactones, hydroxyphenyl valeric acids, tri-/monohydroxyphenyl propionic acids, tri-/monohydroxyphenyl acetic acids, and tri-/monohydroxy benzoic acids.[36]

In support of the *in vitro* batch fermentation findings, GTI treated OB produced a higher antioxidant activity in the soluble fraction compared to OB + GTE during colonic fermentation in a SHIME model, which simulated long-term exposure to the conditions of the PC and DC. The higher antioxidant capacity in the soluble fractions of GTI treated OB and OB + GTE in the PC, compared to the DC, can be explained by the fact that catechins are rapidly metabolized in the PC, similar to carbohydrates,[37] as well as the reduced stability of (poly) phenols at higher pH in the DC (pH 6.6–6.9) compared to the PC (pH 5.6–5.9). In the PC, the antioxidant capacity tended to decrease with OB + GTE, while it increased with GTI treated OB over time. This might be related to the continuous release of catechins and the simultaneous production of metabolites with higher antioxidant capacity, including pyrogallol and 3,4-DHAA. The increase in antioxidant capacity in the insoluble fraction during fermentation of GTI treated OB might be linked to the biosorption of (poly)phenols by microorganisms, which makes the soluble (poly)phenols insoluble by physically adsorbing them. There was also an increase in the weight of the insoluble fraction over time (data not shown), which may indicate the physical adsorption of phenolic compounds. The adsorption of (poly)phenols by microorganisms, particularly yeast cells, has been reported before.[38] Black tea (poly) phenols, which contain higher molecular weight, polymeric (poly)phenols, have been reported to have a stronger binding affinity to yeast cells than green tea (poly)phenols, mostly in the form of monomers, leading to a higher antioxidant activity during digestion.[38] GTI treated OB includes the polymerized form of catechins, similar to black tea (poly)phenols, which can be adsorbed by microorganisms during colonic fermentation.

The SCFA production during batch fermentation indicated that dietary fiber and antioxidant compounds, either bound or in the free form, stimulated the SCFA formation. In particular, the high concentration of bound (poly)phenols in GTI treated OB resulted in a higher SCFA production during the first 24 h of fermentation. On the other hand, SCFA concentration did not change in the PC with OB during fermentation in the SHIME model system. The difference compared to the *in vitro* batch fermentation system could be explained by the fact that the SHIME model included other carbon sources such as pectin, arabinogalactan, xylan, and glucose in the basal diet along with OB, whereas no additional carbon sources other than OB were included in the *in vitro* batch fermentation system. The marginal increase in the amount of fermentable carbohydrates through OB feeding in the SHIME model might not cause any significant effects on SCFA production. The slight increase in SCFA production observed in the DC with OB treatment, rather than the PC, could be attributed to the transfer of dietary fiber and protein to the DC, where they are fermented by the gut microbiota. The higher SCFA concentration in the DC compared to the PC in the model system with dietary fiber feeding has been reported before.[39,40] Since SCFA cannot be produced directly from the aglycon, stimulation of SCFA production by (poly)phenols in both the GTI treated OB and OB + GTE during long term exposure suggested that the presence of phenolic compounds has a positive impact on the gut microbiota in both the PC and the DC. An increase in butyric acid production by GTI treated OB might be an important finding, as butyric acid plays a crucial role in maintaining intestinal health. It is used as an essential energy source for intestinal epithelial cells, promotes epithelial cell proliferation and differentiation, and exhibits anti-inflammatory properties.[41] Additionally, butyric acid strengthens the gut mucosal barrier by stimulating the expression of tight junction proteins and the production of antimicrobial peptides in the intestinal mucosa.[42]

Consistent with our findings, increased SCFA production has been reported in the colon of mice fed a high fat diet containing OB compared to the control group (high fat diet) as well as the groups fed high fat diet containing oat flour or oatmeal.[43] Another study demonstrated the positive effects of OB, but not isolated β-glucan and (poly)phenols, highlighting the importance of the dietary fiber combined with (poly) phenols for the gut microbiota.[44] The significant effects of dietary fiber bound (poly)phenols in OB on SCFA production, rather than dietary fiber alone, have been shown in mouse models fed a high fat diet.[3] The promoted SCFA formation is associated with changes in the gut microbiota. However, while the microbiota profiles differed from those of the control samples, no differences were observed between mice fed dietary fiber alone or dietary fiber bound (poly)phenols in the same study.[3] When free phenolics combined with dietary fiber were compared to dietary fiber bound (poly)phenols, it was observed that certain probiotics, such as *Lachnospiraceae bacterium*, *Bifidobacterium pseudolongum*, and *Clostridium butyricum*, were enriched by bound (poly)phenols, suggesting their role in enhancing the benefits of dietary fiber bound (poly) phenols in OB for gut health.

Regarding the effects of tea (poly)phenols on the gut microbiota, black tea and oolong tea (poly)phenols have shown a greater capacity to produce SCFA than green tea (poly)phenols, which also positively affect the gut microbiota, by stimulating SCFA production, depending on the dosage.[45] This effect has been attributed to the lower absorption of fermented tea (poly) phenols, which interact with the bacterial community that reacts with residual carbohydrates to produce SCFA.[46] In our case, during the preparation of GTI treated OB, green tea (poly)phenols polymerized under slightly alkaline conditions

and bound to OB protein residues attached to the dietary fiber. These polymerized (poly)phenols stimulate the SCFA production in the colon through a mechanism like that of fermented tea.

5. Conclusion

This study demonstrated that enhancing the bound antioxidant capacity of OB through GTI treatment prior to digestion positively affects antioxidant release during digestion and colonic fermentation. The antioxidant capacity of GTI treated OB significantly increased in both the soluble and insoluble fractions after digestion, with 85% of its antioxidant capacity transferred to the colon, where it was fermented by the gut microbiota. During fermentation, catechins that were polymerized onto OB were continuously metabolized in a different way than in OB + GTE, leading to an increase in antioxidant capacity in the soluble fraction. Notably, the antioxidant capacity was strongly correlated with 3,4-DHAA and pyrogallol concentrations, which were higher in GTI treated OB compared to GTE alone and OB + GTE. This study is the first to report the superior antioxidant capacity of pyrogallol among catechin metabolites. The insoluble fraction of GTI treated OB retained substantial antioxidant capacity after 48 h. This may be critical for colon health by preserving antioxidant capacity in distal segments of the colon, including the rectum, a region with a high incidence of colorectal cancer. By establishing a reducing environment, it could potentially lower the risk of cancer. Long-term feeding with GTI treated OB demonstrated superior antioxidant capacity in both the PC and the DC compared to OB + GTE. Regarding gut microbiota metabolites, both bound and free antioxidants stimulated SCFA production. However, the enhanced bound antioxidant capacity of GTI treated OB led to greater butyric acid production compared to free green tea (poly)phenols in OB + GTE. These findings highlight the potential of high bound antioxidant capacity to contribute to the prevention of oxidative stress related colon diseases, such as IBD and colon cancer. Further research is warranted to explore these effects *in vivo*.

Author contributions

Conceptualization: E. D. C., V. G., and E. C.; methodology: E. C.; validation: E. D. C.; investigation: E. D. C.; resources: E. C.; data curation: E. D. C.; writing—original draft preparation: E. D. C.; writing—review and editing: E. C. and V. G.; visualization: E. D. C.; supervision: E. C.; project administration: E. C. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

The data supporting this article have been included as part of the ESI.†

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