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Production of short-chain fatty acids in polyphenol-rich foods by *in vitro* human digestive system

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ABSTRACT

The study examines how specific polyphenols influence *in vitro* human digestion, focusing on their effects on short-chain fatty acids (SCFAs) profiles and antioxidant capacities. Aronia, Cornelian cherry, green tea, and Turkish coffee were digested, and changes in SCFAs and antioxidants were analyzed. Results showed variations in SCFAs levels before and after digestion, with Turkish coffee displaying the lowest acetic acid levels post-digestion (16 ± 0.4 mg/100 g) and green tea showing the highest propionic acid levels (742 ± 19.6 mg/100 g). Cornelian cherry exhibited the greatest increase in butyric acid levels after digestion (4.7 ± 0.12 mg/100 g). Additionally, Turkish coffee showed the highest increase in total phenolic content (TPC) post-digestion, while Cornelian cherry had the highest increase in total antioxidant capacity (TAC). Overall, the findings suggest that polyphenols may positively impact digestion and potentially exhibit prebiotic effects.

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INTRODUCTION

Polyphenols constitute the predominant category among secondary metabolites devoid of energetic properties, encompassing approximately 8,000 distinct compounds that have been identified[1,2,3]. Polyphenols, including flavonols, flavones, flavanones, flavanols, phenolic acids, lignans, and stilbenes, harbor bioactive compounds associated with advantageous physiological impacts, including potential anticancer and anti-inflammatory attributes[4,5].

Referred to as 'essential elements for longevity,' polyphenols play a significant role in health[6]. While numerous studies in the literature document the bioactivity of polyphenols, understanding of the mechanisms driving their health effects is limited due to their poor oral bioavailability[7].

The absorption and bioavailability of dietary polyphenols in the body determine their health benefits[8]. Bioactive compounds undergo transformations as they progress through the gastrointestinal (GI) tract, leading to the generation of various metabolites. In the colon, certain polyphenols undergo fermentation mediated by the gut microbiota, consequently leading to elevated concentrations of short-chain fatty acids (SCFAs). This process specifically affects the microbiota utilized by the host[9]. SCFAs generated within the colonic environment during the fermentation of dietary fiber and specific food components serve various functions with positive effects on health[10,11].

Polyphenols act as metabolic prebiotics[12]. Phenolics that remain unabsorbed in the colon exhibit effects similar to "prebiotics"[13]. Typically, dietary polyphenols exhibit limited bioavailability, with approximately 90-95% evading absorption in the intestine and instead reaching the colon[14]. Unabsorbed molecules undergo biotransformation, being deconjugated, depolymerized, and metabolized into phenolic metabolites with reduced molecular weight, which are then absorbed by colonic microbiota[15].

There is increasing interest in the enzymatic modification of phenolic compounds mediated by lactic acid bacteria (LAB), with several studies showcasing the capability of diverse LAB strains to carboxylate, demethylate, de-esterify, and glycosylate dietary polyphenols[16,17]. LAB can be used to convert polyphenols into bioavailable and bioactivated compounds[18].

Polyphenols, found in legumes, cereals, vegetables, olives, fruits, and various other dietary sources, significantly contribute to overall dietary intake[19]. Notable examples of polyphenol-rich foods, distinguished by their unique flavors, cultural significance, and rich polyphenolic content, include Aronia (*Aronia melanocarpa*), Turkish coffee (*Coffea arabica*), green tea (*Camellia sinensis*), and Cornelian cherry (*Cornus mas* L.).

The aim was to analyze the polyphenols found in Cornelian cherry, Aronia, green tea, and Turkish coffee during *in vitro* digestion and to evaluate their effects on SCFAs, along with their antioxidant properties.

METHODOLOGY

Chemicals

Methanol (product code: 106009), Folin–Ciocalteu reagent (product code: 109001), ammonium acetate (NH_4Ac) (product code: 101116), sodium carbonate (Na_2CO_3) (product code: 106392) and Copper (II) chloride ($CuCl_2$) (product code: 102739) were obtained from Merck (Darmstadt, Germany). Alpha-amylase (1.5 U/mg, from Aspergillus oryzae powder) (product code: 86250),), pancreatin (from porcine pancreas, meeting 8 × USP specifications) (product code: P7545), pepsin (≥250 U/mg solid, from porcine gastric mucosa, lyophilized powder) (product code: P7000), bovine serum albumin, KCl (product code: 58221), lipase (100–500 U/mg protein, from porcine pancreas Type II) (product code: L3126NaCl (product code: S9888), $CaCl_2{\cdot}2H_2O$ (product code: 223506), urea, uric acid, mucin, $NaHCO_3$ (product code: S6014), acetonitrile (ACN) (product code: 34851), bile salts mixture, neocuproine (Nc) (product code: N1501), Trolox (6-hydroxy-2,5,7,8–22 tetramethylchroman-2-carboxylic acid), gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The sample preperation

The fresh Cornelian cherry and Aronia fruits purchased for the study were processed into a puree using a 32-mm pore pulper machine. The lyophilization processes were carried out using the G-Ray 125 freeze-dry machine through sublimation[20]. Turkish coffee and green tea were purchased in boxes. For the Turkish coffee, 6 g of coffee was mixed with 65 mL of water and thoroughly blended before being brewed using a coffee machine[21]. Green tea, consisting of 1.5 g, was prepared by adding 200 mL of boiling water and thoroughly mixing.

In vitro digestion procedure

The method outlined by Lee et al. was slightly adapted for *in vitro* digestion. Each sample containing 5 g of polyphenols (Aronia, Turkish coffee, green tea, and Cornelian cherry) was placed in 50 mL Falcon tubes[22]. *In vitro* human digestion was simulated by adding solutions mimicking those in the oral, gastric, intestinal digestion (Figure 1).

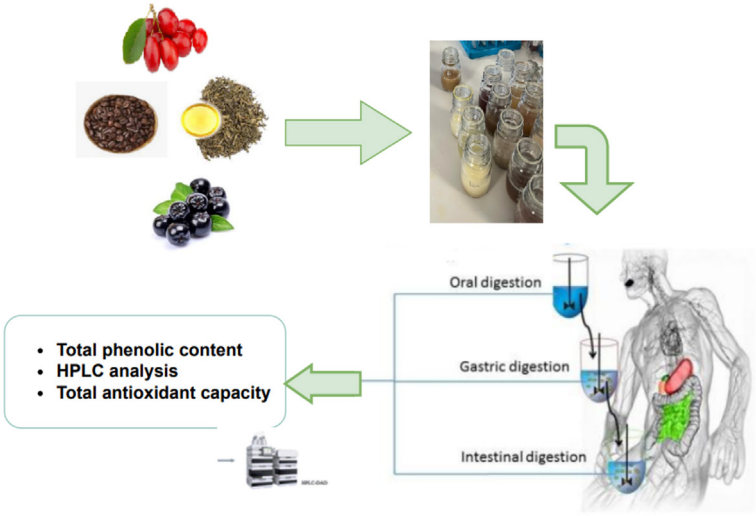

Figure 1. Dynamic changes in polyphenol products of Aronia, Cornelian cherry, green tea, Turkish coffee during the *in vitro* gastrointestinal digestion

For the oral medium, NaCl, urea, uric acid, α-amylase, and mucin were dissolved in deionized water, with the pH adjusted to 6.8 ± 0.2. The gastric medium included HCl, $CaCl_2.H_2O$, bovine serum albumin, pepsin, and mucin, with a pH of 1.5 ± 0.02. The small intestine medium contained KCl, $CaCl_2.2H_2O$, bovine serum albumin, pancreatin, and lipase, adjusted to pH 8.0 ± 0.2. Lastly, the bile solution was composed of $NaHCO_3$, $CaCl_2.2H_2O$, bovine serum albumin, and bile, with a pH of 7.0 ± 0.2. In each case, deionized water was used to make up the volume, and HCl or NaOH was used to adjust the pH[22].

Five mL of salivary solution were added to the samples and incubated in a shaking water-bath (5 min, 37°C). Then, 12 mL of gastric juice was added to the samples and incubated at the same temperature for 30 min. Subsequently, 12 mL of duodenal juice and 6 mL of bile juice were added to the mixture obtained after the addition of gastric juice. The mixture underwent an incubation period in a shaking water-bath (2 hours, 37°C)[22].

The liquid agar for *Escherichia coli (E. coli)* was prepared by mixing 2.5 g of Mueller–Hinton broth with 100 mL of deionized-distilled water (DDW), while the liquid medium for *Lactobacillus plantarum* (*L. plantarum)* involved combining 5.5 g of Lactobacilli MRS Broth with 100 mL of double-distilled water (DDW). Frozen (-80°C*) E. coli* and *L. plantarum* were heated to 37°C. For 1% of the *E. coli* and *L. plantarum* stocks, 1 mL was added to 100 mL of suitable sterile liquid medium. The *E. coli* and *L. plantarum* liquid medium solutions were incubated at 37°C for 12 hours to activate them. For activated *E. coli* and *L. plantarum*, 100 mL of sterile liquid medium was again inoculated and incubated at 37°C for an additional 12 hours. *E. coli* was allowed to grow for 24 hours, and *L. plantarum* for 72 hours at 37°C. After incubation, the final number of *E. coli* and *L. plantarum* colonies was log 10^8-10^{10} colony-forming units (CFU). For the large intestine digestion phase, 38 mL of liquid agar containing *E. coli* and *L. plantarum* solutions were added to samples that had undergone small intestine digestion. The samples were then incubated (4 hours, 37°C)[22].

HPLC analysis

The prepared samples were filtered through a 0.45-µm syringe tip cellulose acetate filter, transferred into 2-mL amber screw-cap vials, and then subjected to HPLC. The HPLC analysis for SCFAs determination, following the method by De Baere et al., was adapted for this study[23]. A Shimadzu Nexera-i HPLC system equipped with a Shimadzu DAD detector was utilized for separation of acetic acid, butyric acid, and propionic acid. The mobile phase consisted of methanol: water: acetonitrile (42:56:2 v/v/v), with detection at 210 nm. An Inersil ODS-3 column (5µm, 4.6x250 mm) was used in a column oven (30°C) with a flow rate (0.8 mL/min).

Total phenolic content analysis

The Folin-Ciocalteu method was employed to determine the total phenolic content (TPC) of the samples[24]. Pre-digestion samples underwent extraction with an extraction solution, followed by centrifugation. Then, 0.1 mL of the centrifuged samples was mixed with 0.75 mL of 6% Na_2CO_3 and 0.75 mL of Folin reagent. Post-digestion, samples were again extracted and centrifuged, and the process was repeated similarly. After incubating in the absence of light at room temperature (90 min), absorbance (760 nm) was determined using a UV-visible spectrophotometer (Shimadzu UV-1700 UV–Vis, Japan). Absorbance values were compared to a gallic acid calibration curve (mg/L-1 gallic acid equivalent [GAE]).

Total antioxidant capacity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was used to evaluate the Total Antioxidant Capacity (TAC), with Trolox as the standard. Each assay was conducted in triplicate for the samples. The procedure followed the method outlined by Karunakaran and Kumaran[25]. Initially, 3.9 mL of 0.1 mM DPPH in methanol was combined with 100 µL of sample extract or standard solution. The blank sample comprised 100 µL of an 80% methanol solution and 3.9 mL of the DPPH solution. Following incubation at room temperature in the absence of light (30 min), absorbance was determined utilizing a UV-visible spectrophotometer, referencing against a blank measurement (at 517 nm).

Data analysis

Analyses were conducted using Minitab 18 software, involved applying one-way analysis of variance (ANOVA) and Tukey post hoc tests to determine any significant differences ($p<0.05$). Triplicate analyses were conducted.

RESULTS and DISCUSSION

The effect of polyphenols on SCFAs

The production of SCFAs by microbiota in colon is essential for various physiological processes, extending beyond the intestinal environment to affect peripheral tissues following absorption[26]. Acetic, propionic, and butyric acid are the primary fermentation products, significantly contributing to the overall SCFAs pool. Acetic acid serves as an essential energy substrate, being metabolized and absorbed by organs. Propionic acid exhibits potential in reducing fatty acid levels in both the liver and plasma, thereby inhibiting cholesterol synthesis. Butyric acid maintains the immune function of the intestinal mucosa and suppresses cytokine production[27].

In this study, Aronia, Cornelian cherry, green tea, and Turkish coffee were subjected to analysis for their acetic, propionic, and butyric acid contents pre- and post-*in vitro* digestion. The data depicted in Table 1, demonstrated variations in the levels of SCFAs among these polyphenol-rich foods. Prior to digestion, Cornelian cherry and green tea exhibited the lowest acetic acid values, while Aronia displayed the highest. Upon *in vitro* digestion, Turkish coffee showed the lowest acetic acid levels, with a notable increase observed in green tea. Similarly, the initial propionic acid levels were lowest in Cornelian cherry and highest in Turkish coffee, with a substantial increase noted in green tea post-digestion. As for butyric acid, Cornelian cherry and green tea had the lowest initial values, whereas Aronia displayed the highest. Following digestion, Turkish coffee exhibited the lowest butyric acid levels, while Aronia demonstrated the most significant increase.

Table 1. Content of acetic, propionic and butyric acids in different types of polyphonels

Polyphonels	Acetic Acid (mg/100g)		Propionic Acid (mg/100g)		Butyric Acid (mg/100g)	
	Pre-digestion	Post-digestion	Pre-digestion	Post-digestion	Pre-digestion	Post-digestion
Aronia	0.53 ± 0.03^{a}	66.5 ± 1.8^{b}	0.24 ± 0.00^{c}	285 ± 7.5^{b}	2.1 ± 0.05^{a}	4.7 ± 0.12^{a}
Cornelian Cherry	0.19 ± 0.00^{c}	62.4 ± 1.65^{b}	0.15 ± 0.00^{d}	279 ± 7.1^{b}	0.18 ± 0.00^{c}	3.7 ± 0.09^{b}
Green Tea	0.17 ± 0.01^{c}	177 ± 4.7^{a}	0.32 ± 0.01^{b}	742 ± 19.6^{a}	0.17 ± 0.04^{c}	3.7 ± 0.09^{b}
Turkish Coffee	0.47 ± 0.02^{b}	16 ± 0.4^{c}	1.30 ± 0.04^{a}	58.5 ± 1.5^{c}	0.39 ± 0.01^{b}	1.14 ± 0.00^{c}

Outcomes are represented ± standard deviation (n=3). The distinct symbols within the same column displayed indicate a significant difference in mean values (ANOVA, Tukey's test, $p<0.05$).

The investigation into the digestion process conducted *in vitro* demonstrated an increase in the acetic, butyric, and propionic acid content of these polyphenol-containing foods compared to their levels before digestion. Phenolic compounds have a limited absorption in the intestine, with 5 to 10% of the total ingested polyphenols undergoing absorption. Complex phenolics that remain unabsorbed in the small intestine undergo biotransformation by resident microbiota in the colon into smaller molecular weight metabolites, facilitating potential absorption[15]. Throughout this procedure, SCFAs are generated, accompanied by notable alterations in the composition of gut microbiota[15]. During *in vitro* gastrointestinal digestion, Aronia, green tea, coffee, and Cornelian cherry exhibited elevated levels of SCFAs (propionic, butyric, acetic acid). This observation may be connected to the effects of processes such as digestion, absorption, and metabolism on the bioavailability[28]. Moreover, gut microbiota exerts a crucial influence on the biotransformation of polyphenols, a process vital for their bioavailability[29,30]. Polyphenols may promote an increase in SCFAs production by exerting a prebiotic effect[31]. It has been found that unabsorbed phenolic compounds and their derivatives in the colon possess 'prebiotic-like' effects[32]. *In vitro* gastrointestinal digestion of Aronia, green tea, coffee, Cornelian cherry exhibited prebiotic potential by promoting the production of SCFAs. Recent studies by Moorthy et al. and Alves-Santos et al. support this study's findings, confirming that polyphenol intake affects gut microbial composition and enhances host health by promoting homeostasis, exhibiting prebiotic properties[31,33]. Polyphenolic compounds, including anthocyanins and

phenolic acids, are noted to promote fermentation, leading to an elevation in SCFAs concentration[34,35]. In the study, green tea was found to contain elevated levels of acetic and propionic acids, whereas Aronia demonstrated increased concentrations of butyric acid following digestion. In line with this study, Rha et al. suggest that specific stable polyphenols found in green tea have higher bioaccessibility in the gastrointestinal system, and their health-regulating effects are based on interactions with gut microbes[36]. The antioxidant properties of green tea and Aronia polyphenols may have created a synergistic effect.

The results of our *in vitro* digestion experiments reveal that polyphenol-rich foods significantly influence the digestive system, aligning with existing literature that highlights their potential to enhance SCFAs production and exert prebiotic effects. This finding corroborates previous studies, such as those by Sorrenti et al. and Dou et al., which have demonstrated that polyphenols can positively modulate gut microbiota and fermentation processes, thereby supporting gastrointestinal health and function[37,38]. Our data extends these observations by confirming that diverse polyphenol sources, including those analyzed in our study, contribute to increased SCFA levels and exhibit prebiotic properties, reinforcing the role of dietary polyphenols in promoting beneficial microbial activity and overall digestive health.

The effect of polyphenols on TAC and TFC

The evaluation and comparison of TAC and TPC of polyphenols pre- and post-digestion revealed significant alterations (Figure 2). Aronia exhibited the highest TPC values initially, while green tea had the lowest. Turkish coffee showed the most considerable increase in TPC post-digestion, whereas green tea and Cornelian cherry displayed the lowest increments. Regarding TAC, Aronia had the highest initial value, while Cornelian cherry and green tea had the lowest. Post-digestion, Aronia demonstrated the highest increase in TAC, whereas Turkish coffee showed the lowest enhancement.

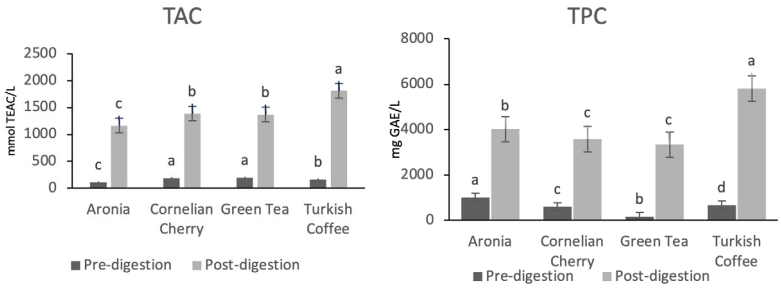

Figure 2. TPC and TAC values of the polyphenols before and after *in vitro* digestion. The distinct symbols displayed indicate a significant difference.

The findings indicated that TAC and TPC levels of polyphenols were higher following *in vitro* digestion in comparison to pre-digestion levels. The increased TAC and TFC of polyphenols subsequent to *in vitro* digestion might be associated with enzymatic hydrolysis in the stomach and intestines as well as the increase in content[39]. Particularly noteworthy was the substantial enhancement observed in both TPC values and TAC of coffee following gastrointestinal processing. These results align with prior research by Campos-Vega et al., which suggests that colonic fermentation enhances the antioxidant capacity of polyphenols derived from coffee grounds through the release of phenolic compounds[40]. Furthermore, coffee's active compounds can be metabolized by gut bacteria, potentially enhancing their antioxidant properties and beneficial effects.

Vamanu et al. endorse the notion that the phenolic acids present in coffee grounds are instrumental in its observed antioxidant properties[41]. The evaluation of antioxidant activities in coffee indicates that the active compounds possessing antioxidant capabilities withstand complete neutralization by digestive enzymes within the GI tract, facilitating their passage to the colon and maintenance of their bioactive effects, as evidenced in the study by de Cosío-Barron et al.[42]. This highlights the high bioaccessibility of polyphenolic compounds during digestion.

Aronia, green tea, Cornelian cherry, and coffee polyphenols on SCFAs production are emerging. Preclinical and *in vitro* studies suggest potential synergistic effects on gut health. This study explores the antioxidant effects of these polyphenols, with digestion enhancing their bioaccessibility. These polyphenols also demonstrate prebiotic activity by increasing the production of butyric, acetic, and propionic acids. Gastrointestinal digestion releases bound phenolic compounds, boosting TPC and TAC. Understanding the nutritional value and potential applications of these polyphenols in functional foods provides valuable insights for consumers and the food industry.

STATEMENT OF ETHICS

This study does not require any ethical approval.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Design: MY, BİPL. Acquisition of data: ÖFM, EYS, EO. Analysis of data: MY, EO. Drafting of the manuscript: EO. Critical revision of the manuscript: MY, BİOK, EO. Statistical analysis: EO. Supervision: MY, BİOK.

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