DOI: 10.26599/FSHW.2025.9250820

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Dietary supplements of algal polysaccharides promote ellagic acid biotransformation targeted on gut bacteria

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ABSTRACT: Ellagic acid (EA) could be converted into urolithins to exert health-promoting effects by intestinal flora, but the biotransformation capacity varied among individuals. Strategies targeted on gut microflora modulation could be a novel method to obtain more urolithins beyond the inter-individual variability. The study aimed at investigating the impacts of four algal polysaccharides (APs) supplements on EA biotransformation. Results showed that simple APs supplements would increase certain urolithin productions, and APs-EA solid dispersions supplement improved urolithins contents from 4.25 μmol to 6.16-6.79 μmol. The enhancement was related to the proliferation of the EA-metabolic-related bacteria like *Gordonibacter*, *Faecalibacterium*, *Sutterella*, *Dialister*, *Enterocloster* and etc. and increasing the bioaccessibility of EA to the bacteria. Furthermore, mono-/co-culture fermentation further verified that *Gordonibacter urolithinfaciens* was greatly proliferated by simple APs and EA solid dispersions supplements. EA/UPP 1-1 showed the best performances in urolithins productions, and the total urolithins contents increased by 3.78 times compared to control EA group. These results showed dietary supplements could improve biotransformation ability by influencing the EA-metabolic-related bacteria and the access to substrates.

Keywords: Ellagic acid; Polysaccharides supplements; Biotransformation process; Gut bacteria

1. Introduction

Gut microflora is a complex ecosystem which plays vital roles in host normo- and pathophysiology. However, genetic and environmental factors, including exercise, dietary habits, receipt of antibiotics and etc., could cause a high variability in gut microflora from individual to individual [1]. For instance, previous data have showed that the *Bifidobacterium* spp. were more abundant in healthy children and might gradually decrease until adulthood [2]. Another example revealed that huge differences in the microflora-associated variables were found between non-drinkers and alcohol consumer cohorts, and a *Bifidobacterium* ASV was most frequently enriched among alcohol consumers [3]. Gut microflora participates in nutrient regulation and many metabolic pathways [4]. As a result, the inter-individual variability of the gut bacteria hinted that different people may receive a heterogeneous response upon the metabolism when consuming same dietary

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Received 27 April 2025
Received in revised from 26 May 2025
Accepted 16 July 2025

substances. An *in vitro* fermentation experiment found that a wide disparity in the kind and content of metabolites was observed in the elderberry anthocyanins when incubated with three single gut microbial strains [5]. There were substantial interindividual differences in the metabolic profiles of (+)-catechin, with 5-(3′,4′-dihydroxyphenyl)-*γ*-valerolactone being the major contributor, suggesting that the bioavailability of catechin highly depended on gut microbiota [6]. Curcumin could be metabolized into a number of active metabolites based on the inter-individual intestinal microbiota, including hexahydrocurcumin, tetrahydrocurcumin, octahydrocurcumin and etc [7].

Ellagic acid (EA) is an herbal naturally occurring polyphenol available in various fruits and nuts with a wide range of health promoting effects [8]. However, its bioavailability is extremely limited due to the hydrophobicity. Urolithins, one of the dibenzopyran-6-one derivatives with different hydroxyl group substitutions, are the microbial metabolites of the unabsorbed EA. As compared to the precursor, urolithins possessed higher oral bioavailability, indicating that the metabolites were primary drivers for health benefits rather than EA. Therefore, regulation targeted on gut microbiota-mediated biotransformation of EA was of great significance to achieve higher bioactive responses. According to the previous study, the production process of urolithins exerted great interindividual variability due to the heterogeneity in gut microflora [9]. For example, a study with 35 healthy Chinese youth reported that 54.3% volunteers could converted EA into urolithin A, 31.4% volunteers could converted EA into isourolithin A and urolithin B, while 14.3% volunteers could not produce urolithins depending on the gut microbiota composition of each person [10]. Similar tendency was observed in a Spanish study involved 249 individuals, and the results also found that the *Coriobacteriaceae* family might be the genera to discriminate the urolithin metabotypes [11]. The biotransformation process of EA was complex *in vivo*, and only a few strains were discovered to be capable to transform EA into some certain urolithins (mainly urolithin A) in a relatively low converting rate with the information on the crucial metabolic enzymes remained unclear until now [12-14]. Besides, significant differences in the physicochemical property and biological activities of urolithins were observed owing to the molecular structural discrepancy among urolithins [15]. Consequently, bioactive responses of individuals to the EA supplement greatly depended on their biotransformation ability and the production profiles of urolithins.

Dietary intervention is considered as a productive strategy to promote the microbiome-mediated EA biotransformation process. A study investigated twenty healthy participants consumed 1000 mg of pomegranate extract for four weeks, and found the urolithin production profiles of volunteers were related to the microflora composition changed by the daily supplement, indicating that pomegranate extract could act as the prebiotics to promote urolithins productions [16]. Polysaccharides possessed numerous biological benefits for gut health, especially regulating intestinal bacteria and producing metabolic prebiotics [17-19], which could be desired dietary supplements to alter EA metabolism. Our previous studies lucubrate four algal bioactive polysaccharides (APs). The results showed that the anti-digestive APs displayed commendable regulation effects of intestinal flora and combinations with natural biological macromolecules greatly improved the water dispersion behaviors of EA [20-23]. Hence, we came up a hypothesis in current study that APs as the

exogenetic dietary supplement could adjust the biotransformation process of EA and achieve more urolithins productions through altering the gut bacteria and adjusting the bio-accessibility of EA to bacteria. *In vitro* fecal fermentation experiment was executed. Then, the metabolite production, microbiome, and the in-depth factors affecting the biotransformation process were explored. Furthermore, mono-/co-culture fermentations were carried out to investigate the adjustment mechanism. The experiment was executed referred to Scheme 1. The results lay a foundation to support that dietary supplements to regulate the microflora could be an effective strategy to enhance expectable health benefits of EA beyond the inter-individual variability in public.

2. Method and materials

2.1 Material and reagent

Sargassum fusiforme, *Scagassum*, *Undaria Pinnatifida* and *Fucus vesiculosus* were bought from Qingdao Fuxingxiang Import & Export Co., Ltd. (Shandong, China). EA (purity ≥ 96%), urolithin A (purity ≥ 98%), urolithin C (purity ≥ 95%), urolithin M5 (purity ≥ 95%) and fructooligosaccharide were purchased from Shanghai Yuanye Bio-technology Co., Ltd. (Shanghai, China). Urolithin M6 (purity ≥ 92%), urolithin M7 (purity ≥ 95%) and isourolitin A standard (purity ≥ 98%) were purchased from Weikeqi Bio-technology Co., Ltd. (Sichuan, China). 1-Methyl-2-pyrrolidinone (NMP, purity was ≥ 98%) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd (Shanghai, China). LC-MS grade acetonitrile (ACN) and methanol was purchased from Fisher Scientific (Loughborough, UK). Formic acid was obtained from Aladdin (Shanghai, China). Ultrapure water was generated using a Milli-Q system (Millipore, Bedford, USA)

Strain: *Gordonibacter urolithinfaciens* BNCC 373913 (*G. uro*), *Venillonella atypica* BNCC 379565 (*V. aty*), *Enterococcus faecalis* BNCC 102668 (*E. fae*), *Lachnospiraceae bacterium* BNCC 354474 (*L. bac*) and *Clostridium sp* BNCC 195293 (*C. sp*) were purchased from BeNa Culture Collection (BNCC, China).

2.2 Preparations of APs

APs were acquired and characterized by our previous study [20-22]. Briefly, the seaweeds were thoroughly washed with running tap water to remove the sediments. After drying at 40 ℃ in the oven, the samples were crushed and sifted through a 40 mesh sieve to obtain powders. Then powders with distilled water (1:20; w/v) were prepared to extract APs at 90 ℃ for 2 h, and then centrifugated at 2000g for 20 min, four-folds absolute ethyl ethanol precipitation, dialysis and freeze-drying. The polysaccharides from *Sargassum fusiforme*, *Scagassum*, *Undaria Pinnatifida* and *Fucus vesiculosus* were named SFP, SCP, UPP and FVP, respectively. The structural characterization of APs was determined according to the previous study and the results were shown in Supplement Table S1.

2.3 Preparations and characterization of EA solid dispersion

Solid dispersion is one of the delivery systems to improve the solubility and dispersity of poorly water-soluble drugs. EA solid dispersions were prepared referred to our previous study with minor modifications. Briefly, APs were fully dissolved in deionized water at 4 ℃ overnight. Then quantitative EA was added to APs solution, and the final concentration of mixture was 1.5% (w/v). The mixed sample was

ground by a planetary ball mill (Changsha Tencan Powder Technology Co., ltd., China) with a polyurethane-milling tank (250 mL) for 24 h, and the milling speed was 200 r/min. After the completion of milling treatment, the solid dispersions were freeze-dried. The solid dispersions were named based on the APs and the ratios of EA/APs. Their water dispersion behaviors were determined referred to the previous study, and the results were shown in Fig. S1.

2.4 APs supplement to EA biotransformation in vitro

In vitro fermentation was carried out to investigate whether APs supplement affected EA biotransformation through alteration of gut bacteria. Concretely, EA basal medium was consisted of 37.0 g/L brain heart infusion, 0.7 g/L L-cysteine, 1.0 mL/L 1% resazurin solution and 1 g/L EA. Then, FVP, SFP, SCP, UPP and fructooligosaccharide (as the prebiotics control) were severally supplemented to EA basal medium at 10 g/L to obtain different supplement medium. The preparations were executed under anoxic conditions at 37 ℃, and the media were split 9 mL to vitreous anaerobic tubes. The tubes containing media were autoclaved at 121 ℃ for 20 min.

Then ten volunteers (five females and five males, regular diets without any antibiotics for >3 months) were recruited to donate their fecal samples. Fecal samples with sterilized saline (feces: saline = 1:9 (w/v)) were mixed and homogenized. The mixtures were filtered by sterile gauze to collect fecal suspension. 10 mL of fermentation system was inoculated with 10 % fecal bacteria (1 mL) under the anaerobic conditions. The fermentation groups were as followed: CK group referred to that the fecal bacteria inoculated in the EA basal media with no other supplement, FVP group referred to that the fecal bacteria inoculated in the EA basal media with FVP supplement, SFP group referred to that the fecal bacteria inoculated in the EA basal media with SFP supplement, SCP group referred to that the fecal bacteria inoculated in the EA basal media with SCP supplement, UPP group referred to that the fecal bacteria inoculated in the EA basal media with UPP supplement, and FOS group referred to that the fecal bacteria inoculated in the EA basal media with fructooligosaccharide supplement. The aliquots were incubated in a 37 ℃ incubator for 5 days anaerobically in quintuplicate (n = 5). At the end of fermentation, the pH of samples was detected by the S400 pH meter (Mettler Toledo, China), the OD_{600} of samples was measured by a microplate reader (Shanghai Flash Spectrum Biotechnology Co. ltd, China) with the wavelength of 600 nm, and the reducing sugar contents were determined by the previous method.

2.5 EA solid dispersion supplement to EA biotransformation in vitro

Another 5-day *in vitro* fermentation was carried out to investigate the impacts of accessibility from substrate to microorganisms on EA biotransformation. Basal medium was consisted of 37.0 g/L brain heart infusion, 0.7 g/L L-cysteine and 1.0 mL/L 1% resazurin solution. Then, EA (1.0 g/L), EA/UPP 3-1 (1.3 g/L), EA/UPP 1-1 (2.0 g/L), EA/UPP 1-3 (4.0 g/L) and physical mixture (2 g/L, physical mixture was prepared by fully blending equal EA and UPP by a vortex for 1 min) were severally supplemented to basal medium to obtain different solid dispersion supplement medium. The split and autoclaving of media and the preparations of another fecal suspension were the same as Section 2.4.

Next, 10 mL of fermentation system was inoculated with 10 % fecal bacteria (1 mL) under the anaerobic conditions. The fermentation groups were as followed: EA group referred to that the fecal bacteria inoculated in the basal media with EA supplement, EA/UPP 3-1 group referred to that the fecal bacteria inoculated in the basal media with EA/UPP 3-1 supplement, EA/UPP 1-1 group referred to that the fecal bacteria inoculated in the basal media with EA/UPP 1-1supplement, EA/UPP 1-3 group referred to that the fecal bacteria inoculated in the basal media with EA/UPP 1-3 supplement, and PM 1-1 group referred to that the fecal bacteria inoculated in the basal media with physical mixture supplement. The aliquots were incubated in a 37 ℃ incubator for 5 days anaerobically in quintuplicate (n = 5). After fermentation, the broths were collected to determine the urolithin productions and bacteria profiles.

2.6 Determination of chemical composition in broths

The contents of urolithins were determined using HPLC system (Thermo Fisher Scientific, USA) with an Acclaim120 C18 column (4.6 mm × 250 mm, 5 μm, Thermo Fisher Scientific, USA) at 30 ℃ and a UV detector according to the previous study.

The contents of lactic acid were detected by a lactic acid assay kit (Nanjing Jiancheng, China). The contents of short chain fatty acids (SCFAs) were determined as followed: 250 μL of broth was suctioned and fully mixed with 1 mL methanol by ultrasonic processing for 30 min. Then the mixed suspension was maintained at 4 ℃ overnight. The supernatant was collected after centrifugation at 12000g for 15 min, and the sufficient anhydrous Na_2SO_4 was added to remove the water in samples. Then the sample was centrifuged and the supernatant was filtered using a 0.22 μm PTFE membrane for GC-MS detection (Thermo Fisher Scientific, USA). A TP-WAX capillary column (30 m × 0.25 mm i.d. × 0.32 μm film thickness, Thermo Fisher Scientific, USA) was utilized for the separation. The injection volume was 1 μL. Helium used as a carrier gas with the flow rates of 1 mL/min. The oven program was adopted at an initial temperature 100 ℃ for 15 min, increased to 150 ℃ at a rate of 5 ℃/min, increased to 240 ℃ at a rate of 30 ℃/min, and maintained at 240 ℃ for 8 min. The temperatures of the flame ionization detector and injector were 250 ℃.

2.7 Gut microbe analysis by 16S rRNA amplicon sequencing

16S rRNA amplicon sequencing was performed on bacterial DNA extracted from the fermentation broths by the Illumina platforms (Illumina, USA). After extraction, Nanodrop (Thermo, USA) and electrophoresis with 1.2% agarose gel were used to quantify the DNA. The hypervariable V3 and V4 regions were amplified using the 338F (5'-CCTACGGRRBGCASCAGKVRVGAAT-3') and 806R (5'-GGACTACNVGGGTWTCTA ATCC-3') primer pair. The amplied DNA was sequenced by MiSeq (Illumina, USA) according to the protocol. Raw paired-end reads from the MiSeq platform were initially filtered, denoised and merged with QIIME2 (Ver. 2019.4). The composition and taxonomic classification of genome in each sample was generated using R software (Ver. 4.4.1). The overall compositional changes of intestinal flora were analyzed by principal coordinates analysis (PCoA) using the Bray-Curtis distance. Clustering analysis based on euclidean distance was performed to compare species composition differences

among samples using the heatmaps. The relationship between the relative abundance of microorganisms and biochemical profiles was revealed by correlation analysis.

2.8 Mono-culture fermentation

Strain activation: The Columbia blood agar medium was prepared with 23 g/L peptone special, 1 g/L starch soluble, 5 g/L NaCl, 10 g/L agar and 5% defidrinated sheep blood. The final pH of the medium was adjusted to 7.3. The sterile medium was inoculated with *G. uro*, *V. aty*, *E. fae*, *L. bac* and *C. sp*, respectively. And incubated in an anaerobic bag at 37℃ for 3 days. Then added sterile water to the plate, used coating rod to scrape bacterial moss, make bacterial suspension and transferred to sterile BHI fluid medium. After cultured at 37℃ for 18 hours, the bacteria were diluted to an optical density measured at a wavelength of 600 nm value around 0.3 for subsequent experiments.

Strain proliferation: The basic medium consisted of 5 g/L casitone, 1.25 g/L yeast extract, 0.45 g/L NaCl, 0.045 g/L $MgSO_4 \cdot 7H_2O$, 0.045 g/L $CaCl_2$, 0.225 g/L K_2HPO_4, 0.225 g/L KH_2PO_4 and 0.5 mg/L resazurin. APs/fructooligosaccharide/glucose were severally added as the carbon source to basic medium to investigate the proliferation of strains. According to the supplements to the media, the groups were divided in to Blank group (no other supplement), FVP group (FVP supplement, 10 g/L), SFP group (SFP supplement, 10 g/L), SCP group (SCP supplement, 10 g/L), UPP group (UPP supplement, 10 g/L), FOS group (fructooligosaccharide supplement, 10 g/L) and GLU group (glucose supplement, 10 g/L). The activated *G. uro* suspension (100 μL) and sterile fluid medium with APs (100 μL) were added in 96 well microtiter plates and placed at 37 ℃ in anaerobic environment. The OD_{600} values were detected at 0, 12, 18, 30, 36 and 48 h.

EA metabolism by strains: The media and grouping situation were prepared referred to Section 2.4 and Section 2.5. Then the activated strain suspension (1 mL) was added to vitreous anaerobic tubes (containing 9 mL corresponding sterile fluid medium). The aliquots were incubated in a 37 ℃ incubator for 5 days anaerobically in quintuplicate (n = 3). At the end of fermentation, the urolithin productions in broths were detected by HPLC method.

2.9 Co-culture fermentation

The activated strains with the OD_{600} around 0.3 in Section 2.8 were equivalently mixed, and the groups and media were prepared referred to Section 2.4 and Section 2.5. Then the mixed suspension (1 mL) was added to vitreous anaerobic tubes (containing 9 mL corresponding sterile fluid medium). The aliquots were incubated in a 37 ℃ incubator for 5 days anaerobically in quintuplicate (n = 3). At the end of fermentation, the urolithin productions in broths were detected by HPLC method.

2.10 Statistical analysis

The data was analyzed by SPSS (version 23.0) software using a one-way analysis of variance (ANOVA) test followed by Duncan's test to calculate the differences. Results were expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Effects of APs supplement on the growth of gut bacteria

The changes of pH, OD_{600} and reducing sugar reflected the fermentation process. As shown in Fig. 1A-B, the initial pH of the basic media (CK group) was neutral but others decreased slightly because of the addition different acidic polysaccharides to media. After 5-day anaerobic fermentation, the pH of all groups decreased due to the carbohydrate degradation by gut bacteria. Specifically, the greatest reduction of pH was observed in FOS group ($-\Delta pH = 3.04 \pm 0.02$), followed by UPP group ($-\Delta pH = 0.77 \pm 0.05$) ($P < 0.05$), indicating acidic compounds produced in broths. OD_{600} value was usually used to represent the turbidity of solutions. In current study, the bacteria and hydrophobic EA were both suspending in fluid media. Thus, the OD_{600} value reflected the microbial growth and the dispersity of EA in fermentation broth, which was showed in Fig. 1C. The initial bacterial concentration was standardized in all groups, but the initial OD_{600} values varied among groups. The initial OD_{600} values of FVP group, SCP group and SFP group were significantly higher than other groups ($P < 0.05$). It suggested that the interactions between the above APs and EA would affect the dissolution behavior of EA, leading to more chances for EA to contact with the suspending bacteria at the initial stage. To clearly compare the bacteria growth among the groups, the ΔOD_{600} values were showed in Fig. 1D. The ΔOD_{600} values were all positive, indicating the promotion of intestinal microorganisms and consumption of poor soluble polyphenols, and the SFP group exhibited the best effect, followed by FVP, SCP, UPP group. While there was no obvious difference in ΔOD_{600} values between CK group and FOS group ($P < 0.05$). Combined with the results of urolithin productions in Table 1, few urolithins were generated in FOS group, hinting that the proliferation effect of fructooligosaccharide targeted on the bacteria which could not participate in EA metabolism and with the consumption of nutrition in medium after 5-day fermentation, these bacteria went to death phase. The changes of reducing sugar content could indicate the consumption of carbohydrates by the microbial community. In Fig. 1E-F, the -Δreducing sugar contents of CK group, FVP group, SFP group, SCP group, UPP group, and FOS group were 10.62 mg/mL, 10.06 mg/mL, 11.27 mg/mL, 10.92 mg/mL, 11.74 mg/mL and 20.71 mg/mL, respectively. On the one hand, the gut bacteria could hydrolyze carbohydrate to produce reducing sugars. On the other hand, the microflora utilized reducing sugars for their growth, which was consistent with previous studies. These changes during the fermentation would suggest that of APs/FOS supplement to the media would change the bacteria growth.

Table 1 Effects of APs supplements on urolithin productions in fermentation broths. Data are expressed as mean ± SD (n = 5). Significant differences ($P < 0.05$) are indicated with different letters. Nd. means not detected.

Group	Contents (μmol)						
	Urolithin M5	Urolithin M6	Urolithin M7	Urolithin C	Urolithin A	Isourolithin A	Urolithin B
CK	0.15 ± 0.12 ^{a}	0.55 ± 0.23 ^{a}	0.26 ± 0.04 ^{a}	1.05 ± 0.31 ^{ab}	1.64 ± 0.27 ^{a}	1.88 ± 0.34 ^{a}	0.90 ± 0.11 ^{a}
FVP	0.08 ± 0.03 ^{a}	0.29 ± 0.13 ^{b}	Nd.	1.10 ± 0.26 ^{b}	Nd.	4.06 ± 0.13 ^{d}	Nd.
SFP	0.11 ± 0.02 ^{a}	0.32 ± 0.06 ^{b}	Nd.	2.23 ± 0.12 ^{c}	Nd.	0.37 ± 0.13 ^{b}	0.53 ± 0.05 ^{b}
SCP	0.32 ± 0.06 ^{b}	0.35 ± 0.08 ^{ab}	0.15 ± 0.07 ^{b}	1.12 ± 0.22 ^{b}	1.01 ± 0.33 ^{b}	3.06 ± 0.62 ^{c}	0.48 ± 0.01 ^{b}
UPP	0.27 ± 0.09 ^{b}	0.35 ± 0.11 ^{ab}	0.16 ± 0.05 ^{b}	0.74 ± 0.11 ^{a}	1.03 ± 0.22 ^{b}	1.58 ± 0.40 ^{a}	0.85 ± 0.12 ^{a}
FOS	Nd.	Nd.	Nd.	Nd.	Nd.	Nd.	Nd.

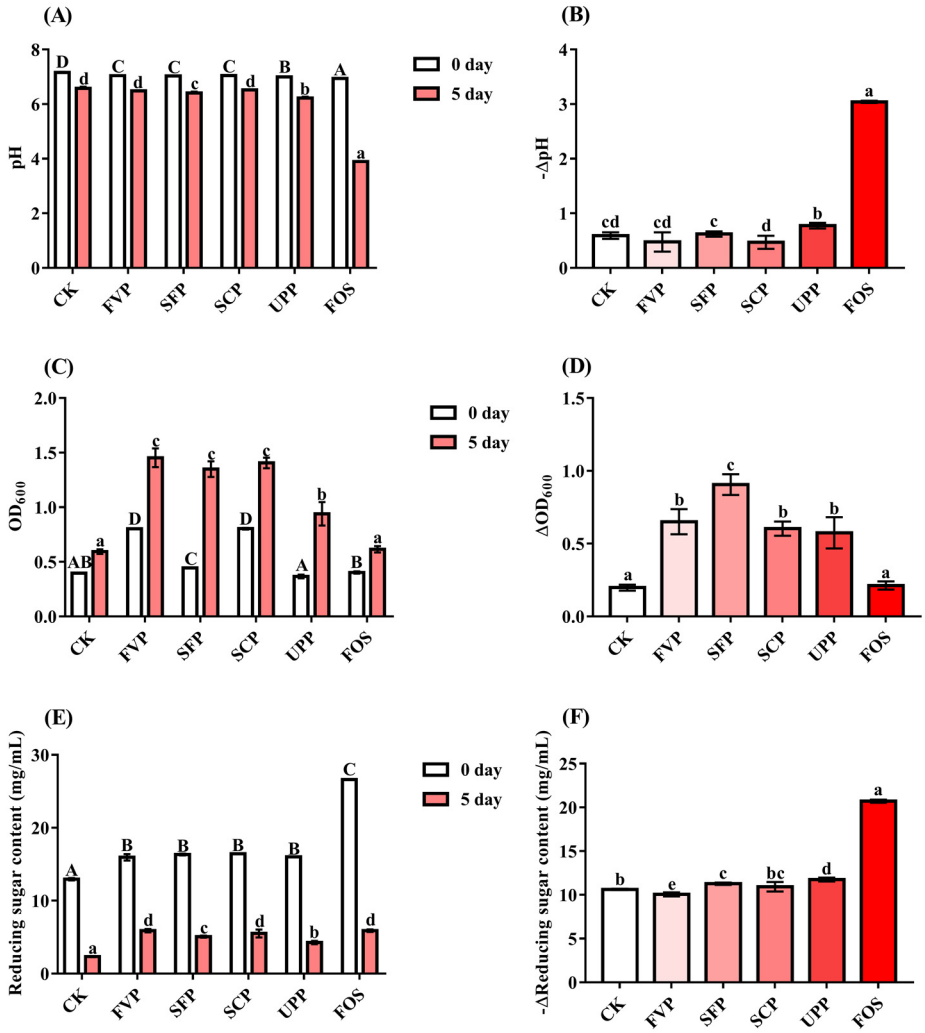


Fig. 1 Effects of APs supplement on pH (A-B), OD_{600} (C-D) and reducing sugar contents (E-F). Data are expressed as mean ± SD (n = 5). Significant differences ($P < 0.05$) are indicated with different letters.

3.2 Effects of APs supplement on urolithin productions

Urolithins were the metabolites of EA by gut bacteria. Table 1 showed the urolithin contents in broths. In CK group, urolithin M5, urolithin M6, urolithin M7, urolithin C, urolithin A, isourolithin A and urolithin B were found after 5-day anaerobic fermentation. In current study, three final urolithins in the metabolic pathway were all detected in CK group, indicating that the volunteers who donated the fecal samples covered the common metabotypes. The levels of urolithin A, isourolithin A and urolithin B were relatively higher, which meant that 5-days fermentation brought the biotransformation process of EA into the latter stage. FOS was a common prebiotic with the proliferation effect on beneficial bacteria and inhibition effect on harmful bacteria, contributing to usual use as the positive control in fecal fermentation test. However, the current result showed that no urolithin was detected in FOS group. The reason might be that the large proliferations of other bacteria and some unexpected secondary metabolites caused by the addition of FOS suppressed the colonization and growth of strains participated in the metabolic pathways from EA to urolithins. In Table 1, when compared to CK group, the isourolithin A levels in FVP group and SCP group were significantly increased ($P < 0.05$), the urolithin C level in SFP group was notably elevated ($P < 0.05$), and the urolithin M5 levels of SCP group and UPP group significantly increased ($P < 0.05$). Both urolithin M7 and urolithin A were

not found in FVP and SFP group, and no urolithin B was detected when FVP adding to the media. These results showed that APs addition considerably altered the metabolism process of EA.

3.3 Effects of APs supplement on organic acid productions

SCFAs are regarded as one the most important microbial metabolites, which play an essential role in maintaining the dynamic balance of intestinal flora and energy metabolism [24]. Fig. S2A-F showed the SCFA contents in fermentation broths. In all the groups, acetic acid and propionic acid accounted for the major proportion of total SCFAs. Among the groups, UPP group produced the most contents of acetic acid, propionic acid, butyric acid and isovaleric acid, contributing to significantly highest total SCFA contents ($P < 0.05$). When compared with CK group, the butyric acid level of SFP group significantly increased, and the valeric acid levels of FVP group and SCP group notably elevated ($P < 0.05$). After 5-day fermentation, the pH value of FOS group was the lowest, while SCFA contents were also lowest among the groups. Same tendency was found in previous study, where FOS group exhibited the lower pH but produced lower SCFAs when compared to polysaccharide samples throughout the fermentation process [25]. The results indicated that the low pH environment in FOS group might be attributed to the productions of other acidic compounds. In Fig.4A-B, results showed that the large proliferation of *Lactobacillaceae* occurred in FOS group, thus the lactic acid contents were also determined in the current study. Fig. S2E showed the lactic acid contents of the broths, and a significantly elevation was observed in the FOS group when compared to CK group, manifesting that FOS supplement to the fermentation media could proliferate the *Lactobacillaceae* and stimulate the accumulation of lactic acid. In summary, the organic acid production was greatly affected by the addition of carbohydrates and might in turn regulate the intestinal microflora to adjust the metabolism of EA.

3.4 Effects of EA solid dispersions on EA biotransformation process

EA was converted into urolithins by gut bacteria, but the conversion degree and efficiency were largely limited by little exposure to microorganisms due to hydrophobicity of EA. Therefore, improving the water dispersion behaviors of EA could be a feasible strategy to promote EA biotransformation. For example, Rocío et al. demonstrated that 1% DMSO would enhance the solubility of EA in medium and lead to more metabolic intermediate productions [26]. However, organic solvents were unacceptable in the food manufacturing industry. APs were nontoxic and edible biological macromolecules with high viscosity and biocompatibility, which could be a desired transport vehicle of EA. Our previous study also showed that AP could increase the solubility and dispersity of EA by form edible EA solid dispersions, and the EA solid dispersions improved the *in vitro* EA biotransformation process due to the advance of microbial accessibility in EA dispersion [23]. In the preliminary experiment, we prepared four AP-EA SDs by ball milling and their characteristics were detected. Fig. S1A was the observation of AP-EA SDs and EA at the concentration of 0.1% (w/v), and the picture showed that precipitations were observed in the EA, FVP-EA SD, SFP-EA SD and SCP-EA SD solutions. Fig. S1B showed that UPP-EA SD exhibited the best performance in forming stable EA solid dispersions and enhancing its dispersity. UV-vis spectra (Fig. S1C) showed that the hyperchromic effects occurred in AP-EA SDs, supporting that AP-EA SDs could improve the EA dispersity. Considering the above results, UPP was

selected to further study the impacts on EA biotransformation process *in vitro* fecal fermentation by preparing three ratios of EA/UPP (1/3, 1/1, 3/1) SDs through water ball milling method.

In Fig. 2, significant differences were observed in urolithin productions among groups. As compared to EA group, EA/UPP solid dispersions greatly enhanced the contents of urolithin M5, urolithin M6 and urolithin C ($P < 0.05$). Among the EA/UPP solid dispersions, EA/UPP 3/1 showed the weaker capacity to suspend EA in fluid media, which might result in the lower contents in urolithin M5, urolithin M6 and urolithin M7. The results indicated that the stably dispersible substrate in media would increase the accessibility to suspending bacteria and consequently improve the metabolite productions. To avoid the effects of UPP supplement in solid dispersions on the fermentation, physical mixture of EA and UPP at the equal quantity (PM 1-1) was prepared to added to the basic media and fermented as the control. When compared to EA/UPP group, the levels of urolithin M5, urolithin M6 and urolithin C in PM 1-1 group were significantly decreased ($P < 0.05$), suggesting that the EA/UPP solid dispersions would notedly improve EA biotransformation process mainly due to their ability to enhance EA dispersity. EA/UPP SDs improved total urolithins contents from 4.25 μmol to 6.16-6.79 μmol. These results showed that EA/UPP solid dispersions could largely increase urolithin productions by enhancing more chances for EA to be exposed to active bacteria.

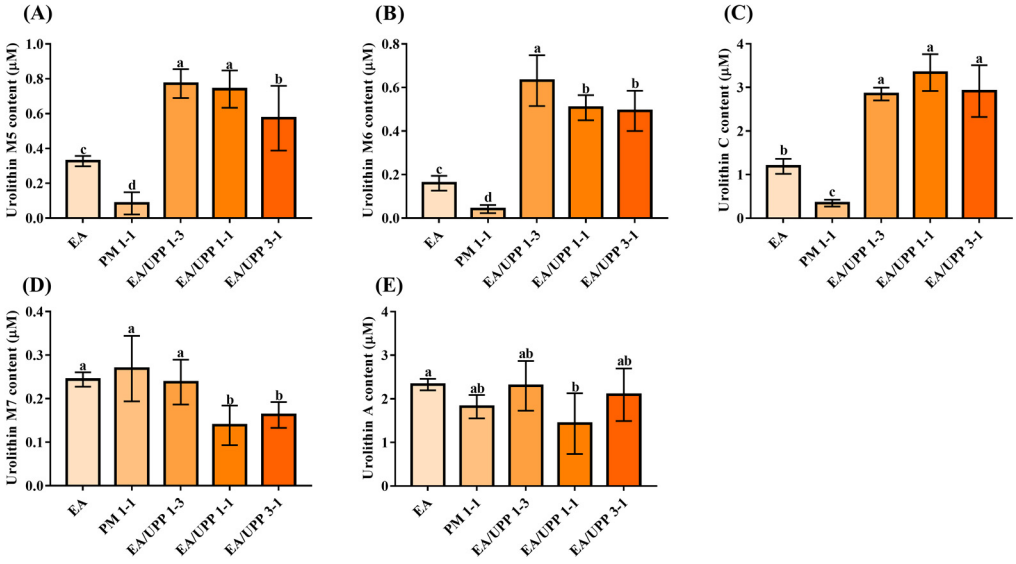


Fig. 2 Effects of EA solid dispersion supplements on urolithin M5 (A), urolithin M6 (B), urolithin C (C), urolithin M7 (D) and urolithin A (E) contents in fermentation broths. Data are expressed as mean ± SD (n = 5). Significant differences ($P < 0.05$) are indicated with different letters.

3.5 Gut bacteria analysis

16S rRNA gene sequencing analysis was executed in order to investigate the potential impacts of APs/EA solid dispersions supplements on the gut microflora reconstruction.

3.5.1 Effects of APs supplement on gut microflora

It was apparent that the relative microbial profile changed in Fig. 3A-B. Owing to the different carbohydrate supplements to the culture media, the bacterial compositions of the groups differed substantially. At the family level, the relative abundance of *Ruminococcaceae*, *Streptococcaceae* and *Enterococcaceae*

significantly increased in UPP, SCP, SFP and FVP group, while the relative abundance of *Veillonellaceae* and *Lachnospiraceae* markedly decreased as compared to CK group. Besides, the relative abundance of *Bacteroidaceae* and *Enterobacteriaceae*_A observably elevated in SCP, SFP and FVP group, and the relative abundance of *Fusobacteriaceae* increased in SCP and FVP group. Particularly, the bacterial compositions displayed an extreme variation in FOS group. Concretely, the relative abundance of *Lactobacillaceae*, *Bifidobacteriaceae*, *Streptococcaceae* and *Peptostreptococcaceae* increased sharply, but the relative abundance of *Veillonellaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Enterobacteriaceae*_A and *Ruminococcaceae* decreased significantly. Similar tendency was noticed at the genus level. UPP supplement remarkably increased the relative abundance of *Faecalibacterium*, SCP and FVP supplements increased the relative abundance of *Streptococcus*, *Escherichia* and *Fusobacterium*_A, and SFP significantly increased the relative abundance of *Klebsiella*. In addition, FOS notedly increased the relative abundance of *Lactobacillus*, *Limosilactobacillus*, *Bifidobacterium* and *Streptococcus*.

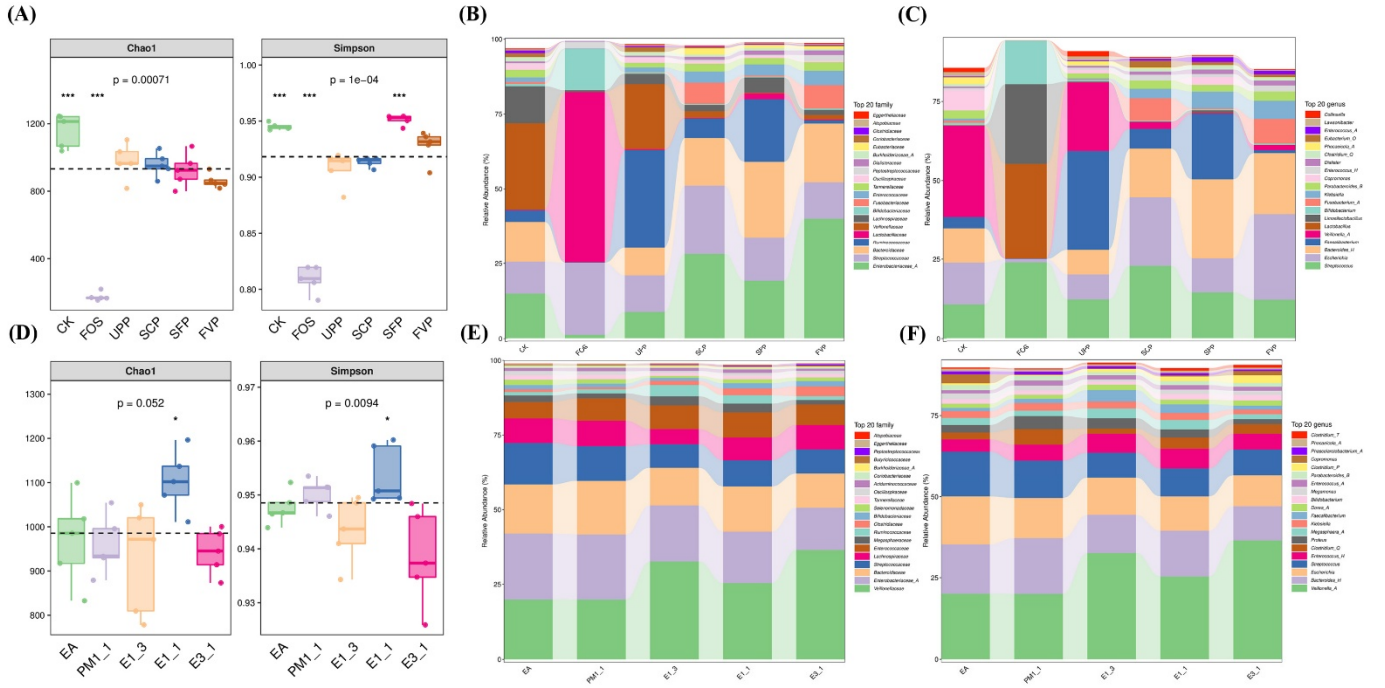


Fig. 3 α-Diversity (A, D), and relative abundance of family (B, E) and genus (C, F) in fermentation broths of different experimental groups. Data are expressed as mean ± SD (n = 5). PM1_1 referred to PM 1-1 group, E1_3 referred to EA/UPP 1-3 group, E1_1 referred to EA/UPP 1-1 group, E3_1 referred to EA/UPP 3-1 group.

In Fig. 3C, Chao1 richness and Shannon index were calculated to evaluate the microbial diversity. Higher values of Chao 1 and Shannon index severally suggested that samples kept greater species richness and microbial diversity. As compared to CK group, the Chao1 and Shannon index dramatically decreased in FOS group, manifesting that the microbial richness and diversity were both lower after 5-day fermentation with FOS. It might result from that the large proliferations of *Lactobacilus*, *Limosilactobacillus* and *Bifidobacterium* and over-accumulation of organic acid to acidic microenvironment inhibited the microflora growth. Interestingly, the *α*-diversity of UPP, SCP, SFP and FVP group slightly decreased when compared to CK group with no significant differences. The reason might be that the 5 days fermentation could bring the biotransformation process of EA into the latter stage but the majority of microbes went through the

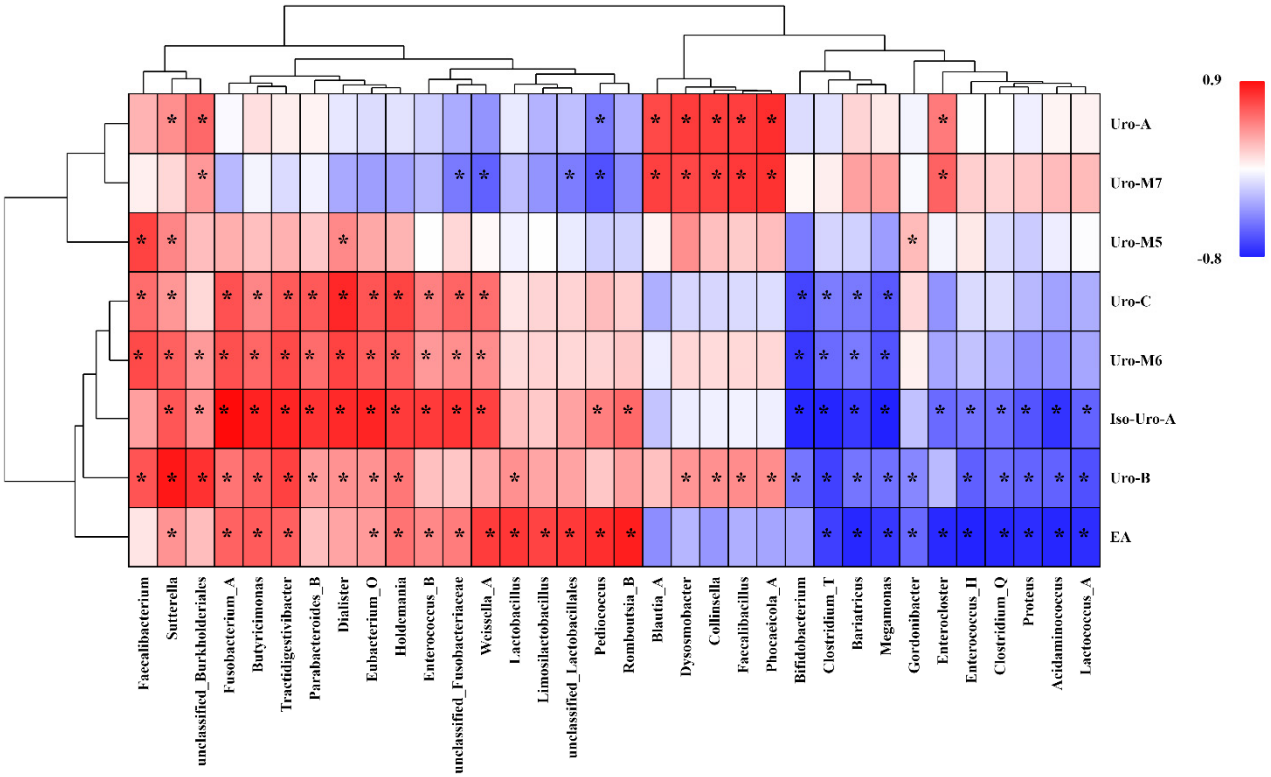
logarithmic and stable growth phase and turned into the declining phase. APs and FOS possessed gut microflora proliferation and regulation capacity, and their supplements would accelerate the microbe growth stage, thus leading to the α-diversity reductions after 5-day fermentation as compared to CK group. These results revealed that APs supplement could modulate the intestinal bacteria.

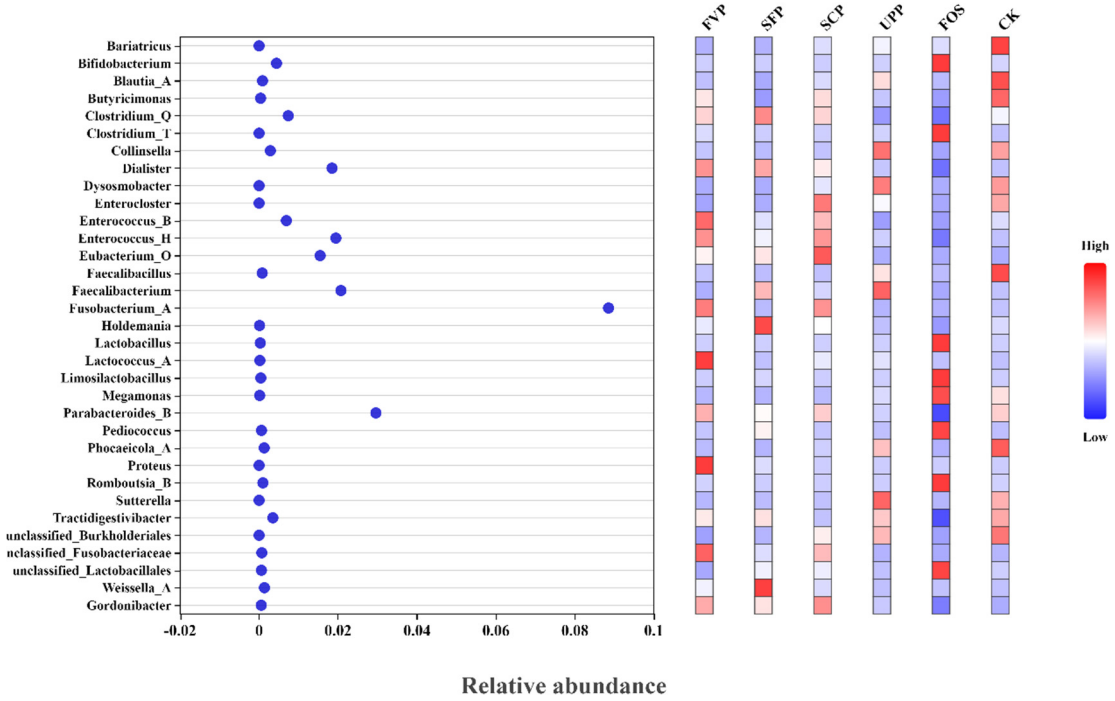
3.5.2 Effects of EA solid dispersions supplement on gut microflora

Fig. 3D-E revealed the relative microbial abundance in fermentation groups with different additive forms of EA. All groups shared same gut flora compositions, while their relative abundances were slightly different. At the family level, *Streptococcaceae* and *Lachnospiraceae* significantly decreased in PM 1-1 group, EA/UPP 1-3 group, EA/UPP 1-1 group and EA/UPP 3-1 group as compared to EA group. The relative abundance of *Ruminococcaceae* and *Veillonellaceae* markedly increased in EA/UPP 1-3 group, EA/UPP 1-1 group and EA/UPP 3-1 group. At the genus level, EA/UPP 1-3 and EA/UPP 1-1 supplements remarkably increased the relative abundance of *Faecalibacterium*, *Megasphaera*_A. and *Veillonella*_A, and significantly decreased the relative abundance of *Streptococcus*, *Bacteroides*_H and *Escherchia* when compared to EA group. In Fig. 3F, the Chao1 richness and Shannon index of EA/UPP 1-1 group were significantly elevated ($P < 0.05$), indicating that EA/UPP 1-1 supplement could enhance the microbial richness and diversity. As a result, the groups with different additive form of EA exhibited minor difference in gut microflora profiles than experimental groups with different carbohydrate supplements, while considerable differences in urolithin productions among the groups. It hinted that pivotal bacteria participated in EA metabolism and urolithin productions might occupy a non-dominant position. Therefore, the relationships between the bacteria changes and EA biotransformation process were further investigated by Spearman's correlation analysis.

3.6 Correlation between urolithin profiles and gut microflora

According to previous studies, the information of the key microorganism and enzymes during the EA biotransformation still remained unclear, leading to the difficulty to full utilization of EA and mass production of urolithins [8]. To further investigate the potential relationship between urolithin productions and gut microflora, Spearman's correlation was used in current study to explore EA-metabolic-related bacteria and partial results (correlation coefficient $|r| > 0.7$, FDR adjusted $P < 0.05$) were showed in Fig. 4A. Results showed that suspending EA contents were significantly associated with the relative abundance of *Sutterella*, *Fusobacterium*_A, *Butyricimonas*, *Tractidigestivibacter*, *Eubacterium*_O, *Holdemania*, *Enterococcus*_B, unclassified_*Fusobacteriaceae*, *Weissella*_A, *Lactobacillus*, *Limosilactobacillus*, unclassified_*Lactobacillales*, *Pediococcus* and *Romboutsia*_B, and negatively related with the relative abundance of *Clostridium*_T, *Bariatricus*, *Megamonas*, *Gordonibacter*, *Enterocloster*, *Enterococcus*_H, *Clostridium*_Q, *Proteus*, *Acidaminococcus* and *Lactococcus*_A, suggesting that the above bacteria might participate in EA metabolism. For one thing, more suspending EA meant more chances to selectively proliferate bacteria which used EA as the substrate. For another thing, proliferation of the EA-metabolic-related bacteria would utilize EA to produce urolithins, leading to the decrease of EA.


0.9
-0.8
Uro-A
Uro-M7
Uro-M5
Uro-C
Uro-M6
Iso-Uro-A
Uro-B
EA
Faecalibacterium
Sutterella
unclassified_Burkholderiales
Fusobacterium_A
Butyricimonas
Tractidigestivibacter
Parabacteroides_B
Dialister
Eubacterium_O
Holdemania
Enterococcus_B
unclassified_Fusobacteriaceae
Weissella_A
Lactobacillus
Limosilactobacillus
unclassified_Lactobacillales
Pediococcus
Romboutsia_B
Blautia_A
Dysosmobacter
Collinsella
Faecalibaculum
Phocaeicola_A
Bifidobacterium
Clostridium_T
Bariatricus
Megamonas
Gordonibacter
Enterocloster
Enterococcus_H
Clostridium_Q
Proteus
Acidaminococcus
Lactococcus_A


Bariatricus
Bifidobacterium
Blautia_A
Butyricimonas
Clostridium_Q
Clostridium_T
Collinsella
Dialister
Dysosmobacter
Enterocloster
Enterococcus_B
Enterococcus_H
Eubacterium_O
Faecalibaculum
Faecalibacterium
Fusobacterium_A
Holdemania
Lactobacillus
Lactococcus_A
Limosilactobacillus
Megamonas
Parabacteroides_B
Pediococcus
Phocaeicola_A
Proteus
Romboutsia_B
Sutterella
Tractidigestivibacter
unclassified_Burkholderiales
unclassified_Fusobacteriaceae
unclassified_Lactobacillales
Weissella_A
Gordonibacter
-0.02
0
0.02
0.04
0.06
0.08
0.1
Relative abundance
FVP
SFP
SCP
UPP
FOS
CK
High
Low

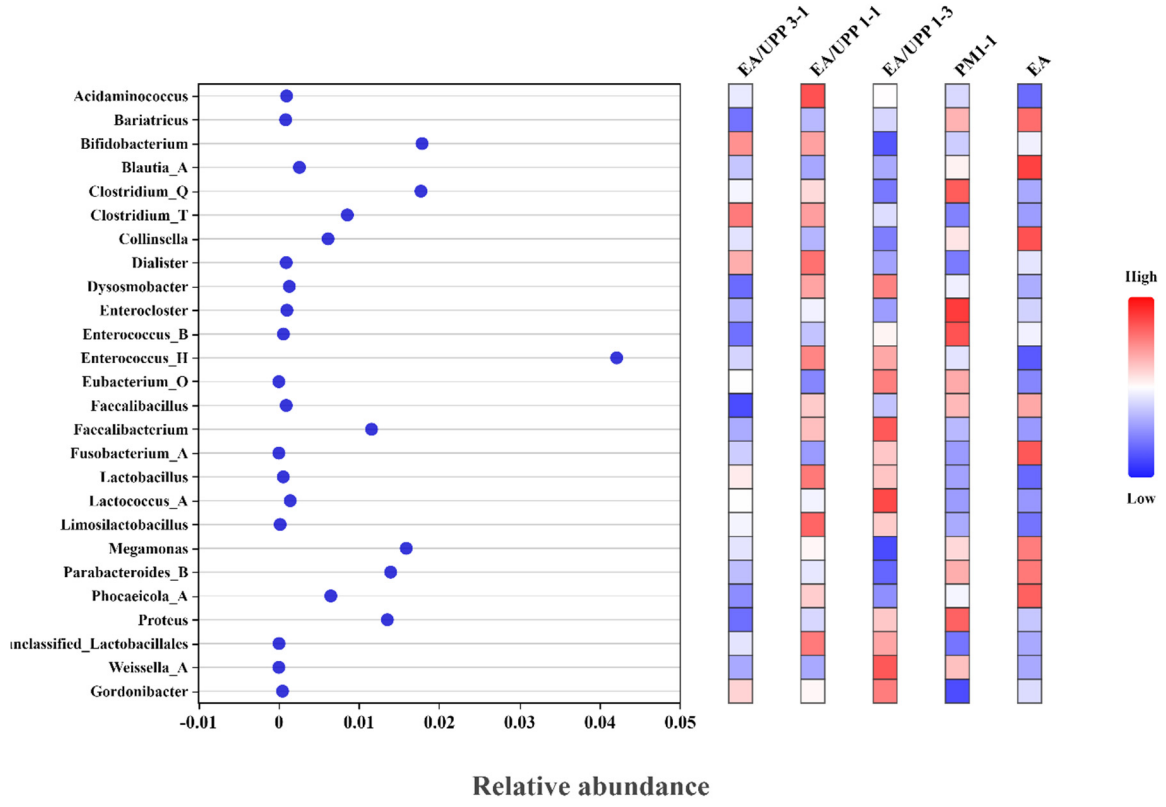


Fig. 4 Spearman's correlation between urolithin profiles and gut microflora (A), and the relative abundance of related bacteria among different groups (B-C). Only the significant strong correlations were drawn in the heatmap (|r| > 0.7, * FDR adjusted $P < 0.05$)

Ulteriorly, urolithin M5 contents showed a strongly positive relationship with the relative abundance of *Faecalibacterium*, *Sutterella*, *Dialister* and *Gordonibacter*, indicating the above bacteria might participate in the productions of urolithin M5. During EA metabolic pathway, urolithin M5 was firstly obtained through the lactone-ring cleavage and decarboxylation, and it was the succeeding substrate to acquire other urolithins. Our previous study showed that urolithin M5 exhibited better biological effects than other urolithins in *in vitro* experiments, suggesting that urolithin M5 was expected to become the prime drives to exert health-promoting functions when administering EA. Therefore, promotion of the biotransformation to obtain more urolithin M5 would be beneficial to enhance the biological effects of EA. In Fig. 4B, FVP and SCP supplements markedly increased the relative abundances of *Dialister* and *Gordonibacter*, while SFP and UPP supplements notedly enhanced the relative abundances of *Faecalibacterium* and *Sutterella*. In Fig. 4C, the relative abundances of *Faecalibacterium*, *Dialister* and *Gordonibacter* were elevated in EA/UPP solid dispersion groups as compared to EA group. These results showed that both APs supplement and EA solid dispersion intervention could proliferate the above bacteria to promote urolithin M5 productions.

In Fig. 4A, urolithin M6, urolithin C, isourolithin A and urolithin B shared similar correlations, which was opposite tendency of urolithin M7 and urolithin A. It indicated that urolithin M6, urolithin C, isourolithin A and urolithin B might be included in the same catabolic pathway by a series of certain strains, while the productions of urolithin M7 and urolithin A would be contributed to other strains. The contents of urolithin

M6, urolithin C, isourolithin A and urolithin B were strongly positively correlated with the relative abundance of *Sutterella*, *Fusobacterium*_A, *Butyricimonas*, *Tractidigestivibacter, Eubacterium*_O and *Holdemania*. The contents of urolithin M7 and urolithin A were positively related with the relative abundance of *Blautia*_A, *Dysosmobacter*, *Collinsella*, *Faecalibacillus* and *Phocaeicola*_A. In recent decades, researchers were devoted to identify the strains related to EA biotransformation. Previous study showed that three human intestinal microflora, *Gordonibacter urolithinfaciens*, *Gordonibacter pamelaeae* and *Ellagibacter isourolithinifaciens*, could involve in the metabolic pathways of EA [27]. Another study described an intestinal bacterium *Lactococcus garvieae* FUA009 could produce urolithin A from EA with high safety [14]. *Enterocloster* was also reported to be a urolithins-producing bacterium. A study assessed that the capacity of *Ellagibacter* and *Enterocloster* to colonize the intestine of rats and convert urolithin non-producers into urolithin-producers, and the results showed that the bacterial strains effectively improved the biotransformation ability [28]. A novel urolithin called urolithin G were detected when incubation with *Enterocloster* species [29]. However, the strains could not produce the downstream products and the transformation ratios were relatively low, and their enzymes and related genes were yet to be characterized. In Fig. 4B-C, it suggested that APs supplement and EA solid dispersions intervention could increase the abundances of *Lactococcus*, *Gordonibacter* and *Enterocloster*, resulted in more urolithin productions during the fermentation (Table 1, Fig. S2).

Except the reported strains, other potential EA-metabolic-related bacteria could be concluded from Fig. 4A. *Dialister* belonged to the order *Firmicutes*, and previous studies manifested that *Dialister* was found to be closely associated with the pathogenesis of major depressive disorder by probably affecting the SCFAs productions [30, 31]. *Agathobaculum*, *Dysosmobacter* and *Coprococcus* were butyrate-producing gut bacteria isolated from human feces, and their abundances were claimed to be related to urolithins production [32, 33]. *Clostridium* was a common genus of the metabolism of several phenolic compounds, and previous study suggested that the bacterium involved in urolithin production [26]. In current study, their relative abundances were strongly corresponded with urolithin contents, and Fig. 4B-C showed dietary supplement would increase their abundance, indicating that these bacteria might be responsible for urolithin productions. *Enterococcus* spp. was a type of lactic acid bacteria found in the intestinal flora of human and animals. For one thing, *Enterococcus* spp. could be applied as a probiotic to involve in the modulation of the immune system. For another thing, owing to the virulence and multi-drug resistance, *Enterococcus* spp. sometimes acted as the opportunistic pathogens [34]. It was proved that *Enterobacteriaceae* possessed the proinflammatory factor and participated in intestinal dysmetabolism. The overgrowth of the bacteria could cause the pathogenesis and progression in inflammatory bowel diseases [35]. The results hinted that the patients with inflammatory or pneumonia diseases might receive better reactions on the EA biotransformation ability when *Enterococcus* overgrew in their gut. Collectively, the results showed that some bacteria like *Gordonibacter*, *Faecalibacterium*, *Sutterella*, *Dialister*, *Enterocloster* and etc. might be EA-metabolic-related bacteria, and dietary supplement to proliferate these bacteria could be a useful method to realize high utilization of EA.

3.7 Mo-/Co-culture fermentation

Considering the correlation analysis, five purchasable commercial strains *G. uro*, *V. aty*, *E. fae*, *L. bac* and *C. sp* were used in mono-/co-culture fermentation to further verify whether these bacteria involve in EA metabolism and assess the impacts of dietary polysaccharides supplements on their promotion and transformation capacity. Firstly, results showed that in mono-culture fermentation, only *G. uro* possessed the capability of EA metabolism. *G. uro* had become a merchantable type strain in recent years, and *G. uro* inoculation could not transform EA into downstream products, where only urolithin M5, urolithin M6 and urolithin C were detected in current fermentation broths [36]. According to the detected metabolites, the biotransformation process was speculated to be that EA firstly removed a lactone ring to form urolithin M5 by lactonase and decarboxylase, then removed hydroxyl group at C-4 position to produce urolithin M6 by 4-pyrocatechol-dehydroxylase, and finally removed hydroxyl group at C-10 position to produce urolithin C by 10- pyrocatechol-dehydroxylase.

In Fig. 5A, there were only few urolithin M5 detected in FOS group, suggesting that FOS supplement could not improve the urolithin productions. The highest urolithin M5 content was observed in SCP group, while no other urolithins were found, indicating that SCP supplement only prevented the metabolic pathway from urolithin M5 to urolithin M6. The total urolithins content in UPP group was significantly increased as compared to CK group ($P < 0.05$), while urolithin M6 content in SFP group was notedly higher than other groups. Fig. 5B expressed the OD_{600} values of the broths during the fermentation to reflect the growth of *G. uro*, and the results showed that UPP supplement greatly improved the proliferation of strain, which was accordant with the productions of urolithins. In Fig. 5C, the levels of urolithins were increased in EA/UPP solid dispersion groups, suggesting that the suspending EA would allow more chances for *G. uro* to get access to the insoluble substrate and lead to more urolithin productions. A previous study found that oral supplementation of EA and *G. uro* could alter the dominant microflora by increasing the abundances of *Akkermansia*, *Lactobacillus* and *Bifidobacterium*, and finally enhance EA-to-urolithin bioconversion [37].

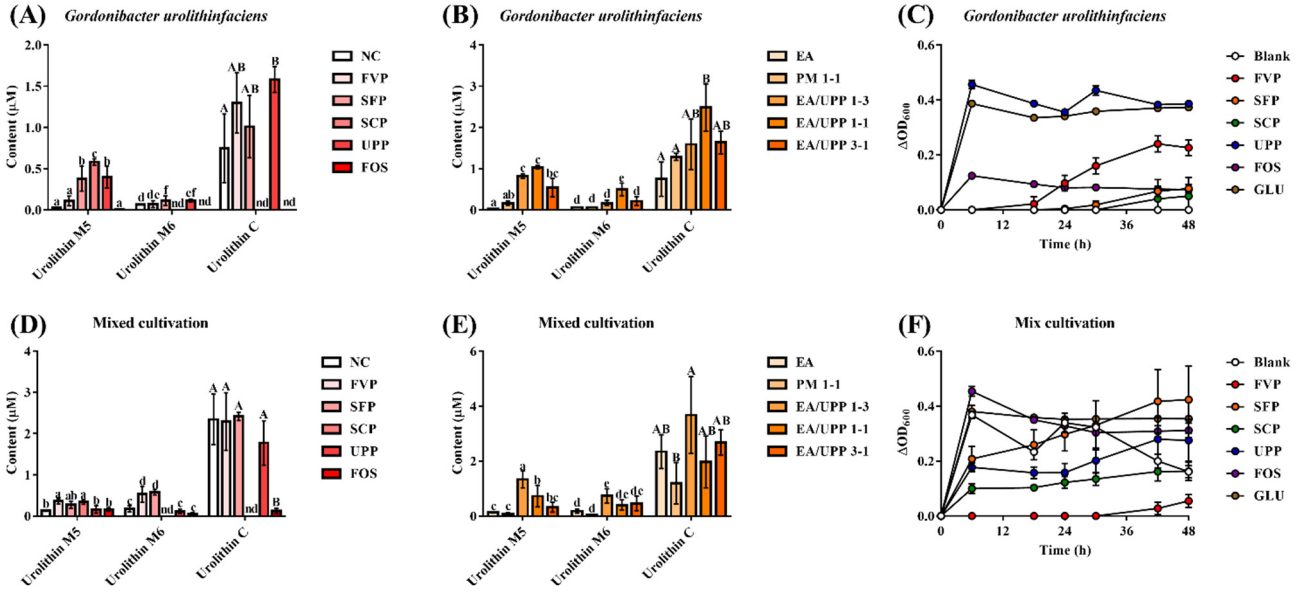


Fig. 5 Effects of APs supplements (A, C, D, F) and EA solid dispersion supplements (B, E) on urolithin productions by *G. uro* in fermentation broths. Data are expressed as mean ± SD (n = 3). Significant differences ($P < 0.05$) are indicated with different letters. Nd. means not detected.

Fig. 5D-F showed the urolithins productions and the bacteria proliferation during the co-culture fermentation. Similar to the results in mono-culture fermentation with *G. uro*, the levels of urolithins were increased in EA/UPP solid dispersion groups. Besides, the conversion rate of each group was increased in co-culture fermentation, suggesting that these strains (*V. aty*, *E. fae*, *L. bac* and *C. sp*) would not straightly participate in urolithins production process, but the microbial interactions among these strains contributed to affecting EA metabolism and urolithin productions.

3.8 Illustration of APs supplements to affect EA biotransformation process

Based on the above results, it was found that the urolithins productions in EA/UPP SDs were significantly higher than those in UPP group, both in fecal microbiota fermentation and in mono-/co-culture fermentation where every fermentation sample contained same initial content of EA. Owing to that the bacteria which participated in the biotransformation of EA did not take the dominant stage, it was difficult to obtain more metabolites with the low abundance of bacteria and hydrophobic EA even with the supplement of biological polysaccharides. The bioconversion improvement by the algal polysaccharides was still limited by the poor accessibility of EA to the critical bacteria. As a whole, that the bioconversions to urolithins were largely enhanced by EA-APs solid dispersions to increase the accessibility of EA to the bacteria, and partly improved by APs supplements to proliferate EA-metabolic-related bacteria (Fig. 6). The current results showed that exogenetic dietary supplement could be a useful way to adjust the gut microbiota-mediated bioconversion of EA. More supplement targeted on the proliferations of EA-metabolic-related bacteria could be explored in further study.

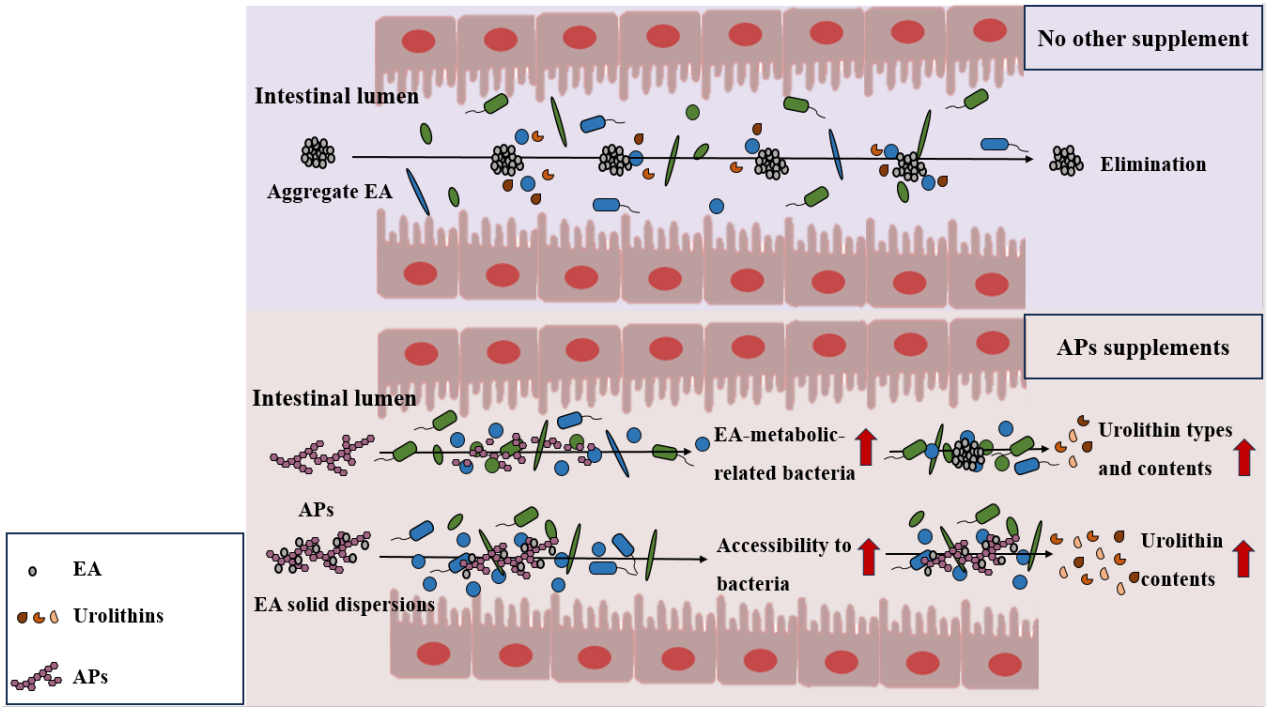


Fig. 6 Overview illustration of APs supplements to affect EA biotransformation process.

4. Conclusion

In conclusion, the current study systematically dissected the interactions among dietary polysaccharides supplements, EA biotransformation process and the intestinal microflora. Results showed that isourolithin A

levels in FVP group and SCP group were significantly increased, urolithin C level in SFP group was notably elevated, and urolithin M5 levels of SCP group and UPP group significantly increased, while urolithins contents were increased in EA solid dispersions groups during the 5-day *in vitro* fermentations. The 16s rRNA gene sequencing analysis indicated that both APs and EA solid dispersions supplements could adjust composition of gut microflora. Furthermore, the correlation analysis indicated that some crucial bacteria like *Gordonibacter*, *Faecalibacterium*, *Sutterella*, *Dialister*, *Enterocloster* and etc. might be EA-metabolic-related bacteria and involve in urolithin productions. The results showed that dietary supplements could proliferate these bacteria and improve EA biotransformation capacity. Ulteriorly, mono-/co-culture fermentation tests reflected that SFP, UPP and EA/UPP solid dispersions showed growth-promoting effects of related strains (especially *G. uro*) and improved urolithins productions. The results confirmed that nutrition strategies targeted on gut microflora modulation could be a novel method to regulate the urolithins productions and receive expectable health benefits beyond the inter-individual variability. More EA-metabolic-related bacteria would be verified and potential mechanism of APs supplements to affect EA biotransformation should be investigated in further study.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledge

This work was financially supported by Guangdong Basic and Applied Basic Research Foundation (No. 2024A1515010576), Guangdong Basic and Applied Basic Research Foundation (No. 2022B1515120083), Program for scientific research start-up funds of Guangdong Ocean University (No. 060302042304, 060302042309), Key-Area Research and Development Program of Guangdong Province (No. 2021B0707060001).

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