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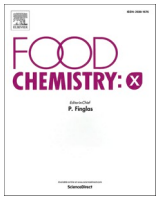

Fructo-oligosaccharide enhanced bioavailability of polyglycosylated anthocyanins from red radish via regulating gut microbiota in mice

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ABSTRACT

The anthocyanins from red radish (ARR) rich in polyglycosylated pelargonidin glucosides were used as pigment. However, bioavailability of anthocyanins was considered at low level. This work examined the intensive effects of fructo-oligosaccharide (FOS) on ARR bioavailability. Pelargonidin, cyanidin and pelargonidin-3-glucoside showed higher level in serum of mice fed with FOS together with ARR for 8 weeks than that fed with only ARR. Co-ingestion of FOS and ARR more effectively elevated the hepatic antioxidant activity by increase in total antioxidant capacity and activities of superoxide dismutase and glutathione peroxidase when compared with intake of ARR. FOS also markedly increased pelargonidin level in cecum of mice. 16S RNA sequencing found that *Bacteroides* genus play an important role in FOS elevating bioavailability of ARR. Fecal bacteria transplantation verified the positive effects of FOS on ARR bioavailability. These results suggested that combined ingestion of FOS and ARR is effective strategy for bioactivity of ARR.

1. Introduction

Anthocyanins are secondary metabolites and the main reason for the colors of red, blue and purple in many fruits, vegetables, flowers, and grains (Charron, Clevidence, Britz, & Novotny, 2007). Red radish (*Raphanus sativus* L.) is native to Fuling district of Chongqing city in China, and its flesh usually showed red or reddish violet due to the fact that it is rich in anthocyanins (Park et al., 2011; Park et al., 2016; Li et al., 2020). Our previous study found that red radish mainly contained 24 types of polyglycosylated anthocyanins, of which pelargonidin-3-(feruloyl)diglucoside-5-(malonyl)glucoside (P3FD5MG), pelargonidin-3-(p-coumaroyl)diglucoside-5-(malonyl)glucoside (P3PD5MG), and pelargonidin-3-(caffeoyl)diglucoside-5-(malonyl)glucoside (P3CD5MG) were the most abundant (Li, Gong, Ma, Xie, Wei, & Xu, 2022a). These polyglycosylated anthocyanins were not only more stable than monoglycosylated anthocyanins due to their complex structure and acyl groups (He et al., 2022), but also showed similar capacities with monoglycosylated anthocyanins in aspect of antioxidant and protect cardiovascular system (Li et al., 2021; Li et al., 2022b; Wang, Sun, Cao, Wang, Li, & Wang, 2010). From a nutritional perspective that it is crucial to understand the metabolism of such compounds to assess their true biological relevance (He et al., 2022). However, anthocyanins of red radish (ARR) are large molecules and highly water-soluble that have been considered low bioavailability for animals and humans (McGhie, & Walton, 2007). Additionally, gut microorganisms could metabolize anthocyanins to phenolic acids, which also have multiple physiological activities (Jokioja, Percival, Philo, Yang, Kroon, & Linderborg, 2021). The phenolic acids might separate from acylated glycosidic groups (Jokioja, Percival, Philo, Yang, Kroon, & Linderborg, 2021), and it also could degrade from the aglycone (Braga, Murador, de Souza Mesquita, & de Rosso, 2018). Therefore, gut microbiota is a potential target for regulating ARR metabolism.

Fructo-oligosaccharide (FOS) are basically oligosaccharides of fructose possessing a single glucose moiety or bound to sucrose, and its sweetness is 0.4–0.6 times sweeter than sucrose (Bali, Panesar, Bera, & Panesar, 2015). Although FOS cannot be digested by mammals, it could promote proliferation of probiotic bacteria and inhibit the growth of harmful microorganisms (Bali, Panesar, Bera, & Panesar, 2015).

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Recently, some studies reveal that FOS could enhance absorption and anti-dyslipidemia capacity of tea flavonoids (Li, Zhang, & Zhao, 2019); and absorption of genistein and daidzein (Uehara, Ohta, Sakai, Suzuki, Watanabe, & Adlercreutz, 2001); and metabolism and anti-diabetes activity of quercetin-3-O-β-glucoside (Phuwamongkolwiwat, Suzuki, Hira, & Hara, 2014). Uehara, Ohta, Sakai, Suzuki, Watanabe and Adlercreutz (2001) speculated these effects of FOS related to regulating gut microbiota. Therefore, FOS might also augment the health benefits of ARR by regulating metabolism.

The aim of current study was to investigate the effects of FOS on bioavailability and antioxidant activity of ARR. Thus, mice were fed with ARR together with or without FOS for 8 continuous weeks. Anthocyanidins, anthocyanins and phenolic acids in serum and cecum content were measured by ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS). Hepatic antioxidant parameters were assessed by commercial kits. Colonic microflora was analyzed by sequencing 16S rRNA.

2. Materials and methods

2.1. Materials and chemicals

Cyanidin (C, pure > 98%), pelargonidin (P, pure > 96%), cyanidin-3-glucoside (C3G, pure > 95%), fructo-oligosaccharide (FOS, pure > 95%) and pelargonidin-3-glucoside (P3G, pure > 95%) were purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). This company also supplied the superoxide dismutase (SOD, ID: R22262) and glutathione peroxidase (GSH-P*x*, ID: R21876) detection kits. Gallic acid (GA, pure > 98%), protocatechuic acid (PA, pure > 98%), p-hydroxybenzonic acid (PHBA, pure > 99%) and caffeic acid (CA, pure > 98%) also obtained from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). Commercial kits for total antioxidant activity (*T*-AOC, ID: BC1315) were purchased from Solarbio Life Science Co. Ltd. (Beijing, China). Other chemicals applied in current work were analytically pure.

2.2. Extraction and identification of ARR

One hundred grams of red radish (particle, 2 × 2 × 2 mm) were dipped 2 times (*m: V*) of water at least 3 h. The extracting solution was collected and residues were further extracted twice. All the extracting solution was concentrated at 45 °C under vacuum rotary evaporator (RV-10, IKA Works Guangzhou, Guangzhou, China). To purify anthocyanins, the concentrated solution was successively dialyzed at 3500 Da and 500 Da according to our previous method (Li et al., 2022c). Vacuum freeze-dryer (Scientz-10 N, Bingbo Scientz Biotechnology Co. Ltd., Ningbo, China) was used to dry concentrated solution for obtain the ARR powder. The qualification and quantification of the individual ARR anthocyanins were performed according to previously described procedures (Li et al., 2022c).

2.3. Animal and administration

During adaptively feeding, male Kunming mice (about 20 ± 2 g, 4 weeks old) freely intake normal diet and tap water. After one week, mice were randomly divided into 3 groups (eight mice for each group) that respectively named as control, ARR and FOS + ARR. The control, ARR, and FOS + ARR mice were daily and respectively administered with 0.4 mL (intragastrically) of normal saline (0.9%), ARR water solution (25 mg/kg·bw), and mixed solution of ARR (25 mg/kg·bw) and FOS (25 mg/kg·bw) for 8 continuous weeks. The faeces from the ARR and FOS + ARR groups were collected and stored at −80 °C. Then, faeces were homogenized with 9 times water. The homogenate was centrifuged at 3000 × *g* for 15 min, and then the supernatant was collected for fecal bacteria transplantation (FMT).

Sixteen mice were divided into two FMT groups (called ARR-FMT, and FOS + ARR-FMT) with 8 mice for each group. The FMT groups were adaptively fed for 4 weeks. All these mice were daily and intragastrically treated with 0.4 mL of mixed antibiotics (1 mg/mL ampicillin, 1.6 mg/mL sulfamethoxazole, 0.32 mg/mL trimethoprim) for building pseudo-intestinal germ-free mice at the sixth week (Turer et al., 2008). Fecal bacteria, that respectively from the ARR and FOS + ARR groups mice, were used to administered intragastrically to the mice from ARR-FMT and FOS + ARR-FMT groups during the seventh week to the eighth week. All the mice were administered with 0.4 mL of ARR water solution at 25 mg/kg·bw at the last administration. After 2 h, the mice were fully anesthetized by the inhalation of isoflurane and then sacrificed by cardiac puncture. Blood was drawn in tubes and immediately centrifuged at 3000 × *g* for 15 min in order to obtain serum. Liver, cecum, and colon were collected and stored at −80 °C.

All the mice received humanistic care that were executed according to the Guidelines of Experimental Animal Administration published by the Publishing House of Science and Technology of Shanghai (Eighth Edition, ISBN-10:0–309-15396–4). The Committee on Care and Use of Laboratory Animals of the Fourth Military Medical University in China approved the experimental protocol (20220324).

2.4. Measurement of anthocyanins in serum and cecal contents

The original anthocyanins and their derivatives from phase II metabolites were analyzed. Serum (150 μL) was mixed with 100 μL of enzyme (6 U/mL β-glucuronidase, EC 3.2.1.31; 124 U/mL sulfatase, EC 3.1.6.1) to release the original anthocyanins from their phase II metabolites (Li, Huang, Gao, & Yang, 2016). Simultaneously, another 150 μL of serum was mixed with 100 μL of water to replace enzyme for revealing the free anthocyanins contents. After incubation at 37 °C for 1 h, 260 μL of acetonitrile was added to sediment protein. Then, the mixture was centrifuged at 12000 rpm for 15 min to obtain supernatant by miniature centrifuge (TGL-16B, Shanghai Dingke Scientific Instrument Co., LTD, Shanghai, China).

One hundred milligram of cecal content was mixed with 100 μL of water and 200 μL of acetonitrile. The supernatant was collected by centrifuging the mixture at 12000 rpm for 15 min. The supernatant from cecal sample or serum sample was filtrated by ultrafiltration membrane for further UHPLC-QqQ-MS/MS analysis.

Anthocyanins in 5 μL of sample were chromatographically separated by ZORBAX SB-C18 column (100 × 2.1 mm i.d., 1.8 μm, Agilent, Waldbronn, Germany) at 35 °C, that was equipped in an Agilent 1290 Infinity UHPLC system equipped with a 6460C triple quadrupole mass spectrometry system (Agilent, Santa Clara, California, USA). The mobile phase was composed of 0.1% formic acid–water (phase A) and 0.1% formic acid-acetonitrile (phase B), respectively. The velocity of mobile phase was 0.2 mL/min with a gradient elution program: 95%-95% A for 0–0.2 min; 95%-55% A for 0.2–10 min; 55%-5% A for 10–10.2 min; 5%-5% A for 10.2–11.2 min; 5%-95% A for 11.2–11.5 min; 95%-95% A for 11.5–15 min. The operating parameters for electrospray ion source (ESI) were follows: positive ion mode at 350 °C, and the flow velocity of nitrogen was 10 L/min. The parameters of fragmentor voltage and collision energy respectively were 140 V and 30 V. The information of molecular ion and fragment ion for anthocyanins were referred in our previous reports (Table S1; Li, Gong, Ma, Xie, Wei, & Wu, 2022a; Li et al., 2022c). The contents were calculated according to standard curves of real C, C3G, P and P3G.

2.5. Antioxidant activity of liver

Liver tissue (0.5 g) was homogenized together with 4.5 mL of normal saline. The homogenate was centrifuged at 3000 × *g* for 15 min. Supernatant was collected for further analysis of *T*-AOC, SOD, and GSH-P*x* according to the relevant instruction book.

2.6. 16S rRNA gene amplification and MiSeq sequencing

The preparation and sequencing of 16S rRNA of colonic microorganism were conducted by a commercial service (Shanghai Ling En Biotechnology Co., LTD, Shanghai, China). After extraction of DNA, the extracted DNA was detected by 1% agarose gel electrophoresis. Barcoded amplicons from the V3-V4 region (515F, 5′-GTGYCAGCMGCCGCGGTAA-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′) of 16S rRNA genes were generated. PCR products of the samples in a group were mixed and detected by 2% agarose gel electrophoresis. PCR products were recovered by gel cutting using AxyPrepDNA gel recovery kit (AXYGEN) and eluted with Tris-HCl buffer. The amplification result was verified by 2% agarose gel electrophoresis. The PCR products were quantified with the QuantiFluor™-ST blue fluorescence Quantification system (Promega) based on the preliminary results of electrophoresis. The samples were mixed according to the sequencing volume requirements of each sample. Sequencing was executed by Illumina PE250. PE reads were first spliced according to overlap relation, and sequence quality was controlled and filtered at the same time. OTU clustering analysis and species taxonomy analysis were performed after samples were distinguished at 97% sequence identity.

2.7. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Student *t*-test and Tukey's multiple comparison test were used to assessed significant difference between two groups, and among more than two groups, respectively. $P < 0.05$ was considered as significant difference. The statistical analysis was carried out by 'agricolae' package (de Mendiburu, & Simon, 2015). The R package, mixOmics (https://mixomics.org/), was applied to perform principal component analysis (PCA) and sparse partial least square (sPLS). Corrplot (https://127.0.0.1:17195/library/corrplot/doc/corrplot-intro.html), a R package, was used to execute correlation analysis.

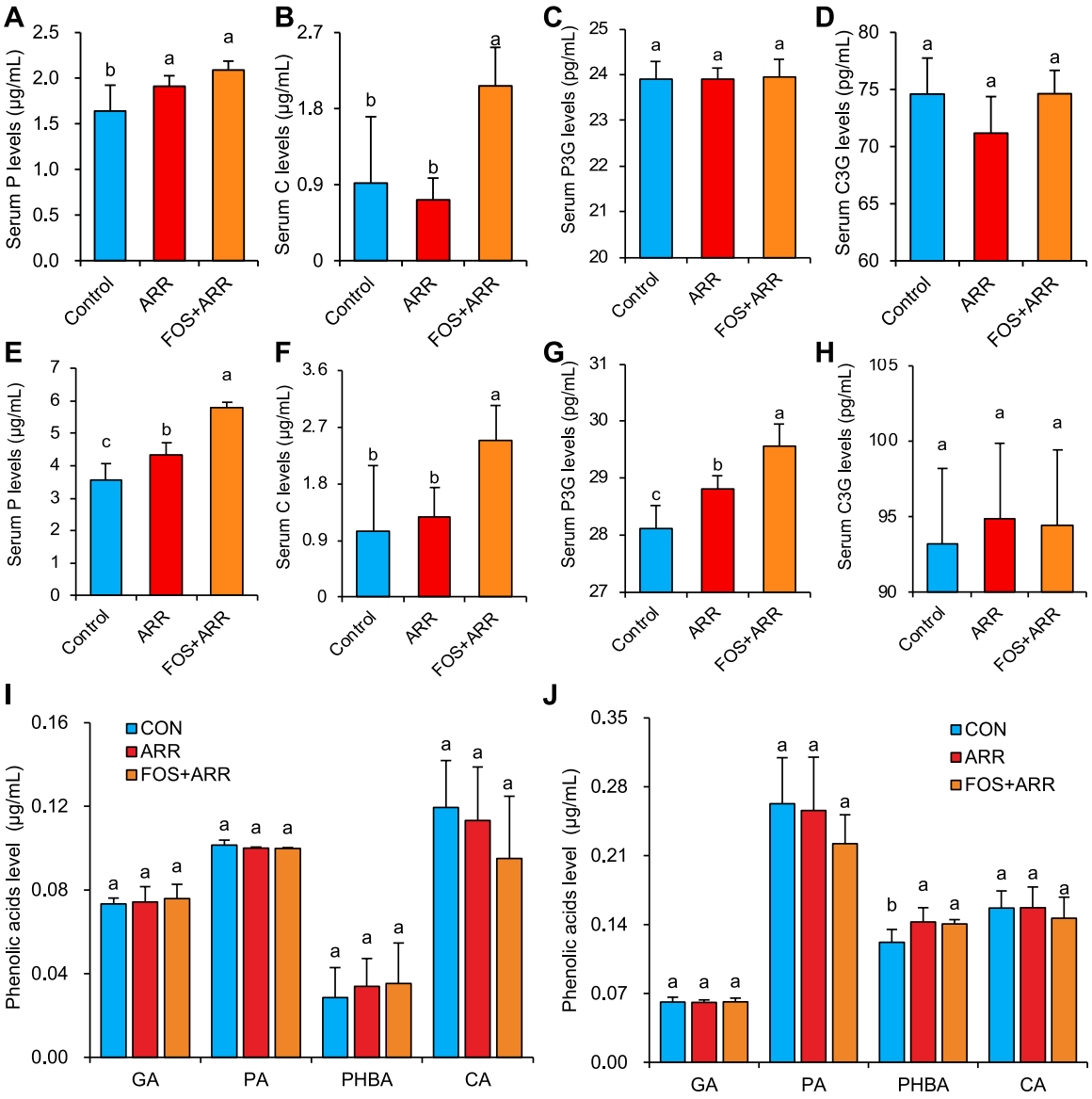

Fig. 1. The serum anthocyanidin, anthocyanins and phenolic acids concentrations in mice fed with anthocyanins of red radish (ARR) together with or without fructo-oligosaccharide (FOS) for 8 weeks. (A-D): serum concentrations of free anthocyanidin and anthocyanins; (E-H): serum total concentrations of free and derivative anthocyanidin and anthocyanins; I: serum concentrations of free phenolic acids; J: serum total concentrations of free and derivative phenolic acids. P: pelargonidin; C: cyanidin; P3G: pelargonidin-3-glucoside; C3G: cyanidin-3-glucoside; GA: gallic acid; PA: protocatechuic acid; PHBA: p-hydroxybenzoic acid; CA: caffeic acid. [a-c]Different alphabet represented $P < 0.05$, which was analyzed by Tukey's multiple comparison post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Serum levels of anthocyanins and phenolic acids

UHPLC-QqQ-MS/MS analysis found 2 anthocyanidins and 17 anthocyanins in the extractive from red radish, and pelargonidin-3-diglucoside-5-(malonyl)glucoside (P3D5MG), pelargonidin-3-(feruloyl) diglucoside-5-(malonyl)glucoside (P3FD5MG) and pelargonidin-3-diglucoside-5-glucoside (P3D5G) were the main individuals (Table S1, Figure S1). Similar result has been showed in our previous report (Li et al., 2022c). It has been found that anthocyanins can be absorbed into blood by active transport using sodium-dependent glucose cotransporter (SGLT1), SGLT2 and bilitranslocase transporters (Kamiloglu, Capanoglu, Grootaert, & Van Camp, 2015). However, these main anthocyanins in ARR were not found in serum of mice treated by ARR with or without FOS for 8 weeks. This may be that anthocyanins with complex glycoside structure are more difficult to be transported by cell membrane transporters (Manolescu, Oprea, Mititelu, Ruta, & Farcasanu, 2019). However, P3G and C3G, two simple anthocyanins, could be transported by these transporters (Kamiloglu, Capanoglu, Grootaert, & Van Camp, 2015). In addition, P and C, two anthocyanidin aglycones, can also transfer into cell via fast passive diffusion (Manolescu, Oprea, Mititelu, Ruta, & Farcasanu, 2019). Thus, their serum levels usually depend on how much they are in the gut. Although the column of FOS together with ARR treatment was higher than that of the only ARR treatment, there was no marked difference between the two groups (Fig. 1A). Additionally, the serum free C levels in mice treated FOS + ARR for 8 weeks were significantly higher than that in mice treated ARR for 8 weeks (Fig. 1B). These findings suggested that FOS might enhance the bioavailability of P and C which might have high contents in gut. Similarly, FOS also elevated the bioavailability of genistein and daidzein, that were also two flavonoid aglycones likes P and C (Uehara, Ohta, Suzuki, Watanabe, & Adlercreutz, 2001). However, there were no markedly difference among the serum levels of free C3G and P3G in mice (Fig. 1C-D).

Phase II metabolism of enterocyte and hepatocyte could induce derivatization of anthocyanins (Xu et al., 2021). Thus, original anthocyanins were released from Phase II derivatization by β-glucuronidase and sulfatase hydrolyzation. Next, the content of anthocyanins in the hydrolyzed sample was called as total content. As shown in Fig. 1E-G, FOS + ARR fed mice had the significantly higher serum total P, C and P3G levels than the control and ARR groups mice. However, the serum total C3G level showed no significant difference among the three groups (Fig. 1G). These results of hydrolyzed samples strongly supported that FOS enhanced bioavailability of P, C and P3G. Unfortunately, mice serum showed no other anthocyanins with complex molecular structure that originally existed in red radish, such as P3D5G, P3D5MG and P3FD5MG (Table S1). This might be that gut microbiota generate some enzymes to break of glycoside linkages of ARR and even cleavage of the anthocyanin heterocycle (Fernandes, Faria, Calhau, Freitas, & Mateus, 2013).

Phenolic acids were the important metabolites of anthocyanins by microorganism (Jokioja, Percival, Philo, Yang, Kroon, & Linderborg, 2021; Braga, Murador, de Souza Mesquita, & de Rosso, 2018). Protocatechuic acid (PA), caffeic acid (CA), gallic acid (GA) and p-hydroxybenzoic acid (PHBA) has been reported as the main metabolites by gut microorganism after anthocyanin consumption (Chen et al., 2017; Aura et al., 2005). However, the serum GA, PA and CA levels in original or hydrolyzed samples had no significant difference among all the groups mice (Fig. 1I, J). Although FOS + ARR-treated mice showed lower level than ARR-treated mice, there was no significant difference between ARR-treated and FOS + ARR-treated groups (Fig. 1J). In any case, these phenolic acids levels in serum were distinctly lower than the serum anthocyanins levels (Fig. 1). Therefore, anthocyanins were the main reason of the antioxidant *in vivo* rather than the phenolic acids produced from anthocyanins by the gut microbes, because the content of anthocyanins was more than that of the phenolic acids. Totally, we suggested that treatment of FOS + ARR might more beneficial to antioxidant than the individual treatment of ARR in mice because it caused higher serum anthocyanins levels.

3.2. Hepatic antioxidant activity

Previous studies found that both FOS and anthocyanins could prevent the abnormal increase of bodyweight in unbalanced diet-fed mice (Wu et al., 2013). However, ingestion of FOS and/or ARR for 8 weeks did not cause prominent changes in the bodyweight of mice when compared with the mice fed with normal diet (Figure S2). Matsukawa, Matsumoto, Shinoki, Hagio, Inoue & Hara (2009) also reported that diet including 1.5% FOS did not significantly changed the body weight of rat. This may indicate that ARR have no toxic side effects on normal organisms. It is more important that ARR presented explicit antioxidant activity in cells and *in vitro* chemical models (Wang, Sun, Cao, Wang, Li, & Wang, 2010; Li et al., 2022b). Moreover, our previous study found that the antioxidant and anticancer activities of pelargonidin glycosides were weaker than that of pelargonidin (Li et al., 2021). Thus, FOS increased serum P and C levels might be conducive to enhance health. Fig. 2A indicated that feeding of ARR showed higher *T*-AOC and activities of SOD and GSH-P*x* in mice liver, when comparing the treatment with normal saline. This was the first time to reported that ARR had antioxidant activity *in vivo*. Although treatment of FOS + ARR caused slightly increase of hepatic *T*-AOC level in mice, there was no significant difference (Fig. 2A). Interestingly, FOS + ARR fed mice showed the highest SOD and GSH-P*x* activity in liver of mice (Fig. 2A). It has been reported that co-feeding of non-digestive stachyose or soluble soybean polysaccharides together with genistein could enhance the hepatic antioxidant activity of mice (Lu, Li, & Yang, 2017; Li, Li, Han, Huang, Lu, & Yang, 2016). Additionally, stachyose and soluble soybean polysaccharides (SSPS) could also increase serum levels of genistein and dihydro-genistein that was an intestinal microbial metabolite of genistein (Lu, Li, & Yang, 2017; Li, Li, Han, Huang, Lu, & Yang, 2016). However, it cannot confirm that the enhancing of hepatic antioxidant activity was due to elevation of serum genistein levels and/or its intestinal microbial metabolites. Thus, we further analyzed the correlation between the hepatic antioxidant parameters and serum anthocyanins and phenolic acids contents. As shown in Fig. 2B, C, anthocyanins showed stronger positive correlation with antioxidant parameters than phenolic acids, except C3G. Moreover, the strongest positive correlation also presented between hepatic antioxidant parameters and serum free GA, free PHBA, total PA, total PHBA and total CA levels, respectively (Fig. 2C). These findings verified our speculation that improving bioavailability of anthocyanins was a key reason for the co-ingestion of FOS and ARR enhancing antioxidant activity. Additionally, phenolic acids as metabolites of anthocyanins might also contribute to some of antioxidant activity.

3.3. Cecal levels of anthocyanins and phenolic acids

It is well known that mice differ from humans in that their cecum is an important metabolic organ. Accordingly, the contents of anthocyanins and phenolic acids in cecum were analyzed by UHPLC-QqQ-MS/MS for explaining ARR metabolism in the gut. We found that cecum contained some anthocyanins with complex molecular structure, which existed in original red radish and not found in serum samples, such as P3CD5G, P3FD5G and P3PD5G (Fig. 1, 3, and S3; Table S1). This might be that acylated anthocyanins were more difficult to be absorbed during digestion than nonacylated ones (Charron et al., 2009). Oki, Suda, Terahara, Sato, & Hatakeyama (2006) found that only two acylated anthocyanins, cyanidin-3-(6′-caffeoyl)-sophoroside-5-glucoside and peonidin-3-(6′-caffeoyl)-sophoroside-5-glucoside, were absorbed after ingestion of purple sweet potato beverages. The unabsorbed acylated anthocyanins kept in the cecum and large intestine for further

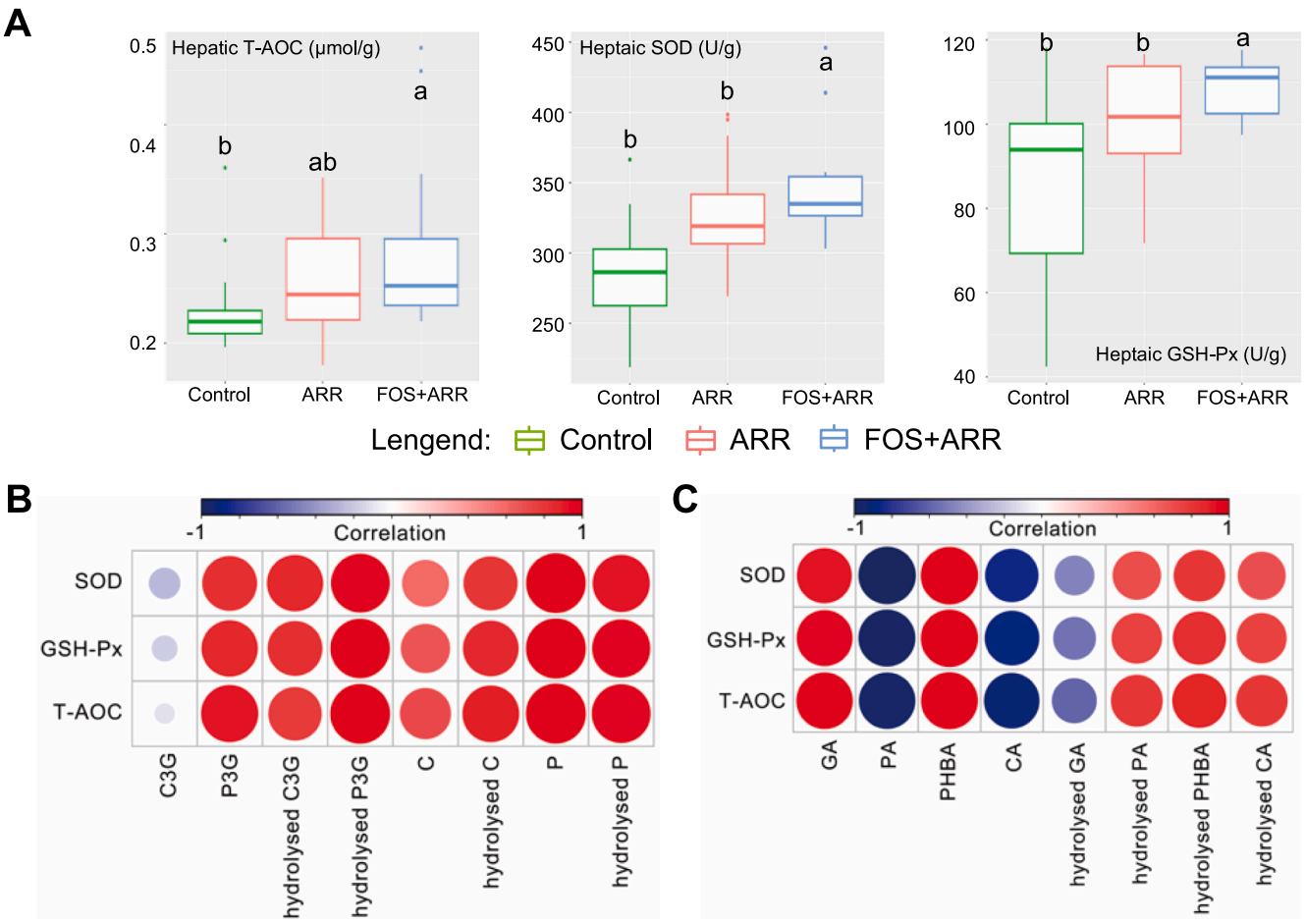

Fig. 2. The effects of FOS and ARR on hepatic antioxidant activity in mice. (A): *T*-AOC, total antioxidant capacity; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase. (B): the correlation between serum anthocyanins contents or phenolic acids and hepatic antioxidant indexes. [a-b]Different alphabet represented $P < 0.05$, which was analyzed by Tukey's multiple comparison post-test.

metabolism. In addition, polyglycosylated and acylated anthocyanins were more stable than the monoglycosylated and nonacylated ones (He et al., 2022). Accordingly, polyglycosylated and acylated anthocyanins were difficult to degrade and thereby be retained in the gut.

Interestingly, the contents of pelargonidin glucosides and cyanidin glucosides were 10 times and hundreds of times lower than the contents of relevant aglycone (P and C), respectively (Fig. 3A-H, Figure S3). Krga and Milenkovic (2019) suggested that gut microbiota was a main reason for anthocyanin hydrolyzing to anthocyanidin, which are mainly represented by bacterial species from the genera *Bifidobacterium*, *Bacteroides*, *Eubacterium* and *Clostridium*. Accordingly, we speculated that a large number of pelargonidin glucosides in ARR were hydrolyzed to P aglycone by β-glucosidase in cecum (Xu, Li, Xie, Mo, & Chen, 2021). Moreover, FOS + ARR-treated mice showed higher cecal P level than the control and ARR-treated mice (Fig. 3A). Matsukawa, Matsumoto, Shinoki, Hagio, Inoue, & Hara (2009) also found that co-ingestion of FOS and quercetin-3-glucoside significantly elevated the quercetin content in cecum and faeces of rat. Although stachyose or SSPS also elevated the serum genistein level, they inhibited the production of genistein characteristic metabolites in the gut (Lu, Li, & Yang, 2017; Li, Li, Han, Huang, Lu, & Yang, 2016). The reason might be that nondigestible saccharides restrained the degradation of quercetin aglycone in gut (Matsukawa, Matsumoto, Shinoki, Hagio, Inoue, & Hara, 2009). Accordingly, there was more pelargonidin glucosides hydrolyzed to aglycone in cecum of FOS + ARR-fed mice than ARR-fed mice.

Compared with ARR treatment, co-treatment of FOS and ARR did not caused significant difference in cecal P3G, P3CD5G, P3G5G, P3FD5G, P3PD5G, C3GR and C3G levels (Fig. 3C-H, Figure S3). However, these anthocyanins were not found in the control group mice, which were fed with ARR for only one time (Fig. 3C-H). We speculated that this might be because of anthocyanins, which have complex molecular structures, are too low level in the gut to be detected by current methods. Our results indicated that the hydrolyzation of pelargonidin glucosides in gut was fast.

Although FOS could inhibit degradation of flavonoid aglycones, there were some flavonoid aglycones still be degraded in gut of FOS + ARR-fed mice. The cecal GA and PA levels in FOS + ARR group mice were significantly higher than that in ARR-treated group mice. Although CA had a higher cecal level than GA, PA and PHBA, there was no markedly difference for CA among the three groups mice. These results seem to contradict our previous theories that FOS might inhibit degradation of pelargonidin. For this reason, we think that FOS also inhibited the degradation of the original phenolic acid in ARR extract, and thereby caused higher cecal phenolic acids levels in FOS + ARR-fed mice than that in ARR-fed ones. In addition, these phenolic acids might also separate from acylated glycosidic groups of ARR that are rich in phenolic acid-acylated pelargonidin glycosides (Jokioja, Percival, Philo, Yang, Kroon, & Linderborg, 2021; Li et al., 2022a, 2022b). Totally, FOS increasing ARR bioavailability was closely related to FOS regulating metabolism of intestinal flora.

3.4. Correlation of ARR bioavailability and gut microbiota

There were 4416, 3717, and 3036 OTUs detected in colon of the

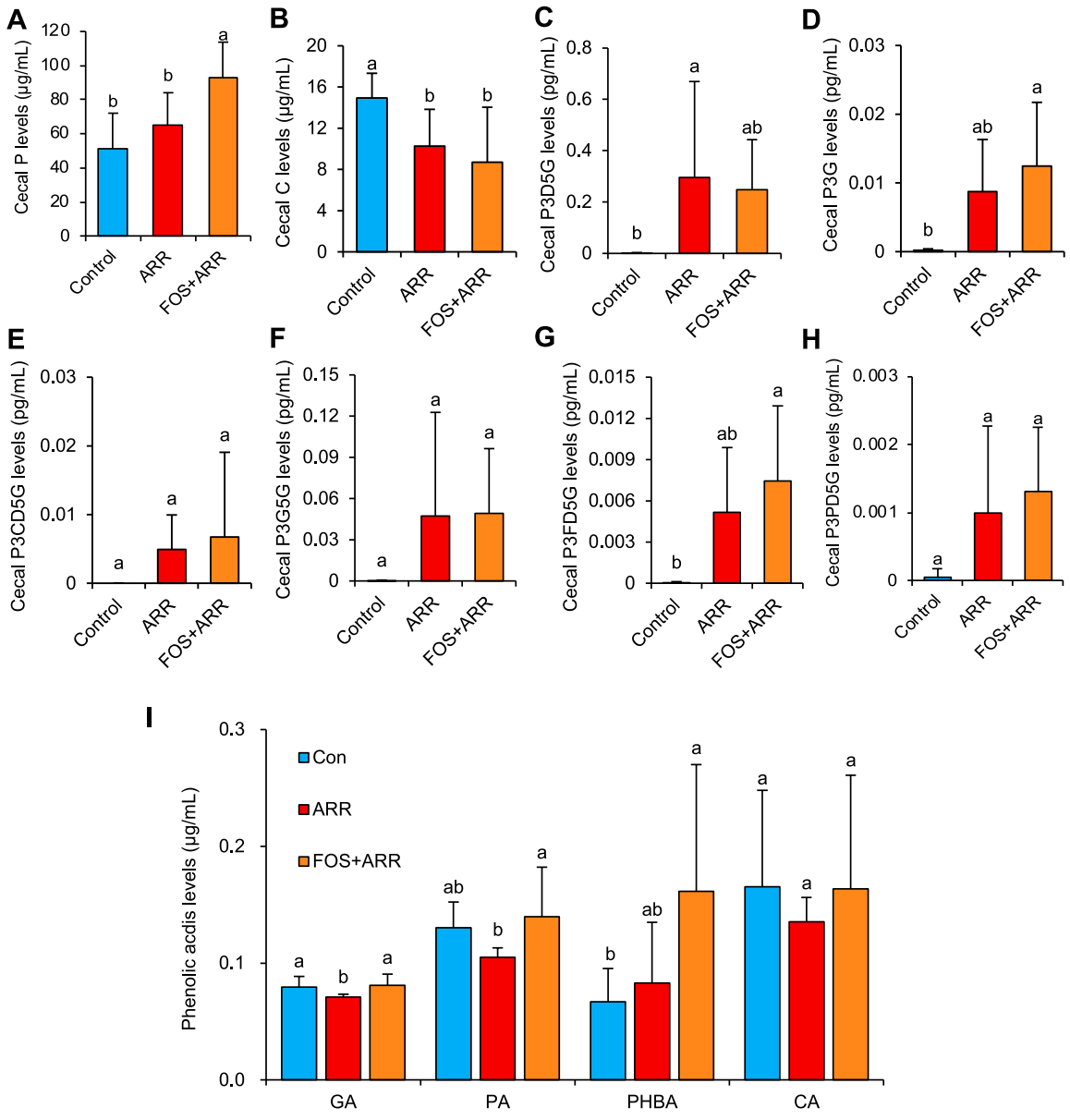

Fig. 3. The cecal free anthocyanidin, anthocyanins and phenolic acids concentrations in mice fed with anthocyanins of red radish (ARR) together with or without fructo-oligosaccharide (FOS) for 8 weeks. P3D5G: pelargonidin-3-diglucoside-5-glucoside; P3CD5G: pelargonidin-3-(caffeoyl)diglucoside-5-glucoside; P3PD5G: pelargonidin-3-(p-coumaroyl)diglucoside-5-glucoside; P3FD5G: pelargonidin-3-(feruloyl)diglucoside-5-glucoside; P3G5G: pelargonidin-3-glucoside-5-glucoside. [a-c]Different alphabet represented $P < 0.05$, which was analyzed by Tukey's multiple comparison post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control, ARR, and FOS + ARR groups mice, respectively. As shown in Fig. 4A, the 2nd principal component of PCA model distinguished the control group from FOS + ARR groups. The control and FOS + ARR groups were placed on the sides of ARR group. It was well known that FOS as an important prebiotic could significantly change in the composition of the colonic microbiota (Roberfroid, 2000). Additionally, it has found that anthocyanins from *Lycium ruthenicum* and purple carrot presented activity to regulate intestinal microecology (Zary-Sikorska, Fotschki, Fotschki, Wiczkowski, & Juskiewicz, 2019; Peng et al., 2019). Accordingly, our results could also suggest that ingestion of ARR together with or without FOS resulted in significant changes of the gut microbiota. We also found that *Firmicutes*, *Bacteroidota* and *Verrucomicrobiota* were the main phylum of gut microbiota in the colon (Fig. 4B). The relative abundance of *Firmicutes* and *Bacteroidota* phylum respectively decreased and increased from the control, ARR to FOS + ARR groups. Some studies found that Firmicutes/Bacteroidetes (F/B) proportion associated with health, such as body weight control and energy metabolism (Koliada et al., 2017; Xue et al., 2016). As can be seen in Fig. S4A, the relative abundance of *Lactobacillus* and *Akkermansia* genus accounted for 12.5%-21.3% and 7.3%-14.6%, respectively. However, the administrations of ARR and FOS + ARR did not change the two genus significantly.

To reveal the correlation between colonic gut microbiota and cecal anthocyanins, we executed sPLS analysis. The distribution of each group in sPLS model (Fig. S4B), is very similar to gut microbiota in the PCA model (Fig. 4A). This result indicated that the sPLS model selected variables can reconstruct the original model well. Furthermore, the clustered image maps from the sPLS models showed that the relative abundance (RA) of *Bacteroides, Akkermansia and Alistipes* genus was positive correlation with cecal levels of multiple anthocyanins (Fig. 4C). In previous report, *Bacteroides* genus has been confirmed was a main reason for anthocyanin hydrolyzing to anthocyanidin (Krga &

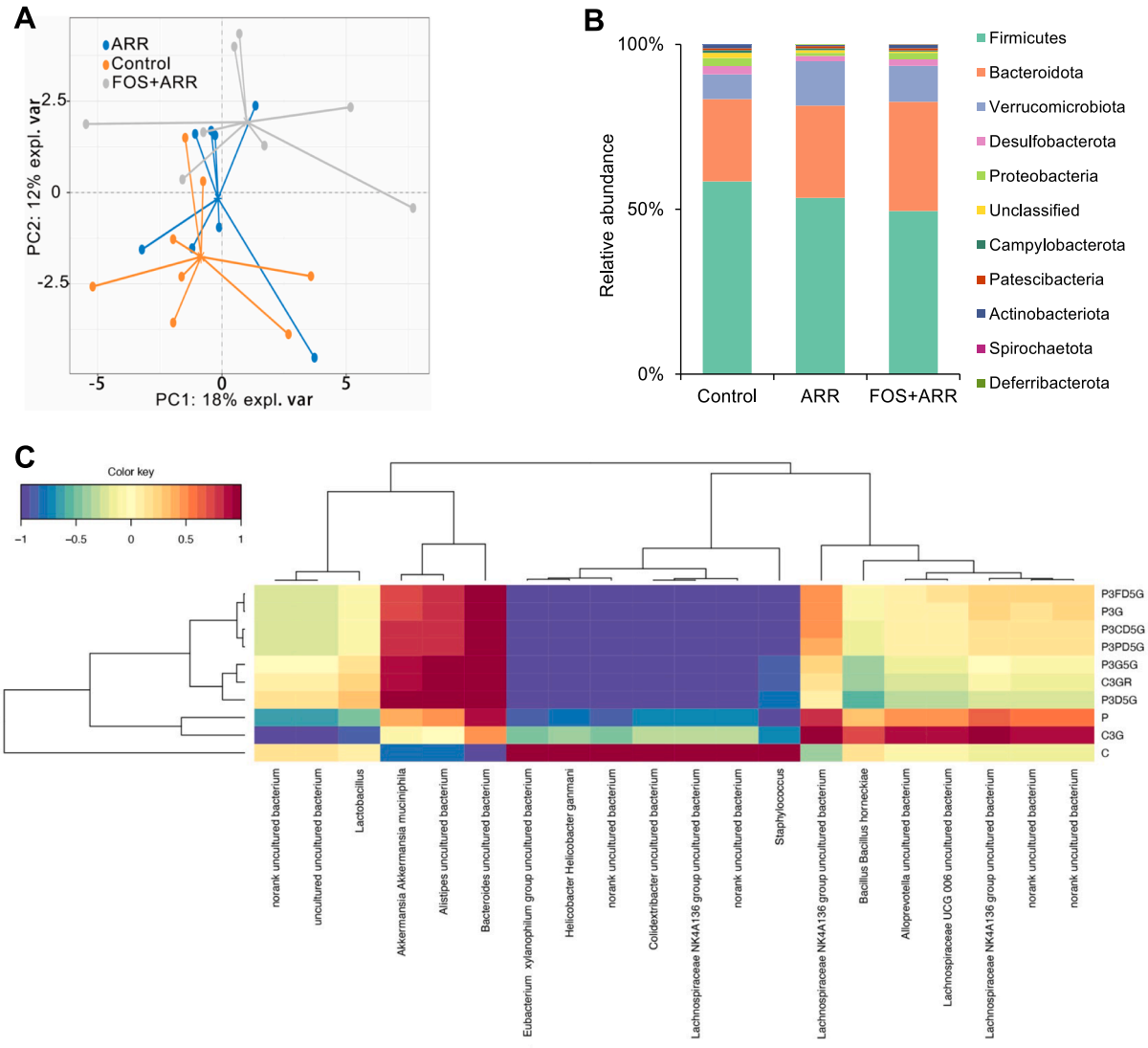

Fig. 4. Co-ingestion of FOS and ARR changed the gut microbial composition in mice. (A): the Score plot of PCA for relative abundance (RA) of intestinal microbes at genus level. (B): the RA of intestinal microbes at phylum level. (C): the CIM of sPLS analysis for RA of gut microbes and cecal anthocyanins levels.

Milenkovic, 2019). Although there is no literature directly showing that *Akkermansia* and *Alistipes* genus affects metabolism of anthocyanin, anthocyanins do have the ability to increase the RA of *Akkermansia* and *Alistipes* genus (Luo, Fang, Yuan, Jin, & Guo, 2019; Peng et al., 2020). Thus, the positive correlation between ARR and *Akkermansia* or *Alistipes* genus because ARR promote the proliferation of *Akkermansia* or *Alistipes* genus, while not *Akkermansia* or *Alistipes* genus regulated ARR metabolism. These results suggested that *Bacteroides* genus might play a bridge role between FOS elevating the bioavailability of ARR.

3.5. FMT verified the role of gut microbiota during FOS regulated ARR metabolism

To verify the role of gut microbiota in FOS regulating ARR metabolism, the fecal microbiota from ARR and FOS + ARR groups mice were transplanted to pseudo-sterile mouse gut. Although the serum free C3G level in the two receptor group mice was no significant difference, the receptor mice for FOS + ARR group showed higher serum free P, P3G and C levels than the receptor mice for ARR group (Fig. 5A-D). Moreover, the serum total P3G level in the mice that received gut microbiota from FOS + ARR group was significantly higher than that in the mice that received gut microbiota from ARR group (Fig. 5E). The serum total C3G, C and P levels in FOS + ARR-FMT group were slightly higher than that in ARR-FMT group despite the fact that the difference have no statistical significance (Fig. 5F-H). Interestingly, the gut microbiota shaped by fecal bacteria from FOS + ARR group caused 40% higher cecal P levels than the gut microbiota formed by ARR group (Fig. 5I). Additionally, similar difference was found in the cecal C3G levels (Fig. 5J). However, there was no visible difference for the cecal C and C3GR levels between the two FMT groups (Fig. 5K-L). These results confirm that gut microbiota plays a key role in FOS increasing ARR bioavailability.

4. Conclusions

Current study results preliminarily demonstrated that FOS could enhance the antioxidant activity of ARR via elevate their bioavailability. Further analysis suggests that the underlying mechanism involves that FOS regulated gut microbiota to promote hydrolyzation of pelargonidin glucosides with complex molecular structure to pelargonidin and simple glucosides, which were easily absorbed into the blood. These findings suggested that co-ingestion of FOS and ARR might is an effective

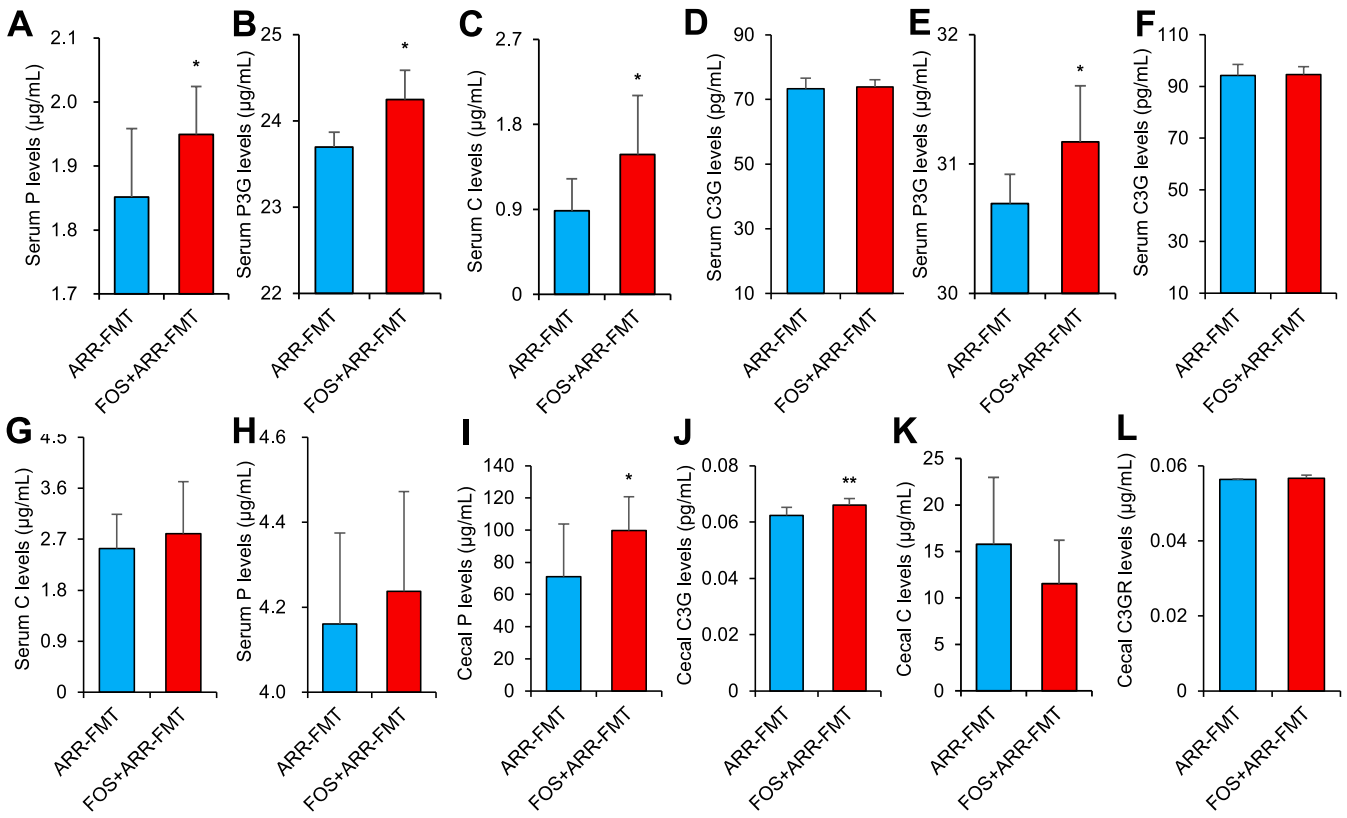

Fig. 5. The serum free (A-D) and total (E-H) anthocyanidins and anthocyanins levels, and cecal anthocyanidins and anthocyanins contents (I-L). * $P < 0.05$, ** $P < 0.01$, which was analyzed by *t*-test.

strategy for using bioactivity of ARR. Furthermore, our results can also extensive application of FOS as a potential enhancer for anthocyanin bioactivity in functional foods.

CRediT authorship contribution statement

Wenfeng Li: Conceptualization, Supervision, Funding acquisition, Project administration, Methodology, Formal analysis, Data curation, Investigation, Writing – original draft. **Wanjie Zhang:** Project administration, Data curation, Formal analysis, Writing – review & editing. **Xin Fan:** Investigation, Methodology. **Hai Xu:** Investigation. **Hong Yuan:** Methodology. **Yimeng Wang:** Investigation. **Rui Yang:** Investigation. **Hua Tian:** Investigation. **Yinmei Wu:** Investigation. **Hongyan Yang:** Resources, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100765.

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