Systematic evaluation of polyphenols composition and antioxidant activity of mulberry cultivars subjected to gastrointestinal digestion and gut microbiota fermentation

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Abstract:

In this study, mulberry cultivars were found to have abundant bioactive compounds such as phenolics (100.97–586.23 mg gallic acid equivalents/100 g fresh weight), flavonoids (16.38–368.16 mg rutin equivalents/100 g fresh weight) and procyanidins (4.20–121.56 mg catechin equivalents/100 g fresh weight) after *in vitro* digestion. HPLC-TOF-MS analysis revealed that digested mulberry cultivars contained multiple phenolic compounds including cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. After gut microbiota fermentation, the contents of anthocyanins were increased initially, then decreased with time, and some anthocyanin metabolites (such as 3,4-Dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, etc.) were detected. Our further cellular study indicated that mulberry (Hanguo) possessed reactive oxygen species scavenging capacity after gut microbiota fermentation. Our results indicated that the new cultivar Hanguo contained abundant polyphenols and exhibited potent antioxidant property after *in vitro* digestion and gut microbiota fermentation compared with other mulberry cultivars, which could be recommended as a dietary source of functional foods.

Keywords: Mulberry | Polyphenol | *In vitro* digestion | Gut microbiota fermentation | Antioxidant capacity

Article:

1. Introduction

The human body is constantly exposed to free radicals, either produced through endogenous biochemical processes or external lifestyle factors. Superoxide anion radical ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl ($^{\bullet}OH$) and alkoxyl ($RO^{\bullet}$) are major reactive oxygen species (ROS), which are produced during the cell metabolism. ROS are associated with the pathogenesis and progression of various diseases, including diabetes, cancer, and cardiovascular diseases (Kaneto et al., 2010, Sugamura and Keaney, 2011, Trachootham et

al., 2009, Xie et al., 2018). Accumulating evidence showed that antioxidants in food or supplements exhibit protection against ROS-induced damage (Genkinger et al., 2004, John et al., 2002). A proper diet including an increase in the proportion of fruits and vegetables in the daily diet can mitigate or hinder the oxidative stress-mediated complications in humans. These potential benefits could be due to the abundant availability of phenolics in fruits and vegetables (Genkinger et al., 2004, Li et al., 2018, Su et al., 2018).

Berry fruits have been reported to contain abundant antioxidant components, including anthocyanins, procyanidins and other phenolics (Del Rio et al., 2010, Gowd et al., 2017, Mikulic-Petkovsek et al., 2012, Xu et al., 2019). In fact, some dietary polyphenolics are susceptible to metabolism by gastrointestinal and colon tract before their absorption in specific organs, which can undergo a structural modification or degradation (Bouayed et al., 2012, Brown et al., 2014). Thus, the biological activities of these functional components may change upon exposure to digestive conditions. The antioxidant property of berry fruits was extensively investigated, but research on the effect of gastrointestinal digestion and human gut microbiota fermentation on polyphenols and their antioxidant activity is limited. More accurate results regarding bioavailability and bioaccessibility of food sources can be achieved by *in vivo* studies using animal models and human volunteers. However, *in vivo* studies are time-consuming and expensive to perform. In this regard, *in vitro* gastrointestinal digestion and human gut microbiota fermentation methods could provide a more convenient way to study the composition of bioactive components and their biological activities after ingestion (Cardinali et al., 2011, Tagliazucchi et al., 2010).

Mulberry (Morus spp.) is a nutrient-rich berry fruit, which has been reported to contain abundant polyphenols (Natic et al., 2015, Hu et al., 2018, Xie et al., 2019). Some studies showed that mulberry extracts have potent antioxidant, antidiabetic, antitumor, neuroprotective, and hypolipidemic activities (Chan et al., 2013, Huang et al., 2008, Jeong et al., 2010, Kang et al., 2006, Liu et al., 2009, Wang et al., 2013, Yang et al., 2010), which are mainly associated with phytochemicals in mulberry fruit, including anthocyanins, procyanidins and other phenolics (Arfan et al., 2012, Bae and Suh, 2007, Ozgen et al., 2009). Though there are many studies on mulberry extract, systematic investigation on the bioactivity of mulberry cultivars using *in vitro* gastrointestinal digestion and human gut microbiota fermentation models are limited.

Hence, the purpose of the present study was to evaluate the antioxidant activity and bioactive components of multiple mulberry cultivars after *in vitro* gastrointestinal digestion and human gut microbiota fermentation. The *in vitro* antioxidant capacities of different mulberry cultivars were evaluated according to ABTS (2-2′azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) and FRAP (ferric reducing antioxidant power) methods. HPLC-TOF-MS was used to identify the phenolic components in digested and fermented mulberry cultivars. Furthermore, the cell model (H_2O_2-induced ROS) was employed to evaluate the cellular antioxidant capacity (ROS scavenging capacity) of digested and fermented mulberry cultivars.

2. Materials and methods

2.1. Chemicals and materials

ABTS (2-2′azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), TPTZ (2,4,6-tripyridy-S-triazine), DCFH-DA (2′,7′-dichlorofluorescin diacetate), gallic acid, citric acid, quercetin-*3*-*O*-rutinoside (rutin), cyanidin-3-*O*-rutinoside, catechin, pepsin, pancreatin and bile salts were obtained from Sigma Chemicals (St. Louis, MO, USA). Folin–Ciocalteu reagent, Vitamin C, vanillin, HPLC-grade acetonitrile and methanol were purchased from Aladdin (Shanghai, China). All other reagents used were of analytical grade unless otherwise stated.

2.2. Mulberry fruits

Mulberry fruits were collected from the same orchard under the same agronomical and environmental conditions in Zhejiang province, China in May 2015, and stored at −80 °C. A total of 11 mulberry cultivars were analyzed, including two white and nine black mulberry cultivars. Zhenzhubai and Xiaodianhong are white mulberry cultivars (Morus atropurpurea Roxb). The remaining 9 cultivars are black mulberry cultivars, including Morus alba L (Mengsi, Tianquan, Riben, and Hanguo), Morus atropurpurea Roxb (Taiwanguosang, Da 10, Guangdong, and Zhongshenyihao) and Morus multicaulis Perr (Hongguo). These cultivars were characterized as new mulberry cultivars (Mengsi, Hanguo, Riben, Zhongshenyihao, Guangdong, and Xiaodianhong) and common mulberry cultivars (Da 10, Hongguo, Tianquan, Taiwanguosang, and Zhenzhubai). Each mulberry cultivar was plucked randomly from several different plants and categorized into three groups for uniformity in color and shape.

2.3. *In vitro* gastrointestinal digestion and gut microbiota fermentation

Eleven mulberry cultivars were employed in the gastrointestinal digestion study as described previously, with slight modifications (Bao et al., 2018, Zhang et al., 2017). Briefly, 10 g of homogenized mulberry fruit was diluted with 10 mL of distilled water followed by mixing with porcine pepsin solution (3000 U/mg, 2 mg, 4 mL). The mixture was adjusted to pH 2.0 followed by the incubation for 2 h at 37 °C using a shaking water bath (100 r/min) to simulate gastric digestion. For intestinal digestion, the pH of the mixture was changed to 6.0 with 1 mol/L sodium bicarbonate followed by the addition of 4 mL of pancreatic juice (contains 20 mg of pancreatin and 125 mg of bile salts). Afterward, the pH was adjusted to 7.4 with 1 mol/L sodium hydroxide and incubated at 37 °C for 2 h. After that, the volume of the digested mulberry cultivars was immediately made up to 30 mL with distilled water and then centrifuged at 3000 g for 10 min at 4 °C. The supernatant was collected, filtered (using 0.22 μM Millipore filter) and finally preserved at −80 °C for further investigation.

The gastrointestinal digested mulberry homogenate was used for the *in vitro* study of human gut microbiota fermentation following by the previous report with slight modifications (Gowd et al., 2018). Fecal samples were collected from three healthy donors who frequently consumed a healthy diet and did not take any antibiotics for at least three months prior to the study, and the samples were instantly taken to the anaerobic chamber. Afterward, the samples were mixed and homogenized using carbonate phosphate buffer (0.2 M and pH 5.5), further diluted (1%) with phosphate buffer, and filtered through 1 mM sieve. Then, the gastrointestinal digested mulberry was taken into sterilized tubes and then mixed with fecal slurry. Finally, the mixture was incubated at 37 °C in an anaerobic chamber (gas phase: N_2/ CO_2/ H_2 = 85/10/5, v/v/v) with constant stirring for 0, 2, 6, 12, 24, and 48 h.

2.4. *In* vitro antioxidant assay

2.4.1. ABTS method

The ABTS method (Bao et al., 2016) was employed to investigate the antioxidant capacity of mulberry cultivars. Briefly, 20 μL of the digested sample was mixed with 700 μL of $ABTS^{\cdot+}$ solution and instantly incubated for 6 min in the dark. The absorbance was measured at 734 nm. The antioxidant capacity of digested mulberry cultivar was determined as mg vitamin C equivalents/100 g fresh weight (FW).

2.4.2. FRAP method

Ferric reducing antioxidant power (FRAP) method (Chen et al., 2013) was applied to determine the antioxidant activity of digested mulberry cultivars. To begin the analysis, 20 μL of the digested mulberry cultivar was incubated with 700 μL FRAP solution at 37 °C for 30 min, and the absorbance was read at 593 nm. Vitamin C was used as a standard. The antioxidant capacity of the sample was expressed as mg vitamin C equivalents/100 g fresh weight (FW).

2.5. Determination of total phenolic, flavonoid and procyanidin contents

Total phenolic contents (TPC) were detected by Folin–Ciocalteu assay (Bao et al., 2016). Data were presented as mg gallic acid equivalents (GAE)/100 g fresh weight (FW). The colorimetric assay was used to measure total flavonoid contents (TFC) (Do et al., 2014). The data were expressed as mg rutin equivalents (RE)/100 g fresh weight (FW). Procyanidins were measured according to the previous method (Hirth, Preiss, Mayer-Miebach, & Schuchmann, 2015). The results were expressed as mg catechin equivalents (CE) per 100 g fresh weight (FW).

2.6. Analysis of phenolic compounds by HPLC-TOF-MS

The quantification of phenolic compounds in the mulberry sample was analyzed by HPLC-DAD (Dionex UltiMate 3000, ThermoFisher Scientific, USA) with Promosil C18 (4.6 × 250 mm, 5 μm) column. The mobile phase A was 1.5% formic acid aqueous solution and mobile phase B was acetonitrile containing 0.1% formic acid. A linear gradient program: 0–15 min from 95 to 87% (A), 15–20 min from 87 to 85% (A), 20–25 min from 85 to 70% (A), 25–28 min 70% (A), 28–32 min from 70 to 55% (A), 32–35 min 55% (A), 35–40 min from 55 to 10% (A), 40–45 min 10% (A), 45–55 min from 10 to 95% (A), then 95% (A) for 5 min was used. The procedure was operated at 30 °C with a flow rate at 0.8 mL/min and the injection amount was 10 μL. Phenolic compounds were detected at 280 and 520 nm. Standards were used for quantification. The qualitative analysis of phenolic compounds in digested mulberry was operated by a Triple-TOF Mass Spectrometry System (AB SCIEX, Triple-TOF 5600plus Framingham, Massachusetts, USA) using Promosil C18 (4.6 × 250 mm, 5 μm) column. The mobile phase A was 0.1% formic acid aqueous solution and mobile phase B was acetonitrile containing 0.1% formic acid. A linear gradient program: 0–5 min 5% (B), 5–25 min from 5 to 16% (B), 25–33 min from 16 to 30% (B), 33–35 min from 30 to 90% (B), 35–40 min 90% (B), 40–45 min from 90 to 5% (B), then 5% (B) for 5 min was used. The procedure was operated at 30 °C with a flow rate at 0.8 mL/min, and the

injection amount was 10 μL. The mass spectrometer was set for ion source with minimum range MS 100 and maximum at 2000 in a negative ion mode detection. The wavelength for UV detector was set to 280 nm. The gas temperature was set at 550 °C, and the capillary voltage was 4500 V. The compounds were identified according to ion molecular mass and MS_2 data.

2.7. Cell culture

Human intestinal epithelial cell line Caco-2 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Caco-2 cells were grown in DMEM medium (Gibco), supplemented with 10% of new calf serum, 0.1 mg/mL streptomycin and 100 U/mL penicillin. Caco-2 cells were incubated in a humidified incubator containing 5% CO_2 and 95% air at 37 °C (Chen, Su, Xu, Bao, & Zheng, 2016).

2.8. Determination of cellular ROS

Caco-2 cells were seeded into 24-well cell culture plates at a density of 2.5×10^4 cells/well. After incubation for 24 h, mulberry sample (0.5 mg/mL, 1 mg/mL or 2 mg/mL, respectively) was added to each well for another 24 h. Then H_2O_2 (500 μmol/L) was added to each well. After incubation for 24 h, the media was removed, cells were washed with PBS. 10 μmol/L DCFH-DA was added to each well, followed by incubation for 2 h at 37 °C. Then cells were rewashed with PBS. Cellular fluorescence was immediately evaluated by an inverted fluorescence microscope. The fluorescence intensity of DCF was calculated by fluorescent images from five different microscopic fields through ImageProPlus6.0 (Media Cybernetics, Inc., Singapore) (Chen et al., 2013).

2.9. Statistical analysis

All data were obtained from at least three independent experiments and expressed as mean ± standard deviations (SD). Statistical differences of the results were performed by One-way ANOVA, with the multiple range significant difference (Duncan) test ($p < 0.05$) using SPSS 22.0. Principal Component Analysis (PCA) was tested using The Unscrambler X (version 10.1).

3. Results and discussion

3.1. Comparison of antioxidant activity of digested mulberry cultivars

In our previous study (Bao et al., 2016), we evaluated the antioxidant activity of non-digested mulberry cultivars, and found that most of the mulberry fruits extracts showed potent antioxidant activity. However, fruits undergo a digestion process before absorption, which may change the bio-accessibility of phytochemicals and their bioactivity. In order to evaluate the antioxidant activity of mulberry fruits more appropriately, we built an *in vitro* digestion model before assessment of the bioactivity. In this work, *in vitro* antioxidant assays (ABTS and FRAP) were performed to evaluate the antioxidant activity of digested mulberry cultivars. The results of *in vitro* antioxidant activity of digested mulberry cultivars were presented in Table 1. The $ABTS^{\cdot+}$ scavenging activity of digested mulberry cultivars ranged from 217.01 (Zhenzhubai) to 1270.48 (Hongguo) mg VCE/100 g FW. White mulberry showed less level of antioxidant

activity than black mulberry cultivars. The antioxidant activity of some digested black mulberry cultivars (Da10, Taiwanguosang, Mengsi, Riben, and Zhongshenyihao) was better than the corresponding non-digested cultivars. In contrast, Hongguo, Tianquan and Guangdong cultivars showed weaker antioxidant capacity than non-digested cultivars. In the case of mulberry cultivars, there was no significant change in the antioxidant activity before and after digestion. White mulberry cultivars (Zhenzhubai, 217.01 mg VCE/100 g FW; Xiaodianhong, 217.24 mg VCE/100 g FW) showed the better antioxidant capacity after *in vitro* digestion compared to non-digested samples (Zhenzhubai, 73 mg VCE/100 g FW; Xiaodianhong, 51 mg VCE/100 g FW) (Bao et al., 2016). According to FRAP assay (Table 1), the antioxidant capacity of digested mulberry cultivars was ranged from 11.92 (Zhenzhubai) to 319.40 (Hanguo) mg VCE/100 g FW. After simulating gastrointestinal digestion, white mulberry cultivars showed poor antioxidant activity compared with black mulberry cultivars, which is consistent with the results of ABTS. In comparison to the FRAP assay, digested white mulberry cultivars possessed better antioxidant activity than the corresponding non-digested cultivars, while all digested black mulberry cultivars showed less antioxidant capacity than their non-digested mulberry cultivars (Bao et al., 2016). Based on the ABTS and FRAP assays, the new cultivar Hanguo and the common cultivar Hongguo showed better antioxidant activity than other mulberry cultivars.

3.2. Determination of phenolic components in digested mulberry cultivars

According to the above results, we found that mulberry cultivars exhibited good antioxidant properties after simulating *in vitro* digestion. Therefore, we further determined the bioactive substances in digested mulberry cultivars. The total phenolic contents (TPC) were ranged from 100.97 (Zhenzhubai) to 586.23 (Hanguo) mg GAE/100 g FW, and the total flavonoid contents (TFC) were from 16.38 (Xiaodianhong) to 368.16 (Hanguo) mg RE/100 g FW (Table 1). After *in vitro* digestion, TPC and TFC of white mulberry fruits (TPC, 100.97 – 101.51 mg GAE/100 g FW; TFC, 16.38 – 17.11 mg RE/100 g FW) were significantly lower than black mulberry cultivars (TPC, 307.20 – 586.23 mg GAE/100 g FW; TFC, 71.33 – 368.16 mg RE/100 g FW). In addition, black mulberry fruits contained high content of procyanidins with the range from 15.73 (Tianquan) to 121.56 (Hanguo) mg CE/100 g FW, while low levels of procyanidins were found in white mulberry cultivars (Xiaodianhong, 4.38 mg CE/100 g FW; Zhenzhubai, 4.20 mg CE/100 g FW). In our previous study (Bao et al., 2016), we found that the content of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside in mulberry cultivars were 125–334 mg/100 g FW and 25–150 mg/100 g FW, respectively. After *in vitro* digestion, mulberry anthocyanins (cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside) were significantly degraded compared to non-digested mulberry cultivars (Bao et al., 2016). The contents of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside in digested mulberry cultivars ranged from 0.0009 to 2.9967 mg/100 g FW and 0.0004–1.9440 mg/100 g FW. The structure of anthocyanin is stable under acidic condition and become change at the alkaline condition (Castaneda-Ovando, Pacheco-Hernandez, Paez-Hernandez, Rodriguez, & Galan-Vidal, 2009). Therefore, the alkaline environment of intestine could significantly affect the structure of anthocyanins during the digestion process. After *in vitro* digestion, TPC of most mulberry cultivars were decreased compared to non-digested mulberry cultivars (Bao et al., 2016), except for Hongguo cultivar. TFC was also decreased in most digested mulberry cultivars while increased in common mulberry cultivar (Da 10) and new mulberry cultivar (Hanguo). On the contrary, procyanidins were decreased significantly in all mulberry cultivars (Bao et al., 2016). Taken together, Hanguo (new cultivar) was found to

possess the most abundant bioactive components (TPC, 586.23 mg GAE/100 g FW; TFC, 368.16 mg RE/100 g FW; procyanidins 121.56 mg CE/100 g FW; cyanidin-3-*O*-glucoside 2.9967 mg/100 g FW) after simulating digestion followed by Mengsi and Hongguo (Table 1).

In order to further elucidate the relationship between antioxidant activity and bioactive components, the Pearson's correlation coefficient was applied to antioxidant activity (ABTS and FRAP), phenolics, flavonoids, procyanidins and anthocyanins (cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside). The results were presented in Table S1. According to ABTS assay, antioxidant activity was closely associated with phenolics (0.689, $p < 0.05$), flavonoids (0.587, $p < 0.05$), procyanidins (0.843, $p < 0.01$), cyanidin-3-*O*-glucoside (0.823, $p < 0.01$) and cyanidin-3-*O*-rutinoside (0.697, $p < 0.05$). According to FRAP assay, the antioxidant capacity showed positive and significant correlation with phenolics (0.850, $p < 0.01$), flavonoids (0.961, $p < 0.01$), procyanidins (0.938, $p < 0.01$), cyanidin-3-*O*-glucoside (0.800, $p < 0.01$) and cyanidin-3-*O*-rutinoside (0.737, $p < 0.01$). Cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside were the main phenolic compounds in mulberry fruits. Therefore, the composition of anthocyanins is likely to play a key role in the antioxidant capacity of mulberry cultivars after *in vitro* digestion.

3.3. Identification of phenolic compounds in digested mulberry

Table 2 summarized the HPLC-TOF-MS/MS characterization of digested mulberry. Chloro adduct of sucrose (*m*/*z* 377) at 2.94 min was determined based on the MS/MS data and gallic acid at 2.99 min were detected at *m*/*z* 215 with MS^2 fragment ions (*m*/*z* 59) (da Costa et al., 2016, Pei et al., 2015). Compound 3 showed a $[M-H]^-$ ion at *m*/*z* 191 with MS^2 fragment ions (*m*/*z* 85) was identified as quinic acid (Quirantes-Pine et al., 2013). The mass data at *m*/*z* 367 and MS^2 fragment ion at *m*/*z* 205 were identified as 3-*O*-feruloylquinic acid according to the previous study (Jaiswal, Matei, Subedi, & Kuhnert, 2014). Compounds with a molecular ion at *m*/*z* 191 and *m*/*z* 329 and retention time at 4.25 and 10.61 min were identified as citric acid and vanillic acid hexoside (Rodriguez-Perez, Quirantes-Pine, Fernandez-Gutierrez, & Segura-Carretero, 2013). The mass data at *m*/*z* 353 and MS^2 fragment ion at *m*/*z* 191 were identified as 3-*O*-Caffeoylquinic acid (Natic et al., 2015). Neochlorogenic acid was identified according to the MS data at 353 and MS^2 fragment ion at *m*/*z* 191 (Fu et al., 2014). The Ion at *m*/*z* 447 and 593 with fragment *m*/*z* at 284 corresponded to the loss of one hexoside or rutinoside were identified as cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside (Sun, Lin, & Chen, 2012). Compound with a molecular ion at *m*/*z* 353 with MS^2 fragment ions at *m*/*z* 173 was identified as chlorogenic acid (Fu et al., 2014). The identification of the $[M-H]^-$ ion at *m*/*z* 771, *m*/*z* 609 and *m*/*z* 463 were derivatives of quercetin according to the typical fragment ion at *m*/*z* 300 (Natic et al., 2015). Three quercetin derivatives were identified as quercetin-3-*O*-rutinoside-glucoside (*m*/*z* 771), quercetin-3-O-rutinoside (*m*/*z* 609) and quercetin-3-*O*-hexoside (*m*/*z* 463) at 22.97, 32.25 and 33.26 min, respectively. Cholic acid was also found in digested mulberry cultivars according to the MS data at 407 and MS^2 fragment ion at *m*/*z* 343 (Bozzetto et al., 2017).

Table 1. The antioxidant capacity (ABTS and FRAP) and phenolic contents of *in vitro* gastrointestinal digested mulberry cultivars.

	Common cultivars					New cultivars					
	Da 10	**Hongguo**	**Tianquan**	**Taiwan guosang**	**Zhenzhubai**	**Mengsi**	**Hanguo**	**Riben**	**Zhongshen yihao**	**Guangdong**	**Xiaodianhong**
ABTS (mg VCE/100 g FW)	530.8 ± 5.84d	1270.48 ± 12.29a	337.18 ± 4.49 g	304.06 ± 1.29 h	217.01 ± 4.08i	656.44 ± 4.58c	850.85 ± 19.78b	537.98 ± 21.81d	468.3 ± 3.69e	357.22 ± 6.45f	217.24 ± 1.87i
FRAP (mg VCE/100 g FW)	92.24 ± 3.73f	210.07 ± 5.38b	54.15 ± 3.06 g	160.16 ± 6.65d	11.92 ± 1.33 h	178.63 ± 5.1c	319.40 ± 6.14a	132.39 ± 3.74e	90.70 ± 3.32f	128.72 ± 3.32e	13.80 ± 0.50 h
Total phenolic contents (mg GAE/100 g FW)	307.20 ± 4.47 g	457.85 ± 8.58c	205.36 ± 4.77 h	329.72 ± 8.76f	100.97 ± 1.55i	509.63 ± 11.49b	586.23 ± 4.15a	389.35 ± 7.92d	314.54 ± 5.88 g	347.87 ± 4.53e	101.51 ± 3.53i
Total flavonoid contents (mg RE/100 g FW)	88.84 ± 6.46f	157.11 ± 2.89c	71.33 ± 3.20 g	164.47 ± 6.52bc	17.11 ± 0.78 h	163.62 ± 3.61bc	368.16 ± 14.11a	166.29 ± 8.23b	112.93 ± 3.00e	126.98 ± 2.69d	16.38 ± 0.74 h
Procyanidins (mg CE /100 g FW)	21.91 ± 2.00f	104.34 ± 3.16b	15.73 ± 0.93 g	31.72 ± 0.91e	4.20 ± 0.79 h	60.37 ± 0.75c	121.56 ± 2.28a	45.60 ± 0.60d	22.38 ± 3.03f	58.40 ± 1.33c	4.38 ± 0.43 h
Cyanidin-3-*O*-glucoside (mg /100 g FW)	0.0020 ± 0.0000e	2.7828 ± 0.0574b	0.0009 ± 0.0000e	0.0009 ± 0.0000e	ND	2.5023 ± 0.1129c	2.9967 ± 0.0871a	1.6927 ± 0.2186d	0.0025 ± 0.0002e	0.0100 ± 0.0002e	ND
Cyanidin-3-*O*-rutinoside (mg CGE/100 g FW)	0.0005 ± 0.0000e	1.1797 ± 0.0821c	0.0004 ± 0.0000e	0.0004 ± 0.0000e	ND	1.9440 ± 0.3887a	1.5472 ± 0.2118b	0.0927 ± 0.0036d	0.0010 ± 0.0000e	0.0016 ± 0.0000e	ND

The values are expressed as mean ± SD (n = 3); VCE, vitamin C equivalents; ND = Not Detected. GAE, gallic acid equivalents; RE, rutin equivalents; CE, catechin equivalents; CGE, cyanidin 3-*O*-glucoside equivalents; FW, fresh weight. Values in the same line with different lower-case letters are significantly different at $P < 0.05$ according to Duncan the test.

Table 2. Identification of phenolic compounds of *in vitro* gastrointestinal digested mulberry by LC-MS^2.

No	Rt(min)	MS	MS^2	Formula	Tentative identification
1	2.9378	377.0857	179.0557(100), 161.0449(60), 341.1100(35), 215.0325(10)	$C_{12}H_{22}O_{11}Cl^-$	Chloro adduct of sucrose
2	2.9908	215.0335	59.0162(100), 71.0158(100), 89.0250(100), 87.0114(50)	$C_7H_6O_5$	Gallic acid
3	3.0818	191.0569	191.0560(100), 85.0301(40), 93.0354(15), 171.0286(10)	$C_7H_{12}O_6$	Quinic acid
4	3.2445	367.1050	367.1052(100),205.0500(40),277.0727(30),143.0351(20), 79.0556(15)	$C_{17}H_{20}O_9$	3-*O*-feruloylquinic acid
5	4.2497	191.0209	87.0103(100), 111.0101(65), 85.0286(33), 85.0413(33), 67.0315(32), 57.0442(32)	$C_6H_8O_7$	Citric acid
6	10.6089	329.0878	329.0892(100), 167.0358(30), 109.0313(30)	$C_{14}H_{17}O_9$	Vanillic acid hexoside
7	16.8204	353.0879	191.0559(100), 179.0342(55), 135.0453(30)	$C_{16}H_{18}O_9$	3-*O*-Caffeoylquinic acid
8	21.5913	447.0927	284.0324(100),285.0399(70),447.0936(30),283.0252(20), 47.0082(10), 240.0431(10)	$C_{21}H_{20}O_{11}$	Cyanidin-3-*O*-glucoside
9	22.1719	353.0876	191.0558(100)	$C_{16}H_{18}O_9$	Neochlorogenic acid
10	22.7856	593.1503	593.1537(100), 284.0331(100), 285.0402(70)	$C_{27}H_{31}O_{15}$	Cyanidin-3-*O*-rutinoside
11	22.9151	353.0879	173.0451(100),191.0558(70),179.0346(68), 135.0449(62), 93.0359(15)	$C_{16}H_{18}O_9$	Chlorogenic acid
12	22.9732	771.1986	771.2050(100), 609.1482(45), 462.0800(20), 300.0354(20)	$C_{33}H_{40}O_{21}$	Quercetin-3-*O*-rutinoside-glucoside
13	32.3482	609.1454	609.1505(100), 300.0277(80), 301.0358(50)	$C_{27}H_{30}O_{16}$	Quercetin-3-*O*-rutinoside
14	33.2572	463.0876	300.0280(100), 301.0365(55), 271.0253(35)	$C_{21}H_{20}O_{12}$	Quercetin-3-*O*-hexoside
15	37.9196	407.2795	407.2815(100), 343.2655(24), 345.2810(18), 289.2181(16)	$C_{24}H_{40}O_5$	Cholic Acid

Table 3. The antioxidant capacity (ABTS and FRAP) and phenolic contents of *in vitro* gut microbiota fermented mulberry cultivars.

	Hanguo (Black mulberry)						Zhenzhubai (White mulberry)					
Time (h)	**0**	**2**	**6**	**12**	**24**	**48**	**0**	**2**	**6**	**12**	**24**	**48**
ABTS (mg VCE/100 g FW)	846.59 ± 30.21b	929.36 ± 18.53a	763.62 ± 7.17c	694.15 ± 8.21d	679.48 ± 7.93d	608.96 ± 4.94e	230.38 ± 12.11B	255.85 ± 5.32A	147.45 ± 3.89C	78.37 ± 1.37E	91.04 ± 1.91D	92.09 ± 1.28D
FRAP (mg VCE/100 g FW)	385.35 ± 3.79c	321.23 ± 7.85e	355.01 ± 21.17d	428.51 ± 6.36a	426.73 ± 7.44a	402.72 ± 7.96b	10.83 ± 0.64A	7.32 ± 0.59C	10.80 ± 0.68A	11.40 ± 1.76A	8.90 ± 0.47BC	9.61 ± 0.74AB
Total phenolic contents (mg GAE/100 g FW)	635.12 ± 13.22d	620.93 ± 14.38e	901.23 ± 6.05c	934.70 ± 2.85b	960.42 ± 3.88a	543.03 ± 3.00f	102.77 ± 2.02A	100.08 ± 1.55B	99.53 ± 1.91B	88.86 ± 2.45D	91.89 ± 1.46C	91.17 ± 1.19CD
Total flavonoid contents (mg RE/100 g FW)	395.90 ± 6.77b	407.56 ± 11.93ab	418.50 ± 11.15a	416.76 ± 1.36a	416.04 ± 12.64a	408.16 ± 16.05ab	22.63 ± 3.21A	18.15 ± 4.66B	16.93 ± 6.00BC	12.35 ± 2.00D	14.72 ± 4.84CD	13.38 ± 4.88D
Procyanidins (mg CE /100 g FW)	95.36 ± 0.73e	86.68 ± 0.81f	114.72 ± 1.31c	131.01 ± 1.20a	125.76 ± 1.48b	112.51 ± 0.58d	3.35 ± 0.37CD	6.26 ± 1.31B	1.33 ± 0.38D	3.46 ± 1.99CD	4.21 ± 0.66C	8.89 ± 1.47A
Cyanidin-3-*O*-glucoside (mg /100 g FW)	46.19 ± 1.24c	37.4 ± 0.87d	56.23 ± 3.24b	68.06 ± 2.24a	46.19 ± 1.68c	29.13 ± 0.73e	ND	ND	ND	ND	ND	ND
Cyanidin-3-*O*-rutinoside (mg CGE/100 g FW)	27.39 ± 0.64b	23.08 ± 1.11c	41.17 ± 1.05a	40.55 ± 2.19a	27.39 ± 0.83b	18.67 ± 0.57d	ND	ND	ND	ND	ND	ND
3,4-Dihydroxybenzoic acid (mg /100 g FW)	6.22 ± 0.21c	6.55 ± 0.21c	7.24 ± 0.12b	7.43 ± 0.65b	9.1 ± 0.33a	5.53 ± 0.39d	0.93 ± 0.03A	0.49 ± 0.01B	0.17 ± 0.00D	0.15 ± 0.00D	0.26 ± 0.00C	0.26 ± 0.01C
2,4,6-trihydroxybenzoic acid (mg /100 g FW)	41.37 ± 0.87a	4.82 ± 0.09e	7.71 ± 0.18d	2.94 ± 0.12f	8.27 ± 0.28c	30.72 ± 0.35b	3.01 ± 0.12D	3.46 ± 0.14C	2.56 ± 0.09E	6.13 ± 0.25B	14.21 ± 0.28A	1.29 ± 0.05F
2,4,6-trihydroxybenzaldehyde (mg /100 g FW)	7.23 ± 0.35f	46.44 ± 0.58a	42.85 ± 1.21b	23.91 ± 0.94c	14.56 ± 0.25d	10.36 ± 0.51e	3.19 ± 0.11A	1.92 ± 0.17B	1.84 ± 0.04B	1.03 ± 0.01D	0.32 ± 0.01E	1.49 ± 0.03C
Catechol (mg /100 g FW)	1.46 ± 0.02a	0.70 ± 0.01c	0.27 ± 0.01e	0.54 ± 0.02d	0.54 ± 0.03d	1.29 ± 0.05b	0.54 ± 0.01A	0.33 ± 0.00B	ND	ND	0.27 ± 0.01C	0.18 ± 0.00D
Catechin (mg /100 g FW)	6.56 ± 0.22a	43.4 ± 1.23c	57.39 ± 1.59b	33.44 ± 1.04d	18.86 ± 0.75e	4.66 ± 0.32f	54.96 ± 2.06B	64.5 ± 2.87A	15.18 ± 1.12E	9.3 ± 0.37F	37.83 ± 1.39C	31.22 ± 1.22D
Chlorogenic acid (mg /100 g FW)	1.14 ± 0.04b	0.32 ± 0.01e	0.18 ± 0.01f	0.52 ± 0.01c	0.43 ± 0.01d	5.25 ± 0.24a	1.15 ± 0.02CD	1.59 ± 0.03B	0.94 ± 0.05E	1.18 ± 0.03C	2.09 ± 0.08A	1.13 ± 0.02D
Caffeic acid (mg /100 g FW)	0.17 ± 0.01d	0.50 ± 0.01b	0.41 ± 0.01b	1.20 ± 0.04a	0.22 ± 0.00c	0.23 ± 0.00c	0.38 ± 0.00B	0.68 ± 0.01A	ND	ND	ND	ND
Gallic acid (mg /100 g FW)	0.27 ± 0.01b	0.21 ± 0.01c	0.20 ± 0.00c	0.33 ± 0.02a	0.28 ± 0.00b	0.30 ± 0.01a	0.18 ± 0.00D	0.41 ± 0.01A	0.21 ± 0.01C	0.2 ± 0.00C	0.44 ± 0.02A	0.37 ± 0.00B
Ferulic acid (mg /100 g FW)	1.63 ± 0.18a	1.10 ± 0.05c	1.17 ± 0.02c	1.38 ± 0.03b	1.58 ± 0.05a	1.62 ± 0.05a	0.74 ± 0.02C	1.32 ± 0.03A	0.89 ± 0.02B	0.75 ± 0.02C	0.61 ± 0.01D	0.76 ± 0.02C
p-Coumaric acid (mg /100 g FW)	0.05 ± 0.00d	0.14 ± 0.00b	0.05 ± 0.00d	0.10 ± 0.00c	0.18 ± 0.01a	0.14 ± 0.00b	0.19 ± 0.01E	0.85 ± 0.02C	1.08 ± 0.03A	0.80 ± 0.01D	0.97 ± 0.01B	0.81 ± 0.00D
Coumarin (mg /100 g FW)	27.28 ± 1.15a	29.59 ± 2.15a	26.91 ± 1.48a	26.35 ± 0.98a	26.87 ± 1.42a	27.56 ± 1.22a	1.78 ± 0.10B	1.04 ± 0.08D	0.94 ± 0.02D	1.38 ± 0.03C	2.15 ± 0.11A	0.37 ± 0.01E

The values are expressed as mean ± SD (n = 3). ND = Not Detected; VCE, vitamin C equivalents; GAE, gallic acid equivalents; RE, rutin equivalents; CE, catechin equivalents; CGE, cyanidin 3-*O*-glucoside equivalents; FW, fresh weight. Values in the same line with different lower-case/capital letters are significantly different at $P < 0.05$ according to the Duncan test.

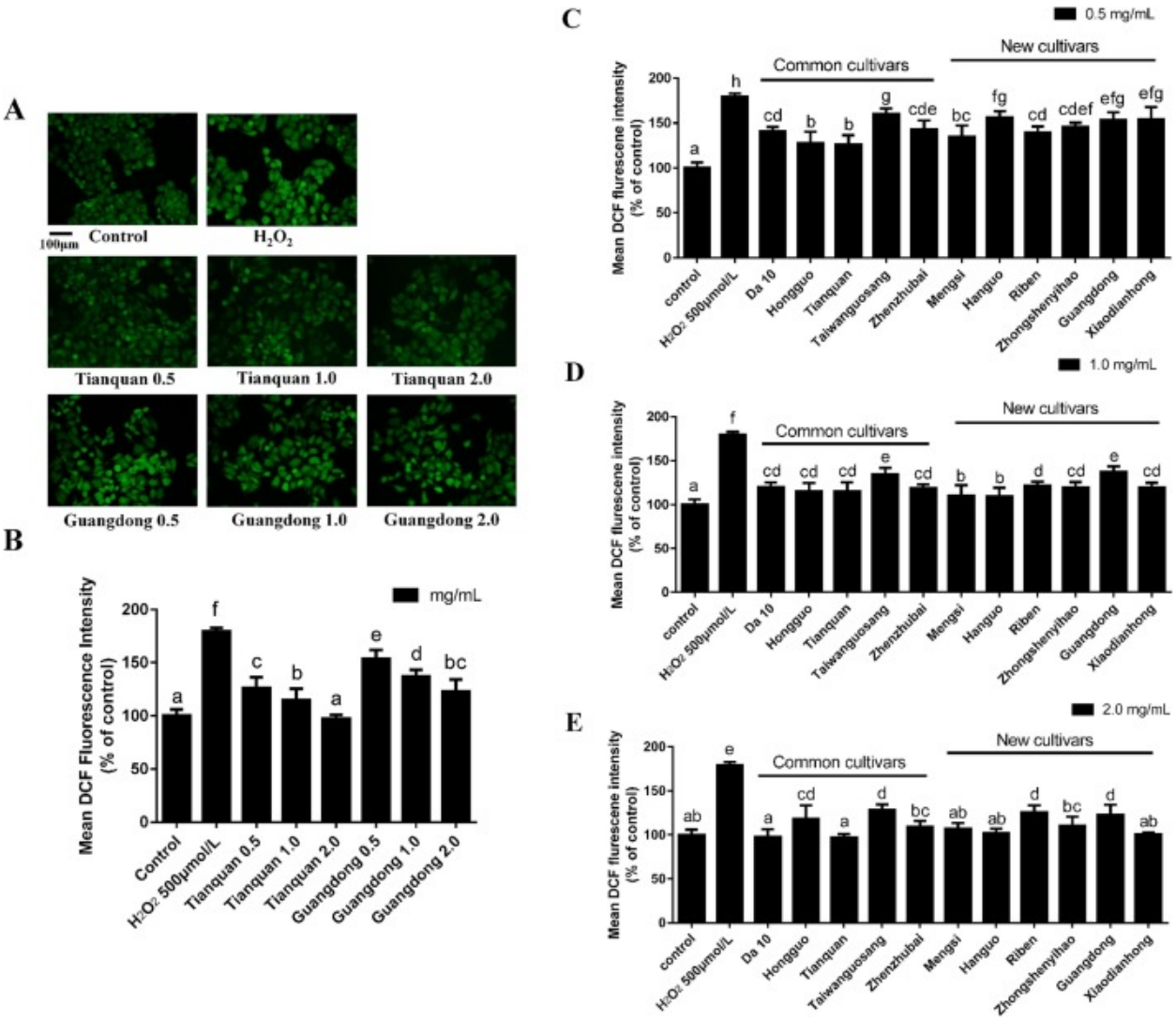

Fig. 1. Cellular radical scavenging activity of digested mulberry cultivars. (A) Caco-2 cells were treated with 500 μmol/L H_2O_2 in the presence or absence of different concentrations of digested mulberry cultivars (0.5 mg/mL, 1.0 mg/mL or 2.0 mg/mL) for 24 h, cells were labelled with 10 μmol/L DCFH-DA. (B, C, D, and E) Fluorescence intensities of DCF were detected. The results were expressed according to (A) (means ± SD; n = 5). Lower-case letters in each column indicate significant differences at $p < 0.05$ by Duncan test.

3.4. Effects of gut microbiota fermentation on antioxidant capacity and phenolic contents of mulberry cultivars

Among mulberry cultivars, black mulberry (Hanguo) and white mulberry (Zhenzhubai) were used to further investigate the effect of human gut microbiota fermentation on antioxidant capacity and phenolic contents. The results representing antioxidant activity are presented in Table 3, black mulberry (Hanguo) showed potential antioxidant activity than white mulberry (Zhenzhubai). According to ABTS assay, after 2 h of gut microbiota fermentation, the $ABTS^{\cdot+}$ scavenging activity of all mulberry samples was increased. Then the $ABTS^{\cdot+}$ scavenging activity of Hanguo samples (metabolites collected at 2, 6, 12, 24 and 48 h fermentation) was

decreased with time, while that of Zhenzhubai samples were lowest at 12 h fermentation. According to FRAP assay, ferric reducing capacity of mulberry samples (metabolites collected at 0, 2, 6, 12, 24 and 48 h fermentation) showed irregular fluctuations.

Phenolic compounds of mulberry samples (metabolites collected at 0, 2, 6, 12, 24 and 48 h fermentation) were analyzed by HPLC and represented in Table 3 and Fig. S1. The total phenolic contents (TPC) of Hanguo were increased to 960.42 mg GAE/100 g FW at 24 h, then significantly decreased to 543.03 at 48 h, while that of Zhenzhubai metabolites collected at 2, 6, 12, 24 and 48 h fermentation were less than that of at 0 h fermentation. The total flavonoid contents (TFC) of Hanguo were first increased and then decreased. TFC of Zhenzhubai metabolites collected at 2, 6, 12, 24 and 48 h fermentation were<0 h fermentation and was lowest (12.35 mg RE/100 g FW) at 12 h fermentation. The highest procyanidins was 31.01 mg CE /100 g FW at 12 h fermentation in Hanguo, and 8.89 mg CE /100 g FW at 48 h fermentation in Zhengzhubai. The contents of anthocyanins (cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside) were increased at 0 h when compared with digested mulberry (Hanguo cultivar), which may be due to the change of pH. The structure of anthocyanin is changed under different pH conditions and is stable under acidic conditions(Castaneda-Ovando et al., 2009). As the fermentation time increases, the contents of anthocyanins were increased initially, then decreased with time in Hanguo metabolites. Based on identified phenolic metabolites, a possible structural transformation of mulberry phenolics was summarized in Fig. S2. The degradation of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside to cyanidin could be produced through bacteria's enzymatic action. Some studies revealed that the most important enzyme that contribute to this biotransformation was β-glucosidase, which is secreted by gut microbiota such as *Bifidobaterium* spp., *Lactobacillus* spp., *Eubacterium ramulus* and *Clostridium saccbarogumia* (Cheng et al., 2016, Fernandes et al., 2015, Hanske et al., 2013).

As shown in Table 3 and Fig. S1, phenolic compounds, such as 3,4-Dihydroxybenzoic, 2,4,6-trihydroxybenzoic acid and 2,4,6-trihydroxybenzaldehyde were identified in fermented mulberry samples. When fermentation reached at 2 h, the contents of some phenolics (2,4,6-trihydroxybenzoic acid, catechin, caffeic acid and p-coumaric acid, and etc.) were significantly decreased, while some phenolics (2,4,6-trihydroxybenzaldehyde, chlorogenic acid, ferulic acid, and etc.) were significantly increased in Hanguo cultivar. As shown in Fig. S2, the formation of caffeic acid and p-coumaric acid could be due to the degradation of chlorogenic acid and ferulic acid. The increment of 2,4,6-trihydroxybenzaldehyde could be attributed due to the degradation of 2,4,6-trihydroxybenzoic acid. When fermentation reached at 6 h, the content of 3,4-Dihydroxybenzoic acid was increased in Hanguo cultivar, which could be derived from cyanidin. When fermentation reached at 12 h, the contents of 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, and catechin were significantly decreased in Hanguo cultivar. The increment of catechol could be attributed due to the degradation of catechin (Fig. S2). At 24 h fermentation, the contents of 3,4-Dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, ferulic acid, and *p*-Coumaric acid were significantly increased in Hanguo cultivar. Some phenolic metabolites, such as cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, and Chlorogenic acid were decreased in Hanguo cultivar (Fig. S2). After 48 h fermentation, the content of 2,4,6-trihydroxybenzoic acid was increased, while cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, 2,4,6-trihydroxybenzaldehyde were found to decrease in Hanguo cultivar at the same time (Fig. S2). The content of catechol was fully degraded at 6 and 12 h, then detected again at 24 and 48 h

fermentation in Zhenzhubai cultivar. The contents of caffeic acid were increased at 2 h, then fully degraded at 6, 12, 24, and 24 h in Zhenzhubai cultivar. At 0, 2, 24, 48 h fermentation, Zhenzhubai contained higher catechin contents than Hanguo.

Based on our previous study, it was clear that cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside were the main phenolic compounds available in mulberry fruit (Bao et al., 2016). Mulberry anthocyanins can undergo a structural modification during the fermentation process which produced the various phenolic metabolites. After *in vitro* digestion, mulberry anthocyanins (cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside) were significantly degraded due to the alkaline condition of intestinal digestion. During gut microbiota fermentation, the contents of anthocyanins were increased initially due to the acidic condition of fermentation. Subsequently, anthocyanins were degraded into some phenolics, including 3,4-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, etc. Pearson's correlation coefficient analysis was applied among antioxidant capacity and bioactive compounds of fermented mulberry cultivars. The results were presented in Table S2. According to ABTS assay, antioxidant activity was significantly ($p < 0.01$) associated with phenolics (0.882), flavonoids (0.956), procyanidins (0.897), cyanidin-3-*O*-glucoside (0.894), cyanidin-3-*O*-rutinoside (0.887), 3,4-dihydroxybenzoic acid (0.927), 2,4,6-trihydroxybenzaldehyde (0.783), ferulic acid (0.748), p-coumaric acid (0.864) and coumarin (0.965). According to FRAP assay, the antioxidant capacity showed positive and significant correlation ($p < 0.01$) with phenolics (0.952), flavonoids (0.990), procyanidins (0.992), cyanidin-3-*O*-glucoside (0.942), cyanidin-3-*O*-rutinoside (0.926), 3,4-dihydroxybenzoic acid (0.974), ferulic acid (0.824), p-coumaric acid (0.845) and coumarin (0.981), which is consistent with ABTS assay. These results suggest that the composition of anthocyanins and their metabolites are likely to play a key role in the antioxidant capacity of mulberry cultivars after gut microbiota fermentation.

3.5. Potent cellular ROS scavenging ability of mulberry cultivars

To further evaluate the antioxidant activity of mulberry cultivars after *in vitro* digestion, human intestinal Caco-2 cells were employed to build oxidative stress cell model. Hydrogen peroxide (H_2O_2) is known as an unstable chemical substance with high oxidative ability, which could induce intracellular redox interference (da Silveira Vargas, Soares, Ribeiro, Hebling, & De Souza Costa, 2014). In this study, we employed H_2O_2 to build a ROS overproduction cell model. Fluorescence probe DCFH-DA was used to observe the cellular ROS level. Fig. 1 clearly indicated that the fluorescence intensity of the H_2O_2-treated group (500 μmol/L) was increased to 179% compared with the control group (normalized to 100%). As shown in Fig. 1B, when cells were pre-treated with digested mulberry cultivars (0.5, 1.0 or 2.0 mg/mL concentrations), the fluorescence intensity of Tianquan and Guangdong group was decreased compared to H_2O_2-treated group in a dose-dependent manner. At the concentration of 2 mg/mL, digested Tianquan cultivar (97%) showed better ROS scavenging activity than Guangdong cultivar (123%). As shown in Fig. 1C, 0.5 mg/mL of common cultivars (Hongguo (128%) and Tianquan (126%) showed the best ROS scavenging capacity. When the concentration of digested mulberry cultivars was raised to 1 mg/mL (Fig. 1D), some new cultivars (Mengsi, 110%; Hanguo, 109%) also exhibited a significant ROS scavenging ability. Moreover, 2 mg/mL of most mulberry cultivars showed the best antioxidant capacity among different concentrations. At the concentration of 2 mg/mL, common mulberry cultivars (Da 10 and Tianquan) and new mulberry

cultivars (Mengsi and Hanguo) potently scavenged ROS, and the mean fluorescence intensities were 98%, 97%, 107%, and 102% respectively (Fig. 1E). Moreover, white mulberry cultivars (Zhenzhubai, 109%; Xiaodianhong, 100%) also showed comparable ROS scavenging capacity at 2 mg/mL concentration.

Since mulberry cultivars possessed potential ROS scavenging activity after *in vitro* gastrointestinal digestion, we further studied the antioxidant activity of gut microbiota fermented mulberry samples. We chose gut metabolites collected at 0, 24 and 48 h of fermentation for this study. As shown in Fig. 2, the fluorescence intensity of the H_2O_2-treated group (500 μmol/L) was increased to 173% compared with the control group. 1.0 mg/mL of all fermented mulberry samples showed better antioxidant capacity than the concentration of 0.5 mg/mL. The ROS scavenging activity of Zhenzhubai was decreased after gut microbiota fermentation. 1.0 mg/mL of Hanguo cultivar (24 h fermentation) showed potent ROS scavenging capacity (101%), while reduced at 48 h fermentation.

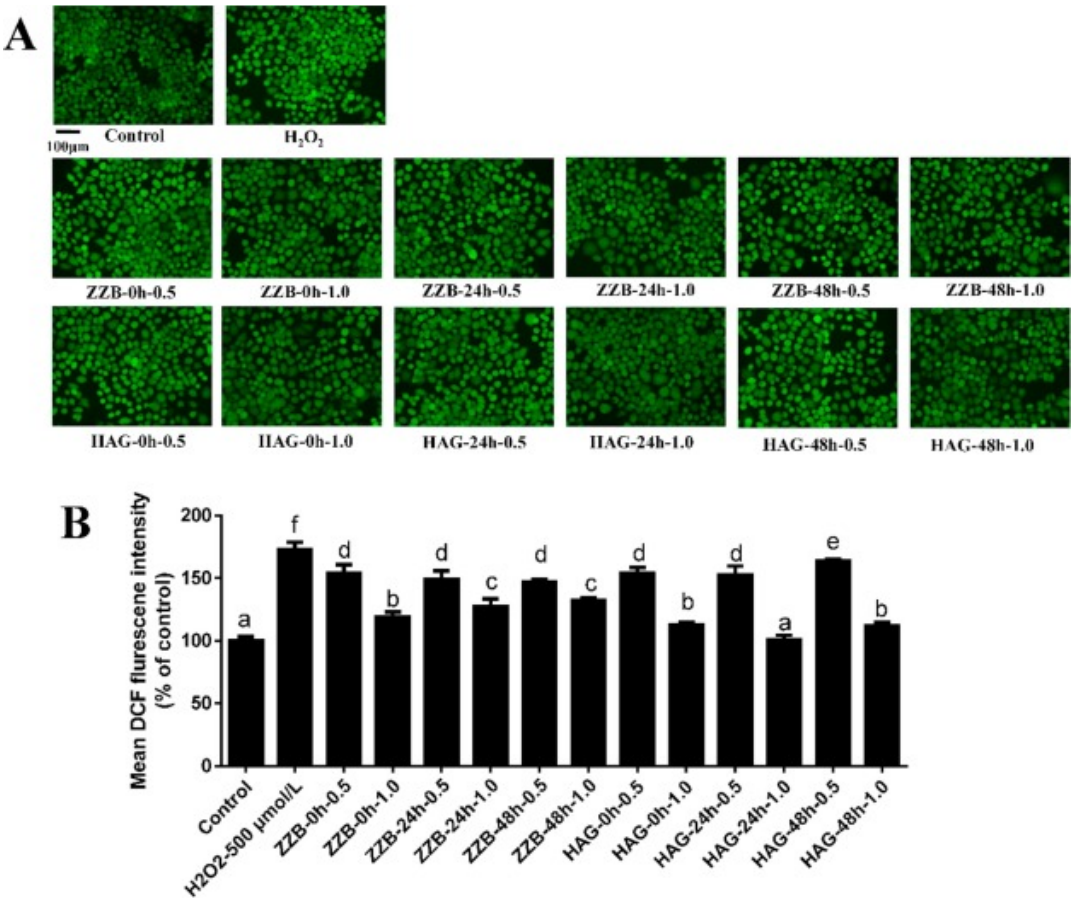

Fig. 2. Cellular radical scavenging activity of fermented mulberry cultivars. (A) Caco-2 cells were treated with 500 μmol/L H_2O_2 in the presence or absence of different concentrations of fermented mulberry cultivars (0.5 mg/mL or 1.0 mg/mL; gut metabolites collected at 0 h, 24 and 48 h fermentation) for 24 h; cells were labeled with 10 μmol/L DCFH-DA. (B) The results were expressed according to (A) (means ± SD; n = 5). Lower-case letters in each column indicate significant differences at $p < 0.05$ by Duncan test. ZZB, Zhenzhubai cultivar; HAG, Hanguo cultivar.

In the present study, our results indicated that mulberry metabolites included cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, 3,4-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, catechin, etc. We found that the digested and fermented mulberry samples could effectively suppress the outbreak of reactive oxygen species (ROS). Therefore, the possible mechanisms involved in the antioxidant effect of mulberry metabolites could be attributed to their properties of suppressing the formation of ROS or scavenging ROS rapidly before they can attack biologically essential molecules (Bernatoniene and Kopustinskiene, 2018, Choi et al., 2007, Kim et al., 2018). There is increasing evidence showing that antioxidants (including anthocyanins, catechin, etc.) act as a cellular signaling messenger to regulate the level of antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT)) (Chen et al., 2012, Demir et al., 2011) and non-enzymatic antioxidants (glutathione, GSH) (Zhu, Jia, Wang, Zhang, & Xia, 2012). In addition, some studies indicated that antioxidants (including anthocyanins, 3,4-Dihydroxybenzoic acid, catechin, etc.) could activate Keap1/Nrf2 signaling pathway, which could increase the gene expression of antioxidant enzymes and maintain cellular redox balance (Hwang et al., 2011, Varì et al., 2011).

3.6. Principal component analysis

Principal component analysis (PCA), a statistical method, is used to explain differentiation between samples, which can enhance discovery on the variable components that mainly influence the sample differences and similarities (Barbu, Neagu, & Dragan, 2015). PCA scores plots allow visual representation of any difference of mulberry cultivars in antioxidant activity and bioactive compound after *in vitro* digestion. In this study, we used PCA to analyze the data among different digested mulberry cultivars, with respect to phenolics, flavonoids, procyanidins, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, ABTS, FRAP and ROS scavenging activity (ROS-H_2O_2). A model with two-components was obtained from PCA, which explained 99% of the total variance (Fig. 3). The PCA was performed on the whole set of the total variance with 88% in the first principal component (PC1) and 11% in the second (PC2) (Fig. 3). Fig. 3A showed the classification of scores plot of 11 mulberry cultivars after *in vitro* digestion. Parameters with high loadings (positive or negative) characterized the major differences among varieties. Based on the loadings for PCA (Fig. 3B), PC1 was positively linked with phenolics, flavonoids, and FRAP. PC2 was correlated with phenolics and flavonoids. Cyanidin-3-*O*-glucoside (C3G), cyanidin-3-*O*-rutinoside (C3R), ROS- H_2O_2-0.5, ROS- H_2O_2-1.0 and ROS-H_2O_2-2.0 are overlapped in the Fig. 3B, which indicated that these variable components have a similar effect on the sample differences and similarities. The new cultivar Hanguo, located in the upper right-hand quadrant, was indicated to have higher contents of bioactive components (phenolics, flavonoids, and procyanidins) and better antioxidant activity than most mulberry cultivars after *in vitro* digestion. The common cultivar Hongguo was located in the lower right-hand quadrant, exhibiting better antioxidant activity according to ABTS assay. White mulberry cultivars (Zhenzhubai and Xiaodianhong) are overlapped on the far-left quadrant showed poor antioxidant capacity and few bioactive components after *in vitro* digestion. The location of Tianquan, Da 10 and Zhongshenyihao in the lower right-hand quadrant suggest better ROS scavenging activity. Taiwanguosang (common cultivar) and Guangdong (new cultivar) had higher phenolic contents, as well as lower antioxidant activity compared with other mulberry cultivars. New cultivar Mengsi showed good antioxidant capacity and abundant bioactive

components. Riben (new cultivar) was close to the origin, which represented a few bioactive components and limited antioxidant ability.

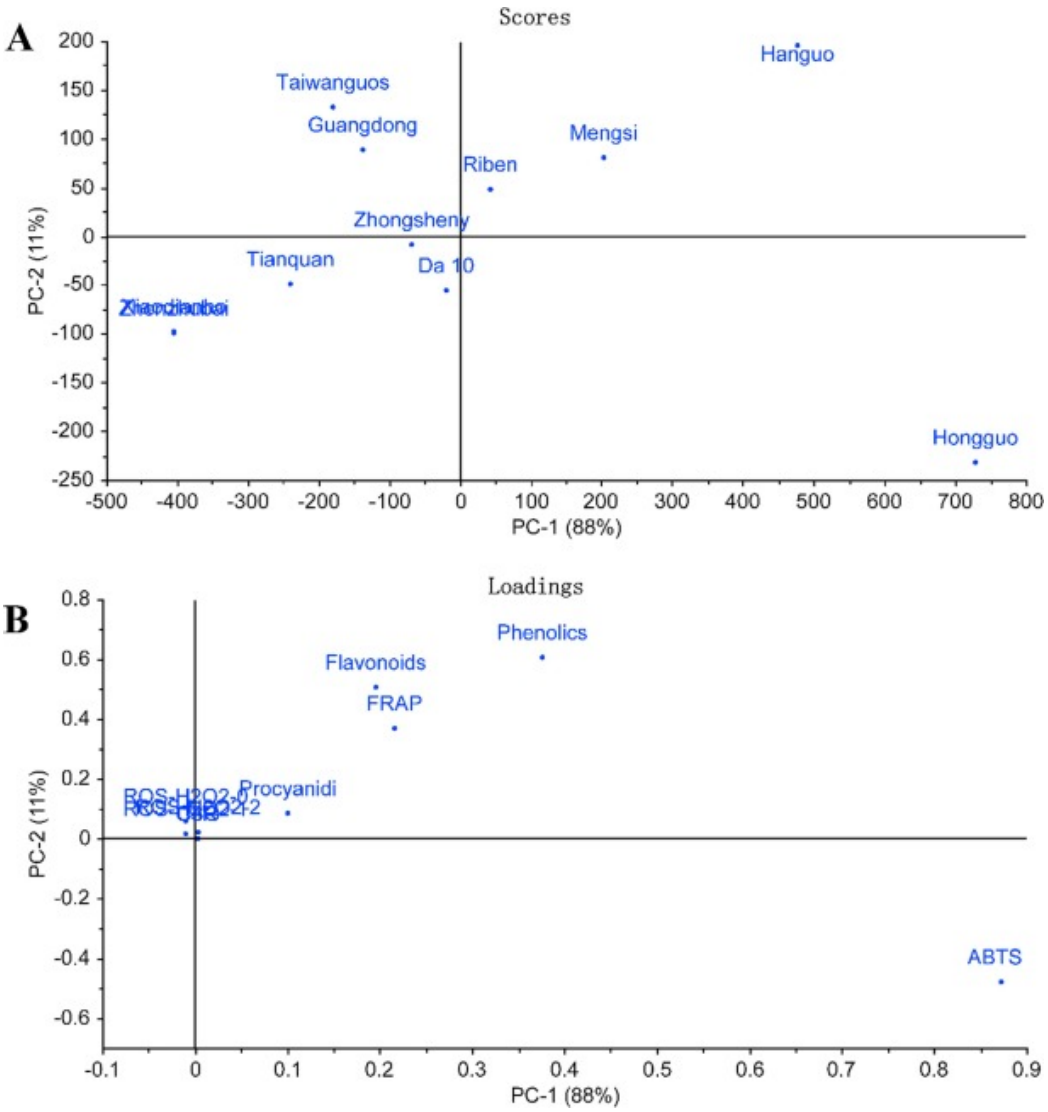

Fig. 3. Principal component analysis (PCA) of digested mulberry cultivars. (A) PC scores plot of the digested mulberry cultivars; (B) loadings plot of the digested mulberry cultivars. cyanidin-3-*O*-glucoside (C3G); cyanidin-3-*O*-rutinoside (C3R); ROS-H_2O_2-0.5 (cells were treated with 500 μmol/L H_2O_2 in the presence of 0.5 mg/mL digested mulberry cultivars); ROS- H_2O_2-1.0 (cells were treated with 500 μmol/L H_2O_2 in the presence of 1.0 mg/mL digested mulberry cultivars); ROS- H_2O_2-2.0 (cells were treated with 500 μmol/L H_2O_2 in the presence of 2.0 mg/mL digested mulberry cultivars).

As shown in Fig. S3, The PCA was performed on the whole set of the total variance with 94% in the first principal component (PC1) and 4% in the second (PC2). PC1 was positively linked with ABTS, phenolics, flavonoids, FRAP, and procyanidins. On the other hand, PC2 was correlated with ABTS and phenolics. The fermented Hanguo samples, located in the right-hand quadrant, were indicated to have higher contents of bioactive components and better antioxidant activity than fermented Zhenzhubai samples (located in the left-hand quadrant). After 2 h of

fermentation, the antioxidant activities of Hanguo and Zhenzhubai increased according to ABTS. Regarding Hanguo cultivar, the content of total phenolics increased with time at 2, 6, 12 and 24 h of fermentation, then decreased at 48 h of fermentation. For Zhenzhubai cultivar, the content of total phenolics increased with time at 2, 6 and 12 h of fermentation. The 24 h and 48 h fermented samples were overlapped, which means there were no significant differences between the two samples. In summary, PCA results indicated that among all the tested cultivars, Hanguo (new mulberry cultivar) possessed abundant bioactive compounds, potent *in vitro* antioxidant activity and cellular ROS scavenging capacity.

4. Conclusion

In this study, mulberry cultivars were found to have abundant bioactive compounds such as phenolics, flavonoids, procyanidins and anthocyanins after *in vitro* gastrointestinal digestion and gut microbiota fermentation. The bioactive components of mulberry cultivars were changed followed by gut microbiota fermentation at 0, 2, 6, 12, 24 and 48 h. Some anthocyanin metabolites (such as 3,4-Dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, and etc.) were detected in fermented black mulberry (Hanguo cultivar). All tested mulberry cultivars showed effective ROS scavenging activity after *in vitro* gastrointestinal digestion and gut microbiota fermentation. Among tested mulberry cultivars, new cultivar Hanguo had higher phenolic contents, showed better *in vitro* antioxidant activity and stronger cellular ROS scavenging capacity compared with most mulberry cultivars. Henceforth, our findings suggest new mulberry cultivar Hanguo as a dietary source to be considered as a functional food.

5. Ethics statements

Our research did not include any human subjects and animal experiments.

Declaration of Competing Interest. The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material
Supplementary data to this article can be found at https://doi.org/10.1016/j.jff.2019.05.017.

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