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Increasing Antioxidant Activity of Procyanidin Extracts from the Pericarp of *Litchi chinensis* Processing Waste by Two Probiotic Bacteria Bioconversions

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ABSTRACT: *Litchi chinensis* pericarp from litchi processing waste is an important plant source of A-type procyanidins, which were considered a natural dietary supplement because of their high biological activity *in vivo*. Litchi pericarp oligomeric procyanidins (LPOPCs) did not selectively modify the growth of *Streptococcus thermophilus* and *Lactobacillus casei*-01 at concentrations of 0.25 and 0.5 mg/mL, and it was demonstrated that the two strains could transform procyanidins during their log period of growth by two different pathways. *S. thermophilus* was able to metabolize procyanidin A2 to its isomer, and *L. casei* could decompose flavan-3-ols into 3,4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, *m*-coumaric acid, and *p*-coumaric acid. The total antioxidant capability (T-AOC) of LPOPCs before and after microbial incubation was estimated, and the results suggested that probiotic bacteria bioconversion is a feasible and efficient method to convert litchi pericarp procyanidins to a more effective antioxidant agent.

KEYWORDS: *Litchi pericarp, oligomeric procyanidins, bioconversion, probiotic bacteria, T-AOC*

INTRODUCTION

Litchi (*Litchi chinensis* Sonn.) is a subtropical fruit with high commercial value, which is widespread in the south of China. Since 2005, its cultivated area was more than 6×10^5 ha, and its yield was more than 1.3×10^6 tons per year. Many studies have demonstrated that the processing byproducts of litchi fruit, such as flowers, pericarp, and seeds, all have antioxidant properties.[1−3] It was also reported that the pericarp of litchi had been used as a traditional medicine with hemostatic and acesodyne functions in ancient time.[4] Litchi pericarp procyanidin extract was effective for prevention and treatment of hyperuricemia and/or gout[5] and showed a protective effect against cardiovascular diseases (proven by our lab). However, the pericarp of litchi, which accounts for 15% of the fresh weight of the fruit, becomes desiccated and turns brown at ambient temperature within 2 or 3 days pastharvest and is often thrown away as waste.[6] Therefore, the recovery of antioxidants from the litchi processing byproduct is of great importance, not only because of their significant pharmacological properties but also because it could apply and exploit a large amount of the litchi industry wastes to maximize the available resources and result in an expanded market for litchi products. Consequently, their environmental impact can also be reduced.

In fact, the main functional factors in litchi pericarp were identified as A-type procyanidins,[1,7] such as procyanidn A2, epicatechin-(4β→8, 2β→O→7)-epicatechin, and A-type procyanidin trimer, epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin. Recently, increasing interest has been brought about in those proanthocyanidins because of their antioxidant, anti-inflammatory, inhibition of platelet aggregation, and antimicrobial activities,[8] which are associated with the prevention of cancer and cardiovascular diseases.[9] However, the use of these important polyphenols is still limited, not only because A-type procyanidins are less studied[10] but also because their molecular mass is larger than the phenolic acids, causing a lower bioavailability in the human body.[11,12]

Actually, most polyphenols in food cannot be absorbed in their native forms, which must be modified by intestinal enzymes or hydrolyzed by the colonic microflora during metabolism, and to different extents, the polyphenols influenced the composition of the gut microbiota.[13] Subsequent reactions in the catabolism of flavan-3-ols comprise C-ring opening, followed by lactonization, decarboxylation, dehydroxylation, and oxidation, among others.[8,14] In the case of galloylated monomeric flavan-3-ols (ECG and EGCG), the microbial catabolism usually starts with the rapid cleavage of the gallic acid ester moiety by microbial esterases, giving rise to gallic acid, which is further decarboxylated into pyrogallol.[15] Galloyl-esterase, decarboxylase, and benzyl alcohol dehydrogenase activities of *Lactobacillus plantarum* led to the formation of gallic acid, pyrogallol, and catechol from grape seed polyphenols, respectively.[16] Thus, gut bacteria may play a major role in the production of new phenolic compounds, possessing better bioavailability and higher *in vitro* and *in vivo* antioxidant activities than polyphenol parents.[17,18]

Probiotic, drinkable, and enriched yogurts are currently formulated with fruit or vegetable pieces rich in bioactive natural polyphenolic compounds,[19] together with *Streptococcus thermophilus*, which is widely used as a starter culture and a part

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of the yogurt probiotic system.[20] Such food products are claimed to have beneficial effects on human health[21] and possess antimicrobial activities. Furthermore, polyphenolic extracts of concentrations up to 40 mg/125 g of yogurt had no effect on the viability of *S. thermophilus*. Probably, unforeseen complexation between antioxidant polyphenols and lactic acid bacteria (LAB) took place during the fermentation process or in the internal gastrointestinal (GI) tract.

Studies demonstrated in both humans and animals that the growth of potential beneficial bacteria, such as *Lactobacillus* and *Bifidobacteria* increased after the administration of monomeric flavan-3-ols from green tea,[22] wine polyphenols,[23] or black-currant extract powder.[24] Therefore, polyphenols appear to have potential to confer health benefits via modulation of the gut microbiota and exerting prebiotic effects.[25] However, there are a limited number of studies about the capacity of potential probiotics, such as LAB and bifidobacteria, to metabolize polyphenol compounds. The increasing antioxidant effect of probiotic bacteria on the procyanidins (especially A-type procyanidin) is even less reported now.

The objective of this study was to increase the antioxidant activity of A-type procyanidin extracts from the pericarp of litchi processing waste by probiotic bacteria. We evaluated the individual effect of the main flavan-3-ol compounds and oligomeric procyanidins from litchi pericarp (LPOPCs) on the growth of *S. thermophilus* and *L. casei*-01 derived from a daily diet. The ability of bacteria to convert procyanidin compounds *in vitro* was also characterized.

MATERIALS AND METHODS

Chemicals and Materials. (−)-Epicatechin (EC), (+)-catechin (CC), 3,4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid (4-HPA), *m*-coumaric acid, and *p*-coumaric acid [high-performance liquid chromatography (HPLC) grade] were purchased from Sigma Chemical Co. (St. Louis, MO).

Fruit of litchi (*L. chinensis* Guiwei) were obtained from Guangzhou, China, and arrived in the laboratory within 24 h postharvest. The fruits were peeled quickly, and the pericarp was stored at −18 °C prior to extraction in 3 days.

Strains and Growth Conditions. *S. thermophilus* used in this study was isolated from the starter cultures of Danisico Co., kindly supplied by the Institute of Wuhan Guangming Dairy Research (Wuhan, China). *L. casei*-01 was kindly offered by Chr. Hansen Company (Beijing, China). *S. thermophilus* and *L. casei* strains were routinely cultured at 37 °C in M17 and MRS broth, respectively.

Single colonies of *S. thermophilus* and *L. casei* were incubated in M17 and MRS fluid nutrient medium for 18 h. Then, the growth of the two LAB was monitored in corresponding medium with or without different concentrations of flavan-3-ol extracts and inoculated (1%) with an overnight culture (about 10^7 cfu/mL) of each strain. Bacteria growth for 48 h at 37 °C under aerobic conditions was measured at 2 h intervals by assessing optical density at 600 nm (OD_{600}) using a 722N vis spectrophotometer (Lengguang Technology Co., Shanghai, China). The data collected were fitted to determine maximum specific growth rates (μ_{max}) depending upon the method by Augustin et al. to calculate the resistance of the strains on the polyphenols.[26] Moreover, the pH change of different cultures was recorded. Appropriate controls were used by incubating strain without flavan-3-ol extracts or medium supplemented with those extracts non-inoculated with the strain.

Extraction of Flavan-3-ol Extracts from Litchi Percarp. Frozen litchi pericarp fragments were extracted to obtain the LPOPCs according to the method by Li et al.[27] The main compounds, such as monomeric flavan-3-ols (CC and EC) and procyanidin dimer A2 [epicatechin-(4β→8, 2β→O→7)-epicatechin], were purified by a Toyopearl HW-40s column (200 × 16 mm inner diameter, Tosoh Chemical Co., Tokyo, Japan) from prepared LPOPCs. Moreover, the contents of EC, CC, and A2 in LPOPCs were about 275, 35, and 280 mg/g, respectively. LPOPCs were added as filter-sterilized solutions to the final medium at doses of 0.25, 0.5, and 1 mg/mL. EC, CC, and A2 were incubated in the liquid medium at final concentrations of 0.25 and 0.5 mg/mL, respectively.

Analysis of Flavan-3-ols and Oligomeric Procyanidins after Bioconversion. To study the interaction process of the LAB strains and antioxidant polyphenols (flavan-3-ols and procyanidins), the products of the two strains and polyphenols were collected at 2, 8, 14, 20, 30, and 48 h in the presence of 0.5 mg/mL LPOPCs during incubation. Cultures were centrifuged (4000 rpm for 10 min), and the supernatants were filter-sterilized by 0.45 μm pore-size filters and kept at −20 °C until HPLC analysis. Moreover, to know the conversion ratio of microflora, the metabolites produced from EC, A2, and LPOPCs after 30 h of incubation were quantified. The concentrations of flavan-3-ols in culture were at 0.5 mg/mL (about 1724 and 836 mmol/L for EC and A2, respectively). All of the samples were analyzed in triplicate.

About 6 mL of nutrient solution, containing oligomeric procyanidins and their microbial metabolites, was extracted with 5 times the volume of ethyl acetate. The resulting supernatant was dried under vacuum and redissolved in 3 mL of methanol, which was determined by an Agilent 1100 series HPLC and liquid chromatography−electrospray ionization−multi-stage mass spectrometry (LC−ESI−MS^n) system (Agilent Technologies Co., Ltd., Santa Clara, CA) to examine the phenolic acids produced after fermentation.

A ZORBAX Eclipse XDB-C_{18} column (150 × 4.6 mm, 5 μm particle size, Shimadzu Co., Kyoto, Japan) was used, and the mobile phases were (A) 0.4% (v/v) aqueous acetic acid and (B) acetonitrile. Elution conditions were as follows: a linear gradient from 5 to 35% B in 40 min, from 35 to 50% B in 5 min, from 50 to 80% B in 5 min, and from 80 to 5% B in 5 min, at a flow rate of 1.0 mL/min. The absorbance of the fluent was monitored at 280 nm using a diode array detector (DAD). The mass fragmentation experiments were performed on an ESI mass spectrometer with a negative-ion mode. Orifice voltage, − 30 V; spray voltage, 4 kV; gas flow rate, 20 mL/min; and heat capillary temperature, 325 °C. The mass scale was defined from m/z 100 to 1200.

Total Antioxidant Capability (T-AOC) of LPOPCs before and after Metabolism by *S. thermophilus* and *L. casei*. The T-AOC of LPOPC before and after metabolism by *S. thermophilus* and *L. casei* was estimated by the total antioxidant capacity assay kit (A015, 50T, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The fermentation liquid containing metabolized LPOPCs was prepared using the same method as described previously, and the final solution was centrifuged at 30 h after incubation to obtain the antioxidant agent. As a control, 0.5 mg/mL LPOPCs were added in the corresponding fermented culture without conversion, comparing the T-AOC of the procyanidin extract before and after microbial metabolism.

RESULTS AND DISCUSSION

Effect of the Main Flavan-3-ols and LPOPCs on the Growth of *S. thermophilus* and *L. casei*. The growth response of *S. thermophilus* and *L. casei* to the main flavan-3-ols (CC, EC, and A2) and LPOPCs is shown in Table 1. The most common response in the two LAB strains was inhibition of growth by LPOPC extracts at several concentrations from 0.25 to 1 mg/mL. However, the resistant extent varied from strain to strain.

The maximum specific growth rates (μ_{max}) of *S. thermophilus* indicated a dose-dependent effect with LPOPCs negatively, suggesting that procyanidins inhibited the growth of *Streptococcus*. However, the *S. thermophilus* strain showed its maximum OD_{600} in the presence of 0.5 mg/mL LPOPCs compared to the control (Figure 1a). Except for 0.5 mg/mL oligomeric procyanidins, another two doses of LPOPCs

Table 1. Growth Response of *S. thermophilus* and *L. casei* to the Presence of Main Flavan-3-ols and LPOPCs[a]

strain	source		control	LPOPCs			EC		CC		A2	
				0.25 mg/mL	0.5 mg/mL	1 mg/mL	0.25 mg/mL	0.5 mg/mL	0.25 mg/mL	0.5 mg/mL	0.25 mg/mL	0.5 mg/mL
S. thermophilus	fermented milk	max OD	0.532	0.423	0.665	0.159	0.366	0.434	0.359	0.334	0.221	0.192
		μ_{max}	0.151	0.126	0.108	0.097	0.171	0.182	0.141	0.148	0.039	0.048
		final pH	4.21	4.41	4.19	6.15	4.33	4.21	4.29	4.13	5.09	5.12
L. casei	vegetable	max OD	1.791	1.743	1.305	0.361	1.788	1.807	1.776	1.537	1.76	1.184
		μ_{max}	0.191	0.192	0.181	0.074	0.218	0.203	0.236	0.208	0.128	0.119
		final pH	3.33	3.32	3.33	3.32	3.31	3.33	3.26	3.33	3.34	3.34

[a]OD was measured at a wavelength of 600 nm. The growth rate (μ_{max}) was calculated for strains grown using Microsoft Excel 12.0 obtained by triplicate.

intervene with the acid formation of the strain, resulting in a higher final cultivar pH and a lower OD_{600} value. Differently, 1 mg/mL LPOPCs inhibited the growth of *L. casei* mostly (Figure 1c), while 0.25 mg/mL LPOPCs did not affect the growth rate of the microflora significantly, referring to the control. The final pH of the *Lactobacillus* liquor fermentation was maintained the same as that containing procyanidins, implying that the carbohydrate metabolic pathway of this strain was not affected by litchi pericarp procyanidins notably.

EC, the main monomer in litchi pericarp procyanidins, revealed a better promoting growth effect on both strains than CC and A2, especially for *L. casei* (panels b and d of Figure 1). However, procyanidin A2 prolonged the lag phase and shortened the log phase of the two strains to different extents at concentrations of 0.25 and 0.5 mg/mL. Even so, the lactose metabolism of *S. thermophilus* was restrained by A2 seriously, which did not happen on *L. casei*. Thus, more research should be performed to study the effect of procyanidins on the mirobial carbohydrate metabolism and the intervention mechanism.

Metabolism and Bioconversion of Flavan-3-ols and LPOPCs by Bacteria. According to the growth experiment, the two LAB were considered to be flavan-3-ol- and oligomeric-procyanidin-resistant. Thus, more analysis was carried out to demonstrate that the resistant strains were able to metabolize these polyphenols. After pre-processing, HPLC pictures of interactive products of *L. casei* strain and LPOPCs were recorded in Figure 2. Obviously, the flavan-3-ol composition of LPOPC was kept stable at the beginning 2 h of incubation (the original LPOPC chromatograph was from Figure 4a); however, it led to a remarkable decrease in the response of EC (peak 2) and A2 (peak 4) from 2 to 14 h, which exactly corresponded to the log period of the strain growth. During this process, CC (peak 1) and A-type procyanidin trimer [peak 3, epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin] also decreased, but to a lesser extent. In other words, the composition of the LPOPCs was transformed through a sizable decrease in the EC monomer and a concomitant increase in the relative amounts of procyanidin dimer and trimer. Furthermore, the concentration of LPOPCs was not significantly changed by the growth of microflora in the next period from 14 to 48 h, indicating that the metabolism of LPOPCs by the *L. casei* strain mostly happened during the log phase and probably decomposed by the endoenzyme of the strain.

Likely, a similar conclusion was obtained from the *S. thermophilus* strain on the conversion of LPOPCs. The amounts of flavan-3-ols and oligomeric procyanidins were greatly altered during 2−14 and 20−30 h, corresponding to the rising phase of the *Streptococcus* growth (Figure 3). However, the conversion of A-type procyanidin trimer (peak 3) varied, decreasing during the log period but recovering to its original content from 20 h to steady time.

To identify the microbial metabolism on the flavan-3-ols in litchi pericarp, we analyzed the metabolites of LPOPCs, EC, and A2 at 30 h after incubation with *S. thermophilus* and *L. casei* (Figure 4). All of the strains grew steady and metabolized to other forms in a great measure at this time point. It was found that the metabolism on the CC monomer by the two strains was much less obvious than EC from HPLC results (panels d−f of Figure 4). However, the consumption of EC by microflora depended upon the concentration of the compound. The transformation ratio of EC at 0.25 mg/mL in cultivar was significantly higher than that at 0.5 mg/mL. LC−MS^2 analysis

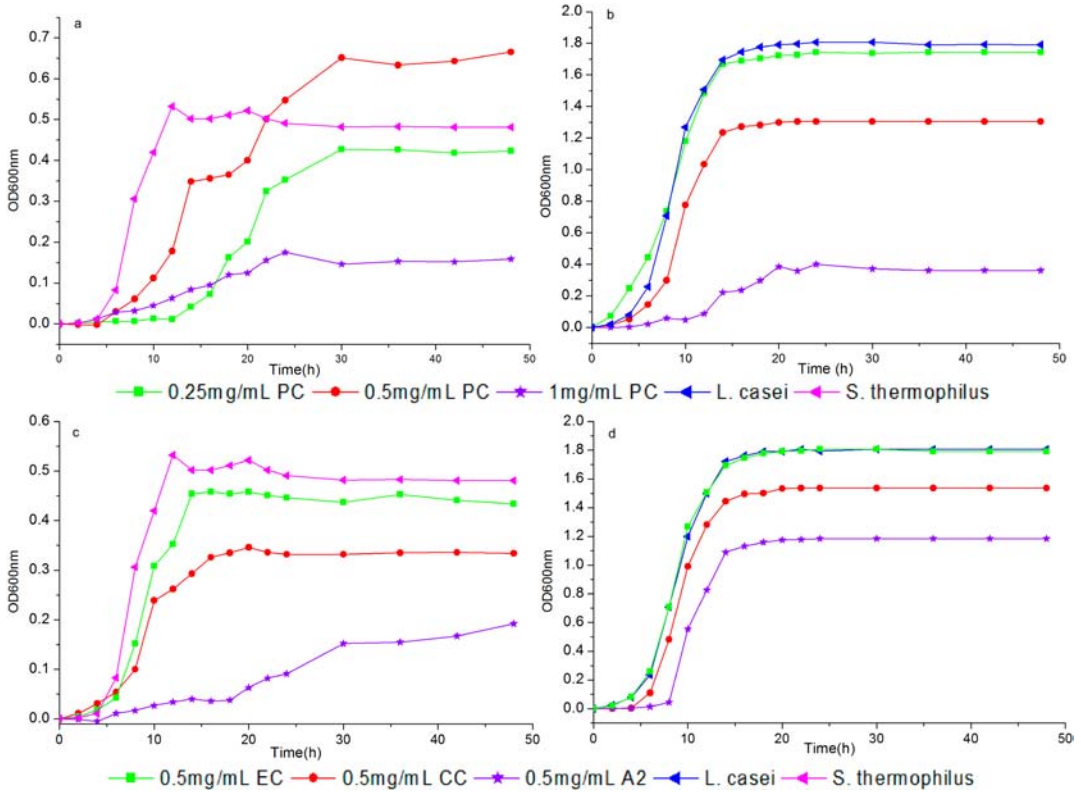

Figure 1. Growth of *S. thermophilus* and *L. casei* in MRS and M17 medium with (a and b) 0.25, 0.5, and 1 mg/mL LPOPCs and (c and d) 0.5 mg/mL EC, CC, and A2 during 48 h of incubation at 37 °C.

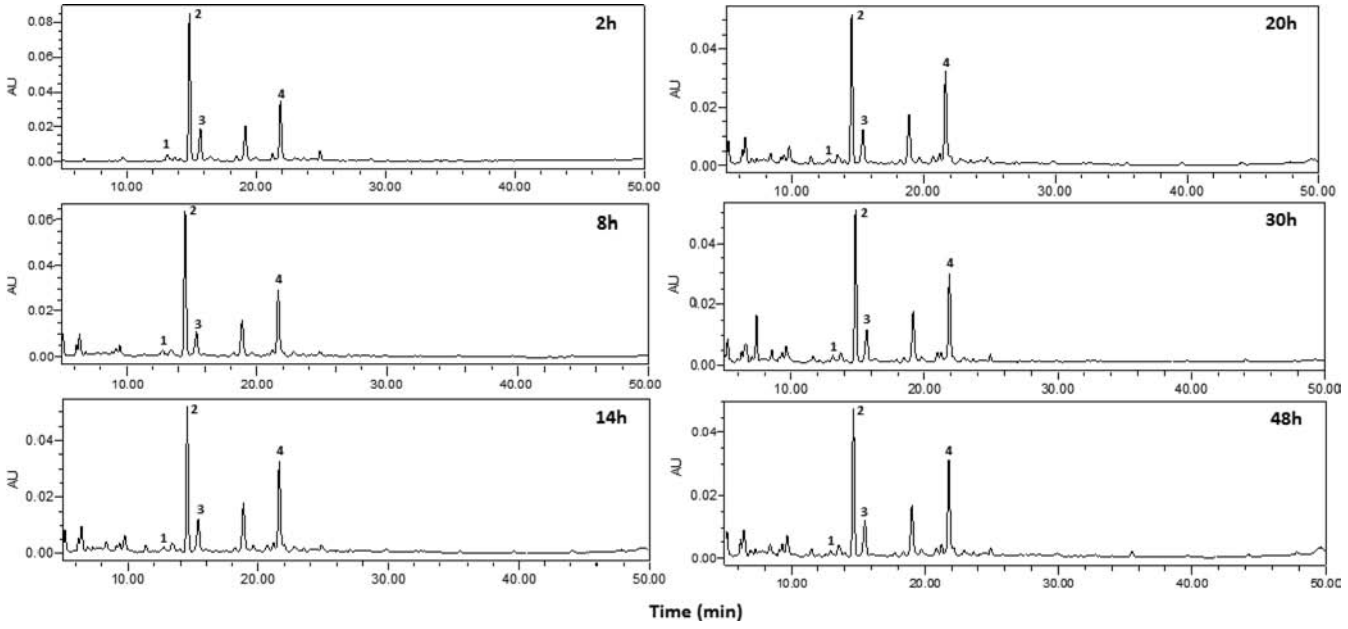

Figure 2. HPLC graphs of LPOPCs during fermentation with *L. casei* at 2, 8, 14, 20, 30, and 48 h. Peaks 1−4 were CC, EC, procyanidin trimer, and A2, sequentially.

of the metabolites of A2 indicated that the dimer was transformed by *S. thermophilus* and *L. casei* depending upon two different ways (panels g−i of Figure 4). *Streptococcus* conversed A2 into its isomer, another A-type procyanidin dimer (575/423, 449; parent ion/product ion), and the transformation ratio was nearly 50% (peak 5 or A-2 in panels b and h of Figure 4), suggesting an isomerase activity of *S. thermophilus* on the A-type procyanidin dimer. This reaction was maintained during the whole log phase of the strain until 30 h. *L. casei* degraded A2 to many small molecular phenolic acids or other compounds, corresponding to its tannase, demethylation, dehydroxylation, and decarboxylation activities probably. All of these polyphenol metabolites possessed less molecular weight and demonstrated stronger polarity than A2, which will promote its absorption through the gut barrier *in vivo*.[28]

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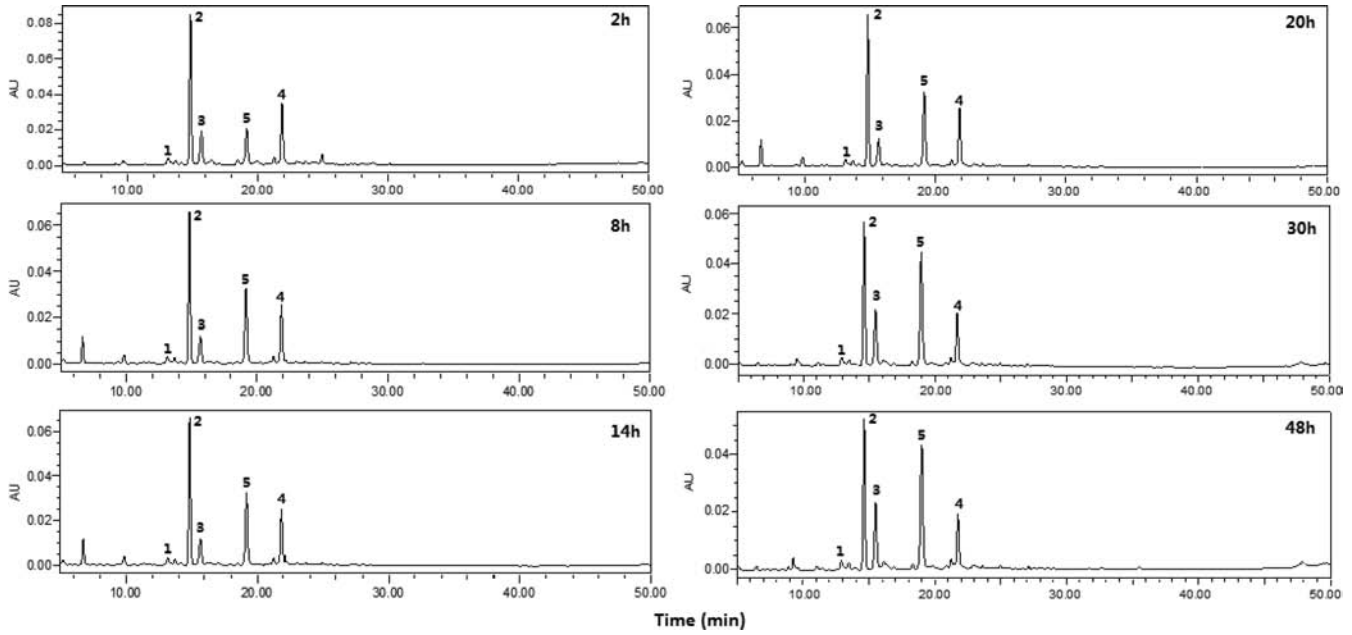

Figure 3. HPLC graphs of LPOPCs during fermentation with *S. thermophilus* at 2, 8, 14, 20, 30, and 48 h, respectively. Peaks 1−5 were CC, EC, procyanidin trimer, A2, and unknown A-type procyanidin dimer, sequentially.

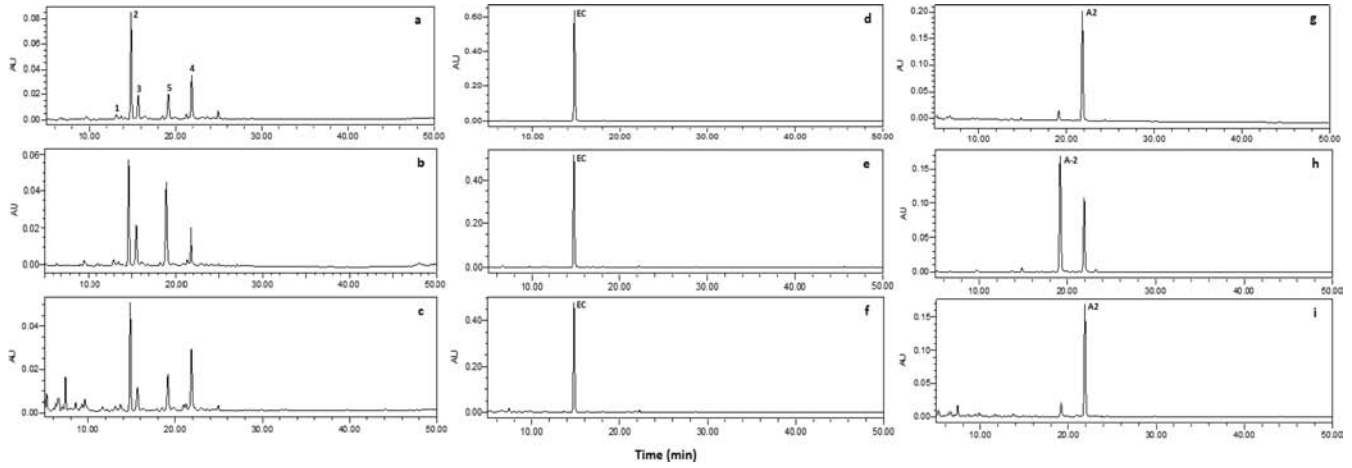

Figure 4. Metabolite analysis of (a−c) LPOPCs, (d−e) EC, and (g−i) A2 by *S. thermophilus* and *L. casei* strains 30 h after incubation with HPLC: (a) LPOPC control (1 mg/mL), (b) LPOPC metabolites of *S. thermophilus*, (c) LPOPC metabolites of *L. casei*, (d) EC control (1 mg/mL), (e) EC metabolites of *S. thermophilus*, (f) EC metabolites of *L. casei*, (g) A2 control (1 mg/mL), (h) A2 metabolites of *S. thermophilus*, and (i) A2 metabolites of *L. casei*.

Table 2. Phenolic Acid Metabolites of EC, A2, and LPOPCs by *L. casei*-01

				concentrations in zymotic fluid (nmol/mL)			
compound produced	retention time (min)	parent ion (m/z)	product ion (m/z)	EC[a]	A2[a]	LPOPCs[a]	blank
3,4-hydroxyphenylacetic acid	9.1	180.8	162.6 (H_2O loss)	69.23 ± 3.32	59.89 ± 0.53	49.77 ± 2.17	ND
4-HPA	18.7	164.8	146.6 (H_2O loss)	268.50 ± 6.13	231.51 ± 8.94	57.87 ± 2.32	ND
m-coumaric acid	17.1	162.7	118.8 (CO_2 loss)	41.82 ± 2.25	29.02 ± 0.33	45.30 ± 1.96	ND
p-coumaric acid	20.6	162.8	118.8 (CO_2 loss)	7.94 ± 0.63	6.17 ± 0.12	8.15 ± 0.58	ND
total				387.49 ± 10.28	326.59 ± 9.24	161.09 ± 6.17	

[a]Concentrations of phenolic acids converted from EC, A2, or LPOPCs by *L. casei*-01. The results were all expressed as means ± standard deviation (SD) (n = 3).

Among the phenolic metabolites screened, 3,4-hydroxyphenylacetic acid (181/163; parent ion/product ion), 4-HPA (165/147; parent ion/product ion), *m*-coumaric acid (163/119; parent ion/product ion), and *p*-coumaric acid (163/119; parent ion/product ion) were observed significantly after the incubation of all LPOPCs, EC, and A2 with *L. casei*-01, in comparison to the control (no extract) and the blank (no bacteria) (Table 2). However, none of these phenolic metabolites screened matched the chromatographic and tandem mass spectrometry (MS/MS) features of the

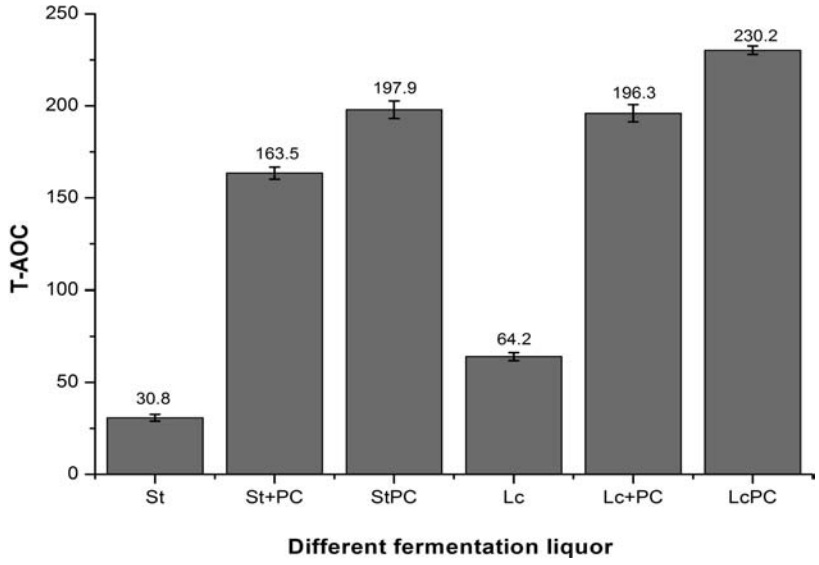

Figure 5. T-AOC of LPOPCs before and after metabolism by *S. thermophilus* and *L. casei* (StPC, LcPC, St + PC, and Lc + PC) and T-AOC of the two LAB fermentation cultures (St and Lc) (n = 3): St, *S. thermophilus* fermentation liquor; St + PC, LPOPCs in St liquor without metabolism; StPC, LPOPCs in fermentation liquor after metabolism by *S. thermophilus*; Lc, *L. casei* fermentation liquor; Lc + PC, LPOPCs in Lc liquor without metabolism; and LcPC, LPOPCs in fermentation liquor after metabolism by *L. casei*. All samples were significantly different from that of the control ($p < 0.01$).

metabolites from *S. thermophilus* on flavan-3-ols, further proving the conclusions above that the metabolic pathway of the two LAB was totally different from each other. Because of the effect of *L. casei*-01, about 26.2% (mol/mol) EC was degraded, referring to the chromatographic results in Figure 4, in which 22.4% (mol/mol) was metabolized to phenolic acids (Table 2). Moreover, 4-HPA was the most important product and composed more than 75% (mol/mol) of the total phenolic acids. However, nearly 20.5% (mol/mol) A2 was catabolized to small molecules, in which a majority of the dimer [about 18.8% (mol/mol) of the origin] was transformed to the same phenolic acids as EC, resulting from inner C−O−C bond fragmentation and C-ring opening, followed by lactonization, decarboxylation, dehydroxylation, and oxidation reactions, as well as others.[8] Additionally, 4-HPA produced from EC and A2 was much higher than that derived from LPOPCs, suggesting that it was easier for *Lactobacillus* to transform the flavan-3-ols with a lower degree of polymerization. It was also implied that *L. casei*-01 expressed lower enzyme activity to decompose procyanidin polymers than that on the degradation of upper and lower units or opening the benzenic ring of flavan-3-ol compounds.

T-AOC of LPOPCs before and after Metabolism by *S. thermophilus* and *L. casei*. As we know, the T-AOC of the procyanidin metabolites corresponded to the activity of samples reducing Fe^{3+} to Fe^{2+}, which was similar to the assay of ferric reducing antioxidant power (FRAP). According to the result, it was obvious that the T-AOC of LPOPCs was significantly enhanced after metabolism by the two strains, which were improved about 25% compared to the original. The normal fermentation liquid of *S. thermophilus* and *L. casei* all had antioxidant activity (Figure 5), because of the galactose metabolism of *Streptococcus* and the glucose metabolism of *Lactobacillus* in medium. LPOPCs possessed their antioxidant ability because of flavan-3-ols and procyanidin oligomers.

As discussed above, relative amounts of procyanidin dimer and trimer in LPOPCs were promoted and small molecules, such as phenolic acids, were produced after microbial incubation. It was demonstrated that the antioxidant capacities of dimeric and trimeric procyanidins were higher than monomers.[27] Phenolic acids (di- and monohydroxylated phenylproponic, phenylacetic, and benzoic acids and derivatives) formed from the subsequent catabolism of flavan-3-ols exhibited better bioavailability and higher biological activities than procyanidin parents,[17,18] such as radical-scavenging,[29] anti-inflammatory,[30] and antiproliferative[31] activities. Consequently, the positive effect of the two probiotic bacteria on the antioxidant activity of procyanidin extracts from litchi pericarp could be explained easily. Reports indicated that agricultural waste extracts have been applied successfully for industrial use of natural antioxidants from the large quantities of plant residues.[32,33] Thus, the results that we obtained suggested that the LAB transformation was another feasible method to convert litchi pericarp material from litchi processing waste to a more effective natural antioxidant that possesses health-promoting benefits, such as anti-aging and anticarcinogenic properties.

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Notes

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