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Effect of Microbial Fermentation on Content of Statin, GABA, and Polyphenols in Pu-Erh Tea

KEE-CHING JENG,[†,‡] CHIN-SHUH CHEN,[§] YU-PUN FANG,[§] ROLIS CHIEN-WEI HOU,[||] AND YUH-SHUEN CHEN*[,⊥]

Department of Education and Research, Taichung Veterans General Hospital, and Institute of Medical Technology and Department of Food Science and Biotechnology, National Chung-Hsing University, Taichung, Taiwan, Republic of China, Jenteh Junior College of Medicine and Nursing Management, Miaoli, Taiwan, Republic of China, and Department of Food Nutrition, HungKuang University, Taichung, Taiwan, Republic of China

Besides cancer prevention, the hypolipidemic effects of tea have been well studied in animals and humans. Recently, statin has been identified in Pu-erh tea extract. Clinical trials have confirmed that statin decreases the incidence of major coronary and cerebrovascular events and this may be due to its hypolipidemic and antiinflammatory effects. Since a good Pu-erh tea needs longer storage (10 years or more) of fermentation to enhance the flavor and fragrance, we screened microorganisms from two Pu-erh teas, 20 and 25 years old. Species of fungi and bacteria strains that contributed to a good taste of Pu-erh tea were isolated. The effect of fermentation was investigated by inoculating fresh tea leaves with individual strains of isolated microorganisms. Results showed that statin, total polyphenol content, and the scavenging activities of α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals increased during fermentation. Tea leaves inoculated with *Streptomyces bacillaris* strain R9 had the highest polyphenol content (3.3 mg/100 g) and scavenging ability to DPPH radicals (92%). *Streptomyces cinereus* strain Y11 was equally good for polyphenol content but yielded the highest amount of statin (1012 ng/g) after 42 days of fermentation. Interestingly, the statin content of fresh tea leaves fermented with strain R9 or Y11 after 180 days was much higher (4- and 8-fold, respectively) than that of the 25-year-old Pu-erh tea (513 ng/g) as measured by the HPLC method. Similarly, these two strains also increased the content of γ-aminobutyric acid (GABA) 5.7- and 4.7-fold in tea fermented for 180 days as compared with the fresh leaves (1270 μg/g) and that were higher than that of the Pu-erh tea (4900 μg/g). Taken together, the present results indicate that tea short-term fermented with *S. bacillaris* or *S. cinereus* enhances the color and content of statin, GABA, and polyphenols.

KEYWORDS: Pu-erh tea; *Streptomyces bacillari*; *Streptomyces cinereus*; fermentation; polyphenols; statin; GABA

INTRODUCTION

Tea is the most consumed drink in the world after water. Green tea is a nonfermented tea and contains more catechins than black tea or oolong tea. Recent human studies suggest that green tea not only contributes to reducing the risk of cardiovascular disease and some forms of cancer but also offers other benefits such as better oral hygiene, lower hypertension, reduction in body weight, antibacterial and antiviral activity, and antifibrotic effects (*1–3*). Recently, statins have been identified in Pu-erh tea (*4*). Statins are a group of hydroxymethylglutaryl-coenzyme A reductase inhibitors that prevent cardiovascular disease (CVD) and mortality in patients and reduce the relative risk of major coronary events and major cerebrovascular events in the population without CVD (*5*). Since association between high plasma cholesterol and cardiovascular risk is well established, statin's lowering LDL–cholesterol effects may be responsible for its therapeutic effects in primary and secondary CV diseases (*6*). An animal study has demonstrated that the body weights of rats and their plasma triglyceride, cholesterol, and LDL–cholesterol levels have been significantly reduced by feedings of Pu-erh, oolong, black, and green tea leaves to the animals (*7*).

* To whom correspondence should be addressed. Telephone: +886-4-2631-8652ext 1600. Fax: +886-4-2472-9568. E-mail: chenys@sunrise.hk.edu.tw.

[†] Taichung Veterans General Hospital.

[‡] Institute of Medical Technology, National Chung-Hsing University.

[§] Department of Food Science and Biotechnology, National Chung-Hsing University.

[||] Jenteh Junior College of Medicine and Nursing Management.

[⊥] HungKuang University.

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Table 1. Storage Age and Statin and GABA Contents of Pu-erh Teas

sample	storage years	statin (ng/g dw)[a]	GABA (μg/g dw)
Qizibing	10	312 ± 11	3000 ± 150
rare Pu-erh	20	327 ± 11	4150 ± 200
level 5 bulk	12	257 ± 10	4600 ± 250
Yiwuchuan	6	130 ± 3	1250 ± 70
Tuo tea	12	216 ± 7	3700 ± 150
Ta-Huang-In	20	412 ± 11	2750 ± 150
Ta-Hon-In	25	513 ± 13	4670 ± 220
fresh tea	0	13 ± 13	1270 ± 100

[a] The brand name of Pu-erh tea from the tea market indicates its source and the quality. Green bulk was 12 years old and was not studied for its statin and GABA contents. Statin and GABA were determined by HPLC methods as described in Materials and Methods.

Pu-erh tea has reddish to brownish red color and fragrance that becomes more prominent as fermentation continues and leaves age. In recent years, studies investigating health benefit effects of Pu-erh tea have shown effects on antioxidant, anticancer, lowering cholesterol, blood pressure, and blood sugar, and improving bacterial flora in the intestines (*8–13*).

Different teas have different chemical ingredients and hence different properties. Also, teas gathered and collected in different seasons differ in their chemical composition. Generally, green tea has the highest proportion of soluble ingredients and black tea the least since it is completely fermented. Polyphenol has been well studied in the extract of Pu-erh tea (*8*, *11*, *14*). Other Pu-erh tea's water-soluble components, such as alkaloids, proteins and free amino acids, carbohydrates, pigments, lipids, minerals, vitamins, and aromatic compounds are less reported. Among these, γ-aminobutyric acid (GABA) has received more attention because it acts as a relaxant and can boost immunity in humans (*15*).

Pu-erh tea is traditionally produced by fermenting leaves at room temperature for a long time, i.e., dry-stored tea. In order to hasten aging, tea is covered with a wet cloth during fermentation, named wet-stored tea. Although wet-stored tea made by this method has a shorter fermentation period, it can harbor undesirable microorganisms either harmful to the body or those that restrict the growth of beneficial microorganisms that enhance the taste of tea. The microorganisms that predominate in Yunnan Pu-erh tea undergoing fermentation are *Aspergillus niger*, *Aspergillus gloucu*, and species of *Penicillium*, *Rhizopus*, *Saccharomyces*, and *Bacterium*. *A. niger* is the most predominant, followed by *Saccharomyces* spp. There are very few *Bacillus* spp. (*16*, *17*). Because the quality of Pu-erh tea is closely related to the postfermentation process, we isolated microbes from Ta-Huang-In and Ta-Hon-In, which are well-known high-quality teas. Batches of tea leaves were inoculated with individual strains of the microbes. Tea leaves were assessed for contents of statin, polyphenols, and γ-aminobutyric acid (GABA) and free radical scavenging activity at regular intervals during fermentation. The best microorganism strain for the fermentation process would be identified as enhancing the quality of tea.

MATERIALS AND METHODS

Source of Pu-erh Teas. Eight Pu-erh teas of different dry-storage ages were used in this study (**Table 1**). The imported teas were kindly supplied by Changhai Tea Store, Taichung, Taiwan. Green teas from the "High mountain" and "Jade mountain" grade were obtained from the Taichung local supermarket.

Screening and Isolation of Microbes from Pu-erh Tea. Samples of different teas, 5.0 g each, were washed with 45 mL of sterilized water, and then 1 mL was plated onto PCA agar (Plate Count Agar; Difco, France). The plates were incubated in an incubator (Firstek Scientific, Taiwan) at 37 °C for 1 week (each sample was replicated three times). In another experiment, samples, 2.0 g each, were washed in sterile water, and after adding 225 mL of sterile water, each sample was homogenized in a blender (CF-15BS; XinGuang Mechanic, Taiwan). In this case also, 1 mL from each homogenized sample was incubated as before (three replications). A selection from different colonies of microorganisms from both of the sources, namely, the wash water and the homogenized leaves, was subcultured using inoculation loops and then incubated for 1 week at 37 °C on NB (nutrient broth; Difco, France). Pure cultures were obtained by subculturing one more time and incubated for 1 week before sending them to the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan) for identification.

Selection of Microbes That Contribute to the Taste of Pu-erh Tea. Samples, 300 g each, of fresh tea leaves were sterilized in an autoclave (TOMY SS-325, Tokyo, Japan) at 121 °C for 15 min and then inoculated under a laminar flow hood (Zao-Xin Inc., Taipei, Taiwan) with the selected isolate (10^4 cfu of microbe/g). The lots were fermented under controlled conditions (at 37 °C under 70% relative humidity) for 7 months, after which time the samples were assessed for flavor and quality by sensory evaluation. Nine assessors were trained to taste eight Pu-erh teas of various ages (**Table 1**) and obtained a sensory scale (0–9) with 12 descriptive categories of aroma, flavor, taste, and aftertaste for the sample inoculated with 24 and 30 microbial isolates of Ta-Hon-In and Ta-Huang-In, respectively.

Analysis of Functional Quality. The Pu-erh tea experimental samples inoculated with different strains from isolates were compared for their antioxidative contents. Samples were analyzed 7 days after inoculation and five times and up to 42 days at 7 day intervals. For statin and GABA content, the experiment was extended to longer periods of time (6–12 months).

Measurement of the Color of Tea Infusion. Tea was brewed from each sample, 3 g each, by flushing the samples with 150 mL of hot water (70 °C) and letting the leaves brew for 15 min. The infusion was cooled to room temperature and its color was measured, Hunter ΔL, Δa, Δb, and total color difference (ΔE), with a spectrophotometer (Nippon Denshoku Co., Tokyo, Japan). Reference samples were totally transparent, and $X = 91.96$, $Y = 93.97$, and $Z = 110.41$ were taken as standard values. In testing, the penetrating mode was adopted, and each test was repeated three times to obtain an average value before the total color difference (ΔE) was calculated.

Polyphenol Content. Total polyphenol content was determined by a procedure using 2% Na_2CO_3 solution and 50% Folin–Ciocalteu's reagent. The tea infusion mixture was reacted for 30 min at room temperature before the absorbance at 750 nm was read. A standard curve was obtained from the gallic acid standard (Sigma-Aldrich Chemie GmbH, Munich, Germany) liquor in five different chroma (*18*).

Superoxide Scavenging Activity. The scavenging activities of tea and tea infusion were measured by a nonenzymatic system (*19*). Briefly, a superoxide negative radical is produced when phenazine methosulfate (PMS) reacts with dihydronicotinamide adenine dinucleotide (NADH). The superoxide negative radical reacts with nitro blue tetrazolium (NBT) to form a diformazan compound, which has a maximum absorbance at 560 nm. A sample decrease of the absorption value indicates the capacity to capture superoxide negative radical. Scavenging activity (%) = (1 − absorbance of sample/absorbance of control without sample) × 100.

DPPH-Radical Scavenging Activity. The capacity to scavenge DPPH radicals was assessed by the method described previously (*18*). Samples were ground to a powder and passed through a 60 mesh sieve. The fine, oven-dried powder, in lots of 10, 50, 150, and 200 mg, was placed into separate small beakers. To each beaker was added 10 mL of hot (70 °C) water. After 5 min, the infusion was filtered through filter paper to obtain the sample liquor. The required quantity of DPPH (α,α-diphenyl-β-picrylhydrazyl; Sigma) to calculate the final value of chroma of 1 mM was dissolved in methanol; 0.5 mL of such freshly prepared DPPH solution was added to the solution of tea liquor in methanol (25 μL of sample liquor diluted to 4 mL with methanol) and thoroughly mixed. After 30 min, absorbance was measured at 517 nm

and the scavenging activity calculated as follows: DPPH radical scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100.

HPLC Analysis of Statins. The method of statin analysis was modified from the procedure recently reported (*4*). The analyses were carried out with a Hitachi L-6200 HPLC (Hitachi Instruments Inc., Tokyo, Japan) using an isocratic solvent system consisting of acetonitrile, deionized water, and acetic acid (70/30/1 v/v) as the mobile phase at a flow rate of 1 mL/min. The stationary phase was a Supelco analytical C18 column (4.6 mm × 25 cm, 5 μm) (Bellefonte, PA), and the sample injection volume was 20 μL. A Hitachi L-7420 UV–vis detector (238 nm) was used to detect statins. The statins in Pu-Erh tea liquors were identified by comparison of retention time, molecular absorption spectra, and mass spectra of the unknown peaks with statin standards. Absorption spectra were obtained on a photodiode array detector (Model 996; Waters Associates, Milford, MA), and mass spectra were resolved on a VG platform II LC-MS (Micromass Co., Cheshire, U.K.).

GABA Content. Tea liquor was prepared as described above with 200 mg of dry tea powder. The derivatives of each sample were examined by methods described previously (*20–22*). Samples of standard tea liquor (1 mL each) were placed in glass tubes to which was added 0.6 mL of 0.1 M buffer and 1 mL of 0.3% 2-hydroxynaphthaldehyde (the derivatizing reagent; TCI, Japan). The tubes were placed for 10 min in a water bath maintained at 80 °C and then taken out and cooled to room temperature. Enough methanol was then added to give a final volume of 5 mL. The guard and analytical column used in HPLC analysis was LiChrosper100 RP18 (5 μm, 4.0 mm i.d. × 15 cm). The mobile phase was comprised of MeOH and H_2O (62:38), the flow speed was 1.0 mL/min, the detection wavelength was 330 nm, and the injection amount was 20 μL. GABA standard liquor (Sigma) was prepared by diluting GABA with pure water to different strengths (10, 50, 100, 150, and 200 μg/mL) to obtain different chroma values. The derivatization reaction was observed with GABA liquor at five values of chroma. Each sample was tested three times, and the average value of the absorbance at different values of chroma was calculated.

Cell Culture. The microglia BV-2 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 units/mL penicillin, and 100 mg/mL streptomycin at 37 °C under 5% CO_2. Confluent cultures were passed using trypsinization. For experiments, cells were washed twice with warm DMEM (without phenol red) and then treated in serum-free medium. In all experiments, cells were treated with tea extract dissolved in methanol. The culture supernatant was collected for the nitric oxide (NO) assay, and the cell extracts were prepared by adding lysis buffer and sonicated.

Nitric Oxide Assay. Nitrite from the culture supernatant, measured by Griess reaction, was taken as a measure of NO production. The absorbance at 540 nm was determined using a microplate reader (spectraMAX 340; Molecular Devices, Sunnyvale, CA).

iNOS Assay. Inducible nitric oxide synthase (iNOS) protein expression from the cell extracts was measured by an ELISA kit (R&D, Minnespolis, MN). The absorbance at 450/570 nm was determined using a microplate reader (spectraMAX).

Statistical Analysis. All data were expressed as the mean ± SD. For single variable comparisons, Student's *t*-test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Thirty strains were obtained from Ta-Huang-In and 24 from Ta-Hon-In in the preliminary screening; these included *Penicillium*, *Rhizopus*, *Saccharomyces*, *Aspergillus*, *Actinoplane*, *Streptomyces*, and *Bacterium*. After fermentation for 7 months, seven and eight microbial strains of Ta-Hon-In and Ta-Huang-In, respectively, that enhanced the taste were selected by nine trained assessors with sensory evaluation (data not shown). These 15 strains contributed to the Pu-erh tea's characteristic taste and flavor. Strains that led to a bitter taste, astringency, and a smell of grass, mold, or soil were discarded. The selected 15 strains were labeled R1, R3, R4, R7, R8, R9, and R15 for Ta-Hon-In (Big red seal) and Y2, Y8, Y11, Y13, Y21, Y23, Y28, and Y29 for Ta-Huang-In (Big yellow seal). The strains were identified by FIRDI, Taiwan, as belonging to *Actinoplanes* and *Streptomyces*. The species represented were *Actinoplanes kinshanensis*, *Actinoplanes pallidoaurantiacus*, *Actinoplanes purpeobrunneus*, *Actinoplanes aurantiacus*, *Streptomyces bacillaris*, *Streptomyces cavourensis* ssp. *cavourensis*, and *Streptomyces cinereus*. Strain R9 (*S. bacillaris*) and strain Y11 (*S. cinereus*) were selected for further study due to their higher antioxidative contents.

Table 2. Tea Color from Various Pu-erh Teas

sample	color scale		
	Δ*L*	Δ*a*	Δ*b*
Qizibing	59.30 ± 0.12	21.58 ± 0.17	51.33 ± 0.09
rare Pu-erh	72.66 ± 0.04	25.11 ± 0.20	74.13 ± 0.17
level 5 bulk	55.14 ± 0.18	33.82 ± 0.16	73.23 ± 0.14
Yiwuchun	93.10 ± 0.07[a]	0.44 ± 0.12[a]	23.79 ± 0.08[a]
Ta-Huang-In	74.20 ± 0.11	19.29 ± 0.18	70.56 ± 0.15
Ta-Hon-In	74.66 ± 0.04	19.93 ± 0.15	72.55 ± 0.11
Tuo tea	56.13 ± 0.06	30.47 ± 0.11	72.19 ± 0.14
R9 (42 day)	82.27 ± 0.07[a]	8.05 ± 0.04[a]	42.43 ± 0.05[a]
Y11 (42 day)	78.00 ± 0.12	17.02 ± 0.05	74.84 ± 0.10
fresh tea	65.05 ± 0.08	2.87 ± 0.05[a]	40.56 ± 0.24[a]

[a] Values are the mean ± SD from triplicate analysis.

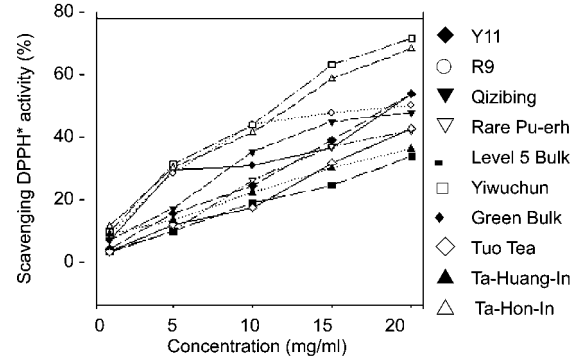

Figure 1. Scavenging capabilities of the DPPH radical by Pu-erh teas and experimental samples. Infusions of R9 and Y11 samples from 42 days of fermentation had middle-ranged scavenging DPPH capabilities.

Color of Tea Infusion. The colors of eight Pu-erh teas and experimental samples were compared. The difference can be attributed to different tea species, microbes, duration of fermentation, and different ingredients used in the process of fermentation. There was no difference in color values of Δ*L*, Δ*a*, and Δ*b* from tea infusion between the old Pu-erh teas (more than 10 years) and the fresh tea inoculated with the Y11 strain (**Table 2**). This suggests that microbial fermentation of fresh tea might enhance the tea color comparable to that of old Pu-erh teas (Δ*a*/Δ*b* ranges: 0.25–0.45).

Scavenging DPPH Activity. Preparation of Pu-erh tea infusions from various quantities of tea leaves (10, 50, 100, 150, and 200 mg) was assessed for the capacity of scavenging DPPH radicals. The result is shown in **Figure 1**. Yiwuchun and Ta-Hon-In had the highest capacity of scavenging DPPH radicals among all Pu-erh teas, and R9 and Y11 samples were somewhere in the middle.

Statin, GABA, and Total Polyphenol Content. The content of statin, GABA, and total polyphenols was increased with the duration of fermentation (**Tables 3–5** and **Figure 2**). Fresh

Table 3. Effect of Fermentation on the Statin Content from Experimental Teas Inoculated with Different Microbial Strains

	statin content (ng/g dw)[a]					
strain	7 days	14 days	28 days	42 days	90 days	180 days
Y2	16 ± 1	23 ± 2	49 ± 3	91 ± 2	87 ± 2	133 ± 1
Y8	43 ± 3	112 ± 5	149 ± 5	518 ± 11	1068 ± 17	2142 ± 51
Y11	53 ± 2	98 ± 5	432 ± 4	1012 ± 13	3512 ± 47	4160 ± 82[b]
Y13	41 ± 2	109 ± 4	192 ± 5	193 ± 11	329 ± 17	316 ± 15
Y21	39 ± 3	81 ± 3	101 ± 6	171 ± 13	162 ± 11	193 ± 14
Y23	27 ± 2	91 ± 2	123 ± 6	212 ± 6	158 ± 9	183 ± 6
Y28	53 ± 3	92 ± 6	151 ± 4	321 ± 5	316 ± 14	396 ± 9
Y29	41 ± 2	73 ± 3	99 ± 5	212 ± 6	252 ± 11	301 ± 12
R1	53 ± 2	163 ± 5	212 ± 8	496 ± 19	913 ± 21	1912 ± 52
R3	63 ± 2	112 ± 3	201 ± 6	312 ± 11	812 ± 16	1021 ± 31
R4	23 ± 1	34 ± 2	112 ± 3	111 ± 4	92 ± 3	94 ± 2
R7	57 ± 2	133 ± 4	211 ± 5	368 ± 12	519 ± 21	921 ± 31
R8	32 ± 1	36 ± 3	91 ± 5	67 ± 2	71 ± 3	106 ± 5
R9	67 ± 3	112 ± 4	198 ± 6	782 ± 16	1923 ± 26	2183 ± 62
R15	38 ± 2	91 ± 3	163 ± 4	318 ± 7	497 ± 11	512 ± 32
control	13 ± 1	14 ± 1	12 ± 1	14 ± 1	13 ± 1	15 ± 2

[a] Statin was determined by the HPLC method as described in Materials and Methods. Data represent the mean ± SD from triplicate analysis. All samples were significantly different from the control (fresh tea leaves), $p < 0.01$. [b] The highest statin content among all strains.

Table 4. Effect of Fermentation on Total Polyphenol Content from Experimental Teas Inoculated with Different Microbial Strains

	total polyphenol content (mg/g)[a]					
strain	7 days	14 days	21 days	28 days	35 days	42 days
Y2	13.1 ± 0.1	17.5 ± 0.2	18.4 ± 0.3	21.2 ± 0.1	23.1 ± 0.1	25.8 ± 0.1
Y8	15.3 ± 0.2	17.6 ± 0.1	19.0 ± 0.1	20.0 ± 0.3	23.1 ± 0.1	26.1 ± 0.1
Y11	15.2 ± 0.1	16.9 ± 0.1	21.9 ± 0.1	25.1 ± 0.1	28.5 ± 0.1	31.9 ± 0.1
Y13	10.3 ± 11	15.8 ± 0.1	21.2 ± 0.1	22.1 ± 0.1	23.5 ± 0.2	24.9 ± 0.1
Y211	1.2 ± 0.1	14.6 ± 0.4	18.1 ± 0.1	21.1 ± 0.1	21.3 ± 0.2	21.5 ± 0.1
Y23	16.7 ± 0.1	19.5 ± 0.1	19.7 ± 0.1	21.1 ± 0.1	23.7 ± 0.1	26.3 ± 0.1
Y28	14.9 ± 0.1	15.4 ± 0.2	19.3 ± 0.1	22.2 ± 0.1	23.6 ± 0.1	25.1 ± 0.1
Y29	10.0 ± 0.1	15.8 ± 0.1	17.7 ± 0.1	23.0 ± 0.1	24.5 ± 0.1	25.9 ± 0.2
R1	19.5 ± 0.1	21.5 ± 0.1	21.9 ± 0.1	26.6 ± 0.1	27.7 ± 0.1	28.7 ± 0.1
R3	16.3 ± 0.1	16.6 ± 0.1	18.7 ± 0.1	21.0 ± 0.1	22.1 ± 0.2	23.3 ± 0.1
R4	17.7 ± 0.1	20.1 ± 0.1	20.4 ± 0.2	21.8 ± 0.1	23.8 ± 0.1	25.7 ± 0.1
R7	17.6 ± 0.1	20.1+0.1	22.3 ± 0.1	24.8 ± 0.1	27.0 ± 0.1	29.3 ± 0.1
R8	16.3 ± 0.1	16.7 ± 0.2	17.7 ± 0.1	18.1 ± 0.2	19.5 ± 0.1	21.0 ± 0.1
R9	11.4 ± 0.1	16.7 ± 0.1	21.6 ± 0.1	24.8 ± 0.1	28.8 ± 0.1	32.9 ± 0.1
control	10.0 ± 0.1	10.3 ± 0.1	10.1 ± 20	10.2 ± 0.2	0.1 ± 0.2	10.0 ± 0.2

[a] Polyphenols were determined by the method described in Materials and Methods. Data represent the mean ± SD from triplicate analysis. All samples were significantly different from the control (fresh tea leaves), $p < 0.01$.

leaves inoculated with either the R9 strain or the Y11 strain had the highest content of statin, GABA, and total polyphenols. With extended fermentation of 180 days, R9 and Y11 samples produced 4- and 8-fold, respectively, more statin than that of the 25-year-old Ta-Hon-In Pu-erh tea (513 ng/g). Similarly, samples of tea fermented with R9 and Y11 produced 5.7- and 4.7-fold more GABA than that of the fresh tea leaves. This is a new finding of improving tea's health benefit by fermentation. Our results also agree with a previous study that the phenolic compound of a tea extract is greatly influenced by fermentation (*23*).

Antioxidant Activity. Human cells suffer oxidation damage following prolonged and constant exposure to free radicals and reactive oxygen. Oxidative damage to cells causes cancer, aging, neuropathy, arteriosclerosis, and diabetes (*24*, *25*). Tea leaves contain many components, such as vitamin C and vitamin E, β-carotene, catechins, and other polyphenols that can eliminate free radicals; among them, catechins show very high antioxidative capacity, which is thought to be closely related to its antitumor properties (*3*, *26*). In addition, water extracts of Pu-erh tea decrease nitric oxide (NO) production of LPS-induced RAW 264.7 macrophages (*8*). The antioxidative effect of tea leaves following fermentation with different strains is shown in **Table 4**. Superoxide scavenging activity did not differ greatly due to either strains or fermentation period (data not shown). The capacity of scavenging DPPH radicals increased 4-fold with the duration of fermentation. The extract from various teas had a similar effect on the inhibition of NO production and iNOS expression in microglia BV-2 cells (**Table 6**). With the longer duration of fermentation, the capacity of scavenging DPPH radicals and inhibition of iNOS protein and NO production were all increased significantly (**Figures 1** and **3**). This suggests that statin contributed the additional effect of inhibition of NO activity (*27*, *28*)

Table 5. Change of DPPH-Radical Scavenging Activities of Experimental Teas during Fermentation

	DPPH radical scavenging activity (%)[a]					
strain	7 days	14 days	21 days	28 days	35 days	42 days
Y2	20 ± 0.6	35 ± 0.6	41 ± 0.3	59 ± 0.4	63 ± 1.0	80 ± 0.6[b]
Y8	14 ± 0.5	34 ± 0.6	58 ± 0.6	80 ± 0.6	86 ± 0.1	91 ± 0.1
Y11	20 ± 0.8	64 ± 1.5	85 ± 0.6	90 ± 0.1	92 ± 0.1	93 ± 0.1
Y13	25 ± 0.5	45 ± 0.3	56 ± 0.9	59 ± 0.6	79 ± 0.1	81 ± 0.1
Y21	14 ± 0.7	39 ± 1.9	52 ± 0.6	60 ± 0.4	71 ± 0.1	82 ± 0.1
Y23	19 ± 0.6	34 ± 0.5	65 ± 0.3	68 ± 0.7	76 ± 0.1	84 ± 0.1
Y28	23 ± 0.3	31 ± 0.2	66 ± 1.1	77 ± 0.5	81 ± 0.6	87 ± 0.1
Y29	23 ± 0.6	35 ± 0.6	66 ± 1.0	74 ± 0.6	78 ± 0.1	81 ± 0.6
R1	33 ± 0.6	42 ± 0.8	62 ± 0.3	75 ± 0.6	89 ± 0.1	92 ± 0.1
R3	22 ± 0.3	47 ± 1.0	54 ± 0.9	71 ± 0.2	82 ± 0.1	87 ± 0.1
R4	12 ± 0.5	28 ± 0.2	59 ± 0.6	63 ± 0.6	78 ± 0.1	83 ± 0.1
R7	32 ± 0.4	58 ± 0.9	80 ± 0.1	90 ± 0.1	89 ± 0.1	92 ± 0.1
R8	20 ± 0.0	36 ± 0.2	49 ± 0.7	58 ± 0.1	62 ± 0.1	76 ± 0.7
R9	22 ± 0.1	58 ± 0.4	87 ± 0.1	91 ± 0.6	92 ± 0.1	92 ± 0.1
R15	30 ± 0.2	33 ± 0.8	66 ± 1.1	79 ± 0.1	91 ± 0.6	91 ± 0.6
control	21 ± 0.7	25 ± 1.8	24 ± 0.7	24 ± 1.9	22 ± 0.9	25 ± 1.1

[a] DPPH-radical scavenging activities were determined by the method described in Materials and Methods. [b] Data represent the mean ± SD from triplicate analysis. All samples were significantly different from the control (fresh tea leaves), $p < 0.01$.

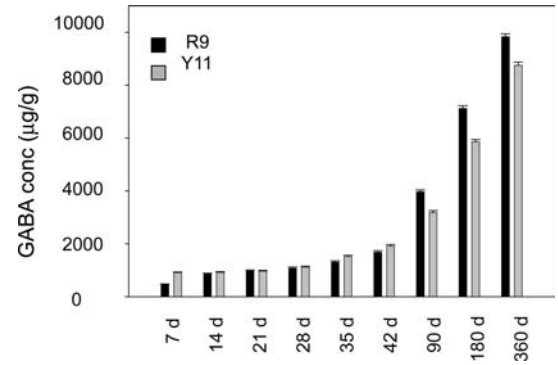

Figure 2. Effect of fermentation with strains Y11 and R9 in tea leaves on γ-aminobutyric acid (GABA) content. Both strains showed a time-dependent increase of GABA content.

GABA is one of the major inhibitory neurotransmitters in the central nervous system and is known to mediate presynaptic inhibition of primary afferent fibers in the motor system and may also be involved in postsynaptic forms of motor neuron inhibition (*29*). Amino acid neurotransmitters are critical for the function of the central nervous system and play an important role in brain function and neurological disease (*30*). In recent a study, GABA-enriched soy product was reported to exert an antihypertensive effect in rats (*31*). GABA occurs naturally in many kinds of foods at low levels, whereas in fermented food products, GABA levels are higher. GABA is used as a functional

Table 6. Inhibition of LPS-Induced Nitrite Production and iNOS Expression by Tea Extracts

group[a]	nitrite (μM)	iNOS (units)
control	1.9 ± 0.8	0.31 ± 0.12
LPS	43.7 ± 4.6	7.31 ± 0.83
methanol	41.1 ± 4.6	7.02 ± 0.92
green tea (1)	29.0 ± 8.1[b]	3.04 ± 0.47[b]
green tea (2)	29.7 ± 6.4[b]	4.40 ± 0.58[b]
Yiwuchun	25.5 ± 6.6[b]	3.20 ± 0.35[b]
level 5 bulk	26.1 ± 8.2[b]	6.80 ± 0.72
Tuo tea	25.8 ± 6.7[b]	6.10 ± 0.45
Tai-Huang-In	25.3 ± 7.1[b]	3.90 ± 0.46[b]
Tai-Hon-In	25.0 ± 5.9[b]	4.30 ± 0.52[b]
R9	8.5 ± 2.2[c]	1.2 ± 0.34[c]
Y11	11.4 ± 2.4[c]	1.3 ± 0.21[c]

[a] Teas were listed in **Table 1**. Green tea (1) was from the High mountain tea and green tea (2) from the Jade mountain tea. Dried tea leaves (10 g) were extracted by 400 μL of methanol. Three microliters of each extract was added to BV-2 cells (5×10^6/mL) in the presence of LPS (10 ng/mL) stimulation and cultured for 16 h (iNOS) or 24 h (NO production) at 37 °C under 5% CO_2 atmosphere. Extracts of R9 and Y11 samples were prepared from 180 days of fermentation. [b] $p < 0.05$ as compared with the vehicle control (methanol). [c] $p < 0.01$ as compared with Pu-erh teas.

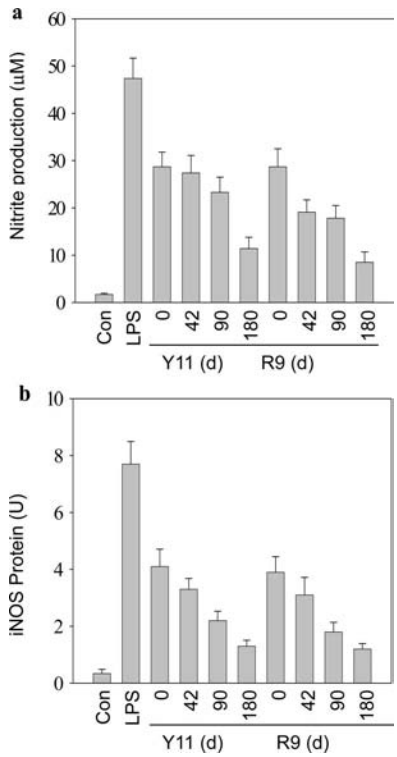

Figure 3. Effect of fermentation time of tea leaves on nitric oxide production (a) and the iNOS protein expression (b) by lipopolysacharide-(LPS-) stimulated BV-2 microglia cells. Extracts from R9 and Y11 samples inhibited LPS-induced iNOS and NO significantly ($p < 0.01$), and samples from 180 days (d) differed from other fermentation periods ($p < 0.01$).

food ingredient in Japan because of its health benefits (*15*). Present results showed that GABA contents of samples inoculated with strains R9 and Y11 were significantly higher than that in seven Pu-erh teas, and they were increased 5.7- and 4.7-fold, respectively over fresh leaves after fermentation for 180 days (**Table 1** and **Figure 2**).

CONCLUSION

Microorganisms from Pu-erh tea, which could enhance the taste, were isolated and identified. Fresh tea leaves were inoculated with these strains and when fermented up to 42 days increased antioxidant capacity. Samples inoculated with R9 (*S. bacillaris*) had the highest total polyphenol content and maximum capacity to scavenge the DPPH radical; similarly, Y11 (*S. cinereus*) had the highest content of statin and GABA, since statins have been confirmed by clinical trials to prevent CVD. Therefore, the tea fermentation process with *S. bacillaris* or *S. cinereus* producing high statin and total polyphenol content might have health benefits.

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