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Antibacterial activity and phytochemical profile of fermented *Camellia sinensis* (fuzhuan tea)

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ABSTRACT

Fuzhuan tea is a traditional preparation of *Camellia sinensis* L. (Theaceae) from Hunan, China, that is fermented with the fungus *Eurotium cristatum*. Metabolomic analysis was performed on fuzhuan tea extracts and compared to extracts of non-fermented green teas using ultra-performance liquid chromatography/time of flight-mass spectrometry (UPLC-ToF-MS). Principal component analysis revealed a unique phytochemical profile between the two types of tea with the largest separation visible along the third principle component, which accounted for 12.4% of dataset variation. Spectral comparison of significantly different tea metabolite features allowed tentative identification of flavonoids including catechins, fatty acid amides, and other lipids and polysaccharides. Fuzhuan tea extracts, at a concentration of 5 mg/mL or less, reduced the growth of enteric pathogens *Shigella sonnei* and *Staphylococcus aureus* by 50%, and had a minimum inhibitory concentration (MIC) of 0.625 mg/mL against *S. aureus*. These results support a distinct phytochemical profile associated with fermented fuzhuan tea compared to non-fermented green teas that warrant further investigation for novel compounds with antimicrobial bioactivity.

1. Introduction

Dark teas, such as pu'er and fuzhuan, are produced by fungal fermentation of *Camellia sinensis* L. (Theaceae) leaves (Xu et al., 2011). Fuzhuan tea, native to the Hunan province of China, is consumed as an ethno-botanical remedy for treating food-borne illnesses, dysentery, and aiding general nutrition and digestion (Fu et al., 2011; Ling et al., 2010; Wu, Ding, Xia, & Tu, 2010; Xu et al., 2011). Fuzhuan tea has been shown to stimulate pancreatic amylase and protease in vitro (Yu, 2009) and inhibit the growth of food-borne pathogens *Bacillus cereus*, *Bacillus subtilis*, *Clostridium perfringens*, and *Clostridium sporogenes* (Mo, Zhu, & Chen, 2008), providing evidence for digestive and intestinal health protective actions.

Several reports suggest that the fermentation process results in a final product with a unique phytochemical profile compared to other types of tea. Targeted analysis of catechins, commonly associated with the antioxidant and cancer fighting properties of green tea, showed their in fuzhuan tea following fermentation (Wu et al., 2010). Several organic acids, including oxalic, α-keto-glutaric, L-malic, succinic and ascorbic acid were also decreased by fermentation. However, organic acids such as lactic, acetic, and citric acids, which are fermentation products of beneficial lactic acid bacteria, increased with fuzhuan tea fermentation (Wu et al., 2010). Taken together, this suggests that fuzhuan tea has a unique phytochemical profile that is, in part, due to the microbial fermentation employed during its production. As fuzhuan tea is produced by microbial fermentation of green tea leaves, which have been dried and pan fried to inhibit oxidative enzymes (Weir et al., 2012), we chose to compare phytochemical profiles of fuzhuan tea extracts to green tea extracts. Unlike many of the previous studies that examined the chemical composition of fuzhuan tea, we have focused our efforts on obtaining a chemical fingerprint of the hot water extracts of the tea that are equivalent to what is directly consumed.

Non-targeted metabolomics is a rapidly advancing field applied to a variety of phytochemical and food-based investigations (Capozzi and Bordoni, 2012). Specifically, our lab has previously utilized this technique to analyze genetic differences between cultivated rice (*Oryza sativa*) varieties (Heuberger et al., 2010). In this study, we used metabolomics to compare global compound profiles of fuzhuan tea to non-fermented green tea, and investigated their potential antimicrobial bioactivity.

Abbreviations: PLS-DA, partial least squares discriminate analysis; MIC, minimum inhibitory concentration; ANOVA, analysis of variance.

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2. Materials and methods

2.1. Reagents

Rifampicin (Sigma, St. Louis, MO), a broad spectrum antibacterial agent, was used as a positive control in bacterial inhibitory assays.

2.2. Bacterial strains and culture conditions

Staphylococcus aureus NCTC 8325, *Pseudomonas aeruginosa* PA01, *Escherichia coli* Op50 and *Shigella sonnei* were kindly provided by Dr. Jorge Vivanco (Colorado State University). *Salmonella enterica* Typhimurium was a generous gift from Dr. Andres Vazquez-Torres (University of Colorado). Bacterial cultures were maintained on Luria-Bertani broth without antibiotics and grown at 37 °C.

2.3. Tea

Fuzhuan tea from Hunan Province in China (provided by Golden Flower Trading Company, Inc., Lone Tree, CO) was used in both loose leaf (n = 2) and dried hot-water extract forms (n = 3). Loose leaf green tea (n = 6) was analyzed from the Zhejiang, Hangzhou, Anhui, Henan, and Yunnan provinces of China, with the addition of a white tea sample from Hunan province (Happy Lucky's Teahouse, Fort Collins, CO) (Table 1). For simplicity, the white tea was grouped with the green teas for metabolomics analysis. Fuzhuan and green tea loose leaf extracts were prepared by steeping 7 g of tea leaves in 200 mL of 100 °C water for 1 h, gently shaking. One gram (equal to 7 g of loose leaves) of concentrated, freeze-dried extracts was re-dissolved in 200 mL of 100 °C water. All extracts were then filtered and freeze dried, re-suspended in methanol, and used for metabolomics and antibacterial assays. For initial analyses, all fuzhuan tea samples were compared with multiple samples extracted from a single type of green tea (n = 6); subsequent analyses were conducted using all tea samples (n = 11).

2.4. Ultra-performance liquid chromatography/time of flight-mass spectrometry (UPLC-ToF-MS)

Injections (1 μL) were performed on a Waters Acquity UPLC system, with separation performed using a Waters Acquity UPLC C8 column (1.8 μM, 1.0 × 100 mm); the gradient was from solvent A (95% water, 5% methanol, 0.1% formic acid) to solvent B (95% methanol, 5% water, 0.1% formic acid). Injections were made in 100% A, which was held for 0.1 min, ramped to 40% B in 0.9 min, to 70% B over 2 min, and to 100% B over 8 min. Mobile phase was begun at 100% B for 6 min and returned to starting conditions over 0.1 min, before re-equilibrating for 5.9 min. For the duration of the run, flow rate was held at a constant 140 μL/min. The column was held at 50 °C, and samples were held at 5 °C.

Column eluent was infused into a Micromass Q-Tof Micro MS fitted with an electrospray source. Data was collected in positive ion mode, scanning from 50 to 1000 at a rate of 1 scan per second with 0.1 s inter-scan delay. Calibration was performed prior to sample analysis via infusion of sodium formate solution, with mass accuracy within 5 ppm. The capillary voltage was held at 2200 V, the source temperature at 130 °C, and the desolvation temperature at 300 °C at a nitrogen desolvation gas flow rate of 400 L/h. The quadrupole was held at collision energy of 7 V.

Peak detection and normalization were conducted by converting raw data files into cdf format using Waters Data Bridge software. The feature detection and alignment were performed using the XCMS Online program, which is a metabolomics software, and R, the statistical and graphing software program (Smith, Want, Tong, Abagyan, & Siuzdak, 2006; R Development Core Team, 2011). Raw peak areas were normalized to total ion signal in R, and the normalized dataset was averaged by injection replicate previous to statistical analysis. Metlin and KEGG databases were next used to match the *m/z* for metabolite identification. Identification confidence scores were assigned as previously described by the Chemical Analysis Working Group Metabolomics Standards Initiative (Sumner et al., 2007).

2.5. Bacterial growth inhibition assay

Bacteria were cultured and grown in Luria-Bertani (LB) media (Sigma, St. Louis, MO). Tea (10 mg/mL) and rifampicin (positive control) stocks were added and serially diluted in triplicate to 96-well plates for a total volume of 100 μL in each well. Bacterial cultures were diluted to an OD_{600} of 0.2 in sterile, 10 mM magnesium sulfate and added to treatment or control wells at a final concentration of 5×10^6 cells/mL and incubated for 24 h at 37 °C. Dosages ranged from 0.625 mg/mL (1:8 v/v) to 5 mg/mL (1:1 v/v) for both fuzhuan and non-fermented green tea. Bacterial density was measured at OD_{600} and growth inhibition was expressed as percent of the negative control (10 mM magnesium sulfate). Experiments were conducted in triplicate.

2.6. Statistical analysis

Multivariate analyses and subsequent ANOVA were conducted in the software program R (Vienna, Austria) on the averages of duplicate injections of five fuzhuan tea samples and six non-fermented green tea samples (n = 11). In addition, a Student's *t*-test was used to compare metabolites between the averages of duplicate injections of samples from five lots of fuzhuan tea and non-fermented green tea samples (n = 6). Bacterial bioassays were analyzed by two-way ANOVA and Bonferroni post-hoc testing using GraphPad Prism (v 5.0, GraphPad Software, Inc., La Jolla, CA).

Table 1
The tea type, origin in China, and source included in data analysis.

Tea	Type	Preparation	Originating Province	Source
Fuzhuan	Fermented	Loose leaf	Hunan	Golden Flower Trading Company
Fuzhuan	Fermented	Compressed coin	Hunan	Golden Flower Trading Company
Fuzhuan	Fermented	Compressed coin	Hunan	Golden Flower Trading Company
Fuzhuan	Fermented	Concentrated extract	Hunan	Golden Flower Trading Company
Fuzhuan	Fermented	Concentrated extract	Hunan	Golden Flower Trading Company
Dragonwell-Long Jing	Non-fermented green	Loose leaf	Zhejiang	Happy Lucky Tea House
Emerald Forest	Non-fermented green	Loose leaf	Zhejiang	Happy Lucky Tea House
Tea Forest	Non-fermented green	Loose leaf	Yunan	Happy Lucky Tea House
Monkey King	Non-fermented green	Loose leaf	Anhui	Happy Lucky Tea House
Xiayang Maojin	Non-fermented green	Loose leaf	Henan	Happy Lucky Tea House
Jasmine Silver Needle	Non-fermented green	Loose leaf	Hunan	Happy Lucky Tea House

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3. Results

3.1. Antibacterial activity of fermented fuzhuan tea and non-fermented green tea

Aqueous tea extracts were assessed for in vitro bacterial growth inhibition. Fuzhuan tea dose dependently and significantly inhibited growth of *S. sonnei* with a minimum inhibitory concentration (MIC) of 5.0 mg/mL as compared with negative treated controls ($p < 0.01$, Fig. 1A). Non-fermented green tea also significantly inhibited *S. sonnei* viability, with a MIC of 0.625 mg/mL ($p < 0.05$, Fig. 1A). No significant differences were observed between the activity of fuzhuan tea and non-fermented green tea against *S. sonnei*.

When incubated for 24 h with *S. aureus*, all doses of the fermented fuzhuan tea and non-fermented green tea extracts demonstrated greater than 50% growth inhibition, significantly different from the negative control ($p < 0.01$, Fig. 1B). There were also significant differences in growth inhibition between fuzhuan and non-fermented green tea at the 5 and 1.25 mg/mL concentrations ($p < 0.05$, Fig. 1B). No growth inhibitory activity was observed against *E. coli*, *S. enterica* Typhimurium, or *P. aeruginosa* (data not shown).

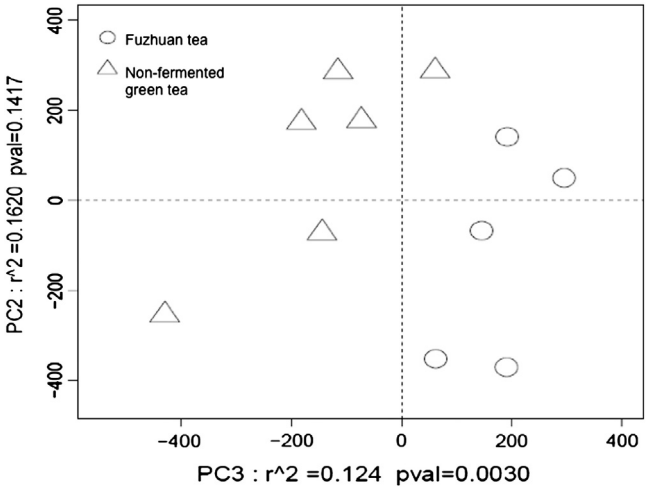

Fig. 2. Principal component analysis of aqueous extracts of fuzhuan tea and non-fermented green tea. Metabolite profiles were detected by LC-ToF-MS, n = 11, average of duplicate injections. Component 2 (16.2%) and component 3 (12.4%) are plotted, labeled PC2 and PC3, and component 3 showed a significant distinction ($p = 0.003$) between tea types.

3.2. Metabolite profiling of fuzhuan tea and green tea

Principal component analysis (PCA) revealed that fuzhuan tea and green tea phytochemical profiles exhibit maximal separation along the third principal component, which accounted for 12.4% of the total variation in the dataset (Fig. 2). To determine which metabolite features differed between the fuzhuan tea and green tea samples, ANOVA was performed using R, and results were summarized graphically as a function of retention time and mass (*m/z*) (Fig. 3). In this plot, circles represent statistically different metabolite features and the larger the circle size, the smaller (more significant) the p value (Fig. 3). The pattern reveals that the features distinguishing the fermented and non-fermented teas were relatively polar metabolites as determined by their early elution times. The *m/z* ratio of 50–300 may be associated with compound classes including fatty acid amides, sugars, organic acids, phenolics, and certain alkaloids. The *m/z* ratio of 450–700 may be associated with larger compounds such as catechins, flavonoids, and triterpenoids, while the *m/z* ratio of 700–900 may be polysaccharides, lipids, flavonoid glycosides, peptides, and saponins (Fig. 3) (Mensack, Fitzgerald, Lewis, & Thompson, 2010).

To further distinguish specific metabolites that differed between a selected set of samples from the fuzhuan tea (n = 5) and green tea (n = 1), we applied a partial least squares discriminate analysis (PLS-DA). Fig. 4 highlights analytes that are present at significantly higher concentrations in fuzhuan tea (bottom left) than in a subsample of the green tea ($p < 0.05$); specific compound candidates from this analysis are presented in Table 2.

3.3. Chemical contents of fermented fuzhuan tea

Some candidate compounds, including catechins, glycosylated flavonoids, and fatty acid amides were identified by spectral comparisons. The *m/z* 744.1905 is consistent with the mass of a glycosylated flavonoid and was found in significantly higher amounts in fuzhuan tea (Table 2, $p < 0.001$). Another glycosylated flavonoid is a likely candidate for the ion observed at 5.40 min and 786.2271 *m/z* (Table 2). The candidate glycosylated flavonoid that corresponds to *m/z* 889.2480 was also significantly more abundant in fuzhuan tea compared to green tea (Table 2, $p < 0.001$). Two candidate compounds that correspond to catechins were detected (Table 2). One of them was tentatively identified as epigallocatechin (Wu et al., 2010), with an observed molecular ion at *m/z* 307.0849, and was 1.52-fold more abundant in green tea than fuzhuan tea; however, this difference was not statistically significant (Table 2). The other candidate compound, epicatechin gallate, at *m/z* 459.0912 was 8.14-fold higher in non-fermented green tea than fuzhuan tea (Table 2, $p < 0.001$). Arrows in Fig. 4 indicate candidate fatty acid amides that were higher in fuzhuan tea compared to replicate extractions from a sample of Xiayang Maojin green tea (Table 1, Fig. 4). Table 2

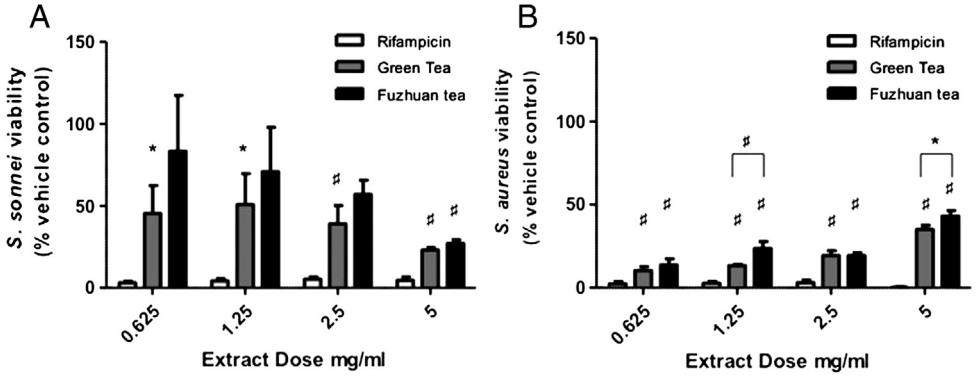

Fig. 1. Growth inhibitory effects of both fuzhuan tea and green tea extracts on viability of *Shigella sonnei* (A) and *Staphylococcus aureus* (B) following a 24 h incubation. Rifampicin (a commonly used antibiotic) was used as a positive control at doses of 31.3, 62.5, 125, and 250 μg/mL. Statistical significance is denoted by * ($p < 0.05$) or ♯ ($p < 0.01$). Bacterial inhibition by each tea type and treatment was compared to the control (100% viability) by two-way ANOVA with a Bonferroni post-hoc test for detection of significant differences.

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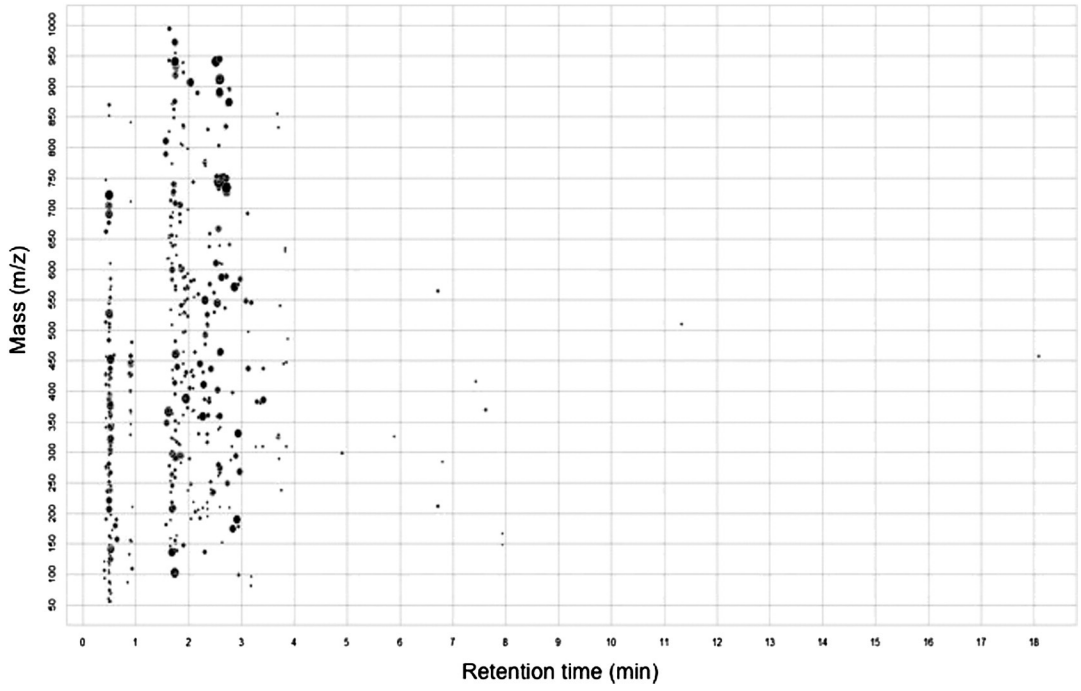

Fig. 3. Metabolome differences between fermented and non-fermented tea. Metabolite profiles of aqueous tea extracts were analyzed for statistically significant differences by ANOVA. Tea samples (n = 11) were plotted as a function of their metabolite features, retention time and mass to charge ratio (*m*/*z*). Circles represent significantly different components with increasing circle sizes, to higher levels of significance (lower p value) between fuzhuan tea and non-fermented green tea ($p < 0.01$).

shows the *m*/*z* and retention times for what we have putatively identified as dodecanamide, linoleamide, and stearamide.

4. Discussion

Our data revealed that fermented fuzhuan tea was biologically active against the growth of Gram-negative *S. sonnei*, as well as Gram-positive *S. aureus*. These findings concur with previously published reports demonstrating antibacterial activity of a fuzhuan tea and green extracts against both Gram-negative and Gram-positive bacteria (Chan, Soh, Tie, & Law, 2011; Ling et al., 2010; Mo et al., 2008). We also report that the pattern of fuzhuan tea inhibition in the *S. sonnei* assay was dose-dependent. Green tea in this assay did not appear to inhibit growth in this manner, and was most effective at the highest concentration tested (Fig. 1A). In the *S. aureus* assay, fuzhuan tea became less bacteriostatic at higher concentrations (Fig. 1B). It is possible that when the extract was added at higher concentrations, some compounds act as growth substrates for the bacteria, thus outweighing inhibitory effects imparted by

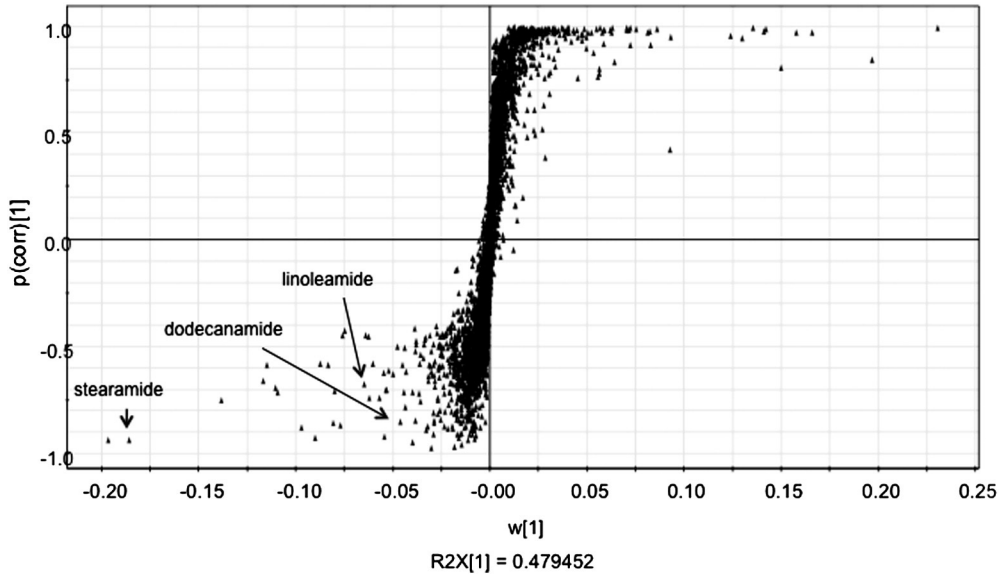

Fig. 4. Partial least squares discriminate analysis (PLS-DA) S-plot. This plot shows a binary comparison of averaged values from five samples of fuzhuan tea versus one representative sample of non-fermented green tea. Metabolite features in the bottom left quadrant indicate higher levels in the fuzhuan tea sample, and metabolite features in the upper right indicate higher levels in the green tea sample. Compounds that were tentatively identified as fatty acid amides (arrows) were significantly different between tea types as determined by Student's *t*-test ($p < 0.05$).

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Table 2
List of candidate metabolites that differ between fuzhuan tea and non-fermented green tea.

Candidate ID	Class	*m/z*	Retention time (min)	Fold-change	p value
Dodecanamide[a]	Fatty acid amide	200.207	4.89	1.59	<0.05
Glycosylated flavonoid[a]	Flavonoid	786.227	5.40	2.78	<0.01
Linoleamide[a]	Fatty acid amide	280.270	5.51	7.20	<0.05
Stearamide[a]	Fatty acid amide	284.288	7.74	1.49	<0.05
Glycosylated flavonoid[a]	Flavonoid	743.191	2.57	51.77	<0.001
Glycosylated flavonoid[a]	Flavonoid	889.248	2.58	35.54	<0.001
Epigallocatechin[a,b]	Flavonoid	307.085	1.63	1.52 (higher in green tea)	NS
Epicatechin gallate[a,b]	Flavonoid	459.091	1.73	8.14 (higher in green tea)	<0.001
Caffeine[a,b]	Alkaloid	195.088	1.85	1.07	NS

NS — not significant.
[a] Level 3 metabolite identifications (Sumner et al., 2007).
[b] Also previously reported in fuzhuan tea (Wu et al., 2010).

tea antimicrobial compounds. Fuzhuan tea's moderate activity against both a Gram-positive and Gram-negative pathogen suggests that it may contain previously unexplored, novel antimicrobial compounds and merits assessment of activity against a suite of human pathogens.

We report several flavonoids that are present in significantly higher amounts in fuzhuan tea as compared with non-fermented green tea. Flavonoids are widely investigated for a range of bioactivity, and a flavonoid-rich fraction of *Eugenia uniflora* has recently been shown to be active against sepsis in a mouse model (Rattmann et al., 2012). This report suggests that flavonoids may be responsible for the antibacterial activity reported here.

We report significantly increased amounts of fatty acid amides in the aqueous extract form of fermented fuzhuan tea compared to non-fermented green tea. Plant-derived fatty acid amides have previously showed antibacterial activity and are in early stages of investigation for potential roles in plant-microbe signaling (Kim, Chapman, & Blancaflor, 2010). This may have relevance to the antibacterial activity we describe. However, fatty acid amides are also used as slip agents in a variety of commercial applications and have been reported as common LC–MS contaminates (Cooper & Tice, 1995). We failed to observe significant differences in these compounds in a broader sample size, and therefore cannot rule out the possibility that they are contaminants introduced during handling and processing of the different types of tea.

Metabolite profiling has previously been shown to be a powerful approach for the investigation of global chemical changes in fermented foods (Ryan et al., 2011), therefore we chose this approach to explore chemical differences between fuzhuan tea and green tea. Fermented foods in general are gaining attention for nutritional and therapeutic benefits (Borresen, Henderson, Kumar, Weir, & Ryan, 2012). Here, we provide evidence that fermented fuzhuan tea significantly differs in chemical compound contents than its non-fermented counterpart. As tea is heavily consumed worldwide, these data have important implications for advancing the medicinal utility of fermented fuzhuan tea.

5. Conclusions

Our data support previous observations from traditional uses of fuzhuan tea for medicinal and prophylactic properties against enteric pathogen and diarrheal infections.

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