



## High Levels of Major Components and Antioxidant Activity of Fermented Tea Treated with *Lactococcus lactis subsp. cremoris*

Kieko Saito<sup>1,2\*</sup> and Yoriyuki Nakamura<sup>2</sup>

<sup>1</sup>School of Food and Nutritional Sciences, University of Shizuoka, Suruga, Shizuoka, 422-8526, Japan.

<sup>2</sup>Tea Science Center, University of Shizuoka, 52-1 Yada, Suruga, Shizuoka, 422-8526, Japan.

### Authors' contributions

This work was carried out in collaboration between both authors. Author KS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author YN managed the analyses of the study. Both authors read and approved the final manuscript.

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### ABSTRACT

Tea is a popular drink all over the world and has been attracting attention for its beneficial health effects. We developed a fermented tea by processing it with an exopolysaccharides-producing lactic acid bacterium, *Lactococcus lactis subsp. cremoris*, in order to manufacture high-quality tea with a physiological function. *Lactococcus lactis subsp. cremoris* was added to tea leaves (*Camellia sinensis*) and fermented for two weeks. To examine the progress of fermentation, we determined the change in pH as well as the contents of ascorbic acid and folic acid in the extract of leaves. Decreases in ascorbic and folic acids were identified, but pH only slightly changed during fermentation, showing a slower development of fermentation with lactic acid bacteria. Furthermore, we analyzed the extract's components, such as catechins, amino acids, including theanine as the major amino acid, and caffeine. Although there were some fluctuations in contents, no significant change was seen over a period of two weeks. Fermentation had no effect on the degradation of these components, suggesting that they may be relatively stable. To investigate a potential physiological function, antioxidant activity was measured using 1,1-Diphenyl-2-picryl-hydrazyl,

\*Corresponding author: E-mail: [saitok@u-shizuoka-ken.ac.jp](mailto:saitok@u-shizuoka-ken.ac.jp);

(DPPH). Consequently, the results showed that the activity of the extracts was unaffected by fermentation until the seventh day, when it began to increase. Our results suggested that the fermented tea developed in this study, which maintained its key components of catechins, theanine and caffeine, exhibit a physiological function as a processed tea and a novel food material.

**Keywords:** *Fermented tea; Camellia sinensis; lactic acid bacteria; antioxidant activity; Exopolysaccharides (EPS); catechin; theanine.*

## 1. INTRODUCTION

Tea (*Camellia sinensis*) has been regarded and used as a medicine since ancient times. Moreover, its health benefits have recently been demonstrated scientifically, and thus it is consumed worldwide [1]. Various types of teas can be manufactured as products (e.g. drink and food material) depending on how they are processed after harvesting. Green tea is non-oxidized leaves (generally called non-fermented tea), while oolong tea (generally called semi-fermented tea) is partially oxidized and black tea (generally called fermented tea) is fully oxidized by polyphenol oxidase and peroxidase in the manufacturing process. Although their characteristics and ingredients are slightly different, they are all popular and exhibit their own individual physiological function [1]. In addition to these teas, others are fermented by microorganisms. Among those, the most famous is Pu-erh tea, also known as post-fermented tea or dark tea, which originally came from Yunnan, China. In its production, it undergoes microbial fermentation with bacteria such as *Aspergillus* or *Rhizopus* [2,3]. Throughout the world, various fermented teas are made using microorganisms such as yeast, fungus or lactic acid bacteria (LAB), with each having its own characteristics [4,5]. These fermented teas should be considered as traditional foods. However, their contents are often unbalanced, and their quality is typically not uniform because they may not be made with a single microorganism, or they may be made by a method that relies on specific experience or tradition. One Japanese fermented tea, Awaban-cha, is fermented with LAB, a microorganism popular for its health benefits. It is also, however, fermented by several microorganisms in addition to the main LAB [4].

Recently, we developed a fermented tea using only an LAB that is resistant to catechins, *Lactobacillus plantarum*, which is derived from a plant. However, its components, including the catechins, decomposed during fermentation, while its antioxidant activity was maintained [5]. To develop a novel fermented tea with high

quality and health benefits, we focused on using *Lactococcus lactis subsp. Cremoris* (*L. cremoris*), which was originally isolated from yoghurt traditionally produced in Scandinavian countries [6,7]. During its growth and metabolism, *L. cremoris* produces exopolysaccharide (EPS), a water-soluble long-chain polysaccharide that exhibits physiological effects such as antibacterial, anti-mutagenic, and antitumor activity as well as immune regulation, cholesterol lowering, and regulation of gastrointestinal function [8,9]. Moreover, EPS protects cells from environmental stress through the effects of water retention, osmotic pressure resistance, and antimicrobial resistance [10]. In this study, we employ *L. cremoris* to develop fermented tea and determine its main component. In addition, its antioxidant activity is discussed.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Materials

The chemicals used in this experiment were purchased from Sigma-Aldrich, Mo., USA. Authentic reagents (Wako Pure Chemicals Industries, Ltd., Japan) were obtained to determine the concentrations of the main components of the tea using an automatic amino acid analyzer and high-performance liquid chromatography (HPLC, Agilent 1100, Agilent Technologies, Palo Alto, Calif., USA). Freshly plucked tea leaves (*Camellia sinensis* L. cv. 'Yabukita') were washed and dried at 50°C for 24 hours.

### 2.2 Production of Fermented Tea

After autoclaved, 100 g of dried tea leaves were mixed with 100 mL of distilled water (DW), and was added an LAB, *Lactococcus lactis subsp. cremoris* CF-4 ( $1.7 \times 10^8$  cells/mL) (Konno Co., Ltd., Akita, Japan). Then, the mixture was packed into an anaerobic airtight container and fermented at 25°C for 2 weeks under a shaded condition [5]. At 0, 2, 7, and 14 days, the mixture was stored at -30°C for analysis.

### 2.3 Preparation of Fermented Tea Extract

The fermented tea samples were dried using a vacuum freeze dryer (FD-80, EYLA, Tokyo, Japan), finally, the water content of the sample was less than 1%, and then milled for 30 seconds to make a powder. Next, 1 g of the powder was added to 100 mL DW, which was heated and kept at 70°C for 1 hour to make an extract. The extract was spun down, and the supernatant was collected and then filtered with a 0.4-µm membrane for use in the following experiments [5].

### 2.4 Analysis of pH, Ascorbic Acid and Folic Acid

The extract of fermented tea leaves was measured by pH meter (Horiba, Ltd., Japan). To determine ascorbic acid content, the tea extract was pretreated and applied to high-performance liquid chromatography (HPLC) adopting a silica column (4.6 i.d. x 100 mm, 5 µm, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan). The mobile phase for the detection was ethyl acetate:n-hexane:acetic acid:DW (60:40: 5:0.5) at a flow rate of 1.0 mL/min at 40°C. Each peak was identified by comparing the UV-Vis spectral characteristics at 495 nm and retention times with those of a commercial standard. A microbiological assay was conducted for analysis of folic acid using *Lactobacillus rhamnosus* ATCC 7469 [11–13]. The microbiological method was adopted from AOAC method given Official Status by AOAC (Method 992.05, 2002) and AACC (AACC Method 86-47).

### 2.5 Analysis of Catechins, Amino Acids and Caffeine

We analyzed catechins, amino acids and caffeine as previously described [5]. Briefly, Catechins and caffeine were analyzed using HPLC (Agilent 1100, Agilent Technologies, Palo Alto, Calif., USA) equipped with a C18 column (4.6 i.d. x 150 mm, 5 µm, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan). The HPLC column was maintained at 30°C in an oven. The mobile phase for the detection was 0.1 M sodium dihydrogen phosphate buffer:acetonitrile (87:13) at a flow rate of 1.0 mL/min. The reagents were purchased from Sigma-Aldrich (St. Louis, Mo., USA), and HPLC-grade reagents were used for the analysis. Each peak was identified by comparing the UV-Vis spectral characteristics and retention times with those of commercial standards. The concentration of amino acids in

the extract was analyzed using an L-8500 automatic amino acid analyzer (Hitachi Co. Ltd., Tokyo, Japan), which is a dedicated instrument for ion-exchange chromatography via the method of post-column derivatization using ninhydrin reagents that contain sodium borohydrate and propylene glycol monomethyl. The analytical column was a Hitachi HPLC Packed column (ion-exchange resin, 4.6 mm i.d., 60 mm length, 3 µm particle size). Throughout the elution program, the flow rate for buffer solutions was 0.35 mL/min. The flow rate for ninhydrin solution was 0.30 mL/min. All buffers were purchased from Wako Pure Chemicals Industries, Ltd., Japan, as a whole package. Detection was by spectrophotometry at 570 and 440 nm with the ninhydrin reaction.

### 2.6 Determination of Antioxidant Activity

The stable free radical DPPH (1,1-Diphenyl-2-picryl-hydrazyl, Sigma-Aldrich, St. Louis, MO, USA) was used to estimate the antioxidant activity of the fermented tea. 1.5-ml aliquot of DPPH solution (0.1 mM, in 95% ethanol) was mixed with 100 µL of tea extract. Standard green tea extract (*Camellia sinensis* L. cv. 'Yabukita') was used as a control. The mixture was shaken vigorously and left to stand for 20 min at room temperature. The absorbance at 517 nm of the DPPH solution was measured using a spectrophotometer (Bio-Spec, Shimadzu, Kyoto, Japan). The antioxidant activity was determined as DPPH radical scavenging activity, which was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1 - (\text{absorbance of sample} / \text{absorbance of control})] \times 100$$

### 2.7 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (ANOVA).

## 3. RESULTS AND DISCUSSION

### 3.1 Fermented State

Generally, the progress of fermentation by an LAB involves a significant decrease in pH due to the production of lactic acid [14]. In this study, the pH did not dramatically decrease over 14 days as shown Fig. 1. However, through typical fermentation processes the amount of ascorbic acid has dramatically decreased [15,16]. Since it

was not reported that *L. cremoris* consumes ascorbic acid to grow, the result showed that the fermentation might proceed without extreme changes in pH. In addition to the ascorbic acid degradation, folic acid clearly decreased for 14 days in Table 1. Most LABs consume folic acid as a growth factor. Therefore, a decrease in folic acid indicates the growth of LAB.

The results in Table 1 showed that the fermentation with *L. cremoris* progressed slowly, at least for 14 days. Mild fermentation may involve a property of EPS, which is the product of *L. cremoris*. It has been reported that the EPS production is related to fermentation conditions (pH, temperature, etc.) and it also depends on bacteria [17]. The pH of yogurt prepared with *L. cremoris* is moderate, leading to a soft taste [18]. Again, EPS has very good characteristics that are useful for LAB as well as human health [19]. The function of *L. cremoris* with EPS requires further study, but *L. cremoris* may exhibit a unique action during fermentation.

### 3.2 Stability of Main Components

Analysis of the important components of the extract such as caffeine, catechins, and amino acids, including theanine, were performed using samples on 2, 7, and 14 days (Fig. 1). These components exhibit excellent taste in palatability as well as having health benefits. Among these components, catechins account for more than 10% in tea leaves, which gives tea its astringency, theanine is around 2%, which provides a delicious taste (called *umami*), and caffeine is around 3%, which supplies bitterness.

Since catechins are the most abundant component in tea leaves, they play an important role with their many physiological functions [1]. The main catechins are (-) Epigallocatechingallate (EGCG), (-) Epigallocatechin (EGC), (-) Galliccatechin (GC), (-) Galliccatechingallate (GCG), (-) Epicatechingallate (ECG), (-) Epicatechin (EC), (+) Catechin (C), and (-) Catechingallate (CG). In addition, there are other various small amounts of catechin derivatives. EGCG is found in the highest amounts in tea leaves. Fig. 1 indicated the change in each catechin content during fermentation for 14 days. These catechins decreased slightly on day 2 and increased on day 7, but no significant change was seen on day 14. Previous studies have reported that catechin degrades to a smaller molecule by fermentation [5,20–22]. In particular, it has been reported that

within a catechin's structure, the gallate groups were most easily eliminated [23]. EGCG and ECG decompose into EGC and EC, respectively, in the early stages of fermentation. Consequently, the composition ratio of each catechin changed; for example, the amount of EC was greater than that of EGCG. However, in this study, no obvious degradation was observed in any catechin, and, moreover, there was no dramatic decrease in EGCG, which was the most likely to decompose. Fig. 2.(A) showed the proportion of each catechin remained almost the same for 14 days, indicating that the catechins contained in the extract are extremely stable. The increase in EGCG on day 7 seems to be due to other factors, and further investigation is needed; however, EGCG was maintained in the content without any reduction in fermentation for 14 days, showing that fermented tea may exhibit a similar physiological function to that of standard green tea. EGCG is the most powerful molecule in tea catechins, and it exhibits antimutagenic, anticancer, antiarteriosclerotic, antibacterial, and antiatherosclerotic effects [1]. Consequently, fermented tea rich in EGCG appears to be beneficial for human health.

Tea contains a large amount of amino acids that express themselves in delicious flavor, contributing significantly to the good taste of tea. The amount of amino acids fluctuated slightly, but not dramatically, over 14 days, showing that each amino acid was relatively stable under fermentation [Fig. 1.(B)]. In addition, the proportion of each amino acid remained nearly the same over the 14 days [Fig. 2.(B)]. In particular, theanine, consisting of more than 50% of the amino acids in tea leaves, was quite stable. Theanine ( $\gamma$ -ethylamide-L-glutamic acid) is an extremely rare amino acid in nature, and it has psychoactive properties because it is readily absorbed and permeates the blood-brain barrier to function in the brain [24]. This function leads to reduced mental and physical stress and improved cognition [25–29]. These results suggest that this fermented tea has the effect of improving brain function.

Caffeine did not significantly change except for a slight increase on day 7, thus keeping relatively stable for 14 days as shown in Fig. 1(C). This stability of caffeine was consistent with the results of previous studies [5]. While caffeine has some side effects, it was reported to enhance the physiological function of catechins through synergistic effects [30,31] and to improve cognition and boost one's mood in combination

with theanine [32]. The caffeine contained in this fermented tea might play a similar role to that of standard green tea in supplying a physiological function.

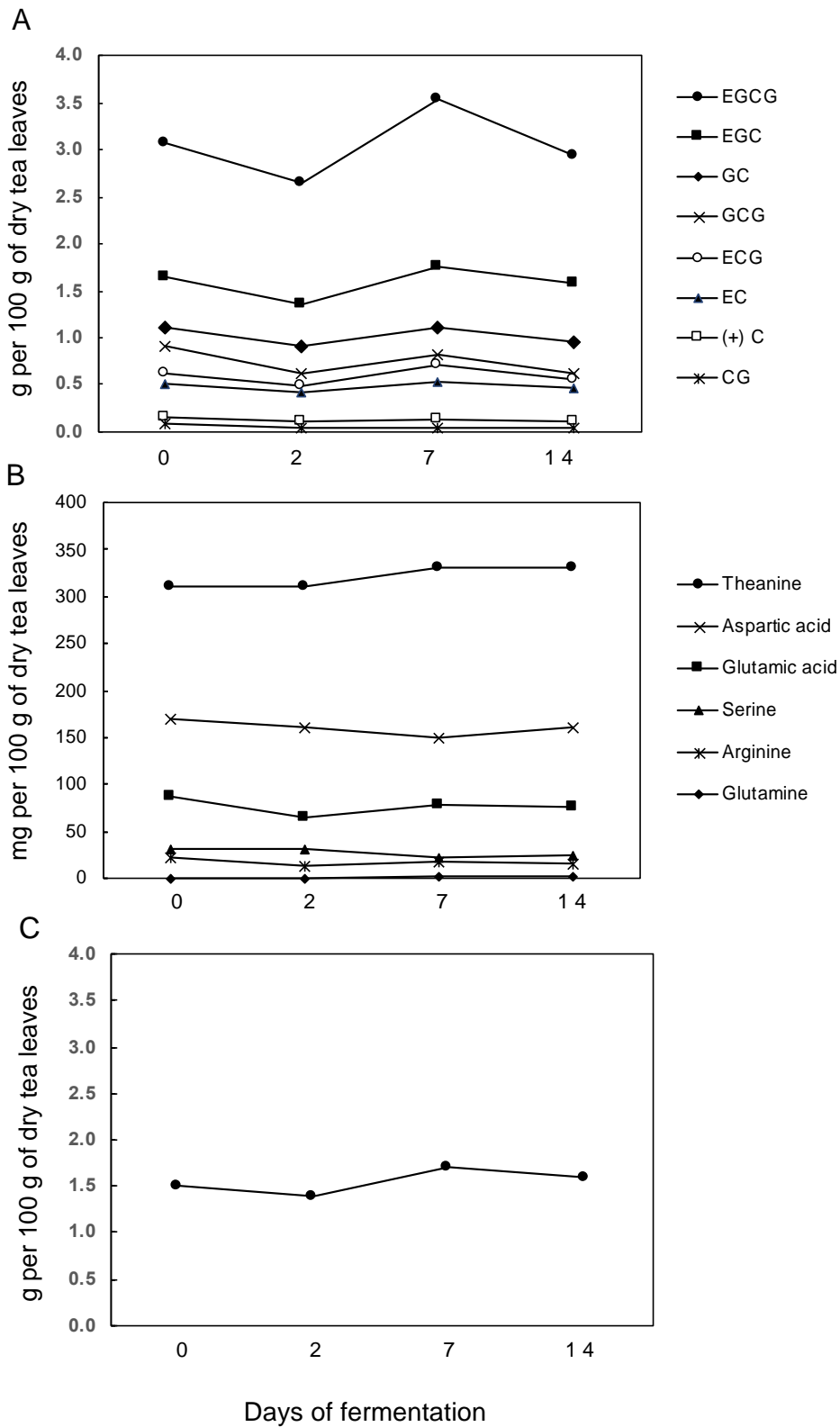
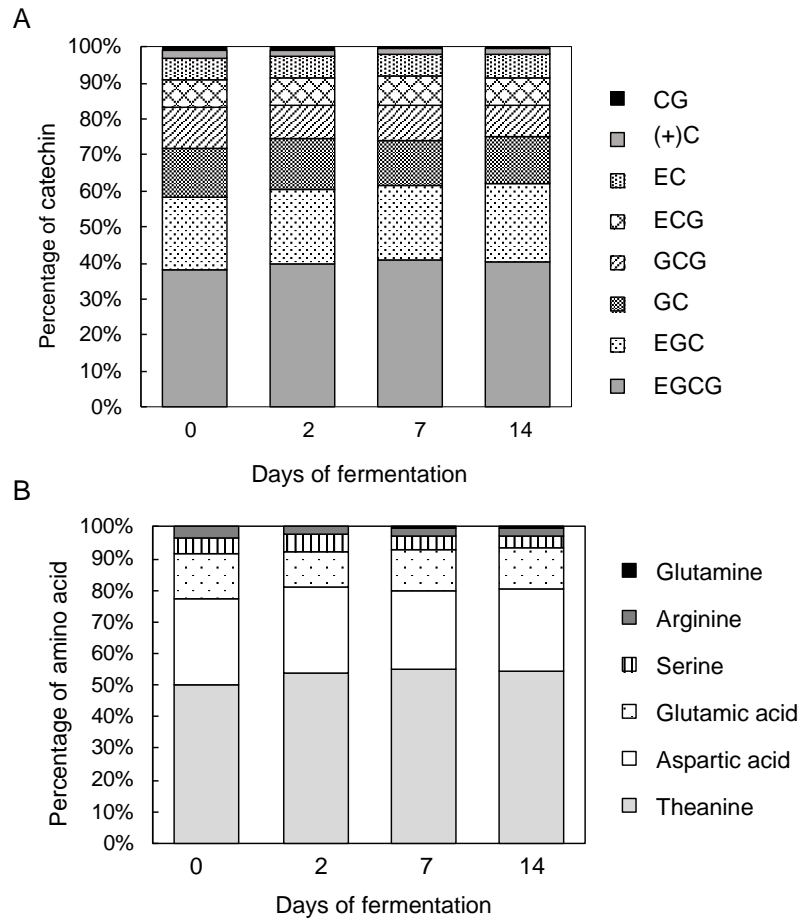
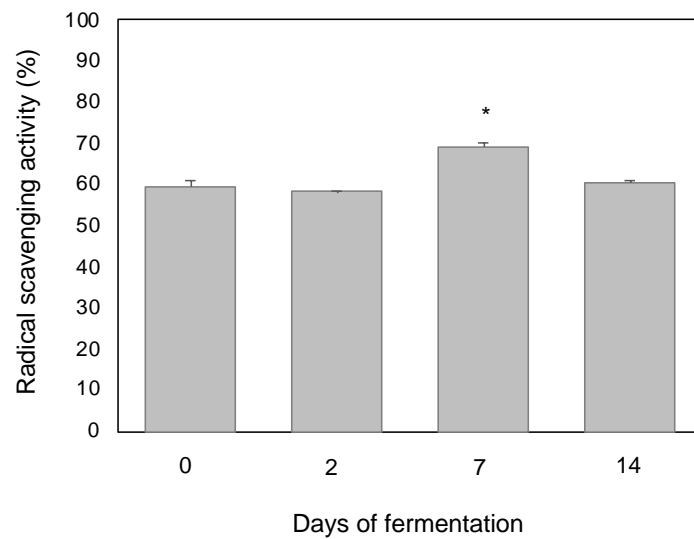


Fig. 1. Changes in catechin, amino acids and caffeine in the extract of fermented tea during fermentation: (A) Catechin, (B) Amino acids, (C) Caffeine



**Fig. 2. Changes in the proportion of catechins and amino acids in the extract of fermented tea during fermentation: (A) Catechins, (B) Amino acids**



**Fig. 3. Change in antioxidant activity of the fermented tea during fermentation. The antioxidant activity was determined as DPPH radical scavenging activity of the tea extract. Data shown are mean±SEM (n=3), \*p<0.05**

**Table 1. Change in pH, ascorbic acid and folic acid contents during fermentation**

	Days of fermentation			
	0	2	7	14
pH	5.48	5.48	5.45	5.43
Total ascorbic acid (mg/100 g)	33	11	4	2
Folic acid (mg/100 g)	0.6	0.46	0.41	0.36

### 3.3 Effect of Fermentation on Antioxidant Activity of the Fermented Tea

We determined the antioxidant activity of the fermented tea's extract using a DPPH method that was accurate and convenient (Fig. 3). Consequently, this activity did not decrease during fermentation. On the contrary, a significant increase was seen on day 7. It is well known that catechins exhibit a strong antioxidant activity. The catechins were not decomposed by fermentation, including the most effective molecule, EGCG, which also increased on day 7 [Fig. 1.(A)]. The antioxidant activity on day 7 may be deeply involved in the performance of EGCG. There was no dramatic decrease in pH for 14 days (Table 1), which may lead to stable antioxidant activity because, generally, antioxidant activity is inhibited by lower pH [33]. Although the mechanism of EGCG contributing to the increase on day 7 is unclear, this phenomenon is definitely interesting for the antioxidant activity of fermented tea. *L. cremoris* could play an important role in increasing other antioxidants and suppressing degradation, since microbial fermentation has provided special qualities and special active compounds that possess powerful antioxidant activities [34,35]. In this study, we employed a characteristic LAB, *L. cremoris*, that produces EPS and that might affect a antioxidant activity. Since there is no report on the effect of EPS derived from LAB on fermented tea but only on yogurt, further research is needed to identify the mechanism that activates antioxidant or the relevant effective molecules.

### 4. CONCLUSION

We developed fermented tea using *L. cremoris*, a unique lactic acid bacterium that produces EPS. This tea's fermentation progressed slowly. Its main components, i.e. catechins, theanine, and caffeine, were extremely stable and did not dramatically degrade over 14 days. The tea's antioxidant activity was also maintained stably, and a significant increase in antioxidant activity was seen on day 7 of fermentation.

This fermented tea prepared with *L. cremoris* may have many unique functions and multiple beneficial effects on human health, in addition to the function provided by standard tea leaves.

### CONSENT

It is not applicable.

### ETHICAL APPROVAL

It is not applicable.

### ACKNOWLEDGEMENTS

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### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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