

# Microbial composition of four kinds of tea with different degree of oxidation

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Master Thesis

Food Technology and Nutrition



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## Popular science summary

Nowadays, tea is becoming more and more popular worldwide due to its attractive taste and health benefits on the human body. There is a variety of tea which can be mainly divided into four types depending on different degrees of oxidation. Generally speaking, green tea has the lowest degree of oxidation while Light oolong and Dark oolong are semi-oxidized with degrees of oxidation ranging from 10 % to 29 %, 60 % to 70 % respectively. Dark tea is completely oxidized with 100 % oxidation degree. The chemical components of tea such as phenolic substances have been well explored and studied. Nevertheless, a very few studies have been performed focusing on the microbial composition of tea as well as its health effects. Therefore the analysis of microbial composition of different kinds of tea has been performed.

In this study, four representative kinds of tea have been studied: Tie guan yin, Light oolong tea, Dark oolong and Pu-erh (oxidation degree from low to high). Firstly, the microbial contents of four kinds of tea were analyzed under normal condition (without brewing). Fully oxidized Pu-erh tea is the only tea that has undergone the fermentation process and has the highest bacterial counts as well as the most diverse bacterial composition, whereas Dark oolong rarely showed any microbial content. Most of the identified bacteria belonging to the family *Bacillaceae*, *Staphylococcaceae* and *Paenibacillaceae* are soil-dwelling (naturally occur in soil) bacteria or a part of skin flora. After that, four kinds of tea were brewed at 90 °C and the microbial analysis indicated that no survived microorganisms could be detected in the brewed tea except for Pu-erh. More surprisingly, a potential probiotic *Bacillus coagulans* was found as the major bacteria in the brewed Pu-erh tea. Some of these *Bacillus coagulans* isolates were brewed directly with hot water. The result showed that *Bacillus coagulans* could not survive at 90 °C without the protection of Pu-erh tea leaves. These isolates were also analyzed using RAPD (Random Amplified Polymorphic DNA) method. The result showed that most of them have different band pattern. Nevertheless, further investigations regarding strain type and characteristic of these *Bacillus coagulans* isolates are required.

In conclusion, the fermented Pu-erh tea can provide beneficial bacteria (*Bacillus coagulans*) in addition to polyphenols to human body.

## **Abstract**

In this study, four representative kinds of tea have been studied: Tie guan yin, Light oolong tea, Dark oolong and Pu-erh (oxidation degree from low to high). The microbial contents of four kinds of tea were analyzed under normal condition (without brewing) and after brewing. Fully oxidized Pu-erh tea is the only tea that has undergone the fermentation process and has the highest bacterial counts as well as the most diverse bacterial composition, whereas Dark oolong rarely showed any microbial content. Most of the identified bacteria belonging to the family *Bacillaceae*, *Staphylococcaceae* and *Paenibacillaceae* are soil-dwelling (naturally occur in soil) bacteria or a part of skin flora. The microbial analysis of four kinds of tea brewed at 90 °C indicated that no survived microorganisms could be detected in the brewed tea except for Pu-erh. Moreover, a potential probiotic *Bacillus coagulans* was found as the major bacteria in the brewed Pu-erh tea. Some of *Bacillus coagulans* isolates were brewed directly with hot water. The result showed that *Bacillus coagulans* could not survive at 90 °C without the protection of Pu-erh tea leaves. These isolates were also analyzed using RAPD (Random Amplified Polymorphic DNA) method. The result showed that most of them are different strains.

**Keywords:** Tea, oxidation degree, microbial composition, fermentation, brewing, probiotics, *Bacillus coagulans*

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# 1. Introduction

Tea is one of the most widely consumed beverages all over the world. It plays an important role in the world's history. Tea was first used and treated as a herbal medicine in southwestern China (mainly in the Yunnan province) and consumed only for medical purposes (Heiss and Heiss, 2011). After that, tea was becoming more and more popular as a recreational beverage in China due to its unique aroma and good taste while it was introduced and spread to western countries during the 16<sup>th</sup> century (Weinberg, 2001). It was demonstrated by *Alan Macfarlane* in 2004 that the consumption of tea in the world equals to the combination of all other drinks including soft drink, coffee and alcohol (Macfarlane, 2004). It has also been proven by a lot of studies that tea has a variety of health benefits and functions on the human body (Weinberg and Bealer, 2001; Mo, *et al.*, 2010). The most significant ones are antimicrobial and antiviral activity, antioxidants ability and anticancer function (Chen, *et al.*, 2010). Tea is so popular not only because of its aroma and good taste, but also because of the health benefits on the human body. Nowadays no other drinks could occupy tea's place in the aspect of culture, market, function and taste.

A traditional tea is made of cured leafs of the evergreen tea plant *Camellia sinensis* and it can be mainly divided into four types depending on the processing method. There are white tea, green tea, oolong tea, black tea and dark tea (post-fermented tea) respectively. Generally, white tea and green tea are barely oxidized; oolong tea is oxidized partially whereas black tea is completely oxidized. Post-fermented tea, also known as dark tea, is fermented by specific microorganisms (Liu, 2005). The chemical components of tea such as phenolic substances have been very well studied recently. Nevertheless, a very few studies have been performed focusing on the microbial component of tea as well as its health effects. Therefore, this study was aimed to analyze the microbial component of different kinds of tea (green tea, oolong tea, Pu-er tea). Since tea is usually prepared by brewing with hot water (around 90 °C) or even boiling water (100 °C), a large amount of microorganisms will likely be killed by this procedure. The possibility of finding surviving beneficial bacteria in brewed tea is also of interest. Additionally, the influence of household tea-making method (brewing with hot water) on bacterial component of tea was also investigated in this study.

## 2. Background

### 2.1 Tea processing

As mentioned above a traditional tea can be divided into many different categories depending on the way it is processed. The tea that has undergone different process differs a lot in regards to taste, aroma, content of nutrients and microbial composition.

As tea processing method has such a significant impact on its qualities and properties, a great care has to be taken on every step during the production. Although a majority of tea manufacturers, especially some traditional Chinese tea manufacturers, produce tea by their own experience (Abe, *et al.*, 2008), different manufacturer as well as different type of tea still shares a highly similar processing system including several irreplaceable steps with very small variations (Li and Ling, 2007).

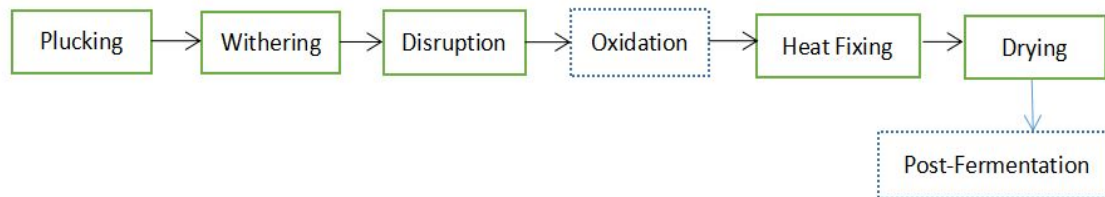


Figure 1. Basic steps of tea processing, dotted line indicates additional steps

As can be seen in Figure 1, plucking is the initial step of the processing. Generally, plucking can be done by either hand or machine but a high quality tea usually requires manual-picking as it reduces the maximal amount of bad leaves and tea sprouts (Ravichandran and Parthiban, 1998). After plucking, the fresh tea leaves will be withered by sun exposure or by storage in a cool breezy place. Redundant water in the tea leaves is removed by withering. This step is also considered as the start of oxidation since the tea leaves are slightly oxidized by catalysis of enzyme (Li and Ling, 2007). Disruption is a step where the tea leaves are bruising, rolling and crushing with a machine (Varnam and Sutherland, 1994). This will cause breakage of tea leaf cells which enables the contact between oxidative enzymes and various phytochemical constituents, e.g. polyphenol oxidase and polyphenol, thus resulting in enzymatic oxidation (Selena, *et al.*, 2010). The amount of oxidation will be enlarged significantly by adapting a suitable temperature and moisture to the disrupted tea leaves. The catechins are oxidized to complex tannins, theaflavins and thearubigins which contribute to the dark color as well as the unique taste and aroma of tea (Liang, *et al.*, 2003). Thus, the degree of oxidation is in direct relation to the sensory quality of tea. Generally speaking, with a higher degree of oxidation, the color of tea will be darker and the taste will be mellower. However, not all types of tea require oxidation in their production. For white and green tea, oxidation is actually undesired so that the degree of oxidation needs to be kept in a minimal range. This is done by heat fixing which is usually accomplished by frying tea leaves in a pan or a wok. The heat can reduce the moisture level and inactivate oxidative enzymes so that the oxidation process can be stopped (Selena, *et al.*, 2010). Afterwards, the tea leaves are sun-dried or baked before being packed for sale on the market.

Post-fermentation is an essential process for fermented tea. After heat fixation of tea

leaves, selected microorganisms such as mold is artificially introduced to the tea and *Aspergillus sp.* is indicated to be the main microbe in this process (Selena, *et al.*, 2010). Meanwhile, humidity and temperature is carefully controlled and adjusted to the optimal level for the fermentation. The microorganisms will catalyze exo-oxidation in tea leaves which can oxidize phenolic substances more thoroughly than the oxidation catalyzed by enzymes (Xie, *et al.*, 2009). This is also referred to the secondary oxidation which produces various fermentation-derived compounds that contributes to the deep color and distinctive taste of the fermented tea (Selena, *et al.*, 2010). The fermentation process can last from several months to a few years. It is also believed that the longer the fermentation lasts, the better the quality of fermented tea will be.

Recent research has pointed out that fermented tea has several unique qualities and functions e.g. anti-obesity effect and cholesterol-lowering effect, contributed by specific microorganisms other than unfermented tea (Mo, *et al.*, 2008; Kuo, *et al.*, 2005). However, the microorganism composition of both unfermented tea and fermented tea has not been well explored. The potential of unidentified microorganisms existing could also affect the quality of tea. In this study, four representative kinds of tea have been studied: green tea (Tie guan yin), Light oolong tea, Dark oolong and dark tea (Pu-erh) respectively.



Figure 2. Four types of tea with different degree of oxidation from low to high. A: Tie guan yin B: Light oolong tea C: Dark oolong tea D: Pu-erh tea. As can be seen in the figure the color of tea is becoming darker with the increased degree of oxidation.



## 2.2 Characteristics of tea

### 2.2.1 Green tea

Green tea is the most well-known unfermented tea famous for its unique refreshing aroma and taste as well as its health effects. It has the lowest oxidation level since the heat fixing is applied as soon as the tea leaves are picked and disrupted. Because of this, the chemical contents e.g. polyphenols, amino acids, vitamins of fresh tea leaves are retained (Graham, 1992). Catechins are considered as the major compounds that contribute to health beneficial effects of green tea. Numerous researches have demonstrated that tea catechins have various functions such as antioxidant, antibacterial, antiviral and anticancer activities (Clement, 2009). The green tea extracts are the component of the first admitted botanical drug in the United States approved by the Food and Drug Administration (Chen, *et al.*, 2008).

There are a lot of studies about the antibacterial effects of catechins. Catechins have a strong effect on inhibiting toxins production and activity of certain pathogens. For instance, the hemolytic activity of the pathogenic toxin thermostable direct hemolysin can be inhibited by catechins (Sachie, *et al.*, 1989). Moreover, the production of vero cytotoxin by *Escherichia coli* O157:H7 can be completely inhibited with a relatively low level of catechins (Yukiko and Akiko, 2005). It has also been reported that catechins can inhibit the growth and viability of spore-forming bacteria e.g. *Clostridium* and *Bacillus* (Yukiko and Mayumi, 1989). In Yukihiko Hara-Kudo's study, the number of *Clostridium botulinum* spores decreased after being incubated with catechins in the media. Therefore, the risk of having certain pathogenic bacteria in green tea is low. It is also worth mentioning that catechins have no significant impact on or will even slightly improve the growth of some health beneficial bacteria e.g. *Lactobacillus spp.*, *Bifidobacterium spp.* and *Eubacterium rectale* (Xin, *et al.*, 2013). However, as green tea has not undergone any microbial fermentation, the microbial composition may be less diverse.

### 2.2.2 Oolong tea

Oolong tea is an unfermented semi-oxidized tea originating from southern China. The oxidation period of oolong tea is relatively short (from hours to several days) and the oxidation is stopped at a certain degree depending on the qualities of tea desired (Nabarun, *et al.*, 2007). The oxidation can be terminated by heat fixing procedure as described in section 2.1. Generally, the degree of oxidation in Light oolong tea can be somewhere between 10 % and 29 % while in Dark oolong this can range from 60 % to 70 % (Zhen, 2003).

The catechin contents of different type of tea can be seen in Table 1. Unlike green tea, catechins in oolong tea have been partially enzymatically oxidized to polymeric compounds e.g. theaflavins and thearubigins by polyphenol oxidase and peroxidase (Kim, *et al.*, 2013). These two secondary polyphenols in oolong tea synergistically contribute to the brown color as well as the astringent but rich taste

(Weerawatanakorna, *et al.*, 2015). Additionally, there is increasing evidence shows that theaflavins and thearubigins have both both *in vivo* and *in vitro* antibacterial activity (Bandyopadhyay, 2005). It has been reported that theaflavin-3,3'-digallate is capable to inhibit the growth of pathogenic fungus *Candida albicans* and *Cryptococcus neoformans* (Koech, *et al.*, 2014). The synergistic activity of different tea polyphenols including catechins, theaflavins and thearubigins against certain pathogens has been proven recently (Koech, *et al.*, 2014). It is also believed that the variety of tea polyphenols improves antifungal and antimicrobial activities synergistically. Since oolong tea contains a mixture of polyphenols and oxidation-derived phenolic substances, it is suspected to have stronger antimicrobial activity than other teas (Hu, *et al.*, 2002).

Table 1. The different type of tea and catechins contents (Weerawatanakorna, 2015)

Types of tea	Process	Total catechins (% w/w)
Pu-erh tea	Microbial fermentation	6.07 ± 0.18
Dark oolong tea	Partial oxidation	7.49 ± 0.22
Light oolong tea	Partial oxidation	8.05 ± 0.18
Green tea	Less oxidation	14.57 ± 1.08

### 2.2.3 Pu-erh tea

Pu-erh tea is the most representative microbial fermented tea mainly produced in the Yunnan province of China (Abe, *et al.*, 2008). The health effects of Pu-erh tea such as anti-obesity, anti-oxidation and anti-allergy have been well studied (Abe, *et al.*, 2008). Recently the microbial fermented tea has drawn more and more attention as it is believed that microorganisms in tea can provide more unique functions other than tea polyphenols (Xiao, *et al.*, 2015).

The microorganisms involved in tea fermentation mainly include fungus and yeasts e.g. *Aspergillus niger*, *Aspergillus glaucus*, *Saccharomyces spp.*, among which the *Aspergillus niger* is dominating. Moreover, a slight amount of *Bacillus spp.* has been involved in the fermentation (Chen, 2012). *Aspergillus niger* is essential in the developing of functional compounds as well as some volatile compounds in Pu-erh tea (Abe, *et al.*, 2008). Polyphenols such as catechins are microbially oxidized thus Pu-erh tea has the least amount of catechins as shown in Table 1. However, the mechanisms of microorganisms in tea fermentation have still not been very well explored as Pu-erh tea is manufactured in empirical procedures and the fermentation

is carried out in a natural way (Abe, *et al.*, 2008; Xiao, *et al.*, 2015).

### 2.3 *Bacillus coagulans*

*Bacillus coagulans* is a gram-positive species belonging to the genus *Bacillus* which has been surprisingly found in the Pu-erh tea. *Bacillus coagulans* was first indicated as *Lactobacillus sporogenes* because of its features of *Lactobacillus* such as forming lactic acid. Nevertheless, it was eventually decided as *Bacillus coagulans* in the genus *Bacillus* since it shows characteristics of *Bacillus* such as spores-bearing ability (Hartemink, 2007). *Bacillus coagulans* is usually considered and used as a probiotic. It has also been qualified by the European Food Safety Authority in the QPS list (Qualified Presumption of Safety) and approved by the United States Food and Drug Administration as GRAS (Generally Recognized As Safe) bacterium (EFSA, 2015; U.S. Food and Drug Administration, 2015).

There are many studies indicating the health effects of *Bacillus coagulans*. Among them, the effect of lowering total serum cholesterol and the prevention of diarrhea have been tested and proven on human studies (Sanders, *et al.*, 2003; La Rosa, *et al.*, 2003). Other effects such as improvement of gastrointestinal and vaginal microflora and enhancement of immune response to virus have also been demonstrated. Despite of this, *Bacillus coagulans* has not been widely applied on human food or medicinal products as the data of clinical tests is not abundant (Vecchi and Drago, 2006).

## 3. Methods and Materials

### 3.1 Preparations

Essential materials (media or buffer) for microbial analysis of tea are prepared before the experiment:

- Bacteriological peptone water (Recipe can be found in Appendix)
- Tryptic soy agar (TSA) (Fluka Analytical, SIGMA-ALDRICH, Spain)
- Violet red bile dextrose agar (VRBD) (Fluka Analytical, SIGMA-ALDRICH, Spain)
- MRS agar (Merck, Merck kGaA, Germany)
- Malt extract agar (Scharlau, Scharlab. S. L, Spain)
- Malt extract broth (Fluka Analytical, SIGMA-ALDRICH, France)

- Rogosa agar (Millipore, Merck, Germany)
- Tryptic soy broth (TSB) (Fluka Analytical, SIGMA-ALDRICH, India)
- MRS broth (Merck, Merck KGaA, Germany)
- Hogness freezing media (Recipe can be found in Appendix)
- Master mix (Recipe can be found in Appendix)

### 3.2 Microbial analysis of tea

In this study, four kinds of tea with different degree of oxidation: Tie guan yin, Light oolong, Dark oolong and Pu-erh were analyzed regarding colony count and microbial composition. Firstly, tea was homogenized with bacteriological peptone water at room temperature and cultured on agar media for further bacterial isolation. Then the brewing method had been carried out; tea was brewed with 90 °C water before being cultured on agar media. Furthermore, randomly isolated bacteria samples originated from the brewed tea were heat treated by 70 °C and 90 °C water. The survivability of isolated bacteria samples at certain temperatures were checked by seeing the reduction regarding bacterial counts after heat treatment.

Six samples were taken for both the “before brewing tea” and “brewed tea” sessions. Duplicates were made when culturing samples on agar media.

#### 3.2.1 Culturing before brewing tea

Tie guan yin, Light oolong, Dark oolong and Pu-erh were purchased in a local tea store in Sweden. 10 g of each kind of tea was sampled by a sterilized spoon and mixed with 90 ml bacteriological peptone solution in a stomacher bag and stored in the cold room (4 °C) for 1 hour. All the samples were then put in the ultrasonic bath (Millipore, US) for 5 min followed by a homogenization process carried out in a stomacher (Seward, Stomacher 400, United Kingdom) at high speed for 2 min. 1 ml of the solution of each sample was diluted serially from -1 to -3 with 9 ml bacteriological peptone solution tubes. After that, the diluted samples were spread and cultured on five different media: TSA, MRS, VRBD, Rogosa and malt. They were further incubated under the specific condition as shown in Table 2 before being taken out for colony count.

Table 2. Temperature and time of incubation for different media

Tryptic soy agar (TSA)	30 °C	3 days
MRS agar	37 °C	3 days
Rogosa agar	37 °C	3 days
malt extract agar	15 °C	7 days
Violet red bile dextrose agar (VRBD)	37 °C	1 day

After colony counting, two random colonies from each plate were picked and isolated on new plates with corresponding media for the purpose of purification. Pure single colonies were picked from isolated plates and incubated in Tryptic soy broth (TSB) tubes (for bacteria from TSA and VRBD), MRS broth tubes (for bacteria from MRS and Rogosa) and malt extract broth tubes (for bacteria from malt) with corresponding time and temperature. Then all the incubated samples were centrifuged at 6000 rpm for 5 minutes in a centrifuge machine (Centrifuge 5804, Eppendorf, Germany) and the supernatant from each sample was poured off. The remaining bacteria mass was then mixed with Hogness freezing media by using a vortex machine (Vortex-Genie 2, Scientific Industries, USA) before being stored in a -80 °C freezer for later use.

### 3.2.2 New Batch

A new batch of teas including Tie guan yin, Light oolong and Dark oolong were purchased from the same tea store where the old batch were bought and the same experiment method described in section 2.1 had been carried out on the new batch.

### 3.2.3 Tea Brewing

In this part, four kinds of tea Tie guan yin, Light oolong, Dark oolong (from the old batch) and Pu-erh were brewed. 10 g of each tea were taken and brewed with 300 ml autoclaved sterilized tap water in a beaker at 90 °C for 2 minutes (followed the instruction on the tea bag). After 2 minutes, half of the tea water (150 ml) was separated from the tea leaves by pouring it to another beaker. Both tea water with leaves inside and tea water without leaves inside were cooled down to room temperature before being spread and plated on different media (TSA, MRS, VRBD, Rogosa and malt). After being incubated for a certain time, two random colonies on each plate were picked then purified on new plates. The following step was incubating pure bacteria in broth and preserving them with Hogness freezing media in -80 °C freezer

as described above.

### 3.2.4 Back brewing

In this experiment, the bacteria originated from Rogosa plates of the previous experiments (section 3.2.3) had been analyzed. All the isolated bacteria samples that have been preserved in the -80 °C freezer were thawed first. Then 10 µl loops were used in order to transfer bacteria to MRS broth. Each isolated bacteria sample was transferred into three MRS broth tubes, incubating at 37 °C for 2 days. Afterwards, all tubes were centrifuged for 5 minutes at 6000 rpm in a centrifuge machine (Centrifuge 5804, Eppendorf, Germany) and the supernatant of each sample was poured off. The three tubes of each isolated bacteria sample had been divided into three different groups: the first group was brewed with sterilized tap water at 90 °C while the second group was treated in the same way but the temperature was 70 °C. For the third one room temperature sterilized tap water was added as a negative control. The first and second group were vortexed directly after addition of water with specific temperature, holding for 1 minute before being cooled down to room temperature by using ice. The cooled samples were then spread on MRS plates for incubation. The diluted samples were spread on MRS plates for incubation. The colonies were counted after three days' incubation.

## 3.3 Sequencing of bacteria

### 3.3.1 DNA Extraction

The bacterial isolates were taken out from the freezer and thawed. Each sample was spread on its corresponding media where the bacteria originated from. After being incubated for a certain time, the bacteria mass on the plate was collected by using a 10 µl loop. Half a loop of pure bacteria was taken and mixed with 1 ml autoclave sterilized Milli-Q water (Millipore, Milli-Q water system, France) in a 1.5 ml PCR tube. Then 8-10 sterilized small glass beads were added to each tube and afterwards all the tubes were put into a shaker (Mixer 5432, Eppendorf, Germany) for 30 minutes' shaking. After that, all the DNA extracted samples were stored at 4 °C until further use.

### 3.3.2 Polymerase chain reaction (PCR)

For polymerase chain reaction, the master mix solution was made. Nucleic-free water was added first, followed by an addition of PCR buffer (Qiagen, Denmark) and dNTP

mix. After that, two primers: the forward primer ENV1 (5'-AGA GTT TGA TII TGG CTC AG -'3) and the reverse ENV2 (5'-CGG ITA CCT TGT TAC GAC TT -3') were added to the solution. The last added reagent was Taq polymerase (Qiagen, Denmark). The recipe of the master mix solution of PCR can be found in Appendix in details.

The DNA extracted samples were centrifuged at 14800 rpm for 1minute in a small centrifuge machine (Heraeus Pico 21 Centrifuge, Thermo Scientific, Waltham, USA). The supernatant was taken and mixed with the master mix solution before being put in the PCR machine (Mastercycler gradient, Eppendorf, Germany) and run with program TOPTAQ25. A negative control was also included, made by using nucleic-free water instead of the DNA sample supernatant to mix with the master mix solution. All PCR samples were stored at 4 °C until further use.

### 3.3.3 Random Amplified Polymorphic DNA (RAPD)

Twelve bacterial isolates originated from the after brewing Pu-erh tea were chosen for RAPD analysis. Unlike polymerase chain reaction, the primer used in the master mix solution of RAPD was P73 (5'-ACG CGC CCT-3'). The procedures of RAPD were basically the same as PCR except that RAPD products had undergone Program FILE 1 in RAPD machine. The recipe of the master mix solution of RAPD can be found in Appendix in details.

### 3.3.4 Gel electrophoresis

The gel electrophoresis was performed to both PCR and RAPD products. The agarose gel was made by mixing agarose pure grade powder (Electran, VWR, Belgium) with TAE buffer (Bio-Rad, USA). The PCR or RAPD products were mixed with 6X orange DNA loading dye (QIAGEN GmbH, Hilden, Germany) before being loaded in the agarose gel. Then, the gel electrophoresis was run at 120V for 60 minutes driven by a power source (Bio-Rad, Power Pac 300, USA). Afterwards, the gel was dyed in Gelred (Biotium, Hayward, USA) solution for 20 minutes to get stained. Finally, the stained gel was observed and analyzed in an UV chamber (UV transilluminator, UVP, US).

### 3.3.5 Data analysis

The PCR samples were sent to Eurofins Genomics (Ebersberg, Germany) for further analysis of sequences. The outcomes were edited using a software BioEdit (Ibis Biosciences, US). All edited data were then uploaded on BLAST website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for identification.

### 3.4 Statistical Analysis

All the bacterial count results were transformed into log CFU/g and presented as mean values of duplicated samples. The Mann-Whitney test was implemented in order to test a statistically significant difference between two different samples. The software Sigmaplot 13.0 (Systat Software Inc., US) was used to carry out the test. The Mann-Whitney test is also indicated as “Rank Sum Test” which is a nonparametric procedure that does not assume normality. In other words, the normality test will fail among all comparisons since the sample size (6) in this study is too small to assume normality. A p-value of 0.05 was considered statistically significant change.

## 4. Results

### 4.1 Before brewing tea

The bacterial count results (log CFU/g) of before brewing Tie guan yin, Light oolong, Dark oolong and Pu-erh on TSA, MRS, Rogosa and malt media are shown in Table 3. As can be seen, Pu-erh has the highest bacterial counts on all media while Dark oolong has the lowest bacterial counts with the same value (1.00 log CFU/g) on all media. Similarly, Table 4 shows the bacterial count results (log CFU/g) of new batch of tea including Tie guan yin, Light oolong and Dark oolong on the same media as described above. However, no colony could be detected on VRBD media for all types of tea. Thus no result of VRBD media is shown or compared in this section. The count results of specific experimental trials can be found in Table in Appendix.

#### 4.1.1 Tryptic soy agar (TSA)

The pairwise comparisons of the bacterial counts between each type of tea on the same medium have been implemented and the results are represented in plot figures. More specifically, four kinds of tea from the old batch have been compared with each other in pairs whereas three kinds of tea from the new batch have also been compared in the same way. Last but not least, the comparisons between the same type of tea from both old and new batch *i.e.* Tie guan yin to new Tie guan yin, Light oolong to new Light oolong and Dark oolong to new Dark oolong have been performed.



Table 3. The bacterial counts (log CFU/g) of four kinds of tea: Tie guan yin, Light oolong, Dark oolong and Pu-erh on TSA, MRS, Rogosa and malt medium. The results are shown in median with range (25 % - 75 %).

	Tie guan yin	Light oolong	Dark oolong	Pu-erh
TSA	2.87 (2.70-3.02)	2.93 (2.73-3.07)	<1.00 (1.00-1.00)	4.71 (4.55-4.87)
MRS	1.94 (1.00-2.18)	<1.00 (1.00-1.00)	1.00 (1.00-1.17)	4.72 (4.46-4.82)
Rogosa	<1.00 (1.00-1.00)	<1.00 (1.00-1.00)	<1.00 (1.00-1.00)	4.88 (4.51-5.20)
malt	1.00 (1.00-2.07)	1.00 (1.00-1.25)	1.00 (1.00-1.17)	2.97 (1.92-3.73)

Table 4. The bacterial counts (log CFU/g) of three kinds of new tea: Tie guan yin, Light oolong and Dark oolong on TSA, MRS, Rogosa and malt agar. The results are shown in median with range (25 % - 75 %).

	New Tie guan yin	New Light oolong	New Dark oolong
TSA	3.61 (3.34-3.77)	3.51 (3.44-3.53)	<1.00 (1.00-1.00)
MRS	1.70 (1.52-2.04)	1.00 (1.00-1.17)	<1.00 (1.00-1.00)
Rogosa	<1.00 (1.00-1.00)	<1.00 (1.00-1.00)	<1.00 (1.00-1.00)
malt	1.70 (1.52-1.77)	2.71 (2.13-2.86)	<1.00 (1.00-1.00)

As shown in Figure 3, the bacterial counts of tea on TSA media are present in the grey plot boxes with a value range 25 % - 75 %. In the old batch, the significant difference can be found between each type of tea except for Tie guan yin and Light oolong. It is the same in the new batch that no significant difference can be found between Tie guan yin and Light oolong. Nevertheless, there are significant differences between two groups: Tie guan yin and new Tie guan yin, Light oolong and new Light oolong. All the p-values of the comparisons of bacterial counts are shown in Table in Appendix.

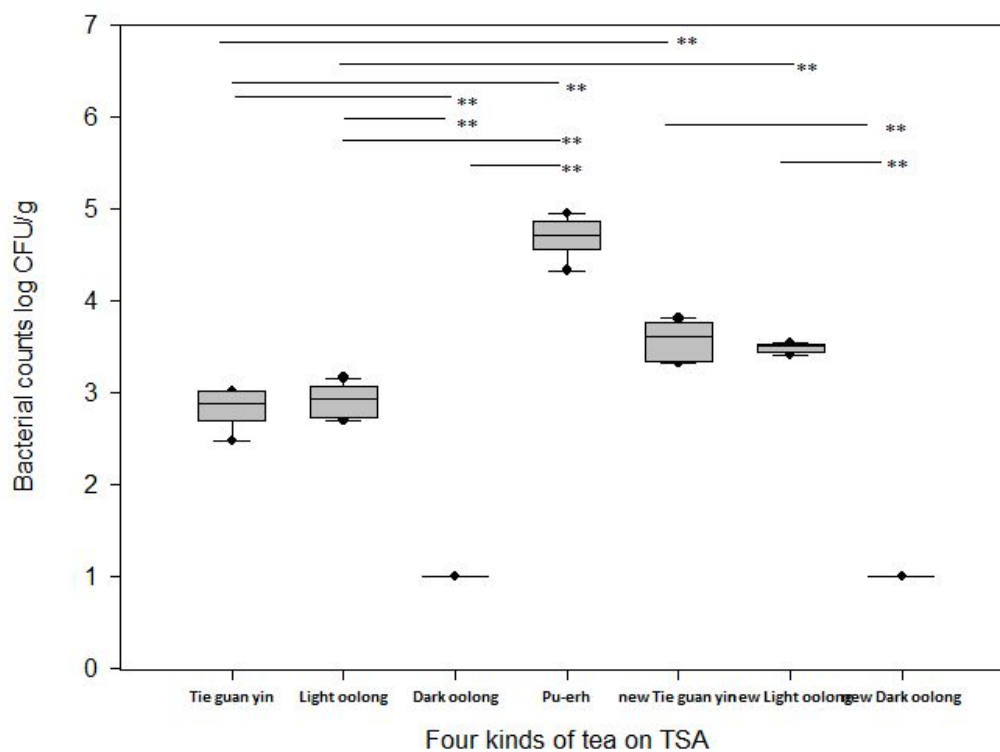


Figure 3. Comparison of bacterial counts on TSA agar between Tie guan yin (old and new), Light oolong (old and new), Dark oolong (old and new) and Pu-erh.

a. \* means  $p < 0.05$

b. \*\* means  $p < 0.01$

Only Pu-erh and Tie guan yin have been investigated regarding the bacterial composition on TSA. As Table 5 indicates, the identified bacteria of Pu-erh mainly belong to the family *Bacillaceae* while *Pseudomonadaceae* is the second dominant bacterial family among all the samples. Analogously, Table 6 shows that the dominating bacteria of Tie guan yin originated from TSA also belong to the *Bacillaceae* family. In addition to that, bacteria belonging to the family *Staphylococcaceae* and *Paenibacillaceae* have also been identified.

Table 5. Identified bacteria of Pu-erh tea on TSA medium. (TP refers to TSA of Pu-erh tea)

Sample	Identified bacteria	Family	Similarity
TP1	<i>Paenibacillus</i>	<i>Paenibacillaceae</i>	99
TP2	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	100

TP3	<i>Pseudomonas fluorescens</i>	<i>Pseudomonadaceae</i>	100
TP4	<i>Staphylococcus warneri</i>	<i>Staphylococcaceae</i>	100
TP5	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	100
TP6	<i>Virgibacillus</i>	<i>Bacillaceae</i>	100
TP7	<i>Bacillus cereus</i>	<i>Bacillaceae</i>	100
TP8	<i>Bacillus</i>	<i>Bacillaceae</i>	100
TP9	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	100
TP10	<i>Bacillus</i>	<i>Bacillaceae</i>	99
TP11	<i>Bacillus</i>	<i>Bacillaceae</i>	99
TP12	<i>Bacillus subtilis/tequilensis</i>	<i>Bacillaceae</i>	100
TP13	<i>Pseudomonas fluorescens</i>	<i>Pseudomonadaceae</i>	100
TP14	<i>Bacillus</i>	<i>Bacillaceae</i>	100
TP15	<i>Bacillus oleronius</i>	<i>Bacillaceae</i>	99
TP16	<i>Ornithinibacillus bavariensis</i>	<i>Bacillaceae</i>	100

Table 6. Identified bacteria of Tie guan yin tea on TSA medium. (TT refers to TSA of Tie guan yin tea)

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
TT1	<i>Bacillus thuringiensis</i>	<i>Bacillaceae</i>	100
TT2	<i>Staphylococcus warneri/pasteuri</i>	<i>Staphylococcaceae</i>	100
TT3	<i>Bacillus safensis/pumilus</i>	<i>Bacillaceae</i>	100
TT4	<i>Staphylococcus capitis</i>	<i>Staphylococcaceae</i>	99
TT5	<i>Bacillus</i>	<i>Bacillaceae</i>	99
TT6	<i>Bacillus</i>	<i>Bacillaceae</i>	97
TT7	<i>Lysinibacillus sphaericus</i>	<i>Bacillaceae</i>	99

TT8	<i>Paenibacillus polymyxa</i>	<i>Paenibacillaceae</i>	100
TT9	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
TT10	<i>Bacillus pumilus</i>	<i>Bacillaceae</i>	99
TT11	<i>Lysinibacillus sphaericus</i>	<i>Bacillaceae</i>	99
TT12	<i>Bacillus subtilis/tequilensis</i>	<i>Bacillaceae</i>	100
TT13	<i>Paenibacillus</i>	<i>Paenibacillaceae</i>	99

#### 4.1.2 MRS

On MRS medium, all the other three kinds of tea in the old batch have significant differences when compared to Pu-erh as Figure 4 illustrates. In the new batch, significant differences can be found between two groups: Tie guan yin and Light oolong, Tie guan yin and Dark oolong. However, no significant difference can be detected between the old and new batch on MRS medium.

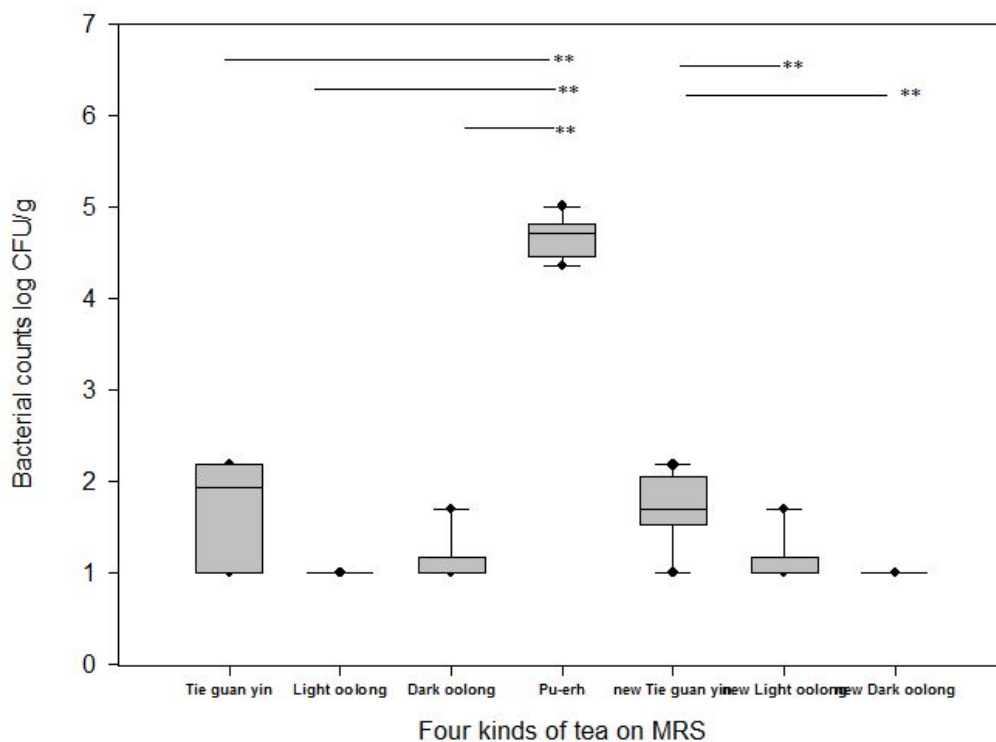


Figure 4. Comparison of bacterial counts on MRS medium between Tie guan yin (old and new), Light oolong(old and new), Dark oolong(old and new) and Pu-erh.

a. \* means  $p < 0.05$

b. \*\* means  $p < 0.01$

All the identified bacteria of Pu-erh and Tie guan yin originated from MRS medium belong to the family *Bacillaceae*. As can be seen in Table 7, 12 out of 13 samples show a high similarity to be the species *Bacillus coagulans*. While in Tie guan yin, all analyzed samples indicate the same species *Bacillus coagulans* (see Table 8).

Table 7. Identified bacteria of Pu-erh tea on MRS medium (MP refers to MRS of Pu-erh).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
MP1	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
MP2	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	96
MP3	<i>Bacillus</i>	<i>Bacillaceae</i>	95
MP4	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
MP5	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
MP6	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	96
MP7	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	95
MP8	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	97
MP9	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
MP10	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	97
MP11	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
MP12	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	97
MP13	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99

Table 8. Identified bacteria of Tie guan yin tea on MRS medium (MT refers to MRS of Tie guan yin).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
MT1	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
MT2	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	96

MT3	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
MT4	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	90

#### 4.1.3 Rogosa

As can be clearly seen in Figure 5, the bacterial counts of all kinds of tea on Rogosa are under the detection limit (bacterial counts <1 log CFU/g) except for Pu-erh. Thus significant differences can only be found in the old batch when comparing other tea with Pu-erh. The identified bacteria of all the 20 analyzed samples are *Bacillus coagulans* as Table 9 indicates.

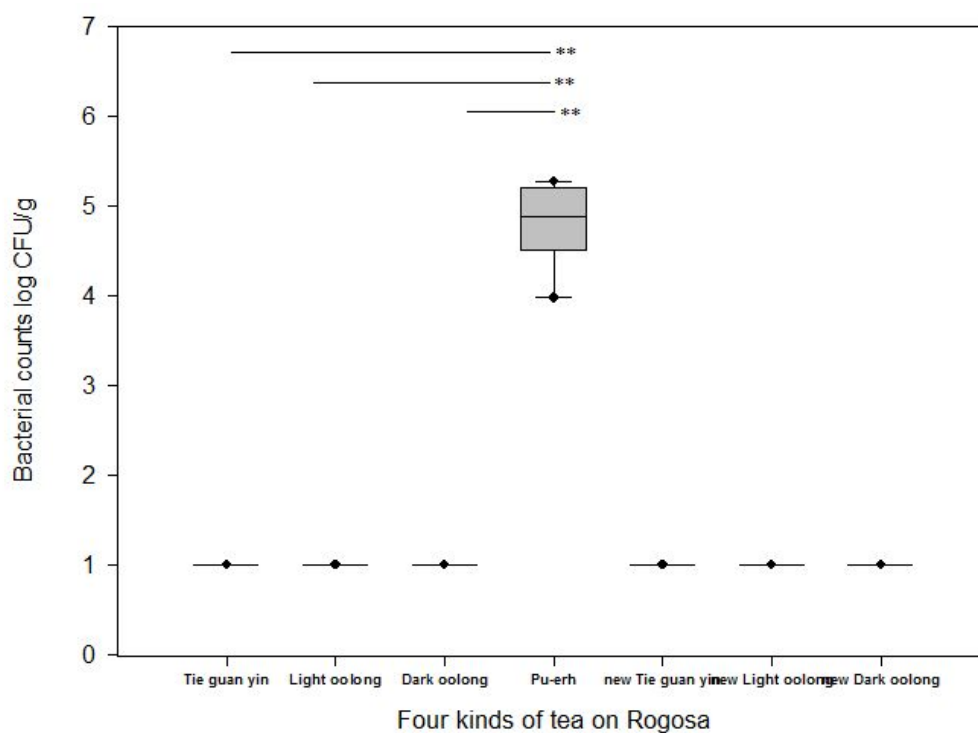


Figure 5. Comparison of bacterial counts on Rogosa agar between Tie guan yin (old and new), Light oolong (old and new), Dark oolong (old and new) and Pu-erh.

a. \* means  $p < 0.05$

b. \*\* means  $p < 0.01$

Table 9. Identified bacteria of Pu-erh tea on Rogosa media (RP refers to Rogosa of Pu-erh).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
RP1	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP2	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP3	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	100
RP4	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP5	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP6	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
RP7	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	97
RP8	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP9	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	96
RP10	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	95
RP11	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP12	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP13	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
RP14	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
RP15	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP16	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	100
RP17	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	100
RP18	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
RP19	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	100
RP20	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99

#### 4.1.4 malt extract agar

When it comes to malt extract agar medium, no significant difference can be detected in the pairwise comparisons between Tie guan yin, Light oolong and Dark oolong in the old batch. However, there are significant differences between these three kinds of tea in the new batch when compared with each other in pairs. Besides, the comparison between old and new Light oolong also shows a significant difference (see Figure 6). Table 10 indicates that the identified bacteria mainly belong to the family of *Pseudomonadaceae*. Other bacterial families e.g. *Bacillaceae* and *Paenibacillacea* have also been identified but with fairly low ratios (2 out of 11 and 1 out of 11 respectively).

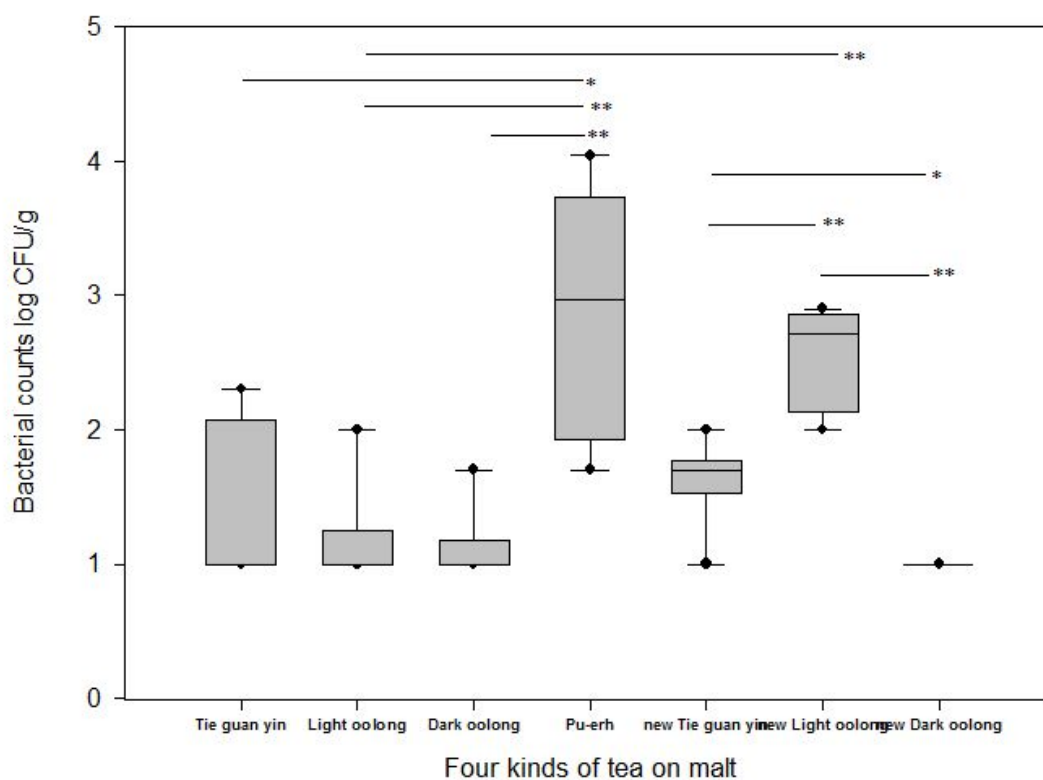


Figure 6. Comparison of bacterial counts on malt medium between Tie guan yin (old and new), Light oolong (old and new), Dark oolong (old and new) and Pu-erh.

a. \* means  $p < 0.05$

b. \*\* means  $p < 0.01$



Table 10. Identified bacteria of Pu-erh tea on malt media (MAP refers to malt of Pu-erh).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
MAP1	<i>Pseudomonas fluorescens</i>	<i>Pseudomonadaceae</i>	100
MAP2	<i>Pseudomonas fluorescens</i>	<i>Pseudomonadaceae</i>	100
MAP3	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	99
MAP4	<i>Bacillus subtilis</i>	<i>Bacillaceae</i>	100
MAP5	<i>Bacillus shackletonii</i> <i>/vietnamensis</i>	<i>Bacillaceae</i>	98
MAP6	<i>Paenibacillus cineris</i>	<i>Paenibacillaceae</i>	100
MAP7	<i>Pseudomonas fluorescens</i>	<i>Pseudomonadaceae</i>	100
MAP8	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	100
MAP9	<i>Pseudomonas poae</i>	<i>Pseudomonadaceae</i>	100
MAP10	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	100
MAP11	<i>Pseudomonas fluorescens</i>	<i>Pseudomonadaceae</i>	100

#### 4.2 After brewing tea

After being brewed with water at 90 °C, the microbial contents can be only found in Pu-erh tea. In other words, no surviving microorganisms could be detected in the brewed tea except for Pu-erh. The bacterial count results of after brewing Pu-erh are shown in Table 11. Brewed Pu-erh has the highest bacterial counts (3.70 log CFU/g) on TSA medium while the lowest bacterial counts (1.00 log CFU/g) can be found on malt medium. The comparisons of bacterial counts between before and after brewing Pu-erh on the same type of medium have also been carried out. As shown in Figure 7, there are significant differences between before and after brewing Pu-erh on all types of media.

Table 11. The bacterial counts (log CFU/g) of before and after brewing Pu-erh tea on TSA, MRS, Rogosa and malt agar. The results are shown in median with range (25 % - 75 %).

	Pu-erh	Brewed Pu-erh
TSA	4.71 (4.55-4.87)	3.70 (3.51-3.88)
MRS	4.72 (4.46-4.82)	3.45 (2.94-4.09)
Rogosa	4.88 (4.51-5.20)	2.24 (1.00-3.61)
malt	2.97 (1.92-3.73)	1.00 (1.00-2.74)

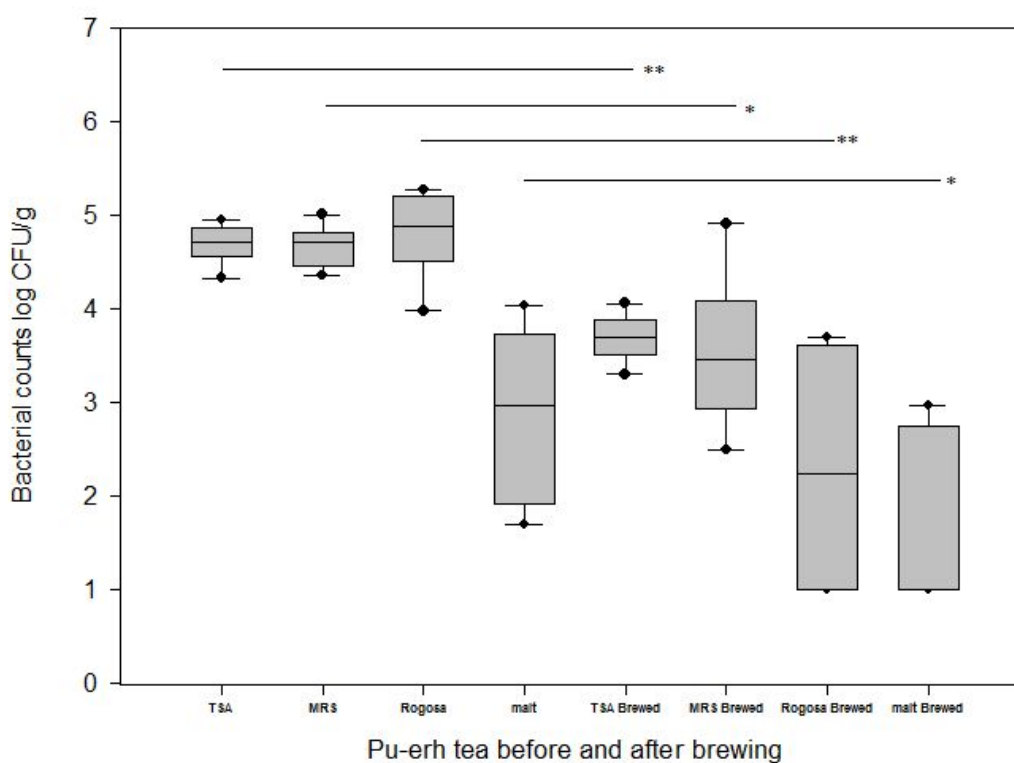


Figure 7. Comparison of bacterial counts on TSA, MRS, Rogosa and malt medium between before and after brewing Pu-erh tea.

a. \* means  $p < 0.05$

b. \*\* means  $p < 0.01$

The identified bacteria of after brewing Pu-erh tea on four types of media are listed in Table 12-15. According to Table 12, TSA medium has the most diverse results among all media regarding microbial composition where bacteria belong to *Bacillaceae*

family, *Paenibacillaceae* family and *Staphylococcaceae* family could be detected. Nevertheless, only 2 out of 28 samples are indicated as *Staphylococcaceae*. On MRS and Rogosa media which are more selective, *Bacillus coagulans* is the only species that could be identified (see Table 13-14). The results in Table 15 reveal that 4 out of 5 samples on malt media are identified as *Bacillaceae*, whereas the one remaining shows a high similarity of *Staphylococcaceae*.

Table 12. Identified bacteria of after brewing Pu-erh tea on TSA media (HTP refers to heated Pu-erh on TSA).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
HTP1	<i>Paenibacillus pueri</i>	<i>Paenibacillaceae</i>	99
HTP2	<i>Staphylococcus capitis</i>	<i>Staphylococcaceae</i>	100
HTP3	<i>Bacillus thermoamylovorans</i>	<i>Bacillaceae</i>	99
HTP4	<i>Paenibacillus barengoltzii</i>	<i>Paenibacillaceae</i>	100
HTP5	<i>Virgibacillus halophilus</i>	<i>Bacillaceae</i>	99
HTP6	<i>Paenibacillus</i>	<i>Paenibacillaceae</i>	99
HTP7	<i>Bacillus ruris</i>	<i>Bacillaceae</i>	100
HTP8	<i>Virgibacillus halophilus</i>	<i>Bacillaceae</i>	99
HTP9	<i>Staphylococcus capitis</i>	<i>Staphylococcaceae</i>	99
HTP10	<i>Paenibacillus pueri</i>	<i>Paenibacillaceae</i>	98
HTP11	<i>Paenibacillus pueri</i>	<i>Paenibacillaceae</i>	95
HTP12	<i>Bacillus thermoamylovorans</i>	<i>Bacillaceae</i>	100
HTP13	<i>Bacillus thermoamylovorans</i>	<i>Bacillaceae</i>	99
HTP14	<i>Bacillus</i>	<i>Bacillaceae</i>	100
HTP15	<i>Bacillus</i>	<i>Bacillaceae</i>	99
HTP16	<i>Bacillus oleronius</i>	<i>Bacillaceae</i>	100
HTP17	<i>Paenibacillus barengoltzii</i>	<i>Paenibacillaceae</i>	100
HTP18	<i>Bacillus</i>	<i>Bacillaceae</i>	99

HTP19	<i>Bacillus</i>	<i>Bacillaceae</i>	99
HTP20	<i>Paenibacillus</i>	<i>Paenibacillacea</i>	99
HTP21	<i>Bacillus</i>	<i>Bacillaceae</i>	99
HTP22	<i>Bacillus aquimaris</i>	<i>Bacillaceae</i>	99
HTP23	<i>Paenibacillus</i>	<i>Paenibacillacea</i>	99
HTP24	<i>Paenibacillus</i>	<i>Paenibacillacea</i>	99
HTP25	<i>Bacillus oleronius</i>	<i>Bacillaceae</i>	99
HTP26	<i>Paenibacillus pueri</i>	<i>Paenibacillacea</i>	99
HTP27	<i>Bacillus thermoamylovorans</i>	<i>Bacillaceae</i>	100
HTP28	<i>Bacillus shackletonii</i> / <i>vietnamensis</i>	<i>Bacillaceae</i>	99

Table 13. Identified bacteria of after brewing Pu-erh tea on MRS media (HMP refers to heated Pu-erh on MRS).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
HMP1	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HMP2	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	97
HMP3	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	95
HMP4	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98
HMP5	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	95
HMP6	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HMP7	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HMP8	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	98

Table 14. Identified bacteria of after brewing Pu-erh tea on Rogosa media (HRP refers to heated Pu-erh on Rogosa).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
HRP1	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP2	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP3	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP4	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	96
HRP5	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	97
HRP6	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP7	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP8	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP9	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP10	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP11	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP12	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP13	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99
HRP14	<i>Bacillus coagulans</i>	<i>Bacillaceae</i>	99

Table 15. Identified bacteria of after brewing Pu-erh tea on malt media (HMAP refers to heated Pu-erh on malt).

<b>Sample</b>	<b>Identified bacteria</b>	<b>Family</b>	<b>Similarity</b>
HMAP1	<i>Bacillus shackletonii</i>	<i>Bacillaceae</i>	99
HMAP2	<i>Bacillus shackletonii/aquimaris</i>	<i>Bacillaceae</i>	99
HMAP3	<i>Staphylococcus warneri/pasteuri</i>	<i>Staphylococcaceae</i>	100
HMAP4	<i>Bacillus shackletonii/vietnamensis</i>	<i>Bacillaceae</i>	99
HMAP5	<i>Bacillus</i>	<i>Bacillaceae</i>	99

### 4.3 Back brewing

The bacterial counts are all below the detection level (bacterial counts < 1 log CFU/g) after 13 isolated bacteria samples originated from Rogosa medium have been brewed with water at 90 °C. However, all the isolated bacteria samples showed the survivability after being brewed at 70 °C. The bacterial count results can be seen in Figure 8. Although all the isolates could survive, the bacterial counts still decreased significantly after being brewed at 70 °C. The highest and lowest bacterial reductions are 5.68 log CFU/g (sample HRP11) and 3 log CFU/g (sample HRP6) respectively. It is also worth mentioning that all examined isolates are *Bacillus coagulans* (see Table 14).

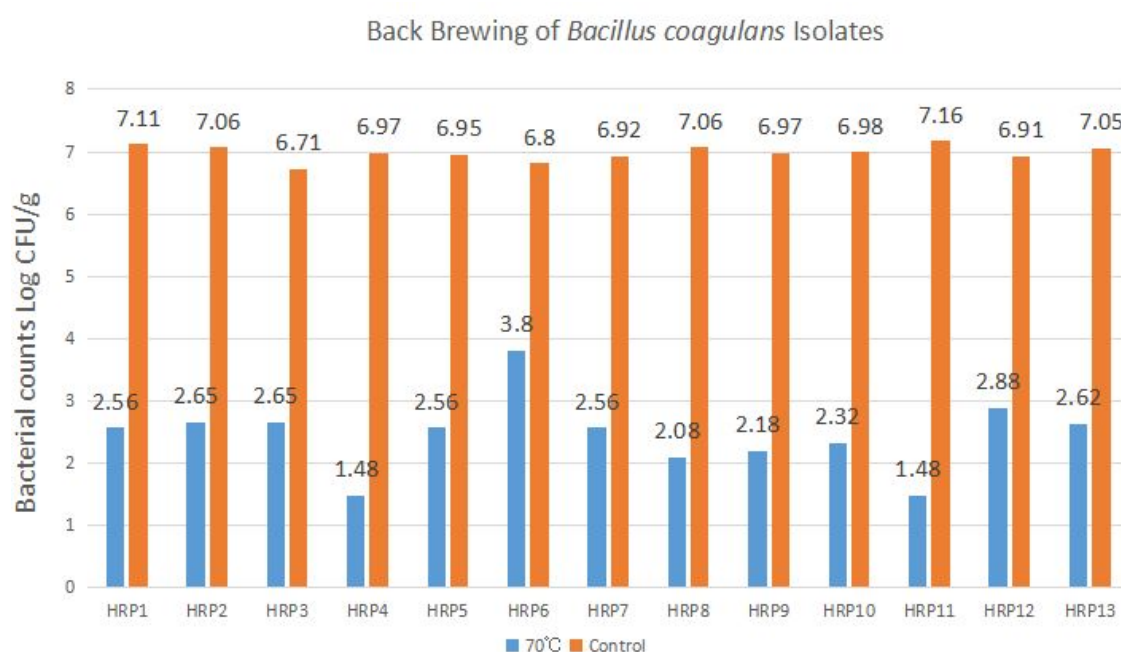


Figure 8. Bacterial counts of 13 isolated bacteria samples originated from brewed Pu-erh. Blue bars indicate “brewed at 70 °C” group while red bars indicate control group (HRP refers to heated Pu-erh on Rogosa).

### 4.4 RAPD (Random Amplified Polymorphic DNA)

As described in 4.2, all isolated bacteria samples (HPR1-13) are the species *Bacillus coagulans*. Thus the similarity of different samples regarding DNA bands pattern indicates a same strain type belonging to *Bacillus coagulans* and vice versa. Figure 9 presents the results of RAPD products after gel electrophoresis. Most of the RAPD products show different DNA bands patterns whereas few of them also share highly similar DNA bands patterns. More specifically, sample HRP3 and HRP4, HPR9 and

HPR10, HPR11 and HPR12 have the same DNA bands patterns on the gel.

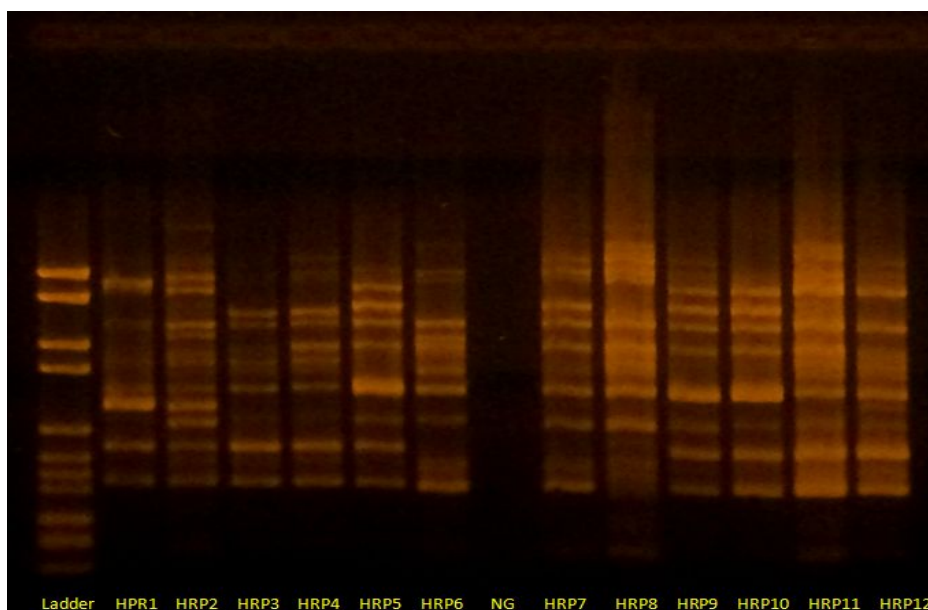


Figure 9. DNA bands of RAPD products after gel electrophoresis (HRP refers to heated Pu-erh on Rogosa, NG refers to negative control).

## 5. Discussion

The absence of bacterial colonies on violet red bile dextrose agar (VRBD) media of all the tea samples indicating that there was no, or just a minor amount of bacteria belonging to the family *Enterobacteriaceae* existing in the analyzed tea samples. The bacterial counts of all types of tea on tryptic soy agar (TSA) media have the relatively high values except for Dark oolong (below detection limit, bacterial counts < 1.00 log CFU/g). The reason for this is that TSA as a general culture medium can support the growth of a wide range of bacteria.

As described in section 4, no significant differences could be found between Tie guan yin and Light oolong on any examined media (TSA, MRS, Rogosa, malt). One possible factor causing this is the similar degree of fermentation (10 % - 29 %) in both kinds of tea (Zhen, 2003). As can be seen in Table 5, the identified bacteria of Tie guan yin on TSA belong to the family *Bacillaceae*, *Staphylococcaceae* and *Paenibacillaceae*. It is worth mentioning that, among these identified bacteria species, *Bacillus thuringiensis*, *Bacillus pumilus*, *Lysinibacillus sphaericus* and *Paenibacillus polymyxa* are bacteria which naturally occur in soil (Priest, 1993; Hu, *et al.*, 2008;

Sadhana and Silvia, 2009) while *Staphylococcus warneri* / *capitis* is commonly considered as part of the human skin flora (Kloos and Schleifer, 1975). The characteristics of these bacteria can clearly explain the reason why they occurred in the tea. It is obvious that the tea derived these bacteria during the production by contacting with soil and human skin. The microbial contents of Tie guan yin on *Lactobacillus* selective MRS agar have also been investigated. All the identified bacteria indicate the species *Bacillus coagulans* which previously belonged to the genus *Lactobacillus*.

Unlike Tie guan yin, when it comes to Dark oolong, it rarely showed any colony no matter what kind of medium it was cultured on. In other words, there was no or just a minor amount of bacteria existing in the examined Dark oolong. As described before, Dark oolong has a relatively high degree of oxidation (60 % - 70 %). Since the secondary polyphenols such as theaflavins and thearubigins derived from enzymatic oxidation can inhibit the growth of fungi and bacteria synergistically (Hu, 2002), it is suspected that the antimicrobial activity of Dark oolong has been improved significantly due to the synergistic activity of different polyphenols in this tea. However, the reason for the rareness of microorganisms in Dark oolong needs further investigations.

Among all types of tea, Pu-erh tea has the highest bacterial counts on all examined media (TSA, MRS, Rogosa, malt). The Mann-Whitney test also indicated significant differences in regard to bacterial counts between Pu-erh and the other three types of tea on all the media (see Figure 3-6). Pu-erh as the only tea that has undergone the fermentation process has also the most diverse microbial content. The identified microorganisms in Pu-erh tea include a variety of soil-dwelling bacteria which have also been identified in Tie guan yin. In addition to that, the species *Pseudomonas fluorescens* belonging to the family *Pseudomonadaceae* was found in Pu-erh. Likewise, *Pseudomonas fluorescens* is a bacteria that can be normally found in soil (Molloy, *et al.*, 2013). As described above, *Bacillus coagulans* was first referred to as *Lactobacillus sporogenes* due to its features of the genus *Lactobacillus* such as forming lactic acid. Therefore, *Bacillus coagulans* has been identified as the only species originated from both *Lactobacillus* selective MRS and Rogosa agar in Pu-erh tea.

According to Michiharu *et al.* (2008), the fermentation temperature of Pu-erh tea is kept at around 50 °C and the water content of the tea leafs was gradually reduced to approximately 30 %. This temperature is also optimal for the growth of some *Bacillus* species such as *Bacillus coagulans* and *Bacillus subtilis* (Hartemink, 2007). Therefore, it is highly probable that a variety of identified bacteria got the proper environment



for growth during the tea fermentation. It has also been noticed that most of the identified bacteria are the spores-forming *Bacillus*. The spores produced by *Bacillus* can protect the bacteria from extreme environments such as chemical and physical treatments. A temperature higher than 121 °C associated with a high pressure is required in order to kill the spores formed by *Bacillus* (Yukiko and Akiko, 2005). This can, to some extent, explain why *Bacillus* can still survive in the Pu-erh tea after the long time storage (around 10 years).

*Aspergillus spp.* is the main microorganism involved in the fermentation of Pu-erh tea (Mo, *et al.*, 2012). According to a previous study, other than *Aspergillus niger*, *Blastobotrys adenivorans* has also been recognized in Pu-erh tea (Abe, *et al.*, 2008). However, in this case, no fungi like colonies could be detected in the Pu-erh tea even on malt extract agar (which is the only kind of agar medium allows for fungi to grow) and the sequencing result also indicate the absence of fungi. The reason for this is that 16S rRNA sequencing technique was implemented. Eukaryotic cells fungi cannot be identified using this technique (instead 18S rRNA sequencing is needed to identify fungi). On the other hand, it is highly probable that *Aspergillus niger* and *Blastobotrys adenivorans* (or other fungi, yeasts, molds) were killed by certain environmental factors during the storage. Another possible factor is the antagonistic effects of *Bacillus* species on molds. It has been proven that some *Bacillus* species have strong antagonistic activity on inhibiting the mycelial growth of *Trichoderma spp* (Wan, *et al.*, 2008).

After brewing at 90 °C, no survived microorganisms could be detected in the brewed tea except for Pu-erh. Significant differences between before and after brewing Pu-erh could be found on all types of media (see Figure 7). The survived bacteria mainly belong to the families *Bacillaceae* and *Paenibacillaceae*. Specifically, the noted species *Paenibacillus pueri* was found on TSA while *Bacillus coagulans* was still the only bacteria identified on MRS and Rogosa. The bacteria *Paenibacillus pueri* is a species that has been commonly recognized in Pu-erh tea. It had survived from the high temperature brewing due to its endospore-forming characteristic which can provide the protection for the bacteria from extreme environments (Kim, *et al.*, 2009).

As the fermentation process goes on, a floating cellulose layer will be formed on the surface of the tea leaf due to the interaction of microorganisms present in the tea. The cellulose layer will also get thicker with a longer fermentation period (Nguyen, *et al.* 2015). Therefore, the cellulose layer of Pu-erh tea is another possible “barrier” for the bacteria in addition to the spores produced by the bacteria itself. The result of back brewing is another evidence supporting the “barrier” effect: all the isolates (*Bacillus coagulans*) originated from the Pu-erh without the protection of tea leaves could only

survive at 70 °C instead of 90 °C. To be more specific, in the “back brewing” experiment, all the isolates (*Bacillus coagulans*) were exposed to hot water at 90 °C and 70 °C directly while no colony was found of isolates brewed at 90 °C (see Section 4.3). However, all these isolates were originated from Pu-erh tea brewed at 90 °C. This indicates all these isolates could only survive at 90 °C when attached to the Pu-erh tea leaves. There are two reasons for this phenomenon: firstly, it is well known that spores of bacteria formed and developed in nature are usually stronger than spores of bacteria formed in laboratory. They are able to survive in extreme environments especially high temperature. Whereas spores formed in laboratory are fragile thus cannot protect bacteria properly; secondly, it is highly probable that the cellulose layer formed on the Pu-erh tea leaves can protect microorganisms from environmental stresses other than high temperatures. It might also be the reason why the Pu-erh tea has the highest bacterial counts no matter it was brewed or not. Nevertheless, the effects of the cellulose layer of Pu-erh tea on microorganisms need further investigations.

## 6. Conclusion

It was unexpected that live bacteria could be detected in the dry and long-time stored tea. More specifically, fully oxidized Pu-erh tea contains the highest number of bacteria and most diverse bacterial community. Most of the identified bacteria belonging to the family *Bacillaceae*, *Staphylococcaceae* and *Paenibacillaceae* are soil-dwelling (naturally occur in soil) bacteria or a part of skin flora. However, Dark oolong with a fermentation degree between 60 % and 70 % rarely showed any microbial content.

After brewing (90 °C), no survived microorganisms could be detected in the brewed tea except for Pu-erh. Although significant differences could be found between before and after brewing Pu-erh tea, the brewed Pu-erh still showed a relatively high bacterial count especially on TSA and MRS agar. More surprisingly, a potential probiotic *Bacillus coagulans* was found as the major bacteria in the brewed Pu-erh tea. 13 *Bacillus coagulans* isolates originated from the brewed Pu-erh tea were also brewed directly with hot water. It turned out that *Bacillus coagulans* could not survive at 90 °C without the protection of Pu-erh tea leaves. These isolates were also analyzed using RAPD (Random Amplified Polymorphic DNA) method. The result showed that most of them have different strain type. Nevertheless, further investigations regarding strain type and characteristic of these *Bacillus coagulans* isolates are needed.

To sum up, this study, to some extent, proved that the fermented Pu-erh tea can provide health beneficial bacteria (*Bacillus coagulans*) in addition to polyphenols to human body when drinking it. It is also recommended tea should be brewed with hot water (around 90 °C) before consuming due to the fact that most of the potential pathogens will be killed while beneficial bacteria will remain (in Pu-erh).

## **Acknowledgement**

For my dear supervisors Åsa Håkansson and Elisabeth Uhlig, thank you so very much for all your help and patience. I really enjoyed the great benefit of your instructions. It was my honor to have such great supervisors like you.

Also for those people who worked with me, you all are really nice and helpful. It has been a great experience working with you all.

Finally, I would like to thank Göran Molin. Thank you for being my examiner in your busy time.

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## Appendix

### Recipes

Table 16. Hogness freezing media (200 ml)

K <sub>2</sub> HPO <sub>4</sub>	0,17 g
KH <sub>2</sub> PO <sub>4</sub>	0,04 g
Tri-natrium-citrat-dihydrat	0,3 g
MgSO <sub>4</sub> x7H <sub>2</sub> O	0,05 g
Glycerol 99,5 %	24,3 ml
Water	175 ml

Table 17. Peptone water, 1 L

Bacterial peptone	1 g
NaCl	8,5 g

Table 18. Master mix for 1 sample 25 µl (no coral load)

H <sub>2</sub> O	18,375 µl
Buffer	2,5 µl
dNTP mix	0,5 µl
ENV1	0,5 µl
ENV2	0,5 µl
Taq pol	0,125 µl

**p-values**

Before brewing

Table 19. p-values of different kinds of tea on TSA

<b>TSA</b>	TIE	Loolong	Doolong	Pu-erh	New TIE	New Light oolong	New Doolong
TIE	-	0.589	0.002	0.002	0.002	-	-
Loolong	0.589	-	0.002	0.002	-	0.002	-
Doolong	0.002	0.002	-	0.002	-	-	1.000
Pu-erh	0.002	0.002	0.002	-	-	-	-
New TIE	0.002	-	-	-	-	0.394	0.002
New Light oolong	-	0.002	-	-	0.394	-	0.002
New Doolong	-	-	1.000	-	0.002	0.002	-

Table 20. p-values of different kinds of tea on MRS

<b>MRS</b>	TIE	Loolong	Doolong	Pu-erh	New TIE	New Light oolong	New Doolong
TIE	-	0.065	0.093	0.002	0.818	-	-
Loolong	0.065	-	0.699	0.002	-	0.699	-
Doolong	0.093	0.699	-	0.002	-	-	0.699
Pu-erh	0.002	0.002	0.002	-	-	-	-
New TIE	0.818	-	-	-	-	0.041	0.015
New Light oolong	-	0.699	-	-	0.041	-	0.699
New Doolong	-	-	0.699	-	0.015	0.699	-

Table 21. p-values of different kinds of tea on Rogosa

<b>Rogosa</b>	TIE	Loolong	Doolong	Pu-erh	New TIE	New Light oolong	New Doolong
TIE	-	1.000	1.000	0.002	1.000	-	-
Loolong	1.000	-	1.000	0.002	-	1.000	-
Doolong	1.000	1.000	-	0.002	-	-	1.000
Pu-erh	0.002	0.002	0.002	-	-	-	-
New TIE	1.000	-	-	-	-	1.000	1.000
New Light oolong	-	1.000	-	-	1.000	-	1.000
New Doolong	-	-	1.000	-	1.000	1.000	-

Table 22. p-values of different kinds of tea on malt

<b>malt</b>	TIE	Loolong	Doolong	Pu-erh	New TIE	New Light oolong	New Doolong
TIE	-	0.589	0.589	0.015	0.485	-	-
Loolong	0.589	-	0.937	0.004	-	0.002	-
Doolong	0.589	0.937	-	0.002	-	-	0.699
Pu-erh	0.015	0.004	0.002	-	-	-	-
New TIE	0.485	-	-	-	-	0.002	0.015
New Light oolong	-	0.002	-	-	0.002	-	0.002
New Doolong	-	-	0.699	-	0.015	0.002	-

After brewing

Table 23. p-values of before and after brewing Pu-erh tea on four media

Pu-erh	TSA	MRS	Rogosa	malt
TSA Brewed	0.002	-	-	-
MRS Brewed	-	0.041	-	-
Rogosa Brewed	-	-	0.002	-
malt Brewed	-	-	-	0.041