Comparative Quantitative Analysis of Probiotic Bacteria During Puer Tea Pile Fermentation

Shuang Li, Zhongyuan Li, Cuixia Feng and Tongcun Zhang

1 Introduction

Puer tea (puer shucha) is a very popular post-fermented tea in China because of the distinctive color, savoury flavours and aromas [1]. Different to non-fermentation tea, puer tea chooses large leafs of *Camellia sinensis* as raw material, and has a special manufacturing process called "pile fermentation", which is a solid-state fermentation (SSF) with turned over once several days until the color of the tea was changed into rufous [1]. The pH of tea pile is acidic and the center temperature could reach to 60 °C [2]. Complex biological transformations are implemented by a symbiosis of many fermenting microbes (including fungi, yeast and bacteria) and their kinds of extracellular enzymes, and ultimately the unique flavours and aromas of puer tea are formed at the end of pile fermentation [1].

Modern clinical medicine has shown that puer tea displays a variety of health care functions for human body, such as lowering blood pressure [3], preventing cardiovascular disease [4] and moderating the risk of cancer [5]. These benefits are not only in relation to the secondary metabolites themselves in puer tea, but also associated with these fermenting microbes. The specific identity of the microbial population present in puer tea has been the focus on attention, but to date, the majority of studies have relied on culture-based analysis. Previous study have shown fungi, yeast and bacteria were all found in puer tea, fungi *Candida* and *Aspergillus niger* are the dominant microbe in fermentation process [6, 7].

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However, the culture-based analysis is limited, because only about 1% of the environmental microbes can be uncultured and reliance on the phenotypic traits could lead to misidentification [8, 9]. Recently, a preliminary metagenomic study of puer tea reveals that bacteria are the dominant microbes in pile fermentation [2]. Especially, some probiotic bacteria are also found in puer tea pile fermentation process [10, 11]. Probiotic bacteria are reported to confer many health benefits such as preventing food allergy, gut inflammation and immunomodulation, and also potentially provide anticarcinogenic activity, which plays important role in many fermented food like kimchi [12] and cheese [13]. However, the knowledge of the species, amount and function analysis of probiotic bacteria in puer tea fermentation is limited. To gain a more comprehensive insight into the probiotic bacteria in puer tea fermentation, in this study, specific real-time quantitative PCR assays (qPCR) were developed to accurately determine the distribution and population sizes of the main probiotic bacteria including *Enterococcus* spp., *Bacillus* spp., *Lactococcus* spp., *Bifidobacterium* spp. and *Lactobacillus* group, in addition, the change patterns of these probiotic bacteria during puer tea pile fermentation were also investigated.

2 Materials and Methods

2.1 Puer Tea Sample Collection and Genomic DNA Extraction

Puer tea samples used in this study were provided by Xinghai Tea Factory in Yunnan province, China. At this factory, puer leaf samples were turned over once a week in pile fermentation process to ensure the homogeneity of the fermented tea. About 100 g puer tea samples of 0, 15, 30, 45 fermentation days were separately obtained from the same tea pile, all samples were frozen in liquid nitrogen within 5 min, transported to the laboratory on dry ice and then stored at −70 °C until used. To measure the pH of different tea samples, 1 g of each sample was firstly suspended in 9 ml distilled water and then mixed continuously for 30 min at room temperature (about 25 °C) [2], and pH of the supernatant for each tea sample was measured using a glass pH electrode (Starter 2000, Ohaus, USA). To normalize for different tea samples, 1 mg DNA of plasmid pEGFP-1 harboring enhanced green fluorescent protein (EGFP) encoding gene (*egfp*) which is used as reference gene were firstly added to each tea sample (5 g) and grinded completely with tea by liquid nitrogen before DNA extraction. To confirm the absence of *egfp* in puer tea, *egfp* was amplified by PCR using the gene specific primers EGFP-F/EGFP-R (Table 1) and genomic DNA of puer tea without adding plasmid pEGFP-1. The grinded tea samples were washed by 20 ml washing buffer according to Lyu et al. [2], and the supernatant was incubated for 5 min in 65 °C, and the strain deposits were collected by centrifugation at 6000×*g* for 10 min, and genomic DNA of tea were extracted following a protocol specific for high-molecular-weight

environmental DNA [14]. The genomic DNA was analyzed by 1% (w/v) agarose gel electrophoresis and the purity of DNA were determined from the absorbance ratios of A_{260}/A_{280} by Ultrospec 2100 pro UV/visible spectrophotometer (Amersham Biosciences, Uppsala, Sweden).

2.2 Primers Design and qPCR Condition

The specific qPCR primers of universal bacteria and specific-genus qPCR primers of *Bifidobacterium* spp., *Enterococcus* spp., *Bacillus* spp., *Lactococcus* spp. and *Lactobacillus* group (including *Leuconostoc* spp., *Pediococcus* spp., *Aerococcus* spp. and *Weissella* spp.) were summarized in Table 1. The specific qPCR primers for gene *egfp* were designed by the Primer3 program [15]. Strains *Bifidobacterium bifidum*, *Enterococcus faecium* A1, *Bacillus subtillis*, *Lactococcus lactis* NZ9000 and *Lactobacillus plantaru*m CGMCC No. 8198 obtained in our laboratory were used as the reference strain for each specific-genus qPCR primer, and *Escherichia coli* harboring pEGFP-1 was used as the reference strain for *egfp*. The specificity of each qPCR primer was further verified by product sequencing and melting curve analysis. To avoid the mutual contamination with other primers, each pair primer was assayed with the other genus reference strains by PCR method and analyzed by agarose gel electrophoresis. The optimal qPCR reaction mixture consisted of 10 μl SYBR Green qPCR mastermix (DBI Bioscience, Shanghai, China), 1 μl template, 0.4 μl 50 × ROX Reference Dye, 0.5 μl PCR forward/reverse primer (10 μmol l^{-1}), and 7.6 μl double distilled H_2O using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA), and the optimal qPCR condition for each qPCR primer was 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s.

2.3 Standard Curves Generation and Estimation of the Copy Numbers of Universal Bacteria and Probiotic Bacteria

The PCR products of each primer with the responding reference strain were purified by DNA product purification kit (TIANGEN Biotech, Beijing, China), ligated with PMD19-T vector (TaKaRa, Dalian, China) and sequenced by Genewiz (Beijing, China), the correct recombinant plasmids were used as the standard plasmid for the following standard curves generation. The standard plasmids for each qPCR primer were extracted by Plasmid Mini Kit (Omega Bio-tek, USA) and quantified by A_{260} measurements. 10-fold dilution series of about 10^1 to 10^7 copies for each target standard plasmid DNA were applied for qPCR analysis. The copy numbers of *Bifidobacterium* spp., *Enterococcus* spp., *Lactobacillus* group, *Bacillus* spp., *Lactococcus* spp. and universal bacteria during tea fermentation were quantified by

Table 1 qPCR primers for Universal bacteria and probiotic bacteria used in this study

Target	Name of primers	Sequences for primers (5′-3′)	Product size (bp)	Efficiency (%)	Reference
Universal bacteria	Bacteria-F	CCTACGGGAGGCAGCAG	193	104.94	[18]
	Bacteria-R	ATTACCGCGGCTGCTGG			
Enterococcus spp.	Ent-F	CCCTTATTGTTAGTTGCCATCATT	144	101.83	[19]
	Ent-R	ACTCGTTGTACTTCCCATTGT			
Lactobacillus group[a]	Lacb-F	AGCAGTAGGGAATCTTCCA	431	94.28	[20, 21]
	Lacb-R	CACCGCTACACATGGAG			
Lactococcus spp.	Lacc-F	GCGGCGTGGCTAATACATGC	305	95.22	[22]
	Lacc-R	CTGCTGCGTCCCGTAGGAGT			
Bacillus spp.	Bac-F	ACGCCGTAAACGATGAGT	424	96.89	[23]
	Bac-R	GTGTGTAGCCCAGGTCATAA			
EGFP	EGFP-F	ACAAGACCCGCGCCGAGGTGAA	253	94.83	This study
	EGFP-R	TCGCCGATGGGGGTGTTCTGCT			

[a] *Lactobacillus* group including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Aerococcus* and *Weissella* but not *Enterococcus* or *Streptococcus*

qPCR with each target qPCR primer. The PCR condition and reaction mixture were the same as mentioned above. Fluorescent products were detected at the last step of each cycle. Each sample was assayed in triplicate. The populations of universal bacteria and other genera at different time points were calculated by threshold cycle values based on each standard curves after normalization against gene *egfp*.

2.4 Statistical Analysis

Correlations between/among pH and amount of universal bacteria and *Enterococcus* spp., *Bacillus* spp., *Lactococcus* spp., *Bifidobacterium* spp. and *Lactobacillus* group were tested with Pearson's correlation analysis using the SAS software package, version 8.01 (SAS Institute Inc., Cary, NC). Data were considered significantly correlated for P values of <0.05 and very significantly correlated for P values of <0.01.

3 Results and Discussion

In order to investigate the species and abundance of probiotic bacteria, qPCR is a very precise method which could detect not only the microbe with very low amount but also those uncultured microbe [16]. The template DNA with high quality and concentration is an important factor for the accuracy and validity of qPCR. In this study, puer tea were firstly washed by the washing buffer to reduce the interference of impurity substance in tea for genomic DNA extraction, and the results showed these genomic DNA of puer tea at 0, 15, 30 and 45 days all have high quality with A_{260}/A_{280} ratio of approximately 1.8 (Fig. 1a), and the DNA concentrations of them were 660, 990, 2250, and 2758 μg ml^{-1}, respectively. According to Fig. 1f, Gene *egfp* was verified as an exogenous gene to puer tea, it was added before genomic DNA extraction and used as the reference gene for quantitative analysis, which could usefully normalize the genomic DNA extraction efficiency and reduce technical variations between each tea sample of four time points, this method was also mentioned in our previous study [17]. The high specificity of qPCR primers for each genus and *egfp* was also essential for qPCR assay. Agarose gel electrophoresis of the PCR products in Fig. 1 showed that only one single DNA band for each corresponding genus's reference strain with the expected lengths (Table 1), and no DNA bands for each primer with any other reference strains as DNA template (Fig. 1). The melt curves analysis of these primers showed their high specificity without interference (data not shown) and DNA product sequences were verified belong to the responding genus or *egfp*. With the standard plasmid as template, standard curve equation of cycle threshold values versus plasmid DNA concentrations for each genus primer were generated as shown in Table 2, the resulting

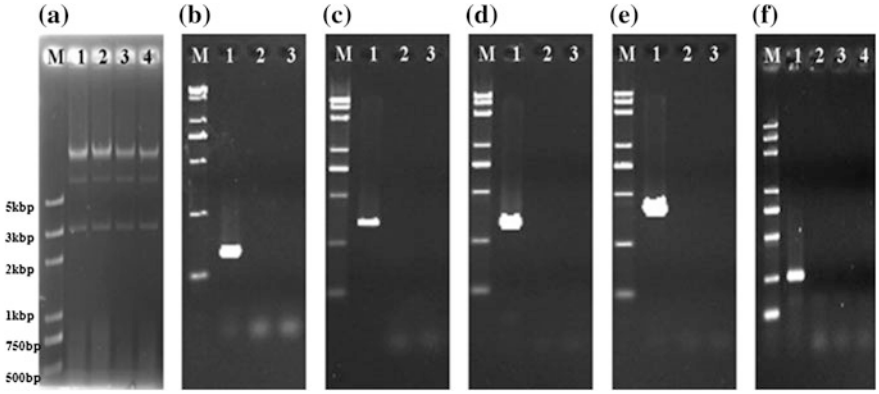

Fig. 1 Verification by agar gel electrophoresis of genomic DNA of puer tea (**a**) at 0 day (*lane 1*), 15 days (*lane 2*), 30 days (*lane 3*), 45 days (*lane 4*) and the specificity of qPCR primers for *Enterococcus* spp. (**b**), *Lactobacillus* spp. (**c**), *Lactococcus* spp. (**d**), *Bacillus* spp. (**e**) and gene *egfp* (**f**). *Lane M* standard molecular size marker; **b–f** *lane 1* PCR product with each reference strain as template; *lane 2* PCR product with sterile water as template; *lane 3* PCR product with other reference strains as template. *Lane 4* PCR product with genome DNA of tea samples without pEGFP-1 as template to evaluate the feasibility of gene *egfp* as the internal reference gene

linear regression analysis suggested the PCR efficiencies ranged from 94.83 to 104.94% (Table 1).

The copy numbers for universal bacteria and other probiotic bacteria were obtained from the threshold cycle values based on standard curves after normalization to that of gene *egfp*. The results showed that the population of universal bacteria was 7.56×10^9–3.45×10^{10} copy g^{-1} in the puer tea fermentation. Among the five genera detected, *Enterococcus* spp. showed 3.43×10^4–6.25×10^5 copy g^{-1}, and *Lactococcus* spp. showed 1.68×10^9–2.78×10^9 copy g^{-1}, which has much more copy numbers than the other two genera *Lactobacillus* spp. (4.26×10^3–5.17×10^4 copy g^{-1}) and *Bacillus* spp. (2.21×10^3–2.31×10^5 copy g^{-1}) according to Fig. 2. These four genera probiotic bacteria all belong to the phylum Firmicutes, which is 20.23% of the total microbe in puer tea fermentation by metagenomic sequencing analysis and played the major role in pile fermentation [2]. Genera *Enterocooccus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Weissella* and some of *Bacillus* like *Bacillus coagulans* are the main genera belong to probiotic bacteria [18]. These results suggested that probiotic bacteria are involved in puer tea fermentation, which could produce variety of polyphenol oxidase and peroxidase, which could change catechin polyphenols into benzene quinone, and then further changed into polypeptide with red color, and benefit for improving the quality of puer tea and reduce the fermentation time [10]. Only *Bifidobacterium* spp. was not detected during the whole fermentation process, and also there were no reports about the *Bifidobacterium* spp. in puer tea, although *Bifidobacterium* spp. belonged to the phylum Actinobacteria which is up to 30.08% of the microbe

Table 2 Standard curve equation of qPCR primers of universal bacteria and probiotic bacteria (*Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Bacillus* spp. and gene *egfp*) used in this study

Strains	Standard curve equation	Variance (R^2)
Bacteria	Y = −3.1072X + 38.145	0.9968
EGFP	Y = −3.523X + 40.147	0.9955
Entq	Y = −3.2788X + 39.549	0.996
lacq	Y = −3.4946X + 39.844	0.995
laccoq	Y = −3.4214X + 44.329	0.997
Bacq	Y = −3.3987X + 41.503	0.9959

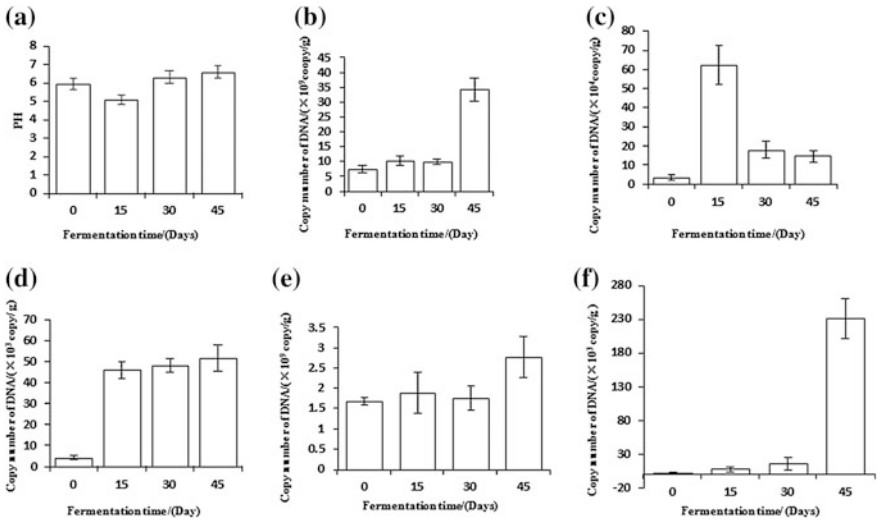

Fig. 2 pH and copy numbers of universal bacteria and bacteria and four genera at different fermentation time. pH (**a**), universal bacteria (**b**), *Enterococcus* spp. (**c**), *Lactobacillus* spp. (**d**), *Lactococcus* spp. (**e**), and *Bacillus* spp. (**f**). *Error bars* represent standard deviations of triplicate measurements

in puer tea fermentation [2]. It might because *Bifidobacterium* spp. as an obligate anaerobe bacterium, could not survive in the SSF because the pile fermentation environment is aerobic.

Among the four genera, family *Bacillaceae* was previously reported to be the main predominant bacteria in the last stage of puer tea fermentation by 16S rRNA clone library analysis [19]. *Lactobacillus* was made up to 30% in the black tea [11]. *Enterococcus* spp. was identified in puer tea by 16S rRNA clone library [19] and *Enterococcus camelliae* sp. nov. was isolated from another fermentation tea from Tailand [20]. But there was no report about *Lactococcus* in puer tea to date. However, based on populations of these four genera in present study, we interestingly found that *Lactococcus* spp. and *Enterococcus* spp. owned larger

population (about 100 folds) than *Lactobacillus* spp. and *Bacillus* spp., which suggested *Lactococcus* spp. and *Enterococcus* spp. were the main probiotic bacteria and play important role in this puer tea fermentation. This inconsistent result with previous studies might be due to the use of different puer tea material and production condition.

The pH profiles of puer tea sample were acidic during the tea fermentation (Fig. 2a), the initial pH of the raw material was 6.17, and decreased to pH 5.07 after 15 days' pile fermentation, and increased to pH 6.28 at 30 days and finally reached to pH 6.58 at the end of the pile fermentation. During the four different time points, universal bacteria and four genera also showed different change patterns throughout fermentation process (Fig. 2). Universal bacteria had a trend of slowly, continually and simply increasing from 7.56×10^9 to 3.45×10^{10} copy g^{-1} during 45 days fermentation (Fig. 2b). Different to the universal bacteria, *Bacillus* spp. increased slightly from 2.21×10^3 to 1.55×10^4 copy g^{-1} at the first 30 days, but was found to significantly increase to the maximum in the last 15 days with 2.31×10^5 copy g^{-1}, which are 104 folds of the abundance of initial stage (Fig. 2f) which is consistent to the previous reported [19], The other three genera displayed much more varied change in the 45 days fermentation. *Lactobacillus* spp. increased quickly to the maximum copy numbers (4.62×10^4 copy g^{-1}) in the first 15 days, and then continue increased to 5.17×10^4 copy g^{-1} at the end of fermentation which is still about 12 folds of that of the initial stage (Fig. 2d). *Enterococcus* spp. showed a similar increased trend with *Enterococcus* spp. in the first 15 days by about 18 folds from 3.43×10^4 to 6.25×10^5 copy g^{-1}, but then decreased sharply to 1.49×10^5 copy g^{-1} at the end of the fermentation which is almost the same to that of the initial stage (Fig. 2c). Different to the other genera, *Lactococcus* spp. firstly maintained from 1.68×10^9 to 1.90×10^9 copy g^{-1} in first 30 days, and then increased rapidly to 2.78×10^9 copy g^{-1} (Fig. 2e). Previous study has also shown that the bacteria, yeast, mold were all changeable during the fermentation process [2], which suggests that complex changes are happened in fermenting microbe community during the puer tea fermentation.

Although Pearson's correlation analysis suggested that there are no significantly correlation between pH values and the amount changes of universal bacteria and *Enterococcus* spp., *Bacillus* spp., *Lactococcus* spp. and *Lactobacillus* group (data not shown) during the fermentation, but at the end of the fermentation with pH 6.58, *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp. were all decreased and only *Bacillus* spp. reached to the high abundance, which might be due to *Bacillus* spp. is much adaptable to neutral pH, but *Enterococcus* spp., *Lactobacillus* spp. and *Lactococcus* spp. prefer to the acidic environment. In addition, the correlation among the abundance changes of universal bacteria and *Enterococcus* spp., *Bacillus* spp., *Lactococcus* spp. and *Lactobacillus* group were also not significantly. The synergism and antagonism among the different groups of microbes and even among different genera within the same group the microbe interactions may be another affect factor for complex change patterns.

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