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Design, optimization and verification of 16S rRNA oligonucleotide probes of fluorescence in-situ hybridization for targeting *Clostridium* spp. and *Clostridium kluyveri*

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Running title: *Clostridium* spp. and *C. kluyveri* FISH probes

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Abstract: Fluorescence in-situ hybridization (FISH) is a common and popular method to investigate microbial communities in natural and engineered environments. In this study, two specific 16S rRNA-targeted oligonucleotide probes CLZ and KCLZ were designed and verified to quantify the genus *Clostridium* and the species *Clostridium kluyveri*. The optimal concentration of hybridization buffer solution for both probes CLZ and KCLZ was 30% (w/v). The specificity of the designed probes was high by using pellets from the pure reference strains. Feasibility was tested using the samples of Luzhou-flavor liquor manufacturing cellar. The effectiveness of detecting target cells appears to vary widely in different environments. In pit mud, the detection effectiveness of the target cell by probe CLZ and KCLZ was 49.11% and 32.14%, respectively. The quantitative analysis by FISH technique of microbes in pit mud and fermented grains showed consistency with the results detected by qPCR and PCR-DGGE techniques, which showed that the probes CLZ and KCLZ were suitable to analyze the biomass of *Clostridium* spp. and *C. kluyveri* during liquor fermentation. Therefore, this study provides a method for quantitative analysis of *Clostridium* spp. and *C. kluyveri* and monitoring their community dynamics in microecosystems.

Keywords: Fluorescence in-situ hybridization, 16S rRNA, specific oligonucleotide probe, *Clostridium, Clostridium kluyveri*
Introduction

Chinese liquor is one of the most famous distilled spirits in the world, thanks to its characteristic aroma endowed by unique brewing techniques [1, 2]. Among different types of Chinese liquor, one important feature for Luzhou-flavor liquor is the strongest dependence on the quality of pit mud (PM). PM is a special kind of clay which provides a suitable habitat for the growth of brewing microbes. Similar to many other niches, such as soil and sludge, the composition of the microbial community in PM is very complex. Various methods, including both culture-dependent and independent measurements, were used to explore the microbial diversity and their successions during the process in the last three decades. It was reported previously and confirmed by independent methods that microbial diversity was closely connected with pit ages [3, 4]. In the last three decades, molecular biology technologies (PCR-based, Fluorescence in-situ hybridization (FISH), etc.) were used to investigate the characteristics of microbial communities in PM [5, 6]. The results from these studies suggest that Clostridium plays an important role in the process of brewing. Hexanoic acid, one of the major metabolites of the brewing process, is the precursor of ethyl hexanoate. Ethyl hexanoate has a fruity, floral, sweet aroma and is one of the main volatile compounds in Luzhou-flavor liquor as well as other types of Chinese liquor [7-11]. Therefore, the abundance of Clostridium, especially Clostridium kluyveri, in PM and fermented grains as well as their activities was deemed as one of the important evaluation criteria for bioactivity [5, 6, 8]. Clostridium, including more than 100 species, belonging to anaerobic bacteria, and the majority of them was uncultured so far [9, 10], apart from a few of species, such as Clostridium spp. and C. kluyveri [12, 13]. Therefore, it has been paid close attention to investigate the characteristics of
*Clostridium* during the process of brewing [11, 14, 15]. However, it was not yet conductive to estimate the evolution of microbial community in this process quantitatively and directly, to *in situ* screen functional *Clostridium* in PM, and to analyze the impact of process on microbial community in PM. The qPCR method was used to determine the copies of the 16S rRNA gene of *C. kluyveri* in PM quantitatively [12]. However, it was still difficult to accurately characterize the composition of *Clostridium* in complex systems due to the influence of various factors, such as DNA extraction, amplification efficiency and inability to distinguish activity. FISH with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments [16]. The advantages of FISH are the ability of allowing the cell of specific microbes group of both cultured and uncultured state being visualized, identified, enumerated and located [17]. It has been used to characterize the archaeal community in complex environments [18, 19]. In our previous researches, the microbial communities in PM were characterized by FISH using various order-specific oligonucleotide probes [20-24]. The composition of four orders methanogenic archaeal (methanosarcinales, methanobacteriales, methanomicrobiales and methanococcales) in various aged PM were also estimated quantitatively [25]. These results suggested that FISH can be used to evaluate the community diversity and their dynamics during the Chinese liquor production, in which choosing the appropriate oligonucleotide probes is the critical step.

The aim of this study was to develop two specific oligonucleotide probes CLZ and KCLZ targeting *Clostridium* spp. and *C. kluyveri*, respectively. Moreover, the concentration of hybridization was optimized, the feasibility and sensitivity of genus and species were examined. The validation of specificity and accuracy of these probes was then carried out. Using PM
spiked with reference strain, real time qPCR, and PCR-DGGE techniques, good effectiveness of these probes has been proved.

Materials and Methods

Microbial strains and culture conditions

Microbial strains used in present research were purchased from China center of industrial culture collection (CICC), except Lactobacillus casei (ATCC 393) (purchased from American type culture collection). These strains included three strains of Clostridium and two strains of non-Clostridium. C. kluyveri (CICC 8022) was cultivated in ethanol/acacetate/succinate/yeast extract (EASY) medium according to culture method described by Weiner et al. [26]. Clostridium butyricum (CICC 10350) was cultivated in medium composed of 5.0 g corn steep flour, 20 g glucose, 1 g (NH₄)₂SO₄, 1.24 g NaHCO₃, 0.2 g MnSO₄·H₂O, 0.2 g MgSO₄·H₂O, 0.2 g MgSO₄·7H₂O, 0.02 g CaCl₂ per liter at 35°C in an anoxic environ. TGY broth (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) was used for Clostridium perfringens (CICC 22949) vegetative growth (initial pH 7.2-7.4). MRS (QDRS BIOTEC, QingDao, China) broth or agar medium was used for L. casei (ATCC 393) vegetative growth (initial pH 6.2-6.4). Bacillus subtilis (CICC 20633) was cultivated in tryptic soy broth or agar (Beijing Borunlaite Science & Technology, Co., Ltd, China).

Sampling and pretreatment

These samples were obtained from the cellar in the Luzhou Laojiao Co., Ltd (Luzhou City, Sichuan Province, China), which has been used to brew liquor for more than 300 years.
Sampling was performed according to the stratified random method described by Ding et al. [27]. The samples obtained from the pit mud, fermented grains and mixture (composed of Daqu powder, fermented grains, rice hull and broken sorghum) were abbreviated as PM, BFG and SFG, respectively. SFG were streamed for distilling liquor and liquefying and saccharifying starch. These samples were packaged in sterile polyethylene bag and immediately stored at -20°C until analysis.

These samples were pretreated prior to examination according to the method described by Li et al. [22].

**Probes design**

Specific oligonucleotide probes for genus *Clostridium* and species *C. kluyveri* were designed according to the method previously described [28, 29]. Sequence manipulations were carried out by Primer Premier 6.0 (Premier Biosoft international, Canada, 2002. 3). The sequences were initially aligned with 16s rRNA gene sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov) by MEGA 6.0 (www.megasoftware.net) and adjusted manually. Specific oligonucleotide probes for genus *Clostridium* and species *C. kluyveri* shown in Table 1, was authorized to Sangon Biotech (Shanghai, China) for synthesis.

**Fluorescence in-situ hybridization**

FISH experiments with CLZ and KCLZ probes were performed as described previously [24, 30]. The pretreated pit mud was fixed in a 4% freshly prepared paraformaldehyde at 4°C for 16 h and then suspended in PBS buffer. After mixed intensively, the pellets were collected.
by centrifugation (10,000 g, 4°C, 10 min, Anke, GL-20G-II), resuspended in 10 mL of sodium pyrophosphate solution (3.8 mM). The suspension was diluted to 10^7-10^8 cfu/mL, and 20 µL of this dispersed sample was doped onto a poly-L-Lysine-coated slide. The slide was air-dried at 37°C for 2 h, and then dehydrated by successive passages through 50%, 80% and 100% ethanol, respectively (each for 3 min). The slides were incubated at 37°C for 30 min in 50 µL lysozyme solution (10 mg/mL), washed with sterile distilled water, and then dehydrated and air-dried by the process described above. Hybridization was performed at 42°C for 2 h with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% (w/v) SDS) containing the designed probes (KCLZ, CLZ), in which different concentrations of formamide was added. For EUB338 (5’-GCTGCCTCCGTAGGAGT-3’) probe, the optimized concentration (20% w/v) was employed as related research has already been reported previously [31]. Slides were washed twice in 50 mL of prewarmed washing buffer (20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% (w/v) SDS, 0.3 M NaCl) at 48°C for 30 min, and subsequently washed with distilled water and air-dried in the dark after hybridization. Finally, the slides were mounted in an anti-fade solution, and observed using a fluorescence microscope (BX-51, Nikon, Japan) with a WG filter for Cy3 (excitation 534-558 nm).

After optimizing the formamide concentration, FISH experiment was performed using firstly the pure culture of reference strains listed above and the mixture of these pure cultures, respectively. The pure culture of C. kluyveri (CICC 8022) was then added into a PM sample. Meanwhile, the mixtures of pure culture were added into other PM samples with different volumes (0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL and 1 mL). After sample pretreatment, the samples were added onto the slides and put into the microscope for FISH counting analysis. Fifteen
fields for each sample were randomly counted using FISH 3.0 software (Imstar, France). The fluoro-stained cell concentration was calculated as follows:

\[ N = n \times \frac{s_1}{s_2} \times \frac{v_1}{v_2} \times D \]

Where \( N \) is the cell number in 1 g of dried pit mud (cells/g), \( n \) is the mean number observed in a field, \( s_1 \) is the spreading area (μm²), \( s_2 \) is the field area (μm²), \( v_1 \) is the spreading volume (μL), \( v_2 \) is the total volume (μL) and \( D \) is the dilution rate.

The samples originated from pit, including PM, BFG and SFG, were pretreated, FISH were carried out according to the process described above using hybridization buffer containing various probes in previous articles [22]. The fluoro-stained cell concentration was calculated.

Quantitative characterization of total bacteria and \textit{C. kluyveri} based on qPCR analysis

Whole genomic DNA was extracted according to the methods of Zhou \textit{et al.} [32]. SybrGreen qPCR Master Mix (Toyobo life science, Shanghai, China) was used for all measurements. The qPCR reaction volume was fixed at 20 μL, containing 10 μM of each primer, 2×reaction buffer and 2 μL of DNA. The primers were 357F (5’-CTACGGGAGGCAGCAG-3’)/517R (5’-ATTACCGCGGCTGCTGG-3’) and CloKly1F (5’-GAGGGAGCAAATCTAAAAACTGC-3’)/CloKly1R (5’-CCTCCTTGGTAGACTACGGACTT-3’) for the amplification of eubacteria and \textit{C. kluyveri}, respectively. Amplification occurred in a Stepone plus™ Real-Time PCR systems (Thermo Scientific, Waltham, MA. US) using the following program: 3 min at 95°C, 45 cycles of 15 s at 95°C, 20 s at 57°C and 30 s at 72°C.

The PCR product of primers specifically for eubacteria and \textit{C. kluyveri} was then amplified by conventional PCR using primer 338F (5’-ACTCCTACGGGAGGCAGCAG-3’)/518R (5’-
ATTACCGCGGCTGCTGG-3') and Clokly1F/CloKly1R, respectively, according to the
methods described by Lee et al. [33], and were denoted as pGEM-C and pGEM-D. A 10-fold
serial dilution series of the pGEM-C and pGEM-D, ranging from $1 \times 10^5$ to $1 \times 10^9$ copies/μL,
was used to construct the calibration curve for *C. kluyveri* and total eubacteria, respectively.
The concentration of the plasmid was measured using a fluorometer and the corresponding copy
number was calculated using the following equation [34]:

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23} (\text{copy/mol}) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 (\text{g/mol/bp})}$$

Where Avogadro’s number is $6.02 \times 10^{23}$ copies/mol, and the average molecular weight of
one DNA base pair (bp) is 660 g/mol. The $C_T$ values obtained from analyzing serially diluted
standard solution are plotted versus the logarithm of the corresponding initial concentrations.
The $C_T$ values were plotted against the logarithm of their initial template copy numbers. A
linear regression of the plot forms the calibration curve so that the concentration of target
sequence in an unknown sample can be estimated by interpolation.

Characterization of the microbial community based on PCR-DGGE analysis

Whole genomic DNA was extracted by the same method as mentioned before. A nested
PCR was employed, and all PCR primers used in this study derived from a previous study [23].
Before DGGE analysis, PCR products were examined by electrophoresis on 1% agarose gels.
The DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) was used for
sequence-specific separation of PCR amplified fragments, and DGGE was performed according
to the previously reported protocols [30]. Representative bands observed on the DGGE profiles
were excised, eluted at 4°C in ultrapure water, and then re-amplified. PCR products were
purified with a universal PCR purification kit (Tiangen, Beijing, China) and sent to a
commercial sequencing company for cloning and sequencing (Sangon, Shanghai, China). The
sequences were then compared with 16S rRNA gene sequences available in the GenBank by
BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and RDP CLASSIFIER
(http://rdp.cme.msu.edu/index.jsp) searches to identify their closest phylogenetic relatives.

The unique sequences obtained from this study were deposited in GenBank with the
following accession numbers: MG729405-MG729417 and MG729419-MG729437 (the 16S
rRNA V3 region sequences of eubacteria).

**Data analysis**

All assays were conducted in triplicate, and the results were expressed as mean ± standard
deviations. Analysis of variance (ANOVA) was used to test the significance of each assay.
Results were considered to be statistically significant at p < 0.05. Community diversity was
determined using Quantity One Software, which was used to covert individual DGGE lanes to
densitometric profiles. Simpson dominance index (D), Species richness (S), Evenness index (J)
and the Shannon-Wiener index (H) were determined based on the number and relative
intensities (RIs) of bands, and were calculated using ZZSTAT V2010.

**Results and Discussion**

To design a specific oligonucleotide probe for the phylogenetic *Clostridium* group,
BLAST and RDP CLASSIFIER was used to align and identify the obtained 16S rRNA
sequences. A target site was identified in almost all available sequences of the *Clostridium*
group, including *Clostridium* genus and *C. kluyveri* species from subgroup. An evolutionary
distance tree of the *Clostridium* (Appendix) outlines the different *Clostridium* groups and the
targets of probes used in the study. The KCLZ target site, with an adenosine-thymine wobble at position, and the CLZ target site, with an adenosine-guanine wobble at position were listed in Appendix. The specificity and coverage of the new probes were evaluated and tested by SILVA [35]. The results revealed high specificity for both probes: the probe KCLZ exhibited a coverage of 4.3% and a specificity of 100% for the species “C. kluyveri”, the probe CLZ demonstrated a coverage of 31.7% and a specificity of 100% for the genus “Clostridium”.

**Exploring the feasibility of quantitative determining genus and species based on CLZ and KCLZ probe**

Three environmental and six methodological factors should be taken into account for the detection of bacteria by FISH technique [36]. In our present experiment, these environmental factors (type of ecosystem, bacteria growth rate and dominant phylogenetic group) were little to choose. Of these methodological factors (fixative, fluorochrome, hybridization temperature, formamide concentration in hybridization buffer, NaCl concentration in wash solution and counting method), formamide concentration in hybridization buffer and NaCl concentration in wash solution are crucial to hybridization efficiency [37]. The hybridization of pretreated pit mud sample with both probes were performed by the hybridization buffer containing 10%, 20%, 30%, 40%, 50%, 60% and 70% of formamide, respectively. The result showed that 30% of formamide was the most efficient, which was similar to the results by EUB338 [24]. As shown in Fig. 1A, compared with 30% of formamide contained in the hybridization buffer, the quantity of detected target microorganism (C. kluyveri) was lower, and the intensity of fluorescence background was weaker in the cases of 10% and 20% formamide content in buffer. Formamide
decreased the melting temperature by weakening the hydrogen bonds, thus enabling lower temperature to be used with high stringency. Therefore, enhancing the formamide concentration increases the specificity of the hybridization advisably, but once it exceeds its optimal concentration and could brought about a drastic drop of bound probe and signal intensity [38]. We have observed similar tendency in our result: a signal intensity drop when 50% or 60% of formamide was contained in buffer. Meanwhile, the effect of formamide concentration on the hybridization efficiency of probe CLZ was similar to that of probe KCLZ. As shown in Fig. 1B, the quantity of detected target microorganism (Clostridium) was lower with 10%, 20% and 40% of formamide concentration, and the intensity of fluorescence background was also weaker in these cases. Both the hybridization signals of target and the intensity of fluorescence background were enhanced with 50%, 60% or 70%. Under the condition of the hybridization buffer of 30% formamide, the fluorescence signals of target were strong and evenly distributed, and the fluorescence background was relatively moderate, which can accurately quantify the number of Clostridium in complex systems. Otherwise, the quantity of detected target microorganism by CLZ probe was higher than KCLZ probe.

Validating the specificity and accuracy

FISH experiments using CLZ and KCLZ probes, respectively, for the pretreated pure reference strains pellets, were performed. The results confirmed the genus-specificity of CLZ and species-specificity of KCLZ probe. Using CLZ probe, it only hybridized with Clostridium, for example C. kluyeri, C. butyricom and C. perfringens, but cannot hybridize with L. casei and B. subtilis, as shown in Fig. 2.
The detected result of the spiked pit mud with the pure culture of *C. kluyeri* \((1.12 \times 10^9\text{ cells/mL})\) was shown in Table 2. It indicates that the recovery rates of various probes were different: 83.05%, 49.11% and 32.14% for EUB338, CLZ and KCLZ, respectively. The efficiency of KCLZ probe was slightly lower compared to that of EUB338 and CLZ probe, which may be caused by different probes behavior, such as the position of the probe’s target. As we known, EUB338, an eubacterial probe used in exploring the microbial abundance in complex systems, was also an overall median of 56% in enriched culture, ranged from 1% to 100% by the influence of various factors [36]. Furthermore, the increased trend in the spiked samples had been revealed, the difference between the spiked quantity and the detected quantity was not significant.

The sensitivity of the probes had been verified by spiking pure cultures into pit mud. As shown in Fig. 3, the quantity of *Clostridium* in the samples spiked increased with volume of the culture that former was a mixture, which included three strains of *Clostridium*’s (The proportion of *C. kluyeri*, *C. butyricom* and *C. perfringens* was 32.21%, 29.98% and 37.81% in the mixture of pure culture, respectively) and later was the pure of *C. kluyeri*.

These probes were used to quantify *Clostridium* spp. and *C. kluyeri* in pit mud samples taken from three different pits, which had all the same age. They were also used for the samples of BFG and SFG from the same pit. As shown in Table 3, no significant difference between the samples in the same niches (pit mud) was found for the results detected by various types of used probes, included EUB338, CLZ and KCLZ probe, but the discrepancy between the samples originated from different phase in the same pit was observed. BFG was regarded as a micro-aerobic phase structured with a large number of voids, and the properties differed from
PM’s. Compared with PM, it may not be suitable for propagating *Clostridium* spp. and *C. kluyeri*. Therefore, the concentration of both in BFG was lower than ones of pit mud’s, similar to the result obtained by Ding, *et al.* [27]. Nevertheless, the proportion of *Clostridium* spp. and *C. kluyeri* in SFG decreased from 36.9% to 27.1%, and 17.9% to 12.3%, respectively, although the quantity of eubacterium and the microbes detected by the CLZ and KCLZ probe were slightly lower than that in BFG. It may be caused by various factors, such as added *Daqu* powder, loss of moisture, etc. It was noteworthy that *C. kluyeri* was detected by KCLZ probe even if the fermented grains were placed in aerobic environment for more than four hours, and then streamed at atmospheric pressure for more than 60 min. Similar result, the thermal death pure culture of *E. coli* by autoclaved, was also observed in our preliminary test. But the fluorescence intensity was weak and the number of cells was low through FISH technique. The possible reason was that *E. coli* was more resistant to thermal than other bacilli, it just led to lower cell activity and no death. It is one of major features that FISH exhibits to detect the physiologically active bacteria in relatively nutrient-rich environments [39]. One can speculate that, due to the thermal denaturation of RNA, the hybridization activity with probe decreased, but no target loss for FISH analysis. Therefore, these nonholonomic RNA residual in the samples may explain the overestimation of specific-target genus and species. However, the result shows that the efficiency of designed species-specific probe was suitable to characterize quantitatively the abundance of *C. kluyeri* in the sample.

**Validating based on quantitative analysis of qPCR**
The 16S rRNA gene copies of *C. kluyeri* and eubacteria in pit mud and fermented grains samples were quantitatively analyzed by using specific primers CloKly1F/CloKly1R and universal primer 357F/517R based on the 16S rRNA gene fragment of *C. kluyeri*. As shown in Fig. 4, the correlation coefficient ($R^2$) values of the linear regression analysis based on the result of the calibration curves constructed for eubacteria and *C. kluyeri* were 0.998 and 0.997, and the amplification efficiency (E) values were 0.966 and 0.940 according to the formula described by Knutsson, *et al.* [40]. The values of eubacteria quantity were $2.87 \times 10^8$ copies/g, wet, $6.31 \times 10^7$ copies/g, wet, and $1.37 \times 10^8$ copies/g, wet, while the ones of *C. kluyeri* quantity were $8.16 \times 10^6$ copies/g, wet, $7.88 \times 10^4$ copies/g, wet, and $1.52 \times 10^4$ copies/g, wet in PM, BFG and SFG samples, respectively. The magnitude order of them was consistent with the results detected by FISH technique, but the abundance of *C. kluyeri* was present at the level of 2.843%, 0.125% and 0.011%, by contraries, ones identified by FISH were 21.463%, 17.857% and 12.258%, in PM, BFG and SFG, respectively. On the whole, the difference of *C. kluyeri* abundance feature among these microsystems was confirmed by the result of qPCR. Relative to the conventional PCR, qPCR is the most sensitive and precise method [41], it has been widely applied in many fields, such as ecology, diagnostics, etc. However, its precision has been also limited due to some inherent limitation and biases of PCR. Of these influencing factor [42], one of the major reasons why specific-groups was quantitatively overestimated was nonviable amplified DNA. For Chinese Luzhou-flavor liquor brewing process, overestimation is a particular problem since the pit mud has been used, and the fermented grains was partly recycled into the pit. Therefore we can assert that it could be impossible to quantify target-genus
and species in such complicated systems even if the estimation error derived from nonviable DNA could be mitigated by various developed approaches [43, 44].

Validating based on PCR-DGGE analysis

In order to assess qualitatively the accuracy of the designed probe, analysis based on PCR-DGGE for PM, BFG and SFG was performed. DGGE profiles of the eubacteria are shown in Fig. 5A. Based on the analysis of DGGE profiles, diversity indices were determined. The results show that the difference of the microbial diversity index between PM, BFG and SFG was significant (As shown in Table 4). The Dominance index (D) of PM’s eubacteria was higher than BFG and SFG. D value and Evenness index (J) of BFG were lower than SFG’s; as for the other index such as species richness (S) and Shannon-winner index (H), an opposite tendency can be observed between these two samples (BFG and SFG). For investigating the eubacterial community structure in PM, BFG and SFG, representative bands from the PCR-DGGE were carefully excised, recovered and sequenced, and the results were shown in Table 5. The eubacterial DGGE pattern of the 16S rDNA V3 regional gene fragment amplicons showed that different numbers of bands were detected in different samples. The similarity of all band sequences was nearly >98% comparing with those available in the GenBank database. All eubacterial 16S rRNA sequences fell into three phyla (Proteobacteria, Bacterodetes and Fimicutes), comprising thirteen genera that were affiliated with six orders in five classes. Class α-proteobacteria (order Rhodospirillales, bands 6, 7, 8, 20, 21, 27, 28, 29, 31 and 32), class γ-proteobacteria (order Enterobacteriales bands 1, 2, 19 and 22) and proteobacterium (band 23) belonged to Proteobacteria. And then, Firmicutes included class Bacilli and class Clostridia,
which involved three orders: Clostridiales, Bacillales and Lactobacillales. Of then, Clostridiales (bands 4, 9, 13, 14, 17, 18, 30 and 31), Bacillales (band 5) and Lactobacillales (band 15) were affiliated with Firmicutes. Besides, Bacterodetes had class Flavobacteriia, then, Flavobacteriia included order Flavobacteriales (band 3). Of these bacteria, Acetobacter spp., Clostridium spp. and Enterobacter spp. were dominant. As shown in Fig. 5B, all eubacteria were detected in PM, and the abundance of Clostridium was 22%, calculated based on the rate of their relative intensity detected (IR’s value). Clostridium genera was also detected in fermented grain samples, and the abundance were 16% and 14% in BFG and SFG samples, respectively. The difference of Clostridium spp.’s abundance between these samples was also similar with FISH’s. Species C. kluyveri was also identified in PM, BFG and SFG. In fact, C. kluyveri was often used to convert alcohol into a mixture of acetic, butyric and caproic acids with methane bacteria [45], as one of important contributors to form representative aroma and flavor compounds in Luzhou-flavor type liquors.

In conclusion, the specific Clostridium spp. and C. kluyveri probes set CLZ and KCLZ, based on the available 16S rRNA genes, were designed and verified by experimental tests. The specificity of designed probes was tested using the pure reference strains pellets. Combined with FISH technique, the Clostridium diversity of PM and fermented grains samples was successfully investigated, and shown the same regularity. The results were confirmed by qPCR and PCR-DGGE analysis on the quantititative results of microbes in the cellar. Overall, this study provides a method on quantitative analysis of the Clostridium and monitoring their community dynamics in various fermentation systems.
Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References


Figure legends

**Fig. 1.** Epifluorescence micrographs of samples hybridized with KCLZ (A) and CLZ (B) probes in the hybridization buffer of optimal (30%) concentration.

**Fig. 2.** Epifluorescence micrographs of the pure reference strains hybridized with CLZ and KCLZ probes.

**Fig. 3.** The effects of different volume of the mixtures of pure culture on the *Clostridium* and *C. kluyveri* in the pit mud.

**Fig. 4.** Calibration curve of the real-time PCR for quantifying the 16S rRNA gene copies of *C. kluyveri* (A) and eubacteria (B); 16S rRNA gene copies of *C. kluyveri* (C) and eubacteria (D) in the samples obtained by qPCR.

**Fig. 5.** DGGE profiles of the eubacteria microbes (A) from V3 region of 16S rRNA obtained from 3 samples and the corresponding relative intensities (%) of the eubacteria microbes (B) in different samples. The sample numbers 1-3 stand for PM, BFG and SFG, respectively.
Table 1 Oligonucleotide sequence and targeted microbes of probes designed in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target group</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
</table>
| KCLZ  | Clostridium kluyveri | 5’-CY3-
|       |                    | CCTGCACACCCTTACGCCAGTAATTCGGAACCA-3’   |
| CLZ   | Clostridium        | 5’-CY3-GGCTACCTTGTTACGACTTCACCCA-3’    |
Table 2  Concentration of microbes in blank samples and spiked samples.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Blank samples ($\times 10^9$ cells/g)</th>
<th>Spiked samples ($\times 10^9$ cells/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLZ</td>
<td>1.06±0.09</td>
<td>1.61±0.12</td>
</tr>
<tr>
<td>KCLZ</td>
<td>0.87±0.10</td>
<td>1.23±0.09</td>
</tr>
<tr>
<td>EUB338</td>
<td>1.81±0.41</td>
<td>2.74±0.33</td>
</tr>
</tbody>
</table>
Table 3 Concentration of microbes in the different pit mud samples of the same aged of pit, BFG samples and SFG samples.

<table>
<thead>
<tr>
<th>Probes types</th>
<th>$1^a (\times 10^7$</th>
<th>$2^b (\times 10^7$</th>
<th>$A^c (\times 10^7$</th>
<th>BFG$(\times 10^7$</th>
<th>SFG$(\times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>264</td>
<td>281</td>
<td>205</td>
<td>0.84</td>
<td>1.55</td>
</tr>
<tr>
<td>CLZ</td>
<td>102</td>
<td>98</td>
<td>73</td>
<td>0.31</td>
<td>0.42</td>
</tr>
<tr>
<td>KCLZ</td>
<td>65</td>
<td>67</td>
<td>44</td>
<td>0.15</td>
<td>0.19</td>
</tr>
</tbody>
</table>

$^a$, $^b$, and $^c$ are pit mud samples taken from different pits with same pit age, respectively.
Table 4 Diversity indices of eubacteria in the samples calculated from the DGGE banding patterns shown in Fig. 5A.

<table>
<thead>
<tr>
<th>Type</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PM</td>
</tr>
<tr>
<td>Eubacteria Dominance index ($D$)</td>
<td>0.053</td>
</tr>
<tr>
<td>Species richness ($S$)</td>
<td>21</td>
</tr>
<tr>
<td>Evenness index ($J$)</td>
<td>0.983</td>
</tr>
<tr>
<td>Shannon-winner index ($H$)</td>
<td>2.993</td>
</tr>
</tbody>
</table>
## Table 5 Eubacteria sequence alignment with BLAST and RDP search tools.

<table>
<thead>
<tr>
<th>Type</th>
<th>Band no.</th>
<th>Closest relatives</th>
<th>Accession no.</th>
<th>Identity (%)</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eubacteria</strong></td>
<td>1</td>
<td><em>Enterobacter</em> sp.</td>
<td>KX914564.1</td>
<td>100</td>
<td><em>Enterobacter</em></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Enterobacter</em> sp.</td>
<td>KY065507.1</td>
<td>100</td>
<td><em>Enterobacter</em></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Chryseobacterium</em> sp.</td>
<td>KY117480.1</td>
<td>100</td>
<td><em>Chryseobacterium</em></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Uncultured <em>Clostridium</em> sp.</td>
<td>LC055608.1</td>
<td>100</td>
<td><em>Clostridium</em></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Staphylococcus</em> sp.</td>
<td>KX881396.1</td>
<td>100</td>
<td><em>Staphylococcus</em></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><em>Acetobacter pasteurianus</em></td>
<td>KX424632.1</td>
<td>98</td>
<td><em>Acetobacter</em></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Uncultured <em>Rhodospirillaceae bacterium</em></td>
<td>LT625193.1</td>
<td>99</td>
<td>bacterium</td>
</tr>
<tr>
<td></td>
<td>8,21,27,28,29</td>
<td><em>Acetobacter pasteurianus</em></td>
<td>CP015168.1</td>
<td>99</td>
<td><em>Acetobacter</em></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td><em>Blautia</em> sp.</td>
<td>LT631509.1</td>
<td>99</td>
<td><em>Blautia</em></td>
</tr>
<tr>
<td></td>
<td>10,12</td>
<td>Uncultured bacterium</td>
<td>EU234163.2</td>
<td>100</td>
<td>bacterium</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Uncultured <em>Lachnoanaerobaculum</em> sp.</td>
<td>KU359740.1</td>
<td>100</td>
<td><em>Lachnoanaerobaculum</em></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td><em>Clostridium autoethanogenum</em></td>
<td>CP012395.1</td>
<td>100</td>
<td><em>Clostridium</em></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Uncultured <em>Ruminococcus</em> sp.</td>
<td>KX672523.1</td>
<td>99</td>
<td><em>Ruminococcus</em></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Uncultured <em>Lactobacillus</em> sp.</td>
<td>KU674948.1</td>
<td>100</td>
<td><em>Lactobacillus</em></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Uncultured bacterium</td>
<td>KJ853484.1</td>
<td>99</td>
<td>bacterium</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td><em>Clostridium kluyveri</em></td>
<td>LC149721.1</td>
<td>99</td>
<td><em>Clostridium</em></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Uncultured <em>Clostridium</em> sp.</td>
<td>JX575865.1</td>
<td>99</td>
<td><em>Clostridium</em></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td><em>Enterobacter ludwigi</em></td>
<td>KX959985.1</td>
<td>100</td>
<td><em>Enterobacter</em></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td><em>Acetobacter tropicalis</em></td>
<td>KX424645.1</td>
<td>99</td>
<td><em>Acetobacter</em></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td><em>Enterobacter asburiae</em></td>
<td>KU724380.1</td>
<td>100</td>
<td><em>Enterobacter</em></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td><em>Gamma proteobacterium</em></td>
<td>LC168577.1</td>
<td>100</td>
<td>proteobacterium</td>
</tr>
<tr>
<td></td>
<td>24,25</td>
<td>Uncultured <em>Caloramator</em> sp.</td>
<td>KX672807.1</td>
<td>100</td>
<td><em>Caloramator</em></td>
</tr>
<tr>
<td></td>
<td>26</td>
<td><em>Gluconobacter</em> sp.</td>
<td>KX578017.1</td>
<td>100</td>
<td><em>Gluconobacter</em></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Uncultured <em>Clostridium</em> sp.</td>
<td>LC036237.1</td>
<td>100</td>
<td><em>Clostridium</em></td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>Uncultured <em>Ruminococcus</em> sp.</td>
<td>KX672726.1</td>
<td>99</td>
<td><em>Ruminococcus</em></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td><em>Acetobacter nitrogenifigens</em></td>
<td>KC763598.1</td>
<td>100</td>
<td><em>Acetobacter</em></td>
</tr>
</tbody>
</table>
Fig. 2
The volume of mixed-culture solution (mL)

\[ y = 0.2255x + 8.4953 \]
\[ R^2 = 0.9868 \]

The volume of pure-culture solution (mL)

\[ y = 0.2847x + 8.2213 \]
\[ R^2 = 0.9466 \]

**Fig. 3**
4.

Fig. 4

A. 
\[ y = -3.475x + 39.967 \]
\[ R^2 = 0.998 \]

B. 
\[ y = -3.407x + 37.318 \]
\[ R^2 = 0.997 \]

C. 

![Graph showing Clostridium kluwyveri 16S rRNA gene copies](image)

D. 

![Graph showing total eubacteria 16S rRNA gene copies](image)
Fig. 5