

Identification and enumeration of *Clostridium* spp. In sufu

Xu Qiong¹, Liu Yang^{1*}, Yu Yi¹, Zhang Nana¹, Wang Yue¹, Zhao Lei¹, Weng Shiyu¹

¹Shanghai Institute of Quality Inspection and Technical Research, Shanghai, China

Abstract. In this study, *Clostridium* spp. was counted in 49 Chinese commercial sufu samples, and 25 samples were detected *Clostridium* in all samples: the detection rate was 51.02%. About 93.87% of the samples contained *Clostridium* at low levels (log CFU/g < 3.0) and one sample had over 4.12lg CFU/g indicating potential hazard to consumers. The 16S rDNA identification was carried out on the genus *Clostridium*, and the isolates were confirmed as *Clostridium botulinum*, *Clostridium sporogenes*, *Clostridium beijerinckii* and *Clostridium bifermentans*, while *Clostridium butyricum*, *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium bifermentans* were the species detected in the sufu samples by high-throughput sequencing. Based on these results, the research on the number and distribution of clostridium and the possible risks of biotoxins in fermented food needs to be strengthened. To our knowledge, this is the first study to isolate and identify *Clostridium* spp. in sufu.

1 Introduction

Sufu is a kind of traditional fermented bean curd with more than 1000 years of history in China. It is a cheese-like, creamy, soft product made from tofu and fermented by microorganisms. It is also known as “Chinese cheese”^[1]. The manufacturing of Sufu is mostly hand work in a semi-open environment without heat sterilization. Sufu may be contaminated with microbes from the air. This would influence Sufu quality and even increase hazards and risks in food safety^[2].

In the previous work, we found that the relative abundance of *clostridium* in one sufu sample was 7.12% by high-throughput sequencing^[3]. *Clostridia* are gram-positive, spore-forming, anaerobic bacteria which are considered to be the principal organisms responsible for late blowing^[4]. A number of cases of botulism have been reported for sufu products. In 2017, a botulism outbreak was associated with by eating homemade stinky tofu and self-cured soybean products^[5] and in 1998, also a homemade sufu caused an outbreak^[6]. Inoculation studies with the causative strain showed the growth of *Clostridium botulinum* and toxin formation in the sufu.

The aim of the study was to count, isolated and identify *Clostridium* spp. in sufu samples. Illumine-based sequencing with *clostridium* was to study the diversity of the species. The identification was performed by 16S rDNA sequencing, and high throughput sequencing was used to analyze the bacterial flora in sufu samples.

2 MATERIALS AND METHODS

2.1 Sufu samples

A total of 47 sufu samples were collected from China. Sufu samples were collected from markets in different regions of China (Table1)

Table1 sufu samples collected from variances of china

sample code	production location	sample code	production location
C1	Beijing	B1	Hunan
C2	Beijing	B2	Beijing
C3	Zhejiang	B3	Zhejiang
C4	Anhui	B4	Jiagnsu
C5	Zhejiang	B5	Anhui
C6	Zhejiang	B6	Anhui
C7	Anhui	B7	Sichuan
C8	Shandong	B8	Sichuan
C9	Heilongjiang	B9	Guangdong
C10	Sichuan	B10	Guangdong
C11	Jilin	B11	Guangdong
C12	Anhui	B12	Jiagnxi
C13	Shandong	B13	Guagnxi
H1	Hunan	B14	Sichuan
H2	Beijing	B15	Guangxi
H3	Beijing	B16	Beijing
H4	Zhejiang	B17	Fujian
H5	Jiagnsu	B18	Sichuan
H6	Guangdong	B19	Sichuan
H7	Tianjin	B20	Guangxi
H8	Beijing	B21	Guangxi
H9	Shanghai	B22	Zhejiang
		B23	Jiangxi

Xu qiong (1987-) (ORCID: 0000-0001-5323-0756), E-mail: xuqiong@sqi.org.cn

* Corresponding author: Liu yang(1980-)(ORCID:0000-0002-5665-034X), E-mail: liuyang@sqi.org.cn

B24	Jiangxi
B25	Zhejiang
B26	Jiagnsu
B27	Shanghai

2.2 Microbiological sampling

The content of *Clostridium* was analysed according to GB 4789.13-2012. In brief, 25 g of sample were transferred to nine times the volume (approximately 225 ml) of BPW and homogenised in a stomacher for one minute. A 10-fold serial dilution in 0.1 per cent (v/v) peptone water (Luqiao) was prepared and 1.0 ml from each dilution was poured onto a Differentia Reinforced Clostridial Agar (DRCA) agar (Haibo) on a petri dish measuring 9 cm in diameter. An additional 10 ml DRCA was poured as an overlay. All agar plates were incubated in an anaerobic atmosphere generated by the use of MARK II (ANOXOMAT) at 37°C for 48±3 hours. The number of *Clostridium* was expressed as log CFU/g.

2.3 DNA extraction and PCR amplification

2.3.1 DNA extraction

DNA was isolated from 1ml liquid cultures and from several colonies obtained from agar plates. The DNA was extracted with MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0(TaKaRa) according to the manufacturer's instructions. The extracted DNA was frozen at -20°C or directly subjected to PCR analysis.

Sufu genomic DNA was extracted from each sample using Modified CTAB method was used: three samples of the same batch were taken and 50 mL Sufu soup was poured from each sample, totaling 150 mL. After mixing, the mixed sample soup was centrifuged for 10 min at 9000 rpm, then the supernatant was removed, and the sediment was added with 10 mL of sterile water. The supernatant was removed by centrifugation at 9000 rpm for 10 min. The supernatant was added with 800µL lysozyme (10mg/mL) and the sediment was bathed in water at 37 C for 1 h. Then 40 µL protease K and CTAB lysate were added to the sediment, and the water bath was held at 56 C for 2 hours. Tris saturated phenol/trichloromethane/isoamyl alcohol with the ratio of 25:24:1 was added to the cracking solution of each tube. The mixture was centrifuged at 13000 rpm for 10 min. The supernatant was put into the new centrifugal tube, added with trichloromethane: isoamyl alcohol (24:1) of same volume, mixed well, centrifuged at 13000 rpm, for 10 min. The supernatant was moved to the new centrifugal tube and mixed with 2-fold volume of anhydrous ice ethanol. The supernatant was kept for about 30 minutes, then centrifuged at 13000 rpm, for 2 minutes. The supernatant was discarded and precipitated 2-3 times with 70% ethanol. The precipitation was placed at room temperature for natural air drying. After air drying, 50 L TE solution was added to dissolve the precipitation.

2.3.2 Illumina MiSeq sequencing

Purified DNA was used as the template for PCR amplification for the bacterial 16S rRNA genes. The V1-V3 variable regions of the bacterial 16S rRNA gene were amplified by the primer pair 27 F (5'-AGAGTTTGTAT CCTGGCTCAG-3') and 515R (5'-ATTACCGCGGCTGCTGG -3') , and the internal transcribed spacer 2 (ITS 2) region of fungi amplicons was amplified with forward primer ITS2F (5'-GCATCGATGAAGAACGCAGC-3') and reverse primer ITS2R (5'- TCCTCCGCTTATTGATATGC-3') primers. The PCR conditions were as follows: initial denaturation at 94°C for 2 minutes; denaturation at 94°C for 30 seconds, annealing at 60°C for 50 seconds; extension at 72°C for 45 seconds, amplification for 40 cycles; preservation at 4°C. The quality of the amplified PCR products was checked by electrophoresis in 1 % agarose gel. Each primer was binded different barcodes and the same adaptors. PCR amplicons were purified and sequenced on the IlluminaMiSeq platform.

2.4 Processing of high-throughput sequencing data[7]

2.4.1 Pre-process

FASTQC(<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) is used for the quality control to check on the quality of raw data. Because the data is generated by Illumina, the universal adaptor from the raw data should be removed by CUTADAPT (<http://code.google.com/p/cutadapt/>). Pair end reads are joined together using FLASH (<https://sourceforge.net/projects/flashpage/files/>). Reads are de-multiplexed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline. First, using "split_libraries_fastq.py" which performs demultiplexing of Fastq sequence data where barcodes and sequences are contained in two separate fastq files above (common on Illumina runs) to get a fasta file which contains the sequence of 16S rRNA and a fasta file of the quality of the sequence file. Removing the chimera sequences in the data. Using "identify_chimeric_seqs.py" which identify chimeric sequences in input FASTA file with usearch61 algorithm to identify the chimera and using "filter_fasta.py" module to remove the chimeric sequences.

2.4.2 Clean data Analysis

Sequences were grouped into operational taxonomic units (OTUs) at 97% sequence similarity using the Greengenes reference database. OTUs that did not cluster with known taxa at 97% identity or higher in the database were clustered de novo and not de novo (UCLUST)^[8].

2.5 Statistical analysis

All experiments were based on a completely randomized block designs, and were performed in triplicate. Results were presented as mean±standard deviation(SD) of replicated measurements.

Table 2. Microbiological composition of commercial sufu (log CFU/g sample)

Sample	<i>Clostridium</i>	Sample	<i>Clostridium</i>	Sample	<i>Clostridium</i>
H1	<1	C1	<1	B1	1.66±0.21
H2	2.02±0.30	C2	<1	B2	<1
H3	2.06±0.46	C3	2.98±0.16	B3	<1
H4	2.45±0.34	C4	<1	B4	<1
H5	<1	C5	1.26±0.24	B5	<1
H6	<1	C6	1.10±0.17	B6	<1
H7	1.64±0.19	C7	<1	B7	<1
H8	1.10±0.17	C8	1.83±0.56	B8	<1
H9	1.39±0.12	C9	1.00±0.00	B9	3.02±0.59
		C10	3.15±0.11	B10	<1
		C11	<1	B11	1.98±0.55
		C12	<1	B12	1.10±0.17
		C13	4.12±0.03	B13	1.08±0.19
				B14	<1
				B15	1.23±0.40
				B16	<1
				B17	<1
				B18	2.04±0.15
				B19	<1
				B20	1.00±0.00
				B21	1.00±0.00
				B22	2.80±0.37
				B23	<1
				B24	<1
				B25	<1
				B26	2.04±0
				B27	1.00±0

From Table 2, 51.02% of the samples contained *Clostridium* spp. were detected in 49 Sufu samples. Among them, the detection rate of red sufu, white sufu and gray sufu was 66.67%, 44.44% and 58.85% respectively. (24 products, no isolates could be made with the present methodology. The number of *Clostridium* spp. varied between 1.00 and 4.12 log CFU/g. About 93.87% of the samples contained *Clostridium* at low levels(log CFU/g < 3.0), but sample B9, C10 and C13 contained 3.02±0.59, 3.15±0.11 and 4.12±0.03 log CFU/g respectively.

Bacterial colonies were isolated and purified from the growing and counting plate specimens and identified by 16S rDNA. By Blast comparison, the suspected bacteria were mainly identified as: *Clostridium beijerinckii*, *Clostridium bifermentans*, *Clostridium botulinum* and *Clostridium sporogenes*.

3 RESULTS AND DISCUSSION

3.1 Microbiological analysis

3.2 Illumina MiSeq Sequencing analysis

The bacterial flora in the sufu samples was analyzed. A total of 14 samples were found to be *Clostridium* by high-throughput sequencing, as shown in Table 3.

The relative abundance of *Clostridium* genus in the samples was very low (< 1%), except for samples B15, H8 and C7. The most relative abundance of *Clostridium* in B15 samples was 6.9972%. *Clostridium butyricum*, *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium bifermentans* were the species detected in the sufu samples by high-throughput sequencing. Dominant bacteria in each sample have certain differences: relative abundance of *Clostridium butyricum* in sample H8 is 95.07%. Relative abundance of *Clostridium Bifermentans* in sample C7 is 69.96%. In sample B15, *Clostridium perfringens* relative abundance is 67.52%. However, 43.24% of B22 were still unable to identify specific species.

Clostridium botulinum and *Clostridium bifermentans* were detected by High throughput sequencing: the results are consistent to 16s rDNA sequencing. *Clostridium perfringens* is a common pathogen, high

relative abundance was found in B15, B22 and C5 samples, respective 67.52%, 29.73% and 56.52%.

Table 3. Relative abundance of *Clostridium* in sufu samples

	<i>Clostridium</i> spp	<i>Cl.butyricum</i>	<i>Clostridium;Other</i>	<i>Cl.botulinum</i>	<i>Cl.perfringens</i>	<i>Cl.bifermentans</i>
B7	0.01%	100%	0	0	0	0
B10	0.01%	100%	0	0	0	0
H1	0.06%	100%	0	0	0	0
B15	7.00%	0.34%	5.04%	26.58%	67.52%	0.51%
B22	0.18%	16.22%	43.24%	10.81%	29.73%	0
H2	0.02%	100%	0	0	0	0
H3	0.01%	100%	0	0	0	0
H5	0.02%	0%	100%	0	0	0
H7	0.01%	100%	0	0	0	0
H8	2.13%	95.07%	1.57%	2.24%	1.12%	0
C5	0.16%	8.70%	4.35%	30.43%	56.52%	0
C6	0.01%	0	50.00%	0	0	50.00%
C7	2.14%	2.93%	17.95%	6.59%	2.56%	69.96%
C8	0.10%	30.00%	30.00%	20.00%	20.00%	0

However, We did not identify *Clostridium perfringens* in the all samples by 16S rDNA, similar to *Clostridium butyric*. The results showed that there were some differences between the results of high-throughput sequencing and traditional microbial culture identification methods. Because of the unsuitable culture conditions, some microorganisms cannot be screened by traditional methods.

Due to the special production process of sufu, it is possible to be contaminated by *Clostridium*. In this study, the *Clostridium* species in the samples were counted, and the virulence types of the isolates were not studied in depth. In addition, because most of the *Clostridium* is fastidious bacteria, the culture cycle is long and the spores are easy to form, so the common bacterial identification methods are not easy to obtain satisfied results. There may still be other species of *Clostridium* that have not been cultured. However, it is necessary to pay attention to the existence of these bacteria in sufu. The number, distribution and biotoxin of *Clostridium* need to be strengthened.

REFERENCES

1. K H Steinkraus. Handbook of indigenous fermented foods. 2nd ed., rev. and expanded.[J]. Microbiology, 1996,**62**,3,131.
2. Yu Shizhe, Fan Yi, Han Beizhong, et al. Analysis of microorganism during the production and processing environment of low-salt sufu[J]. China Brewing, 2017,**36**,12,18-22.
3. XU Qiong, LIU Yang, QU Qinfeng, et al. High-throughput sequencing analysis of bacterial diversity in red sufu from different regions[J]. Food Science, 2020,**10**,41,110-116.

4. Cremonesi P, Vanoni L, Silveti T, et al. Identification of *Clostridium beijerinckii*, *Cl. butyricum*, *Cl. sporogenes*, *Cl. tyrobutyricum* isolated from silage, raw milk and hard cheese by a multiplex PCR assay[J]. Journal of Dairy Research, 2012,**79**,3,318-323.
5. Chang Wenguang, Liu Yi, Liu Peng, et al. Clinical analysis of 5 cases of botulinum toxin B poisoning [J]. Journal of Clinical Neurology, 2017,**1**,
6. Li Bangchuan, Chen Nanjun, Li Yong. An investigation report of botulism caused by homemade fermented bean curd [J]. PLA Journal of Preventive Medicine, 1998.
7. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data[J]. Nat Methods, 2010,**4**,5,335-336.
8. C E R. Search and clustering orders of magnitude faster than BLAST[J]. Bioinformatics, 2010,**26**,19,2460-2461.