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Research Article

Application of Nucleic Acid Reference Material for Rapid Detection of *Cronobacter sakazakii* (*Cronobacter* spp.) in *Flammulina velutipes*

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Cronobacter sakazakii (*C. sakazakii*) widely exists in the environment and is a common foodborne pathogenic microorganism. It can cause meningitis, bacteremia, and necrotizing enterocolitis in infants and elderly people with low immunity. Therefore, the development of the rapid detection method for foodborne pathogen *C. sakazakii* is very important for food safety control. This study has developed a kind of genomic nucleic acid reference material as a positive reference for the rapid detection of *C. sakazakii* in the edible fungus *Flammulina velutipes*. First, the genomic nucleic acids were extracted from the standard strain of *C. sakazakii*, and the nucleic acids were lyophilized. The uniformity test result of the lyophilized powder showed that there were no significant differences between samples. In short-term stability tests at 4°C and 37°C for 14 days, the properties of the nucleic acid reference material were good, and in long-term stability tests at 4°C and −20°C for 8 months, the samples are still stable. To verify the application of this nucleic acid reference material, it was used as a positive control template for PCR detection and applied to the rapid detection of *C. sakazakii* in *F. velutipes*. It was found that 13 of the 15 *F. velutipes* samples were positive for *C. sakazakii*. As far as we know, this is the first attempt to detect *C. sakazakii* in *F. velutipes* using the PCR method with nucleic acid reference material as the positive reference. This study is helpful in promoting the extensive and in-depth application of nucleic acid reference materials in foodborne pathogens detection.

1. Introduction

Foodborne pathogens are pathogenic bacteria that can cause food poisoning or use food as a medium [1]. They threaten human health and can cause serious food safety problems [2]. There are many types of food pathogens, such as diarrheagenic *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, and *Enterobacter sakazakii*. *Cronobacter sakazakii*, previously called *Enterobacter sakazakii*, is an facultative anaerobic Gram-negative Bacillus. This pathogenic bacterium could be isolated from a wide variety of foods and has been detected in infant milk powder, meat, water, vegetables, and other foods [3, 4]. Infants and young children are high-risk groups of *Cronobacter* infections [3, 5]. The infections mainly cause bacteremia, meningitis, and necrotizing enterocolitis, and the fatality rate is as high as 50%–80% [6]. Edible fungi may be contaminated by foodborne pathogens during planting, harvesting, packaging, and transportation. It is reported that pathogenic bacteria, such as *L. monocytogenes*, have been

detected in edible fungi [7, 8]. *Cronobacter* spp. also could be detected in dried edible macrofungi samples [9]. A recent study showed that the detection rate of *Cronobacter* spp. is 17.54% (37 positive in 211 samples) in *Flammulina velutipes* from 44 cities in China [10], indicating that the detection of *F. velutipes* contamination should be strengthened to avoid food safety issues.

The traditional detection method of food pathogenic microorganisms is to conduct biochemical identification after enrichment and cultivation of bacteria in the tested sample [11]. However, this detection method has cumbersome steps and a long time-consuming process, which cannot meet the requirements of rapid foodborne pathogen detection. Therefore, the establishment of accurate, sensitive, rapid, and specific detection methods for the detection of food pathogenic microorganisms could play an important role in the prevention and control of food poisoning events. At present, a variety of rapid detection methods have been developed, these methods are mainly polymerase chain reaction (PCR) method and loop-mediated isothermal amplification (LAMP) method based on molecular biology [12], and enzyme-linked immunosorbent assay (ELISA) based on immunology [13]. Among them, the most commonly used methods based on molecular biology are ordinary PCR and fluorescent quantitative PCR [1, 14]. And ordinary PCR is widely used in food inspection department because of its easiness to operate with simple steps, short time-consuming, and easy to conduct [1]. The common point of these detection methods is that they require DNA reference materials as reaction templates. The template can be genomic DNA of pathogenic bacteria or plasmid carrying characteristic virulence genes. So the preparation of nucleic acid reference materials of standard strains of foodborne pathogens as positive control substances could play an important role in rapid detection of foodborne pathogens.

In order to provide a positive reference material for the rapid detection of *C. sakazakii* in food inspection, the study of ready-to-use *C. sakazakii* nucleic acid reference materials was conducted in this paper, and its application in detection of *F. velutipes* was also performed. The research results not only provides a positive reference material but also greatly shortens the detection time, saves the cost, and improves the detection accuracy.

2. Materials and Methods

2.1. Strains and Culture Condition. *Cronobacter sakazakii* FC4146 (CMCC 45404) was obtained from National Institute for Food and Drug Control of China. For genome extraction, the bacterial strains were cultured in BHI, and for enrichment of *C. sakazakii*, the samples to be detected were cultured in modified lauryl sulfate tryptose broth-vancomycin medium (mLST-Vm).

2.2. Preparation of the Genomic DNA Reference Materials of C. sakazakii

2.2.1. Genomic DNA Extraction of C. sakazakii. The bacterial strain was cultured in BHI for 12–16 h, and the bacteria were collected by centrifugation at 8,000 rpm for 10 min at 4°C. The genome extraction was conducted according to the manufacturer's protocol of EasyPure Bacteria DNA Kit (TransGEN, Beijing), and the genome samples were stored at −20°C.

2.2.2. Determination of Genomic DNA Concentration and Purity. One microliter of genomic DNA sample was applied to Thermo NanoDrop 2000/2000 c to measure the purity and concentration of genomic DNA.

2.2.3. Detection of Characteristic Sequence of C. sakazakii. The characteristic sequence of *C. sakazakii* (16S–23S) was determined according to determination of *Enterobacter sakazakii* (*Cronobacter* spp.) from dehydrated powdered milk for export. Part 2: the PCR method with some modifications (SN/T 1632.2-2013; the People's Republic of China Entry-Exit Inspection and Quarantine Industry Standard, 2013). The primers for amplification of 16S–13S were forward primer, GGGTTGTCTGCGAAAGCGAA, and reversed primer, GTCTTCGTGCTGCGAGTTTG. The PCR reaction system (Table S1) and PCR condition (Table S2) are shown in the supplementary materials.

2.2.4. Detection of the Integrity and Purity of Genomic DNA of C. sakazakii. After PCR, 5 μL of genomic DNA was loaded onto a 1% agarose gel (containing dye GoldView), and the electrophoresis was conducted at 175 V constant pressure for 25 min, in the buffer of 1 × TBE. The integrity and purity of genomic DNA was observed under UV detection in a gel imager.

2.3. PCR Specificity and Sensitivity Evaluation. For specificity evaluation, the genomic DNA of *Escherichia coli* O157 : H7 (CMCC 44939), *E. coli* (CICC 10003), *Staphylococcus aureus* (CMCC 26301), *Salmonella enteritidis* (CMCC 50957), *Bacillus amyloliquefaciens* (CCTCC M2013098), *Bacillus subtilis* (CGMCC No. 14221), *Lactobacillus casei* (CGMCC No. 14249), *Enterococcus faecium* (ATCC 29212), *Alcaligenes faecalis* (CCTCC M2017497), and *Listeria monocytogenes* (CMCC 54008) were extracted according to the protocol of EasyPure® Genomic DNA Kit (TransGEN, Beijing), and the genomic DNA were used as templates to amplify the 16S–23S with the same method above, and sterilized water was used as the negative control. The PCR products were analyzed by 1.0% of agarose gel electrophoresis.

For sensitivity evaluation, the genomic DNA of *C. sakazakii* were diluted with sterilized water with gradients of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. And the diluted samples were applied as templates to amplify 16S–23S. The negative control used sterilized water as template in the reaction. The assessment of PCR products was conducted by 1.0% of agarose gel electrophoresis.

2.4. Preparation of Lyophilized Powder of Genomic DNA Reference Material of C. sakazakii. The genomic samples of *C. sakazakii* were extracted and diluted to 20 ng/μL; then, the samples were sent to Chinese Academy of Inspection and Quarantine for lyophilization, with 300 μL of genomic DNA each ampoule. The lyophilized powder was stored at −20°C.

2.5. Homogeneity Determination of Genomic DNA Reference Material of C. sakazakii. Among the 200 bottles of genomic DNA reference materials, 12 bottles were randomly selected, and 300 μL of sterile ddH_2O was added to hydrate the lyophilized powder; then, 1 μL sample was used to determine the concentration, and each sample was tested under the same condition twice. The test data were processed by SPSS with single factor statistical analysis. And the characteristic sequence 16S–23S was determined for each sample.

2.6. Short-Term Stability Test of Genomic DNA Reference Material of C. sakazakii. The short-term storage stability tests were conducted under the condition of 4°C and 37°C. Forty-two bottles are randomly selected and tested three samples on 1, 3, 5, 7, 9, 11, and 14 days at 4°C and 37°C, respectively. The concentration and the characteristic sequence 16S–23S were determined.

2.7. Long-Term Stability Test of Genomic DNA Reference Material of C. sakazakii. Thirty bottles of genomic DNA samples were selected randomly from the lyophilized powder samples, and 15 samples were stored at 4°C, and the other samples were stored at −20°C. The concentration and characteristic 16S–23S sequence amplification tests were conducted at 7 time points in the first, second, fourth, sixth, eighth, and tenth months, respectively.

2.8. Application of Genomic DNA Reference Material of C. sakazakii in Edible Fungus Flammulina velutipes

2.8.1. Sampling of Edible Fungus Flammulina velutipes. Thirty samples of fresh *Flammulina velutipes* were collected from supermarkets and traditional retail markets in July 2020 from Jinan, China. While the samples were obtained, the analyses were managed immediately.

2.8.2. Determination of C. sakazakii in the Edible Fungus Flammulina velutipes. The needle mushroom (*Flammulina velutipes*) was obtained from the market and put into the sterile sealed bag in the low temperature insulation box. As the sample arrived at the laboratory, the detection was started to avoid additional pollution. The needle mushroom was taken out from the bag in the aseptic operation table, cut into pieces, and placed into the mLST-Vm enrichment medium. The enrichment of *C. sakazakii* was conducted according to the National Food Safety Standard of China Food microbiological examination: *Enterobacter sakazakii* for powdered infant formula [15]. After enrichment of bacteria, the culture was treated by boiling waterbath for 10 min to release the genomic DNA. Then, the genomic DNA was taken as the template to amplify the characteristic 16S–23S sequence. The PCR products were analyzed by 1% agarose gel electrophoresis.

2.8.3. Verification of the Detection of C. sakazakii in the Edible Fungus Flammulina velutipes. The *Flammulina velutipes* samples that were positive for the 16S–23S sequence of *C. sakazakii* detected by PCR were identified according to the method of the National Food Safety Standard of China Food microbiological examination: *Enterobacter sakazakii* for powdered infant formula [15]. First, 1 loop of the enriched culture was taken and streaked into two chromogenic *C. sakazakii* agar plates and cultivated at 37°C for 24 h. Then, at least 5 suspicious colonies were chosen; if there were less than 5, all the suspicious colonies were taken, inoculated them on the TSA plate, and incubated at 25°C for 48 h. Then, the yellow suspicious colonies on the TSA plates were conducted by biochemical identification in the *C. sakazakii* identification reagent strip.

3. Results

3.1. Preparation of Genomic DNA of C. sakazakii. The genomic DNA of *C. sakazakii* was extracted following the steps in the kit instructions, and the concentration and purity of DNA samples were determined using an ultraviolet spectrophotometer (Table 1), and the integrity was analyzed by agarose gel electrophoresis (Figure 1). The concentration of the genomic DNA of *C. sakazakii* was approximately 50 ng/μL, and the purity index A260/A280 was more than 1.7, indicating that the sample was not contaminated by protein. Also on the agarose gel, it could be observed that the genomic DNA band is single and bright, no protein and RNA contamination, consistent with the purity test results, showing that the purity and concentration of the extracted DNA are good, meeting the requirements of subsequent experiments.

3.2. Specificity Analysis of Primers of 16S–23S. The specificity verification of the primers of the characteristic sequence 16S–23S was performed, and the result is shown in Figure 2. There was a bright band (282 bp) on the gel while using the genomic DNA of *C. sakazakii* FC4146 as the template and applying primers of 16S–23S, but no bands could be observed when using other bacterial genomic DNA detected, indicating that the primers for amplifying the sequence of 16S–23S of *C. sakazakii* is specific.

3.3. Sensitive Analysis of Primers of 16S–23S Amplification of C. sakazakii. The sensitive analysis of the primers for characteristic sequence 16S–23S was detected. The genomic DNA was diluted by gradient dilution, and PCR reaction was conducted using the diluted DNA as template and applying the primers of 16S–23S, and each gradient concentration was detected twice. As shown in Figure 3, the bands of PCR products were observed when the template content was greater than or equal to 0.004 ng, illustrating that the

Table 1: The concentration and purity of genomic DNA of *C. sakazakii*.

Tube no.	1	2	3	4
C (ng/μL)	51.0	49.7	51.2	52.6
A260/A280	1.78	1.77	1.78	1.80

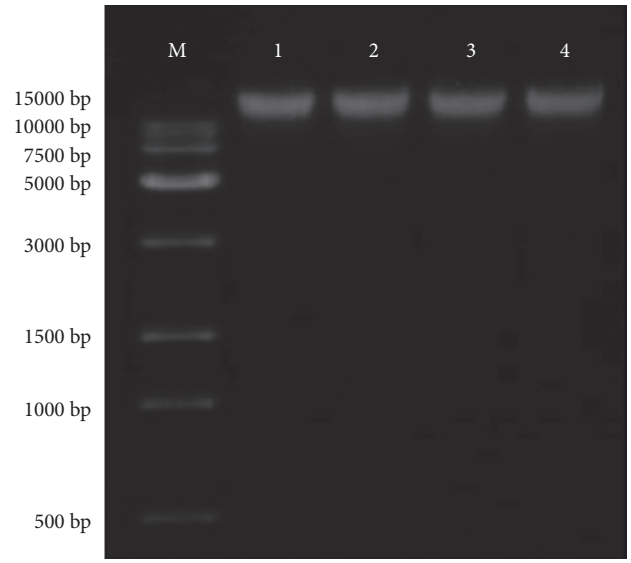

Figure 1: Agarose gel electrophoresis analysis of the genomic DNA of *C. sakazakii*. *M*, trans15K DNA marker; lanes 1–4, genomic DNA of *C. sakazakii*.

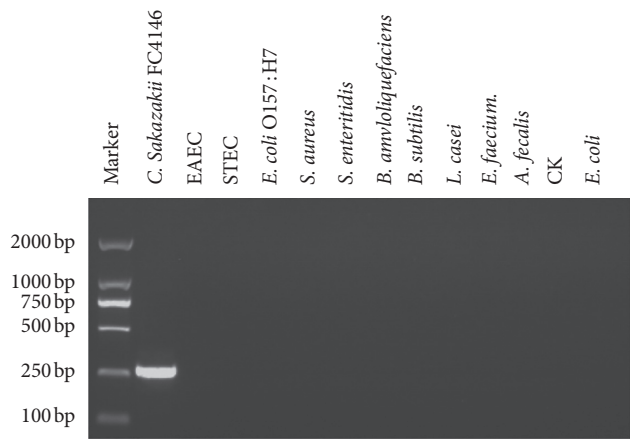

Figure 2: Specificity analysis of 16S–23S PCR result.

detection sensitivity of primers of 16S–23S was as low as 0.004 ng, also showing that the detection limit of the primers applied was at the 4 pg level using genomic DNA of *C. sakazakii* as the template.

3.4. Uniformity Analysis of Genomic DNA Reference Materials of C. sakazakii. After the genomic samples were extracted, the samples were divided into 300 μL each bottle, 6 μg in total for lyophilization, and the total number of samples was 200 bottles. Each bottle is an indivisible whole unit, so 15 samples were randomly selected for uniformity

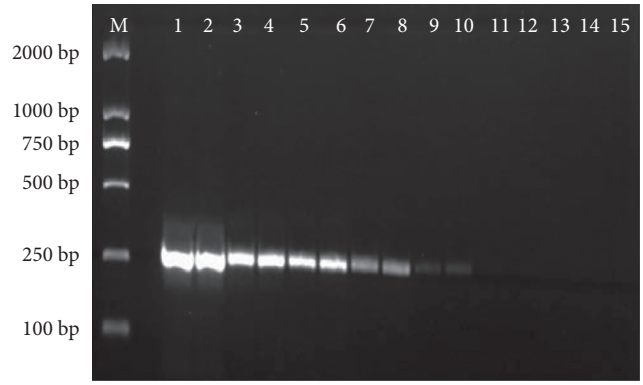

Figure 3: Sensitivity analysis of primers for 16S–23S. *M*, DNA marker D (100–2000 bp); lanes 1-2, 40 ng (10^0); lanes 3-4, 4 ng (10^{-1}); lanes 5-6, 0.4 ng (10^{-2}); lanes 7-8, 0.04 ng (10^{-3}); lanes 9-10, 0.004 ng (10^{-4}); lanes 11-12, 0.0004 ng (10^{-5}); lanes 13-14, 0.00004 ng (10^{-6}); and lane 15, blank control.

Table 2: Statistical analysis of homogeneity detection of genomic DNA reference material of *C. sakazakii*.

Tube no.	Content 1 (μg)	Content 2 (μg)	Content 3 (μg)
1	6.06	5.94	6.18
2	5.85	6.03	5.94
3	5.97	5.85	6.06
4	6.06	5.94	5.85
5	6.06	5.91	6.03
6	5.91	6.00	6.06
7	6.00	5.94	6.06
8	6.12	5.97	5.94
9	6.06	5.88	6.09
10	6.06	6.12	5.97
11	5.91	6.09	6.09
12	6.00	5.91	6.06
13	5.79	6.09	6.09
14	5.88	5.85	6.24
15	6.09	5.91	6.15
Mean (μg)		6.00 ± 0.1	
F value		0.289	
F critical value		$F_{0.05}$ (14, 30) = 2.04	

inspection. The contents were detected using a UV spectrophotometer, and the statistical analysis of the detection result was conducted by the variance analysis method of one-way ANOVA. Three individual tests were performed for each sample. As shown in Table 2, the average content for 15 samples was 6.00 μg, and the variance of the determination results showed that with 95% confidence probability, *F* value, 0.289, was less than the *F* critical value, 2.04, indicating that there is no significant difference between samples. And the characteristic sequence 16S–23S amplification test showed that the PCR products were positive and had a single band on the agarose gel for each test (Figures 4 and 5). Both the results indicate that the uniformity of the sample meets the requirements of the reference material.

3.5. Short-Term Stability Analysis of Genomic DNA Reference Material of C. sakazakii. The genomic DNA reference

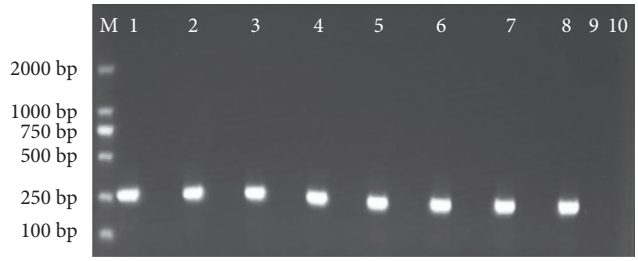

Figure 4: The PCR test for genomic DNA reference materials of no. 1–8. *M*, DNA marker D (100 ~ 2000 bp); lanes 1–8, PCR results of no. 1–8, respectively; lane 9, negative control; and lane 10, blank control.

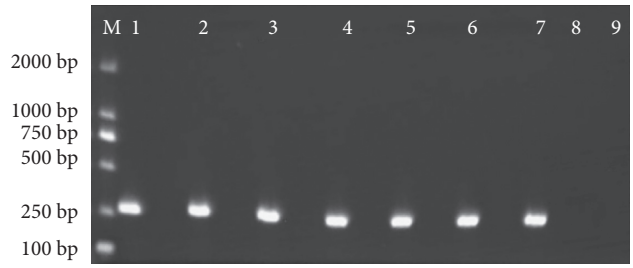

Figure 5: The PCR test for genomic DNA reference materials of no. 9–15. *M*, DNA marker D (100 ~ 2000 bp); lanes 1–7, PCR results of no. 9–15, respectively; lane 8, negative control; and lane 9, blank control.

materials were stored at 4°C and 37°C for a period of 1, 3, 5, 7, 9, 11, and 14 days, respectively; then, the concentration of each sample was detected, and the contents of genomic DNA were calculated, and statistical analysis by one-way ANOVA was conducted. The *F* value (0.485) was less than the critical value (1.53) at 95% confidence, and the contents changes of nucleic acid reference material are shown in Figure 6, showing that the genomic nucleic acid reference material was stable for 14 days stored at 4°C or 37°C. Also, PCR result displays a single bright specific band on the agarose gel using the genomic DNA as the template to amplify 16S–23S sequence after stored at 4°C and 37°C, respectively (Figure 7), indicating that the property of genomic DNA reference materials is good.

3.6. Long-Term Stability Analysis of Genomic DNA Reference Material of C. sakazakii. After the genomic DNA reference materials were lyophilized, they were stored at −20°C. For each test of the long-term stability test, 3 bottles of samples were randomly taken out of the lyophilized samples. At the 1st, 2nd, 4th, 6th, and 8th month, a total of 5 time points were sampled and tested. The content changes among the samples at different time points are shown in Figure 8. The *F* value of 0.696 was less than the critical value of 1.66 at 95% confidence, showing that the genomic DNA reference material was stable for 8 months stored at 4°C or −20°C. Figure 9 shows the results of PCR of the characteristic sequence 16S–23S of each sample. The characteristic gene test is positive, and the bands of PCR products were single, indicating that the sample is stable for 8 months.

3.7. Detection of Foodborne Pathogen C. sakazakii in Flammulina velutipes. Fifteen batches of flesh *Flammulina velutipes* were purchased from supermarkets and traditional retail markets spread over Jinan. The samples were detected immediately while they arrived in the laboratory. The results showed that 16S–23S fragments of *C. sakazakii* were positive in all tested samples from 8 supermarkets (Figure 10) and 5 positive of 7 samples from traditional retail markets (Figure 11). In total, the detection rate was 86.7%.

3.8. Verification of the PCR Detection of C. sakazakii in Flammulina velutipes. The positive samples were verified according to the National Food Safety Standard of China Food microbiological examination: *Enterobacter sakazakii* (GB 4789.40-2010; National Standard of the People's Republic of China, 2010). As shown in Figure 12, the colonies of no. 1 bacterium isolated from *Flammulina velutipes* on TSA plate were yellow and on the chromogenic *Enterobacter sakazakii* agar plate were blue-green (other samples were not shown). The biochemical identification results are shown in Table 3, which were consistent with the positive control of *C. sakazakii* FC4146. All the results indicated that the bacterium detected in *Flammulina velutipes* is *C. sakazakii*.

4. Discussion

In this study, genomic DNA reference materials were prepared by genomic DNA extraction and lyophilization. The uniformity analysis showed that the samples have no significant differences among samples, and they were stable at 4°C or 37°C for 14 days, which could meet the requirements of long-distance transportation. The samples keep good as long as 8 months while stored at 4°C or −20°C. The reference materials were used as the positive control in the detection of *Cronobacter sakazakii* of edible fungus *Flammulina velutipes*. This reference material is suitable for the verification, confirmation, and evaluation of PCR detection methods of *Cronobacter* spp. in food and other samples, quality control of the detection process, personnel assessment, evaluation of laboratory detection capabilities, and interlaboratory comparisons. Also, this reference material could be used as a quantitative tool for *Cronobacter* spp. using other detective methods. For example, a standard curve could be formed based on this reference material using fluorescence quantitative PCR analysis, and the pathogenic bacteria content of the sample to be tested

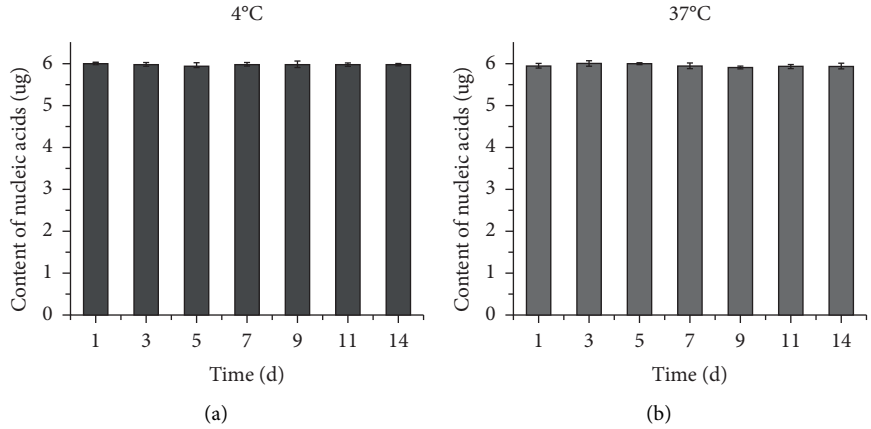

Figure 6: The short-term stability test of changes in 14 days of genomic nucleic acid reference material of *C. sakazakii*.

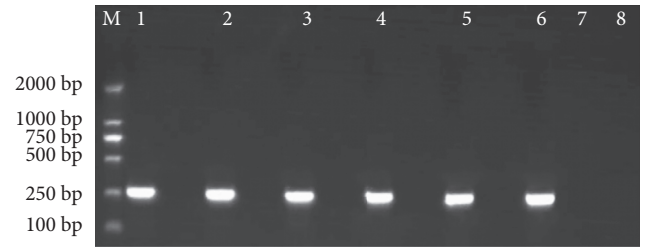

Figure 7: The PCR analysis of the short-term test of genomic DNA reference materials for 14 days. *M*, DNA marker D (100 ~ 2000 bp); lanes 1–3, PCR results of 3 samples stored at 4°C, respectively; lanes 4–6, PCR results of 3 samples stored at 37°C, respectively; lane 7, negative control; and lane 8, blank control.

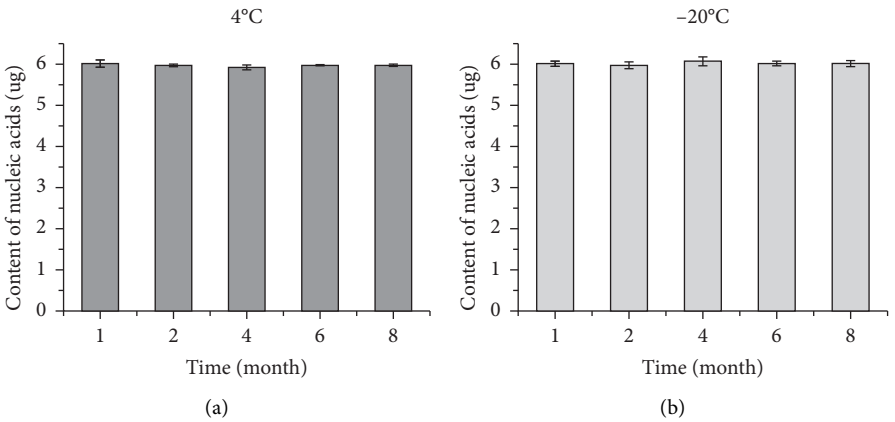

Figure 8: The long-term stability test of changes in 8 months of genomic nucleic acid reference material of *C. sakazakii*.

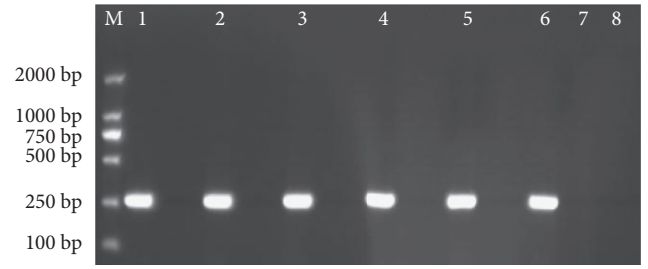

Figure 9: The PCR analysis of the long-term test of genomic DNA reference materials for 8 months. *M*, DNA marker D (100 ~ 2000 bp); lanes 1–3, PCR results of 3 samples stored at −20°C, respectively; lanes 4–6, PCR results of 3 samples stored at 4°C, respectively; lane 7, negative control; and lane 8, blank control.

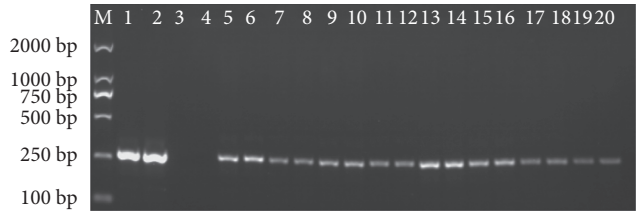

Figure 10: PCR detection of 16S–23S of *C. sakazakii* in *Flammulina velutipes* collected from 8 supermarkets. *M*, DNA marker D (100 ~ 2000 bp); lanes 1-2, positive control; lane 3, negative control; lane 4, blank control; and lanes 5–20, PCR results of 8 samples, respectively; each sample was tested twice.

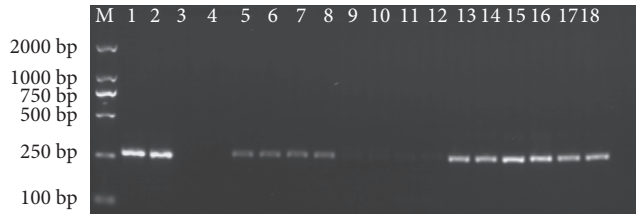

Figure 11: PCR detection of 16S–23S of *C. sakazakii* in *Flammulina velutipes* collected from 7 traditional retail markets. *M*, DNA marker D (100 ~ 2000 bp); lanes 1-2, positive control; lane 3, negative control; lane 4, blank control; and lanes 5–18, PCR results of 7 samples, respectively; each sample was tested twice.

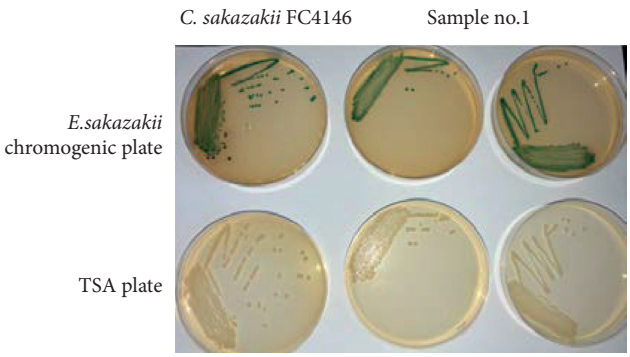

Figure 12: The colonies of *C. sakazakii* and the no. 1 bacterium detected in *Flammulina velutipes*.

Table 3: The biochemical identification of the bacterium detected in *Flammulina velutipes*.

Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	*C. sakazakii* FC4146
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-lysine decarboxylase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citric acid hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	+

can be judged according to the standard curve applied in the same analysis method. No matter what the PCR method is, there are limitations; sometimes, false-positives are very common using PCR methods. In actual food detection applications, PCR detection methods can effectively and quickly exclude a large number of negative result samples, saving time and cost. A positive PCR result is only a preliminary result. In the end, culture and biochemical identifications are required to determine whether the sample contains the target pathogen, as we show in our study above. The development of this research is conducive to the popularization and application of genomic nucleic acid reference materials in rapid foodborne microbial detection.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Huandie Yang and Danyang Guo are co-first authors. Huandie Yang and Danyang Guo conducted the experiments; Leilei Chen and Alexander Suvorov offered the program funding; Qingxin Zhou, Junhua Wang, and Xiaofeng Gao revised the manuscript; Jinyu Yang and Leilei Chen directed the work; Jinyu Yang wrote the manuscript; and Xiangyan Chen supervised the work.

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Supplementary Materials

Table S1. PCR system for detection of characteristic 16S–23S sequence of *C. sakazakii*. Table S2. PCR reaction program for detection of characteristic 16S–23S sequence of *C. sakazakii*. (*Supplementary Materials*)

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