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Species-Level Monitoring of Key Bacteria in Fermentation Processes Using Single-Nucleotide Resolved Nucleic Acid Assays Based on CRISPR/Cas12

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ABSTRACT: Microorganisms can determine the flavor and quality of fermented food, such as Baijiu, which is produced via Daqu fermentation. Therefore, monitoring key microorganisms during fermentation is important for ensuring high-quality fermented food. Here, we report a single-nucleotide resolved nucleic acid assay based on the CRISPR/Cas12 system, enabling the quantification of *Bacillus amyloliquefaciens*, a key microorganism in Daqu fermentation at the species level. The assay employs an amplification-refractory mutation system derived from PCR to analyze minor genetic differences between different *Bacillus* species. The utilization of CRISPR/Cas12 further guaranties the specificity of identifying the PCR amplicon and enables the quantification of *Bacillus amyloliquefaciens* via end-measurement fluorescence. Compared to conventional qPCR, the assay allows for species-level detection of bacteria, thus enabling the precise detection of the *Bacillus* strain that yields high-level 2,3,5,6-tetramethylpyrazine. The assay promises the precise monitoring of bacterial growth and contribution to flavor during Daqu fermentation, thus facilitating fermented food quality control.

KEYWORDS: *fermented food, CRISPR/Cas12, food quality, Baijiu, Bacillus amyloliquefaciens*

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INTRODUCTION

Fermented food accounts for approximately one-third of the human diet globally.[1] For example, in 2020, 11.7 billion liters of traditional fermented milk were consumed. Worldwide retail sales of liquor were estimated to be around £300 billion in 2019. Microorganisms play a crucial role in the production of fermented food, and their impact on the characteristic flavor and quality is significant.[2,3] Liquor is a well-known fermented food, and its flavor and quality depend on the fermentation process via microorganisms, especially the growth of key microorganisms.[4] High-temperature Daqu, a saccharifying agent and starter made from wheat, is essential in brewing liquor. It provides microorganisms, flavor substances, and nutritional substrates for the brewing process.[5−7] Liquor is brewed by solid-state fermentation utilizing high-temperature Daqu as a saccharifying and fermenting agent.[8] Daqu plays a vital role in providing functional microflora and precursors for flavor substances in liquor.[9−11] *Bacillus* is a dominant species in high-temperature Daqu.[12,13] Specifically, *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*), *Bacillus subtilis* (*B. subtilis*), and *Bacillus licheniformis* (*B. licheniformis*) are the main *Bacillus* species.[14,15] Among them, *B. amyloliquefaciens* is capable of producing unique components such as 2,3,5,6-tetramethylpyrazine (TTMP, $C_8H_{12}N_2$) during liquor brewing processes.[16] TTMP is a bioactive alkaloid used as a conventional drug to treat cardiovascular and cerebrovascular diseases,[17] and it is also utilized as an important additive to impart a special flavor characterized by pleasant notes of toast, grilled meat, and nuts.[18] In Kweichow Moutai flavor liquor, TTMP is a functional substance and a key characteristic aroma component,[14,19,20] with levels significantly higher than those in other flavored liquors. Functional Daqu inoculated with *B. amyloliquefaciens* can increase the levels of TTMP in liquor.[16] Moreover, Daqu inoculated with *B. amyloliquefaciens* exhibits a higher capacity for ester production, and the content of esters significantly impacts the quality of liquor. Therefore, monitoring the growth of key microorganisms, such as *B. amyloliquefaciens*, is crucial for improving the quality and flavor of liquor.

The plate counting method is traditionally used to identify and detect microorganisms.[21] This involves growing colonies on plates and identifying species through morphological observation. However, it can be challenging to distinguish species with high morphological similarity, and the process is time-consuming. Although 16S rDNA identification can be used to identify species, DNA sequencing is a lengthy and expensive process. Quantitative real-time PCR (qPCR) is commonly used for quantitative detection of nucleic acids.[22,23] Nevertheless, conventional qPCR can hardly discriminate some species that are with only single nucleotide differences while differ in the performance during the fermentation

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process. For example, the gene sequences of *B. amyloliquefaciens* and *B. subtilis* are highly similar, but *B. amyloliquefaciens* is advantageous in producing high-level TTMP during the Daqu fermentation process.[24,25] Hence, the development of a rapid, simple, and single-nucleotide-resolved nucleic acid assay for detecting microorganisms is highly demanded for monitoring fermentation quality.

The emergence of CRISPR/Cas advances the assays for nucleic acid detection. The CRISPR/Cas system consists of two main components: clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (CAS) proteins.[26−28] This adaptive immune system evolves in prokaryotes.[29,30] Among the CRISPR/Cas systems, Cas12 can bind and target double-stranded DNA (dsDNA) using CRISPR guide RNA (gRNA) as a guide, resulting in nonspecific cleavage activity on single-stranded DNA (ssDNA).[31,32] Cas12/gRNA can specifically recognize nucleic acid amplicons, and by designing a ssDNA reporter, it has been demonstrated to improve the specificity and quantification performance of PCR or other isothermal amplification-based nucleic acid assay. The potential applications of the CRISPR/Cas12 system in food safety and quality analysis, including the detection of foodborne microorganisms, adulteration, and traceability, have been explored.[33,34] For example, the coupling of isothermal amplification with Cas12 was employed to detect *Salmonella* and estimate its colonization in chicken.[35] A visual detection based on Cas12 and nicking enzyme-assisted amplification were reported for the analysis of *Salmonella* on the egg surface.[36] Cas12-based nucleic acid assays are also explored to detect the authentication of halal food and wheat fungal diseases.[37−39] However, the utilization of the CRISPR/Cas12 system in monitoring microorganisms during fermentation processes remains unexplored.

Here, based on CRISPR/Cas12, we reported a single-nucleotide resolved nucleic acid assay, enabling the identification and quantification of key bacteria, in the fermentation process at the species level. Mismatched primers, designed through the amplification-refractory mutation system (ARMS), enable the detection of minor genetic variations among different *Bacillus* species. The CRISPR/Cas12 system is designed to identify the amplified products for improving the specificity and enables the signal readout by end-measurement of fluorescence, thus eliminating the use of real-time fluorescence PCR instrument. The CRISPR/Cas12-based ARMS assay has been applied to monitor the growth of *B. amyloliquefaciens* in Daqu fermentation, which contains many other Bacillus species. The assay enables the quantification of key microorganisms at the species level, which can serve as competitive tools for fermented food quality control.

MATERIALS AND METHODS

Materials and Reagents. Phi 29 DNA polymerase (10 U/μL) (cat.10123955) and T7 RNA polymerase (20 U/μL) (cat. 32108-03) were purchased from Thermo Fisher Scientific (Waltham, USA). LbCas12 with 10× NEB buffer (cat.32108-B), DNase I with 10× reaction buffer, ribonucleotide solution mix (rNTPs), and deoxynucleotide solution mix (dNTPs) were obtained from New England Biolabs (Ipswich, MA). Platinum SYBR Green qPCR SuperMix-UDG w/ROX was provided by Thermo Fisher Scientific (Waltham, USA). Molecular biology-grade H_2O was used in all solutions (New York, USA). Agarose, 6× loading buffer, 50× TAE buffer, and 10,000× GelRed dye were purchased from Beijing DingGuo Biotechnology Co., Ltd. (Beijing, China). 2,3,5,6-Tetramethylpyrazine (CAS: 1124-11-4, chromatographic grade) was purchased from Beijing PuTianTongChuang Biotechnology Co., Ltd. qPCR was performed in 96-well plates by the Real-Time System CFX connect (Bio-Rad, USA). The Ezup Column Bacteria Genomic DNA Purification Kit (cat.H723KA0902) was purchased from Sangon Biotech (Shanghai, China). The DNA reporter was purified by HPLC. Primers and oligonucleotide sequences used were customized by Sangon Biotech (Shanghai, China). The sequence lists are provided in the Supporting Information (Table S1).

Bacteria Culture. Bacterial cultures of *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) (SICC 1.2905), *Bacillus subtilis* (*B. subtilis*) (ATCC 6051), *Bacillus licheniformis* (*B. licheniformis*) (ATCC 14580), *Bacillus cereus* (*B. cereus*) (ATCC 14579), *Bacillus thuringiensis* (*B. thuringiensis*) (CICC 23420), and *Bacillus aerophilus* (*B. aerophilus*) (CGMCC 8840) were grown in a liquid culture medium. To prepare 1 L of liquid culture medium, 10 g of peptone, 5 g of yeast extract, and 10 g of NaCl were added, and the mixture was autoclaved for 20 min before use. The bacteria were cultured in the medium for 18 h at 37 °C on a rotary oscillator and counted using a plate counting method.

DNA Extraction. DNA was extracted using the Ezup column bacteria genomic DNA purification Kit. Briefly, 1 mL of bacterial solution was centrifuged at 7200*g* for 1 min at room temperature to collect the bacteria at the bottom. The bacteria were transferred to a 1.5 mL centrifuge tube, and 100 μL of buffer digestion and 80 μL of lysozyme were added to the tube, which was then incubated at 37 °C for 30 min. After centrifugation at 8000 g for 30 s, 100 μL CE buffer was added and incubated for 3 min. The solution was centrifuged at 10,000*g* for 2 min, and then, the supernatant was collected. The extracted DNA was stored at −20 °C until further use.

Preparation of gRNA. To prepare the gRNA, the transcription reaction was performed in a 30 μL volume, consisting of 10 μL of 10× phi 29 DNA polymerase reaction buffer (10 U/μL), 10 μL of promoter (10 μM), and 10 μL of the DNA template for gRNA synthesis (10 μM). The solution was mixed and then annealed at 90 °C for 3 min, followed by incubation at room temperature for 30 min. Next, 3 μL of phi 29 DNA polymerase (1 U/μL) and 2 μL of dNTP mix (10 μM) were added, and the mixture was incubated at 30 °C for 30 min. Subsequently, 20 μL of 5× transcription buffer, 2 μL of T7 RNA polymerase (20 U/μL), 2 μL of rNTP mix (25 μM), and 41 μL of H_2O were added. The solution was then incubated at 37 °C overnight. To remove any remaining DNA, 2 μL of DNase I was added and the solution was incubated at 37 °C for 2 h. Finally, the product was heated at 75 °C for 10 min, and the gRNA was stored at −20 °C until use.

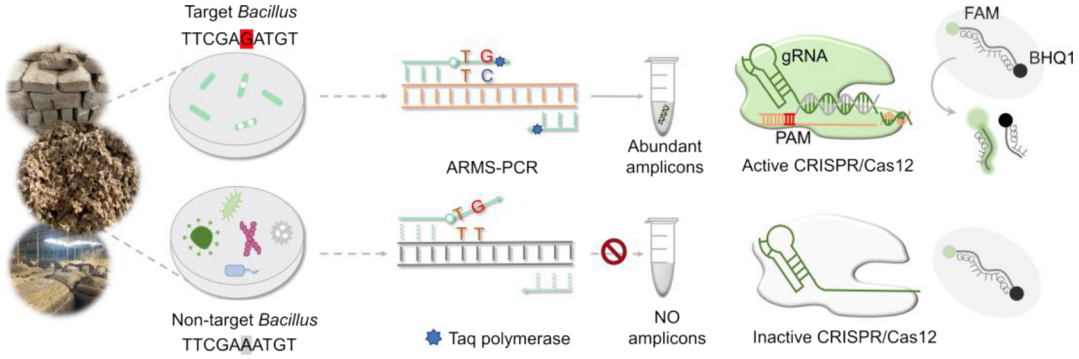
Detection of *B. amyloliquefaciens*. PCR was performed in a 25 μL volume containing 1 μL of extracted DNA, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 12.5 μL of Taq PCR Mix, and 9.5 μL of H_2O. The PCR program involved an initial denaturation step at 95 °C for 1 min, followed by 25 amplification cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The PCR products were stored at 4 °C. For detection based on CRISPR/Cas12, a 38 μL reaction volume was prepared to contain 4 μL of 10 × NEB buffer, 0.4 μL of LbCas12 (10 μM), 4 μL of gRNA (10 μM), 4 μL of reporter (5 μM), 1 μL of PCR products, and 24.6 μL of H_2O, followed by incubation at 37 °C for 1 h. The fluorescence signal was then detected using a microplate reader Synergy H1 (BioTek, USA) with an excitation wavelength of 480 nm and an emission range of 510 to 600 nm.

qPCR Test of *B. amyloliquefaciens*. To detect *B. amyloliquefaciens* using qPCR, a 20 μL reaction mixture was prepared to contain 10 μL of SYBR Green qPCR SuperMix, 1 μL of extracted DNA, 0.8 μL of forward primer (2.5 μM), 0.8 μL of reverse primer (2.5 μM), and 7.4 μL of H_2O. The qPCR program involved an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles at 95 °C for 15 s, 60 °C for 15 s, and 75 °C for 45 s.

Detection of *B. amyloliquefaciens* in Daqu. To detect *B. amyloliquefaciens* in Daqu, a solid-state fermentation medium was prepared by mixing 10% rice husk and 90% wheat flour, which was then steamed for 30 min and cooled. The starter was added at a ratio of 1%, and the mixture was weighed and bottled. To inoculate *B. amyloliquefaciens*, 3% (w/w) of the bacteria was added to the medium.

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Scheme 1. Schematic Diagram of the CRISPR/Cas12-based ARMS Assay for Monitoring Key Bacteria in the Fermentation Process



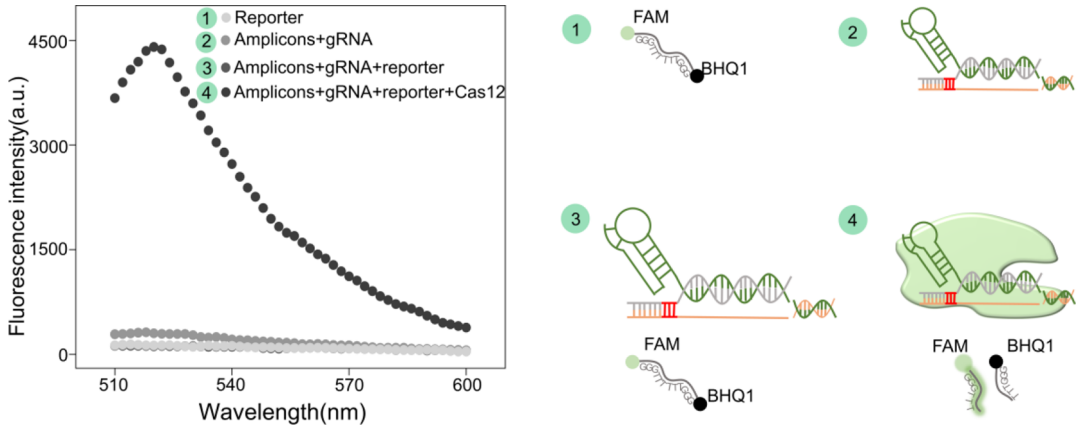


Figure 1. Demonstration of the feasibility of the assay for nucleic acid detection. The fluorescence response was detected using an excitation wavelength of 480 nm, and the emission ranged from 510 to 600 nm.

The medium was fermented in a rotary oscillator by gradient heating, with incubation at 37 °C for the first three days, 45 °C for the fourth day, and 60 °C from the fifth to eighth day. Total DNA was extracted using the Ezup column soil DNA extraction kit, and the DNA concentration was measured using either the reported method or qPCR.

Gel Electrophoresis. The gel was prepared by mixing 1.5 g of agarose, 1 mL of 1× TAE buffer, 3 μL of GelRed dye, and 49 mL of H_2O. The gel mixture was then heated in a microwave oven for 3 min. A reaction system consisting of 5 μL of PCR products and 1 μL of gel loading buffer was used. Gel electrophoresis was carried out at 120 V for 30 min, and the gel was imaged using a Gel Doc XR+ system (Bio-Rad).

Heat-Resistance Test. The absorbance of bacterial medium was measured at a wavelength of 600 nm. Culture medium of *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. subtilis*, and *B. aerophilus* was heated in a water bath at 60 °C for 3 h. The control group was incubated at 37 °C. ΔA is defined as the absorbance difference between the bacteria cultured at 37 and 60 °C.

Analysis of TTMP. The TTMP was tested using HPLC-VWD. A solid-state medium was inoculated with 3% w/w of *B. subtilis*, and the fermentation conditions were the same as *B. amyloliquefaciens*. After fermentation, a mixture of 5 g of Daqu, 0.25 g of CaCl, and 50 mL of 57% ethanol was prepared in a tube at 37 °C. The samples were then extracted by ultrasonic treatment for 20 min and centrifugated at 8000*g* for 10 min. The supernatant was filtered through a 0.22 μm membrane. The material of the 0.22 μm membrane is nylon 66, and the injection volume was 1.5 mL. The HPLC-VWD analysis was performed using an Agilent 2000 under the following conditions: chromatographic column-Agilent TC-C18 (4.6 mm × 250 mm, 0.5 μm); detector-VWD detection device; detection wavelength-278 nm; mobile phase-aqueous solution (with 0.05% trisodium chloride): methanol was 3:7; and flow rate-1 mL/min.

RESULTS AND DISCUSSION

Working Principle. The CRISPR/Cas12-based ARMS assay detects bacterial DNA with single-nucleotide resolution by activating the ribonuclease of CRISPR/Cas12 using the ARMS-PCR amplicon (Scheme 1). The ARMS technique introduces mismatched bases at the 3′ end of the primer to enhance the specificity of the reaction. One or two artificial mismatched bases are added to the primer, which ensures that the last base at the 3′ end of the primer matches exactly with the target species but not with other species. This enables the amplification of the fragments of the target species while preventing the amplification of non-target species. To further improve the specificity of recognition, CRISPR/Cas12 was designed to identify the PCR amplicon. The target PCR amplicon was specifically recognized using the designed gRNA, which activated the Cas12 protein to cleave the ssDNA reporter. The ends of the ssDNA reporter were modified with a 6-carboxy fluorescein (FAM) and a Black Hole Quencher-1(BHQ1). When the PCR amplicon amplified from target genes was present, Cas12 can target and bind the dsDNA under the guidance of gRNA to form the Cas12/gRNA complex. The complex could cut the DNA reporter gene, thus recovering the fluorescence of the reporter. The abundance of bacteria was determined by the end measurement of the fluorescence intensity. Among *Bacillus*, the genome of *B amyloliquefaciens* has the most similar genome sequence

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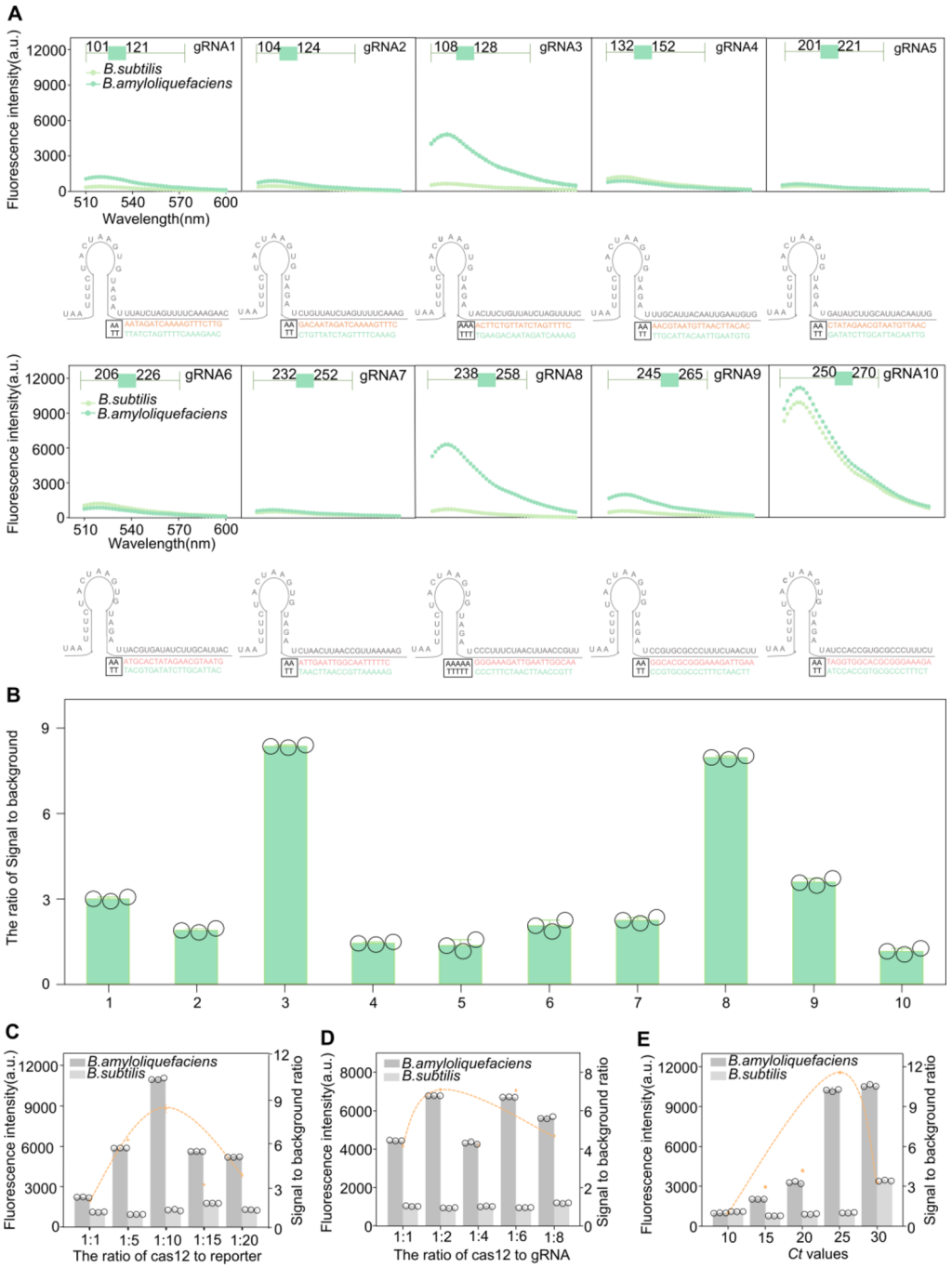


Figure 2. Detection of single-nucleotide variation and optimization of the assay. (A) Fluorescence spectra of the assay's response to *B. amyloliquefaciens* and *B. subtilis* using gRNA targeting in different sites. (B) Discrimination ratio of *B. amyloliquefaciens* to *B. subtilis* using the designed gRNAs. (C) Fluorescence intensity and signal-to-background ratio with different ratios of Cas12 protein and reporter. (D) Fluorescence intensity and signal-to-background ratio with different ratios of Cas12 protein and gRNA. (E) Fluorescence intensity and signal-to-background ratio via PCR with different cycle numbers. The fluorescence response was detected using an excitation wavelength of 480 nm, and the emission ranged from 510 to 600 nm. The error bars indicate the standard deviation of three parallel measurements. The data in B–E represent mean ± standard deviation (n = 3).

compared to that of *B. subtilis*. The ARMS PCR primer for *B. amyloliquefaciens* was designed locating the site of the genome of *B. amyloliquefaciens* and *B. subtilis* that differs in single nucleotide. Contributing to its high specificity that can eliminate the recognition of other *Bacillus*, the assay enables the detection of *B. amyloliquefaciens* in Daqu during fermentation at the species level.

Fluorescence tests were conducted to verify the assay's working principle. The DNA reporter initially produced a low fluorescence intensity of 129 due to the quenching effect of BHQ1 on FAM (Figure 1). The addition of gRNA to the target gene did not result in an increase in fluorescence intensity. The simultaneous presence of Cas12 protein, gRNA, and target DNA resulted in a remarkable increase in fluorescence intensity from 322 to 4452. This suggests that

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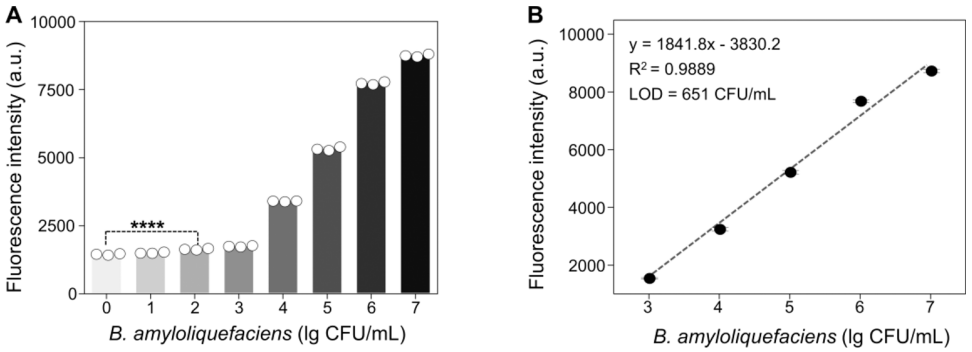


Figure 3. Quantification of *B. amyloliquefaciens* using the CRISPR/Cas12-based ARMS assay. (A) Fluorescence intensity of the assay response to different concentrations of *B. amyloliquefaciens* (10^0–10^7 CFU/mL). (B) Linear relationship between the concentration of *B. amyloliquefaciens* and fluorescence response. The fluorescence response was detected using an excitation wavelength of 480 nm, and the emission ranged from 510 to 600 nm. The data presented in A and B represent the mean ± standard deviation (n = 3). Statistical significance was determined using a two-tailed unpaired Student t test, ****, $P < 0.0001$.

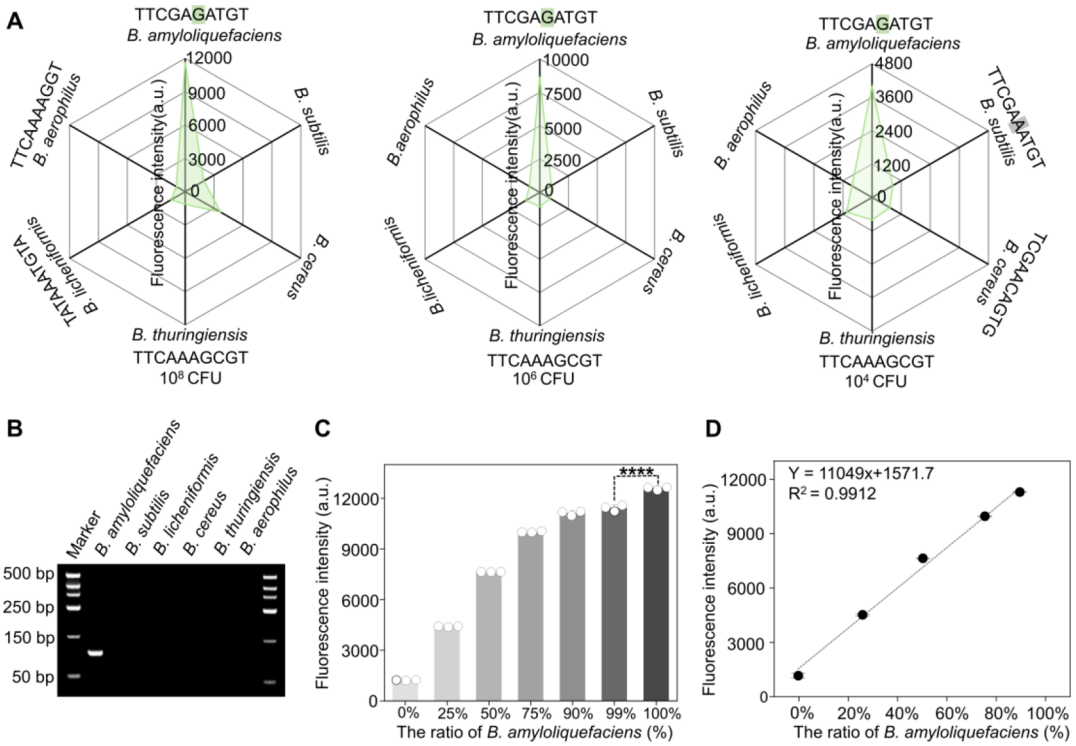


Figure 4. Detection of *B. amyloliquefaciens* at the species level using the CRISPR/Cas12-based ARMS assay. It includes fluorescence intensity of different concentrations of *Bacillus* (A), gel electrophoresis of PCR products amplified from different *Bacillus* (B), fluorescence intensity of different ratio of *B. amyloliquefaciens* (0%∼100%) in a mixture of *B. subtilis* and *B. amyloliquefaciens* (C), and a linear relationship between the proportion of *B. amyloliquefaciens* and fluorescence intensity (D). Statistical significance was evaluated using a two-tailed unpaired Student t test, ****, $P < 0.0001$.

the Cas12 protein was activated by the target DNA and cleaved the DNA reporter, thus resulting in high fluorescence intensity. Therefore, CRISPR/Cas12 coupling with the DNA reporter can serve as the tool for detecting the double-stranded DNA amplicons that are amplified from bacterial genome via PCR.

Selection of gRNAs. The designed primers of 23 S DNA were selected for sequencing *B. amyloliquefaciens* (Table S1), and the amplified sequences were compared to the NCBI database (http://www.ncbi.nlm.nih.gov/ blast) to identify potential matches or similarities with other *Bacillus*. The sequences of 23S DNA of *B. subtilis* were found to be highly similar to that of *B. amyloliquefaciens*, making it difficult to distinguish between the two *Bacillus* species using the qPCR method. To overcome this limitation, the ARMS technique was used to design primers that could distinguish between the two species. Additionally, the CRISPR/Cas12a system was utilized to recognize the PCR amplicon. The CRISPR system utilizes gRNA, which consists of a conserved sequence of the stem-loop structure and a 20 bp spacer region that is complementary to the target DNA. The complementary T nucleotide-rich space neighboring motif (PAM) (TTTN, N is A, T, G, or C) is required for Cas12/gRNA recognition. Based on these sequence requirements, 10 gRNAs were designed (Figure 2A). The different gRNA sites can dramatically affect the fluorescence response of the assay. The signal to

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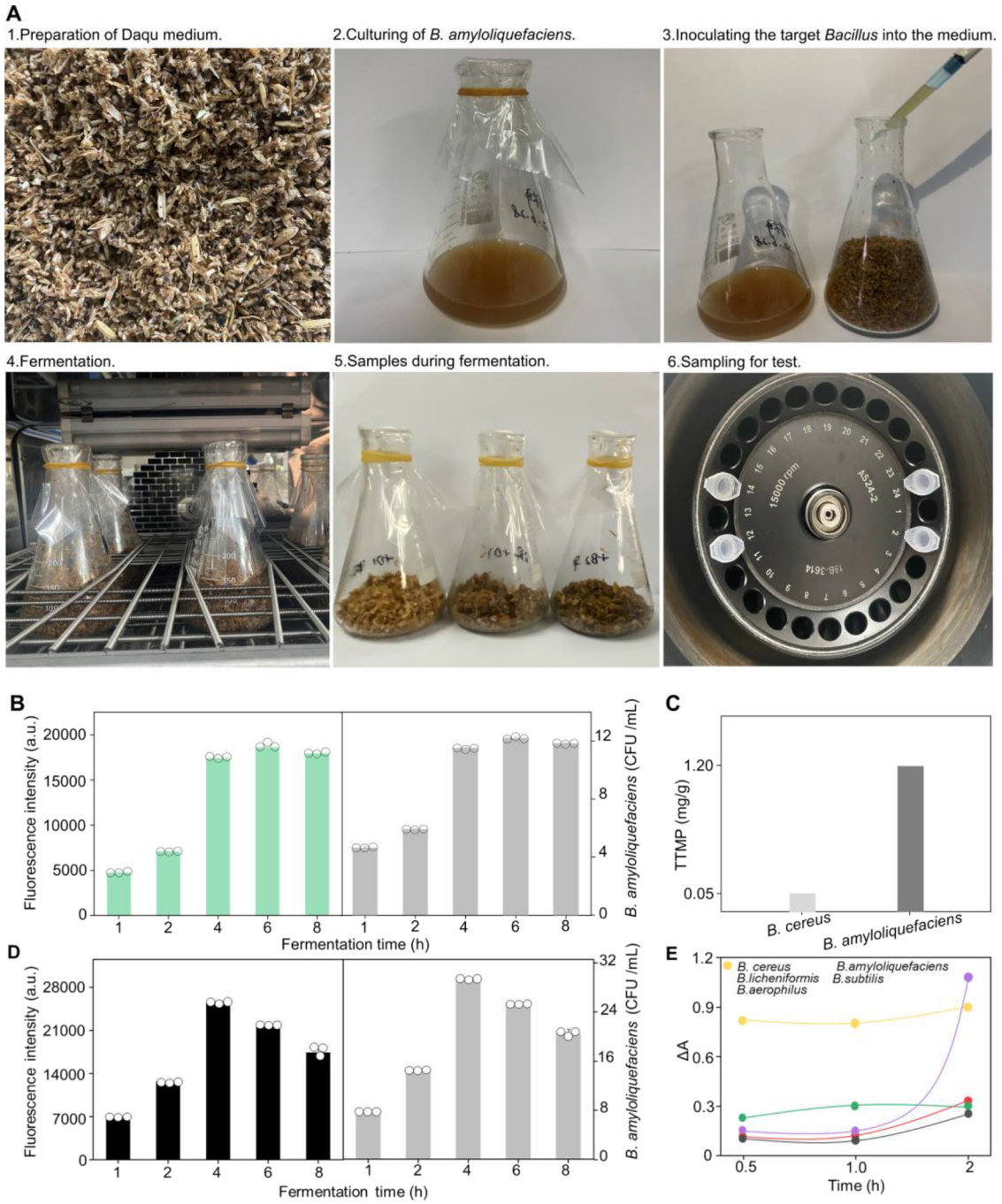


Figure 5. Detection of *B. amyloliquefaciens* in Daqu. (A) Workflow for the detection of *B. amyloliquefaciens* in Daqu using a CRISPR/Cas12-based assay. (B) Fluorescence intensity and the number of *B. amyloliquefaciens* in Daqu detected by CRISPR/Cas12-based tests. (C) Comparison of the level of 2,3,5,6-tetramethylpyrazine in Daqu with *B. amyloliquefaciens* or *B. cereus* after fermentation. (D) Fluorescence intensity and the colony numbers of *B. amyloliquefaciens* in Daqu detected by conventional PCR. (E) Comparison of ΔA of the five bacteria. ΔA is defined as the absorbance difference of the bacteria under the condition of 37 and 60 °C. The fluorescence signal was detected with an excitation wavelength of 480 nm, and the emission ranged from 510 to 600 nm. Data in B and E represent mean ± s. d. (n = 3).

background (S/B) ratio, defined as the ratio of the fluorescence intensity of *B. amyloliquefaciens* to *B. subtilis*, was used to determine the optimized gRNA site. gRNA3, when selected as the identified loci, yielded the highest S/B ratio of 8.27 (Figure 2B). The slightly higher S/B ratio compared to gRNA8 could be attributed to the presence of A bases in the space between G-rich sequences, resulting in its more efficient activation. Conversely, other gRNA sites had lower S/B ratios, possibly due to the complex secondary structure of the target sequences, making it challenging for the gRNA to bind. Consequently, gRNA3 was selected for the follow-up experiments.

Optimization of Assaying Conditions. To optimize the CRISPR/Cas12 system's response, the concentrations of gRNA and the DNA reporter were optimized. The maximum S/B ratio of 8.42 was achieved at a Cas12 protein to reporter ratio of 1:10. However, increasing the reporter concentration led to a higher background fluorescence intensity and a

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decrease in the S/B ratio (Figure 2C). The highest S/B ratio of 7.11 was observed at a Cas12 protein to gRNA molar ratio of 1:2 (Figure 2D).

Additionally, the concentration of amplicons was affected by the number of PCR cycles. The optimal number of cycles was determined to be 25, which resulted in a maximum S/B ratio of 10.67 (Figure 2E). PCR cycles below 25 resulted in less amplification, while exceeding 25 can increase background fluorescence and reduce the S/B ratio.

Quantification of *B. amyloliquefaciens*. The sensitivity of the assay for quantifying *B. amyloliquefaciens* was evaluated by preparing bacterial solutions ranging from 10^0 to 10^7 CFU in a sequential dilution. The results demonstrated that the fluorescence intensity gradually increased with increasing concentrations of bacterial solutions from 10^0 to 10^7 CFU (Figure 3A). A linear relationship between the concentration of *B. amyloliquefaciens* and fluorescence intensity was observed within the range of 10^3−10^7 CFU ($y = 1841.8x - 3830.2$, $R^2 = 0.9889$) (Figure 3B). The limit of detection (LOD) was calculated as 651 CFU, based on the concentration corresponding to three times the standard deviation of the control group without *B. amyloliquefaciens*.

Species-Level Detection of *B. amyloliquefaciens*. In this study, the specificity of the assay was tested by using other *Bacillus* species commonly found in Daqu, including *B. subtilis*, *B. cereus*, *B. thuringiensis*, *B. aerophilus*, and *B. licheniformis*, as non-target *Bacillus* under optimized conditions. *B. amyloliquefaciens* was used as the target *Bacillus*, and the assay was tested at different concentrations (10^8, 10^6, and 10^4 CFU) of *Bacillus* to consider the variable abundance of *Bacillus* in actual samples (Figure 4A). The results showed that in each concentration, the addition of *B. amyloliquefaciens* yielded the highest fluorescence intensity, while other *Bacillus species* were close to the background fluorescence intensity. The specificity of the assay was also confirmed by gel electrophoresis (Figure 4B). Furthermore, to evaluate the sensitivity of the assay, *B. amyloliquefaciens* and *B. subtilis* were mixed in different proportions as samples. *B. subtilis*, a non-target species with high similarity, was used for mixing. The ratio of *B. amyloliquefaciens* varied between 0 and 100% (0, 25, 50, 75, 90, 99, and 100%). The fluorescence intensity increased with the increase of the proportion of *B. amyloliquefaciens* (Figure 4C). A good linear relationship was observed between the fluorescence intensity and the proportion of *B. amyloliquefaciens* in the range of 10−100% ($y = 11{,}049x + 1571.7$, $R^2 = 0.9912$) (Figure 4D). To verify the results, the assay was also tested using ARMS-qPCR (Figure S2). Both the CRISPR/Cas12-based assay and ARMS-qPCR were able to detect other *Bacillus* in proportions as low as 1%. However, the CRISPR/Cas12-based assay eliminates the need for a costly real-time fluorescence quantitative PCR analyzer.

Detection of *B. amyloliquefaciens* in Daqu. The simulation of fermentation experiments was performed to evaluate the feasibility of detecting bacteria during fermentation. The operation flow of simulated fermentation is shown in Figure 5A. *Bacillus*, which can withstand high temperature, is one of the most common microorganisms in Daqu fermentation. *B. amyloliquefaciens* is the key bacteria present in Daqu during fermentation, which not only produces functional substance (like TTMP) but also produces esters and aromas to enhance the quality and flavor of the liquor. Solid-state fermentation was simulated in the presence of *B. amyloliquefaciens* and *B. cereus*, and it was found that the TTMP content was 24 times higher in the presence of *B. amyloliquefaciens* than that in the presence of *B. cereus* (Figures 5C and S3). Therefore, monitoring key microorganisms at the species level, such as *B. amyloliquefaciens*, is crucial to control the quality of fermented food.

During the 8-day fermentation process, the fluorescence intensity gradually increased with fermentation time (0−4 days), indicating that *B. amyloliquefaciens* grew vigorously. The highest colony number was achieved on the sixth day, reaching 10^{12} CFU, and the quantity of *B. amyloliquefaciens* remained stable during days 4−8 (Figure 5B). Conventional PCR was used to quantify *B. amyloliquefaciens* during the fermentation process (Figure 5D). According to the measurement using conventional PCR, the number of bacteria dropped sharply on the fourth day when the temperature was 45 °C. However, based on the measurement using the CRISPR/Cas12-based ARMS assay, the reduction of *B. amyloliquefaciens* did not occur. This result drives us to investigate the effect of temperature on the proliferation rate of these *Bacillus* species. To estimate heat resistance of the bacteria, ΔA is defined as the absorbance difference of the bacteria under the condition of 37 and 60 °C. The ΔA of *B. amyloliquefaciens* was lower than that of other bacteria, indicating that high temperature poses less effect on the growth of *B. amyloliquefaciens*. Therefore, *B. amyloliquefaciens* was more heat-resistant than other bacteria commonly present in Daqu (Figure 5E). High-temperature intolerant bacteria died when the fermentation temperature rose to 60 °C, leaving only the heat-resistant bacteria, *B. amyloliquefaciens*, to survive under high temperature conditions. Therefore, the colony number of heat-resistant *B. amyloliquefaciens* only decreased slightly after 60 °C, as indicated by the assay. However, the conventional PCR method was unable to achieve specific amplification, and it measured that the colony number of different *Bacillus* together decreased sharply at 60 °C. This result indicates that the actual growth of *B. amyloliquefaciens* can be detected by the CRISPR/Cas12-based ARMS assay. *B. amyloliquefaciens* plays a more important role in producing functional substance and characteristic aroma components (such as TTMP) than other *Bacillus*. Therefore, the assay has the potential to detect the actual growth of key microorganisms in the fermentation process and more precisely indicate the fermentation situation.

In summary, a highly specific method for monitoring key microorganisms during fermentation at the species level was developed by integrating ARMS-PCR with the CRISPR/Cas12 system. The dual recognition based on ARMS and CRISPR/Cas12 allowed for the detection of bacterial genes and single-nucleotide difference with high specificity. The CRISPR/Cas12-based ARMS assay was able to distinguish *B. amyloliquefaciens* from other *Bacillus*, enabling its detection at the species level. Furthermore, it yielded high sensitivity that can detect *B. amyloliquefaciens* with a limit of detection of 651 CFU and detect 1% *B. amyloliquefaciens* in a mixture of *B. amyloliquefaciens* and *B. subtilis*. The developed assay enabled the precise quantification of *B. amyloliquefaciens* during the fermentation of Daqu. The results showed that *B. amyloliquefaciens* grew rapidly during 0−4 day and remained steady at day 6 to 8, producing abundant TTMP. In contrast, the conventional PCR method, which measures the colony numbers of different *Bacillus*, cannot differentiate them and therefore may cover up the actual proliferation of *B. amyloliquefaciens* during fermentation. Overall, the CRISPR/Cas12-based ARMS assay, enabling species-level monitoring of

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key microorganisms, is a highly competitive tool for precisely controlling the quality of fermented food.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c04775.

Oligonucleotide sequences of primers and different gRNAs, gel electrophoresis analysis of the PCR products corresponding to the number of PCR cycles, Ct values of the qPCR test of different *Bacillus*, and standard curve for measuring TTMP using HPLC (PDF)

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Chilton, S. N.; Burton, J. P.; Reid, G. Inclusion of fermented foods in food guides around the world. *Nutrients* **2015**, *7*, 390−404.

(2) Şanlier, N.; Gökcen, B. B.; Sezgin, A. C. Health benefits of fermented foods. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 506−527.

(3) Marco, M. L.; Sanders, M. E.; Gänzle, M.; Arrieta, M. C.; Cotter, P. D.; De Vuyst, L.; Hill, C.; Holzapfel, W.; Lebeer, S.; Merenstein, D.; Reid, G.; Wolfe, B. E.; Hutkins, R. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on fermented foods. *Nat. Rev. Gastroenterol. Hepatol.* **2021**, *18*, 196−208.

(4) Wang, L.; Zhong, K.; Luo, A.; Chen, J.; Shen, Y.; Wang, X.; He, Q.; Gao, H. Dynamic changes of volatile compounds and bacterial diversity during fourth to seventh rounds of Chinese soy sauce aroma liquor. *Food Sci. Nutr.* **2021**, *9*, 3500−3511.

(5) Sakandar, H. A.; Hussain, R.; Farid Khan, Q.; Zhang, H. Functional microbiota in Chinese traditional Baijiu and Mijiu Qu (starters): A review. *Food Res. Int.* **2020**, *138*, No. 109830.

(6) Mao, J.; Liu, X.; Gao, T.; Gu, S.; Wu, Y.; Zhao, L.; Ma, J.; Li, X.; Zhang, J. Unraveling the correlations between bacterial diversity, physicochemical properties and bacterial community succession during the fermentation of traditional Chinese strong-flavor Daqu. *LWT* **2022**, *154*, 112764−112773.

(7) Zheng, X.-W.; Tabrizi, M. R.; Nout, M. J. R.; Han, B.-Z. Daqu—A traditional Chinese liquor fermentation starter. *J. Inst. Brewing* **2011**, *117*, 82−90.

(8) Li, P.; Lin, W.; Liu, X.; Wang, X.; Luo, L. Environmental factors affecting microbiota dynamics during traditional solid-state fermentation of Chinese Daqu starter. *Front. Microbiol.* **2016**, *7*, 1237.

(9) Sun, W. N.; Xiao, H. Z.; Peng, Q.; Zhang, Q. G.; Li, X. X.; Han, Y. Analysis of bacterial diversity of Chinese Luzhou-flavor liquor brewed in different seasons by Illumina Miseq sequencing. *Ann. Microbiol.* **2016**, *66*, 1293−1301.

(10) Wang, X.; Ban, S.; Hu, B.; Qiu, S.; Zhou, H. Bacterial diversity of Moutai-flavour Daqu based on high-throughput sequencing method. *J. Inst. Brewing* **2017**, *123*, 138−143.

(11) Li, H.; Lian, B.; Ding, Y.; Nie, C.; Zhang, Q. Bacterial diversity in the central black component of Maotai Daqu and its flavor analysis. *Ann. Microbiol.* **2014**, *64*, 1659−1669.

(12) Wang, X.-D.; Qiu, S.-Y.; Li, P.; Ban, S.-D. Analysis of microbial community structure in traditional and automated Moutai-flavor Daqu. *J. Am. Soc. Brew. Chem.* **2019**, *77*, 140−146.

(13) Zuo, Q.; Huang, Y.; MinGuo. MinGuo, Evaluation of bacterial diversity during fermentation process: a comparison between handmade and machine-made high-temperature Daqu of Maotai-flavor liquor. *Ann. Microbiol.* **2020**, *70*, 57.

(14) Wang, Z. M.; Wang, C. T.; Shen, C. H.; Wang, S. T.; Mao, J. Q.; Li, Z.; Ganzle, M.; Mao, J. Microbiota stratification and succession of amylase-producing *Bacillus* in traditional Chinese Jiuqu (fermentation starters). *J. Sci. Food Agric.* **2020**, *100*, 3544−3553.

(15) Vilela, D. M.; Pereira, G. V.; Silva, C. F.; Batista, L. R.; Schwan, R. F. Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (Coffea arabica L.). *Food Microbiol.* **2010**, *27*, 1128−1135.

(16) Zhang, W.; Si, G.; Du, H.; Li, J.; Zhou, P.; Ye, M. Directional design of a starter to assemble the initial microbial fermentation community of baijiu. *Food Res. Int.* **2020**, *134*, No. 109255.

(17) Guo, M.; Liu, Y.; Shi, D. Cardiovascular actions and therapeutic potential of tetramethylpyrazine (active component isolated from Rhizoma Chuanxiong): Roles and Mechanisms. *Biomed. Res. Int.* **2016**, *2016*, No. 2430329.

(18) Zhang, L.; Huang, J.; Zhou, R.; Wu, C. Evaluating the feasibility of fermentation starter inoculated with *Bacillus amyloliquefaciens* for improving acetoin and tetramethylpyrazine in Baoning bran vinegar. *Int. J. Food Microbiol.* **2017**, *255*, 42−50.

(19) Zhao, T.; Ni, D.; Hu, G.; Wang, L.; Chen, S.; Xu, Y. 6-(2-Formyl-5-methyl-1H-pyrrol-1-yl)hexanoic acid as a novel retronasal burnt aroma compound in soy sauce aroma-type Chinese Baijiu. *J. Agric. Food Chem.* **2019**, *67*, 7916−7925.

(20) He, G.; Dong, Y.; Huang, J.; Wang, X.; Zhang, S.; Wu, C.; Jin, Y.; Zhou, R. Alteration of microbial community for improving flavor character of Daqu by inoculation with *Bacillus velezensis* and *Bacillus subtilis*. *LWT* **2019**, *111*, 1−8.

(21) Xia, X.; Yang, H.; Cao, J.; Zhang, J.; He, Q.; Deng, R. Isothermal nucleic acid amplification for food safety analysis. *TrAC, Trends Anal. Chem.* **2022**, *153*, No. 116641.
(22) Johansson, A. H.; Bejai, S.; Niazi, A.; Manzoor, S.; Bongcam-Rudloff, E.; Meijer, J. Studies of plant colonisation by closely related Bacillus amyloliquefaciens biocontrol agents using strain specific quantitative PCR assays. *Antonie van Leeuwenhoek* **2014**, *106*, 1247−1257.
(23) Rotolo, C.; De Miccolis Angelini, R. M.; Pollastro, S.; Faretra, F. A TaqMan-based qPCR assay for quantitative detection of the biocontrol agents *Bacillus subtilis* strain QST713 and *Bacillus amyloliquefaciens* subsp. plantarum strain D747. *BioControl* **2016**, *61*, 91−101.
(24) Li, Z.; Fernandez, K. X.; Vederas, J. C.; Gänzle, M. G. Composition and activity of antifungal lipopeptides produced by *Bacillus spp*. in daqu fermentation. *Food Microbiol.* **2023**, *111*, No. 104211.
(25) Zhang, W.; Si, G.; Rao, Z.; Li, J.; Zhang, X.; Mei, J.; Wang, J.; Ye, M.; Zhou, P. High yield of tetramethylpyrazine in functional Fuqu using *Bacillus amyloliquefaciens*. *Food Biosci.* **2019**, *31*, No. 100435.
(26) Dorman, C. J.; Bhriain, N. N. CRISPR-Cas, DNA Supercoiling, and nucleoid-associated proteins. *Trends Microbiol.* **2020**, *28*, 19−27.
(27) Hille, F.; Richter, H.; Wong, S. P.; Bratovic, M.; Ressel, S.; Charpentier, E. The biology of CRISPR-Cas: Backward and forward. *Cell* **2018**, *172*, 1239−1259.
(28) Koonin, E. V.; Makarova, K. S.; Zhang, F. Diversity, classification and evolution of CRISPR-Cas systems. *Curr. Opin. Microbiol.* **2017**, *37*, 67−78.
(29) Mojica, F. J. M.; Diez-Villasenor, C.; Soria, E.; Juez, G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, bacteria and mitochondria. *Mol. Microbiol.* **2000**, *36*, 244−246.
(30) Makarova, K. S.; Wolf, Y. I.; Iranzo, J.; Shmakov, S. A.; Alkhnbashi, O. S.; Brouns, S. J. J.; Charpentier, E.; Cheng, D.; Haft, D. H.; Horvath, P.; Moineau, S.; Mojica, F. J. M.; Scott, D.; Shah, S. A.; Siksnys, V.; Terns, M. P.; Venclovas, C.; White, M. F.; Yakunin, A. F.; Yan, W.; Zhang, F.; Garrett, R. A.; Backofen, R.; van der Oost, J.; Barrangou, R.; Koonin, E. V. Evolutionary classification of CRISPR−Cas systems: a burst of class 2 and derived variants. *Nat. Rev. Microbiol.* **2020**, *18*, 67−83.
(31) Gootenberg, J. S.; Abudayyeh, O. O.; Kellner, M. J.; Joung, J.; Collins, J. J.; Zhang, F. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* **2018**, *360*, 439−444.
(32) Paul, B.; Montoya, G. CRISPR-Cas12a: Functional overview and applications. *Biomed. J.* **2020**, *43*, 8−17.
(33) Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* **2018**, *360*, 436−439.
(34) Liang, M.; Li, Z.; Wang, W.; Liu, J.; Liu, L.; Zhu, G.; Karthik, L.; Wang, M.; Wang, K.-F.; Wang, Z.; Yu, J.; Shuai, Y.; Yu, J.; Zhang, L.; Yang, Z.; Li, C.; Zhang, Q.; Shi, T.; Zhou, L.; Xie, F.; Dai, H.; Liu, X.; Zhang, J.; Liu, G.; Zhuo, Y.; Zhang, B.; Liu, C.; Li, S.; Xia, X.; Tong, Y.; Liu, Y.; Alterovitz, G.; Tan, G.-Y.; Zhang, L.-X. A CRISPR-Cas12a-derived biosensing platform for the highly sensitive detection of diverse small molecules. *Nat. Commun.* **2019**, *10*, 3672−3681.
(35) Xia, X.; Ma, B.; Zhang, T.; Lu, Y.; Khan, M. R.; Hu, Y.; Lei, C.; Deng, S.; He, Q.; He, G.; Zhang, K.; Deng, R. G-quadruplex-probing CRISPR-Cas12 assay for label-free analysis of foodborne pathogens and their colonization in vivo. *ACS Sens.* **2021**, *6*, 3295−3302.
(36) Bai, L.; Wang, L.; Huang, S.; Bai, R.; Lv, X.-C.; Sun, L.; Zhang, F.; Xu, X. Rapid, visual, and sequence-specific detection of *Salmonella* in egg liquid with vis-NEAA, a CRISPR/Cas12 empowered new strategy. *J. Agric. Food Chem.* **2022**, *70*, 2401−2409.
(37) Peng, L.; Zhou, J.; Yin, L.; Man, S.; Ma, L. Integration of logic gates to CRISPR/Cas12a system for rapid and sensitive detection of pathogenic bacterial genes. *Anal. Chim. Acta* **2020**, *1125*, 162.
(38) Wu, Y.; Dong, Y.; Shi, Y.; Yang, H.; Zhang, J.; Khan, M. R.; Deng, S.; He, G.; He, Q.; Lv, Y.; Deng, R. CRISPR-Cas12-based rapid authentication of halal food. *J. Agric. Food Chem.* **2021**, *69*, 10321−10328.
(39) Mu, K.; Ren, X.; Yang, H.; Zhang, T.; Yan, W.; Yuan, F.; Wu, J.; Kang, Z.; Han, D.; Deng, R.; Zeng, Q. CRISPR-Cas12a-based diagnostics of wheat fungal diseases. *J. Agric. Food Chem.* **2022**, *70*, 7240−7247.

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