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ORIGINAL PAPER

Establishment of a CRISPR-Cas12a based electrochemical detection method for *Burkholderia gladioli* and its subspecies *cocovenenans* in fresh noodles and tremella

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Abstract
Burkholderia gladioli pv. *cocovenenans* is a pathogenic bacterium of concern due to its potential threat to food safety. This study presents a rapid electrochemical detection method based on dual recombinase-aided amplification (RAA) coupled with CRISPR-Cas12a for the sensitive and specific identification of *B. gladioli* and its toxigenic subspecies *B. gladioli* pv. *cocovenenans* in food. The 16 S rDNA and *bonA* genes were selected as species- and subspecies-specific targets, respectively, and corresponding CRISPR-Cas12a reaction systems were established. An electrochemical biosensor incorporating a gold electrode functionalized with single-stranded DNA probes was constructed, and its specificity and sensitivity were evaluated using artificially contaminated fresh noodles and tremella samples. The method precisely distinguished *B. gladioli* and its toxigenic *cocovenenans* subspecies within 1 h, achieving a limit of detection limit of 10^2 CFU/g in both food matrices. This strategy provides a rapid and field-deployable approach for distinguishing non-toxigenic and toxigenic *B. gladioli* strains in food, supporting timely screening and food safety monitoring.

Keywords *Burkholderia gladioli* pv. *cocovenenans* · Food safety · ECRISPR-Cas detection · Electrochemical detection

Introduction

The foodborne pathogen *Burkholderia gladioli* pv. *cocovenenans* is often associated with the contamination of spoiled foods such as cereal-based products and rehydrated tremella (Anwar et al. 2017; Li et al. 2019). In recent years, outbreaks caused by this bacterium have occurred frequently in Asia. One of the most severe incidents was the 2024 collective poisoning at the "Baolin Teahouse" in Taipei, which resulted in seven deaths. This pathogen produces bongkrekic acid, a potent toxin that inhibits mitochondrial oxidative phosphorylation in mammals, leading to severe poisoning or death (Shi et al. 2019). Bongkrekic acid is colorless, tasteless, and highly heat-stable, making it difficult to eliminate through cooking and allowing it to persist as a threat during food processing and distribution (Anwar et al. 2017). Given its serious impact on public health and food safety, the development of reliable detection methods for both this pathogen and its toxin have become a critical challenge in food safety monitoring (Guo et al. 2022; Li et al. 2021a, b).

The current Chinese national food safety standard GB 4789.29–2020 specifies detection procedures of *B. gladioli* pv. *cocovenenans*. However, this method is highly complex, involving biochemical identification and toxicity assays that require 15–20 days to complete. In particular, the toxicity assay requires toxin fermentation of suspected strains followed by administration of the fermented culture to mice, making the process not only time-consuming but also costly and ethically constrained. Such a prolonged process cannot meet the urgent need for rapid responses to contaminated events, as affected products may reach consumers during the testing period, preventing timely intervention (Wang et al. 2022). Therefore, establishing a rapid, accurate, and efficient detection approach for *B. gladioli* pv. *cocovenenans* is

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essential to improve monitoring timeliness and emergency response capability.

The clustered regularly interspaced short palindromic repeats-CRISPR associated proteins (CRISPR-Cas) system has emerged as a powerful molecular tool due to its rapid response, high sensitivity, and specificity. When combined with electrochemical sensors, CRISPR-Cas techniques have been increasingly used in the development of biosensors for detecting foodborne pathogens, clinical samples, and viruses (Gootenberg et al. 2018; Zhuang et al. 2022). In our prior work, we have developed a dual-gene detection assay integrating recombinase-aided amplification (RAA) with CRISPR-Cas12a/Cas13a, enabling simultaneous detection of *B. gladioli* at the species level and *B. gladioli* pv. *cocovenenans* at the subspecies level (Yao et al. 2024). This approach aimed to differentiate bongkrekic acid–producing *cocovenenans* from non–bongkrekic acid–producing *B. gladioli* to allow risk-based classification of contaminated foods. However, the results indicated that CRISPR-Cas13a was less effective for detecting bacterial DNA targets compared with CRISPR-Cas12a. Moreover, dual RAA was performed on DNA extracted from suspected bacterial strains in food samples, followed by stepwise detection using two CRISPR-Cas12a systems. The first reaction confirmed the presence of *B. gladioli* at the species level. Only when the first reaction was positive, the second reaction was carried out to identify *B. gladioli* pv. *cocovenenans* at the subspecies level (Fig. 1). This workflow could also achieve the goal of effective risk stratification of contaminated foods.

In this study, we integrated dual RAA, the CRISPR-Cas12a system, and electrochemical biosensor technique to establish a rapid, convenient, and highly specific method for nucleic acid detection. This method allows precise differentiation between non-toxic *B. gladioli* and toxic *B. gladioli* pv. *cocovenenans* in food matrices, and is suitable for field-deployable testing, providing an efficient and practical tool for food safety monitoring and risk management.

Materials and methods

Bacterial strains

Burkholderia gladioli strains CICC 10,574, CICC 25,088, CICC 25,089, CICC 25,108, CICC 25,126, CICC 25,131, and CICC 25,132 were obtained from the China Center of Industrial Culture Collection (CICC). Additional *B. gladioli* (ATCC 33664, ATCC 49839, ATCC 18302 and D50076) were obtained from the American Type Culture Collection (ATCC) and Shanghai Microbial Culture Collection Center (SHMCC). *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15,442 were obtained from ATCC. Other bacterial strains were isolated from food or environmental swab samples and stored at -80°C. Details of all strains and their bongkrekic acid-producing characteristics are listed in Table 1.

Culture conditions and DNA template preparation

All *B. gladioli* strains were grown on potato dextrose agar medium at 37°C for 2 days, while *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *S. typhimurium* ATCC 14,028, and *P. aeruginosa* ATCC 15,442 were cultured on nutrient agar medium under the same conditions. For the specificity test, a heat lysis method was used. A single colony of each strain was transferred into a sterile 1.5 mL centrifuge tube containing 50 µL of elution buffer (EB) (Qiagen Inc.,Valencia, CA, USA), incubated at 100°C for 10 min, and then centrifuged at 13,500 × g for 10 min. The resulting supernatant

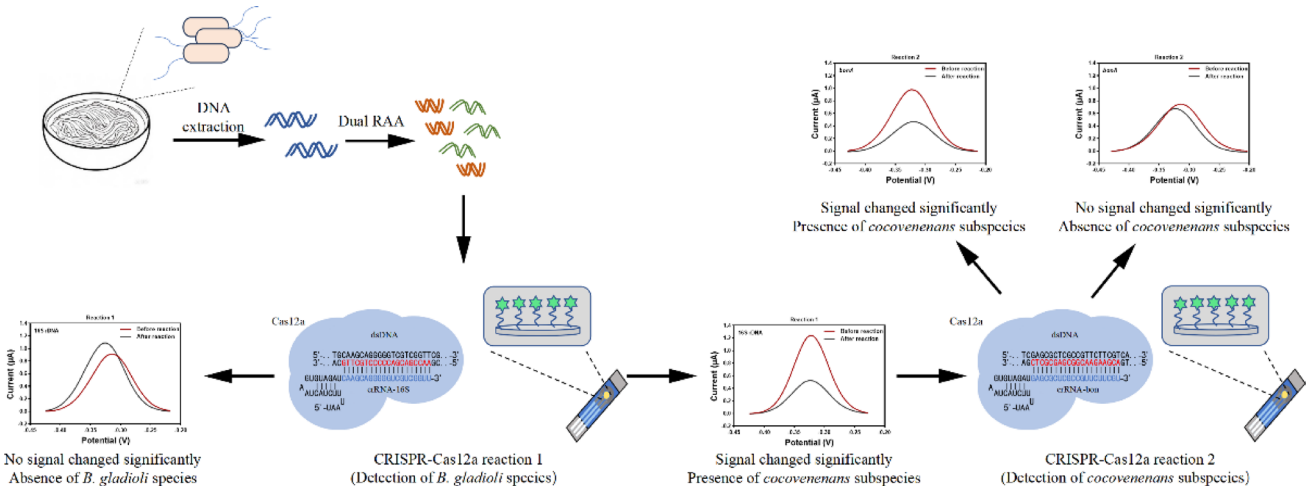


Fig. 1 Schematic illustration of the dual RAA-CRISPR-Cas12a electrochemical method for the stepwise detection of *B. gladioli* and its subspecies *cocovenenans* in food samples

Table 1 Bacterial strains sources and toxigenic characteristics

Strain name	Sample source	Bongkrekic acid production
RB2109	Rice	–[a]
PB2208	Swab sample	–
NB2207	Rice noodles	–
NB2207-2	Rice noodles	–
PB2207	Swab sample	+
RB2210	Rice	+
RB2210-2	Rice	+
PB2107	Swab sample	+
PB2208-2	Swab sample	+
PB2109	Swab sample	+
PB2108	Swab sample	+
PB2109-2	Swab sample	+
Burkholderia gladioli ATCC 18,302	From CHMCC	–
Burkholderia gladioli ATCC 33,664	From ATCC	+
Burkholderia gladioli ATCC 49,839	From CHMCC	+
Burkholderia gladioli CICC 10,574	From CICC	–
Burkholderia gladioli CICC 25,088	From CICC	–
Burkholderia gladioli CICC 25,089	From CICC	–
Burkholderia gladioli CICC 25,108	From CICC	+
Burkholderia gladioli CICC 25,126	From CICC	+
Burkholderia gladioli CICC 25,131	From CICC	+
Burkholderia gladioli CICC 25,132	From CICC	+
Burkholderia gladioli D50076	From CHMCC	–
Burkholderia cepacia CICC 23,648	From CICC	/
Escherichia coli ATCC 8739	From ATCC	/
Staphylococcus aureus ATCC 6538	From ATCC	/
Salmonella typhimurium ATCC 14,028	From ATCC	/
Pseudomonas aeruginosa ATCC 15,442	From ATCC	/

[a]"+" represents a positive bongkrekic acid result. "–" represents a negative result. "/" represents the absence of bongkrekic acid-producing genes

was used for subsequent detection, and all experiments were performed with three biological replicates.

For the sensitivity test in real food samples (fresh noodles and rehydrated tremella), *B. gladioli* ATCC 49,839 was cultured in potato dextrose broth medium at 37 °C and 220 r/min for 36 h. Cultures were then serially diluted with physiological saline to concentrations ranging from 10^6 to 10^2 CFU/mL, and 100 µL of each dilution was added to 1 g of the food sample. *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 were cultured in nutrient broth at 37 °C and 220 r/min for 16 h, and then diluted with physiological saline to a concentration of 10^5 CFU/mL. 100 µL of each suspension was added to the food samples mentioned above as interfering bacteria. A negative control sample was prepared by adding only the interfering bacteria (without *B. gladioli*) to 1 g of the food sample. Following homogenization, DNA was extracted using the DNeasy Mericon Food Kit (Qiagen Inc.,Valencia, CA, USA) for subsequent detection. The concentration and purity (A230/A260 and A260/A280 ratios) of the extracted DNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation and characterization of MB-ssDNA modified gold electrodes

In this study, gold electrodes (Changsha Sunjeen Electronic Technology Co, Ltd, Changsha, China) were used without polishing or grinding, so these steps were omitted. Electrodes were rinsed three times with distilled water and dried. The preparation of Methylene blue-modified single-stranded DNA (MB-ssDNA) probe-modified gold electrodes was performed following the method of Li et al. with slight modification (Li et al. 2021a, b). The MB-ssDNA probe was diluted to 10 µM in RNase-free water and reduced with 10 µM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 37 °C in the dark for 2 h to break disulfide bonds. After reduction, 8 µL of MB-ssDNA solution was dropped onto the working electrode and incubated at 37 °C in the dark for 4 h. The electrode was then rinsed three times with distilled water, dried, and further modified by adding 8 µL of 2 mM 6-mercapto-1-hexanol (MCH) in 10 mM Tris buffer, followed by incubation for 1 h. Finally, the electrode underwent triple rinsing with distilled water, followed by drying and storage for subsequent CRISPR-Cas12a detection.

Modified gold electrodes were characterized using cyclic voltammetry (CV) assay and electrochemical impedance

spectroscopy (EIS) assay with 2.5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl as the redox probe. CV assay was performed within a potential range of − 0.8 V to 0.8 V, with a potential step of 10 mV and a scan rate of 0.05 V/s. EIS assay was conducted over a frequency range of 10^{-1} Hz to 10^4 Hz.

The storage stability of the modified gold electrodes was evaluated using square wave voltammetry (SWV) assay following the procedure detailed below. Three modified gold electrodes were stored at 4 °C and tested after storage periods of 0, 7 and 20 days. The resulting current peaks were compared to assess electrode stability over time.

Dual recombinase-aided amplification (RAA) and CRISPR-Cas12a detection reaction

Primers, probes, crRNAs and amplification product are listed in Table 2. Primers and probes were synthesized from Sangon Biotech (Shanghai) Co., Ltd. Dual RAA amplification was carried out using a commercial recombinase aided amplification kit (Jiangsu Qitian Biotechnology Co., Ltd, Wuxi, China). The 45 µL reaction mixture contained 25 µL of buffer V, 2 µL of each primer (RAA-16 S-F, RAA-16 S-R, RAA-bon-F, RAA-bon-R), 10 µL of double-distilled water (ddH_2O), 1 µL of DNA, and 5 µL of magnesium acetate. The mixtures were incubated at 37 °C for 20 min in an Applied Biosystem™ Veriti™ 96-well Fast Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA), using ddH_2O in place of DNA as the negative control. The amplification products were RAA-16 S and RAA-bon fragments. After purification using the phenol-chloroform method, 5 µL of the supernatant was collected for detection by agarose gel electrophoresis.

DNA templates for crRNA-16 S and crRNA-bon were amplified via overlapping extension PCR using the pairs crRNA-16 S-F/crRNA-16 S-R and crRNA-bon-F/crRNA-bon-R, respectively. The crRNAs were then transcribed in vitro and purified using a high-yield T7 RNA transcription kit (EZassay Biotechnology Co., Ltd, Shenzhen, China) according to the manufacturer's manual. In brief, the 50 µL reaction mixture contained 5 µL of 10× T7 Reaction Buffer, 2 µL of T7 RNA Polymerase Mix, 4 µL of each ATP, CTP, GTP, and UTP (100 mM), 1 µL of RNase inhibitor, and 1 µg of DNA templates. The volume was adjusted to 50 µL with RNase-free ddH_2O. The mixture was then incubated at 37 °C for 2 h. After that, a premix containing 10 µL of DNase I Buffer and 1 µL of DNase I was added and the

Table 2 Sequences of primers, probes and amplification products in this study

Name	Sequence (5′–3′)
Sequences of primers and probes	
ssDNA probe	FAM[a]-TTATTATT-BHQ1[b]
MB-ssDNA probe	MB[c]-AAAAAAAAAAAAAAAAAAAA-SH[d]
crRNA-16 S	UAAUUUCUACUAAGUGUAGAUCAAGCAGGGGGUCGUCGGUU
crRNA-bon	UAAUUUCUACUAAGUGUAGAUGAGCGCUCGCCGUUCUUCGU
RAA-16 S-F	CGTTTGTCATTGAAGATTGGTGGAGGCAGAC
RAA-16 S-R	CTCAGTCGGTTAGAGCACCGTCTTGATAAGG
RAA-bon-F	CTGCATTACCGCACGCCCAACGAGCACTTCG
RAA-bon-R	CGGGCGATATTCCTCGATCACCAGATGCGC
crRNA-16 S-F	TAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATCAA GCAG
crRNA-16 S-R	AACCGACGACCCCCTGCTTGATCTACACTTAGTAG
crRNA-bon-F	TAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATGAG CGCT
crRNA-bon-R	ACGAAGAACGGCGAGCGCTCATCTACACTTAGTAG
Sequences of amplification products	
RAA-16 S	CGTTTGTCATTGAAGATTGGTGGAGGCAGACGGGATCG*AACCG ACGACCCCCTGCTTG*CAAAGCAGGTGCTCTCCCAGCTGAGCTA TGCCCCCATACAGAGACCTCTCAGGTTTCTACCGCCAGACAAT TGGTGGGTCTGGTTGGATTCGAACCAACGACCCCCGCCTTATC AAGACGGTGCTCTAACCGACTGAG[e]
RAA-bon	CTGCATTACCGCACGCCCAACGAGCACTTCGATTTC*GAGCGCT CGCCGTTCTTCGT*CAACACGGTCCTGCAGCCCTGGCCGGCCGA CGATGGCCGGCCGCGGCGCGCCGCGGTCAGCTCGTTCGGTATC AGCGGCACCAACGCGCATCTGGTGATCGAGGAATATCGCCCG

The underlines represent crRNA binding sites

[a] FAM = 5-Carboxyfluorescein

[b] BHQ1 = Black hole quencher 1

[c] MB = Methylene blue

[d] SH= Thiol group

mixture was incubated at 37 °C for 1 h to remove the DNA templates. The transcribed RNA was then purified by ethanol precipitation. The concentration and purity (A230/A260 and A260/A280 ratios) of the crRNAs were determined using a NanoDrop spectrophotometer. The crRNAs were aliquoted and stored at -20 °C. Prior to use, an aliquot was diluted to the desired concentration using RNase-free water.

For fluorescence-based detection of the 16 S rDNA, the CRISPR-Cas12a reaction mixture included 2 µL of 10 × Cas12a reaction buffer, 0.5 µL of ssDNA probe (10 µM), 1 µL of crRNA-16 S (10 µM), 1 µL of LbaCas12a (1 µM; EZassay Biotechnology Co., Ltd, Shenzhen, China), 1 µL of DNA, and RNase-free water to a final volume of 20 µL. For *bonA* gene detection, crRNA-16 S was replaced with crRNA-bon. Reactions were carried out at 37 °C for 30 min in a LightCycler480 II real-time fluorescent quantitative PCR instrument (Roche Diagnostics (Shanghai) Ltd., Shanghai, China), with FAM fluorescence recorded every minute. Cleavage efficiency was expressed as $\Delta F = F_1 - F_0$, where F_0 and F_1 are the fluorescence intensity at 0 and 30 min, respectively. The experiments were performed with three biological replicates.

For quantitative PCR (qPCR) detection, primer-probe sets targeting the 16 S rDNA gene and the *bon* gene cluster were adopted from previous studies (Lin et al. 2020; Wang et al. 2022). Each 25 µL reaction mixture contained 12.5 µL of Premix Taq (Takara Bio, Inc., Dalian, China), 1 µL each of forward primer and reverse primer, 0.65 µL of probe, and nuclease-free water added to volume. Amplification was performed on a LightCycler 480 II system (Roche Diagnostics, Shanghai, China) under the following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 5 s and 60 °C for 40 s, with fluorescence acquisition at 60 °C. The experiments were performed with three biological replicates.

For electrochemical detection, the ssDNA probe was omitted from the above CRISPR-Cas12a reaction mixture. SWV assay was performed by adding 40 µL of 0.1 M PBS buffer onto the MB-ssDNA modified gold electrode. SWV parameters were as follows: potential range, -0.5 V to 0 V; potential step, 5 mV; pulse amplitude, 25 mV; frequency, 25 Hz. The peak current before adding the CRISPR-Cas12a reaction mixture was recorded as I_0. After washing three times with ddH_2O and drying, the electrode was incubated with the CRISPR-Cas12a reaction mixture at 37 °C for 40 min, and the peak current was recorded as I_1. Cleavage efficiency was calculated as the relative change in current ($\Delta I\% = (I_0 - I_1)/ I_0$). The MB signal variation for the 16 S rDNA target was defined as ΔI% (16 S rDNA), and that for the *bonA* gene was defined as ΔI% (*bonA*). All SWV curves were baseline-corrected using PSTrace 5 software. The experiments were performed in triplicate as technical replicates.

Results

Establishment of a dual RAA-CRISPR-Cas12a reaction system

To establish a dual RAA-CRISPR-Cas12a reaction system capable of distinguishing between non-bongkrekic acid-producing *B. gladioli*, bongkrekic acid-producing *B. gladioli* pv. *cocovenenans* and non-target strains, we first performed a specificity test using a quantitative fluorescence PCR instrument. A total of 23 *B. gladioli* strains and 4 non-target bacterial strains were tested. The bongkrekic acid-producing characteristics of the 23 *B. gladioli* strains had been previously confirmed by HPLC-MS/MS in our earlier study (Yao et al. 2024). Among these, 14 strains were *B. gladioli* pv. *cocovenenans* and 9 were non-bongkrekic acid-producing *B. gladioli*. Details of the strains and their bongkrekic acid-producing characteristics are listed in Table 1.

Genomic DNA from each strain was extracted from each colony by heat lysis, and the resulting supernatant was amplified by dual RAA and subsequently analyzed using CRISPR-Cas12a reaction 1 and reaction 2, targeting the 16 S rDNA and *bonA* genes, respectively. The fluorescence curves of both reactions for the amplified products of each strain are shown in Fig. 2a. For quantitative comparison, the change in FAM fluorescence intensity (ΔF) over 30 min was calculated for each sample (Fig. 2b). In the 16 S rDNA assay, ΔF values of all 23 *B. gladioli* strains were significantly higher than those of non-target strains and the negative control ($p < 0.05$), indicating that reaction 1 effectively recognized the *B. gladioli* species. In contrast, in the *bonA* assay, ΔF values of the 14 *B. gladioli* pv. *cocovenenans* strains were significantly higher than those of the 9 non-bongkrekic acid-producing *B. gladioli* strains, non-target strains and the negative control ($p < 0.05$), demonstrating that reaction 2 specifically recognized the *cocovenenans* subspecies.

These results collectively indicated that the established dual RAA-CRISPR-Cas12a method can accurately differentiate non-bongkrekic acid-producing *B. gladioli*, bongkrekic acid-producing *cocovenenans* subspecies and non-target bacterial strains.

Specificity test of dual RAA-CRISPR-Cas12a electrochemical method

To achieve rapid and field-deployable detection independent of fluorescence quantitative PCR instrument, an electrochemical detection method based on signal transduction was developed. MB-ssDNA probes were immobilized on gold electrodes, and their surface modification was characterized using cyclic voltammetry (CV) assay and electrochemical

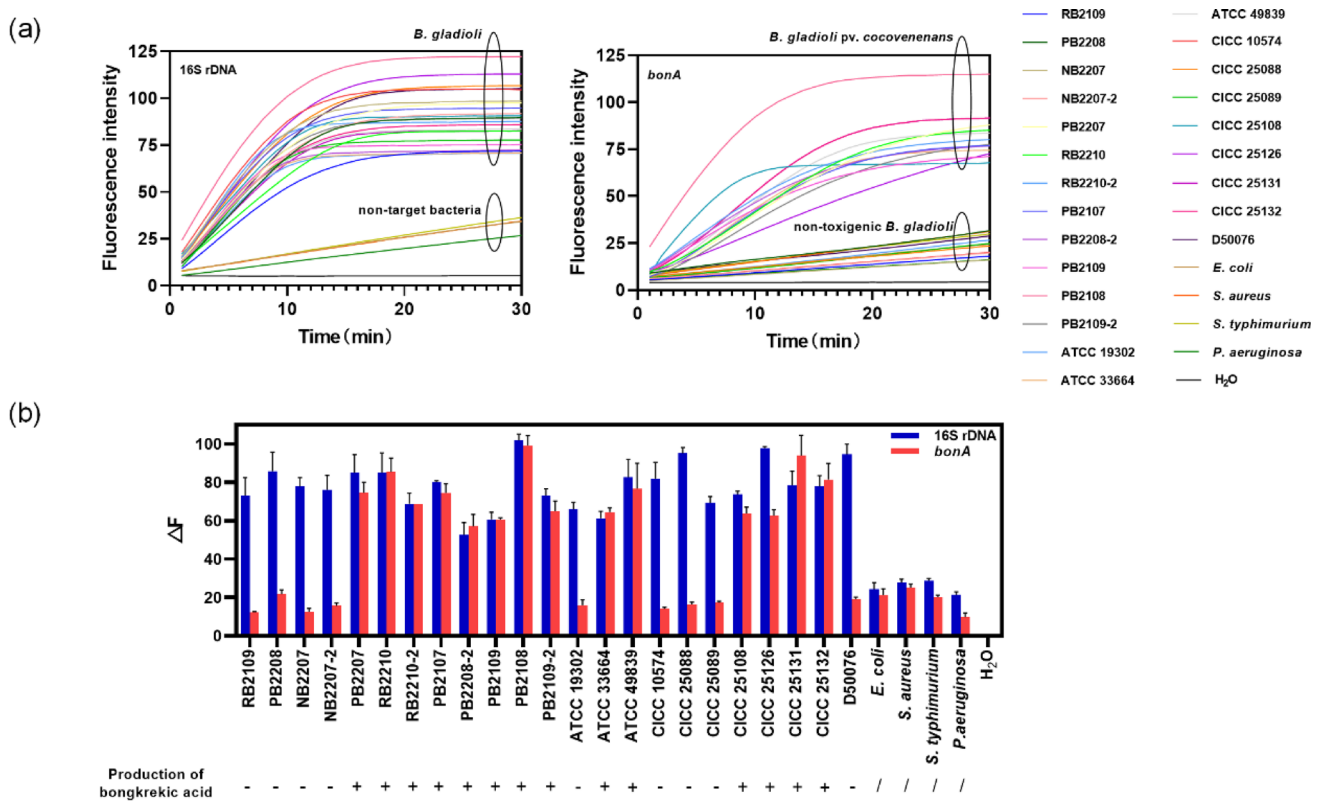


Fig. 2 Specificity analysis of the dual RAA-CRISPR-Cas12a method. **a** Fluorescence curves of CRISPR-Cas12a reaction 1 and 2 for detecting 16 S rDNA and *bonA* respectively. **b** Statistical results of the specificity analysis for 23 *B. gladioli* strains, 4 non-target bacterial strains and a negative control. ΔF represents change in fluorescence intensity. Bongkrekic acid production was determined by HPLC-MS/MS (Yao et al. 2024). "+" represents a positive bongkrekic acid result. "–" represents a negative result. "/" represents the absence of bongkrekic acid-producing genes

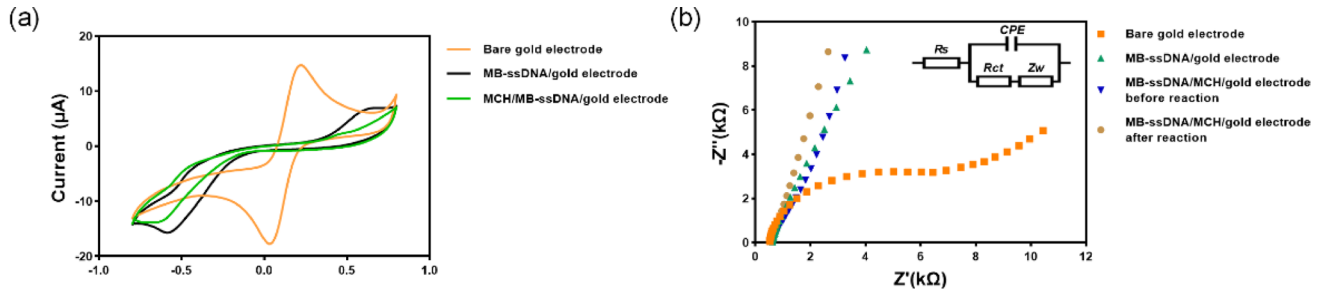


Fig. 3 Characterization of surface modifications on gold electrodes using **a** cyclic voltammetry (CV) assay and **b** electrochemical impedance spectroscopy (EIS) assay. In the equivalent circuit diagram (insert in **b**), "*Rs*", "*Rct*", "*CPE*" and "*Zw*" represent the solution resistance, charge transfer resistance, constant phase element, and Warburg impedance, respectively

impedance spectroscopy (EIS) assay. As shown in Fig. 3a, the bare gold electrode displayed well-defined reversible redox peaks. A pronounced decrease in peak current was observed following MB-ssDNA probes immobilization, due to electrostatic repulsion between the negatively charged DNA probes and the negatively charged $[Fe(CN)_6]^{3-/4-}$ in the electrolyte solution (Jia et al. 2021), indicating successful immobilization of MB-ssDNA probes on the working electrode surface. Subsequent treatment with MCH led to a further decrease in peak current, indicating effective surface passivation of the working electrode (Zheng et al. 2020). The EIS results, based on the Randles equivalent circuit (insert in Fig. 3b), showed that the bare gold electrode exhibited a complete semicircular pattern, whereas the MB-ssDNA modified electrode displayed an incomplete but semicircular trend. The simulated circuit model indicated a relatively large charge transfer resistance (Rct), which may account for the incomplete semicircle. We have evaluated the storage stability of the MB-ssDNA–modified electrode at 4 °C. After storage for 7 and 20 days, the average signal

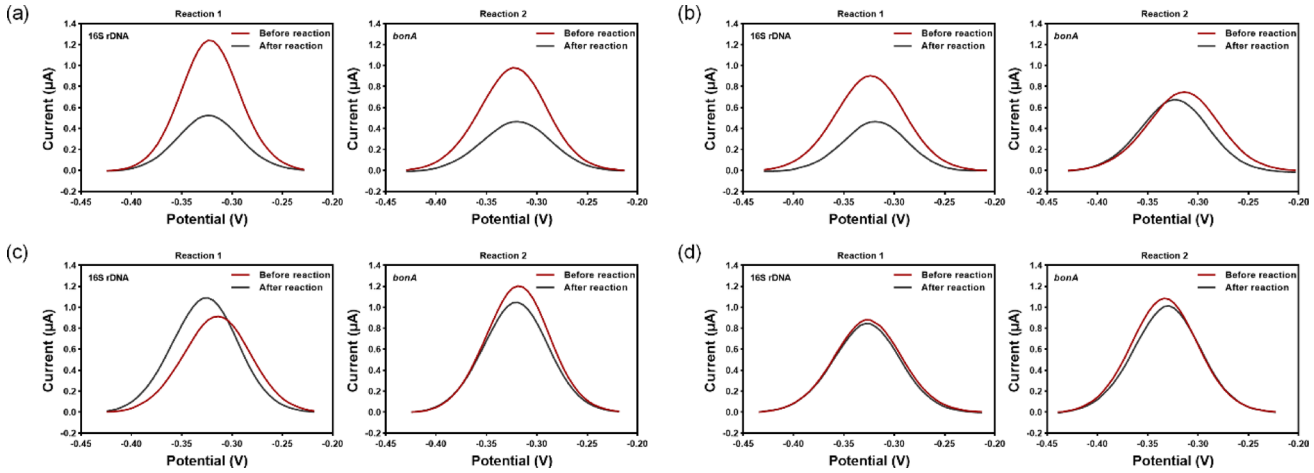


Fig. 4 Electrochemical detection of **a***B. gladioli* pv. *cocovenenans* (ATCC 49839), **b** non-toxigenic *B. gladioli* (CICC 10574), **c** non-target bacterial strain (*S. aureus*), and **d** negative control (H_2O). Each panel shows two CRISPR-Cas12a electrochemical reactions. Reaction 1 detects 16 S rDNA (left) and reaction 2 detects *bonA* (right), with two SWV curves for each reaction representing measurements before and after reaction

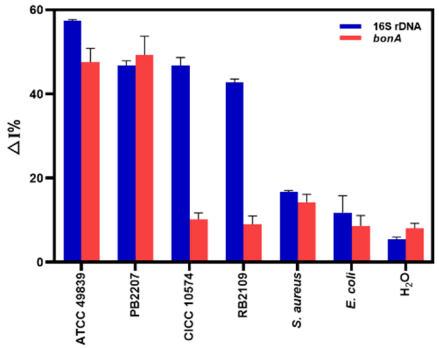


Fig. 5 Specificity analysis of the dual RAA-CRISPR-Cas12a electrochemical detection method. ΔI% represents relative change in current

remained at 91.5% and 80.1% of the initial signal, respectively, indicating that the electrode exhibits good stability for short-term use (Fig. S1).

To evaluate the specificity of the dual RAA-CRISPR-Cas12a electrochemical detection method, two strains each were selected for testing from non-toxigenic *B. gladioli* (CICC 10574 and RB2109), toxigenic *B. gladioli* pv. *cocovenenans* (ATCC 49839 and PB2207) and non-target bacterial strains (*E. coli* and *S. aureus*). Distilled water served as the negative control. Detection of the 16 S rDNA and *bonA* genes was performed on two separate electrode sensors and signal changes were determined by square wave voltammetry (SWV) assay. As shown in Fig. 4a–d, an MB redox peak appeared around −0.32 V (vs. Ag/AgCl). During electrochemical detection of the 16 S rDNA and *bonA* genes, a significant decrease in MB signal was observed when the CRISPR-Cas12a system recognized the target DNA, whereas only minor changes occurred in the absence of the targets. The relative change in signal was quantified as ΔI%.

As shown in Fig. 5, for bongkrekic acid-producing *B. gladioli* pv. *cocovenenans* strains ATCC 49,839 and PB2207, both ΔI% (16 S rDNA) and ΔI% (*bonA*) were significantly higher than in non-target bacterial strains and the negative control ($p<0.05$). In contrast, for non-toxigenic *B. gladioli* strains CICC 10,574 and RB2109, only ΔI% (16 S rDNA) was significantly higher than that of non-target bacterial strains and the negative control ($p<0.05$), whereas ΔI% (*bonA*) showed no significant difference ($p>0.05$). The results demonstrated that the dual RAA-CRISPR-Cas12a electrochemical detection method can specifically distinguish non-toxigenic *B. gladioli*, toxigenic *B. gladioli* pv. *cocovenenans*, and non-*B. gladioli* strains.

Sensitivity test of dual RAA-CRISPR-Cas12a electrochemical method

To evaluate the performance of the dual RAA-CRISPR-Cas12a electrochemical assay in real food matrices, fresh noodles and rehydrated tremella were selected as representative substrates for sensitivity testing. Artificially contaminated food samples were prepared by inoculating suspensions of *B. gladioli* pv. *cocovenenans* strain ATCC 49,839 into the food matrices at concentrations ranging from 10^5 to 10^1 CFU/g. To simulate naturally contaminated food conditions, *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 were used as interfering bacteria and added to the food matrices at a concentration of 10^4 CFU/g. As shown in Fig. 6, for both food matrices, ΔI% (16 S rDNA) and ΔI% (*bonA*) were significantly higher at bacterial concentrations of 10^5-10^2 CFU/g compared with 10^1 CFU/g, the negative control and the blank control ($p<0.05$). For fresh noodles, ΔI% (16 S rDNA) and ΔI% (*bonA*) were linearly

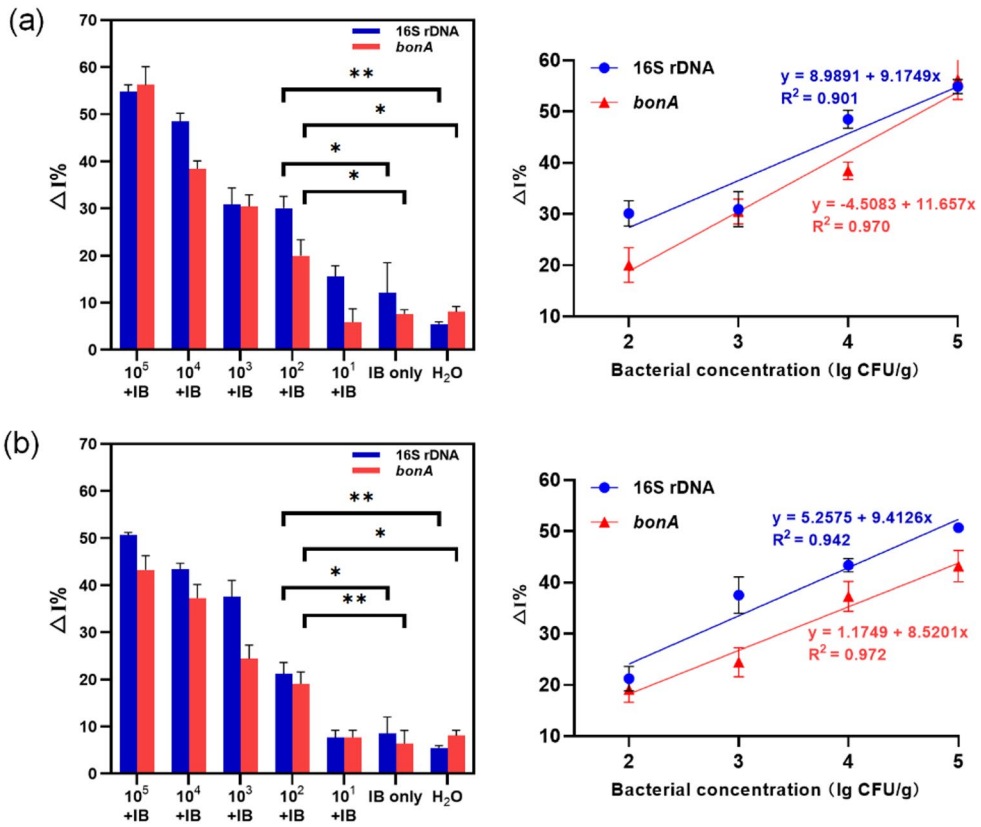


Fig. 6 Sensitivity analysis of the dual RAA-CRISPR-Cas12a electrochemical detection method in artificially contaminated food samples, **a** fresh noodles and **b** tremella. IB represents interfering bacteria. ΔI% represents relative change in current. "*" represents $p<0.05$ and "**" represents $p<0.01$

related to the logarithms of *B. gladioli* ATCC 49,839 bacterial concentrations, this were mapped with the equation ΔI% (16 S rDNA) = 8.9891 + 9.1749 $lgC_{B.\ gladioli}$, and ΔI% (*bonA*) = -4.5083 + 11.657 $lgC_{B.\ gladioli}$, furthermore the R^2 were calculated to be 0.901 and 0.970 respectively. While for tremella, ΔI% (16 S rDNA) = 5.2575 + 9.4126 $lgC_{B.\ gladioli}$, and ΔI% (*bonA*) = 1.1749 + 8.5201 $lgC_{B.\ gladioli}$, with R^2 were 0.942 and 0.972 respectively. Therefore, the limit of detection of the dual RAA-CRISPR-Cas12a electrochemical method for both fresh noodles and tremella was determined to be 10^2 CFU/g.

Discussion

Burkholderia gladioli pv. *cocovenenans* is a highly pathogenic foodborne pathogen that poses a significant public health risk due to its potential to cause food poisoning incidents. Rapid and accurate detection methods are urgently needed to identify contaminated foods before they reach consumers and to prevent poisoning incidents. Traditional culture-based methods, such as those described in the Chinese national food safety standard GB 4789.29–2020, are time-consuming and complex. In contrast, nucleic acid-based approaches are faster and more convenient, and several methods have been developed to meet this need.

Quantitative PCR (qPCR), one of the most widely used nucleic acid detection technique, offers simplicity, rapidity and specificity, but its sensitivity is limited, with a detection limit around 10^2 CFU/mL for bacterial suspensions (Jiang et al. 2023; Wang et al. 2022). In this study, we also evaluated the sensitivity of qPCR assays using artificially contaminated food samples, employing species-specific and subspecies-specific primer-probe sets reported by Lin et al. (2020) and Wang et al. (2022), respectively. The results showed that the detection limit in both fresh noodles and tremella matrices was 10^3 CFU/g (Fig. S2). The CRISPR-Cas system, with its collateral cleavage activity, can improve sensitivity by 2 to 3 orders of magnitude (Jiang et al. 2023). Zheng et al. (2023) developed a species-specific method combining recombinase polymerase amplification (RPA) with CRISPR-Cas12a, achieving a detection limit of 10^1 CFU/mL for bacterial suspension. In our previous study, the RAA-CRISPR-Cas12a/Cas13a method reached a comparable detection limit of 10^0 CFU/mL for *B. gladioli* pv. *cocovenenans* suspension and 10^2-10^3 CFU/g in food matrices (Yao et al. 2024). However, we observed that CRISPR-Cas13a produced weaker signals than CRISPR-Cas12a when detecting DNA in food matrices, likely due to matrix effects on RNA transcription. To overcome this limitation, we adopted an alternative strategy using dual RAA amplification followed by detection with two independent

CRISPR-Cas12a systems targeting the 16 S rDNA and *bonA* genes. Additionally, an electrochemical biosensing technique was integrated, enabling rapid on-site detection with a limit of 10^2 CFU/g for fresh noodles and tremella, which is comparable to the detection limit obtained in our previous study.

Most currently available methods focus either on species-level identification of *B. gladioli* or on subspecies-level detection of *B. gladioli* pv. *cocovenenans*. For instance, Wang et al. (2022) established a qPCR method for the subspecies, while Zheng et al. (2023) developed an RPA-CRISPR-Cas12a method for the species. However, many of these methods were developed using a limited number of available *cocovenenans* strains, which may not fully represent the genetic diversity within this subspecies. Moreover, the mechanism of bongkrekic acid production remains incompletely understood, and mutations or deletions in target genes may increase the risk of false-negative results. Species-specific detection strategies that assume all detected *B. gladioli* strains are toxigenic may lead to inaccurate risk assessment, particularly when non-toxigenic strains are present.

Thus, the dual RAA-CRISPR-Cas12a electrochemical method developed here allows stepwise detection to discriminate between toxigenic and non-toxigenic *B. gladioli* (Fig. 1). If no signal change occurs in reaction 1 targeting 16 S rDNA, the food can be considered free of *B. gladioli*, and *bonA* detection is unnecessary. If a signal change is observed, *bonA* detection is performed in reaction 2. Absence of a signal change indicates a non-toxigenic strain, whereas a signal change suggests the presence of toxigenic *B. gladioli* pv. *cocovenenans*. This stepwise detection approach enables rapid identification of *B. gladioli* at the species level while also providing an indication of its toxigenic potential, thereby facilitating timely follow-up confirmation and enabling prompt responses to potentially contaminated product.

Nevertheless, it should be noted that gene-based detection provides an indirect indication of toxigenicity. Although the *bonA* gene is a key component of the bongkrekic acid biosynthesis gene cluster and has been shown to correlate with toxin production in previously characterized strains, its presence does not necessarily guarantee active toxin synthesis under all conditions. Therefore, while the stepwise detection strategy can reduce the risk of overlooking potentially toxigenic strains and enables rapid on-site screening, confirmation by direct bongkrekic acid detection remains necessary in practical food safety investigations.

We also performed quantitative analysis by evaluating the relationship between ΔI% and the logarithm of bacterial concentration. However, the correlations obtained were moderate, indicating that further optimization will be required to improve the quantitative performance of the system. Nevertheless, the proposed method is well suited for rapid preliminary screening in food safety monitoring.

Conclusions

In this study, we developed a rapid, convenient, and highly specific dual RAA-CRISPR-Cas12a electrochemical detection method capable of accurately differentiating non-toxigenic *Burkholderia gladioli*, toxigenic *B. gladioli* pv. *cocovenenans*, and non-target bacterial strains. This method allows field-deployable detection in food matrices within 1 h, overcoming the limitation of conventional qPCR assays. Specificity tests confirmed specific identification of target species and subspecies, while sensitivity test in fresh noodles and tremella demonstrated a detection limit of 10^2 CFU/g. Overall, this method provides a practical approach for rapid preliminary screening in food safety monitoring and may support timely surveillance and risk management of *B. gladioli* contamination in food.

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Data availability The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

References

Anwar M, Kasper A, Steck AR, Schier JG (2017) Bongkrekic acid-a review of a lesser-known mitochondrial toxin. J Med Toxicol 13(2):173–179. https://doi.org/10.1007/s13181-016-0577-1

Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F (2018) Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science 360(6387):439–444. https://doi.org/10.1126/science.aaq0179

Guo YX, Mei CH, Ruan Z, Li BS (2022) The latest research progress of *Pseudomonas cocovenenans* subsp. *farinofermentans* and its toxin. J Chin Cereals Oils Assoc 37(1):196–202

Jia F, Liu D, Dong N, Li YY, Meng SY, You TY (2021) Interaction between the functionalized probes: The depressed efficiency of dual-amplification strategy on ratiometric electrochemical aptasensor for aflatoxin B1. Biosens Bioelectron 182:113169. https://doi.org/10.1016/j.bios.2021.113169

Jiang H, Li Y, Lv XF, Deng YL, Li XQ (2023) Recent advances in cascade isothermal amplification techniques for ultra-sensitive nucleic acid detection. Talanta 260:124645. https://doi.org/10.1016/j.talanta.2023.124645

Li JH, Zhou L, Long CY, Fang L, Chen QC, Chen QX, Liang JH, Yang Y, Zhu HM, Chen ZH, Gao SP, Li ZM, Li Q, Huang Q, Zhang YH (2019) An investigation of bongkrekic acid poisoning caused by consumption of a nonfermented rice noodle product without noticeable signs of spoilage. J Food Protect 82(10):1650–1654. https://doi.org/10.4315/0362-028x.Jfp-19-121

Li BF, Huang YQ, Lei LB, Ma R, Duan XJ, Cui BL (2021a) The contamination and detection methods of *Pseudomonas cocovenenans* subsp. in food. Guangdong Chem Ind 48(6):145–146

Li F, Ye Q, Chen M, Zhou B, Zhang J, Pang R, Xue L, Wang J, Zeng H, Wu S, Zhang Y, Ding Y, Wu Q (2021b) An ultrasensitive CRISPR/Cas12a based electrochemical biosensor for Listeria monocytogenes detection. Biosens Bioelectron 179:113073. https://doi.org/10.1016/j.bios.2021.113073

Lin J, Fang CY, Lu JF, Yun DH, Zhao QQ, Wang JS (2020) Detection of Pseudomonas cocovenenans subsp. farinofermentans by fluorescence real-time PCR method. J Food Saf Qual 11(11):3538–3544. https://doi.org/10.19812/j.cnki.jfsq11-5956/ts.2020.11.031

Shi RJ, Long CY, Dai YD, Huang Q, Gao YZ, Zhang NP, Chen YC, Liu SP, Ma QG, Quan L, Zhang YH, Luo B (2019) Bongkrekic acid poisoning: severe liver function damage combined with multiple organ failure caused by eating spoiled food. Legal Med 41:101622. https://doi.org/10.1016/j.legalmed.2019.07.010

Wang XW, Chen J, Chen GP, Huang JF, Wang YY, Li BF, Yang GW (2022) Developing a novel method detecting *Burkholderia gladioli* pv. *cocovenenans* by real-time fluorescent PCR. Food Sci Technol 47(1):330–335. https://doi.org/10.13684/j.cnki.spkj.2022.01.003

Yao XR, Luo MS, Huang JZ, Zhou LJ, Zhang BB, Liang ZS, Li XY (2024) Rapid dual-gene detection of *Burkholderia gladioli* and subspecies *cocovenenans* in fresh noodles and tremella using CRISPR-Cas12a/Cas13a. J Aoac Int 108(1):116–122. https://doi.org/10.1093/jaoacint/qsae084

Zheng XX, Li L, Zhang LN, Xie L, Song XR, Yu JH (2020) Multiple self-cleaning paper-based electrochemical ratiometric biosensor based on the inner reference probe and exonuclease III-assisted signal amplification strategy. Biosens Bioelectron 147:111769. https://doi.org/10.1016/j.bios.2019.111769

Zheng JL, Liu L, Li XM, Xu ZL, Gai ZQ, Zhang X, Lei HT, Shen X (2023) Rapid and Simple Detection of Burkholderia gladioli in Food Matrices Using RPA-CRISPR/Cas12a Method. Foods 12(9):1760 https://doi.org/10.3390/foods12091760

Zhuang XW, Yang XQ, Cao B, Sun HM, Lv XY, Zeng CJ, Li FG, Qu B, Zhou SS, Cui FY, Zhou Q (2022) Review-CRISPR/Cas systems: Endless possibilities for electrochemical nucleic acid sensors. J Electrochem Soc 169(3):037522. https://doi.org/10.1149/1945-7111/ac5cec

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