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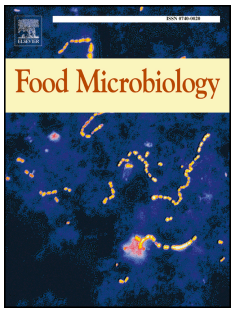

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1 *Pyrococcus furiosus* Argonaute based *Alicyclobacillus acidoterrestrsis* detection in fruit juice

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Abstract: *Alicyclobacillus acidoterrestris* is the major threat to fruit juice for its off-odor producing characteristic. In this study, *Pyrococcus furiosus* Argonaute (*Pf*Ago), a novel endonuclease with precise DNA cleavage activity, was used for *A. acidoterrestris* detection, termed as PAD. The partially amplified 16S rRNA gene of *A. acidoterrestris* can be cleaved by *Pf*Ago activated by a short 5'-phosphorylated single strand DNA, producing a new guide DNA (gDNA). Then, *Pf*Ago was activated by the new gDNA to cut a molecular beacon (MB) with fluorophore-quencher reporter, resulting in the recovery of fluorescence. The fluorescent intensity is positively related with the concentration of *A. acidoterrestris*. The PAD assay showed excellent specificity and sensitivity as low as 10^1 CFU/mL, which can be a powerful tool for on-site detection of *A. acidoterrestris* in fruit juice industry in the future, reducing the economic loss.

Keywords: *Alicyclobacillus acidoterrestris*, *Pyrococcus furiosus* Argonaute, nucleic acid detection, fluorescence, fruit juice

Introduction

Alicyclobacillus acidoterrestris is an acidophilic, thermophilic, spore-producing, rod-shaped bacterium, which has been identified as the main contamination in commercial fruit juice (Neggazi et al., 2023). The spore is highly resistance to heat, which survive pasteurization and subsequently germinate in juice matrix (Wahia et al., 2021). Moreover, *A. acidoterrestris* have wide soluble solid content growth range of 5.4-16.2 °Brix, allowing it to persist and metabolize in a diversity of juice(Splittstoesser et al., 1994). The main metabolic characteristic of *A. acidoterrestris* is to utilize the juice components to produce 2-methoxyphenol and halophenols, resulting in "medical, phenolic and antiseptic" off-flavor in fruit juices (Pornpukdeewattana et al., 2020). As *A. acidoterrestris* is more resistant to heat than pectin methylsterease, a pasteurization indicator, therefore, *A. acidoterrestris* has been suggested as reference microorganism to design pasteurization process and to determine the quality of fruit juice. However, in most cases, the identification of juice spoilage caused by *A. acidoterrestris* remain challenge, since it is not related to gas production or acidity and turbidity alteration (Wahia et al., 2022). And observable minimal sediment and perceptible off-odor only appear when the contamination level of *A. acidoterrestris* in fruit juice reach the critical concentration of 10^5 CFU/mL (Cai, Yuan, et al., 2015). Therefore, necessary means should be developed to efficiently detect *A. acidoterrestris* in fruit juice to reduce spoilage-related economic loss.

Plate counting method are traditionally internationally acceptable approach in juice industry for the detection of *A. acidoterrestris* (Henczka et al., 2013). Although it is economical and accurate, the time-consuming and labor-intensive features have limited its widespread application. Therefore, some novel methods have emerged to enhance efficiency of the identification of *A. acidoterrestris*

(Sourri et al., 2022). Indirect methods including GC-MS (Huang et al., 2015) and electronic nose (Wahia et al., 2020) can rapidly confirm the contamination of *A. acidoterrestris* in juice by monitoring the levels of off-flavors. However, the relevance between cell number of *A. acidoterrestris* and concentration of off-flavors are ambiguous, resulting in the reduced accuracy of these approaches. In order to improve the specificity, enzyme-linked immunosorbent assay (ELISA) was proposed for the detection of *A. acidoterrestris*(Li et al., 2014; Li et al., 2013; Shi et al., 2021). Owing to the selective recognition of antibody to surface protein or whole cell, the cross-reaction with non-target was significantly eliminated. However, the limit of detection (LOD) of ELISA could not reach a relatively low level. In contrast, nucleic acid-based detection method such as polymerase chain reaction (PCR) and real-time PCR showed excellent sensitivity as low as several cells benefit for the powerful amplification effect(Li et al., 2021; Wang, Yue, et al., 2021). However, the reliance on professional equipment and the lack of endpoint detection have restricted their flexibility in application. To further broad their applicability especially in on-site test, amplification assays combined with programmable nuclease such as CRISPR (clustered regularly interspaced short palindromic repeats) associated proteins (Cas) have been developed(Chen et al., 2022; Márquez-Costa et al., 2023; Wang et al., 2019). Owing to the specific recognition and cleavage feature of CRISPR/Cas system, these assays showed enhanced specificity and sensitivity. And the detection result can be recognized by portable ultraviolet (UV) lamp, smart phone or test strip(Song et al., 2022; Zhou et al., 2022).

The *Pyrococcus furiosus* Argonaute (*Pf*Ago) is a novel endonuclease which can perform single-strand DNA (ssDNA) cleavage activated by a small 5'-phosphorylated ssDNA as guide DNA (gDNA)(Swarts et al., 2015). The activated *Pf*Ago can precisely cleave the phosphodiester bond

between the 10th and 11th nucleotide of target DNA counting from 5'-end of gDNA(Enghiad and Zhao, 2017). In most cases, the gDNA must be strictly complementary to the target DNA, endowing *Pf*Ago with highly specificity to identify single base mutation(Zhao et al., 2022). Compared with Cas nucleases, the activity of *Pf*Ago do not rely on the protospacer-adjacent motif (PAM) and the *Pf*Ago can be guided by DNA rather than RNA. Therefore, the application of *Pf*Ago is more flexible and more suitable for multiple targets detection(Ye et al., 2022). Even so, only a few studies explored the potential of *Pf*Ago in the DNA-based target detection(He et al., 2021; Li et al., 2023; Wang, Yang, et al., 2021; Wang, He, et al., 2021; Yang et al., 2023).

In this work, we developed a *Pf*Ago based *A. acidoterrestris* detection (PAD) method. In this assay, only one gDNA was used to trigger the stepwise cleavage activity of *Pf*Ago where a new gDNA could be generated from the former gDNA guided cleavage process, which in turn, guided a new round of cleavage. Finally, a reporter strand with fluorophore and quencher was cut off, leading to the recovery of fluorescence and the fluorescence intensity is positively correlated to the concentration of *A. acidoterrestris* (Figure 1). The PAD is sensitive and specific, which showed excellent applicability in fruit juice.

2. Materials and methods

2.1 Bacterial strains and Chemicals

A. acidoterrestris (ATCC 49025) and other bacterial strains used in the experiment were purchased from American Type Culture Collection (ATCC, US) previously and stored in our laboratory. All the oligonucleotides and molecular beacon (MB) used in this experiment (Table 1) were synthesized by Sangon Biotech Co. Ltd. (China). The plasmid pET-23a containing *Pf*Ago gene (pET-23a-*Pf*Ago) was kindly provided by Dr. Wang Fei (Hubei University, China). The T4 Polynucleotide Kinase was

purchase from New England Biolabs (US). The TIANamp Bacterial DNA Kit was purchased from Tiangen Biotech Co. Ltd., (China). The juice samples were collected from the local market.

2.2 Expression and purification of *Pf*Ago

The plasmid pET-23a-*Pf*Ago was first transformed into *Escherichia coli* BL21 (DE3) pLysS and cultured at 37 °C overnight. Then the culture was inoculated in LB medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol and cultured at 37 °C. When the OD_{600nm} reached 0.6-0.8, isopropyl β-d-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, followed by incubated at 18 °C for 17 h to induce the over-expression of *Pf*Ago. The cell was harvested and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH=8.0), followed by lysed by ultrasonic disruptor. Cell lysates were centrifuged at 12000×g for 15 min to collect supernatants. After treatment with heating at 75 °C for 30 min, the *Pf*Ago in the supernatants were purified with Ni-NTA resin affinity purification column following manual's instruction (Figure S1). The protein sample was concentrated using dialysis bag and resuspended in storage buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.5 mM $MnCl_2$, 15% (v/v) glycerol, pH 8.0) and stored at -80 °C for further use.

2.3 Genomic DNA extraction and DNA amplification

The genomic DNA of *A. acidoterrestris* was extracted with the TIANamp Bacterial DNA Kit according to the instruction. The PCR mixture in a final volume of 20 μL containing 2 μL of the extracted genomic DNA, 2 μL of primers (10 μM for forward primer and reverse primer), 10 μL 2×Taq premix and 6 μL sterilized water was prepared. The PCR reaction was performed as follow: initial denaturation at 94 °C for 4 min, 30 thermo-cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extending at 72 °C for 30 s, then keeping at 72°C for 10 min. The products

were stored at -20 °C for further use.

2.4 *Pf*Ago based for *A. acidoterrestris* detection

The gDNA was first phosphorylated by T4 polynucleotide kinase before use. The PCR products (5 μL) was mixed with 1.5 μM *Pf*Ago, 0.5 μM gDNA, 2 μM MB and nuclease-free water to a final volume of 20 μL. The mixture was then kept at 95°C for 30 min and the fluorescence intensity was recorded.

3. Results and discussion

3.1 Principle of PAD

In the PAD system, the 16S rRNA gene of *A. acidoterrestris* was selected as the detection target. The amplification zone is located in the 89-169 site of the 16S rRNA gene, which have been proved with high specificity in the detection of *A. acidoterrestris* in previous study (Wang et al., 2014). In the presence of *A. acidoterrestris* in the sample, this representative region of 16S rRNA gene can be exponentially amplified. The resultant fragment is specifically recognized by *Pf*Ago guided by the designed gDNA, which is strictly complementary to the special region (112-129 site of the 16S rRNA gene) of one strand of the amplicon. Subsequently, the cleavage activity of *Pf*Ago is triggered, resulting in the breakage of the strand at the specific site. Moreover, this process generated a new 5'-phosphorylated ssDNA because of the hydrolysis of phosphodiester bond, which can serve as a new gDNA to guide *Pf*Ago for another round of cleavage. The target of the second round of cleavage can be the designed MB or the other strand of the amplicon. In the first case, the MB forms hairpin structure to make FAM fluorophore at one end of MB proximity to BHQ1 quencher at another end of MB. And the sequence of MB loop is designed to be complementary to the new gDNA. Therefore, the MB could be cleaved by *Pf*Ago guided by the new gDNA, bringing FAM away from BHQ1,

leading to the recovery of fluorescence. In the second case, owing to the complementary of two strands of the amplicon, the new gDNA can guide *Pf*Ago to cut the other strand. Therefore, the PCR amplicon is divided into two pieces finally.

3.2 Feasibility and optimization of PAD

The feasibility of the proposed PAD assay for the detection of *A. acidoterrestris* was verified firstly. As shown in Figure 2A and 2B, in the presence of gDNA and *Pf*Ago, the fluorescence intensity of the system changed significantly and strong fluorescence can be observed under UV light. While in the absence of gDNA, there was no obvious fluorescent signal showed and the system remained non-luminance, which means *Pf*Ago cannot be activated without gDNA. In the meantime, as shown in Figure 2C, the amplicon was split into two pieces when the gDNA and *Pf*Ago co-existed in the system simultaneously. While in other cases, the amplicons remained intact. These phenomena proved the second scenario described in the principle of PAD. All the results demonstrated the developed assay was feasible for *A. acidoterrestris* detection.

To achieve the best performance of PAD, the parameters which influence the detection system were optimized. As shown in Figure 3A, when the concentration of gDNA was 0.5 μM, the fluorescence intensity of the system reached highest. While when the concentration of gDNA was lower or higher than 0.5 μM, the fluorescence intensity reduced. Similarly, the fluorescence intensity enhanced with the concentration of *Pf*Ago increased, reaching plateau at the *Pf*Ago concentration of 1.5 μM (Figure 3B). Then, the fluorescence intensity decreased slightly when the concentration of *Pf*Ago exceed 1.5 μM. These phenomena indicated that the ratio of *Pf*Ago to gDNA should be kept in a reasonable range so that *Pf*Ago can be activated maximumly(Xun et al., 2021). Therefore, 0.5 μM and 1.5 μM were chosen as the optimal concentration of gDNA and *Pf*Ago in this work, respectively. Next, the

concentration of MB was optimized. As shown in Figure 3C, the fluorescence intensity reached maximum when the concentration of MB was 2 μM. And the fluorescence intensity could keep in relative high level at the concentrations above 2 μM but with some fluctuations. To reduce cost, 2 μM was selected as the best MB concentration for PAD assay. Under these optimal reaction conditions, the fluorescence signal of the detection system response upon time was tested. As shown in Figure 3D, the fluorescence intensity increased rapidly within the first 30 min and reached equilibrium gradually in the 30-50 min time frame. Therefore, 30 min was determined to be the most suitable reaction time for PAD.

3.3 Performance of PAD

To verify the specificity of PAD method, different bacteria including other species of *Alicyclobacillus* genus and common foodborne pathogens were tested. As shown in Figure 4A, there was a clear difference in fluorescence intensity between positive and negative samples where fluorescent signal was only appeared in the presence of *A. acidoterrestris*. Thus, it can be concluded the developed PAD method has an excellent specificity which can distinguish not only distinct bacterial genera, but also different species of *Alicyclobacillus* genus. This superior specificity can be attributed to two reasons. One reason is that the amplified region is highly variable among *Alicyclobacillus* genus and the designed primers can only amplify the region in the genome of *A. acidoterrestris*(Wang et al., 2014). The other reason is that the designed gDNA is strictly complementary to one strand of amplicons where a single nucleotide mismatch between gDNA and complementary sequence could reduce the cleavage activity of *Pf*Ago (Xun et al., 2021). Therefore, the negative samples could not be detected by the PAD method.

The sensitivity of the PAD method was determined using a serial concentration of *A. acidoterrestris*

with 10^6-10^0 CFU/mL. As shown in Figure 4B, the fluorescence intensity was still high at the bacterial concentration of 10^1 CFU/mL. But there was no significant difference in fluorescence intensity between 10^0 CFU/mL of *A. acidoterrestris* and the control group. Therefore, the limit of detection (LOD) of PAD was determined to be 10^1 CFU/mL. The sensitivity of PAD is better than most of the currently available nucleic acid-based detection assays such as PCR, real-time PCR, LAMP (loop-mediated isothermal amplification) and SPIA (single primer isothermal amplification) (Table 2). This excellent sensitivity also can be ascribed to two reasons. One reason is that the PCR procedure could effectively amplify low DNA concentration to detectable level. The second reason is the powerful target recognition and cleavage capacity of *Pf*Ago which could detect DNA as low as femtomolar level (Xun et al., 2021). Therefore, the two-stage amplification greatly enhanced the detection sensitivity of PAD assay. Moreover, the delivered results of PAD assay can be simply detected by portable ultraviolet lamp, which do not need agarose gel electrophoresis, gel imaging system or real-time PCR instrument. Therefore, compared with PCR and real-time PCR (Table 2), the result analysis of PAD is cheaper and more convenient. And compared with the isothermal amplification methods such as LAMP and SPIA, the design of primers and gDNA of PAD system is simpler. In addition, the PAD assay has strong anti-interference ability owing to the precise nucleic acid recognition and cleavage capacity of *Pf*Ago that the false-positive and high background value scenario often present in the above-mentioned isothermal amplification methods can be effectively avoided. In a word, the proposed PAD assay is simpler, more sensitive and accurate than the listed assays in Table 2.

3.4 Application of PAD in juice samples

To verify the application capacity of PAD in real food samples, fruit juices such as apple juice,

orange juice and kiwi juice were first artificially contaminated by *A. acidoterrestris*. Sample 1-4, 6-9, 11-14 represent different juices contaminated with *A. acidoterrestris* in the level of 10^2-10^5 CFU/mL, respectively. And sample 5, 10, 15 represent un-contaminated juices. Then these samples were detected using the developed sensing system. As shown in Figure 5 A, all positive samples can be recognized by PAD with obvious fluorescence intensity difference compared to the negative samples. The results were consistent with the PCR detection that a clear band with the length of 81 bp can be seen in all positive samples and no band was showed in negative samples (Figure 5B). The results highlighted the potential of PAD assay in the detection of real samples.

4. Conclusion

Here, a novel method named PAD was proposed for the detection of *A. acidoterrestris*. This method took advantage of the powerful stepwise cleavage ability of *Pf*Ago which could be activated and guided as long as 5'-phosphorylated ssDNA existed in the system and the cleavage process could continuously proceed until the final target is depleted. Therefore, only one guide input could induce the output of obvious fluorescence signal in PAD. Compared with CRISPR/Cas based detection methods, PAD uses DNA as guide which is more stable and cheaper than RNA. The method can detect *A. acidoterrestris* as low as 10^1 CFU/mL without cross-reaction with non-targets. The high sensitivity and specificity endow PAD with great potential in real sample application. However, the requirement of PCR amplification is the main drawback of PAD which restrict its application in on-site detection. The future work will focus on the integration of isothermal amplification techniques to overcome the disadvantage and develop convenient on-site detection system. All in all, this work provides a novel proof-of-concept that all nucleic acid containing creatures such as virus, pathogens and cancer cells can be identified based on this principle.

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Declaration of interest

The authors declare that they have no conflicts of interest in relation to this study.

Reference

Barrios Eguiluz, K.I., Salazar-Banda, G.R., Funes-Huacca, M.E., Alberice, J.V., Carrilho, E., Spinola Machado, S.A., Avaca, L.A., 2009. Sequence-specific electrochemical detection of Alicyclobacillus acidoterrestris DNA using electroconductive polymer-modified fluorine tin oxide electrodes. Analyst 134, 314-319.

Cai, R., Wang, Z., Yuan, Y., Liu, B., Wang, L., Yue, T., 2015. Detection of Alicyclobacillus spp. in Fruit Juice by Combination of Immunomagnetic Separation and a SYBR Green I Real-Time PCR Assay. Plos One 10.

Cai, R., Yuan, Y.H., Wang, Z.L., Guo, C.F., Liu, B., Liu, L.P., Wang, Y.T., Yue, T.L., 2015. Precursors and metabolic pathway for guaiacol production by Alicyclobacillus acidoterrestris. International Journal of Food Microbiology 214, 48-53.

Chen, J., Ma, X., Yuan, Y., Zhang, W., 2011. Sensitive and rapid detection of Alicyclobacillus acidoterrestris using loop-mediated isothermal amplification. Journal of the Science of Food and Agriculture 91, 1070-1074.

Chen, Y., Xu, X., Wang, J., Zhang, Y., Zeng, W., Liu, Y., Zhang, X., 2022. Photoactivatable CRISPR/Cas12a Strategy for One-Pot DETECTR Molecular Diagnosis. Analytical Chemistry 94, 9724-9731.

Connor, C.J., Luo, H.L., Gardener, B.B.M., Wang, H.H., 2005. Development of a real-time PCR-based system targeting the 16S rRNA gene sequence for rapid detection of Alicyclobacillus spp. in juice products. International Journal of Food Microbiology 99, 229-235.

Enghiad, B., Zhao, H., 2017. Programmable DNA-Guided Artificial Restriction Enzymes. ACS Synthetic Biology 6, 752-757.

He, R., Wang, L., Wang, F., Yang, J., Yu, X., Wang, Y., Liu, Z., Li, C., Ma, L., 2021. Combination of ultrashort PCR and Pyrococcus furiosus Argonaute for DNA detection. Analyst 147, 35-39.

Henczka, M., Djas, M., Filipek, K., 2013. Optimisation of a direct plating method for the detection and enumeration of Alicyclobacillus acidoterrestris spores. Journal of Microbiological Methods 92, 1-8.

Huang, X.C., Guo, C.F., Yuan, Y.H., Luo, X.X., Yue, T.L., 2015. Detection of medicinal off-flavor in apple juice with artificial sensing system and comparison with test panel evaluation and GC-MS. Food Control 51, 270-277.

Jang, J.H., Kim, S.-J., Yoon, B.H., Ryu, J.-H., Gu, M.B., Chang, H.-I., 2011. Detection of Alicyclobacillus Species in Fruit Juice Using a Random Genomic DNA Microarray Chip. Journal of Food Protection 74, 933-938.

Li, H., Chen, H., Liu, B., Cai, R., Jiang, N., Yue, T., Wang, Z., 2021. Establishment of quantitative PCR assays for the rapid detection of Alicyclobacillus spp. that can produce guaiacol in apple juice. International Journal of Food Microbiology 360.

Li, J., Huang, R., Xia, K., Liu, L., 2014. Double antibodies sandwich enzyme-linked immunosorbent assay for the detection of Alicyclobacillus acidoterrestris in apple juice concentrate. Food Control 40, 172-176.

Li, J., Xia, K., Yu, C., 2013. Detection of Alicyclobacillus acidoterrestris in apple juice concentrate by enzyme-linked immunosorbent assay. Food Control 30, 251-254.

Li, Y., Kou, J., Han, X., Qiao, J., Zhang, W., Man, S., Ma, L., 2023. Argonaute-triggered visual and rebuilding-free foodborne pathogenic bacteria detection. Journal of Hazardous Materials 454.

Luo, H., Yousef, A.E., Wang, H.H., 2004. A real-time polymerase chain reaction-based method for rapid

and specific detection of spoilage Alicyclobacillus spp. in apple juice. Letters in Applied Microbiology 39, 376-382.

Márquez-Costa, R., Montagud-Martínez, R., Marqués, M.-C., Albert, E., Navarro, D., Daròs, J.-A., Ruiz, R., Rodrigo, G., 2023. Multiplexable and Biocomputational Virus Detection by CRISPR-Cas9-Mediated Strand Displacement. Analytical Chemistry 95, 9564-9574.

Neggazi, I., Colas-Meda, P., Vinas, I., Garza, S., Alegre, I., 2023. Occurrence of Alicyclobacillus acidoterrestris in pasteurized and high hydrostatic pressure-treated fruit juices and isolates' characterization. International Journal of Food Microbiology 396.

Pornpukdeewattana, S., Jindaprasert, A., Massa, S., 2020. Alicyclobacillus spoilage and control - a review. Critical Reviews in Food Science and Nutrition 60, 108-122.

Shi, Y., Zhou, L., Qu, X., Yue, T., Yuan, Y., 2021. Targeting the cell wall: Preparation of monoclonal antibody for accurate identification of Alicyclobacillus acidoterrestris in apple juice. Food Control 121.

Song, J., Cha, B., Moon, J., Jang, H., Kim, S., Jang, J., Yong, D., Kwon, H.-J., Lee, I.-C., Lim, E.-K., Jung, J., Park, H.G., Kang, T., 2022. Smartphone-Based SARS-CoV-2 and Variants Detection System using Colorimetric DNAzyme Reaction Triggered by Loop-Mediated Isothermal Amplification (LAMP) with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Acs Nano.

Sourri, P., Tassou, C.C., Nychas, G.-J.E., Panagou, E.Z., 2022. Fruit Juice Spoilage by Alicyclobacillus: Detection and Control Methods-A Comprehensive Review. Foods 11.

Splittstoesser, D.F., Churey, J.J., Lee, C.Y., 1994. GROWTH-CHARACTERISTICS OF ACIDURIC SPOREFORMING BACILLI ISOLATED FROM FRUIT JUICES. Journal of Food Protection 57, 1080-1083.

Swarts, D.C., Hegge, J.W., Hinojo, I., Shiimori, M., Ellis, M.A., Dumrongkulraksa, J., Terns, R.M., Terns, M.P., van der Oost, J., 2015. Argonaute of the archaeon Pyrococcus furiosus is a DNA-guided nuclease that targets cognate DNA. Nucleic Acids Research 43, 5120-5129.

Wahia, H., Fakayode, O.A., Mustapha, A.T., Zhou, C., Dabbour, M., 2022. Application and potential of multifrequency ultrasound in juice industry: Comprehensive analysis of inactivation and germination of Alicyclobacillus acidoterrestris spores. Critical Reviews in Food Science and Nutrition.

Wahia, H., Zhou, C., Fakayode, O.A., Amanor-Atiemoh, R., Zhang, L., Mustapha, A.T., Zhang, J., Xu, B., Zhang, R., Ma, H., 2021. Quality attributes optimization of orange juice subjected to multi-frequency thermosonication: Alicyclobacillus acidoterrestris spore inactivation and applied spectroscopy ROS characterization. Food Chemistry 361.

Wahia, H., Zhou, C., Mustapha, A.T., Amanor-Atiemoh, R., Mo, L., Fakayode, O.A., Ma, H., 2020. Storage effects on the quality quartet of orange juice submitted to moderate thermosonication: Predictive modeling and odor fingerprinting approach. Ultrasonics Sonochemistry 64.

Wang, B., Wang, R., Wang, D., Wu, J., Li, J., Wang, J., Liu, H., Wang, Y., 2019. Cas12aVDet: A CRISPR/Cas12a-Based Platform for Rapid and Visual Nucleic Acid Detection. Analytical Chemistry 91, 12156-12161.

Wang, F., Yang, J., He, R., Yu, X., Chen, S., Liu, Y., Wang, L., Li, A., Liu, L., Zhai, C., Ma, L., 2021. PfAgo-based detection of SARS-CoV-2. Biosensors & Bioelectronics 177.

Wang, L., He, R., Lv, B., Yu, X., Liu, Y., Yang, J., Li, W., Wang, Y., Zhang, H., Yan, G., Mao, W., Liu, L., Wang, F., Ma, L., 2021. Pyrococcus furiosus Argonaute coupled with modified ligase chain reaction for detection of SARS-CoV-2 and HPV. Talanta 227.

Wang, Z., Cai, R., Yuan, Y., Niu, C., Hu, Z., Yue, T., 2014. An immunomagnetic separation-real-time PCR system for the detection of Alicyclobacillus acidoterrestris in fruit products. International Journal

of Food Microbiology 175, 30-35.
Wang, Z., Yue, T., Yuan, Y., Zhang, Y., Gao, Z., Cai, R., 2021. Targeting the vanillic acid decarboxylase gene for Alicyclobacillus acidoterrestris quantification and guaiacol assessment in apple juices using real time PCR. International Journal of Food Microbiology 338.
Xun, G., Liu, Q., Chong, Y., Guo, X., Li, Z., Li, Y., Fei, H., Li, K., Feng, Y., 2021. Argonaute with stepwise endonuclease activity promotes specific and multiplex nucleic acid detection. Bioresources and Bioprocessing 8, 46.
Yang, L., Guo, B., Wang, Y., Zhao, C., Zhang, X., Wang, Y., Tang, Y., Shen, H., Wang, P., Gao, S., 2023. Pyrococcus furiosus Argonaute Combined with Recombinase Polymerase Amplification for Rapid and Sensitive Detection of Enterocytozoon hepatopenaei. Journal of Agricultural and Food Chemistry 71, 944-951.
Yang, Y., Yang, Q., Ma, X., Zhang, Y., Zhang, X., Zhang, W., 2017. A novel developed method based on single primer isothermal amplification for rapid detection of Alicyclobacillus acidoterrestris in apple juice. Food Control 75, 187-195.
Ye, X., Zhou, H., Guo, X., Liu, D., Li, Z., Sun, J., Huang, J., Liu, T., Zhao, P., Xu, H., Li, K., Wang, H., Wang, J., Wang, L., Zhao, W., Liu, Q., Xu, S., Feng, Y., 2022. Argonaute-integrated isothermal amplification for rapid, portable, multiplex detection of SARS-CoV-2 and influenza viruses. Biosensors & Bioelectronics 207.
Yuan, N., Zhang, Y., Xu, H., Zhou, Z., Lu, X., Chen, T., Yang, Q., Tan, J., Zhang, W., 2020. Development of the Saltatory Rolling Circle Amplification Assay for Rapid and Visual Detection of Alicyclobacillus acidoterrestris in Apple Juice. Journal of Agricultural and Food Chemistry 68, 4538-4545.
Zhao, C., Yang, L., Zhang, X., Tang, Y., Wang, Y., Shao, X., Gao, S., Liu, X., Wang, P., 2022. Rapid and Sensitive Genotyping of SARS-CoV-2 Key Mutation L452R with an RPA-PfAgo Method. Analytical Chemistry.
Zhou, B., Ye, Q., Li, F., Xiang, X., Shang, Y., Wang, C., Shao, Y., Xue, L., Zhang, J., Wang, J., Ding, Y., Chen, M., Wu, Q., 2022. CRISPR/Cas12a based fluorescence-enhanced lateral flow biosensor for detection of Staphylococcus aureus. Sensors and Actuators B-Chemical 351.

341 Figure legends

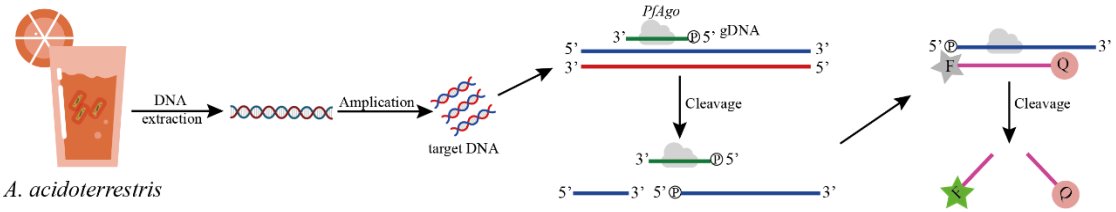
342 Figure 1. Schematic illustration of PAD.

343 Figure 2. Feasibility of PAD. (A) Fluorescent spectra of different sample. (B) Fluorescent image of
344 different sample under 365 nm UV lamp. (C) Gel image of different sample. "+" represents in the
345 presence of *Pf*Ago or gDNA. "-" represents in the absence of *Pf*Ago or gDNA.

346 Figure 3. (A) Optimization of gDNA concentration in the PAD system. (B) Optimization of *Pf*Ago
347 concentration in the PAD system. (C) Optimization of MB concentration in the PAD system. (D)
348 Optimization of reaction time in the PAD system.

349 Figure 4. (A) Specificity of PAD. (B) Sensitivity of PAD.

350 Figure 5. (A) Detection of *A. acidoterrestris* in fruit juice samples using PAD assay. (B) Gel image
351 of fruit juice samples tested by PCR assay. Sample 1-4, 6-9, 11-14 represent different juices
352 contaminated with *A. acidoterrestris* in the level of 10^2-10^5 CFU/mL, respectively. Sample 5, 10,
353 15 represent un-contaminated juices.



A. acidoterrestris

Figure 1.

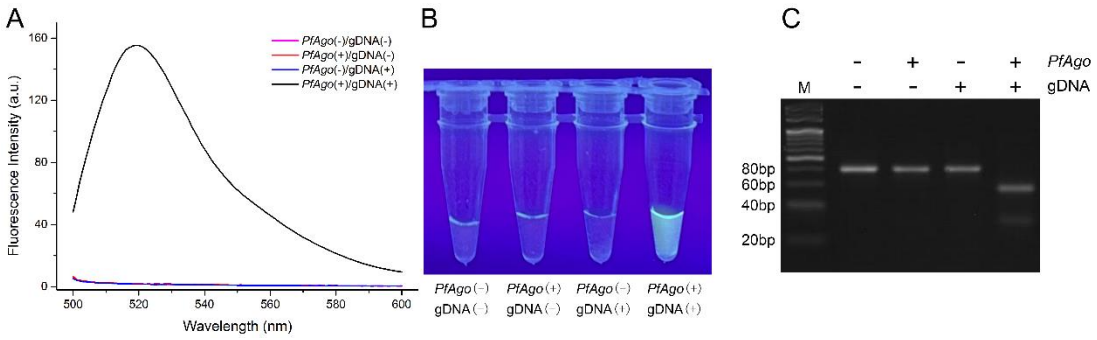
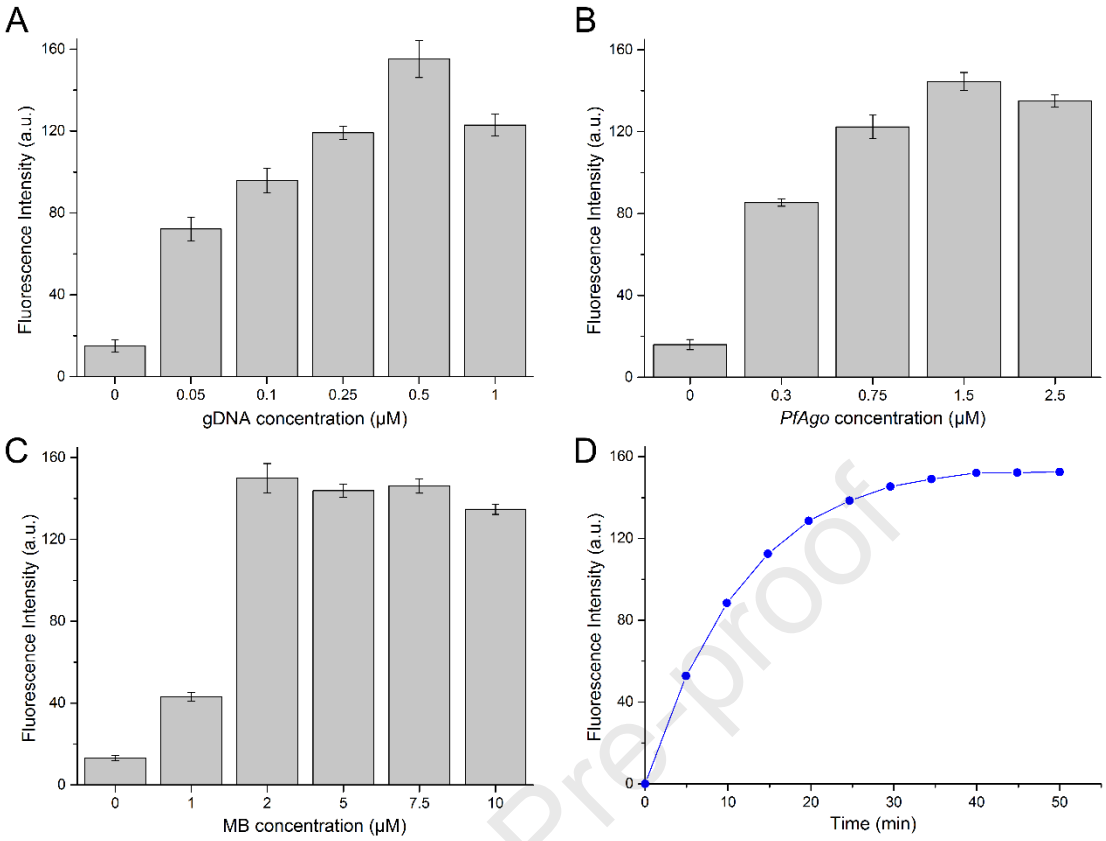
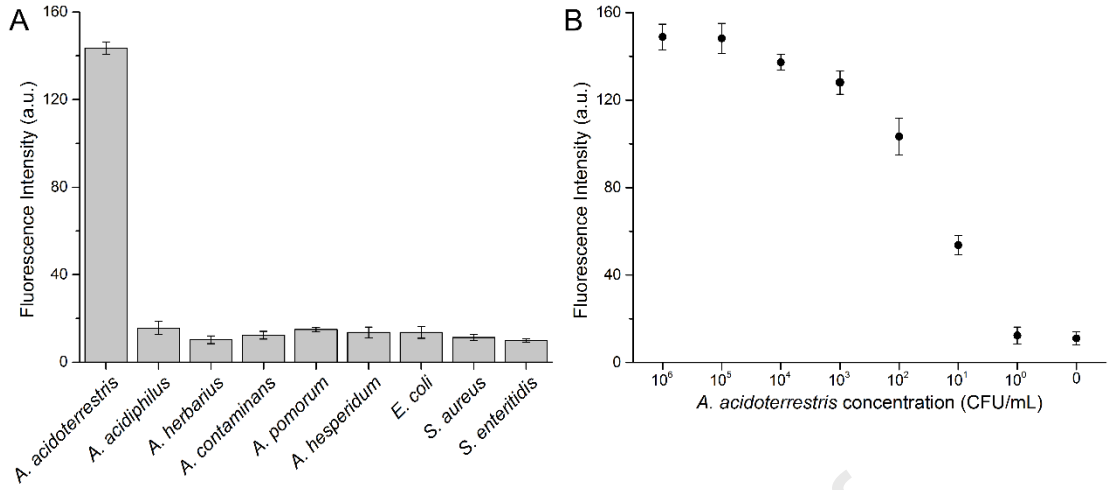


Figure 2.



358

359 Figure 3.



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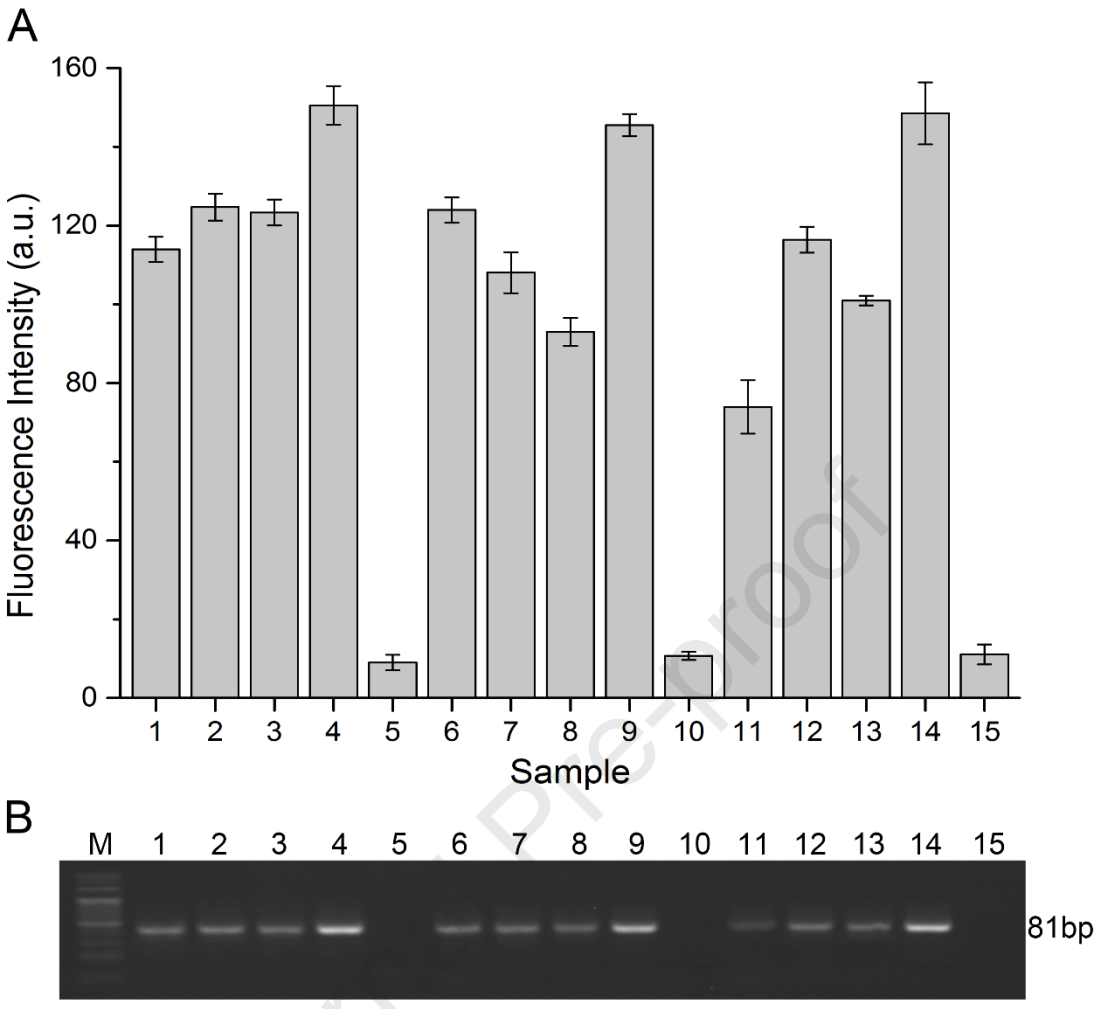
361 Figure 4.



362

363 Figure 5.

364 Table 1. oligonucleotide sequences

Type	Description	Sequence (5'-3')
Primers	16S rRNA-Forward	TGAGTAACACGTGGGCAATCTG
	16S rRNA-Reverse	CTACCCGTGTATTATCCGGCAT
gDNA	guide DNA of *Pf*Ago	CTACCCGTGTATTATCCGGCAT
Molecular beacon	MB	FAM-cgcaccCAATCTGCCTTTCAGACTggtgcg-BHQ1

365

366 Table 2. Comparison of different nucleic acid detection method for *A. acidoterrestris*

Method	LOD (CFU/mL)	Specificity	Reference
Quantitative PCR	2.6×10^2	Yes	(Li et al., 2021)
Real-time PCR	<10	No	(Luo et al., 2004)
	<100	No	(Connor et al., 2005)
IMS[a] real-time PCR	<10	Yes	(Wang et al., 2014)
	2.8×10^1	No	(Cai, Wang, et al., 2015)
Asymmetric nested RT-PCR[b] coupled with electrochemical detection	2	Not report	(Barrios Eguiluz et al., 2009)
Random genomic DNA Microarray	2×10^3	No	(Jang et al., 2011)
LAMP[c]	2.25×10^1	Yes	(Chen et al., 2011)
SRCA[d]	4.5	Yes	(Yuan et al., 2020)
SPIA[e]	4.8	Yes	(Yang et al., 2017)
PAD	10^1	Yes	This work

367 [a] Immunomagnetic separation.

368 [b] Reverse transcription PCR.

369 [c] Loop-mediated isothermal amplification.

370 [d] Saltatory rolling circle amplification.

371 [e] Single primer isothermal amplification.

- A novel nucleic acid detection method based on argonaute for *A. acidoterrestris* was developed.
- The assay showed excellent specificity and sensitivity with a limit of detection of 10^1 CFU/mL.
- The assay exhibited good performance in real sample analysis.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: