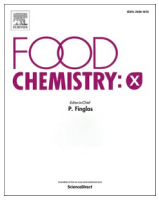
Food Chemistry: X 24 (2024) 101886

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Real-time fluorescence growth curves for viable bacteria quantification in foods

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ARTICLE INFO

Keywords:
Bacterial quantification
Food safety control
SYBR green
Real-time
Fluorescence growth curve

ABSTRACT

Here, for the first time, we used a membrane permeable fluorescent nucleic acid stain (SYBR Green) to trace the in-vivo DNA replication during bacterial binary fission. Such stain did not influence the growth of bacteria. Nor did the bacteria degrade the stain, enabling the fluorescent microplate reader to monitor sensitively the growth of the bacteria. Hence, a real-time fluorescence growth curve (RTFGC) method was put forward for the sensitive quantification of viable bacteria in foods. Using *E. coli* O157:H7 as a bacteria model, the RTFGC method could quantify bacteria within the range of 10 to 1×10^6 cfu/mL, with a linear correlation coefficient R^2 of 0.997. It was found that melting curve was unique for a particular bacterial strain, which could be used for contamination identifications. Good practicability of the RTFGC in quantifying *E. coli* O157:H7 from tap water, juices, and milks was demonstrated.

1. Introduction

Bacteria widely exist on Earth, and they often live symbiotically with other organisms, including human beings. These commensal bacteria are associated with the homeostasis of their hosts in a quantity-dependent manner (Jin et al., 2022; Sy et al., 2023). The quantitative imbalance can lead to the conversion of bacteria from symbiotic microorganisms to pathogenic ones. Therefore, the quantification of bacteria has great significance in food pathogen detection and microbiological research. Currently, plate cultivation combined with colony counting is still the most commonly used method for bacterial quantification. However, this method has a low throughput for large-scale testing, and it needs intensive labor for gradient dilution and plating of the samples (He et al., 2024; Liu et al., 2023).

In the past twenty years, many culture-independent methods have been developed, such as enzyme-linked immunosorbent assay (ELISA) (Hu et al., 2024; Luo, Li, & Li, 2024), Fow cytometry (FCM) (He, Hong, Zhang, Wu, & Yan, 2020; Lian, He, Chen, & Yan, 2019; M. Wang et al., 2023), surface-enhanced Raman scattering (SERS) (Shen et al., 2022; *P.* Wang, Liu, Li, Li, & Li, 2024), lateral flow assay (LFA) (He, 2023; Liao, Luo, Byeon, & Park, 2024; Liu et al., 2024), and gold nanoparticle colorimetric detection (X. Li & Wu, 2024; J. Wang et al., 2023). However, the quantification of bacteria with a concentration lower than 100 cfu/mL remains a great challenge. For instance, without further enrichment, FCM and colorimetric assays can hardly detect bacteria lower than 1000 cfu/mL. Although several recently developed method could lower the limit to 10 cfu/mL (Guo et al., 2021; Kumarajith, Powell, & Breadmore, 2024; S. Wang et al., 2022; Xiao et al., 2023; Zhang et al., 2021), most of these techniques have a narrow linear range of approximately to 2–3 orders of magnitude for bacterial quantification. For the detection of bacteria, there is a sensitivity/linear-range paradox remained to be solved.

Alternatively, real-time fluorescence quantitative PCR (qPCR) has become a widely used method for quantifying bacteria in various environments through DNA amplification (Jerome et al., 2022; Lei, Chen, & Zhong, 2021; Yan et al., 2023). QPCR has many advantages, particularly in terms of sensitivity and a wide linear range of detection, with linear ranges of about 5–7 orders of magnitude (Li et al., 2024; Sun et al., 2024). Exponential amplification of the target enables qPCR to probe DNA/RNA-containing biological samples at extremely low concentrations (as low as 1 copy/mL). However, it does not enable the discrimination between DNA arising from live or dead bacteria, and the necessary extraction and purification of genomic DNA leads to bias in bacterial quantification (He et al., 2017; Lima, Franca, Muzny, Taylor, &

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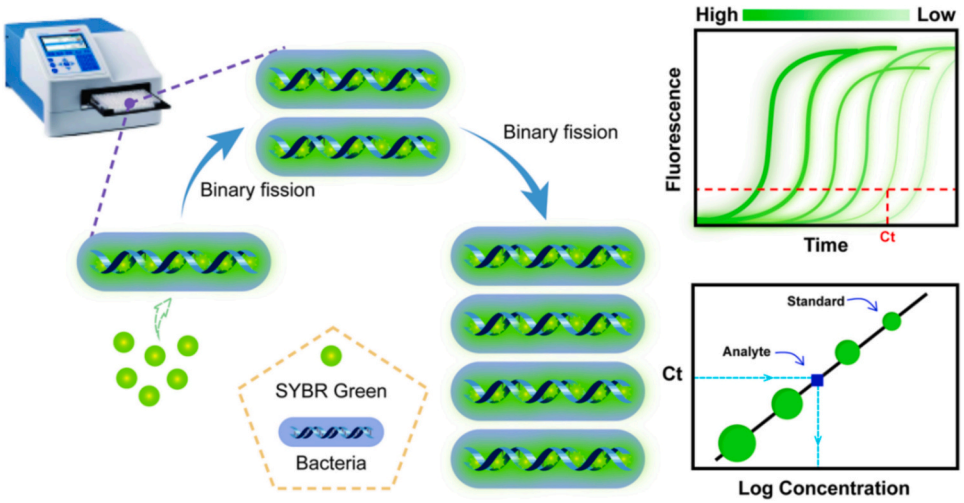
https://doi.org/10.1016/j.fochx.2024.101886
Received 31 July 2024; Received in revised form 29 September 2024; Accepted 6 October 2024
Available online 9 October 2024



Scheme 1. Schematic illustration of the RTFGC for bacterial quantification.

Cerca, 2022).

Inspired by the qPCR principle, here we established a real-time fluorescence growth curve (RTFGC) method for the sensitive quantification of viable bacteria. A membrane permeable fluorescent nucleic acid stain (SYBR Green) was used to trace the in-vivo DNA replication during bacterial binary fission, as shown in Scheme 1. Such stain did not influence the growth of bacteria. Nor did the bacteria degrade the stain, enabling the fluorescent reader to monitor sensitively the growth of the bacteria for quantification. The RTFGC not only had the advantages of qPCR for high sensitivity, high throughput and a wide linear range of quantification, but also enabled the selective detection of viable bacteria in food samples. Additionally, the RTFGC could also perform drug-resistance analysis to determine both the minimal inhibitory concentration (MIC) and sub MIC of the bacteria.

2. Experimental section

2.1. Reagents and bacterial strain

SYTO 9, SYTO 62, and SYBR Green were purchased from Thermo Fisher Scientiffc (Waltham, USA). DAPI, PI, Reddot, EB, PI, and M9 medium were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). PBS, ciprofloxacin, and norfloxzcin were purchased from Solarbio Life Sciences (Beijing, China). Bacterial strains *E. coli* O157:H7, *P. aeruginosa*, and *A. baumannii* were cultured at 37 °C and 200 rpm/min in M9 Media supplemented with proline (1150 mg/L), alanine (890 mg/L), serine (1050 mg/L), aspartic acid (1330 mg/L), glutamic acid (1470 mg/L), and glycine (750 mg/L). *S. aureus* was cultured at 37 °C and 200 rpm/min in LB medium, and L. *casei* was cultured at 37 °C in de Man, Rogosa, and Sharpe (MRS) broth (Baisi biotechnology, Hangzhou, Zhejiang, China). Bacterial-free drinking water, milk, and apple juice were purchased from the Wal-Mart Store located in Chaoyang Road, Nanning, Guangxi province, China.

2.2. Preparation of bacteria

All the bacteria strains were cultured in medium and harvested at an optical density at 600 nm (OD_{600}) of 0.8 by centrifugation at 5000 rpm for 5 min, as described previously (Sun et al., 2021). Cells were washed with PBS and resuspended to an OD_{600} of 1.0 (containing approximately 1×10^9 cfu/mL). The suspensions were subjected to plate counting to quantify the bacterial concentrations. The bacterial cells were diluted by PBS to prepare standard solutions.

2.3. Optimization and selection of stain for viable bacteria staining

A 100 μL aliquot of *E. coli* O157:H7 suspension (~1×10^8 cfu/mL) was pipetted into a 1.5 mL Eppendorf tube. Another 100 μL aliquot of stain solution (2×) was added to the suspension to obtain a final concentration of 1× (the final dilution suggested by the reagent providers). The suspension was incubated at room temperature for 5 min and subjected to fluorescence spectral scanning by a fluorescence spectrophotometer (Perkin Elmer FL8500). The FL intensity at 520 nm for SYTO 9, 535 nm for SYBR Green, 450 nm for DAPI, 620 nm for PI and GelRed, 461 nm for Reddot, and 600 nm for EB was recorded.

To investigate the stability of the stains in the bacterial solution, a 100 μL aliquot of bacterial suspension (~10^8 cfu/mL) was pipetted into a 300 μL quartz cuvette. Then another 100 μL aliquot of stain solution (2×) was added to the quartz cuvette, which was then covered by a lid to protect the solution from other bacterial contamination. The quartz cuvette was placed at room temperature for hours, during which fluorescence scanning was carried out at every set intervals.

2.4. The influence of stain on the growth of bacteria

A 10 μL aliquot of bacteria suspension (~1×10^8 cfu/mL) was inoculated into 4 mL medium containing SYBR Green (1×). The bacteria was cultured at 37 °C and 200 rpm/min for 48 h, during which the bacteria were sampled at every set intervals for plate counting and high performance liquid chromatography (HPLC) analysis. For HPLC analysis, 20 μL bacterial sample was diluted with 980 μL water. The diluted fermentation broth was filtered through a nitrocellulose membrane (pore size of 0.22 μm), and the filtrate was analyzed by a HPLC (STI-501, Shanghai Yitian, Shanghai, China) with a C18 chromatographic column as stationary phase and methanol-water of 1:1 as mobile phase.

2.5. RTFGC for bacterial quantification and resistance analysis

A 15 μL aliquot of *E. coli* O157:H7 suspension ($10^{-1} \times 10^7$ cfu/mL) was pipetted into a qPCR tube. Another 15 μL aliquot of M9 medium (2×) containing SYBR Green (2×) was added to the suspension. The qPCR tube was placed in a fluorescent reader (StepOnePlusTM Real-Time PCR Instrument, Thermo Fisher Scientific, Waltham, USA) for testing. The amplification program was set as follows: 37 °C, 30 min (binary fission); 90 cycles. For milk and juice, the samples were diluted 10 times with water, followed by the steps as described above. For bacterial resistance analysis, a 15 μL aliquot of *E. coli* O157:H7 suspension (1×10^6 cfu/mL) was pipetted into a qPCR tube. Another 15 μL aliquot of M9 medium (2×) containing SYBR Green (2×) and antibiotic

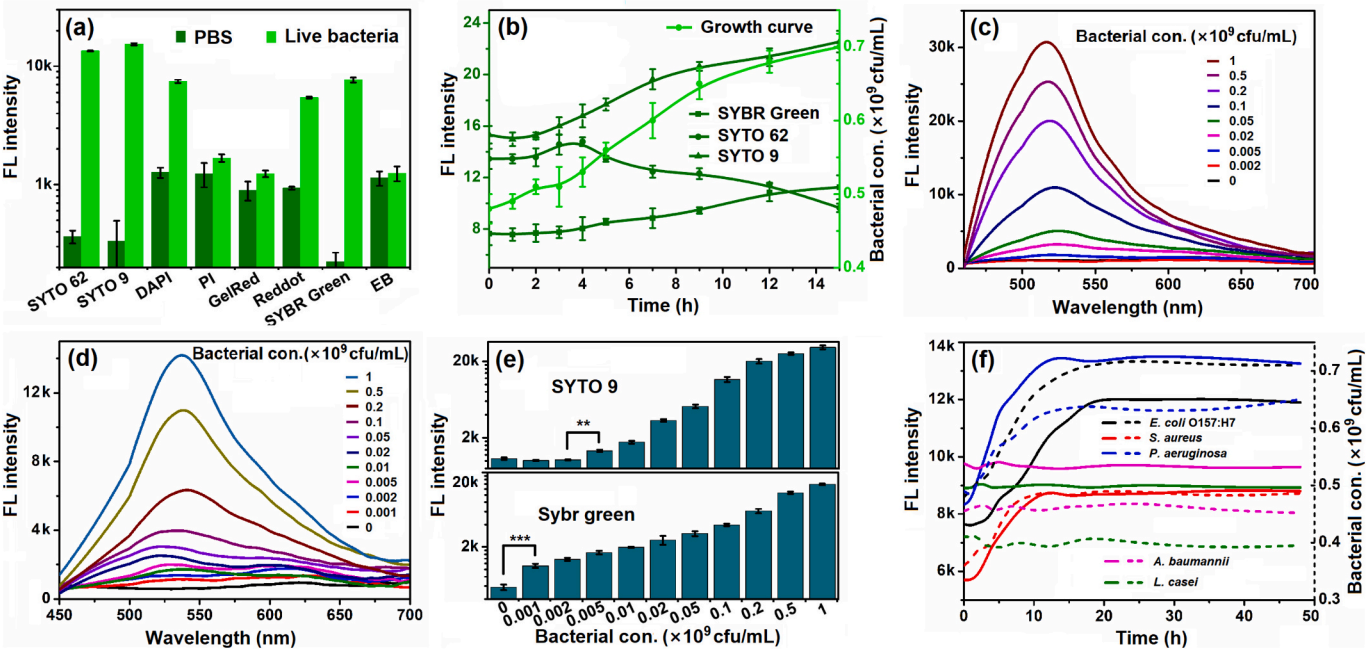


Fig. 1. Fluorescent nucleic acid stains for viable bacteria staining. (a) FL of live bacteria (*E. coli* O157:H7) incubated with different stains. PBS was used as controls. The stains concentrations and their emission wavelengths were provided in the method section. (b) Dynamic FL change of the bacteria/stain solutions, which was incubated at room temperature for 15 h. The bacteria reproduced slowly during the incubation. (c-d) FL spectra of the bacteria stained with SYTO 9 (c) or SYBR Green (d), with bacterial concentration ranging from 0.001×10^9 to 1×10^9 cfu/mL. (e) Comparison in FL intensity of the bacteria between the two stains. (f) Dynamic FL change of the bacteria/stain solutions for different bacterial strains. The solid lines represent FL intensity and dotted lines represent bacterial concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(2×) was added to the suspension, followed by the steps as described above. Traditional broth dilution method was carried out as followings. A 200 μL aliquot of bacteria suspension (~1×10^7 cfu/mL) was inoculated into 2 mL M9 medium. The bacteria was cultured at 37 °C and 200 rpm/min for 48 h, during which the bacterial solution were photographed at every set intervals for nephelometric analysis by naked eyes.

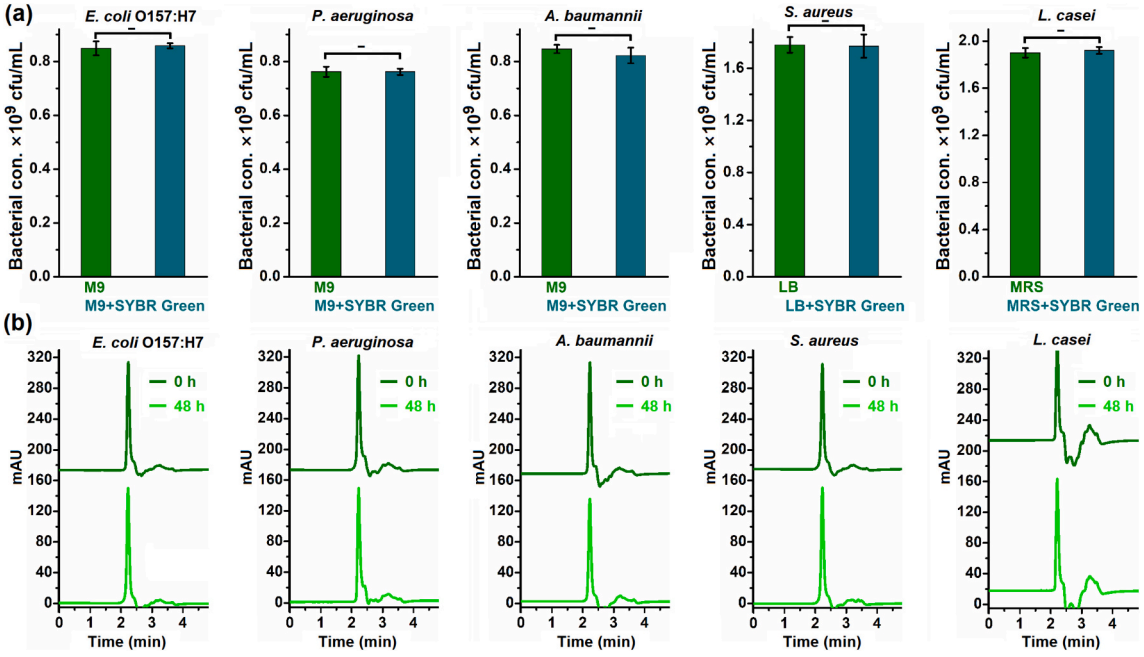


Fig. 2. The influence of SYBR Green on the growth of bacteria. (a) Comparison of the biomass between the bacteria cultured with and without SYBR Green for 12 h. The normal mediums are M9 medium for *E. coli* O157:H7, *P. aeruginosa*, and *A. baumannii*, LB medium for *S. aureus,* and MRS medium for L. *casei.* "–" represents no obvious difference between the two groups. (b) HPLC analysis of the fermentation broths for different times. The absorption peaks at 2.3 min represented SYBR Green content. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

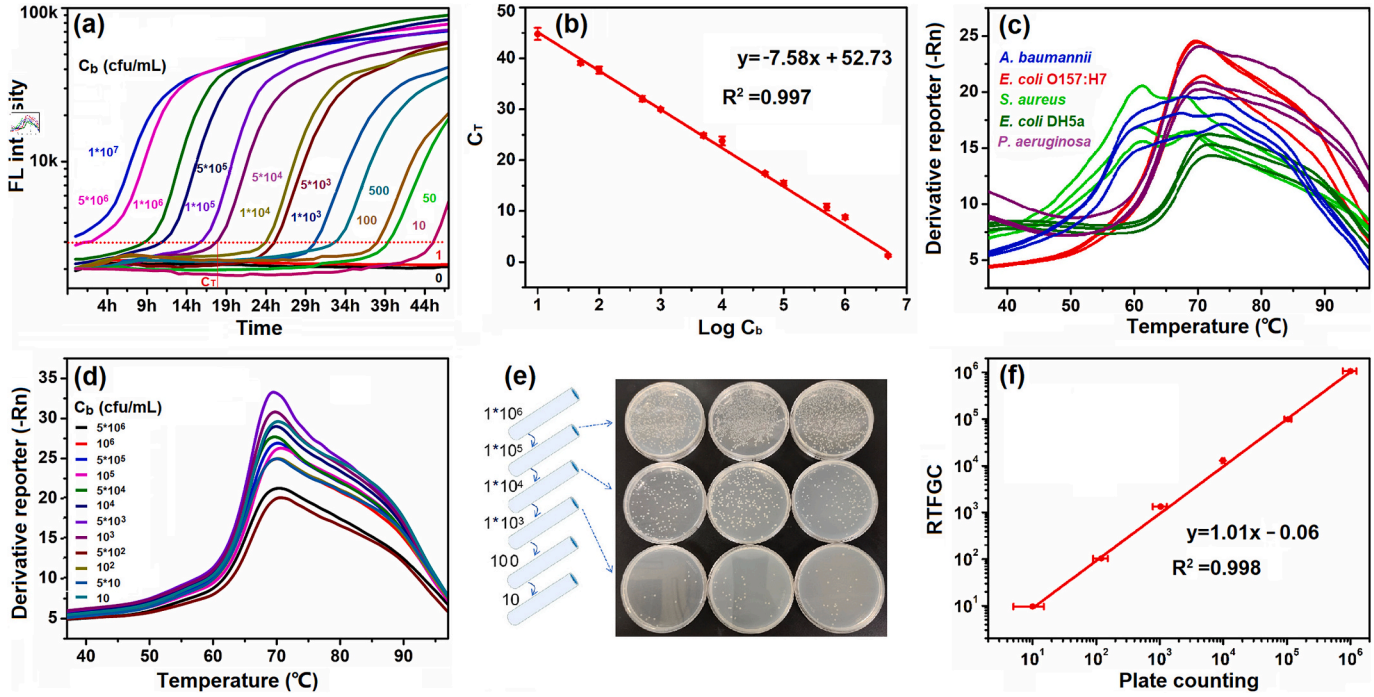


Fig. 3. RTFGC for bacterial quantification. (a) Representative amplification curves for standard bacterial (*E. coli* O157:H7) solutions. The red-dotted line represents the fluorescent threshold. (b) Linear analysis of the relationship between the Ct value and the logarithm of bacterial concentration (C_b). (c) Melting curves of different bacteria. (d) Melting curves of *E. coli* O157:H7 for different concentrations, after the proliferation and corresponded to the samples in Fig. 3a. (e) Plate counting for the standard bacterial (*E. coli* O157:H7) solutions. (f) Linear analysis of the relationship between the quantification results of RTFGC and plate counting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Fluorescent nucleic acid stains for viable bacteria staining

Firstly, we used eight fluorescent nucleic acid stains (SYTO 62, SYTO 9, DAPI, PI, GelRed, Reddot, SYBR Green, and EB) to compare their staining capacity for live bacteria. All these stains are commercially available and have been widely used for DNA staining; the fluorescence (FL) of the stains is very weak in aqueous solution, but their FL increases after binding to DNA. Using *E. coli* O157:H7 as a model bacteria, PI, GelRed, and EB had weak FL, as shown in Fig. 1a. Suggested these three stains were unable to penetrate into the live bacteria (Chen et al., 2024; Khalef, Lydia, Filicia, & Moussa, 2024). In the history of biological evolution, the formation of a biological membrane enables the cells to selectively intake small molecules through passive or active transportation (He et al., 2024). In active transportation, molecules move against a concentration and ATPs are required, thus keeping the toxicants outside the cells. In contrast, passive transportation as Brownian motion allows intracellular transport of small molecules from high to low concentrations, which does not consume ATPs and may not affect the bacterial growth. Here, SYTO 62, SYTO 9, and SYBR Green were suggested to be passively transported from intracellular to extracellular regions. Therefore, they were chosen as the candidates for in-vivo DNA staining, since they met all the requirements of weak background for PBS (as controls), high FL for live bacteria, and no need for ATPs.

To trace the in-vivo DNA replication of bacteria, the stains should be stable and have extreme resistance to the metabolic degradation of the bacteria. Hence, the three candidate stains were incubated with live bacteria for a period of time to investigate their stability, as shown in Fig. 1b. Both SYBR Green and SYTO 9 showed increased FL with the proliferation of the bacteria, indicating their good stability in the bacterial cells. Inversely, SYTO 62 showed decreased FL due to the metabolic degradation of the stain by the bacteria. We further compared the sensitivities in sensing live bacteria between SYTO 9 and SYBR Green, as shown in Fig. 1c–e. The SYBR Green showed better sensitivity than SYTO 9, as the former could differentiate 10^6 cfu/mL bacteria from the background. The SYBR Green was incubated with five different bacterial strains for 50 h to further investigate the stability of the stain, as shown in Fig. 1f. Good stability of the SYBR Green for tracing the in-vivo DNA replication of bacteria was demonstrated.

3.2. The mutual effects of SYBR green and the growing bacteria

There is large heterogeneity in genome and metabolome of bacteria between different types or strains. This heterogeneity created the current bacterial diversity and plays important roles in biological evolution, ecological environment balance, and the development of resistant bacteria (Lian et al., 2019). Such heterogeneity also enables the bacteria to metabolize and degrade various organic matters, ranging from small-molecule dyes to high-molecular polymers. Here, five commonly used bacterial strains were employed to study the metabolism of SYBR Green by the bacteria, and investigate the effects of SYBR Green on the growth of the bacteria. All the bacterial strains were cultured in both normal mediums (for controls) and normal-SYBR Green mediums (normal medium supplemented with SYBR Green). As shown in Fig. 2a, SYBR Green did not influence the growth of all the tested bacteria. HPLC analysis of the fermentation broths of the bacteria again demonstrated the good stability of the SYBR Green in the bacterial solution. These results indicated that SYBR Green did not influence the growth of bacteria. Nor did the bacteria degrade the stain, enabling the stain to be used for sensitively monitoring the growth of the bacteria.

3.3. Real-time fluorescence growth curves for bacterial quantification

To establish the RTFGC method, standard solutions with bacterial concentrations (C_b) ranging from 1 to 1×10^7 cfu/mL were used for evaluating the accuracy and specificity of the tests. We defined Ct value as the culture time needed to reach the FL threshold set by the

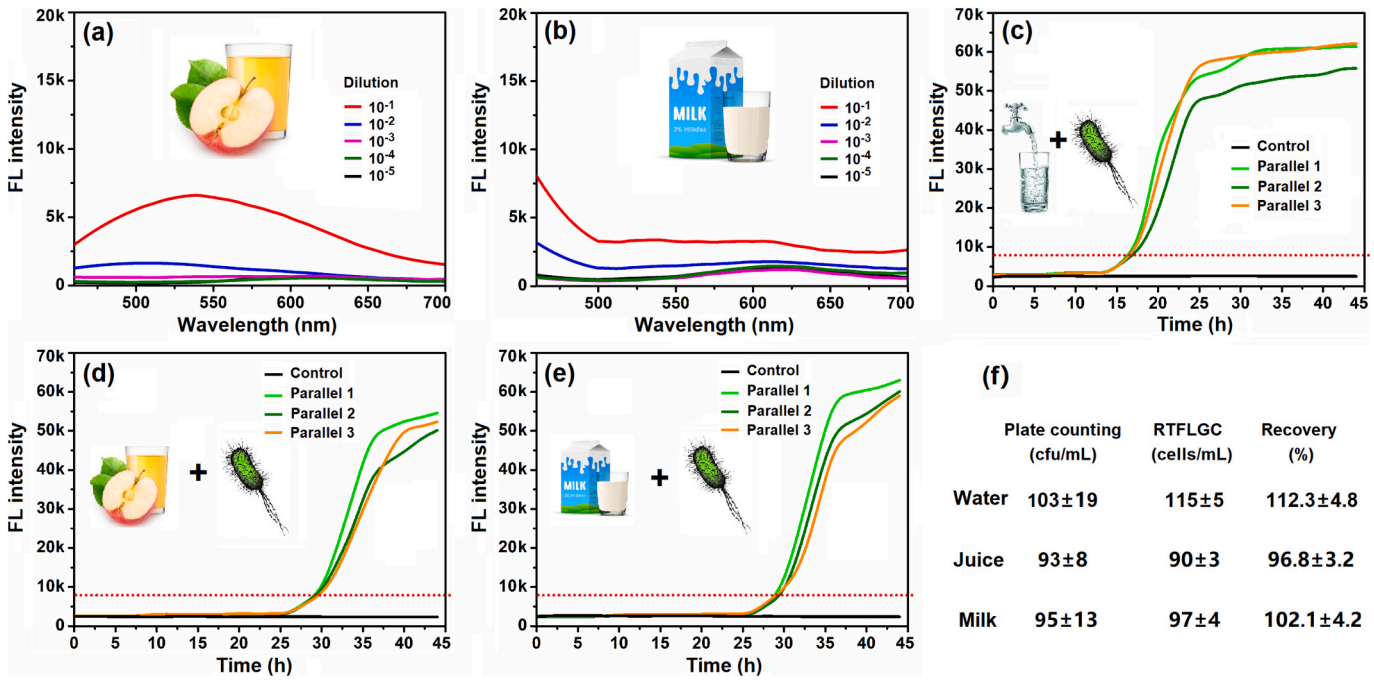


Fig. 4. RTFGC for real-sample detection. (a–b) FL spectra of apple juice (a) and milk (b) for different dilutions. (c–e) Real-time FL growth curves for drinking water (c), apple juice (d), and milk (e) spiked with 10^2 cfu/mL bacteria. The samples without bacteria were used as controls. Three replications were carried out for each samples. (f) Recovery of the RTFGC method, taking plate counting for references.

fluorescent reader. As shown in Fig. 3a and Fig. 3b, the Ct value decreased with the increase of the bacterial concentration. Good linear relationship between the Ct value and the logarithm of C_b was obtained in a wide range (from 10 to 1 × 10^6 cfu/mL), with a correlation coefficient R^2 of 0.997.

In qPCR assays, thermodynamic analysis are widely used to get the melting curve of the DNA duplexes as well as their T_m values for nonspecific amplification analysis (Liang, Luo, Lin, Zhou, & Jiang, 2009; Ruijter et al., 2019). As DNA polymorphisms are the bases of bacterial diversity, here we tried using melting curve to characterize different bacteria, as shown in Fig. 3c. It was found that the melting curves were unique for each bacterial strain, indicating it could be used for contamination identifications. Fig. 3d showed the melting curves of the standard solutions of *E. coli* O157:H7 after the amplification, with initial concentration ranging from 10 to 1 × 10^6 cfu/mL. Their shapes were consistent and in agreement with that of *E. coli* O157:H7 in Fig. 3c, indicating no contamination happened during the bacterial proliferation. Plate counting, as a gold standard method for bacteria quantification, was conducted to compare the precision between the RTFGC and plate counting methods, as shown in Fig. 3e–f. There was a good correlation between the bacterial concentration measured by RTFGC and plate counting, with a correlation coefficient R^2 of 0.998. Compared with plate cultivation for bacterial quantification, the RTFGC showed better repeatability, especially for detecting bacteria lower than 1000 cfu/mL. As different types of bacteria have different growth rates and trends in qPCR, the RTFGC method could not accurately quantify a mixed bacterial sample based on a non-selective medium. Hence, a selective medium may favor the quantification of a specific bacterial type, and the RTFGC could identify if the sample were contaminated with more than one type of bacteria through melting curves analysis.

3.4. Practicability of the RTFGC for real-sample detection

Bacterial contamination can occur in a wide range of food products such as milk and fruit juices, which also possess DNA and can be stained by nucleic acid stains (He et al., 2020). This complicated food composition may produce high FL background and disturb the FL signal of the bacteria. Fig. 4a and b show representative FL backgrounds for apple juice and milk of different doses. Both apple juice and milk still showed high FL backgrounds even after they were diluted 10 times by the water. These background were relieved by diluting the samples 100 times by the water. Therefore, for the foods such as milks and juices, samples must be diluted to a final dilution of 100 times. Compared with the complicated sample pretreatment for qPCR and plate cultivation, such dilution is economical and easy to perform.

To further investigate the practicability of the as-developed RTFGC for real-sample detection, tap water, orange juice, and milk were spiked with *E. coli* O157:H7 with a concentration of about 100 cfu/mL. Both RTFGC and plate counting were carried out to evaluate the bacterial recovery. For the milk and orange juice, dilution was need to minimize the FL background of the drinks, as described in the method section. Fig. 4c–e showed the FL growth curves of the samples conducted with three repetitions. Good repeatability of using Ct value to imply the bacterial concentration was demonstrated. By using plate counting as a reference, the bacterial recoveries for all the drinks exceeded 90 %, as shown in Fig. 4f. There was no significant difference in detection results between the two methods. However, the RTFGC demonstrated a much better performance for its labor-saving operation and good repeatability in quantifying bacteria of low concentrations. Compared with other culture-independent methods, such as ELISA, fow cytometry, qPCR, and SERS, the RTFGC showed advantages by increasing the detection sensitivity while broadening the linear range of detection to 6 orders of magnitude.

3.5. The use of RTFGC for drug-resistance analysis

Drug-resistance analysis has great significance in microbiological research and antimicrobial drug development. Broth dilution, disk diffusion, and *E*-test are the most widely used methods for antibiotic susceptibility tests to determine the minimal inhibitory concentration (MIC) (Benamrouche, Lazri, Tali-Maamar, & Rahal, 2014). However, they can not draw the scope of subinhibitory concentrations (sub-MIC), which shapes the selection window of the antibiotic for resistant bacteria. Such selection window plays an important role in the optimization

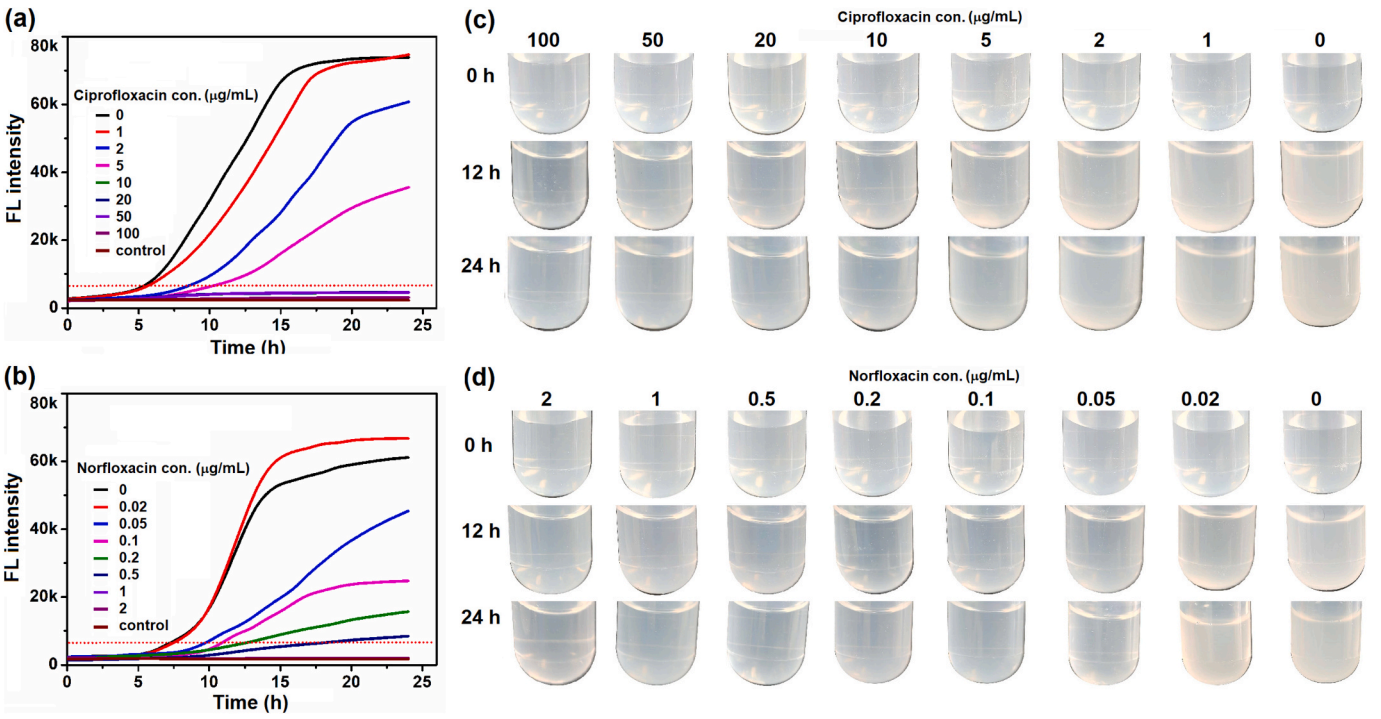


Fig. 5. RTFGC for drug-resistance analysis. (a) and (b) Real-time FL growth curves for bacteria in the presence of ciprofloxacin (a) and norfloxzcin (b) of different concentrations. (c) and (d) Broth dilution method for drug-resistance analysis. The images showed the turbidity of the broth after cultivation for 12 or 24 h in the presence of ciprofloxacin (c) and norfloxzcin (d) of different concentrations.

of therapy regimen and the direction of rational drug use to prevent the rapid spread of resistant bacteria (Sanz-Garcia, Hernando-Amado, & Luis Martinez, 2022; Z. Wang et al., 2024). Here, the antibiotics ciprofloxacin and norfloxzcin were used to test practicability of the RTFGC for drug-resistance analysis, as shown in Fig. 5a and b. For ciprofloxacin, the MIC was determined to be 10 μg/mL, excess of which the FL growth cure of the bacteria could not reach the FL threshold. The sub-MIC of ciprofloxacin was 1–5 μg/mL, which decreased the growth velocity of the bacteria and shaped the selection window of the antibiotic for resistant bacteria. For norfloxzcin, the MIC (1 μg/mL) was ten times lower than that of ciprofloxacin, and its sub-MIC ranged from 0.05 to 0.5 μg/mL. In contrast, traditional broth dilution method for drug-resistance analysis was hard to determine exactly the MIC and sub-MIC, as shown in Fig. 5c and d. It is worth noting that the MIC and sub-MIC of an antibiotic could be affected by various factors such as the bacterial strain and bacterial concentrations used for testing. The analysis results here are just to demonstrate the successful application of the RTFGC method for drug-resistance analysis.

4. Conclusion

Summary, we put forwarded a real-time fluorescence growth curve (RTFGC) method for the sensitive quantification of viable bacteria. For the first time, we used SYBR Geen to trace the in-vivo DNA replication during bacterial binary fission. Such stain did not influence the growth of bacteria. Nor did the bacteria degrade the stain, enabling the fluorescent microplate reader to monitor sensitively the growth of bacteria. Inspired by the qPCR principle, we defined Ct value as the culture time needed to reach the FL threshold set by the fluorescent reader. Good linear relationship between the Ct value and the logarithm of bacterial concentration was obtained in a wide range. We also found that the melting curves were unique for each bacterial strain, and it could be used for contamination identification. The as-developed RTFGC method was applied to the quantification of *E. coli* O157:H7 in foods such as water, milk, and apple juice. In addition, the RTFGC was successfully applied for drug-resistance analysis to determine both the minimal inhibitory concentration (MIC) and sub MIC of the bacteria. Compared with traditional methods such as plate counting, ELISA, SERS and FCM, the RTFGC not only had the advantages of high sensitivity, high throughput and a wide linear range of quantification, but also enabled the selective detection of viable bacteria in food samples. It is believed that RTFGC has many potential applications in monitoring the fermentation process, assessing the antibiotic susceptibility, and studying the effect of prolonged storage on bacterial viability.

CRediT authorship contribution statement

Yajing Chen: Writing – original draft, Investigation. **Yanlin Chen:** Investigation. **Siying Tang:** Data curation. **Biao Tang:** Data curation. **Shengbin He:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grants 32360245) and Guangxi Natural Science Foundation (Grants 2023GXNSFAA026044), for which we are most grateful.

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