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Research article

Development of Quantitative PCR Method for Quantifying *Streptococcus thermophilus* Growth During Batch Culture

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Abstract

Streptococcus thermophilus is a thermophilic lactic acid bacterium that is commonly used in food fermentation. However, quantifying bacterial populations using conventional culture-dependent assays is laborious. The genome-based method has been recognized as an alternative rapid method. In this study, we developed a method to use a specific gene of *S. thermophilus*, the glucokinase gene (*GlcK*), by quantitative PCR (qPCR). A 139-bp PCR product was successfully cloned and used to generate a DNA standard curve by plotting the threshold cycle (Cq) versus log DNA concentration of plasmid DNA, with an amplification efficiency of 97.2%. Coefficient of variation was calculated by considering both Cq and bacterial cells enumerated by plate counts, which indicated a log CFU/mL (1.69-6.56) and log DNA copies (2.07-6.03). This linear relationship revealed a quantitative curve (R^2 = 0.989) with a detection limit of range from 2.07 to 6.03 log copies per reaction. In terms of efficiency and repeatability, the relative standard deviations (RSDs) were in the range of 92-110% and 0.01%, respectively. Lastly, we used the developed qPCR method to determine the growth curves of bacterial cells and the specific growth rate (μ) during batch culture for 24 h. The established method facilitated the determination of specific growth rates, showing a specific growth rate of 0.57 h^{-1} with lactose supplementation and 0.30 h^{-1} in the absence of lactose. Hence, qPCR-based methods facilitated reliable quantification of *S. thermophilus* during fermentation.

Keywords: *Streptococcus thermophilus*; glucokinase gene (*GlcK*); quantitative PCR; probiotic; batch culture

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1. Introduction

Streptococcus thermophilus, a Gram-positive thermophilic bacterium, is involved in the production of yoghurt and other dairy products (Roux et al., 2022). During fermentation, it rapidly converts lactose into lactic acid, lowering the pH and producing exopolysaccharides that contribute to the desired texture and rheology of the final product (Alexandraki et al., 2019). Furthermore, it has the potential to function as a probiotic, a type of living bacteria that, when consumed in sufficient quantities, can provide a variety of health benefits to the host (Sánchez et al., 2017; Li et al., 2022). Since probiotics and fermentation have been studied, culture-based methods (e.g., agar plating with selective media and optical density measurement) are commonly used to determine prebiotic properties. However, these methods are labor-intensive and time-consuming and not suitable for determining in mixed culture (Chioccioli et al., 2014; Davis, 2014; Dan et al., 2023). Therefore, the developed quantitative method is very useful to monitor lactic acid bacteria (LAB), in particular, *S. thermophilus,* as a starter for fermented products, ensuring consistent quality and accurate monitoring during batch fermentation.

The genome-based method has gained recognition as a rapid alternative method. So far, PCR-based methods, especially quantitative PCR (qPCR), are mainly used for the detection, identification, and quantification of pathogens or beneficial microbes (Li et al., 2024).This technique combines the amplification of a target DNA sequence or PCR product (amplicon) with the determination of the concentration of that amplicon in the reaction by observing fluorescence intensity at the end of each cycle (Higuchi et al., 1992; Kim, 2001). It is faster, more sensitive, and more specific than culture-based approaches and enables high-throughput assays (Toley et al., 2015). qPCR is a useful tool in probiotic studies for identification, quantification (Cleusix et al., 2010; Sheu et al., 2010; Catone et al., 2024) and growth analysis (Cotto et al., 2015; Zhao et al., 2022). So far, some reports have identified several gene targets for qPCR-based detection and quantification of LAB species such as *Lactobacillus* and *Bifidobacterium*. These included 16sRNA sequences (Fan et al., 2021) and some specific gene sequences; *Tuf* (elongation factor Tu) (Shi et al., 2022), *Xfp* (xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Cleusix et al., 2010), *HolA (*delta subunit of DNA polymerase III) (Zhao et al., 2022) and *PheS* (α-subunit of phenylalanyl-tRNA synthase (Zhao et al., 2023; Catone et al., 2024). In fact, 16S rRNA has often been used to identify *S. thermophilus* (Fan et al., 2021), but the sequence homology of the gene among the LAB species was high, showing an unreliable method of strain-specific identification. Furthermore, the majority of target genes developed for LAB were not relevant to the monitoring of bacterial growth during fermentation of *S. thermophilus*.

In this study, a specific gene, glucokinase *(GlcK*), a key enzyme in glucose metabolism, catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P) using ATP. This reaction initiates the glycolytic pathway, leading to the conversion of glucose into pyruvate and the generation of ATP (Cáceres et al., 2007). Here, a rapid and efficient qPCR method targeting the *GlcK* gene was developed for the detection and quantification of *S. thermophilus*. This method was validated for specificity, repeatability, and precision. This work presents a new genomic approach to quantify the cell density of a specific strain and to describe the microbial growth during batch culture.

2. Materials and Methods

2.1 Bacterial strain and culture condition

Streptococcus thermophilus strain TISTR 894 was cultured and maintained using MRS broth. Before use, a single colony was cultured on MRS (Merck, USA) agar plates at 40°C for 48 h and then transferred to MRS liquid media under the same conditions with shaking at 180 rpm as an inoculum for further study.

2.2 Bacterial genomic DNA extraction

Bacterial culture was collected, and genomic DNA was extracted from the cell pellet using the GF-1 Nucleic Acid Extraction kit as described in the Vivantis's instruction manual (Vivantis, Germany). DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermofisher, USA) by measuring absorbance at 260 and 280 nm, following standard procedures (Sambrook & Russel, 2001).

2.3 PCR and gel electrophoresis

PCR reaction was performed in a total volume of 10 µL containing 5 µL of DreamTaq Green PCR Master Mix (Thermofisher, USA) (2x), 2 µL of ddH_2O, 1 µL of each primer, and 1 µL of a diluted 10x DNA template. The parameters for the reaction were as follows: initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; with final extension at 72°C for 15 min. The primers used to amplify *GlcK* were forward primer (5'-ACTGGGTGAACGTTGGGTTG-3') and reversed primer (5'-ACCAATTT CCCCACCAGCAC-3'). The presence and sizes of PCR products were verified by 1% agarose gel electrophoresis using a 100 bp DNA ladder (Solis BioDyne, USA).

2.4 Gene cloning using TOPO cloning kit

The PCR products were cloned into a TOPO vector using the pCR™II-TOPO® Vector System Cloning Kit (Invitrogen, USA). The resulting recombinant TOPO vector was transformed into competent *E. coli* DH5α cells via the heat shock method and cultured on Luria-Bertani (LB) agar plates (Merck, USA) containing chloramphenicol at 34 µg/mL for 24 h. Plasmid DNA was isolated using the GF-1 Plasmid DNA Extraction Kit (Vivantis, Germany). The isolated plasmid was confirmed to contain the *GlcK* gene by PCR analysis using the *specific* primers. The recombinant plasmid containing the *GlcK* gene was confirmed by sequencing analysis (Macrogen, Inc).

2.5 Quantitative PCR assay

Quantitative PCR experiments were performed in five replicates using a Real-Time Thermal Cycler CFX96 (Bio-Rad, USA). Each 20 µL reaction mixture contained 5 µL of ddH_2O, 2 µL of EvaGreen (Biotium, USA), 1 µL of template DNA (10 ng), and 1 µL of each forward and reverse primer. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 38 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 40 s, and extension at 72°C for 30 s. A melting curve analysis was performed at the end of each cycle.

2.6 Standard curves and amplification efficiency

2.6.1 Calibration of the *GlcK* plasmid standard curve

The threshold cycle (Ct) was plotted against the logarithm of known DNA concentrations using a series of 10-fold dilutions (copy number ranged from $3x10^3$–$3x10^8$) of a plasmid containing the *GlcK* gene, using real-time PCR to create the *GlcK* plasmid standard curve.

The equation of plasmid copy = [6.02 x 10^{23} (copy number/mol) x plasmid DNA amount (g)] / [plasmid DNA length (bp) x 660 (g/mol/bp)] was used to determine the copy numbers of the plasmid concentration as described by Lee et al. (2006). To find gene copy number, a scatter plot of the Cq readings against logarithmic plasmid concentrations was created using linear equation CT = (log10 starting copy) x slope + y-intercept (Whelan et al., 2003). The slope value of the linear curve was adopted to calculate the amplification efficiency (E); E= ($10^{-1/slope}$) (Rasmussen, 2001).

2.6.2 Sensitivity and repeatability test

Quantitative PCR amplification was performed using serial dilutions of three initial nucleic acid concentrations (10, 5, and 2 ng/μL). All tests were performed in three replicates following the real-time PCR procedure as described above, and a standard curve was generated by plotting the Cq value against the logarithmic DNA concentration. The reaction efficiency was estimated using the formula provided in Section 2.6.1. The repeatability was carried out with three replicates by using three initial DNA concentrations (0.1, 1, and 10 ng/μL). The repeatability of the assay was assessed by calculating the relative standard deviation (RSD) of the replicated Cq values at each concentration. RSD was calculated as (standard deviation/mean) x100. The RSDs were then compared among the different concentrations. The value should be less than or equal to 1% (Allen & Rutan, 2011).

2.7 Correlation between qPCR and cell density measurement

Streptococcus thermophilus culture was initiated and grown in MRS medium at 40°C until it reached an optical density or OD_{600nm} of 0.8 and was subsequently diluted to a final OD_{600nm} of 0.1 before used. Ten milliliters of this culture were then divided. The first 5 mL was used for viable cells counting using serial dilution plating on MRS agar (ranging from 10^{-1} to 10^{-6}) and another 5 mL was used for genomic DNA extraction for qPCR (as described in Section 2.5). Finally, the calibration curve was generated by plotting the log DNA copy numbers against the log CFU counts.

2.8 The growth determination of *S. thermophilus* in batch culture

To determine the bacterial growth using qPCR, bacterial cells were cultured in MRS medium of 1/4 strength with 1% of lactose and without lactose, and incubated at 40°C, and 180 rpm. Subsequently, the density of cells and gene copy numbers were monitored and collected at intervals of 2 h to determine bacterial growth for 24 h. The density of cells was measured using OD_{600} absorbance readings, and the number of gene copies was evaluated using qPCR. The calculation of the nonlinear curve fitting regression relationship between the density of cells and gene copy number was performed and then the specific growth rate (μ, h^{-1}) was calculated based on the equation of μ= (lnX-lnX0)/t (Monod, 1949).

3. Results and Discussion

3.1 Gene cloning and DNA sequencing analysis

In this study, a cloned *GlcK* gene for quantify *S. thermophilus* growth during culturing using qPCR was developed. We successfully amplified a 139-bp PCR product from the *S. thermophilus* genome with specific primers and optimized PCR conditions (Figure 1). The sequence analysis identified the gene as the *GlcK* gene of *S. thermophilus* (accession no. CP113237.1) by using BLAST search in the NCBI database. Following amplification, the *GlcK* gene was cloned into the TOPO vector system, verified by PCR (Figure 1) and sequencing. A standard curve was then established using real-time PCR with serially diluted this plasmid DNA.

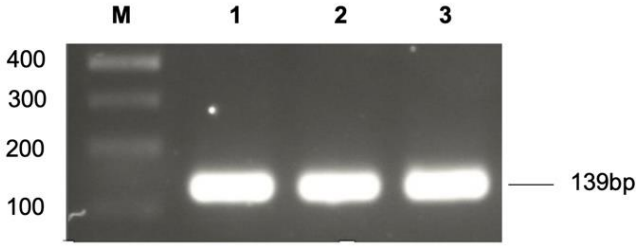

Figure 1. Amplification of 139 bp-PCR product was analyzed by gel electrophoresis. (M; 100 bp DNA ladder, and 1, 2 and 3 correspond to the *S. thermophilus* genome, and selected clones of the recombinant plasmid with *GlcK* gene, respectively).

3.2 Quantification of gene amplicon using qPCR

We employed the *GlcK* gene as a specific reference gene to construct a standard curve of five 10-fold serial dilutions of plasmid DNA ranging from 3×10^4 to 3×10^8 copies, which were analyzed using real-time PCR. Figure 2A illustrates the consistent amplification curves of ten-fold serial dilutions, whereas melting curve analysis of the PCR product, as presented in Figure 2B, revealed a single peak. This result confirmed the specificity of the PCR primers, as it demonstrated that they did not form primer dimers or amplify regions outside of the gene target (Adams, 2006).

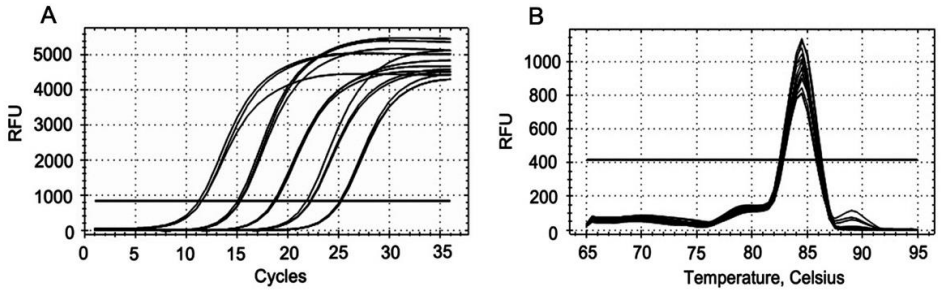

Figure 2. Amplification curve of standard (A) and melting curve (B) of series diluted plasmid DNA using qPCR analysis

Next, a standard curve was established by plotting the Cq against the logarithm of the plasmid gene copy number per dilution. The findings indicated a linear correlation coefficient of R^2 = 0.9978 and an efficiency value of 97.2% for the standard curve of the *GlcK* gene, indicating that the quantification of *S. thermophilus* cells would allow accurate bacterial DNA quantification (Figure 3). This validated standard curve was subsequently used in the next experiment.

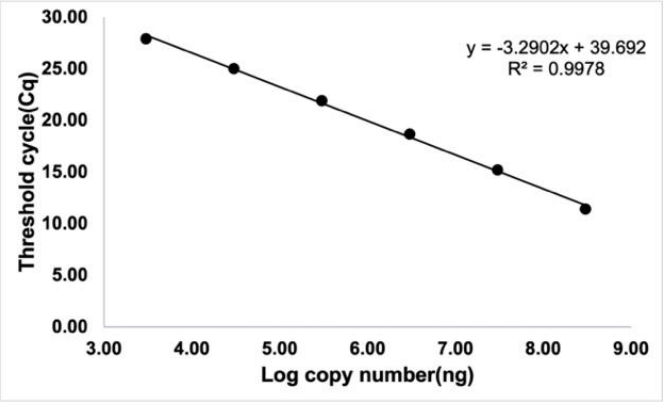

Figure 3. Calibration curve of Cq values and *GlcK* gene copy number

3.3 Performance of qPCR in quantification

An evaluation of qPCR amplification efficiency, including sensitivity and repeatability, was performed. Sensitivity was determined using serial dilutions of the *GlcK* plasmid DNA (10, 5, 2 ng/µL). The calibration curve of gene copy number versus plasmid DNA concentration showed a strong linear correlation, with efficiencies of 105%, 92.0%, and 110% and corresponding R^2 values of 0.9686, 0.9921 and 0.9887, respectively (Figure 4). Our findings indicated a reliable and accurate result, with efficiencies within 90-110% as reported by Broeders et al. (2014). Furthermore, we also evaluated qPCR reproducibility. Here, PCR inhibitors and DNA extraction efficiency were addressed (Pancza et al., 2021). It was seen that the method worked very well to measure bacterial DNA levels ranging from 2 to 10 ng/µL, with a slope close to -3.3 (Figure 4). The result suggests that there were no PCR inhibitors in the reaction because the average slope value was in between -3.1 and -3.6 (Adams, 2006; Elizaquível et al., 2013). Next, the DNA extraction used in this study demonstrated a consistent DNA concentration of 23.1±0.90 ng/µL from the bacterial culture at an OD600 of 0.1 to ensure the precision of the qPCR analysis. Due to the use of a single bacterial culture, this study had no issues with DNA efficiency or PCR efficiency. However, food samples with complex matrices, such as those high in protein, lipids, and colloids, present challenges to DNA extraction and qPCR sensitivity. Fan et al. (2021) demonstrated that a consistent DNA concentration was achieved by a 15- fold dilution of yogurt and milk combined with the addition of proteinase K and 8% of SDS in a DNA extraction procedure.

To further validate the method, we assessed its reliability by testing three different DNA concentrations (0.1, 1, and 10 ng/L). Our findings showed high repeatability with a 0.01% RSD for Cq averages across DNA concentrations (Table 1). This consistent

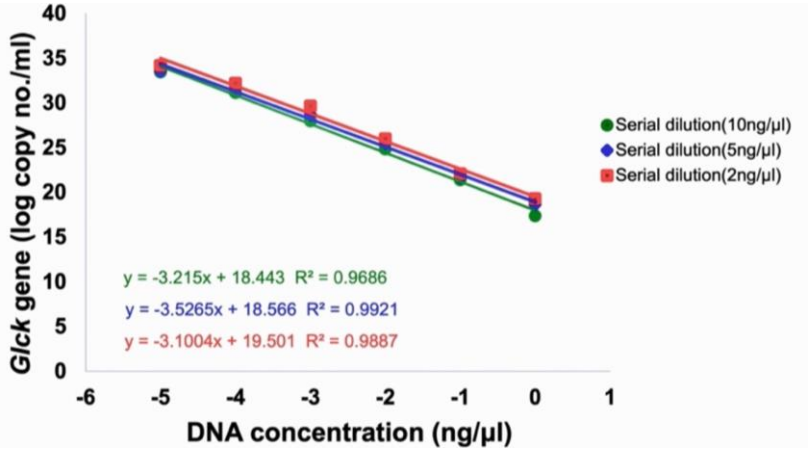

Figure 4. Calibration curves and sensitivity of the qPCR assay derived from DNA concentrations of the *GlcK* gene of 10, 5 and 2 ng/µL, respectively

Table 1. The repeatability of the qPCR method was assessed by calculating the relative standard deviation (RSD) of three different DNA concentrations.

DNA Concentrations (ng/µL)	Cq					Mean Cq	RSD (%)
10	17.19	17.37	17.51	17.4	17.41	17.38	0.01
1	20.37	20.13	20.56	20.28	20.38	20.34	0.01
0.1	23.34	23.4	23.68	23.61	23.46	23.5	0.01

performance indicated high repeatability of the method, even at low DNA levels, which highlighted the method's sensitivity and reliability for detecting in the range of DNA concentrations. This method also proved for the accurate quantification of bacterial DNA in food and fermentation processes Zhao et al., (2022).

3.4 Correlation of colony forming unit (CFU/mL) and gene copy number

The linear regression was calculated using logarithm mean values of the abundance of bacteria and gene amplicons (Figure 5.). This revealed a significant correlation between total plate count and qPCR, with a coefficient of determination $R^2 = 0.9890$. These findings confirmed a good correlation between the quantity of the *GlcK* gene and the numbers of bacterial cells. For example, at 10^{-1} and 10^{-6} dilutions, the number of bacterial cell quantification using the traditional plate count method was 6.56±0.06 and 1.69±0.07 log CFU/mL, while qPCR showed 6.03±0.03 and 2.07±0.01 log copy number/mL. Although qPCR values were slightly less than the plate count method, this may be due to DNA extraction efficiency or the presence of non-viable cells, which are detectable by qPCR but not cultivation. Similarly, previous studies indicated that the 16S rRNA gene standard calibration curve had a high amplification efficiency of 98.42% (Barer et al., 2015). Also, there was a strong linear relationship ($R^2 = 0.9981$) between the number of copies of this

gene and the number of cells. This allowed for reliable measurements with sensitivity of 15.1 CFU/mL/reaction (Chen et al., 2020).

Through the development of a linear relationship, Cq values were directly converted to CFU/mL, covering a range of 1.69 to 6.56 log CFU/mL, which corresponded to 2.07 to 6.03 log DNA copy number (Figure 5 and Table 2). Hence, the qPCR method could detect bacterial cells in the range of 10^2 to 10^6 CFU/mL. Moreover, the calibration curve offered a sufficient dynamic range to quantify bacterial cell growth in batch culture. The results exhibited a strong linear relationship between Cq values and bacterial counts throughout the bacterial growth phrase, indicating that the method is reliable for measuring bacterial growth during the lag, logarithmic, and stationary phases during batch fermentation as described by Chen et al. (2021). This approach has been developed for detecting and quantifying bacterial cells in meat and dairy products (Kawasaki et al., 2005; Fan et al., 2021). In this study, the *GlcK* gene of *S. thermophilus* proved suitable as quantitative maker, enabling accurate monitoring of the bacterial growth in batch culture.

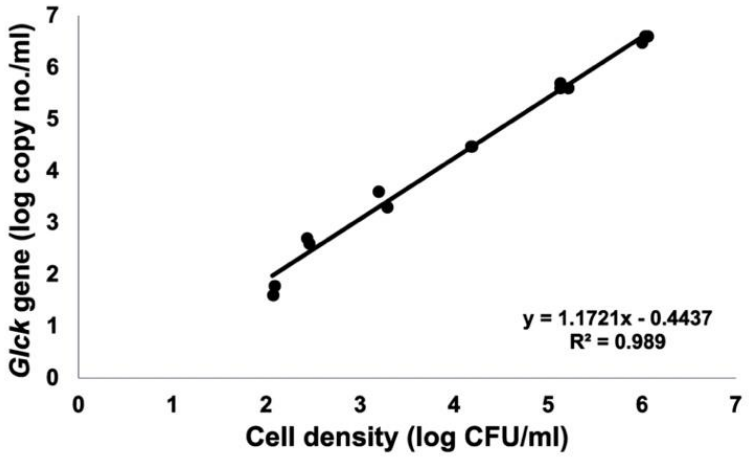

Figure 5. The correlation of quantitative method was evaluated by qPCR (gene copy number) and total plate count method (CFU/mL).

Table 2. Quantification of S. *thermophilus* using the total plate count (log CFU/mL) and qPCR (log gene copy number) method

Dilutions	Log CFU/mL	Log Copy Number/mL
10^{-1}	6.56±0.06	6.03±0.03
10^{-2}	5.63±0.05	5.16±0.04
10^{-3}	4.52±0.06	4.18±0.01
10^{-4}	3.50±0.14	3.21±0.05
10^{-5}	2.63±0.05	2.53±0.12
10^{-6}	1.69±0.07	2.07±0.01

3.5 Application of qPCR assay in bacterial growth determination

Finally, we applied the calibration curve to determine the growth in batch culture. Here, the bacterial cells were cultured in MRS medium, both with and without lactose, at 40°C for 24 h. The bacterial cell growth was quantified using the validated calibration curve of the *Glck* gene (Figure 5). Here, the qPCR results clearly exhibited a similar pattern growth trend along with OD measurements (Figure 6) in either lactose-supplemented or lactose-free cultures which showed that the microbial growth reached the stationary growth phase in 24 h (Figures 6A-C). At 24-hour period, the growth curves for *S. thermophilus* measured by absorbance and qPCR showed a typical microbial growth pattern, as shown in Figure 6. Lactose supplementation revealed a rapid growth with log phase (from 2 to 4 h) compared to lactose-free medium (from 4 to 8 h). Next, specific growth rates were determined from the qPCR-derived microbial growth curves. During the log phase, these were found to be 0.30 h^{-1} (without lactose) and 0.57 h^{-1} (with lactose) (Figures 6A&B). Of these, qPCR was successfully used to determine kinetic growth in single and dual cultures of *E. coli* K12 and *Pseudomonas putida* as described by Oliveira et al. (2009) and Cotto et al. (2015).

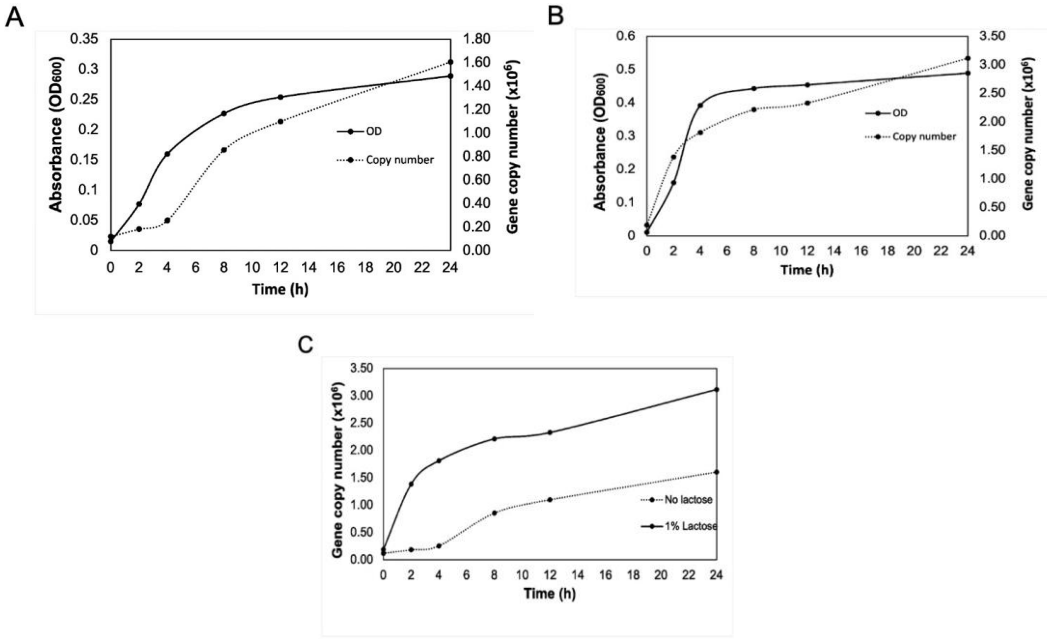

Figure 6. Growth determination of *S. thermophilus* in batch culture for 24 h using cell density and qPCR (A) without lactose and (B) supplementation with 1% of lactose; (C) quantification of bacterial growth using qPCR

Our findings demonstrated that the *GlcK* gene can serve as an alternative marker for monitoring the *S. thermophilus* enumeration and growth state in batch culture. The method allows for strain-specific biomass quantification in pure culture validation. Notably, the method could be extended to the enumeration of *S. thermophilus* in various sources and environments, for example, in meat, dairy products, and fecal samples as well as the bacterial kinetic growth analysis of mixed culture (Cotto et al., 2015).

While qPCR provides a reliable method for identifying and quantifying bacterial cells, it does not distinguish between live and dead cells in culture. Alternatively, qPCR can be coupled with pretreatment using propidium monoazide (PMA). PMA is a membrane-permeable dye that selectively penetrates the cell membrane, irreversibly binds to DNA, and prevents PCR amplification. The PMA-qPCR offers a rapid and quantitative approach for viable bacterial cell numbers in various applications (Fittipaldi et al., 2012; Chen et al., 2021; Shi et al., 2022).

4. Conclusions

To summarize, a method to evaluate bacterial growth on the prebiotic test using quantitative PCR was successfully developed in this study. The *GlcK* gene was used as the target DNA sequence to design specific primers for the detection of *S. thermophilus*, thereby replacing the conventional culture-dependent assays. Hence, this qPCR-based methods facilitated reliable quantification of *S. thermophilus* during growth, revealing a good correlation between the log CFU/mL and the log DNA copies. These results supported the potential use of this method as an accurate, repeatable, and rapid alternative method to quantify bacterial growth and its application could be extended to the bacterial community in dairy products.

5. Acknowledgements

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6. Authors' Contributions

AO designed and supervised the research. LY and NT performed the experiments; NS, LY, and SC analyzed the data. LY and AO wrote the manuscript, which SC, NS, and AO reviewed and edited. All authors read and approved the manuscript.

7. Conflicts of Interest

The authors declare no competing interests.

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