Food Control 168 (2025) 110889

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Development, evaluation and application of propidium monoazide (PMA) based methodologies on viable cell quantification of *Pediococcus acidilactici* in rice noodles products

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ARTICLE INFO

Keywords:
Pediococcus acidilactici
PMA based methodologies
Viable cell quantification
Rice noodles products
Low temperature

ABSTRACT

Pediococcus acidilactici provides a special taste during dough fermentation, but its improper presence will accelerate the spoilage of rice noodles products. In this study, propidium monoazide (PMA) based methodologies on viable cells quantification of *P. acidilactici* were developed, evaluated and applied in rice noodles products. Prior to qPCR and RT-qPCR detection, PMA treatment was included to effectively remove the residual DNA from membrane-compromised (dead) cells. Firstly, considering the influence of food component and PMA on qPCR result determination, 4 standard curves corresponding Ct value to *P. acidilactici* cell number were established in 4 different rice noodle products, respectively. The 4 standard curves were subsequently applied for viable cell quantification of *P. acidilactici*. Secondly, mimicking the storage conditions of rice noodles products and growth states of *P. acidilactici*, planktonic and biofilm (early, mediate, mature) cells in 4 types of rice noodles products stored at −20 °C and 4 °C were taken into consideration to evaluate the efficiency of PMA-qPCR for viable cell quantification. Thirdly, stress response gene expression in *P. acidilactici* was monitored by PMA-RT-qPCR and compared with culturable and viable cell number quantification. The PMA-qPCR method with specific standard curves for 4 types of rice noodle products was applicable for viable cell quantification of *P. acidilactici* in planktonic and biofilm (early, mediate, and mature) states. The planktonic and biofilm cells of *P. acidilactici* maintained high and stable in both culturable and viable cell number in different growth states, rice noodles products, and low temperature conditions, but acquired significantly different gene expression levels. The findings provided viable cells quantification methods and an alarm for *P. acidilactici* contamination risk in instant rice noodles products.

1. Introduction

According to global statistics, approximately a quarter of food is contaminated with mycotoxins every year (Tournas & Niazi, 2018). For rice noodle products, once the raw materials are contaminated by fungi, they are highly likely to be mildewed in the further process of production, processing, storage, transportation, and sales, leading to the occurrence of mycotoxin poisoning events (Salman & Mudalal, 2022). In addition, during the process of raw and auxiliary materials pretreatment, dough preparation, molding, sterilization, etc., insufficient

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https://doi.org/10.1016/j.foodcont.2024.110889
Received 29 June 2024; Received in revised form 30 August 2024; Accepted 16 September 2024
Available online 17 September 2024

hygiene operation and handling, incomplete cleaning for machinery and equipment, as well as ineffective sterilization processes, have facilitated microbial contamination (Lüdin et al., 2018; Ming, 2010, pp. 1746–1750). According to previous reports on the microbial contamination of commercially available instant rice noodles products, food spoilage bacteria such as *Pediococcus acidilactici*, and food poisoning bacteria such as *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, are the leading causative microorganisms unqualified quick-frozen rice noodles products (İncili, Karatepe, & İlhak, 2020; Miao et al., 2017; Patterson, Venkitanarayanan, & Kariyawasam, 2014; Wang, Kalchayanand, Bono, Schmidt, & Bosilevac, 2012). Growth of these microorganisms and production of various metabolites or bacterial toxins can cause food spoilage and poisoning (Ceuppens et al., 2011), especially for instant rice noodles products where additional food materials are added, such as meat, poultry, eggs, aquatic products, vegetables, sugar, oil and other auxiliary materials, which further increases the risk of cross-contamination and complicates the microbial proliferation (Zhu, Jackson, & Wang, 2017). As a major food spoilage microorganism in rice noodle products, *P. acidilactici* utilizes carbohydrates to produce lactic acid and becomes a common dough fermentation starter (Ahn, Kim, & Kim, 2017), which is often used in the fermentation process of food, such as yogurt, kimchi, bread, etc., so as to give food a special taste (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). However, under a large variety of conditions (particularly low-oxygen, low--temperature and acidic environments), metabolic process of *P. acidilactici* produces various substances including lactic acid, acetic acid, diacetyl, etc., which disrupts the food quality (resulting in products with a pungent sour taste and mucus) and further leads to food spoilage (Choyam, Srivastava, Shin, & Kammara, 2019; Rawat & Research, 2015). Collectively, microbial growth and proliferation is a leading concern in food quality and safety for rice noodle products, and thus their accurate monitoring is essentially important for further control and prevention of such microorganisms.

Up-to-date, culture-based methodologies are still the “golden standards” for the detection of food borne microorganisms. However, such methodologies depend on the culturability of microbial cells, and thus its incapability in detecting culturability compromised microorganisms leads to “false-negative” results which increases the risk of food quality and safety. In 1982, Xu et al. discovered a special state of bacteria and named it “Viable but Non-culturable" (VBNC) (Xu et al., 1982). VBNC bacterial cells cannot grow in regular culture medium, but have a complete cell membrane structure and maintain a certain metabolic activity, thus causing food safety hazards (Dong et al., 2020; Liu, Zhou, et al., 2017). A variety of unfavorable environmental factors can induce bacteria to enter a VBNC state, such as low temperature, low nutrient condition, high osmolarity, low pH and even UV irradiation (Cunningham, O’Byrne, & Oliver, 2009; Foster, 1999; Guan et al., 2021; Guo et al., 2019; Ramaiah, Ravel, Straube, Hill, & Colwell, 2002). Amongst, low temperature has been proved to be the most common way to induce the entry of the VBNC state. *E. coli* had been found entering the VBNC state and produce a certain amount of Shiga toxin in a low temperature storage environment (Wei & Zhao, 2018). The number of *E. coli* in the VBNC state in the quick-frozen beef balls had been identified as high as 10^7 colony forming unit (CFU)/mL. The VBNC state of *S. aureus* and *E. coli* had been successfully induced by low temperature combined with oligotrophic conditions. Through low temperature and subculture of beer, *Lactobacillus delbrueckii* could enter into the VBNC state and lead to cloudy spoilage of beer by producing excessive lactic acid, acetic acid and diacetyl (Liu, Li, et al., 2017). As concluded, the VBNC state formation ability of foodborne microorganisms highlights the importance to identify both culturable and viable cell numbers in food systems.

Quantitative polymerase chain reaction (qPCR) had been proposed as an effective method to quantify total bacterial cell number and used to determine foodborne pathogen and spoilage bacteria (Moser, Berthoud, Eugster, Meile, & Irmler, 2017). However, as a relative quantification method based on DNA abundance, qPCR fail to differentiate viable cells due to the residual DNA from dead cells. False positive detection is its major issue. To differentiate viable and dead cells in studies of VBNC state, some methods have been developed based on viability assays such as respiratory and metabolic activity measurement, cytoplasmic membrane integrity, immunological tests, fluorescent-based hybridization, and molecular methods for DNA and RNA hybridization probes in PCR and reverse transcriptase PCR (Junyan Liu, Liang Yang et al., 2023). Amongst, direct DNA extraction and PCR followed by propidium monoazide (PMA) treatment is one of practical methods for assessment of viable cells from dead cells (Zhong & Zhao, 2018). Prior to PCR detection, PMA treatment was included to effectively remove the residual DNA from membrane-compromised (dead) cells. Considering the limitation of PCR, more DNA amplification techniques including isothermal amplification (loop-mediated isothermal amplification (LAMP), cross priming amplification (CPA), polymerase spiral reaction (PSR), etc.) and qPCR have been developed to overcome the complication and not able to be quantification, respectively (Guan et al., 2021; Han et al., 2020; Junyan Liu, Tengyi; Huang et al., 2023; Liu, Xiang, Huang, Xu, Liu, et al., 2024; Liu, Xiang, Huang, Xu, Ma, et al., 2024; Zhao et al., 2024). However, such methods still have significant limitations, Firstly, these methods have not been applied in the detection of *P. acidilactici*, except for a PMA-CPA assay which has been used to detect VBNC *P. acidilactici* under freezing conditions (Guan et al., 2021). Secondly, PMA combined with isothermal amplification techniques including PMA-CPA fail to enable quantitative detection of viable cells. Thirdly, most studies on the development and application of PMA-qPCR have focused on its specificity and sensitivity but omitted the complication of specific food products and the real existing status (biofilm) of bacterial cells in food.

Thus, considering the above limitation of PMA-based methods, in this study, a PMA-qPCR method was developed and evaluated to effectively quantify viable planktonic and biofilm cells in specific food products. Firstly, considering the influence of food component and PMA on qPCR result determination, 4 standard curves corresponding Ct value to *P. acidilactici* cell number were established in 4 different rice noodle products, respectively. Secondly, 4 different bacterial states *P. acidilactici* might experience in 4 rice noodle products, as well as 2 storage temperature (−20 °C and 4 °C) were taken into consideration to evaluate the efficiency of PMA-qPCR for viable cell quantification. Thirdly, stress response gene expression in *P. acidilactici* was monitored by RT-qPCR and compared with culturable and viable cell number quantification.

2. Materials and methods

2.1. Bacterial strain

The *P. acidilactici* strain used in this study was previously stored as glycerol stock at −80 °C. The strain was streaked on de Man, Rogosa, and Sharpe (MRS) agar (Huankai, Guangzhou, China) and cultured at 37 °C for 24 h. Subsequently, a single colony was inoculated into 2 mL of MRS broth and cultured overnight at 37 °C with shaking at 200 rpm. Overnight culture was subcultured in fresh MRS broth for 4∼6 h to acquire log phase culture.

2.2. Standard growth curve for planktonic and biofilm states

For planktonic state, culture with cells in log phase was used and centrifuged at 5000 rpm for 5 min to remove liquid medium. The cells were washed twice with saline and resuspended in MRS broth, with concentration adjusted to 10^3 CFU/mL. The cells were incubated at 37 °C with 200 rpm shaking and CFU counting was performed every 2 h within 24 h. For biofilm state, log phase liquid culture with concentration adjusted to 10^6 CFU/mL was added in a sterile 6-well plate and incubated at 37 °C without shaking for 8 h, 24 h and 72 h, respectively. The supernatant was removed subsequently and saline was used in triplicate to remove residual planktonic cells. The biofilm cells were

scratched out by pipette tips and subjected to CFU counting.

2.3. Rice noodle products and artificial contamination

A total of 4 common rice noodles products were selected for further study, including steamed bun (SB), rock crystal (RC), rice noodle (RN) and turnip cake (TC) (Likoufu, China). For artificial contamination of *P. acidilactici* strain, 25 g of rice noodle products were used for each experiment. For both planktonic and biofilm states, initial inoculations were adjusted to a final concentration of 10^7 CFU/g at the volume of 1 mL based on the standard growth curve established and added into 25 g of rice noodle products. After artificial contamination, all rice noodle products were stored at −20 °C and 4 °C. The artificial contamination was performed in triplicate.

2.4. Sample processing and CFU counting

The changes in bacterial growth in the artificially contaminated rice noodle products were recorded every 3 days. Sample processing was performed according to the Chinese national standard on food microbiology test. In brief, 25 g of rice noodle products were added into 225 mL of sterile saline, followed by homogenizing at 9000 rpm for 1 min. For biofilm samples, ultrasonication (setting as 50%, 125 W, 20 kHz) for 5 s with 5 s interval for 3 times using Ultrasonic Homogenizer Q125 (Qsonica, USA) was performed to disrupt biofilm structure. Culturable cell determination was performed by CFU counting at 21 different time points, which were every 3 days since start point (0 d) to 60 d. The homogenized rice noodle products were serial diluted and plated on MRS agar in triplicated.

2.5. PMA treatment and DNA extraction

To remove residual dead cells, 10 μL of PMA reagent was added into 500 μL of the food matrix-removed sample to meet a final concentration at 10 μg/mL (Hennessy et al., 1996). Optimization of the PMA concentration was conducted by preparation of treatment solution at different PMA concentrations (0, 1, 3, 5, 10, 20, 50 and 100 mg/mL) and 10 μg/mL was verified to be the optimum concentration which is in consistent with our previous study (Liu, Zhou, et al., 2017). The tube was incubated for 5 min at approximately 25 °C in the dark. Subsequently, incubation under a 500 W halogen lamp was required at a distance of 15 cm for 15 min, ensuring that PMA solution was fully cross-linked to DNA from dead cells. The treated samples were adapted to centrifugation at 5000 rpm for 10 min upon rapid bacterial genomic DNA extraction. The genomic DNA from viable cells was extracted using a bacterial rapid DNA extraction kit (Dongsheng, Guangzhou, China). The extracted DNA was frozen at −20 °C until usage.

2.6. Establishment of standard curve for PMA-qPCR

Considering the difference caused by 4 rice noodle products, standard curve was firstly established for PMA-qPCR. Gene *pheS* was used as amplification targets with specific primers designed with Primer Premier 5.0 and primer-BLAST (Table 1). PMA treated rice noodle products with a cell concentration varying from 10^2 to 10^8 CFU/g (10 fold serial dilution) were subjected to DNA isolation. The DNA samples from different rice noodle products with different cell concentrations were used for qPCR. The standard curves were established with bacterial cell concentration (log CFU/g) as X-axis and Ct value from qPCR as Y-axis.

Table 1
qPCR and RT-qPCR primers sequences.

Primer Name	Primer Sequence (5′-3′)	Primer Length (bp)
pheS-F	GGACGATGCAACTCATTCGC	20
pheS-R	ACGCAGGCGAACTTCAAAAC	20
16s rRNA-L.c-F	AGCCGACCTGAGAGGGTAAT	20
16s rRNA-L.c -R	TTGCTCCATCAGACTTGCGT	20
atpA-F	GAAACCAAGGCTGGAGACGTT	21
atpA-R	GACGAACTCCGGAATGGAAGT	21
dnak-F	ACGTTTGAAGGACGCCGCTGA	21
dnak-R	AATGGTCCGGCTTCGCCAGC	20

2.7. Application of PMA-qPCR on quantification of viable cells in rice noodle products

The developed PMA-qPCR method as above was further applied to quantify viable cells number of *P. acidilactici* strain from 4 rice noodle products, in planktonic and biofilm (3 different time points representing maturity) states. PMA-qPCR was performed every 12 days including 0, 12, 24, 36, 48 and 60 days during storage at −20 °C and 4 °C. In comparison, CFU counting was also conducted. Both PMA-qPCR and CFU counting were performed in triplicate.

2.8. RNA extraction and reverse transcription (RT)

1 mL of homogenized and PMA treated artificially contaminated rice noodle products were pipetted into a 1.5 mL centrifuge tube and subjected to centrifugation at 12,000 rpm for 1 min to collect the bacterial cells. Then, 1 mL of Total RNA Isolation Reagent was added, following repeatedly pipetting to fully disperse the cells in the extraction reagent. The tube was vortexed for 1 min every 3 min at room temperature within 30 min to ensure sufficient sample lysis. 200 μL of chloroform was added, followed by vortexing for 30 s, and incubating at room temperature for 3 min. After centrifugation at 12,000 rpm and 4 °C for 15 min, the sample was divided into an upper aqueous phase and a lower organic phase, with a buffy coat layer in the middle. Then, 450 μL of the upper colorless aqueous phase containing total RNA was carefully pipetted into a new centrifuge tube. An equal volume of ice-cold isopropanol was added and mixed by inversion for several times, followed by precipitation at room temperature for 10 min. After centrifugation at 12,000 rpm and 4 °C for 10 min, RNA precipitates appeared at the bottom of the tube. The supernatant, was carefully discarded and 1 mL of 75% ethanol (prepared with diethyl pyrocarbonate (DEPC)-treated water) was added to mix the samples by vortexing or inverting. The tube was centrifuged at 7500 rpm for 5 min at 4 °C and supernatant was discarded. To fully remove the residual organic solvent in the sample, the cell pellet was washed again with 1 mL of 75% ethanol. Subsequently, after brief centrifugation, the residual ethanol in the centrifuge tube was carefully aspirated. The RNA was resuspended in 25 μL of DEPC-treated water. The centrifuge tubes, pipette tips and related consumables used in the above process were free of nuclease contamination.

The concentration of each RNA sample was determine using a nanodrop. A final concentration of 0.1–2 μg was add in the reaction system, with 4 μL of 5 × RT MasterMix, 1 μL of 20 × Oligo dT & Random Primer, and RNase free H_2O. The RT reaction was conducted at conditions including 25 °C for 10 min, 55 °C for 45 min, and 85 °C for 5 min. The obtained cDNA was stored at −20 °C for subsequent RT-qPCR experiments.

2.9. PMA-RT-qPCR on stress response gene expression in rice noodle products

To assess the stress response gene expression in rice noodle products, housekeeping gene *16S rRNA* was used as the selected reference gene and *atpA* and *dnak* were used as the target genes. Primers were synthesized by Guangzhou Ige Biotechnology Co., Ltd upon designing using primer blast tool in NCBI databased and further confirmation by software Primer Premier 5 (Table 1).

The RT-qPCR reaction system included 10 μL of 2 × qPCR mix, 2 μL of 10-fold diluted cDNA template (20 ng), 0.4 μL each of forward and reverse primers (final concentration at 0.5 μM), and nuclease free dH_2O to reach 20 μL. The reaction program was set as pre-denaturation for 2

min at 95 °C, denaturation for 15 s at 95 °C, annealing at for 30 s 60 °C, and extension at for 30 s 72 °C, with a total of 45 cycles. Each experimental group was set in triplicate to ensure accuracy. The relative quantification method was used for gene expression differences, and the $2^{-\Delta\Delta Ct}$ calculation was used for relative changes in gene expression (Schmittgen & Livak, 2008).

2.10. Statistical analysis

The experimental data were shown as the mean ± standard deviation (SD) from at least 3 replicates. All datasets were tested using two-way ANOVA. Results were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Standard curve of PMA-qPCR in different rice noodle products

In order to accurately correspond the Ct values in PMA-qPCR to viable cell number, 4 standard curves were established for *P. acidilactici* strain in 4 different types of food sample (SB, RC, MF, and TC), respectively (Fig. 1). Bacterial cells change their growth rate to survive and persistence in different food, which are changeable and complicated environmental conditions to bacteria. For the four standard curves (Fig. 1), the detection range of viable cell number was 10^2~10^8 CFU/g. Since the presence of PMA dyes delays the Ct value in PMA-qPCR, high bacterial concentration might exceed the detection limit (Xiao, Tian, Yu, & Wu, 2013). For qPCR, the premise of whether the standard curve is credible depends on a suitable amplification efficiency. An amplification efficiency close to 100% is optimum to ensure result repeatability. In practice, the amplification efficiency of qPCR is generally 90–105%. The results in this study acquired 4 standard curves with the linear regression coefficients >0.99, and the amplification efficiency at 92%–104%, resulting in high quality in reliability and repeatability. The 4 standard curves were subsequently applied for viable cell quantification of *P. acidilactici* in planktonic and biofilm (early, mediate, and mature) states.

3.2. PMA-qPCR for viable cells quantification of P. acidilactici in planktonic state

Mimicking the storage conditions of rice noodle products and growth states of *P. acidilactici*, planktonic and biofilm (early, mediate, mature) cells in 4 types of rice noodle products (SB, RC, RN, TC) stored at −20 °C and 4 °C were included in this study. Cell numbers were monitored within 60 days by PMA-qPCR in comparison with traditional CFU counting. Concerning planktonic cells of *P. acidilactici*, they could survive in the 4 rice noodle products within 60 days at both 4 °C (Fig. 2A) and −20 °C (Fig. 2B). The culturable cell numbers of *P. acidilactici* maintained stable and high (approximately 10^7 CFU/g) at 4 °C regardless of food type (Fig. 2A), indicating the high risk of *P. acidilactici* contamination at relevant conditions. The survival rate of *P. acidilactici* differed among food types at −20 °C, possibly, due to the deference in food component. Maintaining at high levels in SB and TC, the culturable cell number decreased in RC (to 10^5 CFU/g at Day 60) and to a lower level (10^3 CFU/g) in RN (Fig. 2B). By comparing the major component of the rice noodle products, the least nutrient was present in RN (mostly carbohydrate and protein), possibly contributing to the significantly reduced cell numbers. It is noteworthy that planktonic *P. acidilactici* is capable of surviving in rice noodle products within 60 days during low temperature storage.

PMA-qPCR was applied in the viable cell quantification of *P. acidilactici* planktonic state at Day 0, 12, 24, 36, 48, and 60 (Fg.2C-2J). Similar to culturable cell number, the viable cell number of *P. acidilactici* maintained stable and high (approximately 10^7 cell/g) at 4 °C regardless of food type (Fig. 2C, E, 2G, 2I). Relatively consistent to the trend of survival rate of *P. acidilactici* in different food types, viable cell numbers maintained high levels in SB (Fig. 2D) and TC (Fig. 2J) at −20 °C but showed decreasing in RC (Fig. 2F) and RN (Fig. 2H). Importantly, significant differences between culturable and viable cell numbers were identified in RN at all time points, indicating the entry into the VBNC

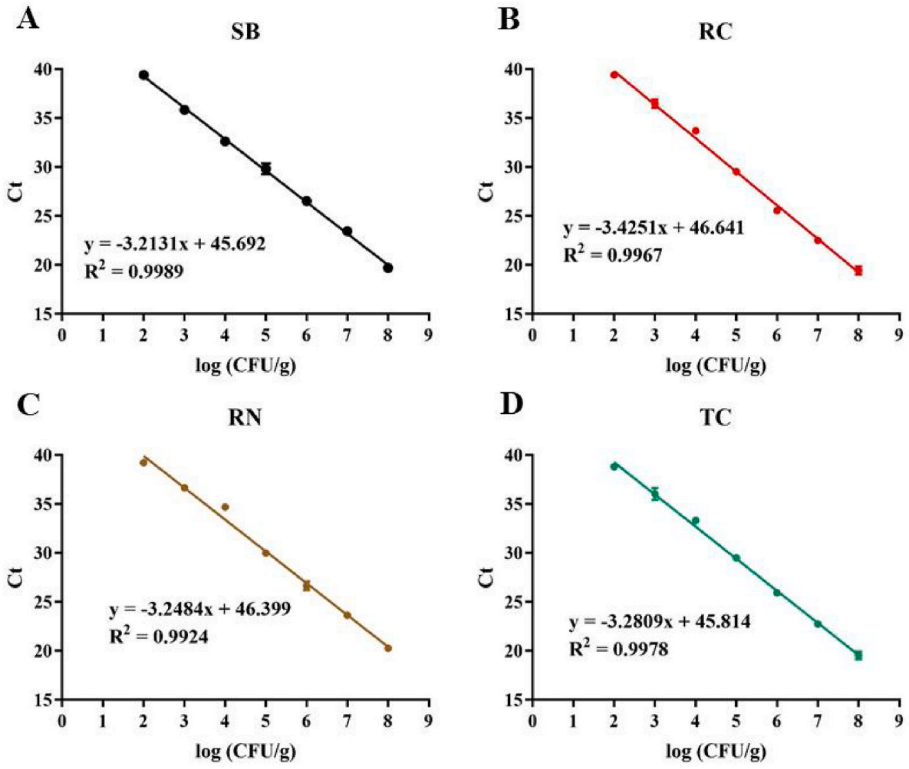

Fig. 1. The standard curve of PMA-qPCR for *P. acidilactici* in 4 food samples including steamed bun (A), rock crystal (B), rice noodle (C) and turnip cake (D).

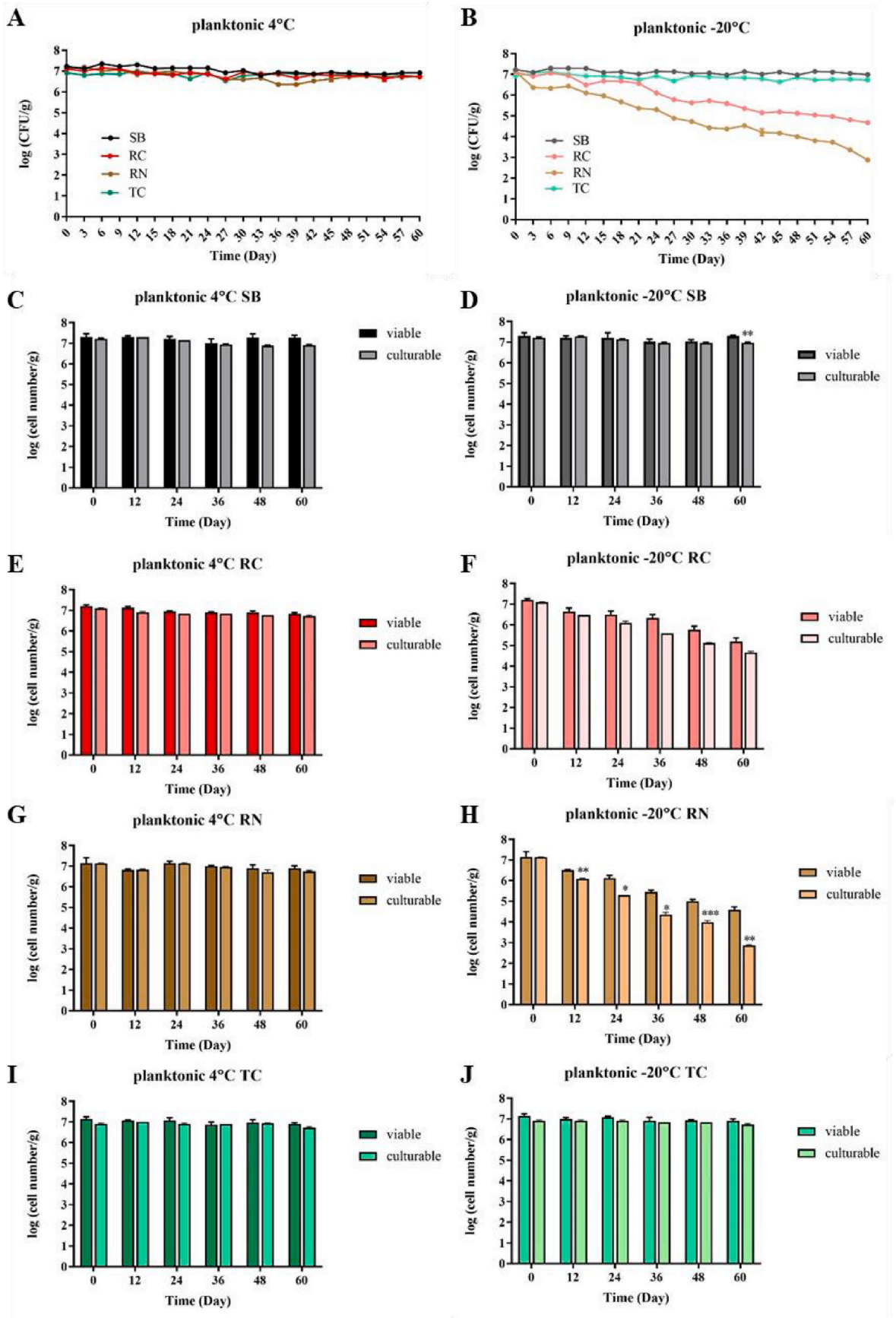

Fig. 2. CFU counting for culturable cell quantification (A, B) and PMA-qPCR for viable cells quantification (C–J) of *P. acidilactici* in planktonic state at 4 °C and −20 °C in steamed bun (C, D), rock crystal (E, F), rice noodle (G, H) and turnip cake (I, J). The culturable cell number was compared to the corresponding viable cell number at each time point. The marks *, **, ***, and **** at the top of the bars represent p-value <0.05, <0.01, <0.001, and <0.0001, respectively.

state of partial *P. acidilactici* cells (approximately 10^4 cell/g VBNC cells at Day 60) with the induction of −20 °C in RN (Fig. 2H). Temperature and nutrient availability are key factors influencing the induction of the VBNC state in bacteria. Extreme temperatures are well-documented triggers for the VBNC state. Bacteria exhibit heightened sensitivity to temperature fluctuations, which can significantly impact their viability and culturability (Noor, Zahidul Islam, Munshi, & Rahman, 2013). For example, *Vibrio vulnificus*, exposed to 5 °C in artificial seawater microcosms, became non-culturable after 24 days, though metabolic activity persisted up to 32 days (Linder & Oliver, 1989). Similarly, *Listeria monocytogenes* entered into the VBNC state after 42 days at 10 °C in a broth model (Gurresch, Gerner, Pin, Wagner, & Hein, 2016), and *E. coli* O157 transitioned to VBNC after 80 days at −20 °C in LB broth (Wei & Zhao, 2018). High temperatures also induce the VBNC state rapidly. *Legionella pneumophila* strains lost culturability at 55 °C, 60 °C, and 70 °C after 3–8 h, 60 min and <2 min respectively, with some cells surviving at these extremes, while *E. coli* O157 became non-culturable at 50 °C after 2 h (Fu et al., 2020). Low temperatures have been known as a common inducing factor for VBNC state (J. Liu, Huang, et al., 2023). However, the failure of 4 °C in all food types and −20 °C in SB, RC, and TC to induce the VBNC state of *P. acidilactici* suggested that food components also affect the VBNC state formation of foodborne bacteria. Nutrient availability differences in food components further complicated the VBNC state formation. Such observation had been proved in our previous study on the effect of environmental conditions on the formation of the VBNC state of *P. acidilactici* (Li et al., 2020) as well as plenty other studies. *Vibrio parahaemolyticus* entered into the VBNC state in approximately 12 days at 4 °C under nutrient-poor conditions (Mizunoe, Wai, Ishikawa, Takade, & Yoshida, 2000), and *Aeromonas hydrophila* entered into the VBNC state after 7 weeks in nutrient-depleted distilled water at 4 °C (Mary, Chihib, Charafeddine, Defives, & Hornez, 2002). Similarly, *Vibrio tasmaniensis* and *Vibrio shiloi* entered into the VBNC state after 157 days and 126 days, respectively, in artificial seawater at 4 °C (Vattakaven, Bond, Bradley, & Munn, 2006). Extended starvation resulted in these cells also shifting from rod-shaped to spherical forms, accompanied by extensive bubbling, production of polymeric substances, and increased membrane roughness (Vattakaven et al., 2006).

3.3. PMA-qPCR for viable cells quantification of P. acidilactici in early biofilm state

Early (8 h) biofilm cells are attached cells whose matrix has not been robustly formed. Early biofilm cells of *P. acidilactici* could also survive in the 4 rice noodle products within 60 days at both 4 °C (Fig. 3A) and −20 °C (Fig. 3B). The culturable cell numbers of *P. acidilactici* maintained stable and high (approximately 10^7 CFU/g) at 4 °C regardless of food type (Fig. 3A). The survival rate of *P. acidilactici* was maintained at high levels in SB and TC (10^7 CFU/g), relatively lower in RC (10^6 CFU/g), and decreased to a lower level (10^4 CFU/g) in RN (Fig. 3B). The different major components of the rice noodle products possibly contributed to the variation in survival rate of *P. acidilactici*. It is noteworthy that early biofilm state *P. acidilactici* is capable of surviving in rice noodle products within 60 days during low temperature storage.

PMA-qPCR was applied in the viable cell quantification of *P. acidilactici* early biofilm state at Day 0, 12, 24, 36, 48, and 60 (Fg.3C-3J). Similar to culturable cell number, the viable cell number of *P. acidilactici* remain unchanged (approximately 10^7 cell/g) at 4 °C in all rice noodle products (Fig. 3C, E, 3G, 3I). Consistent with the trend of survival rate of *P. acidilactici* in different food types, viable cell numbers are 10^6 to10^7 cell/g in SB (Fig. 3D), RC (Fig. 3F), and TC (Fig. 3J) at −20 °C but lower to 10^4 cell/g at Day 60 in RN (Fig. 3H). Significant differences between culturable and viable cell numbers were identified in RN at Day 48 and Day 60, indicating the entry into the VBNC state of partial *P. acidilactici* cells (approximately 10^5 cell/g) with the induction of −20 °C in RN for more than 48 days (Fig. 3H). The entry of biofilm cells into the VBNC state had been observed in bacteria including *S. aureus*, *Lactobacillus plantarum*, *L. monocytogenes*, *E. coli*, and *Pseudomonas aeruginosa* (Fu et al., 2021; Mangiaterra et al., 2020; Olszewska & Białobrzewski, 2019; Pasquaroli et al., 2014), but rarely in food systems and induced by low temperature. In this study, this is the first report of *P. acidilactici* biofilm cells entering into the VBNC state.

3.4. PMA-qPCR for viable cells quantification of P. acidilactici in mediate biofilm state

Mediate (24 h) biofilm cells are developing cells whose matrix has been partially formed. Mediate biofilm of *P. acidilactici* have stronger structure thus could survive at high cell number (10^6 to10^7 cell/g) in the 4 rice noodle products within 60 days at both 4 °C (Fig. 4A) and −20 °C (Fig. 4B). Less than 1 log culturable cell number change of mediate biofilm of *P. acidilactici* was observed in RN at −20 °C (Fig. 4B). Mediate biofilm state *P. acidilactici* has high capability of surviving in rice noodle products within 60 days during low temperature storage.

PMA-qPCR was applied in the viable cell quantification of *P. acidilactici* mediate biofilm state at Day 0, 12, 24, 36, 48, and 60 (Fg.4C-4J). Insignificant decrease in viable cell number of *P. acidilactici* mediate biofilm was identified in most time points in the 4 rice noodle products within 60 days at both −20 °C and 4 °C. Minor decease in viable cell number (less than 1 log cell/g) was recorded in few samples including Day 24 in SB at 4 °C, Day 12 and Day 48 in RN at 4 °C, Day 60 in RC at −20 °C, and Day 24 in RN at −20 °C. The results indicated that small amounts of mediate biofilm cells of *P. acidilactici* have entered into the VBNC state induced by lower temperature storage in SB, RN, and RC. Attention should be paid to the possibility of robust biofilm cells entering into the VBNC state in rice noodle products, potentially posing risk to false negative detection and difficulty in eliminating such cells.

3.5. PMA-qPCR for viable cells quantification of P. acidilactici in mature biofilm state

Mature (72 h) biofilm cells are well-organized cells whose matrix has been robustly formed. Acquiring robust matrix and structure, mature biofilm of *P. acidilactici* could survive at high cell number (around 10^7 cell/g) in the 4 rice noodle products within 60 days at both 4 °C (Fig. 5A) and −20 °C (Fig. 5B), except for that in RN at −20 °C (10^5 to 10^7 cell/g). PMA-qPCR was applied in the viable cell quantification of *P. acidilactici* mature biofilm state at Day 0, 12, 24, 36, 48, and 60 (Fg.5C-5J). The viable cell number of *P. acidilactici* mature biofilm was stable (10^7 cell/g) in most time points in the 4 rice noodle products within 60 days at both −20 °C and 4 °C. Although mature biofilm cells of *P. acidilactici* have the highest capability of surviving in rice noodle products within 60 days during low temperature storage, they are less likely to enter into the VBNC state. Different from planktonic cells and early and mediate biofilms, mature biofilms have developed robust biofilm matrix, which allows the dynamic biofilm mode of life (Flemming & Wingender, 2010). The matrix protects cells in the biofilm from desiccation, biocides, antibiotics, heavy metals, ultraviolet radiation, host immune defences and many protozoan grazers (Flemming & Wingender, 2010). Also, extracellular polymeric substances immobilize biofilm cells, keeping them in long-term close proximity and serve as a nutrient source. Thus, with the protection of robust biofilm matrix, mature biofilms form stable and dynamic community which block stresses which induce the formation VBNC state. Above all, the PMA-qPCR method with specific standard curves for 4 types of rice noodle products was applicable for viable cell quantification of *P. acidilactici* in planktonic and biofilm (early, mediate, and mature) states.

3.6. PMA-RT-qPCR on stress response gene expression in rice noodle products

Low temperatures and possible food environments (pH, nutrient, oxygen) pose stress to *P. acidilactici* strain survival. The stress response

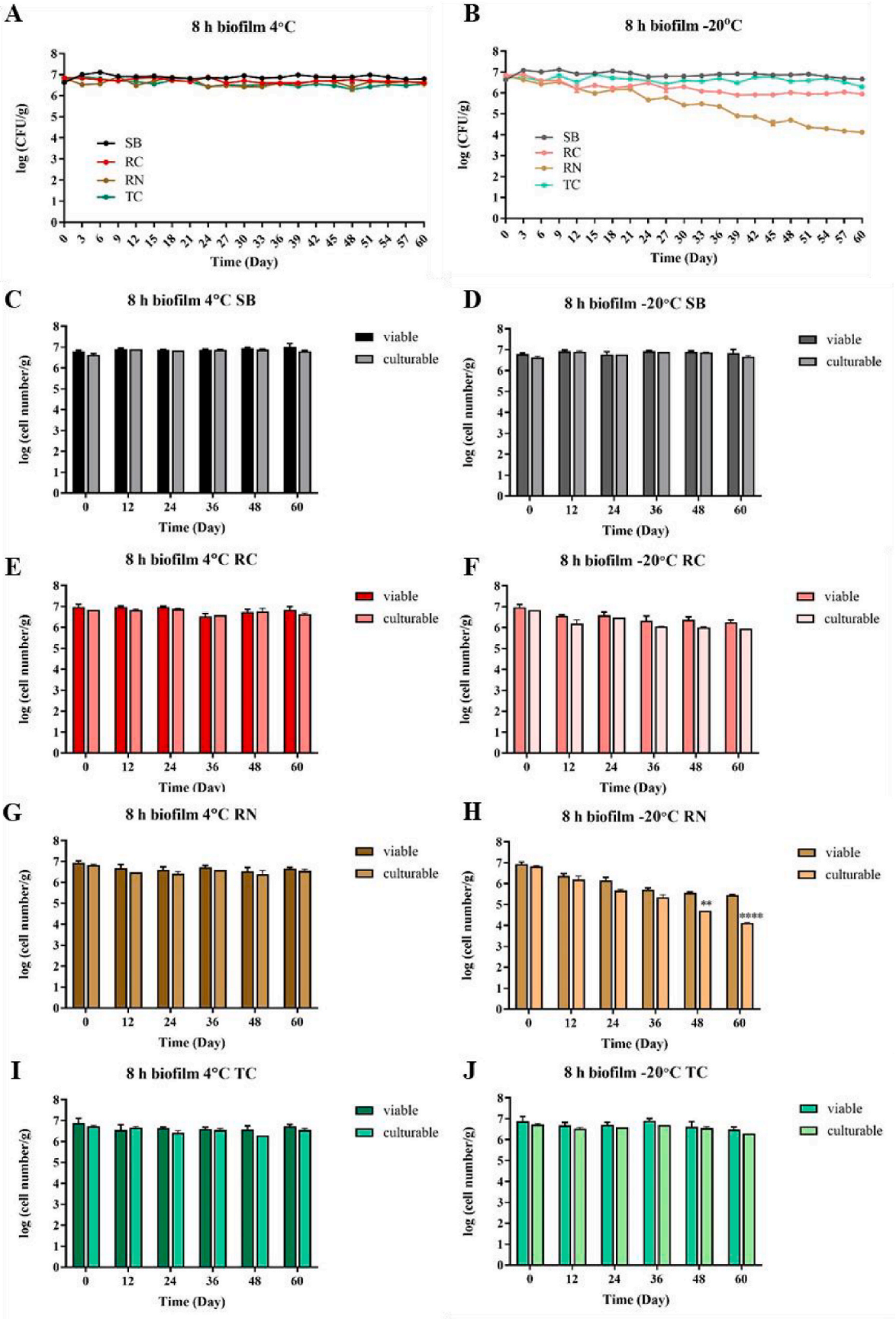

Fig. 3. CFU counting for culturable cell quantification (A, B) and PMA-qPCR for viable cells quantification (C–J) of *P. acidilactici* in early biofilm state at 4 °C (A) and −20 °C (B) in steamed bun (C, D), rock crystal (E, F), rice noodle (G, H) and turnip cake (I, J). The culturable cell number was compared to the corresponding viable cell number at each time point. The marks *, **, ***, and **** at the top of the bars represent p-value <0.05, <0.01, <0.001, and <0.0001, respectively.

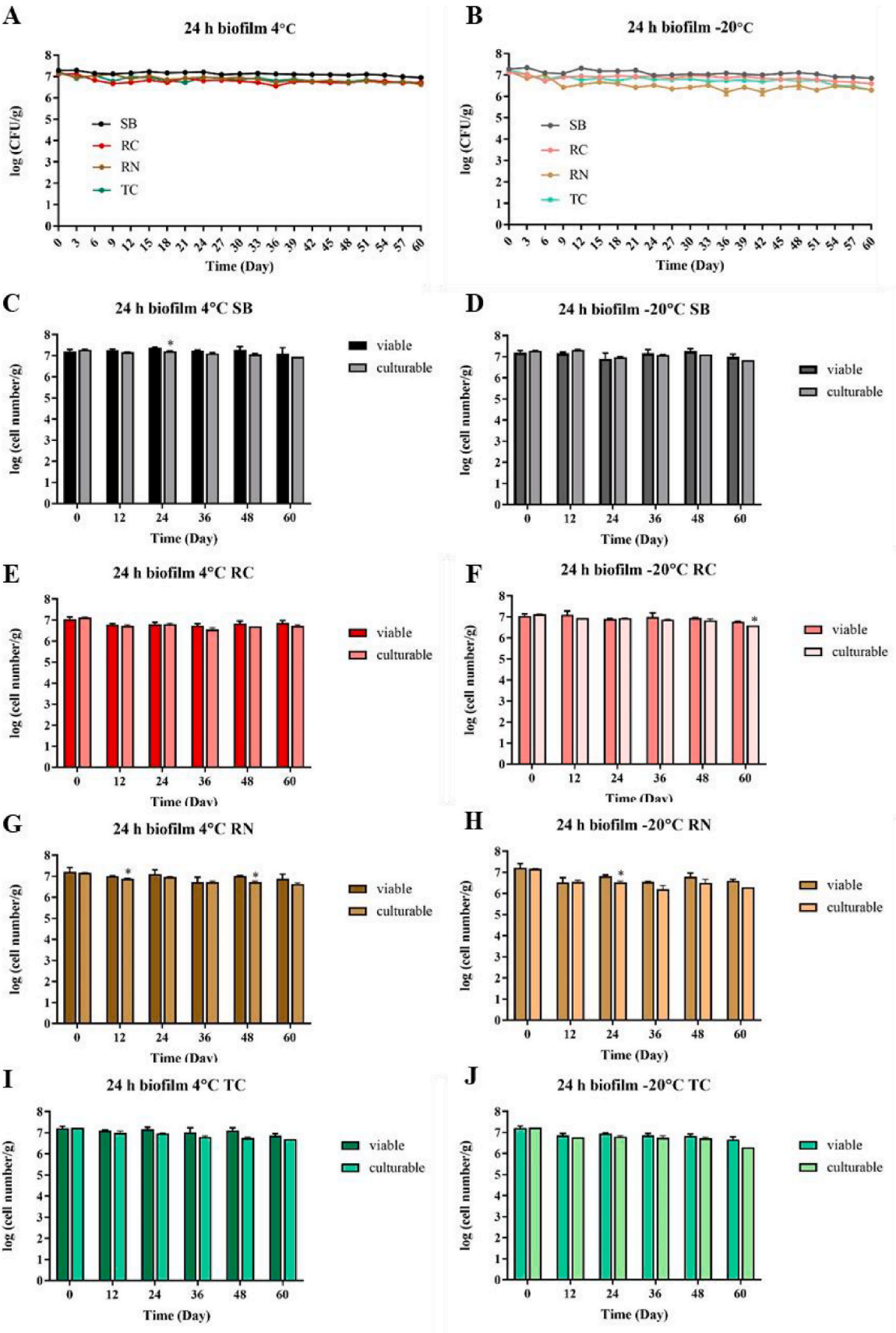

Fig. 4. CFU counting for culturable cell quantification (A, B) and PMA-qPCR for viable cells quantification (C–J) of *P. acidilactici* in mediate biofilm state at 4 °C (A) and −20 °C (B) in steamed bun (C, D), rock crystal (E, F), rice noodle (G, H) and turnip cake (I, J). The culturable cell number was compared to the corresponding viable cell number at each time point. The mark * at the top of the bars represents p-value <0.05.

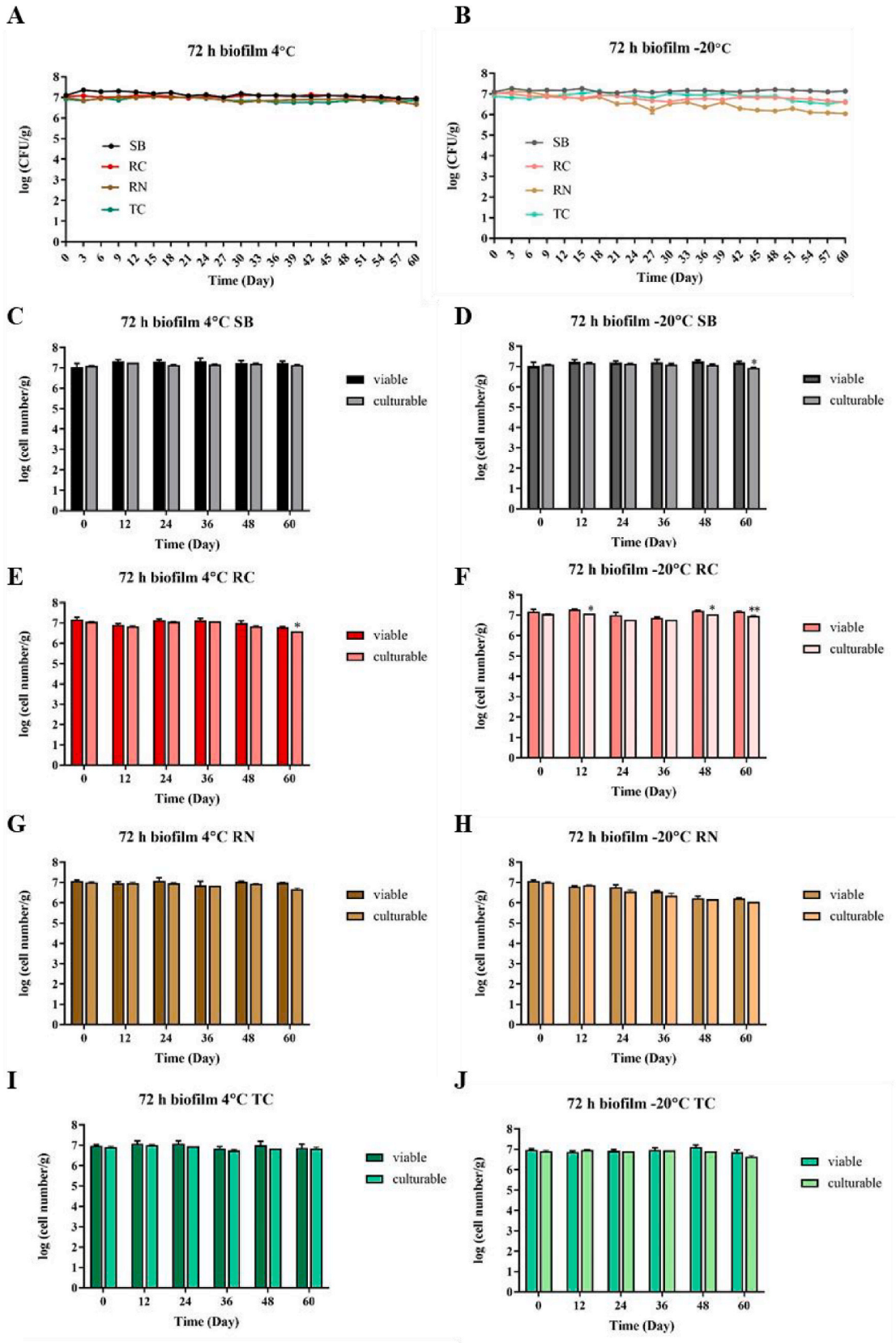

Fig. 5. CFU counting for culturable cell quantification (A, B) and PMA-qPCR for viable cells quantification (C–J) of *P. acidilactici* in mature biofilm state at 4 °C (A) and −20 °C (B) in steamed bun (C, D), rock crystal (E, F), rice noodle (G, H) and turnip cake (I, J). The culturable cell number was compared to the corresponding viable cell number at each time point. The marks * and ** at the top of the bars represent p-value <0.05 and < 0.01, respectively.

gene expression level of viable cells is an indicator of cell reaction to various conditions. In this study, 2 stress response genes *atpA* and *dnaK* were selected to examine the stress response strategies of *P. acidilactici* strain in 4 different types of food sample (SB, RC, MF, and TC) at 4 °C and −20 °C (Fig. 6). The multi-subunit F1F0-ATPase encoded by the *atpA* gene is a major class of pH-tolerant enzymes. Related studies have shown that the expression of *atpA* gene is affected by changes in ambient temperature (Wallenius, Uuksulainen, Salonen, Rautio, & Eerikäinen, 2011). The *dnaK* gene activates the expression of heat shock protein DnaK under external stress conditions, such as temperature, heavy metals, oxidants, acid treatment, to ensure cell survival (Aertsen et al., 2004; Cotter & Hill, 2003). A PMA-RT-qPCR assay was developed to accurately examine the gene expression level in viable cells and correspond to viable cell number (determined by PMA-qPCR) by removing residue DNA from dead cells using PMA upon RT-qPCR.

According to the results, expression of *atpA* gene expression remained relatively unchanged or up-regulated at Day 12 in most groups except for early biofilm in SB at 4 °C (Figs. 6C-1), mature biofilm in SB at −20 °C (Figs. 6H-1), and mature biofilm in TC at 4 °C (Figs. 6G-4), implying that *P. acidilactici* cells experienced and responded to pH and low temperature stress with the up-regulation of *atpA* gene until Day 12. On the contrary, significant down-regulation of *atpA* gene were identified in all groups since Day 24, suggesting the *P. acidilactici* cells had adapted to the pH in food environments and low temperatures from Day 24 to Day 60. Whole transcriptomic profiles of *V. parahaemolyticus* in three phases (exponential, early stationary phase and VBNC state) had shown *atpA* gene was significantly downregulated in the VBNC state compared with exponential and early stationary phase (Meng, Alter, Aho, & Huehn, 2015). After Day 24, more *P. acidilactici* cells showed reduced viability and entered into the VBNC state, which possibly connect with the downregulation of *atpA* gene.

More diversities were obtained in the expression of *dnaK* gene, possibly due to its responsiveness to various stress conditions. The only common point of *dnaK* gene expression in all groups is that it was down-regulated at Day 48 and Day 60. At Day 12, *dnaK* gene was unchanged or down-regulated in most groups, except for early (Figs. 6D-1) and mediate biofilm in SB at −20 °C (Figs. 6F-1), mature biofilm in SB at 4 °C, early biofilm in RC at 4 °C (Figs. 6C-2) and −20 °C (Figs. 6D-2), mediate biofilm in RC at −20 °C (Figs. 6F-2), mature biofilm in RC at 4 °C (Figs. 6G-2), early biofilm in RN at −20 °C (Figs. 6D-3), mature biofilm in RN at −20 °C (Figs. 6H-3), early biofilm in TC at −20 °C (Figs. 6D-4), mediate biofilm in TC at 4 °C (Figs. 6E-4) and −20 °C (Figs. 6F-4). The *dnaK* gene in 3 groups including mature biofilm in SB at 4 °C (Figs. 6G-1), mature biofilm in RC at 4 °C (Figs. 6G-2), and early biofilm in RN at −20 °C (Figs. 6D-3) showed continuous up-regulation at Day 12, Day 24, and Day 36. Besides, the *dnaK* gene was up-regulated at both Day 24 and Day 36 in 10 groups including mediate biofilm in SB at 4 °C (Figs. 6E-1), planktonic in RC at 4 °C (Figs. 6A-2) and −20 °C (Figs. 6B-2), mature biofilm in RC at −20 °C (Figs. 6H-2), planktonic in RN at 4 °C (Figs. 6A-3) and −20 °C (Figs. 6B-3), mediate (Figs. 6E-3) and mature biofilm in RN at 4 °C (Figs. 6G-3), planktonic in TC at −20 °C (Figs. 6B-4), and mature biofilm in RN at 4 °C (Figs. 6G-4). Specially, the *dnaK* gene was down-regulated in all time points in 3 groups including early biofilm in SB at 4 °C (Figs. 6C-1), mature biofilm in SB at −20 °C (Figs. 6H-1), and planktonic in TC at 4 °C (Figs. 6A-4). Heat shock protein-related gene *dnaK* had been reported highly overexpressed in the VBNC cells of *Salmonella Montevideo* during storage in red pepper powder at 30 °C with 40% relative humidity (Lee, Kim, & Kang, 2022). However, in this study, *dnaK* gene was upregulated at early to mid-term time points (ranging from Day 12 to Day 36) when viable cell numbers started to decrease, but downregulated at later time points (ranging from Day 48 to Day 60) when viable cell numbers reached the lowest point and VBNC state formed. The connection indicated the upregulation of *dnaK* gene possibly only happened at the start point of the VBNC state formation. The later time points of VBNC state formation could be related with other co-worker genes of *dnaK*, considering DnaK is an ATP-dependent chaperone that works together with the co-chaperone DnaJ and the nucleotide exchange factor GrpE (Shabayek & Spellerberg, 2017).

Overall, PMA-RT-qPCR assay was efficient in determining stress response gene expression of *P. acidilactici* cells in rice noodle products. The *atpA* gene is possibly more responsive for the pH and low temperature stress at early time point (Day 12), while the *dnaK* gene is responsive to more stresses in complicated food systems.

3.7. Comparison from CFU to viable cells to gene expression

Examining the cell status in different rice noodles products aids in the understanding of food spoilage caused by *P. acidilactici* and guides downstream control strategy development. CFU counting is the conventional method to determine cell number, which is more accurately culturable cell number. CFU counting had been mistakenly used to determine viable cell number and most International Organization for Standardization (ISO) standards for enumeration of safety indicators and detection of microbes are based on CFU counting (Ferone, Gowen, Fanning, & Scannell, 2020). Upon the understanding on cell status including VBNC state, we have the awareness that CFU counting is specifically for determining culturable cell number. Concerning viable cell number, direct viable count (DVC) was the firstly used through the application of nalidixic acid which inhibits DNA synthesis and causes cell elongation and expand (Dong et al., 2020). However, DVC cannot be applied in all microbial species as their response to nalidixic acid differs and cell elongation and expand does not always occur during nutrient uptake. In addition, rough observation on cell size and estimate cell numbers are used as result determination, thus it cannot be used as species specific determination method (Dong et al., 2020). Cell membrane integrity based fluorescent dyes with LIVE/DEAD BacLight Bacterial Viability kit as representative have also been widely used to determine viable cell number in combination with fluorescent microscopy or flow cytometry (Kumar & Ghosh, 2019; Stiefel, Schmidt-Emrich, Maniura-Weber, & Ren, 2015). However, one biggest disadvantage of fluorescent dyes is lacking specificity. In this study, PMA-qPCR with specific standard curves corresponding to each rice noodles products was developed to determine viable cell number of *P. acidilactici* with growth states including planktonic and biofilms (early, mediate, mature). Upon PMA treatment prior to DNA extraction, viable cells are easily identified as species level and relatively quantified by qPCR with standard curves (Kibbee & Örmeci, 2017). Our results have tested the efficiency of PMA-qPCR in determine viable cell numbers of *P. acidilactici* in 4 growth states, 4 rice noodles products, and 2 low temperature conditions. In addition, a PMA-RT-qPCR assay was developed on determining the stress response gene expression in rice noodle products. Although with stable culturable and viable cell numbers in different growth states, rice noodles products, and low temperature conditions, *P. acidilactici* acquired significantly different gene expression levels.

4. Conclusion

In this study, PMA based methodologies on viable cells quantification of *P. acidilactici* were developed, evaluated and applied in rice noodles products. Firstly, considering the influence of food component and PMA on qPCR result determination, standard curves corresponding Ct value to *P. acidilactici* cell numbers were separately established according to 4 specific rice noodle products, obtaining the linear regression coefficients >0.99 and the amplification efficiency at 92%–104%, demonstrating high quality in reliability and repeatability. Then, the established PMA-qPCR methods were further applied for viable cell quantification of *P. acidilactici* in planktonic and biofilm (early, mediate, and mature) states in the 4 corresponding types of rice noodle products. As found, planktonic and biofilm (early, mediate, mature) cells of *P. acidilactici* are capable of surviving in rice noodle products within 60

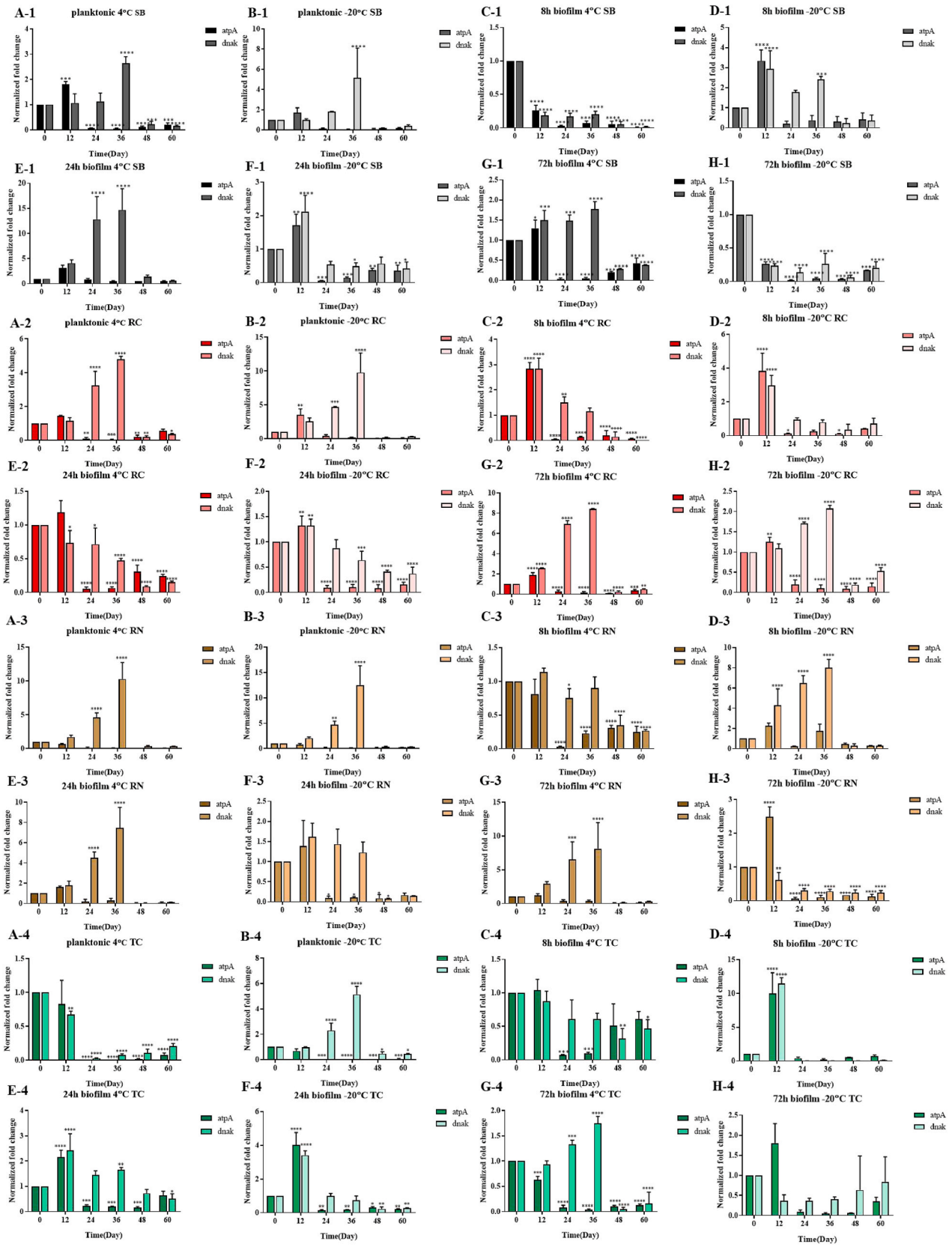

Fig. 6. PMA-RT-qPCR on *atpA* and *dnaK* gene expression of *P. acidilactici* in food samples in steamed bun (A-1 to H-1), rock crystal (A-2 to H-2), rice noodle (A-3 to H-3) and turnip cake (A-4 to H-4) at 4 °C and −20 °C. The normalized fold change of *atpA* and *dnaK* genes at 12 h, 24 h, 36 h, 48 h, and 60 h were each compared to the corresponding value at 0 h. The marks *, **, ***, and **** at the top of the bars represent p-value <0.05, <0.01, <0.001, and <0.0001, respectively.

days during low temperature storage. Similar to culturable cell number, the viable cell number of *P. acidilactici* maintained stable and high (approximately 10^7 cell/g) at 4 °C regardless of food type and cell status (planktonic and biofilm). Significant differences between culturable and viable cell numbers were identified in certain rice noodle products (mostly RN) at later time points (Day 48 and 60), indicating the entry into the VBNC state of partial *P. acidilactici* cells. Mature biofilms, followed by mediate and early biofilms were less likely to enter into the VBNC state compared to planktonic cells. In addition, stress response gene expression in *P. acidilactici* was monitored by PMA-RT-qPCR and compared with culturable and viable cell number quantification. The planktonic and biofilm cells of *P. acidilactici* maintained high and stable in both culturable and viable cell number in different growth states, rice noodles products, and low temperature conditions, but acquired significantly different gene expression levels. The *atpA* gene is likely to be responsive for the adaptation to pH changes at early time point (Day 12), while the *dnaK* gene is responsive to various stresses in complicated food systems.

As concluded, in this study, we have developed viable cells quantification methods for *P. acidilactici* and validated their applicability in different rice noodle products. Then based on these methods, we have further revealed the growth and proliferation of *P. acidilactici* during storage of rice noodle products, including changes in culturable, VBNC and total viable cells, as well as expression of key genes for stress response. The findings may provide essentially important information and guidance in future control and prevention of food spoilage bacteria *P. acidilactici* contamination in quick-frozen rice noodles products.

CRediT authorship contribution statement

Zhenbo Xu: Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Feifeng Zhong:** Writing – original draft, Investigation, Formal analysis, Data curation. **Aijuan Xu:** Methodology. **Yuting Luo:** Investigation. **Thanapop Soteyome:** Writing – review & editing, Resources. **Gamini Seneviratne:** Writing – review & editing, Supervision. **Lei Yuan:** Validation. **Junyan Liu:** Writing – original draft, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgement

This work was supported by the National Natural Science Foundation of China [grant numbers, 32202199], Guangdong Major Project of Basic and Applied Basic Research [grant numbers, 2020B0301030005], Guangdong Basic and Applied Basic Research Foundation Natural Science Foundation [grant numbers, 2023A1515012332], Guangzhou Basic and Applied Basic Research Project [grant numbers, 2023A04J1446], Guangdong Province Higher Education Featured Innovation Project [grant numbers, 2023KTSCX053], the Open Research Fund of State Key Laboratory of Biological Fermentation Engineering of Beer [grant numbers, K202105], Guangzhou Science and Technology Commissioner Research grant [grant numbers, 2024E04J1244], Jiangmen Science and Technology Commissioner Research grant [grant numbers, 2023760100130007729], and the 111 Project [grant numbers, B17018].

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