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The survival and enterotoxin gene expression of *Staphylococcus aureus* planktonic and biofilm cells in quick-frozen food

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ABSTRACT

Food safety issues caused by food-borne pathogenic microorganisms during low-temperature food storage have attracted broad attention. In the current study, *Staphylococcus aureus* cells in planktonic and different stages of biofilm formation were inoculated into four types of quick-frozen rice and flour products. The changes in culturable cell number of *S. aureus* in the artificially contaminated food samples stored at 4 °C and −20 °C were recorded for 60 days. Next, the PMA-qPCR method was used to explore the viable cell number and determine whether low temperature induced *S. aureus* enter into the viable but non-culturable (VBNC) state. Finally, the expression changes of enterotoxin gene *seb* in *S. aureus* were determined by RT-qPCR during food sample storage. Results showed that the culturable cell number of *S. aureus* was not significantly changed during 60 days except for the decreased culturable cell number in the 8 h biofilm cells in MF stored at −20 °C. In addition, *S. aureus* cells did not enter into the VBNC state during low temperature storage. Moreover, the *seb* gene was continuously expressed in most samples although with difference in expression level. The findings provided an alert for the risk of *S. aureus* contamination in quick-frozen rice and flour products.

1. Introduction

Due to their convenience and time-saving, quick-frozen rice and flour products have become popular for consumers in daily life (Ji et al., 2007; Park et al., 2020; Potluri et al., 2018). However, at the end of 2011, major brands in China's frozen food industry, including Sanquan, Sinian and Wanchai Ferry, were successively announced that their quick-frozen rice and flour products (for example dumplings and wonton) were contaminated with *Staphylococcus aureus*, significantly influencing quick-frozen food industry in China. According to the data monitored by the Jilin Provincial Center for Disease Control and Prevention in 2016–2019 in China, 8 in 13 types of food were detected with *S. aureus*, and the detection rate of *S. aureus* in quick-frozen rice and flour products was as high as 9% (Wang et al., 2020). In China, 12.5% of ready-to-eat foods collected from 24 cities in 2011–2014 were tested positive for *S. aureus* (Yang et al., 2016). According to European Food Safety Authority (European Food Safety. et al.) sampling of food samples from five countries in 2017, 40 of the 645 samples tested were positive

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Table 1
Design of the RT-qPCR primers sequences.

Name	Sequence	Length (bp)
femA-F	CTGAGCGATAACAGCATT	18
femA-R	TGCGTTACCCAGAAATAC	18
16s rRNA-S.a-F	GCCGTAAACGATGAGTGCTAA	21
16s rRNA-S.a-R	CGAATTAAACCACATGCTCCA	21
seb-F	ATTACTGTTCGGGTATTTG	19
seb-R	TTCATAAGGCGAGTTGTT	18

for staphylococcal enterotoxins (European Food Safety A. European Food Safety A. & Ctr Dis Prevention Control E, 2018). In the United States, approximately 0.24 million of food poisoning cases caused by Staphylococcus occur each year (Scallan et al., 2011). Although a report from the United States Centers for Disease Control and Prevention indicated that *S. aureus* has fallen from the number one common pathogen causing foodborne illness to the fifth most known pathogen, *S. aureus* is still considered as one of the most common foodborne illnesses and poses a major threat to public health (Centers for Disease Control and Prevention, 2011).

One of the major features contributing to the high detection rate of *S. aureus* in quick-frozen food is its persistence at low temperature. It has been reported *S. aureus* is capable of surviving at 10 °C in sliced cooked chicken breast for at least 480 h (Rodriguez-Caturla et al., 2012). At lower temperature (4 °C), studies have shown decreased and varied survival rate for different *S. aureus* strains but certain amount of cells remain viable within 4 weeks (Suo et al., 2022). The survival of *S. aureus* at different temperatures (4 °C, −3 °C, −11 °C, −18 °C) has also been determined (Suo et al., 2014). Approximately 10^3~10^5 cells/mL were able to survive for 100 days. Undoubtedly, the highest survival rate was observed at 4 °C, followed by −3 °C, −11 °C, and −18 °C. At the temperature lower to −20 °C which is the most common for frozen food storage, *S. aureus* could still survive (Saklani et al., 2020). The survival of *S. aureus* has been determined to depend on inoculum level. *S. aureus* inoculated at 10^3 CFU/g in seafood at −20 °C remained stable after 60 days of storage (Saklani et al., 2020). Thus, *S. aureus* has strong survival rate at low temperature in different types of food.

S. aureus isolates carrying enterotoxin genes have been frequently isolated from food samples (Haghi et al., 2021). In a *S. aureus* prevalence investigation in China, 60% of quick-frozen dumplings were positive for *S. aureus* and 10.3%–38.5% of strains carried enterotoxin genes (*sec*, *seg*, *sej*, *see*, *sea*, and *seb*) (Hao et al., 2015). Enterotoxins are considered to be responsible for staphylococcal food poisoning (Angeles Argudin et al., 2010). Amongest, SEA and SEB are the most common toxins related to food poisoning (Angeles Argudin et al., 2010). SEB in more efficient at traversing the epithelial barrier than SEA (Pinchuk et al., 2010) and is stable to heat, proteolytic digestion, a wide pH range (Pinchuk et al., 2010), thus easy to produce and distribute. In addition, a very small amount (0.004 μg/kg) is effective at inducing symptoms and a dose of 0.02 μg/kg could be lethal (Pinchuk et al., 2010). Studies have shown the expression of enterotoxin genes or the production of enterotoxins differ in different *S. aureus* isolates and environments (including temperature) (Abolghait et al., 2020; Almutawif et al., 2019; Kataoka et al., 2016; Lopes et al., 2021; Wu & Su, 2014). Decreased *seb* gene expression or enterotoxin production have been frequently determined at low temperatures (8 °C, 12 °C, −20 °C, 4 °C) (Abolghait et al., 2020; Almutawif et al., 2019; Kataoka et al., 2016; Lopes et al., 2021; Wu & Su, 2014). However, enterotoxin production could still be observed after −20 °C storage for 4 weeks (Kataoka et al., 2016; Wu & Su, 2014), indicating the high risk posed by *S. aureus* contamination in frozen food.

The capability of *S. aureus* to enter into the VBNC state at low temperature exacerbate the risk (Yan et al., 2021). Studies have confirmed that a variety of unfavorable environmental factors can induce the entry of bacteria into the viable but non-culturable (VBNC) state, including low temperature, oligotrophy, high salt, low/high pH, and UV irradiation (Cunningham et al., 2009; Foster, 1999; Guo et al., 2019; Ramaiah et al., 2002). Among them, low temperature is the most common way to induce VBNC state formation (Li et al., 2020). Bacteria in the VBNC state cannot form colony in conventional agar media, resulting in false negative detection by conventional culturing based methods, but they maintain integrity of cell membrane structure (Lahtinen et al., 2008). Meanwhile, VBNC cells remain certain level of metabolic activity to ensure virulence gene expression and metabolites transportation (Ou et al., 2021). Therefore, as invisible contaminant, VBNC state bacteria poses a huge threat to food safety (Xu et al., 2020). The entry of *S. aureus* into the VBNC state was not found until 2010 (Masmoudi et al., 2010). The traditional process of verifying the existence of VBNC bacteria is to determine the viable cell number after the cells are nonculturable (CFU = 0). In fact, VBNC state is often formed earlier than the time point when CFU is 0. In addition, although stresses in food systems and their processing and storage environments have been proved to capable of inducing VBNC state, bacterial VBNC state formation in real food system needs further exploration.

Moreover, *S. aureus* has a strong biofilm forming ability, and its biofilm formed on pipes and equipments due to improper situation process is the main cause of contamination in the processing of meat, egg and dairy products, seafood and other food types (Bevilacqua et al., 2017; Chmielewski & Frank, 2003). The biofilm formation of *S. aureus* has been reported to enhance after cold stress (4 °C, −20 °C) even for long term (4–24 weeks) storage (Qiao et al., 2020; Qiao et al., 2021). However, the majority of VBNC studies use planktonic cells as initial inoculation, overlooking the actual status (biofilm) in which bacteria survive in food processing and storage.

The focus of the current study was on the survival (culturability and viability) of *S. aureus* in four types of quick-frozen rice and flour products under refrigeration (4 °C) and freezing (−20 °C) conditions, and to explore whether *S. aureus* could enter into the VBNC state in such conditions. Also, *S. aureus* enterotoxin gene *seb* expression level change during low temperature storage were monitored.

2. Materials and methods

2.1. Study design

Stored at low temperature (4 °C as fridge storage and −20 °C as freezer storage), 4 types of rice and flour products, including steamed bread (MT), crystal cake (SJB), rice flour (MF) and carrot cake (LBG), were selected as food substrates to explore the survival of *S. aureus*. Firstly, simulating the microbial cell status on real food surface, *S. aureus* cells in planktonic state, as well as early biofilm (8 h) and mature biofilm (24 h and 72 h) states, prepared in a 6-well plate culture model, were used to artificially contaminate the 4 types of food. Secondly, artificially contaminated food substrates were stored at low temperature for 60 days, with culturable and viable cell numbers determined during the whole process. For the real-time quantitative detection of viable cell number, the propidium monoazide (PMA)-quantative polymerase chain reaction (qPCR) method was used to establish a standard curve. The Ct value from PMA-qPCR was correspond to cell number base on the standard curve. Thirdly, the enterotoxin gene *seb* expression changes were monitored.

2.2. Microbial strains and culturing conditions

The *S. aureus* strain 22822 was isolated from First Affiliated Hospital of Guangzhou Medical University and stored in 30% glycerol at −80 °C. In order to obtain fresh cell culture, the *S. aureus* strain was streaked out from glycerol stock onto tryptic soy agar (TSA) plate and grown at 37 °C for 24 h. Then, a single colony on TSA agar was inoculated into 2 mL tryptic soy broth (TSB) and grown overnight at 37 °C with shaking at 200 rpm.

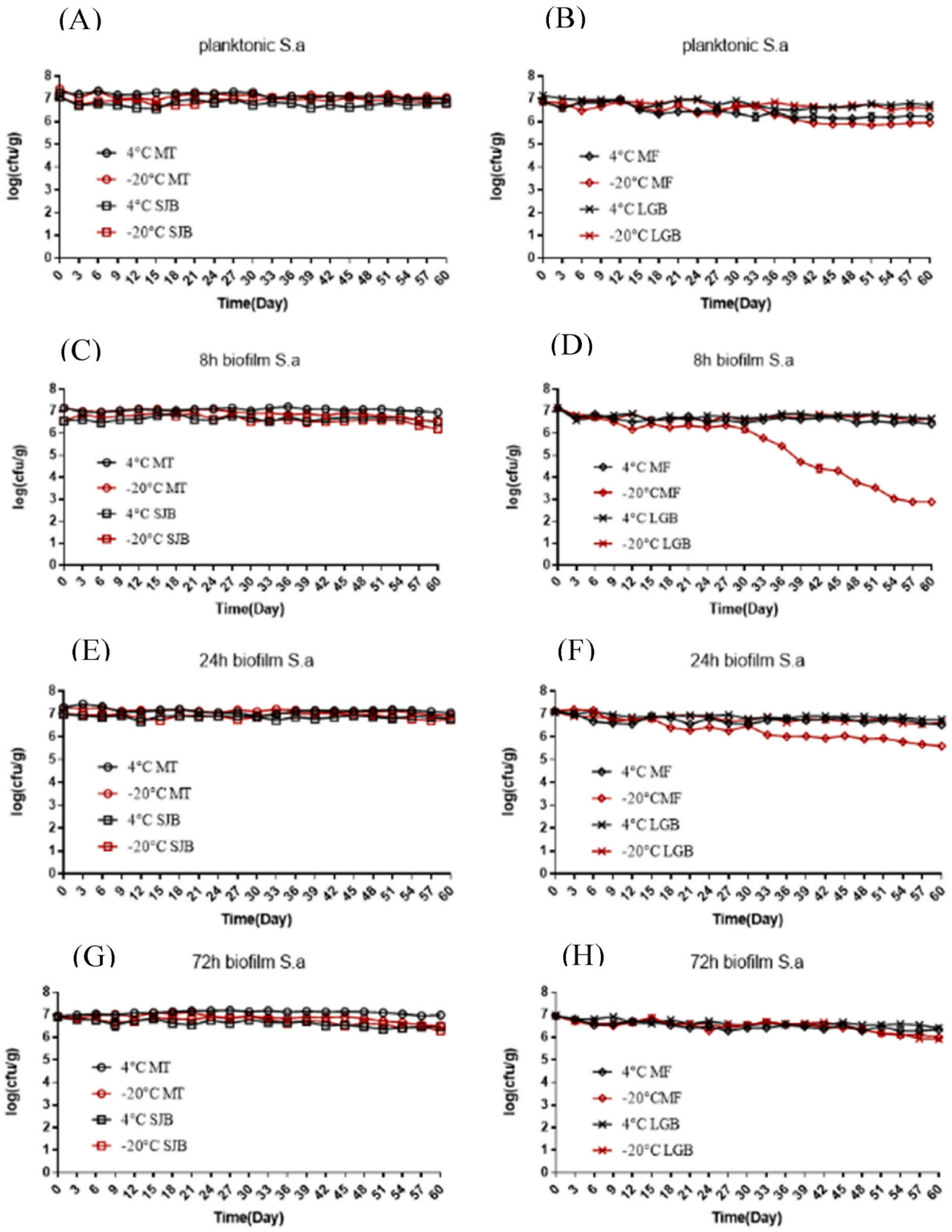

Fig. 1. The culturable cell number of *S. aureus* in different types of rice and flour food products including MT (A, C, E, G), SJB (A, C, E, G), MF (B, D, F, H) and LBG (B, D, F, H) at 4 °C and −20 °C in planktonic state (A, B) and in 8 h (C, D), 24 h (E, F), and 72 h (G, H) biofilm state.

2.3. S. aureus sample preparation and artificial contaminaton

For the bacteria in the planktonic state, log phase bacterial culture was centrifuged for 5 min at 5000 rpm to get rid of supernatant, and the obtained planktonic cells (~10^7 CFU/g) were inoculated into 25 g of food samples.

For bacteria in the biofilm state, overnight cultured bacterial solution was diluted to 10^6 CFU/mL in a sterile 6-well plate, and cultivated for 8 h, 24 h and 72 h, respectively. Then the biofilm cells were washed with saline three times to remove planktonic cells on the biofilm surface. Approximately, 10^7 CFU/g of biofilm cells were inoculated on 25 g of food samples.

The food samples artificially contaminated with planktonic and biofilm cells, respectively, were stored at low temperature (4 °C and

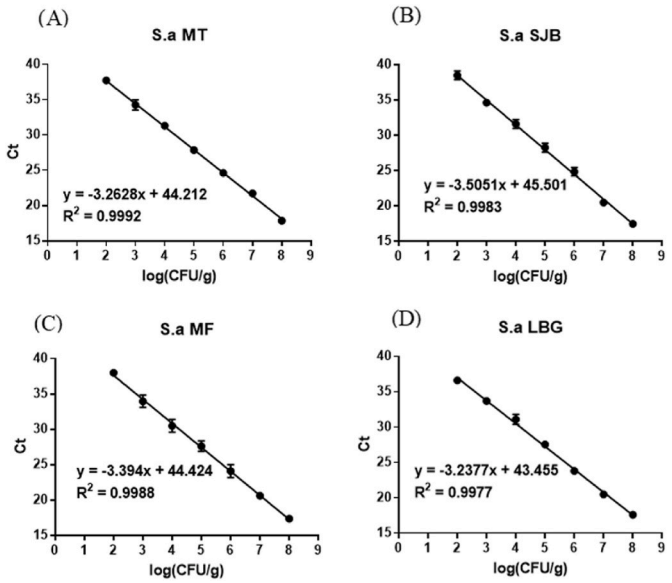

Fig. 2. The standard curve of *S. aureus* cell number and Ct value correlation in MT (A), SJB (B), MF (C) and LBG (D) for PMA-qPCR experiment.

−20 °C) for 60 days to mimic food storage conditions. At certain time points, 25 g of food samples were placed in 225 mL of sterile saline and homogenized at 9000 rpm for 1 min before CFU counting and further treatment.

2.4. PMA-qPCR

The samples from the food matrix were diluted to 10~10^8 CFU/g bacterial liquid (10-fold serial dilution), which was then reacted with PMA. 500 μL of the sample was added into a centrifuge tube, and 10 μL of PMA working solution was added to a final concentration of 10 μg/mL (Liu et al., 2017). The sample was incubated in dark for 5 min at room temperature, followed by incubation for 15 min under a 500 W halogen lamp at a distance of 15 cm, so that the PMA reagent and DNA are fully cross-linked. The treated sample was centrifuged at 5000 rpm for 10 min for bacterial genomic DNA extraction using a bacterial rapid DNA extraction kit (Dongsheng Biotech Co., Ltd, Guangzhou, China).

femA was selected as the determination gene of *S. aureus* to monitor viable cell number in qPCR. Primers (Table 1) were designed using NCBI primer blast combined with Primer Premier 5 and synthesized by Guangzhou Ige Biotechnology Co., Ltd. The DNA samples from PMA treat cells were adapted to qPCR assay. The qPCR reaction system included 10 μL of qPCR Mastermix (2 ×), 0.4 μL of upstream and downstream primers (F/R), respectively, 2 μL of DNA template (10-fold diluted), and DEPC-treated water to make up the volume to 20 μL. The qPCR program was set as pre-denaturation for 2 min at 95 °C, denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C, with 45 cycles. Three parallel controls were set for each experimental group to ensure the accuracy of the experiment.

2.5. Culturable and viable cell number curves establishment

The culturable bacterial cell number of was obtained every 3 days within 60 days storage period by CFU counting. After the homogenized food sample was shaken evenly, 20 μL was taken into 180 μL sterile saline for multiple gradient dilutions, and then 10 μL of each gradient was removed from the drop plate. The single colonies were counted after culturing at 37 °C for 12–18 h. Three parallel experiments were performed for each gradient. For the experimental group whose culturable number was reduced to 0, 1 mL of food homogenate was used for plate counting.

The standard curve for PMA-qPCR was used to quantify viable bacterial cells. Firstly, the amplified Ct value of each sample at different concentrations in 4 types of food samples was obtained by RT-qPCR. Secondly, 4 standard curves were established with Ct value as Y-axis and cell concentrations as X-axis for 4 types of food samples, respectively. Lastly, the corresponding standard curve was applied to obtain the number of viable bacterial cells. Viable cell number was determined at day 0, 12, 24, 36, 48 and 60 during storage. The VBNC bacterial cell number of is calculated by the viable bacterial cell number subtracting the number of culturable bacteria.

2.6. RT-PCR assay

The RNA from each sample were extracted using total RNA isolation reagent following the instruction. RNA concentration and quality were examined by Nanodrop 2000. According to the concentration of the total RNA extracted, its final amount in the reaction system was adjusted to be 0.1–2 μg. 4 μL of 5 × RT Master Mix, 1 μL of Oligo dT (20 ×) and Random Primer, were included in the reaction system, which was made up to 20 μL with RNase free H_2O. The reverse transcription reaction program was set as follows: 10 min at 25 °C, 45 min at 55 °C, followed by 5 min at 85 °C, and the reverse transcription reaction was terminated after cooling the cDNA at 4 °C, and the cDNA sample was stored at −20 °C until further qPCR experiments. The housekeeping gene *16S rRNA* was selected as an internal reference gene and *seb* as a target gene (primer sequences listed in Table 1). The RT-qPCR reaction was set up as the same as described above. Express levels of *seb* at day 12, 24, 36, 48 were compared to those at day 0 by using the ΔΔCt method.

3. Results

3.1. Survival of S. aureus during low temperature storage

Exploring the survival of food-borne microorganisms in food matrix is of great significance for predicting food safety and quality. In this study, the culturable cell numbers of planktonic and biofilm *S. aureus* in MT, SJB, MF and LBG during storage at 4 °C and −20 °C were determined (Fig. 1). For planktonic cells (Fig. 1A and B), *S. aureus* could maintain stable culturable cell number in the four rice and flour products at 4 °C and −20 °C. For biofilm cells (Fig. 1C–H), *S. aureus* in the rice and flour products could maintain a stable culturable cell number at 4 °C. But in −20 °C storage environment, the culturable cell number of *S. aureus* 8 h biofilm in MF decreased significantly from day 30 to day 60. And the culturable cell number of 24 h biofilm cells of *S. aureus* in MF were slightly lower than that of other groups from the 15th day, while the culturable cell numbers of 8 h and 24 h biofilm of *S. aureus* in SJB, LBG and MT remained basically unchanged during storage for 60 days. Besides, the culturable cell number of 72 h biofilm of *S. aureus* also basically maintained stable in four rice and flour products.

3.2. Validation of VBNC cells by PMA-qPCR

After PMA treatment of *S. aureus* with different final concentrations (10~10^8 CFU/g) in four types of food samples, DNA was extracted for qPCR experiment. According to the bacterial concentration in the sample and the Ct value obtained by PMA-qPCR, four standard curves were established for four types of rice and flour products, respectively (Fig. 2). The results showed that the linear regression coefficients of the four standard curves were all greater than 0.99, and the amplification efficiency ranged from 92% to 104%, indicating that the four standard curves had good repeatability and reliability, and could be used for viable cell number quantification.

To explore whether *S. aureus* cells enter into the VBNC state in the storage of low temperature conditions, the PMA-qPCR technique was used to determine the viable cell number within 60 days. The VBNC cell number was calculated by the viable cell number subtracting culturable

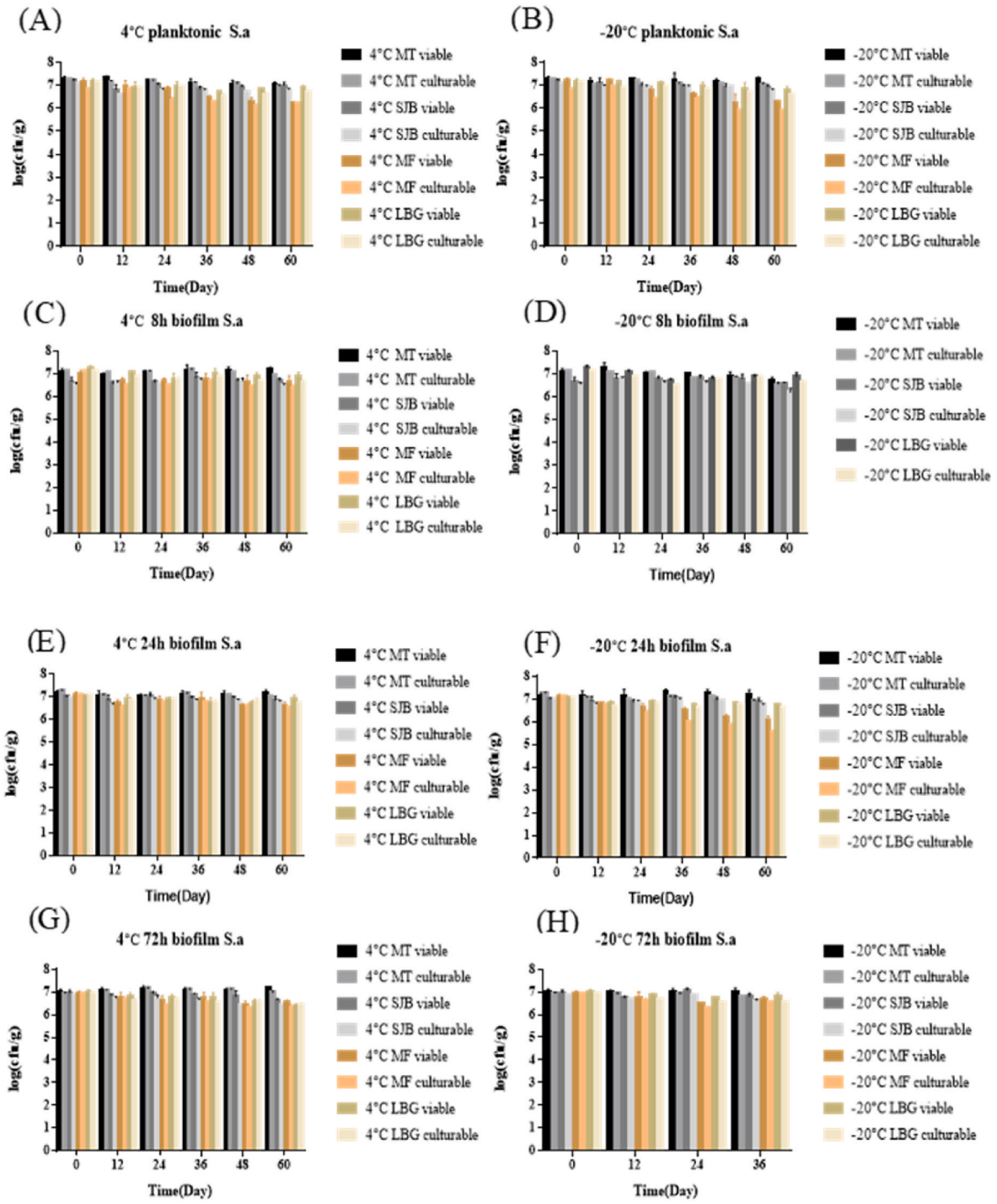

Fig. 3. The viable and culturable cell numbers of *S. aureus* in different types of rice and flour food including MT, SJB, MF and LBG at 4 °C (A, C, E, G) and −20 °C (B, D, F, H) in planktonic state (A, B) and in 8 h (C, D), 24 h (E, F), and 72 h (G, H) biofilm state.

cell number. For the sample group in which the culturable cell number did not change significantly, the difference between the viable cell amount and the culturable number of *S. aureus* was much less than one (Fig. 3). Therefore, the entry of *S. aureus* cells into the VBNC state was not recognized in this process.

3.3. Changes in the expression level of S. aureus enterotoxin gene seb

With *16s rRNA* as reference gene and *S. aureus* cultures at day 0 as control, the expression levels of enterotoxin gene *seb* in flour types of food samples artificially contaminated with planktonic and 8 h, 24 h, 72 h biofilm cells of *S. aureus* were monitored (Fig. 4). For planktonic cells (Fig. 4A), the expression levels of *seb* were mostly increased at day 12, 24, 36, and 48, except for those in MT at 4 °C at day 24 and 36. Similar results were shown in 8 h biofilm cells, the expression levels of *seb* were increased at all time points with expression in MT at 4 °C at day 24, 36, and 48 (Fig. 4B). More variation appeared in 24 h and 72 h biofilm cells among different food samples and storage temperatures (Fig. 4C–D). Decreased expression was observed in MT at 4 °C and −20 °C for either 24 h or 72 h biofilm cells at all time points. Decreased expression was also determined in groups including 24 h biofilm cells in LBG at −20 °C, 72 h biofilm cells in SJB and MF at −20 °C. Noteworthy, no *seb*

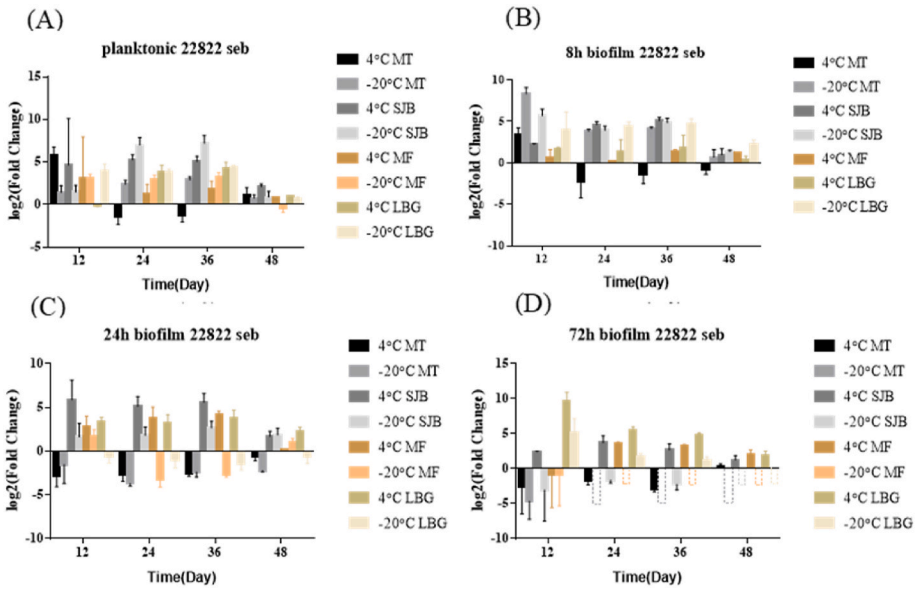

Fig. 4. Expression level of *seb* genes in *S. aureus* planktonic cells (A) and 8 h (B), 24 h (C), and 72 h (D) biofilm cells.

expression was determined in 72 h biofilm cells in MT and MF at −20 °C since day 24. In contrary, higher *seb* expression levels were recorded in 24 h and 72 h biofilm cells in SJB, MF, and LBG at 4 °C, as well as 24 h biofilm cells in SJB at −20 °C and 72 h biofilm cells in LBG at −20 °C. Overall, *seb* gene was expressed in most groups, indicating the ability of *S. aureus* 22822 to express enterotoxin gene *seb* in rice and flour products at low temperature storage.

4. Discussion

S. aureus is a gram-positive bacterium with thicker cell walls and higher mechanical strength, so it can better resist external stress changes. Many studies have shown that *S. aureus* can better cope with the stress caused by low temperature and cold stress, mainly because the continuous expression of cold shock protein ensuring *S. aureus* continuously adapt to low temperature survival (Bai et al., 2022; Suo et al., 2022). However, the studies on the survival of *S. aureus* in real food samples, especially in quick-frozen rice and flour products storing at low temperatures are rare (Abolghait et al., 2020; Almutawif et al., 2019; Kataoka et al., 2016; Saklani et al., 2020; Wu & Su, 2014). Moreover, limited studies have considered biofilm cells of *S. aureus*, which are supposed to have higher resistance to external stresses (Qiao et al., 2020; Qiao et al., 2021). In this study, we selected four types of rice and flour products (MT, SJB, MF, and LBG) and mimicked their storage conditions (4 °C as fridge storage and −20 °C as freezer storage) to test the survival of *S. aureus* planktonic and biofilm cells (8 h, 24 h, and 72 h). Surprisingly, the culturable cell numbers of *S. aureus* remain stable (10^6–10^7 CFU/mL) in most groups within 60 days except for 8 h and 24 h biofilm cells in MF at −20 °C. Similar studies have shown *S. aureus* is capable of surviving at 4 °C with 10^3~10^5 CFU/mL for 100 days (Suo et al., 2014) and at −20 °C with 10^3 CFU/g in sea food for 60 days (Saklani et al., 2020). It has been proofed to be hard to maintain high cell numbers (10^6 CFU/mL) at low temperature (Saklani et al., 2020). However, our results showed the ability of *S. aureus* 22822 to persist at 4 °C and −20 °C at a high cell number for 60 days. Considering the fact that *S. aureus* may enter into VBNC state at low temperature (Yan et al., 2021), a PMA-qPCR technique was developed to evaluate the VBNC state formation in our experimental model. To ensure accuracy, four standard curves were built in four rice and flour products, respectively. However, no VBNC cells were assessed, suggesting low temperature (4 °C or −20 °C) storage in four types of rice and flour products could not induce the formation of VBNC state for *S. aureus*. In a recent study, *S. aureus* entered into the VBNC state after 72 days of induction citric acid (a common food additive) buffer at −20 °C (Yan et al., 2021). The discrepancy in strain, storage substrates and time might cause the difference in the behavior of *S. aureus* cells.

The expression of *S. aureus* enterotoxin genes has high nutritional requirements (Angeles Argudin et al., 2010; Pinchuk et al., 2010). The complex nutritional environment of rice and flour products might enable *S. aureus* to survive and express enterotoxin genes. At present, most discussions on the suitable growth conditions of *S. aureus* and the expression of enterotoxin genes are based on the laboratory growth media. Some studies have found that the enterotoxin genes of *S. aureus* are expressed although at a decreased level at low temperature environments (8 °C,12 °C, 4 °C, −20 °C) (Abolghait et al., 2020; Almutawif et al., 2019; Kataoka et al., 2016; Lopes et al., 2021; Wu & Su, 2014). It is noteworthy that *S. aureus* enterotoxin has strong heat resistance, and its protein structure is difficult to be destroyed even under the condition of heating at 100 °C for 30 min (Tsutsuura et al., 2013). Therefore, the contamination of *S. aureus* in quick-frozen rice and flour products and the accumulation of enterotoxins may cause serious food safety incidents. In this study, the expression levels of enterotoxin gene *seb* were monitored at day 12, 24, 36, and 48 during the storage of *S. aureus* planktonic and 8 h, 24 h, 72 h biofilm cells in four types of rice and flour products at 4 °C and −20 °C, respectively. The *seb* gene expression changes were similar in planktonic cells and 8 h biofilm cells, while the changes in 24 h and 72 h biofilm cells showed a difference pattern. It suggested the different cell behavior in planktonic/early-biofilm cells and mature biofilm cells. Significantly, the overall continuous expression of *seb* gene in most groups indicated the high possibility for *S. aureus* to produce enterotoxin SEB when remaining in quick-frozen rice and flour products.

5. Conclusion

In this study, we have taken several factors (biofilm, VBNC state, enterotoxin gene) in to consideration to examine the survival of *S. aureus* in quick-frozen rice and flour products. Firstly, artificially contaminated food sample models were built by inoculating *S. aureus* cells in planktonic and different stages (8 h for early, 24 h for proliferation and 72 h for maturity) of biofilm states into four types of rice and flour products (MT, SJB, MF, LBG) and stored at 4 °C and −20 °C to mimic their storage environments, respectively. The changes in culturable cell number were recorded for 60 days, showing the persistence of *S. aureus* in rice and

flour products at low temperatures. Secondly, a PMA-qPCR technique was developed with four standard curves to examine the viable cell number thus determine the existence of VBNC cells. However, our artificially contaminated food sample models were not able to induce the VBNC state formation. Thirdly, pathogenic enterotoxin gene *seb* expressions were monitored in planktonic and biofilm cells in the food samples. Although changes in *seb* expression were recorded in *S. aureus* cells in different states and food samples, the overall continuous *seb* gene expression in combination with stable cell numbers indicated high risks may be posed by *S. aureus* contamination in quick-frozen rice and flour products.

Credit author statement

Zhenbo Xu: Conceptualization, Writing – review & editing, Project administration, Funding acquisition. Yuting Luo: Methodology. Yuzhu Mao: Software. Tengyi Huang: Validation, Visualization, Writing – original draft. Chunyun Qu: Formal analysis. Junyan Liu: Investigation, Writing – original draft, Funding acquisition. Thanapop Soteyome: Resources. Gamini Seneviratne: Data curation. Gongliang Liu: Supervision. Birthe V. Kjellerup: Supervision. Lei Yuan: Validation, Writing – review & editing. Qin Ma: Visualization, Writing – review & editing.

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.

Acknowledgments

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