Application of propidium monoazide quantitative real-time PCR to quantify the viability of *Lactobacillus delbrueckii* ssp. *bulgaricus*

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ABSTRACT

In this study, a combination of propidium monoazide (PMA) and quantitative real-time PCR (qPCR) was used to develop a method to determine the viability of cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02 (*L. bulgaricus*) that may have entered into a viable but nonculturable state. This can happen due to its susceptibility to cold shock during lyophilization and storage. Propidium monoazide concentration, PMA incubation time, and light exposure time were optimized to fully exploit the PMA-qPCR approach to accurately assess the total number of living *L. bulgaricus* ND02. Although PMA has little influence on living cells, when concentrations of PMA were higher than 30 µg/mL the number of PCR-positive living bacteria decreased from 10⁶ to 10⁵ cfu/mL in comparison with qPCR enumeration. Mixtures of living and dead cells were used as method verification samples for enumeration by PMA-qPCR, demonstrating that this method was feasible and effective for distinguishing living cells of *L. bulgaricus* when mixed with a known number of dead cells. We suggest that several conditions need to be studied further before PMA-qPCR methods can be accurately used to distinguish living from dead cells for enumeration under more realistic sampling situations. However, this research provides a rapid way to enumerate living cells of *L. bulgaricus* and could be used to optimize selection of cryoprotectants in the lyophilization process and develop technologies for high cell density cultivation and optimal freeze-drying processes.

Key words: *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02, propidium monoazide, quantitative real-time PCR, live/dead cells

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* (*L. bulgaricus*) is one of the most important bacteria in dairy fermentation and has been used as a component of starter cultures for the production of yogurt and also Italian and Swiss varieties of cheese. However, in industrial production of starters, *L. bulgaricus* strains are subjected to preservation techniques for storage such as freezing and vacuum freeze-drying. These treatments can influence the viability and functionality of *L. bulgaricus* starter cultures and the fermented products made using such treatments (Shao et al., 2014). Cold storage can induce the bacteria to enter a viable but nonculturable (VBNC) state. In the VBNC state, cells are still alive and metabolically active, but the loss of culturability means the cells fail to grow using conventional culture techniques. Many kinds of gram-positive and -negative bacteria (more than 60 species) have been studied for their likelihood of entering into the VBNC state (Salma et al., 2013). However, most research has focused on pathogenic bacteria with very few studies on lactic acid bacteria (LAB) and other probiotics. An effective method is needed to determine the viability of potentially living cells in the VBNC state.

Nucleic acid intercalating dyes, such as propidium monoazide (PMA) and ethidium monoazide (EMA), are widely used in the differentiation and enumeration of living from dead microorganisms (Tomás et al., 2009; Andorrà et al., 2010; Fittipaldi et al., 2010; Desfossés-Foucault et al., 2012; Elizaquível et al., 2012; Sánchez et al., 2012; Álvarez et al., 2013; Villarreal et al., 2013). Cell membrane integrity is a critical indicator for the evaluation of viability in bacteria. Nucleic acid intercalating dyes enter cells with damaged membranes (i.e., dead cells) and then intercalate into the cells' DNA. The presence of a photo-induced azide group enables these dyes be covalently crosslink when exposed to strong visible light. This photolysis process transforms the azide group to a highly reactive nitrene radical (Fittipaldi et al., 2012), which restricts PCR amplification of DNA. In contrast, viable cells with intact cell

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membrane cannot be penetrated by the DNA-bound dyes. The characteristics of the DNA-binding dyes make it possible to discriminate between living and dead cells, and by combining DNA-binding dyes with quantitative real-time PCR (qPCR) the direct quantification of the living cells (including those in the VBNC state) in samples can be achieved. Combining DNA-binding dyes with qPCR could resolve the problem that nonculturable cells fail to be counted using traditional plate-counting methods and the fact that you can get positive DNA amplification from dead cells, suggesting they are alive. For the DNA-intercalating dyes PMA and EMA, Nocker et al. (2006) demonstrated that PMA displayed a major advantage over EMA, which was more permeable through living cells. Moreover, PMA-qPCR can determine viable counts faster than plate counting which takes approximately 36 to 48 h to obtain the results.

The ability to precisely evaluate viability and enumerate living *L. bulgaricus* in starter cultures is important to our understanding of the loss of viability and functionality over time and could contribute to the development of strategies for preventing loss of viability and ensuring the efficient and successful manufacture of fermented dairy products. In this research, lethal conditions and dye incubation conditions (PMA concentration, PMA incubation time, and light exposure time) were optimized to allow the use of the PMA-qPCR approach to accurately assess the total viable counts of *L. bulgaricus* strain ND02.

MATERIALS AND METHODS

Bacterial Strains, Culture Medium, and Growth Conditions

Lactobacillus delbrueckii ssp. *bulgaricus* strain ND02 and *Lactobacillus plantarum* strain P-8 were obtained from the culture collection at the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02 was originally isolated from naturally fermented yak milk from Qinghai province; it was the predominant strain in the sample (Sun et al., 2010). The control strain of *L. plantarum* P-8 was isolated from naturally fermented yogurt in Inner Mongolia, China (Zhang et al., 2015).

Lactobacillus delbrueckii ssp. *bulgaricus* ND02 was activated at 37°C for 24 h by growing it in sterilized 10% (wt/vol) skim milk (121°C for 7 min). The activated cultures were propagated in de Man, Rogosa, Sharpe broth (MRS, Oxoid CM0359, Thermo Fisher Scientific, Waltham, MA) or on MRS agar (Oxoid, CM0361,

Thermo Fisher Scientific) which were sterilized at 121°C for 15 min. Two percent inoculum was used and incubated at 37°C for 12 h. *Lactobacillus plantarum* P-8 was grown in the same way and used as a control for qPCR.

Lethal Conditions for *L. bulgaricus* ND02

Lactobacillus delbrueckii ssp. *bulgaricus* ND02 was activated as described earlier and 2% of the activated culture was inoculated into 30 mL of sterile MRS broth and incubated at 37°C until the cells reached the exponential growth phase. When the exponential phase was attained, the cells were concentrated ($5,116 \times g$ at 4°C for 10 min) and washed 3 times in 0.85% NaCl. The pellet was then resuspended in a range of different volumes of 0.85% NaCl. Optical density of the bacterial suspensions at 600 nm (OD_{600}) and bacterial counts were determined to establish the correlation between OD_{600} value of the suspension and the number of bacteria.

Cells from the exponential growth phase were centrifuged ($5,116 \times g$ at 4°C for 10 min) and washed 3 times in 0.85% NaCl. The OD_{600} of the suspension was adjusted with 0.85% NaCl to provide a bacterial count corresponding to approximately 1×10^6 cfu/mL as measured using optical density. Thirty Eppendorf [Eppendorf (Shanghai) International Trade Co., Ltd., Shanghai, China] tubes (1.5 mL) were prepared and 1 mL of the suspended cells were added to each tube. These tubes were subjected to a constant temperature of 75°C in a water bath for variable periods of time to determine how long it took for the cells to be killed. Every minute one incubating tube was taken out, cooled for 5 min, and the viable count of cells determined using plate culture. This allowed us to assess the lethal time of *L. bulgaricus* ND02. The experiment was repeated 3 times.

Determining Light Exposure Conditions for Cross-Linking

One milligram of PMA (Biotium Inc., Fremont, CA) was dissolved in 100 μ L of dimethyl sulfoxide to achieve a concentration of 10 mg/mL and stored at -20°C in darkness. Cells from the exponential growth phase were harvested as described previously, adjusted to a concentration of 1×10^6 cfu/mL in 0.85% NaCl, and killed using the conditions determined in the previous experiment. The PMA was added to 1-mL samples of heat-treated suspensions in Eppendorf tubes ($n = 21$) to achieve final concentrations of 10 μ g/mL and maintained in darkness. The Eppendorf tubes containing a

mixture of PMA and dead cells were each sealed with parafilm, vortexed for 15 s, and placed on ice. The Eppendorf tubes were then placed 12 cm from a 650-W halogen light source (to enable PMA covalent crosslinking). Tubes were exposed for 0, 5, 10, 15, 20, 25, and 30 min ($n = 3$ for each time period), respectively, before assessment.

Total genomic DNA from 1 mL of light-exposed treated cells or 1 mL of *L. plantarum* P-8 at a concentration of 9.9×10^9 cfu/mL of viable cells were extracted using a DNA extraction kit (Qiagen, Valencia, USA) following the manufacturer's instructions. According to these instructions, DNA was dissolved in 130 μ L of TE (Tris-EDTA buffer solution), resulting in a corresponding count of 7.6×10^7 cfu/ μ L of DNA. The DNA from *L. plantarum* P-8 was diluted to 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 cfu/ μ L of DNA, respectively, before evaluation.

All qPCR assays were done in a 48-well plate using StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) in accordance with the manufacturer's instructions. Primer pairs for the qPCR used in our study were *Lactobacillus*-specific primers, LacF (5'-CTTGTAACACACCGCCCGTCA-3') and LacR (5'-CTCAAAACTAAACAAAGTTTC-3'), with a length of 170 bp and melting temperature of 58°C (Dubernet et al., 2002). The qPCR reactions were done using SYBR Premix Ex Taq II (Takara, Dalian, China). The samples were tested in triplicate. The reactions were done in 20- μ L volumes containing 10 μ L of $2 \times$ SYBR Premix Ex Taq, 0.8 μ L of forward primer (10 μ M), 0.8 μ L of reverse primer (10 μ M), 0.4 μ L of $50 \times$ ROX Reference Dye II, 2 μ L of DNA, and 6 μ L of dH₂O. The DNA concentrations were adjusted to approximately 100 ng/ μ L by adding dH₂O. Sterile ultrapure water was substituted for DNA in the negative control for detecting possible contamination. The qPCR reaction conditions were 90°C for 20 s, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 40 s.

Relative normalized fluorescence change (ΔR_n) was plotted against time (PCR cycle number) to facilitate real-time assessment of each PCR. The calculated cycle threshold (Ct) provides an arbitrary cut-off point at which a Ct value could be assigned for each individual sample.

Relationships between Ct and bacterial counts were determined using the StepOne Software v2.2 (Applied Biosystems). Statistical analysis of the routine data was done using ANOVA in the software package Data Processing System (DPS) 6.55 (Zhejiang University and Hangzhou Rui Feng Information Technology Co. Ltd., Hangzhou, China). Figures presented in this paper were plotted using software GraphPad Prism 5.01

(GraphPad Software Inc., La Jolla, CA). All results were expressed as averages $\pm$ standard deviation.

Determining the Best Concentration of PMA

The concentration of PMA required needs to be sufficient to fully inhibit PCR of dead cells while at the same time not restricting PCR of living cells. Five, 10, 15, 20, 30, 40, or 50 μ g/mL of PMA were added to living cells to determine the highest concentration of PMA that could be used before inhibition of PCR of the living cells, and 2, 4, 6, 8, 10, 15, and 20 μ g/mL of PMA were added to dead cells to determine the lowest concentration of PMA needed to fully inhibit PCR of dead cells. The experiment was done in the same way as above with 3 replicates of each sample; the light exposure time was the optimal value as determined in the previous experiment.

qPCR of Living and Dead Cells in Combination with Intercalating PMA

Results from the thermal death time, light exposure conditions, and concentration of PMA experiments described above allowed selection of optimal conditions for distinguishing between living and dead cells. These were then verified in a final experiment using mixtures of living and dead cells at known concentrations.

Aliquots of 500 μ L of dead cells (sampled from the exponential growth phase and heat-treated under optimal conditions to ensure zero viability as described previously) at a concentration equivalent to 2×10^6 cfu/mL were placed in 1.5-mL centrifuge tubes. Five hundred microliters of living cells at a concentration of either 2×10^6 , 2×10^5 , 2×10^4 , 2×10^3 , or 2×10^2 cfu/mL were added to each of the 1.5-mL centrifuge tube containing 500 μ L of dead cells. Three replicate tubes were used for each dead and living cell combination. Each mixture of living and dead cells was evaluated using the optimal PMA concentrations and light regimen conditions determined in the experiments described earlier. In addition, there were 3 controls. The first control consisted of 1 mL of every concentration of living cells without any dead cells being added and was evaluated with optimal PMA and light exposure conditions. The second control was 1 mL of dead cells at a concentration equivalent to 1×10^6 cfu/mL and evaluated with optimal PMA and light exposure conditions. The third control was either living cells or dead cells at a concentration equivalent to 1×10^6 cfu/mL but evaluated without PMA and light exposure.

Furthermore, a reciprocal experiment was run alongside using the same design and conditions but where

Figure 1. Lethal conditions for *Lactobacillus bulgaricus* ND02. When incubated at 75°C in a water bath, viable counts of bacteria decreased with time and fell to zero after 17 min. Error bars represent standard deviation.

different concentrations of dead cells (concentrations equivalent to 2×10^6 , 2×10^5 , 2×10^4 , 2×10^3 , and 2×10^2 cfu/mL) were added to a single concentration of living cells rather than the other way round. There were 3 controls. The first control consisted of 1 mL of every 10-fold dilution of dead cells without any living cells being added and was evaluated with optimal PMA and light exposure conditions. The second control was living cells at a concentration of 1×10^6 cfu/mL and evaluated with optimal PMA and light exposure conditions. The third control was either living cells or dead cells at concentrations equivalent to 1×10^6 cfu/mL but evaluated without PMA and light exposure.

RESULTS

Determining Lethal Conditions for *L. bulgaricus* ND02

A growth curve for *L. bulgaricus* ND02 was produced and showed that *L. bulgaricus* ND02 entered the exponential phase 4 h after inoculation. Characteristics of exponential growth were exhibited between 4 and 9 h after inoculation. After 9 h of inoculation, *L. bulgaricus* ND02 entered the stationary phase. In the experiments described here, bacteria that had been fermented for 7 h were used to determine PMA intercalation conditions. Optical density and viable counts were 0.62 and 8×10^6 cfu/mL, respectively. Concentrations of 1×10^6

cfu/mL of *L. bulgaricus* ND02 in 0.85% NaCl were used to determine the heat treatment (75°C) times necessary to ensure zero viability.

When incubated at 75°C for 17 min, viable counts of bacteria fell to zero (Figure 1). None of the viable cells can be detected in longer time of incubation. To guarantee 100% mortality of bacteria, 75°C for 20 min was selected as the thermal death time.

Determining Light Exposure Conditions for Cross-Linking

The relationship among Ct value, quantity of DNA, and bacterial counts is necessary to determine the actual number of bacteria participating in PCR done on unknown samples. For this a standard curve of the quantity of DNA at known concentrations of bacterial cells is required. A standard curve was produced of DNA from *L. plantarum* P-8 at different concentrations (10^2 – 10^6 cfu/μL of DNA) against Ct values determined using StepOne Software v2.2.2 (Figure 2). The calculated Ct provided an arbitrary cutoff point at which a Ct value could be assigned to individual samples in the experiments. The Ct value is the cycle number needed for fluorescence signal to intersect the threshold line. A negative linear relationship exists between the Ct value of template and logarithm of starting copy number of the nucleic acid target. The standard curve is the plot of Ct value versus target gene concentration.

The Ct value increased with a decreasing amount of template. Based on this standard curve, the number of PCR-positive dead bacteria after different periods of light exposure could be calculated. As light exposure time increased the number of PCR-positive dead bacteria decreased (Figure 3). The number of PCR-positive dead bacteria declined sharply after 15 min of exposure to intense light from the equivalent of 8.76×10^5 to 6.9×10^4 cfu/mL. When exposed to light for longer than 20 min, we found no significant change in the number of PCR-positive dead bacteria ($P > 0.05$), which were recorded as equivalent to 7.7×10^3 and 5.2×10^3 cfu/mL. The Ct value of the control with no PMA or light exposure treatment was 17.347, corresponding to bacteria counts equivalent to 1.54×10^6 cfu/mL, which was not significantly different to the control (Ct value of 17.408 equivalent to 1.38×10^6 cfu/mL) that was treated with PMA but not exposed to light ($P > 0.05$), indicating the necessity for intense light for PMA-crosslinking. To ensure the crosslinking reaction was effective, 20 min of light exposure was selected for use in subsequent experiments.

Determining the Best Concentration of PMA

We conducted 2 experiments to determine the concentration of PMA necessary to inhibit PCR of genes from dead bacteria without influencing PCR on living bacteria. The PCR-positive counts of living bacteria were relatively constant when concentrations of PMA were lower than 20 $\mu\text{g/mL}$, and then decreased at higher concentrations of PMA (Figure 4). At concentrations of PMA of 50 $\mu\text{g/mL}$, PCR-positive counts of living bacteria fell to an equivalent of 5.1×10^5 cfu/mL, illustrating that PMA had negatively influenced PCR of genes from living cells.

In the experiment on effects of PMA on PCR of dead bacteria, as the concentration of PMA increased, the PCR-positive counts of bacteria decreased (Figure 5). The PCR-positive dead bacteria counts were only equivalent to 6.1×10^3 cfu/mL (Ct value of 21.527) when the concentration of PMA was 10 $\mu\text{g/mL}$, showing 99% inhibition of the PCR reaction for dead bacteria. When concentrations of PMA were 15 and 20 $\mu\text{g/mL}$, the number of PCR-positive dead bacteria were

Figure 2. Standard curve of the quantity of DNA isolated from *Lactobacillus plantarum* P-8 at known concentrations of bacterial cells. Standard curve was produced of DNA from *L. plantarum* P-8 at different concentrations (10^2 – 10^6 cfu/ μL of DNA) against cycle threshold (Ct) values determined using StepOne Software ver. 2.2.2 (Applied Biosystems, Carlsbad, CA).

Figure 3. Light exposure conditions for cross-linking of propidium monoazide to DNA of *Lactobacillus bulgaricus* ND02. Ct = cycle threshold. Error bars represent standard deviation. Color version available online.

Figure 4. Effects of propidium monoazide (PMA) on PCR of living bacteria. Error bars represent standard deviation.

equivalent to only 1.26×10^3 (Ct value of 22.457) and 2.1×10^2 cfu/mL (Ct value of 23.107), respectively. We found no significant difference in PCR reaction ($P < 0.05$) between concentrations of PMA of 10 and 15 $\mu\text{g/mL}$. Taking into account the results from the 2 experiments, a PMA concentration of 10 $\mu\text{g/mL}$ was selected for subsequent experiments, as it inhibited PCR of genes from dead bacteria but had little influence on detection of living bacteria.

qPCR of Mixtures of Living and Dead Cells Treated with Optimal Conditions

Here we verified the ability of qPCR and optimal conditions of PMA and light to distinguish between living and dead cells using mixtures of living and dead cells at known concentrations. The relationship between Ct value and bacteria counts detected by qPCR was determined when different concentrations of living cells were added to a standard number of dead cells (Figure 6). With increasing concentrations of living cells being added to dead cells, the black (blue) columns show a downward trend, which was similar in the gray (dark pink) columns, illustrating that Ct values were not affected by the addition of dead cells when they had been treated with PMA and light exposure (Figure 6).

The relationship between Ct value and bacteria counts detected by qPCR was determined when dif-

ferent concentrations of dead cells were added to a standard number of living cells (Figure 7). There was no significance difference ($P < 0.05$) among the experimental groups, indicating that Ct values did not change significantly with the addition of different dilutions of dead cells. These results showed that PMA and light exposure could effectively inhibit PCR of dead cells, making it possible to count only living cells using qPCR in the samples where living cells were mixed with dead cells.

DISCUSSION

Lactobacillus delbrueckii ssp. *bulgaricus* is vulnerable to the cold, which interferes with rod morphology. However, preparation and preservation of starter cultures of *L. bulgaricus* are always done at a low temperature, which could lead to the bacteria entering in to the VBNC state (VBNC data for ND02 not shown in this paper). In the VBNC state, bacteria may still be alive but unable to grow under standard cultivation conditions. Many factors, including temperature, salinity, lack of nutrients, low pH, low oxygen content, low light, low ventilation, and the physiological age of the culture, may induce the bacteria to enter the VBNC state (Cunningham et al., 2009; Oliver, 2010; Chen et al., 2012; Prakash et al., 2013). Activity of bacteria in the VBNC state can be recovered using nutrients, such

Figure 5. Effects of propidium monoazide (PMA) on PCR of dead bacteria. Error bars represent standard deviation.

Figure 6. The relationship between cycle threshold (Ct) value and bacterial counts of mixtures of different concentrations of living cells and a standard number of dead cells. Black (blue) columns show Ct values against the total number of dead cells and 10-fold dilutions of living cells evaluated using optimal propidium monoazide (PMA) and light conditions. Gray (dark pink) columns show Ct values against 10-fold dilutions of living cells without dead cells evaluated using optimal PMA and light conditions. Column (C) (red) shows Ct values against 1×10^6 cfu/mL of dead cells evaluated using optimal PMA and light conditions, and columns (A) (green) and (B) (gray) show the relationship between Ct values and 1×10^6 cfu/mL of live and dead cells, respectively, which were not treated with PMA and light exposure. Error bars represent standard deviation. Color version available online.

as AA (Dhif and Bakhrouf, 2008; Pinto et al., 2011). The presence of cells in the VBNC state results in difficulties in accurately determining the number of living and functioning bacteria in starter cultures and makes it difficult to control the quality of fermented products such as yogurt, cheese, and milk beverages.

A combination of qPCR, a quantitative molecular biology method, and the chemical, PMA, was used to quantify the number of living *L. bulgaricus*. Previous research has reported that this method was useful for discriminating living from dead cells in other species of probiotics. Kramer et al. (2009) found that PMA

treatment followed by real-time PCR analysis was a promising approach for monitoring the quality and stability of probiotics such as *Lactobacillus acidophilus* and *Bifidobacterium lactis*. Oketič et al. (2015) compared plate counting and culture-independent PMA-qPCR for enumeration of *Lactobacillus gasseri* and *L. bulgaricus* and found that the values determined by qPCR of non-PMA-treated bacteria were higher than those obtained by qPCR of PMA-treated bacteria or plate counting. From published studies the methods associated with PMA coupled with qPCR for enumeration of LAB were varied (Kramer et al., 2009; Villarreal et

Figure 7. The relationship between cycle threshold (Ct) value and bacterial counts of mixtures of different concentrations of dead cells and a standard number of living cells. Black (dark blue) columns show Ct values against total number of living cells and 10-fold dilutions of dead cells evaluated using optimal propidium monoazide (PMA) and light conditions. Gray (orange) columns show Ct values against 10-fold dilutions of dead cells without living cells evaluated using optimal PMA and light conditions. Column (C) (light blue) shows Ct values against 1×10^6 cfu/mL of live cells evaluated using optimal PMA and light conditions, and columns (A) (green) and (B) (gray) show the relationship between Ct values and 1×10^6 cfu/mL of live and dead cells respectively, which were not treated with PMA and light exposure. Error bars represent standard deviation. Color version available online.

al., 2013; Ganesan et al., 2014; Oketič et al., 2015), although Fujimoto et al. (2011) found that the conditions for combined qPCR with PMA were simply optimized.

However, systematic research on the conditions required for the use of DNA-intercalating dyes on different species of LAB is insufficient. We consider that the type of sample, PMA concentration, light exposure condition, species of bacteria, and the target gene may all influence the efficiency of cross linkage of PMA to DNA. In the highly cited research of Nocker et al. (2006), the samples used were pure genomic DNA and bacterial cultures. However, the types of sample used in

DNA-intercalating research are different types of food, such as meat, vegetables, juices, dairy products, fish, and wine (Elizaquível et al., 2012), which may influence the efficiency of PMA chelation with DNA. From the study of Nocker et al. (2006) the optimal PMA incubation time and concentration for optimal genomic DNA yield was different depending on the bacteria under evaluation (i.e., *Escherichia coli* and *Streptococcus sobrinus*). Nocker et al. (2006) found that at a PMA concentration of $3 \mu M$, DNA yield from dead cells of *E. coli* was different to the yield from *S. sobrinus* with the same incubation time; this demonstrated that *E.*

coli was easier to be chelated by PMA than *S. sobrinus*. This may be explained by the fact that different species of bacteria have genomes of different size and sequence and different genes may be used for the qPCR. In the case of the Nocker et al. (2006) study both species were pathogens and the *stx1* gene was used for qPCR. The target genes used in PMA-qPCR studies of other LAB were not the same. For example, the 16S rRNA gene has been used in *Lactococcus*, *Lactobacillus*, and *Bifidobacterium* research (Kramer et al., 2009; Villarreal et al., 2013; Ganesan et al., 2014). In contrast, Desfossés-Foucault et al. (2012) used *tuf*, *tal*, and 16S rRNA genes for PMA-qPCR enumeration of *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Lactococcus* sp., and *Bifidobacterium animalis*. Those authors suggested that melting curves of amplicons display one peak, indicating that specific amplification is required for each primer set. In our research we used *Lactobacillus*-specific primers for qPCR amplifications and found Ct values of 17 to 25, which were similar to the Ct values of 17 to 21 found in the study of Desfossés-Foucault et al. (2012). This indicated specific amplification by the *Lactobacillus*-specific primers. The 16S rRNA gene is a component of prokaryotic ribosomes, and can be used as a universal target gene for bacterial enumeration in qPCR. On the basis of the research, different specific target genes can also be chosen for qPCR for selective enumeration for particular species of bacteria.

When applying qPCR to PMA-treated bacteria, several conditions need to be considered. In this research, the lethal conditions for *L. bulgaricus* ND02, the light exposure time for DNA binding and the PMA concentration were all optimized, demonstrating that PMA had little influence on living cells. However, when concentrations of PMA were higher than 30 µg/mL, counts of PCR-positive living bacteria did decrease by a magnitude of 10⁶ to 10⁵ cfu/mL when enumerated by qPCR. Consequently, PMA concentration should be studied further to fully inhibit PCR of dead cells without influencing the living cells. Mixtures of living and dead cells were used as the samples for enumeration by PMA-qPCR, demonstrating that the method was feasible and effective in quantifying the number of living *L. bulgaricus* among dead cells.

CONCLUSIONS

This research provides a way to determine the quality of a starter culture and whether *L. bulgaricus* in storage may have entered in to the VBNC state. Combining the PMA and qPCR methods can be used for enumeration of living cells and could be a quick way to optimize the cryoprotectants used in lyophilization (technology for high cell density cultivation and freeze-drying). We

suggest that several conditions still need to be considered and studied before PMA-qPCR methods can be accurately applied in the enumeration of living cells under more realistic situations, but this study is a step toward that.

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