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Development of a propidium monoazide-polymerase chain reaction assay for detection of viable *Lactobacillus brevis* in beer

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ABSTRACT

The spoilage of beer by bacteria is of great concern to the brewer as this can lead to turbidity and abnormal flavors. The polymerase chain reaction (PCR) method for detection of beer-spoilage bacteria is highly specific and provides results much faster than traditional microbiology techniques. However, one of the drawbacks is the inability to differentiate between live and dead cells. In this paper, the combination of propidium monoazide (PMA) pretreatment and conventional PCR had been described. The established PMA-PCR identified beer spoilage *Lactobacillus brevis* based not on their identity, but on the presence of *horA* gene which we show to be highly correlated with the ability of beer spoilage LAB to grow in beer. The results suggested that the use of 30 μg/mL or less of PMA did not inhibit the PCR amplification of DNA derived from viable *L. brevis* cells. The minimum amount of PMA to completely inhibit the PCR amplification of DNA derived from dead *L. brevis* cells was 2.0 μg/mL. The detection limit of PMA-PCR assay described here was found to be 10 colony forming units (CFU)/reaction for the *horA* gene. Moreover, the *horA*-specific PMA-PCR assays were subjected to 18 reference isolates, representing 100% specificity with no false positive amplification observed. Overall the use of *horA*-specific PMA-PCR allows for a substantial reduction in the time required for detection of potential beer spoilage *L. brevis* and efficiently differentiates between viable and nonviable cells.

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Introduction

Limited ranges of bacteria are capable of spoiling beer owing to the presence of ethanol (0.5–10% w/w), high carbon dioxide content (approximately 0.5% w/v), relatively low pH (3.8–4.7), extremely reduced concentration of oxygen (<0.1 ppm), lack of nutrition and the antibacterial effects of hop bitter compounds. Among the most problematic beer spoilers are several species of the gram-positive genera lactobacilli and pediococci.[1,2] *Lactobacillus brevis* appears to be the most frequently isolated beer spoilage *Lactobacillus* species in beer and breweries. More than half of the bacterial incidents were caused by this species.[1] It is one of the best-studied beer spoilage bacteria and grows optimally at 30 °C and pH 4–6.

Current methods of detecting beer spoilage bacteria are time-consuming. Therefore, the brewer requires a rapid, accurate method as a quality control tool for screening samples before release into the marketplace. To shorten the detection time, several molecular methods have been developed for the detection of beer spoilage bacteria particularly *L. brevis*, based on techniques such as the polymerase chain reaction (PCR).[3,4] However, one of the drawbacks is the inability to discriminate between live and dead cells due to the persistence of DNA after cell death. Ethidium monoazide (EMA) and propidium monoazide (PMA) were applied prior to PCR analysis to circumvent this problem, allowing a live/dead discrimination of bacteria.[5–8] The intercalating dye can enter bacteria with damaged cell membranes and covalently bind to genomic DNA upon exposure to light. The bound DNA cannot be amplified by PCR, thus preventing the detection of dead cells.[8] Although EMA/PMA-PCR has been known for several years, its applications in the brewing industry are scarce.

Beer spoilage *L. brevis* is generally resistant to hop compounds and thus can spoil beer.[1,2] It is thought that *L. brevis* undergoes a multi-factorial hop adaptation process involving changes in metabolism and morphology, as well as the more energy-dependent multidrug transporter, hop-efflux mechanisms.[9] The known beer spoilage-specific genetic markers for these bacteria are *hitA*,[10] *horA*,[11] and *horC*,[3,12] with *hitA* and *horC* recently being shown to be less well associated with ability to spoil beer.[4] Another beer spoilage related gene, *bsrA*, was recently found to be a marker for predicting beer spoilage ability of *Pediococcus* isolates.[13] The wide and exclusive distributions of *horA* in various beer spoilage *L. brevis* isolates indicate the possibility of species-independent detection of beer spoilage *L. brevis* with the genetic marker.[4,14] The hop resistance gene, *horA*, was originally identified on a 15.0 kb plasmid, designated as pRH45.[2] This plasmid was carried by a strong beer spoilage *L. brevis* strain ABBC45. pRH45 was initially recognized as a plasmid, the copy number of which multiplied with the hop adaptation of *L. brevis* ABBC45.[2] Consequently, the aim of this study was to investigate the applicability of PMA-PCR targeting the *horA* gene to discriminate between viable and nonviable *L. brevis* not to amplify other bacteria.

Materials and methods

Bacterial strains

A list of the bacterial species tested is provided in Table 1, with the strains comprising 13 *L. brevis* and 5 non-lactic acid bacteria (5 species). All these strains employed in this study were isolated and stored in our laboratory previously.[15] Among them, the lactic acid bacteria (LAB) were grown anaerobically in de Man Rogosa Sharpe (MRS) broth (Oxoid, UK) at 26 °C for 5 days, while the non-LAB were incubated at 37 °C and maintained in Luria-Bertani (LB) broth (Oxoid, UK) for 24 h.

The beer spoilage ability was investigated using the traditional "growth in beer test" described as Deng et al.[16] Approximately 10^2 cells mL^{-1} of each strain were inoculated onto the apical surface of commercial bottled lager beers (filter-sterilized, 4.5% vol/vol alcohol, pH 4.8, around 9 bitterness units) under sterile conditions at room temperature. Bottle headspaces were flushed with CO_2 at a flow rate of 120 mL/min for approximately 3 min to remove the air. These bottles were then tightly recapped with metal lids and incubated at 26 °C and examined regularly for visible growth for up to 1 month. Bacteria capable of growing in either beer were considered to be beer-spoilers. The ability of these 18 isolates to grow in beer was recorded in Table 1 for direct comparison with the results on presence or absence of *horA* gene.

Inactivation of bacterial cells

The bacteria were heated at 65 °C in a water bath for 30 min. The resulting heat-treated samples were cooled to room temperature and the absence of viable cells determined by the passive dye exclusion method[16] using a Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, USA). Two fluorescent dyes SYTO 9 and propidium iodide (PI) were used following the manufacturer's instructions to evaluate cell membrane integrity in this kit. Cell samples were stained with the mixture of SYTO 9 (5 μM final concentration) and PI (30 μM) in 0.5 M sodium phosphate buffer at pH 7.0, and incubated in the dark at room temperature for 20 min. The stained cells were analyzed under the Guava easyCyte 8HT flow cytometer (Guava Technologies Inc., USA) using blue line excitation at 488 nm. Results are expressed as the number of viable cells per milliliter of the samples.

DNA isolation and PCR assays

Genomic DNA were extracted from bacterial strains by using the TIANamp Bacteria DNA kit (Tiangen Biotech, China) according to the manufacturer's instructions. The primer pairs specific to *horA* were designed as described by Haakensen et al.[4] The sequences of forward and reverse primers are 5′-ATCCGGCGGTGGCAAATCA-3′ and 5′-AATCGCCAATCGTTGGCG-3′ respectively, and amplify a 335-bp segment in the conserved region of the *horA* gene. [15]

Table 1 – Bacterial strains, presence of genes and ability to grow in beer.

Isolates	Origins	*horA*[a]				Growth (days)[b]
		1:1[c]	1:99	1:999	0:1	
Lactic acid bacteria						
L. brevis CTT	Brewery	+	+	+	–	+ (5)
L. brevis DY	Brewery	+	+	+	–	+ (6)
L. brevis WCK	Brewery	+	+	+	–	+ (3)
L. brevis CN086	Brewery	+	+	+	–	+ (4)
L. brevis 2013-17	Brewery	+	+	+	–	+ (5)
L. brevis 86719	Brewery	+	+	+	–	+ (4)
L. brevis CN3	Brewery	+	+	+	–	+ (6)
L. brevis C598	Brewery	+	+	+	–	+ (3)
L. brevis C663	Pickled cabbage	–	–	–	–	–
L. brevis CGMCC 1.2028	Cured meat	–	–	–	–	–
L. brevis CGMCC 1.3847	Milk	–	–	–	–	–
L. brevis CGMCC 1.1945	Wine	–	–	–	–	–
L. brevis CGMCC 1.2561	Wine	–	–	–	–	–
Non-lactic acid bacteria						
Bacillus subtilis CGMCC 1.3376	Soil	–	–	–	–	–
Staphylococcus aureus CGMCC 1.1809	Milking machine	–	–	–	–	–
Enterococcus gallinarum CGMCC 1.9125	Unknown	–	–	–	–	–
Escherichia coli O157:H7 CGMCC 1.2386	Human feces	–	–	–	–	–
Salmonella enterica CGMCC 1.10603	Chicken	–	–	–	–	–

[a] Determined by *horA*-specific PMA-PCR.
[b] The detection time is shown in parenthesis (days).
[c] Different mixtures of live and heat-killed cells in 1:1, 1:99, 1:999, and 0:1 ratios were subjected to *horA*-specific PMA-PCR analysis, respectively.
+: visible turbidity in beer.
–: no visible turbidity in beer.

Taq DNA polymerase and reaction mixtures were supplied as a kit (TaKaRa Ex Taq, Takara Bio, Japan). PCR reactions were carried out in a PTC-100 Thermocycler (MJ Research, USA), and the particular cycling profile were performed as previously described.[4] Amplicons were detected by electrophoresis in 2.0% agarose gels containing ethidium bromide. Digital images were obtained using a Spectroline Model EAS-1000 Electronic Archival System (Spectronics Corp., USA). NIH Image 1 .61 software was then used for relative quantitation of DNA bands. The mean values of the fluorescence intensities of bands were derived from triplicate independent assays. To check for DNA amplification failure and to confirm the reliability of the PCR assay, PCR was performed with a synthetic DNA molecule name internal control. The number of copy of the internal control is very low. When the concentration of the target sample is high, the competition between the two PCR can conduct of the inhibition of the internal PCR product. Thus, a PCR is positive when we can detect the target PCR product (*horA*); a PCR is negative when we are not able to detect the target PCR product but able to detect the PCR internal control product. The internal control DNA yielded a 706 bp fragment after PCR amplification. The primers for the internal control are a confidential part of the now purchased kit from Tiangen Biotech, China.

Determination of the maximum concentration of PMA that does not inhibit PCR from L. brevis CTT

PMA (catalogue no. 40013, Biotium Inc., Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide and added to the suspensions of *L. brevis* CTT at a density of 10^2 colony forming units (CFU)/mL to final concentrations of 1, 5, 10, 20, 30, 50, 75, and 100 μg/mL, respectively. Samples were then incubated for 10 min in the dark at room temperature before being placed in an iced cooling box. Subsequently, the tubes were exposed for 10 min unless otherwise indicated to an LED (light emitting diode) light source (470 nm) at a distance of 15 cm to activate and photolyse the PMA. After photo-induced cross-linking of PMA, cells were pelleted at 10,000 × *g* for 5 min prior to DNA extraction.

Determination of the minimum concentration of PMA inhibiting PCR amplification from dead L. brevis

After heat treatment of *L. brevis* CTT culture (approximately 10^2 CFU/mL) in microcentrifuge tubes, the intercalating dye PMA was added to cell suspensions to final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 3.0 μg/mL, respectively. The tubes were then placed in dark followed by agitation at room temperature for 10 min to allow the PMA to penetrate the heat-treated dead cells. They were further exposed to the LED light as described above for viable cells.

Optimization of light exposure time to active and photolyse PMA

Cell suspensions of *L. brevis* CTT containing a total of 10^2 CFU in microcentrifuge tubes were treated with PMA at a final concentration of 3.0 μg/mL as described above followed by immediate removal to the dark for 10 min. The tubes were

then placed in an iced cooling box and subsequently exposed to light for 1, 5, 10, 15, and 20 min respectively at a distance of 15 cm.

Sensitivity of horA-specific PCR for detection of L. brevis CTT

The detection limit of PCR assays targeting the *horA* was ascertained by minimal viable cell number of bacteria. About 10^2 cells mL^{-1} of *L. brevis* CTT were inoculated onto 100-mL bottled beers as described in "growth in beer test". After incubation at 26 °C for 10 days, the suspensions of *L. brevis* CTT were then diluted with beer for serial 10-fold, ranging from 10^4 to 10^8 CFU/mL (1 μL included in the PCR reaction). A negative control was performed using beer instead of bacterial culture. DNA isolation and PMA-PCR were conducted as described above.

Specificity of PMA-PCR assays targeting the horA gene

According to the previous results of "growth in beer test", the isolates capable of growing in beer were grown anaerobically in lager beer at 26 °C for 10 days as described above, whereas others were grown in LB broth at 37 °C for 24 h. The viable and heat-killed cells of each isolate presented in Table 1 were then mixed in 1:1, 1:99, 1:999, and 0:1 ratios corresponding to 50%, 99%, 99.9%, and 100% nonviable cells, respectively. The total number of viable plus nonviable cells in the 1 mL volumes was kept constant at 10^5. The live/dead mixtures were treated with PMA at a final concentration of 3.0 μg/mL for 10 min in the dark, and then exposed to the halogen lamp at a distance of 15 cm for 10 min as described above. The resulting bacterial cells were further subjected to DNA extractions and PMA-PCR analysis as aforementioned.

Statistical analysis

Student's *T*-test was used to determine statistically significant differences between the mean of the log of genomic targets derived from PCR and the mean of the log of CFU derived from viability assays with the use of SAS system for windows 6.12 software. A confidence interval at the 99% level ($p < 0.01$) was considered in all cases.

Results

The maximum concentration of PMA not inhibiting the PCR amplification of DNA from viable cells

When the culture of *L. brevis* CTT were treated with the PMA at a concentration of 30 μg/mL or less, no significant inhibition of amplification of the target DNA occurred in the PMA-PCR procedure (Fig. 1A). Statistically significant ($p < 0.01$) reductions in amplification of target DNA from viable cells occurred when the concentration of PMA was above 30 μg/mL. Increasing concentrations of PMA above 30 μg/mL resulted in proportional decreases in the amplification of target DNA from viable cells. Notable inhibition of amplification occurred when the PMA concentration was 100 μg/mL (Fig. 1A).

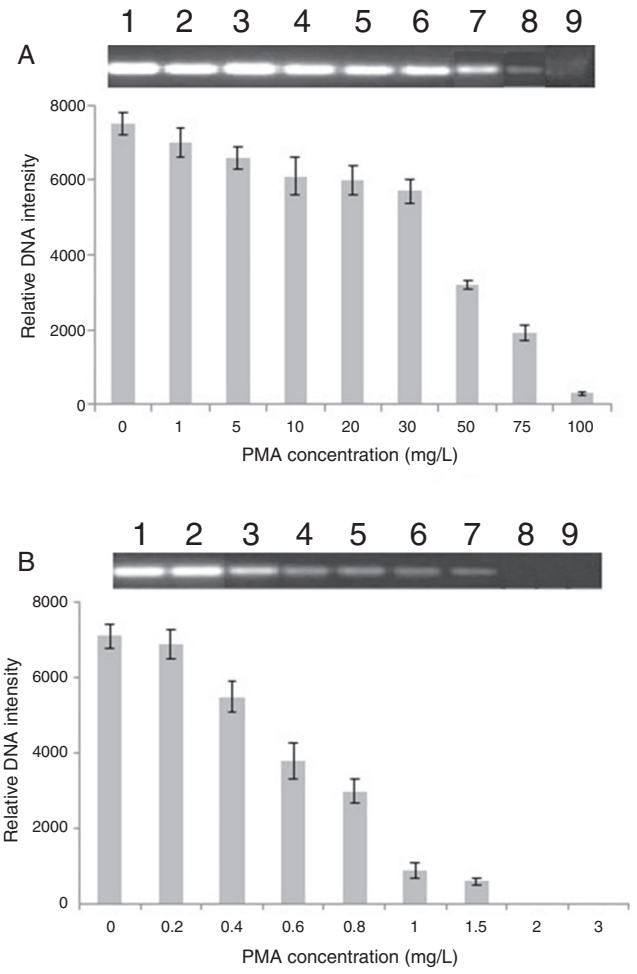

Fig. 1 – (A) Determination of the maximum amount of PMA not inhibiting the PCR amplification of DNA from viable *L. brevis* CTT. Lanes 1–9, varying concentrations of PMA (0, 1, 5, 10, 20, 30, 50, 75, and 100 mg/L, respectively). (B) Determination of the minimum amount of PMA to inhibit the PCR amplification of DNA from heat-killed *L. brevis*. Lanes 1–9, varying concentrations of PMA (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 3.0 mg/L, respectively). Microcentrifuge tubes containing a total of 10^2 viable or nonviable cells were treated with different concentrations of PMA in these two tests, respectively. Top: typical agarose gel image of PCR amplified products. Bottom: bar graphs of fluorescence intensity of corresponding DNA bands derived from PCR with respect to corresponding concentrations of PMA.

The minimum concentration of PMA required to inhibit the amplification of DNA from nonviable cells

The amplification of target DNA derived from heat-killed cells of *L. brevis* CTT was completely inhibited when such cells were subjected to treatment with PMA at a concentration of 2.0 μg/mL or higher (Fig. 1B). In contrast, target DNA from the nonviable cells was amplified when the PMA concentration was 0.2 to 1.5 μg/mL. The 2.0-μg/mL concentration of PMA that inhibited the amplification of DNA from the heat-killed cells is well below the level (>30 μg/mL) that resulted in the inhibition

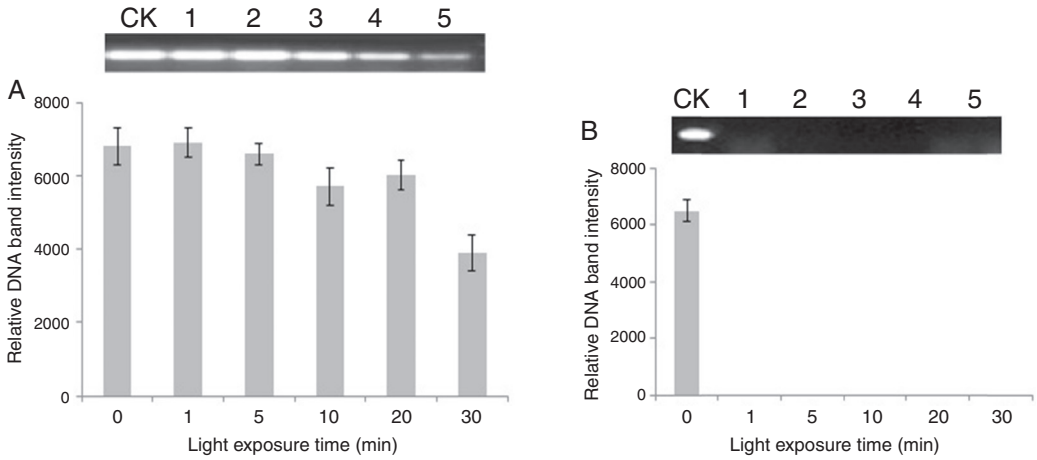

Fig. 2 – Effects of the light exposure time to achieve complete photolysis of free PMA in suspensions of live and heat-killed *L. brevis* CTT. Suspensions (1 mL) containing a total of 10^2 viable or nonviable cells were treated with PMA (3.0 μg/mL). The tubes were then exposed to the halogen light for 1 to 20 min at a distance of 15 cm. (A) Top, typical agarose gel image of PCR amplified products derived from DNA of viable cells. Lane CK: viable cells without PMA treatment as a control, lanes 1–5, exposure time for photolysis of free PMA (1, 5, 10, 15, and 20 min, respectively). (B) The same protocol as with (A) except with dead cells.

of amplification of DNA derived from viable cells. PMA at a concentration of 3.0 μg/mL should be therefore ideally suitable for discrimination of DNA from a mixed population of viable and nonviable beer spoilage *L. brevis* CTT by the PMA-PCR.

The optimal light exposure time after treatment of live and dead cells with PMA

The suspensions of *L. brevis* CTT at a density of 1×10^5 CFU/mL were exposed to 3.0 μg/mL PMA as described above. The free PMA in the suspensions of *L. brevis* cells were then exposed to the halogen lamp for 1, 5, 10, 15, and 20 min, respectively. Inactivation of free PMA was achieved with light exposure from 1 min to 20 min (Fig. 2A), which was reflected in the absence of a decrease in DNA amplification with each of these light exposure times compared to the amplification of DNA from the control cells not treated with PMA ($p < 0.01$). Light exposure of dead cells similarly treated with PMA for 1 min or longer completely prevented amplification (Fig. 2B). Light exposure for 1 min or longer was therefore found to be satisfactory for selective amplification of target DNA from the viable cells. Finally, a light exposure period of 10 min was chosen for our subsequent standardized assay to ensure inactivation of free PMA which is capable of binding to DNA following cell lysis and thereby preventing amplification of target DNA from the viable cells.

Sensitivity of horA-specific PCR for detection of L. brevis CTT

The sensitivity of *horA*-specific PCR assays was ascertained by minimal viable cell number of *L. brevis* CTT. As can be seen in Fig. 3, the detection limit of PCR assays was found to be 10 CFU/reaction (PCR was positive for sample containing 10^4 CFU/mL, with 1 μL included in the reaction system) for the *horA* gene.

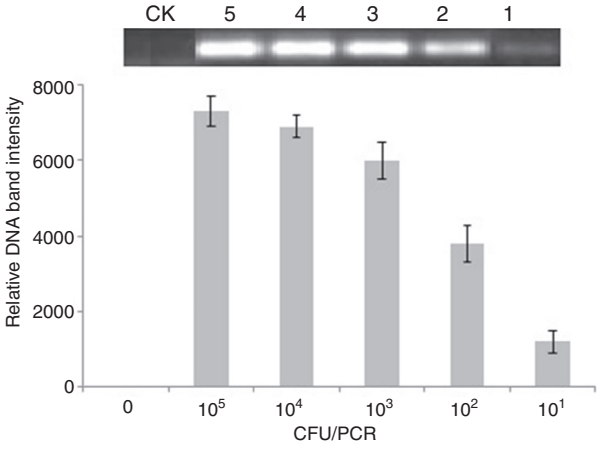

Fig. 3 – Sensitivity of PMA-PCR assays targeting the *horA* gene for detection of *L. brevis* CTT. The detection limit was evaluated with serially diluted cells suspensions containing between 10^4 and 10^8 viable *L. brevis* cells (namely 10^1 to 10^5 CFU/PCR). The number of viable cells is increased decimally from line 1 (10^5 CFU/PCR) through lane 5 (10^1 CFU/PCR). Lane CK: beer as a negative control.

Differentiation between live and dead cells in mixed samples by PMA-PCR

The established *horA*-specific PCR assays were applied for differentiation between viable and nonviable cells of the beer spoilage *L. brevis* and non-spoilage bacteria listed in Table 1. When the DNA from a constant number of total bacteria was 1×10^5 mL^{-1} derived from different ratios of viable and nonviable cells, the PCR amplification of target DNA from the dead cells was effectively inhibited by 3.0 μg/mL of PMA as expected (data not shown). Eight of these strains were detected to be

positive for *horA* by PMA-PCR as shown in Table 1. The fluorescence was not influenced by the presence of the DNA from the dead cells, even when the heat-killed cells constituted 99.9% of the total cell population. This observation is in accord with the results of growth in beer. All the 8 *horA*-positive isolates were capable of growing in beer tested, showing the *horA*-specific PMA-PCR had a very high specificity for detecting the beer spoilage *L. brevis* (Table 1).

Discussion

In our work, the observation that 100% of *horA* PCR-positive LAB isolates could grow in beer reinforces the fact that the *horA* PCR accurately detected *L. brevis* capable of rapidly causing beer spoilage. The *horA* gene has homology to adenosine triphosphate (ATP)-binding transporter to export *trans*-isohumulone, preventing its accumulation in the intracellular space.[17] The gene *horA* is considered as a significant and effective predictor of beer spoilage capability.[9]

Sami et al. initially described a PCR assay designed to amplify the ATP-binding cassette (ABC) region of *horA*.[11] But the *horA* PCR primers described by Sami et al. are not optimal, and a low PCR cycle number must be used to avoid non-specific amplification of non-*horA*, ABC-containing genes. In 2006, Suzuki et al. attempted to improve on the *horA* primers of Sami et al.[9] The *horA*-specific PCR method developed by Suzuki et al. allows more specific and sensitive determination of the beer-spoilage ability of *L. brevis*.[9] Subsequently, Haakensen et al. designed a multiplex PCR to detect four putative beer-spoilage-associated genes (*horA*, *hitA*, *horC*, and ORF5) that included the 16S rRNA gene as an internal control.[4] Their data further indicated that assaying for the presence of *horA* was highly accurate in predicting the beer-spoilage potential of *L. brevis* isolates.[4] However, some inactivated cells may be detected with these above *horA*-specific PCR methods as no differentiation is made between DNA from viable or nonviable cells. This is a considerable weakness of the conventional PCR methodology as it can lead to false-positive results.[18,19] A promising strategy to avoid this issue relies on the use of nucleic acid intercalating dyes, such as PMA or EMA, as a sample pretreatment prior to PCR.

PMA is able to enter cells with compromised cell walls and intercalate into DNA of dead cells. On light exposure a covalent DNA-PMA complex is formed, however, this bound DNA cannot be PCR amplified. Here we have found that PMA treatment prior to PCR generally reduces the signal from dead cells. In this study, the detection limit was found to be 10 CFU/PCR reaction which corroborated the earlier findings.[9] Evaluation of the efficacy of PMA-PCR and EMA-PCR on mixtures of viable and dead cells thus demonstrated that PMA is effective in selectively allowing PCR amplification from viable cells when in the presence of DNA from dead cells.[5,6] In contrast, EMA was shown to significantly inhibit PCR amplification from viable cells, as well as from dead cells. Although an intact cell membrane should be an effective barrier to EMA because of the charge of the molecule, various reports have also suggest that EMA may penetrate viable cells of some bacterial species.[6,8,20] The first report of EMA inhibiting PCR amplification from viable cells was made for the bacterium *Anoxybacillus*.[21] Nocker et al.[6] subsequently reported that although EMA and PMA were equally efficient in preventing PCR amplification from dead *Staphylococcus aureus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Mycobacterium avium* and *Streptococcus sobrinus* cells, EMA also caused inhibition of PCR amplification from viable cells of these species. These studies indicate that EMA cannot be considered to be membrane impermeable for all bacterial species. PMA appears to have the advantage over EMA of not penetrating membranes of viable cells, yet effectively entering damaged or dead cells.[5] The enhanced selectivity of PMA is most likely due to the higher charge of the PMA molecule (PMA has two positive charges, while EMA has only one), and the greater impermeability through the intact cell membrane.[6,22]

In spite of the demonstrated efficiency of the PMA-PCR for the prevention of amplification from dead *L. brevis* cells in pure culture, the application of this method to real food matrices such as brewery products in the beer production process still requires further evaluation. It would also be of interest to apply the developed PMA-PCR on other species of beer spoilage bacteria in order to ensure that the method is applicable as an entity. Additionally, the assays described in this work allow qualitative viable/dead differentiation in pure cultures of *L. brevis*. For future applications, however, the *horA*-specific PMA-PCR method in this study should be adapted for quantitative viable/dead differentiation in mixed bacterial cultures by using quantitative real-time PCR. Real-time PCR is the most widely applied technology for direct quantification of cells in mixed samples. Real-time PCR is increasingly being used for direct detection and quantification of pathogens in foods and environmental or clinical samples.[23–25] In 2007, Haakensen et al. described a quantitative real-time PCR assay without the pretreatment of PMA/EMA for the specific detection of *horA* gene.[26] This is the first report on the quantitative detection of beer-spoilage LAB. The *horA* real-time PCR developed by Haakensen et al. was proved to be an effective tool for rapid, accurate detection and quantitation of *L. brevis* in beer.[26]

In conclusion, the treatment of samples containing beer spoilage *L. brevis* with PMA prior to PCR has great potential for reducing the false-positive signal from nonviable cells. By specifically targeting organisms capable of beer spoilage through combined use of PMA and *horA*-specific PCR, brewery quality control laboratories will be able to make rapid and accurate predictions regarding the potential beer spoilage outcome of contamination by *L. brevis*.

Conflicts of interest

The authors declare no conflicts of interest.

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