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Optimised loop-mediated isothermal amplification (LAMP) for reliable detection and quantification of lactic acid bacteria in red and white wines and musts

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ABSTRACT

This study presents an improved loop-mediated isothermal amplification (LAMP) procedure for the quick, inexpensive, and precise identification and quantification of lactic acid bacteria (LAB) in white and red wines and in grape musts. Custom primers targeting the *16S rRNA* gene were developed to enhance specificity. Key advancements include a mobile phone-based semi-quantification approach using hydroxy naphthol blue (HNB), endpoint quantification using a microplate reader, and a real-time quantitative LAMP (qLAMP) assay using EvaGreen®. Moreover, PMA-qLAMP was optimised to differentiate viable from non-viable bacterial cells. A major advantage arises from the fact that all red and white wine and must samples examined exhibited consistent behaviours. The LAMP improvements described provide wineries with accessible tools for microbial quality control by themselves, offering a practical alternative to traditional methods requiring sophisticated instrumentation.

KEYWORDS: qLAMP, lactic acid bacteria, *16S rRNA*, PMA-LAMP, wine, grape must

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, rod-shaped microorganisms that produce lactic acid as the final product of fermentation (König & Fröhlich, 2017) and play an essential role in the secondary fermentation of wine, particularly malolactic fermentation. The principal genera include *Lactobacillus*, *Oenococcus*, and *Pediococcus*, among others (Liu *et al.*, 2014). Zheng *et al.* later reclassified the *Lactobacillus* genus into 25 genera (Zheng *et al.*, 2020). While many LAB contribute positively to wine stability and organoleptic qualities, certain strains are known to cause spoilage through the production of undesirable metabolites, such as volatile acidity, biogenic amines, and compounds responsible for mousy taint or buttery off-flavours (Bartowsky, 2009).

Traditional detection techniques, including culture-based plate counting and microscopy, are still widely used with some drawbacks. Plate-based methods require extended incubation times and are more prone to errors because of the presence of viable but non-culturable (VBNC) cells. Although microscopy is quicker, it does not possess the sensitivity needed to detect lactic acid bacteria (LAB) at the low concentrations, usually present in wine matrices (Fugelsang & Edwards, 2007).

Molecular approaches provide enhanced sensitivity, specificity, and speed, allowing for the distinction between culturable and non-culturable cells. Among these methods, loop-mediated isothermal amplification (LAMP) has proven to be a promising alternative method for the detection and quantification of microorganisms. In comparison to polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR), LAMP requires minimal equipment. LAMP is more tolerant to common inhibitors present in wine (*e.g.*, ethanol and polyphenol), and enables visual result detection through changes in turbidity and dye colour (Notomi *et al.*, 2015; Wong *et al.*, 2018).

The research work aims to develop and optimise a fast LAMP-based protocol for the swift detection and quantification of LAB in both white and red wines, as well as grape musts. The main goals of this study include: (i) to design primers specific for the LAB that target the *16S rRNA* gene, (ii) to enable visual detection of LAMP products through colorimetric dyes, (iii) semi-quantitative evaluation utilising a mobile phone application, (iv) quantifying endpoint products of LAMP using a microplate reader, and (v) the incorporation of propidium monoazide (PMA) for selective quantification of living cells. Although only representative data are presented herein, all methods were systematically validated across both red and white wine and must samples, which displayed comparable performance characteristics.

MATERIALS AND METHODS

1. Chemicals and reagents

The following reagents were employed to optimise the LAMP assays for the detection and quantification of lactic acid bacteria: Bst DNA polymerase 2.0 (New England, BioLabs), magnesium sulphate ($MgSO_4$), 10× isothermal amplification buffer, deoxynucleotide triphosphates (dNTPs) (NZYtech, Lisbon, Portugal), custom-designed primers (Metabion, Munich Germany), hydroxy naphthol blue (HNB) (Metabion, Munich Germany), neutral red (ZellBio GmbH, Blaubeuren Germany), gold nanoparticles (AuNPs) 5 nm citrate-stabilised (Sigma-Aldrich, Tres Cantos, Madrid), EvaGreen dye (20×, BIOTIUM), TEN buffer, TE buffer, and propidium monoazide (PMA; Sigma-Aldrich).

2. Bacterial strains and culture conditions

Ten bacterial strains were employed, comprising nine LAB species and one *Acetobacter* species (Table 1). Additionally, some yeast and other bacteria were used; however, the data are not presented. LAB were cultivated in MRS medium, except *Oenococcus oeni* L33 and *Acetobacter aceti* CECT 298^T, which were cultured in MLO medium (Zúñiga *et al.*, 1993). The strains were inoculated into commercial red (Bobal) and white (Chardonnay) wines and corresponding grape musts. Cell concentrations were verified using a haemocytometer, and all experiments were conducted in triplicate.

TABLE 1. Bacterial strains used in this research work are listed with their origin.

Bacterial strains	Origin
Lactiplantibacillus plantarum 5458	Enolab collection
Lentilactobacillus hilgardii 5W	Manca de Nadra (Cerela, Argentina)
Pediococcus parvulus 4247	Enolab collection
Liquorilactobacillus mali 4438	Enolab collection
Latilactobacillus sakei CECT 906^T	Enolab collection
Lentilactobacillus buchneri ST2-A	M. Medina (CIT-INIA, Spain)
Lentilactobacillus hilgardii 4496	Enolab collection
Oenococcus oeni 4485	Enolab collection
Lactococcus lactis subsp. *lactis* MG 1363	Manuel Zúñiga (IATA CSIC, Spain)
Acetobacter aceti CECT 298^T	Enolab collection

3. Cell preparation and washing

Cell suspensions from lab media were only washed twice with Milli-Q water; cells from white wine and musts were additionally washed with 10 % TEN buffer (0.1 M Tris-HCl, pH 7.5, 0.05 M EDTA, and 0.8 M NaCl), followed by twice in Milli-Q water and finally suspended in TE buffer. Cell suspensions from red wine and red grape must were washed with 10 % TEN buffer supplemented with 2 % polyvidone (PVP) and twice with Milli-Q water to eliminate phenolic interference. The genomic DNA extraction was achieved by vortexing with 0.5 mm glass beads for 10 minutes (Soares-Santos *et al.*, 2018).

4. Primer design and *in silico* validation

Primers specific to LAB were designed using the Genome-based LAMP Primer Design tool (GLAPD): (http://cgm.sjtu.edu.cn/GLAPD/online/) (Jia *et al.*, 2019), targeting conserved regions of the *16S rRNA* gene of lactic acid bacteria. For this purpose, the *Lactiplantibacillus plantarum* genome sequence was used as a reference genome, and *Oenococcus, Lactobacillus,* and *Pediococcus* species were used as the target group in GLAPD. Yeast and some acetic acid bacteria (*A. aceti* and *Gluconobacter oxydans*) were used in the background group for LAB primer generation. Each set included two outer primers (F3 and B3) and two inner primers (FIP and BIP). Primer specificity was confirmed via sequence alignment using MEGA X software against reference genomes of LAB and non-LAB strains.

5. LAMP reaction optimisation

To minimise nonspecific amplification, various concentrations of gold nanoparticles (0, 1, 3, and 5 µL) were evaluated. The optimal condition, yielding the clearest distinction between positive and negative reactions, was determined to be 5 µL per 25 µL LAMP reaction. Reactions were conducted at 62 °C for 30 minutes in a heat block.

Each LAMP reaction mixture (25 µL) comprised: 1.4 mM dNTPs, 0.2 µM each outer primer, 1.6 µM each inner primer, 8 mM $MgSO_4$, 1× buffer, 5 µL gold nanoparticles, 0.32 U/µL Bst polymerase, 7.5 µL extracted DNA, and sterile Milli-Q water (negative control).

6. Visualisation of LAMP products

Colorimetric detection was performed using HNB (120 µM) and neutral red (100 µM) dyes, and also by turbidity in the absence of any dye. Colour changes were assessed by visual inspection and with the HNB enhance app (https://colorimetry.net/hnb-app/) to assist interpretation. Additionally, gel electrophoresis (2 % agarose) was used to confirm amplicon formation.

7. Semi-quantitative detection via smartphone application

Semi-quantification was achieved by tracking the change in red colour intensity (RGB values) every five minutes during amplification using the "What a color?" app (https://apps.apple.com/us/app/what-a-color/id1229503218). A reduction in red intensity correlated with increasing DNA amplification (Figure 1).

8. Endpoint quantification via microplate reader

Endpoint measurements were obtained by transferring 20 µL of LAMP product into 364-well microtiter plates. Optical density was recorded using a FLUOstar OPTIMA plate reader (BMG LABTECH) with an excitation filter at 340–10 nm. A standard curve was constructed using LAB suspensions ranging from 10^2 to 10^8 CFU/mL in the same matrix as the samples (red or white grape musts and wines).

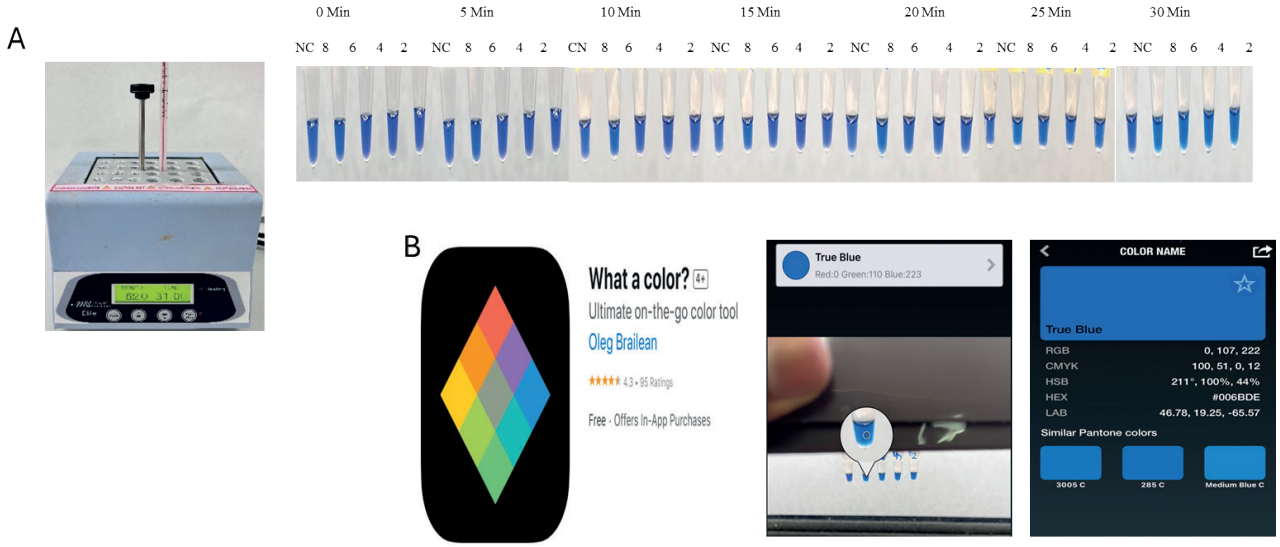


FIGURE 1. Schematic diagram of the LAMP reaction with HNB, utilising a mobile app for semi-quantification. A) Various concentrations of cells, 8, 6, 4, 2, corresponding to 10^8, 10^6, 10^4, and 10^2 cells/mL, were used in the LAMP reaction, conducted on a heat block at 60 °C for 30 minutes. Pictures were taken every 5 minutes for a total duration of 30 minutes. B) A mobile app ("What a color?") was used to analyse the red components' colour differences.

9. Real-time qLAMP amplification

Quantitative LAMP (qLAMP) was conducted on a StepOne™ real-time PCR system. The reaction mix was identical to the standard LAMP, with the substitution of EvaGreen (1.5 µL) as the fluorescence dye. Amplification curves and melt profiles were generated to assess specificity and quantify LAB concentrations.

10. Differentiation of viable cells using PMA-qLAMP

To distinguish viable from dead cells, LAB cultures were heat-killed (100 °C for 30 minutes). PMA (50 µM) was added to cell suspensions (6.3×10^6 CFU/mL), incubated in the dark for 15 minutes, then photoactivated using a PhAST Blue LED system (465–475 nm, 30 minutes). PMA-treated and untreated samples were then subjected to qLAMP under the same conditions as above.

RESULTS

1. Primer design and validation

Two primer sets targeting conserved regions of the *16S rRNA* gene were designed using the GLAPD software. These comprised outer primers LAB-F3 and LAB-B3 and inner primers LAB-FIP and LAB-BIP, optimised for high specificity to LAB. Sequences of the four LAB general primers are: LAB-F3 (AGTGAGTGGCGAACTGGT), LAB-B3 (GCCGATTACCCTCTCAGGT), LAB-FIP (GCGG TCCAAGTTGTTATGCGGTGAGTAACACGTGGG AAACCT), and LAB-BIP (ATCATTTTGGATGGTCCCGC GTGCCATGGTGAGCCGTTA). Sequence alignment using MEGA X confirmed a high identity between the designed primers and the LAB strains employed in this study, while non-target organisms, including *A. aceti*, showed negligible alignment, as shown in Figure S1. This *in silico* validation was further corroborated by successful amplification in LAMP assays, with LAB strains showing robust product formation on gel electrophoresis, whereas non-LAB controls did not amplify (Table 2), including several species of yeasts and acetic acid bacteria (data not shown).

2. Optimisation of LAMP reaction conditions

Due to a high tendency to produce non-specific amplification, the influence of gold nanoparticles (AuNPs) was assessed. AuNPs were evaluated at volumes of 0, 1, 3, and 5 µL. At 5 µL, the negative control consistently showed no amplification, while positive samples maintained high efficiency, suggesting this as the optimal concentration (Figure 2). This improvement reinforces the role of AuNPs in reducing non-specific amplification, thus improving reaction specificity.

3. Visual detection of amplification products

Multiple detection approaches were evaluated for LAMP amplicon visualisation in both white and red musts and wines. HNB produced a distinctive colour change from violet to blue in the presence of amplification, attributable to magnesium pyrophosphate formation. Neutral red provided a secondary confirmation via a pH-dependent colour shift from red to pink (Figures 3B1 and 3C1). These results were visually intensified using the HNB-enhance software tool, improving discrimination between positive and negative samples (Figures 3B2 and 3C2). In addition, amplified samples exhibited visible turbidity in the absence of any dye (Figure 3A). Gel electrophoresis confirmed successful amplification by displaying the characteristic ladder-like banding pattern (Figure 3D). While only representative data are shown in the figures, all methods were validated across both red and white wines and musts. The performance of the assay remained consistent across matrices (data not shown).

TABLE 2. The sequence identity results of the designed primers with the selected species sequence were obtained by using the Mega X software. *Acetobacter aceti*, a species of acetic acid bacteria, was used as the negative control, showing the lowest sequence identity score in the table.

Primers	Bacterial strains									
	Lactiplantibacillus plantarum 5458	*Lentilactobacillus hilgardii* 5W	*Pediococcus parvulus* 4247	*Liquorilactobacillus mali* 4438	*Latilactobacillus sakei* CECT 906^T	*Lentilactobacillus buchneri* ST2-A	*Lentilactobacillus hilgardii* 4496	*Oenococcus oeni* 4485	*Lactococcus lactis* subsp. *lactis* MG 1363	*Acetobacter aceti* CECT 298^T
LAB-F3	100 %	83.3 %	94.4 %	83.3 %	88.8 %	83.3 %	83.3 %	83.3 %	77.7 %	66.6 %
LAB-FIP	100 %	80.4 %	87.8 %	85.3 %	85.3 %	85.3 %	82.9 %	75.6 %	85.3 %	60.9 %
LAB-B3	100 %	100 %	100 %	94.4 %	100 %	100 %	100 %	61.1 %	72.2 %	66.6 %
LAB-BIP	97.5 %	87.5 %	87.5 %	85 %	82.5 %	80 %	87.5 %	70 %	77.5 %	62.5 %
Average %	99.3 %	87.8 %	92.4 %	87 %	89.1 %	87.1 %	88.4 %	72.5 %	78.1 %	64.1 %

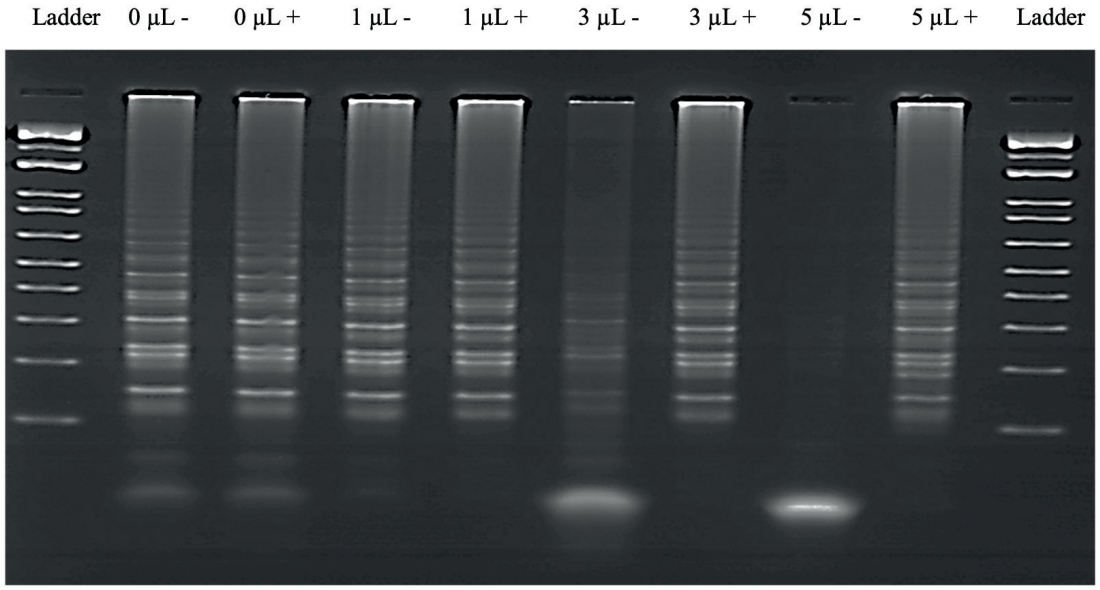


FIGURE 2. The effect of different concentrations of AuNPs on LAMP specificity for wine samples detection and quantification. Five microliters of AuNPs suppress false positives, as shown in the gel. "–" shows the negative control, and "+" shows the positive.

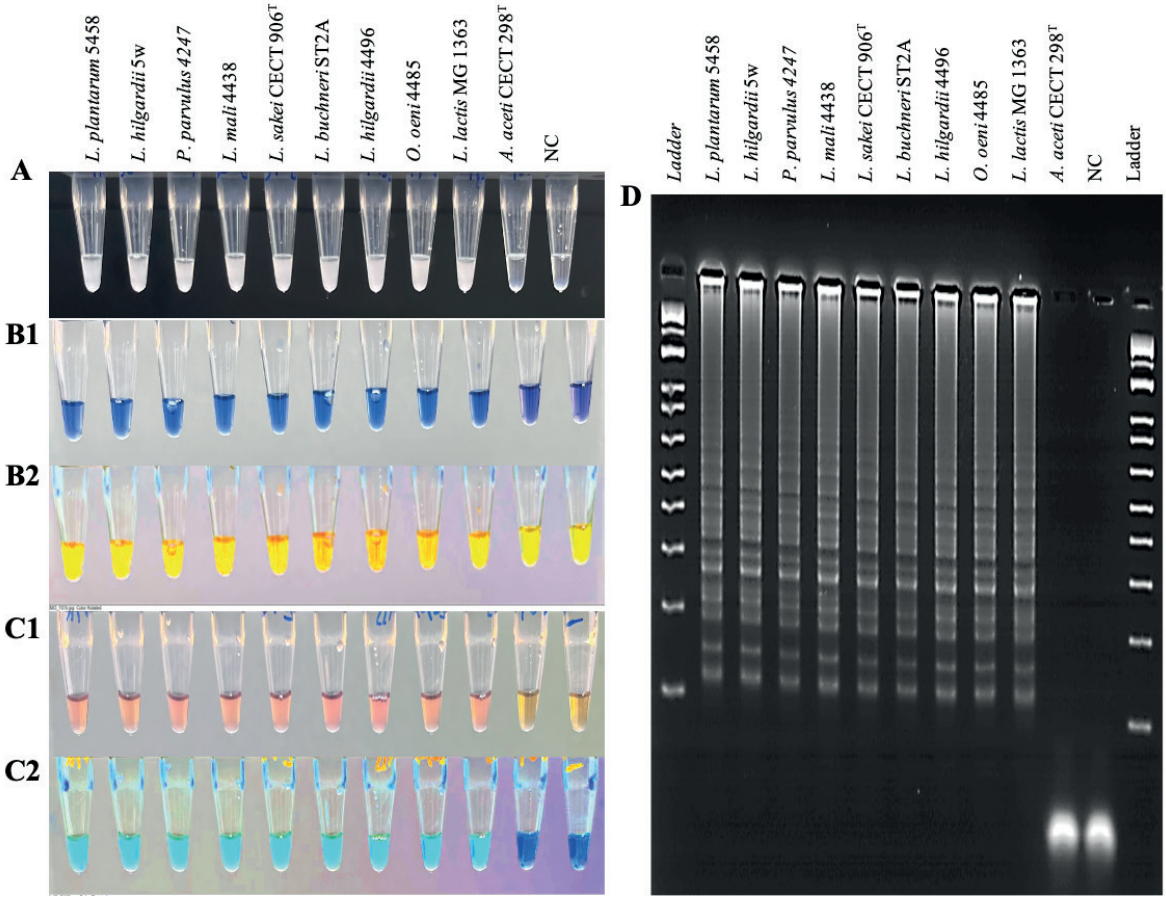


FIGURE 3. The LAMP reaction of lactic acid bacteria with HNB was conducted at 62 °C for 30 minutes. A) The turbidity of the LAMP reaction: a clear solution without precipitation indicates a negative result, while turbidity with precipitation indicates a positive result. B) Colorimetric analysis of HNB (up) and a colour-stretch filter image (down) of the LAMP product was enhanced using the HNB enhance app. C) The LAMP reaction was visualised using neutral red (up) and a colour-rotate filter (down) from the HNB enhance app. D) The LAMP reaction of lactic acid bacteria was analysed using 2 % agarose gel electrophoresis.

NC = Negative control

4. Semi-quantitative assessment using a mobile application

A novel semi-quantification method based on HNB colour intensity and RGB values was developed. A clear inverse relationship between the red component intensity and incubation time was observed in positive samples (Figure 4). This sigmoidal behaviour was consistent across matrices, including white and red wine and musts. No colour change was observed in negative controls, validating the specificity of the assay.

5. Endpoint quantification via microplate reader

Endpoint quantification was successfully achieved using optical readings of the LAMP amplicons following an HNB-based colorimetric reaction. Standard curves generated from serial dilutions of LAB ranging from (10^2 to 10^8 CFU/mL) showed a clear dose-response relationship, with higher bacterial concentrations yielding greater optical density (Figure 5). This provides a valuable and low-cost alternative to real-time fluorescence-based quantification.

6. Real-time quantitative LAMP (qLAMP)

Quantification of LAB using EvaGreen-based real-time LAMP showed reliable amplification kinetics. Amplification time (Tt) was inversely correlated with initial bacterial concentration (Figure 6A), and melt curve analysis demonstrated high specificity (Figure 6B). Linear regression of standard dilutions (10^2 to 10^8 CFU/mL) yielded an excellent correlation ($R^2 > 0.98$), affirming the method's quantitative potential (Figure 7).

7. PMA-qLAMP for differentiating viable and dead cells

To distinguish live cells from dead, PMA-qLAMP was employed. Without PMA, both live and heat-killed cells produced similar amplification signals. However, following PMA treatment, Tt values for dead cells were significantly delayed, or amplification was entirely inhibited, while live cells amplified normally (Figure 8). This confirms the PMA-qLAMP assay's capability to selectively quantify viable LAB cells in complex wine and must matrices.

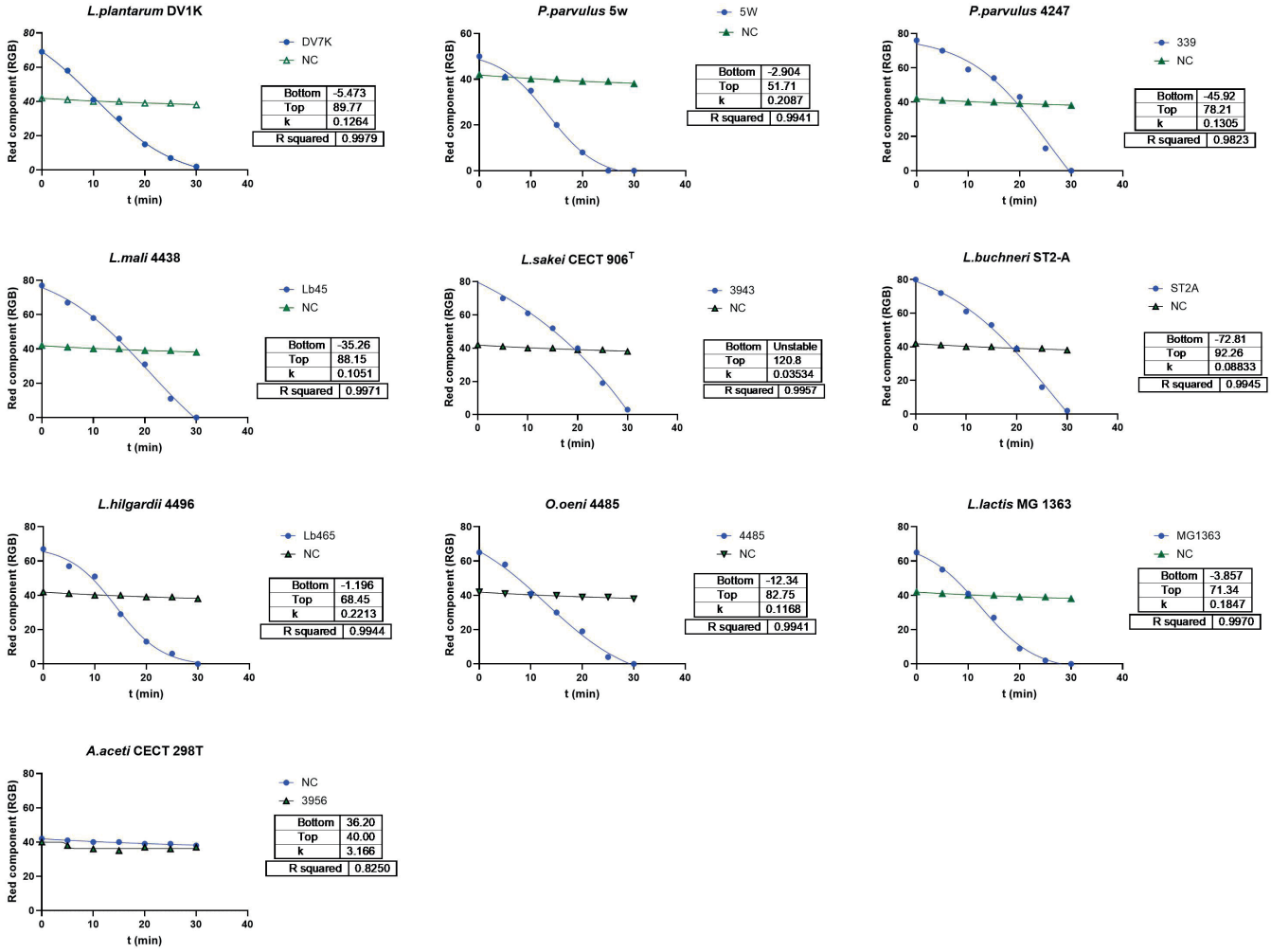


FIGURE 4. The graphs illustrate the colour intensity over the amplification time for lactic acid bacteria extracted from wine, compared to negative controls (without DNA and with *A. aceti*) derived from semi-quantification of LAMP products using a mobile app called "What a color?". A decrease in colour intensity is observed in the positive sample over time, while no intensity change is noted in the negative control. These LAMP reactions were performed at 62 °C for 30 minutes.

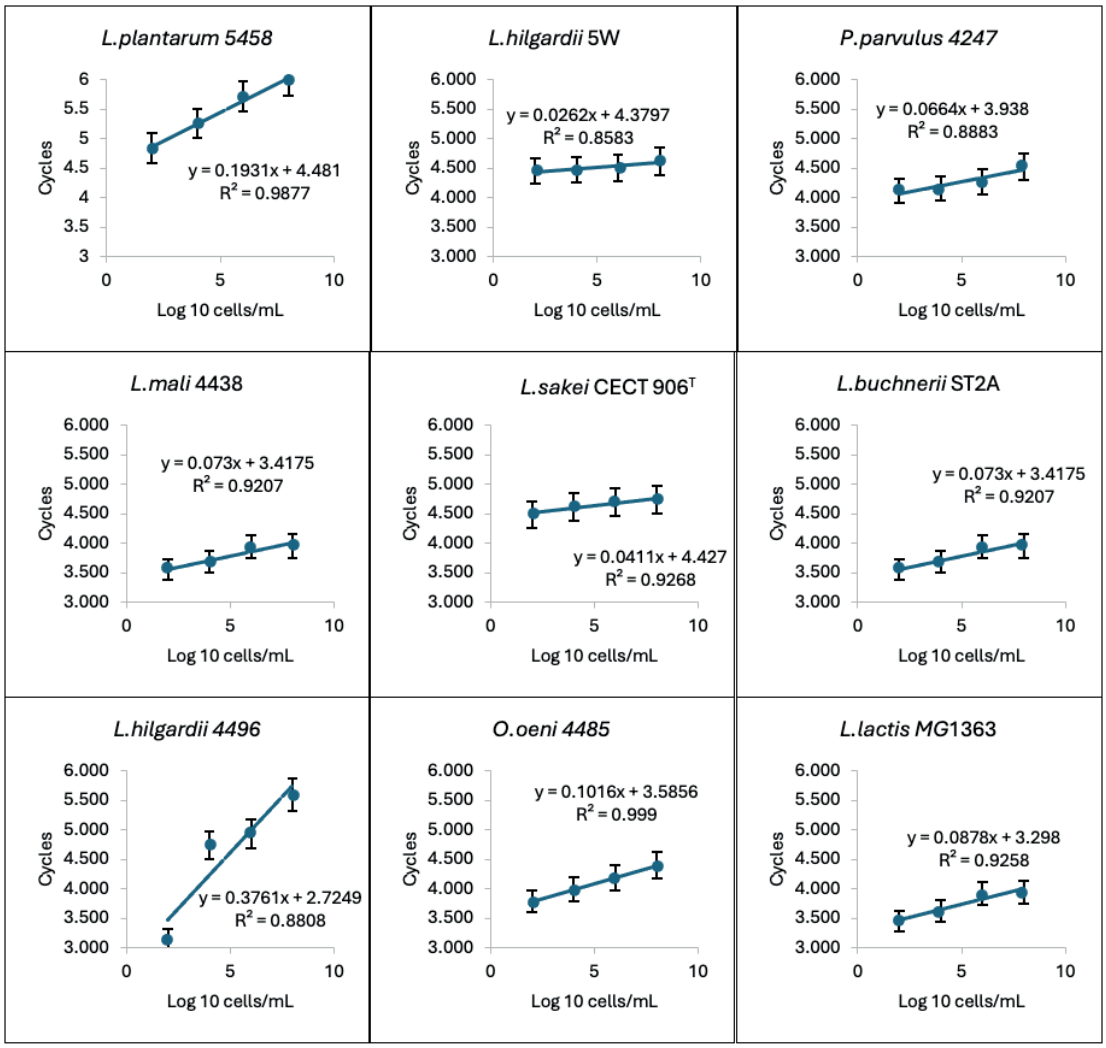


FIGURE 5. The endpoint results of the LAMP product from grape musts with HNB were evaluated using a microplate reader (OPTIMA). The standard curve was generated based on the number of cycles and included at least four dilutions of each bacterial strain. LAMP reactions for each sample were duplicated, with standard deviations less than < 0.85.

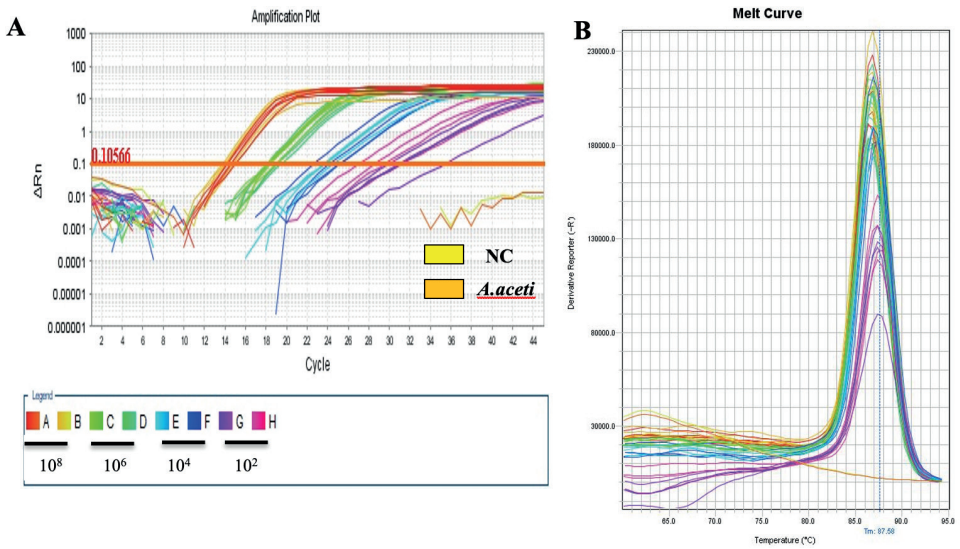


FIGURE 6. EvaGreen® fluorescent-based qLAMP with different dilutions of lactic acid bacteria, A) Amplification plots showing ΔRn *vs* cycles; B) Melting curve analysis of lactic acid bacteria amplified product in qLAMP. Straight lines are used as a negative control, and *A. aceti* shows no amplification.

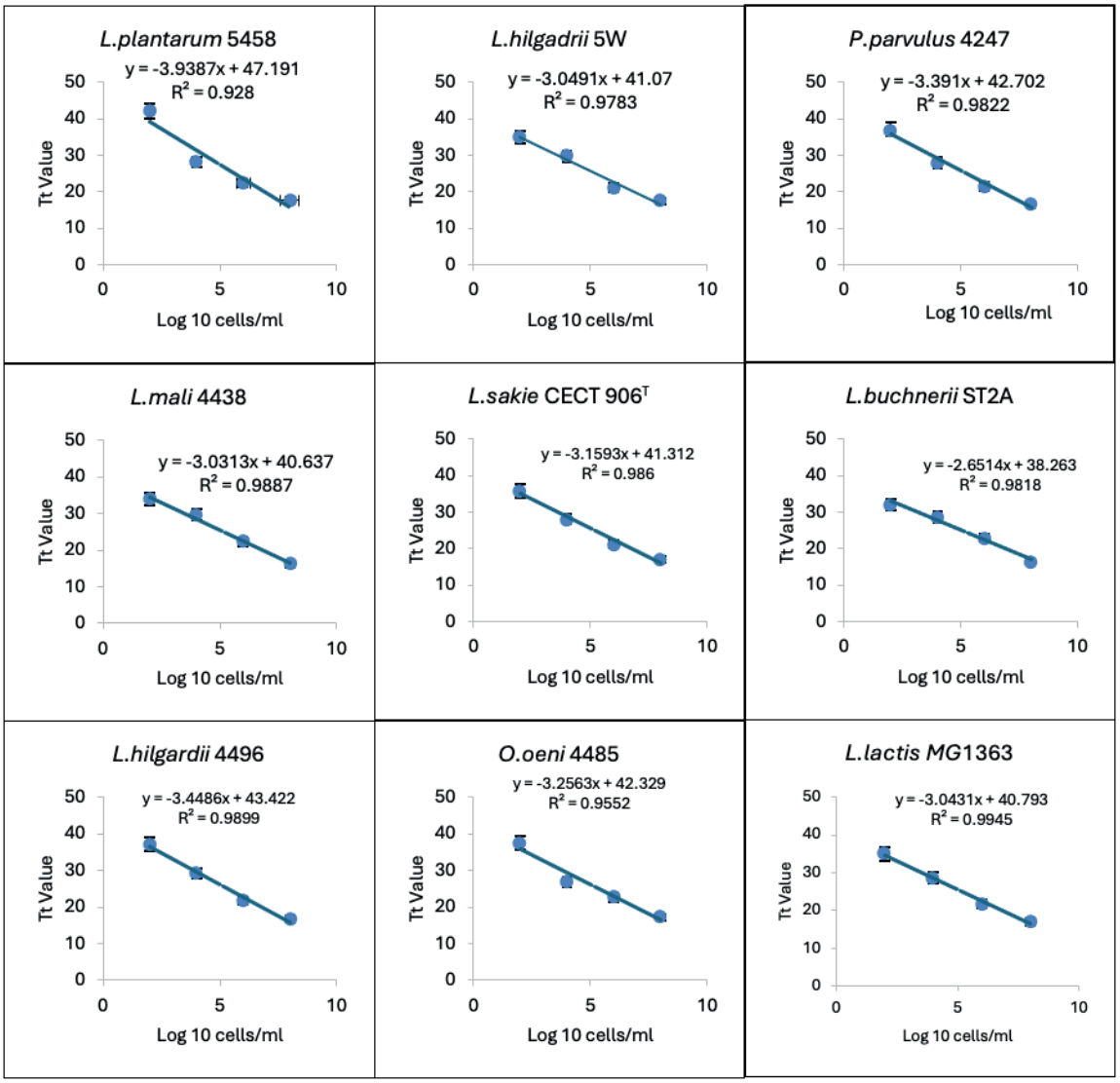


FIGURE 7. The standard curves of lactic acid bacteria in wine were obtained using qLAMP EvaGreen with different dilutions. Tt values are the average of replications.

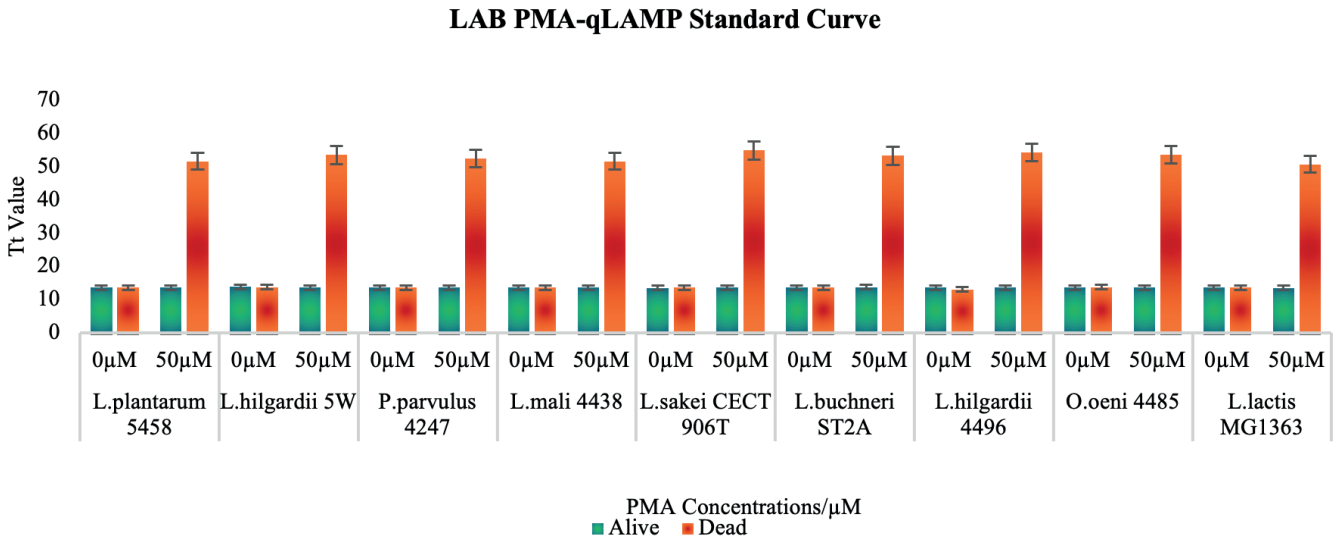


FIGURE 8. Differentiation of wine live cells from dead cells by PMA-qLAMP: one set of cell mixtures was treated with PMA (propidium monoazide), while the other set was not treated before DNA extraction. Each bar represents the average threshold cycle (Tt) values from triplicate experiments.

8. Comparison of the methods

This study analysed four different methods, highlighting their differences in speed, accuracy, sensitivity, and cost. LAMP semi-quantification using a mobile app is fast and low-cost; however, it lacks precision. In contrast, endpoint quantification offers greater accuracy after the reaction but does not provide real-time results. Real-time LAMP enables precise and dynamic monitoring with high sensitivity, although it requires specialised equipment. Additionally, PMA-LAMP exclusively detects viable cells, making it useful for assessing microbial viability, but it necessitates extra sample preparation (see Table 3).

DISCUSSION

Lactic acid bacteria (LAB) are integral to malolactic fermentation and overall wine microbiota. However, certain strains can adversely impact wine quality, contributing to faults such as volatile acidity, mousiness, and excessive diacetyl production (Bartowsky & Pretorius, 2009; Grainger, 2021). Consequently, wine quality depends on the early detection and quantification of lactic acid bacteria. Most important traditional methods, like culture assay and microscopy, have limitations; they require a lot of time, low sensitivity, and failure to detect viable but non-culturable cells (Fugelsang & Edwards, 2007). Molecular methods such as qPCR are accurate but require costly instrumentation and are influenced by common wine inhibitors, such as ethanol and polyphenols (Khan *et al.*, 2018). The LAMP-based approaches described in this study overcome many obstacles effectively. One of the most important novel features of this study was its use of gold nanoparticles (AuNPs) to enhance specificity. LAMP is prone to primer dimmer or non-specific amplification because of the long inner primers used (Meagher *et al.*, 2018), but the incorporation of AuNPs blocked non-specific single-stranded amplification, reducing false positive results (Ye *et al.*, 2018).

The colour-based visual detection with hydroxy naphthol blue (HNB) and neutral red dyes allows positive and negative reactions to be easily differentiated. Colour changes of HNB, enhanced by digital image processing, eliminated the need for gel electrophoresis, thereby facilitating the application of this approach in the wine industry. The measurement results of the turbidity and colourimetry were entirely coincidental with the assessments of gel electrophoresis, confirming the appropriateness of these low-supplied alternative methods.

Three different approaches for quantification were tested. The on-site semi-quantification method using a smartphone-based application is a simple, rapid, and inexpensive method for non-specialist users. Although it may be affected by variables (camera settings, lighting, etc.), this method demonstrated consistent results and acts as an available screening tool. The endpoint quantification method using a microplate reader offers a balanced solution regarding both cost and accuracy. It allows consistent and repeatable measurement without the complexity associated with real-time systems. Quantitative LAMP using EvaGreen fluorescence produced the most accurate and sensitive results, with amplification time directly correlating with bacterial cell concentrations. Real-time PCR instrumentation is well-suited for extensive laboratory use due to its rapidity, precision, and resistance to inhibitors.

In addressing the critical distinction between live and dead cells, PMA-qLAMP enabled selective amplification of the only live LAB cells. This is particularly important in winemaking, where the presence of dead cells could otherwise lead to false positives in molecular assays. By integrating photo-activated PMA treatment, we successfully inhibited DNA amplification from dead cells while maintaining sensitivity for viable ones, consistent with previous findings (Hu *et al.*, 2022; Yan *et al.*, 2017).

This study successfully improved the loop-mediated isothermal amplification (LAMP) technique for detecting and quantifying LAB in complex matrices, including both red and white wines and grape musts. Although specific examples are showcased, all experiments were conducted on a range of samples, yielding consistently similar results.

TABLE 3. Comparison of all the methods developed and evaluated in this study, summarising their principles, target specificity, detection limits, cost, and time for detecting lactic acid bacteria (LAB) in must and wine samples.

Parameter	Semi-quantification using a mobile app	Endpoint point quantification using a microplate reader	Real-time quantitative LAMP amplification (qLAMP)	PMA-LAMP
Principle	Visual or qualitative assessment based on signal intensity	Measurement of amplified DNA after reaction completion	Continuous monitoring of DNA amplification using fluorescent dyes	Differentiate live/dead cells
Specificity	Moderate	High	Very high	Very high excludes dead cells
Sensitivity	Moderate	High	Very high	Very high includes live cells
Detection limit (CFU/mL)	10^6	10^4	10^2	10^2
Cost	Low	Moderate	High	Much higher
Time	Short	Moderate	Short to moderate	Moderate
Application	Field testing and Point of Care (POC)	Laboratory screening and high-throughput studies	Diagnostics, research, and precise measurement	Food safety, environmental monitoring, and clinical viability testing

Collectively, the advancements discussed here greatly improve the reliability, versatility, and applicability of LAMP for monitoring LAB. The variations in methodology allow users, from field technicians to analytical laboratories, to select a protocol that suits their resource capabilities and sensitivity needs. These innovations have immediate applicability in the wine industry for microbial monitoring and quality assurance.

CONCLUSION

This study developed an optimised suite of LAMP-based techniques for detecting and quantifying lactic acid bacteria in both white and red grape musts. Custom-designed primers based on the *16S rRNA* gene showed high specificity for LAB. Furthermore, gold nanoparticles improved LAMP reaction accuracy by reducing the non-specific amplification. Multiple visualisation and quantification strategies were validated. A mobile application-based colorimetric method allowed semi-quantification at the lower resource settings, where the microplate-based endpoint quantification method offered a reliable mid-range option for quantification. Real-time qLAMP using EvaGreen fluorescence provided high precision and sensitivity, suitable for laboratory-scale implementation. The PMA-qLAMP assay resolved an important gap in molecular diagnostics by differentiating viable from non-viable LAB cells in wine.

In the context of the wine industry, these innovations provide greater flexibility while dramatically reducing costs and increasing scalability. They allow rapid microbial monitoring and could enhance proactive management of fermentation and spoilage risks. The methods developed herein will have broad applicability and represent a significant step forward in the practical use of LAMP in oenological microbiology.

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