

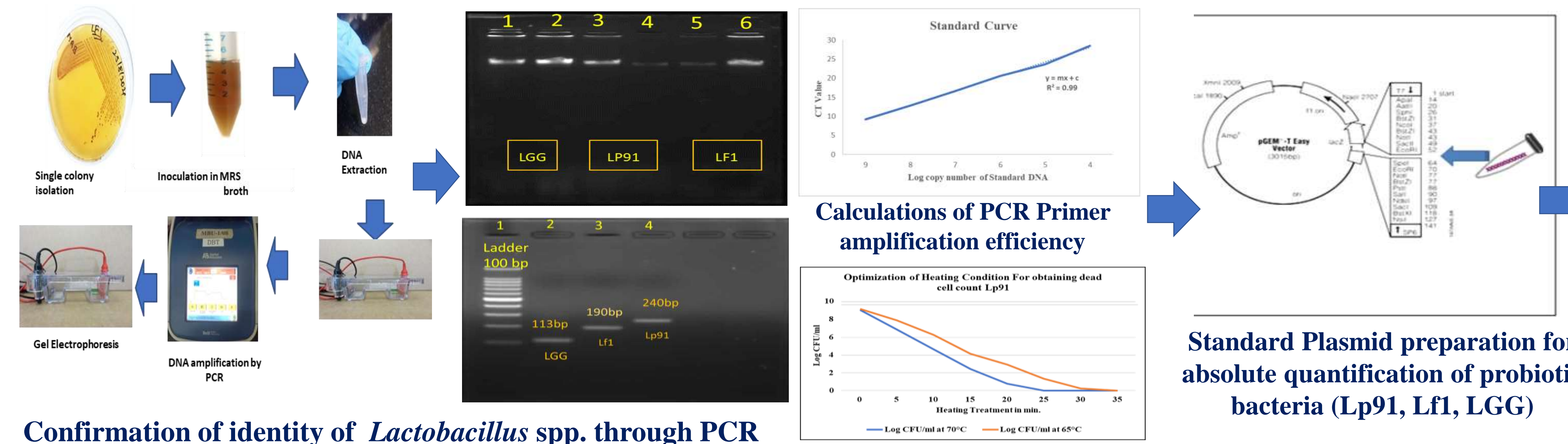
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Introduction

- Currently, the probiotic products consist of both live and dead microorganisms (*Lactobacillus* spp), showing no relationship with the number of viable *Lactobacillus* spp. present in the product. Since probiotics must be consumed alive to have health benefits, it is necessary to distinguish alive from the dead.
- In order to deliver crucial benefits, it is extremely desirable that the viable counts of probiotics in the finished product be at least 10^8 cfu/ml.
- However, traditional plating and molecular methods used, have time constraint and are unable to differentiate live and dead bacteria. Hence, in the present study, attempts have been made to develop Propidium Monoazide (PMA) assisted Real Time PCR-based Assay For Rapid Quantification Of Probiotic *Lactobacillus* Spp.

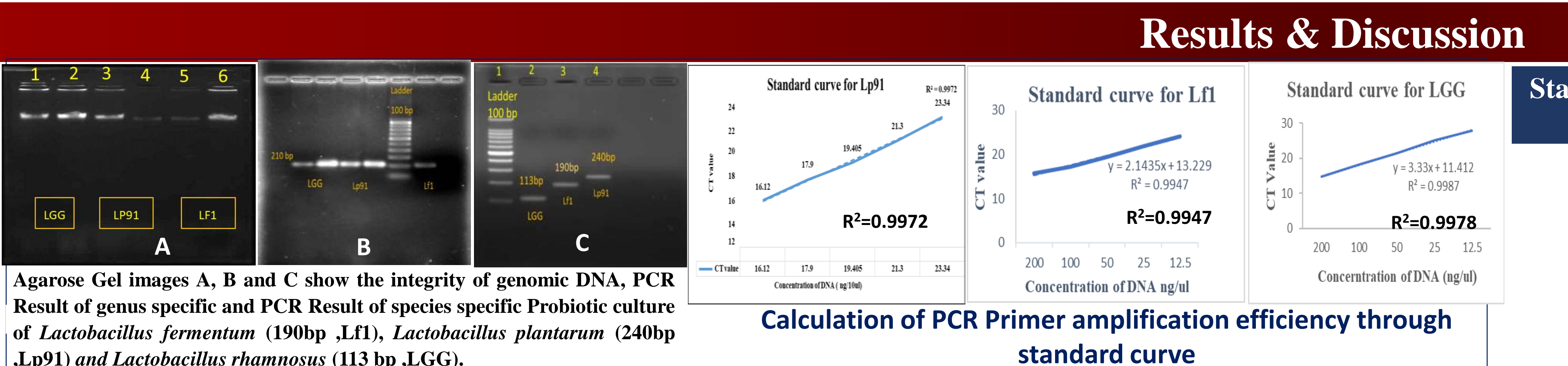
Methodology



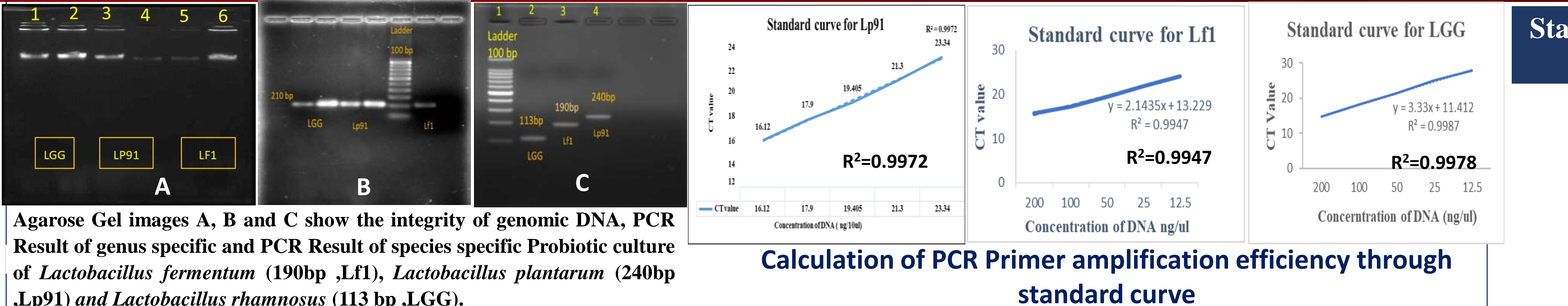
Objectives

- Objective 1: Development of PMA-qPCR based assay for quantification of probiotic *Lactobacillus* spp**
 - Activity 1: Preparation of standard plasmid to construct for absolute quantification of targeted probiotic bacteria
 - Activity 2: Optimization of time-temperature conditions to prepare heat-killed (dead) cells of probiotic bacteria
 - Activity 3: Optimization of condition for PMA-qPCR.
 - Development of PMA-qPCR based assay for quantification of probiotic *Lactobacillus* spp. in saline system
- Objective 2: Evaluation of developed assay in milk and dairy products**
 - Activity 1: Optimization of DNA extraction protocol from Probiotic Fermented milk and Probiotic Dahi
 - Activity 2: Absolute Quantification of Probiotic *Lactobacillus* spp. in Fermented milk and Probiotic Dahi & Milk

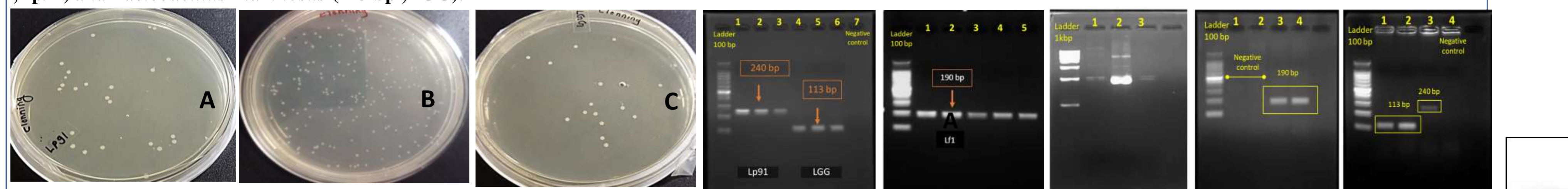
Confirmation of identity of *Lactobacillus* spp. through PCR



Results & Discussion

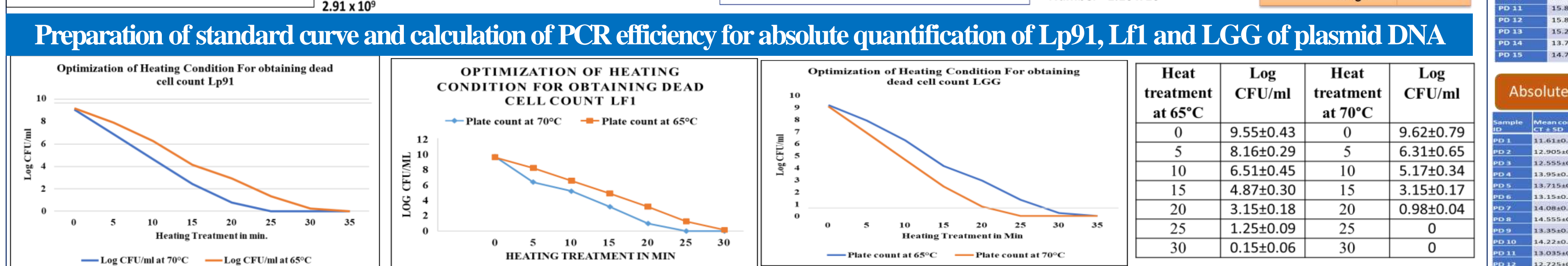
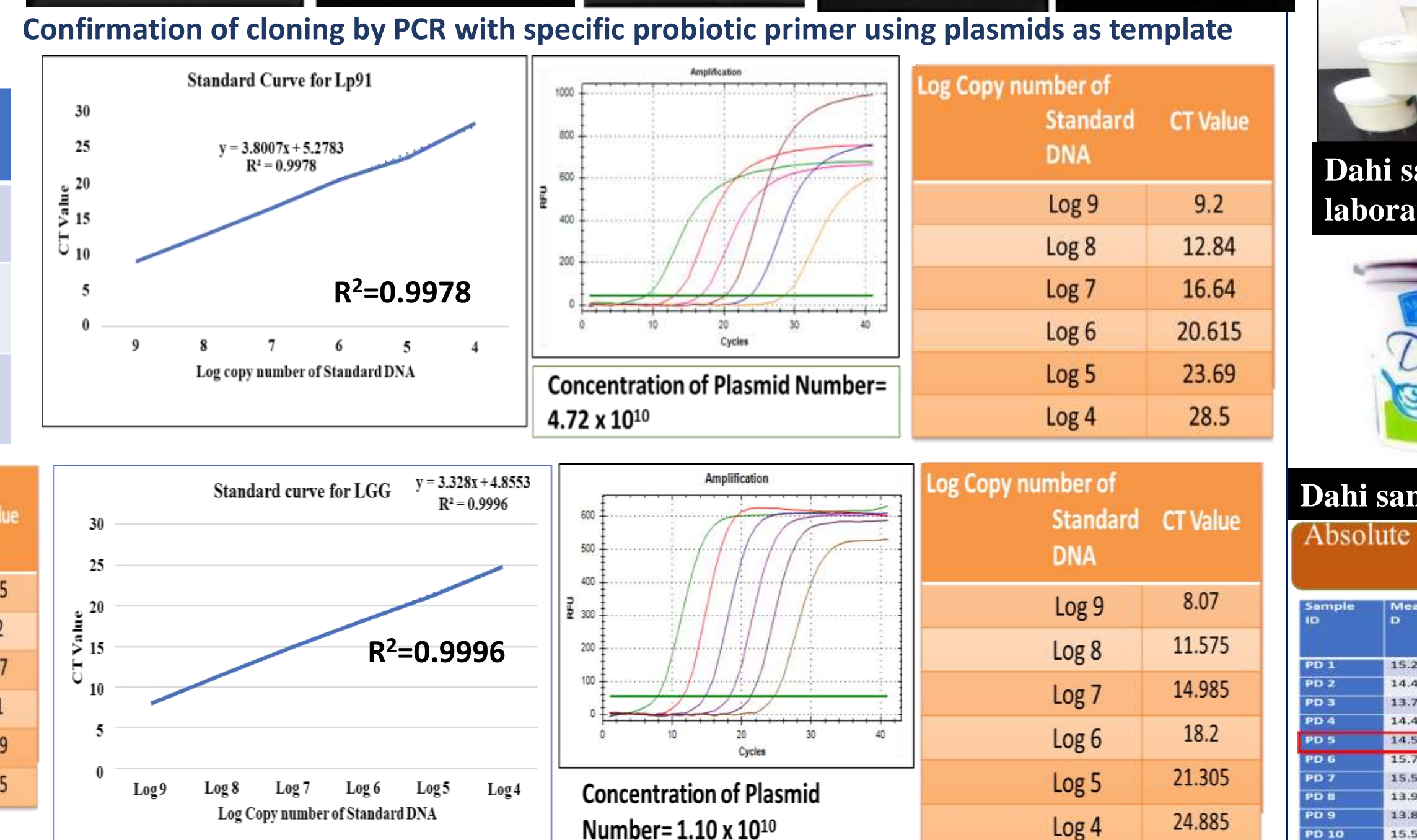


Calculation of PCR Primer amplification efficiency through standard curve

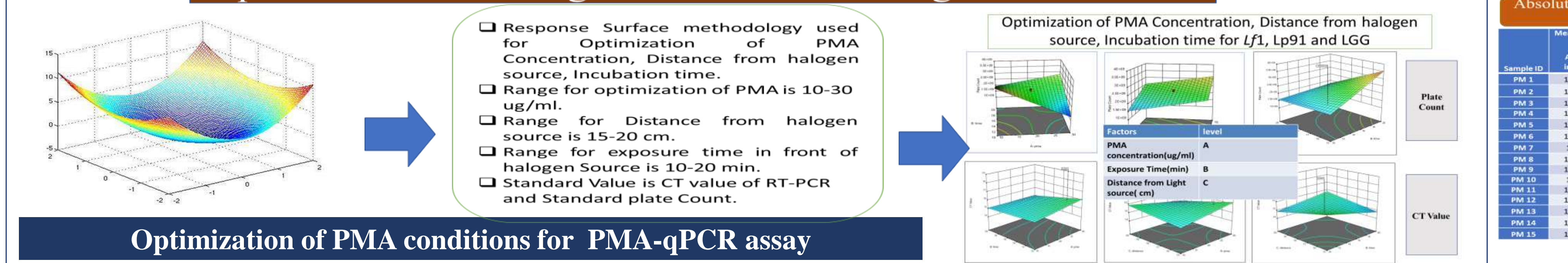


A, B, C represents Clones of target DNA (Lp91, Lf1, LGG) respectively.

S.No.	Name of Probiotic Lactobacillus species	Plasmid copy number
1	<i>Lactiplantibacillus plantarum</i> 91	4.72×10^{10}
2	<i>Limosilactobacillus fermentum</i> 1	2.91×10^{10}
3	<i>Lactocaseibacillus rhamnosus</i> GG	1.10×10^{10}



Optimization of Killing Method for Obtaining the Dead Cells



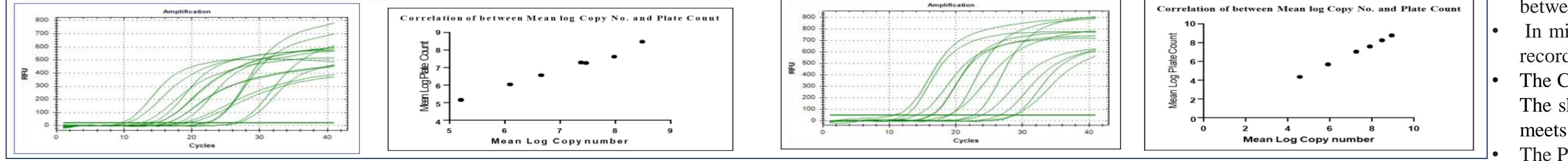
Optimization of PMA conditions for PMA-qPCR assay

Absolute Quantification of *Limosilactobacillus fermentum* 1 in saline.

Sample ID	Mean $C_T \pm S.D$	Mean log Copy No.	Plate Count
Saline 0 min	10.37 \pm 0.04	8.49 \pm 0.05	8.47 \pm 0.22
Saline 3 min	12.6 \pm 0.06	7.98 \pm 0.02	7.62 \pm 0.09
Saline 6 min	14.77 \pm 0.11	7.47 \pm 0.01	7.26 \pm 0.15
Saline 9 min	16.46 \pm 0.04	7.38 \pm 0.02	7.29 \pm 0.18
Saline 12 min	19.57 \pm 0.07	6.66 \pm 0.04	6.56 \pm 0.08
Saline 15 min	21.98 \pm 0.02	6.10 \pm 0.06	6.03 \pm 0.12
Saline 18 min	25.82 \pm 0.03	5.21 \pm 0.07	5.15 \pm 0.17

Absolute Quantification of *Lactiplantibacillus plantarum* 91 in saline.

Sample ID	Mean $C_T \pm S.D$	Mean log Copy No.	Plate Count
Saline 0 min	12.38 \pm 0.02	8.29 \pm 0.01	8.19 \pm 0.12
Saline 3 min	14.06 \pm 0.05	7.80 \pm 0.01	7.65 \pm 0.23
Saline 6 min	16.33 \pm 0.08	7.39 \pm 0.04	7.31 \pm 0.15
Saline 9 min	18.78 \pm 0.04	6.55 \pm 0.02	6.55 \pm 0.22
Saline 12 min	23.81 \pm 0.02	6.10 \pm 0.01	5.90 \pm 0.15
Saline 15 min	28.94 \pm 0.06	5.23 \pm 0.01	5.15 \pm 0.09



Concluding remarks

The assay could be an appropriate alternative for the existing time-consuming and laborious conventional pour plating methods for routine monitoring of probiotic count in probiotic dairy products at the designated threshold level as prescribed by FSSR.

Acknowledgements

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- The author also thank the MBU laboratory students and staff for helping in carrying out the research work.

Key message

The developed PMA-qPCR assay can be applied on the industrial scale as it is rapid and precise method for quantification of probiotic bacteria.

Accuracy: The closeness of the agreement between a test result and the accepted reference value

Accuracy: 0.91

Precision: %CV = (standard deviation of measurements / mean) x 100

Precision: 2%

Biassness: 1%

Accuracy: The closeness of the agreement between a test result and the accepted reference value

Accuracy: 0.86

Precision: %CV = (standard deviation of measurements / mean) x 100

Precision: 3%

Biassness: 1%

Precision of <10 and Accuracy of not less than 70% is very good for any method.

- The developed assay was able to quantify viable *Lactobacillus* spp. in buffer system with strong positive Pearson correlation of 0.999 between mean copy number of bacterial DNA and mean viable cell count.
- In milk matrix (e.g., fermented milk, Dahi), the correlation coefficient between developed assay and conventional plating method was recorded as 0.876-0.998.
- The CT value of Lf1 and Lp91 was approximately >14.65 and 14.7, respectively showed at less than 8 log copy no. of probiotic bacteria. The shelf life evaluation of probiotic dahi has showed 7 days of shelf life at 4°C with a log count of 8.09 and 8 log copy number that meets the FSSAI regulatory limits.
- The PMA-qPCR approach demonstrated an accuracy of over 86% in detecting *Lactobacillus* spp., with a precision range of 2-3%.
- Developed PMA-qPCR based assay is sensitive, accurate, precise, less biased, repeatable and reproducible method for the detection of probiotic *Lactobacillus* spp. in probiotic dairy products.