Full Length Article

VIABILITY PCR TO DETECT THE MOST-PROBABLE-NUMBER OF VIABLE PROBIOTIC BACTERIA IN COMMERCIAL PREPARATIONS

B. Vasanthi[1], K. Nirmala[2], K.G. Tirumurugaan*[3], Kumar Rounak[4], A. Alagumaruthanayagam[5], G. Dhinakar Raj[6] and M. Raman[7]

Zoonoses Research Laboratory
Centre for Animal Health Studies
Tamil Nadu Veterinary and Animal Sciences University
Chennai, Tamil Nadu, India

ABSTRACT

The polymerase chain reaction (PCR) modification that enables molecular diagnosis and detection viable cells from diverse samples is based on the Viability PCR (V-PCR). In this study, we optimized V-PCR using a candidate *L. plantarum* probiotic strain and the technique performed efficiently in detecting live cells from an admixed suspension of live and dead cells. Application of the V-PCR on different probiotics strains (*B. coagulans, L. plantarum and L. fermentum*) also revealed a strong positive correlation in its performance across the strains tested even with an admixture of varied concentration of live and dead cells and different dilutions respectively (r=0.93 to 0.98). We obtained five and four commercial probiotics available for humans and animal use respectively from the market and tested for the recovery of total viable bacteria by agar pour plate method and also the count of viable bacteria by V-PCR. All commercial preparations when tested by the pour plate method recovered their listed viable counts except for the probiotic B and F, and C & G revealed lower counts (<1 to 2 log, and <3 to 4 log respectively) than the the manufacturer's claims. A semi quantification approach using arbitrary density units with the universal 16S rRNA PCR amplicons following V-PCR to quantify the viable organism count from these commercial probiotic products also revealed a similar counts ($r^2 = 0.7$). Screening several commercial probiotic preparations indicated for human and animal health will enable us to develop this technique as a routine monitoring tool to determine the viability at the point of use rather than at manufacturer's site.

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*Corresponding author, email: tirumurugaan.k.g@tanuvas.ac.in; kgtanuvas@gmail.com

[1]ABCEP Trainee, Translational Research Platform for Veterinary Biologicals (TRPVB), Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India

[2]Junior Research Fellow, Translational Research Platform for Veterinary Biologicals, TANUVAS, Chennai, India

[3]Professor and Head, Zoonoses Research Laboratory , TANUVAS

[4]Senior Research Fellow, ABCEP Programme, Translational Research Platform for Veterinary Biologicals, TANUVAS, Chennai, India

[5]Post Doctoral Fellow, Translational Research Platform for Veterinary Biologicals, TANUVAS, Chennai, India

[6]Professor and Head, Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, India

[7]Project Director (i/c) (Rted), TRPVB, TANUVAS, Chennai, India

INTRODUCTION

Microorganisms include bacteria, fungi, protoctists, and viruses that are abundant in any ecosystem, with vast information available on bacterial species. To date about 159,000 species of bacteria have been identified (less than 5% of total existence) to be distributed across extreme habitats. For example, the number of bacteria inhabiting the various sites of the human/animal body is known to be approximately 100 trillion which is approximately 10 fold that of the host cells (Wu and Lewis, 2013). Noteworthy to mention in this context is the gut microorganisms in human and different animal species, the vital role they play in maintaining health and their influence on the immune system (Tremaroli and Bäckhed, 2012). Studies on the gut microbiome have enabled us to identify a collection of probiotic microorganisms in the market shelves whose administration in recommended amounts has resulted in health benefits. Even though the exact roles of probiotics are unclear, it is proposed that they are more likely to alter the function and the composition of the microbiota for health benefits (Weichselbaum, 2009; Bäckhed and Fraser, 2012; Sanders and Guarner, 2013). The worth of the global market of probiotics, supplements, and food was valued at $48.88 billion in 2019 and expected to grow at a CAGR of 6.7% from 2020 to 2027 to reach $76.7 billion by 2027.

The commercially available probiotics for human and veterinary application include Lactic acid bacteria (LAB), *Bacillus, Peptostreptococcus, Clostridium butyricum, Fusobacterium, Eubacterium* and *Bifidobacteria* with several inherent properties (Fioramonti and Theodorou, 2003). Molecular methods targeting nucleic acids (16S ribosomal RNA) such as polymerase chain reaction (PCR) have also enabled easier specific identification and classification. A combination of the 16S rRNA gene detection with the genus/species specific primer will reduce the effort in screening a large population of unknown organisms. In this context, the reported universal primer pair LbLMA1/ R16-1 amplifies a 213bp from the *Lactobacillus* genus and helps to identify 23 different *Lactobacilli* strains corresponding to three phylogenetic groups and 21 species of various origins with fermentative groups (Dubernet *et al.,* 2002).

The word 'probiotic' ('pro bios') reflects only viability or life (Lahtinen, 2012), but a major concern on the commercial probiotic products is the total count of the organisms that is viable at the time of consumption. Studies indicate the beneficial properties of viable products rather than using non-viable products. The crucial or critical counts to be used vary for each species of probiotics; with a general recommendation of 10^9 to 10^{10} colony forming units (CFU) as a daily dosage (Rupa, 2012; Tsuchiya et.al., 2004), used heat-inactivated probiotics in their study to conclude subjective development of the symptoms in 80% of the patients, compared to 40% in the viable group. A recent study on the recovery of viable bacteria by culture dependant approach from commercial probiotic brands for oral health indicated only one brand could be recovered within one log of the producer's starting amount of bacteria while the others resulted in a three-log decrease in the count (Bannas and

Popp, 2013). In addition, there is no existing regulatory requirement that insists on the count of the viable bacteria to be present in commercial preparations at the point of use.

Viability PCR (V-PCR) a modification of PCR to differentiate viable from dead cells could be an alternative to culture-based confirmation of microorganisms. This methodology was introduced as a fast and powerful tool to detect and quantify viable organisms from various sources and applies to various fields such as clinical microbiology, infectious disease evaluation, pathogen detection, health risk assessment of environmental and food samples. V-PCR uses a cell-permeable photoreactive dye that preferentially cross links to double-stranded DNA using the azide group with high affinity in membrane-compromised cells (usually dead cells). Upon exposure of such cells to strong visible light (of specific wavelength), it converts into a nitrene radical that is highly reactive to any organic molecule in its vicinity and hence strongly inhibit amplification (DeTraglia *et al.,* 1978; Nocker and Camper, 2006). Two major azide dyes namely Ethidium Mono Azide (EMA) and Propidium Monoazide (PMA) have been successfully used in various applications to detect viable microorganisms that include bacterial vegetative cells (Bae and Wuertz, 2009; Agusti *et al.,* 2010) bacterial spores (Rawsthorne *et al.,* 2009) fungi (Vesper *et al.,* 2008) viruses (Graiver *et al.,* 2010; Sanchez *et al.,* 2012) yeast (Andorrà *et al.,* 2010; Shi *et al.,* 2012) and protozoa (Brescia *et al.,* 2009; Fittipaldi *et al.,* 2011).

With potential applicability of the V-PCR and documented reports on variation in the counts of the live organisms in the commercial probiotic preparations, this study was planned to optimize V-PCR for detecting live and dead organisms from probiotic cultures and assess the viable load from commercial products available for human and veterinary applications at the market shelf.

MATERIALS AND METHODS

Bacterial strains and other molecular biologicals

Lactobacillus plantarum an in-house probiotic strain was used for all the optimization studies. Commercial probiotic preparations from the market shelf included different combinations of probiotic organisms and the details are listed in (Table 1). Other reagents include the culture media the *Lactobacilli* deMan, Rogosa and Sharpe (MRS) (Hi Media Laboratories, Mumbai) and Tryptic Soy Agar (TSA) (Difco laboratories, NJ) with 5% Sheep‘s blood, Lysozyme and Proteinase K (MP Biomedicals, CA); Propidium Mono Azide (PMA) (Biotium, CA); Emerald Amp GT PCR master mix (Takara, Japan) and primers for PCR (synthesized from Sigma, India and Eurofins, India).

Preparation of probiotic cultures with different viable and dead counts

One milliliter of the above standard culture (a single colony up scaled from the standard culture) with 10^9 CFU/ml was transferred into five tubes. Culture volumes of 100 µl, 200 µl, 400 µl, 600 µl, and 800 µl were removed from each tube, exposed to 90^{o}C for 20 min to heat kill the cells and were transferred back to the respective

Table 1. Viable bacteria recovered from commercial probiotic preparations by pour plate method

Commercial Probiotic Preparation	Listed count	Total Viable Count (by CFU on TSA agar plates)
Products for use in Humans		
Probiotic A (Tablet)	**1.66 x 10^8 spores**	6.73E+07
Streptococcus faecalis	:→ 60 million	(± 3.73E+05)
Lactobacillus sporogens	:→ 100 million	
Clostridium butyricum	:→ 2 million	
Bacillus mesentricus	:→ 1 million	
Probiotic B (Sachet-0.5g)	**8.3 x 10^7 spores**	2.70E+07
Streptococcus faecalis	:→ 30 million	(±3.6E+05)
Lactobacillus sporogens	:→ 50 million	
Clostridium butyricum	:→ 2 million	
Bacillus mesentricus	:→ 1 million	
Probiotic C (Tablet)	**Not less than**	6.07E+08
Lactobacillus acidophilus	**1.25 x 10^9 spores**	(± 3.45E+05)
Lactobacillus rhamnosus		
Bifidobacterium longum		
Saccharomyces bouolardii		
Probiotic D (Tablet)	**Not < 1.50 x 10^8Spores**	3.00E+06
Lactobacillus acidophilus		(± 1.47E+04)
Probiotic E (Powder)	**Not < 1.50 x 10^8Spores**	5.5E+07
Lactobacillus sporogenes		(± 6.3E+05)
Products for use in Animals		
Probiotic F (Powder)	**2.2 x 10^7 CFU/g**	1.80E+07
Bacillus subtilis		(± 2.82E+05)
Probiotic G (Powder)	**10^{12} CFU/g**	2.00E+07
Lactobacillus acidophilus		(± 2.82E+07)
Lactobacillus casei		
Lactobacillus bulgaricus		
Streptococcus lactis		
Bacillus subtilis		
Saccharomyces cerevisiae		
Probiotic H (Powder)	**3 x 10^7 /g**	3.37E+ 06
Lacobacillus viable spores		(± 1.4E+05)
Probiotic I (Powder)	**10^{12} CFU/g**	2.00E+ 07
Selective strains of 13 essential species of direct-fed microbials (Lacobacillus sp., Bacillus sp., Acetobactor sp. and Saccharamyces sp.)		(± 1.40E+06)

*All the probiotic preparations were obtained from the market shelf and the composition of the probiotic preparations has been mentioned leaving the trade name. * TSA was supplemented with 5% sheep blood to support the growth of most of the bacteria.*

original tubes to result in probiotic cultures with known counts of viable and dead cells. These cultures were centrifuged at 10000 rpm for 10 min, resuspended in 400µl saline, and processed for V-PCR. Ten-fold serial dilution of live cells 10^9 until 10^6 CFU/ml was also processed for V-PCR. Both the procedures were also repeated with *Bacillus coagulans* and *L. fermentum* cultures and the efficiency of the V-PCR in determining the counts of viable bacteria were also correlated.

Recovery of viable bacteria from commercial probiotic preparations

The commercial probiotic preparations probiotics for animal and human use as listed in (Table 1); 1g in the case of powder & the complete preparation in case of a tablet were re-suspended in 10 ml of nutrient broth and revived for an hour at room temperature (RT). A standard serial dilution and plating technique in TSA with 5% sheep blood was performed to determine the total count of viable bacteria in each probiotic preparation tested (Jeffrey et.al., 2013) using the formula [(No. of colonies x dilution factor) / volume of culture plated]. The total viable count in the different commercial probiotic preparations was correlated with the listed counts and also the counts determined by V-PCR.

Viability PCR to detect live and dead probiotic organism

PMA treatment of cultures

PMA was used in this study to selectively modify DNA from dead cells that had compromised membrane integrity, while leaving DNA from viable cells intact. A final concentration of 50 µM was added to the bacterial suspension (in 400 µl sample volume) from a stock solution 200mM and incubated for 5 min at RT before they were subjected to photolysis.

Photolysis

Photolysis was performed using an indigenously built thermally stable blue Light Emitting Diode (LED) light source to link PMA to dsDNA. The device provides a high-density wavelength (4 units) for 20 min to nick the dye-bound nucleic acids with high affinity. Following photolysis, the samples were centrifuged at 10,000 rpm for 10 min, the supernatant discarded and the pellet was used for DNA extraction.

DNA extraction

PMA treated samples (live cells, dead cells, bacterial standards with mixtures of viable and dead cell counts and commercial preparations) were further processed for extracting DNA using high salt method (Kumar and Ramadass, 2001). The PMA treated bacterial pellet (from different samples) was re-suspended in 200 µl of TKM buffer (10 mM TrisHCl, 10 mM KCl, 10 mM $MgCl_2$) with 80 µl lysozyme (10 mg/ml), 40 µl of Proteinase K (10 mg/ml) and incubated at 37ºC for 30 min. To this suspension, 50 µl of 10% sodium dodecyl sulfate (SDS) and 250 µl of 6M Sodium Chloride were added and mixed thoroughly. The samples were centrifuged at 12,000 rpm for 10 min; the supernatant was mixed with an equal volume of isopropyl alcohol, incubated at room temperature for 10 minutes and centrifuged at 12,000 rpm for 10 min to precipitate the DNA. The DNA

precipitate was washed with 500 µl of 70% ethanol and re-suspended in LTE (10mM TrisHCl, 1mM EDTA) buffer by incubating in a 65ºC water batch for 10 min. The concentration of the DNA across the samples were determined in Multimode Reader (Tecan infinite M200 PRO, Switzerland)

Polymerase chain reaction (PCR) to detect live and dead bacteria and semi-quantification

An equal volume (3 µl aliquot) of diluted DNA sample from the different samples was used in a PCR with the *Lactobacilli* genus-specific primers LbLMA1F – 5' CTCAAAACTAAACAAAGTTTC - 3' and R16-R – 5' CTTGTACACACCGCCCGTCA 3'(Dubernet *et al.,* 2002) and the universal primer targeting 16S gene namely U16RTF 5' ACTCCTACGGGAGGCAGCAGT 3' and U16SRTR 5' TATTACCGCGGCTGCTGGC 3' to detect the live and dead bacteria. The PCR cycling conditions included 5 min at 94ºC followed by 30 cycles of 45 sec at 94 ºC, 45 sec at 60 ºC, and 45 sec at 72ºC; a final extension at 7 min at 72 ºC. The products were visualized after electrophoresis in a 1.5% agarose gel with Tris Acetate buffer. For a semi-quantitative approach, the PCR reaction was stopped at 25 cycles, the amplicons electrophoresed, image documented and band intensity measured in arbitrary units using Image J software (available at http://imagej.nih.gov/ij/index.html). Linear regression was developed for the arbitrary densitometry units (ADU) to the different standard counts of live and dead cells and used to predict viable bacterial counts in unknown samples.

V-PCR to quantify viable organism count from probiotic preparations

The commercial probiotic products (listed in Table 1) were re-suspended in 10 ml of PBS and 1 ml of the suspension was processed for V-PCR to detect viable organisms employing the universal 16S primer. The PCR amplicons were electrophoresed in a 1.5 % gel and the band intensity was measured in the Image J software. The ADU for each probiotic preparation was used in the linear regression curve developed with a mixture containing standard counts of viable and dead bacteria to predict the viable count and the results were compared with the count obtained by plate method.

RESULTS AND DISCUSSION

Recovery of viable bacteria from commercial probiotic preparations

Four different commercial probiotics from the market shelf were tested for the total viable bacterial count employing TS agar with 5% sheep's blood that supports the growth of most of the bacterial species. The preparation test that had *Lactobacillus* sp. as a constituent was also tested on MRS agar to selectively look at the count of viable *Lactobacillus* species and there was a great variation in the no of bacteria recovered. The manufacturers claim on the number of viable bacteria in the commercial preparations that were tested in this study ranged from roughly 83 million (8.3×10^7) to 1.66 billion (1.66×10^8) indicated for human use and 3 x 10^7 to 10^{12} CFU/g of the powder indicated for animal use (Table 1). The probiotic B and F had the highest total viable count of 2.7E+07 ($\pm$3.60E+05) and

1.80E+07 (± 2.82E+05) respectively while probiotic G and Probiotic I had the lowest viable count of 2.00E+07 (± 2.82E+07) and 2.00E+07 (± 1.4E+06) respectively in comparison with the listed counts (Table 1). Except for probiotic B (human preparation) and probiotic F (veterinary preparation) all the other preparations resulted in overall lower counts than listed (ranging from 2 to 4 logs less).

Optimizing the V-PCR with pure cultures of *Lactobacillus sp*.

The candidate strain *L. plantarum* was confirmed for its characteristics namely growth in selective agar (MRS), gram-positive rods and also the amplification of a 213 bp product by PCR with the *Lactobacillus* genus-specific primers LbLMA1-rev and R16-1 (Dubernet *et al.,* 2002) (Fig. 1). A quick survey of the probiotic preparations revealed a maximum concentration of billion cells (10^9) in the final product for use (except for two preparations that had 10^{12} CFU/g). Hence, all the optimization experiments were performed with 10^9 cells of bacteria that matched to scale number 4 of McFarland standard turbidity. The optimization experiments included treatment with different concentrations of PMA, the intensity and time of exposure in the photolyser, a combination of both PMA and photolyser before subjecting the samples to DNA extraction and subsequent amplification before arriving at a concentration of 50µm PMA and 4 units of the blue LED treatment (data not shown). Treatment to 50 µm PMA for 5 min at room temperature (Fig. 2A and 2B) or exposure to only 4 units of blue LED for 20 min without PMA treatment (Fig. 2C and 2D) slightly reduced the amplification intensity in V-PCR which could not visibly differentiated both live and dead bacteria respectively. However, a combination of 50 µM PMA final concentration at room temperature for 5 min and exposure to high-density wavelength of the blue LED (4 units) for 20 minutes significantly reduced the amplification intensity of the 213bp V-PCR amplicon across live and dead cells (Fig. 2E). Hence, incubation with 50µm PMA for 5 min at room temperature followed by a 20 min exposure to blue LED was the optimal condition in which the amplification of DNA from dead *L. plantarum* cells was suppressed (more than 99.9%) with no significant inhibition in the amplification of DNA live cells. Using the above-established conditions, our preliminary results for the repeatability of V-PCR were evaluated with live and heat-killed *L. plantarum* cells on 6 independent occasions at the maximum cell density of 10^9. The arbitrary densitometry units (AD units) for the V-PCR on live and dead cells were 169.51± 33.23 and 20.89±7.41 respectively (Table 2). Application of V-PCR on different probiotic strains (*B. coagulans, L. plantarum and L. fermentum*) with mixtures of different concentrations of live and dead cells was found to be linear with an R^2 value of 0.9 across all the three strains (Fig. 3A and 3B). The V-PCR also revealed a strong positive correlation in its performance across the three strains tested both in an admixture of different concentration of live and dead cells and also at different dilutions respectively (r=0.93 to 0.98 for V-PCR in (Fig. 3A) when tested within a narrow range between 2 E+08 to 1E+09 and r=0.84 to 0.96 for V-PCR in (Fig. 3B) at a wider concentration range between 10^5 to 10^9CFU/ml).

Table 2. Repeatability of the V-PCR method

Sample	Live *L. plantarum* cells		Heat-killed *L. plantarum* cells	
	Log $_{10}$ CFU/ml *	AD units	Log $_{10}$ CFU/ml *	AD units
Untreated control	9		9	
PMA + Photolysis replicate				
1	9	103.24	9	10.96
2	9	186.13	9	27.49
3	9	185.13	9	27.08
4	9	171.31	9	22.97
5	9	191.77	9	24.67
6	9	179.97	9	12.19
Mea AD units ± SD	**169.51± 33.23**		**20.89 ±7.41**	

** The Log 10 CFU/ml of the L. plantarum cultures were fixed based on the McFarland turbidity standard; AD units – Arbitrary densitometry units (determined using ImageJ)*

Table 3. Comparison of the listed counts Vs the viable count determined by plate method and V-PCR

Commercial Probiotic	Listed Count (CFU)	Viable count (CFU/ml)	V-PCR AD units	Predicted CFU/ml
		Products for use in Humans		
Pro A	1.66E+08*	6.73E+07	229.82	8.92E+08
Pro B	8.30E+07*	2.70E+07	39.13	3.37E+07
Pro C	Not < 1.25E+09*	6.07E+08	220.92	8.68E+08
Pro D	Not < 1.5E+08*	3.00E+06	24.21	4.16E+06
Pro E	Not < 1.5E+08*	5.50E+07	33.81	5.22E+07
		Products for use in Animals		
Pro F	2.20E+07	1.80E+07	35.39	3.51E+07
Pro G	1.00E+12**	2.00E+07	29.45	3.04E+07
Pro H	3.00E+07*	3.37E+06	24.27	4.46E+06
Pro I	1.00E+12**	2.00E+07	33.78	3.70E+07

* These preparations include spore forming organisms and hence should be more stable

** These preparation included *Saccharomyces sp*. and the counts of the individual probiotic organism in the consortia are not mentioned. The lower viable count by plate and V-PCR (V-PCR has not been optimized for yeast) could be due to higher levels of the yeast in the preparation.

Note: Except for one commercial preparation indicated for use in humans (Probiotic B) / animals (Probiotic F) there is one to two log lower CFU measured by plate method or predicated by V-PCR in comparison with the listed counts.

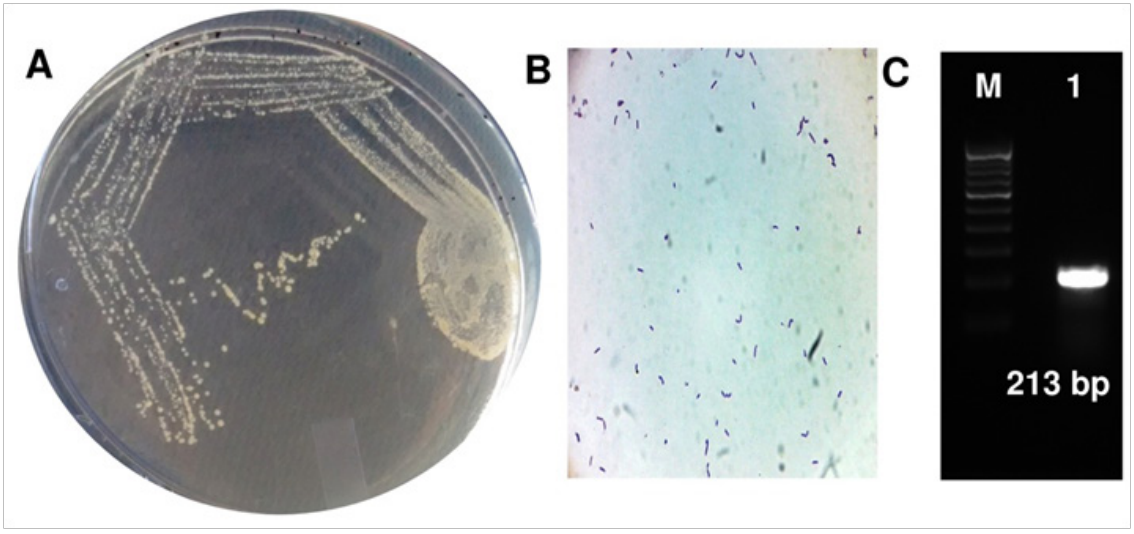

Fig. 1. Candidate probiotic strain (*L. plantarum*). The candidate probiotic *L.plantarum* (ORO-CUTETM) was characterized for its (A) growth in specific media deMan, Rogosa and Sharpe (MRS agar); (B) gram-positive rods; and (C) a 213 bp PCR amplicon with *Lactobacillus* genus-specific primer pair

V-PCR for semi-quantification from commercial probiotic products

To develop an approach for semi quantification and the influence of live/dead organism's ratio in a sample on V-PCR was evaluated. From 5 tubes each with 1 mL of 10^9 CFU/ml, volumes of 100, 200,400,600, 800 µl respectively were removed, heat-killed and replaced into the respective tubes to result in serial dilutions of live and dead. The above dilution resulted in a viable cell count of 10^8 to 10^2 CFU/ml admixed with dead cells. The V-PCR method showed good linearity, i.e., arbitrary density unit's proportional to the viable *L. plantarum* concentration with the 180 bp amplicon of the universal primer than with the 213 bp of the *Lactobacilli* genus-specific primer (Fig. 4A, 4B and 4C). The signal in the V-PCR gradually decreased with decreasing concentrations of viable *L. plantarum* cells. The arbitrary density unit's (AD units) ranged from 213 to 35 and 190 to 56 for the above dilution of cells with universal primer (U16SRTF / U16SRTR) and *Lactobacillus* primer (LbLMA1F / R16-R) respectively. Plots of the cell concentration for the proportion of viable and dead *L. plantarum* cells present in mixtures to the arbitrary density units are provided in the graph in (Fig. 4).

The probiotics preparations indicated for human (Prob. A, B, C, D & E) and animal use (Prob. F, G, H & I) available at the market shelf resulted in a 180bp amplicon of different intensity with the universal 16S rRNA primer pair. The listed counts in the products A, B,

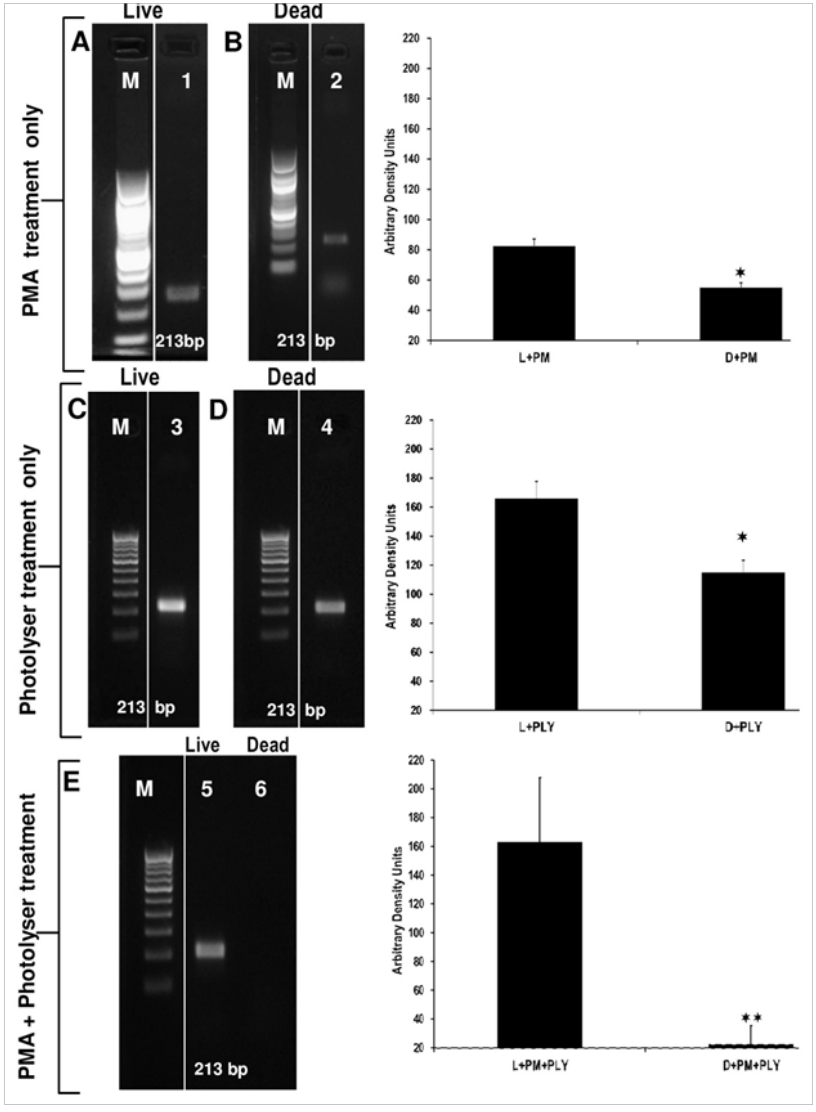

Fig. 2: Optimization of viability PCR (V-PCR) with pure cultures of *Lactobacillus plantarum* a candidate probiotic strain (Orocute™) was grown overnight in MRS broth, turbidity adjusted to 10^9 CFU/ml using McFarland standard and used for optimizing viability PCR. A & B; Effect of PMA treatment only on live & dead cells respectively: C & D Effect of photolyser exposure only on live & dead cells respectively and E: Effect of PMA and photolyser treatment on live & dead cells respectively. M - 100 bp DNA ladder: Lanes 1, 2, 3, 4 & 5 amplification of 213 bp Lactobacillus genus-specific amplicon. *Note: Complete absence of PCR amplification in dead cells following PMA and photolysis treatment (lane 6) indicating an optimal condition for V-PCR.*

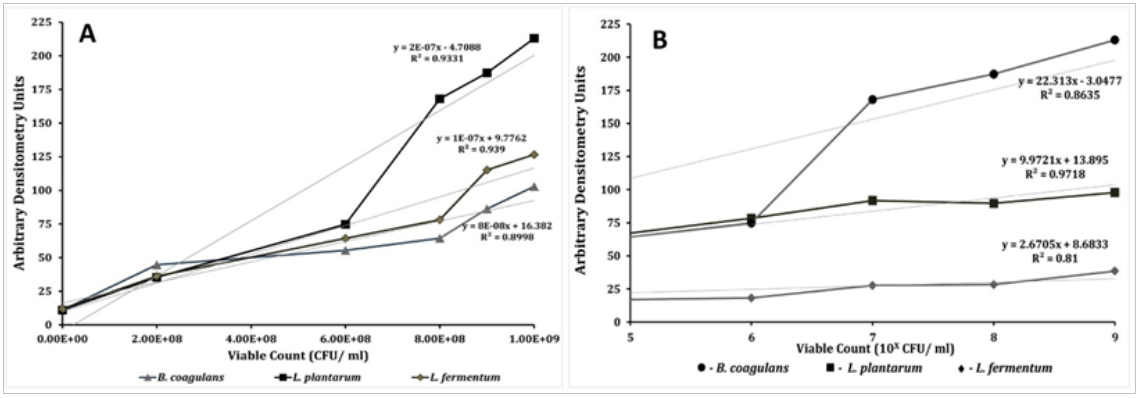

Fig.3: V-PCR in a mixture of different counts of live and dead cells across different probiotic strains (*B. coagulans, L. plantarum and L. fermentum*). The performance was tested at different CFU counts of live bacteria in a culture with total counts were maintained at 10^9 CFU/ml. **A)** 2 x 10^8 to 1 x 10^9 CFU of live bacteria and **B)** 10^5 to 10^9 CFU of live bacteria. *Note: The performance of V-PCR was found to be linear with an R2 value of 0.9 across all three strains.*

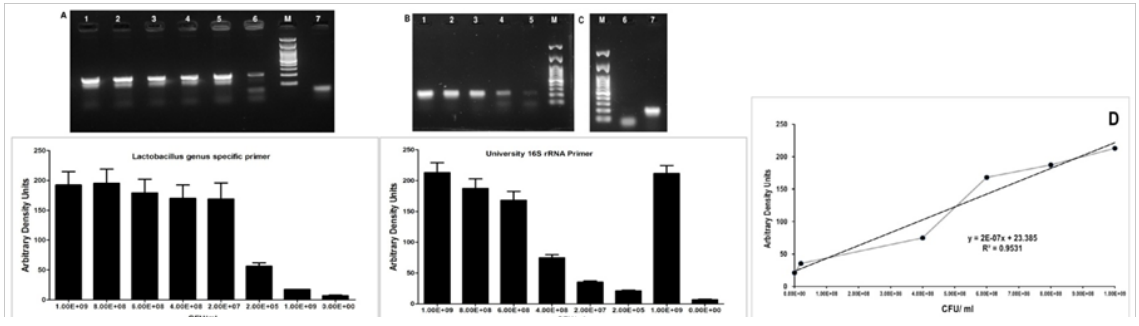

Fig. 4: Viability PCR for semi-quantification of live and dead probiotic organisms from standard cultures. V-PCR was performed on 5 tubes each with 1 mL of 10^9 CFU/ml, volumes of 100,200,400,600,800µl respectively were removed, heat-killed and replaced into the respective tubes to result in serial dilutions of live and dead. The above dilution resulted in a viable cell count of 10^8 to 10^2 CFU/ml admixed with dead cells. **A**) V-PCR 213 bp amplicon with Lactobacillus genus-specific primer (Lanes 1,2,3,4,5,6 & 7); **B)** V-PCR 180 bp amplicon with universal 16S rRNA primer (Lanes 1,2,3,4,5); **C)** V-PCR reaction in dead and live cells (Lanes 6,7); **D)** Linear regression curve generated using the AD units of the PCR amplicon with Universal primer **(B)** to be used for semi-quantification. *Note: The good linearity (r2=0.9531) the band intensity of the 180 bp PCR in the complex mixture of viable and dead bacteria admixture*

C, D & E for human use were 1.66E+08, 8.3E+07, Not < 1.25E+09, Not < 1.5E+08 and Not < 1.5E+08 respectively. The viable count for these products as determined by plate count vs V-PCR were 6.73E+07 vs 8.92E+08, 2.70E+07 vs 3.37E+07, 6.07E+08 vs 8.68E+08, 3.00E+06 vs 4.16E+06, 5.50E+07 vs 5.22E+07 respectively (Table 3). The listed counts in the products F, G, H & I for animal use were 2.20E+07, 1.00E+12, 3.00E+07 and 1.00E+12 respectively. The viable count for these products as determined by plate count vs V-PCR were 1.80E+07 vs 3.51E+07, 2.00E+07 vs 3.04E+07, 3.37E+06 vs 4.46E+06, 2.00E+07 vs 3.70E+07 respectively (Table 3). The viable counts by plate method and the V-PCR had an acceptable correlation (r^2 = 0.70) and only two of the probiotic preparations B and F showed viable organisms correlating to the listed counts of the manufacturer. All the other preparations tested revealed a one to two log reduction in the CFU in comparison with the listed counts both by the plate and the V-PCR method.

PCR a widely applied molecular technique for specific detection of target nucleic acid is the method with several reported modifications to enable its application in microbial diagnostics. However, there has been a problem in its application to differentiate genome amplification from viable and non-viable cells. V-PCR is a technique to suit such a purpose as it enables detection of target nucleic acid and differentiates its source to be either viable or dead cells. In this study, we have optimized V-PCR for discriminating viable from non-viable probiotic bacteria and also its preliminary application in quantifying the viable organism in commercial probiotic preparations from the market shelf.

The important criterion in a viable bacterium is its membrane integrity (Josephson *et.al.,* 1993), and this characteristic feature has been targeted in a wide variety of applications including V-PCR. The intact membranes from live cells can exclude DNA-binding dyes such as EMA and PMA that easily enters dead cells with compromised membrane. The combination of such dyes with PCR selectively differentiates viable cells from mixed bacterial population. This approach to differentiate viable and dead cells of pathogens such as *E. coli*, *Salmonella sp.*, *Listeria sp.* and *Campylobacter jejuni* was initially employed (Rudi et al., 2005). Despite the advantages of V-PCR and its application, the challenges include its utility to different samples and the two above-listed dyes which have specific advantages and disadvantages. In addition, the other factors to be considered are the dye concentration, incubation conditions, light source, presence of a high number of dead cells, suspended solids, pH of the mix and length of the target genes.

In the context of useful microbes, a huge classification of 'microorganisms' that possess an inherent property of viability "the probiotics" have been administered for several health applications in humans and animals. Cell viability and activity are pre-requisites to determine the potential mechanisms for probiotic action and hence many of the clinical studies have been performed with viable probiotics and are in the dose range of 10^9 to 10^{10} CFU. Thus, the dehydrated strains of probiotics available in the market

shelf prescribed for several purposes should meet this essential criterion. However, there is no regulatory document that stresses the necessity of probiotic preparations to maintain the minimum required viable count to produce the claimed beneficial effects. A recent study on probiotics used for oral health provides data with scientific evidence to prove that only one brand could be recovered within one log of the manufacturer's listed concentration (Jeffrey *et al.,* 2013).

We procured five and four different health-related human and animal probiotic brands respectively (single and multi-strain) with available expiration date limits from the local store and tested them within a week to look at the viable count and did not aim to address the ability of these brands to accomplish the health claims of the manufacturer's. Storage conditions were as mentioned on the label (usually cool, dry place for all the brands). All the human probiotic brands had spore-forming bacteria in the consortia and hence assumed to be more stable. One of the human probiotics (Prob. B) and two of the probiotics indicated for animal use (Prob. G & I) also included *Saccharomyces sp.* as a part of the consortia. All the above strains grow very well on blood agar. The listed manufacturer's claim for the commercial probiotic preparations that were tested in this study ranged from roughly 83 million (8.3×10^7) to 1.66 billion (1.66×10^8) indicated for human use and 3 x 10^7 to 10^{12} CFU/g of the powder indicated for animal use (Table 1). For the number of viable bacteria listed in the commercial preparations, only Prob. B and Pro. F met the manufacturer's claims (a viable count of 2.07E+08 and 1.80E+07 to the list count of 8.30E+07 and 2.20E+07 respectively). All the other commercial preparations tested for their viable count recovered one to two logs less than the listed viable count and the except for Prob. C and Prob. G yielding three to four logs less. The observed lower viable counts in the Prob.C and Prob. G could be explained due to the availability of *Saccharomyces sp.* in these preparations as the viable count protocol followed favored bacterial growth. The reduction in the viable count could also be due to the decreased stability of the *Lactobacillus* species in the preparations as the viable counts were lower in the selective MRS agar (data not shown). With this information as a background, we hypothesized that V-PCR can help in getting quick information on the viable count, and in this study, preliminary attempts to look into the performance and application of V-PCR to determine viable organisms from probiotic preparations at the market shelf.

We used the candidate probiotic strain *L. plantarum* to optimize the V-PCR assay. The candidate strain was confirmed to *Lactobacillus sp*. by its growth characteristics in MRS agar, gram-positive, rods and amplification of the 213 bp amplicon (Dubernet *et al.,* 2002). In this approach PMA dye, which is identical to propidium iodide (PI) except for the azide group has been used to cross-link the dye to DNA upon light exposure (Nocker and Camper, 2006).The extensive utilization of PI to identify dead cells in a mixed population and the higher charge in PMA (two charges) are the basic reasons for the choice of PMA in our V-PCR approach. The V-PCR approach used 50 µM concentration of PMA for 5 minutes (Fittipaldi *et al.,* 2012) at room temperature as earlier studies of 100 µM resulted only in

minimally reduced colony counts and choose primers that resulted in smaller size amplicon *Lactobacilli* genus-specific 213 bp amplicon (Dubernet *et al.,* 2002) and 180 bp universal amplicon (Clifford *et al.,* 2012) since a better differentiation was observed as reported in earlier studies. Exposure of live and dead *L. plantarum* cells to 50 µM PMA alone resulted in arbitrary density units (ADU) of 82.62 and 55 respectively indicating a 32% reduction in the amplification of the 16S RNA target in dead cells. Photolyser application to live and dead cells on the contrary without PMA treatment provided ADU of 165.8 and 114.7 respectively indicating a 31% decrease in 16S RNA target in dead cells. However, a combination of PMA (50 µM) and photolyser (20 min at 4 wavelengths) resulted in an ADU of 162.98 and 22.33 respectively in live and dead cells thereby resulting in an 87% decrease in amplification of the 16S RNA target which can be visibly appreciated and also helped in easy detection of viable cell population and this optimized conditions were used for further studies.

The efficiency of the V-PCR method also was found to be affected by the ratio of live and dead cells in the sample. A critical ratio of 10^4 to 10^3 live/ dead was found to provide a linear relationship between the Ct number and the number of viable cells in earlier studies (Wang et.al., 2009; Chen and Chang, 2010). In this study, we prepared an admixture of viable and dead cells as listed in the methods to result in a suspension of 10^8 to 10^2 viable CFU/ml admixed with 10^2 to 10^8 dead CFU/ml respectively. The V-PCR method revealed good linearity ($r^2 = 0.9531$) with the ADU ranging from 213 to 35 with the universal primer pair. A plot of the proportion of viable cell concentration to ADU for the universal primer was used to determine the viable count of the probiotic organisms from commercial probiotic products.

Many studies even though reported consistencies between plate counts and V-PCR in detecting several pathogens but the same has not been applied to screen commercial probiotic preparations. Hence V-PCR was used as a preliminary attempt in screening commercial probiotic preparations from market shelves indicated for human and animal use. A good correlation was found in our study in the optimization of the V-PCR with the candidate *L. plantarum* with 10^9 cells (a total count found in many of the probiotic products) that were performed on 6 different batches of the culture on different occasions. The predicted CFU/ml based on the V-PCR ADU revealed a similar count as determined by the plate method except for the Prob.A and Prob.C in which the counts were similar to that of the listed counts of the manufacturer. The results of the V-PCR correlated well with the approved method of culture-based viable count determination. The discrepancy reported in some studies is due to the presence of sublethally injured cells due to stress exposure that can temporarily compromise membraneintegrity and allow low dye uptake leading to a higher count in the V-PCR (Nocker *et al.,* 2007). To overcome this and also to potentially minimize the discrepancy we performed a short incubation in recovery medium (30 min. incubation) before determining the count by plate or the V-PCR method as already reported (Andorrà .*et al.,* 2010; Shi *et al.,* 2012).

CONCLUSION

In this study, we have optimized a V-PCR for its ability to differentiate live and dead also probiotic bacteria and developed a semi-quantitative approach for determining the viable organism count in commercial probiotic products. Application of V-PCR to probiotics will allow screening and monitoring the status of the commercial products at the market shelf as there exists no regulatory requirement on to the viable counts in these preparations at the user point in our country. Screening and application of this assay with several probiotic preparations indicated for human and animal health can enable us to develop this technique as a routine tool for monitoring the viability at the point of use rather than at the manufacture's site.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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