

Full Length Research Paper

# Differential cultivation and enumeration of *Lactobacillus plantarum* (PTCC 1058) in the presence of yogurt starter bacteria

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This study was aimed at assessing the different selective media and incubation conditions for achieving an appropriate method in the isolation and enumeration of *Lactobacillus plantarum* PTCC 1058, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in probiotic yogurt. The yogurt samples containing 1% of *L. plantarum* PTCC 1058 were prepared using YoFlex Express cultures (containing *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) and stored at 4°C. After 24 h, they were cultivated in seven different agar media including: de Man, Rogosa and Sharpe (MRS) agar, MRS-Bile agar, acidified MRS agar (pH = 5.2), yogurt-lactic-agar (YL), Elliker's lactic-agar (EL), M17 medium and *S. thermophilus* (ST) agar as pour plate and surface plate culture, and were incubated in aerobic and anaerobic conditions at three different incubation temperatures: 15, 37 and 45°C, for 48 to 72 h. For more practical examination, *L. plantarum* PTCC 1058 and yogurt bacteria were enumerated every week over 6 weeks. Also, the ability of *L. plantarum* PTCC 1058 and yogurt bacteria to grow in concentration of 0.5 to 5% of bile salts were investigated. The results indicated that both *L. plantarum* PTCC 1058 and *L. bulgaricus* could grow in low concentration ( $\leq 2\%$ ) of bile salts. So, MRS-Bile-agar medium was not suitable for differentiation of *L. plantarum* PTCC 1058 and yogurt bacteria. The best condition for differentiation of *L. plantarum* PTCC 1058 from yogurt bacteria was cultivation on MRS medium and incubation at 15°C for 72 h. YL and EL media and aerobic incubation at 37°C for 48 h were suitable for enumeration of *L. bulgaricus*, and surface plate culture in YL, M17 and ST media and aerobic incubation at 45°C for 48 h was useful for enumeration of *S. thermophilus* in yogurt.

**Key words:** *Lactobacillus plantarum* PTCC 1058, probiotic yogurt, differential culture, yogurt starter bacteria.

## INTRODUCTION

Probiotics are live microorganisms that have beneficial health effects on the host when they are in adequate administration (FAO/WHO, 2001). Lactic acid bacteria are common microorganisms used as probiotics (Lourens-Hattingh and Viljoen, 2001). Lactobacilli

represent a large group of lactic acid bacteria. *Lactobacillus* genus contains many species that are often classified as probiotics, such as *Lactobacillus plantarum* (Versalovic and Wilson, 2008). *L. plantarum* is a facultative hetero-fermentative bacterium and is one of the main microorganisms responsible for fermentation in fermented products with plant origin (Cebeci and Gurakan, 2003). *L. plantarum* is introduced as a probiotic species with abundant and proven health effects on human, such as cholesterol-lowering activities in blood

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and antimicrobial effects against certain group of pathogenic microorganisms due to its ability to produce the special bacteriocin called plantaricin (Cebeci and Gurakan, 2003; Sirilun et al., 2010). *L. plantarum* is also used in the production of probiotic dairy products like yogurt.

Numerous studies have been conducted to evaluate the different selective media for cultivation and enumeration of *L. plantarum*, such as *L. plantarum* selective medium (Bujalance et al., 2006), and de Man, Rogosa and Sharpe (MRS) agar with modified pH (DeMan et al., 1960). Many previous studies have been carried out for differential culture and enumeration of various probiotic species in yogurt and other fermented milk products (Talwalkar and Kailasapathy, 2003; Castele et al., 2005; Antunes et al., 2007; Kailasapathy et al., 2008; Sacccaro et al., 2012). The standard method of culturing probiotic lactobacilli is pour plate on MRS-agar medium, followed by aerobic or anaerobic incubation at 37°C for at least 72 h (Iranian standards, 2008). For differential cultivation and enumeration of yogurt starter bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*), many rather complicated media have been developed such as yogurt-lactic-agar (Matalon and Sandine, 1986; Marth and Stele, 2001), Elliker's lactic-agar (Elliker et al., 1956), MRS agar (pH = 5.2) (for *L. bulgaricus*) (Sacccaro et al., 2012), M17 medium and *S. thermophilus* medium (for *S. thermophilus*) (Terzaghi and Sandine, 1975; Dave and Shah, 1996).

In some products, like probiotic yogurt, differentiation of probiotic species from other bacteria is important. However, since yogurt contains bacteria of starter culture which are commonly called "yogurt starter bacteria," the conditions for the isolation and enumeration of probiotic species is difficult. Hence, there are no specific standards for isolation and enumeration of *L. plantarum* in the presence of yogurt bacteria unlike other probiotic species such as *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacteria* (Iranian standards, 2008). Therefore, we attempted to assess the selective media and different incubation conditions to obtain an appropriate method for differential culture and enumeration of this probiotic species in combination with yogurt bacteria.

## MATERIALS AND METHODS

### Preparation of probiotic culture

The commercial culture of *L. plantarum* 1058 PTCC as pure and freeze-dried was prepared from Pasteur Research Institute of Iran and activated by several microbial passages in MRS broth and MRS agar media. Next, the culture was inoculated in MRS-broth and incubated at 37°C for 48 h. For isolation of pure bacterial mass, 1 ml of cultured medium was transmitted into the 1.5 ml sterile micro-tubes and was centrifuged in 11000 × g relative centrifugal force (RCF) for 10 min. Bacterial sediment was washed several times with sterile saline and used for manufacturing probiotic yogurt.

### Manufacture of probiotic yogurt containing *L. plantarum* PTCC 1058

Yogurt samples were produced in Pegah dairy plant (Tabriz, Iran) using commercial starter culture; YoFlex Express (Christian Hansen, Denmark) containing two yogurt bacterial species (*S. thermophilus* and *L. delbrueckii* spp. *bulgaricus*). After inoculation of milk with (2% w/v) yogurt starter culture, 1% (v/v) of microbial suspension containing  $5.6 \times 10^9$  CFU/ml of *L. plantarum* PTCC 1058 was added in to the milk and then the inoculated milk was filled in 100 ml poly styrene containers and incubated at 43°C for 3 - 4 h (until reach to pH = 4.6). Yogurt samples were stored at 4°C for 24 h after preparation.

### Cultivation and enumeration of *L. plantarum* PTCC 1058 and yogurt bacteria

Different media and incubation conditions were evaluated for isolation and enumeration of *L. plantarum* PTCC 1058 and yogurt bacteria. For this purpose, yogurt samples were cultured as surface plate culture and pour plate culture after preparation of serial dilutions in seven different agar media including: MRS agar (Merck, Germany), MRS bile-agar, acidified MRS agar (pH = 5.2), yogurt lactic agar (Himedia, India), Elliker's-lactic-agar, *S. thermophilus* (ST) agar and M17 (Merck, Germany) media, and were incubated at three different times; 15, 37 and 45°C in two aerobic and anaerobic conditions for 48 to 72 h. Anaerobic condition was prepared by the use of gas pack C and anaerobic jar. For more practical examination, *L. plantarum* PTCC 1058 and yogurt bacteria were enumerated every week over 6 weeks of storage period at ≤10°C. All procedures were performed in triplicate.

Each of the media used in this study were prepared according to the manufacturer's instruction. For preparation of MRS bile agar, 0.5% (w/v) of bovine bile salts (Ox Bile, Merck, Germany) was added to the MRS-agar base medium. Yogurt lactic agar was made by adding (7% w/v) skim milk (11% dry matter) to the lactic agar base medium, while for preparation of Elliker's Lactic-agar, 50 µg/ml of 2,3,5-triphenyltetrazolium chloride and 1% (v/v) of Tween 80 were added to the lactic agar base medium (Matalon and Sandine, 1986).

### Evaluation of bacterial growth in different concentrations of bile salts

The ability of *L. plantarum* PTCC 1058 and yogurt bacteria to grow in different concentrations of bile salts: 0.5 to 5% (w/v) in MRS broth medium was investigated. In brief, 1% of each of yogurt starter bacteria and *L. plantarum* PTCC 1058 were inoculated separately in different concentrations of bile salts and incubated in both aerobic and anaerobic conditions at 37°C for 48 h. Then the growth of bacteria was determined based on the visual determination of turbidity of tubes compared with the control tube (not containing cultured MRS broth medium).

## RESULTS AND DISCUSSION

### Effect of bile salts on the growth of *L. plantarum* PTCC 1058 and yogurt bacteria

The results of the growth of *L. plantarum* PTCC 1058 and yogurt bacteria in different concentrations of bile salts are shown in Table 1. The ability of bacteria to grow in bile salts was similar in both aerobic and anaerobic

**Table 1.** The growth of *L. plantarum* PTCC 1058 and yogurt starter bacteria in different concentration of bile salts after 48 h incubation at 37°C in aerobic condition.

Bacterial culture	Concentration of bile salts (%)						
	0	0.5	1	2	3	4	5
<i>L. plantarum</i>	+	+	+	+/-	-	-	-
<i>L. bulgaricus</i>	+	+	+	+/-	-	-	-
<i>S. thermophilus</i>	-	-	-	-	-	-	-

+: Full growth (high turbidity); +/- : weak growth (low turbidity);  
-: lack of growth (no turbidity).

incubation. Based on the Iranian standard (no. 11325), the growth of yogurt starter bacteria is inhibited by the use of bile salts, hence MRS bile agar medium is recommended for enumeration of one probiotic species in the presence of yogurt starter bacteria (Iranian standards, 2008). According to our results, *L. plantarum* PTCC 1058 was not able to grow in concentration above 2% of bile salts. Beside, yogurt starter bacteria were able to grow in low concentrations of bile salts ( $\leq 2\%$ ); therefore, MRS-bile-agar medium was not useful for differential culture of *L. plantarum* PTCC 1058 in yogurt (Table 1).

#### The growth of *L. plantarum* PTCC 1058 and yogurt bacteria on different media and conditions

The results of the growth of *L. plantarum* PTCC 1058 and yogurt starter bacteria in different media and incubation conditions are shown in Table 2. Generally, the growth of bacteria in aerobic and anaerobic incubation conditions was nearly similar, while the bacterial growth was mostly affected by incubation temperature. The surface plate culture and incubation in aerobic conditions showed the best results for the growth of yogurt bacteria. *L. plantarum* PTCC 1058 could grow on MRS, acidified MRS, yogurt lactic agar and Elliker's lactic agar media, but it was not able to grow on the other tested media. However, a few colonies were seen on MRS bile agar at 37°C. Moreover, *L. plantarum* PTCC 1058 was not able to grow at 45°C, but it was able to grow at 15°C in addition to 37°C. In contrast, *L. bulgaricus* and *S. thermophilus* were able to grow at 37 and 45°C without any growth at 15°C (Table 2). *L. bulgaricus* could grow on all of the tested media except MRS bile agar; however, its growth on yogurt lactic agar and Elliker's lactic agar media was better than other media. *S. thermophilus* showed a better growth on Yogurt lactic agar, M17 and ST agar media without any growth on other media.

#### Enumeration of *L. plantarum* PTCC 1058 and yogurt bacteria in yogurt

The results of enumeration of *L. plantarum* PTCC 1058 and yogurt bacteria in different selective media and in

probiotic yogurt are respectively shown in Tables 3 and 4. The mean number of *L. plantarum* PTCC 1058, *L. bulgaricus* and *S. thermophilus* in yogurt samples were respectively, 9.40, 8.0 and 8.23 log cfu/g after 24 h storage at 4°C. However, the mean numbers of mentioned bacteria decreased to 6.05, 1.89 and 6.19 log cfu/g, respectively, after 6 weeks storage at  $\leq 10^\circ\text{C}$ . Our results are in agreement with findings of Shah (2000) who reported that *L. bulgaricus* showed more reduction in the number and its count declined to  $<10^5$  log cfu/g after 2 - 3 weeks of storage in combination with *S. thermophilus*, *L. acidophilus* and *Bifidobacterium* spp. In another study, Saccaro et al. (2012) indicated that the counts of *L. bulgaricus* decreased to 5.68 log cfu/g after 2 weeks storage at  $\leq 10^\circ\text{C}$  in fermented milk products, while the number of *S. thermophilus* was 6.50 log cfu/g in the same condition. The count of *L. plantarum* PTCC 1058 was reduced to  $< 7$  log cfu/g after 6 weeks storage in yogurt, which is not sufficient for its probiotic function (Ouweland and Salminen, 1998). It was reported that the acid produced by the yogurt starter bacteria during the fermentation, could influence the viability of some probiotic bacteria (Marafon et al., 2011).

#### The macroscopic morphology of the bacterial colonies on different media

The morphology of *L. plantarum* PTCC 1058, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* colonies is shown in Figure 1. The appearance of *L. plantarum* PTCC 1058 colonies in MRS medium were large, convex round and white after 72 h incubation at 15 and 37°C (Figure 1c). The smaller round pink colonies (sometimes with a red spot) in Elliker's Lactic-agar medium were produced after 48 to 72 h incubation at 37°C, in both aerobic and anaerobic conditions (Figure 1a). *L. bulgaricus* could grow in Elliker's Lactic-agar medium and produce the large red colonies in this medium after incubation at 37 or 45°C for 48 to 72 h (Figure 1d); however, *S. thermophilus* could not grow in this medium under no circumstances. Meanwhile, our findings are in contrast with Matalon and Sandine (1986) who reported that *S. thermophilus* produced tiny and red colonies in Elliker's Lactic-agar medium, and *L. bulgaricus* raise white colonies in this medium. However, they reported that this characteristic is variable depending on bacterial strains. *L. bulgaricus* produced the larger, creamy white colonies with the opaque white halo around them in yogurt lactic agar after 24 to 48 h incubation at 37°C, whereas *S. thermophilus* raised very small, shiny colonies with whitish grey color in this medium (Figure 1b). Moreover, our findings are in agreement with the results of Matalon and Sandine (1986) who reported that *S. thermophilus* produced tiny and white color colonies in yogurt lactic agar medium, but the colonies of *L. bulgaricus* in this medium are larger, white color with opaque halo around the colonies. In some cases, the

**Table 2.** The growth of *L. plantarum* PTCC 1058 and yogurt traditional bacteria in different culture media and incubation conditions.

Media (agar)	Temperature of incubation (C)	Bacterial culture		
		<i>L. plantarum</i>	<i>L. bulgaricus</i>	<i>S. thermophilus</i>
MRS	15	+	-	-
	37	+	+	-
	45	-	+	-
MRS B	15	-	-	-
	37	+/-	+/-	-
	45	-	-	-
MRS A	15	+	-	-
	37	+	+	-
	45	-	+	-
EL	15	+	-	-
	37	+	+	-
	45	-	+	-
YL	15	+	-	-
	37	+	+	+/-
	45	-	+	+
M17	15	-	-	-
	37	-	+/-	+
	45	-	+/-	+
ST	15	-	-	-
	37	-	+/-	+
	45	-	+/-	+

The culture method was the surface plating method and incubation time was 48 - 72 h. MRS, de Man, Rogosa and Sharpe agar; B, bile; A, acidified (pH=5.2); EL, Elliker's lactic agar; YL, yogurt lactic agar; ST, *Streptococcus thermophilus* agar; +, full growth; -, no growth on any plates; +/-, growth of a few colonies on plates.

**Table 3.** Counts (log<sub>10</sub> cfu/g) of *L. plantarum* PTCC 1058 and yogurt starter bacteria on different selective media after 24 h storage at 4°C.

Bacterial culture	Media (agar)						
	MRS	MRS B	MRS A	EL	YL	M17	ST
<i>L. plantarum</i>	9.55 ± 0.3	ng	9.46 ± 0.4	9.37 ± 0.2	9.25 ± 0.2	ng	ng
<i>L. delbrueckii</i>	8.05 ± 0.4	ng	7.92 ± 0.2	8.12 ± 0.1	8.15 ± 0.3	7.93 ± 0.4	7.88 ± 0.3
<i>S. thermophilus</i>	ng	ng	ng	ng	8.27 ± 0.2	8.19 ± 0.1	8.23 ± 0.2

MRS, de Man, Rogosa and Sharpe agar; B, bile; A, acidified (pH = 5.2); EL, Elliker's lactic agar; YL, yogurt lactic agar; ST, *Streptococcus thermophilus* agar; ng - No growth on any plates. The counts are mean of three readings in each trial, N = 3 (Mean ± SE).

halo around the colonies of *L. bulgaricus* was not seen or was unclear, and could be associated with inappropriate concentration of skim milk, technical problems in preparation of medium and inappropriate incubation conditions.

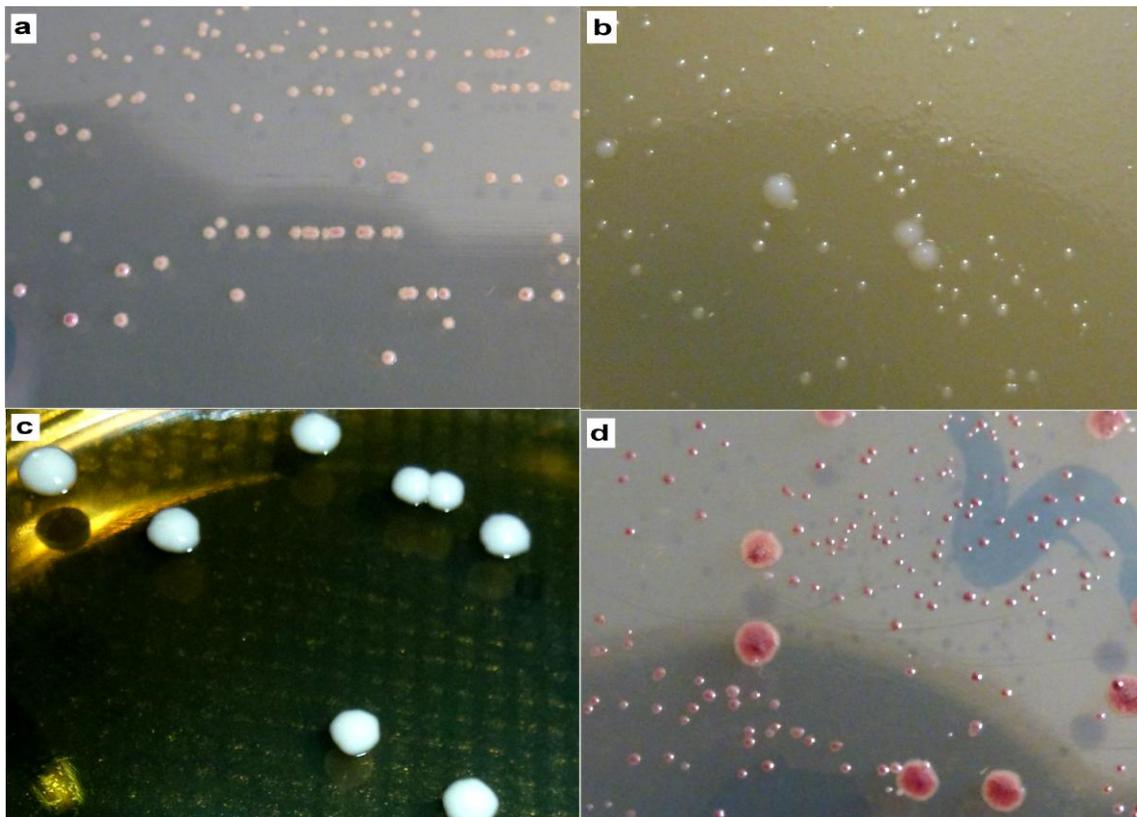
## Conclusions

Based on the results of this research, it was concluded that the best conditions for differential culture and enumeration of *L. plantarum* PTCC 1058 in the presence

**Table 4.** Counts (log<sub>10</sub> cfu/g) of *L. plantarum* PTCC 1058 and yogurt starter bacteria in probiotic yogurt after 1 to 6 weeks storage at ≤10°C.

Bacterial culture	Media (agar)	Storage time (weeks)					
		1	2	3	4	5	6
<i>L. plantarum</i>	MRS	8.74 ± 0.2	7.63 ± 0.1	7.44 ± 0.2	7.07 ± 0.5	6.77 ± 0.1	6.20 ± 0.2
	YL	8.63 ± 0.12	7.52 ± 0.3	7.37 ± 0.7	6.87 ± 0.1	6.42 ± 0.5	6.05 ± 0.01
	EL	8.55 ± 0.4	7.43 ± 0.1	7.32 ± 0.4	6.79 ± 0.1	6.35 ± 0.2	5.92 ± 0.5
<i>L. delbrueckii ssp. bulgaricus</i>	YL	7.34 ± 0.4	6.32 ± 0.002	5.65 ± 0.2	5.46 ± 0.3	4.30 ± 0.4	2.07 ± 0.8
	EL	7.25 ± 0.1	6.05 ± 0.4	5.27 ± 0.6	5.19 ± 0.5	4.17 ± 0.1	1.95 ± 0.03
	MRS	7.18 ± 0.5	5.96 ± 0.5	5.16 ± 0.1	5.08 ± 0.1	3.92 ± 0.1	1.67 ± 0.9
<i>S. thermophilus</i>	YL	8.23 ± 0.01	7.66 ± 0.3	7.46 ± 0.2	7.30 ± 0.4	6.30 ± 0.9	6.20 ± 0.7
	M17	8.20 ± 0.4	7.59 ± 0.1	7.42 ± 0.8	7.29 ± 0.9	6.25 ± 0.2	6.18 ± 0.09
	ST	8.21 ± 0.06	7.63 ± 0.6	7.40 ± 0.3	7.32 ± 0.2	6.28 ± 0.4	6.20 ± 0.1

MRS, de Man, Rogosa and Sharpe agar; YL, yogurt lactic agar; EL, Elliker's lactic agar; ST, *Streptococcus thermophilus* agar. counts are mean of three readings in each trial, N=3 (Mean ± SE).



**Figure 1.** *L. plantarum* PTCC 1058 colonies with pink color and red spots on Elliker's lactic-agar medium (a), *L. bulgaricus* large colonies and *S. thermophilus* tiny colonies on yogurt lactic agar medium (b), white colonies of *L. plantarum* PTCC 1058 on MRS-agar medium (c), large red colonies of *L. bulgaricus* and small pink colonies with red spot of *L. plantarum* PTCC 1058 on Elliker's lactic-agar medium (d).

of yogurt starter bacteria is culturing in MRS agar medium and incubation at 15°C for at least 72 h. For differential culture of *L. bulgaricus*, culturing in yogurt

lactic agar and Elliker's lactic agar media, and incubation at 37°C for 48 to 72 h are suitable. The best condition for differential culture of *S. thermophilus* was culturing in

yogurt lactic agar, M17 and ST agar media, and aerobic incubation at 45°C for 48 to 72 h.

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