

Research Article

Viability of Probiotics (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) in set type yogurt made from buffalo milk

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Abstract

Yogurt is a well-known popular food that contains probiotics, such as *Lactobacillus acidophilus*, this strain is commonly incorporated in yogurt in some countries. Probiotics of yogurt found to have anti-oxidative properties and boost the immune system. Unfortunately in Pakistan there is no trend of accumulation of probiotics in dairy foods. Present study has been designed to incorporate probiotics in yoghurt, to study the best storage temperature and adequate level of probiotics for best yogurt production. The effect of refrigerated storage temperature at 4, 5 and 6°C on the viability after addition of probiotics (*Lactobacillus acidophilus* & *Bifidobacterium bifidum*) in set type yogurt was observed for 28th day's to identify the best storage temperature for probiotics. The viability of the probiotic micro-organisms were analyzed at 07 days interval. Results showed that storage at 4°C showed the highest viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* probiotics and the lowest viability was observed when yogurt was stored at 6°C.

Keywords: Probiotics; Set yogurt; Shelf life; Storage temperature; Viability

Introduction

Yogurt is a product obtained by coagulation of milk with lactic acid fermentation in the presence of microbes such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [1]. These bacterial species bear symbiotic interactions while growing in milk and develop unique characteristics [2]. To acquire an excellent efficiency for lactic acid production while fermentation, a unique combination of Lactic acid Bacteria (LAB) species is required. However, certain species

of LAB that are indigenous to human gastrointestinal tract (GIT) are being used that could survive the high acidity and low pH of gastrointestinal tract (GIT). These cultures must also be viable in the finished product till its claimed shelf life. The final product must contain more than or equal to 10⁸ organisms/gram to meet the criteria as established by National Yogurt Association [2]. The consumer is becoming more diet and health conscious, so the use of probiotic products is also increasing day by day [3].

Yogurt is known quite well because of its therapeutic properties [4]. Yogurt is inoculated worldwide with probiotics like *Lactobacillus acidophilus*. Probiotic yogurt consumption has been found efficient to cure many health issues like diarrhea, enhancing lactose intolerance, urinary and genital tract infections, enhanced cholesterol levels in serum, limiting allergens, promoting anti-carcinogenic activity and enhancing resistance towards diseases [5]. Furthermore, probiotics of yogurt have also been found to have anti-oxidative properties and boost the immune system [6]. Along with the beneficial health properties, yogurt and its probiotics must be economical, should not alter the taste and aroma of yogurt adversely, the shelf life of product must not be affected during its acidification [7]. Storage temperature is a major issue which critically effects yogurt activity; in order to get desirable benefits from probiotic yogurt there should be use of sufficient level of probiotics and adequate storage temperature conditions. Therefore, present study was designed to identify the best storage temperature and adequate level of probiotics use for best yogurt production. By setting the refrigerated temperature there will be maximum growth of beneficial bacteria and hence better results will be obtained.

Materials and Methods

Procurement of samples

Buffalo milk procured from B-block UVAS Ravi campus Pattoki, while probiotic cultures procured from local market, Lahore.

Materials

Lactobacillus MRS Agar, *Bifidobacterium* Selective Media, M17, Anaerobic Jar, Anaerobic Sachet were also obtained from market.

Standardization of milk

Buffalo milk yoghurt with different combinations of cultures was prepared. Milk first standardized up to 15% total solids and

3.5% fat. For standardization of yoghurt skim milk powder was added and pasteurized at 82°C, for 5 minutes, then cooled down to 43°C, starter culture was added according to the treatments plan. All yogurt batches were incubated at 43°C for 3 – 3.5 hours. Throughout this period pH was monitored and at 4.6 pH, the batches were shifted to the blast chilling room at 1-2°C; and then stored at 4, 5 and 6°C [8].

Starter culture

Pure DVS culture, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Chr-Hanson) were procured from local market, Lahore and used in the experiment for yogurt manufacturing. Cultures were prepared and maintained according to the instructions given by the manufacturer.

Culture media for enumeration

MRS bile agar, M17, (anaerobic sachet) gas packed media were used for enumeration of bacteria. Samples of yogurt were taken and plates were prepared using their respective media and after that these plates were incubated at 37°C for the period of 1-3 days according to the instructions by the manufacturer. For *L. acidophilus* these plates were incubated aerobically while for *B. bifidum* plates were incubated anaerobically in gas packed environment. After standardization and pasteurization of milk the cultures were mixed in 1 liter of milk separately. After that above cultured mixed milk was taken and used for further culturing of yogurt according to the method described above and incubated at 42°C for 3-3.5 hours at pH of 4.6 after pasteurization. A total of 4 treatments were prepared in which 1st treatment contained DVS 100% (B₀), 2nd contained 50% DVS and 50% *Lactobacillus acidophilus* (B₁), 3rd contained 50% DVS and 50% *Bifidobacterium bifidum* (B₂) and 4th contained 50% DVS, 25% *Lactobacillus acidophilus* and 25% *Bifidobacterium bifidum* (B₃). All samples were placed at 4,

5 and 6°C to check viability of bacteria for 28 days.

Statistical analysis

Quality comparison of all prepared yoghurt samples was statistically analyzed by use of Three-way Analysis of Variance (ANOVA) under Completely Randomized Design (CRD). Duncan's Multiple Range (DMR) Test (SAS 9.4 Statistical Software) was used for significant difference comparisons [9].

Results

Yogurt is most popular food in the world due to its wonderful health benefits for humans. It contained several important microorganisms that are very helpful for human gut and immunity. These probiotics give strong immune system and provides several beneficial effects for cardiac patients. Viability of probiotics is important consideration in order to get desired health benefits. In present study, yogurt was prepared using different cultures such as control group (DVS 100%) represents B0, *Bifidobacterium bifidum* 50% with DVS 50% represents to B1, *Lactobacillus acidophilus* 50% with DVS 50% represents B2 and *Bifidobacterium bifidum* 25% + *Lactobacillus acidophilus* 25% with DVS 50% represents B3. Also 3 different temperatures were used to study the shelf life and chemical nature of yogurt that were 4, 5 and 6°C (T1, T2 and T3) respectively. The effect of variation in the physico-chemical nature of yogurt was also studied across four weeks. The CFU count/ viability was also studied using different cultures in yogurt.

Yogurt cultures (Treatments)

Lactobacillus acidophilus was more stable than *Bifidobacterium bifidum* and yogurt culture bacteria. *Lactobacillus acidophilus* showed greater viability, low viability loss percentage than other probiotics because it can survive at low pH, low acidity as compared to *Bifidobacterium bifidum* and

yogurt culture bacteria. The pH value in B2 is high which means good stability of *Lactobacillus acidophilus* than B1, B3 and B0 respectively (Table 1).

Similarly in terms of acidity% treatment B2 showed low acidity as compared to other three treatments (Table 2). Our results are in agreement with [10], in their study they revealed that *Bifidobacterium bifidum* is less acid tolerant and less viable at low pH. They concluded that *Lactobacillus acidophilus* can survive up to 4.0 pH but *Bifidobacterium bifidum* cannot survive below 5 pH. So at low pH and high acidity *Lactobacillus acidophilus* showed maximum viability than other probiotics [10]. Our results also showed same trend (Fig 2).

Yogurt containing *Lactobacillus acidophilus* showed less increase in the syneresis as compared to others as shown in (Table 3). The reason behind the low syneresis value of *Lactobacillus acidophilus* was its acid tolerance ability which is high as compared to other microorganisms. *Lactobacillus acidophilus* survives at low pH and high acidity, so it had less syneresis. *Bifidobacterium bifidum* showed less syneresis value and it was high for control (B0) [11]. Due to high tolerance of *Lactobacillus acidophilus* the production of lactic acid was less, this might be due to exopolysaccharides [12].

The Syneresis of yogurt of all treatments increased with the increase in storage time from day 0 to day 28. For first week it was lower because of low activity of lactic acid bacteria and low CO₂ production. But at day 28 it was maximum (Fig. 3) as lactic acid production was high and pH was low. [11] in their study also revealed that increase in syneresis directly related to increase in acidity and decrease in pH. They showed that syneresis value for first 2 weeks increased slowly but at the end of 4th week it was high, as acidity was high.

Table 1. Effect of storage temperature and storage period on pH of yogurt

Temperature	Storage (Days)	Treatments			
		Bo	B1	B2	B3
T1	0	4.5667 ^a	4.5833 ^a	4.5833 ^a	4.5867 ^a
	7	4.5433 ^a	4.4600 ^b	4.4700 ^b	4.4800 ^b
	14	4.4700 ^b	4.3300 ^c	4.3200 ^d	4.3567 ^e
	21	4.1733 ^d	4.2200 ^{c^d}	4.2133 ^{d^e}	4.1867 ^f
	28	4.0333 ^f	4.0800 ^e	4.1033 ^e	4.0700 ^g
T2	0	4.5767 ^a	4.5700 ^a	4.5767 ^a	4.5800 ^a
	7	4.5300 ^a	4.4167 ^c	4.4400 ^c	4.4500 ^c
	14	4.4533 ^b	4.2633 ^c	4.3100 ^d	4.3233 ^e
	21	4.2033 ^d	4.1200 ^{d^e}	4.1567 ^e	4.2033 ^{e^f}
	28	4.0200 ^f	4.0667 ^e	4.0867 ^f	4.0700 ^g
T3	0	4.5667 ^a	4.5667 ^a	4.5833 ^a	4.5800 ^a
	7	4.5000 ^b	4.4267 ^b	4.4200 ^c	4.4167 ^d
	14	4.2833 ^c	4.2967 ^c	4.3000 ^d	4.2733 ^f
	21	4.0933 ^e	4.0900 ^{d^e}	4.1067 ^{e^f}	4.1367 ^{e^f}
	28	4.0000 ^f	4.0433 ^d	4.0533 ^f	4.0533 ^g
P-Value		<0.01			

Different superscripts indicate significance of a treatment according to the temperature *days interaction

Bo = DVS 100% (Control)

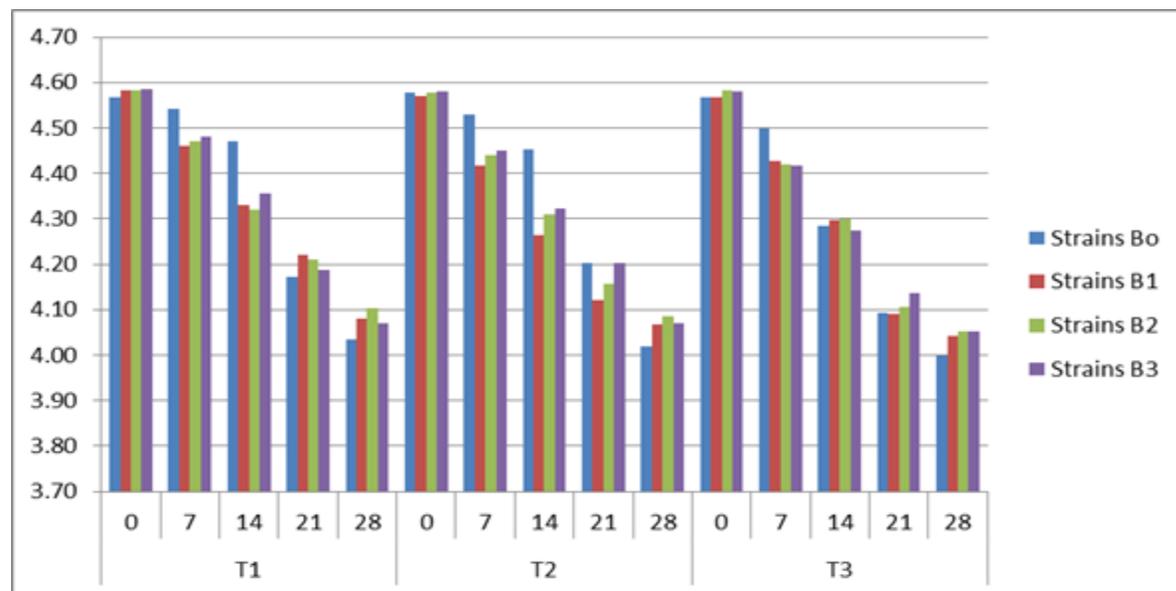
B1 = *Bifidobacterium bifidum* 50% + DVS 50%

B2 = *Lactobacillus acidophilus* 50% + DVS 50%

B3 = DVS 50% + *Bifidobacterium bifidum* 25% + *Lactobacillus acidophilus* 25%

Whereas T1 = 4°C, T2 = 5°C, T3 = 6°C

pH was decreased with the increase of temperature and passage of time (Fig. 1)



Whereas: T1 = 4°C, T2 = 5°C, T3 = 6°C

Figure 1. Results showed that storage temperature and storage days had significant effect (P-value <0.05) on pH of yogurt

Table 2. Effect of storage temperature and storage period on Acidity% of yogurt

Temperature	Storage	Treatments			
		Bo	B1	B2	B3
T1	0	0.9100 ^c	0.9133 ^c	0.9087 ^f	0.9033 ^f
	7	0.9133 ^c	0.9117 ^c	0.9127 ^f	0.9113 ^{ef}
	14	0.9233 ^c	0.9167 ^d	0.9117 ^f	0.9161 ^{ef}
	21	0.9233 ^c	0.9237 ^{de}	0.9447 ^d	0.9227 ^e
	28	0.9267 ^c	0.9627 ^e	0.9233 ^f	0.9287 ^e
T2	0	0.9233 ^c	0.9333 ^b	0.9307 ^e	0.9320 ^{cd}
	7	0.9333 ^c	0.9433 ^b	0.9417 ^d	0.9370 ^d
	14	0.9433 ^c	0.9533 ^{bc}	0.9317 ^e	0.9393 ^d
	21	0.9533 ^{bc}	0.9553 ^{bc}	0.9380 ^e	0.9433 ^c
	28	0.9633 ^{bc}	0.9587 ^c	0.9530 ^d	0.9533 ^c
T3	0	0.9733 ^{ab}	0.9633 ^{ab}	0.9633 ^{bc}	0.9633 ^{ab}
	7	0.9833 ^b	0.9733 ^b	0.9733 ^c	0.9760 ^{ab}
	14	0.9933 ^b	0.9817 ^b	0.9833 ^b	0.9900 ^b
	21	1.0167 ^b	0.9917 ^b	0.9920 ^a	0.9953 ^a
	28	1.0933 ^a	0.9967 ^a	0.9950 ^a	0.9983 ^a
P-Value		<0.01			

Different superscripts indicate significance of a treatment according to temperature *days interaction

Bo = DVS 100% (Control)

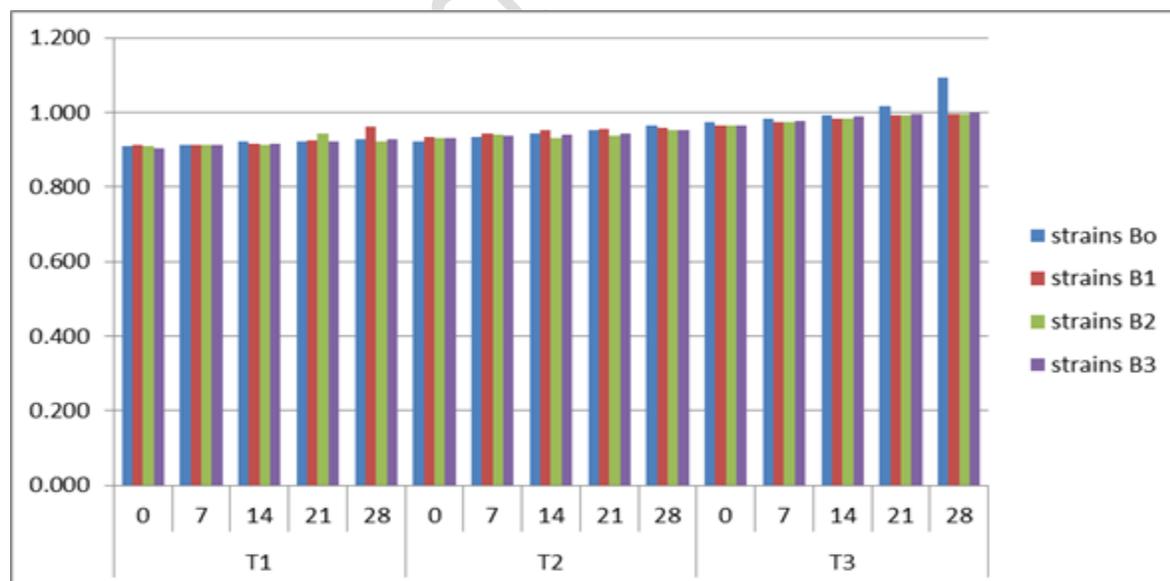
B1 = *Bifidobacterium bifidum* 50% + DVS 50%

B2 = *Lactobacillus acidophilus* 50% + DVS 50%

B3 = DVS 50% + *Bifidobacterium bifidum* 25% + *Lactobacillus acidophilus* 25%

Whereas T1 = 4°C, T2 = 5°C, T3 = 6°C

Acidity% increased with the increase of temperature and passage of time (Fig 2).



Whereas: T1 = 4°C, T2 = 5°C, T3 = 6°C

Figure 2. Results showed that storage temperature and storage days had significant effect (P-value <0.05) on Acidity% of yogurt

Table 3. Effect of storage temperature and storage period on syneresis (%) of yogurt

Temperature	Storage	Treatments			
		Bo	B1	B2	B3
T1	0	49.0333 ^g	48.6333 ^g	48.2333 ^g	48.3333 ^g
	7	49.2667 ^g	48.9067 ^{fg}	48.4333 ^f	48.4333 ^{fg}
	14	49.6333 ^{fg}	48.9467 ^{fg}	48.5333 ^{ef}	48.5000 ^f
	21	49.8333 ^f	49.0667 ^f	48.6333 ^f	48.6333 ^f
	28	50.0333 ^{ef}	49.2333 ^{ef}	48.9333 ^{ef}	48.8333 ^{ef}
T2	0	50.3333 ^e	49.3333 ^e	49.0333 ^e	49.0333 ^e
	7	50.5333 ^{de}	49.4333 ^e	49.1333 ^e	49.3333 ^{de}
	14	50.6333 ^{de}	49.6333 ^{de}	49.3333 ^d	49.5333 ^d
	21	50.8333 ^d	49.8333 ^{de}	49.4667 ^{cd}	49.7333 ^d
	28	50.9333 ^d	49.9333 ^{de}	49.6333 ^{cd}	49.9333 ^{cd}
T3	0	51.0333 ^{cd}	50.0333 ^d	49.9333 ^c	50.0333 ^c
	7	51.2333 ^c	50.2333 ^{cd}	50.0333 ^{bc}	50.3333 ^{ab}
	14	51.3333 ^b	50.4333 ^c	50.1333 ^{ab}	50.4333 ^b
	21	51.5333 ^a	50.7333 ^b	50.2333 ^b	50.5667 ^b
	28	51.6333 ^a	50.9333 ^a	50.3333 ^a	50.7333 ^a
P-Value		<0.01			

Different superscripts indicate significance of a treatment according to temperature *days interaction

Bo = DVS 100% (Control)

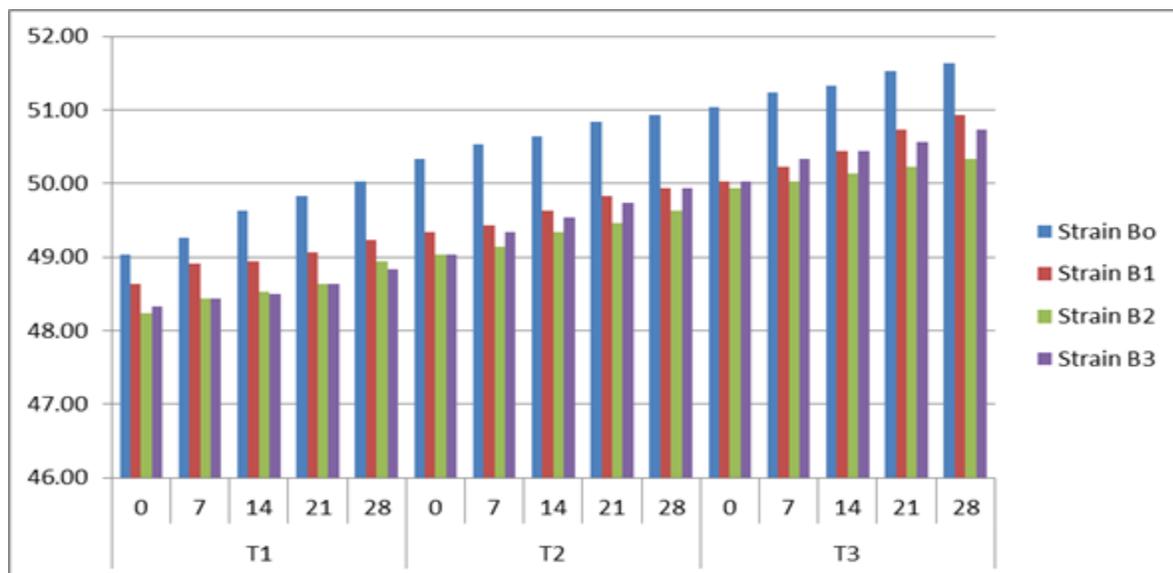
B1 = *Bifidobacterium bifidum* 50% + DVS 50%

B2 = *Lactobacillus acidophilus* 50% + DVS 50%

B3 = DVS 50% + *Bifidobacterium bifidum* 25% + *Lactobacillus acidophilus* 25%

Whereas T1 = 4°C, T2 = 5°C, T3 = 6°C

Syneresis increased with the increase of temperature and passage of time (Fig 3).



Whereas: T1 = 4°C, T2 = 5°C, T3 = 6°C

Figure 3. Results showed that storage temperature and storage days had significant effect (P-value <0.05) on Syneresis of yogurt.

The CFU/ viability in case of B2 was high than B3, B1 and B0. *Lactobacillus acidophilus* showed more viability than other three treatments because of synergistic relationship of *Lactobacillus acidophilus* with yogurt bacteria (Table 4) and (Fig 4). Yogurt bacteria made synergism with the probiotics hence there was less lactic acid production and less acidity, so viability loss percentage was low. On the other hand *Bifidobacterium* being less acid tolerant and less survived at low pH even below pH 5 had high viability loss percentage as compared to *Lactobacillus acidophilus* [13].

Temperature

By maintaining proper temperature which is necessary for optimum growth of probiotics in yogurt, the therapeutic as well as economic benefits of yogurt will be achieved. Hence present study was designed

and the results indicated that the CFU values of temperatures (T1-T3) varied significantly. The results of CFU of yogurt made from buffalo milk are demonstrated in (Fig 4). Maximum CFU was observed for T1 while minimum CFU was observed for T3. Reason for survival at low temperature is that, at low temperature the production of lactic acid by *Lactobacillus bulgaricus* is low so both *Lactobacillus acidophilus* and *Bifidobacterium bifidum* showed greatest viability at low temperature like 4°C. In the same case *Lactobacillus acidophilus* and *Bifidobacterium bifidum* showed low viability at high temperature as mentioned in the study 5°C and 6°C, as on these temperatures the production of lactic acid bacteria high which retarded the growth of these probiotics and ultimately reduced number of viable count [14].

Table 4. Effect of Storage temperature and storage period on Probiotic viability (log cfu/ml) of yogurt

Temperature	Storage	Treatments			
		Bo	B1	B2	B3
T1	0	7.1683 ^a	7.3433 ^a	7.4633 ^a	7.3167 ^a
	7	7.1200 ^b	7.2067 ^b	7.4133 ^b	7.2300 ^b
	14	6.7867 ^c	6.9333 ^c	6.7933 ^c	6.7983 ^c
	21	6.5083 ^d	6.5333 ^d	6.7000 ^d	6.5317 ^d
	28	6.1750 ^{de}	6.2333 ^{de}	5.5733 ^e	5.8550 ^e
T2	0	7.1683 ^a	7.3433 ^a	7.4633 ^a	7.3183 ^a
	7	7.0450 ^{ab}	7.0667 ^b	7.4133 ^b	7.1950 ^b
	14	6.7733 ^c	6.8333 ^c	6.7333 ^c	6.7917 ^c
	21	6.2400 ^{de}	6.2133 ^{de}	6.2333 ^{de}	6.2067 ^{de}
	28	5.3717 ^e	5.4667 ^e	5.6333 ^e	5.5217 ^e
T3	0	7.1733 ^a	7.3433 ^a	7.4667 ^a	7.3333 ^a
	7	7.0150 ^b	7.0833 ^{ab}	7.4267 ^b	7.2383 ^b
	14	6.6783 ^c	6.7333 ^{cd}	6.6333 ^d	6.6783 ^{cd}
	21	6.0333 ^{de}	6.1667 ^{de}	6.4167 ^{de}	6.2750 ^{de}
	28	5.3433 ^e	5.3333 ^e	6.0333 ^{de}	5.6567 ^e
P-Value		<0.01			

Different superscripts indicate significance of a treatment according to temperature *days interaction

Bo = DVS 100% (Control)

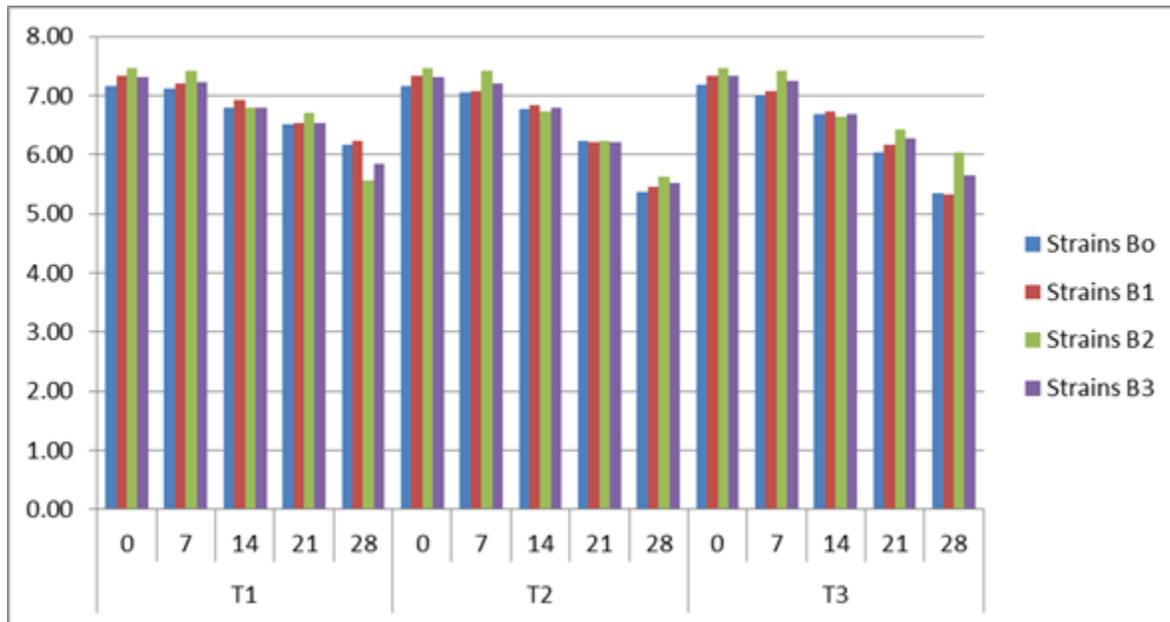
B1 = *Bifidobacterium bifidum* 50% + DVS 50%

B2 = *Lactobacillus acidophilus* 50% + DVS 50%

B3 = DVS 50% + *Bifidobacterium bifidum* 25% + *Lactobacillus acidophilus* 25%

Whereas T1 = 4°C, T2 = 5°C, T3 = 6°C

Viability decreased with the increase of temperature and passage of time (Fig 4).



Whereas: T1 = 4°C, T2 = 5°C, T3 = 6°C

Figure 4. Results showed that storage temperature and storage days had significant effect (P value <0.05) on Viability of yogurt.

pH at 6°C was low as compared to 5°C and 4°C because of high activity of lactic acid bacteria at increased temperature (Fig 1). At 6°C there was more lactic acid formation and more viability loss so at that temperature there was low pH at 6°C. *Lactobacillus acidophilus* and *Bifidobacterium bifidum* showed better survival rate at low storage temperature because of less production of lactic acid in the yogurt and low acidity (Fig 2). At high temperature metabolic activity of cell high and death rate of bacterial cell increased due to this reason at high temperature viability loss increased as compared to low temperature 4 and 5°C [15].

The Syneresis of yogurt of all treatments increased (Fig 3) with the increase of temperature. (Fox and Cagan 2017) in their study also revealed that increase in syneresis directly related to increase in acidity and decrease in pH. They also concluded that increase in temperature increased the syneresis as with increase in temperature,

pH decreased and acidity increased [11]. When temperature increases viscosity decreases because of high metabolic activity of microorganisms and high lactic acid formation [16].

Shelf life

At first week of storage the viability loss was low as compared to 3rd and 4th week (Fig 4). [13] in study also revealed that there was decrease in viability of probiotics observed across the weeks. They detected that with the increase of storage time the viability of probiotics decreased and its viability percentage loss increased. This was due to increased activity of *Lactobacillus bulgaricus* and antagonistic relationship of yogurt bacteria with these probiotics [13].

The decrease of pH and increase in acidity% of yogurt of all treatments (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* and yogurt culture bacteria) occurred as the time passed. Maximum pH was observed at week 1 and lowest pH was observed at week 4. So from week 1-4 pH of yogurts decreased. This

is due to high lactic acid production from lactose by these bacteria. They produced lactic acid, and production was maximum after 4 weeks of storage. The lactose of yogurt was utilized by lactic acid bacteria which converted it to lactic acid and as a result pH decreased and acidity increased. This occurred might be due to buffering action of proteins [17].

Conclusion and Recommendations

Probiotics are beneficial for human health, so their viable count should meet the required quantity in order to get good results. Various probiotics are available in the market, present study suggests *Lactobacillus acidophilus* is best among all these probiotics. *Lactobacillus acidophilus* gave maximum viable count than yogurt culture and *Bifidobacterium bifidum*. Maximum viability of probiotics was obtained when these probiotics stored at 4°C and afterwards their viability decreased due to increased metabolic activity of the microbes. Viability loss percentage for both strains was maximum when yogurt stored at 6°C on day 28th. So for probiotic yogurt, optimum conditions for better quality, sensory properties and to enhance shelf life it must be stored at 4°C. If this strain will use with yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) then there will be best therapeutics as well as quality results will be obtained.

Authors' contributions

Conceived and designed the experiments: S Inayat, Performed the experiments: M Bilal, Analyzed the data: S Manzoor, Contributed reagents/ materials/ analysis tools: MH Lashari & A Hassan, Wrote the paper: S Inayat.

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