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DEVELOPMENT AND EVALUATION OF PROBIOTIC YOGURTS CONTAINING LACTOBACILLUS ACIDOPHILUS AND STREPTOCOCCUS THERMOPHILUS

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ABSTRACT

Probiotics, which means "for life", have been used for centuries as natural components in health promoting food. They have a long history of association with dairy products because some of the same bacteria that are associated with fermented dairy products also make their home in different sites in the human body. Activation and Growth kinetics of the probiotic microorganisms using standardized microbiological techniques in their respective broths i.e. deMannRogosa Sharpe Agar (MRS) for *L.acidophilus* and Nutrient Agar for *S.thermophilus*. It was found that *L.acidophilus* had long lag phase of 12 hrs whereas *S.thermpohilus*started growing within 6 hrs of inoculation. Two types of Probiotic Yogurt were prepared with low fat milk one containing *L.acidophilus* and other containing *L.acidophilus* along with *S.thermophilus.* Different inoculum rates (0.5%, 1.0% and 1.5%) were used. Physiochemical properties of yogurt [Brix, pH; acidity (%lactic acid); Brix acid rates; total plate count]was done over the period of three days under the constant temperature of 25±2°C. Palatable acidity was maintained even after 72 hr of storage period. The fermented product were standardized after formulation and evaluated organoleptically. Among the products yogurt containing single stain of *L.cidophilus*at 1.5%inoculum rate was most accepted with overall acceptability of 8.37±0.12. In case of yoghurt contain both the strains of *L.acidophilus* and *S.thermophilus* yogurt with 1.0% had highest score of 8.30±0.11. Probiotic fermented food products can play a dual role in transforming milk into a diverse array of fermented dairy products (yogurt, cheese, kefir, etc.), and contributing to the important role of colonizing bacteria. A dairy product containing probiotics makes a healthy "functional food package" along with its therapeutic properties.

Keywords: Probiotics, Kinetics, *L.acidophilus*, Microbiological, Organoleptically.

INTRODUCTION

Probiotics found in food products and dietary supplements are one of the good bacteria. Probiotics, which means "for life", have been used for centuries as natural components in health promoting food. The original observation of the positive role played by certain bacteria was first introduced by a Russian scientist Noble Laureate Elie Metchnikoff. Dairy foods fermented with specific probiotic bacteria can produce modest reduction in the total LDL cholesterol levels and blood pressure. There are various experiments that suggest a range of potentially beneficial medicinal use of probiotics in CHD, peptic ulcers, irritable bowel syndrome (Survarna and Boby, 2005).

Lactobacillus acidophilus (meaning *acid-loving milk-bacterium*) is a species in the genus *Lactobacillus*. *L. acidophilus* is a homo-fermentative species, fermenting sugars into lactic acid, which grows readily at rather low pH values (below pH 5.0) and has an optimum growth temperature of 30 °C (86 °F). *L. acidophilus* occurs naturally in the human and animal gastrointestinal tract, mouth, and vagina (www.ebi.ac.uk).When organisms such as lactobacillus acidophilus are used medicinally then the term "probiotic" is used (National Institute of Health, 2010). *Streptococcus thermophilus* is a Gram-positive facultative anaerobe. It is a cytochrome-, oxidase- and catalase-negative organism that is nonmotile, non-spore forming and homofermentative. It is also classified as lactic acid bacteria (LAB). *Streptococcus thermophilus* is used, along with *Lactobacillus* spp., as a starter culture for the manufacture of several important fermented dairy foods, including yogurt and Mozzarella cheese (US

Probiotics.org, 2008).

A dairy product containing probiotics makes a healthy "functional food package". In addition to the vitamins, calcium, other minerals, and protein obtained from milk products. Modern research has suggested healthful properties of fermentation-derived peptides and butyric acid found in some dairy products. Amongst the probiotic effects attributed to LAB, as assimilation (removal) of cholesterol and deconjugation of bile acids in the small intestine may be important in lowering the blood cholesterol concentration, consequently in reducing the risks of Coronary Heart Disease (CHD) (Ljungh and Wadström, 2010).Consumption of three or more servings of dairy products each day has been associated with lower levels of obesity, and hence lower incidence of hypertension and heart disease (Shah, 2001). The DASH (Dietary Approaches to Stop Hypertension) diet also recommends three servings of low fat dairy products. Considering all these findings, dairy products combined with probiotic bacteria results into improved health status (US Probiotics.org, 2008).

MATERIALS AND METHODS

PREPARATION OF STOCK CULTURES OF PROBIOTIC ORGANISMS

LACTOBACILLUS ACIDOPHILUS

The lyophilized cells of *Lactobacillus acidophilus* were transferred to sterilized reconstituted skimmed milk (5%) under aseptic conditions. The inoculated skim milk was incubated at 37°C for 48 h in an incubator. A loopful of *Lactobacilli* growth from the skim milk was streaked on MRS agar plate and again incubated for 24h at 37°C. After the colonies developed, a single pure colony was picked up and its gram stain slide was examined microscopically. These *Lactobacilli* cultures were found to be gram positive and rod shaped. Stock cultures of the *Lactobacilli* were maintained on MRS agar by stab inoculation. After incubating at 37°C for 12h, the stock culture tubes were stored in refrigerator (4°C). For maintenance the cultures were transferred regularly after 3 weeks intervals. Activation of culture was done by three successive transfers at 24h intervals in MRS broth.

STREPTOCOCCUS THERMOPHILUS

The lyophilized cells of *Streptococcus thermophilus* were transferred to sterilized reconstituted skimmed milk (5%) under aseptic conditions. The inoculated skim milk was incubated at 37°C for 48 h in an incubator. A loopful of *Streptococci* growth from the skim milk was streaked on Nutrient agar plate and again incubated for 24h at 37°C. After the colonies developed, a single pure colony was picked up and its gram stain slide was examined microscopically. These *Streptococci* cultures were found to be gram positive and coccus shaped. Stock cultures of the *Streptococci* were maintained on Nutrient agar by stab inoculation. After incubating at 37°C for 12h, the stock culture tubes were stored in refrigerator. For maintenance the cultures were transferred regularly after 3 weeks intervals. Activation of culture was done by three successive transfers at 24h intervals in Nutrient broth.

STAINING OF MICRO ORGANISMS

PREPARATION AND FIXING OF BACTERIA

The glass slide was washed with soap and water to remove all dirt and grease. The wet slide was dried and a loopful of solid culture was placed over the clean dried slide. A drop of water was placed on the slide and thoroughly mixed with culture. The smear was dried by holding the slide high above the flame. This was done 3 times to fix the smear. The area of the film was marked at the back slide of the slide with the marker.

GRAM STAINING

The fixed smear was covered with crystal vio for 30 seconds. The stain was washed off with gentle flow of tap water and the smear was then covered with gram's iodine for 60 seconds and the slide was again rinsed with tap water. The rinsed slide was decolorized with 95 per cent ethyl alcohol for 10-15 seconds. The reaction of alcohol was stopped by rinsing the slide under gentle flow of tap water. The smear was lastly covered with saffranin for 20 seconds again washed off with tap water. The slide was blot dried with filter paper and observed under high power and oil immersion objectives.

PREPARATION OF GROWTH CURVE

METHOD USED FOR COUNTING BACTERIA

In the present investigation Optical density and spread plate technique was used to determine number of organisms in a given food sample. Optical density was measured at 600 nm on Bausch and Lomb spectronic- 20 and a measured amount of suspension was spread over desired medium in a petridish.

OPTICAL DENSITY

The respective sterilized broth was analyzed for growth in terms of increase in optical density at 600 nm on Bausch and lombspectronic – 20. For blank uninnoculated sterilized broth was used.

SPREAD PLATE METHOD

The sample was diluted quantitatively. One gram or one ml of sample was diluted step-wise through a series of tubes containing known amount of sterile water. One ml of bacterial suspension was added to 9 ml of sterile water blank with a sterile 1.0 ml delivering pipette. Further dilution upto 10^{-10} were made using fresh pipettes for each. The dilution (0.1ml) was spread plated in sterilized petriplates. The petriplates should be prepared with desired medium one day before spread plating. These plates were incubated at desired temperature. The colonies were counted and multiplied by the dilution factor to obtain the viable count per ml in the original suspension.

GROWTH CURVE

The sterilized broth (100 ml) was inoculated with loopful of unknown probiotic organism. Initial count was taken by taking 0.1ml from the bacterial suspension and spread plating of the same was done. The count was taken after 4 h intervals during day time for 3 days. The number of dilutions increased with increase in time of incubation. The plates containing colones between 30- 300 were selected and multiplied with the dilution factor and the growth curve was plotted against log number of cells and time intervals.

PREPARATION OF PROBIOTIC YOGURT

STANDARDIZATION OF PROBIOTIC YOGURT

Milk was standardized to 3.5-4.0 per cent fat and was heated to 70°C and then two-stage homogenized at 65°C. The homogenized milk was then pasteurized and cooled to 43°C. Milk was then inoculated with starter culture of *Lactobacillus acidophilus* (0.5%; 1.0%; 1.5%) in case of first set of samples. In case of another set *Streptococcus thermophilus* and *Lactobacillus acidophilus* were added at different rates (0.5:0.5; 1.0:1.0; 1.5:1.5) to the yogurt. Inoculated milk was poured into cups and incubated at 42±1^{0}C for 3hrs and 30 mins. The cups containing yogurt were immediately transferred to the refrigerator and stored at 4-7°C as shown in the fig.1. The prepared product was evaluated for physiochemical parameters viz., appearance, setting, cut surface, pH and acidity. The prepared product was subjected to organoleptic evaluation and supplemented to the experimental group.

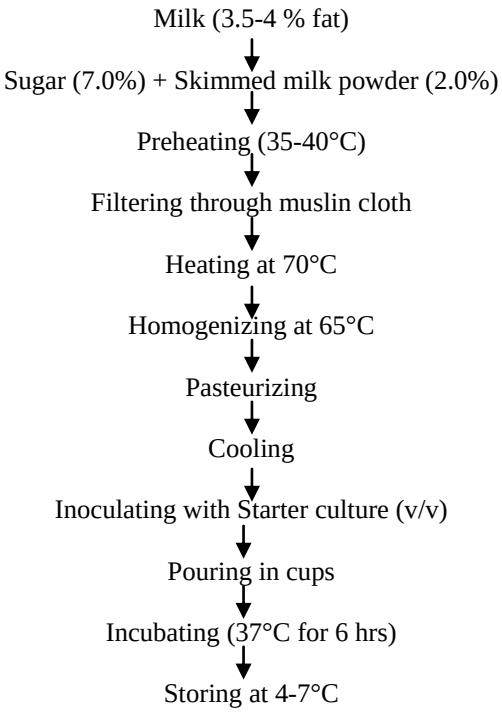

Fig.1: Steps for making Probiotic Yogurt

EVALUATION OF PREPARED PROBIOTIC YOGURT

CHEMICAL EVALUATION

The pH, acidity (%), TSS (°B), Brix acidity ratio, was measured followingAOAC (1980).

TOTAL PLATE COUNT

This was enumerated by diluting the samples decimally and spread plating 1ml aliquots on nutrient agar and incubating at 30°C for 48 after which the plates were counted using a colony counter (Macfaddin, 1980).

ORGANOLEPTIC EVALUATION

The samples of different probiotic yogurts were evaluated for organoleptic qualities on the basis of color, appearance, texture, taste, aroma and overall acceptability by a panel of judges. Consumer acceptance for the products was evaluated on a nine point hedonic scale (Amerine *et.al.,* 1965) with following scale.

Scale	Sensory Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

STATISTICAL ANALYSIS

The data on all the parameters of organpleptic evaluation was done by the mean standard error, F-ratio and their statistical significance was ascertained using a computer programme package (Cheema and Singh, 1990).

RESULTS AND DISCUSSIONS

KINETICS OF LACTOBACILLUS ACIDOPHILUS AND STREPTOCOCCUS THERMOPHILUS IN RESPECTIVE BROTHS

Growth kinetics of two bacteria namely *Lactobacillus acidophilus* and *Streptococcus thermophilus* was performed in MRS agar and Nutrient agar respectively. The growth during the fermentation was studied with respect to optical density, and viable cell count. The physiochemical properties of the yogurt prepared was studied with respect to pH, Acidity (% Lactic acid), TSS (° Brix) and Brix acid ratio.

As depicted in the Table 1 in case of *Lactobacillus acidophilus* the Growth curves of optical density and viable cell count (log10 no. of cells per ml.) showed a definite pattern with first a long lag phase of 12 hrs followed by exponential growth up to 60 hrs as indicated by sharp increase in optical density and viable cell count. Whereas in case of *Streptococcus thermophiles*

as in table 2, there was a short lag phase of 3 hrs followed by exponential growth up to 48 hrs the stationary phase for both the stains was shortened and not much increase in cell no. was observed. Viable cell count started decreasing 72 hrs onwards showing death phase. Decrease in yield of growth and acceleration of bacterial death may be due to inadequate supply of nitrogenous substances, vitamins, concentration of dissolved oxygen, and concentration of insoluble solids (Pandove, 2007 and Nazni *et.al.*, 2014). Physicochemical properties of the Yogurt like decrease in pH, brix acid ratio and increase in acidity was also observed along with the organoleptic evaluation of the two different formulated probiotic yogurts.

Table1: Growth of *Lactobacillus acidophilus* in MRS Broth and MRS Agar in terms of optical density and cell count

Incubation time (hrs)	Optical density at 600nm	Log10 no. of cell per ml
0	0.00	0
3	0.05	0
6	0.05	0
9	0.06	0
12	0.06	0
24	0.07	0.6
27	0.10	1.09
30	0.15	1.61
33	0.20	1.94
36	0.26	2.15
48	0.32	2.23
57	0.72	2.45
60	0.80	2.70
72	0.85	2.80

Growth Conditions
Bacterial Culture – *Lactobacillus acidophilus*
Incubation temperature - 37±2°C
Inoculum Concentration – 0.5% v/v

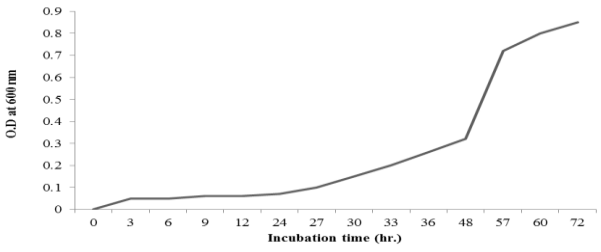

Fig.2: Growth of Lactobacillus acidophilus in MRS Broth in terms of optical density

PHYSIOCHEMICAL PROPERTIES OF PROBIOTIC YOGURTS

PROBIOTIC YOGURT PREPARED ONLY FROM *LACTOBACILLUS ACIDOPHILUS*

As given in Table 3 the physico-chemical characteristics of Yogurt prepared with 0.5%v/v inoculums concentration was pH 3.84at 0 hr and decreased to 3.81 at the 72 hr., % Acidity increased from 0.54 at 0 hr. to 0.96 at 72 hr, TSS did not vary much from 7.1 to 7.0 but the Brix acid ratio decreased from 13.15 at 0 hr to 7.29 at 72 hr as seen the Table 3. When the inoculums concentration was increased to 1.0%v/v the pH was 3.88 at 0 hr. decreasing to 3.79 at 72 hr, the % acidity also increased to 0.89 by the 72 hr. from 0.51, TSS almost remained constant at 7.1 and the Brix acid ratio decreased from 14.12 at 0 hr. to 7.98 at 72 hr. On increasing the inoculums concentration to 1.5%v/v again decrease in pH was noticed at 72 hr (3.79) from 0 hr. (3.88), % acidity increased from 0.48 to 0.63 within a period of 72 hr, TSS remained nearly constant to 7.0, and Brix acid ratio decreased from 15.21 (0hr) to 11.11 (72hr.). In all the cases the growth conditions were kept constant at 25±2°C.

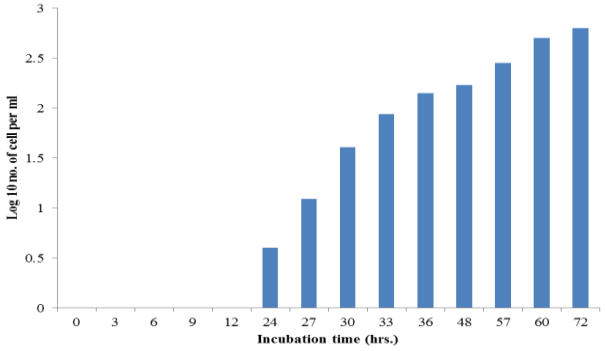

Fig.3: Growth of Lactobacillus acidophilus in MRS Agar in terms of cell count

Table2: Growth of *Streptococcus thermophilus* in Nutrient Broth and Nutrient Agar in terms of optical density and cell count

Incubation time (hrs)	Optical density at 600nm	Log10 no. of cell per ml
0	0.00	0.00
3	0.06	1.09
6	0.15	1.60
9	0.25	1.94
12	0.40	2.19
24	0.60	2.30
27	0.64	2.39
30	0.67	2.45
33	0.69	2.62
36	0.70	2.80
48	0.74	3.11
57	0.84	3.20
60	0.90	3.35
72	0.89	3.40

Growth Conditions
Bacterial Culture – Streptococcus*thermophilus*
Incubation temperature - 37±2°C
Inoculum Concentration – 0.5% v/v

PROBIOTIC YOGURT PREPARED FROM *LACTOBACILLUS ACIDOPHILUS* AND *STREPTOCOCCUS THERMOPHILUS*

The physico-chemical characteristics of Yogurt prepared with 0.5%v/v inoculum concentration of both stains each was pH 3.78at 0 hr and decreased to 3.72 at the 72 hr., % Acidity increased from 0.51 at 0 hr. to 0.83 at 72 hr, TSS did not vary much from 7.5 to 7.0 but the Brix acid ratio decreased from 14.70 at 0 hr to 8.433 at 72 hr (Table 2). When the inoculums concentration was increased to 1.0%v/v each the pH was 3.82 at 0 hr. decreasing to 3.66 at 72 hr, the % acidity also increased to 0.89 by the 72 hr. from 0.44, TSS almost changed from 8.0 to 7.0 within 72 hrs and the Brix acid ratio decreased from 18.18 at 0 hr. to 7.86 at 72 hr. On increasing the inoculums concentration to 1.5%v/v again decrease in pH was noticed at 72 hr (3.64) from 0 hr. (3.90), % acidity increased from 0.35 to 0.66 within a period of 72 hr, TSS varied from 6.8 to 7.0, and Brix acid ratio decreased from 22.85 (0hr) to 10.61 (72hr.) as shown in table 4. In all the cases the growth conditions were kept constant at 25±2°C.

ORGANOLEPTIC EVALUATION OF PROBIOTIC YOGURTS

PROBIOTIC YOGURT CONTAINING SINGLE STAIN *LACTOBACILLUS ACIDOPHILUS*

The mean score of the acceptability trials of the formulated yogurt by the expert panel of 10 judges using nine hedonic scales are presented in the Table 5. Three samples of yogurt were prepared using different inoculum concentration of the stain (*L.acidophilus*) and normal commercially available curd was used as standard. The mean score of colour was highest for A3 (1.5% v/v) was 7.87 which was liked very much. The mean scores for appearance and flavor was 8.00 and 7.60 which was highest when compared to others samples. The mean score of texture and taste was also highest with in case of A3 i.e. 7.97 and 8.13 respectively in comparison to other samples (A1 and A2) as well as control. The overall acceptability was also highest 8.37, and was liked extremely. In case of samples with lower inoculum concentration decreased the mean score than A3 and the overall acceptability also less than sample A3. Therefore the male subjects at risk CHD were supplemented with A3, containing 1.5% v/v inoculums concentration of *Lactobacillus acidophilus*.

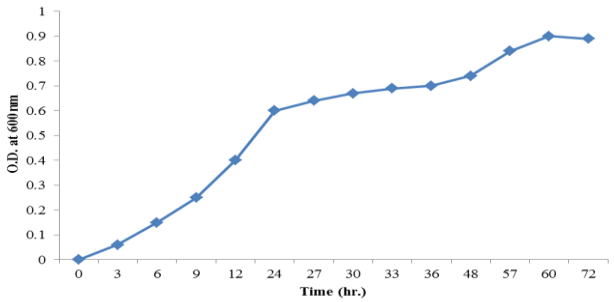

Fig.4: Growth of *Streptococcus thermophilus* in Nutrient Broth in terms of optical density

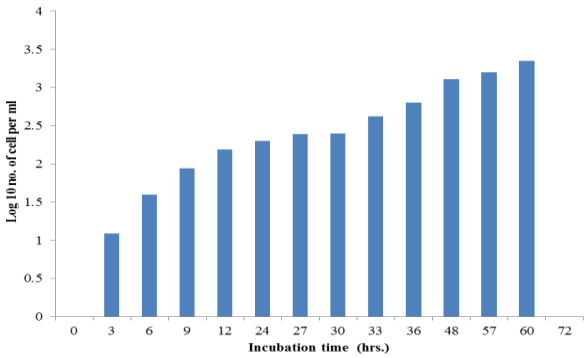

Fig.5: Growth of Streptococcus thermophilus in Nutrient Agar in terms of cell count

Table3: Effect of Storage on Physiochemical characteristics of Yogurt prepared from – *L. acidophilus*

Inoculum Concentration – 0.5% v/v					
Incubation time (hrs)	**pH**	**Acidity %**	**TSS (°B)**	**Brix Acid Ratio**	**Total Plate count**
0	3.84	0.54	7.1	13.15	$7.53x10^7$
24	3.85	0.64	7.3	11.41	$7.27x10^7$
48	3.83	0.77	7.0	9.10	$6.77x10^8$
72	3.81	0.96	7.0	7.29	$8.27x10^7$
Inoculum Concentration - 1.0 % v/v					
0	3.86	0.51	7.2	14.12	$6.73x10^8$
24	3.87	0.57	7.1	12.46	$7.50x10^9$
48	3.85	0.70	6.9	9.86	$8.23x10^8$
72	3.77	0.89	7.1	7.98	$9.17x10^8$
Inoculum Concentration - 1.5 % v/v					
0	3.88	0.48	7.3	15.21	$3.54x10^9$
24	3.83	0.51	7.2	14.12	$5.92x10^9$
48	3.87	0.57	7.0	12.29	$8.78x10^9$
72	3.79	0.63	7.0	11.11	$4.99x10^9$

Growth Conditions
Bacterial Culture - *Lactobacillus acidophilus*
Incubation temperature - 25±2°C

PROBIOTIC YOGURT CONTAINING TWO STAINS *LACTOBACILLUS ACIDOPHILUS* AND *STREPTOCOCCUS THERMOPHILUS*

The mean score of the acceptability trials of the formulated yogurt by the expert panel of 10 judges using nine hedonic scales are presented in the Table 5.Three samples of yogurt were prepared using different inoculum concentration of the stains (*L.acidophilus* and *S. thermophilus*) and normal commercially available curd was used as standard. The mean score of colour was highest for B2 (1.0% v/v each) was 8.57 which was extremely liked. The mean scores for appearance and flavor was 8.23 and 7.83 which was highest when compared to others samples. The mean score of texture and taste was also highest in case of B2 i.e. 8.57 and 8.10 respectively in comparison to other samples (B1 and B3) as well as control. The overall acceptability was also

highest 8.30, and was liked extremely. In case of samples with inoculum concentration 0.5 % v/v i.e. B1 and 1.5% v/v i.e. B3 decreased the mean score when compared to B2 and the overall acceptability also less than sample B2 (table 6). The same observation was reported by Nazni *et.al.,* 2014.

Table 4: Effect of Storage on Physiochemical characteristics of Yogurt Prepared from– *L. acidophilus* and *S.thermophilus*

Inoculum Concentration - 0.5 % v/v each					
Incubation time (hrs)	**pH**	**Acidity %**	**TSS (°B)**	**Brix Acid Ratio**	**Total Plate count**
0	3.78	0.51	7.5	14.70	$6.50x10^8$
24	3.83	0.57	7.5	13.16	$8.27x10^8$
48	3.85	0.64	6.8	10.62	$8.47x10^8$
72	3.72	0.83	7.0	8.433	$8.77x10^8$
Inoculum Concentration – 1.0 % v/v each					
0	3.82	0.44	8.0	18.18	$7.43x10^9$
24	3.90	0.54	7.5	13.88	$8.51x10^9$
48	3.84	0.61	6.9	11.31	$8.23x10^9$
72	3.66	0.89	7.0	7.86	$7.27x10^9$
Inoculum Concentration - 1.5 % v/v each					
0	3.90	0.35	8.0	22.85	$5.54x10^9$
24	3.75	0.40	6.9	17.25	$6.72x10^{10}$
48	3.75	0.45	6.8	15.11	$8.21x10^{10}$
72	3.64	0.66	7.0	10.61	$8.33x10^9$

Growth Conditions

Bacterial Culture - *Lactobacillus acidophilus and Streptococcus thermophilus*

Incubation temperature - 25±2°C

Table 5: Organoleptic Scores of Probiotic Yogurt (Mean ± SE)

Sample	Color	Appearance Acceptability	Flavor	Texture	Taste	Overall
Control	7.50±0.17	7.60±0.13	6.77±0.14	7.73±0.13	6.30±0.19	6.87±0.12
A1	7.63±0.14	7.30±0.13	6.97±0.11	7.07±0.14	7.16±0.11	7.33±0.11
A2	7.60±0.12	7.57±0.12	7.30±0.09	7.60±0.14	7.60±0.12	7.63±0.08
A3	7.87±0.14	8.00±0.16	7.60±0.11	7.97±0.13	8.13±0.12	8.37±0.12
B1	7.87±0.12	7.67±0.12	7.16±0.11	7.80±0.13	7.30±0.10	7.37±0.09
B2	8.17±0.14	7.90±0.13	7.73±0.12	8.07±0.10	8.10±0.11	8.30± 0.11
B3	8.50±0.12	8.23±0.13	7.83±0.10	8.53±0.11	7.97±0.13	8.00±0.11
F-Ratio	6.44**	5.69**	11.66**	11.85**	24.00**	24.77**
CD at 5%	.39	.38	.33	.36	.38	.31

Amerin*et al* (1965)

** Significant at 5% level

Control - Normal Curd

A1 = 0.5% *Lactobacillus acidophilus,* A2 = 1.0% *Lactobacillus acidophilus,* A3 = 1.5% *Lactobacillus acidophilu,* B1 = 0.5% *Lactobacillus acidophilus* + 0.5% *Streptococcus thermophilus,* B2 = 1.0% *Lactobacillus acidophilus* + 1.0% *Streptococcus thermophilus* B3 = 1.5% *Lactobacillus acidophilus* + 1.5% *Streptococcus thermophilus*

CONCLUSION

Use of yogurt containing probiotic strains like Lactobacillus acidophilus and Streptococcus thermophilus should be encouraged as it helps to improve lipid profile and could be easily incorporated in our daily diet along with meals. A dairy product containing probiotics is a safe alternative immunotherapeutic agent and healthy "functional food package" in addition to the vitamins, calcium, other minerals, and protein obtained from milk products. People should be encouraged to consume probiotic yogurt as it is natural, safe, has no side effects and economical alternative to the usually used hypolipidemic drugs.

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