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Compatibility Assessment of Yoghurt Starters with Indigenous Isolates of *Lactobacillus acidophilus for* Development of Synbiotic Yoghurt by Checking Different Attributes like Contact Inhibition, Titrable Acidity, Viable Counts and pH

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A B S T R A C T

Yogurt is produced by culturing with the lactic-acid producing bacteria *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. Additional probiotic cultures can be added for the purpose of conferring their presumptive health benefits. Different starter strains *Streptococcus thermophilus* NCDC 74 (ST-74), *Streptococcus thermophilus* NCDC 311 (ST-311), *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDC 9 (LB-9) and *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDC 305 (LB-305) were comparatively evaluated for the compatibility with probiotics *lactobacillus acidophilus* NCDC 13 (LA-13), *Lactobacillus acidophilus* NCDC 291(LA-291) to find out a desirable combination for the development of synbiotic yoghurt, by checking different attributes like contact inhibition, titrable acidity, viable counts and pH. LA-13 shows more increment in the count during co-culturing with LA-291. Although LA-291 has more acid producing ability than LA-13, there is no considerable difference between the titrable acidity of these two. Further the increment in the count of LA-13 was higher with the combination LB-09 + ST-74 than all other combinations. This study suggest the importance of incorporation of starter strains during preparation of synbiotic yoghurt with selected *Lactobacillus acidophilus* isolate as it increases the viability of probiotic strain during storage of food product and also improves the product characteristics.

Introduction

Probiotic cultures have been exploited extensively by the dairy industry as a tool for the development of novel products. Especially yogurt, are frequently used as probiotic delivery systems. According to the standards of identity listed in the United States Code of Federal Regulations (CFR), yogurt (in section 21 CFR part 131.200) is produced by culturing with the lactic-acid producing bacteria *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus. Additional* cultures known as "probiotics" may be added for the purpose of conferring their presumptive health benefits. *L. bulgaricus* and *S. thermophilus* are used as the main yoghurt starters, and probiotics are added as

supplements. Probiotics used in commercial products today are mainly members of the genera *Lactobacillus* and *Bifidobacterium*.

Some probiotics (especially lactic acid bacteria) are fastidious microorganisms, and are susceptible to environmental conditions, such as organic acid accumulation, high temperature, hyper osmotic pressure, low water activity, high redox potential (presence of oxygen) and the presence of inhibitory substances (Lourens-Hattingh and Viljoen, 2002). Consequently, survivability and stability of probiotics have become a marketing and technological challenge for the industry. Therefore, in order to obtain their desirable functional properties, probiotics need to be delivered in sufficient numbers with high viable rate (Talwalker and Kailasapathy, 2004). Yogurt starter cultures may enhance the growth and survival of probiotics by producing growth-promoting substrates or by reducing the oxygen content in milk (Saarela *et al.*, 2000; Vinderola *et al.*, 2002). *S. thermophilus* is a facultative anaerobic microorganism, which reduces the redox potential in milk by the consumption of oxygen by NADH oxidase (an enzyme catalyzed NADH + H^+ with the presence of O_2 to NAD and hydrogen peroxide) and pyruvate oxidase (an enzyme catalyzed pyruvate with the presence of O_2 to acetate and CO_2) (Teraguchi *et al.*, 1978). Accordingly, this can create an anaerobic condition stimulating the growth of *L. bulgaricus* and probiotics in yoghurt (Lankaputhra and Shah, 1996). In addition, *L. bulgaricus* possess proteolytic enzymes that liberate amino acids such as valine, glycine, and histidine that are essential to some probiotics (*lactobacilli* species) (Tamime, 2005).

Since the efficacy of probiotics is related to the viable number at the time of consumption, they must survive during the processing, storage and transit through the gastrointestinal tract. Yoghurt starters possess some benefits during fermentation. Hence the aim of this study is to comparatively evaluate the compatibility of starter strains with the *lactobacillus* strains to find out a desirable combination for the development of synbiotic yoghurt.

Materials and Methods

Bacterial strains and growth conditions

The probiotic strains *Lactobacillus acidophilus* NCDC 13 (LA-13), *Lactobacillus acidophilus* NCDC 291(LA-291) and starter strains *Streptococcus thermophilus* NCDC 74 (ST-74), *Streptococcus thermophilus* NCDC 311 (ST-311), *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDC 305 (LB-305) and *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDC 09 (LB-09) used in the present investigation were obtained from the National Collection of Dairy Cultures (NCDC), Dairy Microbiology Division, National Dairy Research Institute (NDRI), Karnal, India. Freeze dried lactic cultures were activated in chalk litmus milk at 37°C for 24 hr. The yoghurt starters, *Streptococcus thermophilus* strains were activated and maintained in M17 medium (Himedia, Mumbai, India), while the *Lactobacillus bulgaricus* strains were activated and maintained in RCA medium (Himedia, Mumbai, India), and sub-cultured monthly. Before use, the lactic cultures were subcultured twice in de Man Rogosa Sharpe (MRS) broth (Himedia, Mumbai, India) and the yoghurt starters in their respective mediums.

Compatibility of *L. acidophilus* strains with yoghurt starters

The compatibility of probiotic *L. acidophilus* strains with yoghurt cultures was evaluated by well-diffusion agar assay and further confirmed by growth and activity studies by co-culturing in skim milk.

Well-diffusion agar assay

The starter cultures whose inhibitory actions have to be determined were grown in 10% reconstituted skim milk at 37°C for 24 hr. Cell-Free Supernatants (CFS) were obtained by centrifugation at 3300 X g for 20 min at 5°C. Sterilization of CFS was done by filtration through 0.45 µm pore filter. Twenty millilitre of MRS agar (for *L. acidophilus*), M17 agar (for *S. thermophilus*) and RCA (for *L. bulgaricus*) was melted, tempered to 45°C and vigorously mixed with 200 µl of an overnight culture and poured into petri dishes. After setting, wells of 5 mm in diameter were made and 50 µl of CFS of the respective strain was placed into each well. The plates were kept in the refrigerator for proper diffusion and incubated aerobically or anaerobically at 37°C for 48 hr. After incubation, the plates were examined for clear zones around well. Experiments were replicated three times (Vinderola *et al.*, 2002).

Compatibility in relation to growth and activity

The two probiotic *L. acidophilus* strains were inoculated in co-culture with two combinations of yoghurt starters as enlisted in table 1. Sterlized 12% reconstituted skim milk was used in the medium for growth. The yoghurt starters were inoculated (2%) in the ratio of 1:1 and probiotic *L. acidophilus* strains (1%) were used as controls. All the samples were incubated at 42°C for 4 hr. The samples were analyzed for pH, titrable acidity and individual counts before and after the incubation period.

Analysis of samples

pH

The pH of the samples was measured using a Digital pH meter (Model CP901, centaury) after calibrating with pH 4.0 and pH 7.0 standard buffers.

Titrable acidity

The acidity of the samples in terms of percent lactic acid was determined as per the method described in IS : 1479 (1960).

Selective enumeration of organisms

One ml of the sample was diluted with 9 ml of 0.1 % peptone water and mixed uniformly with a vortex mixture. Subsequent serial dilutions were prepared and viable numbers enumerated using pour plate technique. The counts of *S. thermophilus* were enumerated on M17 agar after incubation aerobically at 37°C for 48 hr (Terzaghi and Sandine, 1975). RCA agar adjusted to pH 5.3 and anaerobic incubation at 37°C for 72 hr was used for the selective enumeration of *L. bulgaricus* (Dave and Shah, 1997 b). Maltose-MRS agar was used for the enumeration of *L. acidophilus*, as *L. bulgaricus* did not utilize maltose. Incubation was carried out anaerobically at 37°C for 48 hr (Hull and Roberts, 1984).

Results and Discussion

Evaluation of compatibility of *L. acidophilus* strains with yoghurt starters

In order to select a suitable combination for the manufacture of yoghurt containing *L. acidophilus,* the interactions among the organisms was checked by well diffusion agar assay and the effect on the growth and acid production was studied by co-culturing them in skim milk.

Well-diffusion agar assay

A total of four strains of yoghurt starter bacteria (two of each *S. thermophilus* and *L. bulgaricus)* and two probiotic *L. acidophilus*

strains were tested for interactions among them using cell-free supernatants (CFS) obtained from skim milk cultures. Four behaviours were observed with this methodology: complete inhibition (a clear absence of growth around the well), weak inhibition (partial inhibition around the well), absence of inhibition and more growth around the well.

Effect of yoghurt starters on *L. acidophilus* strains

The effect of CFS obtained from yoghurt starters on the growth of *L. acidophilus* strains is presented in table 2. As evident from the table, the Lb-09 completely inhibited the growth of LA-291, whereas ST-74 showed a partial inhibition of growth of LA-291. Both the yoghurt starters Lb-09 and ST-74 did not affected the growth of LA-13, which suggests its compatibility with these starters. It was found that the yoghurt starters Lb-305 and ST-311 had no effect on the growth of both the probiotic strains. Vinderola *et al.,* (2002) also observed a weak inhibition by *L. bulgaricus* supernatant on the growth of *L. acidophilus* strains.

Dave and Shah (1997a) suggested that the possible factors affecting the viability of *L. acidophilus* strains could be the antagonism by yoghurt strains or the higher concentration of hydrogen peroxide produced by *L. bulgaricus.* The partial inhibition of ST-74 on the probiotic strain LA-291 may be due to the higher acid production by the former organism.

Effect of probiotic strains on yoghurt starters

Table 3 shows the effect of cell-free supernatants of *L. acidophilus* strains on the growth of *L. bulgaricus* and *S. thermophilus* strains. The probiotic strain LA-291 showed a partial inhibition on the growth of LB-305, but had no effect on the growth LB-09, whereas the strain LA-13 showed absence of interaction towards both the *L. bulgaricus* strains. The cell-free supernatants of both probiotic strains had a stimulatory effect on the growth of *S. thermophilus* strains, as more growth was observed around the well. *L. acidophilus* was shown to produce bacteriocin against several strains of *L. bulgaricus, L. helveticus* etc. (Dave and Shah, 1997a). Vinderola *et al.,* (2002) also reported a complete growth inhibition of *L. Bulgaricus* A61 when cultured in cell-free supernatant of *L. Acidophilus* CNRZ 1881. The growth stimulation of *S. Thermophilus* strains may be attributed to some growth factors produced by *L. Acidophilus* strains during the growth in skim milk for 24 hr.

Compatibility in relation to growth and activity

The growth and activity of probiotic strains in pure culture and in combination with yoghurt cultures was studied in order to select the more compatible cultures.

Growth compatibility of *L. acidophilus* strains with yoghurt starters LB-09 and ST-74

The increment in count of the individual cultures, attained after co-culturing is depicted in table 4. The probiotic strain LA-291 recorded an increment in count by 1.85 log cycles in culture, but the count decreased to the extent that it was not detectable during co-culturing with yoghurt starters. The increase in the count of LA-13 following an incubation period of 4 hr was 1.88 log cycle in pure culture and 1.07 log cycle in combination. In general, the growth of *L. acidophilus* was found to decrease during co-culturing with yoghurt starters. This is in agreement with the result obtained by Oliveira *et al.,* (2001), who

reported that the mixed cultures resulted in lower counts of probiotic strains and it could be explained by a mechanism of nutritional competition.

The increment in count of *L. bulgaricus* did not show much difference in presence of *L. acidophilus* strains. The increase in count was 1.41 log cycle with ST-74 and slightly declined to the extent of 1.10 to 1.30 log cycle during co-culturing with *L. acidophilus* strains. On the contrary the counts of *S. thermophilus* showed a slight increase (1.52 to 1.75 log cycle), while used in combination with probiotic strain, which confirm the synergistic action between them as observed in well-diffusion agar assay. The less growth of *L. bulgaricus* in presence of *L. acidophilus* strains can also be attributed to the completion of nutrients.

Figure 1 represents titrable acidity in terms of lactic acid after an incubation period of 4 hr ranged from 0.31 to 0.43% in cases of pure cultures of *L. acidophilus* and 0.91 to 0.96% (Table 4) in case of combination which suggests that the *L. acidophilus* is the slow acid producer as compared to yoghurt starters and require long incubation period. The pH also showed similar pattern with the lowest value (4.51-4.63) in case of combination when compared to pure cultures (6.16-6.33). Similar results have been reported by Oliviera *et al.*, (2001). The time needed to attain the maximum acidification rate was found to be longer for pure cultures of *L. acidophilus* than that with mixture cultures. Shah (2001) reported that it was necessary to add yoghurt starters to attain the desired characteristics in the finished probiotic yoghurt.

Growth compatibility of *L. acidophilus* strains with yoghurt starters LB-305 and ST-311

Table 5 presents the growth and activity of *L. acidophilus* strains during co-culturing with yoghurt starter combination LB-305 and ST-311. As evident from the data, the increment in the count of LA-291 declined to 0.70 log cycle, while that of LA-13 declined to 0.73 log cycle during co-culture. This again suggests that co-culturing reduces the count of *L. acidophilus* (Oliviera *et al.*, 2001). The increment in the count of LB-305 found to be lowest (1.21 log cfu/ml) when co-cultured with LA-291. This confirms the result obtained in well-diffusion agar assay that LB-305 was partially inhibited by LA-291. There was no significant difference in the increment in count of LB-305 in presence of NCDC 13. Co-culturing with *L. acidophilus* did not exhibit much difference in the count of ST-311.

Table.1 The combinations of yoghurt starters and probiotic *L. acidophilus* strains used for compatibility studies

Sl. No.	Yoghurt Starters	Probiotic *L. acidophilus* Strains	Incubation Temperature (°C)	Incubation Time (hr)
1.	ST-NCDC 74 (1%)	LA-NCDC 291 (1%)	42	4
	LB-NCDC 09 (1%)			
2.	ST-NCDC 74 (1%)	LA-NCDC 13 (1%)	42	4
	LB-NCDC 09 (1%)			
3.	ST-NCDC 311 (1%)	LA-NCDC 291 (1%)	42	4
	LB-NCDC 305 (1%)			
4.	ST-NCDC 311 (1%)	LA-NCDC 13 (1%)	42	4
	LB-NCDC 305 (1%)			

Table.2 Effect of cell-free supernatants of yoghurt starters on probiotic *L. acidophilus* strains

Probiotic Strains	Cell-Free Supernatants (CFS)			
	LB-09	**LB-305**	**ST-74**	**ST-311**
LA-291	*	–	×	–
LA-13	–	–	–	–

***** = Complete inhibition, x = Weak inhibition, – = No effect.

Table.3 Effect of cell-free supernatants of probiotic *L. acidophilus* strains on yoghurt starters

Yoghurt Starters	Cell-Free Supernatants (CFS)	
	LA-291	**LA-13**
LB-09	–	–
LB-305	×	–
ST-74	+	+
ST-311	+	+

+ = More growth around well, × = Weak inhibition, — = No effect.

Table.4 Growth Compatibility of *L. acidophilus* Strains with Yoghurt Starters *L. bulgaricus* NCDC 09 and *S. thermophilus* NCDC 74

Culture Combination	Increment in count after incubation (log cfu/ml)			
	LB-09	ST-74	LA-291	LA-13
LA- 291 (1%)	–	–	1.85	–
LA- 13 (1%)	–	–	–	1.88
LB- 09 (1%) + ST- 74 (1%)	1.41	1.52	–	–
LB- 09 (1%) + ST- 74 (1%) + LA- 291 (1%)	1.30	1.75	ND	–
LB- 09 (1%) + ST- 74 (1%) + LA- 13 (1%)	1.10	1.73	–	1.09

ND=Not Determined

Table.5 Growth Compatibility of *L. acidophilus* Strains with Yoghurt Starters *L. bulgaricus* NCDC 305 and *S. thermophilus* NCDC 311

Culture Combination	LB-305	ST-311	LA-291	LA-13
LA-291 (1%)	–	–	1.94	–
LA-13 (1%)	–	–	–	1.82
LB-305 (1%) + ST-311 (1%)	1.52	2.16	–	–
LB-305 (1%) + ST-311 (1%) + LA-291 (1%)	1.21	1.95	0.70	–
LB-305 (1%) + ST-(1%) + LA-13 (1%)	1.39	2.18	–	0.73

Fig.1 Effects on Titrable Acidity & pH in Different Culture Combinations. A= LA- 291 (1%), B= LA- 13 (1%), C= LB- 09 (1%) + ST- 74 (1%), D= LB- 09 (1%) + ST- 74 (1%) + LA- 291 (1%), E= LB- 09 (1%) + ST- 74 (1%) + LA- 13 (1%)

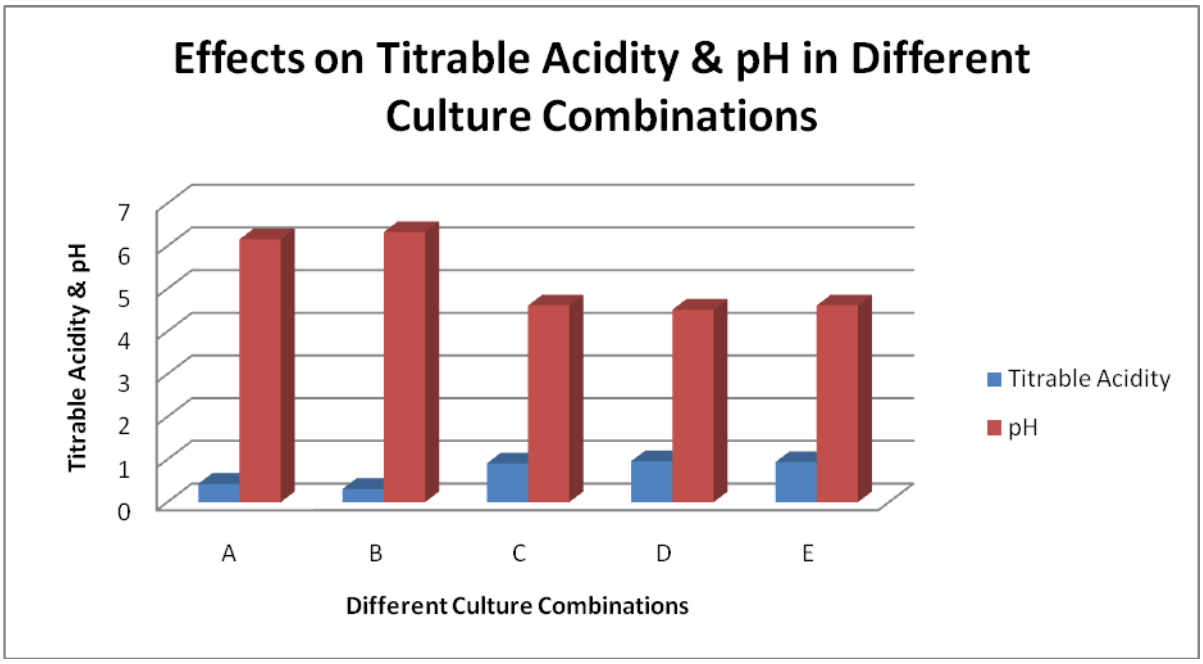

Fig.2 Effects on titrable acidity & pH in different culture combinations. A= LA- 291 (1%), B= LA- 13 (1%), F= LB-305 (1%) + ST-311 (1%), G= LB-305 (1%) + ST-311 (1%) + LA-291 (1%), H= LB-305 (1%) + ST-311 (1%) + LA-13 (1%)

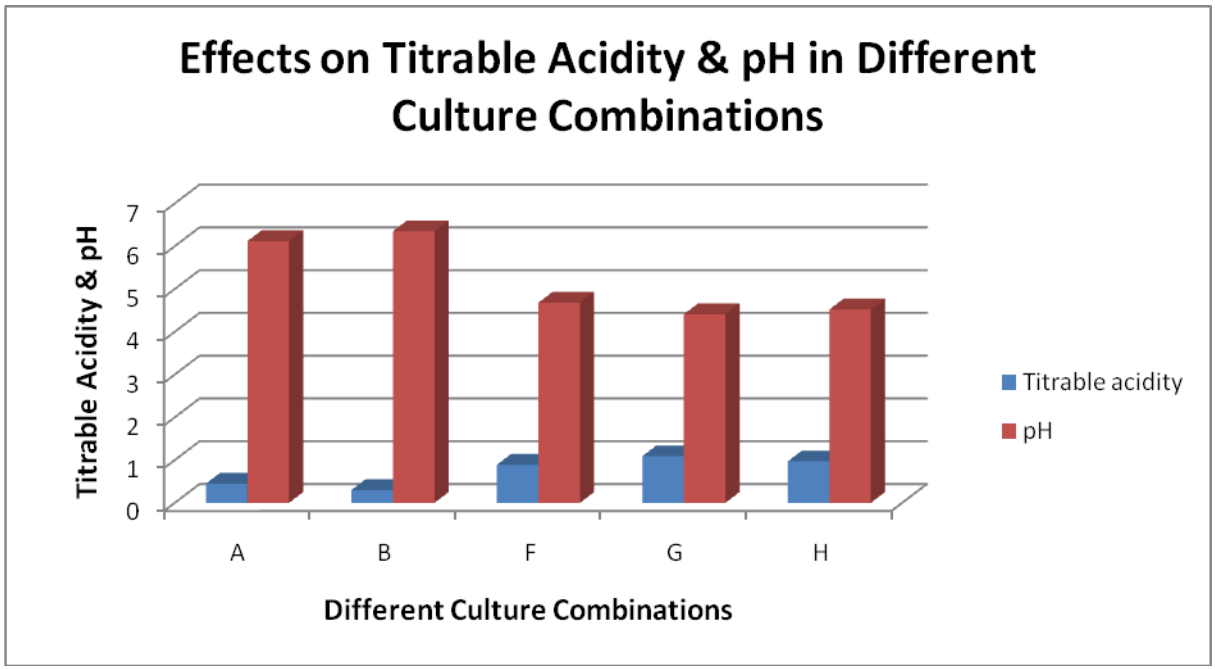

Figure 2 represents titrable acidity was found to be varying from 0.30-0.45% lactic acid in case of pure cultures and from 0.89 to 1.09% lactic acid in case of culture combinations. The highest titrable acidity was recorded for the combination of NCDC 305 + 311 + 291. The pH of the fermented samples also showed similar trends for the lowest pH (4.41) for the culture combination containing LA-291. Among the pure culture also, LA-291 produced more acidity than LA-13. These results showed that LA-291 is a better acid producer as compared to LA-13.

This study concludes as follows:

Selection of suitable combination

The compatibility studies showed that *L. acidophilus* NCDC 13 is more compatible than *L. acidophilus* NCDC 291. Although LA-291 has more acid producing ability than LA-13, there is no considerable difference between the titrable acidity of these two. Therefore, with respect to the increment in the count during co-culturing LA-13 was selected as the probiotic strain for further studies. Further the increment in the count of LA-13 was higher (1.09 log cfu/ml) with the combination LB-09 + ST-74. Also LB-09 + ST-74 are the defined and the commonly used yoghurt starters. Therefore the starter culture combination LB-09 + ST-74 and *L. acidophilus* NCDC 13 was selected for yoghurt making.

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Abbreviations: CFU, Colony Forming Units

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