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Effect of Lactic Acid Microbial Ratio of Yoghurt Starter Culture in Yoghurt Fermentation and Reduction of Post Acidification

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Abstract

The microbial ratio of the bulk yoghurt starter cultures determines the strength of proto cooperation among the lactic acid bacteria which can build up the proper chemical and physical structure of the yoghurt and control the post-acidification effect. Therefore, this attempt is to find out the best microbial ratio of the yoghurt starter culture which can produce good quality yoghurts and control post acidification. The isolated *L. bulgaricus* and *S. thermophilus* strains from bulk cultures were used to prepare new starter cultures by changing *L. bulgaricus*, *S. thermophilus* ratios as 1:1, 1:1.5 and 1.5:1 and then *S. thermophilus* and *L. bulgaricus* growth pattern during the yoghurt incubation period was determined and compared that with the typical lactic acid fermentation growth curve. Then each culture added yoghurt sample was stored under the cooling conditions for 15 days and then checked pH, acidity like chemical changes, physical changes of the yoghurt and sensory properties to select the best microbial ratio. The results revealed when *L. bulgaricus* and *S. thermophilus* ratio was 1:1, their growth patterns are similar to the typical lactic acid growth curve as two exponential phases separated from stationery phase which reveals the efficient proto cooperation among two bacteria and higher metabolic potentials of them which is able to release metabolites into the fermenting medium in right time to develop standard yoghurt qualities as texture, color, taste and pH. Furthermore, the results revealed end of the 15 days pH of yoghurt was 4.4 which are suitable to maintain the good quality of yoghurt with high shelf life. But when *L. bulgaricus* population is high in the yoghurt starter culture, post acidification was high, and it deforms the yoghurt structure due to syneresis. And, when *S. thermophilus* population is high in the yoghurt starter culture, sensory qualities of the yoghurt were not up to the standards.

Keywords: Proto cooperation; Post acidification; Bulk culture; Syneresis; Exponential phase

Introduction

Yoghurt is a coagulated milk product which is produced due to the fermentation activity of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* like lactic acid bacteria in the milk and milk products [1]. Lactic acid bacterial collection which use in the yoghurt production is called yoghurt starter culture and most of the industrial yoghurt starter cultures are defined mixed cultures [2]. These defined cultures can be DVS (Direct vat set cultures) and bulk cultures [3]. Defined yoghurt starter cultures consist with various kinds of microorganisms as *Streptococcus thermophilus*, *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Streptococcus thermophilus Lactobacillus lactis* and *Bifidobacterium* sp.

Proto cooperation among lactic acid bacteria gives proper chemical and physiological characters to the yoghurt [4]. Proto cooperation is stimulation of each other's growth by exchanging metabolites produced by each of these lactic acid bacteria [5]. In fact, *S. thermophilus* utilizes amino acids in milk and produce folic acid. This folic acid stimulates the growth of *L. bulgaricus*. Metabolic activity of each of these bacteria enhances their growth rates due to the available compound in the medium and produced compounds to the medium. As a result of these metabolic potentials, growth curve of *S. thermophilus* consists with two exponential phases separated from transition phase (Figure 1). Due to the created favorable environment by *S. thermophilus*, *L. bulgaricus* starts their exponential growth [6]. For well proto cooperation, microbial ratio of the lactic acid bacteria in the yoghurt starter culture is very important. As well as, proper proto cooperation controls the post acidification when yoghurt store under the cooling temperature for long time.

Survival ability of lactic acid bacteria in cooling temperature of the yoghurt creates excess acidity in the yoghurt and it is called post acidification [7]. Due to the high acidity development in the yoghurt, it causes some leakages of whey proteins which are called syneresis [8]. These qualitative defects in the yoghurt structure reduce the shelf life of the yoghurt sample. Post acidification depends on the type of strain, microbial ratio in the yoghurt starter culture, the storage temperature, and the storage time. Post acidification manipulation can be done changing the microbial ratio and it is increased the shelf life of the yoghurt.

Sri Lankan yoghurt producers basically depend on the bulk yoghurt starter cultures. The microbial ratio of the bulk yoghurt starter culture determines the quality and shelf life of the yoghurt. The better proto cooperation among the lactic acid bacteria build up the proper chemical and physical qualities in the yoghurt and control the post-acidification effect of the yoghurt. Therefore, this attempt is to select the best lactic acid microbial ratio which can produce yoghurt with proper chemical and physical properties and low post acidification to enhance the shelf life of yoghurt.

Method and Materials

Determination of LB: ST ratio of currently using bulk culture

L. bulgaricus to *S. thermophilus* ratio of the currently using bulk culture of Milco (pvt) Ltd in Sri Lanka was determined following serial dilution and pour plate method using modified Elliker medium.

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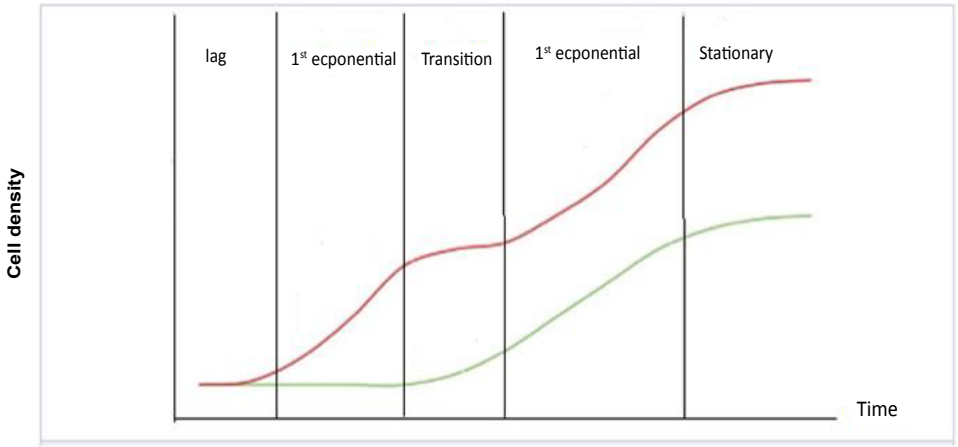

Figure 1: Growth phases in the typical co-fermentation of *S. thermophilus* (red) and *L. bulgaricus* (green).

Isolation of *L. bulgaricus* and *S. thermophilus*

L. bulgaricus and *S. thermophilus* bacterial species were isolated from the currently using bulk culture in the milk factory. Moreover, *L. bulgaricus* in the bulk yoghurt starter culture was isolated into modified MRS medium and *S. thermophilus* was isolated into modified M17 medium. Then pure cultures were prepared.

LB1: ST1 ratio, LB1.5: ST1.5 ratio and LB1:ST1.5 yoghurt starter cultures production

L. bulgaricus and *S. thermophilus* were separately inoculated into autoclaved skim milk samples and incubated at 41°C for 24 hrs to take high dense cultures for LB1:ST1 ratio yoghurt culture preparation. After both samples reached to 0.5 OD level, 50 ml of each sample was added into 4.95 L of autoclaved skim milk sample and it was incubated at 41°C for 24 hrs and after the incubation period colony count was taken and it was kept under cooling conditions for 10 hrs period.

Same method was followed up to prepare LB1.5:ST1 ratio culture only by changing the mixing volumes as 60 ml from *Lactobacillus bulgaricus* and 40 ml from *Streptococcus thermophilus*. The sample was kept at 41°C for 24 hrs and then it was kept in cooling room for 10 hrs time period.

Same method was followed to produce LB1:ST1.5 ratio culture only by changing the mixing volumes as 40 ml from *Lactobacillus bulgaricus* and 60 ml from *Streptococcus thermophilus*. The sample was kept at 41°C for 24 hrs and then it was kept in cooling room for 10 hrs time period.

Final microbial ratio of each new yoghurt starter culture after the incubation period was determined following the serial dilution method.

Determination of *L. bulgaricus* and *S. thermophilus* population growth during the incubation period

Each yoghurt starter culture was added to the yoghurt mixture and then filled the mixture into the yoghurt cups and finally, they were kept at 42°C for 4 hrs incubation period. During the incubation period, in every 30 minutes time intervals samples were collected. Every sample taken in the time intervals were tested to take colony count of *L. bulgaricus* and *S. thermophilus* by following the serial dilution and pour plate method.

Testing of post acidification in yoghurt under cooling storage conditions

The produced yoghurts using three yoghurt starter cultures were kept in 4°C cooling condition for 15 days. Then pH and acidity testing were done on 4th and 8th day of refrigerator storage. And, sensory evaluation was conducted on 4th and 8th day of refrigerator storage to check sensory qualities.

Statistical analysis

The results were analyzed for statistical significance by ANOVA, and Turkey honest significant using SPSS version 25. Coefficients, ANOVA tables, and significance ($p<0.05$) were computed using Statistical version 5.0.

Results

Determination of LB: ST ratio of bulk culture

Modified Elliker medium was used for microbial enumeration of bulk culture since *L. bulgaricus* and *S. thermophilus* growth was weak in Elliker medium. Proper growth of *L. bulgaricus* was observed in lactose added medium and *S. thermophilus* growth was high in sucrose added medium. Therefore, Elliker medium was modified to obtain a proper colony count by adding lactose: sucrose 1:1 ratio. Further to get good results, skim milk powder was added into the medium.

The microbial ratio of currently using bulk culture in the factory just after the incubation was LB1.5:ST1. Further results revealed *L. bulgaricus* population density was comparatively higher than that of *S. thermophilus*. Usual process in the yoghurt production factory is just after the incubation all the bulk culture samples keep under the cooling conditions for 10 hours of period. Therefore, 10 hrs cooled samples were checked to find out is there any effect from the cooling process to the LB: ST ratio. The enumeration results revealed after the cooling process LB: ST ratio is 1.6:1. The results revealed after the cooling process there was a slight elevation of the *L. bulgaricus* population. But statistically there is no significant difference among before cooling and after cooling results of colony counts of *L. bulgaricus* and *S. thermophilus*. Hence, 10 hrs like short period cooling conditions does not affect to the microbial ratio of the bulk culture statistically. But this is the starting point of the post acidification due to the continuously increasing population density of lactic acid bacteria.

Microbial growth within the incubation period of yoghurt

After addition the LB: ST 1:1 yoghurt starter culture to the yoghurt mixer, four hours incubation period required to get set the yoghurt. *L. bulgaricus* to *S. thermophilus* ratio fluctuation was observed within these four hours incubation period. Within the first thirty minutes time period there was no sign of population growth of two bacterial strains. *S. thermophilus* started their exponential growth after 30 minutes and it was continued for another 60 minutes time period. After that they showed stationary phase until 150 minutes time duration and at the 150 minutes again, they started their second exponential phase (Figure 2). According to these results, *S. thermophilus* has shown exponential growth in two places in their growth curve. When *S. thermophilus* reached to stationary phase *L. bulgaricus* started their exponential growth. According to these results end of the incubation period when yoghurts transfer for storage under cooling conditions their LB: ST ratio is still 1:1. End of the incubation period, yoghurt pH was 4.7.

Currently using bulk starter culture consists with LB: ST 1.5:1 ratio. In LB to ST 1.5:1 ratio yoghurt starter culture, initially *L. bulgaricus* population density is high compare to the *S. thermophilus*. Within the first thirty minutes, there was no growth rate in both microbial strains as same as in Figure 2. Although, after 30 minutes *S. thermophilus* started its exponential growth phase, their growth rate was very low compare the growth rate of Figure 2. After 60 minute it started its stationary phase and then again second exponential growth was started. The second exponential growth is also in low rates. Until second exponential growth phase of *S. thermophilus*, *L. bulgaricus* was having slow growth rate, but after this phase, *L. bulgaricus* showed rapid growth (Figure 3). After that *L. bulgaricus* had shown continuous population growth until end of the incubation period. End of the incubation period yoghurt consist with LB: ST 2:1 microbial ratio when it was ready to transfer for the storage under cooling temperature. PH of this set yoghurt just after incubation was 4.5.

After adding LB: ST 1:1.5 yoghurt starter culture into yoghurt mixture within the incubation period of the yoghurt there was a remarkable fluctuation in the *L. bulgaricus* and *S. thermophilus* bacterial population. Although within 30 to 60 mins *S. thermophilus* showed rapid growth, after the 60 mins their population growth rate slowed down (Figure 4). After 120 mins they reached into stationary phase and then again, they came to their second exponential growth. Compare to first exponential growth phase of *S. thermophilus* second exponential growth is high. After 30 min *L. bulgaricus* initiated their

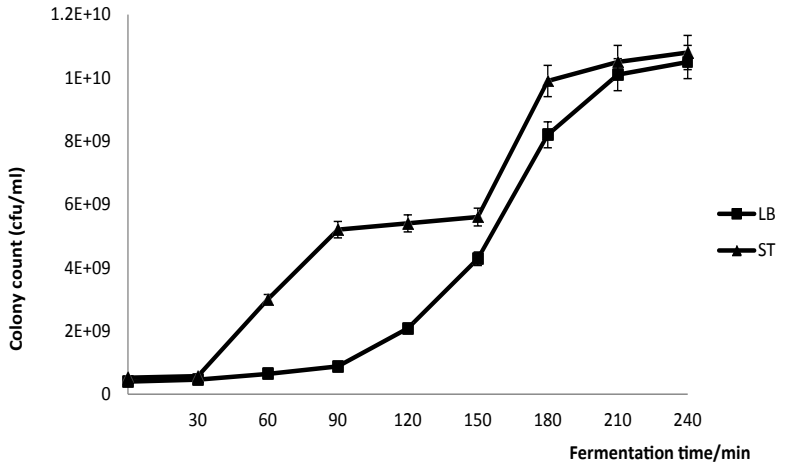

Figure 2: Colony counts of yoghurt samples within 4 hours incubation period after adding the yoghurt starter culture.

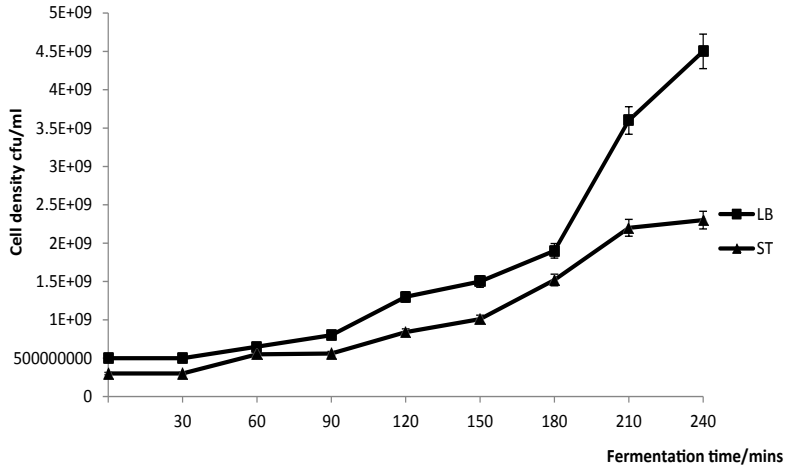

Figure 3: Colony counts of yoghurt samples within 4 hours incubation period after adding the yoghurt starter culture.

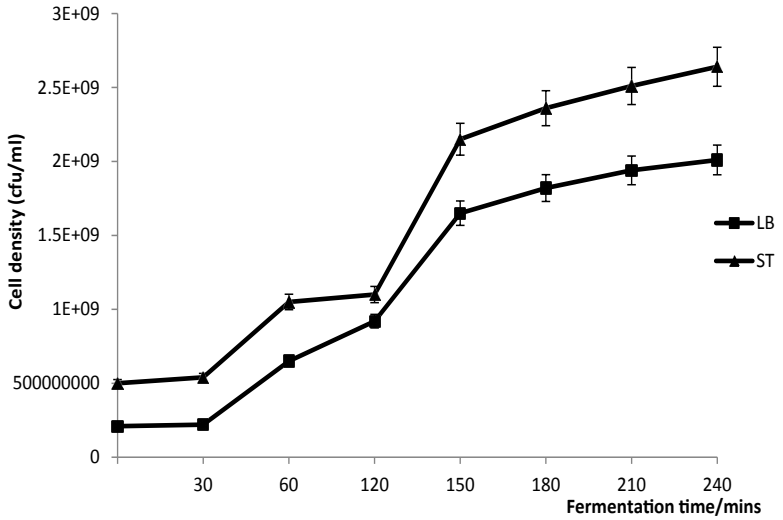

Figure 4: Colony counts of yoghurt samples within 4 hours incubation period after adding the yoghurt starter culture.

rapid growth and continuously they were in their exponential growth. At the end of the incubation period LB: ST ratio in the yoghurt was 1:2. PH of the yoghurts was 4.6.

Determination of post-acidification effect of newly produced bulk culture samples

According to the statistical analysis there is a significance difference in between mean pH values of three types of yoghurts during the 15 days period of storage (Figure 5). Hence the results revealed, microbial ratio of the yoghurt starter culture effects to the pH fluctuations of the yoghurts when they store under the refrigerator conditions.

At the end of incubation period (Day 1) pH was comparatively similar. After 15 days, pH of LB1.5:ST1 yoghurt starter culture added sample was 3.8 and it was the lowest pH value compares to other two cultures added yoghurt samples. These results revealed; the highest post acidification occurred in *L. bulgaricus* initial population high samples. *L. bulgaricus* to *S. thermophilus* 1:1 ratio was the best microbial ratio which was able to control post acidification of the yoghurt.

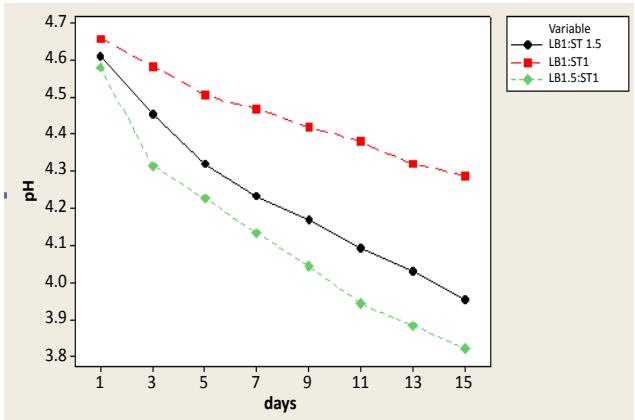

Figure 5: pH fluctuation of different types of starter culture added yoghurt during the refrigerator storage.

Sensory analysis results of yoghurt produced by adding different yoghurt starter cultures

According to the sensory analysis results of LB1:ST1 culture added yoghurt samples, 4th and 8th days of storage under the refrigerator conditions showed more than 70 ranked customer preferences on color, flavor, texture, sour taste and sweetness like physical characters (Table 1). But for other two cultures added yoghurt samples the ranks were below 50 (Figure 6). When *L. bulgaricus* starting population, density was high in the yoghurt starter culture, these physical character ranks were even below 35. The data revealed the shelf life of this LB1.5:ST1 added yoghurt samples were less than 8 days. Furthermore, when *S. thermophilus* density was high also showed deteriorated physical characters in the yoghurt in 4th and 8th storage days which had ranks below 50. Therefore, the best microbial ratio which can keep under cooling conditions without any defect to yoghurt texture, color, taste and flavors like characters is LB1:ST1.

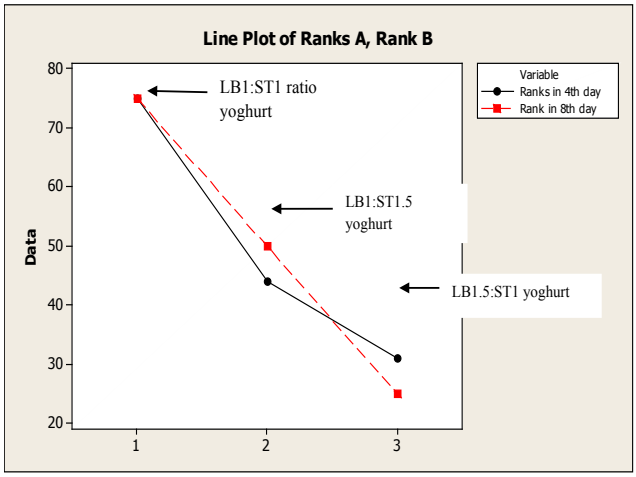

Figure 6: Overall rank belongs to three types of yoghurt cultures added yoghurts.

Discussion and Conclusion

In LB:ST 1:1 ratio culture *S. thermophilus* have shown two

Sample	Color		Flavor		Texture		Sour taste		Sweetness		Overall	
	4th	8th	4th	8th	4th	8th	4th	8th	4th	8th	4th	8th
LB:ST 1:1	74	75.0	72.5	74.5	71.5	74.5	71.0	75.0	74.5	75.0	75.0	74.5
LB:ST 1:1.5	44	47.5	46.0	45.0	44.0	43.5	46.5	43.5	41.0	46.0	44.0	43.0
LB:ST 1.5:1	33	27.5	31.5	30.0	34.5	32.0	32.5	27.5	34.5	29.0	31.0	32.5

Table 1: Rank of qualities of three types of yoghurt in 4th and 8th day of storage under the cooling condition.

exponential growth phases separated by the transitional phase with slow growth rate which are almost like the growth curve of typical lactic acid fermentation curve [9]. While the growth curves of LB:ST 1:1.5 followed up the features of typical fermentation growth curve, LB:ST 1.5:1 totally deviated from it. Finally, different microbial ratios in yoghurt cultures gave different qualities to the yoghurts. These results revealed proto cooperation in between *L. bulgaricus* and *S. thermophilus* depends on the microbial ratio of the yoghurt starter culture.

According to the population growth results of LB:ST 1:1 added yoghurt sample, *S. thermophilus* showed two exponential growth phases in the growth curve which were separated from a transition phase. The first exponential growth phase of *S. thermophilus* is due to the favorable neutralizing pH in the medium and more effective utilization of the amino acid and trace elements highly available in the medium. *Lactobacillus bulgaricus* is unable to start amino acid utilization due to lack of stimulating genes. Therefore, *L. bulgaricus* purine biosynthesis starts after getting stimulation from the formic acid and folic acid produced by *S. thermophilus* [10]. Therefore, after *S. thermophilus* reached to exponential growth *L. bulgaricus* started their log phase due to suitable environment. Moreover *L. bulgaricus* prefers to grow in low oxygen medium. Since, *S. thermophilus* is an aerobic microorganism, it uses more oxygen and as a result of that it creates low oxygen medium which is stimulation for *L. bulgaricus* growth [11]. But after this exponential phase, *S. thermophilus* showed a transition phase due to the lack of enough amino acid in the medium. Milk consist with low amino acid amount, after utilizing all these amino acids by *S. thermophilus*, they were unable to produce amino acids from milk due to lack of exoprotease enzyme to produce oligopeptides and amino acids from milk [12]. In here *L. bulgaricus* was in their exponential phase and they can produce oligopeptides and amino acids from milk which are the growth requirement of *S. thermophilus*. As a result of supplement of required amino acid amount to the medium *S. thermophilus* started their second exponential growth phase while supporting the exponential growth of *L. bulgaricus*. Within this second exponential phase *S. thermophilus* was able to synthesize exopolysaccharides (EPS) which were the contributors of aroma enhancements of the yoghurt [13]. Furthermore, acetoin and diacetyl compounds produced by *S. thermophilus* responsible for the flavor of the yoghurt. And due to low pH coagulated milk protein gave proper viscosity to the yoghurt. At the end of this growth phase yoghurt acquired their quality. Due to low pH *S. thermophilus* activity was slowed down and *L. bulgaricus* grown continuously. As a result of this, post acidification started during the storage under cooling condition. But LB:ST 1:1 culture showed comparatively low post-acidification due to the proper proto cooperation. As a result of this at the end of 15 days pH of the yoghurt samples was 4.4. This acidity level avoided the syneresis and due to that it increased the shelf life of the yoghurt. Sensory analysis results revealed color, flavor, texture, sour taste and sweetness like physical characters of the yoghurt showed statistically above 70 ranks [14]. Therefore, manipulation of *L. bulgaricus* growth by *S. thermophilus* controls the post acidification during the cooling storage and avoids syneresis. Result is high shelf life of the yoghurt samples [15-18].

Growth curve of LB:ST 1.5:1 was deviated from typical growth curve. *S. thermophilus* showed exponential growth with low growth rates and after the 30 minutes *L. bulgaricus* showed continuous growth rate until end of the incubation period. Due to low *S. thermophilus* growth, yoghurt quality was not good up to the standard level. Post acidification results were high compare to LB:ST 1:1 ratio. Since *S. thermophilus* provided the entire requirement for the growth of *L. bulgaricus*, they showed high growth rate and result of this was high pH in the medium [19,20]. Therefore, *S. thermophilus* population was getting low due to the unfavorable conditions as low pH and low temperature in the medium, but *L. bulgaricus* population was getting high due to the surviving ability in low pH, low temperature and low oxygen [14]. As a result of post acidification effect in 4th and 8th day's yoghurt physical characters were below 40. Therefore, post acidification causes syneresis effect to the yoghurt structure.

S. thermophilus of LB:ST 1:1.5 yoghurt culture added yoghurt mixture showed two exponential phases. First exponential growth rate was low compare to the second one. Here since *S. thermophilus* starting population was high, they used existing amino acid in the medium quickly and due to the running off of substrate their metabolic potential decreased and immediately they reached to stationary phase until getting enough amino acid substrate. Within their transition period *L. bulgaricus* started their role and produced more amino acid. Then onwards *S. thermophilus* started their second exponential phase with high rate compare to first phase. Due to the efficient second exponential phase yoghurt consisted with proper quality, but taste was close to sweetness than the sour taste which was deviated from the preference of Sri Lankan customers. But during the 15 days of storage period, these yoghurts reached to 4 pH level through the post acidification effect due to high activation of lactic acid bacteria. This high acidic environment increased whey protein leakage and due to this yoghurt quality deteriorated and rank of it was below 40 [21].

All in all the results revealed the best proto cooperation among lactic acid bacteria is achieved when *L. bulgaricus* to *S. thermophilus* ratio is 1:1. This yoghurt starter culture is able to provide good chemical and physical properties to the yoghurt while reducing the post acidification under refrigerator storing condition. This provides good texture, aroma and flavor to the yoghurt and it helps to popularize the product among customers. When *L. bulgaricus* population density is high it causes high post acidification to the yoghurt during the storage period which makes defects in yoghurt structure. As well as when *S. thermophilus* is high it initially effects to the yoghurt taste and then during the storage period it causes some post acidification defects.

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