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Prevalence of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* stability in commercially available yogurts in Sri lanka

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ABSTRACT

Aims and Objectives: Common starter cultures found in fermented milk products are *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The viability of these bacteria is important in order to gain health benefits. It is important to investigate the stability of commercially available yogurts with respect to starter cultures and the quality. **Materials and Methods:** Yogurt samples were collected from highly marketed different brands designated as A, B, C, D, E, F, G and H from different areas from Sri Lanka. MRS and M17 agar were used to enumerate *L. bulgaricus* and *S. thermophilus* respectively and a pH change was measured. **Results:** The pH values decreased significantly and only two of these products maintained 10^6 cfuml^{-1} viable count of *L. bulgaricus* till the end of the shelf life. All products showed the highest number of *S. thermophilus*. **Conclusion:** The pH of the yoghurts significantly change with the storage. For optimum benefits, the yogurt products should be consumed within seventh to fourteenth day from its manufacturing date. Only two of the yoghurt products maintained 10^6 cfuml^{-1} viable count of *L. bulgaricus* till the end of the expiry

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INTRODUCTION

The word "yogurt" is Turkish in origin and it is a fermented milk product produced by bacterial fermentation of milk. Among the number of dairy products marketed, most widely encountered one is yogurt. It can be a cheap and effective tool to improve the nutritional status and the health of people in developing countries.[1,2] In Sri Lanka, milk is a food item with high cultural value. Sri Lanka has a unique food culture with a long history with the historical writings and archeological sources provide witnesses for this.[3-5] Our ancestors used cow's milk in preparation of "pasgorasa" (five products from milk) namely milk, curd, butter milk, ghee and butter. It is one of the most consumed and most popular food product around the world. Yogurt is collectively well-known for its distinctive taste, relative ease to produce, health benefits, and versatility. Cow's milk is most commonly used to make yoghurt in Sri Lanka.

Yogurt is fermented when bacteria ferment the sugar lactose ($C_{12}H_{22}O_{11}$) into lactic acid ($C_3H_6O_3$). During the production of yogurt, the lactose sugar is broken down to the glucose and galactose by the lactase enzyme produced by bacteria. Further, processing of glucose and galactose results in the end products of lactic acid and acetaldehyde.[6] Several types of bacteria can ferment lactose. Traditionally, yogurt is manufactured using *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp.* as starter cultures. These two exhibit a symbiotic relationship during the fermentation of yogurt.[7] In recent years some yogurt products have been reformulated to include live strains of *Lactobacillus* strains such as *L. acidophilus*, and species of *bifidobacterium* in addition to the conventional yogurt organisms, *S. thermophilus* and *L. bulgaricus*.[3]

The consumption of live microbial supplements with presumptive health benefits on human physiology, the so-called probiotics, has become a common practice. Probiotic bacteria positively impact on the immune system and on

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the composition and functioning of the gut microbiota. Furthermore, the production of vitamins has been claimed among the causal relationships of the healthy benefits of probiotics.[4]

Viability and activity of the bacteria are important considerations for the concept of probiotic, because the bacteria must survive in the food during shelf life and during transit through the acidic conditions of the stomach, and should resist degradation by hydrolytic enzymes and bile salts in the small intestine. It is essential that products sold with any health claims meet the criterion of a minimum 10^6 cfu/ml bacteria at the expiry date, because the minimum therapeutic dose per day is suggested to be 10^8–10^9 cells.[8]

The objectives of this study are to evaluate the microbial count during the shelf life of yogurt to find out whether sufficient numbers of bacterial cells survive throughout the shelf life of the product and to observe the changes of pH with the shelf life with respect to the viability of bacteria in yogurt.

MATERIALS AND METHODS

Eight commercial fermented yogurt brands i.e. A, B, C, D, E, F, G and H (seven full fats and one non fat) were selected from Sri Lankan market during 2013. Yogurts were obtained in the market on the 2nd, 7th, 14th, 21st day of production from all the brands of yogurts with 3 samples from each. Samples were refrigerated and the descriptions of the yogurt samples were given in the Table 1.

The pH of yoghurt samples was measured at 1, 7, 14 and 21 days of storage by using a pH meter with a glass electrode which was calibrated with pH 7.0 and 4.0 standard buffers at room temperature.

MRS medium (OXOID, United Kingdom) was rehydrated in distilled water and the pH of the medium was adjusted to 5.4 by using hydrochloric acid (0.1M HCl).

M17 agar (OXOID, United Kingdom) was prepared according to manufacturer's instructions. Sterilized lactose solution 12.5 ml was added to 250 ml sterile M17 agar and mixed before pouring the plates at 40°C. Bacteriological peptone diluents were prepared by dissolving 15 g in 1 L of distilled water. All the media were sterilized at 121°C for 15 min.

Each yogurt sample was homogenized and 0.5ml were dissolved in 4.5 ml of Peptone water. After uniform mixing, subsequent serial decimal dilutions up to 10^{-9} were prepared in 4.5 ml of sterile peptone water. The count of *L. bulgaricus* and *S. thermophilus* were enumerated according to Daniela Saccaro by using MRS agar pH 5.4 and M17 agar respectively.[5] Probiotic bacteria were isolated from yoghurt samples using the spread plate technique. Dispersed 0.25 ml from the first dilution around the center of the plate and Sterile L-shaped spreader was used to spread the inoculum evenly around the plate. Each sample was plated thrice in both MRS and M17 media and plates were incubated aerobically at 37°C for 2 days.

Colonies of bacteria were isolated from the incubated plates. Plates containing 20 to 200 colonies were enumerated, and the counts were expressed as log 10 cfu/ml of the product. Isolated bacteria were stained with grams stain and were tested for catalase activity.

RESULTS

The initial pH values of fermented yogurt products ranged from 4.25 - 4.50 (Table 1). In general, the pH of all the tested products decreased gradually from the production day to the end of storage period. The difference in pH was significant between the date of production day and the end of storage period. However E and F products showed significant increase in pH (4.26 to 4.41 and 4.22 to 4.38 respectively). Brand G yogurt was an exception since it was the only product which had pH value close to 4.0 at the expiry day.

At the end of the incubation period colonies appeared in different numbers on both MRS and M17 plates. Counts of bacteria after 2 days of incubation were given (Table 2). It was expressed as number of colony forming units at different dilutions. All the bacteria were Gram positive bacteria and the isolates coming from MRS plates were all bacilli with long and rounded ends. They appeared mostly as a chain of 3-4 cells or single. The isolates coming from M17 plates were all cocci with spherical or ovoid morphology. They appeared mostly as pairs or forming chains. All the organisms were catalase negative.

As shown in Table 2, the viable counts of the bacteria were fluctuated in all the products during storage period. *S. thermophilus* seemed to grow more rapidly than *L. bulgaricus* during the storage of all samples. Among all the products A, F and H recorded the highest number of *L. bulgaricus* count (7.15, 6.48 and 6.32 log cfu ml^{-1}, respectively) and D, G and E products show minimum count of *L.bulgaricus* (2.95, 4.39 and 4.52 log cfu ml^{-1}, respectively) on the production day. On the other hand, C, D, and H recorded the highest number of *S. thermophilus* count (9.00, 8.90 and 8.71 log cfu ml^{-1}, respectively) and A, B and E products of minimum count (8.18, 8.41 and

Table 1: Changes of pH of Yogurt samples during the shelf life

Product	1st day	7th day	14th day	21st day	EXD
A	4.25±0.006	4.27±0.01	4.29±0.00	4.28±0.01	4.22±0.006
B	4.36±0.006	4.35±0.01	4.34±0.02	4.27±0.006	4.20±0.006
C	4.48±0.006	4.25±0.00	4.23±0.006	4.25±0.005	4.20±0.01
D	4.43±0.01	4.28±0.005	4.33±0.01	4.38±0.006	4.27±0.01
E	4.44±0.006	4.40±0.01	4.35±0.00	4.26±0.01	4.41±0.006
F	4.32±0.00	4.27±0.005	4.25±0.00	4.22±0.01	4.38±0.006
*G	4.25±0.006	4.15±0.006	4.05±0.00	-	-
*H	4.50±0.00	4.39±0.01	4.34±0.06	-	-

*Yogurts with two weeks Shelf life. The counts are mean of three readings in each trial, that is N=3

Table 2: The Counts of Isolates from MRS and M17 culture Plates (log cfu ml^{-1})

Product	1st day		7th day		14th day		21st day		EXD	
	MRS	M17	MRS	M17	MRS	M17	MRS	M17	MRS	M17
A	7.15±0.212	8.18±0.106	6.15±0.212	8.29±0.163	7.39±0.58	7.91±0.014	6.97±0.099	7.88±0.163	7.4±0.056	7.45±0.636
B	4.97±0.191	8.41±0.007	6.85±0.495	9.37±0.177	5.22±0.537	8.6±0.120	5.87±0.035	8.30±0.007	2.39±0.127	9.15±0.212
C	5.45±0.212	9.00±0.063	4.34±0.028	8.73±0.035	2.87±0.042	8.69±0.035	2.15±0.212	8.41±0.01	1.00±0.000	7.88±0.035
D	2.95±0.071	8.9±0.000	4.36±0.092	8.75±0.007	4.68±0.021	8.82±0.014	3.74±0.057	9.01±0.021	2.92±0.035	8.63±0.014
E	4.52±0.056	8.64±0.028	5.32±0.396	8.42±0.056	6.30±0.424	8.11±0.021	5.63±0.212	8.53±0.056	6.20±0.134	8.39±0.021
F	6.48±0.000	8.66±0.021	6.57±0.035	8.65±0.007	5.99±0.219	8.00±0.000	6.00±0.000	7.15±0.212	5.00±0.000	8.22±0.106
G*	4.39±0.127	8.68±0.056	5.54±0.021	8.57±0.156	7.40±0.113	8.53±0.304	-	-	-	-
H*	6.32±0.028	8.71±0.014	5.28±0.028	9.02±0.014	4.00±0.000	8.37±0.042	-	-	-	-

*Yogurts with two weeks Shelf life. The counts are mean of three readings in each trial, that is N=3

8.64 log cfu ml -^{1}, respectively) on the same day. C product showed a significant decrease in viable count of *L. bulgaricus* and finally it was 1.0 log cfu ml^{-1}. However the population of *S. thermophilus* remained above 10^{6} cfu ml^{-1} until the expiry date.

DISCUSSION

The pH of the product needs to be 4.5 or lower to meet legal requirements and to produce good quality yogurts. National Yogurt Association proposed a yogurt standard that requires an acidity of pH 4.6 or lower. According to the Australian Food Standards Code (Standard H8), yogurt must have a pH of ≤ 4.5.[9]

The reduction in pH can be due to the breakdown of lactose into lactic acid. According to Klaver, one of the most constraining drawbacks associated with the use of dietary cultures in fermented milk products is the lack of acid tolerance of some species and strains.[10] When the lactic acid content increases, pH levels correspondingly decrease during fermentation. 'Over-acidification' or 'post production acidification' is due to the decrease in pH after fermentation and during storage at refrigerated temperature.[11]

Different types of products were proposed as carrier foods of microorganisms by which consumers can take in large amounts of cells in order to get the therapeutic effects. Yogurt has long been recognized as a product with many desirable effects for consumers, and it is also important that most consumers consider yogurt to be 'healthy'. The yoghurt cultures including *L. delbrueckii subsp bulgaricus* and *S. thermophilus* are active even at refrigerated temperature and still can produce small amounts of lactic acid by fermentation of lactose which results is noticeable pH decrease. The post-acidification, during storage, was due to b-galactosidase which is still active at 0–5°C.[8] According to Reyhan and Ufuk, the use of industrial starters with low proportion of *L. bulgaricus* allowed the production of yoghurt with a reduced acidity and with lesser risks of post acidification.[12]

Enumeration was performed according to the standard media accepted by the International Dairy Federation of the yogurt species, *L. bulgaricus* and *S. thermophilus* are MRS and M17 agar, respectively. In this study yeast and mould were detected in some of the yogurt samples. It was may be due to the unhygienic environment conditions during the processing.

The survival curves for the starter cultures in the yoghurt were quite different for the different brands tested. Numbers of *L. bulgaricus* decreased faster than those of *S. thermophilus*. Puhan indicated that viability of *S. thermophilus* and *L. bulgaricus* in yogurt was dependent upon pH.[1] Numbers of *S. thermophilus* increased in yogurts with an initial pH greater than 4 until the pH decreased below 4, and the numbers then diminished rapidly. Numbers of *L. bulgaricus* either remained constant or increased for

the first 10-20 days with an initial pH greater than 4 and then decreased. These results were not confirmed by our results in which an increase or decrease in the numbers of *S. thermophilus* and *L. bulgaricus* does not seem to be related to pH. Similar results have been also found by evaluating the microbial quality of yogurts available in the Portuguese market during the shelf-life period.[13] According to Medina and Jordano this was possibly due to manufacturing practices and the strains of starter cultures utilized by the different manufacturers.[14]

In order to obtain the desired therapeutic effects, the yogurt bacteria must be available in sufficient numbers. Recommended lower limit of International Dairy Federation (IDF) for counts in dairy product is 10^6 cfu per one ml.[15] In this regard, Kneifel showed that approximately 80% of commercial yogurts had higher counts of cocci than rods.[11] In several countries have established minimum values of culture bacteria for yogurts and/or fermented milk during shelf life. It was reported that the Fermented Milks and Lactic Acid Beverages Association has introduced a standard of a minimum of $>1x10^7$ cfu/ml or cfu/g viable probiotic cells for fresh dairy products.[15] It is essential that any fermented products should claim this criterion.

As per the study, four out of eight products contained over10^6cfuml^{-1} of *L. bulgaricus* on the production day to the seventh day. Only two of these products maintained 10^6cfuml^{-1} viable count of *L. bulgaricus* till the end of the expiry. One was not reached 10^6cfuml^{-1} until the seventh day but it has reached to 10^6cfuml^{-1} at the end of the expiry. Conversely, all products showed the highest number of *S. thermophilus* (above 10^8cfu ml^{-1}) within a week and two were reduced it is viable count up to 10^7cfuml^{-1}. Three out of eight yogurt brands (A, E and G) were obtained inclusive its minimum therapeutic potential values within its expiry in both the cultures.

The survival of bacteria in fermented dairy products depends on varied factors such as the strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium (e.g. carbohydrate source), final acidity, milk solids content, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen (especially for Bifidobacterium sp.), level of inoculation, incubation temperature, fermentation time and storage temperature.[11,16,17] However, the main factors for loss of viability of organisms have been attributed to the decrease in the pH of the medium and accumulation of organic acids as a result of growth and fermentation.[18,19]

Inoculum size of the bacteria is an important key factor to ensure sufficient viable cells in the final food product. Using of a high level of inoculum will ensure a high cell count at the end of the incubation and survival of the bacteria during storage until consumption.[20]

Actual temperature of storage in the markets is important for bacteria viability in yoghurts. Industrial standards recommend for yoghurts as a conservation temperature should not higher than 8°C. In this study skimmed milk yoghurts showed higher viable numbers than whole yoghurts and this has been reported earlier.[21] According to Vinderola, Bailo and Reinheimer, high concentration of sugars in sweetened yoghurts affected the bacilli viability.[22]

Several studies have been done to evaluate the microbial quality of yogurts available in the market. Nogueira, Albano, Gibbs and Teixira evaluated the Microbial quality of Protuguese yogurts during the shelf life period and it was always within the range of recommended values.[13] In order to achieve the optimum benefits, yogurt should be consumed within one week of their production.[4] Further, the viable *S. thermophilus* and *L. bulgaricus* numbers in the industrial yogurt increases the bioavailability of viable yoghurt microflora. As a result, they showed yoghurts produced by using starter cultures have higher therapeutic and/or antimicrobial properties beside of their organoleptic characteristics.[12]

A number of yoghurt brands commercially available in Sri Lanka have been analyzed and there are variations in the quality of yoghurt samples in the market. It is generally accepted that the yoghurt should contain 10^6 cfu of viable bacteria per ml of yoghurt to obtain the desired therapeutic effects. Based on the results of the study, it can be concluded that the 3 brands (approximately 37.5% of marketed brands) were obtained inclusive its therapeutic potential values within its expiry in both starter cultures. Most of the products contained very low numbers of organisms, especially *L. bulgaricus* which do not fulfill the quality criteria. For optimum benefits, the fermented milk products with live bacteria should be consumed within two weeks of their production date, especially within seventh to fourteenth days from the manufacturing date. Since, Sri Lanka does not specify any requirements regarding the numbers of bacteria in the fermented dairy products, standardization of commercial yoghurt products would be applicable to the development of quality fermented dairy products. The research provided useful information to the dairy industry to develop new technology to ensure the supply of high quality milk products to the consumers.

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Authors Contribution:
JGSR: Concept and design of the study, manuscript preparation critical revision of the manuscript, Concept, critical revision of the manuscript , review of study. Interpretation of study; **WTRP:** Reviewed the literature, collected review of literature , helped in preparing first draft of manuscript. literature search, statistically analyzed and interpreted , prepared first draft of manuscript collected data.
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