

Viable Counts of Lactic Acid Bacteria in Philippine Commercial Yogurts

Short Communication

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Abstract

Viable counts of lactic acid bacteria (LAB) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* claimed to be present in commercially available yogurts in the Philippines were determined. Seven (7) yogurt samples with three (3) replicates per sample namely: NAV, NCY, CYP, NFB, LYB, CYI, and NFJ were examined. The highest *S. thermophilus* count was obtained in LYB with $10.20 \pm 0.06 \log_{10}$ CFU/ml and lowest in CYI with $8.18 \pm 0.02 \log_{10}$ CFU/ml. The highest *L. bulgaricus* count was also found in LYB with $9.96 \pm 0.01 \log_{10}$ CFU/ml while CYI also had the lowest count of $8.15 \pm 0.09 \log_{10}$ CFU/ml. Mean LAB counts of all six (6) yogurt samples were significantly higher ($P \leq 0.05$) than CYI. The ratio of *S. thermophilus* to *L. bulgaricus* in all yogurt samples examined was about 1:1. LYB had the highest titratable acidity of 0.98% lactic acid while CYI had the lowest titratable acidity of 0.35% lactic acid. No correlation was found between viable LAB counts and titratable acidity of yogurt samples. The viable counts of *S. thermophilus* and *L. bulgaricus* in all seven (7) commercial yogurts examined met the prescribed minimum viable count of 10^6 CFU/ml for the suggested therapeutic effects and health benefits for the consumers.

Keywords: Lactic Acid Bacteria; LAB; Viable Counts; Yogurt; *S. Thermophilus*; *L. Bulgaricus*.

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Introduction

Yogurt is traditionally manufactured using *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* as starter cultures. These organisms were claimed to offer some health benefits, though they are not natural inhabitants of the intestine or are unable to survive the conditions in the GI tract. Certain health benefits attributed to yoghurt consumption were due to reports that both yoghurt starter bacteria are proteolytic and thus generate bioactive peptides from milk proteins. Researchers have identified peptides with angiotensin converting enzyme inhibitory (ACEI) activity or antihypertensive peptides from yoghurt and other fermented milks [1]. It is in this sense that these two yoghurt bacteria can be considered probiotic bacteria or bacteria with health beneficial effects. Probiotic bacteria such as *Lactobacillus*

acidophilus, *Bifidobacterium* sp. and *Lactobacillus casei* are incorporated as adjuncts to the usual yoghurt starter bacteria [2]. Lactic acid bacteria (LAB) are the most important probiotic microorganisms typically associated with the human gastrointestinal tract [3]. Numerous commercial products containing LAB have been claimed to have health promoting function [4]. Some LAB products have been proven to be useful as adjuncts in preventing gastrointestinal disorders in humans [5].

Currently, there is an increasing commercial interest to add probiotic bacteria to fermented dairy products. This was due to the hypothesis that the diet may modulate various functions in the body [6-8]. In order to provide the claimed health benefits to humans, the minimum viable count of probiotic bacteria in the fermented milks should be $\geq 10^6$ colony forming units (CFU)/g at the end of the shelf-life of the product [9]. An important parameter in assessing product quality by monitoring viable organisms is the ability to count probiotic bacteria selectively. In order to ensure that minimum number of probiotic bacteria are present in the end-product, rapid and reliable methods for routine enumeration are required. Furthermore, such methods are also essential to monitor possible physiological or biochemical changes in the probiotic bacterial population during the storage of commercial products [10]. Moreover, several studies have revealed that some probiotic products in the market do not meet the minimum viable count of probiotic strain(s), especially in products containing bifidobacteria [11, 12]. The viability of probiotic bacteria in yogurt depends on many factors, among them are strains used, interaction between species present, culture conditions, production of hydrogen peroxide due to bacterial metabolism, final acidity of the product, and the concentrations of lactic and acetic acids. However, the main factors for the loss of viability of probiotic organisms were attributed to the decrease in the pH of the medium and accumu-

lation of organic acids as a result of bacterial growth during the fermentation of the milk [13, 14]. Yogurt with additives such as stabilizers, flavouring ingredients, sweeteners, preservatives, etc., raises some doubts about the actual viable bacteria concentration when the product reaches the consumers [15].

The viability of lactic acid bacteria in commercial yogurts in the Philippines has not been studied. It is questionable whether such products can provide the claimed health benefits if the viable counts of LAB are low. The aim of this study, therefore, is to determine the viable counts in commercial yogurts and verify the presence of LAB claimed to be present in such products. The data from this study will be useful in verifying the claims of different yogurt manufacturers regarding the benefits of live and active cultures in their products.

Materials and Methods

This study was conducted from February to May 2013 in the Dairy Training and Research Institute (DTRI) laboratory, Animal and Dairy Sciences Cluster, College of Agriculture, University of the Philippines Los Baños.

Yogurt Sampling

Seven (7) brands of commercially available yogurts were used in this study as samples. The different yogurt samples were: plain yogurt, frozen yogurt (yogurt ice cream), fruit-flavored yogurt, and fruit-flavored yogurt with jelly. Samples were collected as fresh as possible from grocery stores in Los Baños, Laguna, Philippines. Three (3) samples per brand of yogurt were obtained of the same flavor and of the same manufacturing dates. All samples were stored immediately and maintained at a temperature ranging from 4 to 6°C. Their description, composition and best before dates stated on the product label were carefully recorded.

Plating and Isolation

Plating and isolation were done following the IDF [16] and Karna et al. [17] procedures. Enumeration was carried out using pour plate technique. *S. thermophilus* and *L. bulgaricus* were enumerated on M17 and MRS agar, respectively. Serial dilutions were prepared using peptone diluents. One ml of thoroughly mixed yogurt sample was transferred using a sterile 1 ml pipette to the first tube of 9 ml sterile diluent, which represents the 10⁻¹ dilution. The diluted sample was blended for one minute by using a vortex mixer. One ml of 10⁻¹ dilution was transferred to the second tube of 9 ml sterile diluent to prepare 10⁻² dilution. This operation was repeated until the required dilution was obtained by using fresh and sterile pipettes and diluents.

For counting of *L. bulgaricus*, one ml of the appropriate dilution was transferred into the Petri dishes in triplicates then 12 to 15 ml of MRS agar medium [18] at 45°C was poured into each Petri dish with the appropriate dilution. The content of the Petri dish was mixed carefully by rotating the Petri dish five times clockwise and five times counter-clockwise then allowed to solidify on a level surface. Plates were inverted and incubated anaerobically in a tightly sealed anaerobic jar at 37°C for 72 hours.

For counting *S. thermophilus*, the same diluents were used for pre-

paring serial dilutions. One ml of appropriate dilution was transferred into Petri dish in triplicates then 12 to 15 ml of M17 agar at 45°C was added into each Petri dish containing one ml of appropriate dilution. The content of the Petri dish was mixed carefully by rotating the Petri dish five (5) times clockwise and five (5) times counter-clockwise then allowed to solidify on a level surface. Plates were then inverted and incubated aerobically at 37°C for 48 hours.

Colonies in plates with 25 to 250 colonies were counted and viable counts in CFU/ml were calculated as follows:

$$N = \sum C / [(1.0 \times n1) + (0.1 \times n2)] d$$

where:

N = number of colonies per ml or gram of sample.

∑C = sum of all of the colonies in all plates counted.

n1 = number of plates in the lower dilution counted.

n2 = number of plates in the next higher dilution counted.

d = dilution from which the first counts were obtained.

Morphological Characterization

To verify whether the colonies counted were *S. thermophilus* and *L. bulgaricus*, morphological characterization of typical colonies was done by examining colony growth, Gram reaction, cell morphology (cocci or rods) and by testing their catalase reaction. The Gram staining procedure was done according to Murray et al. [19] while catalase test was done according to Smibert and Krieg [20].

Typical and well isolated colonies were stabbed into MRS agar for *L. bulgaricus* and incubated at their optimum temperature of 37°C for 72 hours. For *S. thermophilus*, typical and well isolated colonies were streaked on M17 agar slants and incubated at 37°C for 48 hours. Isolates were then Gram stained and tested for catalase reaction. Colony confirmation was done for the Gram positive, catalase negative chains of cocci or diplo cocci in the case of *S. thermophilus* and nonspore-forming, Gram positive and catalase negative rods in the case of *L. bulgaricus*.

Acidity Test

Acidity test procedures were done according to APHA [21]. This was done after plating to avoid contamination of the yogurt samples. Acidity was expressed as percent lactic acid and was computed using the following formula:

$$\% \text{ Lactic Acid} = (\text{Volume of NaOH used} \times \text{Normality of NaOH} \times 90/100) / 9 \times 100$$

Statistical Analysis

Completely Randomized Design (CRD) with three replications for each treatment was used. Yogurt samples were considered as the treatments to evaluate the presence of bacteria, their types, numbers and characteristics [22]. Data on the bacterial counts were statistically analyzed using Analysis of Variance (ANOVA). Test of significant differences among the treatment means was analyzed by using the LSD test. Simple linear correlation analysis was used to measure the degree of linear association between titratable acidity and viable LAB counts.

Results and Discussion

LAB counts of yogurt samples

Enumeration of viable LAB counts were done on seven (7) yogurt brands namely: Nestle Acti-V (NAV), Nestle Creamy Yogurt (NCY), Carabest Yoghurt (CYP), Nestle Fruit Selection Yogurt (NFB), Live Yogurt (LYB), Carabest Soft-served Yoghurt Ice Cream (CYI), and Nestle Fruit Selection Yogurt plus Jelly (NFJ). Viable counts were determined two (2) to three (3) weeks prior to the expiration date of the yogurt samples. The mean viable counts of *S. thermophilus* and *L. bulgaricus* are shown in Table 1. *S. thermophilus* counts ranged from $8.18 \pm 0.02 \log_{10}$ CFU/ml to $10.20 \pm 0.06 \log_{10}$ CFU/ml with CYI having the lowest and LYB having the highest viable count.

L. bulgaricus mean viable counts ranged from $8.15 \pm 0.09 \log_{10}$ CFU/ml to $9.96 \pm 0.01 \log_{10}$ CFU/ml with CYI and LYB having the lowest and highest viable counts, respectively. Moreover, the data showed that the mean LAB counts of all six (6) yogurt samples were significantly higher ($P \leq 0.05$) than CYI. The data further showed that all seven (7) yogurt samples met the prescribed minimum viable count of 10^6 CFU/ml or gram [22].

In this study, CYI showed the lowest viable LAB counts which were still more than the prescribed minimum viable count. A minimum viable number of 10^6 CFU per ml or gram was suggested while a viable count of 10^8 CFU/g was recommended to compensate for reduction during passage through the gut [23]. It is generally accepted that at the point of consumption of probiotic products, the probiotic bacterial count should be $>1 \times 10^6$ CFU/ml or gram and that a total of 10^8 to 10^9 probiotic microorganisms should be consumed daily if therapeutic effects are to be realized.

Lower mean LAB counts in CYI were linked to the low temperature of the product being a frozen yogurt. Frozen yogurt environment is not optimum for the survival of bacteria [24]. The freezing process of the mix may cause a loss of 0.5 to 1 log cycle in the viable counts. The fluctuation in temperature, causing large ice crystal formation during re-freezing, may rupture bacterial cells and reduce viability. Moreover, the sugar concentration in the product could have inhibited the growth of yogurt bacteria.

Differences in viable counts were attributed to dissimilarity of manufacturing dates among yogurt samples used, although efforts were exerted to obtain samples of very close manufacturing dates. Furthermore, the viability of yogurt bacteria depends on a number of factors such as interaction between species present, culture conditions, fermentation time and storage conditions, pH of the yogurt (post-acidification during storage), sugar concentration (osmotic pressure), milk solids content, availability of nutrients, the presence of hydrogen peroxide, dissolved oxygen content, buffering capacity, and β -galactosidase concentration in the yogurt [25].

Colony characteristics of LAB isolates

Colony characteristics of *S. thermophilus* and *L. bulgaricus* were studied by picking up the typical, well isolated and representative colony that appeared on the plate. A single colony was aseptically transferred to stab/slant for study of growth pattern of isolates on solid media. Growth of the LAB isolates was observed and cultural characteristics were described in Table 2.

Streptococcus thermophilus colonies on M17 agar plate appeared to be creamy white, about 0.5 to 3.0 mm in diameter, circular, entire, low convex and showed irregular growth on slant. On the other hand, LAB belonging to the genus *Lactobacillus* usually have small size colonies having 2 – 5 mm size with entire margin, convex, smooth, glistening and opaque without pigment [26].

Morphological characteristics of LAB isolates

The LAB isolates were characterized morphologically by Gram staining of 48 hour-old culture in the case of *S. thermophilus* and 72 hour-old culture in the case of *L. bulgaricus*. All LAB isolates were Gram positive. Microscopic examination and observation of the LAB isolates were done under the high power objective (40x). The cells of *S. thermophilus* were circular in shape commonly occurring in chains. They were Gram positive, catalase negative, non-motile, and nonspore-forming.

Lactobacillus delbrueckii ssp. *bulgaricus*, on the other hand, have rod shaped cells but sometimes they are almost coccoid commonly in short chains. Our results were in agreement with that of Holt et al. [27]. *L. bulgaricus* are Gram positive, nonsporing and facultatively anaerobic. Their optimum temperature is 30 – 40°C and

Table 1. Mean viable counts of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

Yogurt samples	Mean viable LAB counts ¹ (\log_{10} CFU/ml)	
	n = 3	
	<i>S. thermophilus</i>	<i>L. bulgaricus</i>
Nestle Acti-V (NAV)	8.93 ± 0.57^a	9.43 ± 0.47^a
Nestle Creamy Yogurt (NCY)	9.25 ± 0.17^a	9.71 ± 0.09^a
Carabest Yoghurt (CYP)	9.07 ± 0.30^a	9.01 ± 0.13^a
Nestle Fruit Selection Yogurt (NFB)	9.57 ± 0.28^a	9.76 ± 0.75^a
Live Yoghurt (LYB)	10.20 ± 0.06^a	9.96 ± 0.01^a
Carabest Yoghurt Ice Cream (CYI)	8.18 ± 0.02^b	8.15 ± 0.09^b
Nestle Fruit Selection Yogurt plus Jelly (NFJ)	9.82 ± 0.32^a	9.74 ± 0.26^a

¹Mean viable counts with the same superscript are not significantly different ($P \leq 0.05$)
n = number of samples examined per yoghurt brand

Table 2. Colony characteristics of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

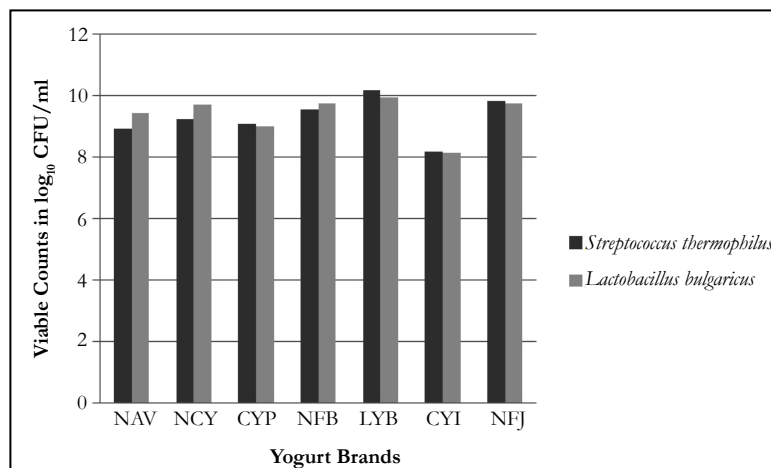
Colony Characteristics	<i>S. thermophilus</i>	<i>L. bulgaricus</i>
Colony Color	Creamy white	Creamy gray
Colony Size	0.5 – 3.0 mm	1.0 – 5.0 mm
Colony Shape	Circular	Circular
Growth on agar slant	Irregular	Irregular
Elevation	Convex	Raised
Margin	Entire	Entire

Table 3. Titratable acidity, viable LAB counts (log₁₀ CFU/ml) and ratio of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in the yogurt samples.

Yogurt sample	% Lactic acid	Mean viable LAB counts ¹ (log ₁₀ CFU/ml)		Ratio (S/L)
		<i>Streptococcus thermophilus</i> (S)	<i>Lactobacillus bulgaricus</i> (L)	
Nestle Acti-V (NAV)	0.75	8.93 ± 0.57 ^a	9.43 ± 0.47 ^a	1:1.06
Nestle Creamy Yogurt (NCY)	0.75	9.25 ± 0.17 ^a	9.71 ± 0.09 ^a	1:1.05
Carabest Yoghurt (CYP)	0.94	9.07 ± 0.30 ^a	9.01 ± 0.13 ^a	1:1
Nestle Fruit Selection Yogurt (NFB)	0.75	9.57 ± 0.28 ^a	9.76 ± 0.75 ^a	1:1.02
Live Yoghurt (LYB)	0.98	10.20 ± 0.06 ^a	9.96 ± 0.01 ^a	1.02 : 1
Carabest Yoghurt Ice Cream (CYI)	0.35	8.18 ± 0.02 ^b	8.15 ± 0.09 ^b	1:1
Nestle Fruit Selection Yogurt plus Jelly (NFJ)	0.65	9.82 ± 0.32 ^a	9.74 ± 0.26 ^a	1:1

¹ Mean viable counts with the same superscript are not significantly different (P ≤ 0.05)

Figure 1. Comparative viable counts of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in different yogurt samples.



their metabolism is fermentative and saccharoclastic, at least half of the end product of carbon is lactate.

Correlation between titratable acidity and viable LAB counts

The values of titratable acidity, viable counts (log₁₀ CFU/ml) of *S. thermophilus* and *L. bulgaricus* and their ratio in different yogurt samples were shown in Table 3. There was no correlation found between *S. thermophilus* (r=0.68) and *L. bulgaricus* (r = 0.67) counts and titratable acidity. Live Yoghurt (LYB) had the highest titrat-

able acidity of 0.98% lactic acid while Carabest Soft-served yogurt Ice Cream (CYI) had the lowest titratable acidity of 0.35% lactic acid. The values obtained for titratable acidity of all yogurt samples except NFJ were above the standard value of 0.7% lactic acid [28]. No federal standards have been established on minimum titratable acidity for frozen yogurts like CYI. In general, the industry practice is to achieve a minimum titratable acidity of 0.30 % lactic acid which was met by CYI with titratable acidity of 0.35 % lactic acid [29].

Comparative Viable LAB Counts

The comparative viable counts of *S. thermophilus* and *L. bulgaricus* in different yogurt samples were shown in Figure 1. The ratio of the yogurt organisms is important because it determines the quality of yogurt. In all the yogurt samples, the ratio of *S. thermophilus* to *L. bulgaricus* in general was almost 1:1 (Table 3 and Figure 1). Our results were in agreement with the study conducted by Mikulec and Niketic [30]. The two organisms have a symbiotic relationship during the manufacture of yogurt with the ratio of *S. thermophilus* to *L. bulgaricus* constantly changing. Both organisms produce lactic acid as the main fermentation product. For proper flavor development, the ratio of *S. thermophilus* to *L. bulgaricus* should be in the range of 1:1 to 3:1 [30].

The present study revealed that four (4) out of seven (7) yogurt samples namely CYP, LYB, CYI and NFJ have mean viable counts of *S. thermophilus* higher than the *L. bulgaricus*. This may be due to the use of a starter with dominant *S. thermophilus* which is common in local market. The use of industrial starters with low proportion of *L. bulgaricus* allows the production of yogurt with a reduced acidity and with lesser risks of post acidification. Moreover, the high concentration of sugars in several types of yogurt might have affected the viability of *Lactobacillus bulgaricus* [15].

It was reported earlier that the starter culture for most yogurt production has a ratio of 50:50 (*S. thermophilus*: *L. bulgaricus*) [30]. Although they can grow independently, the rate of acid production is much higher when used together than when either of the two organisms were grown individually. *S. thermophilus* grows faster and produces both acid and carbon dioxide. The carbon dioxide produced stimulates growth of lactobacilli.

Conclusions

This study was conducted to determine the viable counts and to verify the presence of *S. thermophilus* and *L. bulgaricus* claimed to be present in commercially available yogurts in the Philippines. Seven (7) samples replicated three (3) times per sample namely: Nestle Acti-V (NAV), Nestle Creamy Yogurt (NCY), Carabest Yoghurt (CYP), Nestle Fruit Selection Yogurt (NFB), Live Yogurt (LYB), Carabest Soft-served Yoghurt Ice Cream (CYI) and Nestle Fruit Selection Yogurt plus Jelly (NFJ) were examined in this study. It was shown that the mean viable LAB counts (*S. thermophilus* and *L. bulgaricus*) of all six (6) yogurt samples were significantly higher ($P \leq 0.05$) than CYI. Lower mean LAB counts in CYI were attributed to the low temperature of the product being a frozen yogurt.

Differences in viable counts were also attributed to dissimilarity of manufacturing dates among yogurt samples used, although efforts were exerted to obtain samples of very close manufacturing dates. No correlation was found between *S. thermophilus* and *L. bulgaricus* counts and titratable acidity of yogurt samples. The viable counts of *S. thermophilus* and *L. bulgaricus* in all seven (7) commercial yogurts examined met the prescribed minimum viable count of 10^6 CFU/ml for the suggested therapeutic effects and health benefits for the consumers.

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