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RESEARCH ARTICLE

Viability kinetics of *Lactobacillus casei* Shirota strain in a commercial fermented milk drink during refrigerated storage

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ABSTRACT

Background: One of the most important parameters in a commercial fermented milk drink containing probiotics is the viability of the microorganisms. **Aims and Objectives:** This study described the viability kinetics of *Lactobacillus casei* Shirota strain in a commercial fermented milk drink during refrigerated storage using nonlinear models. **Materials and Methods:** Viability of *L. casei* Shirota strain was monitored during refrigerated storage using standard bacterial plate count method. Several nonlinear mathematical models such as the zero-order, first-order, and second-order kinetic equations were employed in describing the population dynamics. Best-fit models were selected based on prescribed criteria including sum of squares of the error, p-value, and coefficient of determination. **Results:** The viable counts of *L. casei* Shirota strain in the fermented milk samples stored under refrigerated conditions decreased from 3.73×10^8 colony forming units per ml (CFU/ml) to 2.70×10^8 CFU/ml when monitored every 3-4 days interval over 10 different monitoring points within the product's indicated shelf life. The counts significantly differed between the monitoring points that were at least 14 days apart. The lowest viable count was still within the recommended therapeutic dose. The decrease in the bacterial population behaved under a second-order kinetic relationship. **Conclusion:** The viability of *L. casei* Shirota strain in a commercial fermented milk product during refrigerated storage is governed in accordance with the second-order kinetic mechanism.

KEY WORDS: Viability Kinetics; *Lactobacillus casei*; Probiotics; Second-order Kinetics

INTRODUCTION

The increase in awareness on the health benefits of fermented milk products containing probiotic microorganisms has resulted to their increased production and market availability worldwide.[1] One of the probiotics frequently utilized in the manufacture of these fermented milk products belongs to *Lactobacillus* spp.[2] The viability count in the final product between production and expiration dates is one of the most important quantitative parameters of these probiotic microorganisms. For probiotics, the acceptable level is 10^6 colony forming units per milliliter (CFU/ml), while the satisfactory level is 10^7-10^8 CFU/ml.[3,4]

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The viability, survival, and biochemical characteristics of these probiotics in fermented milk products are significantly influenced by several factors such as incubation and refrigeration storage temperature, inoculation rate, heat treatment, mixed probiotic cultures, and even the combined effects of these factors.[5-10] However, no study has been

conducted that assessed the viability kinetics of these probiotics during storage. In this paper, *Lactobacillus casei* Shirota strain found in commercial fermented milk product was the primary microorganism of interest. Although several studies reported the decreasing population count of the microorganism over time,[11] no such quantitative characterization of the kinetic relationship between viable count and time was conducted. Hence, this study aimed to elucidate the viability kinetic profile of *L. casei* Shirota strain in the commercially available fermented milk drink during refrigerated storage using nonlinear models. In particular, cell viability measured as CFU/ml was monitored during refrigerated storage using the standard bacterial plate count method. Several mathematical models such as zero-order, first-order, and second-order equations were examined using regression analysis. Parameters of the models were compared based on prescribed criteria including sum of squares of the error (SSE), *P*-value, and coefficient of determination. Parameter estimates obtained from the kinetic models will provide additional information for better understanding of the population dynamics of the microorganism in a commercially prepared fermented milk product during refrigerated storage.

MATERIALS AND METHODS

The study protocol was reviewed and approved as it adhered to the existing institutional ethical guidelines. Thirty-six 80-ml bottles of fermented probiotic milk drink (Yakult®, Yakult Philippines) of the same manufacturing batch and expiration date (C-AG.7:07, 09 May 2017) were purchased directly from the distributor (Yakult Philippines, Manila). These items were transported and kept per manufacturer's storage instructions with the temperature maintained under 10°C until expiration.[11] Three bottles were monitored within their indicated shelf life every 3-4 days for a total of 31 days.

Each sample was diluted from 10^{-1} to 10^{-6} and inoculated in MRS agar plates (HiMedia Laboratories Pvt. Ltd.). The plates were incubated in a candle jar[12] at 37°C for 48 h (Memmert Incubator INC108MED). Each test was done in triplicate. The viable cell count was monitored every 3-4 days for a total of 31 days. Statistical analyses of the results were performed using analysis of variance and Bonferroni test in the *post hoc* analyses. Nonlinear curves were generated to describe the relationship between viable population and time. Several kinetic models including zero-order, first-order, and second-order rate equations were employed to describe the behavior of viable populations over time. The parameter estimates of these models were obtained using the linearized forms of the nonlinear models (Table 1). Numerical and statistical analyses were performed using Microsoft Excel® and STATA® software at 5% level of significance.

RESULTS

The viable counts of the fermented milk samples stored under refrigerated conditions were monitored every 3-4 day interval

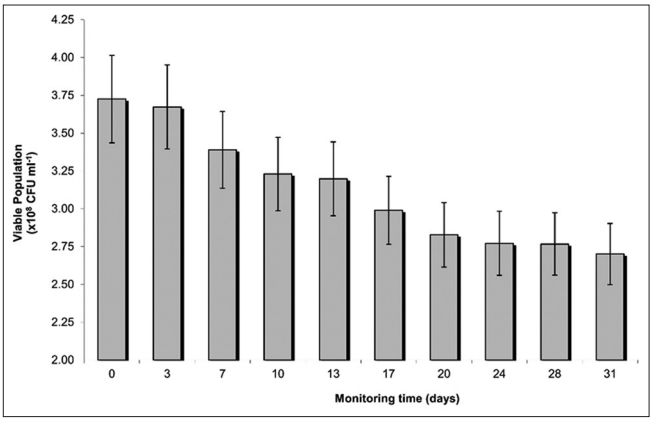

Figure 1: Comparisons of the mean viable *Lactobacillus casei* Shirota strain population in a commercial fermented milk drink during refrigerated storage over varying monitoring time

Table 1: Nonlinear and linearized forms of the viability kinetic models

Kinetic model	Nonlinear model	Linearized form
Zero-order	$\frac{dP_t}{dt}=k_o$	$P_t=k_0t+P_o$
First-order	$\frac{dP_t}{dt}=k_1P_t$	$\log P_t=\log P_o+\frac{k_1}{2.303}\ t$
Second-order	$\frac{dP_t}{dt}=k_2P_t^2$	$\frac{-1}{P_t}=k_2t+\left(\frac{-1}{P_0}\right)$

P_t (CFU/ml): Population count at time t (day), k_o (CFU/ml day^{-1}): Zero-order rate constant, k_1 (CFU/ml day^{-1}): First-order rate constant, k_2 (ml/CFU day^{-1}): Second-order rate constant, P_o: Initial population, CFU: Colony forming unit

Table 2: Comparison on the viability count of *L. casei* Shirota strain in commercial fermented milk drink during refrigerated storage across different monitoring points

Day	Mean±SD (×10^8 CFU/ml)
0	3.726±0.627^{a}
3	3.673±0.602^{ab}
7	3.390±0.551^{abc}
10	3.230±0.526^{abcd}
13	3.198±0.529^{abcde}
17	2.990±0.486^{cdef}
20	2.828±0.462^{cdefg}
24	2.771±0.459^{defgh}
28	2.767±0.446^{defghi}
31	2.701±0.436^{defghi}

Means with the same superscript letters do not differ at 5% level of significance using Bonferroni test, SD: Standard deviation, CFU: Colony forming units, *L. casei: Lactobacillus casei*

over 10 different monitoring points. The bacterial population decreased over time within the product's indicated shelf life (Figure 1). The viable population counts significantly differed between two monitoring points which were at least 14 days apart (Table 2). The mean viable population counts on days 17, 20, 24, 28, and 31 significantly varied from the baseline value (day 0) ($P < 0.05$). Moreover, viability counts on day 3 significantly differed when compared to those of days 17, 20, 24, 28, and 31 ($P < 0.05$), and population counts on day 7 also significantly differed from days 24, 28, and 31 ($P < 0.05$).

Assessment of the kinetic viability of *L. casei* in the commercial fermented milk product revealed that when population *P* was plotted against time *t*, the relationship reflects a zero-order rate mechanism (Figure 2a) as justified by the value of the coefficient of determination close to 1.0 (Table 3). In this zero-order rate kinetics, the half-life of the population is 52 days. However, examination of the linear plots of log *P* against *t* describing the first-order kinetic process revealed better parameter estimates with a half-life of 62 days (Table 3) and generated better fit of the experimental data (Figure 2b). The linear plots of -1/P against *t* (Figure 2c) yielded parameter estimates for the second-order kinetic model with a half-life of 77 days. This second-order kinetic model has the highest coefficient of determination (R^2) with lowest *P* and SSE values (Table 3) suggesting that the viability of *L. casei* during refrigerated storage was governed in accordance with the second-order kinetic rate model (Figure 2d).

Table 3: Parameter estimates, half-life, coefficient of determination, and error analysis of the different viability kinetic models

Kinetic model	Parameter estimates
Zero-order	
k_0	−0.0349
P_0	3.6614
R^2	0.9425
$t_{½}$	52.456
SSE	7.41×10^{-2}
P	3.06×10^{-6}
First-order	
k_1	−0.0111
P_0	3.6797
R^2	0.9547
$t_{½}$	62.432
SSE	5.27×10^{-6}
P	1.17×10^{-6}
Second-order	
k_2	−0.0035
P_0	3.7045
R^2	0.9637
$t_{½}$	77.126
SSE	3.90×10^{-2}
P	4.83×10^{-7}

k_o (CFU/ml day^{-1}): Zero-order rate constant, k_1 (CFU/ml day^{-1}): First-order rate constant; k_2 (ml/CFU day^{-1}): Second-order rate constant, P_o: Initial population, R^2: Coefficient of determination, $t_{½}$: Half-life (days), SSE: Sum of squares of the error, P: *P* value

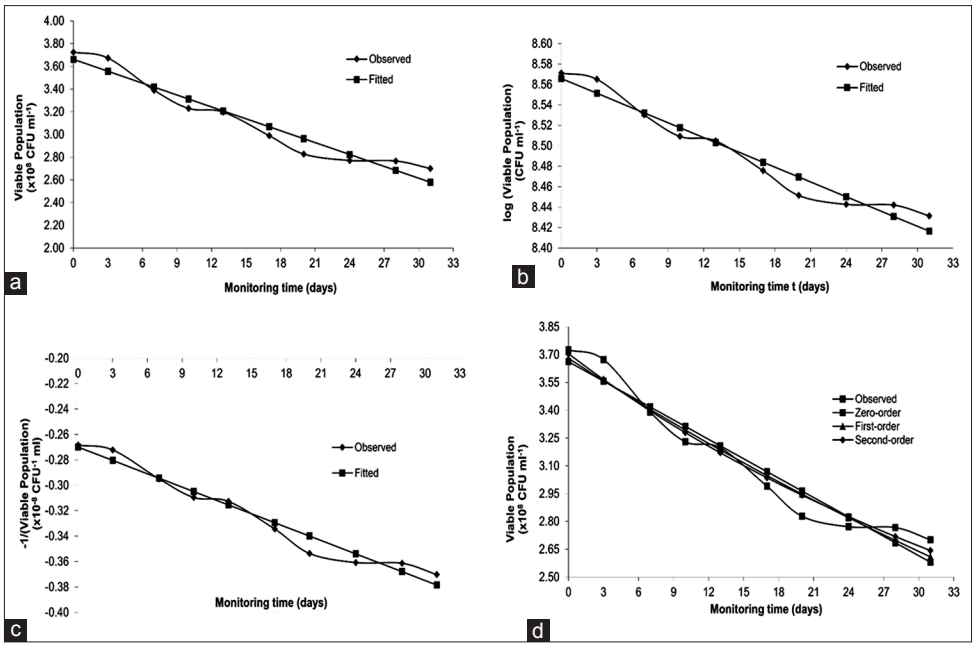

Figure 2: Linear plots between time and (a) viable population in a zero-order kinetic model, (b) log of the viable population in a first-order kinetic model, (c) negative reciprocal of the viable population in a second-order kinetic model, and (d) kinetic profile of the viable population in a fermented milk drink during refrigerated storage

DISCUSSION

Lactic acid bacteria are well utilized in the food industry, particularly in the production of fermented milk products such as yoghurt, sour cream and cheese, and fermented meat and fish among others. Several studies have been conducted on these lactic acid bacteria as probiotic microorganisms including their shelf life, growth and survival during production, resistance to bile salts and acids, and adherence and survival in the human gut.[13-15] One of the successfully commercialized strains is the *L. casei* found in copious amounts in fermented milk products[16] such as Yakult®.[11]

Yakult has been in the Philippines since 1978, and due to its increasing popularity, a factory was established in the Philippines, unlike with most other countries that rely on Japan's production.[17] The bacteria have been shown to remain viable throughout their shelf life when the product was stored under refrigerated conditions, although their numbers decreased slightly over time.[11] This study also identified such decreasing viable bacterial population. The behavior appeared in a nonlinear fashion resembling an exponential decay in accordance with the second-order kinetic model. The viability decreases more slowly in a second-order, compared to that in a first-order rate equation.

Numerous studies have been conducted on the health effects of bacteria found in commercially prepared fermented milk products. *L. casei* Shirota has been found to improve chronic constipation after 2 weeks of intervention of 65 ml per day,[18] and when consumed continuously, it offered full benefits in the improvement of diarrheal cases.[19,20] Moreover, Yakult had been established to have antimicrobial activities against extended-spectrum β-lactamase (ESBL)-producing strains of *Enterobacter aerogenes, Klebsiella pneumoniae, Proteus mirabilis,* and *Escherichia coli*, metallo-β-lactamase - producing strain of *Pseudomonas aeruginosa,* and strains of methicillin resistant *Staphylococcus aureus*.[12] The probiotics inhibited the growth of all the tested multiple drug resistant strains and were bactericidal for all strains except for ESBL-producing *E. coli.* Furthermore, *Lactobacillus* spp. has a role in cancer treatment and prevention of inflammation as a precursor to carcinogenesis. Spent probiotic culture media from *Lactobacillus* spp. isolated from Yakult did not have a significant cytotoxic effect on normal human dermal fibroblasts and THP-1 leukemia cells but were significantly cytotoxic for the HT-29 and HCT116 colon cancer cell lines.[21] When these probiotics are taken in specified doses, these have health benefits beyond the intrinsic basic nutritional contents.[22] As such, several criteria for these probiotics have been established such as the potential to resist chemical processes (bile and gastric acids), to influence metabolic activities, to persist and survive in the human gut, and to modulate the immune response.[14,15]

Although several studies have identified the effects of varying factors on the benefits of probiotics on human health, there is limited information on survival, activity, and dose response. The specific number of probiotic microorganisms that should be ingested has not been well established. It is suggested that 10^8-10^9 CFU of probiotic bacteria per day as the minimum therapeutic dose and at least 10^6 CFU per ml at the expiry date.[3,23] *In vitro* survival studies recommended 10^9-10^{10} CFU daily dose for probiotic effects since 10^6 CFU/ml might be insufficient as not all bacteria reach the intestines alive.[24] The present study identified a range of 2.2-3.0 × 10^{10} CFU per 80-ml bottle of Philippine Yakult monitored for a period of 31 days, which is comparable with the identified approximately 3.0 × 10^{10} CFU per 65-ml recommended dose bottle of Yakult[25] containing at least 1.0 × 10^8 CFU/ml at the expiry date.[11]

Plate counting has been employed in most researches for the assessment of probiotic viability. The complex nature of cell viability includes whether cells are culturable or not in the culture medium and growth conditions used. Cells that are active but not culturable *in vitro* can still have beneficial health effects. These active microorganisms that are not necessarily culturable can convert lactose, produce antibacterial compounds, and assimilate cholesterol, with the nonviable forms adhering to intestinal mucus and modulating certain immune responses.[26,27] Owing to the complex effects of diverse bacterial cell populations, a more advanced, appropriate, and accurate method is recommended to have an accurate quantification of the viable bacterial populations, more accurate parameter estimates, and better descriptions of the population dynamics.

CONCLUSION

Results of the study showed that the counts of *L. casei* Shirota strain significantly differed between the monitoring points that were at least 14 days apart. The viable population still met the minimum therapeutic dose of 10^8 CFU/ml within the intended shelf life of the commercial fermented milk product during refrigerated storage. The kinetic viability profile of the microorganism appeared to be governed in accordance with the second-order kinetic model.

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