Effect of Selected Oligosaccharides on the Viability and Fermentation Kinetics of *Lactobacillus acidophilus* and *Lactobacillus casei* in Cultured Milk

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Abstract: The study aimed to investigate the effect of fructo-oligosaccharides (FOS) on the growth and fermentation kinetics of *Lactobacillus casei* LC-01 (LC) and *Lactobacillus acidophilus* LA5 (LA) in cultured milk. Two commercially available FOS with different degree of polymerization (DP), namely Fibrulose F97 (DP, 2-20) and Fibruline Instant (DP, 3-60) were used at 4% (w/v) and 8% (w/v) respectively during fermentation and storage of cultured milk. Physicochemical properties and acidification kinetic of milk were measured throughout the fermentation. The concentration and DP values of the FOS do not seem to affect the growth of both probiotics during fermentation. Nevertheless, the pH and total soluble solid of milk fermented by both probiotics supplemented with FOS decreased tremendously during fermentation. It is noted that the percentage of lactic acid produced in *L. acidophilus* is higher than *L. casei* owing to the metabolic characteristic of the strain. The kinetic of maximum acidification rate V_{max} of cultured milk was significantly higher with the addition of FOSs at 4%. However, FOS with lower DP seemed to enhance ($p<0.05$) the stability of LA in cultured milk during cold storage, but no significant effect on LC. The results of this work indicate that FOS could significantly improve the survival of probiotics in cultured milk especially during refrigerated storage.

Keywords: Fructo-oligosaccharides, *Lactobacillus acidophilus, Lactobacillus casei, growth,* fermentation kinetic.

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [1]. The global probiotics market is expected to be worth US$ 32.6 billion by 2014, representing an annual growth of 12.6% with the Europe and Asia accounting for nearly 42% and 30% of the total revenues respectively. Dairy products, mainly yoghurts, are the most popular food carriers, and the probiotics used in these products typically are from the *Lactobacillus* (naturally found in the human small intestine) and *Bifidobacterium* (naturally found in the human large intestine) genera [2]. Their benefits to human health include prevention of diarrhea [3], reduction of cholesterol level [4], paediatric atopic dermatitis prevention [5], relief of Irritable bowel syndrome [6] and relief of milk allergy in infants [7]. In order to produce the desired benefits, probiotic bacteria should be present in the product with a minimal concentration of 10^6colony-forming units (CFU) per gram throughout the product shelf life [8]. In views of technological aspects, lactobacilli is a preferred choice to be incorporated into dairy food products as they are facultative anaerobes and tolerable to oxygen exposure during processing, transport and storage [9].

A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host wellbeing and health [10]. It is expected that prebiotics improving health in a similar manner to probiotics, whilst being easier to incorporate into the diet than live microbes [11]. Oligosaccharides such as lactulose, galacto-oligosaccharides, inulin, fructo-oligosaccharides (FOS), xylo-oligosaccharide and other food carbohydrates are some of the well-known examples of prebiotics. There is an obvious potential for a synergetic effect when combining probiotics and prebiotics appropriately, because prebiotics promote the growth and activities of probiotics [12]. Prebiotic FOSs is gaining increasing recognition as agents to modulate the colonic microbiota in humans and animals. They are relatively new functional food ingredients that have great potential as prebiotics, apart from having a number of desirable characteristics, which are beneficial to the health of consumers [13]. Some of these prebiotics selectively stimulate beneficial microbes within the gut microbiota, directly stimulate immunity, protect against pathogens, and facilitate host metabolism and mineral absorption [14].

It has been established that short-chain FOSs are fermented in the proximal colon, thereby leaving the long-chain prebiotics for more distal colonic activity [15]. A number of *in vitro* and *in vivo* studies have confirmed that inulin and long-chain FOSs are fermented into lactic and short-chain carboxylic acids

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[16, 17]. Furthermore, it has been well demonstrated that inulin and FOS selectively stimulate the growth of bifidobacteria or lactobacilli, both of which are considered beneficial to the host [18]. Nagpal and Kaur [19] reported that addition of prebiotics has a significant effect on probiotics, and hence, a combination of suitable *Lactobacillus* strain(s) with a specific prebiotic could be a viable probiotic-based functional food approach in administering the beneficial bacteria *in-vivo.*

By combining the rationale of pro- and prebiotics, the concept of synbiotics is proposed referring to the potential synergy between probiotics and prebiotics [20]. The approach has been reported to enhance the efficiency through improving the growth and activities of *Bifidobacterium* spp. in skim milk with inulin and increase the sustainability of probiotics during cultured milk storage [21]. Up to date, studies in seeking for the appropriate match between prebiotics and probiotics for symbiosis and synbiotic effect are still ongoing. It is noticed that there is a lack of study in which the reference for fermentation kinetic with only a few attempts made using pure culture of *L. acidophilus* [22, 23, 24, 25], *L. casei* [26] and mixed cultures [24, 25]. Therefore, the study aimed to investigate the effect of fructo-oligosaccharides (FOS) with different degree of polymerization (DP) on the growth and fermentation kinetic of *Lactobacillus casei* LC-01 (LC) and *Lactobacillus acidophilus* LA5 (LA) in cultured milk.

2. MATERIALS AND METHODS

2.1. Materials

Lyophilized cultures of *L. acidophilus* LA5 (LA) and *L. casei* LC-01 (LC) were provided by Christian Hansen (Denmark). Chicory fructo-oligosaccharides (FOS), namely Fibrulose® F97 (DP ≤ 20 = 92%) and Fibruline® Instant (DP< 20 = 60%) supplied by Cosucra Groupe Warcoing S.A. (Belgium) were used as prebiotics [27]. Other materials used were Skim milk powder (Sunlac™, Australia), peptone water, MRS broth and MRS agar (Merck, Malaysia).

2.2. Fermentation of Milk

Milk supplemented with 4% (FI4) and 8% (FI8) (w/v) Fibruline Instant and 4% (FS4) (w/v) and 8% (FS8) (w/v) Fibrulose F97 and non-supplemented milk (C) were submitted to the thermal treatment at 90°C for 5 min, followed by immediate cooling in ice water. Prior to the assay, freeze dried cultures were serially transferred 3 times in MRS broth and incubated at 37°C for 48 hours. The lactobacilli cells from overnight (18 hours) culture were harvested by centrifugation at 4000 rpm for 5 min, washed 3 times with 0.85% NaCl and inoculated in the milk to obtain an initial count of 6.1-6.5 Log CFU/ml. Fermentations were conducted in the fermentor Biostat®Bplus (Sartorius) at 37°C under stirring of 60 rpm for 60 hours [25].

2.2.1. Enumeration of L. acidophilus (LA) and L. casei (LC)

The viable count of LA and LC was determined after 12 hours up to 60 hours of fermentation. Briefly, 10 g of samples were withdrawn and suspended into 90 ml of 0.1% (w/v) peptone water followed by homogenization using stomacher. Subsequent serial dilutions were made and viable cell numbers enumerated using the pour plate technique. The counts of LA and LC were enumerated on de Mann Rogosa and Sharpe Agar (MRS, Merck) incubated anaerobically at 37°C for 48-72 hours. Plates containing 25-250 colonies were enumerated and recorded as colony forming unit (CFU/ml) of the sample. All samples were analyzed in triplicate; all experiments were repeated at least twice.

2.2.2. pH and Lactic Acid Content

Samples were withdrawn similarly to the method used for the determination of total viable count. The pH of each sample at every sampling occasion was measured using a HI 9321 Microprocessor pH meter (HANNA Instruments). The pH meter was standardized using reference pH 4.0 and pH 7.0 buffer solutions. Titratable acidity (as % lactic acid) of the cultured milk was determined in triplicate according the AOAC titration method 947.05 using 0.1 M NaOH [28].

2.3. Storage of Fermented Milk

Upon completion, the fermented milk was kept in sterile bottles at 4°C and the survival of the probiotics in the milk was enumerated as described previously.

2.4. Kinetics of Acidification on Fermentation

The fermentation kinetic of acidification was calculated based on the result of titratable acidity throughout the fermentation of milk, following the method described by Oliveira *et al*. [24]. The maximum acidification rate (V_{max}) was calculated as the time variation of pH (dpH/dt) and expressed as pH units min^{-1}. At the end of the incubation, the following kinetic parameters were also recorded: (i) t_{max}, which was the time at which, V_{max} was reached; (ii) $t_{pH5.0,}$ which was the time to reach pH 5.0; and (iii) $t_{pH4.5,}$ which was the time to reach pH4.5.

2.5. Statistical Analysis

Data obtained from the study were analysed using the Statistical Package of The Social Sciences (SPSS) version 17.0. Significant differences among various prebiotic formulation at different kinetic parameters were analysed by Analysis of variance (ANOVA) with mean values compared using the Tukey's test at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Enumeration of Lactobacilli During Fermentation

The changes in Log_{10} count of probiotics during milk fermentation for the first 60 hours are presented in Figure **1**. *Lactobacillus casei* and *Lactobacillus acidophilus* were able to grow in skim milk and their counts were found stable after 24 hours of fermentation. FI4, FI8 and FS8 supplemented fermented milk were found to have slightly higher *L. casei* count (7.7 Log_{10} CFU/ml) than FS4 and the control (7.4 Log_{10} CFU/ml) at 12 hours of fermentation; while the control had slightly higher count than all of the fructo-oligosaccharides (FOSs) added samples after 24 hours of fermentation. However, no significant different ($p>0.05$) were observed. Similarly, after *L. acidophilus* fermented the skim milk for 12 hours, the total count of the control, FS4, FS8, FI4 and FI8 was found not significantly difference ranged between 7.4 to 7.5 Log_{10} CFU/ml. Significant increase of *L. acidophilus* was detected for FS4 supplemented fermentation at 24 hours. However, other formulations with FS8, FI4 and FI8 were found not significantly differed from the control, and this trend continues until the end of fermentation. These results partly concur with the study done by Rodrigues *et al*. [29] where they reported the insignificant growth of probiotics (*Lactobacillus* and *Bifidobacterium*) when comparing cultures in curdled milk with inulin/FOS supplementation and non-supplemented. The significantly higher *L. acidophilus* count for FS4 at the end of fermentation shows the bifidogenic property of Fibrulose F97. Besides, Fibruline Instant also shows minor bifidogenic property during fermentation. Similar results were also reported by Moro *et al.* [30] who found that ingestion of prebiotics had increased the number of *Lactobacilli* significantly in infants. An interesting finding in the current study is that FS4 had slightly higher count (8.5 Log_{10} CFU/ml) than FS8 (8 Log_{10} CFU/ml), indicating that the concentration of the inulin used has great influence on the total number of *Lactobacilli*. However, it has been previously reported that the growth of *L. casei* was higher in reconstituted skim milk supplemented with 1g Raftiline HP than 3g Raftiline HP [31]. Therefore, an optimization on the concentration of the type of prebiotics used is required when applying these into foods.

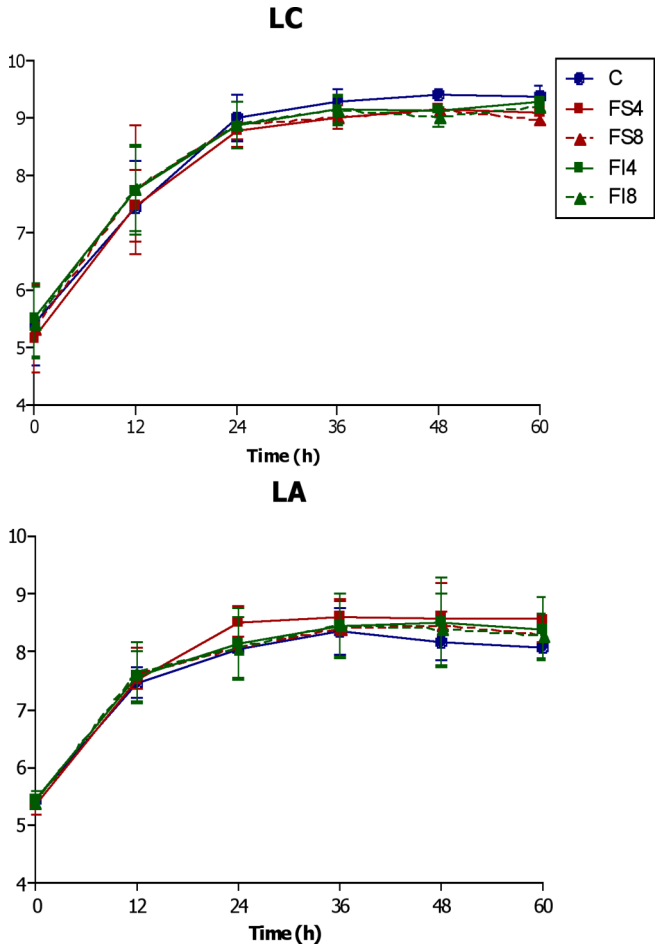

Figure 1: The extent of growth of *Lactobacillus casei* (LC) and *Lactobacillus acidophilus* (LA) for milk supplemented with various prebiotics during fermentation.

[1]Error bars represent standard deviations of replicates.

It is interesting to find that no significant difference ($p>0.05$) was found in between the growth of *L. casei* in fructo-oligosaccharides supplemented or non-supplemented milk samples (Figure **1**). Although the bifidogenic nature of inulin and fructo-oligosaccharides on *Lactobacillus* have been reported elsewhere [32, 33], there are also contradicting reports on inulin for not supporting the growth of *Lactobacillus acidophilus* [31] which perhaps due to the different growth media and the sources of prebiotic used in those studies [34]. Saminathan *et al*. [35] further demonstrated the ability of lactobacilli to utilise FOS is strain dependent. This is probably due to lack of ability for the *Lactobacillus* to produce enzymes that could hydrolyse the long chain fructans such as ß-fructofuranosidase [36].

Nevertheless, the failure of inulin to increase the total number of *L. casei* and *L. acidophilus* could be due to the presence of other carbon source, such as sucrose in the skim milk. Barrangou *et al*. [37] showed that when FOS is present together with readily fermentable sugars (sucrose, glucose) even at lower concentration, the later repressed the expression of msm E in *Lactobacillus acidophilus*, which encodes a transporter associated with fructosidase. Therefore, it is suggested that the occurrence of a regulatory mechanism of preferred carbohydrate utilization pathway lowered the prebiotic effect on both probiotic strains. This further explains the reason of higher prebiotic effect of Fibrulose F97 than Fibruline Instant. It was also reported earlier that *Lactobacillus* selectively ferments shorter oligosaccharides than those longer chains due to the nature of low molecular mass substrates containing more non-reducing ends per unit mass that are prone to rapid attack by probiotic exo-enzymes [38, 39]. On the contrary, Aryana *et al*. [40] suggested that there are no growth differences between the addition of inulins of various chain lengths (average polymerization degree: 4, 10 and 23) into low-fat plain yoghurt. Nevertheless, the results emphasize the understanding on the mechanisms and regulation of prebiotic sugar utilization by probiotic bacteria and targeted commensals, which it is necessary for rational selection and development of effective probiotics and prebiotics cocktails.

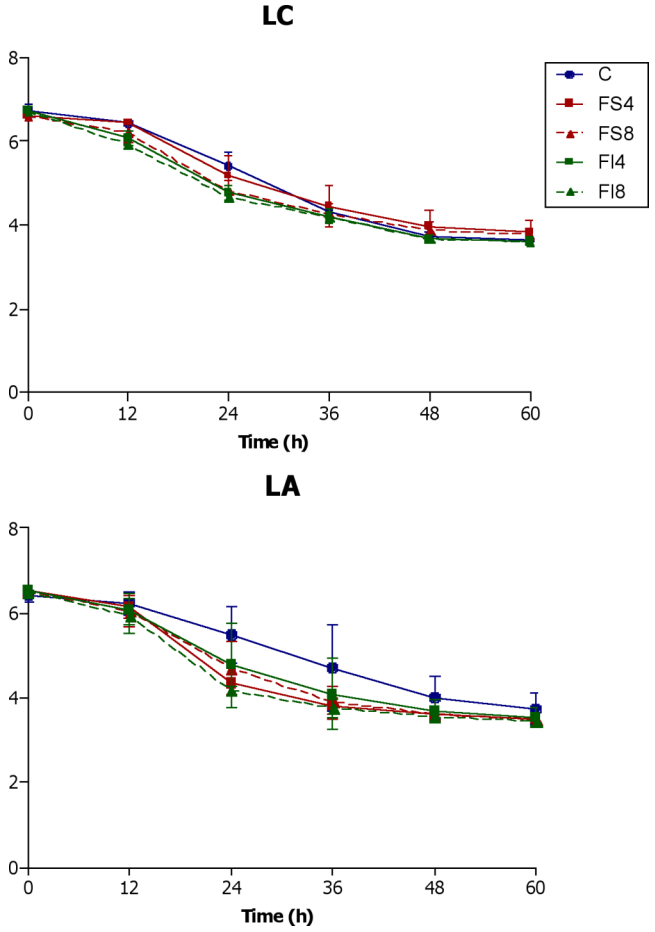

Figure 2: Changes in pH for milk supplemented with various prebiotics during fermentation of *Lactobacillus casei* (LC) and *Lactobacillus acidophilus* (LA).

[1]Error bars represent standard deviations of replicates.

3.2. Changes in pH and Lactic Acid Production During Fermentation

The pH of milk fermented by the two probiotics supplemented with various prebiotics decreased gradually from pH 6.5 to pH 3.9 within 60 hours of fermentation (Figure **2**). Besides, the titratable acidity of fermented milk expressed in percentage of lactic acid increased from 0.1% to 0.8% and 0.9% respectively for *L. casei* and *L. acidophilus* (Figure **3**). In addition, the current study has demonstrated a strong correlation (R^2> 0.9, p<0.05) between the reduction of pH and the production of lactic acid as the main metabolic product by the lactobacilli.

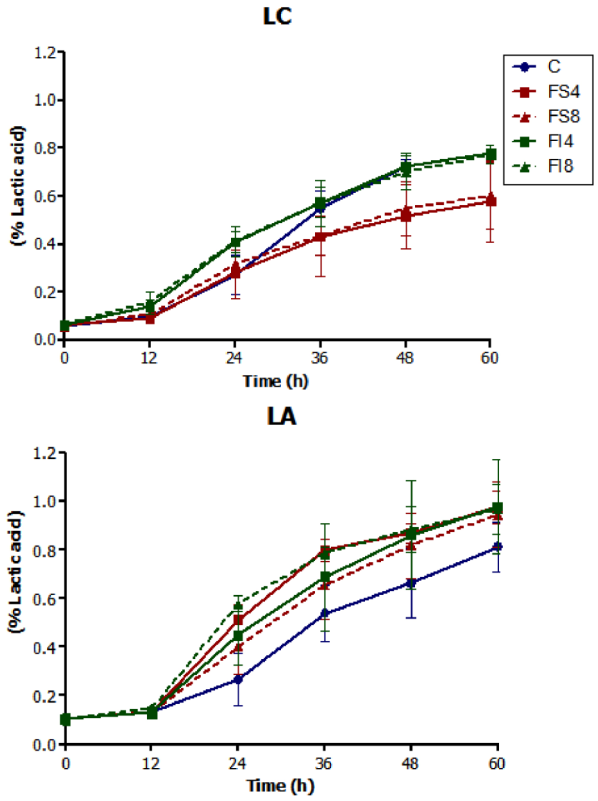

Figure 3: Production of lactic acid for milk supplemented with various prebiotics during fermentation of *Lactobacillus casei* (LC) and *Lactobacillus acidophilus* (LA).

[1]Error bars represent standard deviations of replicates.

For the fermentation of *L. casei*, the production of lactic acid in milk supplemented with 4% and 8%

Fibruline Instant is significantly higher ($p<0.05$) than the control and milk supplemented with Fibrulose F97 at 12 hours and 24 hours respectively. Meanwhile, the percentage of lactic acid produced in *L. acidophilus* fermentation showed no significant difference ($p>0.05$) between milk supplemented with Fibruline Instant and Fibrulose F97. However, it was noticed that the acidification rate of *L. acidophilus* fermented milk was relatively faster than *L. casei* fermentation. The acidification rates had in turn influenced the availability of more favourable simple sugars such as fructose, thus increases the number of lactic acid bacteria. It was found that at pH 4.0, hydrolysis reaction started to occur at the fructo-oligosaccharide chains and the stability of the chains was lowered by protonic activation of the leaving group, thus releasing more simple sugars of fructose or glucose [41]. This was further confirmed by Matusek *et al*. [42] where the decrease of pH would increase the degradation rate of the oligosaccharides. However, some of the current works exhibited contradictory results with those previously reported studies, as the addition of oligosaccharides have no effect on the rate of acidification during fermentation [31, 43]. The variation in the acidification rate reported could be affected by the type and sources of oligosaccharides and different bacterial strains used in the study which directly affects the formation rate of different proportion of organic acids such as lactic acid, pyruvic acid and acetic acid [43]. It is also noted that the percentage of lactic acid produced by *L. acidophilus* is higher than *L. casei* owing to the homo-fermentative characteristics of the strains. *L. acidophilus* produces lactic acid and pyruvic acid as the main metabolites through Embden-Meyerhoff-Parnas (EMB) pathway using NADH as the cofactor and the enzyme lactate dehydrogenase, while *L. casei*, a facultative hetero-fermentative bacterium, able to choose between utilizing EMB pathway as well as 6-phosphogluconate/phosphoketolase pathway that yields the production of other metabolites such as lactate, CO, and ethanol which may explain the slightly lower percentage of lactic acid [44, 45, 46].

3.3. The Effect of Prebiotic on Storage of Cultured Milk

There were no significant differences in pH, titratable acidity and total soluble solid between samples that were kept for one week and four weeks at refrigerated temperature (data not shown). Besides, the viability of *L. casei* was found to be stable (9.1 log CFU/ml) throughout the refrigerated storage (Figure **4**). The result is aligned with the finding by Donkor *et al.* [47] who reported that lactobacilli strain, particularly *L. casei* LAFTI® 26 has good cellular stability in maintaining a constant viability throughout the storage which might have been sustained by the free amino acids in the product. It is further elaborated that the addition of prebiotic may also have sustained the metabolic activity of the probiotic organism throughout cold storage, increasing the concentration of primary metabolites but no detrimental effects caused by the high levels of lactic acid and acetic acid in the media towards the probiotic observed [48]. This phenomenon is not visualized in the cold storage of *L. casei* in the current study due to the viability of the probiotic with added prebiotic is not significant compared to ones in the control.

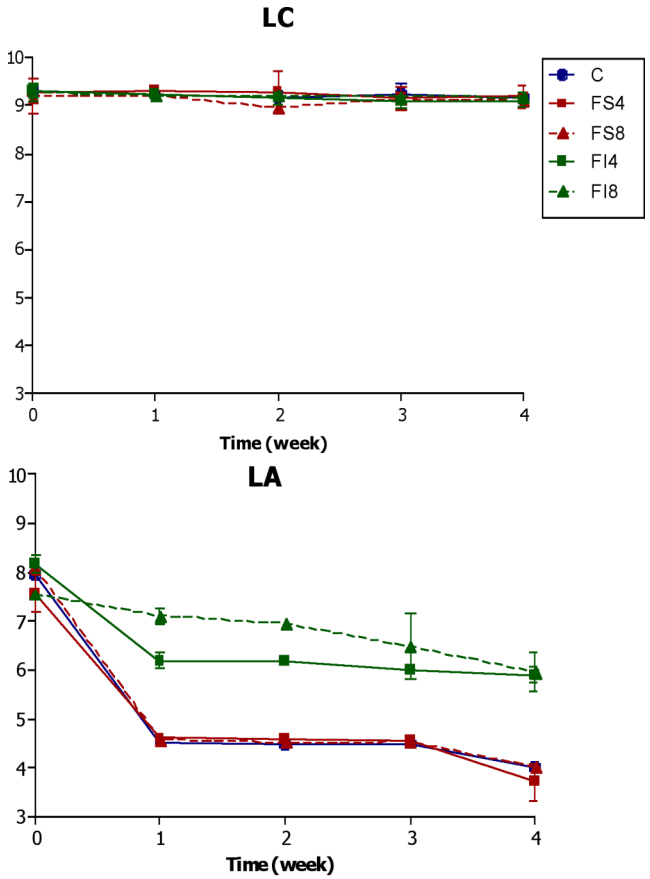

Figure 4: Survivability of *Lactobacillus casei* (LC) and *Lactobacillus acidophilus* (LA) in cultured milk during storage at 4°C.

[1]Error bars represent standard deviations of replicates.

However, the viable count of *L. acidophilus* in non-supplemented milk and milk supplemented with Fibrulose F97 declined by 40% from the initial 7.8 log CFU/ml to 4.5 log CFU/ml within the first week (Figure **4**). The reduction pattern of probiotic viability of FS4 and FS8 are similar to the control for the entire 4 weeks of storage, indicating that Fibrulose F97 was not effective in protecting probiotics from the storage

conditions. At the same time, survivability of *L. acidophilus* in milk supplemented with Fibruline Instant at both concentrations was significantly higher ($p<0.05$) than the control throughout the four weeks of study; with FI8 showing better sustainability than FI4 at first and second week. The viable count of *L. acidophilus* in milk supplemented with Fibruline Instant is maintained at 6 Log CFU/ml throughout the entire storage period from the initial count of 7 Log. The variation in viability of *L. acidophilus* is most probably due to the differences in the degree of polymerizations (DP) between Fibrulose F97 and Fibruline Instant. Fibrulose F97 was reported to contain more short chain oligosaccharides (DP< 20) than Fibruline Instant [27]. The author also stated that as the degree of polymerization increased, residual FOS increased after growth of the strains, and the rate of consumption of FOS decreased. Residual FOS might function as protectant to the probiotic cells from acid injury or promote metabolic activity [49], thus sustaining the viability during low temperature storage. Besides, shorter chain fructo-oligosaccharides (Fibrulose F97) containing primarily fructose chains and fructose chain with terminal glucose bound by α (1 2) bond glycocidic linkages may have undergone rapid degradation in highly acidic environment and by *L. acidophilus* exo-enzymes during fermentation could have remain in low level concentration to be able to function as protectant against acid injury due to low pH [38, 40, 50]. This could be detected as a possible factor when the milk pH decreased rapidly during fermentation as discussed previously, because the decrease of pH will in turn promotes the hydrolysis reaction between bonds of fructo-oligosaccharides [42]. Besides, Hincha *et al*. [51] found that the fructo-oligosaccharides with higher chain length better protects cell membrane lipids. This helps to explain the reason of Fibruline Instant has better protective effect than Fibrulose F97 during low temperature storage.

3.4. The Effect of Prebiotic on Fermentation Kinetic of Milk

It is noted that the V_{max} value for supplemented skim milk is statistically higher than the control sample without prebiotic, particularly Fibruline Instant for both concentrations of 4% (w/v) and 8% (w/v) respectively. Thus, the prebiotic that showed higher acceleration of acidification is Fibrulose F97, showing V_{max} increases by 18% at 4% (w/v) and 43% at 8% (w/v) (Table **1**). In general, both of the prebiotics yielded higher V_{max} value at higher concentration. Meanwhile, these two prebiotics were able to accelerate the fermentation time to achieve pH 5.0, showing 36% time reduction for Fibrulose F97 at 4% (w/v), 28% for 8% (w/v); and 39% at 8% (w/v) Fibruline Instant. It is noted as well that, the study conducted by Oliveira *et al.* [52] showed higher V_{max} for *L. casei* in comparison to *L. acidophilus* with the value of 14.3 ± 0.2.

However, addition of prebiotics into skim milk significantly increases the time to achieve V_{max} (t_{max}) and time to reach pH 4.5. It was also found that increase in prebiotic concentration reduced t_{max} for Fibrulose F97 but vice versa for Fibruline Instant; whilst, increase in prebiotic concentration significantly decreased $t_{pH4.5}$ for both Fibrulose F97 and Fibruline Instant. The results contradicted with the findings by Oliveira *et al.* [24], which showed a significant decrease in t_{max} by 5.5% in 13% (w/v) skim milk supplemented with 4% (w/v) oligofructose. The variation might be resulted from the use of starter culture *Streptococcus thermophilus* coupled with probiotic, instead of only probiotic cultures in the current study.

Table 1: Acidification kinetic parameters of fermentations of milk and milk supplemented with 4% (w/v) Fibrulose F97 (FS4), 8% (w/v) Fibrulose F97 (FS8), 4% (w/v) Fibruline Instant (FI4), and 8% (w/v) Fibruline Instant (FI8), by *Lactobacillus casei*

Prebiotic	V_{max}[A] (10^{-3} pH units/min)	t_{max}[B] (h)	$t_{pH5.0}$[C] (h)	$t_{pH4.5}$[C] (h)
Control[E]	19.03 ± 0.03[d]	3.38 ± 0.17[a]	24.25 ± 0.23[c]	27.67 ± 0.25[a]
FS4	20.45 ± 0.07[d]	8.38 ± 0.18[d]	15.58 ± 0.11[a]	33.54 ± 0.29[c]
FS8	24.04 ± 0.05[b]	4.46 ± 0.29[b]	17.46 ± 0.29[b]	30.50 ± 0.35[b]
FI4	22.40 ± 0.85[c]	4.50 ± 0.24[b]	24.96 ± 0.17[c]	35.92 ± 0.36[d]
FI8	27.13 ± 0.16[a]	6.33 ± 0.34[c]	14.75 ± 0.70[a]	26.79 ± 0.53[a]

Means with different letters within the same column indicates significant difference ($p<0.05$).
[A]Maximum rate of acidification.
[B]Time to reach V_{max}.
[C]Time to reach pH 5.
[D]Time to reach pH 4.5.
[E]Not supplemented skim milk was used as the control.

4. CONCLUSIONS

As conclusion, the prebiotics (FOS) were able to maintain the survival of probiotics significantly. However, the effect of fructo-oligosaccharides in maintaining the viability of *Lactobacillus* during storage varies with the degree of polymerization but no significant effect was observed in promoting the probiotic growth. It is suggested that longer chain prebiotics were used for maintaining the viability of probiotics especially *L. acidophilius* for long storage period. Besides, the results of kinetic parameters particularly V_{max} from this study is useful in elucidating the prebiotic effect on milk fermentation providing new insight in designing functional dairy products in the future.

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