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Probiotic viability, pH and lactic acid concentration of opened commercial probiotic dairy drinks stored at different temperatures and durations

Yun Khoon Liew^{1*} , Kyan Aung², Li Li Chan², Sandhya Baskaran³ and Siew Thong Mak³

Abstract

Background: The commercial cultured milk drinks contain either single or mixed probiotic species and supply in different serving sizes. It is known that different combinations of probiotics might provide the various products' quality in terms of nutritional value during their manufacturing process. However, a lack of information about probiotic viability and physicochemical properties of the opened fermented products for continuous fermentation leads to the driving force in conducting this study. Therefore, four locally available cultured milk drinks (branded Y, F, N and V) with 20 bottles each were aseptically transferred into their respective sterile containers and stored at 4 °C, 25 °C and – 20 °C for 1–13 days. Then, the viable cells were quantified using the drop plate method on de Man, Rogosa and Sharpe (MRS) agar. The pH change was investigated using the calibrated pH meter, and the Enzytec D-/L-Lactic acid kit determined the content of D-lactic acid via spectrophotometer. Eventually, the data were analysed using the statistical tool.

Results: The viability of probiotics in brands Y and V was significantly increased even when stored at – 20 °C and 4 °C with at least 1 log CFU/mL increment. The proliferation of probiotics was moderately influenced by the pH of the opened cultured milk. High content of D-lactate was found in Y- and F-branded products after 13 days of storage. The Y-branded cultured milk drink had the highest content of D-lactate with 0.52 g/L and 0.40 g/L when stored for 13 days at room temperature and 4 °C, respectively.

Conclusions: This study sheds light on the necessity to elucidate the properties of opened probiotic beverages over time, especially when bottled in large quantities. This allows some improvement steps.

Keywords: Probiotic, D-lactic acid, *Lactobacillus*, *Bifidobacterium*

Background

The use of probiotics in fermented food has been practised for several decades, and it is a part of an ancient human diet (Podolsky 2012; Mackowiak 2013). Probiotics are formulated and applied to dairy products and non-dairy products (Prado et al. 2008; Kandyliis et al.

2016; Zheng et al. 2017; Ranadheera et al. 2017). On top of that, the advancement in technology also enables the probiotics to be prepared in the encapsulated form and used for dietary supplement purposes as well (Govender et al. 2014; Nguyen et al. 2016). Of note, the trend of consuming probiotics is increasing among health-conscious consumers and is resulting in the expansion of probiotic companies (Stanton et al. 2001; Granato et al. 2010; Lau et al. 2013; Reid 2015; Shi et al. 2016; Nor et al. 2016; Zheng et al. 2017). The growing demand may be due to some of the general health benefits that probiotics provide, including nutrition, improved gastrointestinal

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health, improved homeostasis of the immune system and reducing symptoms in lactose-intolerant individuals via consumption of non-dairy probiotic drinks (Granato et al. 2010; Kneifel and Salminen 2010; Kechagia et al. 2013; Verhoeven et al. 2013; Reid 2015). Foremost, current studies also show the potential of probiotics in anti-tumour activity or in preventing patients from encountering chemotherapy-related infection diseases (Lee et al. 2004; Osterlund et al. 2007; Kumar et al. 2010; Maroof et al. 2012; Soltan Dallal et al. 2012; Aragón et al. 2014; Lakritz et al. 2014; Chen et al. 2017; Jacouton et al. 2017; Marschalek et al. 2017). All these benefits add to the value of probiotic products in the market.

It is vital that products maintain the appropriate amount of viable probiotics required to confer health benefits (10^7 – 10^9 cells per gram) (Kailasapathy and Chin 2000; Minelli and Benini 2008; Verna and Lucak 2010; Yildiz 2010; Bertazzoni et al. 2013; Castro et al. 2013; Hill et al. 2014). Generally, the probiotics that can be detected in either dairy or non-dairy products include *Bacillus* spp., *Bifidobacterium* spp., *Escherichia coli*, and lactic acid bacteria such as *Lactobacillus* spp., and *Streptococcus* spp. (Yildiz 2010; RoushanZadeh et al. 2014; Minervini et al. 2017). It is known that different bacterial genus and species can adapt differently to a given food environment or storage temperature and period (Stern and Frazier 1941; Nighswonger et al. 1996; Fiorentini et al. 2011; Céspedes et al. 2013; Daneshi et al. 2013; Ferdousi et al. 2013; Mani-López et al. 2014; RoushanZadeh et al. 2014; Lupien-Meilleur et al. 2016; Abdullah and Tulay 2018). Therefore, the viability of probiotics from different products will be affected to varying degrees under the given condition. For example, Shah et al. (1995) have studied the survival of *L. acidophilus* and *B. bifidum* in five distinct commercial yoghurts stored in refrigerator and found that the survivability of each strain varied among the commercial brands of yoghurts. The response of probiotics from different commercial cultured milk products to various pH environments or oxygen content has also been evaluated and can be reviewed elsewhere (Stern and Frazier 1941; Talwalkar and Kailasapathy 2004; McSweeney 2007; Ting and DeCosta 2009; Soccol et al. 2010; Sanhueza et al. 2015). However, no studies have examined the viability of probiotic bacteria in dairy beverages after being opened and stored under different conditions. It is an important knowledge gap that needs to be filled as some of the branded probiotic drinks' volumes can reach approximately 700 mL, which cannot be completely consumed after being opened. Furthermore, the lack of power supply in rural areas of developing countries results in the storage of unfinished probiotic beverages at room temperature. Currently, the growing interest in using dairy probiotic products for homemade

ice cream also leads to the unknown answer regarding the viability of probiotics after hardening and being stored at -20 °C. There are limited studies that focus on commercial fermented milk subjected to frozen storage conditions (O'Brien et al. 2016).

In addition, the probiotics in dairy products also lead to an increase in lactic acid production via fermentation of carbohydrates if they are growing. There are two types of lactic acid isomers: D- and L-lactic acid (Mack 2004; Vitetta et al. 2017). A previous study showed that some lactobacilli (*Lactobacillus acidophilus*) might accumulate D-lactic acid in patients with short bowel syndrome (Satoh et al. 1982; Perlmutter et al. 1983; Caldarini et al. 1996; Ku et al. 2006; Bested et al. 2013). This might enhance the absorption of D-lactic acid into the bloodstream because of its slower metabolism. Subsequently, the increased blood levels of D-lactic acid may be associated with behavioural changes such as anxiety and aggression, and also impaired memory, as highlighted by other researchers (Godey et al. 2000; Petersen 2005; Ku et al. 2006; Sheedy et al. 2009; Htyte et al. 2011; Bested et al. 2013). Therefore, the possibility of accumulation of lactic acid during the storage of probiotic beverages becomes a concern. However, not all lactobacilli will pose the risk of D-lactic acid absorption into the bloodstream. For example, *L. reuteri* has previously been shown to produce no elevation of D-lactic acid in the blood of infants, and *L. casei* subspecies *rhamnosus* or a mixture of probiotics of *B. breve* and *L. casei* was used in treating D-lactic acidosis as well as preventing its recurrence (Gavazzi et al. 2001; Uchida et al. 2004; Connolly et al. 2005; Takahashi et al. 2013). To our knowledge, testing the D-lactic acid content of probiotic dairy products after their manufacture and storage has never been studied before.

Therefore, the objective of this study was to determine the probiotic viability of the opened dairy probiotic beverages branded as Y, E, N and V under different storage conditions. The tested probiotic beverages might comprise monocultures or mixed cultures. The alteration in pH and D-lactic acid content during the storage conditions are also being studied. Finally, we aimed to investigate the correlation between the pH alteration of the opened dairy probiotic beverage and probiotics' total viability during their storage.

Methods

Commercial probiotic dairy drinks

Four different brands of cultured milk containing distinct probiotics were used in this study. A total of 20 bottles of each brand were purchased from local supermarkets and kept at 4 °C but were tested and used on the same day of acquisition. The probiotic products obtained from the supermarket were within 25–31 days of their expiry

date. This was to minimise the confounding factor caused by different manufacturing dates of the products. Due to ethical concerns for reasons of legislative compliance, the brand of the probiotic drinks is not being revealed in this study. The probiotic drink products were instead identified as brands Y, F, N and V. The monoculture of *Lactobacillus casei* could be found in Y-branded cultured milk. The mixed culture of *L. casei* and *L. acidophilus* is contained in V-branded products, while N-branded probiotic dairy drinks also contain *L. acidophilus* but together with *Streptococcus thermophilus*. The F-branded product is another type of multi-species product that comprise *L. rhamnosus*, *Bifidobacterium lactis*, *L. acidophilus* and *S. thermophilus*. All of these samples were aseptically aliquoted into the microcentrifuge tubes which were used in the following experiments.

Storage conditions of opened dairy probiotic drinks

Some of the aliquots from each branded product were immediately subjected to bacterial enumeration and pH measurement. The rest of the aliquots were stored at 4 °C, 25 °C and −20 °C for 1, 5, 9 and 13 days, respectively. Following storage, the samples were subjected to bacterial enumeration and measurement of pH and lactic acid content.

Viable bacterial enumeration of total lactobacilli in opened probiotic dairy drinks

The culture medium used for the bacterial enumeration was de Man, Rogosa and Sharpe (MRS) agar (Oxoid) with its composition as peptone 10.0 g/L; Lab Lemco powder 8.0 g/L; yeast extract 4.0 g/L; glucose 20 g/L; Sorbitan monooleate 1 mL/L; K₂HPO₄ 2.0 g/L; triammonium citrate 2.0 g/L; sodium acetate 5.0 g/L; MgSO₄·7 H₂O 0.2 g/L; MnSO₄·4H₂O 0.04 g/L; and agar 10.0 g/L. The MRS agar and peptone water (0.1%) were sterilised at 121 °C for 15 min via autoclave. Peptone water (0.1%) was used as a diluent for the tenfold serial dilution for the tested samples. Briefly, 100 µL of the stored probiotic dairy drink aliquot was diluted in 900 µL of sterile peptone water (0.1%), and all of the dilutions were plated on MRS agar using the drop plate method. The total bacterial counts, colony-forming unit per millilitre (CFU/mL), were carried out at least in triplicates.

Determination of pH value and D-lactic acid concentration of the opened probiotic dairy drinks after being stored under different conditions

The pH of each probiotic drink aliquot was measured through the calibrated probe that was connected with a pH meter (pH 2700, Eutech Instruments, Singapore) at designated storage time points and temperatures. While D-lactic acid in the filtrate of a probiotic drink aliquot

was quantified by a spectrophotometer (SpectraMax M3, Molecular Devices, USA) based on the instructions from the commercial enzymatic bioanalysis kit “Enzytec D-/L-Lactic acid” (R-Biopharm, Germany). Two absorbance readings (A₁ and A₂, respectively) were measured at 340 nm before and after the addition of the D-lactate dehydrogenase solution. The difference in absorbance was applied for the D-lactic acid concentration (g/L) calculation as suggested by the manufacturer of the enzymatic bioanalysis kit.

Statistical analysis

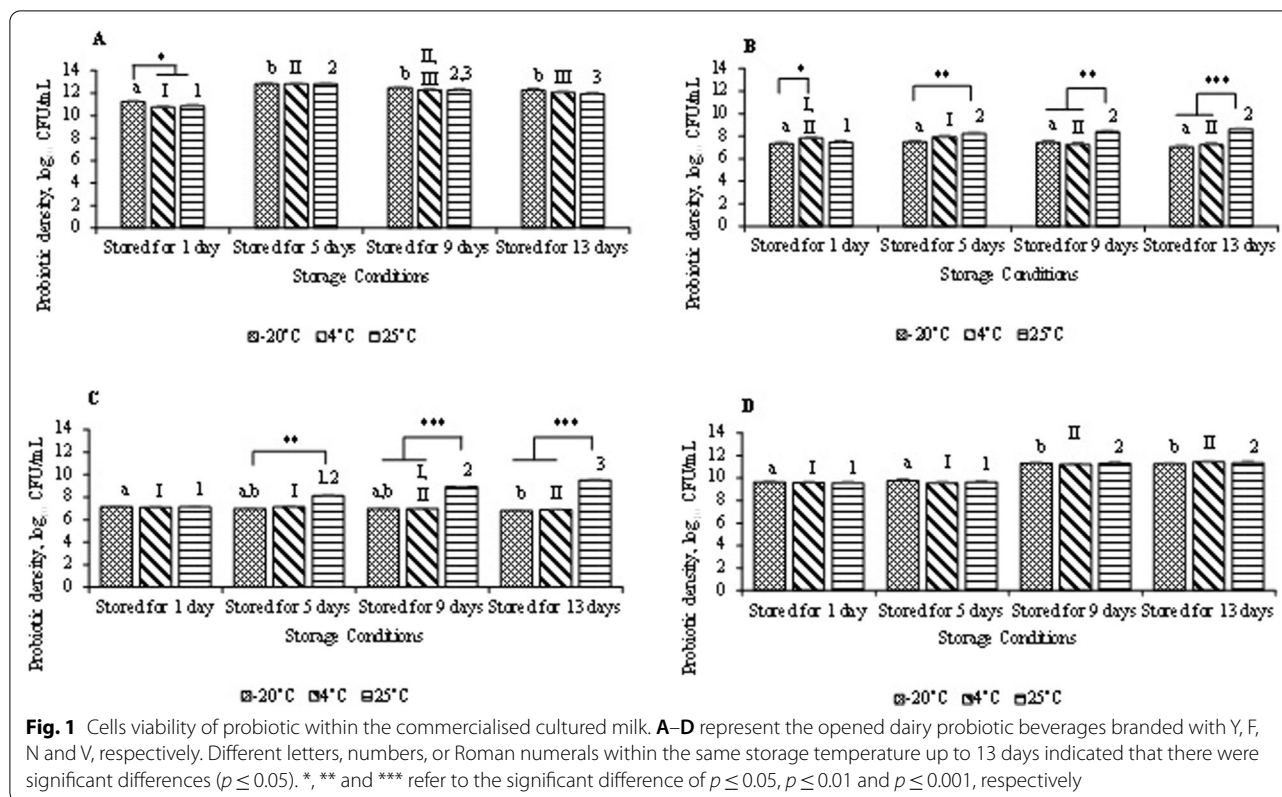
The results obtained from each triplicate were presented as mean values with standard error. The comparison between the data was made by using Student's *t* test, two-way ANOVA, and nonparametric Mann–Whitney test or Kruskal–Wallis test followed by the Dunn–Bonferroni post hoc method. The correlation coefficients between total viable lactobacilli and pH of opened probiotic dairy drinks in the given storage conditions were then evaluated by bivariate Spearman's correlation. Statistical significance by a two-tailed analysis was considered if $p \leq 0.05$ or $p <$ (adjusted p value for significance according to the Dunn–Bonferroni correction). All the statistical analysis was performed using PASW Statistic 18 (IBM SPSS Inc., New York).

Results

Total probiotic viability of different commercial fermented dairy drinks stored under different temperature–time conditions after opening

The initial amount of total viable bacteria in the commercial probiotic dairy drinks branded Y, F, N and V was 11 log CFU/mL, 7.08 log CFU/mL, 7.36 log CFU/mL and 10.05 log CFU/mL, respectively. Remarkably, the low density of probiotics was detected in products F and N.

Following the storage of the aliquot of commercial probiotic dairy drinks, the aliquot of Y- and V-branded products did not show a high variation in their total probiotic density within different storage temperatures at the same period of storage (Fig. 1A and Fig. 1D). In contrast, most of the time, probiotics in the branded F and N dairy drinks showed higher viable counts for the products kept at room temperature compared with those aliquots stored either at refrigeration temperature or under frozen condition (Fig. 1B and Fig. 1C). Upon storage for 13 days at 4 °C, the viability of probiotics in products F and N was significantly reduced, while there was notable growth of probiotics in the products Y and V. It was found that probiotic density of product N was decreased to approximately 6.8 log CFU/mL after the 13th day of −20 °C storage. In contrast, the probiotics in products Y and V were actively grown at −20 °C, where the cultured milk was



completely transformed from a liquid to a solid (Fig. 1A and Fig. 1D).

The pH of opened commercial fermented dairy drinks stored at various temperature–time conditions

All opened probiotic drinks were evaluated for their pH value under different storage conditions. The pH values were correlated with the viable probiotics that were detected during each storage condition for the respective dairy probiotic beverage brands. Data showed that all of the cultured milk products were at a pH less than 4.2 and the variation in the viable counts of total probiotics was significantly negatively correlated with the pH values of the opened commercial cultured milk drinks (Fig. 2). The statistical analysis indicated a weak correlation for the V product ($R = -0.181$; $p = 0.007$) and a moderate negative correlation for the rest ($R = -0.401$ to -0.563 ; $p < 0.0001$).

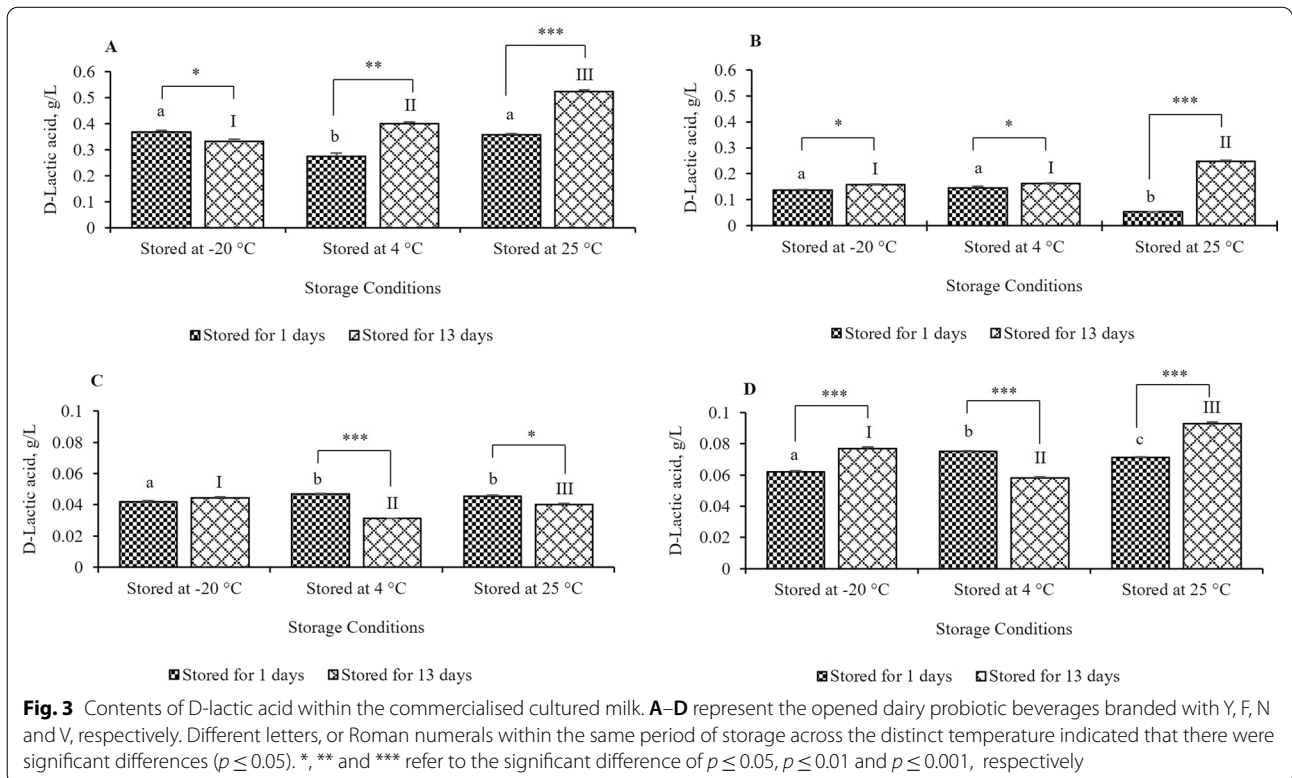
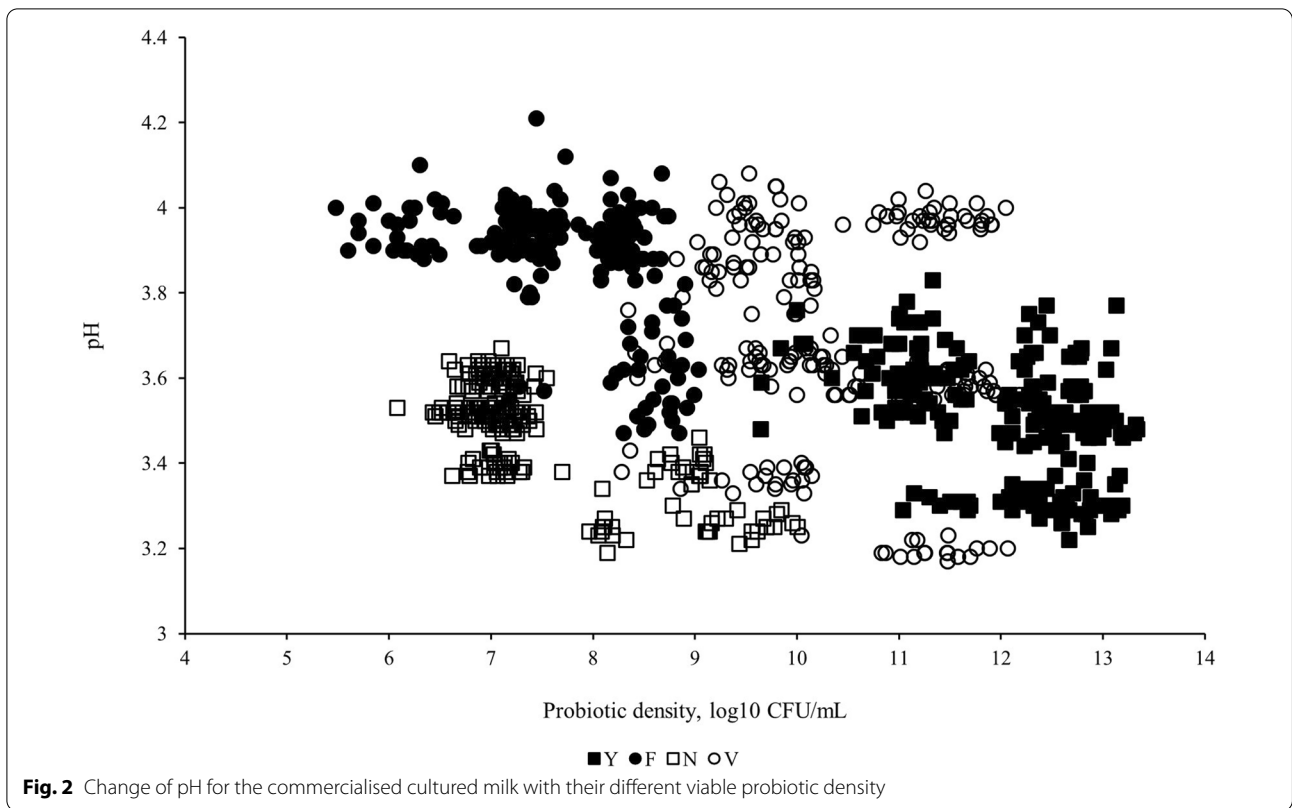
D-lactic acid content of open commercial fermented dairy drinks when stored at different temperature–time conditions

The highest amount of D-lactic acid content (0.52 g/L equivalent to 5.77 mM) was detected in the Y-branded product, especially at the end of the storage period (13 days) at room temperature. On the other hand, the

increase in D-lactic acid was also observed for products F and V after these cultured milk products had been opened and stored at 25 °C for almost 2 weeks (Fig. 3). Besides, a significant increase in D-lactic acid was found for particular brands which were stored at cold temperatures when compared with their one-day storage at low temperatures as well (Fig. 3). However, the increase in D-lactic acid was not observed for N-branded products at any storage conditions and remained at less than 0.05 g/L. In this study, the content of D-lactic acid for the opened Y product was significantly elevated to as high as 4.44 mM and 5.77 mM after 13 days stored in the refrigerator and at room temperature, respectively.

Discussion

As found elsewhere in the market, probiotic dairy beverages contain not only one species or single strain of live culture, but can be formulated with multiple mixed cultures (Shah et al. 2000; Stanton et al. 2001; Tamime et al. 2007; Granato et al. 2010; Wills 2012). The viability of probiotics during storage is strain or species-dependent, and it is also influenced by the manufacturing process or composition of probiotic products. Most of the studies showed that the survival of probiotics could be steadily maintained in the commercial products over the storage duration, although some probiotics might slightly lose



their viability (Shah et al. 2000; Al-Otaibi 2009; Liu 2011; Dharmasena 2012; Haddad 2017; Sumalapao et al. 2017). However, the viability of the opened commercial cultured probiotic milk and their physicochemical properties, especially pH and D-lactic acid, are yet to be examined after being opened and stored for a few days.

The initial quantity of total viable bacteria clearly shows that the probiotic density results varied among the tested commercial probiotic dairy drinks. The low probiotic density in products F and N may be due to the presence of lactic starter culture (*S. thermophiles*). The high fermentation temperature requested by starter cultures was proven to lead to a loss of viability of the probiotic (Fiorentini et al. 2011). However, the tested commercial products were still considered to be able to exert a benefit on the consumer because the probiotics delivered in these four branded products were at the level of at least 10^9 CFU per serving. The impact of probiotics on humans in correlation with their doses has been comprehensively reviewed by others (Kailasapathy and Chin 2000; Yildiz 2010; Bertazzoni et al. 2013; Hill et al. 2014).

We observed in our study that the number of probiotics in aliquots of commercial dairy drinks increased when stored at room temperature. *Lactobacillus* grew continuously at 25 °C, which is not surprising given that the majority of probiotic bacteria are mesophilic and thermophilic bacteria (Chacko et al. 2010; Alabdulkarim et al. 2013; Ranadheera et al. 2017). According to Alabdulkarim et al. (2013), the total viable counts of lactic acid bacteria in fermented goat milk were markedly increased compared to samples stored at a cool temperature. Similar results were reported by Chacko et al. (2010), who investigated the effect of storage conditions such as temperature on the viability of microbes in the different fermented dairy products. A major discovery of the present study is the finding that higher amounts of the probiotic from products Y and V could be detected at −20 °C after 13 days of storage compared to storage for one day at the same temperature. This indicates that certain probiotic strains can actively grow at −20 °C even as the cultured milk changed from a liquid to a solid form. Different factors could contribute to these discrepancies, such as fermented milk formulation, inoculation level of probiotics, and probiotic species. The latter factor would be more considered to explain our findings. The most apparent difference between the branded products regarding probiotic species is that F- and N-branded products do not comprise *L. casei* but contain *L. acidophilus*. In the previous study, a significant decline in probiotic counts of *L. acidophilus* was reported with the increased storage time of cultured buttermilk at 5 °C (Nighswonger et al. 1996). Other groups (Akin et al. 2007; Arslan et al. 2016; Ayar et al. 2018) examined the survivability of *L.*

acidophilus in ice cream and found that *L. acidophilus* was commonly reduced after a certain storage period at −18 °C or −20 °C. In the storage of Y product which contains only *L. casei*, we observed a substantial increase in viable counts of approximately 2 log CFU/ml for the cultured milk aliquots after 5 days of storage at either 4 °C or −20 °C. The result of this study is in agreement with Nematollahi et al. (2016), who demonstrated that the *L. casei* strain could continually grow at 4 °C during cold storage. A similar finding was also found for *L. casei* BL23 and revealed that specific genes were upregulated in order to adapt to cold temperatures (Lee et al. 2015). However, there is no study to show the ability of *L. casei* strains to multiply within the frozen milk products. The ability of probiotics to actively grow at cold temperatures gives new insight into the potential to utilise some of these probiotic strains to produce heat-labile pharmaceutical products under the lowest temperature, such as probiotic-based vaccines.

It is very important to know the viability of probiotics for the opened commercial cultured milk products when stored for the following days of consumption. The physicochemical properties of unfinished probiotic cultured milk products were hypothesised to be influenced by a continuously increasing trend of viable probiotics or a significant decline in probiotic counts. In this study, the pH of all cultured milk products was mildly acidic, and the viable counts of total probiotics from these opened commercial cultured milk drinks showed a negative correlation with pH. The increasing amount of viable probiotics might reflect the increase in enzymatic activity to some extent and results in the accumulation of acidic end products, which leads to a pH decrease in the cultured milk drinks. Generally, during fermentation, yeast or other microbes cause acidification by their metabolic process. As a result, the broth is turned acidic which further accelerates the microbial growth and fermentation rate, as reflected by the results (Majumder et al. 2021). Increasing acidity is a factor which indirectly assures the progress in the fermentation process and growth of microbes. Furthermore, other studies also found that some probiotic strains can tolerate the acidic condition and could continue growing at a pH lower than 4.0 (Sahadeva et al. 2011; Olatunde et al. 2018; Wu et al. 2021). In the present study, the high content of D-lactic acid was detected in the Y-branded product when stored at room temperature for up to 13 days. It noted that the Y product only contained *L. casei*, and this study disagreed with the other study, which mentioned that *L. casei* was only producing L-lactic acid (Uchida et al. 2004). Interestingly, the increase in D-lactic acid in the other tested products at different storage temperatures was also observed in the present study, except for the N-branded

product. *Lactobacillus* spp. is the microorganism among the probiotic mixture that contributes to the fluctuation of D-lactic acid levels in those opened commercial cultured milk products. According to Mayeur et al. (2013), different lactobacilli species can produce varying amounts of D-lactic acid. The distinct types of cultured milk composition among the commercial products were also speculated to affect D-lactic acid production. A study by Garvie (1967) showed that different medium compositions have resulted in various proportions of lactic acid isomers produced by *L. acidophilus*. The interaction of the mixed probiotics in F, N and V products to influence the D-lactic acid production throughout the storage period remains unknown. It is well known that humans do not actively metabolise D-lactic acid. Its accumulation in the gut followed by absorption in the blood may display unwanted symptoms, especially for those facing intestinal failure. In this study, the content of D-lactic acid for the opened Y product was the highest compared to the others, as aforementioned. However, there has not been much research into the effect of D-lactic acid-containing foods on posing a threat to human health. To our knowledge, this level of D-lactic acid for the Y brand is still low and may not cause a significant increase in D-lactic in the blood of a healthy person. As de Vrese and Barth (1991) reported, consumption of yoghurt containing D-lactic acid (1.06 mmol/kg body weight) was safe for healthy adults. One should bear in mind that in patients with short bowel syndrome, it has been suggested to avoid consuming lactobacillus species that can produce D-lactic acid in the colon.

Conclusions

In summary, this study revealed that the growth of probiotics in the sterile, opened cultured milk drinks varied among the brands. The continual rise of viable probiotic counts was detected throughout storage at room temperature. We also demonstrated that probiotics still continuously grew within the frozen aliquot of cultured milk products. Such growth is a unique characteristic of probiotics in the cultured milk drinks of brands Y and V. The increase in viable probiotics during the storage moderately affected the pH of the opened cultured milk products. High content of D-lactate was also found for certain cultured milk brands after 2 weeks of storage. This suggests that energy acquired from lactate oxidation by probiotics to support their growth might have occurred due to the presence of oxygen after commercial products have been opened. This study implies the importance of evaluating the survivability of probiotic bacteria and physicochemical properties not only during production and packaging but also after they have been opened and stored under certain conditions. Consequently, the

information might guide redesigning the probiotic cultured milk beverage when it needs to be bottled in large volumes for consumers where it cannot be finished. The combination effect of probiotic species in assisting in maintaining the stability of viable cells after opening is equally fascinating in exploring the nutrition changes or colour changes and total soluble solid for the commercial cultured milk product stored over the period. On the other hand, the elucidation of the underlying mechanisms for the continual growth of probiotic cells within the frozen milk medium will improve the current large bottled product via genome engineering to optimise the probiotics or milk composition.

Abbreviations

ANOVA: Analysis of variance; CFU: Colony-forming unit; D-lactic acid: Dextrorotatory lactic acid; L-lactic acid: Levorotatory lactic acid; MRS agar: de Man, Rogosa and Sharpe agar; PASW: Predictive analytics software; pH: Potential of hydrogen; SPSS: Statistical Package for the Social Sciences.

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Author contributions

LYK designed the study and revised the manuscript. BS and MST performed the study. AK, CLL, BS and MST drafted the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

No competing interest exists in the research outcome presented in this article.

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