Influence of miracle fruit (*Synsepalum dulcificum*) extract and microencapsulated *Lactococcus lactis* Gh1 on the antioxidant activity and probiotic viability of functional yogurt

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Abstract

Nowadays, functional food market is dominated by dairy-based probiotic products, mainly yogurt. The nutritional values of yogurt can be further enhanced by the inclusion of miracle fruit (*Synsepalum dulcificum*) and potential probiotic *Lactococcus lactis* Gh1. The present work investigated the anti-oxidative capacity and survivability of probiotic strains of six yogurts fortified with *S. dulcificum* pulp extract and encapsulated *L. lactis* Gh1 (in alginate-starch coating agent via extrusion technique). The flavonoid contents (TFC) were not significantly different between yogurts, whereas the phenolic contents (TPC) showed an increasing trend throughout the storage. Among the yogurts, the one supplemented with both *S. dulcificum* and encapsulated *L. lactis* Gh1 showed the highest TFC (1.18 µg QE/mL) and TPC (15.382 µg GAE/mL). The antioxidant assay (DPPH) showed a gradual increase on the first 7 d, but decreased afterward. In comparison, yogurts fortified with *S. dulcificum* demonstrated higher antioxidant activity (± 80% DPPH inhibition) than the plain yogurts (± 50% DPPH inhibition). The viability of starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) drastically increased during the first week (log 8 ~ 10 CFU/mL) especially for yogurts containing free cell *L. lactis*, but subsequently decreased (log 6 ~ 8 CFU/mL). The viability of *L. lactis* Gh1 in yogurts maintained at high count (log 9.43 and 9.04 CFU/mL) throughout 21 d when it was being encapsulated. In general, the fortification of *S. dulcificum* extract with microencapsulated *L. lactis* Gh1 had greatly enhanced the quality and potential benefits of the functional yogurts.

Introduction

Today, probiotic products have begun to gain worldwide interest for promoting health care apart from their good taste (Granato *et al.,* 2020). They are ready-to-eat foods rich in nutritional components with the inclusion of various ingredients such as fruits, plants, collagens, probiotics, and prebiotics (Fazilah *et al.,* 2019; Shafi *et al.,* 2019). Yogurt eating has been associated with several health benefits such as improved lactose metabolism, reduced oxidative stress, ease of diarrhoea, immune system stimulation, aid in digestion process, and protection against cancer (Hassanzadeh-Taheri *et al.,* 2018; Fazilah *et al.,* 2018).

Yogurt is fermented milk using lactic acid bacteria (LAB) as a starter culture. Mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* is commonly used as starter culture for yogurt, but both cannot survive in a sufficient amount in the

digestive tract (Wihansah *et al.,* 2018). To confer health benefit, probiotic bacteria must reach the intestines and remain viable in sufficient numbers at log 6 - 7 CFU/g of products (Fenster *et al.,* 2019). Even though *S. thermophilus* and *L. delbrueckii* are able to grow individually in milk, they can have a symbiotic interaction called "protocooperation" in mixed cultures (Sieuwerts, 2016). *L. delbrueckii* in yogurt produces acids and hydrogen peroxide that may affect other bacteria. Therefore, in formulating probiotic yogurts, the addition of another probiotic strain capable of surviving passage through digestive tract is essential to ensure that the minimum number of viable probiotics is sustained. In addition, cell microencapsulation technique is another alternative for protecting cells to further enhance the survivability of probiotic strains (Fazilah *et al.,* 2019). The easiest microencapsulation technique is via extrusion (Halim *et al.,* 2017). The technique is easy to handle as it is in a form of matrix

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beads or microcapsules, and can be done with any ingredient whether it is hydrophilic, hydrophobic, liquid, or viscous oil. Besides, it does not involve extreme temperatures during bead formation, which makes it best suited for bacteria. Among the commonly used coating agents include k-carrageenan, alginate, cellulose acetate, chitosan, polysaccharide mixtures, and starch, whereas the commonly used solidifying agent is calcium chloride.

Lactococcus lactis species is part of LAB that has the GRAS (Generally Recognised as Safe) status. *L. lactis* has been identified for its beneficial properties for potential applications as a probiotic and starter culture in the food industry (McAuliffe, 2018). Further-more, *L. lactis* also has a high potential to be used as a biopreservative agent to avoid food spoilage (Hwan-hlem *et al.,* 2017). Benkerroum *et al.* (2000) demon-strated that *L. lactis* subs. *lactis* produced a bacteriocin with an excellent inhibitory effect against *Listeria monocytogenes* in jben (Moroccan fresh cheese). Hence, it is interesting to further explore this strain especially on its possible applications in food products.

Synsepalum dulcificum, also known as mira-cle fruit or miracle berry, is an indigenous tropical plant originated from West Africa (He *et al.,* 2016). Miracle fruit is known for its unique ability in convert-ing sour tasting foods into sweet taste. Miraculin, the glycoprotein compound found in the pulp of miracle fruit is responsible for the change of sour to sweet taste (He *et al.,* 2015; 2016). Miraculin binds to the sweet receptor cells of the tongue which activates in acidic condition and suppresses the response of a sour taste in the central nervous system until it is diluted and elim-inated by saliva. The taste modification function gives a great potential for miracle fruit to be exploited in the food industry especially as an alternative sweetener. Moreover, miracle fruit's pulp, seed and stem are also rich in nutrients with antioxidant and antidiabetic prop-erties (Fazilah *et al.,* 2020). However, to date, very little is known about the health benefits of *S. dulcificum* plant extract and its antioxidant activity when incorpo-rated into food products.

The present work thus endeavoured to develop functional yogurts fortified with *S. dulcificum* pulp extract and potential probiotic strain, *L. lactis* Gh1. In this regard, the inclusion of the plant extract and micro-encapsulated probiotic is expected to enhance the nutritional values of the yogurt as such functional food that contains sufficient viable probiotic strains and antioxidant components.

Materials and methods

Plant materials

The pulp, seed, and leaf of *S. dulcificum* plant were obtained from a local farm located in Selangor, Malaysia.

Milk and yogurt starter culture

Pasteurised cow milk (Farm Fresh, Malaysia) was purchased from a local market. Freeze-dried yogurt starter culture (YC-380) containing a mixture (ratio of 1:1) of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was purchased from Chr. Hansen (Hoersholm, Denmark).

Lactococcus lactis Gh1

L. lactis Gh1 was obtained from the culture collection of Bioprocessing and Biomanufacturing Research Centre, Universiti Putra Malaysia (UPM). This LAB strain was isolated from ghara, an Iranian traditional flavour enhancer (Abbasiliasi *et al.,* 2012; Jawan *et al.,* 2018), and was used in two forms; as free cell suspension and microencapsulated cells.

Preparation and extraction of Synsepalum dulcificum

The collected plant samples (age 3 - 5 y) were thoroughly washed with tap water to remove unwanted materials. The seeds were separated from the pulp using a knife, and both were freeze-dried for 48 h by a pilot-scale freeze dryer (Epsilon 1-8D, Martin Christ, Osterode am Harz, Germany). The leaf samples were dried in an oven at 50°C for 24 h. Samples were then grinded using a household blender into a fine powder, and kept in labelled zip lock plastics in a chiller (4°C) until further use.

Water extraction of the plant samples was done following a method described by Amirdivani (2015) with some modifications. The fine powders of pulp, seed, and leaf were added in distilled water in a ratio of 1:10 (w/v). Then, the solutions were incubated in a water bath (50°C) for 16 h, followed by filtration using Whatman's filter paper No. 5. The filtrates were harvested and centrifuged (5810R, Eppendorf AG, Hamburg, Germany) at 6,000 *g*, 4°C for 10 min. The pellets were discarded while the supernatants were stored in 4°C, and used within 3 d.

Antioxidant properties in S. dulcificum plant parts
Total phenolic content (TPC) assay

The TPC assay was performed following the method described by Nordin *et al.* (2019) with some changes. The reaction mixture contained 50 μL of plant extract (or distilled water for control), 250 μL of the Folin-Ciocalteu reagent (R&M Chemicals, UK), and added with 7.5% (w/v) sodium carbonate (Na_2CO_3) (Loba Chemie, India) to make up a total volume of 500 μL. The mixture was mixed with vortexing and

incubated in darkness at room temperature for 1 h. The absorbance was measured at 765 nm (Biochrom Libra S21 visible spectrophotometer, Biochrom Ltd., Cambridge, UK) against absolute methanol (Bumi-Pharma, Malaysia) as blank, and gallic acid standard (Merck, Darmstadt, Germany) was used to calculate phenolic contents in the samples. The results were expressed as mg gallic acid equivalent.

Total flavonoid content (TFC) assay

The TFC assay was performed following the method described by Nordin *et al.* (2019) with some changes. An aliquot of 500 µL extract in an Eppendorf tube was mixed with 500 µL of 2% aluminium chloride (Al_2CO_3) solution (UNI-Chem, UK). The mixture was mixed by vortexing, and allowed to stand at room temperature for 10 min. The absorbance was measured at 435 nm against absolute methanol as blank, and quercetin standard (Merck, Darmstadt, Germany) was used to calculate flavonoid contents in the samples. The results were expressed as mg quercetin equivalent.

DPPH assay

The DPPH (2,2-diphenyl-1-picryl-hydra-zyl-hydrate) assay was performed following the method described by Nordin *et al.* (2019) with some changes. Aliquot of 500 µL extract in 2 mL Eppendorf tube was added with a solution containing 500 µL of 0.2 mM DPPH solution (Merck, Darmstadt, Germany) dissolved in absolute methanol. The mixture was thoroughly mixed by vortexing, and incubated at room temperature in darkness for 60 min. The absorbance was measured at 517 nm and DPPH radical-scavenging activity was calculated using Eq. 1:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{Acontrol - Asample}{Acontrol} \times 100 \qquad \text{(Eq. 1)}$$

where, $A_{control}$ = absorbance of the control reaction (replacing extract with methanol), and A_{sample} = absorbance of sample.

Preparation of L. lactis Gh1
Cell suspension

A single colony of *L. lactis* Gh1 was inoculated in 50 mL of MRS broth (Merck, Darmstadt, Germany) followed by incubation at 30°C for 18 h. 5% (v/v) of the culture was transferred into 95 mL of MRS broth and incubated at 30°C for 16 h. *L. lactis* was harvested upon reaching maximum cell growth (approximately 1.6×10^{10} CFU/mL) by centrifuging at 10,000 rpm, 4°C for 15 min. The cell pellets obtained were then washed with 0.1% (w/v) sterilised buffered peptone

(Fisher Scientific, Pittsburgh, US) water.

Microencapsulation via extrusion technique

Microencapsulation technique was done following a method for the production of microparticles described by Halim *et al.* (2017) with some modifications. Solutions of 2% (w/v) sodium alginate (Fisher Scientific UK, Loughborough, UK) and 2% (w/v) corn starch (Sigma-Aldrich, USA) were separately prepared and autoclaved at 121°C for 15 min together with 0.1 M calcium chloride ($CaCl_2$) (Sigma-Aldrich, USA). Both solutions were mixed and stirred when warm before *L. lactis* Gh 1 suspension was added in a ratio of 1:4 (v/v). The solution was then pumped into 0.1 M $CaCl_2$ through silicone tube (internal diameter 1 mm) using microtube pump MP-3N (Eyela, Tokyo, Japan). The beads formed were further stirred in 0.1 M $CaCl_2$ for 30 min to ensure a complete gelation. Then, the beads were removed from the solution using a sterilised sieve (50 µm) and washed with sterile distilled water.

To determine encapsulation efficiency (EE), the beads were initially disintegrated by vortexing in a 0.1 mol/L phosphate buffer (Merck, Darmstadt, Germany), pH 7.0 to obtain uniformly cloudy solution (Fazilah *et al.,* 2019). The entrapped viable cells were then counted by track plate technique in MRS agar. The EE was calculated using Eq. 2.

$$\text{Encapsulation efficiency} = [\text{Log}_{10} X / \text{Log}_{10} X_0] \times 100 \qquad \text{(Eq. 2)}$$

where, X = number of entrapped viable cells in beads, and X_0 = number of free viable cells before microencapsulation.

Yogurt fermentation

The freeze-dried yogurt starter culture (YC-380) was inoculated in fresh pasteurised cow milk in a ratio of 1:100 (w/v) (Amirdivani, 2015). The mixture was then incubated at 42°C overnight without shaking. The yogurt formed was kept in a chiller (± 4°C) and used as a starter culture within 1 w. Following the method described by Shori and Baba, (2012) six types of yogurt were prepared: (1) C. Plain: control plain yogurt; (2) C. Pulp: control yogurt supplemented with *S. dulcificum* pulp extract; (3) F. Plain: plain yogurt supplemented with free cells of *L. lactis* Gh1; (4) F. Pulp: yogurt supplemented with free cells of *L. lactis* Gh1 and *S. dulcificum* pulp extract; (5) B. Plain: plain yogurt supplemented with microencapsulated of *L. lactis* Gh1 beads; and (6) B. Pulp: yogurt supplemented with microencapsulated of *L. lactis* Gh1 beads

and *S. dulcificum* pulp extract. All yogurts were added with 10 g of yogurt starter culture, and 10 mL of *S. dulcificum* water extract were added into 80 mL of fresh milk. B. Plain and B. Pulp were also added with 3% (w/v) alginate-starch beads, whereas F. Plain and F. Pulp were supplemented with 3% (v/v) free cells of *L. lactis* Gh1. The milks were incubated at 42°C while pH was quantified every 30 min. The same procedure was carried out for control yogurts by adding either distilled water (C. Plain) or *S. dulcificum* extract (C. Pulp). Figure 1 illustrates the example of B. Pulp yogurt formulated from *S. dulcificum* pulp extract and micro-encapsulated of *L. lactis* Gh1 beads. The incubation was stopped once it reached pH 4.5 and the yogurts were kept in a chiller (4°C) for 21 d.

Preparation of yogurt extracts

Aliquots of 10 g yogurt sample was homogenised (by vortexing) with 2.5 mL of sterile distilled water (Amirdivani, 2015). The pH of the yogurt was acidified to 4.0 with 0.1 M hydrochloric acid (HCl) (Merck, Darmstadt, Germany). The yogurts were then heated in a water bath (45°C) for 10 min prior to centrifugation (MiniSpin, Eppendorf AG, Hamburg, Germany) at 7,500 *g*, 4°C for 10 min. The supernatants were harvested, and pH was adjusted to 7 with the addition of 0.1 M sodium hydroxide (NaOH) (Sigma-Aldrich, USA). The yogurt water extracts were re-centrifuged under the same condition, and the supernatants were harvested and kept in -20°C until further analysis.

pH profiling of yogurt samples

Yogurt samples were initially homogenised in distilled water in 1:1 and 1:9 ratio for pH (Fazilah *et al.,* 2019). pH was quantified using a pH meter (HI-2211, Hannah Instruments Ltd., Bedfordshire, UK).

Microbial viable cell counts
Yogurt sample preparation

Yogurt samples (0.1 g) were mixed (by vortex) with 0.9 mL of buffered peptone water. A serial dilution was also prepared using buffered peptone water.

Enumeration of yogurt starter culture and L. lactis Gh1

S. thermophilus was enumerated using M17 agar following the method described by Shori and Baba (2012). M17 agar (Merck, Darmstadt, Germany) was prepared and autoclaved at 121°C for 15 min. The hot agar was allowed to cool (45°C) before the addition of 10% (w/v) sterilised lactose solution. The agar mixture was poured in a Petri dish, and allowed to solidify. The plate was divided into four parts, and 10 μL of diluted yogurt sample was transferred onto each part. The plate was held vertically to allow the sample to flow through the agar surface. The plate was then incubated at 37°C for 48 h. Viable cell count was calculated as colony forming unit (CFU) using Eq. 3:

$$\frac{CFU}{mL} = \frac{Number\ of\ colonies\ formed \times dilution\ factor\ of\ sample}{1\ mL\ of\ sample}$$

(Eq. 3)

L. delbrueckii subsp. *bulgaricus* was enumerated using MRS agar. After autoclaving at 121°C for
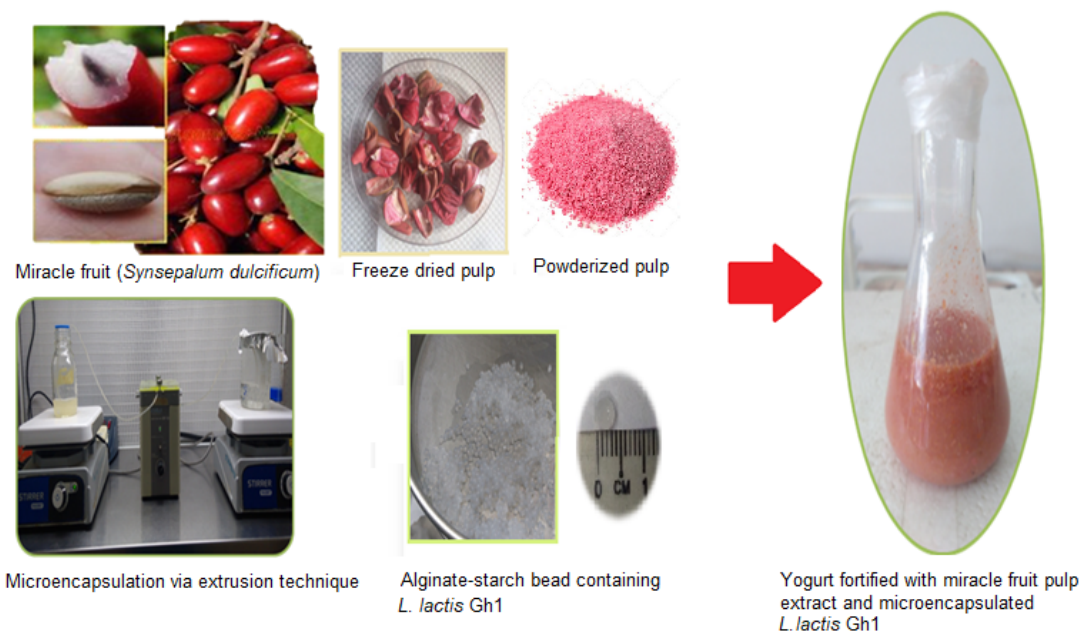

Figure 1. The prepared yogurt fortified with miracle fruit (*S. dulcificum*) pulp extract and micro-encapsulated (via extrusion technique) *L. lactis* Gh1.

15 min, the melted MRS agar was allowed to cool down (45°C) before being poured in a Petri dish. 1 mL of diluted yogurt sample was aliquoted into the molten agar. The plate was then sealed with Parafilm, and incubated at 37°C for 48 h. Viable cell count was calculated as colony forming unit (CFU) (Eq. 3).

L. lactis Gh1 was enumerated on MRS agar using track plate technique as described earlier. The prepared plate was incubated at 30°C for 48 h. Viable cell count was calculated as colony forming unit (CFU) (Eq. 3).

Scanning Electron Microscopy (SEM)

Prior to viewing under SEM, all samples were frozen at -20°C for 2 d and freeze dried (Epsilon 1-8D, Martin Christ, Osterode am Harz, Germany) to further reduce their moisture content. The samples were crushed and mounted on a double stick carbon tape placed on an aluminium stub (Halim *et al.,* 2017). Then, the samples were introduced into the chamber of the sputter coater, and coated with a very thin film of 40 - 60 nm metal gold/palladium. The samples were viewed under SEM at 1,000 and 3,000 magnifications.

Statistical analysis

All experiments were performed on at least three different occasions (*n* = 3), and data were expressed as mean ± standard deviation (SD). Analyses were performed on Graph Pad prism 6 and Microsoft Excel (2013). Statistical analysis was done using the analysis of variance (ANOVA) on Graph Pad prism 6, and $p < 0.05$ was considered significant.

Results and discussion

Antioxidant properties of S. dulcificum

The highest concentration of phenolic and antioxidant activity was found in the pulp of *S. dulcificum*, while the lowest was in the seed. The TPC of the pulp was 15.93 ± 0.002 µg GAE Equiv./g sample, approximately three times higher than that of the seed 5.62 ± 0.024 µg GAE Equiv./g sample). Leaf extract contained 8.98 ± 0.003 µg GAE Equiv./g sample. Meanwhile, for TFC, there was no significant difference between extracts (*p* > 0.05); however, pulp still contained the highest TFC 3.98 ± 0.002 µg QE Equiv./g sample as compared to seed 1.18 ± 0.002 µg QE Equiv./g, and leaf 2.28 ± 0.001 µg QE Equiv./g extracts. These observations are supported by Du *et al.* (2014) who reported higher TFC and TPC values for methanol extract of *S. dulcificum* pulp than in seeds.

The percentage of free radical scavenging of *S. dulcificum* pulp (85.69 ± 0.004%) was significantly higher (*p* < 0.05) than that of the seed (55.08 ± 0.023%) and leaf (76.91 ± 0.005%). The high reduction capability of the pulp could be attributed to the high level of phenolics, which are the stronger reductants in donating electrons (Lewoyehu and Amare, 2019). Based on the antioxidant-rich phytochemicals content, pulp extract was selected and used for subsequent experiments of yogurt preparation.

Characterisation of functional yogurt
pH profile

pH measurement is one of the critical quality controls during the manufacturing of dairy products, especially yogurt (Baros *et al.,* 2019). Generally, reduction in fermentation time with faster acidification period is preferable to efficiently produce yogurt at optimal cost. Most manufacturers have a set pH point between 4.0 and 4.6 to cease the yogurt fermentation. The amount of lactic acid present at this pH level is ideal for yogurt, thus giving it distinctive flavour and texture (Fazilah *et al.,* 2018).

The changes in pH were measured during fermentation at 42°C until it reached approximately 4.5 (Figure 2A). From the beginning of fermentation, yogurts containing *S. dulcificum* (C. Pulp, F. Pulp, and B. Pulp) displayed a lower pH (~ 5.0) than the plain yogurts (C. Plain, F. Plain, and B. Plain) with pH ~ 5.6. Thus, it took a shorter time of 180 to 210 min for the *S. dulcificum* fortified yogurts to reach pH 4.5, whereas the plain yogurts required around 330 to 360 min.
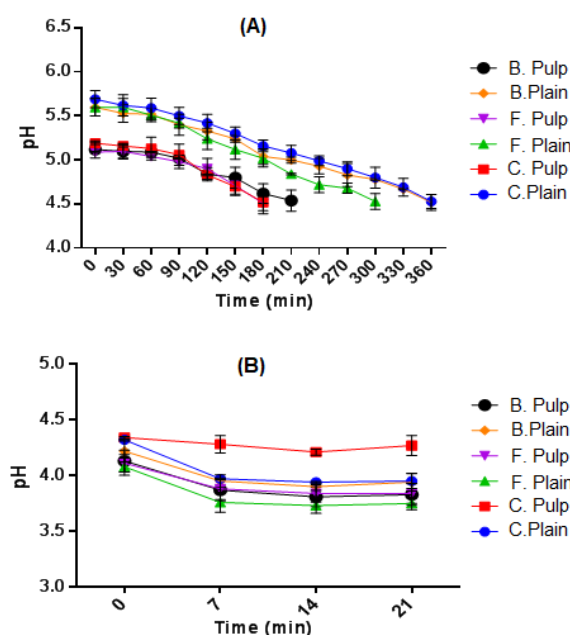

Figure 2. Changes in pH of six different yogurt formulations during (A) fermentation during lactic acid fermentation at 42°C, and (B) cold storage at 4°C. The error bars represent the standard deviation of the means (*n* = 3).

The presence of plant extract often affects the pH as it enriches the metabolic activity of yogurt bacteria (Manzoor *et al.*, 2019). The pH value signifies the free H⁺ concentration generated through the production of organic acids by LAB (Ming *et al.*, 2016). For comparison on culture variation, the free cell yogurts showed a faster pH reduction for F. Pulp and F. Plain than the microencapsulated cell yogurts (B. Plain and B. Pulp) and control yogurts (C. Plain and C. Pulp). The reduction of pH in yogurts containing *S. dulcificum* could also be due to the large amount of vitamin C in the flesh of this fruit (He *et al.*, 2016).

Further, the pH reduction of yogurts continued to decrease throughout the storage (Figure 2B), but not as fast as observed during the fermentation stage (Figure 2A). This indicates that LAB did not produce much acidity under storage conditions, as similarly reported by Tan *et al.* (2018). The decrease of pH values may be resulted from the accumulation of acetic acid, acetaldehyde, citric acid, formic acid, and lactic acid (Østlie *et al.*, 2005).

Antioxidant properties of yogurts

Figure 3A shows the total phenolic content of yogurts during 21 d of storage at 4°C. The TPC in yogurts supplemented with *S. dulcificum* was higher than that in plain yogurts. It was observed that the refrigerated storage had minimal effects on TPC of all yogurts. Similar result was also observed for the TPC value in yogurts fortified with *A. sativum*, while the plain yogurt was not affected during refrigerated storage (Shori and Baba, 2012). On day 0 (after fermentation had been stopped), the F. Pulp showed the highest TPC (11.865 µg GAE/mL), while the F. Plain showed the lowest (4.743 µg GAE/mL). All yogurts showed a drastic increase after 24 h, and continued to gradually increase until day 21. This observation is in contrast with the results reported by Karaaslan *et al.* (2011) on the decrement of TPC in yogurts fortified with grape and callus extracts. They presumed that the reduction of TPC was closely related to the decline of antioxidant activity. In the present work, among microencapsulated and free cells yogurts, the TPC values were approximately the same, however, the microencapsulated cells yogurts showed a greater phenolic content on the 21ˢᵗ day of storage with 11.048 µg GAE/mL for B. Plain, and 15.382 µg GAE/mL for B. Pulp.

As depicted in Figure 3B, the *S. dulcificum* pulp extract exhibited lower flavonoid content when fortified in yogurt formulations. The reduction was correspondent to more than 3.6-fold in comparison to the TFC of pulp extract before added into the yogurt.
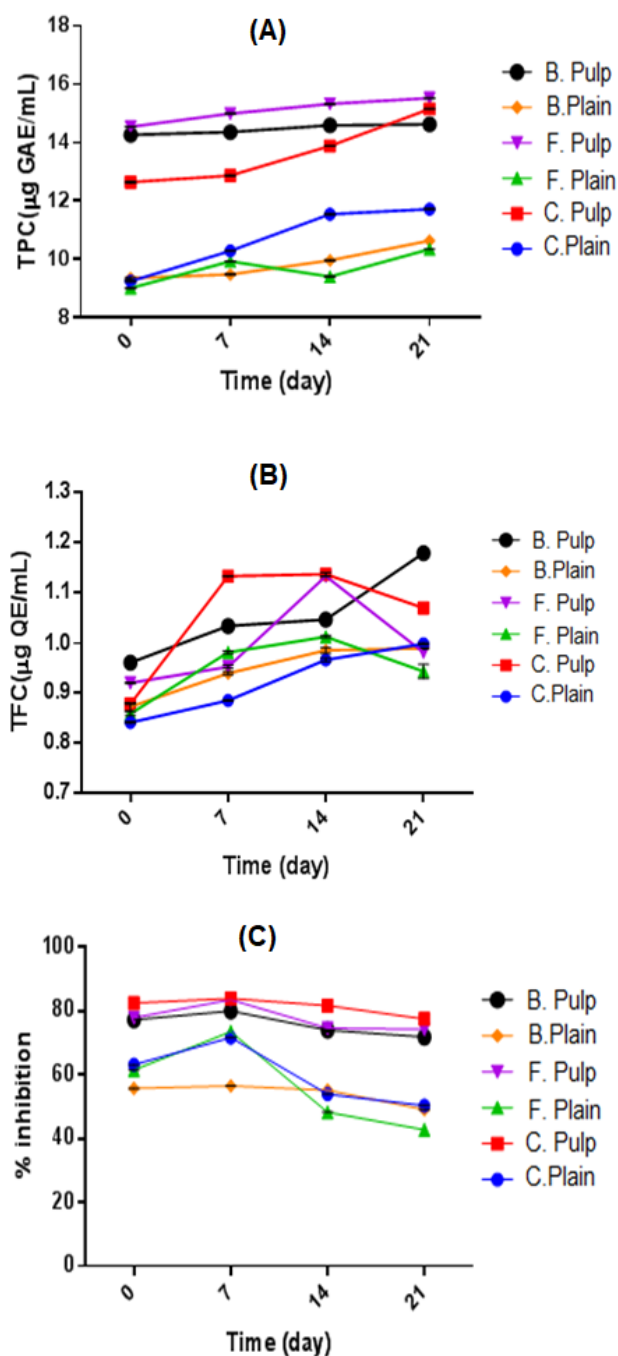

Figure 3. (A) Total phenolic content, (B) total flavonoid content, and (C) percentage of DPPH inhibition of six different yogurt formulations during cold storage at 4°C. The error bars represent the standard deviation of the means ($n = 3$).

The highest TFC (1.18 µg QE/mL) was obtained for the yogurt containing microencapsulated cells and *S. dulcificum* pulp (B. Pulp).

The percentages of DPPH inhibition for all yogurts peaked on day 7 with the highest value recorded by C. Pulp (83.97%), followed by F. Pulp (83.56%), and B. Pulp (80.05%) (Figure 3C). The lowest inhibition was recorded by B. Plain with 56.58% DPPH inhibition. The high DPPH inhibition

after 7 d of storage may be attributed to the metabolically active yogurt bacteria (Papadimitriou *et al.,* 2007). Moreover, the inclusion of plant extracts may have significantly ($p < 0.05$) increased the antioxidant activity as compared to milk alone (Muniandy *et al.,* 2016). Yogurts fortified with *S. dulcificum* (C. Pulp, F. Pulp, and B. Pulp) and plain yogurt (B. Plain) appeared to have a similar trend of steadily increasing during the first 7 d, but started to decrease afterwards. Meanwhile, C. Plain and F. Plain exhibited a sudden increase with a dramatic reduction after day 7 of storage. The decrease in antioxidant activity during storage may be related to the stability of the product. Lactic acid and various herbal extracts have been often reported to significantly affect the stability of the product over time, hence affecting the antioxidant activity during storage (Dabija *et al.,* 2018).

Viability of yogurt starter cultures and L. lactis Gh1

The viability of mixed starter cultures (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) and *L. lactis* Gh1 in six different yogurts were recorded throughout 21 d at 4°C. For all yogurts, the cell count of *S. thermophilus* significantly ($p < 0.05$) increased during the first seven days, but then subsequently decreased (Figure 4A). The highest *S. thermophilus* count was recorded in yogurts fortified with *S. dulcificum* pulp in the range of log 8 ~ 10 CFU/mL, while the lowest was in C. Plain with log 6.36 CFU/mL. The same trend was observed for *L. delbrueckii* (Figure 4B) except for yogurts supplemented with free *L. lactis* Gh1 cells (F. Plain and F. Pulp) that reached their highest on day 14th before started to decline. *S. dulcificum* pulp might have promoted the growth of LAB in yogurt, possibly by providing some sugars for growth. A recent study also showed that adding apple peel polyphenol extract into yogurt ice cream improved the growth of probiotics (Ahmad *et al.,* 2020). Hence, the decrease in viability of *S. thermophilus* and *L. delbrueckii* during prolonged storage may occur due to lack of nutrients. Nevertheless, the total LAB during the 21 d of cold storage still fulfil the standard for the presence of viable bacteria in yogurt products (Halim *et al.,* 2017).

Microencapsulation technique has been reported to enhance the survivability of probiotic bacteria in unfavourable conditions by protecting the live cells within a shell material (Fazilah *et al.,* 2019). Result signifies successful entrapment of *L. lactis* Gh1 viable cells (99.7%) in the beads prepared via extrusion technique. High cell viability (log 9.67 CFU/g) was achieved in the resistant maize
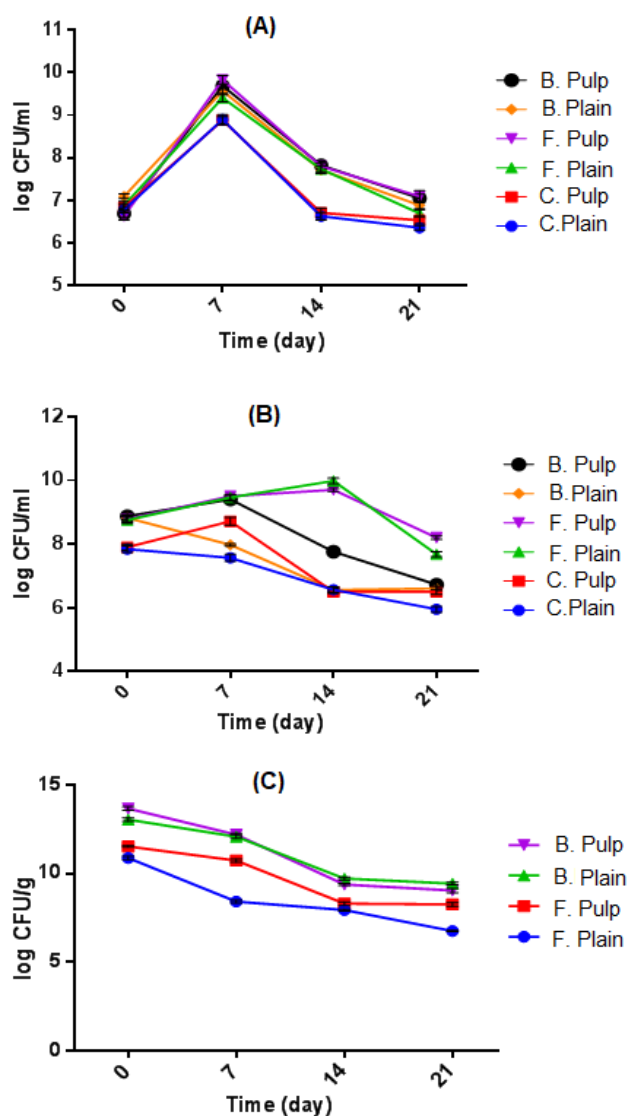
Figure 4. The colony forming unit (log CFU/mL) of yogurt starter culture strains; (A) *Streptococcus thermophilus* and (B) *Lactobacillus delbrueckii* subsp. *bulgaricus*, in six different yogurt formulations, and (C) *Lactococcus lactis* Gh1 in four different yogurt formulations. The error bars represent the standard deviation of the means ($n = 3$).

starch-alginate microcapsules. Starch is known to play a role in preventing the formation of cracked and porous bead surface (Gouin, 2004).

The viability of *L. lactis* Gh1 decreased throughout the storage for all yogurts (Figure 4C). Nevertheless, it was observed that the *L. lactis* Gh1 encapsulated cells had survived better than the free cells. During 21 d of storage, F. Plain showed the lowest *L. lactis* Gh1 viability (log 6.76 CFU/mL) while the highest was observed in B. Plain (log 9.43 CFU/mL). This highlights the advantage of cells microencapsulation in food and dairy industries that can be used as a tool to avoid any detrimental interaction between dairy product matrices with probiotic cells (Kailasapathy and Champagne, 2011). It was also noted that, F. Pulp had higher cell viability as

compared to F. Plain. This suggests that the presence of *S. dulcificum* pulp had a beneficial effect on the cell count and may potentially serve as prebiotic. Nonetheless, further investigation is required before prebiotic attribute of this plant can be ascertained. A similar result has also been reported by do Espírito Santo *et al.* (2012) in which an increase in probiotic viable cells was observed when yogurt was fortified with passion fruit peel powder.

In general, the reduction of viability observed for all three LAB strains throughout the storage was presumably due to the increase in the acidity of the yogurt. The accumulation of acid in the

yogurts may inhibit and become toxic to the LAB. Moreover, the cell viability can also be affected from the contamination by pathogens and the presence of other food spoiling organisms (Meor Hussin *et al.,* 2017). *L. delbrueckii* in yogurt has been reported to secrete essential amino acids that can support the growth of probiotics, but at the same time produce acids and hydrogen peroxide that may inhibit the cells (Shihata and Shah, 2000). Nonetheless, positive effects including enhanced probiotic viability observed from applying the combination of additional microencapsulated potential probiotic strain and plant extract into yogurt emphasised their practical
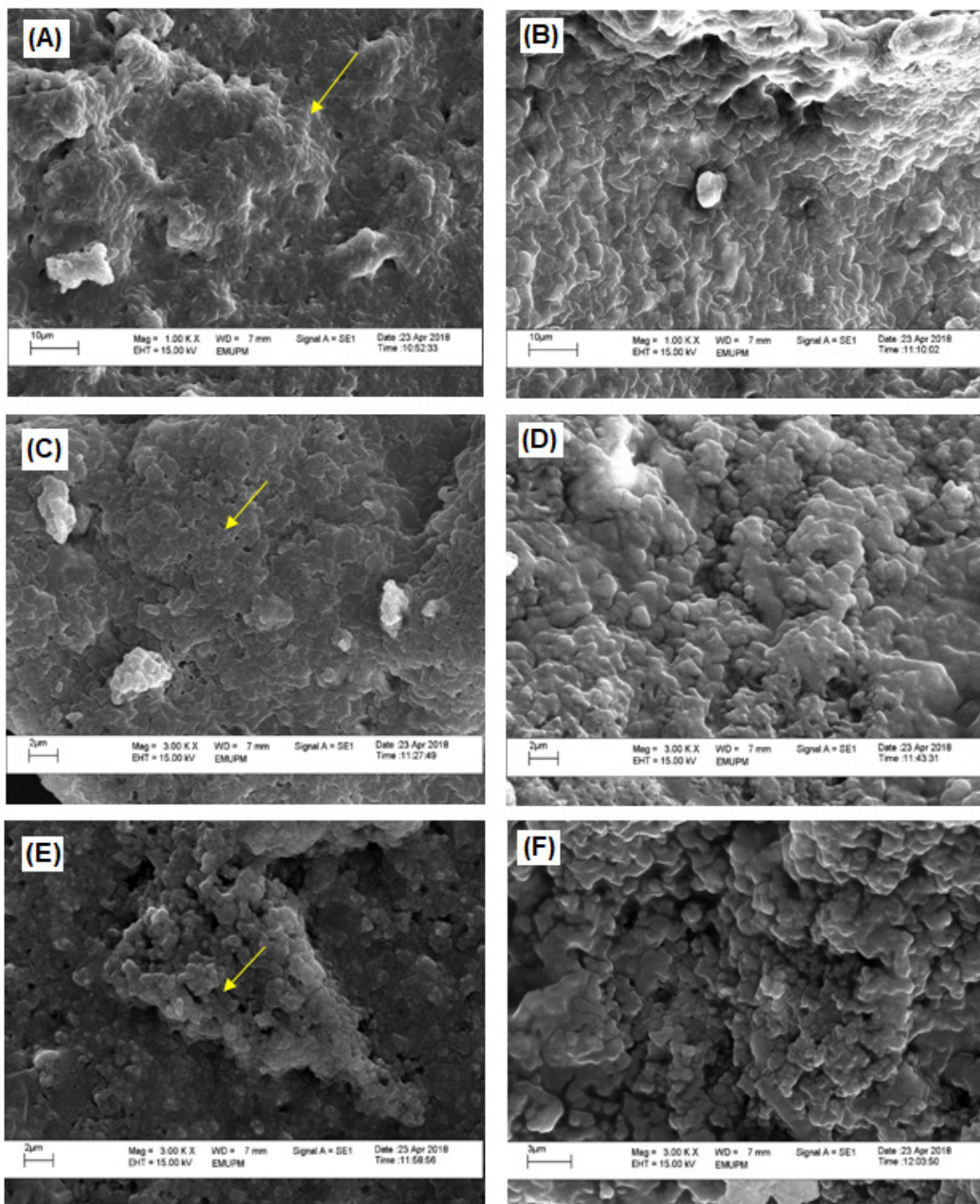

Figure 5. Scanning electron microscopy (SEM) images of seven different yogurt formulations. (A) C. Plain viewed at 1,000× magnification, (B) C. Pulp viewed at 1,000× magnification, (C) F. Plain viewed at 3,000× magnification, (D) F. Pulp viewed at 3,000x magnification, (E) B. Plain viewed at 3,000× magnification, and (F) B. Pulp viewed at 3,000×.

contributions for the development of functional dairy industry (Kailasapathy and Champagne, 2011). In addition, some important aspects of commercial yogurt production such as organoleptic properties must first be ascertained prior to considering pilot run on the potential consumer acceptability (Pinto *et al.*, 2018; Chetachukwu *et al.*, 2019). Since *S. dulcificum* pulp contains a miraculin compound, which is a taste modifying protein that converts sour tasting foods to sweet taste, the undesirable sour taste in yogurts can perhaps be eliminated, thus improving consumers' perception and acceptance.

Yogurt morphology

The differences in microstructure of yogurts were qualitatively inferred from the SEM micrographs. Prior to viewing under SEM, the yogurt samples were freeze-dried to remove moisture content. Freeze drying affects the hydrogen bonds involved in binding the surface layer of protein to the cell wall of yogurt bacteria (Jaya, 2009). This may be the reason of the structural collapse observed in the morphology of all yogurts, making the presence of microencapsulated cells and free cells to not be well distinguished (Figure 5). In freeze dried yogurt, the fat globules present appeared as small holes (Jaya, 2009). Comparing all the plain yogurts (C. Plain, F. Plain, and B. Plain) to the yogurts supplemented with *S. dulcificum* pulp (C. Pulp, F. Pulp, and B. Pulp), many fat globules (marked with yellow arrow) could be seen in the plain yogurts, indicating high content of fat. The spherical particles appeared in the microstructure of all yogurts were formed by compact association of protein. From Figure 5B, 5D, and 5F, yogurts containing *S. dulcificum* pulp exhibited matrices characterised by large, fused casein micelles clusters with comparatively lower porosity than all the plain yogurts (Figure 5A, 5C, and 5E). Similar observation has been previously reported for yogurt containing stevia (Hernández-Rodríguez *et al.*, 2017). Meanwhile, the sputter texture indicates the presence of LAB in yogurts. Yogurts fortified with *S. dulcificum* (Figure 5B, 5D, and 5F) were seen to have more spluttery texture than the yogurts without *S. dulcificum* (Figure 5A, 5C, and 5E).

Conclusion

The data obtained in the present work suggest that *S. dulcificum* pulp extract contains high phenolic and antioxidant activity. The pulp of *S. dulcificum* showed the highest antioxidant activities than the seed and leaf. Therefore, the consumption of *S. dulcificum* pulp could be of great value in supporting healthy ageing and preventing oxidative stress related diseases. The incorporation of microencapsulated *L. lactis* Gh1 cells in yogurt had resulted in a good sustainability of viable cells above the minimum recommended concentration (10^7 CFU/mL) for probiotic products. Overall, the inclusion of *S. dulcificum* pulp extract and potential probiotic strain, *L. lactis* Gh1 into yogurt has the potential to be a new functional food-based product with high nutritional appeal and beneficial properties.

References

Abbasiliasi, S., Tan, J. S., Tengku Ibrahim, T. A., Ramanan, R. N., Vakhshiteh, F., Mustafa, S., ... and Ariff, A. B. 2012. Isolation of *Pediococcus acidilactici* Kp10 with ability to secrete bacteriocin-like inhibitory substance from milk products for applications in food industry. BMC Microbiology 12: article no. 260.

Ahmad, I., Khalique, A., Junaid, M., Shahid, M. Q., Imran, M. and Rashid, A. A. 2020. Effect of polyphenol from apple peel extract on the survival of probiotics in yoghurt ice cream. International Journal of Food Science and Technology 55(6): 2580-2588.

Amirdivani, S. 2015. Inclusion of *Allium sativum* in yogurt and its effects on inhibition of diabetes and hypertension-associated by enzymes *in vitro*. Applied Food Biotechnology 2(3): 29-37.

Baros, R. F., Cutrim, C. S., Costa, M. P., Conte Junior, C. A. and Cortez, M. A. S. 2019. Lactose hydrolysis and organic acids production in yogurt prepared with different onset temperatures of enzymatic action and fermentation. Ciência Animal Brasileira 20: article ID e43549.

Benkerroum, N., Oubel, H., Zahar, M., Dlia, S. and Filali-Maltouf, A. 2000. Isolation of a bacteriocin-producing *Lactococcus lactis* subsp. *lactis* and application to control *Listeria monocytogenes* in Moroccan jben. Journal of Applied Microbiology 89(6): 960-968.

Chetachukwu, A. S., Thongraung, C. and Yupanqui, C. T. 2019. Development of reduced-fat coconut yoghurt: physicochemical, rheological, microstructural and sensory properties. International Journal of Dairy Technology 72(4): 524-535.

Dabija, A., Codină, G. G., Ropciuc, S., Gâtlan, A.-M. and Rusu, L. 2018. Assessment of the

antioxidant activity and quality attributes of yogurt enhanced with wild herbs extracts. Journal of Food Quality 2018: article ID 5329386.

do Espírito Santo, A. P., Perego, P., Converti, A. and Oliveira, M. N. 2012. Influence of milk type and addition of passion fruit peel powder on fermentation kinetics, texture profile and bacterial viability in probiotic yoghurts. LWT 47(2): 393-399.

Du, L., Shen, Y., Zhang, X., Prinyawiwatkul, W. and Xu, Z. 2014. Antioxidant-rich phytochemicals in miracle berry (*Synsepalum dulcificum*) and antioxidant activity of its extracts. Food Chemistry 153: 279-284.

Fazilah, N. F., Ariff, A. B., Khayat, M. E. and Halim, M. 2020. Anti-diabetic properties of *Synsepalum dulcificum* and potential inclusion in functional yogurt. IOP Conference Series Materials Science and Engineering 716: article ID 012004.

Fazilah, N. F., Ariff, A. B., Khayat, M. E., Rios-Solis, L. and Halim, M. 2018. Influence of probiotics, prebiotics, synbiotics and bioactive phytochemicals on the formulation of functional yogurt. Journal of Functional Foods 48: 387-399.

Fazilah, N. F., Hamidon, N. H., Ariff, A. B., Khayat, M. E., Wasoh, H. and Halim, M. 2019. Microencapsulation of *Lactococcus lactis* Gh1 with gum Arabic and *Synsepalum dulcificum* via spray drying for potential inclusion in functional yogurt. Molecules 24(7): article ID 1422.

Fenster, K., Freeburg, B., Hollard, C., Wong, C., Laursen, R. R. and Ouwehand, A. C. 2019. The production and delivery of probiotics: a review of a practical approach. Microorganisms 7(3): article no. 83.

Gouin, S. 2004. Microencapsulation: industrial appraisal of existing technologies and trends. Trends in Food Science and Technology 15(7-8): 330-347.

Granato, D., Barba, F. J., Kovacevic, D. B., Lorenzi, J. M., Cruz, A. G. and Putnik, P. 2020. Functional foods: product development, technological trends, efficacy testing, and safety. Annual Review of Food Science and Technology 11: 93-118.

Halim, M., Mustafa, N. A. M., Othman, M., Wasoh, W., Kapri, M. R. and Ariff, A. B. 2017. Effect of encapsulant and cryoprotectant on the viability of the probiotic *Pediococcus acidilactici* ATCC 8042 during freeze-drying and exposure to high acidity, bile salts and heat. LWT - Food Science and Technology 81: 210-216.

Hassanzadeh-Taheri, M., Hassanzadeh-Taheri, M., Jahari, F. and Hosseini, M. 2018. Effect of yoghurt butter oils on rat plasma lipids, haematology and liver histology parameters in a 150-day study. International Journal of Dairy Technology 71(1): 140-148.

He, Z., Tan J. S., Abbasiliasi, S., Lai, O. M., Tam, Y. J. and Ariff, A. B. 2016. Phytochemicals, nutritionals and antioxidant properties of miracle fruit *Synsepalum dulcificum*. Industrial Crops and Products 86: 87-94.

He, Z., Tan, J. S, Abbasiliasi, S., Lai, O. M., Tam, Y. J., Halim, M. and Ariff, A. B. 2015. Primary recovery of miraculin from miracle fruit, *Synsepalum dulcificum* by AOT reverse micellar system. LWT - Food Science and Technology 64(2): 1243-1250.

Hernández-Rodríguez, L., Lobato-Calleros, C., Ramírez-Santiago, C., Rodríguez-Huezo, M. E. and Meraz, M. 2017. Microstructure and rheology of yogurt added with protein-*L. plantarum*-polysaccharide coacervate and stevia in substitution of milk-fat and sucrose. Revista Mexicana De Ingeniería Química 16(1): 77-89.

Hwanhlem, N., Ivanova, T., Haertlé, T., Jaffrès, E. and Dousset, X. 2017. Inhibition of food-spoilage and foodborne pathogenic bacteria by a nisin Z-producing *Lactococcus lactis* subsp. *lactis* KT2W2L. LWT - Food Science and Technology 82: 170-175.

Jawan, R., Abbasiliasi, S., Mustafa, S., Halim, M. and Ariff, A. B. 2018. *In vitro* properties analysis of potential probiotic of isolated lactic acid bacteria from milk by-product. Borneo Journal of Medical Sciences 1: 61-62.

Jaya, S. 2009. Microstructure analysis of dried yogurt: effect of different drying methods. International Journal of Food Properties 12(3): 469-481.

Kailasapathy, K. and Champagne, C. P. 2011. Trends in the microencapsulation of probiotics for application in dairy products. Agro Food Industry Hi-Tech 22(2): 25-27.

Karaaslan, M., Ozden, M., Vardin, H. and Turkoglu, H. 2011. Phenolic fortification of yogurt using grape and callus extracts. LWT - Food Science and Technology 44(4): 1065-1072.

Lewoyehu, M. and Amare, M. 2019. Comparative evaluation of analytical methods for determining the antioxidant activities of honey: a review. Cogent Food and Agriculture 5(1): article ID 1685059.

Manzoor, S., Yusof, Y. A., Chin, N. L., Tawakkal., I. S. M. A., Fikry, M. and Chang, L. S. 2019. Quality characteristics and sensory profile of stirred yogurt enriched with papaya peel powder.

Pertanika Journal of Tropical Agricultural Science 42(2): 519-533.

McAuliffe, O. 2018. Symposium review: *Lactococcus lactis* from nondairy sources: their genetic and metabolic diversity and potential applications in cheese. Journal of Dairy Science 101(4): 3597-3610.

Meor Hussin, A. S., Mohamad Said, N. H., Che Wan Sapawi, C. W. N. S. and Anzian, A. 2017. The properties of yogurt coconut shake and coconut shake. Food and Applied Bioscience Journal 5(1): 50-68.

Ming, L. C., Halim, M., Abd Rahim, R., Wan, H. Y. and Ariff, A. B. 2016. Strategies in fed-batch cultivation on the production performance of *Lactobacillus salivarius* I 24 viable cells. Food Science and Biotechnology 25(5): 1393-1398.

Muniandy, P., Shori, A. B. and Baba, A. S. 2016. Influence of green, white and black tea addition on the antioxidant activity of probiotic yogurt during refrigerated storage. Food Packaging and Shelf Life 8: 1-8.

Nordin, N. A., Mohd Zin, N. B., Fazilah, N. F, Wasoh, H., Ariff, A. B. and Halim, M. 2019. Phytochemicals, antioxidant and antimicrobial properties of *Senna alata* and *Senna tora* leaf extracts against bacterial strains causing skin infections. Bacterial Empire 2(1): 19-25.

Østlie, H. M., Treimo, J. and Narvhus, J. A. 2005. Effect of temperature on growth of probiotic bacteria in milk. International Dairy Journal 15(10): 989-997.

Papadimitriou, M. N. B., Resende, C., Kuchler, K. and Brul, S. 2007. High Pdr12 levels in spoilage yeast (*Saccharomyces cerevisiae*) correlate directly with sorbic acid levels in the culture medium but are not sufficient to provide cells with acquired resistance to the food preservative. International Journal of Food Microbiology 113(2): 173-179.

Pinto, L. P. F., Silva, H. L. A., Kuriya, S. P., Maçaira, P. M., Oliveira, F. L. C., Cruz, A. G., ... and Freitas, M. Q. 2018. Understanding perceptions and beliefs about different types of fermented milks through the application of projective techniques: a case study using Haire's shopping list and free word association. Journal of Sensory Studies 33(3): article ID e12326.

Shafi, A., Raja, H. N., Farooq, U., Akram, K., Hayat, Z., Naz, A. and Nadeem, H. R. 2019. Antimicrobial and antidiabetic potential of symbiotic fermented milk: a functional dairy product. International Journal of Dairy Technology 72(1): 15-22.

Shihata, A. and Shah, N. P. 2000. Proteolytic profiles of yogurt and probiotic bacteria. International Dairy Journal 10(5-6): 401-408.

Shori, A. B. and Baba, A. S. 2012. Viability of lactic acid bacteria and sensory evaluation in *Cinnamomum verum* and *Allium sativum*-bio-yogurts made from camel and cow milk. Journal of the Association of Arab Universities for Basic and Applied Sciences 11(1): 50-55.

Sieuwerts, S. 2016. Microbial interactions in the yoghurt consortium: current status and product implications. SOJ Microbiology and Infectious Diseases 4(2): 1-5.

Tan, P. Y., Tan, T. B., Chang, H. W., Tey, B. T., Chan, E. S., Lai, O. M., ... and Tan, C. P. 2018. Effects of storage and yogurt matrix on the stability of tocotrienols encapsulated in chitosan-alginate microcapsules. Food Chemistry 241: 79-85.

Wihansah, R. R. S., Arief, I. I. and Batubara, I. 2018. Anti-diabetic potency and characteristics of probiotic goat-milk yogurt supplemented with roselle extract during cold storage. Tropical Animal Science Journal 41(3): 191-199.