International Food Research Journal 31(2): 398 - 416 (April 2024)
Journal homepage: *http://www.ifrj.upm.edu.my*

Characterisation of physicochemical properties, anti-hyperglycaemic effects, and probiotic potentials in fermented pumpkin drink utilising *Lactobacillus mali* isolated from water kefir

[1]*Koh, W. Y., [2]Lim, X. X., [2]Uthumporn, U., [2]Tan, T. C., [1]Kobun, R. and [3]Rasti, B.

[1]*Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia*
[2]*Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM Penang, Penang, Malaysia*
[3]*Australasian Nanoscience and Nanotechnology Initiative, 8054 Monash University, Clayton, 3168 Victoria, Australia*

Article history

Received:
10 February 2022
Received in revised form:
26 July 2023
Accepted:
4 January 2024

Keywords

shelf-life, probiotics, pumpkin, α-glucosidase inhibition, simulated gastrointestinal condition

Abstract

Pumpkin has been reported to be a good probiotic delivery vehicle, but it has a short shelf life, is susceptible to enzymatic browning, and prone to microbial spoilage. Pumpkin could be transformed into a value-added beverage through the fermentation by LAB. In the present work, the probiotic viability, physicochemical, antioxidative, antihyperglycemic, and sensory properties of *Lactobacillus mali*-fermented pumpkin drink (FPD) during eight weeks of refrigerated storage at 4°C were investigated. There were significant reductions ($p < 0.05$) in colour, pH, texture characteristics, and antioxidant properties after four weeks of storage. FPD exhibited total phenolic content ranging from 83.75 to 90.75 mg GAE/mL, total flavonoid content ranging from 47.01 to 50.01 mg QE/mL, FRAP reducing power ranging from 160.76 to 169.76 µmol Trolox equivalent/mL, and DPPH scavenging activity ranging from 52 to 54.56% during storage. FPD's total soluble solids decreased ($p < 0.05$) from 0.60 to 0.02 °Brix, while concentrations of ethanol (0.62 to 1.2%, v/v), lactic acid (0.03 to 0.11 g/L), and acetic acid (0.02 to 0.18 g/L) significantly increased ($p < 0.05$) over eight weeks of storage. *L. mali* strain within FPD maintained its viability ($p > 0.05$) under simulated gastrointestinal conditions, with 8 - 9.1 log CFU/mL for 28 days at 4°C. FPD retained up to 90% α-glucosidase enzyme inhibition throughout the 28 days storage period. Sensory evaluation showed that FPD's attributes remained unchanged ($p > 0.05$) during the initial four weeks of storage, with an overall acceptability score ranging from 6.56 to 6.88. In conclusion, FPD remained stable in terms of physicochemical, antioxidative, and sensory properties, as well as probiotic viability and anti-hyperglycaemic effects during 28 days of refrigerated storage. This highlights FPD's potential as a functional beverage, presenting an innovative method to utilise pumpkin as an effective probiotic carrier.

DOI
https://doi.org/10.47836/ifrj.31.2.11

Introduction

Food-based type-2 diabetes mellitus (T2DM) preventative strategies, such as probiotic foods, are safer, sustainable, cost-effective, and with lesser adverse effects. These strategies are more effective in countering T2DM in its early stage compared to conventional medications due to high patient compliance (Fujita *et al.*, 2017). Furthermore, factors such as increased lactose intolerance, allergic to milk protein, vegetarianism, and high cholesterol content in probiotic dairy products cause a surge in the demand for plant-based probiotic products with no dairy components (Min *et al.*, 2019). Freshly cut, minimally processed products including fruits and vegetables, usually have a shorter shelf-life because they are prone to microbial spoilage (Linares-Morales *et al.*, 2018). Lactic acid fermentation was reported to be capable of improving the sensory and nutritional qualities of fruit and vegetable matrices, at the same time preventing microbial spoilage (Valero-Cases and Frutos, 2017; Linares-Morales *et al.*, 2018).

Probiotics are live microorganisms that can benefit the host by restoring gut microbial balance

*Corresponding author.
Email: weeyin@ums.edu.my

when consumed in adequate numbers (10^6 - 10^7 CFU/mL or CFU/g) (FAO/WHO, 2002). In our previous study, we managed to isolate a probiotic strain, namely *Lactobacillus mali* K8 from water kefir grains, which has α-glucosidase inhibition ability and probiotic potential (Koh *et al.*, 2018b), and has been successfully used as an inoculum in the optimisation and development of a pumpkin-based fermented drink (FPD) (Koh *et al.*, 2018a). Pumpkin (*Cucurbita pepo* L.) contains an abundance of bioactive phytochemicals that were reported to have anti-diabetic (Thanh *et al.*, 2021), anti-hyperglycaemic (Xia and Wang, 2006), antioxidative (Miljić *et al.*, 2021), and anti-inflammatory (Umavathi *et al.*, 2021) properties. The polysaccharides found in pumpkin can serve as prebiotics, a food ingredient that stimulates the activity and/or growth of probiotics (Koh *et al.*, 2018b). However, pumpkin is highly perishable, and with its large size and weight, makes transportation and storage very expensive (Kiharason and Isutsa, 2019). Furthermore, the high content of insulin-dependent sugars is also another limiting factor for restriction in pumpkin consumption among T2DM patients (Koretska *et al.*, 2020).

Low energy-requiring lactic acid bacteria (LAB)-based fermentations can enhance the sensory, functional, and nourishment qualities, extend shelf life, as well as develop value-added products together with opening new market probabilities for pumpkin (Fujita *et al.*, 2017; Koh *et al.*, 2018a; Sun *et al.*, 2022). However, the stability of LAB-fermented drinks during storage is necessary and vital since it ensures that the probiotic functionalities and effectiveness of the products stay alike with those freshly manufactured during storage, even when the products are stored for several weeks (da Costa *et al.*, 2017; Min *et al.*, 2019). The objective of the present work was, therefore, to examine the quality parameters (physicochemical, microbiological, sensory, antioxidative, and antihyperglycemic properties) of FPD together with the non-inoculated control (PD) during cold storage at 4°C for a period of eight weeks.

Materials and methods

Materials

Pumpkins with similar degree of harvest maturity (visual estimation of flesh colour: deep yellow to orange colouration; average weight of 3 - 4 kg) were purchased from a local commercial plantation (Balik Pulau, Penang, Malaysia), and processed into purée (Koh *et al.*, 2018a). Porcine pancreatic α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1), rat intestinal alpha-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), pepsin from porcine stomach mucosa, Amano lipase G from *Penicillium camemberti*, bovine bile, and pancreatin from porcine pancreas were purchased from Merck, Darmstadt, Germany.

Bacterial strains

Lactobacillus mali K8 strain used in the previous work was isolated in our previous work (Koh *et al.*, 2018b).

Inoculum preparation and fermentation

L. mali K8 glycerol stock was aseptically transferred and incubated in a sterile MRS broth at 37°C for 24 h. After incubation, the culture was centrifuged (10,000 *g*, 20 min, 4°C) to collect the cell pellets. Subsequently, the cell pellets were diluted with sterilised saline until the turbidity was similar to No. 0.5 McFarland standard to provide a concentration of 10^8 CFU/mL. A total of 10 mL of 10^8 viable cells were suspended in 90 mL of 40% (w/v) pumpkin purée in distilled water in a 250 mL glass bottle with a lid, and fermented at 35°C for 28 h (Koh *et al.*, 2018a). The control sample (PD) was prepared using the same protocol as earlier described but without *L. mali* inoculation. Both FPD and PD were kept at 4°C for further analysis (weekly intervals, 8-week study period).

Physicochemical and physical properties

Total soluble solids (TSS), colour, pH, and texture properties of FPD and PD over 8-week storage (4°C) were evaluated. The TSS of the samples were measured using a digital refractometer (PAL-1, Atago, Tokyo, Japan). The colours of FPD and PD were measured in terms of *CIE* L*, a*, b* values using a tristimulus colourimeter (model CR-400, Konica Minolta Sensing Inc., Osaka, Japan). A digital pH meter (Mettler Toledo, Geissen, Germany) was used to measure the samples' pH values. The texture properties (consistency, firmness, viscosity, and cohesiveness) were measured using a TA-XT plus texture analyser (Stable Micro System Ltd., London, England).

Antioxidative properties

Carotenoids contents of FPD and PD were determined according to Barkallah *et al.* (2017). The total phenolic and flavonoid contents, radical scavenging activities, and reducing powers of FPD and PD were measured as per described by Syahariza *et al.* (2017) with slight modifications.

Sample preparation

To prepare the test samples, FPD and PD were first subjected to centrifugation (13,000 *g*, 15 min, 4°C). The supernatants were then decanted, filtered with a polytetrafluoroethylene (PTFE) membrane with a pore size of 0.22 µm, placed into screw-capped, amber coloured glass bottles, and stored at -20°C until used for further analysis (Koh *et al.*, 2018a).

Carotenoid content

An aliquot (1 mL) from the prepared sample was centrifuged (3,000 *g*, 10 min), sonicated (65°C, 30 min) in ethanol (1 mL), and again, subjected to centrifugation (10,000 *g*, 5 min). The supernatant was decanted into cuvettes, and the absorbance was read at 470, 653, and 666 nm using a spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan). The carotenoids content was then calculated, converted, and expressed in mg/100 mL using Eq. 1:

$$\text{Carotenoids (mg/100 mL)} = \frac{(1{,}000\times A_{470} - 2.86[15.65\times A_{666} - 7.340\times A_{653}] - 85.9[27.05\times A_{653} - 11.21\times A_{666}])}{245} \quad \text{(Eq. 1)}$$

where, A_{470}, A_{653}, and A_{666} = absorbances read at 470, 653, and 666 nm, respectively.

Total phenolic content (TPC)

The Folin-Ciocalteau spectrophotometric method was undertaken to determine the TPC of the sample. The mixture of Folin-Ciocalteau reagent (0.2 N, 2.5 mL) and the sample solubilised in methanol (2.5 mL) were homogenised for 5 min followed by the addition of sodium carbonate (Na_2CO_3, 7.5% (w/v), 2.5 mL). After the mixture was subjected to incubation (37°C, 30 min), its absorbance was spectrophotometrically measured at 765 nm (UV mini-1240, Shimadzu, Kyoto, Japan). The TPC of the samples were then estimated from a calibration curve prepared with various concentrations of gallic acid, from 0 to 100 mg/100 mL, and was expressed as mg gallic acid equivalent (GAE) per 100 mL sample.

Total flavonoid content (TFC)

The TFC of the sample was determined using the aluminium trichloride colorimetric method. Briefly, the sample (5 mL) was homogenised in aluminium trichloride ($AlCl_3.6H_2O$) methanol solution (2% (w/v), 5 mL) followed by incubation (37°C, 30 min), and their absorbance was subsequently measured using a spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan) at 415 nm. TFC of samples was then estimated based on a calibration curve of quercetin (0 - 60 mg/100 mL), and expressed as mg quercetin equivalent (QE) per 100 mL sample.

DPPH radical scavenging activity (RSA) assay

The free radical scavenging activity (RSA) of the sample was determined using DPPH assay. An aliquot of DPPH solution prepared in methanol (0.2 mM, 1 mL) was combined with the sample (200 µL) and subsequently subjected to incubation under dark condition (37°C, 30 min). The absorbance of the sample was then taken at 517 nm using a spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan). The RSA was expressed as DPPH free radical inhibition (%), using Eq. 2:

$$\text{Inhibition (\%)} = \left[\frac{A_B - A_S}{A_B}\right] \times 100 \quad \text{(Eq. 2)}$$

where, A_B and A_S = absorbances of the blank (DPPH solution) and sample (FPD or PD, and DPPH solution), respectively.

Ferric-reducing antioxidant power (FRAP)

The sample extracts and FRAP reagent were mixed in proportions of 1:20 (v/v), and incubated in the dark for 30 min at 37°C prior to the determination of FRAP assay. The FRAP reagent was freshly prepared by mixing sodium acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM 2,4,6-tri(2-pyridyl)-s-triazine in 40 mM hydrochloric acid), and ferric (III) chloride hexahydrate ($FeCl_3.6H_2O$, 20 mM) at proportions of 10:1:1 ratio (v/v), and kept warm in a water bath (37°C) until used. The absorbance of the reaction mixture was measured using a spectrophotometer (Shimadzu, UV-160A PC, Kyoto, Japan) at 593 nm, quantified against a standard curve of ferrous sulphate (0 - 30 mM/mL) prepared in deionised water, and expressed as mM ferrous sulphate equivalent (mM eq. Fe (II)) per 100 mL sample.

Chromatographic analysis

The sugars (glucose, fructose, and sucrose), ethanol, organic acid (acetic and lactic acids), and phenolic acid (syringic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, caffeic, and *p*-coumaric acids) contents in the samples were determined using high-performance liquid chromatography (HPLC) equipped with a refractive index (RI) detector system (Waters 2414, Waters Corporation, Milford, MA, USA). After centrifuged (6,000 *g*, 15 min, 4°C), the resulting supernatant was filtered through a PTFE syringe filter (pore size: 0.22 µm) and Sep-Pak C_{18} cartridge (pre-activated prior to the use with equal volumes of methanol, air, and water) before sample injection (20 µL/run). Sugars and ethanol were quantified and separated using Waters Sugar Pak (300 × 6.5 mm) column at 80°C and 0.5 mL/min flow rate using $CaNa_2$-EDTA (50 mg/L) solution, whereas organic acids were separated using Agilent ZORBAX SB-C_{18} (4.6 × 250 mm, 5 µm) analytical column at 30°C and 0.8 mL/min flow rate using $(NH_4)_2HPO_4$ solution (0.02 mol/L, pH 2.7), and measured at 210 nm with a UV detector (Model 2487, Waters Corporation, Milford, MA, USA). Furthermore, chromatographic analysis of phenolic acids was performed using photodiode array detector (Model 996), separation module (Model 2690), ZORBAX SB-C_{18} (4.6 × 250 mm, 5 µm) analytical column from Agilent maintained at 37°C, and 1.0 mL/min flow rate with water:acetonitrile (90:10 v/v) with 2% (v/v) acetic acid. The wavelength for syringic, protocatechuic, and *p*-hydroxybenzoic acids was 280 nm, while that for chlorogenic, caffeic, and *p*-coumaric acids was 320 nm.

Anti-hyperglycaemic properties

Sample preparation

The samples (FPD and PD) were prepared in the same way as in the previous section (antioxidative properties).

Determination of α-amylase inhibition

Sample (500 µL) was first mixed with 0.02 M sodium phosphate buffer (500 µL, pH 6.9 adjusted with 0.006 M sodium chloride (NaCl)) followed by the addition of porcine pancreatic α-amylase (0.5 mg/mL, EC 3.2.1.1, ≥ 1,000 units/mg protein). After being subjected to pre-incubation (37°C, 10 min), a solution of 1% starch prepared using the same buffer (500 µL) was added. The reaction mixture was then incubated (37°C, 10 min), followed by the introduction of 3,5-dinitrosalicylic acid (DNS, 1 mL), immersed in a boiling water bath (100°C, 5 min), and undergone a ten-fold dilution in distilled water. As a standard reference, the inhibition ability of acarbose (a standard α-glucosidase inhibitor) was also assessed using the same procedure described earlier. The quantities of released reducing sugars were then measured spectrophotometrically at 540 nm (UV mini-1240, Shimadzu, Kyoto, Japan), and the α-amylase inhibition (%) was determined using Eq. 3 (Nagappan *et al.*, 2017):

$$\alpha\text{-amylase inhibition (\%)} = \left[\frac{A_C - A_S}{A_C}\right] \times 100 \qquad \text{(Eq. 3)}$$

where, A_C and A_S = absorbance of the control (buffer with enzyme) and sample (sample, buffer with enzyme), respectively.

Determination of α-glucosidase inhibition

The sample, *p*-nitrophenyl-α-D-glucopyranoside (10 mM, PNPG, Sigma-Aldrich), and phosphate-buffered saline (0.1 M, PBS, pH 7.4), in proportions of 1:1:1 (25 µL each) were mixed together, aliquoted into a 96-well microplate, and subjected to pre-incubation (37°C, 10 min) followed by the initiation of enzyme reaction *via* the addition of 50 µL of rat intestinal α-glucosidase enzyme solution. After being subjected to incubation (37°C, 30 min), the reaction mixture was then placed in a Tecan microplate reader (Tecan Group Ltd, Mannedorf, Switzerland) to measure its absorbance at 405 nm, and the α-glucosidase inhibition (%) of the sample was calculated using Eq. 4 (Koh *et al.*, 2018a; 2018b):

$$\text{Inhibition (\%)} = \left[\frac{1 - [A_S - A_B]}{A_P - A_N}\right] \times 100 \qquad \text{(Eq. 4)}$$

where, A_S, A_B, A_P, and A_N = absorbance of sample (reaction with sample and enzyme), blank (reaction with sample), positive (reaction with enzyme), and negative controls (reaction without enzyme and sample), respectively.

Survival of L. mali in FPD under simulated gastrointestinal conditions (SGC)

Both simulated gastric and enteric conditions were prepared based on the method proposed by Koh *et al.* (2018a). Briefly, FPD was treated for 2 h in simulated gastric (pH 2.3 - 2.6), followed by 2 h each in simulated enteric with lower pH (5.4 - 5.7) and

higher pH (pH 6.8 - 7.2). Triplicate samples were taken after 2, 4, and 6 h, and *L. mali* survival was enumerated *via* pour-plating on De Man Rogosa Sharpe (MRS) agar. The survival rate (%) of *L. mali* was then determined using Eq. 5:

$$\text{Survival rate (\%)} = \frac{\text{Log CFU N}_1}{\text{Log CFU N}_0} \times 100 \qquad \text{(Eq. 5)}$$

where, N_1 and N_0 = total viable cell count of the *L. mali* after and before treatments, respectively.

Microbiological analysis

The microbiological qualities of the samples were evaluated prior to sensory evaluation. The sample (50 mL) was diluted with sterile quarter-strength Ringer's solution (450 mL) in a stomacher bag, homogenised in a stomacher for 1 min, and further diluted decimally up to 10^6 in the same medium. The yeast and mould count were determined using Potato Dextrose Agar (25°C, 5 d), whereas the presumptive identification and confirmation of total coliform, faecal coliforms, and *Escherichia coli* were conducted on Lactose Broth (37°C, 48 h) and Brilliant-Green Bile Lactose Broth (44.5°C, 48 h). The yeast and mould and coliform counts were then expressed as colony forming units (CFU)/mL, and most probable number (MPN)/100 mL, respectively.

Sensory evaluation

An acceptance test was performed by 50 semi-trained panellists (25 male, 25 female), who were recruited among undergraduate students from the School of Industrial Technology, Universiti Sains Malaysia. Samples (30 mL) were presented individually in a randomised order, and mineral water was provided for the panellists to rinse their mouths before testing each sample. The attributes included appearance (colour), texture (consistency), taste, odour, and overall acceptability were evaluated by the panellists using a 7-point hedonic scale (1 = dislike extremely, 4 = neither like nor dislike, 7 = like extremely).

Statistical analysis

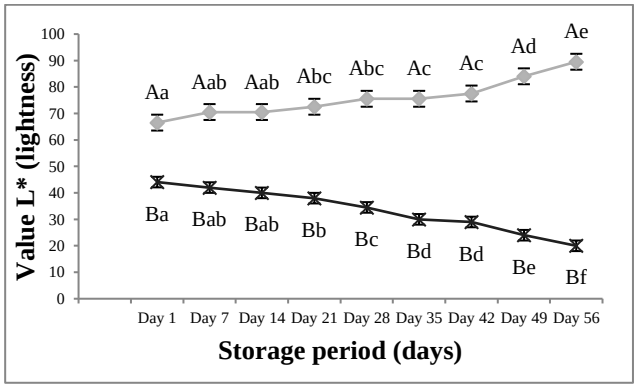
All experiments were performed in triplicate, in which three independent repetitions were performed for each sample, and data were expressed as mean ± standard deviation. Repeated measures ANOVA and Tukey's test were performed using SPSS software (IBM 21.0, Armonk, NY, USA) to test the main effects of storage time and samples on the treatments (simulated gastric and enteric conditions at 0, 2, 4, and 6 h). Paired sample *t*-test was carried out to compare the differences in the mean value for samples (PD and FPD) at 0.05 significant level.

Results and discussion

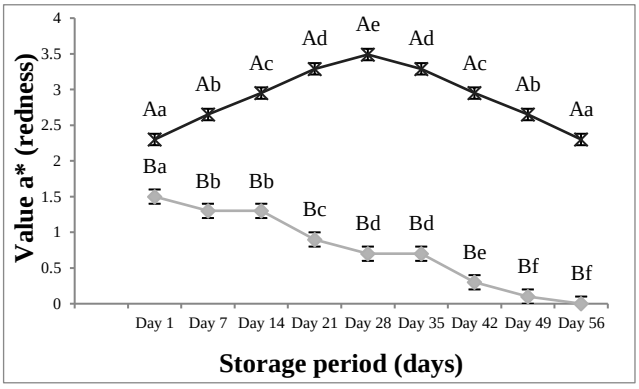
Physicochemical and physical properties

For PD (control), a gradual increase in *CIE* L* ($p < 0.05$) and a decrease in *CIE* a* and b* values ($p < 0.05$) were observed throughout the storage periods (Figures 1a - 1c). This could be linked to the oxidation of pumpkin's natural pigments and other chemical compounds during storage (Kaprasob *et al.*, 2017), resulting in the decrease in colour intensity of PD. Meanwhile, FPD was significantly ($p < 0.05$) darker (lower in *CIE* L* values), and higher in redness (*CIE* a*) and yellowness (*CIE* b*) compared to PD. This suggested that *L. mali* changed the tissue structure of substrate (pumpkin purée) that led to the release of natural pigments, such as carotenoids to the matrix, thus increasing the redness and yellowness, at the same time decreasing the lightness values of FPD (Do and Fan, 2019).

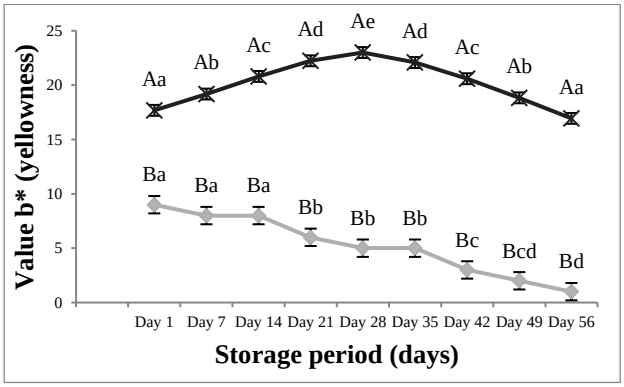
Gradual ($p < 0.05$) decrease in *CIE* L* values of FPD were seen from the beginning to the end of the storage period (Figure 1a). The accumulation of dead probiotic cultures could be the reason that contributed to the darkening of FPD. The loss of viable *L. mali* cells in FPD (Figure 2) resulted in the build-up of lysed or dead bacterial cells, leading to the increased turbidity and colour darkening of FPD during storage (Porto *et al.*, 2018). Furthermore, the decrease in light intensity was most probably attributed to oxidative and non-oxidative reactions of polyphenols (formation of melanoidins) leading to coloured condensation products (Porto *et al.*, 2018). Pumpkins are rich in phenolic compounds. The oxidation of polyphenols by polyphenol oxidase triggered the generation of dark pigments. During storage, FPD which was acidic, inhibited the enzyme activity of polyphenol oxidase and subsequently induced an enzymatic browning of phenolic compounds. While pumpkins have high concentration of soluble sugars and proteins, the heat treatment involved in the preparation of pumpkin purée could degrade the reducing sugars and amino acids thus leading to the Maillard reaction. Both *CIE* a* and b* values of FPD increased notably ($p < 0.05$) during early-stage storage, but then decreased after Week 4 (Figures 1b - 1c). This could be attributed to


Value L* (lightness)
Aa Aab Aab Abc Abc Ac Ac Ad Ae
Ba Bab Bab Bb Bc Bd Bd Be Bf
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

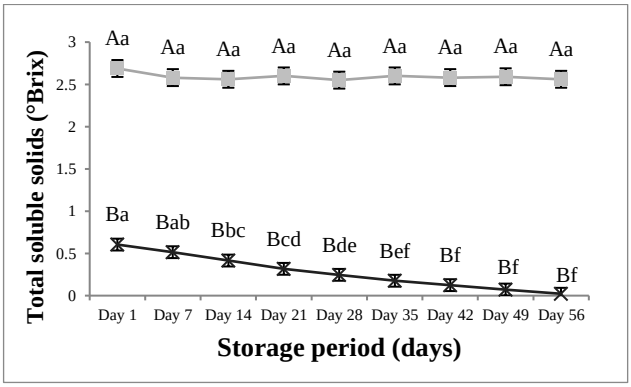
(a)


Value a* (redness)
Aa Ab Ac Ad Ae Ad Ac Ab Aa
Ba Bb Bb Bc Bd Bd Be Bf Bf
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

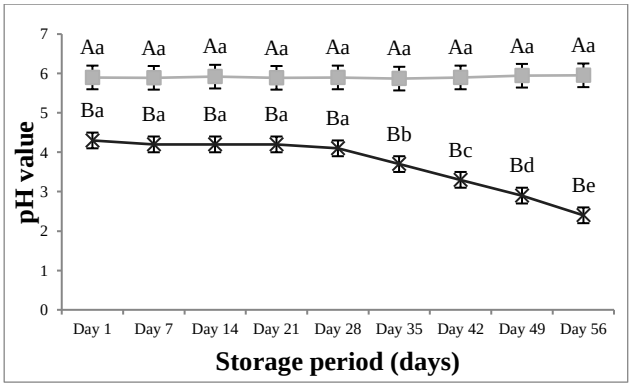
(b)


Value b* (yellowness)
Aa Ab Ac Ad Ae Ad Ac Ab Aa
Ba Ba Ba Bb Bb Bb Bc Bcd Bd
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

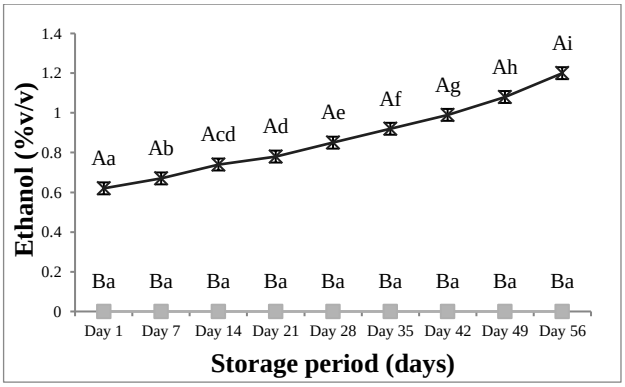
(c)


Total soluble solids (°Brix)
Aa Aa Aa Aa Aa Aa Aa Aa Aa
Ba Bab Bbc Bcd Bde Bef Bf Bf Bf
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

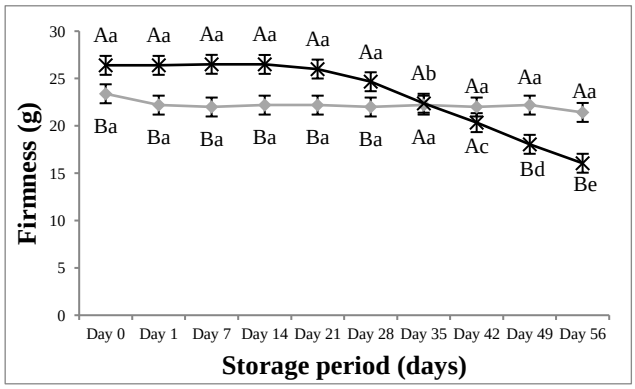
(d)


pH value
Aa Aa Aa Aa Aa Aa Aa Aa Aa
Ba Ba Ba Ba Ba Bb Bc Bd Be
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

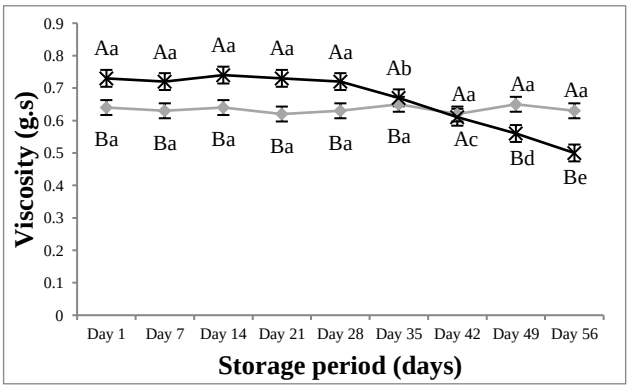
(e)


Ethanol (%v/v)
Aa Ab Acd Ad Ae Af Ag Ah Ai
Ba Ba Ba Ba Ba Ba Ba Ba Ba
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

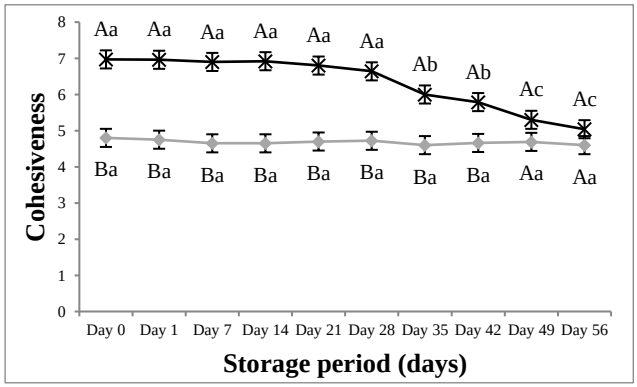
(f)


Firmness (g)
Aa Aa Aa Aa Aa Aa Ab Aa Aa Aa
Ba Ba Ba Ba Ba Ba Aa Ac Bd Be
Day 0 Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

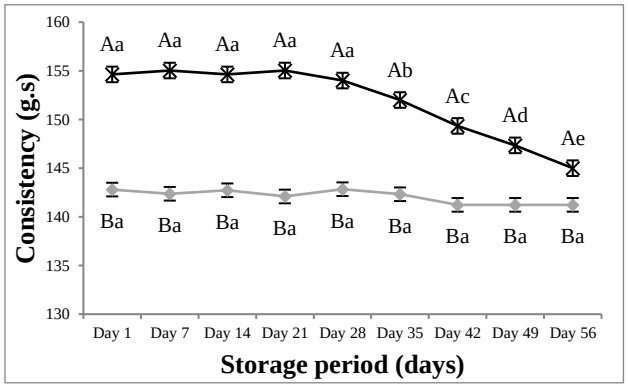
(g)


Viscosity (g.s)
Aa Aa Aa Aa Aa Ab Aa Aa Aa
Ba Ba Ba Ba Ba Ba Ac Bd Be
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

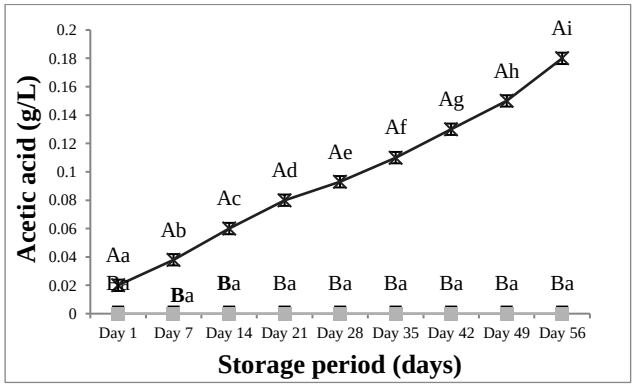
(h)


Cohesiveness
Aa Aa Aa Aa Aa Aa Ab Ab Ac Ac
Ba Ba Ba Ba Ba Ba Ba Ba Aa Aa
Day 0 Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

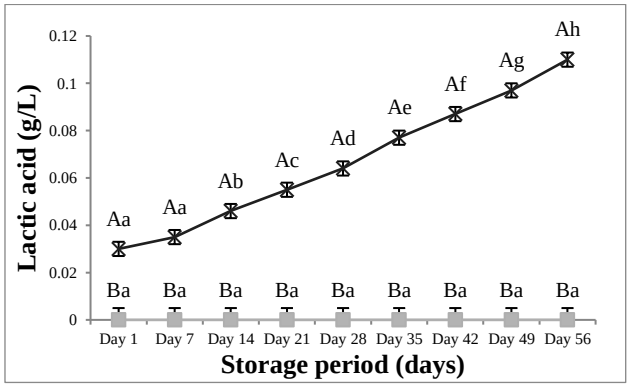
(i)


Consistency (g.s)
Aa Aa Aa Aa Aa Ab Ac Ad Ae
Ba Ba Ba Ba Ba Ba Ba Ba Ba
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

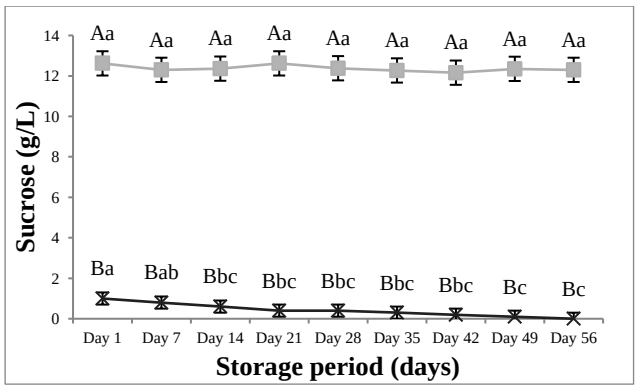
(j)


Acetic acid (g/L)
Aa Ab Ac Ad Ae Af Ag Ah Ai
Ba Ba Ba Ba Ba Ba Ba Ba Ba
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

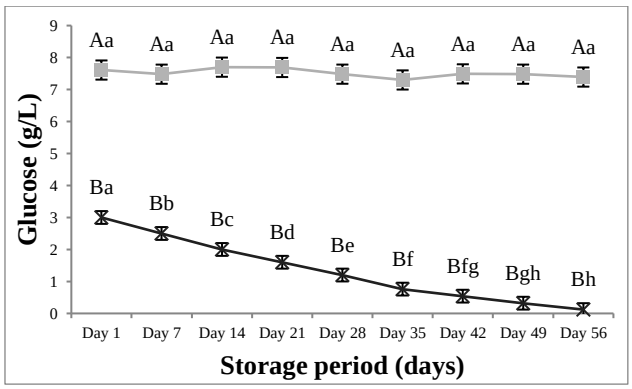
(k)


Lactic acid (g/L)
Aa Aa Ab Ac Ad Ae Af Ag Ah
Ba Ba Ba Ba Ba Ba Ba Ba Ba
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

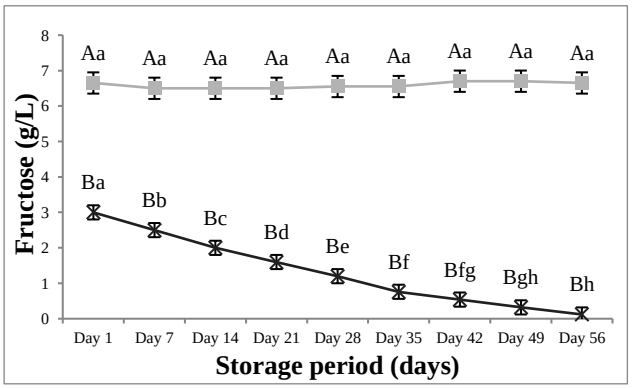
(l)


Sucrose (g/L)
Aa Aa Aa Aa Aa Aa Aa Aa Aa
Ba Bab Bbc Bbc Bbc Bbc Bbc Bc Bc
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

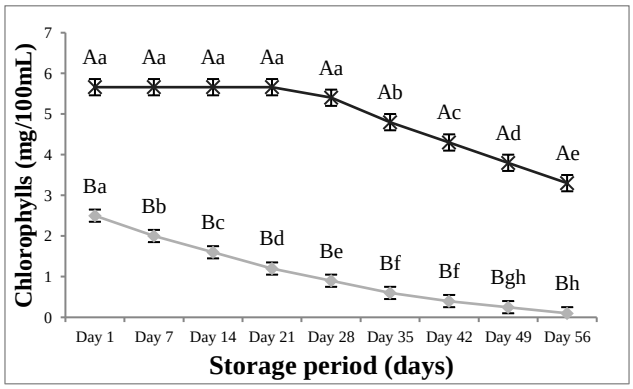
(m)


Glucose (g/L)
Aa Aa Aa Aa Aa Aa Aa Aa Aa
Ba Bb Bc Bd Be Bf Bfg Bgh Bh
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

(n)


Fructose (g/L)
Aa Aa Aa Aa Aa Aa Aa Aa Aa
Ba Bb Bc Bd Be Bf Bfg Bgh Bh
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

(o)


Chlorophylls (mg/100mL)
Aa Aa Aa Aa Aa Ab Ac Ad Ae
Ba Bb Bc Bd Be Bf Bf Bgh Bh
Day 1 Day 7 Day 14 Day 21 Day 28 Day 35 Day 42 Day 49 Day 56
Storage period (days)

(p)

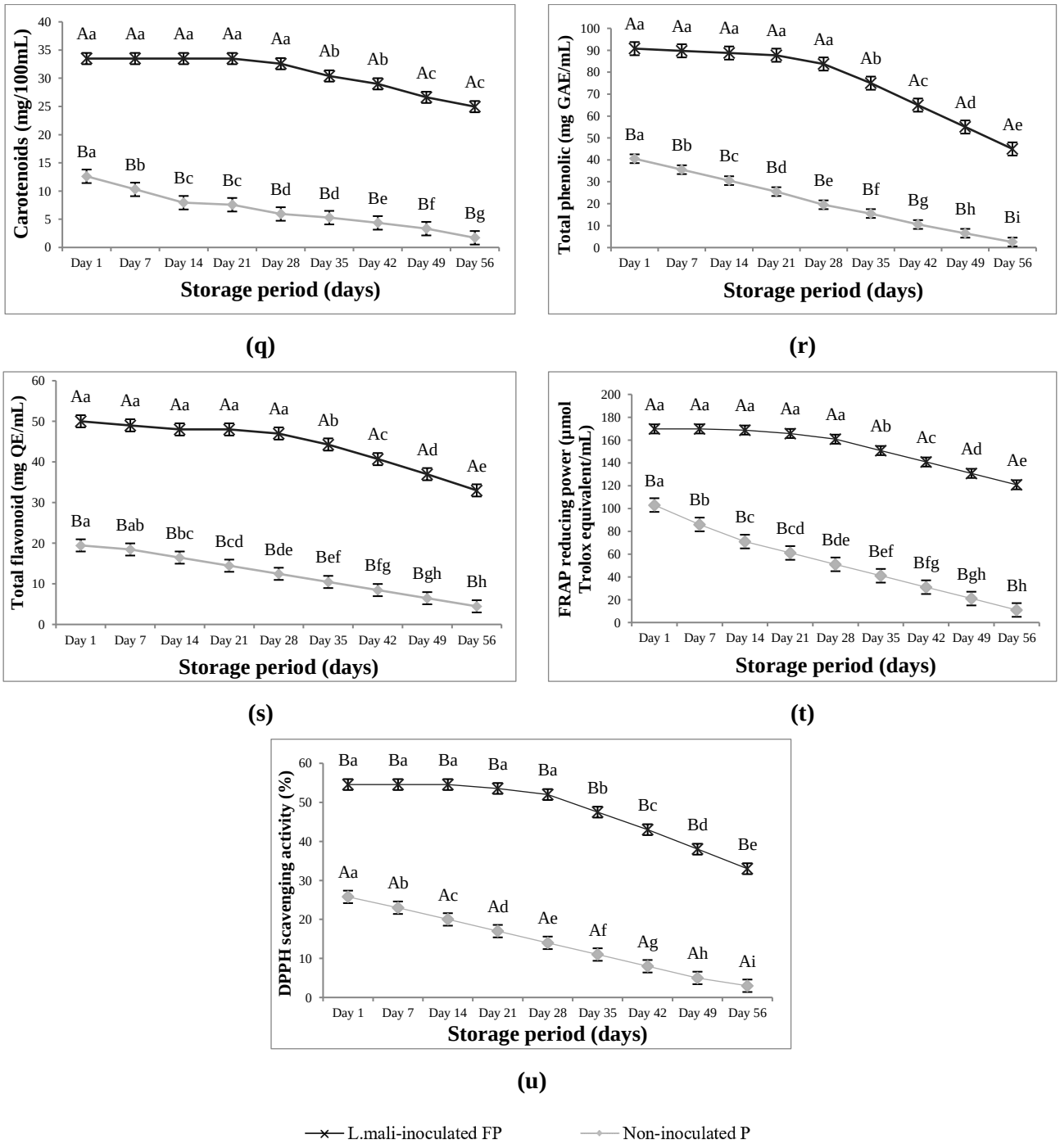
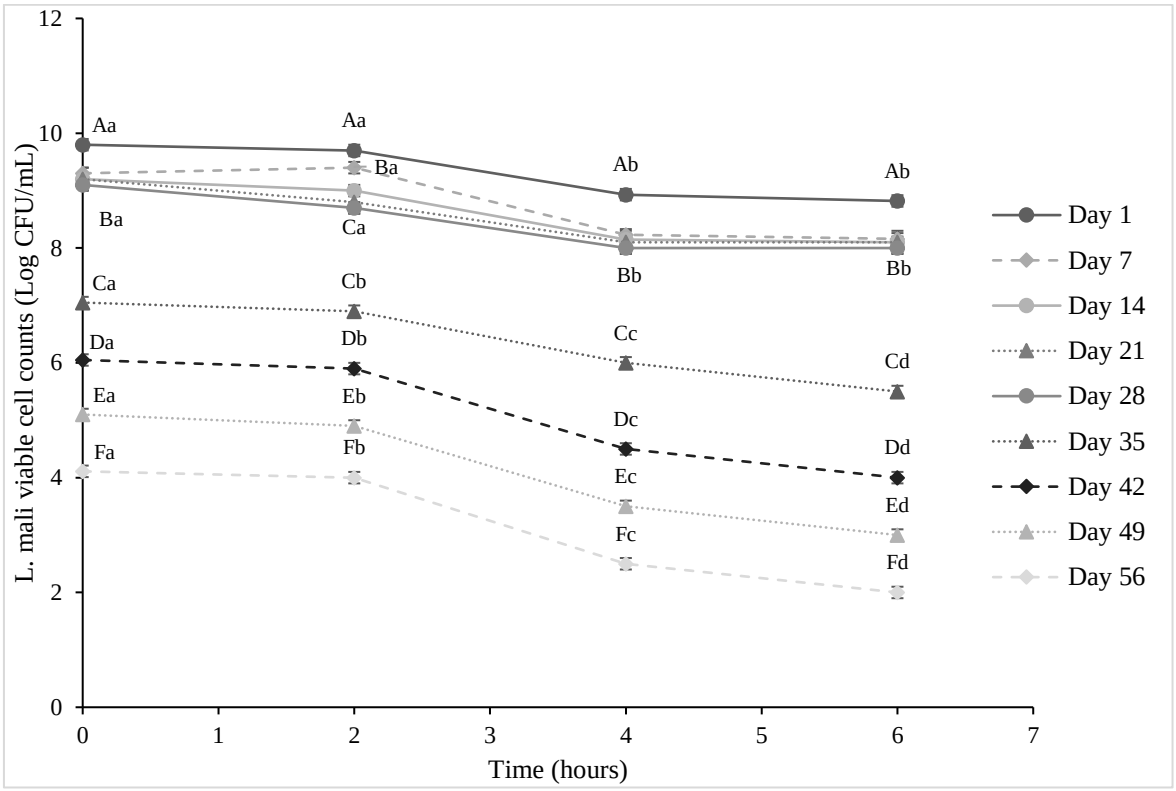
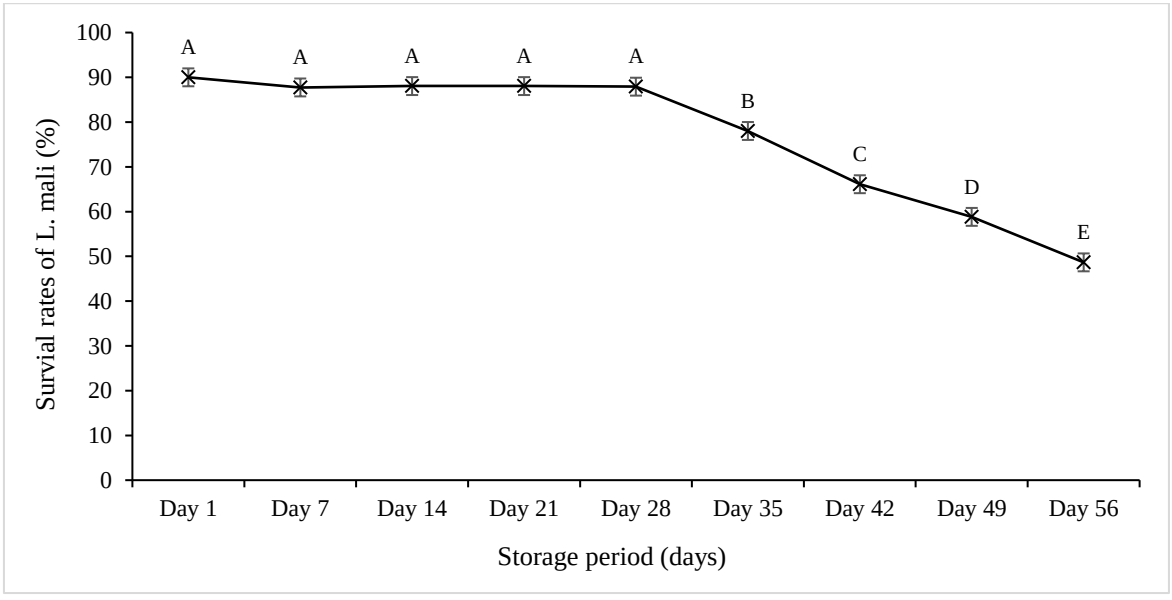


Figure 1. Changes in colour indexes [**(a)** *CIE* L* (lightness), **(b)** *CIE* a* (redness), **(c)** *CIE* b* (yellowness)], **(d)** total soluble solids, **(e)** pH values, **(f)** ethanol contents, texture parameters [**(g)** firmness, **(h)** viscosity, **(i)** cohesiveness, **(j)** consistency], the contents of **(k)** acetic acid, **(l)** lactic acid, **(m)** sucrose, **(n)** glucose, and **(o)** fructose, and antioxidant properties [**(p)** chlorophylls, **(q)** carotenoids, **(r)** total phenolics, **(s)** total flavonoids, **(t)** FRAP reducing power, **(u)** DPPH scavenging activity] for non-inoculated control (PD) and *L. mali*-inoculated fermented pumpkin drinks (FPD) during cold storage (4°C for 8 w). Means with standard deviation (error bars) in the different lines followed by different uppercase letters are significantly different at $p < 0.05$ between samples of the same storage time. Means with standard deviation in the same line followed by different lowercase letters are significantly different at $p < 0.05$ for each sample affected by the storage time.



(a)



(b)

Figure 2. Changes in **(a)** viable cells of *Lactobacillus mali* in *L. mali*-inoculated fermented pumpkin drink (FPD) during cold storage (4°C for 8 w), before (0 h) and during exposure to *in vitro* simulated gastric (2 h) and enteric (4 and 6 h) conditions; **(b)** survival rates of *L. mali* in FPD after simulated gastrointestinal conditions. Means with standard deviation (error bars) in the different lines followed by different uppercase letters are significantly different at $p < 0.05$ between sampling periods. Means with standard deviation in the same line followed by different lowercase letters are significantly different at $p < 0.05$ between different treatments for the same sampling periods.

the isomerisation of *trans*-carotenoid isomers to *cis*-, which led to the colour changes in FPD (Figure 1q) (Do and Fan, 2019). A similar observation was reported in yoghurt supplemented with pumpkin fibre, whereby their redness and yellowness also increased during the initial stage of storage, but decreased after being stored for 7 d (Bakirci *et al.*, 2017). However, the decrease in both the *CIE* a* and b* values after Week 4 was most probably due to the degradation of the carotenoids as shown in Figure 1q.

FPD contained remarkably ($p < 0.05$) lesser TSS values than PD (Figure 1d). This suggested that most of the sugars were consumed by *L. mali* during fermentation. TSS of PD was maintained at a constant level over the whole storage period, but TSS for FPD decreased significantly ($p < 0.05$) (Figure 1d). This could have been due to the depletion in sugars, which were metabolised by *L. mali* during storage (Figures 1m - 1o). The continuous decrease in TSS in FPD during storage suggested that the remaining sugars were utilised during storage, but at a slower rate compared to that during fermentation (Kaprasob *et al.*, 2017). The decrease in TSS observed in FPD after storage was aligned with the findings reported by Managa *et al.* (2021) in their study on lactic acid fermentation of chayote leaf-pineapple smoothies using *L. plantarum* and *Weissella cibaria*. This supported the notion that lactic acid fermentation can occur even under cold temperatures.

When compared to PD, FPD had significantly ($p < 0.05$) lower pH values during storage (Figure 1e). The pH of the FPD samples was maintained at a constant level during the initial storage period, but then decreased significantly ($p < 0.05$) after Week 4. The uniform pH of FPD in the first 4 w of storage could be related to the high buffering effect of substrate (Koh *et al.*, 2018a), as well as organic acid metabolism by *L. mali* (da Costa *et al.*, 2017). The pH value decrease during storage could have been due to post-acidification that occurred at 4°C, thus suggesting that *L. mali* remained viable and active even at this temperature (Figure 2), metabolising simple sugars present in FPD (Figures 1m - 1o) into lactic and acetic acids (Figures 1k - 1l), which contributed to the decreasing pH (Figure 1e). Previously, Setyawardani *et al.* (2019) also reported that certain LAB could grow in refrigerated conditions. In addition, bacterial intracellular enzymes released upon cellular lysis (Figure 2) could also decrease the pH value of the fermented drink (Delgado *et al.*, 2019).

The ethanol content in FPD increased progressively ($p < 0.05$) during storage, whilst no ethanol was detected in PD (Figure 1f). *L. mali* used in the present work were capable of fermenting sugars (Figures 1m - 1o) into metabolites, including acetaldehyde and ethanol. Acetaldehyde was then further dehydrogenised by alcohol dehydrogenase into ethanol (Iglesias *et al.*, 2018). A comparable outcome was documented in a study where pomegranate juice, fermented with immobilised *L. plantarum*, displayed an ethanol content of 1% (v/v) after 4 w of storage (Mantzourani *et al*., 2019). This was aligned with the heterofermentative nature of certain *Lactobacillus* spp. strains, which have been reported to ferment glucose into carbon dioxide, ethanol, lactic, and acetic acids (Szutowska, 2020). For ASEAN countries including Malaysia, the maximum permissible alcohol level for halal food products is only 1% (v/v) (Iglesias *et al.*, 2018). The ethanol content of FPD remained below the maximum permitted limit during 6 w of storage. However, the ethanol content in FPD exceeded the maximum permitted limit on Week 7, and reached up to approximately 1.2% (v/v) when the storage period came to an end, and hence, was no longer suitable for Muslim consumer consumption.

The analysed texture characteristics (cohesiveness, firmness, viscosity, and consistency) of FPD remained at a constant level until Week 4 of storage, and then decreased significantly ($p < 0.05$) (Figures 1g - 1j). The decrease might have been caused by the decreasing pH values that dropped below 4.0 (Figure 1e), which was widely considered unfavourable to the survival of probiotics, including *L. mali*, that produce exopolysaccharides (texturising stabilisers agents) (Barkallah *et al.*, 2017). The subsequent decrease in the texture properties could also be attributed to the enzymes released from lysed bacteria during storage (Figure 2) that resulted in an increase in the solubility and degradation of certain chemical components in FPD, such as fibres, starches, as well as pectin and non-starch polysaccharides (Do and Fan, 2019). Furthermore, the texture characteristics of FPD demonstrated a significantly ($p < 0.05$) higher index than its counterpart during the first 4 w of storage (Figures 1g - 1j). This could be probably due to the presence of active *L. mali* that were attached to protein matrices *via* the exopolysaccharides, resulting in an improved texture profile of FPD (Do and Fan, 2019). The texture profile of FPD was similar to the texture

profile of probiotic orange juice fermented with *L. paracasei* and supplemented with oligofructose reported in a previous study (da Costa *et al.*, 2017).

Organic acid and sugar contents

In the determination of organic acids, the results of HPLC showed that the two predominant organic acids present in FPD were lactic and acetic acids (Figures 1k - 1l), but they were not found in PD. The concentration of lactic and acetic acids in FPD increased significantly ($p < 0.05$) throughout storage. This supported the idea that *L. mali* synthesised lactic and acetic acids *via* carbohydrate metabolism during fermentation and storage (Kaprasob *et al.*, 2017), thus resulting in a lower pH value (Figure 1e). A congruent finding was documented in the study conducted by Mantzourani *et al*. (2022) on pomegranate juice fermentation using immobilised *L. plantarum*. The authors explained that during storage, LAB break down glucose and other carbohydrates present in pomegranate juice, resulting in the generation of lactic and acetic acids. The concentration of acetic acid was higher than the lactic acid content in FPD (Figures 1k - 1l). This could be attributed to the amino acid catabolism or lactic acid metabolism by the LAB (Dongmo Nsogning *et al.*, 2018).

Although pumpkins have a hypoglycaemic effect (Hosen *et al.*, 2021), high insulin-dependent sugar content in pumpkin fruits restricts its consumption among diabetic populations (Koretska *et al.*, 2020). The major sugars in PD and FPD were glucose, fructose, and sucrose (Figures 1m - 1o). FPD was observed to have significantly ($p < 0.05$) lower sugar contents than PD, and these decreased ($p < 0.05$) progressively throughout storage, indicating that most of the sugars were being consumed during fermentation and storage. The sucrose content in FPD was near to zero, indicating that sucrose was degraded *via* reciprocal transformation into glucose and fructose by enzymes (*e.g.* invertase) released by *L. mali*, and further utilised as a carbon source for bacterial growth (Ujiroghene *et al.*, 2019). Besides that, *L. mali* could have also utilised sucrose for extracellular polysaccharides production (Li and Gänzle, 2020), resulting in a greater extent of sucrose reduction (Figure 1m). Furthermore, the total sugar contents detected in FPD decreased to below 3 g/L (Figures 1m - 1o) after 5 w of storage, which might have caused the loss of viable cells (Figure 2). Survival rates of a probiotic strain are proposed to be dependent on the levels of sugar in probiotic foods (Nguyen *et al.*, 2019).

Antioxidant properties

Total phenolic, total flavonoid, chlorophyll, and carotenoid contents were significantly ($p < 0.05$) improved. RSA and ferric-reducing power were also enhanced (Figures 1p - 1u) with the incorporation of *L. mali* in FPD. The antioxidant profile of FPD was found to be comparable with those of yoghurt fortified with *Spirulina platensis* reported in a previous study (Barkallah *et al.*, 2017). The total antioxidant activity in fermented drinks is proposed to be dependent on the strain of bacteria utilised (Skrzypczak *et al.*, 2019). *L. mali* was able to release the antioxidative compounds that were bound to the dietary fibre through its microbial enzymatic activities (Christopher *et al.*, 2021), thus resulting in the increase in antioxidative capacities. Furthermore, carotenoids could be additionally synthesised by the LAB (Kaprasob *et al.*, 2017). The increase in the polyphenolic compounds after LAB fermentation have been reported in previous studies (Kaprasob *et al.*, 2017; Christopher *et al.*, 2021; Darvishzadeh *et al.*, 2021). Moreover, water kefir contained some LAB strains that can scavenge reactive oxygen species (Darvishzadeh *et al.*, 2021). These results were similar to our findings in this study.

The antioxidative properties in FPD were retained during the first initial storage period (Figures 1p - 1u). After 4 w of storage, FPD showed a gradual decrease ($p < 0.05$) in the antioxidant properties. PD, on the other hand, exhibited a sharp decrease in antioxidant characteristics throughout storage. A similar observation was reported by Hashemi *et al*. (2017), whereby their sweet lemon juice supplemented with *L. plantarum* also showed a decrease in the antioxidant properties after 4 w of refrigerated storage. FPD remained to possess over 50% RSA after 4 w of storage, demonstrating overall good free radical scavenging potential of FP (Kaprasob *et al.*, 2017). The maintenance of the antioxidant properties of FP was most likely due to the antioxidative compounds in pumpkin combining or binding with other components, such as sugar or amino acid, thus becoming more stable (Kaprasob *et al.*, 2017). Besides that, glass bottle packaging with reduced headspace and low temperature (4°C) during storage used in the present work might have also helped to conserve the antioxidant content (da Costa *et al.*, 2017). The stability in antioxidative properties

of FPD found in the present work was similar to those of chokeberry juice fermented by LAB, whereby no significant changes in the antioxidative properties during storage at 4°C were reported (Bontsidis *et al.*, 2021). The consequent decrease in these antioxidant properties after week 4 was potentially due to the loss of viable *L. mali* strain in FPD (Figure 2), which might possess the antioxidative ability as discussed earlier. Furthermore, the loss of antioxidants might also have been due to the possible detoxification mechanism of bacteria (*L. mali*) which produced enzymes that degraded simple monocyclic antioxidative compounds, resulting in lower antioxidant contents (Porto *et al.*, 2018; Thanh *et al.*, 2021). In addition, oxidation degradation of certain antioxidative compounds because of residual oxygen in the sample (*e.g.* oxidation and/or isomerisation of carotenoids) may also lead to a reduction in antioxidative properties (Kaprasob *et al.*, 2017).

Based on the results in Table 1, the major phenolic acids that have been detected in both PD and FPD in the first week of storage after production was chlorogenic, *p*-hydroxybenzoic, caffeic, *p*-coumaric, protocatechuic, and syringic acids. The obtained phenolic acid profile of PD and FPD was in accordance with those reported in pumpkin (Kulczyński and Gramza-Michałowska, 2019). FPD contained remarkably ($p < 0.05$) higher phenolic acid content compared to PD. This was most likely due to the breakdown of large polymeric phenolics by *L. mali* into simple monocyclic phenolics during fermentation (Christopher *et al.*, 2021).

Table 1. Phenolic acid profile of *L. mali* fermented drink obtained from pumpkin purée (FPD) and non-inoculated control (PD) on first week of storage after production.

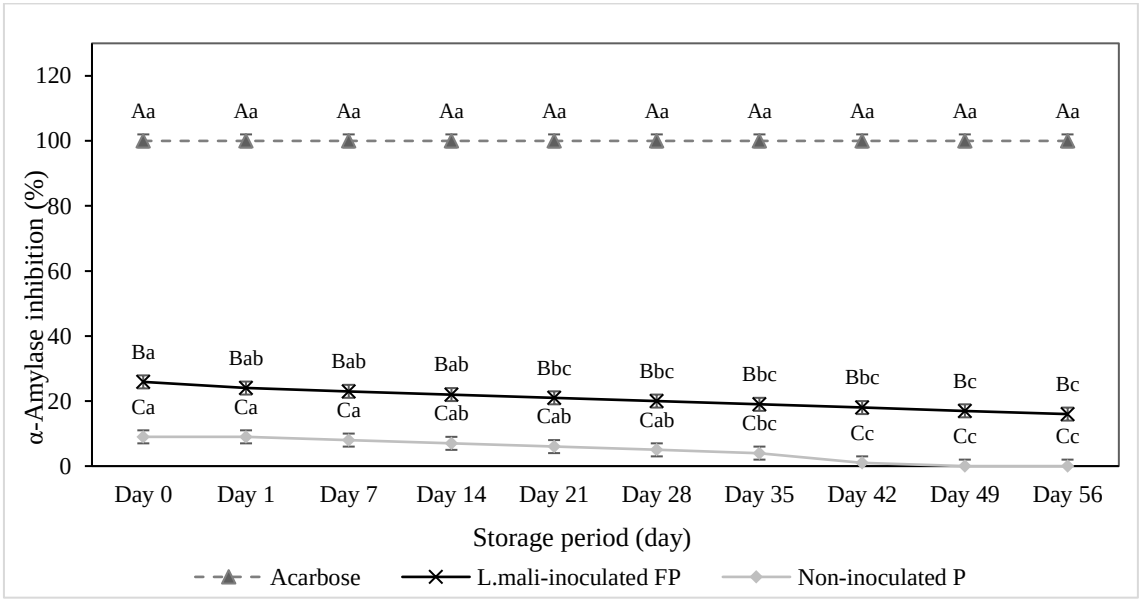
Phenolic compound	FPD	PD
Chlorogenic acid	18.41 ± 0.00^{a}	10.48 ± 0.00^{b}
Caffeic acid	2.33 ± 0.00^{a}	0.69 ± 0.10^{b}
p-Coumaric acid	1.27 ± 0.00^{a}	0.50 ± 0.00^{b}
Protocatechuic acid	11.31 ± 0.03^{a}	5.27 ± 0.05^{b}
Syringic acid	13.38 ± 0.00^{a}	7.13 ± 0.00^{b}
p-Hydroxybenzoic acid	15.49 ± 0.00^{a}	8.41 ± 0.04^{b}

Values are mean ± standard deviation of triplicate ($n = 3$), and expressed in mg/L. Means within a row followed by different lowercase superscripts are significantly different ($p < 0.05$).

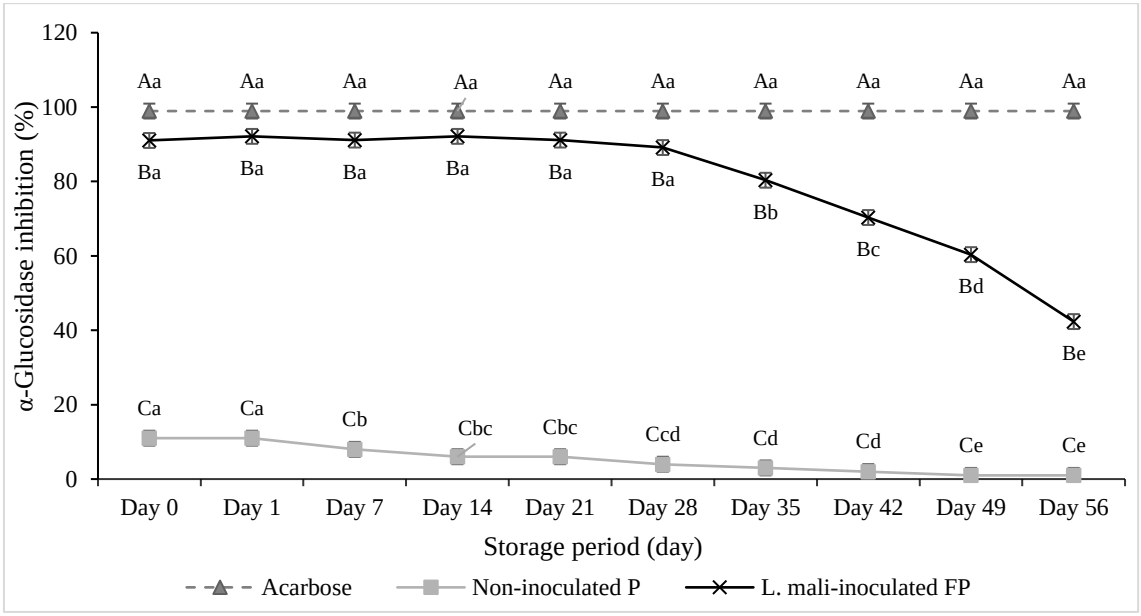
Anti-hyperglycaemic properties

The role of postprandial hyperglycaemia (PH) in T2DM is being increasingly recognised in the medical community. It is known that PH contributes to the increased risk of both micro- and macro-vascular complications in T2DM patients. Synthetic hypoglycaemic agents, such as acarbose, are often associated with adverse health effects. Therefore, researchers have been switching their research focus toward antidiabetic plants, such as pumpkins, as well as natural probiotics products, particularly those with strong α-glucosidase inhibition (Koh *et al.*, 2018a). In the digestion of carbohydrates, pancreatic α-amylase cleaves the α-1-4-glycosidic linkages to release oligosaccharides, whereas α-glucosidase hydrolyses terminal α-1-4-linked glucose to release glucose, into the small intestine (Yusuf *et al.*, 2021). By inhibiting the enzyme activities, the digestion and breakdown of carbohydrates can be slowed down, thus hampering the glucose absorption in the small intestine, which in turn helps to control PH linked to T2DM (Fujita *et al.*, 2017).

By comparing the FPD with its counterpart, the incorporation of *L. mali* significantly ($p < 0.05$) enhanced both the α-amylase and α-glucosidase inhibitory activities (Figures 3a - 3b). This enhancement was most likely due to the metabolic activities of *L. mali* that broke down and released the polysaccharides in pumpkins, which possess inhibitory effects on α-glucosidase enzymes (Koh *et al.*, 2018a; 2018b; Yusuf *et al.*, 2021). The polysaccharide extracted from pumpkins was found to improve blood glucose levels in diabetic mice, and demonstrated strong α-glucosidase inhibition (97.4%) (Thanh *et al.*, 2021). After being stored for 4 w, the α-glucosidase inhibitory activity of FPD was observed to decrease, and it was most likely due to the loss of viable *L. mali* cells (Figure 2). In addition, the decrease in the inhibition percentage of both enzymes in FPD could also have been due to the loss



(a)



(b)

Figure 3. Changes in percentage inhibition of **(a)** α-amylase and **(b)** α-glucosidase for acarbose (1 mM) for non-inoculated control (PD) and *L. mali*-inoculated fermented pumpkin drinks (FPD) during cold storage (4°C for 8 w). Means with standard deviation (error bars) in the different lines followed by different uppercase letters are significantly different at $p < 0.05$ between samples for the same storage time. Means with standard deviation in the same line followed by different lowercase letters are significantly different at $p < 0.05$ for each sample affected by the storage time.

of antioxidant compounds and capacities, which may have potential inhibitory effects (Figures 1p - 1u) as a detoxification mechanism for the bacteria (Kaprasob *et al.*, 2017).

Compared to PD, FPD retained marked inhibitory effects of α-glucosidase in the earliest stage (first 4 w) of storage (> 90%). However, it gradually decreased to moderate inhibition (42%) towards the end of storage (Week 8). This phenomenon suggested that FPD possessed effective anti-hyperglycaemic properties for 4 w. On the other hand, the inhibitory ability of FPD on α-amylase was weaker than on α-glucosidase, ranging between 16.2 - 25.9%. FPD demonstrated slightly stronger α-amylase inhibitory

activity compared to PD, and decreased slowly (but significantly, $p < 0.05$) during storage. Variations of α-amylase and α-glucosidase inhibition were likely dependent on the formation or degradation of simple phenolics with inhibitory activity (Agustinah *et al.*, 2020), and were characterised by their chemical structures and interactions with flavonoids (Fujita *et al.*, 2017). Phenolic compounds with anti-hyperglycaemic properties such as flavonols and flavones were reported to be in abundance in pumpkins (Agustinah *et al.*, 2020). Besides that, the increase in the amount of aglycones liberated by β-glucosidase activity, and the presence of caffeic and chlorogenic acids may also contribute to the inhibition of key enzymes linked to T2DM (Fujita *et al.*, 2017). The major phenolic compound detected in FPD, chlorogenic acid (Table 1), was also detected in pumpkin, and had been proven to be effective against α-glucosidase enzyme inhibitory activity by a non-competitive mechanism, reported in a previous study (Kulczyński and Gramza-Michałowska, 2019). Therefore, the synthesis of chlorogenic acid by LAB fermentation potentially contributed to the higher α-glucosidase inhibitory activity of FPD compared to that of PD.

Compared to a popular oral anti-hyperglycaemic agent, *i.e*. acarbose (100% inhibition), FPD demonstrated strong α-glucosidase inhibition (Figure 3b), but showed a mild α-amylase inhibitory effect (Figure 3a). Based on the findings, FPD appeared to have substantial potential to serve as a safe, and cost-effective therapy for PH linked T2DM with lesser side effects because most of the antidiabetic drugs with high α-amylase inhibition have unwanted effects. Therefore, this dietary strategy involving natural plant-based food and probiotics benefits not only helps to control our blood glucose but also reduces the dependency on synthetic drugs to counter the early stages of T2DM (Koh *et al.*, 2020).

Survival of L. mali in FPD under simulated gastrointestinal conditions

Probiotic cellular stress begins in the acidic environment of the stomach with a pH as low as 1.5, followed by the duodenum, where bile is released. In order for probiotic organisms to exert health benefits to the host, they must colonise the epithelium of the lower gastrointestinal (GI) tract (small intestine and colon) at an adequate amount (between 10^6 - 10^8 CFU/mL). Therefore, probiotic strains need to tolerate and survive the harsh GI conditions (da Silva *et al.*, 2021).

As observed in Figure 2a, FPD stored for not more than 4 w met this criterion as the *L. mali* population in FPD was maintained above 10^6 CFU/mL even after being exposed to SGC for a total of 6 h. However, the *L. mali* viable cell counts decreased significantly ($p < 0.05$) after 4 w of storage, whereby it was observed that the survival of *L. mali* was badly affected, and the viable cell count was below 10^6 CFU/mL after the SGC treatment. Based on these two findings, it can be suggested that FPD possessed only 4 w of probiotic effectiveness. The loss of viable cells after 5 w of storage was most likely due to most of the sugars (carbon source for *L. mali*) in FPD had been consumed (Figures 1m - 1o).

In addition, *L. mali* population was reduced to a greater extent within the enteric transition than the gastric model in the present work. This could be due to the prebiotic polysaccharides and high fibre content in pumpkins that act as a protection for the *L. mali* viability during gastric transit, as well as the high acid-tolerant feature of *L. mali* strain (Koh *et al.*, 2018a; 2018b). In an acidic condition (pH < 4.5), higher energy is required for probiotic cells to keep their intracellular pH constant. As a result, it is often associated with the shortage of ATP for other critical cellular functions and in turn, leads to cellular apoptosis. Cellular homeostasis was disrupted upon bile exposure, which subsequently caused the disintegration of the lipid bilayer and integral protein of the cell membranes, thus resulting in the leakage of cell contents and cellular apoptosis (Dawood *et al.*, 2018). Overall, based on the survival under SGC, FPD was a suitable carrier of *L. mali* (Koh *et al.*, 2018a; 2018b). In general, *L. mali* strain in FPD was conserved at a constant survival rate during the first 4 w of storage, but demonstrated significant ($p < 0.05$) decrease after Week 4 (Figure 2b).

Microbiological quality

The microbiological quality test of FPD was conducted before the sensory analysis to ensure the product is safe for consumption by the sensory panellists. Within the storage period, neither yeast, mould, nor coliform bacteria were observed in any of the FPD samples. This showed that the storage at 4°C for 8 w did not affect the quality of the FPD ,and it was still safe to be consumed. Based on these results, we can also infer that the FPD was processed in a hygienic and sanitary manner (Barkallah *et al.*, 2017).

Sensory evaluation of FPD

The sensory assessment of FPD during storage was conducted using the hedonic test. As shown in Figure 4, the scores for all sensory attributes for FPD stored for 8 w were significantly the lowest, *i.e.* ≤ 2.5 (defined as unacceptable) (Valero-Cases and Frutos, 2017), indicating that the shelf life for FPD is 7 w at 4°C. As evidenced by the overall acceptability score, with a hedonic score near 7 on a 7-point scale, the sensory panellists liked the FPD sample very much during the first 4 w of storage. For FPD stored for 7 and 6 w, the panellists neither liked nor disliked the FPD sample (hedonic value near 4). Subsequently, the panellists disliked the FPD sample (hedonic value dropped to about 3.0) stored for 7 w, and very much disliked the FPD sample stored for 8 w (hedonic score dropped below 2.5). Some of the components in FPD could be metabolised by *L. mali*, and this could have negatively affected the aroma and flavour of FPD, which was characterised as "acid", "bitter", or even "astringent" (Cai *et al.*, 2019).

In addition, the colour of the fermented drink is one of the main quality attributes which influence consumer acceptance (da Costa *et al.*, 2017). The appearance (colour) score decreased gradually after 4 w of storage, which might consequently, negatively affect the overall acceptance of the sensory panellists on the product (Figure 4). The reduction in the appearance score of the FPD due to the increase in the storage duration was supported by the instrumental colour analysis data which indicated the decolourisation in FPD during storage (Figures 1a - 1c). Sugars and organic acids may have contributed to the taste and flavour profile of FPD, which may have enhanced the sensorial acceptance (de Oliveira *et al.*, 2017). However, the acidity can alter the sensory characteristics of the product, as well as decrease the viability of the *L. mali* (de Oliveira *et al.*, 2017). There were numerous factors that contributed to the reduction in sensory acceptance, including the depletion in sugars content in FPD (Figures 1m - 1o), accumulation of organic acids (Figures 1k - 1l), and reduction in pH values (Figure 1e), as a result of post-acidification during storage. Firmer, more consistent, viscous, and cohesive are considered good qualities of fermented drinks, as these textural properties are more accepted and desired by consumers (de Oliveira *et al.*, 2017). Therefore, the reduction in the FPD acceptance may also be related to the loss of texture characteristics, as shown in Figures 1g - 1j.

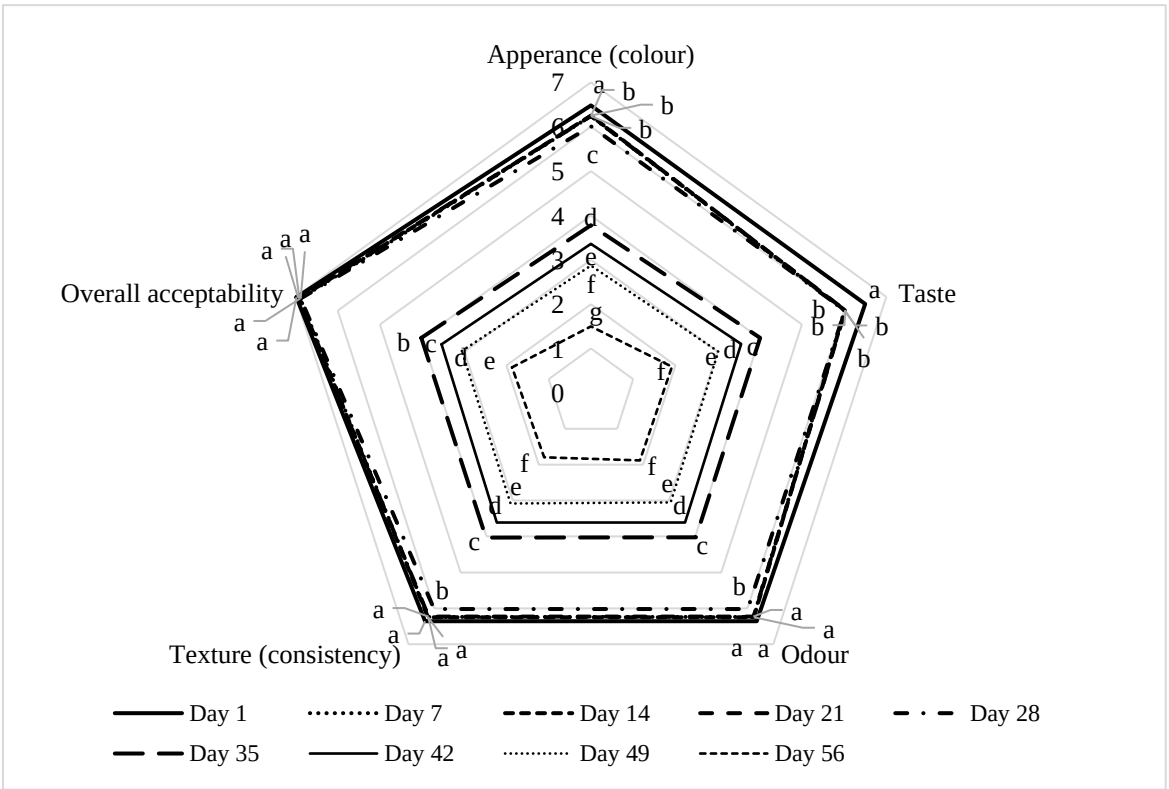


Figure 4. Changes in sensory attributes of *L. mali*-inoculated fermented pumpkin drink (FPD) during cold storage (4°C for 8 w). Means followed by different lowercase letters are significantly different at $p < 0.05$ affected by the storage time.

Conclusion

The fermentation of pumpkin purée with *Lactobacillus mali* was successful in creating a functional beverage, FPD, that maintained its quality for 28 days under refrigerated conditions (4°C). Throughout this storage period, FPD exhibited stability in terms of pH, texture attributes (cohesiveness, firmness, viscosity, and consistency), antioxidant properties, and α-glucosidase inhibitory effects (> 90%). The organoleptic qualities of FPD remained acceptable, and its probiotic counts remained consistent without any contamination from foodborne pathogens. Considering these promising attributes, FPD could be a potential anti-hyperglycaemic beverage, offering a viable option for managing early-stage type 2 diabetes mellitus (T2DM). Further investigations into its long-term stability, efficacy, and clinical applications are warranted to fully unlock its therapeutic potential, and expand its impact on public health.

Acknowledgement

The present work was financially supported by grants: SLB2228, SBK0503 and DN21099provided by Universiti Malaysia Sabah (UMS), Malaysia; extended sincere gratitude to Dr. Norliza binti Julmohammad from UMS.

References

Agustinah, W., Joshua, M. and Shetty, K. 2020. Lactic acid fermentation and bioactive enrichment of functional ingredients in fruits. In Shetty, K. and Sarkar, D. (eds). Functional Foods and Biotechnology - Biotransformation and Analysis of Functional Foods and Ingredients, p. 57-76. United States: CRC Press.

Bakirci, S., Dagdemir, E., Boran, O. S. and Hayaloglu, A. A. 2017. The effect of pumpkin fibre on quality and storage stability of reduced-fat set-type yogurt. International Journal of Food Science and Technology 52(1): 180-187.

Barkallah, M., Dammak, M., Louati, I., Hentati, F., Hadrich, B., Mechichi, T., … and Abdelkafi, S. 2017. Effect of *Spirulina platensis* fortification on physicochemical, textural, antioxidant and sensory properties of yogurt during fermentation and storage. LWT – Food Science and Technology 84: 323-330.

Bontsidis, C., Mallouchos, A., Terpou, A., Nikolaou, A., Batra, G., Mantzourani, I., … and Plessas, S. 2021. Microbiological and chemical properties of chokeberry juice fermented by novel Lactic acid bacteria with potential probiotic properties during fermentation at 4°C for 4 weeks. Foods 10(4): 768.

Cai, W., Tang, F., Zhao, X., Guo, Z., Zhang, Z., Dong, Y. and Shan, C. 2019. Different lactic acid bacteria strains affecting the flavor profile of fermented jujube juice. Journal of Food Processing and Preservation 43(9): e14095.

Christopher, A., Sarkar, D. and Shetty, K. 2021. Improving phenolic-linked antioxidant, antihyperglycemic and antibacterial properties of emmer and conventional wheat using beneficial lactic acid bacteria. Applied Microbiology 1(2): 270-288.

da Costa, G. M., de Carvalho Silva, J. V., Mingotti, J. D., Barão, C. E., Klososki, S. J. and Pimentel, T. C. 2017. Effect of ascorbic acid or oligofructose supplementation on *L. paracasei* viability, physicochemical characteristics and acceptance of probiotic orange juice. LWT – Food Science and Technology 75: 195-201.

da Silva, M. N., Tagliapietra, B. L. and dos Santos Richards, N. S. P. 2021. Encapsulation, storage viability, and consumer acceptance of probiotic butter. LWT – Food Science and Technology 139: 110536.

Darvishzadeh, P., Orsat, V. and Martinez, J. L. 2021. Process optimization for development of a novel water kefir drink with high antioxidant activity and potential probiotic properties from Russian olive fruit (*Elaeagnus angustifolia*). Food and Bioprocess Technology 14(2): 248-260.

Dawood, M. A., Koshio, S. and Esteban, M. Á. 2018. Beneficial roles of feed additives as immunostimulants in aquaculture - A review. Reviews in Aquaculture 10(4): 950-974.

de Oliveira, A., da Silva, I., Dionísio, J., Klososki, S., Januário, J., Pimentel, T. and de Oliveira, J. 2017. Probiotic yoghurt flavored with organic beet with carrot, cassava, sweet potato or corn juice: Physicochemical and texture evaluation, probiotic viability and acceptance. International Food Research Journal 24(1): 359-366.

Delgado, S., Guadamuro, L., Flórez, A. B., Vázquez, L. and Mayo, B. 2019. Fermentation of commercial soy beverages with lactobacilli and bifidobacteria strains featuring high β-glucosidase activity. Innovative Food Science and Emerging Technologies 51: 148-155.

Do, T. V. T. and Fan, L. 2019. Probiotic viability, qualitative characteristics, and sensory acceptability of vegetable juice mixture fermented with *Lactobacillus* strains. Food and Nutrition Sciences 10(4): 412.

Dongmo Nsogning, S., Kollmannsberger, H., Fischer, S. and Becker, T. 2018. Exploration of high-gravity fermentation to improve lactic acid bacteria performance and consumer's acceptance of malt wort-fermented beverages. International Journal of Food Science and Technology 53(7): 1753-1759.

Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO). 2002. Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report. Retrieved on 16 October 2021 from website: http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm

Fujita, A., Sarkar, D., Genovese, M. I. and Shetty, K. 2017. Improving anti-hyperglycemic and anti-hypertensive properties of camu-camu (*Myriciaria dubia* Mc. Vaugh) using lactic acid bacterial fermentation. Process biochemistry 59: 133-140.

Hashemi, S. M. B., Khaneghah, A. M., Barba, F. J., Nemati, Z., Shokofti, S. S. and Alizadeh, F. 2017. Fermented sweet lemon juice (*Citrus limetta*) using *Lactobacillus plantarum* LS5 - Chemical composition, antioxidant and antibacterial activities. Journal of Functional Foods 38: 409-414.

Hosen, M., Rafii, M. Y., Mazlan, N., Jusoh, M., Oladosu, Y., Chowdhury, M., … and Khan, M. M. H. 2021. Pumpkin (*Cucurbita* spp.): A crop to mitigate food and nutritional challenges. Horticulturae 7(10): 352.

Iglesias, M., Echeverría, G., Viñas, I., López, M. and Abadias, M. 2018. Biopreservation of fresh-cut pear using *Lactobacillus rhamnosus* GG and effect on quality and volatile compounds. LWT – Food Science and Technology 87: 581-588.

Kaprasob, R., Kerdchoechuen, O., Laohakunjit, N., Sarkar, D. and Shetty, K. 2017. Fermentation-based biotransformation of bioactive phenolics and volatile compounds from cashew apple juice by select lactic acid bacteria. Process Biochemistry 59: 141-149.

Kiharason, J. W. and Isutsa, D. K. 2019. Shelf-life of pumpkin fruit slices, flour and blended products. International Journal of Food Science and Biotechnology 4(1): 14.

Koh, H. S. A., Lu, J. and Zhou, W. 2020. Structural dependence of sulfated polysaccharide for diabetes management: Fucoidan from *Undaria pinnatifida* inhibiting α-glucosidase more strongly than α-amylase and amyloglucosidase. Frontiers in Pharmacology 11: 831.

Koh, W. Y., Utra, U., Ahmad, R., Rather, I. A. and Park, Y.-H. 2018b. Evaluation of probiotic potential and anti-hyperglycemic properties of a novel *Lactobacillus strain* isolated from water kefir grains. Food Science and Biotechnology 27(5): 1369-1376.

Koh, W., Uthumporn, U., Rosma, A., Irfan, A. and Park, Y. 2018a. Optimization of a fermented pumpkin-based beverage to improve *Lactobacillus mali* survival and α-glucosidase inhibitory activity: A response surface methodology approach. Food Science and Human Wellness 7(1): 57-70.

Koretska, I., Polyovyk, V., Maslikov, M. and Kuzmin, O. 2020. Thermophysical characteristics of frozen semi-finished products for restaurant technology. Ukrainian Journal of Food Science 8: 231-240.

Kulczyński, B. and Gramza-Michałowska, A. 2019. The profile of secondary metabolites and other bioactive compounds in *Cucurbita pepo* L. and *Cucurbita moschata* pumpkin cultivars. Molecules 24(16): 2945.

Li, Q. and Gänzle, M. G. 2020. Host-adapted lactobacilli in food fermentations - Impact of metabolic traits of host adapted lactobacilli on food quality and human health. Current Opinion in Food Science 31: 71-80.

Linares-Morales, J. R., Gutiérrez-Méndez, N., Rivera-Chavira, B. E., Pérez-Vega, S. B. and Nevárez-Moorillón, G. V. 2018. Biocontrol processes in fruits and fresh produce, the use of lactic acid bacteria as a sustainable option. Frontiers in Sustainable Food Systems 2: 50.

Managa, M. G., Akinola, S. A., Remize, F., Garcia, C. and Sivakumar, D. 2021. Physicochemical

parameters and bioaccessibility of lactic acid bacteria fermented chayote leaf (*Sechium edule*) and pineapple (*Ananas comosus*) smoothies. Frontiers in Nutrition 8: 649189.

Mantzourani, I., Terpou, A., Alexopoulos, A., Kimbaris, A., Bezirtzoglou, E., Koutinas, A. A. and Plessas, S. 2019. Production of a potentially synbiotic pomegranate beverage by fermentation with *Lactobacillus plantarum* ATCC 14917 adsorbed on a prebiotic carrier. Applied biochemistry and biotechnology 188: 1096-1107.

Mantzourani, I., Terpou, A., Bekatorou, A. and Plessas, S. 2022. Valorization of lactic acid fermentation of pomegranate juice by an acid tolerant and potentially probiotic LAB isolated from kefir grains. Fermentation 8(4): 142.

Miljić, M., Rocchetti, G., Krstić, S., Mišan, A., Brdar-Jokanović, M., Marcheggiani, F., … and Damiani, E. 2021. Comparative *in vitro* antioxidant capacity and terpenoid profiling of pumpkin fruit pulps from a Serbian *Cucurbita maxima* and *Cucurbita moschata* breeding collection. Antioxidants 10(10): 1580.

Min, M., Bunt, C. R., Mason, S. L. and Hussain, M. A. 2019. Non-dairy probiotic food products: An emerging group of functional foods. Critical Reviews in Food Science and Nutrition 59(16): 2626-2641.

Nagappan, H., Pee, P. P., Kee, S. H. Y., Ow, J. T., Yan, S. W., Chew, L. Y. and Kong, K. W. 2017. Malaysian brown seaweeds *Sargassum siliquosum* and *Sargassum polycystum*: Low density lipoprotein (LDL) oxidation, angiotensin converting enzyme (ACE), α-amylase, and α-glucosidase inhibition activities. Food Research International 99: 950-958.

Nguyen, B. T., Bujna, E., Fekete, N., Tran, A., Rezessy-Szabo, J. M., Prasad, R. and Nguyen, Q. D. 2019. Probiotic beverage from pineapple juice fermented with *Lactobacillus* and *Bifidobacterium* strains. Frontiers in Nutrition 6: 54.

Porto, M. R. A., Okina, V. S., Pimentel, T. C., Garcia, S. and Prudencio, S. H. 2018. Beet and orange mixed juices added with *Lactobacillus acidophilus*. Nutrition and Food Science 48(1): 76-87.

Setyawardani, T., Sumarmono, J. and Widayaka, K. 2019. Effect of cold and frozen temperatures on artisanal goat cheese containing probiotic lactic acid bacteria isolates (*Lactobacillus plantarum* TW14 and *Lactobacillus rhamnosus* TW2). Veterinary World 12(3): 409.

Skrzypczak, K., Gustaw, W., Kononiuk, A., Sołowiej, B. and Waśko, A. 2019. Estimation of the antioxidant properties of milk protein preparations hydrolyzed by *Lactobacillus helveticus* T80, T105 and B734. Czech Journal of Food Sciences 37(4): 260-267.

Sun, X., Zhang, Y., Li, F., Jiao, X., Ma, D., Zhang, L., … and Li, Q. 2022. Effects of lactic acid bacteria fermentation on chemical compounds, antioxidant capacities and hypoglycemic properties of pumpkin juice. Food Bioscience 50: 102126.

Syahariza, Z. A., Torkamani, A. E., Norziah, H. M., Mahmood, W. A. K. and Juliano, P. 2017. Optimisation of pressurised liquid extraction for antioxidative polyphenolic compound from *Momordica charantia* using response surface methodology. International Journal of Food Science and Technology 52(2): 480-493.

Szutowska, J. 2020. Functional properties of lactic acid bacteria in fermented fruit and vegetable juices: A systematic literature review. European Food Research and Technology 246(3): 357-372.

Thanh, T. T. T., Quach, T. T. M., Yuguchi, Y., Nguyen, N. T., Van Ngo, Q., Van Bui, N., … and Ho, C. D. 2021. Molecular structure and anti-diabetic activity of a polysaccharide extracted from pumpkin *Cucurbita pepo*. Journal of Molecular Structure 1239: 130507.

Ujiroghene, O. J., Liu, L., Zhang, S., Lu, J., Zhang, C., Pang, X. and Lv, J. 2019. Potent α-amylase inhibitory activity of sprouted quinoa-based yoghurt beverages fermented with selected anti-diabetic strains of lactic acid bacteria. RSC Advances 9(17): 9486-9493.

Umavathi, S., Keerthika, M., Gopinath, K., Kavitha, C., Romij Uddin, M., Alagumanian, S. and Balalakshmi, C. 2021. Optimization of aqueous-assisted extraction of polysaccharides from pumpkin (*Cucurbita moschata* Duch) and their biological activities. Saudi Journal of Biological Sciences 28(12): 6692-6700.

Valero-Cases, E. and Frutos, M. J. 2017. Development of prebiotic nectars and juices as potential substrates for *Lactobacillus*

acidophilus: Special reference to physicochemical characterization and consumer acceptability during storage. LWT – Food Science and Technology 81: 136-143.

Xia, T. and Wang, Q. 2006. D-chiro-Inositol found in *Cucurbita ficifolia* (Cucurbitaceae) fruit extracts plays the hypoglycaemic role in streptozocin-diabetic rats. Journal of Pharmacy and Pharmacology 58(11): 1527-1532.

Yusuf, D., Nuraida, L., Dewanti-Hariyadi, R. and Hunaefi, D. 2021. *In vitro* antioxidant and α-glucosidase inhibitory activities of *Lactobacillus* spp. isolated from Indonesian kefir grains. Applied Food Biotechnology 8(1): 39-46.