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Assessment of yeast, acetic and lactic acid bacteria isolated from water kefir grains and their application as starter culture in the production of fermented pumpkin-based water kefir beverages in improving gastrointestinal tract digestive tolerance and inhibition against α-glucosidase

[1]Koh, W. Y., [1*]Uthumporn, U., [2]Rosma, A., [1]Effarizah, M. E. and [3]Wan Rosli, W. I.

[1]Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[2]Bioprocess Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[3]School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, 16150 Kota Bharu, Kelantan, Malaysia

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Abstract

Water kefir is the product of a brown sugar solution fermented with water kefir grains. To produce starter cultures for manufacturing commercial water kefir on an industrial scale, six lactic acid bacteria (LAB), three acetic acid bacteria (AAB), and two yeast strains have been isolated from local water kefir grains, and *in vitro* α-glucosidase inhibitory activity has been investigated. Five of the 11 isolates (LAB [K65, K9, K67], AAB [A5], and yeast [Y6]) showed high α-glucosidase inhibitory activity. They were then tested in an *in vitro* gastrointestinal tract tolerance test in which all demonstrated tolerance (40–80% survival rate). These five strains, identified as *Lactobacillus mali*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Gluconobacter hansenii* and *Saccharomyces cerevisiae*; were then used to ferment water kefir beverages. The storage stability of the fermented beverage products was analysed during 28 days of storage at 4°C by measuring α-glucosidase inhibitory activity, microbial cell viability, and microbial survival in the beverage treated with simulated gastric juice. The water kefir beverage sample containing both pumpkin purée and brown sugar fermented using all five strains (10^9 CFU/mL LAB strains, 10^7 CFU/mL yeast strain, and 10^6 CFU/mL AAB strain) was the most suitable formulation because it achieved >50% α-glucosidase inhibitory activity and microbial survival rates of 10^6–10^{10} CFU/mL viable LAB cells, 10^6–10^7 CFU/mL viable AAB cells, and 10^5–10^8 CFU/mL viable yeast cells during storage. Therefore, these five strains have potential to act as starters in the formulation of water kefir, which is an anti-hyperglycaemic beverage that can be used to manage type-2 diabetes mellitus.

Introduction

The role of postprandial hyperglycaemia in type-2 diabetes mellitus (T2D) has been widely recognised. Postprandial hyperglycaemia increases micro- and macrovascular complication risks in T2D patients (Sudhir and Mohan, 2002; van Dijk *et al*., 2011). To manage T2D, synthetic drugs such as acarbose and miglitol are commonly used to reduce blood glucose by inhibiting enzymes from hydrolysing carbohydrate (Dada *et al*., 2016). However, their consumption at relatively high doses can be accompanied by undesirable side effects such as flatulence, diarrhoea and abdominal dilatation. Therefore, an alternative pharmaco-nutritional strategy is needed (Dada *et al*., 2016). Shori and Baba (2013) reported that herb-based yogurt fermented by a mixture of probiotic bacteria was effective at inhibiting the carbohydrate digestive enzyme α-glucosidase, which could serve as an alternative to current drug treatments with higher safety index in the form of functional food to control postprandial hyperglycaemia in T2D. However, probiotic product development requires selection of a compatible probiotic strain or food matrix in order to maintain high cell viability in the product (at least 10^6 CFU/mL) (Muganga *et al*., 2015) so that the probiotic strains can provide health benefits to the hosts.

*Corresponding author.
Email:sapina@usm.my

Pumpkin (*Cucurbita pepo* L.) is nutrient dense and has been used in the treatment of diabetes in many countries (Sedigheh *et al*., 2011). Gutierrez (2016) reported that *C. pepo* possesses anti-diabetic activity, which is likely due to mechanisms such as stimulation of pancreatic β-cell proliferation and inhibition of α-glucosidase activity. Pumpkin has been reported to be a good probiotic delivery vehicle, as its fibre and oligosaccharide content can protect probiotics from adverse food processing and digestive tract conditions (Du *et al*., 2011; Zhao *et al*., 2015; Genevois *et al*., 2016). Furthermore, Genevois *et al*. (2016) found that pumpkin fermented with probiotic-supplemented starter culture demonstrated improved probiotic cell viability and survival in simulated gastrointestinal tract conditions.

The importance of non-dairy probiotic products is increasing worldwide due to lactose intolerance and a growing vegan population (Corona *et al*., 2016). Water kefir is a dairy-free beverage produced by fermenting water kefir grains consisting of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Randazzo *et al*., 2016). Certain strains of bacteria and yeasts isolated from water kefir grains have been recognised as probiotics, and their components have been reported to have hypoglycaemic effects in experimental diabetic rats (Alsayadi *et al*., 2014; Zanirati *et al*., 2015). Hence, a mixture of microorganisms isolated from water kefir grains would allow the formulation of a novel uniform quality product with anti-hyperglycaemic benefit.

To the best of our knowledge, the use of yeast, LAB, and AAB strains isolated from water kefir grains in fermentation of pumpkin-based beverages and the anti-hyperglycaemic potential of such beverages remain underexplored. Therefore, the present work was undertaken to assess the inhibitory potential of yeast, LAB, and AAB strains isolated from water kefir grains against α-glucosidase and the gastrointestinal tract tolerance of these strains. Selected water kefir starter cultures were further used to produce pumpkin-based beverages, and their α-glucosidase inhibition, gastrointestinal tract tolerance, and cell viability were measured throughout a refrigerated storage (4°C) period of 28 days.

Materials and methods

Enzymes

Pepsin from porcine stomach mucosa (Sigma-Aldrich, St Louis, MO, USA; ≥250 units/mg), trypsin from porcine pancreas (Sigma-Aldrich; >10,000 units/mg), and α-glucosidase from *Saccharomyces cerevisiae* Type I (Sigma-Aldrich; ≥10 units/mg) were used.

Microorganisms and culture media

Water kefir grains collected from a cottage industry in Kuala Lumpur, Malaysia (My Kefir World) were used. The water kefir grains were generated by inoculating them in brown sugar water (10% w/v) at 25°C for 72 h, and this procedure was repeated twice. The grains were filtered from the fermented brown sugar water using a plastic kitchen sieve and rinsed with distilled water. Water kefir grains (16 g) were blended with 90 mL sodium chloride (NaCl) solution (0.85% m/v) in a sterile stomacher bag for 5 min. Subsequently, serial 10-fold dilutions were made using phosphate-buffered saline (PBS), and 100 µL of each 10^{-6} and 10^{-7} dilution were separated on their respective agar medium and incubated for 72 h. LAB were grown in De Man-Rogosa-Sharpe agar (MRS; Merck, Darmstadt, Germany) at 37°C, AAB were grown in *Acetobacter* (glucose) agar (AB; HiMedia, Mumbai, India) at 37°C, and yeast were grown in malt yeast agar (YPG; HiMedia) at 28°C. In total, six LAB, three AAB, and two yeast strains were isolated and successively streaked on their respective agar medium three times until a uniform colony was obtained. The LAB, AAB, and yeast isolates were stored at –80°C in the respective MRS broth (MRSBr; Merck) at 37°C, AB broth (ABBr; HiMedia) at 37°C, and YPG broth (YPGBr; HiMedia) at 28°C, and they were sub-cultured every six months. Before every experiment, isolates from frozen stocks were sub-cultured at least once in the respective aforementioned broth at the respective temperature for 24–48 h.

Preparation of microbial cell-free extract and cell-free supernatant

The overnight pure cultures of all 11 isolated microbial strains were centrifuged (12,000 g, 15 min, 4°C), and the pH of the supernatant was adjusted to 7.4 using sodium hydroxide and hydrochloric acid. They were then filtered to obtain the cell-free supernatant (CFS) using a 0.22 µm filter that was kept in an ice bath. The intact cells were washed in PBS three times. The pellet was resuspended in the same buffer and adjusted to 1.5×10^8 CFU/mL using the McFarland standards. Cells were disrupted by ultrasonication for 30 min at 4°C in an ice bath (Elmasonic S 60 (H), Elma, Singen, Germany) to obtain intracellular cell-free extracts (CFE). The cell fractions were removed by centrifugation (12,000 g, 15 min). The clear supernatant was filter-sterilised with a 0.22 µm filter membrane (Zeng *et al*., 2016).

α-glucosidase inhibition by LAB, AAB, and yeast strains

The α-glucosidase inhibitory activities of the CFS and CFE from all 11 isolated microbial strains were determined according to the modified method from Ankolekar *et al*. (2012). The reaction mixture consisted of 100 µL of 1 unit/mL α-glucosidase in 0.1 M phosphate buffer (pH 7.4) and 50 µL of sample supernatants/extracts. The mixture was pre-incubated at 37°C for 10 min. Next, 5 mM p-Nitrophenyl-α-D-glucopyranoside solution (Sigma-Aldrich) (50 µL) in phosphate-buffer (0.1 mol/L, pH 7.4) was added. Subsequently, the mixture was incubated (37°C, 10 min). The reaction system with 50 µL buffer solution instead of the sample was used as the control. Before and after incubation, absorbance at 405 nm was measured using a UV visible spectrophotometer (UV-1700; Shimadzu Co., Kyoto, Japan), and inhibition (%) was calculated as $(A_c - A_s) / A_c \times 100\%$, where A_c was the absorbance of the control and A_s was the absorbance of the test sample.

Tolerance of LAB, AAB, and yeast strains to simulated gastrointestinal tract

Three LAB, one AAB, and one yeast strains were selected due to their better α-glucosidase inhibitory activities as compared to the other six strains. These five selected strains were tested for their *in vitro* gastrointestinal tract tolerance following the modified method of Casarotti and Penna (2015). Simulated gastric juice (pH 2.5), duodenal juice (pH 5.0), and intestinal juice (pH 8.0) were prepared as described in Muganga *et al*. (2015). The cells were harvested from overnight cultures by centrifugation (804 g, 4°C, 15 min). The cells were washed twice with an equal volume of sterile PBS (pH 7.4), and resuspended in simulated gastric juice (pH 2.5). Determination of total viable cell counts was performed after 0 and 3 h of incubation in simulated gastric juice at 37°C, after which 1 mL cell suspension was transferred into 9 mL simulated duodenal juice and incubated (37°C, 2 h). After duodenal juice digestion, the cell suspensions (1 mL) were transferred into 9 mL simulated intestinal juice and incubated at 37°C for 8 h. After the intestinal juice digestion, cell viability was determined. One mL aliquot was serially diluted in a sterile saline solution (0.85% NaCl) and plated on the respective aforementioned agar and incubated at the respective temperature for 72 h. The microbial tolerance in simulated gastric, duodenal, and intestinal juice were expressed as survival (%) and calculated as (β/α) 100, where α was the $\log_{10}$ colony forming units (CFU)/mL of the assayed strain before treatment, and β was the $\log_{10}$ CFU/mL of the same strain after incubation.

Identification of the selected strains

Deoxyribonucleic acid (DNA) from the pure selected five strains was extracted using the NucleoSpin tissue kit (Machery-Nagel, Duren, Germany) following the protocols specified by the manufacturer. The amount and purity of the extracted DNA were determined spectrophotometrically. The pure DNA was sent to the Centre for Chemical Biology, Universiti Sains Malaysia, Penang, Malaysia to be identified by 16S and 18S rRNA sequencing and comparison with the BLAST database using a sequence matching program.

Preparation of pumpkin purée

Fresh pumpkins were purchased from a local farm (Balik Pulau, Penang, Malaysia). The voucher specimens were deposited at the Herbarium, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia under the number 11598. The pumpkin was processed into purée following the method described by Koh *et al*. (2017a) and then packed in sterile stomacher bags (50 g/bag) and immediately stored at –20°C. The purée was stored until further use for the preparation of beverage samples after 24 h of thawing.

Preparation of water kefir beverages

To prepare each inoculum, colonies from cultured MRS, AB, and YPG agar plates were transferred to sterile 10% (w/v) filter-sterilised brown sugar water and incubated at 37°C for bacteria and at 28°C for yeast, for 18–24 h. Viable cells of the pure cultures were enumerated and used as the inoculum to prepare water kefir beverage samples at the following concentrations: 10^9 CFU/mL LAB strains; 10^7 CFU/mL yeast strain; 10^6 CFU/mL AAB strain.

These five strains were combined to form starter cultures of water kefir by taking 1 mL from each starter culture and combining them into a total volume of 5 mL. They were then inoculated into 100 mL formulated water kefir (WK) beverage samples (WKPP [pumpkin purée], WKBS [brown sugar], and WKBSPP [brown sugar and pumpkin purée]) in formulations defined in Table 1. Glass bottles of each sample were incubated at 32°C for 24 h. The strain combination, fermentation ingredients, and fermentation conditions applied were based on the preliminary optimisation tests conducted previously using the response surface methodology approach (Koh *et al*., 2017a). After 24 h incubation, the beverage samples were kept at 4°C for further analysis.

Table 1. Experimental treatments of water kefir beverages preparation.

Treatments	Inoculum	Water kefir (WK) beverages formulation of the substrates
1	*Lactobacillus mali* K65, *Lactobacillus casei* K9, *Leuconostoc mesenteroides* K67, *Gluconobacter hansenii* A5, and *Saccharomyces cerevisiae* Y6	10% w/v brown sugar (WKBS)
		20% w/v pumpkin purée (WKPP)
		20% w/v pumpkin purée + 10% w/v brown sugar (WKBSPP)
2	Non-inoculated	10% w/v brown sugar (BSNIC)
		20% w/v pumpkin purée (PPNIC)
		20% w/v pumpkin purée + 10% w/v brown sugar (BSPPNIC)

Cell viability characterization of water kefir beverages during storage

Cell viability in water kefir beverage samples (WKPP, WKBS, and WKBSPP) prepared according to the formulation in Table 1 were analysed after 0, 2, 7, 14, 21, and 28 days of storage at 4°C. The numbers of viable LAB, AAB, and yeast cells in the beverage samples were determined by the spread plate technique using MRS, AB and YPG agar, respectively. The viable cells were enumerated after incubation at their respective temperature for 72 h. Viable cell counts were expressed as $\log_{10}$ CFU/mL.

Survival of microbial populations in water kefir beverages treated with simulated gastric juice during storage

Tolerance of the selected microbial strains in water kefir beverage samples to simulated gastric juice was determined on days 0, 2, 7, 14, 21, and 28 of storage at 4°C following the procedures described above in the '*Tolerance of LAB, AAB, and yeast strains to simulated gastrointestinal tract'* section.

α-glucosidase inhibition in water kefir beverages during storage

The fermentation broth of the water kefir samples (WKPP, WKBS, and WKBSPP) and non-inoculated (NIC) samples (PPNIC, BSNIC, BSPPNIC) stored for 0, 2, 7, 14, 21, and 28 days at 4°C were centrifuged (5,939 g, 10 min, 4°C). The pH of the supernatants was adjusted (pH 7.4), and the samples were centrifuged again (13,362 g, 10 min, 4°C). The supernatants were then filter-sterilised through a 0.22 μm filter membrane to obtain CFS. Percentage inhibition of α-glucosidase by CFS-WK and CFS-NIC samples was determined as described above in the '*α-glucosidase inhibition by LAB, AAB, and yeast strains*' section.

Statistics

All data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's test (T). A probability value of $p < 0.05$ was used as the criteria for significant differences. In the first stage of data analysis, ANOVA and T were applied for comparison among CFS and CFE microbial isolate samples and among microbial strains. In the second stage of data analysis, ANOVA and T were repeated in separate trials to establish differences among the beverage samples and among the storage periods. The beverage samples studied were organised in columns (variables) and the storage periods were organized into lines (cases). In a separate ANOVA, the storage periods were organised in columns (variables) and the beverage samples studied were organised into lines (cases). All analyses were performed using SPSS 21.0 software (IBM, Armonk, NY, USA).

Results and discussion

α-glucosidase inhibitory activity of LAB, AAB, and yeast strains

Of the six LAB, three AAB, and two yeast strains isolated from water kefir grains in the present work, only five strains demonstrated α-glucosidase inhibitory activity of >15% for CFS and ≥10% for CFE. CFS and CFE of LAB strains (K65, K9, and K67) showed high levels of inhibition as compared to the other strains, followed by AAB strain A5 and yeast strain Y6 (Figure 1). These five strains were selected for further study of their survival in gastrointestinal tract conditions and for their application in producing a water kefir beverage.

Both CFS and CFE of the microbial strains were tested instead of the intact cells because intact microbial cells could not enter the blood; α-glucosidase inhibitory activity depends on active metabolites produced and secreted by the microbial strains into the CFS and CFE (Shori and Baba, 2013; Zeng *et al*., 2016). Results demonstrated that CFS and CFE of LAB, AAB, and yeast isolated from water kefir grains possessed α-glucosidase inhibitory activity that could have beneficial effects on reducing postprandial hyperglycaemia. Inhibition of α-glucosidase enzyme can reduce the increase

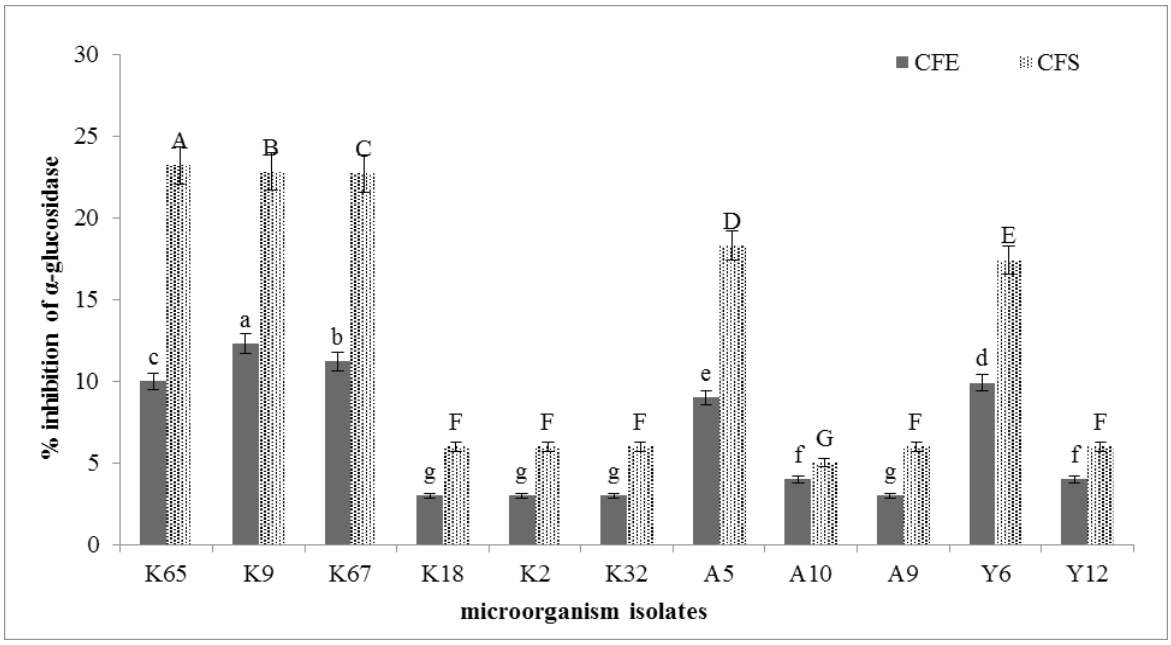

Figure 1. Six lactic acid bacteria (K65, K9, K67, K18, K2 and K32), three acetic acid bacteria (A5, A10 and A9) and two yeasts (Y6 and Y12) isolated from water kefir demonstrated rat intestinal α-glucosidase inhibition (%); mean values with different uppercase indicate significant difference among CFS isolate samples; mean values with different lowercase indicate significant difference among CFE isolate samples. CFE, cell-free extract; CFS, cell-free supernatant. Error bars show the standard deviation of the means, $n = 3$.

in postprandial plasma glucose. Therefore, these strains could serve as potential anti-hyperglycaemic probiotics by alleviating the effects of hyperglycaemia and T2D (Shori and Baba, 2013). Similar findings were reported by Lin *et al*. (2016) who demonstrated that the strains isolated from kefir possessed anti-T2D effects by improving glucose homeostasis, insulin sensitivity and lipid metabolism.

Survival of LAB, AAB, and yeast strains under simulated gastrointestinal tract conditions

All five selected water kefir strains (K65, K9, K67, A5, and Y6) exhibited sufficient survival ability under simulated gastrointestinal tract conditions (Figure 2). These strains were identified by analyses of 16S and 18S rRNA sequences as follows (with GenBank accession number): *Lactobacillus mali* (AB690199.1), *Lactobacillus casei* (AB690196.1), *Leuconostoc mesenteroides* (KT952376.1), *Gluconobacter hansenii* (AB682236.1), and *Saccharomyces cerevisiae* (KC588952.1).

Three hours after incubation in simulated gastric juice, the survival percentage decreased, with strain A5 showing the lowest survival (63.2%) and strain K9 the highest (77.9%) (Figure 2). All five strains exhibited sufficient survival in simulated duodenal juice (40.2–59.3%) and simulated intestinal juice (36.4–48.9%) after 8 h incubation. Therefore, strains K65, K9, K67, Y6, and A5 were considered tolerant to the simulated gastrointestinal tract conditions and were selected for further use as starter cultures in water kefir production.

Resistance to gastrointestinal stress is a key requirement for probiotic strains (Ida Muryany *et al*., 2017). Probiotics however, including those commercially available, often are greatly injured by the acidic environment of the stomach and the presence of bile salts in the duodenum (Succi *et al*., 2017). The acid tolerance of microbial strains can be attributed to the presence of an acid resistance transporter (acid efflux pump) that maintains a constant pH gradient between the extracellular fluid and the cytoplasm of the microbial cells (Illeghems *et al*., 2013). Loss of survival in the duodenal and intestinal juices is likely due to the previous stress of exposure to bile salts, pepsin and trypsin enzymes, which have detrimental effects on microbial cell membrane lipids, thus interfering with cell wall integrity (Muganga *et al*., 2015).

Microbial cell viability in water kefir beverage during storage

Figure 3(A) shows changes in LAB levels during storage of WKBS, WKPP and WKBSPP beverage samples. The levels of LAB gradually decreased in all beverage samples over the storage time at 4°C, with the greatest decrease (≈two log units) detected on day 28. The loss of cell viability could be due to

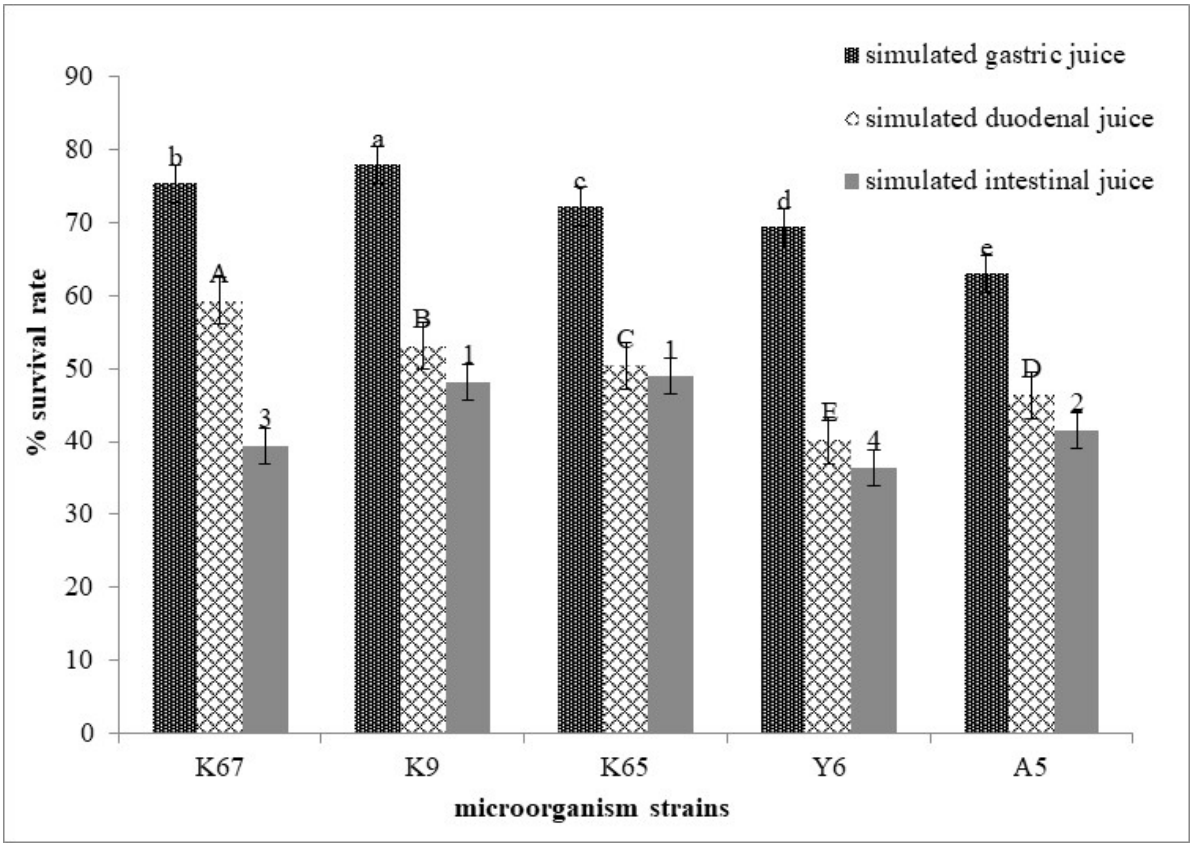

Figure 2. Comparison of five selected water kefir strains survival under simulated gastrointestinal conditions. Mean values with different lowercase indicate significant difference among microbial strains for simulated gastric juice; mean values with different uppercase indicate significant difference among microbial strains for simulated duodenal juice; mean values with different numbers indicate significant difference among microbial strains for simulated intestinal juice. Error bars show the standard deviation of the means, $n = 3$.

lack of sufficient nutrients needed to sustain LAB culture viability at the end of the storage period (Costa *et al*., 2016). The LAB present in the WKBSPP beverage sample had the highest overall microbial load and was one log higher than the LAB content found in WKPP and WKBS because more nutrients (pumpkin purée and brown sugar) were available for microbial cell growth. Some of the factors that affect cell viability during storage are the microbial strains used, pH, dissolved oxygen and hydrogen peroxide content, concentration of metabolites such as lactic and acetic acids, buffering capacity of the medium, storage temperature, and the nature of the added ingredients (Shori, 2016). In this sense, WKPP, which contained pumpkin purée, improved the LAB, AAB, and yeast viability in comparison with WKBS. The findings obtained in the present work are in agreement of Genevois *et al*. (2016) and Du *et al*. (2011), who found that pumpkin could act as a prebiotic that stimulates the growth of probiotic strains. It is herein proposed that the kefir isolates were able to utilise pumpkin as a carbon substrate for their growth during the fermentation process.

In all water kefir beverage samples, cell viability of AAB and yeast showed a significant increase after two weeks of storage (Figures 3(B), (C)). During the third week of storage, the marked increase of AAB and yeast populations in all beverage samples could be due to synergistic interactions among LAB, AAB and yeast. Indeed, LAB ferment carbohydrates (e.g., glucose and fructose) via the homofermentative pathway into lactic acid or via the heterofermentative pathway into lactic acid, acetic acid, or ethanol and carbon dioxide. Heterofermentative LAB can produce bacterial metabolites (e.g., organic matter) that stimulate yeast growth, and the yeast can convert organic matter into ethanol (Illeghems *et al*., 2013). The availability of substrates (such as ethanol, lactic acid, and acetic acid) is favourable for the growth of AAB, and the main activity of AAB is oxidation of ethanol into acetic acid (Wang *et al*., 2012; Illeghems *et al*., 2013). AAB and yeast growth decreased after three weeks of storage (Figures 3(B), (C)) due to high acetic acid concentration caused by acidification (Li *et al*., 2015).

Only the LAB, AAB, and yeast populations in the WKBSPP beverage sample demonstrated viable cell counts above the recommended concentration

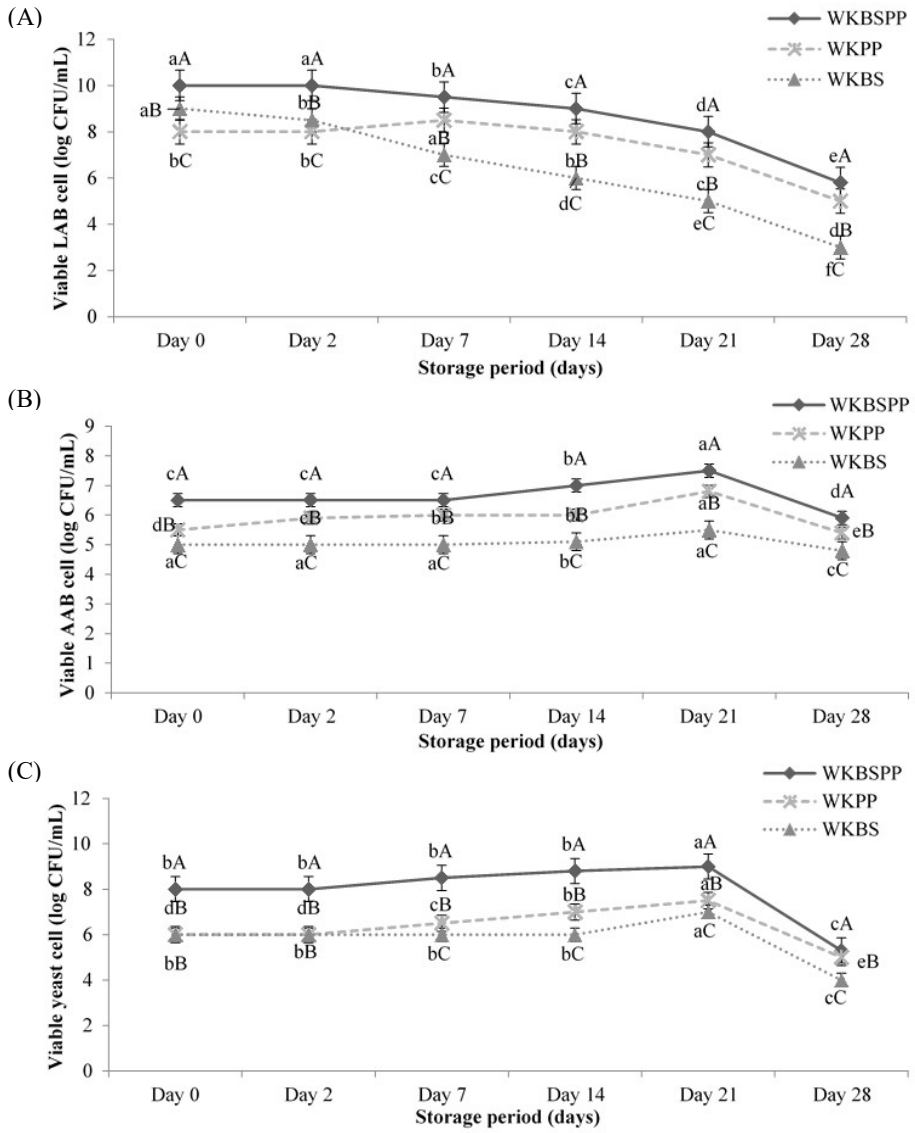

Figure 3. Viability of LAB (A), AAB (B) and yeast (C) in the WKBSPP, WKPP and WKBS during storage at 4°C for 28 days. Error bars show the standard deviation of the means, $n = 3$. Mean values with different lowercase for the same line patterns (same sample) indicate significant difference between storage periods. Mean values with different uppercase for different line patterns (different sample) within the same storage period indicate significant difference between samples as determined by Tukey's test.

of live probiotics (at least 10^6 CFU/mL) in probiotic food products (Muganga *et al*., 2015; Plessas *et al*., 2017) throughout the 21 days of storage. The higher number of viable LAB, AAB, and yeast cells in WKBSPP and WKPP beverage samples as compared to the WKBS beverage sample during storage was most likely due to the presence of pumpkin purée. The pumpkin purée contained oligosaccharides, which could sustain microbial metabolic activities at the high acid condition of the growth media (Koh *et al*., 2017b). The shelf life of WKBSPP beverage kept at refrigerated temperature is proposed to be at least 21 days, as the LAB, AAB, and yeast cell viability in WKBSPP decreased significantly to $\leq 10^6$ CFU/mL by day 28 of storage. This decrease in cell viability at the end of the storage period could be because the nutrients in the beverage samples had been used up by the microorganisms.

α-glucosidase inhibition by CFS-WK and CFS-NIC beverages during storage

Figure 4 shows the α-glucosidase inhibition activity by CFS of WK samples (CFS-WKBS, CFS-WKPP, and CFS-WKBSPP) and NIC samples (CFS-PPNIC, CFS-BSNIC, CFS-BSPPNIC). The results confirmed that some α-glucosidase inhibition activity could be attributed to the presence of pumpkin purée and microbial strains but not the brown sugar, as

indicated by 10.1–19.2% inhibition in CFS-PPNIC and CFS-BSPPNIC but no inhibition in CFS-BSNIC throughout the storage period. On the other hand, low α-glucosidase (30.2–35.3%) inhibitory activities were recorded for the inoculated brown sugar water kefir (CFS-WKBS) sample during storage at 4°C. In contrast, both CFS-WKPP and CFS-WKBSPP samples exhibited high α-glucosidase inhibitory activities (50.1–67.3% and 70.6–79.2%, respectively) during 21 days of refrigerated storage (Figure 4).

The WKBSPP beverage sample had the highest α-glucosidase inhibitory activity among all WK beverage samples, with >70% inhibition during 21 days of storage at 4°C. The microbial fermentative activities (e.g., proteolysis) produce bioactive peptides, which could be potent inhibitors of α-glucosidase (Shori and Baba, 2013). Alsayadi *et al*. (2014) reported the anti-hyperglycaemic activity of water kefir, as they demonstrated that five consecutive weeks of water kefir consumption significantly lowered blood glucose levels in streptozotocin-induced diabetic Wistar rats. In addition, pumpkin purée was found to contain polysaccharides that could inhibit the α-glucosidase enzyme (Wang *et al*., 2016, 2017). Furthermore, Yoshinari and Igarashi (2010) reported that pumpkin paste concentrate contained a relatively abundant amount of trigonelline and nicotinic acid, which suppressed the progression of diabetes and lowered the blood glucose levels in T2D Goto-Kakizaki obese mice.

The α-glucosidase inhibitory activities of all CFS of WK beverage samples decreased gradually throughout the 28 days storage period. The loss of α-glucosidase inhibitory activity in these samples during storage was likely due to the loss of viable cells with the ability to hydrolyse the polysaccharides in pumpkin purée (Muganga *et al*., 2015; Wang *et al*., 2017). Evidence suggests that pumpkin-rich diets could lower postprandial blood glucose variation due to the α-glucosidase inhibition activity of pumpkin

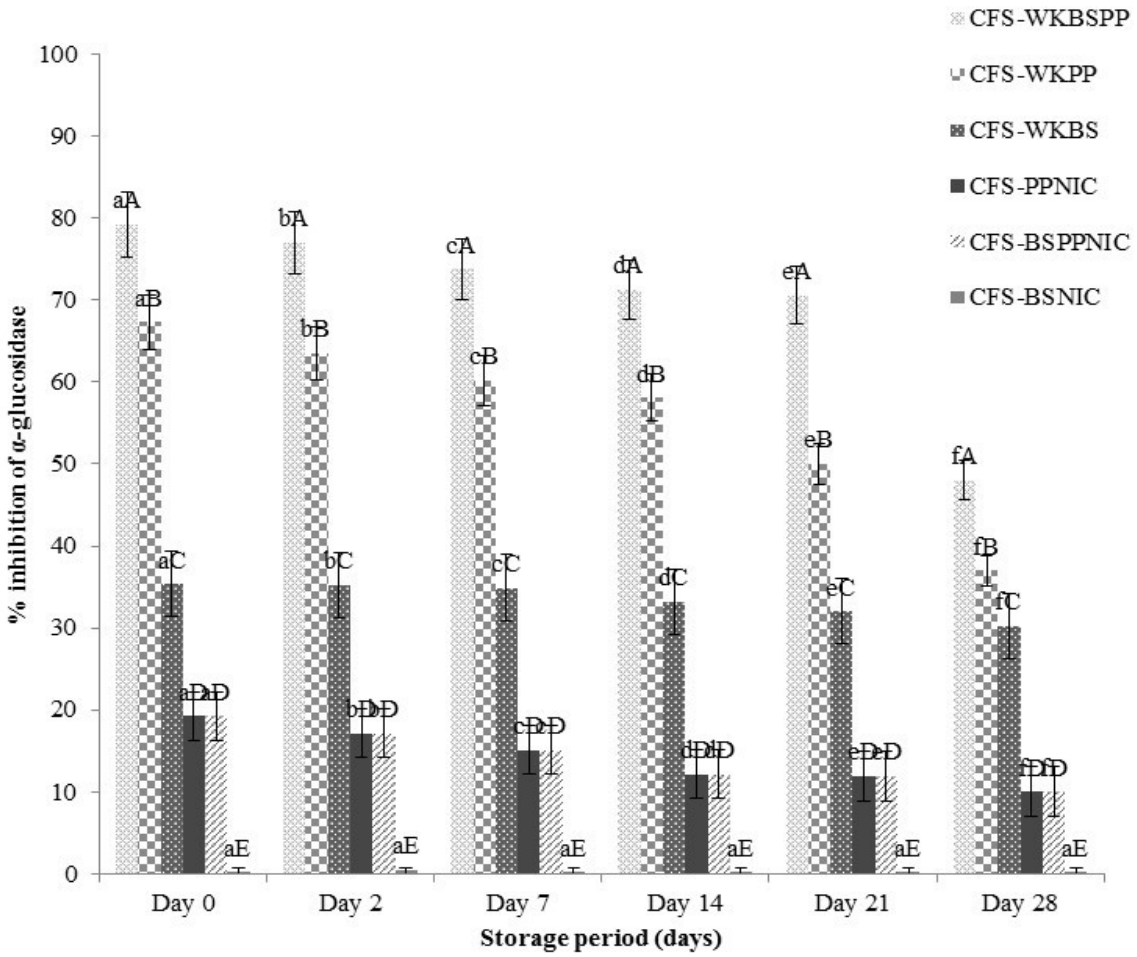

Figure 4. The α-glucosidase inhibition activity in the cell-free supernatants (CFS) of negative controls (CFS-PPNIC, CFS-BSNIC and CFS-BSPPNIC) and water kefir samples (CFS-WKBSPP, CFS-WKPP and CFS-WKBS) during storage at 4°C for 28 days. Means with different letters indicate significant difference. Error bars show the standard deviation of the means, $n = 3$. Mean values with different lowercase for the same bar patterns (same sample) indicate significant difference between storage periods. Mean values with different uppercase for different bar patterns (different sample) within the same storage period indicate significant difference between samples as determined by Tukey's test.

polysaccharides (Jin *et al*., 2013). Moreover, fermentation of pumpkin-based beverages could tremendously enhance the α-glucosidase inhibition of the pumpkin due to the activities of the fermenting microorganisms (Koh *et al*., 2017a). Further investigation however is needed to determine how to prevent loss of α-glucosidase inhibitory activity in WK beverage samples during storage.

Microbial strain survival in water kefir beverages treated with the simulated gastric juice

Resistance to gastric juice is currently the most used *in vitro* test for the evaluation of probiotics in foods (FAO/WHO, 2002). Microbial population survival in all WK beverage samples treated with simulated gastric juice during storage were investigated (Figures 5(A), (B), (C)). Although there was a general decrease in survival of all microorganism populations (LAB, AAB, and yeast) in all WK beverage samples over the storage period, the microbial populations remained high (>60% survival rate) until 21 days of storage; they then sharply decreased until day 28 of storage. This decrease was likely due to the loss of cell viability during subsequent storage (Figure 3).

As observed in Figure 5, WKBSPP beverage sample demonstrated the greatest improvement in microbial strain survival treated with simulated gastric juice as compared to the fresh pure cultures (Figure 2) and other WK beverage samples (WKBS and WKPP). The LAB, AAB, and yeast populations in the WKBSPP beverage sample exhibited good survival under simulated gastric juice conditions and surpassed the minimum effective dose of probiotics needed to obtain a clinical effect in the small bowel (10^6 CFU/mL) (Minelli and Benini, 2008; Muganga *et al*., 2015).

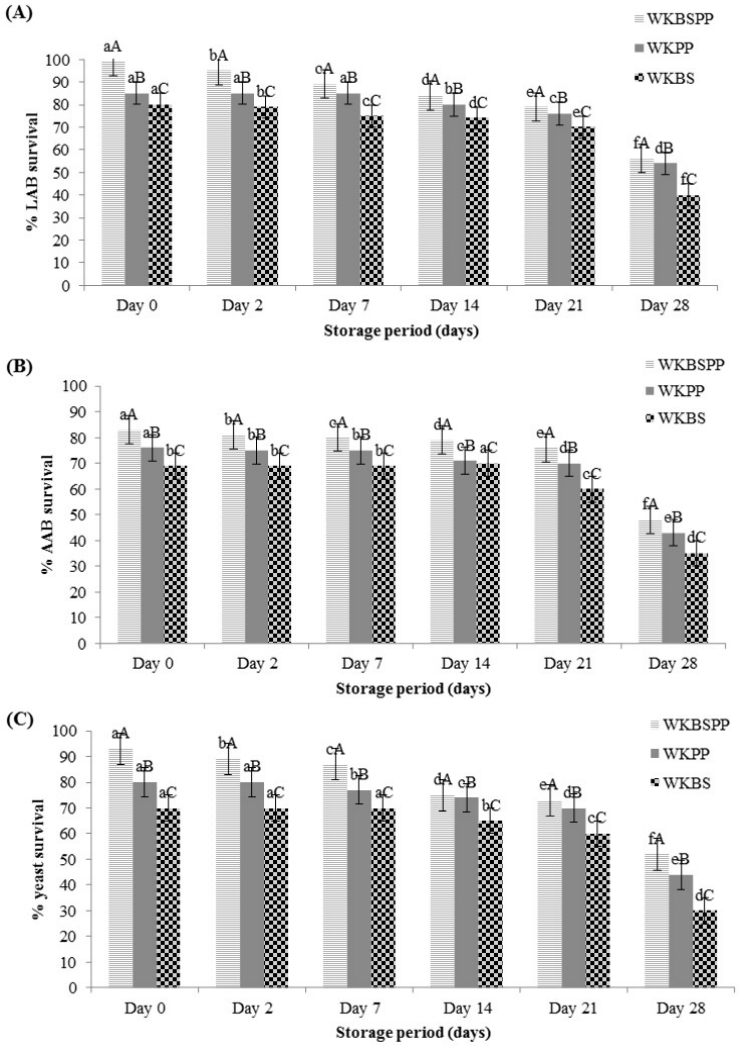

Figure 5. Survival in simulated gastric juice at pH 2.5 and 37°C for 3 h of LAB (A), AAB (B) and yeast (C) strains from WKBSPP, WKPP and WKBS during storage at 4°C for 28 days. Means with different letters indicate significant difference. Error bars show the standard deviation of the means, $n = 3$. Mean values with different lowercase for the same bar patterns (same sample) indicate significant difference between storage periods. Mean values with different uppercase for different bar patterns (different sample) within the same storage period indicate significant difference between samples as determined by Tukey's test.

Factors such as food matrices are important in maintaining the survival of microbial strains in probiotic products. The pumpkin purée could act as a buffer against the acidic condition of simulated gastric juice (Koh *et al*., 2017a). Therefore, it is herein hypothesised that pumpkin purée acted as a prebiotic to improve the gastrointestinal tract digestive tolerance of the water kefir microorganisms in the beverage samples. The oligosaccharides in pumpkin pulp have been shown to have prebiotic potential because they resisted hydrolysis by artificial human gastric juice, thereby stimulating the growth of probiotics and protecting probiotics during beverage processing and gastric transit (Du *et al*., 2011; Genevois *et al*., 2016). In summary, supplementation of water kefir strains (K65, K9, K67, Y6, and A5) with pumpkin purée in the WKBSPP beverage sample increased the survival of LAB, AAB, and yeast in simulated gastric juice as compared to the fresh pure cultures and other WK beverage samples.

Conclusion

The current study demonstrated that five isolated water kefir strains (K65, K9, K67, Y6, and A5) were able to inhibit α-glucosidase and tolerate *in vitro* gastrointestinal tract conditions. Fermentation of all WK beverage samples supplemented with the combination of all five isolated water kefir starter cultures during storage time improved α-glucosidase inhibition and potential probiotic survival in the simulated gastric juice compared to fresh pure cultures. The WKBSPP beverage sample exhibited the highest α-glucosidase inhibition among the WK beverage samples tested. To our knowledge, this has not been reported previously, and the strong and discernible inhibition of α-glucosidase activity for as long as 21 days of storage should be noted. These five water kefir strains could be used as potential probiotics in the formulation of fermented beverages to mitigate the effects of T2D. Further investigations are required to elucidate the anti-hyperglycemic potential of these strains and their fermented beverage products *in vivo* as well as to evaluate the sensory acceptance of the fermented beverage products by consumers before use in commercial applications.

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