Food Research 6 (3) : 324 - 334 (June 2022)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Chemical and microbiological analysis of fermented probiotic watermelon juice

[1]Lani, M.N., [1]MohdMangsor, N.H., [2]Sharifudin, S.A., [1]Abdullah, W.Z.W., [1]MohdIsa, N.S., [1]Jamzuri, M.N.S. and [1,*]MohdMaidin, N.

[1]Department of Food Science, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[2]Enzyme and Fermentation Technology Programme, Food Science and Technology Research Centre, MARDI Headquarters, 43400 Serdang, Selangor, Malaysia

Article history:
Received: 4 June 2021
Received in revised form: 29 July 2021
Accepted: 4 November 2021
Available Online: 16 June 2022

Keywords:
Probiotic watermelon juice,
Functional drink,
Fermentation,
Chemical analysis,
Microbiological analysis

DOI:
https://doi.org/10.26656/fr.2017.6(3).402

Abstract

Fruit juice is an ideal medium in the production of a probiotic drink. Watermelon fruit is a suitable medium for the production of probiotic juice due to its nutritional content. However, its utilisation as a probiotic drink is underutilised. Experiments were conducted to determine the chemical (pH, Brix, titrable acidity) and microbiological changes (lactic acid bacteria (LAB) count) during fermentation of probiotic watermelon juice using different concentrations of *Lactobacillus plantarum, Lactobacillus paracasei* and *Lactobacillus acidophilus* (2%, 5% and 10%) at 30°C for 72 hrs. The results indicated a significant drop in pH of the probioticated watermelon juice (2%, 5% and 10%) using various Lactobacilli strains during 24 hrs of fermentation. Interestingly, 2% probioticated watermelon juice in all their strains of probiotic were higher than 5% and 10% probioticated watermelon juice. The lactic acid content in probioticated watermelon juice was reduced significantly throughout 72 hrs of fermentation, but the watermelon juice without probiotic strains increased only within 24 hrs of fermentation. The LAB count in probioticated watermelon juice (2%, 5% and 10%) maintained a high population count (10^8 to 10^9 CFU/mL) for all probiotic strains throughout fermentation. Since all concentrations studied showed similar results in the chemical and microbiological analyses, 2% concentration is proven to be sufficient for probioticated watermelon juice regardless of the types of *Lactobacillus* strains used.

1. Introduction

Watermelon, also known as *Citrulus lanatus*, is one of the common crops being familiarly known by people all around the globe for its distinctive sweetness as compared to other fruits. As mentioned by Bisognin (2002), watermelon cultivation and consumption exceed that of all other *Cucurbitaceae* plants. Watermelon is a creeping herbaceous plant that belongs to the family *Cucurbitaceae*. Initially, they originated from Southern Africa (specifically in the southern Kalahari region). The fruit of the plant contains 93% water, thus giving its distinctive name "Watermelon" (Ninomiya *et al*., 2020). Due to its large number of cultivations, watermelon has proven its relevance, showing tremendous economic importance and being widely eaten around the world, accounting for about 7% of the world's region devoted to vegetable production, with China producing 67% of the overall quantity made (Zamuz *et al.*, 2021). Aside from its large number of consumptions, watermelon is known for its health benefits. As reported by Dammak *et al.* (2019), watermelon is known because of its low-calorie content, as well as its health benefits, thirst-quenching capacity, good nutritional value, and antioxidant properties. Besides, Romdhane *et al.* (2017) also reported that watermelon is an excellent source of mineral salts (Potassium, Sodium, Iron and Magnesium), vitamins (A, B, C and E), antioxidants such as phenolic compounds and carotenoids, and few specific amino acids such as citrulline and arginine.

Food today has many purposes, it satisfies appetite and provides essential foods for humans, it promotes physical and mental well-being, it improves fitness, and it prevents and/or reduces nutrition-related diseases. Furthermore, in recent years, consumers' perception of the connection between food and health has sparked a surge of interest in "healthy foods", this phenomenon may be due in part to rising healthcare costs, rising life

*Corresponding author.
Email: *nurmahani@umt.edu.my*

eISSN: 2550-2166
Published by Rynnye Lyan Resources

expectancy, and older people's desire for a better quality of life in their later years (Granato *et al.*, 2010). Healthy food, or can also be known as "functional foods" are defined as food that, in addition to its dietary benefits, has additional beneficial effects or functions in the body. Functional foods include those that contain or are processed with bioactive compounds like dietary fibre, oligosaccharides, and active "friendly" bacteria that help keep intestinal bacterial strains in balance. Probiotics are part of a new wave of active ingredients that includes prebiotics, phytonutrients, and lipids, in addition to well-known functional ingredients like vitamins, minerals, and micronutrients.

Watermelon juice, for example, has its own set of advantages due to its high concentration of lycopene, nutrients, and vitamins A, B, and C. The benefit of regular consumption of watermelon juice is it will increase blood concentration of lycopene and beta-carotene (Edwards *et al.,* 2003). Probiotication of watermelon juice with various probiotic lactobacilli has improved the nutritional value of the juice (Sivudu *et al.*, 2014). In their study, *Lactobacillus fermentum* and *Lactobacillus casei* were found capable to grow well in watermelon juice without nutrient supplementation. Both strains produced significantly more titrable acidity expressed as lactic acid at 30°C of fermentation (Sivudu *et al.,* 2014).

Lactic acid bacteria, mainly *Lactobacilli* and *Bifidobacterium* have contributed to more than 90% of probiotic food supplements. Many experiments have been conducted on the method and development of probiotic products fermented with probiotic microbes, in addition to the identification of the health benefits of probiotic bacteria (Pakbin *et al.*, 2014). These LAB strains have been reported to suppress the growth of pathogenic bacteria (Salleh *et al.,* 2014; Salleh *et al.,* 2021). These bacteria include probiotic properties with health benefits (Nuraida, 2015; Ahmad *et al*., 2018).

Fruit and vegetable do not contain any dairy allergens that are suitable to be consumed by wide segments of people that can be one of the vehicles for the delivery and incorporation into the human intestine (Tuorila and Gardello, 2002). For instance, there is a genuine enthusiasm for the production of fruit-juice-based nutritional drinks fortified with probiotic and prebiotic ingredients. Fruit juices have been proposed as an ideal vehicle for practical wellness ingredients because they naturally provide valuable nutrients, have flavour profiles that appeal to people of all ages, and are viewed as nutritious and soothing (Tuorila and Gardello, 2002). The fruits and vegetables are high in functional food ingredients including minerals, vitamins, dietary fibres, and antioxidants, and they are free of dairy allergens, which could discourage some people from eating those (Hasler *et al.*, 2002).

To the best of our knowledge, there is no comprehensive study on the viability of lactic acid bacteria (LAB) strains conducted in watermelon juice during the fermentation process with limited studies on the physicochemical of fermented watermelon juice. Therefore, this study aimed to determine the chemical and microbiological analyses of fermented watermelon juice using commercial probiotic strains during the fermentation process.

2. Materials and methods

2.1 Preparation of watermelon juice

Ripe watermelon fruit was purchased from a local store (Kuala Terengganu) and kept at 4°C prior to use. The sample was then washed and peeled. The watermelon juice was prepared using a fruit juicing machine (National, MJ-68M, Malaysia). The juice produced was further pasteurized until it reaches the temperature of 80°C with a holding time of 15 mins (Mousavi *et al.*, 2011). Pasteurization was done by cooking the juice on the stove and controlling the temperature by using a thermometer.

2.2 Preparation of inoculum

Commercial strains of Lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus paracasei*) were obtained courteously from the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor in the form of freeze-dried. These cultures were originally purchased from UAS Labs, Madison, the USA, which is one of the few fully integrated suppliers of probiotic solutions (http://www.uaslabs.com). The starters were cultured in 100 mL MRS broth for 24 hrs at 30˚C and transferred into a 50 mL centrifuge tube before being centrifuged at 8000 rpm, 15 mins, and 4˚C. The pallet collected was washed with phosphate buffer saline (PBS) with pH 7.3 (Oxoid, UK) and mixed. The mixture was centrifuged again at 8000 rpm, 15 mins and 4˚C. The mixture was then washed again with PBS to be used as inoculum. Before the experiment, the purity of the cultures was confirmed by streaking them on MRS agar. The same single colony on MRS Agar indicated that the cultures were considered pure (Khatoon *et al.*, 2015).

2.3 Optical density

The commercial probiotic cultures (*Lactobacillus plantarum, Lactobacillus acidophilus and Lactobacillus paracasei*) were grown in MRS broth. Fermentation was done in the incubator shaker (PROTECH, SI-50D,

Malaysia) for 48 hrs. The reading of the absorbance of the probiotic cultures was recorded at different time intervals, i.e. 0, 2, 4, 24, 26, 28, 46, and 48 hrs at 560 nm using a spectrophotometer (ThermoSpectronic, GENESYS 20, United States). LAB count was determined at time intervals of 0, 2-, 4-, 24-, and 26-hrs using MRS agar (Hoque *et al.*, 2010).

2.4 Fermentation of watermelon juice with lactic acid bacteria

A fermentation experiment was conducted by using 200 mL transparent glass bottles. A bottle of the sample containing 100 mL of pasteurised watermelon juice without probiotics was treated as a control. The other three bottles containing 100 mL of pasteurized watermelon juice were inoculated with starter cultures. The samples of watermelon juice were inoculated with the same inoculum from each starter culture at three different concentrations of 2%, 5% and 10% v/v. They were then incubated at 30°C for 72 hrs. Samples were taken at 0, 24, 48, and 72 hrs for chemical analysis and microbiological analysis (Khatoon *et al.*, 2015).

2.5 Chemical analysis

2.5.1 pH analysis

The pH of watermelon juice was measured with a Benchtop pH meter (WTW series, Inolab, Germany). The pH was taken at 0, 24, 48, and 72 hrs intervals.

2.5.2 Titrable acidity

Total acidity was expressed as% lactic acid using the formula below. 10 mL of juice samples were added with 10mL of distilled water and the lactic acid percentage was determined by titrating the prepared samples with 0.1 N NaOH using phenolphthalein as an indicator until a light pink colour persists (Akin *et al.,* 2007).

% lactic acid = [(mL of 0.1 M NaOH) × (0.9)] / [sample volume]

2.5.3 Total Soluble Solid

The total soluble solid of the samples was taken as degree Brix by using a Refractometer (Milwaukee, MA871, United States) (Serpen, 2012).

2.6 Microbiological analysis

2.6.1 Determination of LAB count using MRS Agar

Firstly, 10 mL of juice sample was mixed with 90 mL of MRS broth. The sample was incubated for 24 hrs at 30˚C in a CO_2 incubator (Galaxy S, Model: 170-200, United Kingdom). After 24 hrs, 1.0 mL of sample was pipetted into a tube containing 9 mL of saline water (0.85% salt) for appropriate series of serial dilutions. Then, 0.1 mL of the sample was surface plated on de Man, Rogosa and Sharpe (MRS) Agar (Oxoid, UK) plates in triplicates. The plates were then incubated at 30˚C in a CO_2 incubator for 24-72 hrs (Khatoon *et al.,* 2015). Further biochemical tests were carried out for confirmation of LAB.

2.6.2 Biochemical tests for selected bacteria

2.6.2.1 Gram-staining

Gram staining was conducted by using the standard procedure (Smith *et al.*, 2005). Briefly, a loopful of bacteria was transferred aseptically onto a clean glass slide and was heat fixed using a Bunsen burner. The smeared area was flooded with crystal violet for 1 min. Then, it was rinsed with tap water, tilting the slide in order to rinse all stains. After that, the whole slide was covered with Lugol's iodine for 1 min. After 1 min, the washing step was repeated. With the slide slightly tilted, the iodine solution was washed off with 95% ethanol and was treated continuously with alcohol until the washings were pale violet. The slide was rinsed immediately with tap water. The smear was then covered with safranin solution to counterstain for 1 min and was rinsed again with tap water after 1 min. It was then blotted dry with a paper towel using firm pressure. The smear was examined with a compound microscope (Leica, DME, UK) starting from the low power objective and finally the immersion lens (Smith *et al.*, 2005).

2.6.2.2 Catalase test

The procedure was done according to Hitchins and Jinneman, (2011). Briefly, a small amount of colony was taken from isolates and placed on a slide. A few drops of hydrogen peroxide (H_2O_2) reagent were put onto the sample using a Pasteur pipette. The reaction was observed for bubbles formation (positive reaction) whilst no bubbles formation indicated a negative reaction.

2.6.2.3 Oxidase test

A piece of filter paper was prepared and moistens with tetramethyl-p phenylenediamine, a chromogenic reducing agent. A small number of bacteria colonies were rubbed onto the moist paper by using a toothpick. The presence of dark brown-purple colour showed a positive result, while no colour change showed a negative result (Shields and Cathcart, 2013).

2.7 Statistical analysis

All experiments were carried out in triplicate and the results were expressed as mean ± SD (n = 3). Data generated from the experiments were analysed for significance using the one-way analysis of variance, ANOVA. Statistical analysis was obtained using Minitab 14 statistical software and significant differences

($p<0.05$) between means were determined by Fisher's multiple range test.

3. Results and discussion

3.1 Optical density of probiotic lactic acid bacteria

The optical density (560 nm) of 3 commercial probiotic strains of LAB, grown in MRS broth were monitored at different time intervals. The purpose of this experiment was to determine the growth curve of different strains of LAB. Figure 1 shows the absorbance of probiotic lactic acid bacteria before inoculating them into the watermelon juice. MRS broth without any lactic acid bacteria was prepared as a control.

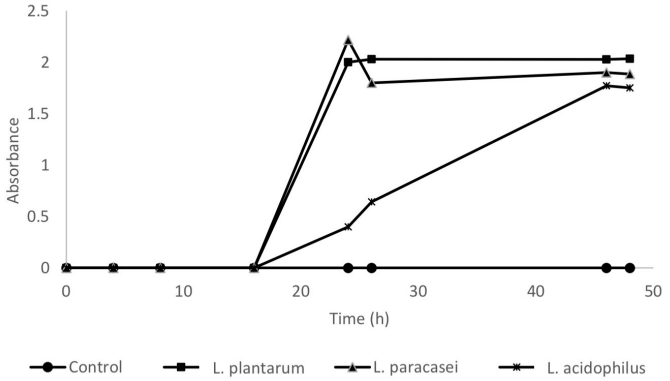

Figure 1. Absorbance at 560 nm for probiotic culture used

In Figure 1, it can be seen that the absorbance for the control sample is 0 for 0 hr until 48 hrs of incubation time. It was confirmed that without LAB, no growth of microbes was observed through optical density at 600 nm. For *L. plantarum*, zero absorbance was observed while *L. paracasei* showed less than 0 absorbance values during the first 4 hrs of incubation. The results then showed a similar trend for both of the strains whereby an increase in absorbance to more than 2 was observed at 24 hrs to 28 hrs of incubation time. However, a slight decrease in absorbance was observed after 46 hrs. For *L. acidophilus*, the absorbance was in the range of zero from 0 h until 28 hrs. An increase in the absorbance to the value of 1 to 2 was only observed at 46 hrs to 48 hrs of incubation. Both *L. plantarum* and *L. paracasei,* reached the maximum absorbance at 24 hrs of incubation in MRS broth while for *L. acidophilus,* the maximum absorbance was at 46 hrs.

In order to support the results obtained from optical density, the total viable count of the LAB was determined as shown in Table 1. From the results obtained, it was determined that by comparing the three strains, *L. acidophilus* had the slowest growth rate while *L. plantarum* and *L. paracasei* shows similar and comparable growth rate. The number of viable cells at maximum absorbance was 2.52×10^9 CFU/mL and 9.00×10^7 CFU/mL for *L. plantarum* and *L. paracasei*, respectively (Table 1). From the results, the most suitable time to inoculate probiotic lactic acid bacteria into the watermelon juice was after 24 hrs of incubation time. It has been suggested previously that the ideal cell count for inoculation is between the range of 10^7 to 10^8 CFU/ mL with an absorbance of 0.6-0.8 (Trontel *et al.,* 2010).

Table 1. LAB Count (CFU/mL) before inoculated into watermelon juice

Time (hrs)	Control	*L. plantarum*	*L. acidophilus*	*L. paracasei*
0	0	5.72×10^5	0	2.40×10^5
2	0	5.32×10^5	0	3.88×10^5
4	0	6.44×10^6	7.9×10^6	9.00×10^5
24	0	2.52×10^9	8.00×10^7	9.00×10^7
26	0	1.93×10^9	1.00×10^5	5.00×10^8

3.2 pH analysis

Figure 2 shows the changes in pH value of probiotic watermelon juice fermented with three strains of LAB namely *L. plantarum* (2A), *L. paracasei* (2B) and *L. acidophilus* (2C) with four different concentrations; 0%, 2%, 5% and 10% (w/v) at time of 0 hr, 24 hr, 48 hr, and 72 hr respectively. The control sample had the highest pH as compared to samples treated with 2%, 5%, and 10% of LAB during the fermentation time. During 0h fermentation time, there was no significant difference ($p>0.05$) of pH between 0%, 2% and 5% concentration of bacteria used but was only noted at 10% concentration. No significant difference ($p>0.05$) was noted in the pH during 24 hrs of fermentation at 0% concentration between the three bacteria used, but there was a significant reduction of pH when the bacteria concentration increased to 2-10% for all three bacteria used at the same fermentation time. At 48 hrs fermentation time, there was no significant difference ($p>0.05$) between the control sample with 2% for samples containing *L. plantarum* but a difference was observed between control with samples containing 2%, 5% and 10% of *L. paracasei* and *L. acidophilus*. During the 72 hrs of fermentation time, the pH of the juices becomes insignificantly different at ($p<0.05$) for all concentrations used. The juice's samples were inoculated with *L. plantarum* at 2%, 5% and 10% showing a rapid decrease in pH at the beginning of the fermentation process which was from 0 hr to 24 hrs and then slowly decreases after 24 hrs of fermentation time. The same trends were also observed in *L. paracasei* and *L. acidophilus*. A rapid decrease in pH at the beginning of fermentation increases the acidity and hence minimizes the influence of spoilage bacteria (Karovica *et al.*, 2003).

From the results obtained, the pH of the watermelon juice was greatly reduced to 3 after 24 hrs up to 72 hrs of fermentation time when 2%, 5% and 10% of lactic acid

bacteria were introduced into the juice. During the fermentation of probiotic juice, the medium pH decreased as a result of the accumulation of organic acids, including lactic acid (Giori *et al.*, 1985). Shukla *et al.*, (2013) investigated the production of probiotic pineapple juice by *L. acidophilu*s and whey. In their study, the pH reduced from 4.36 to 3.87 during fermentation. The same observation was also noted in the present study.

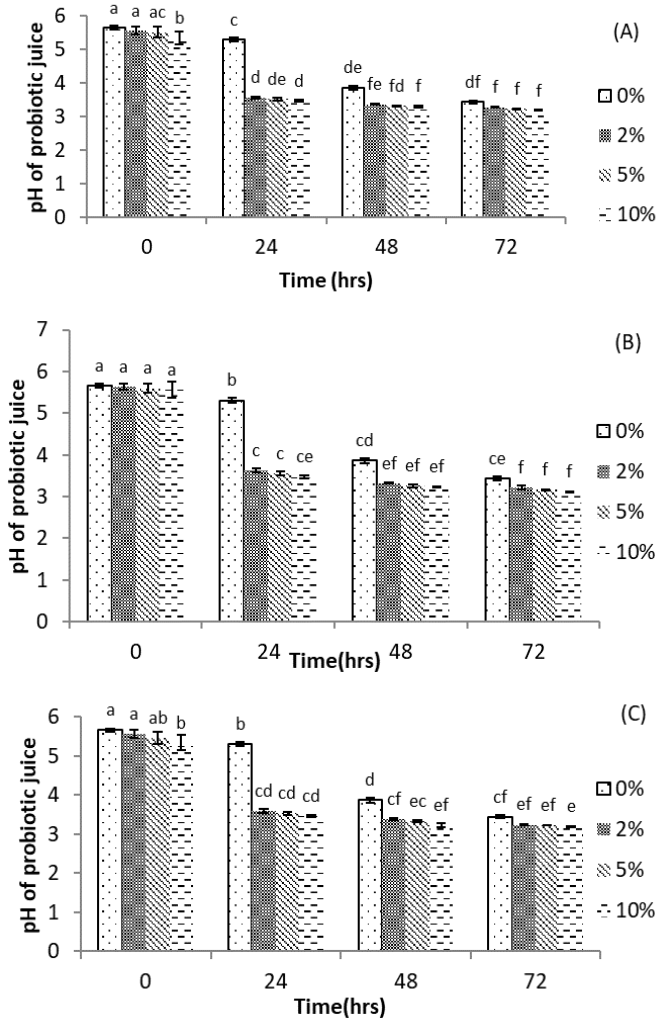

Figure 2. pH of probiotic watermelon juice fermented with (A) *L. plantarum,* (B) *L. paracasei* and (C) *L. acidophilus.* Different letters between groups indicates significant difference at $p<0.05$ (n = 3).

Lactic acid bacteria are generally fastidious on artificial media, but they grow readily in most food substrates and help in lowering the pH rapidly to a point where competing organisms are no longer able to grow. The presence of *Leuconostoc* and *Lactic Streptococci* generally caused the reduction of pH to about 4.0 to 4.5 while some of the *Lactobacilli* to about pH 3.5, before inhibiting their growth. Tolerance to low pH levels and bile salts is vital for bacteria to survive and grow in the gastrointestinal tract, making these the main requirements for bacteria to be considered probiotics. A previous study has demonstrated that *L. plantarum* exhibited good growth at the pH value of 3 to 5. Bacteria are generally sensitive to the stomach's low pH values. However, some LAB can survive and grow at a relatively low pH because they have a system that simultaneously transports lactic acid and protons to the cell's exterior (Ramirez *et al.*, 2013).

3.3 Brix analysis

Figure 3 shows the Brix reading of probiotic watermelon juice fermented with three strains of LAB namely *L. plantarum* (Figure 3A)*, L. paracasei* (Figure 3B) and *L. acidophilus* (Figure 3C) at four different concentrations and fermentation times. In Figure 3A, the reduction of the Brix value was observed during a long fermentation time. This result was expected as the glucose was consumed by the bacteria with an increase in fermentation time. However, it is interesting to note that the Brix reading of watermelon juices treated with 2% probiotic bacteria was higher as compared to the others in all fermentation times. This could be due to the formation of free sugars and amino acids (Salmerón *et al*., 2014). Figure 2B shows the Brix reading of the probiotic watermelon juice inoculated with *L. paracasei*

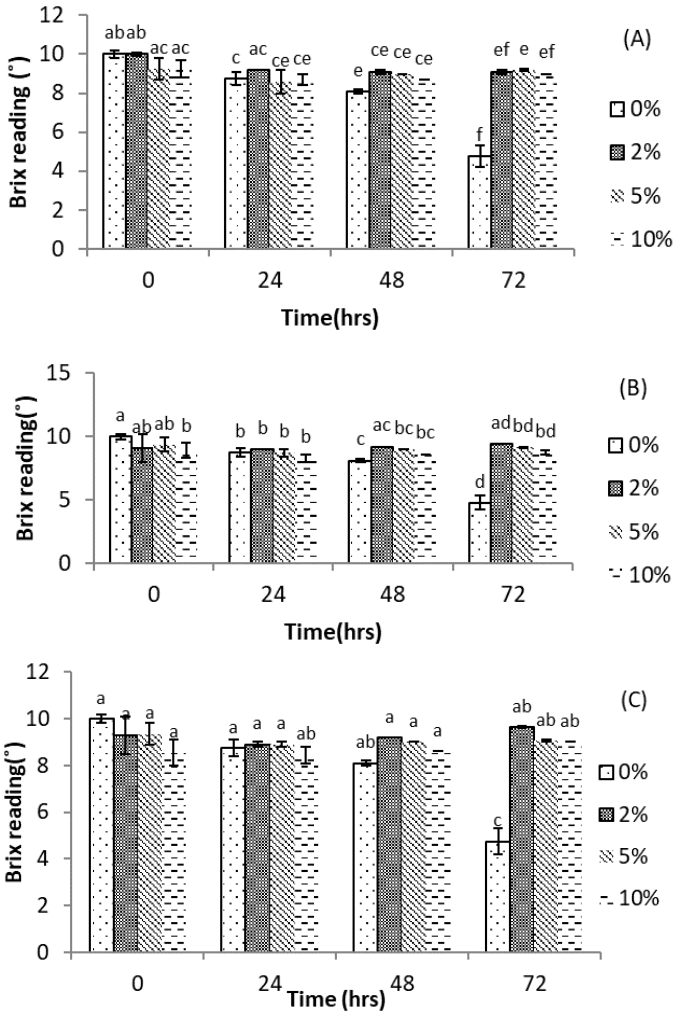

Figure 3. Brix of probiotic watermelon juice fermented with (A) *L. plantarum,* (B) *L. paracasei* and (C) *L. acidophilus.* Different letters between groups indicates significant difference at $p<0.05$ (n = 3).

at various concentrations. In general, there was no significant difference at ($p>0.05$) in the Brix reading between 0%, 2% and 5% concentration of bacteria used. In addition, there was a significant difference in the Brix reading of the juices between 0% and 10% of the bacteria used at 24 hrs fermentation time. Interestingly, a significant ($p<0.05$) increase of Brix value was observed between control and 2% of *L. paracasei* at 48 hrs and 72 hrs fermentation time. The same observation was noted in Figure 2C where no significant changes in Brix value were noted during the first 24 hrs of fermentation time when fermented with *L. acidophilus*. After 24 hrs of fermentation, there was no significant difference ($p>0.05$) in Brix value between 0%, 2%, 5% and 10% concentration of *L. acidophilus* used. The same trend was noted during the 48 hrs fermentation time. However, at 72 hrs fermentation time, there was a significant difference ($p<0.05$) noted on the Brix reading between the juices treated with 0% probiotic bacteria with another three concentrations of 2%, 5% and 10%.

3.4 Titrable acidity

Figure 4 shows the changes in Titrable acidity expressed as% lactic acid of probiotic watermelon juice fermented with 0%, 2%, 5% and 10% (w/v) of LAB namely *L. plantarum* (4A), *L. paracasei* (4B), and *L. acidophilus* (4C) at time 0 hr, 24 hrs, 48 hrs, and 72 hrs.

During 0 hr of fermentation time, there was no significant difference ($p>0.05$) between 5% and 10% concentration for all bacterial strains. However, a significant difference ($p<0.05$) was observed between samples of 0% with each 2%, 5% and 10% concentrations of *L. plantarum* while no significant difference was observed between samples of 0% with 2%, 5% and 10% concentration of *L. acidophilus*. During 24 hrs of fermentation time, no significant difference ($p>0.05$) was observed between samples fermented with 2%, 5% and 10% concentrations of bacteria. However, a significant difference was observed ($p<0.05$) between samples of 0% bacterial concentration with samples of 2%, 5% and 10% concentration respectively for all strains. At 48 hrs of fermentation time, significantly different ($p<0.05$) results were shown by samples with 2% and 5% concentration and also 5% and 10% concentration for *L. plantarum*. However, no significant differences ($p>0.05$) were observed between samples of 0% with each of the samples containing 2%, 5% and 10% concentrations of bacteria for all strains. At 72 hrs of fermentation time, there was no significant difference ($p>0.05$) in lactic acid content for all samples of *L. plantarum* and *L. acidophilus* while a significant difference ($p<0.05$) was observed between the control samples and samples containing 2%, 5% and 10% concentrations of *L. paracasei*.

The lactic acid content in the control (0%) probiotic juice was increased after 24 hrs of fermentation time and then decreased at 48 hrs and 72 hrs of fermentation time while for samples containing 2% bacteria, a decrease in lactic acid content occurred after 24 hrs of fermentation time followed by an increase in percentage at 48 hrs and 72 hrs. For samples containing 5% and 10% of bacteria, an increase in lactic acid content was observed after 72 hrs of fermentation for both samples of *L. plantarum* and *L. paracasei*, while for samples fermented with *L. acidophilus*, a reduction in lactic acid content was observed. The variations in acidity values during the fermentation process are due to the distinct amounts of sugars available within these substrates which were mainly transformed to organic acids by the lactic acid bacteria that lowers the pH hence, thus increasing its acidity (Salmerón *et al*., 2014).

The increasing trend in titrable acidity for samples containing *L. plantarum* and *L. paracasei* during 72 hrs

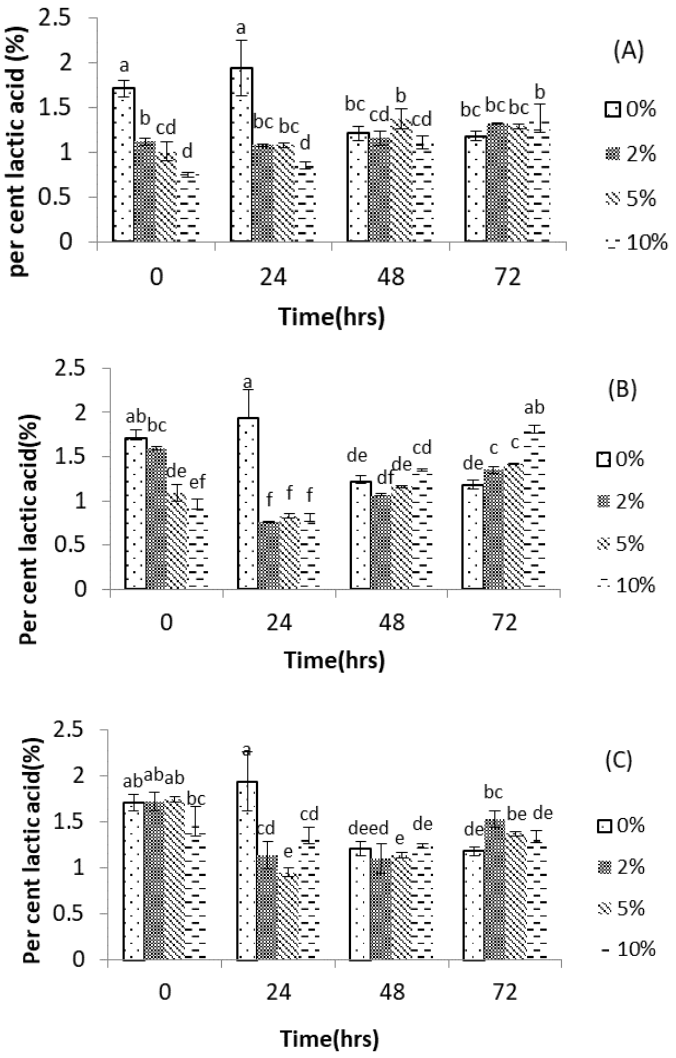

Figure 4. Percentage of lactic acid during fermentation of watermelon juice fermented with (A) *L. plantarum,* (B) *L. paracasei* and (C) *L. acidophilus.* Different letters between groups indicates significant difference at $p<0.05$ ($n = 3$).

of fermentation time with samples containing 10% of bacterial concentration shows the highest lactic acid content indicating the suitability of these strains in the production of watermelon probiotic juice. The results obtained were similar to the findings by Hussain *et al.* 2009) in which the average titrable acidity of probiotic yoghurt was 1.41%. Similar observations were also reported by Tarakci and Erdogan (2003) and Güler-Akin (2005) who observed an increase in total Titrable acidity during the storage period. A study done by Mousavi *et al.* (2011) indicated that lactic acid in pomegranate juice was produced by all the strains (*L. acidophilus* DSMZ 20079*, L. plantarum* DSMZ 20174*, L. delbrueckii* DSMZ 20006*, L. paracasei* DSMZ 15996) and its concentration increased as the fermentation commenced.

From these studies, it can be concluded that the best probiotic juice must contain a high percentage of lactic acid as it indicates the ability of lactic acid bacteria to metabolise sugar in the juice and converts it to lactic acid. The ability to metabolise the natural sugar in the juice shows that the probiotic bacteria could retain their survival and eventually exerts their beneficial properties to the consumers. Besides that, lactic acid can also be used as a preservative (acidifier) and flavour-enhancing agent by the food industry (Papagianni, 2012). Therefore, probiotic watermelon juice with a 10% concentration of *L. plantarum* and *L. acidophilus* was the best probiotic juice.

3.5 Microbiological analysis of probiotic watermelon juice

3.5.1 LAB count in log_{10} (CFU/mL) during fermentation of probiotic watermelon juice

The bacterial cell count was determined in log_{10} (CFU/mL) of probiotic watermelon juice fermented with 0%, 2%, 5% and 10% (w/v) of *L. plantarum, L. paracasei* and *L. acidophilus* during fermentation. The results are displayed in Figure 5. Samples inoculated with 0% cell concentration were treated as controls.

Figure 5 (A) shows the LAB count of probiotic watermelon juice inoculated with *L. plantarum* at 0 hr, 24 hrs, 48 hrs and 72 hrs of fermentation time. From 0 hr of fermentation time up to 72 hrs, no significant difference ($p>0.05$) was observed between samples inoculated with 2%, 5% and 10% of *L. plantarum*, but a significant difference ($p<0.05$) was observed between control samples of 0% bacterial concentrations with inoculated samples at all concentrations. Figure 5 (B) shows the LAB count of the probiotic watermelon juice inoculated with *L. paracasei*. A similar trend of bacterial growth was observed whereby the LAB count of the juice treated with 2%, 5%, and 10% *L. paracasei* was also significantly higher ($p<0.05$) than control during 0 hr, 24 hrs, 48 hrs and 72 hrs of fermentation time. No significant difference ($p>0.05$) of LAB count was observed between samples containing 2%, 5% and 10% from 0h to 72 hrs of fermentation time. The LAB counts of the probiotic watermelon juice inoculated with *L. acidophilus* at different concentrations are shown in Figure 5 (C). A significant difference ($p<0.05$) in LAB counts was observed between the control sample (0% concentration) and inoculated samples of 2%, 5% and 10% of bacteria at 0 hr, 48 hrs and 72 hrs of fermentation time. However, ($p<0.05$) a significant difference in LAB count was only observed between the control sample and the sample containing 2% of bacteria at 24 hrs.

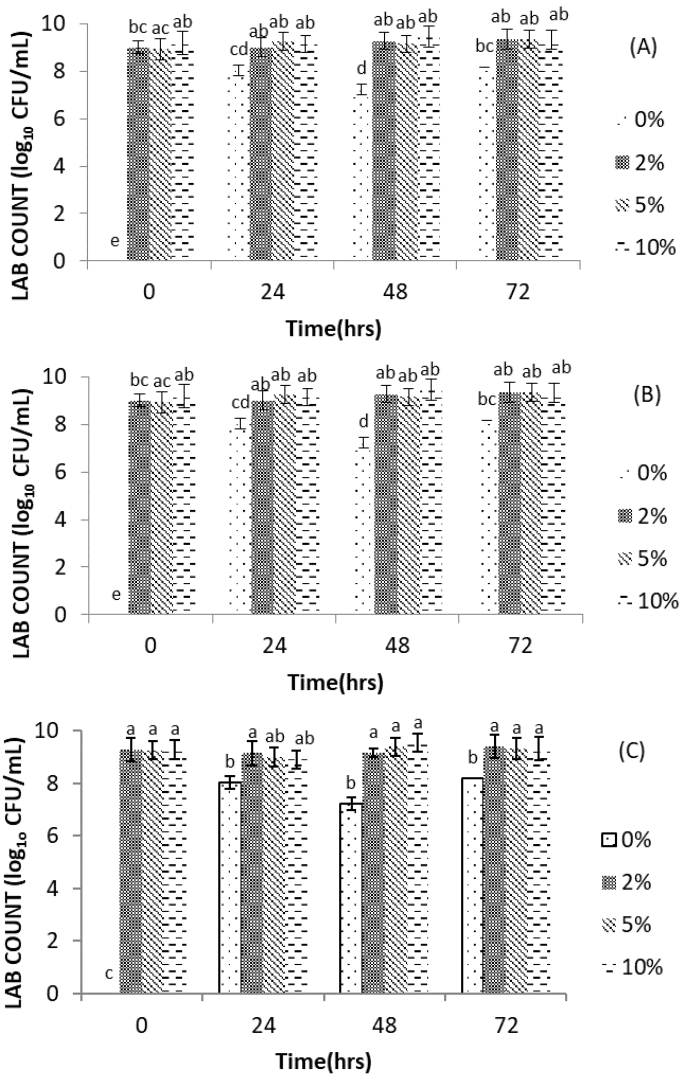

Figure 5. LAB count in log_{10} CFU/mL during fermentation of probiotic watermelon with (A) *L. plantarum,* (B) *L. paracasei* and (C) *L. acidophilus.* Different letters between groups indicates significant difference at $p<0.05$ ($n = 3$).

From the results, the increase in the LAB count of the control sample (0% concentration of bacteria) during 72 hrs of fermentation was due to the presence of naturally occurring lactic acid bacteria. It has been reported in a previous study that a total of 58 acid-producing bacteria were isolated from the young watermelon fruit samples (Chena *et al.,* 2016). In addition, all of the three strains of commercial probiotic

LAB used (*L. plantarum, L. paracasei* and *L. acidophilus*) have a count of between 8 to 9 $\log_{10}$ CFU/mL or has the viability between 10^8 to 10^9 CFU/mL. Different levels of probiotic bacteria in Bio-Yogurts have been recommended and specified, in order to exert the claimed health effects and are considered as probiotic products. The National Yogurt Association (NYA) of the United States specifies that 10^8 CFU/mL of lactic acid bacteria at the time of manufacture are required to use the NYA 'Live and Active Culture' logo on the products containers (Ostlie *et al.,* 2003). In Japan, the Fermented Milks and Lactic Acid Bacteria Beverages Association has specified a minimum of 10^7 CFU/mL of *Bifidobacteria* to be present in fresh dairy products as a standard (Ishibashi *et al.,* 1993). Therefore, maintaining the probiotic bacteria viability and survivability during product manufacturing and storage is a very crucial factor for effective probiotic products.

From the result, three strains of probiotic lactic acid bacteria tested in this study were suitable to be used for producing watermelon based probiotic drinks. The reasons why these cultures were used are because their effectiveness as probiotic cultures has been measured by UAS Laboratories before being used in food products such as clinical studies. According to Collins *et al.* (1998), proper *in vitro* studies should establish the potential health benefits of probiotics prior to undertaking *in vivo* trials. Tests such as acid and bile tolerance, antimicrobial production and adherence ability to human intestinal cells should be performed depending on the proposed health benefits by the UAS Laboratories. To clarify the identity of a probiotic present in the food, it was recommended that the microbial species be stated on the label. If a selection process has been undertaken at the strain level, the identity of the strain should also be included, since the probiotic effect seems to be strain specific. There is a need to accurately enumerate the probiotic bacteria in food products in order to include them on the label. The label should state the viable concentration of each probiotic present at the end of shelf life (Reid *et al.*, 2001).

3.6 Biochemical tests for LAB

Table 2 shows the result of a biochemical test for lactic acid bacteria isolated from the probiotic watermelon juice during the fermentation process. The biochemical test was conducted to confirm the presence of lactic acid bacteria that was introduced into the juice and to ensure that no contamination occurs during the analysis.

From Table 2, all of the lactic acid bacteria were identified as Gram-positive bacteria with a negative result on the catalase and oxidase test. The control was the plate with unknown bacteria that was isolated from the watermelon juice sample. Lactic acid bacteria are identified as Gram-positive, non-spore-forming rods, catalase-negative, usually non-motile, do not reduce nitrate, able to utilize glucose and in the absence of indole (Sheeladevi and Ramanathan, 2011). The control and the three strains of *L. plantarum, L. paracasei* and *L. acidophilus* were subjected to Gram staining and they were examined under a compound microscope. All the strains including the control sample gave blue-purple colour with staining; hence they all were Gram-positive bacteria (Devi *et al.*, 2013). The control (watermelon juice without probiotic lactic acid bacteria) may have natural LAB that is an inhabitant in the watermelon fruits. Based on the previous study, a total of 58 acid-producing bacteria were isolated from the young

Table 2. Results for biochemical tests of probiotic lactic acid bacteria in the watermelon juice

Bacteria		Gram-staining		Catalase test		Oxidase test	
		R1	R2	R1	R2	R1	R2
L. plantarum	0 hrs	+	+	-	-	-	-
	24 hrs	+	+	-	-	-	-
	48 hrs	+	+	-	-	-	-
	72 hrs	+	+	-	-	-	-
L. paracasei	0 hrs	+	+	-	-	-	-
	24 hrs	+	+	-	-	-	-
	48 hrs	+	+	-	-	-	-
	72 hrs	+	+	-	-	-	-
L. acidophilus	0 hrs	+	+	-	-	-	-
	24 hrs	+	+	-	-	-	-
	48 hrs	+	+	-	-	-	-
	72 hrs	+	+	-	-	-	-
Control	0 hrs	ND	ND	ND	ND	ND	ND
	24 hrs	+	+	-	-	-	-
	48 hrs	+	+	-	-	-	-
	72 hrs	+	+	-	-	-	-

R1 = Replicate 1, R2 = Replicate 2, ND = Not Detected

watermelon fruit samples. The total 176 isolates were initially divided into six groups (R1-R6) according to cell morphology and the results of the 16S rDNA RFLP analysis. The results identified group R1 isolates as *L. plantarum*-related species (Chena *et al.,* 2016).

A catalase test was done to test the ability of the organism to produce catalase. The oxidation of flavoproteins invariably results in the formation of hydrogen peroxide as one major product. In addition, this oxidation (and other oxygenation) produce small quantities of an even more toxic radical. In aerobes and aerotolerant aerobes, the potentially lethal accumulation of oxygen is prevented by the enzyme superoxide dismutase which catalyses it to hydrogen peroxide and oxygen. Catalase lies close to the cell membrane. A positive result was detected by the formation of air bubbles and negative results show no air bubbles. The growth of lactic isolates in the MRS medium was clear. Their growth was not accompanied by any appearance of gas bubbles. The total lack of CO_2 gas for all strains tested was an indicator of the homo-fermentative type. The preliminary test makes it possible to confirm the genus of the bacteria as *Lactobacillus* spp. (Holzapfel, 2002).

An Oxidase test was done to detect the presence of cytochrome C and hence the production of oxidase enzyme by the given test organism. Positive results on the oxidase test indicate that the test organism was able to develop purple colour due to oxidation of the reagent after the colony was directly applied with the oxidase reagent. The negative result did not show the purple colour (Hemraj *et al.*, 2013). The negative results of the oxidase test confirm the presence of *Lactobacillus* spp.

4. Conclusion

In conclusion, three different strains of commercial probiotic lactic acid bacteria, *L. plantarum, L. paracasei* and *L. acidophilus* produced different results in the chemical analyses and also microbiological analyses. The pH of all juices samples was significantly changed during the time of incubation but was not affected by bacterial concentration. Moreover, no significant difference ($p>0.05$) was observed in the Titrable acidity of the watermelon juice during the time of incubation but differ with different concentrations of lactic acid bacteria used. However, no significant difference ($p>0.05$) were shown in the Brix reading of the watermelon juice with the function of both times of incubation and different concentration of lactic acid bacteria used. In addition, the duration of incubation time does not significantly affect ($p>0.05$) the LAB count of the juices but was affected by the concentration of the bacteria. Therefore, all three strains are suitable for the development of probiotic watermelon juice. In future, the sensorial aspect could be further studied to confirm the acceptability of this juice.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank MARDI, Serdang for the collaboration with UMT. The authors would like to thank the financial support and assistance of laboratory staff from the Faculty of Fisheries and Food Science, UMT.

References

Ahmad, A., Yap, W.B., Kofli, N.T. and Ghazali, A.R. (2018). Probiotic potentials of *Lactobacillus plantarum* isolated from fermented durian (Tempoyak), a Malaysian traditional condiment. *Food Science and Nutrition*, 6(6), 1370-1377. https://doi.org/10.1002/fsn3.672

Akin, M.B., Akin M.S. and Kyrmacy, Z. (2007). Effects of inulin and sugar levels on the viability of yoghurt and probiotic bacteria and the physical and sensory characteristics in probiotic ice-cream. *Food Chemistry*, 104(1), 93–99. https://doi.org/10.1016/j.foodchem.2006.11.030

Bisognin, D.A. (2002). Origin and evolution of cultivated cucurbits. *Ciência Rural*, 32(4), 715–723. https://doi.org/10.1590/s0103-84782002000400028

Chen, Y.-S., Wu, H.-C., Yu, C.-R., Chen, Z.-Y., Lu, Y.-C. and Yanagida, F. (2016). Isolation and characterization of lactic acid bacteria from xi-gua-mian (fermented watermelon), a traditional fermented food in Taiwan. *Italian Journal of Food Science*, 28(1), 9–14. https://doi.org/10.14674/1120-1770/ijfs.v451.

Collins, J.K., Thornton, G. and O'Sullivan, G.O. (1998). Selection of probiotic strains for human applications. *International Dairy Journal*, 8(5-6), 487-490. https://doi.org/10.1016/S0958-6946(98)00073-9

Dammak, M.I., Salem, Y.B., Belaid, A., Mansour, H.B., Hammami, S., Le Cerf, D. and Majdoub, H. (2019). Partial characterization and antitumor activity of a polysaccharide isolated from watermelon rinds. *International Journal of Biological Macromolecules*, 136, 632–641. https://doi.org/10.1016/j.ijbiomac.2019.06.110

Devi, M., Rebecca, L.J. and Sumathy, S. (2013). Bactericidal activity of the lactic acid bacteria *Lactobacillus delbreukii. Journal of Chemical and*

Pharmaceutical Research, 5(2), 176-180.

Edwards, A.J., Vinyard, B.T., Wiley, E.R., Brown, E.D., Collins, J.K. and Perkins-Veazie, P.A. (2003). Consumption of watermelon juice increase plasma concentrations of lycopene and β-carotene in humans. *Nutrition*, 133(4), 1043-1050. https://doi.org/10.1093/jn/133.4.1043

Giori, G., de Valdez, G., Holgado A. and Oliver, G. (1985). Effect of pH and temperature on the proteolytic activity of lactic acid bacteria. *Journal Dairy Science*, 68, 2160–2164. https://doi.org/10.3168/jds.S0022-0302(85)81085-7

Granato, D., Branco, G.F., Nazzaro, F., Cruz, A.G. and Faria, J.A.F. (2010). Functional Food and Nondairy Probiotic Food Development: Trends, Concepts and Products. *Comprehensive Reviews in Food Science and Food Safety*, 9(3), 292-302. https://doi.org/10.1111/j.1541-4337.2010.00110.x

Güler-Akin, M.B. (2005). The effects of different incubation temperatures on the acetaldehyde content and viable bacteria counts of bio-yogurt made from ewe's milk. *International Journal of Dairy Technology*, 58(3), 174-179. https://doi.org/10.1111/j.1471-0307.2005.00209.x

Hasler, C.M. (2002). Functional foods: Benefits, concerns and challenges - A position paper from the American Council on Science and Health. *Journal of Nutrition*, 132(12), 3772–3781. https://doi.org/10.1093/jn/132.12.3772

Hemraj, V., Diksha, S. and Avneet, G. (2013). A review on commonly used biochemical test for bacteria. *Journal of Life Science*, 1(1), 1-7.

Hitchins, A.D. and Jinneman, K. (2011). BAM: Detection and Enumeration of *Listeria monocytogenes*. Retrieved on September 6, 2019 from FDA Website: http://www.fda.gov/Food/Food Science Research/LaboratoryMethods/ucm071400.htm.

Holzapfel, W.H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology,* 75(3), 197-212. https://doi.org/10.1016/S0168-1605(01)00707-3

Hussain, I., Rahman, A. and Atkinson, N. (2009). Quality Comparison of Probiotic and Natural Yogurt. *Pakistan Journal of Nutrition,* 8(1), 9-12. https://doi.org/10.3923/pjn.2009.9.12

Ishibashi, N. and Shimamura, S. (1993). *Bifidobacteria*: Research and Development in Japan. *Journal of Food Technology*, 47(6) 126, 129–134.

Khatoon, N., Rajinder, K. and Gupta. (2015). Probiotics Beverages of Sweet Lime and Sugarcane juices and its Physiochemical, Microbiological and Shelf-life Studies. *Journal of Pharmacognosy and Phytochemistry*, 4(3), 25-34.

Mousavi, Z.E., Mousavi, S.M., Razavi, S.H., Emam-Djomeh, Z. and Kiani, H. (2011). Fermentation of pomegranate juice by probiotic lactic acid bacteria. *World Journal of Microbiology and Biotechnology*, 27(1), 123–128. https://doi.org/10.1007/s11274-010-0436-1

Ninomiya, M., Itoh, T., Fujita, S., Hashizume, T. and Koketsu, M. (2020). Phenolic glycosides from young fruits of *Citrullus lanatus*. *Phytochemistry Letters*, 40(10), 135–138. https://doi.org/10.1016/j.phytol.2020.09.014

Nuraida, A. (2015). A review: Health promoting lactic acid bacteria in traditional Indonesian fermented foods. *Food Science and Human Wellness*, 4(2), 47-55. https://doi.org/10.1016/j.fshw.2015.06.001

Ostlie, H., Helland, M.H. and Narvhu, J. (2003). Growth and metabolism of probiotics in milk. *International Journal of Food Microbiology*, 87(1-2), 17-27. https://doi.org/10.1016/S0168-1605(03)00044-8

Pakbin, B., Razavi, S.H., Mahmoudi, R. and Gajarbeygi, P. (2014). Producing Probiotic Peach Juice. *Biotechnology and Health Science,* 1(3), 1-5. https://doi.org/10.17795/bhs-24683

Papagianni, M. (2012). Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. *Computational and Structural Biotechnology Journal*, 3(4), e201210003. https://doi.org/10.5936/csbj.201210003

Ramirez, C., Wacher, M.L., Eslava, C.A. and PerezChabela, M.L. (2013). Probiotic potential of thermotolerant lactic acid bacteria strains isolated from cooked meat products. *International Food Research Journal*, 20(2), 991-1000.

Reid, G., Zalai, C. and Gardiner, G. (2001). Urogenital lactobacilli probiotics, reliability and regulatory issues. *Journal Dairy Science*, 84(E. Suppl.), E164-169. https://doi.org/10.3168/jds.S0022-0302(01)70211-1

Romdhane, M.B., Haddar, A., Ghazala, I., Jeddou, K.B., Helbert, C.B. and Ellouz-Chaabouni, S. (2017). Optimization of polysaccharides extraction from watermelon rinds: Structure, functional and biological activities. *Food Chemistry*, 216(2), 355–364. https://doi.org/10.1016/j.foodchem.2016.08.056

Salleh, F., Lani, M.N. and Ismail, N. (2014). Antimicrobial activity of cell-free supernatant of lactic acid bacteria isolated from fermented durian flesh against multiple antibiotic resistances,

Salmonella associated with food poisoning cases in Malaysia. *IOSR Journal of Pharmacy and Biological Science*, 9(6), 60-65. https://doi.org/10.9790/3008-09646065

Salleh, F., Lani, M.N., Tuan Chilek, T.Z., Kamaruding, N.A. and Ismail, N. (2021). Lactic acid bacteria producing sorbic acid and benzoic acid compounds from fermented durian flesh (Tempoyak) and their antibacterial activities against foodborne pathogenic bacteria. *Applied Food Biotechnology*, 8(2), 121-132.

Salmerón, I., Thomas, K. and Pandiella, S.S. (2014). Effect of substrate composition and inoculum on the fermentation kinetics and flavour compound profiles of potentially non-dairy probiotic formulations. *LWT-Food Science and Technology*, 55(1), 240– 247. https://doi.org/10.1016/j.lwt.2013.07.008

Sheeladevi, A. and Ramanathan, N. (2011). Lactic Acid Production Using Lactic Acid Bacteria under Optimized Conditions. *International Journal of Pharmaceutical and Biological Archives*, 2(6), 1686-1691.

Shields, P. and Cathcart, L. (2013). Oxidase Test Protocol. ASM Microbe Library [online]. Retrieved on September 9, 2019 from ASM Microbe Library Website: http://www.microbelibrary.org/library/laboratory+test/3229 oxidase-test-protocol.

Shukla, M., Kumar Y, Admassu S.H. (2013). Development of Probiotic Beverage from Whey and Pineapple Juice. *Journal of Food Processing and Technology*, 4(2), 1-4.

Sivudu, S.N. Umamahesh, K. and Reddy, O.V.S. (2014). Comparative study on Probiotication of mixed Watermelon and Tomato juice by using Probiotic strains of Lactobacilli. *International Journal of Current Microbiology and Applied Sciences*, 3(11), 977-984.

Smith, A.C. and Hussey, M. (2005). Gram Stain Protocols. MicrobeLibrary.org. Retrieved December 18, 2016 from: http://www.microbelibrary.org/asmonly/details.asp?id=1989

Tarakci, Z. and Kucukoner, E. (2003). Physical, chemical, microbiological and sensory characteristics of some fruit-flavored yogurt. *Research Journal of Dairy Science*, 14(2), 10-14.

Trontel, A., Barsic, V., Slavica, A., Santek, B. and Novak, S. (2010). Modelling the effect of different substrates and temperature on the growth and lactic acid production by *Lactobacillus amylovorus* DSM 20531T in batch process. *Food Technology and Biotechnology,* 48(3), 352–361.

Tuorila, H. and Gardello, A.V. (2002). Consumer responses to an off-flavor in juice in the presence of specific health claims. *Food Quality and Preference*, 13(7–8), 561–569. https://doi.org/10.1016/S0950-3293(01)00076-3

Zamuz, S., Munekata, P.E.S., Gullón, B., Rocchetti, G., Montesano, D. and Lorenzo, J.M. (2021). *Citrullus lanatu*s as source of bioactive components: An up-to-date review. *Trends in Food Science and Technology*, 111(3), 208–222. https://doi.org/10.1016/j.tifs.2021.03.002

eISSN: 2550-2166