



## Production of a novel probiotic date juice with anti-proliferative activity against Hep-2 cancer cells

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### Abstract

Date fruit is the ancient discovered and consumed fruit. Worldwide, Egypt is the largest producer of date. Besides its health benefits, many recent studies have proved its antioxidant, anticancer and anti-inflammatory effect. Currently, probiotic fermented fruit juices have a great attention especially for lactose intolerance patients. This study assessed the survival of two probiotics (*Lactobacillus acidophilus* and *L. sakei*) in the date juice. Phenolics characterization was performed by HPLC. Anti-proliferative activity of fermented juice against Caco-2 and Hep-2 cell lines was also evaluated. Under cold storage, *L. sakei* was still live for 2 weeks at  $1.8 \times 10^7$  CFU/mL. In contrast, *L. acidophilus* reduced from  $5.2 \times 10^6$  CFU/mL to below the minimum value of probiotic products just after 1 week. *L. sakei* significantly multiplied the total phenols and antioxidant activity in the juice compared to the unfermented juice. Interestingly, probiotic date juice exhibited an antitumor activity against larynx cell lines (Hep-2) with no activity against Caco-2. Date juice was proved to be suitable for probiotic and as potential larynx anticancer juice. Therefore, the developed juice is recommended to contribute to the available probiotic foodstuffs.

**Keywords:** date juice; non-dairy probiotic product; phenolics multiplication; lactose intolerance; Hep-2 antitumor.

**Practical Application:** Probiotic fermented date juice with antitumor effect was created.

### 1 Introduction

Recently, the priorities of research are focused on developing functional foods and beverages to improve human health. The most widespread functional ingredient is probiotics (Guimaraes et al., 2020). In 2001, FAO and WHO debated the emerging field of probiotics and defining probiotics as “[...] live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al., 2014, p. 506). The consumption of probiotics-enriched food becomes a popular trend due to the scientific evidence about its beneficial effect on the gut micro-flora (Rijkers et al., 2011). Currently, probiotics are widely added to dairy products (Mortazavian et al., 2011; Barros et al., 2020) as well as non-dairy foodstuffs (Shori, 2016). The high capacity of probiotics to provide health benefits is varied according to the probiotic lactic acid strain and the delivery food product (Zucko et al., 2020; Roobab et al., 2020). Probiotics can decrease the weight gain, waist circumference, serum glucose, insulin level and low density lipoprotein-cholesterol, oxidized products in the blood, inflammation markers; and increase glutathione and high-density lipoprotein-cholesterol (Ejtahed et al., 2019; Rezazadeh et al., 2019; Roshan et al., 2019; Salami et al., 2019). Probiotics also showed anti-obesity (Chen et al., 2011), gastrointestinal ecosystems improvement (Reddy & Rivenson, 1993; Tang et al., 2016), anti-cancer (Kumar et al., 2012), anti-oxidative of the lungs, gut, and liver (Vasconcelos et al., 2019) and anti-hyperglycemic effect (Grom et al., 2020).

Cancer is the second cause of death all over the world that reached 9.6 million deaths in 2018. Approximately 70% of cancer deaths appear in low and middle-income countries. Nearly,

30% of cancer deaths are due to several reasons such as: high BMI (body mass index), low intake of fruit and vegetable, lack of exercise, smoking, and alcoholism (World Health Organization, 2018). Colorectal cancer (Caco-2) is the third most common cancer in men and the second common cancer in women worldwide (Dallal et al., 2015). Recently, new cases suffered from larynx cancer (0.8% of total cancer cases number) were also disclosed (World Health Organization, 2019). Probiotics and their metabolites exhibited an anti-cancer activity towards different kinds of cancer. Kwak et al. (2014) reported that, the traditional Korean fermented food by probiotic lactic acid bacteria (*Weissella*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* species) are known to put down the activity of carcinogen-activating enzymes, such as nitroreductase,  $\beta$ -glucosidase, azoreductase, 7- $\alpha$ -dehydrogenase, and  $\beta$ -glucuronidase and inhibit or neutralize the cancer-causing agents and pathogenic microbes. On the other side, the water soluble peptides extracts obtained from buffalo and cow milk Cheddar (prepared by the action of *Lactococcus lactis* subsp. *lactis* and *cremoris*) demonstrated marked growth inhibition activity towards human lung (H-1299) (Rafiq et al., 2018a), colon (HT-29) (Rafiq et al., 2018b) and adenocarcinoma (HCT-116) cancer cell lines (Rafiq et al., 2020) by cell cycle arrest and extensive apoptosis induction.

Dairy products are the common food carrier for probiotics, but the consumption of these products is limited due to increment of vegetarianism and lactose intolerant individuals. Thus, the development of non-dairy probiotic products, including foods based on vegetables (Eliane et al., 2013), cereals (Yahyaoui et al., 2017)

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and fruit (White & Hekmat, 2018; Campos et al., 2019) has been widely invented. The date palm (*Phoenix dactylifera* L.), a tropical and subtropical fruit tree, which belongs to the family *Palmae arecaceae* is broadly grown around the world (El Sohaimy et al., 2015). Egypt is the world's largest producer (1,590,414 tons in 2017) according to FAOSTAT (Food and Agriculture Organization of the United Nations, 2017) and it produces about 20% of the total world production. Semi-dry date represents about 20.4% of the total Egyptian production and Siwi date variety is one of semi-dry dates which represent about 16.9% (Bekheet & El-Sharabasy, 2019). Date fruit is rich in phytochemicals such as carotenoids, polyphenols (e.g., phenolic acids, isoflavons, lignans and flavonoids), tannins and sterols (Al-Alawi et al., 2017). It is considered a moderate source of riboflavin, niacin, pyridoxal and folate as 100 g of dates provide over 9% of the daily (RDA/AI) for adults (Baliga et al., 2011). Many studies have shown that date has antitumor activity as it can stimulate IFN- $\gamma$  RNA expression in cells and increases Th1 immune response (Karasawa et al., 2011; Tang et al., 2013). Date syrup was evaluated as additive to some probiotic dairy products such as milk (Al-Otaibi et al., 2013) and yoghurt (Aleid et al., 2018). However, no reports investigate nor the suitability of non-dairy date juice as a vehicle of probiotic bacteria or the effect of the fermented juice on cancer cell lines.

This study aimed to evaluate the influence of probiotic lactic acid bacteria (*Lactobacillus acidophilus* La5 and *L. sakei* NRRL 1917) fermentation on the physicochemical and sensory properties, phenols profile using HPLC, antioxidant power of the obtained date juice. Furthermore, *in vitro* antitumor activities of probiotic juice towards human larynx carcinoma (Hep-2) and colorectal cancer (Caco-2) cell lines were also tested.

## 2 Materials and methods

### 2.1 Materials

Dimethylsulphoxide (DMSO), RPMI-1640 medium, trypan blue, fetal bovine serum, penicillin/streptomycin antibiotic, Trypsin-EDTA, Folin-Ciocalteu, DPPH and phenolics standards were purchased from Sigma Aldrich Chemical Co. (St. Louis, Mo, USA). de Man Rogosa and Sharpe (MRS) medium was obtained from Merck (Darmstadt, Germany). The commercial minced date fruit (*Phoenix dactylifera* L. var. Siwi) was purchased from the local market and reserved at room temperature. Human tumor carcinoma cell lines (human colon cancer, Caco-2) and (human larynx carcinoma, Hep-2) that used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were maintained by sub-culturing at the National Cancer Institute, Cairo, Egypt.

Two probiotic lactic acid bacteria (LAB) strains were used in this study *i.e.* *Lactobacillus acidophilus* La5 and *Lactobacillus sakei* NRRL 1917. They supplied from Chr. Hansen (Horsholm, Denmark) and the US Department of Agriculture, Northern Regional Research Laboratories (NRRL), respectively. LAB stock cultures were stored frozen at  $-20^{\circ}\text{C}$  in MRS medium (with 20% glycerol) and activated at  $37^{\circ}\text{C}$  in MRS medium before use. The numeration of the LAB cells after fermentation and

during cold storage was performed by the standard plate count method with MRS agar after a proper dilution and expressed as log CFU/mL.

### 2.2 Preparation of inoculum and fermented date juice

The bacterial cultures were grown separately at  $37^{\circ}\text{C}$  for 48 h in the MRS broth to reach  $1.2 \times 10^6$  CFU/mL for *L. acidophilus* La5 and  $1.0 \times 10^7$  CFU/mL for *L. sakei*, then cells were harvested by centrifugation at  $4000 \times g$  for 5 min (Hermle, Z 300, Germany), washed twice then resuspended in sterilized distilled water. Subsequently, the minced date was mixed with distilled water and diluted to 18% TSS (initial pH 4.0). Diluted juice was packed in sterilized glass bottles and pasteurized at  $85^{\circ}\text{C}$  for 10 min, cooled then inoculated with the inoculums (4% v/v, inoculums/ juice) and anaerobically fermented at  $37^{\circ}\text{C}$  for further 48 h. Viability of *L. acidophilus* La5 and *L. sakei* were  $7 \times 10^6$  and  $2.2 \times 10^8$  CFU/mL, respectively. Samples were taken after the fermentation process for chemical and microbiological experiments. The fermented juice bottles were refrigerated at  $4^{\circ}\text{C}$  for 21 days just after the end of fermentation. The viable cell counts, pH, acidity, reducing and total sugars, total phenolics and antioxidant activity of probiotic juice were determined weekly intervals of cold storage.

### 2.3. Physicochemical analysis

#### Total soluble solids, acidity, reducing and total sugar

Total soluble solids (TSS) of date juice were measured with a hand refractometer (Model DR-A1; Atago, Japan), and results were expressed as percent of total soluble solids (%) in juice at  $25^{\circ}\text{C}$  (Khosravi et al., 2019). Total titratable acidity, expressed as mg/100 mL lactic acid equivalents of organic acids, was determined by titrating the diluted date juice samples with 0.1 N NaOH. The pH measurement was accomplished by a pH-meter (Orion Research Incorporated, Boston, USA).

Concentration of reducing sugar in date juice was estimated by the dinitrosalicylic acid method (DNSA) at 540 nm using Spectrophotometer (Unico, UV2000, USA) according to Garriga et al. (2017). Whereas, the total sugar content was determined by Anthrone method at 630 nm according to More et al. (2018). Glucose was used as a standard for both methods.

#### Total Phenolic Compounds (TPC)

Fermented or unfermented date juice content of TPC was determined according to Folin-Ciocalteu method as described by Singleton & Rossi (1965) with some modifications. Half mL of date juice was diluted with 12.5 mL water then, filtrated using filter paper No. 1. One mL of Folin-Ciocalteu reagent (10%, v/v) was added and after 5 min at room temperature, 0.8 mL of 20% (w/v)  $\text{CaCO}_3$  was added to the mixture and vortexed, then allowed to stand for 1 h at room temperature in the darkness. The absorbance was measured at 760 nm using Spectrophotometer. The phenolic compound values were calculated using the standard curve of known concentrations of gallic acid solutions and expressed as milligram gallic acid equivalents (GAE)/100 mL date juice.

### Antioxidant activity

Antioxidant activity of the date juice was determined according to the method of Cam et al. (2014) with some modifications. The stock solution of DPPH was prepared as follows: 2.5 mg of DPPH was dissolved in 100 mL methanol (about 0.0625 mM). 0.1 mL of date juice was diluted with 10 mL water, filtrated using Whatman No. 1 filter paper then, mixed with 3.9 mL of DPPH stock solution. The control sample was DPPH stock solution. The absorbance was measured using Spectrophotometer at 515 nm after incubation in the dark at room temperature for 30 min. The Antioxidant activity as inhibition percent was calculated according to the following Equation 1:

$$\text{Inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

### 2.4 Phenols characterization

High-Performance Liquid Chromatography (HPLC) was used to identify the phenolic compounds in fermented and unfermented (control) date juice after 2 weeks of cold storage. HPLC analyzed with Agilent 1260 infinity HPLC series (Agilent, USA), equipped with a quaternary pump, a Zorbax Eclipse plus C180 column 100 × 4.6 mm i.d., (Agilent Technologies, USA), operated at 30 °C. The separation was carried out using a triple linear elution gradient: A)- HPLC grade water 0.2% H<sub>3</sub>PO<sub>4</sub> (v/v), B) - methanol and C)- acetonitrile. The injected volume was 20 µL. The wavelength of the VWD detector was 284 nm. 24 polyphenol standards such as: kampherol, rutin, gallic acid, catechol, *p*- hydroxyl benzoic acid, caffeine, vanillic acid, caffeic acid, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, ellagic, benzoic acid, *o*-coumaric acid, salicylic acid, chlorogenic and cinnamic acid were used for identification and quantification of the phenolic compounds. The identification of each phenolic compound was done by comparing its peak area (Supplementary material 1) with the calibration curve.

### 2.5 Sensory evaluation

Sensory characteristics including color, sweetness, sourness, odor, consistency or smoothness and overall acceptability were evaluated by 40 untrained panelists of the Food Science Department (20 females and 20 males, aged 25 to 40 y), Cairo University. A 9-point Hedonic scale (0-2 = dislike extremely, 3-4 = dislike slightly, 5 = fair, 6-8 = like moderately, and 9 = excellent) was utilized for such purpose. The rejection limit for the sample recorded 6 during the storage period. The date juice samples were served at room temperature in 50 mL glass cups at zero time and after 1, 2 and 3 weeks of cold storage (Ali, 2018).

### 2.6 Cytotoxicity effect on human cancer cell lines

#### Cell line culture

RPMI-1640 medium was used for culturing and maintenance of the human tumor cell lines. After filter sterilization (0.22 µm), the medium was supplemented with 1% penicillin/streptomycin (100 U/mL penicillin and 2 mg/mL streptomycin) and 10% of fetal bovine serum (FBS).

### Antitumor effect of fermented date juice

The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay according to Vichai & Kirtikara (2006). Cells were seeded in 96-well microtiter plates (at initial concentration, 3 × 10<sup>3</sup> cell/well) in 150 µL fresh medium and allowed to attach the plates. Firstly, fermented and unfermented date juice samples (after 2 weeks of cold storage) were freeze dried (at – 40 °C by Snijders Scientific, Holland). Different concentrations (0, 62.5, 125, 250, 500 µL/mL) of juice were added to the plates and incubated for 48 h. The cells were fixed with 50 µL cold trichloroacetic acid (10% final concentration) for 1 h at 4 °C. The plates were washed with distilled water using automatic washer (Tecan, Germany) and stained with 50 µL SRB dye (0.4% dissolved in 1% acetic acid for 30 min at room temperature). The plates were washed with acetic acid (1%) then air-dried. The dye was solubilized with 100 µL/well of 10 M Tris buffer (pH 10.5) and the absorbance of each well was measured spectrophotometrically at 570 nm with the ELISA microplate reader (Sunrise Tecan reader, Germany). The mean background absorbance was automatically subtracted and the mean values of each juice concentration were calculated. The percentage of cell survival was calculated by the following Formula 2:

$$\text{Surviving fraction} = \text{OD (treated cells)} / \text{OD (control cells)} \quad (2)$$

The IC<sub>50</sub> values (the concentrations of resveratrol required to produce 50% inhibition of cell growth) were also calculated.

### 2.7 Statistical analysis

The experiments were repeated three times (means = 3) and the pooled data were subjected to one-way analysis of variance (ANOVA) with Co-stat software. The Duncan's test at *p* < 0.05 was used to compare means among treatments during storage period.

## 3 Results and discussion

### 3.1 Viability of probiotic bacteria, acidity and pH during cold storage

Based on existing standards and from a health view-point, it is very important to retain the viability and functional activity of probiotic strains throughout the shelf life of the product (Granato et al., 2010). The effect of cold storage (at 4 °C) for 3 weeks on the viability of LAB, acidity and pH of fermented date juice is indicated in Table 1.

Both studied LAB strains in fermented date juice remained viable for three weeks under cold storage. That may be correlated to the high content of β-glucan in dates that protects LAB during refrigeration as noted by Perricone et al. (2015). Results demonstrated that *Lactobacillus sakei* cells reached 2.25 × 10<sup>8</sup> CFU/mL after incorporation and fermentation in date juice and still survive for 2 weeks under cold storage (1.8 × 10<sup>7</sup> CFU/mL) even with low pH. It decreased 3 logarithmic orders after the 3<sup>rd</sup> week; that probably due to cold temperature or organic acids accumulation. On the other hand, viable cells of *L. acidophilus* reduced from 5.2 × 10<sup>6</sup> CFU/mL to below the minimum accepted value (10<sup>6</sup> CFU/mL) of probiotic products just after 1 week storage. After that, the cell viability was stable at 10<sup>4</sup>

**Table 1.** Viability of *L. acidophilus* and *L. sakei*, acidity and pH of fermented and unfermented date juice during refrigerated storage at 4 °C ± SD.

Time (week)	Viability (log CFU/mL)		Acidity (%)			pH		
	<i>L. acidophilus</i>	<i>L. sakei</i>	<i>L. acidophilus</i>	<i>L. sakei</i>	Control	<i>L. acidophilus</i>	<i>L. sakei</i>	Control
zero	6.81 ± 0.11 <sup>aB*</sup>	8.35 ± 0.05 <sup>aA</sup>	0.57 ± 0.05 <sup>bA</sup>	0.45 ± 0.05 <sup>bb</sup>	0.28 ± 0.08 <sup>bc</sup>	4.33 ± 0.06 <sup>aB</sup>	4.26 ± 0.06 <sup>aB</sup>	6.06 ± 0.06 <sup>bA</sup>
1	5.10 ± 0.03 <sup>bb</sup>	7.82 ± 0.07 <sup>ba</sup>	0.69 ± 0.05 <sup>aA</sup>	0.60 ± 0.01 <sup>aB</sup>	0.27 ± 0.00 <sup>bc</sup>	3.96 ± 0.06 <sup>bc</sup>	4.13 ± 0.06 <sup>bb</sup>	6.30 ± 0.10 <sup>aA</sup>
2	4.79 ± 0.04 <sup>cb</sup>	7.25 ± 0.02 <sup>ca</sup>	0.75 ± 0.05 <sup>aA</sup>	0.63 ± 0.06 <sup>aB</sup>	0.33 ± 0.05 <sup>bc</sup>	3.76 ± 0.06 <sup>cc</sup>	4.03 ± 0.06 <sup>bc</sup>	6.10 ± 0.17 <sup>abA</sup>
3	4.32 ± 0.02 <sup>db</sup>	5.71 ± 0.12 <sup>da</sup>	0.72 ± 0.01 <sup>aA</sup>	0.65 ± 0.06 <sup>aA</sup>	0.45 ± 0.00 <sup>ab</sup>	3.43 ± 0.06 <sup>dc</sup>	3.66 ± 0.05 <sup>cb</sup>	5.73 ± 0.06 <sup>ca</sup>

Samples of date juice were fermented at 37 °C for 2 days (zero time) then stored at 4 °C for 3 weeks; \*The experimental values (means and standard deviations for n=3) with capital letters are significantly different between columns, while small letters between rows.

**Table 2.** Estimated TSS % of fermented and unfermented date juice during refrigerated storage at 4 °C ± SD.

Time (week)	TSS %		
	<i>L. acidophilus</i>	<i>L. sakei</i>	Control
zero	18.45 ± 0.00 <sup>aC*</sup>	18.60 ± 0.28 <sup>aB</sup>	18.80 ± 0.00 <sup>aA</sup>
1	18.35 ± 0.00 <sup>bb</sup>	18.40 ± 0.07 <sup>bb</sup>	18.80 ± 0.14 <sup>aA</sup>
2	18.15 ± 0.21 <sup>cd</sup>	18.25 ± 0.07 <sup>cc</sup>	18.75 ± 0.42 <sup>aA</sup>
3	17.80 ± 0.00 <sup>dc</sup>	18.00 ± 0.00 <sup>db</sup>	18.75 ± 0.49 <sup>aA</sup>

Samples of date juice were fermented at 37 °C for 2 days (zero time) then stored at 4 °C for 3 weeks; \*The experimental values (means and standard deviations for n=3) with capital letters are significantly different between columns, while small letters between rows.

**Table 3.** Estimated total and reducing sugar (g/100 mL) of fermented and unfermented date juice during refrigerated storage at 4 °C ± SD.

Time (week)	Total sugar (g/100 mL)			Reducing sugar (g/100 mL)		
	<i>L. acidophilus</i>	<i>L. sakei</i>	Control	<i>L. acidophilus</i>	<i>L. sakei</i>	Control
zero	14.50 ± 0.00 <sup>aC</sup>	15.20 ± 0.00 <sup>aB</sup>	16.20 ± 0.00 <sup>aA</sup>	11.90 ± 0.00 <sup>aC</sup>	12.60 ± 0.00 <sup>aB</sup>	13.49 ± 0.01 <sup>aA</sup>
1	12.03 ± 0.01 <sup>bc</sup>	13.05 ± 0.07 <sup>bb</sup>	16.18 ± 0.05 <sup>aA</sup>	11.05 ± 0.07 <sup>bc</sup>	11.45 ± 0.07 <sup>bb</sup>	13.49 ± 0.07 <sup>aA</sup>
2	11.75 ± 0.07 <sup>cc</sup>	12.80 ± 0.00 <sup>cb</sup>	16.05 ± 0.07 <sup>aA</sup>	10.88 ± 0.07 <sup>cb</sup>	11.00 ± 0.00 <sup>cb</sup>	13.15 ± 0.01 <sup>ba</sup>
3	11.60 ± 0.00 <sup>dc</sup>	12.50 ± 0.00 <sup>db</sup>	15.50 ± 0.21 <sup>ba</sup>	10.50 ± 0.00 <sup>dc</sup>	10.90 ± 0.00 <sup>db</sup>	12.89 ± 0.21 <sup>ca</sup>

Samples of date juice were fermented at 37 °C for 2 days (zero time) then stored at 4 °C for 3 weeks. The experimental values (means and standard deviations for n = 3) with capital letters are significantly different between columns, while small letters between rows.

for the next 2 weeks. This decline may be due to the decrease in the pH from 4.33 to 3.43 after 3 weeks. Sheehan et al. (2007) reported that low pH juices, with range of pH between 2.5 and 3.7, cause an increase in cells sensitivity to stressful conditions. Viability of probiotic lactic acid bacteria in fermented fruits or vegetables juices depends on many factors such as: the nutrients content, pH and acidity. The effect of the refrigerated storage on the viability of probiotics has been studied by many researchers. Viability of *L. casei* in fermented sonicated pineapple juice maintained higher than 10<sup>6</sup> CFU/mL throughout the cold storage for 6 weeks (Costa et al., 2013). However, Daneshi et al. (2013) studied the viability in milk/carrot juice and they observed that all studied LAB strains attained viable cell numbers reduction of less than 1 log CFU/mL. *L. acidophilus* La5 showed a stable level of viable cells (98.8% viability) during storage over 20 days. The viability of other strains including *B. lactis* BB 12, *L. rhamnosus* and *L. plantarum* was 91.9, 90.1 and 88%, respectively. On the other hand, Karbasi et al. (2015) noticed that *L. acidophilus* survived above 10<sup>6</sup> CFU/mL for 4 weeks while *L. rhamnosus* reduced below this value after 2 weeks in cold fermented pomegranate juice. Also, *L. rhamnosus* GR-1 reached viable counts of at least 10<sup>7</sup> CFU/mL in all fruit juice samples (apple cider, orange, and grape) that tested by White & Hekmat (2018) during cold storage. Only the orange juice samples did not have a significant difference ( $p < 0.05$ ) in mean microbial counts over 30 days of storage.

Results in Table 1 also showed a significant ( $p < 0.05$ ) decrease in pH values with a simultaneous increase in acidity in juice incorporated *L. acidophilus* in compared to control juice.

Meanwhile, it could be observed that *L. sakei* decreased the pH after 1 week to 4.13 and remained with no significant difference for the next two weeks. During the cold storage of control sample (without LAB), pH did not show significant change until the second week, but it decreased to 5.73 in 3<sup>rd</sup> week. The acidity increase was also considerably higher in *L. acidophilus* (103%) than *L. sakei* (60%) after the fermentation in comparison to the unfermented juice. Acidity was stable with no significant difference after 1 week of storage in both probiotic fermented juices. These findings are agreed with Mohan et al. (2013) who studied the probiotic tomato juice and found that the acidity increased to 0.65% or higher.

### 3.2 Estimated TSS %

Total soluble solids (%) of the date juice significantly decreased from 18.45 to 17.8% and from 18.6 to 18% in fermented juice samples by *L. acidophilus* and *L. sakei*, respectively during the storage period (Table 2). In contrast, the control sample recorded no-significant change ( $p < 0.05$ ) in TSS during the refrigerated storage. The sugars consumption and the formed acids during fermentation process by *L. acidophilus* and *L. sakei* are the main reasons of TSS reduction during storage.

### 3.3 Estimated total and reducing sugar

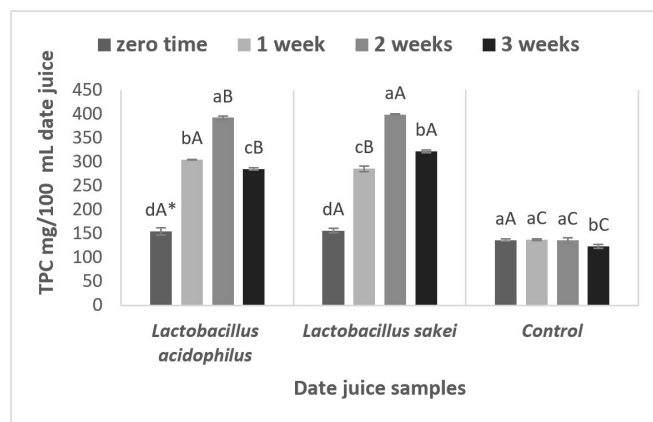
Table 3 revealed that; *Lactobacillus* requires sugar for growth and as the fermentation continues, the sugar content of samples declined gradually. Initially total sugar in control juice

(unfermented juice) was 16.20 g/100mL which was significantly decreased in fermented juice to 14.50 and 15.20 g/100 mL by *L. acidophilus* and *L. sakei*, respectively after the fermentation. The observed differences between *L. acidophilus* and *L. sakei* in sugar content may be due to the ability of *L. sakei* to produce exo-polysaccharides (Ruas-Madiedo & De los Reyes-Gavilan, 2005). Both the total sugar content significantly reduced during the cold storage by 20% and 17.8% of fermented juice by *L. acidophilus* and *L. sakei*, respectively. On the other side, reducing sugar content reduced by 11.8% and 13.5% after 3 weeks of cold storage in fermented juice by *L. acidophilus* and *L. sakei*, respectively.

### 3.4 Effect of the fermentation and cold storage on concentration and profile of the phenolic compounds

The change in the total phenolic compounds concentration (TPC) of the fermented and unfermented (control) date juice during cold storage for 3 weeks is shown in Figure 1. There was no significant difference ( $p < 0.05$ ) between TPC concentrations for all date juice samples at zero time (after 48 h of fermentation process at 37 °C) which ranged between 136.41-156.07 mg/100 mL juice. After 2 weeks of the refrigerated storage, there was no change in TPC content of the control sample (137.26 mg/100 mL date juice). In contrast, the fermentation using *L. acidophilus* or *L. sakei*, impacted a great increase (duplicate the concentration of TPC) which reached to 392.30 and 399.02 mg/100 mL, respectively which considered the highest concentration reached among the storage intervals. Pereira et al. (2017) noted that, in different fruit juices, lactic acid bacteria can synthesize varied phenolic compounds depend on the food composite and the applied strain. Several studies recorded that, the levels of antioxidant compounds, such as polyphenols are increased due to the fermentation by the lactic acid bacteria (Wu et al., 2011; Dimitrovski et al., 2015). For example, phenolic compounds increased by *L. casei* fermentation of cashew apple juice that conferring more health benefits to that functional juice (Pereira et al., 2013). Also, Pontonio et al. (2019) reported that, the fermentation process with *L. plantarum* of pomegranate juice increased the concentration of phenolics which improved the antioxidant activity percent more than 40% compared to the unfermented pomegranate juice.

Table 4 represents the quantity of the identified phenolic compounds of probiotic date juice after two weeks of cold storage as estimated by HPLC. A total of 19 phenolic acids was identified and quantified in the fermented date juice meanwhile; only 15 phenolic acids were identified and quantified in unfermented date juice. Among the phenolic acids, kampherol and gallic acid were identified as the most dominant, recording for the highest percentage of 54.4, 66.6% and 14.56, 10.86% in date juice fermented by *L. acidophilus* and *L. sakei*, respectively. On the other hand, the most dominant phenolic acids in unfermented juice (control) were ellagic, rutin and benzoic acid which recorded the highest percentage of 24.72, 23.62 and 18.05%, respectively. These results are in accordance with Gan et al. (2016) and Kwaw et al. (2018) who observed the ability of *Lactobacillus* strains for increasing the TPC concentration in juice during the fermentation process. It was known that the fermentation process causes the releasing of



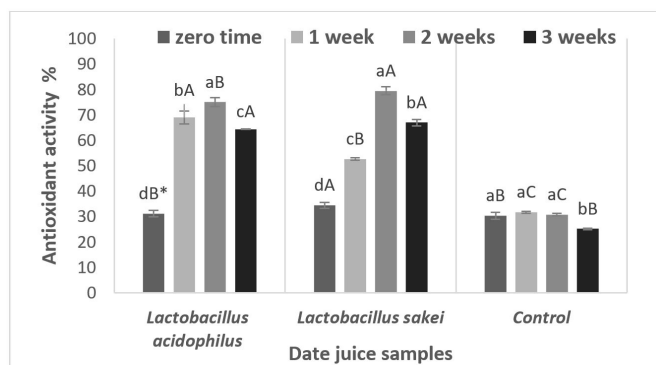
**Figure 1.** Total phenolic compounds (TPC) of fermented and unfermented (control) date juice during refrigerated storage at 4 °C for 3 weeks. \* Capital letters indicate significant differences (Duncan's test,  $p < 0.05$ ) between the samples in each storage time, and small letters indicate significant differences during the storage period in each sample.

**Table 4.** The quantity of the identified phenolic compounds (mg/mL) in fermented and unfermented date juice (control) after refrigerated storage at 4 °C for 2 weeks as estimated by HPLC.

Phenolic compound	Fermented juice		Control
	<i>L. acidophilus</i>	<i>L. sakei</i>	
Pyrogallol	-	-	-
Quinol	1.58	0.40	-
Gallic acid	14.56	10.86	3.55
Catechol	4.03	3.89	-
<i>p</i> -Hydroxy benzoic acid	6.24	4.45	3.76
Caffeine	0.85	0.39	0.28
Chlorogenic	0.97	0.60	0.37
Vanillic acid	1.86	0.74	0.27
Caffeic acid	0.21	0.14	0.13
Syringic acid	0.30	0.25	0.18
Vanillin	2.15	1.84	0.59
<i>p</i> - Coumaric acid	-	-	0.59
Ferulic acid	0.42	0.29	0.31
Benzoic acid	-	-	18.05
Rutin	1.78	1.23	23.62
Ellagic	1.87	0.85	24.72
<i>o</i> -Coumaric acid	0.68	0.49	0.12
Salicylic acid	-	-	-
Cinnamic acid	-	-	-
Myricetin	4.12	3.48	3.49
Quercetin	0.63	0.66	-
Rosemarinic	1.26	1.29	-
Neringein	3.42	3.02	-
Kampherol	54.40	66.64	-

Each phenolic compound was identified by comparing its peak area (supplementary material) with the calibration curve.

microbial enzyme which producing higher chemical compounds from the plant such as flavonoids, tannin, alkaloids, and phenylpropanoid (Nazarni et al., 2016). Also, the fermentation process by *Lactobacillus* strains contributes to the simple



**Figure 2.** Antioxidant activity % of fermented and unfermented (control) date juice during refrigerated storage at 4 °C for 3 weeks. \* Capital letters indicate significant differences (Duncan's test,  $p < 0.05$ ) between the samples in each storage time, and small letters indicate significant differences during the storage period in each sample.

conversion and de-polymerization of phenolic compounds with the high molecular weight by poly-phenoloxidase (Othman et al., 2009). Moreover, natural fermentation using lactic acid bacteria induces the pH reduction thus; several implicated enzymes in the complex polyphenols hydrolysis were activated resulting in the active, simpler and higher polyphenols (Wijayanti et al., 2017). The concentration of TPC was significantly decreased after 3 weeks of storage time in all samples and that may be due to the decreasing of cell viability. Hashemi & Mahmoodi (2017) reported that phenolic compounds can be decreased during storage due to the degradation occurred by enzymes and chemical reactions. Furthermore, the stability of phenolic compounds depends on pH (Kwaw et al., 2017) therefore; the pH decrease in fermented juice could be the cause of phenol stability. It can be concluded that, the fermented date juice had a high content of phenolic compounds than unfermented juice (control). Samples after 2 weeks of refrigerated storage were chosen for testing the antitumor effect as it exhibited the highest phenolic content.

### 3.5 Effect of the fermentation and cold storage on the antioxidant activity %

Antioxidant activity of the fermented and unfermented date juice samples was determined by DPPH and presented in Figure 2. The illustrate results revealed the positive effects of lactic acid fermentation process on the antioxidant activity indicated by the increase in the inhibition of DPPH. There is no significant difference in antioxidant activity of unfermented (control) date juice sample during cold storage for 2 weeks, which ranged between 30.3 to 30.7%. Meanwhile, the antioxidant activity of fermented date juice was higher than the control sample and reached the maximum after 2 weeks of cold storage. It recorded 75.03% in fermented date juice using *L. acidophilus* and 79.46% in fermented date juice using *L. sakei*. These results are in accordance with Zhang et al. (2012) who noticed the change in antioxidant activity after fermentation process that might be affected by the bacterial strain. Marazza et al. (2009) observed that the lactic acid bacteria producing  $\beta$ -glucosidase (including

*Lactobacillus acidophilus*, *L. casei* and *L. plantarum*) has the ability to increase the aglycone during the fermentation process which act as an antioxidant. The results indicate a decrease in antioxidant activity in all samples after 3 weeks of cold storage, it probably due to the reduction of TPC in date juice which strongly correlated with the antioxidant activity.

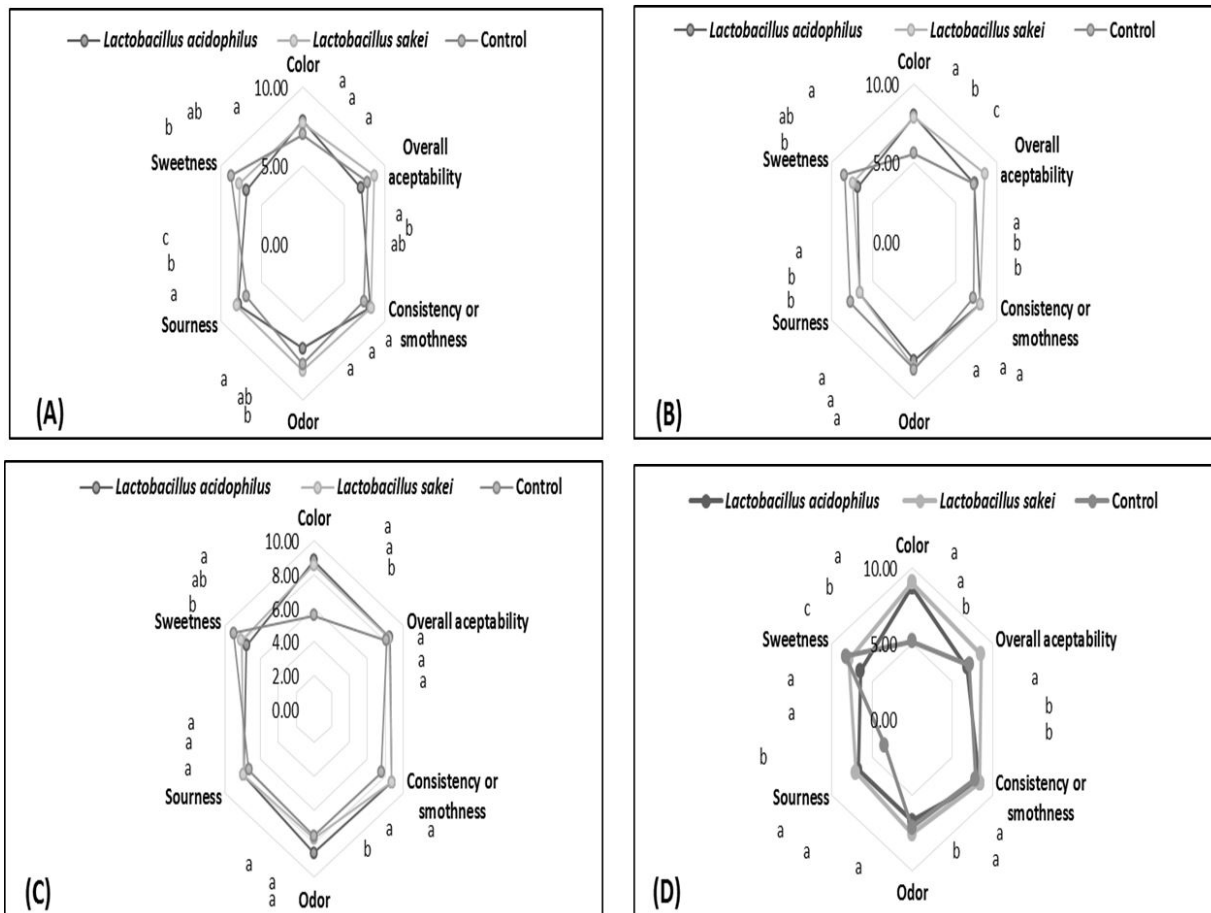
### 3.6 Sensory evaluation

The observed variation of sensory evaluation scores during cold storage for 3 weeks of unfermented (control) and fermented date juice are presented in Figure 3A, B, C and D.

All date juice samples have scores greater than the rejection limit (6) during the storage period (3 weeks), except the color of the control sample at the end of the storage time (score=5.57). It may be due to the chemical reactions or microbial growth occurred during the storage. The results indicated that lactic acid fermentation impacted on the color and taste (sweetness and sourness) of date juice during storage. Those results are in accordance with Kwaw and co-workers (2017) who referred to the correlation between the phenolic compounds and organoleptic properties of food especially TPC that directly affected the color and flavor. That might be referred to duplicate TPC percent formed by fermentation with both strains during the cold storage compared to the unfermented juice. Also, as mentioned before there was a significant decrease in pH during storage in fermented date juice sample than control, which creates a great and acceptable flavor due to the balance, occurred between sweet and sour taste. The overall acceptability of fermented date juice was evaluated by the panelists with scores slightly significant than unfermented juice, during the cold storage.

### 3.7 Anticancer effects of probiotic date juice

Substantial research using human cancer cells/cell lines has demonstrated that probiotics possess anti-proliferative activities in these cells (Yu & Li, 2016). To investigate the anticancer effects of probiotic fermented date juice, two types of human cancer cell lines (Caco-2 and Hep-2) were treated with the freeze dried date juice samples after 2 weeks of cold storage at several concentrations. There were evidence of 50% Hep-2 cell death at 315 and 317  $\mu\text{l/mL}$  after treating with date juice fermented with *L. acidophilus* and *L. sakei*, respectively that was significantly different from the non-treated cells ( $p < 0.05$ ) and control juice. It may be correlated to the higher content of polyphenols and antioxidant activity than the control juice that plays a significant role in cancer control (Rahmani et al., 2014). In contrast; there is no cytotoxicity effect on Caco-2 cell lines observed by date juice fermented with both strains up to 500  $\mu\text{l/mL}$ . These results suggest that date juice fermented by both *Lactobacillus* strains (*acidophilus* and *sakei*) may be preferable as natural and safe laryngeal cancer therapeutic agent. The anticancer activities of various LAB strains including *Lactobacillus*, *Bifidobacterium* and *Lactococcus* have been previously confirmed (Choi et al., 2006; Azam et al., 2014).



**Figure 3.** Sensory evaluation of fermented and unfermented date juice (control) date juice during refrigerated storage at 4 °C for 3 weeks. (A) zero time; (B) 1-week; (C) 2-weeks; (D) 3-weeks. Means (n = 40) followed by different letters indicate significant differences between samples in each storage time.

#### 4 Conclusion

The fermented date juice developed in this study could provide the consumers with phytochemicals because it has an elevated antioxidant activity (150-166% than the unfermented juice). The overall acceptability of the fermented date juice during cold storage was significantly higher than the unfermented juice. In addition, the probiotic fermented date juice using both strains showed a positive effect on larynx human cancer cell line (Hep-2). The obtained probiotic juice is an alternative functional beverage that can contribute to the available probiotic products.

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## Supplementary material

Supplementary material accompanies this paper.

Figure 1. Characterization of phenolic compounds of unfermented date juice (control) using HPLC.

Figure 2. Characterization of phenolic compounds of fermented date juice by *L. acidophilus* using HPLC.

Figure 3. Characterization of phenolic compounds of fermented date juice by *L. sakei* using HPLC.

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