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Nutritionally Enhanced Probioticated Whole Pineapple Juice

Wilawan Palachum [1,2,3], Wanna Choorit [4,5,*] and Yusuf Chisti [6]

1 Department of Medical Technology, School of Allied Health Sciences, Walailak University, Tasala, Nakhon Si Thammarat 80161, Thailand; pwilawan.wp@gmail.com
2 Research Excellence Center for Innovation and Health Products, Walailak University, Tasala, Nakhon Si Thammarat 80161, Thailand
3 Center of Excellence Research for Melioidosis, Walailak University, Tasala, Nakhon Si Thammarat 80161, Thailand
4 Biotechnology Program, School of Agricultural Technology, Walailak University, Tasala, Nakhon Si Thammarat 80161, Thailand
5 Biomass and Oil Palm Center of Excellence, Walailak University, Tasala, Nakhon Si Thammarat 80161, Thailand
6 School of Engineering, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand; Y.Chisti@massey.ac.nz
* Correspondence: cwanna35@gmail.com

Abstract: Nutritionally enhanced probioticated whole pineapple juice (WPJ, comprising juice of pineapple pulp and peel) beverages were produced by fermentation of WPJ with the probiotic bacterium *Lactobacillus plantarum* WU-P19. The 12 h fermented juice contained between 2.1×10^9 and 3.7×10^9 live cells of the probiotic per milliliter, depending on the beverage formulation. The beverage had a pH of around 4.1 and a lactic acid content of ~12.8 g L^{-1}. It had a total sugar (glucose, sucrose, fructose, maltose) content of ~100.2 g L^{-1}. During fermentation, some of the initial glucose and fructose were consumed by the probiotic, but sucrose and maltose were not consumed. The original WPJ was free of vitamin B12, but fermentation enhanced vitamin B12 content (~19.5 mg L^{-1}). In addition, fermentation enhanced the concentrations of vitamins B2, B3, and B6, but the bacterium consumed some of the vitamin B1 originally present. From a nutritional perspective, the final probioticated beverage was a good source of vitamin B12, vitamin C and vitamin B6. In addition, it contained nutritionally useful levels of vitamins B1, B2, and B3. The calorific value of the final beverage was 56.94 kcal per 100 mL. The product was stable during 21-day refrigerated (4 °C) storage.

Keywords: *Lactobacillus plantarum*; *Ananas comosus*; pineapple juice; probiotic beverage; vitamin B12

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1. Introduction

Pineapple (*Ananas comosus* (L.) Merr) is a widely consumed fruit grown in many tropical and subtropical regions [1–3]. Pineapple and its products are known for their pleasant aroma and flavor. The fruit is rich in certain vitamins, minerals, polyphenol antioxidants, and other phytochemicals [1,4]. Approximately 60% of fresh pineapple is edible, resulting in 45–55% of the mass of fresh fruit being discarded as waste in commercial processing operations [5]. The commercial juicing industry generates a significant amount of residue, including peels and trimmings [6]. Pineapple peel is rich in polyphenols [7,8] and its water-insoluble fiber-rich fraction has been shown to improve intestinal function in test animals [9]. Juice extracted from the peels is richer in polyphenols compared to conventional juice expressed solely from the pineapple pulp.

Fruit juices are popular worldwide. Nutritional value of a fruit juice and its palatability may be further enhanced by enriching it with probiotic microorganisms [10]. Due to their naturally present sugars and other nutrients, fruit juices generally support good growth of probiotics [10,11]. Various probioticated beverages have been made from pineapple pulp juice [12–14] and other fruit juices [15–17], but no such beverages have been developed

using whole pineapple juice comprising the pulp and peel juices. Development of a probiotic-rich fermented beverage based on whole pineapple juice, and its characterization in terms of sensory and nutritional properties and storage stability, were the objectives of the present work.

Flavoring agents such as malt extract may be added to the juice before or after fermentation. Potable ethanol may be added after fermentation to make a mildly alcoholic beverage with a broadened appeal. Live microorganisms are a necessary component of a probiotic beverage, but microorganisms are commonly inhibited by ethanol [18,19]. Therefore, any supplementation with ethanol usually occurs after fermentation. Supplementation with ethanol requires care so that the survival of the probiotic during the shelf-life of the product is not impaired. In food products, organic acids and ethanol are among the most common microbial stressors [20] and the effects of one may be accentuated by the other.

Lactobacillus bacteria are well known probiotics that are commonly consumed in widely accepted fermented foods such as yogurt. In principle, some of the *Lactobacillus* bacteria with a well-established track record of safe use in foods can be used to produce probiotic beverages based on fruit juice. Fermentation of juice with a probiotic has the potential to generate nutrients not normally found in the juice and increase its acceptance by modifying flavor and other sensory attributes [14,21,22]. The bacterium *Lactobacillus plantarum* is a well-known probiotic that tolerates organic acids and is commonly used for fermenting plant-based foods [17,23–25]. *L. plantarum* produces diverse enzymes that modify the fermenting juice to generate flavors and aromas [26]. Furthermore, at least some *L. plantarum* strains are able to synthesize vitamin B12 [22,26], an essential vitamin that is deficient in many vegetarian diets [27]. Various other B-group vitamins are also produced by lactobacilli [28,29]. *L. plantarum* WU-P19 [30] is known to produce conjugated linoleic acids [31] and has been confirmed to produce vitamin B12 [23]. In addition, *L. plantarum* WU-P19 has been shown to produce health-promoting short-chain fatty acids from certain prebiotics [32].

This work investigated the nutritional enrichment of whole pineapple juice by fermentation with the probiotic *L. plantarum* WU-P19 to provide a palatable beverage. The changes brought about by fermentation in the key properties of the juice were assessed. The stability of the beverage during refrigerated storage of up to 21 days, was evaluated. The sensory acceptability and nutritional parameters of the probioticated beverage were characterized.

2. Materials and Methods

2.1. Chemicals

The following HPLC-grade vitamin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): retinol (vitamin A, product no. R7632); thiamin (vitamin B1, product no. 47858); riboflavin (vitamin B2, product no. 47861); niacin (vitamin B3, product no. 47865-U); pyridoxine (vitamin B6, product no. 47862); cyanocobalamin (vitamin B12, product no. 47869); ascorbic acid (vitamin C, product no. 47863). Methanol (HPLC grade, 99.9%) and trifluoroacetic acid (TFA, >99.5% protein chemistry grade) were obtained from Thermo Fisher Scientific (Thermo Fisher Scientific, Loughborough, UK), unless specified otherwise.

Carrez I solution was prepared by adding 10.6 g of potassium ferricyanide (Sigma-Aldrich, product no. P3289) to 100 mL of distilled water. Carrez II solution was prepared by dissolving 21.95 g of zinc acetate dihydrate (Sigma-Aldrich, product no. 383058) in 30 mL of 99% (*v*/*v*) acetic acid in 100 mL of distilled water [33]. The Carrex I and II were stored at 4 °C until use.

Aqueous ammonia solution (10% *v*/*v*) (25% aqueous stock solution; Ricca Chemical, Thermo Fisher Scientific, Loughborough, UK) was sterilized by filtration through a 0.45 µm sterile membrane filter. The following solutions were sterilized by autoclaving at 121 °C for 15 min [23]: 0.1 M NaOH, 0.1 M HCl, and 0.85% (*w*/*v*) NaCl.

2.2. Whole Pineapple Juice Preparation

Pineapple (*Ananas comosus* L. Merr.; Dole Tropical Gold®, product of the Philippines, www.dolenz.co.nz) was used for this study. Pineapple fruits (15 fruits) were thoroughly washed with tap water. The fruits were peeled and the peel was separated from the fruit pulp. The pulp was chopped into small pieces and blended in an electric blender (Brabantia BBEK1051, Brabantia Solid Company, Hong Kong, China) at a speed setting of 1 for 5 min. The blended pulp was filtered through two layers of a cotton cheese cloth (0.7 mm pore size), to remove suspended solids.

The pineapple peels (6.75 kg) were pressure cooked (30 min, 80 kPa; Shaffer-Berry pressure cooker; www.shaffer-berry.com). For pressure cooking, the cooker was partly filled with water (250 mL) and the peels (1.7 kg) were placed in a tray above the water level. The cooked peels were cut with a knife into small pieces and blended as above. Sterile water (800 mL) was added to the peels during blending. The blended peel was filtered through cheese cloth, as above, to remove excess fiber. The pineapple pulp juice (6.0 L) and pineapple peel juice (3.0 L) were mixed in a volume ratio of 2:1. The mixed juice (9.0 L) is referred to as fresh whole pineapple juice (WPJ) throughout this work. WPJ was sterilized at 121 °C for 15 min [34]. The sterilized whole pineapple juice was stored at −20 °C until use.

For all work, the sterilized WPJ was adjusted to pH 6.0 by adding sterilized 10.0% v/v ammonia solution (1.70 mL of 10.0% v/v ammonia solution per 100 mL WPJ). Recipes for making fermented WPJ consisted of Recipe 1 (R1), Recipe 2 (R2), and Recipe 3 (R3). Recipe 1 comprised 250 mL sterilized whole pineapple juice. Recipe 2 comprised of 250 mL of sterilized whole pineapple juice supplemented with 2% w/v (5.0 g) sterilized food-grade malt extract (Maltexo, New Zealand; www.maltexo.co.nz). Recipe 3 comprised of 250 mL of sterilized whole pineapple juice supplemented with 5% w/v (12.5 g) sterilized malt extract. According to the manufacturer (Maltexo, New Zealand), the malt extract contained: protein (3.9% w/w), fat (0.1% w/w), dietary fiber (0.7% w/w), and total sugars (42.6% w/w). The latter included maltose (30.5% w/w), glucose (9.9% w/w), sucrose (1.1% w/w), fructose (1.1% w/w), and lactose (<0.1% w/w).

2.3. Bacterial Strain and Culture Medium

Lactobacillus plantarum WU-P19 [30], isolated from a traditional fermented herb [30], was used in this work. WU-P19 had earlier been evaluated as a potential probiotic [30] and was shown to produce conjugated linoleic acids (CLA1 (*cis*-9, *trans*-11-octadecadienoic acid; CLA2, *trans*-10, *cis*-12-octadecadienoic acid) [31] and vitamin B12 [23]. In addition, it was shown to be able to produce health-promoting short-chain fatty acids from xylooligosaccharides (XOS) prebiotics [32]. The bacterium was maintained as a pure culture and stored at 4 °C on MRS agar (de Man, Rogosa and Sharpe agar). Agar cultures were refreshed monthly.

The modified MRS medium was prepared by dissolving the following components in 1 L of distilled water: soya peptone 10 g (peptone from soymeal; Merck, Kenilworth, NJ, USA), yeast extract 5 g (Oxoid, Basingstoke, UK), glucose 20 g (LabServ, Thermo Fisher Scientific), Tween 80 1 g (PanReac AppliChem, Darmstadt, Germany), sodium acetate dihydrate 8.3 g (LabServ, Thermo Fisher Scientific), di-ammonium hydrogen citrate 2 g ('Baker Analyzed' Reagent, J.T. Baker®), manganese sulfate monohydrate 0.055 g (Unilab, Ajax chemicals), magnesium sulfate heptahydrate 0.2 g (LabServ, Thermo Fisher Scientific), potassium dihydrogen phosphate 2.5 g (LabServ, Thermo Fisher Scientific), and dipotassium hydrogen phosphate 2 g (LabServ, Thermo Fisher Scientific). The medium was adjusted to pH 6.0 using sterile 0.1 M NaOH and/or HCl, and sterilized at 121 °C for 15 min. The MRS agar medium was prepared by adding 15 g of agar to 1 L of the above medium prior to sterilization.

2.4. Inoculum Preparation

Strain WU-P19 was twice subcultured in 10 mL of a modified MRS broth (Section 2.3) and incubated anaerobically at 37 °C for 24 h to obtain an active culture. This culture was used to inoculate multiple 250 mL Duran® bottles containing the modified MRS medium at

pH 6.0. The bottles were incubated anaerobically at 37 °C for 18 h. The pH was controlled at 6.0 by adding sterile 0.1 M NaOH or HCl every 2 h, as needed. The bacterial cells were recovered by centrifugation (Sigma 6-16, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 5465× *g*, 4 °C, 10 min, and washed twice with 0.85% (*w*/*v*) NaCl. The washed cell pellet was suspended in whole pineapple juice to initiate a fermentation. The viable cell count just after inoculation was 3.2 × 10^8 CFU mL^{-1}.

2.5. Experimental Setup

2.5.1. Whole Pineapple Juice Fermentation

Whole pineapple juice was fermented as follows: in separate experiments, Recipe 1, Recipe 2, and Recipe 3 were inoculated with *L. plantarum* WU-P19 using the inoculum prepared as in the Section 2.4. Fermentations were carried out 250 mL Duran® bottles. The viable cell count just after inoculation of WPJ was ~3.2 × 10^8 CFU mL^{-1}. The inoculated WPJ was incubated at 37 °C for 12 h under static conditions, except for gentle mixing just prior to sampling every 2 h.

Mixing before sampling ensured a homogenous sample. The following measurements were made on the samples: the viable cell count, the pH, the protein concentration in cell-free juice, concentrations of sugars (glucose, fructose, sucrose, maltose), concentrations of organic acids (lactic acid, citric acid, malic acid), and concentration of total phenolic compounds. The levels of vitamin A, C, B1, B2, B3, B6, and B12 were also determined.

2.5.2. Effect of Ethanol Supplementation and the Storage Period

Only Recipe 2 was selected from among the three fermented whole pineapple juice recipes (R1, R2, and R3; see Section 2.5.1) to test the effects of refrigerated storage, with and without supplemental ethanol, on the beverage properties.

The Recipe 2 fermented product (R2) was supplemented with potable ethanol to 2% *v*/*v* and 4% *v*/*v* levels to obtain the products designated as R2-E1 and R2-E2, respectively. A commercial vodka (40% ethanol by vol; Absolut® vodka, The Absolut Company, New Zealand; www.absolut.com) was used for the supplementation. In beverage formulation R2-E1, the vodka supplementation level was 12.5 mL per 250 mL of the fermented Recipe 2. In formulation R2-E2, 250 mL of fermented Recipe 2 was mixed with 25 mL of commercial vodka. The above referenced Recipe 2 fermented beverage (i.e., R2) without supplementation with any ethanol (0% *v*/*v* ethanol) was used as control. All products were stored at 4 °C for 21 days. During 21 days of refrigerated storage, each product was gently mixed and sampled every 7 days. The samples were used to measure the pH, the viable cell count, total sugar, total acids (lactic acid, citric acid, malic acid), and ethanol.

2.6. Analyses

2.6.1. Viable Cell Count

The concentration of viable cells was determined as colony-forming units (CFU) per unit volume of the fermenting juice. Appropriate decimal dilutions of the fermented whole pineapple juice were prepared by pipetting 10 mL of a sample into 90 mL of 0.85% *w*/*v* NaCl. A 0.1 mL portion of the appropriately diluted sample was spread on the surface of MRS agar medium and incubated anaerobically at 37 °C for 48 h [23]. The number of colonies formed, the volume of the original sample, and the dilution factor were used to calculate the viable cell concentration (CFU mL^{-1}).

2.6.2. Determination of Total Sugar

The total sugar in fermented whole pineapple juice samples was quantified by using the phenol sulfuric acid method [35]. The fermented WPJ samples were centrifuged (5465× *g*, 4 °C, 10 min) and the supernatant was filtered through a sterile membrane filter (0.22 µm pore size, MF-Millipore™) to remove suspended material (pineapple pulp and bacterial cells). The filtered juice (600 µL) was mixed with 5% *w*/*v* of phenol (600 µL) and then 3 mL of 96% *v*/*v* sulfuric acid was added. The mixture was incubated at 25 °C for 30 min. The

spectrophotometric absorbance was measured at 490 nm. A standard curve was prepared by using standard solutions of glucose. The blank was distilled water treated the same way as the sample.

2.6.3. Sugars, Ethanol, and Organic Acids

Samples of the sterilized WPJ and the fermented WPJ were filtered through a membrane filter (0.22 µm pore size) before analysis.

Sugars (glucose, fructose, and sucrose) and ethanol in each fermented sample were determined together by using an HPLC system (TCC-100; Dionex, Sunnyvale, CA, USA) equipped with a refractive index detector (RID; Dionex, USA). A Sugar pak I column (Waters, Milford, MA, USA) was used. The column was maintained at 75 °C. The mobile phase was aqueous CaEDTA (50 mg L^{-1}) at a flow rate of 0.6 mL min^{-1}.

Maltose was measured by using an HPLC system (TCC-100; Dionex, Sunnyvale, CA, USA). With a refractive index detector (RI-101, Dionex/Shodex). A MetaCarb-H plus column (Agilant, Santa Clara, CA, USA) was used at the following conditions: a column temperature of 60 °C; 8.5 mM H_2SO_4 as the mobile phase; a mobile phase flow rate of 0.4 mL min^{-1}. A standard aqueous solution of pure maltose was used for calibration.

Organic acids (citric acid, malic acid, and lactic acid) were determined using an HPLC system (UltiMate 3000, Thermo Fisher Scientific, CA, USA) equipped with a photodiode array detector (UltiMate™ Diode Array Detector, Thermo Fisher Scientific, CA, USA). A Meta Carb-H plus column (Agilant, Santa Clara, CA, USA) was used. The column was maintained at 70 °C. The mobile phase was 8.5 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1}.

2.6.4. Culture pH and Total Nitrogen

The pH was measured using a pH meter (SevenCompact™ pH/ion meter S220, Mettler-Toledo, Columbus, OH, USA). Total nitrogen in the filtrate of the samples was determined by using a total nitrogen analyzer (TOC-L CPN with TNM-L, Shimadzu, Kyoto, Japan; www.shimadzu.com). The measured total nitrogen was corrected for the aqueous ammonia that was added and the corrected value was multiplied by 6.25 to estimate the protein.

2.6.5. Determination of Vitamins

Vitamin A, the B vitamins (B1, B2, B3, B6, and B12), and vitamin C in sterilized WPJ and fermented WPJ samples were analyzed using published methods [22,33]. For the sterilized WPJ samples, a 5.0 mL portion of the homogenized fresh WPJ was mixed with 10.0 mL methanol. The mixture was centrifuged (3000× *g*, 5 min) and the supernatant was filtered through a 0.45 µm nylon membrane filter before analysis by HPLC. For the fermented WPJ samples, a 5 mL portion of the sample was mixed with 0.5 mL of Carrez I and 0.5 mL of Carrez II (see Section 2.1). The mixture was then centrifuged (5465× *g*, 4 °C, 10 min) and the supernatant was filtered (nylon membrane filter, 0.45 µm pore size) before analysis by HPLC.

All vitamins were measured by using a Luna® C18(2) column (5 µm, C18(2), 100 Å, LC Column 150 × 4.6 mm; Phenomenex Inc., Torrance, CA, USA) in a UltiMate™ 3000 Standard Dual HPLC system (Thermo Fisher Scientific Inc.) with an UltiMate™ diode array detector. The column temperature was 30 °C and the total run time was 30 min. Gradient elution of solvents A (0.01% *v*/*v* TFA in water) and B (methanol) was used. The gradient elution parameters were as follows: (1) 95% A and 5% B at the start with a flow rate of 0.6 mL min^{-1}; (2) 95% A and 5% B at 4 min with a flow rate of 0.7 mL min^{-1}; (3) 2% A and 98% B at 10 min with a flow rate of 0.7 mL min^{-1}; (4) 2% A and 98% B at 13 min with a flow rate of 0.7 mL min^{-1}; (5) 100% B at 15 min with a flow rate of 1.3 mL min^{-1}; (6) 100% B at 25 min with a flow rate of 1.3 mL min^{-1}. The measurement wavelengths for the different vitamins were as follows: 320 nm of vitamin A; 262 nm of vitamin C; 253 nm of vitamin B1; 258 nm of vitamin B3; 289 nm of vitamin B6; 290 nm for vitamins B2 and B12. Calibration curves were prepared using standard solutions of the different vitamins and the HPLC peaks were identified by comparison with authentic standards.

2.6.6. Color Measurements

The color of the samples was determined in terms of the spectral parameters L*, a*, and b*. These parameters were measured using Hunterlab Miniscan/EX instrument (10° standard observer, illuminant D_{65}; Hunter Associates Laboratory Inc., Reston, VA, USA). The instrument had been calibrated to a white and black standard. For the measurements, the fermented juice sample was weighed and placed in a clear plastic plate supplied with the instruments. The L*-value showed lightness in the range of black (0) to white (100). The a* showed a hue in the red-green color range with a positive value for redness and negative value for greenness. The b* indicated a hue in the yellow-blue spectral range with a positive value showing yellowness and a negative value indicating blueness [13,36].

2.6.7. Sensory Evaluation

The sensory evaluation protocols used in this work were reviewed and approved by the Human Research Ethics Committee of Walailak University, Thailand (approval no, WUEC-20-253-01). The sensory evaluations followed the method explained by Salmerón et al. [37]. Samples of the fermented beverages were refrigerated (4 °C) immediately after preparation and evaluated by the panelists within 48 h.

In a preliminary sensory evaluation, the three fermented products (R1, R2, and R3; Section 2.5.1) were assessed by 15 untrained panelists. Only the one product (i.e., R2) that was the most accepted by the panelists was carried forward to the next stage. The product R2 and two of its ethanol-containing formulations (i.e., R2-E1 and R2-E2; Section 2.5.2) were reassessed by an expanded panel of 30 untrained individuals. The beverage samples (15 mL) were presented to the panelists at 4 °C in a random order in white plastic cups with a 3-digit identification code. To minimize any residual effects, the panelists rinsed their mouths with water between samples and took all the time they needed to score a sample. The samples were evaluated in terms of appearance, color, odor, taste and overall acceptance. In each assessment category, the participants scored the beverages on a 9-point scale (1 = dislike very much to 9 = like very much).

2.6.8. Nutrition Analysis

The nutritional composition was measured using the methodology of Association of Official Analytical Chemists [38], as follows: Protein (Method 968.06), total sugars (Method 925.35), fat (Method 948.15), total dietary fiber (Method 991.43), ash (Method 942.05), and the minerals profile (Method 984.27). Carbohydrate content was determined according to Sullivan and Carpenter [39]. The calorific value was determined using a bomb calorimeter. The total phenolic content in the fermented beverages was measured using the method of Chen et al. [40] with gallic acid as the standard.

2.6.9. Statistical Analysis

The data are presented as mean values (±standard deviation) of three independent replicates. The data were analyzed by one-way ANOVA and Tukey's test. A value of $p < 0.05$ was taken as a significant difference.

3. Results and Discussion

3.1. Fermentation Profiles of Whole Pineapple Juice

Three probiotic fermented whole pineapple juice (WPJ) products were made: (1) fermented WPJ without additives (product R1); (2) WPJ supplemented with 2% w/v malt extract and then fermented (product R2); (3) WPJ supplemented with 5% w/v malt extract and then fermented (product R3). The probiotic microorganism used in all fermentations was *Lactobacillus plantarum* WU-P19. Except for the juice formulations specified above, all fermentations were identical (anaerobic, 37 °C, initial pH 6.0, initial CFU of 3.2×10^8 mL^{-1}, 12 h total duration). Malt extract was added to formulations R2 and R3 for its distinct flavor and nutrients (e.g., certain B vitamins [41]) that might support growth of the probiotic. The fermentations profiles are shown in Figure 1.

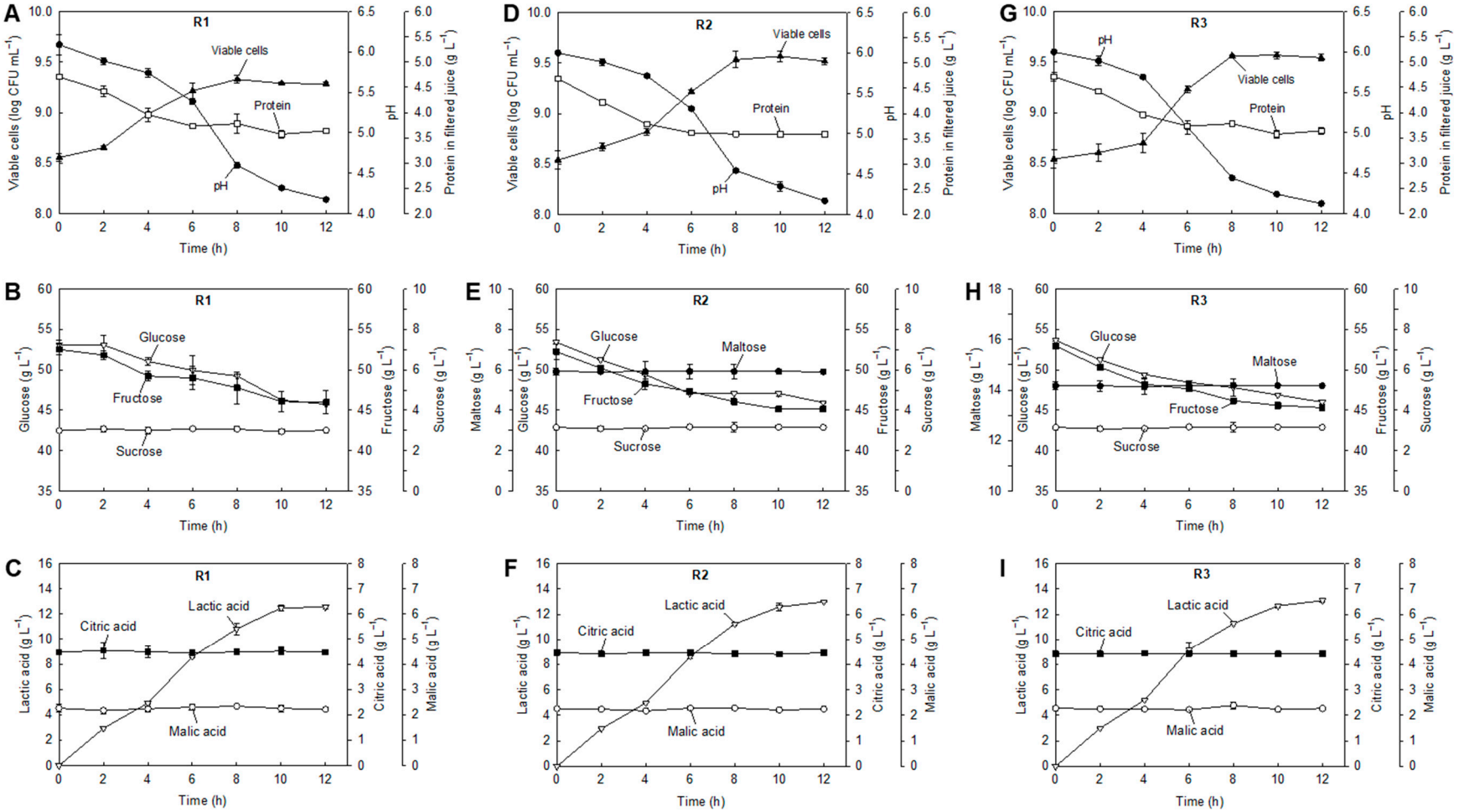

Figure 1. Anaerobic fermentation (37 °C, 12 h) profiles of Recipe 1 (**A**–**C**), Recipe 2 (**D**–**F**), and Recipe 3 (**G**–**I**). For each recipe, the following are shown: the viable cell counts, the pH and the protein in filtered juice (**A**,**D**,**G**); the concentrations of sugars (**B**,**E**,**H**); the concentrations of organic acids (**C**,**F**,**I**). (R1 (Recipe 1), fermented whole pineapple juice without malt extract; R2 (Recipe 2), fermented whole pineapple juice with 2% *w*/*v* of malt extract; R3 (Recipe 3), fermented whole pineapple juice with 5% *w*/*v* of malt extract.).

In all cases, the viable count of the probiotic increased following a typical pattern of an initial phase of slow growth, then exponential growth and then a stationary phase (8–12 h; Figure 1A,D,G). At 8 h, the viable probiotic count was 2.1×10^9 CFU mL^{-1} in R1 (Figure 1A) and 3.7×10^9 CFU mL^{-1} in R2 and R3 (Figure 1D,G). The higher cell count in R2 and R3 was attributed to the additional nutrients provided by the malt extract (Section 2.2; some B vitamins [41]), but the juice without the malt extract supported sufficiently good growth (R1, Figure 1A).

In other studies, pineapple pulp juice without additives has supported growth of the probiotics *Lactobacillus casei* [13], *Bifidobacterium lactis* [42], *Lactobacillus plantarum* [42] and *Lactobacillus acidophilus* [42]. Beneficial effects of adding malt extract to the culture medium have been observed on growth of lactobacilli also in some other fermentations. For example, in milk fermentations addition of 5% *w*/*w* malt extract was found to increase biomass production by *L. casei* and *L. acidophilus* relative to control (no malt extract) [43].

In the present work, the pH of all fermentations declined to around 4.5 by 8 h, the instance of onset of the stationary phase, and ultimately reached around 4.1 at termination (12 h) (Figure 1A,D,G). This decline was associated with the production of lactic acid from glucose and fructose. Thus, in all fermentations, concentration of glucose and fructose declined due to consumption by the bacterium (Figure 1B,E,H) and the concentration of lactic acid increased (Figure 1C,F,I). Glucose (53.1 g L^{-1}), fructose (52.5 g L^{-1}), and sucrose (3.0 g L^{-1}) were the main sugars in WPJ (Figure 1B). The maltose in Figure 1E,H was due to the malt extract added to R2 and R3. The microorganism utilized mainly glucose and fructose, but not sucrose and maltose (Figure 1B,E,H). Both glucose and fructose are known to be metabolized by lactobacilli [44].

By termination at 12 h, all fermentations contained large quantities of residual glucose, fructose and sucrose (Figure 1B,E,H). The bacterium utilized protein as a source of nitrogen but by termination the media contained around 3.6 g L^{-1} of residual protein (Figure 1A,D,G). The protein data in Figure 1A,D,G represent only the protein in the whole pineapple juice, as the data were corrected for protein in malt extract (Section 2.2) and the apparent protein due to the nitrogen in aqueous ammonia used in pH adjustments (Section 2.2). Therefore, at termination (12 h) neither the carbon sources nor the nitrogen sources were fully exhausted. The onset of the stationary phase at 8 h (Figure 1A,D,G) was attributed to the decline in pH to ~4.5. Although *L. plantarum* survives at pH values as low as 3.0–3.5 [23,45,46], many strains cease to grow once the pH declines to ~4.5 [46].

The initial WPJ protein value in all recipes was 4.7 g L^{-1} (0.47% *w*/*v*) (Figure 1A,D,G). This was consistent with the previously reported data of 0.4–0.5% protein in pineapple juice [4,14]. By 12 h the protein content dropped to around 3.6 g L^{-1}. Thus, ~23% of the initial protein in the juice was consumed by the bacterium during growth.

Among organic acids, citric acid and malic acid were neither produced nor consumed (Figure 1C,F,I) relative to the amounts present in WPJ. Citric acid and malic acid are known to be the main organic acids in pineapple juice [3]. Lactic acid did not occur in the WPJ and all of it was produced by bacterial action (Figure 1C,F,I). At termination (12 h), the fermented products contained citric acid at a concentration of between 12.6 and 13.0 g L^{-1} (Figure 1C,F,I).

3.2. Vitamins in the Fermented Whole Pineapple Juice

As in conventional pineapple pulp juice [47], the WPJ contained vitamins A, C and several of the B-group vitamins, but not vitamin B12 (Figure 2). Vitamin A content of the unfermented juice was in the range of 0.67–0.69 μg L^{-1} (Figure 2A). Fresh pineapple fruit contains trace amounts of vitamin A (3 μg/100 g fresh weight) [48,49], but the amount in the WPJ was much lower. There was no degradation or consumption of vitamin A during the fermentation, nor was there any production by the probiotic (Figure 2A). There were no significant differences ($p < 0.05$) in vitamin A levels between fermented R1, R2, and R3 (Figure 2A). Overall, pineapple, its juice and the fermented products (R1–3; Figure 2A)

were not good sources of vitamin A, because their consumption in any reasonable amount would not provide at least 10% of the daily requirement (700–900 µg daily for adults [50]).

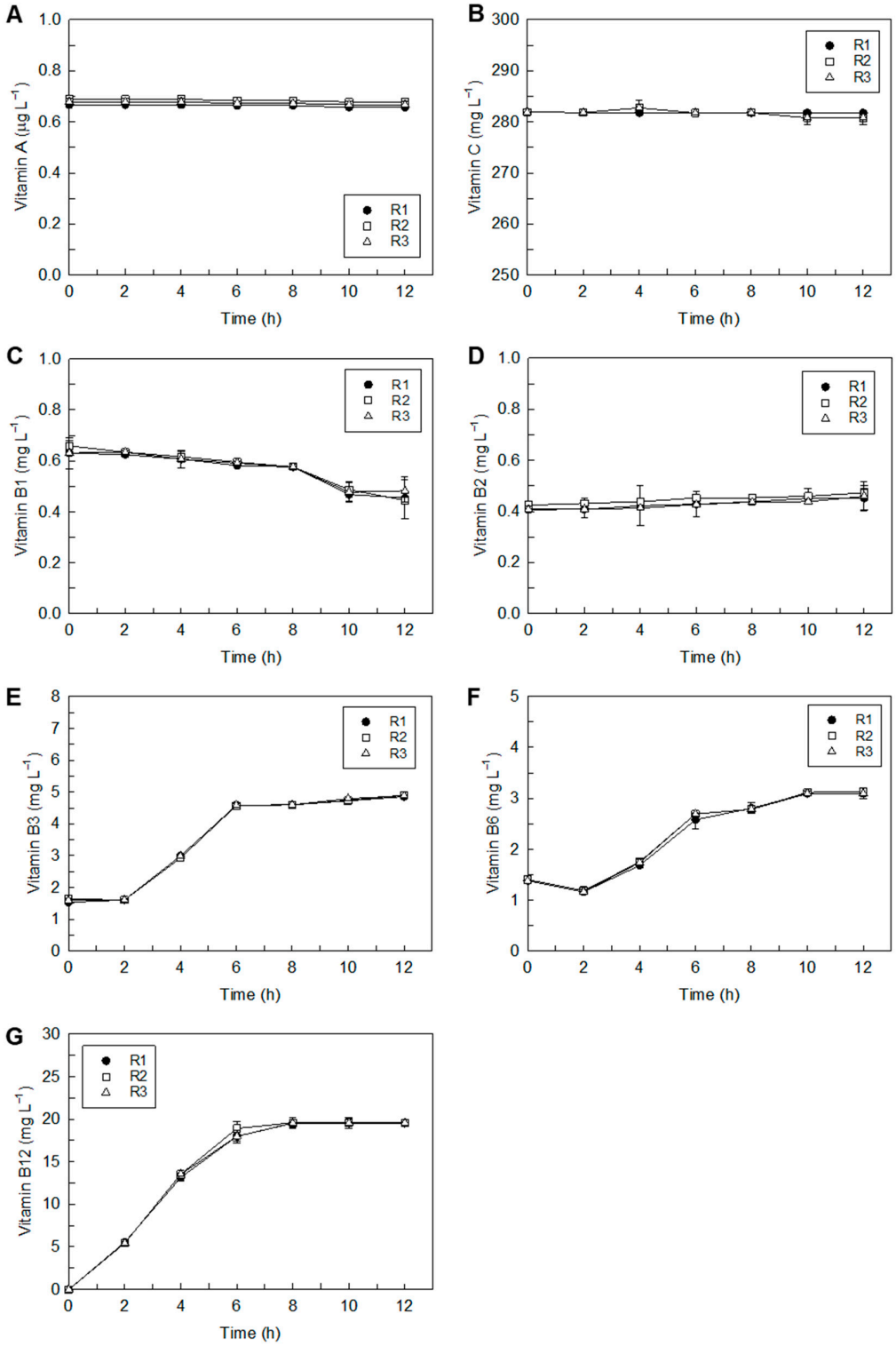

Figure 2. Vitamin concentrations in whole pineapple juice during culture of *L. plantarum* WU-P19: (**A**) vitamin A (retinal); (**B**) vitamin C (ascorbic acid); (**C**) vitamin B1 (thiamin); (**D**) vitamin B2 (riboflavin); (**E**) vitamin B3 (niacin); (**F**) vitamin B6 (pyridoxine); (**G**) vitamin B12 (cyanocobalamin). (R1, fermented whole pineapple juice without malt extract; R2, fermented whole pineapple juice with 2% *w*/*v* of malt extract; R3, fermented whole pineapple juice with 5% *w*/*v* of malt extract). Data are averages ± standard deviations of nine measurements (three replicate samples × three replicate fermentations) of fermented whole pineapple juice (R1, R2, and R3).

Vitamin C levels in fresh pineapple juice range from 92 to 938 mg L^{-1}, although the levels tend to be lower in commercial pineapple juice (e.g., 120–420 mg L^{-1} [51,52]) because of the damage caused by thermal sterilization. In WPJ and its fermented products (R1–3), the quantity of vitamin C was between 280 and 283 mg L^{-1} (Figure 2B), comparable to data published for commercial pineapple juice [51,52]. The fermentation process neither produced, nor destroyed, vitamin C. In other studies, vitamin C in probiotic pineapple juice has ranged from 291 to 710 mg kg^{-1} [12], apparently because some of the probiotics used (*Pediococcus pentosaceus*, *Lactobacillus rhamnosus*, *Pediococcus pentosaceus*) produced the vitamin. As the recommended daily intake of vitamin C for adults is in the range of 75–120 mg, the fermented products R1–3 are excellent potential sources of this vitamin. A 200 mL portion of these products can provide around 50% or more of the daily requirement of vitamin C. Although vitamin C is a known antioxidant, it has been previously shown to have no effect on survival of *L. plantarum* in pineapple juice during refrigerated storage [53].

WPJ contained vitamins B1 (Figure 2C), B2 (Figure 2D), B3 (Figure 2E) and B6 (Figure 2F), but it lacked vitamin B12 (Figure 2G). High levels of vitamins B1, B2, B3 and B6 [47] and a lack of B12 in pineapple fruit have been previously reported. In pineapple juice, the content of various B vitamins has been reported [54,55] to be as follows (per kg): 0.55–0.58 mg B1, 0.2 mg B2, 1.99–3 mg B3, 1 mg B6, and 0 µg B12. The data in Figure 2C–G agreed well with these reports. The amounts of some vitamins in pineapple pulp and the juice can be significantly different [4].

The probiotic fermentation consumed around 29% of the vitamin B1 present originally (Figure 2C). The observed consumption of vitamin B1 was consistent with an earlier report of similar consumption during fermentation of cashew apple juice with *L. plantarum* TISTR 543 [22]. Lactic acid bacteria are known to use small amounts of vitamin B1 for various metabolic processes including growth and production of lactic acid [22]. Consumption of B1 notwithstanding, all the final fermented products contained ~0.46 mg B1 L^{-1} (Figure 2C). The maximum daily requirement of vitamin B1 for an adult is 1.4 mg [56]; therefore, a 250 mL portion of any of the fermented juices (Figure 2C) would meet around 8% of the daily requirement.

Unlike vitamin B1, vitamin B2 was neither consumed nor produced during fermentation (Figure 2D). For all recipes, the initial and final levels of B2 were not significantly different. At termination of fermentation, all products contained ~0.46 mg B2 L^{-1} (Figure 2D). In other studies, certain lactobacilli (*L. plantarum*, *Lactobacillus fermentum* and *Lactobacillus acidophilus*) were shown to produce at least some vitamin B2 [57,58]. Daily requirement of vitamin B2 for an adult is around 1.3 mg, although it is 1.6 mg during lactation [59]. A 250 mL portion of any of the fermented juices of this study (Figure 2D) could provide around 9% of the daily needs of a nonlactating adult.

The probiotic fermentation produced vitamins B3 (Figure 2E) and B6 (Figure 2F). The concentration of vitamin B3 increased threefold during fermentation (Figure 2E) whereas the concentration of vitamin B6 increased ~2.2-fold (Figure 2F). All 12 h fermented products contained around 4.9 mg B3 L^{-1} and 3.1 mg B6 L^{-1} (Figure 2E,F). These results were consistent with earlier work where some lactic acid bacteria were shown to synthesize vitamin B3 [22,60,61]. The maximum daily requirement of vitamin B3 for an adult is around 18 mg as niacin [62]. A 200 mL portion of any of the fermented juices of this study would provide around 5% of the daily requirement and, therefore, the fermented products were not good sources of vitamin B3.

The vitamin B6 content of all final fermented products was around 3.1 mg L^{-1} (Figure 2F). A vitamin B6 production capability has previously been shown in many lactobacilli [60] although *L. plantarum* did not produce a significant quantity of vitamin B6 during a 48 h fermentation of cashew apple juice [22]. Daily requirement of vitamin B6 for adults ranges from 1.5 to 2.0 mg [63]. Thus, all fermented juices of this study could be considered good sources of vitamin B6, as in a 200 mL portion they would provide at least 30% of the required daily intake.

The probiotic fermentation produced vitamin B12 (Figure 2G). The unfermented WPJ lacked vitamin B12 whereas all the final fermented juices contained this vitamin at a concentration of around 19.5 mg L^{-1} (Figure 2G). Exactly the same probiotic bacterium was previously shown to produce vitamin B12 in fermentations of guava pulp [23]. Probiotics such as *L. reuteri* [64,65] and *L. plantarum* [16,66–69], have been shown to produce vitamin B12 in certain other fermented foods.

The maximum daily requirement of vitamin B12 for an adult is around 2.8 μg [70]. Thus, a 200 mL portion of any of the fermented juices of this study (Figure 2G) would provide 1390-fold the required amount of this vitamin. Vitamin B12 is considered safe in large doses as the excess is excreted [23]. Therefore, the probiotic fermented juices of this study were excellent sources of vitamin B12.

3.3. Effect of Ethanol Concentration on Product Stability during Storage

Based on the results of a preliminary sensory analysis (see Section 2.6.7), the most liked probioticated product was the Recipe 2 (R2) fermented juice. Therefore, only R2 was evaluated by itself (as control) and after supplementation with 2% *v*/*v* ethanol (R2-E1) and 4% *v*/*v* ethanol (R2-E2), for the effects of refrigerated storage (4 °C). These products were stored for 21 days.

The profiles of viable probiotic concentration, the pH, total sugar, total acid and ethanol concentration were similar for all recipes (Figure 3A–C). In all cases, the number of viable probiotic cells decreased slightly during 21 days of storage (Figure 3A–C). After 21 days, the product R2 (no ethanol) contained 1.3×10^9 CFU mL^{-1} whereas the most ethanol rich product R2-E2 contained 1.2×10^9 CFU mL^{-1} viable cells. Therefore, ethanol in the concentration range tested, was concluded to have barely any effect on survival of the probiotic. The ethanol concentration barely changed during storage (Figure 3B,C), therefore, the bacterium neither produced nor consumed ethanol during storage.

In all recipes, there was a slight (~2.5%) increase in the total organic acids concentration during 21 days of storage (Figure 3A–C). Consistent with this increase, for all recipes, the pH declined slightly to pH 3.9 on day 21 (Figure 3A–C). There was slight (a maximum of ~0.7%) consumption of total sugar during refrigerated storage (Figure 3A–C). This sugar consumption explained the slight increase in concentration of the organic acids. In all respects (pH, CFU count, total acids, total sugar), all products (R2, R2-E1, R2-E2; Figure 3) were stable during 21 day storage at 4 °C. *L. plantarum* WU-P19 tolerated well the combinations of ethanol concentrations, pH values and total acids concentrations in the fermented whole pineapple juice products. These results were generally consistent with observations in other fruit juices fermented with *L. plantarum*. For example, the viable count of *L. plantarum* NCMIB 8862 in fermented fruit juice (cranberry, pomegranate, lemon, lime) after a 6-week refrigerated storage was around 10^7 CFU mL^{-1} [25]. Similarly, viable concentration of *L. plantarum* LS5 in sweet lemon juice declined from 4.3×10^8 to 1.4×10^7 CFU mL^{-1} after 28-day storage at 4 °C [17]. Although the present work was deliberately restricted to only mildly alcoholic beverage formulations, at least some *L. plantarum* strains are known to grow at 18 °C in media with ethanol concentrations as high as 13% *v*/*v* [71].

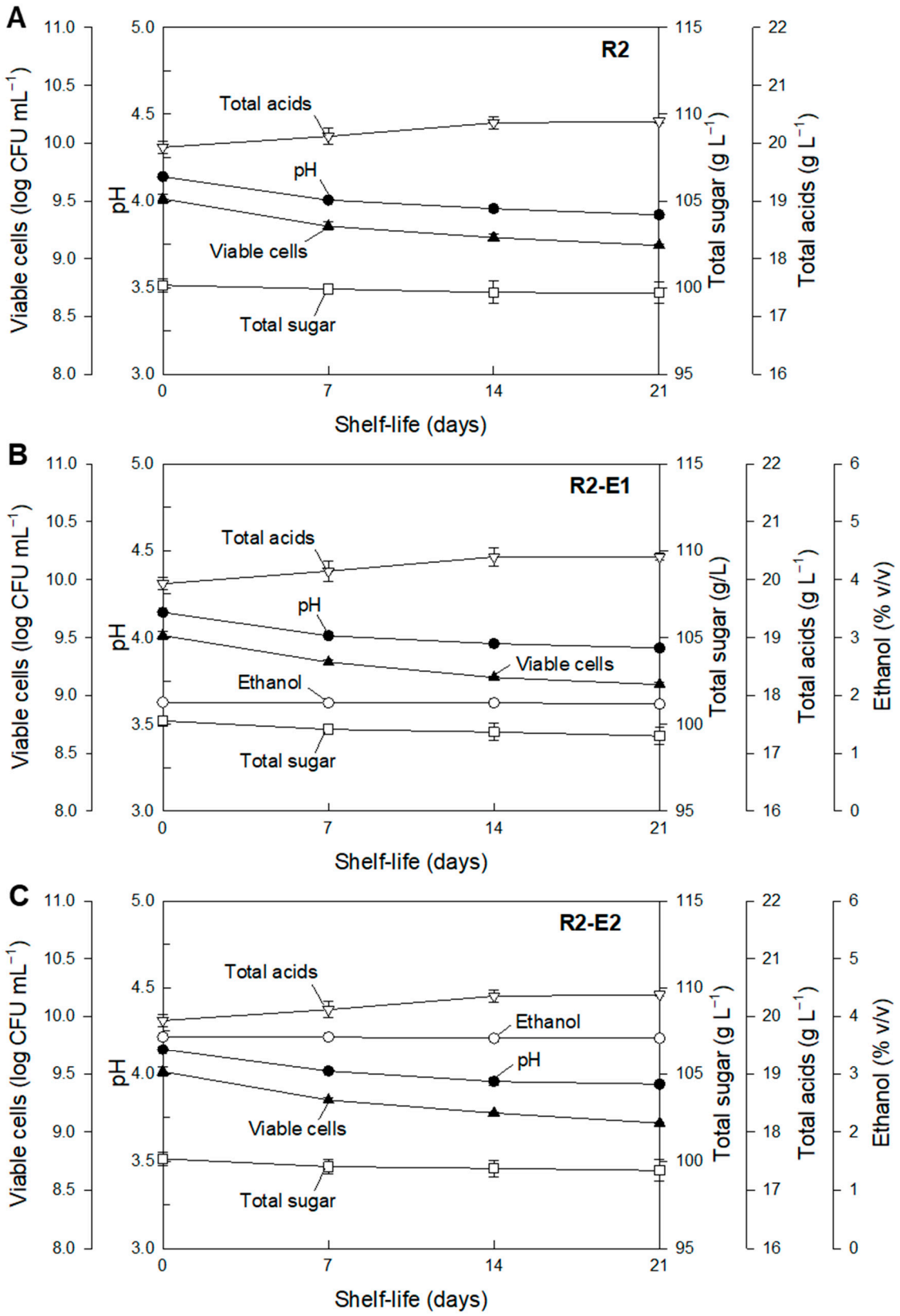

Figure 3. Effects of supplemental ethanol in R2-E1 and R2-E2, on profiles of viable cells, pH, total sugar, and total acids during refrigerated (4 °C) storage: (**A**) R2, fermented whole pineapple juice Recipe 2 (no supplemental ethanol) used as control; (**B**) R2-E1, fermented whole pineapple juice Recipe 2 with 2% v/v supplemental ethanol; (**C**) R2-E2, fermented whole pineapple juice Recipe 2 with 4% v/v supplemental ethanol.

3.4. Color Assessment of the WPJ Recipes

The appearance of the fresh whole pineapple juice (WPJ), the sterilized WPJ, and the freshly fermented products (R1–R3), is shown in Figure 4. The fresh juice was slightly paler in color (Figure 4A) compared to the sterilized juice (Figure 4B) and the fermented products made from the sterilized juice (Figure 4C–E). The slight darkening of the sterilized juice and its fermented products was apparently a consequence of the well-known browning reaction [72] between sugars and proteins during sterilization. No changes in color were observed as a consequence of the fermentation per se.

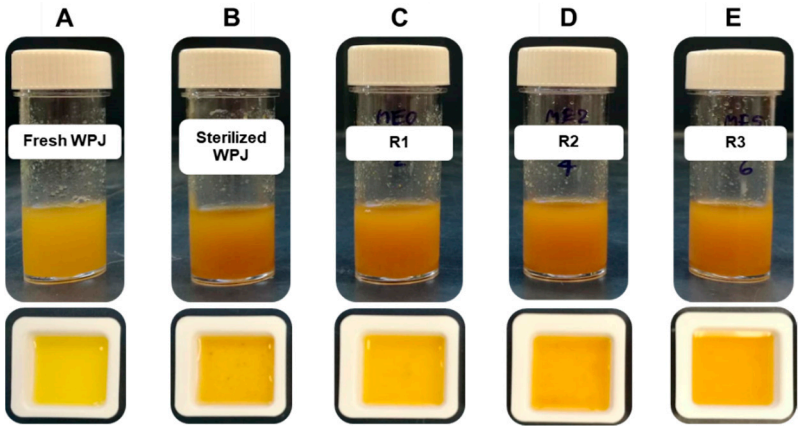

Figure 4. Appearance of the fresh whole pineapple juice (WPJ) prior to sterilization (**A**); the sterilized whole pineapple juice (**B**); the fermented whole pineapple juice Recipe 1 (R1) at 12 h (**C**); the fermented whole pineapple juice Recipe 2 (R2) at 12 h (**D**); the fermented whole pineapple juice Recipe 3 (R3) at 12 h (**E**).

The electronic color parameters (L*, a*, and b*) of the fermented juice products R2, R2-E1 and R2-E2, are shown in Table 1. The data are for various times during refrigerated storage after the fermentation. None of the color parameters varied significantly during storage and, therefore, the products had stable colors and appearances during refrigerated storage for up to 21 days at least (Table 1). Therefore, during refrigerated storage the live probiotic did not alter the color and appearance of the products.

Table 1. Appearance and color parameters of fermented whole pineapple juice R2, R2-E1, and R2-E2 during storage at 4 °C for 21 days.

Recipe [£]	Shelf-Life (day)	Appearance	Color Parameter [¥]		
			L*	a*	b*
R2	0		41.73 ± 0.24 [a]	−0.83 ± 0.06 [a]	8.08 ± 0.25 [a]
	7		42.12 ± 0.28 [a]	−0.80 ± 0.12 [a]	8.14 ± 0.04 [a]
	14		42.08 ± 0.25 [a]	−0.81 ± 0.09 [a]	8.19 ± 0.01 [a]
	21		42.05 ± 0.15 [a]	−0.82 ± 0.11 [a]	8.20 ± 0.08 [a]

Table 1. *Cont.*

Recipe [£]	Shelf-Life (day)	Appearance	Color Parameter [¥]		
			L*	a*	b*
R2-E1	0		41.51 ± 0.18 [a]	−0.86 ± 0.11 [a]	7.49 ± 0.10 [a]
	7		41.81 ± 0.05 [a,b]	−0.74 ± 0.09 [a]	7.65 ± 0.34 [a,b]
	14		41.82 ± 0.06 [a,b]	−0.79 ± 0.08 [a]	7.84 ± 0.07 [b]
	21		41.87 ± 0.13 [b]	−0.80 ± 0.08 [a]	7.85 ± 0.06 [b]
R2-E2	0		41.56 ± 0.10 [a]	−1.03 ± 0.10 [a]	7.24 ± 0.14 [a]
	7		41.79 ± 0.13 [a,b]	−0.98 ± 0.11 [a]	7.48 ± 0.08 [a,b]
	14		41.81 ± 0.16 [b]	−0.99 ± 0.07 [a]	7.65 ± 0.06 [b]
	21		41.89 ± 0.08 [b]	−1.00 ± 0.09 [a]	7.66 ± 0.08 [b]

[£] R2, fermented whole pineapple juice Recipe 2, no added ethanol; R2-E1, R2 supplemented with 2% *v*/*v* of ethanol; R2-E2, R2 supplemented with 4% *v*/*v* of ethanol. [¥] Identical superscript letters within a column of the same recipe indicate an absence of a statistically significant difference based on Tukey's test.

For all products (R2, R2-E1, R2-E2), the L* values on day 0 were statistically identical (Table 1). In all products, the b* values increased slightly but consistently with time during storage (Table 1). This might have been a consequence of the observed decline in pH during storage (Figure 3). There was no discernible visual impact of this slight increase in b*.

3.5. Sensory Analysis

The 30 panelists scored the four beverages as summarized in Figure 5. The beverages were (1) the sterilized whole pineapple juice (control, C); (2) the sterilized WPJ fermented after supplementation with 2% *w*/*v* malt extract (R2); (3) the fermented product R2 supplemented with 2% *v*/*v* ethanol (R2-E1); (4) the fermented product R2 supplemented with 4% *v*/*v* ethanol (R2-E2 (Figure 5).

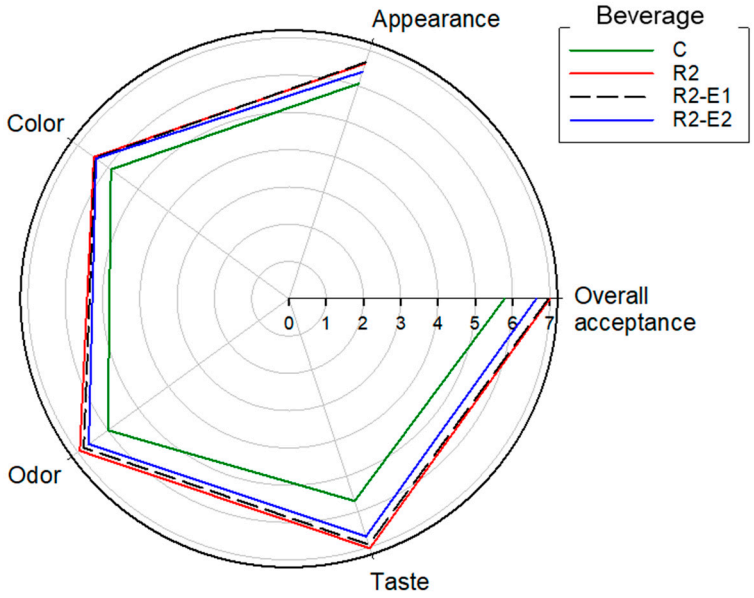

Figure 5. Sensory evaluation scores of the beverages: C, sterilized whole pineapple juice (control, C); R2, fermented whole pineapple juice Recipe 2 (R2); R2-E1, product R2 supplemented with 2% v/v ethanol; R2-E2, product R2 supplemented with 4% v/v ethanol. Data are mean values ± standard deviation (n = 30). Each score was generated by 30 individuals using a 9-point hedonic scale ranging from 1 (dislike very much) to 9 (like very much).

All fermented products had a turbid appearance as a consequence of the suspended probiotic cells (Figure 4D, Table 1). They all had a slight fermented odor and a sour-sweet taste. All fermented products (i.e., R2, R2-E1, and R2-E2; Figure 5) received similar scores in all categories, but they scored distinctly higher than the control (C) (Figure 5). The overall acceptance of the product R2 and its low-ethanol variant R2-E1 were nearly identical, but a little higher than overall acceptance of the more alcoholic product R2-E2 (Figure 5). For the product R2, the mean values of the appearance, color, odor, taste, and overall acceptance were 6.63 ± 1.19, 6.47 ± 1.07, 6.93 ± 0.78, 7.03 ± 1.07, and 7.00 ± 0.95, respectively. In an earlier work, a product made by fermenting a different fruit pulp with exactly the same bacterium as used here, was assessed highly by a panel of tasters [23]. In conclusion, the fermented beverages R2 and R2-E1 were distinctly superior to whole pineapple juice in terms of all sensory parameters.

3.6. Nutrition

Nutritional data for the sterilized whole pineapple juice and the fermented WPJ Recipe 2 (R2) are shown in Table 2. The calorific value of the fermented product was around 8% higher than the initial juice (Table 2) partly because of the malt extract added to the fermented product and also because of the protein and other metabolites contained in the bacterial biomass.

Table 2. Nutritional analysis of the sterilized whole pineapple juice and the fermented whole pineapple juice Recipe 2 (R2).

Test Item	Unit/100 mL [a]	Sterilized Whole Pineapple Juice	Fermented Whole Pineapple Juice Recipe 2 (R2)
Energy	kcal	52.63 ± 0.00	56.94 ± 0.00
Protein	g	0.47 ± 0.01	1.31 ± 0.02
Fat	g	0.27 ± 0.01	0.69 ± 0.02
Total carbohydrate	g	12.07 ± 0.49	11.22 ± 0.17
Total sugars	g	10.84 ± 0.19	10.01 ± 0.04
Total dietary fiber	g	0.70 ± 0.02	0.70 ± 0.01
Ash	g	0.29 ± 0.00	0.31 ± 0.01
Minerals			
Calcium (Ca)	mg	16.20 ± 0.02	16.60 ± 0.01
Magnesium (Mg)	mg	11.30 ± 0.00	12.00 ± 0.00
Potassium (K)	mg	178.00 ± 0.15	178.00 ± 0.10
Sodium (Na)	mg	0.4 ± 0.00	1.8 ± 0.00
Phosphorus (P)	mg	13.1 ± 0.06	16.4 ± 0.05
Iron (Fe)	mg	0.41 ± 0.03	0.38 ± 0.02
Copper (Cu)	mg	0.097 ± 0.00	0.092 ± 0.00
Manganese (Mn)	mg	0.43 ± 0.00	0.63 ± 0.00
Zinc (Zn)	mg	1.76 ± 0.00	1.65 ± 0.00
Vitamins			
Vitamin A (retinol)	µg	0.069 ± 0.00	0.068 ± 0.00
Vitamin C (ascorbic acid)	mg	28.38 ± 0.28	28.07 ± 0.13
Vitamin B1 (thiamin)	mg	0.066 ± 0.00	0.045 ± 0.00
Vitamin B2 (riboflavin)	mg	0.042 ± 0.00	0.047 ± 0.00
Vitamin B3 (niacin)	mg	0.16 ± 0.00	0.49 ± 0.00
Vitamin B6 (pyridoxine)	mg	0.14 ± 0.00	0.31 ± 0.00
Vitamin B12 (cyanocobalamin)	mg	0.00 ± 0.00	1.96 ± 0.00
Total phenolic content	mg GAE [b]	90.97 ± 0.12	135.82 ± 0.15

[a] All analyses were in triplicate. Data are average values ± standard deviations. [b] GAE, gallic acid equivalent.

The nutritional parameters of the sterilized WPJ (Table 2) were generally consistent with the data published for conventional fresh pineapple juice. For comparison, the fresh pineapple juice has the following nutritional characteristics (per 100 g): energy content of 53 kcal; protein content of 0.36 g; total lipid content of 0.12 g; carbohydrate content of 12.87 g; total dietary fiber content of 0.2 g; total sugars content of 9.98 g [55]. All these data are close to the values in Table 2, assuming 100 g to be approximately the same as 100 mL and recognizing that conventional juice was expressed solely from the pineapple pulp whereas WPJ was expressed from both the pulp and the peel. The WPJ (Table 2) was considerably richer in minerals compared to the fresh conventional pineapple juice. For the latter, the content of the key minerals (per 100 g) are as follows: potassium 130 mg, calcium 13 mg, phosphorus 8 mg, magnesium 12 mg, zinc 0.11 mg, and iron 0.31 mg [55]. The vitamin content of sterilized WPJ was comparable to data published for fresh pineapple juice [55] as discussed in Section 3.2.

Relative to the sterilized whole pineapple juice, fermentation enhanced the product R2 in terms of the following (Table 2): total protein (includes the protein in the bacterial cells) by ~2.8-fold; fat (by ~2.6-fold); vitamin B3 (~3.1-fold); vitamin B6 (~2.2-fold); vitamin B12 (to 19.6 mg L^{-1} from zero in the original juice); the content of total phenolics (~1.5-fold increase). Fermentation was the direct cause of the observed enrichment in vitamins as discussed earlier in Section 3.2.

The observed increase in total phenolics (Table 2) was also a consequence of the fermentation. Whether any *L. plantarum* including the strain WU-P19 is able to actually produce phenolics is uncertain; however, substantial evidence suggests the bacterium is able to biochemically modify diverse phenolics extracted from the plant tissue to generate products such as gallic acid [73]. Phenolics are typically measured in terms of gallic acid equivalents (GAE), as in the present study (see Section 2.6.8). Therefore, the observed increase in phenolics (Table 2) may have been a consequence of the production of gallic acid by the action of the bacterium on the phenolics extracted in the WPJ from the pineapple peels. Increased levels of total phenolics in terms of GAE have been observed in fermentation of fruit material by *L. plantarum* [74] as a consequence of modification of the

profile of the plant phenolics. Similar increases in total phenolics have been reported by others [14,16].

4. Conclusions

A nutritionally enriched probioticated fermented beverage could be made using whole pineapple juice and the probiotic *L. plantarum* WU-P19. The probioticated product and its mildly alcoholic variant were superior to whole pineapple juice in terms of all the sensory parameters. The fermented beverage proved to be an excellent purely vegetarian source of vitamin B12, an essential nutrient that is deficient in dairy-free vegan diets. The beverage contained nutritionally useful amounts of other vitamins, including vitamins C, B1, B2, B3 and B6. The probioticated beverages were stable during refrigerated storage for the duration of their 21-day shelf-life. Within this shelf-life, the beverages could provide more than 10^{11} live cells of the probiotic for each 100 mL of the product consumed.

Author Contributions: Conceptualization, W.P. and W.C.; methodology, W.P., W.C. and Y.C.; investigation, W.P.; resources, W.C. and Y.C.; writing—original draft preparation, W.P. and W.C.; writing—review and editing, W.P., W.C. and Y.C.; supervision, W.C. and Y.C.; funding acquisition, W.C., W.P. and Y.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Human Research Ethics Committee of Walailak University (approval WUEC-20-253-01 dated 25 August 2020).

Informed Consent Statement: Informed consent was obtained from all participants of the sensory panels involved in this study.

Data Availability Statement: All data relating to this study are included within this article. Data will be made available on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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