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Production and Optimisation of Fermented Pumpkin-Based Mature Coconut Water Kefir Beverage Using Response Surface Methodology

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Abstract: Fermentation of pumpkin puree and mature coconut water using water kefir grains is a potential method for producing a novel functional non-dairy-based probiotic drink. In the present study, response surface methodology based on Box–Behnken design (RSM-BBD) was used to optimise fermentation temperature and substrates' concentrations. The optimised fermentation temperature, pumpkin puree, and brown sugar concentrations of pumpkin-based mature coconut water kefir beverage (PWKC) were 27 °C, 20%, and 10% *w*/*v*, respectively. The optimised PWKC ($PWKC_{opt}$) obtained an overall acceptability (OA) score of 4.03, with a desirable *Lactobacillus* count (6.41 Log CFU/mL), 0.68% *v*/*v* lactic acid content, 31% of water kefir grains' biomass growth rate, and fermentation time (to reach pH 4.5) of 4.5 h. The optimized beverage, $PWKC_{opt}$, contained 3.26% proteins, 2.75% dietary fibre, 2186.33 mg/L of potassium, 180.67 mg/L phosphorus, and 137.33 mg/L calcium and had a total phenolic content of 89.93 mg GAE/100 mL, flavonoid content of 49.94 mg QE/100 mL, and carotenoid content of 33.24 mg/100 mL, with antioxidant activity (FRAP: 169.17 mM Fe(II)/100 mL, IC_{50} value of DPPH free radicals scavenging activity: 27.17 mg/mL). Water kefir microorganisms in $PWKC_{opt}$ remained stable for at least 56 days at 4 °C. Therefore, $PWKC_{opt}$ might potentially serve as a value-added product, offering a basis for sustainable development within both the coconut and pumpkin industries.

Keywords: acetic acid bacteria; Box–Behnken design; fermented pumpkin; lactic acid bacteria; mature coconut water; yeast

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1. Introduction

The growing demand of consumers for foods that offer more than just basic sustenance has increased interest in the development of fermented functional foods containing probiotics. Probiotics have been defined as 'live microorganisms, that are believed to confer health benefits to the host when administrated in adequate amounts' by the FAO/WHO [1]. Traditionally, dairy products are the predominant vehicle for the delivery of probiotic bacteria. However, the high prevalence of lactose intolerance and the ongoing trend of veganism have led to an increase in the demand for non-dairy alternatives in the probiotic market. According to Euromonitor International [2], approximately 20% of consumers in the world's major markets intend to increase plant origin consumption while decreasing animal origin consumption.

Water kefir, a traditional fermented beverage crafted from sugary water using water kefir grains, offers a viable non-dairy alternative to conventional probiotic dairy products.

Water kefir grains are irregularly shaped and brittle, composed of a grain-like exopolysaccharide matrix made up of a symbiotic culture of lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeasts [3]. The historical origin of water kefir grains is not precisely known, but some of the earliest records indicate that 'Tibi grains' were plucked from the leaves of a Mexican cactus (*Optunia*) [4]. Water kefir brewing is a relatively mild fermentation process (24–72 h, 21–30 °C [3,5]) and hence the bioactive compounds such as vitamins, minerals, and organic acids present in the fermented water kefir brew can be preserved well. Water kefir grains can be retrieved from the fermented medium and used indefinitely by re-inoculating the grains in a new sugar–water medium [6], and therefore, water kefir production is relatively inexpensive. Low-energy water kefir-based fermentations can improve sensory, functional, and nutritional properties, increase shelf life, and generate value-added goods [3].

The consumption of fermented water kefir beverages has also been related to health benefits, such as gastrointestinal beneficial effects and anti-hypertensive, anti-bacterial, anti-microbial, anti-cancer, anti-oxidative, anti-allergenic, anti-asthmatic, anti-tumour, anti-inflammatory, anti-obesity, anti-hyperglycaemia, anti-carcinogenic, cholesterol-lowering, and immune modulation effects, and helps in minimising or preventing oedema, ulcers, and pathogen growth [3,7–10]. Traditionally, a table sugar or brown sugar solution is used as the substrate for water kefir fermentation [3]. However, a sugar solution has a relatively simple nutritional composition and reduces the growth rate and activity of water kefir over time. In order to ensure the health benefits of kefir, studies on fermentation using alternative matrices are necessary. Locally available, nutritious but highly perishable pumpkin and mature coconut water, which are often discarded as waste, have the potential to be used as fermentation substrates in the production of water kefir.

Pumpkin (*Cucurbita* L.), a squash fruit vegetable belonging to the *Cucurbitaceae* family [11], is a significant domestic crop grown worldwide [12], with global production of 22.81 million tons in 2022 [13]. The total production of pumpkin in Malaysia was 20,369 tons in 2022, making it one of the most important agricultural commodities in the country [14]. In the present study, pumpkin is selected as the fermentation substrate over different sources due to its richness in indigestible carbohydrates (such as rhamnose, glucose, arabinose, and galactose) with potential prebiotic properties [15]. Previous studies have demonstrated that the prebiotic potential of pumpkin could enhance the viability of probiotics under adverse food processing and simulated gastrointestinal conditions [16,17]. Pumpkin is also a good source of vitamins, minerals, and carotenoids, which include α- and ß-carotene, and lutein, and is low in fat, which is beneficial for the developed beverage as a functional food [15,18]. However, raw pumpkin is not ready-to-eat, and the processing of pumpkin from cutting to cooking is laborious due to its large volume and mass. Furthermore, freshly cut pumpkin is susceptible to enzymatic browning and microbial spoilage [19]. Fermentation with *Lactobacillus* has been found to reduce anti-nutrients (phytates, tannins, phenolic compounds, and oxalates) in pumpkin [20], produce antimicrobial ingredients to inhibit the growth of pathogenic microorganisms, and generate high antioxidant activity, which can inhibit enzymatic browning in pumpkin [21]. Pumpkin can be transformed into a ready-to-drink, value-added beverage product with prolonged shelf life, enhanced nutritional values, and improved digestibility through the fermentation process [20,21]. Previously, pumpkin has been used as a fermentation substrate in the production of fermented juices using *Lactobacillus casei* [18] and *Lactobacillus rhamnosus* [15]. However, to the best of our knowledge, using pumpkin as a substrate for water kefir fermentation appears to have received limited attention in scientific research.

Coconut (*Cocos nucifera* L.) is a multipurpose perennial crop that is widely grown in tropical regions around the world. Multifaceted uses of coconut have resulted in high global production of 62.41 million tons in 2022 [13]. Among the major coconut-producing countries, Malaysia ranked 11th, with a total production of 604,428 tons in 2022. Coconut is also the fourth essential industrialised crop in Malaysia, after rice, rubber, and oil palm [22]. Coconut water is suitable to be consumed directly as well as to be transformed into different

beverages owing to its high quantities of sugar, amino acids, vitamins and minerals and low fat content [23]. Scientific evidence is growing to support the use of coconut water for health and therapeutic purposes. Coconut water's diverse applications can be attributed to its unique chemical composition, including carbohydrates, vitamins, minerals, amino acids, and phytochemicals [24]. Depending on the age of the coconut upon harvest, coconut water can be classified as young or mature. As a coconut grows, in particular, in the ninth month, the overall sugar level peaks at 2.9% [25], and therefore, young coconut water is generally sweeter than mature coconut water. While the maturity level does not notably affect the nutritional properties, the rise in fat content and decline in water content as the coconut matures can lead to a degradation in flavour [23]. As a result, mature coconut water is disposed of as waste most of the time during the manufacturing of coconut milk, chutney, chips, or cream, resulting in substantial environmental pollution and a significant loss of mature coconut water [26]. For instance, a total of 200,000 tons/year of mature coconut water is discharged down the drain in Thailand [27]. There have been attempts to make various value-added products such as therapeutic drinks [28], refreshment drinks [25], and vinegar [23] using mature coconut water. The flavour of mature coconut water can be overshadowed by the acid–sweetish flavour imparted by other additives [25], and hence, mature coconut water is drinkable after the fermentation process with improved organoleptic properties [28]. A functional beverage produced by fermenting mature coconut water with potential probiotic *Lactobacillus plantarum* DW12 yielded a probiotic count of 8.4 Log CFU/mL [27], surpassing the minimum therapeutic dose of probiotic cells, which is 6 Log CFU/mL. This suggests that mature coconut water is an appropriate medium for delivering probiotic strains [29]. Therefore, employing the water kefir fermentation process to create fermented mature coconut water beverages could mitigate off-flavours present in mature coconut water and potentially improve customer acceptance.

The usage of water with low buffering capacity and calcium concentrations is linked to reduced water kefir grain growth [30]. The calcium content of coconut water constantly rises throughout the maturation process [24]. The protein content of coconut water also increases as it matures, which could be linked to increased stored protein synthesis during the change of liquid watery endosperm into solid white coconut meat [24]. According to Guzel-Seydim et al. [31], the addition of whey protein isolates or modified whey protein to kefir grains could maintain the kefir grain microflora and enhance biomass production. As a result, it could be hypothesised that mature coconut water with high protein and calcium ion contents is more suited for water kefir fermentation. Young coconut water has been extensively studied, but the exploration of mature coconut water as a water kefir fermentation substrate has been relatively limited.

This study used a combination of mature coconut water and pumpkin puree as substrates in the development of a fermented pumpkin-based mature coconut water kefir beverage with water kefir grains as a starter. While a wide range of substrates have been utilised in the preparation of water kefir beverages, the practice of combining multiple substrates is not so common. Mature coconut water is naturally rich in sugars, vitamins, and minerals [23] while pumpkin puree is a good source of indigestible carbohydrates [15]. Utilisation of a combination of mature coconut water and pumpkin puree as substrates could offer a diverse range of nutrients for the kefir microbiota and consequently support the growth of kefir grains and enhance the fermentation process and fermented final product.

Traditional water kefir is manufactured by people in Southeast Asian countries on a household scale, using solid-state fermentation under non- or barely aseptic conditions, which poses a risk of pathogenic or possibly unwanted microbial contamination, requires a long fermentation period, has low acid production and inconsistent flavour, and hence is not applicable for industrial production [32]. Water kefir can be prepared via a consistent method to ensure quality and consistency in the final product. Therefore, in the present study, response surface methodology based on the Box–Behnken design (RSM-BBD) was used to optimise the fermentation temperature and the concentrations of substrates (pump-

kin puree and brown sugar) to obtain a final fermented product (pumpkin-based mature coconut water kefir beverage (PWKC)) with minimum fermentation time (to reach pH 4.5), while achieving maximum overall acceptability (OA) score, water kefir grain biomass growth rate, lactic acid content, and *Lactobacillus* count. The chemical composition, physicochemical properties (total soluble solids, viscosity, pH value, and colour), antioxidative contents and activities, sugar (glucose and fructose) and ethanol contents, and viabilities of presumptive *Lactobacillus*, LAB, AAB, and yeast, sensorial properties, and shelf-life stability of optimised PWKC ($PWKC_{opt}$) were also studied.

2. Materials and Methods

2.1. Materials

Ripe pumpkins (*Cucurbita moschata* Duschene) and mature coconuts (12 months old, Malayan Coconut Tall) were bought from Comfort Organic Farm (Balik Pulau, Penang, Malaysia) and Anba Coconuts (Abu Siti Lane, Penang, Malaysia), respectively. The water kefir grains were purchased from My Kefir World, Kuala Lumpur, Malaysia.

2.2. Chemicals and Reagents

Brilliant green lactose bile broth, chloramphenicol, cycloheximide, de Man Rogosa Sharpe (MRS) agar, lactose broth, Folin–Ciocalteau reagent, modified deoxycholate-mannitol-sorbitol (mDMS) agar, peptone solution, plate count agar (PCA), Ringer's solution, sodium acetate buffer, and yeast extract glucose chloramphenicol (YGC) agar were purchased from Merck (Darmstadt, Germany). Dichloran Rose Bengal Chloramphenicol agar (DRBC) was obtained from Biokar Diagnostics (Beauvais, France). *Lactobacillus-selective* base agar was bought from Neogen Corporation (Lansing, MI, USA). Aluminium trichloride, ferric chloride hexahydrate, and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol and methanol were supplied by HmbG Chemicals (Hamburg, Germany). Sodium carbonate and hydrochloric acid were bought from Systerm ChemAR (Shah Alam, Malaysia) and R&M Chemicals (London, UK), respectively. All chemicals and reagents were of analytic grade and had not undergone any further purification.

2.3. Preparation of Fermentation Substrates

2.3.1. Preparation of Pumpkin Puree

The pumpkins were trimmed, washed, cut into slices, steam-pasteurised to aid in softening the fruits at 121 °C for 20 min in an autoclave (HV-25, Hirayama Manufacturing Co., Saitama, Japan), and subsequently blended into a puree. Once the puree had cooled to room temperature, it was packaged in sterile polyethylene zip-seal bags and stored at −20 °C until use. Before use as the beverage substrate, the pumpkin puree was thawed at 4 °C overnight.

2.3.2. Preparation of Mature Coconut Water

The mature coconuts were washed with distilled water containing 1% household bleach (Clorox, The Clorox Company, Oakland, CA, USA) and rinsed thoroughly with distilled water. After air-drying for 1 h, the coconut water was extracted by drilling the fruit mesocarp with a blade. The extracted mature coconut water was filtered using a muslin cloth and flash pasteurised (15 min at 121 °C) using a lab-scale autoclave (HV-25, Hirayama Manufacturing Co., Saitama, Japan), poured into jars, and cooled to 25 °C.

2.4. Activation of Water Kefir Grains

The mixture of mature coconut water (64% v/v), pumpkin puree (22% w/v), and brown sugar (9% w/v, Malayan Sugar Manufacturing (MSM) Prai Berhad, Seberang Perai, Malaysia) was pasteurised at 121 °C for 20 min using a lab-scale autoclave and left to cool to 25 °C. Subsequently, water kefir grains at 5% (w/v) were added, and the mixture was left

to ferment at 32 °C for 12 h [33]. After fermentation, the water kefir grains were separated from the brew by filtering the mixture through a plastic fine mesh sieve (3 mm pore size).

2.5. Preparation of PWKC

Activated water kefir grains were used directly as inoculum at 5% *w*/*v* to ferment the PWKC beverage samples as shown in Table 1. The fermentation process was considered complete when the pH value of the sample reached 4.5. All the preparation procedures were conducted in a laminar airflow cabinet (Class II Type A2 Biological Safety Cabinet, Esco Labculture, Horsham, PA, USA) to maintain aseptic conditions. The fermented beverage samples were stored in a refrigerator (4 °C) until used.

Table 1. Box–Behnken design (BBD) arrangement of the independent variables (X_1, X_2, and X_3) and their observed responses including the fermentation time to reach pH 4.5 ($T_{pH4.5}$).

Run	X_1 (% *w*/*v*)	X_2 (% *w*/*v*)	X_3 (°C)	$T_{pH4.5}$ (h)	OA (Score)	Biomass Growth Rate (% G)	Lactic Acid (% *v*/*v*)	*Lactobacillus* Count (Log CFU/mL)
1	20.00 (−1)	0.00 (−1)	27.00 (0)	6.36	2.09	45.00	0.80	7.54
2	30.00 (+1)	0.00 (−1)	27.00 (0)	6.15	2.02	40.00	0.62	6.01
3	20.00 (−1)	10.00 (+1)	27.00 (0)	4.56	3.98	30.00	0.64	6.23
4	30.00 (+1)	10.00 (+1)	27.00 (0)	3.64	5.89	22.00	0.65	4.32
5	20.00 (−1)	5.00 (0)	22.00 (−1)	6.00	2.64	42.00	0.73	6.82
6	30.00 (+1)	5.00 (0)	22.00 (−1)	5.45	2.83	38.00	0.64	5.95
7	20.00 (−1)	5.00 (0)	32.00 (+1)	3.00	4.98	15.00	0.54	3.82
8	30.00 (+1)	5.00 (0)	32.00 (+1)	2.25	6.50	10.00	0.32	2.08
9	25.00 (0)	0.00 (−1)	22.00 (−1)	8.08	0.74	55.00	0.82	8.04
10	25.00 (0)	10.00 (+1)	22.00 (−1)	4.76	2.07	31.00	0.65	6.03
11	25.00 (0)	0.00 (−1)	32.00 (+1)	3.64	2.25	20.00	0.40	3.08
12	25.00 (0)	10.00 (+1)	32.00 (+1)	3.00	6.26	15.00	0.45	3.82
13	25.00 (0)	5.00 (0)	27.00 (0)	4.07	4.46	25.00	0.40	4.01
14	25.00 (0)	5.00 (0)	27.00 (0)	4.00	4.46	25.00	0.45	3.26
15	25.00 (0)	5.00 (0)	27.00 (0)	4.00	4.22	24.00	0.45	3.88
16	25.00 (0)	5.00 (0)	27.00 (0)	4.08	4.37	24.00	0.45	3.88
17	25.00 (0)	5.00 (0)	27.00 (0)	4.00	4.23	26.00	0.43	3.12

X_1 = pumpkin puree content (20–30% *w*/*v*); X_2 = brown sugar content (0–10% *w*/*v*); X_3 = fermentation temperature (22–32 °C).

2.6. Experimental Design

The three independent variables studied were concentrations of pumpkin puree (X_1; 20–30% *w*/*v*) and brown sugar (X_2; 0–10% *w*/*v*), and fermentation temperature (X_3; 22–32 °C). The independent variables were optimised using RSM-BBD with Design-Expert 13.0 software (DX13, Stat Ease Inc., Minneapolis, MN, USA), for PWKC production. The three coded levels of variables were −1, 0, and +1 (low, medium, and high) (Table 1). Seventeen experiments were executed following the design matrix, and their response values were obtained. The activated water kefir grains were used directly as inoculum at (5% *w*/*v*) to ferment the PWKC samples at different fermentation temperatures and brown sugar and pumpkin puree concentrations, as shown in Table 1. Five dependent variables, namely (i) fermentation time to reach pH 4.5 ($T_{pH4.5}$, h), (ii) overall acceptability score (OA), (iii) water kefir grains' biomass growth rate (% G), (iv) lactic acid content (%), and (v) *Lactobacillus* count (Log CFU/mL), were assessed.

Each variable's significance level (*p*-value) was established based on Student's *t*-test with $p < 0.05$ indicating a statistically significant difference. Analysis of variance (ANOVA) was employed to determine the significance of the generated models. DX13 software was used to determine the response (Y) of the second-order polynomial equation, the coefficient of determination (R^2), the predicted R-squared and adjusted R-squared, the coefficient of variance (CV), and the probability F-value. The functional (mathematical) relationship

between the three independent variables and the dependent variable was presented by a second-degree polynomial equation (Equation (1)):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y and X_i are the predicted response and independent variables (3 variables), respectively. β_0, β_i, β_{ii}, and β_{ij} are the constant, linear, quadratic, and interaction regression coefficients of the model computed by DX13.

The response surface and contour plots were used to study the effect of two independent variables on dependent variables. An experiment was then conducted using a combination of the optimum predicted values to validate an optimal model.

2.7. Microbiological Safety Analyses

Microbiological safety analyses were conducted following the methods described by Gómez-Aldapa et al. [34] and Cassani et al. [35] prior to hedonic assessment. The sample (50 mL) was diluted with quarter-strength (¼) Ringer's solution (450 mL) and homogenised in a stomacher (1 min; Lab Blender, Seward 400, London, UK). The homogenised samples were diluted (until 10^6) using ¼ Ringer's solution and inoculated onto the corresponding agar or broth for enumeration of total aerobic mesophilic bacteria (PCA Agar, 35 °C for 48 h), filamentous fungi (DRBC agar, 30 °C for 120 h), coliform bacteria, faecal coliforms, and *Escherichia coli* presumptive identification and confirmation (lactose broth and brilliant green lactose bile broth at 37 °C for 48 h and 44.5 °C for 48 h, respectively). The total mesophilic aerobic bacteria and filamentous fungi counts were expressed as CFU/mL whereas coliform count was expressed as the most probable number (MPN)/100 mL.

2.8. Measurement of Responses

2.8.1. Fermentation Time

The fermentation time ($T_{pH4.5}$, h) was determined based on the amount of time for PWKC beverage samples to reach a pH value of 4.5. This pH value was selected as the fermentation endpoint of plant-based water kefir and probiotic yoghurt-like products [36,37]. Throughout the fermentation process, the pH value of PWKC samples was determined using a pH meter (Mettler-Toledo, SevenEasy, Griefensee, Switzerland) at 30 min intervals.

2.8.2. Overall Acceptability

Hedonic assessment of PWKC beverage samples was carried out in sensory booths, and sensory panellists were recruited according to ISO 11136 [38]. Fifty semi-trained panellists (25 males and 25 females, Food Technology undergraduates at School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia) were recruited to evaluate the OA of the beverage samples using a 7-point hedonic scale, where 1 = dislike extremely, 4 = neither like nor dislike, and 7 = like extremely. All the panellists were trained on the products' sensory evaluation, and consent was obtained from the panellists. Purified water was provided to cleanse the palate between samples.

2.8.3. Water Kefir Grain Biomass Growth Rate

The biomass growth rate was determined using Equation (2) to evaluate the effects of culture conditions [39]:

$$G(\%) = \frac{X_{n+1} - X_n}{X_n} \times 100 \quad (2)$$

where G is the growth rate, X_n is the biomass weight after n hours (g), and X_{n+1} is the biomass weight after (n + 1) hours (g).

2.8.4. Lactic Acid Content

The lactic acid content of PWKC beverage samples was evaluated using a high-performance liquid chromatography (HPLC) equipped with a refractive index (RI) detector

system (Waters 2414, Waters Corporation, Milford, MA, USA) [40]. The lactic acid content was then calculated based on the standard curve constructed.

2.8.5. Viability of Presumptive *Lactobacillus*

Presumptive *Lactobacillus* count of PWKC beverage samples was determined according to the method proposed by Laureys and De Vuyst [41], using the spread plate method on *Lactobacillus*-selective base agar supplemented with cycloheximide (0.1 g/L). The colonies were counted after being incubated at 30 °C for 6 days, and results were expressed as Log CFU per mL of beverage samples.

2.9. Characterisation of Optimised Fermented Pumpkin-Based Mature Coconut Water Kefir (PWKC$_{opt}$)

PWKC$_{opt}$ were placed in glass bottles, closed tightly with lids, and stored at 4 °C for further characterisation and shelf-life study in terms of physicochemical (total soluble solids (TSS), pH, viscosity, and colour) and sensory properties (appearance (colour), odour, taste (sourness), texture (consistency), and overall acceptability), and microbiological changes (*Lactobacillus*, lactic acid bacteria, acetic acid bacteria, and yeast counts), measured at days 0, 28, and 56 of storage. The antioxidative properties, proximate and elemental compositions of the PWKC$_{opt}$ were measured at the end of fermentation. Chromatographic analysis of sugars, ethanol, and organic acid contents were conducted during the fermentation process at 0, 2.25, and 4.50 h of fermentation.

2.9.1. Chemical Composition

The proximate composition of PWKC$_{opt}$, i.e., moisture content (AOAC 925.19), ash content (AOAC 923.03), total protein content (AOAC, 955.04), crude lipid (AOAC 960.39), total dietary fibre (AOAC 991.43), and mineral composition (AOAC 985.35), was determined according to AOAC methods [42].

2.9.2. Physicochemical Properties

The physicochemical properties (total soluble solids, viscosity, pH value, and colour) of PWKC$_{opt}$ were determined following the methods described by Hlangwani et al. [43].

Total Soluble Solids

Total soluble solids content of the PWKC$_{opt}$ was measured using a refractometer (PAL-BX/RI, Atago, Tokyo, Japan).

Viscosity

Viscosity of the PWKC$_{opt}$ was measured using a viscometer (DVII + Pro, Brookfield, Middleborough, MA, USA) with LV-4 spindle rotating at 12 rpm. The reading displayed in centipoise (cP) was recorded when the value shown remained constant, with no fluctuation.

pH Value

pH value of the PWKC$_{opt}$ was measured using a pH meter (Orion 4 star A211, Thermo Fisher Scientific, Waltham, MA, USA). The reading was taken once a constant value was observed.

Colour

Colour (CIE L*; lightness, CIE a*; redness, and CIE b*; yellowness) of the PWKC$_{opt}$ was analysed using a colourimeter (Model 45/0-L, HAL, New York, NY, USA).

2.9.3. Antioxidative Contents and Activities

The carotenoid content, total phenolic content, total flavonoid content, ferric reducing antioxidant power, and free radical scavenging activity of PWKC$_{opt}$ were examined using the methods proposed by Barkallah et al. [44] and Wang et al. [45]. PWKC$_{opt}$ was centrifuged (15,000× *g*, 10 min, 4 °C), filtered through a polytetrafluoroethylene (PTFE)

membrane (pore size = 0.22 µm, Merck, Frankfurt, Germany), and stored in a screw-capped, amber-coloured glass bottle at −20 °C until analysis.

Total Carotenoid Content

Sample (1 mL) was centrifuged (3000× *g*, 10 min), sonicated (65 °C, 30 min) in ethanol (1 mL) and centrifuged (10,000× *g*, 5 min) again before its absorbance was taken (UV mini-1240, Shimadzu, Kyoto, Japan). The total carotenoid content of the sample was calculated according to Equation (3) [46] and expressed in mg/100 mL:

$$\text{Totalcarotenoid}(\text{mg/L}) = \frac{(1000 \times A_{470} - 2.86[15.65 \times A_{666} - 7.340 \times A_{653})] - 85.9[27.05 \times A_{653} - 11.21 \times A_{666}])}{245} \quad (3)$$

where A_{470}, A_{653}, and A_{666} are the absorbances taken at 470 nm, 653 nm, and 666 nm, respectively.

Total Phenolic Content (TPC)

The TPC of $PWKC_{opt}$ was measured using the Folin–Ciocalteau method. The sample in methanol (2.5 mL) was homogenised with Folin–Ciocalteau reagent (0.2 N, 2.5 mL) for 5 min followed by the addition of sodium carbonate (2.5 mL, 7.5% *w*/*v*) and incubation at 37 °C for 30 min. The absorbance of the mixture was then spectrophotometrically measured at 765 nm. TPC of the sample was estimated from the gallic acid calibration curve (0–100 mg/100 mL) and expressed as mg gallic acid equivalent (GAE) per 100 mL sample (mg GAE/100 mL).

Total Flavonoid Content (TFC)

Aluminium trichloride colourimetric method was used to determine the TFC of $PWKC_{opt}$. Sample (5 mL) was homogenised in aluminium trichloride methanol solution (5 mL, 2% *w*/*v* aluminium trichloride in methanol) and incubated at 37 °C for 30 min. The absorbance of the mixture was read at 415 nm, and the TFC of the sample was estimated based on the quercetin calibration curve (0–60 mg/100 mL) and quantified as mg quercetin equivalent (QE) per 100 mL sample.

Ferric Reducing Antioxidant Power (FRAP)

Sample (1 mL) was combined with FRAP working solution (20 mL, prepared by mixing sodium acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM) in 40 mM hydrochloric acid, and ferric chloride hexahydrate (20 mM,) at proportions of 10:1:1 ratio (*v*/*v*)) and incubated at 37 °C for 30 min in the dark. The absorbance of the mixture was read at 593 nm and reported as the millimole equivalent of ferrous sulphate per 100 mL sample (mM eq Fe (II)/100 mL) according to the calibration curve of ferrous sulphate in deionised water (0–30 mM/mL).

DPPH Radical Scavenging Ability (RSA)

A mixture of the sample (200 µL) and DPPH solution prepared in methanol (1 mL, 0.2 mM) was incubated at 37 °C for 30 min in the dark and the absorbance of the mixture was taken at 517 nm. The RSA of the sample was calculated according to Equation (4) and expressed as half the maximal inhibitory concentration value (IC_{50}, mg/mL), according to the regression equation (y = 12.246 ln(x) − 9.1955) obtained from the standard curve.

$$\text{Inhibition}(\%) = \left[\frac{A_B - A_S}{A_B}\right] \times 100 \quad (4)$$

where A_B and A_S are the absorbances of the blank (DPPH solution) and sample with DPPH solution, respectively.

2.9.4. Chromatographic Analysis

The sugar (glucose and fructose) content of $PWKC_{opt}$ was quantified using an HPLC equipped with an RI detector (Waters 2414, Waters Corporation, Milford, MA, USA) as previously described by Filip et al. [47], whereas organic acid (lactic, acetic, malic, and tartaric) contents were quantified using an HPLC with UV detector (Model 2487, Waters Corporation, Milford, MA, USA) [40]. Ethanol content (% v/v) was determined using a gas chromatograph (Shidmazu, GC-2010, Milan, Italy) fitted with a flame ionisation detector (Shidmazu, Milan, Italy) and equipped with a ZB-WAX film capillary column (length of 30 m × diameter of 0.25 mm, film thickness of 0.25 µm) (Phenomenex, Torrance, CA, USA) [48]. Identification and quantification of the ethanol, sugars, and acids were performed on samples collected at 0, 2.25, and 4.50 h of fermentation. The sample collected at 0 h was used as a control.

2.9.5. Viability of Presumptive *Lactobacillus*, LAB, AAB, and Yeast

To determine the viability of presumptive *Lactobacillus*, LAB, AAB, and yeast, a total of 10 mL of $PWKC_{opt}$ was homogenised with 90 mL of 1 g/L aqueous peptone solution, and necessary serial dilutions were performed. The diluted sample (100 µL) was spread on the *Lactobacillus*-selective base agar, MRS agar supplemented with cycloheximide (0.1 g/L,), mDMS agar supplemented with cycloheximide (0.1 g/L), and YGC agar supplemented with chloramphenicol (0.1 g/L,) using sterile beads followed by incubation at 30 °C for 48 to 72 h. The viability of presumptive *Lactobacillus*, LAB, AAB, and yeasts was expressed as Log CFU per mL [41,49].

2.9.6. Hedonic Test

Hedonic sensory testing was conducted in sensory booths according to the ISO 11136 standard [38]. A total of 50 untrained panellists (25 males and 25 females, Food Technology undergraduates at School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia) were recruited to evaluate the colour, odour, taste (sourness), texture (consistency), and overall acceptability of $PWKC_{opt}$ using a 7-point hedonic scale. Purified water was served to the panellists, who were instructed to rinse their mouths during the sensory test.

2.10. Identification of Isolate

Genomic deoxyribonucleic acid (DNA) from the dominant cultivable species isolates in the optimised beverage was isolated with the NucleoSpin Tissue kit (Machery-Nagel, Duren, Germany) based on the instructions supplied by the manufacturer. The concentration and purity of the extracted DNA were determined using a UV spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan). The pure genomic DNA was identified by 16S rRNA gene sequencing using universal primers (designed by the Centre for Chemical Biology, Universiti Sains Malaysia, Penang, Malaysia). The sequence similarity and closest phylogenetic relatives were then analysed and matched using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI).

2.11. Statistical Analysis

The analysis of the experimental design and data were performed using Design-Expert 13.0 (DX13, Stat Ease Inc., Minneapolis, MN, USA). The fitness of the polynomial equations was evaluated using the analysis of variance (ANOVA), and the statistical significance of the regression coefficients was tested by Fisher's test (F-test). All analyses were performed in triplicate, and data from characterisation of the $PWKC_{opt}$ were statistically analysed using one-way ANOVA with Tukey's honestly significant difference (HSD) test for post hoc comparisons using the IBM SPSS Statistics software version 27 (IBM, Chicago, IL, USA). The results from hedonic test were analysed using the Kruskal–Wallis test. Differences with p-values less than 0.05 were regarded as statistically significant.

3. Results and Discussion

3.1. RSM Model Fitting

A total of seventeen experimental runs were made to model the empirical relationship between the key factor parameters using the Box–Behnken design (BBD) (Table 1). ANOVA was conducted to confirm the validity of the regression model and determine the effects of the key factor parameters, i.e., pumpkin puree concentration (X_1; % w/v), brown sugar concentration (X_2; % w/v), and fermentation temperature (X_3; °C), on the output response function.

The model for all the output response functions was significant (p-value < 0.05, Table 2). The coefficient of determination (R^2) for each model achieved a desirability of 0.99, indicating the model developed for PWKC fitted the experimental data. All models possessed low correlation variation, non-significant lack of fit ($P_{lof} > 0.05$), and an adequate precision level of ≥4, which indicated that the developed model could predict the response function and the effect of independent variables on dependent variables. The quadratic model was attempted, and the non-significant terms ($p > 0.05$) were excluded from the fitted second-degree polynomial equations. The regression equations presented the extent of the response as a function of the three independent variables and are interpreted in the following sub-sections.

Table 2. Estimated coefficients of the fitted second-order polynomial models for dependent variables.

Term	Regression Coefficients Estimated				
	$T_{pH4.5}$ (h)	**OA (Score)**	**Biomass Growth Rate (% G)**	**Lactic Acid (% *v/v*)**	***Lactobacillus* Count (Log CFU/mL)**
			Intercept		
β_0	4.03	4.35	24.80	0.44	3.63
			Linear		
β_1	−0.30 *	0.44 *	−2.75 **	−0.06 **	−0.76 **
β_2	−1.03 *	1.39 *	−7.75 *	−0.03 ***	−0.54 **
β_3	−1.55 *	1.46 *	−13.25 *	−0.14 *	−1.76 *
			Interaction		
$\beta_1\ \beta_2$	−0.18 **	0.49 *	−0.75 $^{n.s.}$	0.05 ***	−0.10 $^{n.s.}$
$\beta_1\ \beta_3$	−0.05 $^{n.s.}$	0.33 **	−0.25 $^{n.s.}$	−0.03 $^{n.s.}$	−0.22 $^{n.s.}$
$\beta_2\ \beta_3$	0.67 *	0.67 *	4.75 *	0.06 **	0.69 ***
			Quadratic		
β_{11}	0.23 **	0.28 ***	2.72 **	0.11 **	0.91 **
β_{22}	0.92 *	−1.13 *	6.72 *	0.13 *	1.48 **
β_{33}	−0.08 ***	−0.39 **	−1.27 $^{n.s.}$	0.01 $^{n.s.}$	0.13 $^{n.s.}$
R^2	0.9993	0.9979	0.9961	0.9837	0.9796
CV%	1.34	3.05	3.91	5.23	8.37
Adq. Pre.	125.87	66.27	53.49	22.13	20.31
p_{lof}-value	0.1188 $^{n.s.}$	0.5293 $^{n.s.}$	0.1683 $^{n.s.}$	0.1758 $^{n.s.}$	0.4990 $^{n.s.}$
p_m-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Subscripts: 1 = pumpkin puree content (20–30%, w/v); 2 = brown sugar content (0–10% w/v); 3 = fermentation temperature (22–32 °C). CV%: coefficient variation (%); Adq. Pre.: adequate precision; p_{lof}-value: probability of F value for the lack of fit; p_m-value: probability of F value for the model. $T_{pH4.5}$: fermentation time to reach pH 4.5; OA: overall acceptability; % G: water kefir grain biomass growth rate. $^{n.s.}$ non-significant at $p > 0.05$. * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$.

3.2. Effect of Independent Variables on Dependent Variables of RSM Model

The model of second-order regression from the experimental data of the five output response functions: Y_1 = fermentation time to reach pH 4.5 ($T_{pH4.5}$), Y_2 = overall acceptabil-

ity (OA) score, Y_3 = water kefir grain biomass growth rate, Y_4 = lactic acid content, and Y_5 = *Lactobacillus* count (Log CFU/mL) are displayed in Equations (5)–(9), respectively.

$$Y_1 = 4.03 - 0.30X_1 - 1.03X_2 - 1.55X_3 + 0.23X_1^2 + 0.92X_2^2 - 0.08X_3^2 - 0.18X_1X_2 + 0.67X_2X_3 \quad (5)$$

$$Y_2 = 4.35 + 0.44X_1 + 1.39X_2 + 1.46X_3 + 0.28X_1^2 - 1.13X_2^2 - 0.39X_3^2 + 0.49X_1X_2 + 0.33X_1X_3 + 0.67X_2X_3 \quad (6)$$

$$Y_3 = 24.80 - 2.75X_1 - 7.75X_2 - 13.25X_3 + 2.72X_1^2 + 6.72X_2^2 + 4.75X_2X_3 \quad (7)$$

$$Y_4 = 0.44 - 0.06X_1 - 0.03X_2 - 0.14X_3 + 0.11X_1^2 + 0.13X_2^2 + 0.05X_1X_2 + 0.06X_2X_3 \quad (8)$$

$$Y_5 = 3.63 - 0.76X_1 - 0.54X_2 - 1.76X_3 + 0.91X_1^2 + 1.48X_2^2 - 0.44X_1X_2 + 0.69X_2X_3 \quad (9)$$

where X_1 and X_2 are the concentrations of pumpkin puree and brown sugar (% w/v), respectively, and X_3 is the fermentation temperature (°C).

3.2.1. Fermentation Time ($T_{pH4.5}$)

Fermentation time is crucial in development of probiotic products, as it can directly influence both the safety and quality of the final product. An inadequate fermentation time could lead to the proliferation of pathogenic microorganisms, but a prolonged fermentation time could lead to over-acidification that could negatively affect the sensory properties of the product [50]. In the development of probiotic foods, pH 4.5 is frequently chosen to be the fermentation endpoint, as a pH value < 4.6 could inhibit the growth of putrefying and pathogenic bacteria, consequently increasing microbial stability during storage and the shelf life of the product [51]. As shown in Table 1, $T_{pH4.5}$ varied from 2.25 to 8.08 h. The shortest and longest fermentation times were obtained at 32 °C, with a medium containing 30% w/v pumpkin puree and 5% w/v brown sugar (run 8), and 22 °C, with a medium containing 25% w/v pumpkin puree and 0% w/v brown sugar (run 9), respectively. The $T_{pH4.5}$ obtained was similar to the result obtained in the studies conducted by Acevedo-Martínez et al. [52] and Fawzi et al. [53], which reported a fermentation time of 2.25–5.5 h and 8 h to reach a pH value of 4.5 in the fermentation of skim milk supplemented with different grape pomace extracts, using *Streptococcus thermophilus* and *Lactobacillus acidophilus* [52] and broken rice milk, using *Lactobacillus plantarum* ATCC 14917, *Lactobacillus casei* DSM 20011, and *Lactobacillus acidophilus* ATCC20552 [53], respectively.

All the independent variables showed a significant negative linear effect ($p < 0.001$) on $T_{pH4.5}$ (Table 2). The increment of the fermentation temperature and concentrations of pumpkin puree and brown sugar within the studied range resulted in decrements of $T_{pH4.5}$ (Figure 1a–c), with the fermentation temperature in its linear term showing the dominant effect (Figure 1b,c). Microflora in water kefir were principally mesophilic. Mesophilic temperature could lead to higher metabolic activity and facilitate a faster fermentation rate and a higher organic acid production, leading to a shorter fermentation time [54,55].

As shown in Table 2, brown sugar concentration demonstrated a positive interactional effect with fermentation temperature ($p < 0.001$) and a negative interactional effect with pumpkin puree concentration ($p < 0.01$) on $T_{pH4.5}$. As illustrated in Figure 1a,c, decrement of $T_{pH4.5}$ was more significant when fermentation temperature and pumpkin puree and brown sugar concentrations at the upper-end of the studied range were used. The indigestible carbohydrates, sugar in pumpkin puree [16,17], and sucrose [3] in brown sugar were proven to be excellent nutrient/carbon sources and growth media for LAB and yeast strains in water kefir grains. Bengoa et al. [54] and Laureys et al. [55] also reported that nutrient-rich fermentation media accompanied by a mesophilic temperature (25–37 °C) could facilitate faster water kefir microorganisms' fermentation rate.

The quadratic effect of brown sugar concentration positively impacted $T_{pH4.5}$ significantly ($p < 0.001$), where a gradual decrease of $T_{pH4.5}$ was observed when brown sugar concentration increased from 0–8% w/v, as shown in Figure 1a,c. When a higher sugar concentration was used, more carbon was available for fermenting microorganisms' growth, thus facilitating a faster metabolic rate and producing a higher amount of organic acid, and hence, reaching a pH value of 4.5 in a shorter time [55]. However, when more than 8% w/v

brown sugar was used, $T_{pH4.5}$ was observed to increase slightly. A high substrate concentration could exert an osmotic pressure, which prevents the fermenting microorganisms from metabolising, thus prolonging the fermentation time [56]. Pumpkin puree concentration had a positive quadratic effect on $T_{pH4.5}$ ($p < 0.01$). When pumpkin puree concentration increased from 20–26% w/v, the $T_{pH4.5}$ decreased slightly. However, $T_{pH4.5}$ became almost constant when pumpkin puree concentration further increased, indicating that 26% w/v of pumpkin puree contributed to a minimum $T_{pH4.5}$. Pumpkin puree is rich in indigestible polysaccharides such as rhamnose, glucose, arabinose, and galactose and sugar, which could facilitate and support the growth of microorganisms [15]. The negative linear effect ($p < 0.001$) of fermentation temperature on $T_{pH4.5}$ was more significant than that of quadratic effect ($p < 0.05$) (Table 2). A sharp decrease of $T_{pH4.5}$ was observed when fermentation temperature was raised from 22 to 32 °C (Figure 1b,c). Water kefir microorganisms grew faster at the mesophilic temperature (upper end of the studied range), produced more acids, and, hence, reached the fermentation endpoint in a shorter time [54,55].

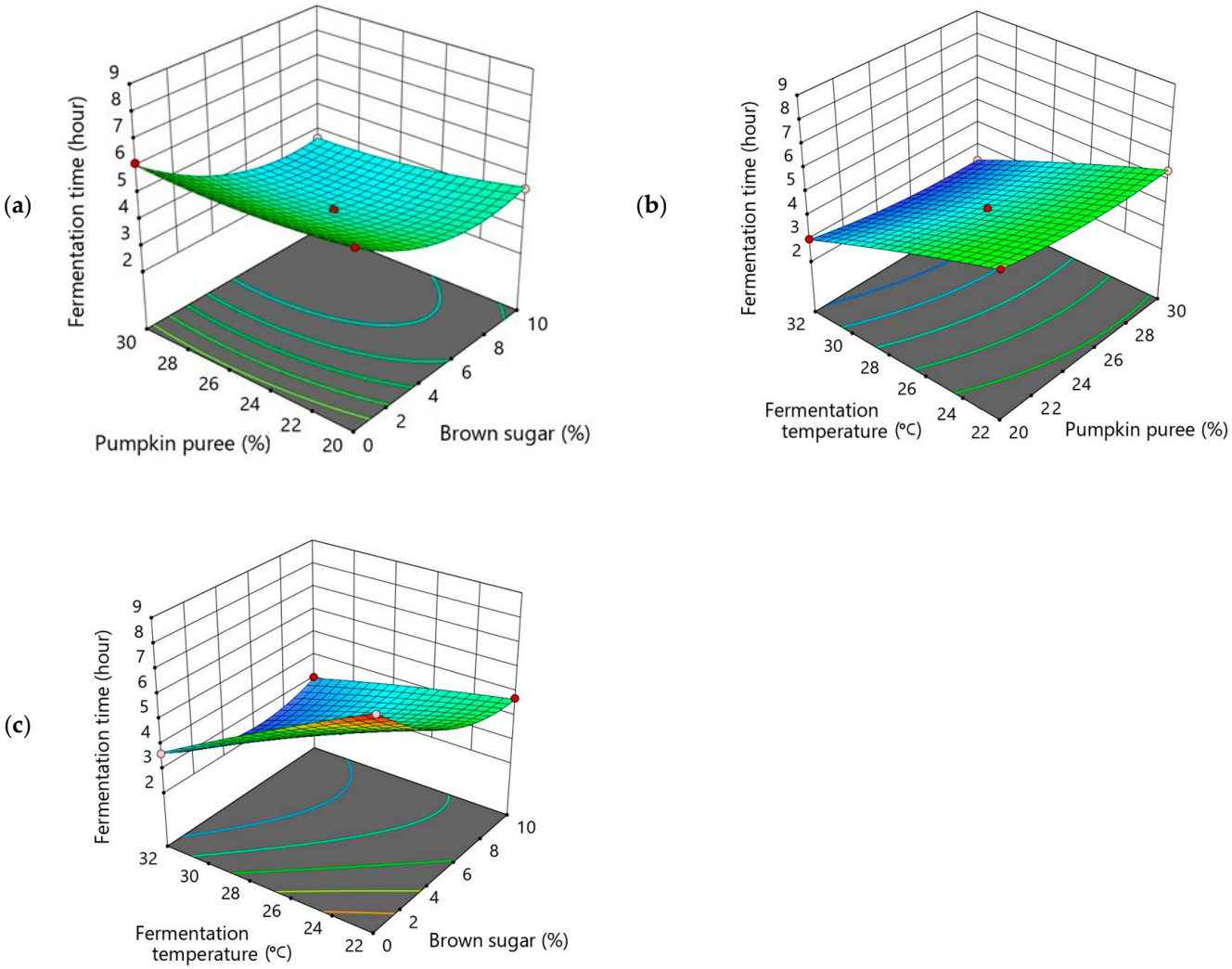

Figure 1. Response surface plots illustrating the interaction effect of (**a**) pumpkin puree and brown sugar content, (**b**) fermentation temperature and pumpkin puree content, and (**c**) fermentation temperature and brown sugar content on the fermentation time required to prepare pumpkin-based mature coconut water kefir beverage (PWKC).

3.2.2. Overall Acceptability (OA)

The adequate level of sensory acceptance of fermented food products has been considered an important factor due to its influence on the overall quality of the product [41,57].

The OA score of PWKC samples falls in the range of 0.74 to 6.50 on a 7-point hedonic Likert scale (Table 1). The PWKC sample containing 25% v/v pumpkin puree and 0% v/v brown sugar fermented at 22 °C (run 9) had the lowest OA score, whereas the PWKC sample containing 30% v/v pumpkin puree and 5% v/v brown sugar fermented at 32 °C scored the highest (run 8). The score obtained was comparable to kefir-like beverages derived from fruits or vegetables with an OA score of 1.5 to 5.0 on a 7-point hedonic Likert scale [56].

Fermentation temperature and pumpkin puree and brown sugar concentrations demonstrated a positive linear effect ($p < 0.001$) on the OA score of PWKC samples, indicating that the OA score can be enhanced by increasing the value of the independent variable as standalone events. The sensory attributes of fermented beverage products could be attributed to fermentation metabolites produced during the fermentation process, such as volatile compounds and organic acids. The production of metabolites during fermentation is depending on the fermentation condition, types and concentration of ingredients used in the production of fermented food products [58]. As illustrated in Figure 2a–c, a marked increase in OA score was observed as fermentation temperature and substrates' concentrations were elevated. When the fermentation temperature and substrates' concentration increased, the metabolism and growth rate of microorganisms during fermentation fasten, available substrates can be utilised to a greater extent, thus resulting in higher production of organoleptic compounds, which contributes to the improvement in the sensory acceptance of fermented beverage sample [59].

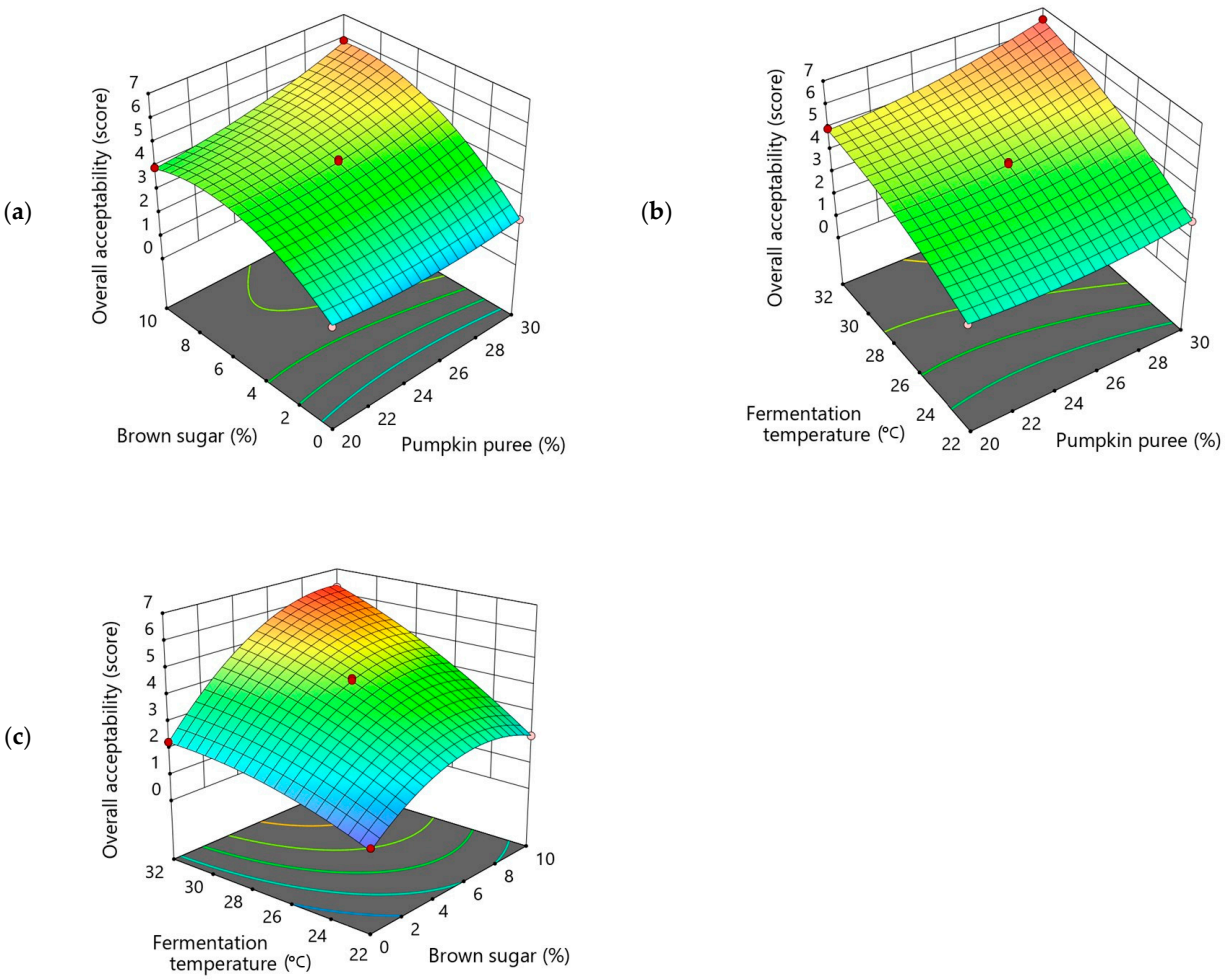

Figure 2. Response surface plots illustrating the interaction effect of (**a**) pumpkin puree and brown sugar content, (**b**) fermentation temperature and pumpkin puree content, and (**c**) fermentation temperature and brown sugar content on the overall acceptability (OA) score of pumpkin-based mature coconut water kefir beverage (PWKC).

The interactional effect between all the independent variables were significantly positive (Table 2, Figure 2a–c), revealing that by increasing fermentation temperature along with brown sugar or pumpkin puree concentration to the upper limit of the studied range, OA scores increased. Pumpkin puree with no additives has a bitter aftertaste [60], whereas mature coconut has a neutral taste, and hence both are unacceptable in terms of sensory properties [23]. Results demonstrated that the water kefir fermentation had improved the sensory qualities of the mature coconut water and pumpkin puree. Under mesophilic conditions with adequate nutrients, water kefir microorganisms, LAB, and yeast could produce several desirable organoleptically-active by-products such as lactic acid, acetic acid, ethanol, carbon dioxide, and an array of aromatic molecules [10]. Previous studies also proved that fermentation could eliminate unpleasant notes and resemble the organoleptic properties of pumpkin puree and mature coconut water [20,21,61].

Brown sugar concentration ($p < 0.001$) and fermentation temperature ($p < 0.01$) demonstrated significant negative quadratic effect, whereas pumpkin puree concentration exerted a moderately significant positive quadratic effect ($p < 0.05$) on the OA score (Table 2). OA score increased with the concentration of brown sugar (0–8% w/v) and became almost constant when more than 8% w/v of brown sugar was used (Figure 2a,c). The addition of sugar in the fermentation medium was effective in stimulating the growth of fermenting microorganisms. LAB and yeast ferment sugars to produce carbon dioxide, ethanol, and some aromatic compounds which give better sensorial properties to the fermented beverages [10]. Excessive sweetness and acidity can lead to a cloying or overly rich taste and a sharp tart flavour, respectively, and adversely affect the sensory experience. As observed in Figure 1b,c, OA scores increased when fermentation temperature increased from 22 to 32 °C. Water kefir microorganisms, which prefer to grow at mesophilic temperatures (25–37 °C), could produce more desirable metabolites and improve the sensory acceptance of the fermented product [54,55]. A gradual increase in OA score was noticed when 20–30% w/v of pumpkin puree was used (Figure 2a,b). Szydłowska et al. [15] reported that readily available nutrients in pumpkin puree favoured the growth and metabolic activities of LAB and yeast and increased the production of flavanols and esters that contribute to better organoleptic properties of the fermented beverages.

3.2.3. Water Kefir Grains Biomass Growth Rate

Culture medium for producing kefir grain biomass must be designed for high biomass output and reduction of production costs to develop economically viable cultures for industrial applications [62]. Water kefir grains' biomass growth rate is useful in studying the factors affecting the grains' growth. The minimum water kefir grain biomass growth rate obtained was 10%, which was found in the medium containing 30% w/v pumpkin puree and 5% w/v brown sugar fermented at 32 °C (run 8). Meanwhile, fermentation at 22 °C in a medium with 25% w/v pumpkin puree and 0% w/v brown sugar (run 9) resulted in the maximum water kefir grain biomass growth rate of 55% (Table 1). This outcome is consistent with observations of Pop et al. [63] that reported the biomass growth rate of kefir grains in skim milk medium ranged from 22.97 to 74.55%.

The water kefir grain biomass growth rate reduced when fermentation temperature and pumpkin puree and brown sugar concentrations rose as standalone events, with temperature in its linear term as the dominant effect (Figure 3a–c). This indicated that fermentation temperature at the lower end limit of the studied range favoured the kefir grains' biomass growth rate. This outcome is consistent with Pop et al. [63], who showed that the optimum temperature for kefir grains to grow was 25 to 28 °C, with the greatest growth rate recorded at 37.93 g/L for 24 h.

The factors influencing kefir grain biomass increment have received little attention. Guzel-Syedim et al. [64] reported that it is critical to utilise proper concentrations of different nutritional carbohydrate substrates to increase the growth rate of kefir grains. The quadratic term of substrates' concentrations demonstrated a significant positive effect on water kefir grains' biomass growth rate (Figure 3a–c). The water kefir grains' biomass

growth rate decreased gradually when 22–26% w/v of pumpkin puree and 0–6% w/v brown sugar concentration were used. Subsequent increase of substrates' concentrations increased the water kefir grains' biomass growth rate. This demonstrated that increasing the amount of pumpkin puree and brown sugar to the top of the studied range could boost the kefir biomass.

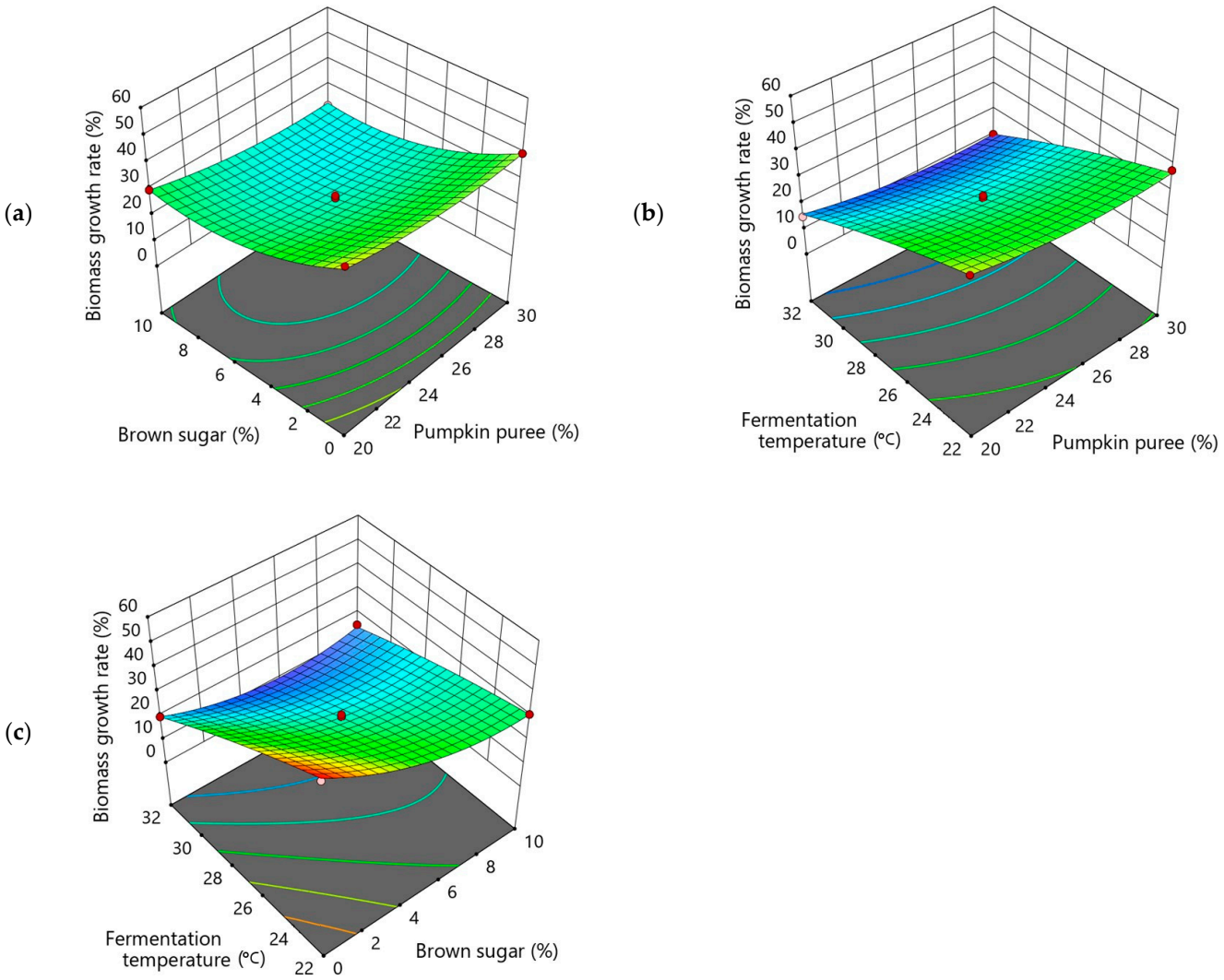

Figure 3. Response surface plots illustrating the interaction effect of (**a**) pumpkin puree and brown sugar content, (**b**) fermentation temperature and pumpkin puree content, and (**c**) fermentation temperature and brown sugar content on the water kefir grains' biomass growth rate.

Figure 3c demonstrates a positive interactional effect between temperature and brown sugar. The rate of biomass growth of the water kefir grains was accelerated by simultaneous increases in fermentation temperature and brown sugar content. The increase in fermentable sugar (glucose, sucrose, and fructose) and temperature enhanced the rate of metabolism for the microbial flora and led to a rise in the kefir grains' biomass growth.

3.2.4. Lactic Acid Content

The distinctive aroma and flavour of water kefir are mainly associated with the organic acid produced by the water kefir microorganisms during fermentation [65]. Lactic acid fermentation occurs during the production of water kefir by *Lactobacillus*, which breaks down sugars to produce energy in the form of adenosine triphosphate [66]. Lactic acid generated during the fermentation process gives the water kefir a tangy or sour flavour [10].

The production of lactic acid could also lead to the development of an acidic environment, which could inhibit the growth of spoilage microorganisms and consequently extend the shelf life of the fermented product [67]. As shown in Table 1, the minimum and maximum lactic acid contents in PWKC are 0.32% v/v (run 8: 30% w/v pumpkin puree, 5% w/v brown sugar, 32 °C) and 0.82% v/v (run 9: 25% w/v, 0% w/v, 22 °C), respectively (Table 1). Previously, Laureys and De Vuyst [68] also reported a lactic acid content of 0.5% v/v in honey beverages fermented by water kefir grains.

Fermentation temperature and pumpkin puree and brown sugar concentrations demonstrated negative linear effect ($p < 0.05$) on the lactic acid content of PWKC samples. Fermentation temperature in its linear term showed the dominant effect. Lactic acid content in PWKC samples decreased when the fermentation temperature increased from 22 to 32 °C (Figure 4b,c). The *Lactobacillus* sp. in PWKC might require specific nutrients and temperature to grow optimally. This behaviour was also reported by Alves et al. [69], who fermented insulin-supplemented coconut extract with water kefir grains. While certain microbial species never linked to water kefir grains cultivated in sugary water were discovered, the usual strains were not present [69]. This result could be supported by Darvishzadeh et al. [7] who studied Russian olive juice and found that the optimal fermentation temperature of the *Lactobacillus* sp. (*Lactobacillus paracasei*, *Lactobacillus parabuchneri*, *Lactobacillus kefiri*, and *Lactobacillus casei*) was 31.2 °C. Although the optimal growth temperature of water kefir grains ranges from 21 to 30 °C [3,5], some *Lactobacilli* are mesophilic (30–40 °C), with an upper limit of 40 °C [70], thereby restricting the *Lactobacilli* growth and consequently lowering the content of lactic acid in PWKC.

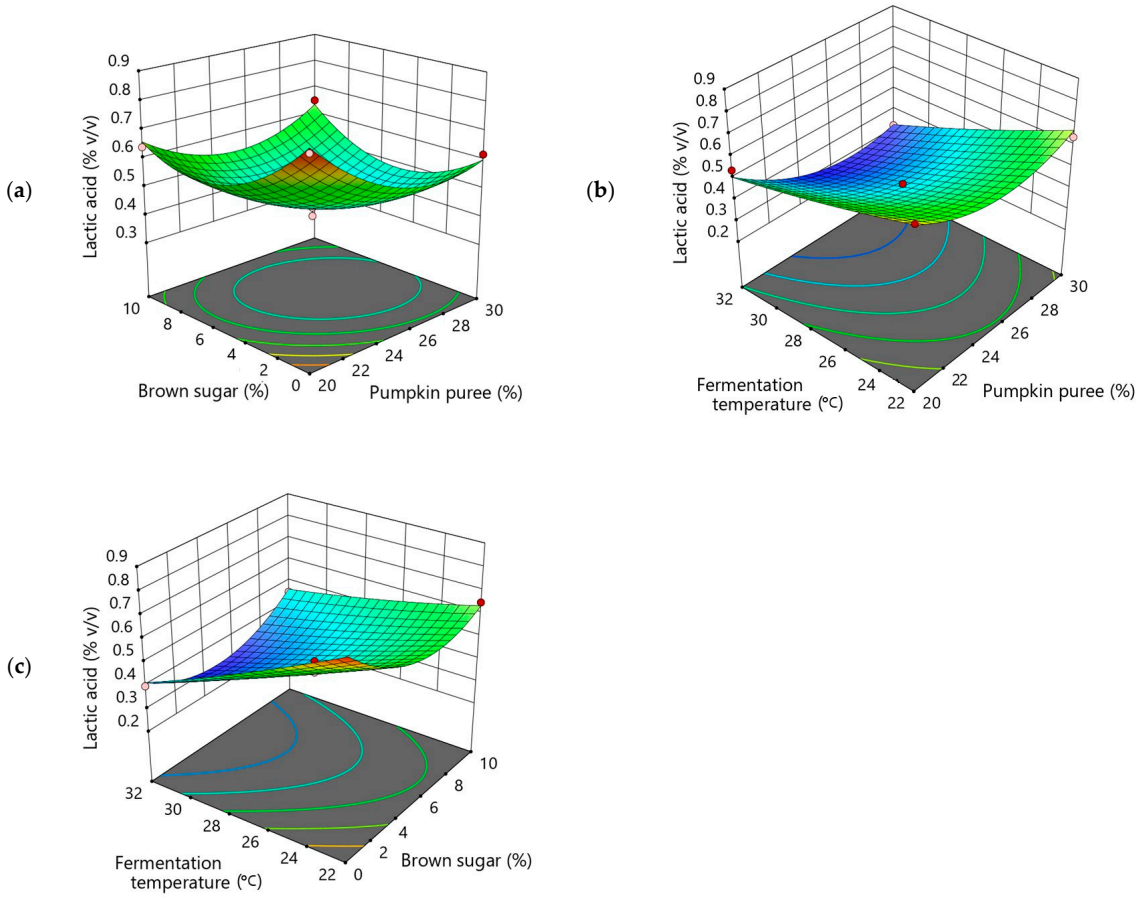

Figure 4. Response surface plots illustrating the interaction effect of (**a**) pumpkin puree and brown sugar content, (**b**) fermentation temperature and pumpkin puree content, and (**c**) fermentation temperature and brown sugar content on the lactic acid content of pumpkin-based mature coconut water kefir beverage (PWKC).

A positive interactional effect between the substrates' concentrations is shown in Figure 4a. When both the pumpkin puree and brown sugar concentrations increased, the lactic acid content of PWKC increased. At the upper limit of the pumpkin puree and brown sugar concentrations, more carbohydrates were available for *Lactobacillus* fermentation and lactic acid conversion. Pumpkin puree (rich in sucrose, glucose, and fructose [16,17]) and brown sugar (consisting mainly of sucrose [3]) are potential carbon sources for lactic acid fermentation. Dimitrovski et al. [18] also reported on the utilisation of glucose and fructose in pumpkin juice and the production of lactic acid by *Lactobacillus casei* 431. Bueno et al. [65], who fermented red pitaya and apple pulp using water kefir grains, also reported on the reduction of sucrose over the fermentation time, along with the production of lactic acid. As observed in Table 2 and Equation (5), the quadratic term of the substrates positively affected the lactic acid content in PWKC. When the concentrations of pumpkin puree and brown sugar increased from 20–26% w/v and 0–6% w/v, respectively, lactic acid content showed a slight decrease (Figure 4a–c). Further increase in the concentrations of both substrates increased the lactic acid content. The pumpkin puree and brown sugar concentrations below 6 and 26% w/v were inadequate to initiate the production of lactic acid.

As observed in Figure 1c, the lactic acid content of PWKC rose when higher concentration of brown sugar and fermentation temperature were used. At the top end of the studied range, the positive interactional effect between brown sugar concentration and fermentation temperature was more pronounced. Fermentation temperature is a crucial factor in determining the growth and fermentative activities of water kefir microorganisms [3,5]. Most of the water kefir *Lactobacilli* in PWKC grew better at a fermentation temperature at the upper limit of the studied range, and thus, when more fermentable nutrients (pumpkin puree and brown sugar) were available, *Lactobacilli* fermented at a faster rate and produced a greater amount of lactic acid. Laureys et al. [71] also found that the metabolism of certain microorganisms in the water kefir grains could be affected by the fermentation temperature and consequently reduced the lactic acid content.

3.2.5. *Lactobacillus* Count

Lactobacillus is the dominant bacterial genera in water kefir beverages [3,5]. The consumption of *Lactobacillus* has been associated with health benefits such as protective effects against pathogenic bacteria, immune response-modulating effects, allergy and cancer prevention effects, oxidative stress reduction effects, and anti-diabetic effects [72]. The count of *Lactobacilli* contained in the final fermented product at the time of consumption is crucial for determining its potential health benefits. Higher counts generally indicate a potentially more potent probiotic food product, as more bacteria are available to exert therapeutic effects on the host. The lowest and highest *Lactobacillus* counts of PWKC obtained were 2.08 Log CFU/mL (run 8: 30% w/v pumpkin puree, 5% w/v brown sugar, fermentation temperature of 32 °C) and 8.04 Log CFU/mL (run 9: 25% w/v, 0% w/v, 22 °C), respectively (Table 1). Our results are comparable to the study conducted by Ozcelik et al. [73], who obtained *Lactobacillus* spp. count varying between 5.72 and 7.94 Log CFU/mL in water kefir grain-fermented fruit juices.

Table 2 and Equation (6) show the negative linear effect ($p < 0.05$) of fermentation temperature and pumpkin puree and brown sugar concentrations on the *Lactobacillus* count. As can be seen in Figure 5b,c, fermentation temperature in its linear term dominantly affected *Lactobacillus* count. *Lactobacillus* count decreased when the fermentation temperature increased (Figure 5a,c), indicating that some of the *Lactobacillus* strains in PWKC preferred the fermentation temperature at the lower end limit of the studied range. This finding can be supported by a study conducted by Dadkhah et al. [74], who used kefir grains to ferment soy milk at 22 °C, and a high *Lactobacillus* count of 8.14 Log CFU/mL was obtained.

Brown sugar concentration in its quadratic term demonstrated a significant positive effect on *Lactobacillus* count (Figure 5a,c). *Lactobacillus* count decreased slightly when the brown sugar concentration increased from 0–6% w/v, but rose with further increase of brown sugar. Water kefir microbes prefer the sucrose in brown sugar compared to other

substrates [3]. When sucrose is adequate, water kefir grains could grow at a faster rate, as more sucrose can be converted into water kefir grain dextran exopolysaccharide by glucansucrases of *Lentilactobacillus hilgardii*, thus resulting in a higher *Lactobacillus* count [6]. Figure 1a,b shows the positive quadratic effects of pumpkin puree concentration on the *Lactobacillus* count. When the pumpkin puree concentration increased from 20 to 26% *w*/*v*, the *Lactobacillus* count decreased. However, when the pumpkin puree concentration was further increased (26–30% *w*/*v*), the *Lactobacillus* count increased. The substitution of sucrose with glucose and fructose has been reported to reduce the water kefir grain growth rate as sucrose is necessary for dextran exopolysaccharide production [10]. Brown sugar concentration and fermentation temperature demonstrated a positive interactional effect ($p < 0.05$) on the viability of *Lactobacillus* (Table 2, Equation (6)). *Lactobacillus* count increased when the concentration of brown sugar and fermentation temperature rose simultaneously (Figure 5c). This indicated that fermentation temperature and brown sugar content at the upper limit of the measured parameter favoured *Lactobacillus* growth in PWKC. When more sugar is available, *Lactobacillus* cell division will be higher, thus resulting in a higher viable count.

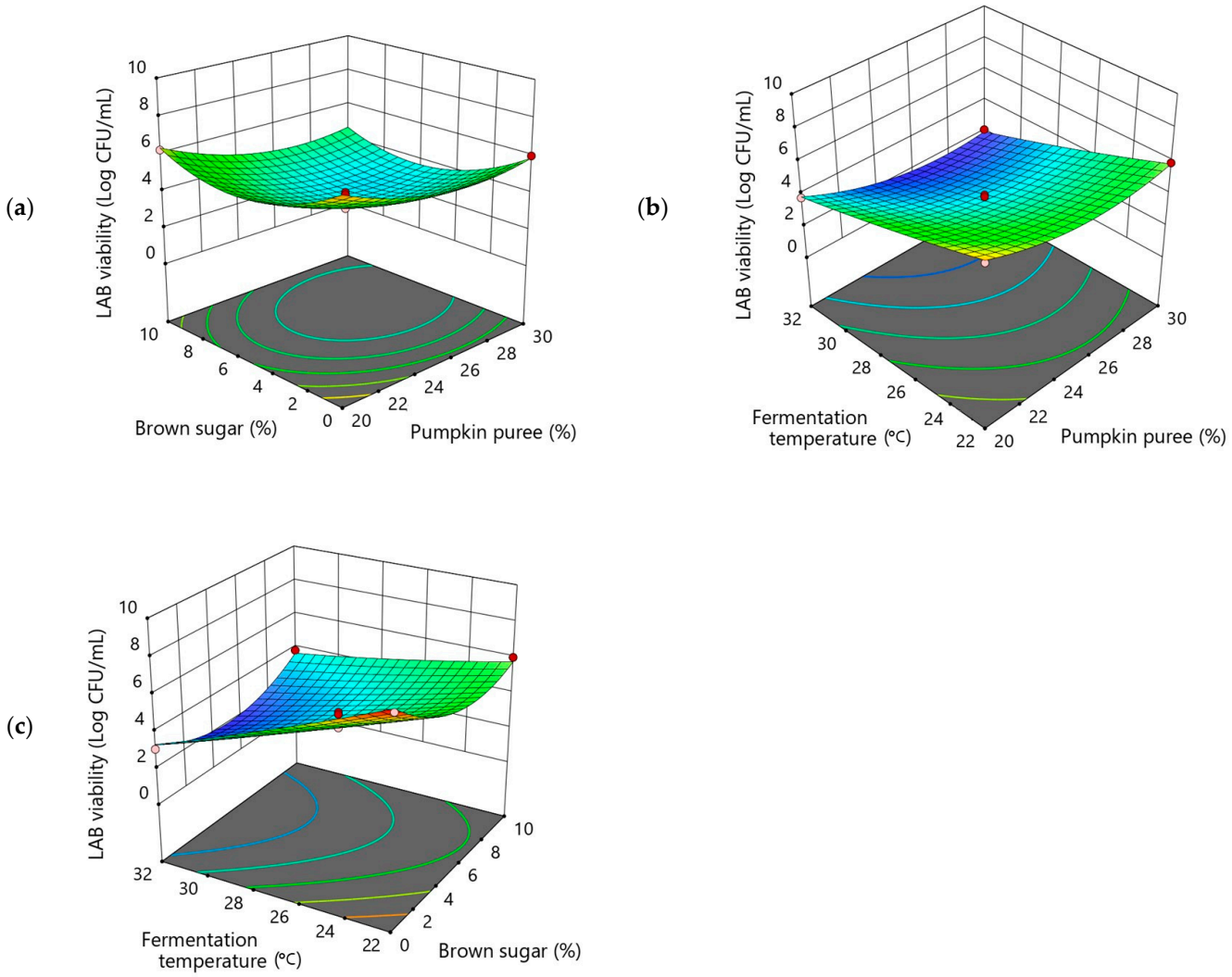

Figure 5. Response surface plots illustrating the interaction effect of (**a**) pumpkin puree and brown sugar content, (**b**) fermentation temperature and pumpkin puree content, and (**c**) fermentation temperature and brown sugar content on the *Lactobacillus* count of pumpkin-based mature coconut water kefir beverage (PWKC).

3.3. Optimisation

The optimal conditions for the $PWKC_{opt}$ obtained in the current study are X_1 = 20.00% w/v, X_2 = 9.99% w/v and X_3 = 27.19 °C. These optimal values were then rounded to 20% w/v pumpkin puree, 10% w/v brown sugar, and a 27 °C fermentation temperature within a range of 1% and 1 °C. Under these optimum conditions, all five responses of $PWKC_{opt}$ were validated by triplicate experimental runs (Table 3).

Table 3. Model predicted, experimental values and the intervals obtained from two-sided confirmation of point predicted by RSM at confidence level of 95% confidence level.

Response Variables	Goal	Predicted Mean [a]	95% PI Low [c]	Observed Value [b]	95% PI High [c]
$T_{pH4.5}$ (h)	Minimize	4.59	4.44	4.54 ± 0.06	4.74
OA (score)	Maximize	4.01	3.73	4.03 ± 0.12	4.30
Biomass growth rate (% G)	Maximize	29.68	26.92	31.00 ± 1.12	32.44
Lactic acid (% v/v)	Maximize	0.66	0.59	0.68 ± 0.03	0.73
Lactobacillus count (Log CFU/mL)	Maximize	6.31	5.32	6.41 ± 0.40	7.30

[a] Desirability for this result was 0.99. [b] Values are the means of three determinations ± standard error. [c] Prediction interval (PI). $T_{pH4.5}$: fermentation time to reach pH 4.5; OA: overall acceptability.

Overall desirability value is a measure that combines multiple response variables into a single metric and is always employed to assess the suitability of the prediction of the optimal value of input parameters corresponding to the output responses [75]. $PWKC_{opt}$ obtained in the current study possessed an overall desirability value of 0.99, which indicated that $PWKC_{opt}$ attained an ideal response value. For instance, the PWKC obtained under the optimal condition required a fermentation time of 4.54 h to reach pH 4.5, which corresponds to previous studies [52,53]. The optimal condition has resulted in a water kefir grain biomass growth rate of 31%, *Lactobacillus* count of 6.41 Log CFU/mL, and lactic acid content of 0.68% v/v, which are consistent with the result obtained in previous studies [63,68,73]. The optimal condition has optimised the water kefir grains' biomass growth, allowed the kefir microbiota to efficiently utilise the nutrients (such as carbohydrates, sugars, vitamins, and minerals) provided in mature coconut water and pumpkin puree, consequently resulting in a high *Lactobacillus* count and producing lactic acid in a concentration that would not adversely affect the sensory characteristics of the final product, PWKC, resulting in an OA score of 4.03 (on a 7-point hedonic Likert scale), which is acceptable and is comparable to other fruit- or vegetable-based kefir-like beverages [76].

3.4. Microbiological Safety Analyses

Microbiological safety analyses were performed on all 17 experimental runs of PWKC before the sensory evaluation test to ensure the absence of food-borne pathogens in the production of PWKC. There were no coliform bacteria or *E. coli* detected in all 17 PWKC samples throughout the entire storage period. These results confirmed that adequate hygienic measures were adopted during the preparation and processing of PWKC. The findings also revealed that PWKC presented a good microbial shelf stability for up to 56 days (4 °C).

Aerobic mesophilic bacteria (2.05×10^{10}–2.46×10^{10} CFU/mL) and filamentous fungi (1.00×10^{2}–1.95×10^{2} CFU/mL) were detected in all 17 PWKC samples during storage, which agreed with previous literature for kefir [77]. While the mixture of mature coconut water, pumpkin puree, and brown sugar was heat-treated (121 °C for 20 min) to reduce microbial load before fermentation, the aerobic mesophilic bacteria and filamentous fungi counts observed in PWKC were attributed to the water kefir grains. LAB in water kefir grains constitute a significant number among the aerobic mesophilic genera. Water kefir grains also contain filamentous fungi living in symbiosis [77].

3.5. Characterisation of the Optimised Pumpkin-Based Mature Coconut Water Kefir Brew (PWK_{Copt})

3.5.1. Chemical Composition

The proximate composition of $PWKC_{opt}$ is depicted in Table 4. Results revealed that the optimised beverage was 89.06% moisture, 2.81% ash, 3.26% protein, 2.75% dietary fibre, 0.14% fat, and 1.98% carbohydrates. According to the literature, pumpkin fruit consists of 91.6% moisture, 1% protein, 0.1% fat, 0.8% ash, 0.5% dietary fibre, and 6.5% carbohydrates [78], whereas mature coconut water is 95.97% moisture, 0.51% protein, 0.06% fat, 0.47% ash, 0.08% dietary fibre, and 2.91% carbohydrates [79]. When comparing the values, $PWKC_{opt}$ was lower in moisture and carbohydrate content but higher in protein, fat, ash, and dietary fibre contents than pumpkin fruit and mature coconut water. During fermentation, kefir microorganisms metabolise nutrients for growth and produce organic compounds and at the same time reduce the moisture and carbohydrate contents. The enzymatic lipolytic and proteolytic activities of kefir microorganisms promote the release of fatty acids and amino acids and subsequently increase the fat and protein contents. Kefir microorganisms could promote an increase in mineral content under certain circumstances, hence resulting in higher ash content [69,80]. The findings indicated that water kefir fermentation could enhance the nutritive values of the beverage. According to Annex of Regulation (EC) No 1924/2006 [81], a beverage product can be claimed as 'fat-free' when the beverage product contains no more than 0.15 g of fat per 100 mL. Therefore, $PWKC_{opt}$ formulated in this study can be characterised as a fat-free beverage product.

Table 4. Major constituents of the optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$).

Parameters	Values
Proximate composition (% wet bases)	
Moisture	89.06 ± 0.14
Ash	2.81 ± 0.05
Protein	3.26 ± 0.13
Dietary fibre	2.75 ± 0.07
Fat	0.14 ± 0.03
Carbohydrate	1.98 ± 0.20
Minerals (mg/L)	
Potassium (K)	2186.33 ± 1.53
Phosphorus (P)	180.67 ± 0.58
Magnesium (Mg)	207.07 ± 2.65
Calcium (Ca)	137.33 ± 2.08
Iron (Fe)	1.37 ± 0.03
Zinc (Zn)	0.23 ± 0.03
Copper (Cu)	0.56 ± 0.04
Selenium (Se)	0.47 ± 0.03
Manganese (Mn)	0.77 ± 0.03

Results are presented as mean ± standard deviation (n = 3).

Nine minerals were detected in $PWKC_{opt}$, with K being the element identified at the highest concentration (2186.33 mg/L), followed by Mg, P, Ca, Fe, Mn, Cu, Se, and Zn (Table 4). The minerals detected in $PWKC_{opt}$ matched the mineral profile of pumpkin fruit reported by USDA [78]. Mineral elements such as K, Na, Ca, Zn, Fe, and Mg were also detected in mature coconut water [24]. K is the predominant mineral in pumpkin fruit (3400 mg/L) [78] and mature coconut water (1960–2400 mg/L) [24]. Results suggested that the mineral profile of $PWKC_{opt}$ was highly influenced by the raw materials (pumpkin puree and mature coconut water) used. The differences between the mineral contents of $PWKC_{opt}$ and raw materials were attributed to the proportions used in the formulations.

3.5.2. Physicochemical Properties

The physicochemical properties (TSS, pH value, viscosity, and colour) of $PWKC_{opt}$ were evaluated (Table 5). The TSS and pH values of $PWKC_{opt}$ were 6.87 °Brix and 4.53, respectively. The TSS of $PWKC_{opt}$ was lower compared to the TSS of pumpkin puree (11.7 °Brix) [82] and mature coconut water (4.0 °Brix) [83] available in the literature. During fermentation, LAB utilised the sugars in pumpkin puree and mature coconut water to produce organic acids, thus resulting in a lower TSS [69]. According to the literature, pumpkin puree and mature coconut water have pH values of 6.30 [82] and 5.50 [83], respectively. The lower pH value of $PWKC_{opt}$ was due to the production of organic acids during the LAB fermentation [10]. Viscosity is a crucial quality characteristic of a fermented product, as it would affect sensory qualities such as flavour release and mouthfeel. $PWKC_{opt}$ had a viscosity of 54.57 cP, which is considered a mildly thick (nectar-like) consistency by the National Dysphagia Diet (NDD). The viscosity of the $PWKC_{opt}$ was comparable with the viscosities of *Lactobacillus plantarum*-fermented fruit juice (31.34–63.86 cP) prepared by Aly et al. [84]. $PWKC_{opt}$ had L*, a*, and b* values of 37.21, 2.24, and 23.83, respectively, indicating its 'yellowy-orange' colour. The colour of the $PWKC_{opt}$ could corresponded to the high total carotenoids content (33.24 mg/100 mL) detected in the $PWKC_{opt}$.

Table 5. The physicochemical properties of the optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$) during 56 days storage period (4 °C).

Physicochemical Properties		Storage Time (Day)		
		0	28	56
TSS (°Brix)		6.87 ± 0.02 [a]	6.87 ± 0.02 [a]	6.84 ± 0.04 [a]
pH		4.53 ± 0.02 [a]	4.55 ± 0.05 [a]	4.43 ± 0.12 [a]
Viscosity (cP)		54.57 ± 0.02 [a]	54.57 ± 0.02 [a]	54.53 ± 0.05 [a]
Colour	L*	37.21 ± 0.01 [a]	37.23 ± 0.12 [a]	37.10 ± 0.90 [a]
	a*	2.24 ± 0.03 [a]	2.24 ± 0.01 [a]	2.15 ± 0.13 [a]
	b*	23.83 ± 0.02 [a]	23.83 ± 0.03 [a]	23.81 ± 0.11 [a]

Results are displayed as mean ± standard deviation (n = 3). Values in a row with different superscript letters are significantly different ($p < 0.05$).

3.5.3. Antioxidative Contents and Activities

$PWKC_{opt}$ was found to have 33.24 mg/100 mL carotenoids, 89.93 mg GAE/100 mL phenols, 49.94 mg QE/100 mL flavonoids, a FRAP antioxidant power of 169.17 mM Fe (II)/100 mL, and half the maximal inhibitory concentration of DPPH free radicals of 27.17 mg/mL (Table 6). Pumpkin fruit is known to contain bioactive phenolic compounds with antioxidant capacities, particularly polyphenols, flavonoids, and carotenoids [85]. For instance, the most common phenolic acids in pumpkin are chlorogenic acid, quercetin, caffeic acid, gallic acid, p-Coumaric acid, and ferulic acid. Flavonoids such as catechin, quercetin, and narginin can also be found in the pumpkin fruit [86]. Antioxidative properties of mature coconut water have also been reported [20,87]. During fermentation, LAB release polyphenol oxidase, which could depolymerise the conjugated, complex phenolic compounds into smaller molecules, thereby increasing the antioxidant content [88,89]. Studies by Ozcelik et al. [73] and Tu et al. [88] revealed that lactic acid fermentation could help in increasing the antioxidant activities of Cornelian cherry, hawthorn, red plum, roseship, and pomegranate juices [73] and soy whey [88], respectively. Fiorda et al. [9] also found that some of the LAB strains (*Lactobacillus satsumensis* and *Leuconostoc mesenteroides*) present in kefir microbiota can scavenge reactive oxygen species.

Table 6. Antioxidant properties of the optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$).

Antioxidative Contents and Activities	Values
Total carotenoid (mg/100 mL)	33.24 ± 0.29

Table 6. *Cont.*

Antioxidative Contents and Activities	Values
Total phenolic (mg GAE/100 mL)	89.93 ± 0.35
Total flavonoid (mg QE/100 mL)	49.94 ± 0.34
FRAP (mM Fe (II)/100 mL)	169.17 ± 0.06
DPPH IC_{50} (mg/mL)	27.17 ± 0.07

Results are presented as mean ± standard deviation (n = 3). GAE: gallic acid equivalents; QE: quercetin equivalents; Fe (II): iron (II) sulphate equivalents; DPPH IC_{50}: half maximal inhibitory concentration of 2,2-diphenylpicryl hydrazine (DPPH) free radicals.

3.5.4. Sugar Content

Sucrose is the main carbon and energy source for the microorganism's growth and fermentation, whereas glucose and fructose are major carbohydrates that are mainly metabolised into organic acids and ethanol. The sucrose in $PWKC_{opt}$ is mainly from the brown sugar added initially to produce $PWKC_{opt}$. At the beginning of the water kefir fermentation, yeast hydrolysed sucrose into glucose and fructose and produced ethanol at the same time. The produced glucose and fructose are the carbon source for LAB, whereas ethanol is utilised by AAB [88]. The composition of glucose and fructose sugars in the $PWKC_{opt}$ was monitored during the fermentation process (Table 7). Prior to the fermentation process, the initial values of glucose and fructose detected in the non-fermented $PWKC_{opt}$ were 38.75 and 27.17 g/L, respectively. Both glucose and fructose contents decreased significantly during fermentation. This finding indicated that water kefir microorganisms were able to metabolise both glucose and fructose presented in the pumpkin puree and mature coconut water. The glucose consumption (22.03 g/L, 56.85% of consumption) was higher than that of fructose (12.30 g/L, 45.27% of consumption). Glucose is always the preferred substrate for water kefir microorganisms over fructose owing to its faster degradation rate [90].

Table 7. The composition of glucose and fructose sugars in the optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$) during the fermentation process.

Fermentation Time (h)	Sugar (g/L)	
	Glucose	Fructose
0	38.75 ± 0.09 ^{c}	27.17 ± 0.08 ^{c}
2.25	23.35 ± 0.05 ^{b}	19.10 ± 0.05 ^{b}
4.50	16.72 ± 0.07 ^{a}	14.87 ± 0.07 ^{a}

Results are displayed as mean ± standard deviation (n = 3). Values in a column with different superscript letters are significantly different ($p < 0.05$).

3.5.5. Organic Acids Content

Sucrose, glucose, and fructose are the major sugars present in pumpkin and mature coconut water. Probiotic strains (*Lactobacillus casei* 431, *Saccharomyces cerevisiae* D254) have been reported to metabolise these sugars through the glycolytic pathway and tricarboxylic acid cycle to produce organic acids in their respective fermented products [18,26] The four main organic acids identified in $PWKC_{opt}$ were lactic acid, acetic acid, malic acid, and tartaric acid (Table 8).

Lactic acid is one of the organic acids formed during lactic acid fermentation, which can help in inhibiting the growth of pathogenic microorganisms and preventing food spoilage [88]. Among the organic acids detected, lactic acid was the most concentrated in $PWKC_{opt}$. The lactic acid content in the $PWKC_{opt}$ increased prominently to 5.80 g/L after fermented for 2.25 h and further to 6.79 g/L when fermentation time reached 4.50 h. The high lactic acid content detected in $PWKC_{opt}$ corresponded to the high viability of LAB (8.57 Log CFU/mL) in $PWKC_{opt}$. LAB metabolised glucose and fructose in pumpkin puree and mature coconut water into lactic acid. This finding is supported by the results obtained in the previous section (sugar content) of this work, where the increment in lactic

acid content was accompanied by the decrement in glucose and fructose contents during fermentation. Lactic acid was also found to be the main fermentation end-product in other studies [65,76].

Table 8. The content of organic acids in the optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$) during the fermentation process.

Fermentation Time (h)	Organic Acid (g/L)			
	Lactic Acid	Acetic Acid	Malic Acid	Tartaric Acid
0	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.01 ^{b}	0.02 ± 0.02 ^{a}
2.25	5.80 ± 0.01 ^{a}	0.08 ± 0.02 ^{a}	0.17 ± 0.01 ^{a}	0.12 ± 0.03 ^{b}
4.50	6.79 ± 0.03 ^{b}	0.13 ± 0.01 ^{b}	0.16 ± 0.02 ^{a}	0.14 ± 0.02 ^{b}

Results are displayed as mean ± standard deviation (n = 3). Values in a column with different superscript letters are significantly different ($p < 0.05$).

Acetic acid plays an important role in producing the fruity flavour and aroma of water kefir beverages [88]. The acetic acid content in $PWKC_{opt}$ increased after fermentation, and higher ($p < 0.05$) acetic acid content was obtained from the 4.5 h-fermentation than that of 2.20 h. Increments of acetic acid could be attributed to the ethanol oxidation by acetic acid bacteria and the anaerobic degradation of lactic acid by heterofermentative LAB [5,73,88].

Malic and tartaric acids were quantified at 0.20 and 0.02 g/L, respectively, at the beginning of the fermentation. The malic and tartaric acids are the organic acids in pumpkin and coconut water [24,91,92]. Malic acid can also be produced during the fermentation of water kefir by yeast metabolism [76]. A fermentation time of 2.25 h was found to reduce the malic acid content but increase the tartaric acid content in $PWKC_{opt}$. LAB could decarboxylate malic acid into lactic acid and CO_2 and metabolise glucose to tartaric acid. Decrements in malic acid and increments in tartaric acid were also observed in the fermentation of coconut water and blueberry juice, using *Lactobacillus casei* L4 [40] and *Lactobacillus plantarum* [93], respectively.

3.5.6. Ethanol Content (EC)

Water kefir is an ethanol and carbon dioxide-containing beverage that has a 'floral' and 'fruity' taste, which are primarily attributed to the yeast sugar fermentation and alcohol dehydrogenase activity of *Lactobacillus* sp. [5]. The ethanol content (EC) in the $PWKC_{opt}$ rose remarkably ($p < 0.05$) during fermentation (Table 6). The increment of EC was accompanied by decrements in glucose and fructose contents (Table 9). Yeast cells in the beverage metabolised the available glucose and fructose into ethanol.

Table 9. The ethanol content in the optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$) during the fermentation process.

Fermentation Time (h)	Ethanol (g/L)
0	0.05 ± 0.00 ^{a}
2.25	0.51 ± 0.03 ^{b}
4.50	0.66 ± 0.02 ^{c}

Results are displayed as mean ± standard deviation (n = 3). Values in a column with different superscript letters are significantly different ($p < 0.05$).

The EC of $PWKC_{opt}$ was 0.66%, and our finding was in accordance with the EC of fruit/vegetable-based water kefir-like fermented beverages ranging between 0.1 and 5.0%, varying depending on the substrate used for the fermentation [94]. In the Association of Southeast Asian Nations (ASEAN) countries, the allowable maximum alcohol level in the final product for alcohol-containing halal food is only 1%. EC of the $PWKC_{opt}$ was less than 1%, and, hence, it can be considered a non-alcoholic beverage [95].

3.5.7. Viability of Presumptive Lactic Acid Bacteria (LAB), *Lactobacillus*, Acetic Acid Bacteria (AAB) and Yeast

Water kefir is known to contain a consortium of microorganisms which have potential health benefits [3]. As shown in Table 10, the viable counts of $PWKC_{opt}$ for LAB, AAB, and yeast are 8.57, 2.08, and 6.29 Log CFU/mL, respectively. Therefore, the $PWKC_{opt}$ complied with the established minimum viable counts (10^7 and 10^4 CFU/mL of viable microorganisms and yeast, respectively) of the Codex definition for kefir and can be characterised as a kefir beverage [96].

Table 10. The microbiological properties of optimised fermented pumpkin-based mature coconut water kefir beverage ($PWKC_{opt}$) during 56 days storage period (4 °C).

Microorganisms' Viability (Log CFU/mL)	Storage Time (Day)		
	0	28	56
Lactic acid bacteria	8.57 ± 0.06 [a]	8.54 ± 0.36 [a]	8.98 ± 0.56 [a]
Lactobacillus	6.41 ± 0.40 [a]	6.65 ± 0.58 [a]	6.75 ± 0.59 [a]
Acetic acid bacteria	2.08 ± 0.08 [a]	2.08 ± 0.06 [a]	2.03 ± 0.32 [a]
Yeast	6.29 ± 0.08 [a]	6.36 ± 0.23 [a]	6.52 ± 0.42 [a]

Results are displayed as mean ± standard deviation (n = 3). Values in a row with different superscript letters are significantly different ($p < 0.05$).

As shown in Table 10, the viable count of the $PWKC_{opt}$ for *Lactobacillus* sp. is 6.41 Log CFU/mL, demonstrating that *Lactobacillus* sp. is the dominant LAB species in the $PWKC_{opt}$. Our finding agreed with the literature, in which LAB in water kefir is dominantly *Lactobacillus* [5]. *Lactobacillus* sp. has the potential to modulate host immunity and inhibit the growth of foodborne pathogenic bacteria [10]. Ozcelik et al. [73] have reported similar results, where *Lactobacillus* sp. is also the predominant LAB species in fermented fruit juices after the kefir fermentation process. The other LAB in water kefir includes *Lactococcus* and *Leuconostoc* [5].

3.5.8. Sensory Evaluation

The sensorial quality of a probiotic product is of crucial importance because it has a collective role in influencing the consumers' preference for the product as well as their purchasing behaviour. The metabolism of probiotic bacteria has been reported to negatively affect the sensory characteristics of the product. Despite the potential health benefits, consumers are unwilling to compromise on the taste of probiotic foods [97]. Therefore, sensory evaluation was performed on the optimised beverage in this study. The hedonic mean scores for appearance (colour), odour, taste (sourness), texture (consistency), and overall acceptability of the optimised beverage are shown in Figure 6. In general, the overall acceptability of sensory panellists on $PWKC_{opt}$ was acceptable (4.03 out of a 7-point hedonic Likert scale). The highest score was found for the appearance (colour) attribute (4.27), indicating that the colour of $PWKC_{opt}$ was the main quality attribute, which influenced the overall acceptance. The sensory panellists gave the lowest score for odour (3.93) among other sensory attributes, indicating that the panellists neither liked nor disliked the sour odour of $PWKC_{opt}$. Overall, despite the sour odour, $PWKC_{opt}$ was well accepted by the panellists. It is common for a fermented beverage to have a sour odour due to the formation of fermentation by-products.

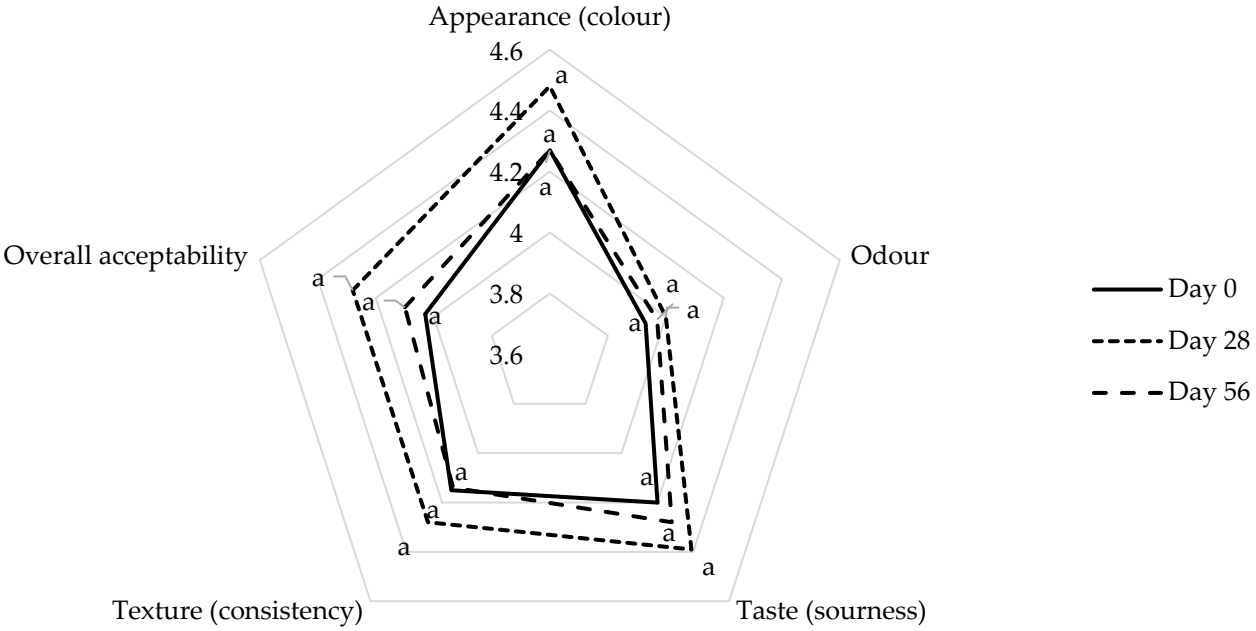

Figure 6. The sensory properties of optimised fermented pumpkin-based mature coconut water kefir beverage during 56 days storage period (4 °C). Results are displayed as mean ± standard deviation (n = 50). Similar superscript letters represent no significant difference ($p > 0.05$) between the attributes of different storage day.

3.5.9. Shelf-Life Study

The shelf-life of $PWKC_{opt}$ in terms of physicochemical (TSS, pH, viscosity, and colour), microbiological (LAB, *Lactobacillus*, AAB, and yeast), and sensorial properties, was conducted on days 0, 28, and 56 during refrigerated storage at 4 °C. In general, the changes of $PWKC_{opt}$ in its physicochemical (Table 5), microbiological (Table 10), and sensorial (Figure 2) properties during refrigerated storage were not statistically significant ($p > 0.05$).

Both the TSS and pH values of $PWKC_{opt}$ were observed to remain constant (TSS: day 0 = 6.87 ± 0.02 °Brix, day 28 = 6.87 ± 0.02 °Brix, day 56 = 6.84 ± 0.04 °Brix, $p > 0.05$; pH value: day 0 = 4.53 ± 0.02, day 28 = 4.55 ± 0.05, day 56 = 4.43 ± 0.012, $p > 0.05$) throughout the storage period (4 °C, 56 days). Results demonstrated that the lactic culture (*Lactobacillus* sp. K8) in $PWKC_{opt}$ could have become metabolically inactive during the refrigerated storage. A low temperature of 4 °C can reduce the cellular fluidity, subsequently, decrease the metabolic rate, and induce the dormant state in most of the probiotic cells [98]. Acevedo-Martínez et al. [52] also reported on the metabolic inactivity of the probiotic strain (*Lactobacillus casei*) in fermented mango juice stored at 4 °C. The TSS and sugar contents of the fermented mango juice were maintained over the 4 weeks of refrigerated storage [52]. The viscosity of $PWKC_{opt}$ ranged from 54.57 ± 0.01 (day 0) to 54.57 ± 0.02 (day 28) and 54.53 ± 0.05 cP (day 56) during the storage. The high stability in the viscosity of the beverage could be attributed to the water-soluble pumpkin pectinic polysaccharides consisting of glucose, rhamnose, arabinose, galactose, and galacturonic acid [15,99]. The polymeric properties of the polysaccharides created a repulsive force counterbalancing attractive van der Waals forces acting on a particle approaching another particle, stabilizing the colloid of liquid, and hence maintaining the viscosity of the beverage [100]. No difference was found in the colour (L*: day 0 = 37.21 ± 0.01, day 28 = 37.23 ± 0.12, day 56 = 37.10 ± 0.90, $p > 0.05$; a*: day 0 = 2.24 ± 0.03, day 28 = 2.24 ± 0.01, day 56 = 2.15 ± 0.13, $p > 0.05$; b*: day 0 = 23.83 ± 0.02, day 28 = 23.83 ± 0.03, day 56 = 23.81 ± 0.11, $p > 0.05$) of $PWKC_{opt}$ during the refrigerated storage time. The result indicated that the natural pigments and polyphenols in $PWKC_{opt}$ were well preserved. This might be attributed to the *Lactobacillus*'s

ability to entrap residual oxygen in the beverage, which in turn prevents the occurrence of the auto-oxidation process that is responsible for the colour degradation [89].

According to Codex Alimentarius, for a product to be considered kefir, it should contain at least 10^7 CFU/mL of viable microorganisms and 10^4 CFU/mL of viable yeast [96]. The viability of LAB (8.57 ± 0.06–8.98 ± 0.56 Log CFU/mL), *Lactobacillus* (6.41 ± 0.40–6.75 ± 0.59 Log CFU/mL), AAB (2.08 ± 0.08–2.03 ± 0.32 Log CFU/mL), and yeast (6.29 ± 0.08–6.52 ± 0.42 Log CFU/mL) remained stable over 56 days of storage. The preservation of high count of *Lactobacillus*, LAB, AAB, and yeast in $PWKC_{opt}$ during storage was thought to be attributed to the availability of nutrients in pumpkin puree. The nutrients available in pumpkin purees, such as dietary fibre, vitamins, minerals, and antioxidants sustained the growth and viability of the water kefir microorganisms in $PWKC_{opt}$ [15]. Similar to the shelf-life study by Genevois et al. [101], it was found that the viability of *Lactobacillus casei* supplemented with powdered pumpkin peel and pulp was constant for 21 days of storage at 8 and 22 °C with counts of more than 10^6 CFU/g. At the end of the storage, $PWKC_{opt}$ contained 8.98 ± 0.56 Log CFU/mL LAB and 6.52 ± 0.42 Log CFU/mL yeast, which complied with the minimum level of total viable counts of microorganisms and yeast in kefir [96]. In addition, according to FAO/WHO [1], a minimum viable probiotics dose of 10^7 CFU/mL in probiotic food at the time of consumption could exert health benefits to the host. Therefore, $PWKC_{opt}$ could confer probiotic effects to the host with a shelf-life of at least 56 days.

After 56 days of storage at 4 °C, panellists did not detect any significant or undesirable changes ($p > 0.05$) in the sensorial properties of $PWKC_{opt}$ during storage (Figure 2). The sensory deterioration of probiotic-fermented products is always correlated with the proteolytic activity of bacteria, high acid production, and degradation of fat and protein. Therefore, the maintenance of sensory profiles indicated that $PWKC_{opt}$ possessed a desirable physicochemical characterisation and considerable proteolysis pattern, which is an acceptable and stable product [102].

3.6. Identification of Selected Isolate

The 16S rRNA gene sequences of dominant cultivable species isolates in $PWKC_{opt}$ was perceived to be a close relative to *Lactobacillus mali*, *Lactobacillus satsumensis*, and *Lactobacillus hordei* (96% similarity) under the accession number of NR_112691.1, LC311746.1, and NR_044394.1, respectively (Table 11). This result proposed that dominant cultivable species isolates belong to the genus *Lactobacillus*, supported by similar phenotypic characteristics to *Lactobacillus* species.

Table 11. Closely-related relatives of dominant cultivable species isolates in the optimised fermented pumpkin-based mature coconut water kefir ($PWKC_{opt}$) were determined based on almost complete 16S rRNA gene sequence.

Closely-Related Relatives (Type Strains)	Max Score	Query Cover	E Value	Similarity
Lactobacillus satsumensis	2215	94%	0	96%
Lactobacillus mali	2215	94%	0	96%
Lactobacillus hordei	2207	92%	0	96%
Lactobacillus oeni	2189	95%	0	95%
Lactobacillus uvarum	2141	94%	0	95%
Lactobacillus aquaticus	2141	94%	0	95%
Lactobacillus sucicola	2130	95%	0	94%
Lactobacillus capillatus	2126	95%	0	94%
Lactobacillus mobilis	2098	93%	0	94%
Lactobacillus vini	2098	93%	0	94%

4. Conclusions

Developing a kefir beverage using non-dairy substrates such as mature coconut water and pumpkin puree is a novel approach for probiotic beverage production. This study

was conducted to determine the optimal fermentation conditions (fermentation temperature, concentrations of pumpkin puree and brown sugar) to minimize fermentation time (to reach pH 4.5) while maximising the overall acceptability (OA) score, water kefir grains' biomass growth rate, lactic acid content, and *Lactobacillus* count of the fermented pumpkin-based mature coconut water kefir. The optimised fermentation condition included a fermentation temperature of 27 °C, 20% *w*/*v* of pumpkin puree, and 10% *w*/*v* of brown sugar, and optimised kefir drink ($PWKC_{opt}$) obtained had an overall acceptability (OA) score of 4.03, 6.41 Log CFU/mL of *Lactobacillus* count, 0.68% *v*/*v* lactic acid content, 31% of water kefir grains' biomass growth rate, and fermentation time (to reach pH 4.5) of 4.5 h. The fermentation process had a positive effect on the nutritional value, physicochemical, antioxidative, and microbiological properties of the beverage, including increases in protein, dietary fibre, minerals, antioxidant activities, organic acid content, and viable count of probiotic microorganisms. $PWKC_{opt}$ is stable for at least 56 days when stored under refrigerated conditions (4 °C). Therefore, $PWKC_{opt}$ has the potential to function as a value-added probiotic beverage product.

However, there are some limitations in this work that should be acknowledged. First, the viabilities of *Lactobacillus*, LAB, acetic acid bacteria, and yeast reported in this work reflected only their estimated counts. Future research should identify and confirm the counts through characterisation tests such as microscopic examination, catalase test, and Gram stain test.

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