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The Effect of Refrigerated Storage on Anti-Diabetic and Antioxidant Potency of Probiotic Yogurt Treated with Some Medicinal Plants

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Abstract: This research aimed to evaluate the effect of the inclusion of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), and *Psidium guajava* (PG) water extracts in yogurt (Y) on phenolic antioxidant-linked α-amylase and α -glucosidase inhibitory activities. Four types of herbal yogurt (CP-Y, IV-Y, LB-Y, and PG-Y) and plain-Y (control) were prepared and stored in disposable plastic containers at 4 °C for 28 days. All samples were analysed for peptide concentration using *O*-phthaldialdehyde, total phenolic content (TPC), 1,1-Diphenyl-2-Picrylhydrazyl radical scavenging activity, and α-amylase and α-glucosidase inhibitory activities (IC_{50}). LB-Y showed the highest peptide concentration and TPC ($p < 0.05$) among all the yogurts during storage. IV-Y showed the highest ($p < 0.05$) radical scavenging activity among all herbal yogurts. The best α-amylase and α-glucosidase inhibitory activity (IC_{50}) for all herbal yogurt was on days 7 and 14 of storage. In conclusion, all herbal yogurts could be considered as a potential functional food with antioxidant and anti-diabetic properties.

Keywords: herbal yogurt; peptide concentration; antioxidant; α-amylase; α-glucosidase

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1. Introduction

Diabetes mellitus (DM) is a growing global health crisis that has increased about 5-fold in the last 10 years, reaching 135 million today and an estimated 300 million by 2025 [1]. DM has been shown to be associated with cardiovascular disease, obesity, and microvascular damage, which eventually leads to the failure of the eyes, kidneys, and nerves [2]. Postprandial hyperglycaemia is a common symptom of type 2 diabetes and is associated with an abnormal rise in blood sugar levels following a meal [3]. This condition is caused when there is inadequate production of insulin or when the body's cells do not respond to the insulin that is produced. As a result, the body fails to convert glucose into energy, and the blood sugar levels rise [3]. Therefore, medication combined with dietary restrictions and exercise is the best strategy for controlling postprandial plasma glucose levels.

The primary source of blood glucose is dietary carbohydrates i.e., starch, which is hydrolysed by α-glucosidases and pancreatic α-amylase [4]. This hydrolysis produces glucose absorbed by the small intestine, thus contributing to the increase in blood glucose. Therefore, management of Type II diabetes can be accomplished by inhibiting the hydrolyzation of carbohydrates by α-glucosidase and α-amylase, which delays the absorption of glucose in the digestive tract. In addition, inhibiting these enzymes not only causes a delay in carbohydrate digestion, but also prolongs overall carbohydrate digestion time, resulting in a reduction in glucose absorption and, therefore, slowing the increase in blood glucose levels after a meal [5].

The majority of medications used to treat type 2 diabetes mellitus (DM) work by reducing the digestion and absorption of the carbohydrates and sugars that cause postprandial hyperglycaemia [6]. These medications primarily function by lowering the activity of α-glucosidases, pancreatic α-amylases, and intestinal disaccharidases, such as sucrase and maltase [6]. However, the side effects of antidiabetic medications such as acarbose and miglitol, which include weight gain, hypoglycaemia, stomach pain, lactic acidosis, and gastrointestinal disturbances, lower their compliance rates and thus their effectiveness [6]. Hence, finding alternative treatments for diabetes is necessary [7].

Free radicals are formed when glucose is auto-oxidized at high levels [8]. These free radicals are responsible for the oxidative damage to DNA, fats, and proteins. Phytochemicals are bioactive components derived from medicinal plants that can lower oxidative stress and modify detrimental biological pathways, therefore ameliorating various chronic disorders [9]. In addition, herbs, spices, and flavonoid-rich foods have therapeutic properties, including antidiabetic and antioxidant activities, and they promote good health without causing any negative side effects [6,7].

Probiotics are "live microbial feed supplements that when administered in adequate amounts, confer a health benefit on the host by improving its intestinal microbial balance" [10]. A heterogeneous group of probiotic bacteria (rods and cocci) is commonly used to produce organic acids during food fermentation, which is known as lactic acid bacteria (LAB; [11]). LAB consists of several genera, including Lactobacillus, Bifidobacterium, Streptococcus, and Enterococcus [12]. These LAB strains have different beneficial effects on the host and have been used for decades in foods for human well-being [5].

The food industry has expanded over the past few decades in response to the rapid shifting of consumers' needs. Consumers of all ages value the health benefits of probiotic foods, which include modification of the immune system, reduction in cholesterol, alleviation from lactose intolerance, faster relief from diarrhoea, restoration of a healthy vaginal microbiota, lowered blood pressure, and lowered glucose levels [9,10]. Fermented milk products such as yogurt contain a variety of nutritional benefits to the human diet [13]. Yogurt's nutritional value comes from the nutrients in milk and the byproducts of probiotic fermentation. Milk proteins released by proteolysis may serve as an important source of a range of bioactive peptides encrypted within the sequence of the native proteins [14]. These bioactive peptides are able to confer a variety of important nutritional and therapeutic benefits to consumers, which include antidiabetic and antioxidant activities [5,14]. Therefore, the objective of this research was to evaluate the effect of the inclusion of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), and *Psidium guajava* (PG) water extracts in yogurt on phenolic antioxidant-linked α-amylase and α-glucosidase inhibitory activities during 28 days of storage. In this paper, we propose a new perspective on CP, IV, LB, and PG yogurt products as complementary strategies to help manage type 2 diabetes.

2. Materials and Methods

2.1. Herbal Water Extract

Illicium verum (leaves), *Psidium guajava* (leaves), *Codonopsis pilosula* (root), and *Lycium barbarum* (fruit) were purchased in dried form from a local Chinese medicinal shop. The dried leaves (IV and PG), root (CP), and fruit (LB) were ground to powder form, placed in an airtight container, and stored at room temperature away from direct sunlight. Each powder (20 g) was homogenized individually in sterile distilled water using a homogenizer, and the volume of up to 200 mL was prepared [13]. The mixture was left incubated overnight in a water bath (70 °C) and then it underwent centrifugation (15 min, 2000 rpm at 4 °C), and the supernatant was used as water herbal extract in the experiment.

2.2. Preparation of Starter Culture

Fresh and pasteurized full cream cow's milk (1 L) was heated up to 41 °C in a 2000 mL beaker. One packet of premix starter culture (Chris-Hansen, Denmark®) consisting of *Lactobacillus acidophilus* LA-5, *Bifidobacterium bifidum* Bb-12, *L. casei* LC-01 and *Streptococcus*

thermophilus Th-4 in a ratio of 4:4:1:1 was added into the milk [15]. This was followed by the addition of one capsule of probiotic mix (BIO-Life Advanced Multi Blend Probiotix®) containing *L. bulgaricus*, *L. rhamnosus*, *B. infantis*, and *B. longum* in a ratio of 1:1:1:1. The starter culture was stirred thoroughly in the milk and incubated overnight at 41 °C.

2.3. Preparation of Yogurt

Four types of herbal yogurt—*C. pilosula* (CP), *I. verum* (IV), *L. barbarum* (LB), and *P. guajava* (PG) yogurts—were prepared. Each type of yogurt was made by the addition of herbal water extract (100 mL) into 850 mL milk containing 50 mL of starter culture [9]. Full cream milk powder (20.0 g) was then added to correct the milk's solid content. The mixture was mixed thoroughly and incubated at 41 °C. All herbal yogurts were incubated at 41 °C in an incubator, and the pH of the yogurts was measured every 30 min until the pH was reduced to 4.5 before being aliquoted into sterile disposable plastic containers and stored at 4 °C for 28 days. Plain yogurt (control) was prepared in a similar procedure, except that the herbal water extract was replaced with distilled water.

2.4. Preparation of Water-Soluble Extract from Herbal Yogurt

Herbal yogurt (100 g) was homogenized with 25.0 mL of sterile distilled water [9]. The herbal yogurt pH was determined, and the pH was acidified to 4.0 with hydrochloric acid (HCl). Coagulation of protein was allowed to occur at 45 °C for 10 min prior to centrifugation (5000× *g*, 10 min at 4 °C) to separate the supernatant from the precipitated proteins. The supernatant was removed and neutralized at pH 7.0 using NaOH (0.5 M). The supernatant was centrifuged again (5000× *g*, 10 min at 4 °C) to remove residual proteins. The water extract was ready to use. The excess supernatants were stored at −20 °C refrigerator.

2.5. O-Phthaldialdehyde (OPA) Assay

In this experiment, 30 µL of a water-soluble extract of the yogurt was added to the cuvette containing 1 mL of freshly prepared OPA reagent [5]. The cuvette was sealed with parafilm and inverted twice. Reading was taken after 2 min at 340 nm. A standard curve of peptide concentration was plotted using tryptone with various concentrations (0.25–1.50 µg/mL).

2.6. Total Phenolic Content (TPC) Assay

The TPC was determined by a method adopted from Shori, [16]. Briefly, 1 mL of each water-soluble extract of the yogurt was shifted to a test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. Then, 0.5 mL of 50% (*v*/*v*) Folin–Ciocalteu reagent was added to each sample and mixed well. After 5 min, 1 mL of 5% Na_2CO_3 was added to the mixture and left to stand for 60 min. The absorbance was taken at 725 nm. The absorbance values were converted to TPC and were expressed in microgram equivalents of gallic acid per millilitre of the sample. Standard curves were established using various concentrations of gallic acid (5–60 µg/mL).

2.7. Antioxidant Activity by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical-Scavenging Activity Assay

To 3 mL of 60 mM DPPH in ethanol, 250 µL of each water-soluble extract of the yogurt was added; the absorbance was monitored at 517 nm after 1 h [5]. The readings were compared with the controls, which contained 250 mL of ethanol instead of the extract. The % inhibition was calculated by the following formula:

$$\text{Inhibition\%} = \frac{\text{Absorbance of control} - \text{Absorbance of extracts}}{\text{Absorbance of control}} \times 100 \quad (1)$$

2.8. Alpha-Amylase Inhibition Assay

Water-soluble extract of the yogurt (500 µL) and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9) with 0.006 M sodium chloride containing 0.5 mg/mL α-amylase solution was incubated at 37 °C for 10 min [5]. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) with 0.006 M sodium chloride was added to each tube at time intervals. The reaction mixtures were then incubated at 37 °C for 10 min. The reaction was stopped with 1.0 mL of dinitrosalicylic acid (DNSA) colour reagent. The test tubes were then incubated in a boiling water bath for 5 min. Subsequently, 1.0 mL of 18.2% tartrate solution was added to each tube after the boiling session before cooling at room temperature. The reaction mixture was then diluted after adding 10 mL of dH_2O. Absorbance was measured at 540 nm using a spectrophotometer (Genesys 10 UV, Thermo Electron Corporation, Waltham, MA, USA). The control was prepared using the same process, except the extract was replaced with 500 µL of buffer solution.

The formula to calculate enzyme inhibition is stated as below:

$$\text{Inhibition\%} = \frac{\text{Absorbance of control} - \text{Absorbance of extracts}}{\text{Absorbance of control}} \times 100 \quad (2)$$

The 50% inhibition value (IC_{50}) for the yogurt extracts used to inhibit α-amylase enzyme activity was obtained by plotting a graph of the percentage of inhibition against three different dosages of yogurt extracts. Besides 500 µL, the other two dosages were 250 µL and 125 µL with 250 µL and 375 µL buffer, respectively.

2.9. Alpha-Glucosidase Inhibition Assay

The analysis of α-glucosidase inhibition was conducted as described by Zahid et al. [17]. Water-soluble extract of the yogurt (500 µL) was mixed with 1000 µL of 0.1 M potassium phosphate buffer (pH 6.90) containing α-glucosidase solution (1.0 U/mL) and incubated in a water bath at 37 °C for 10 min. Then, 500 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M of potassium phosphate buffer (pH 6.90) was added to each cuvette at timed intervals. The reaction mixtures were incubated at 37 °C for 5 min. Before and after incubation, absorbance readings were recorded at 405 nm by a spectrometer. The results were compared to the control that contained 500 µL of buffer solution instead of the extract. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\text{Inhibition\%} = \frac{\text{Absorbance of control} - \text{Absorbance of extracts}}{\text{Absorbance of control}} \times 100 \quad (3)$$

The 50% inhibition value (IC_{50}) for the yogurt extracts used to inhibit α- glucosidase enzyme activity was obtained by plotting a graph of the percentage of inhibition against three different dosages of yogurt extracts. Besides 500 µL, the other two dosages were 250 µL and 125 µL with 250 µL and 375 µL buffer, respectively.

2.10. Statistical Analysis

All data were expressed as mean ± standard deviation. Three experimental batches were prepared in duplicate (n = 3 × 2). Data were analysed using one-way ANOVA using SPSS 14.0. Duncan's new multiple-range and descriptive test was applied to evaluate differences between means. In addition, the difference was deemed significant at the level of $p < 0.05$.

3. Results and Discussion

3.1. Peptides Concentration in Yogurt

The peptide concentration in the control yogurt ranged from 26 to 31 µg/g, with the highest value shown on day 7 (Figure 1). Furthermore, peptide concentration increased significantly ($p < 0.05$) only on day 7 for the plain and herbal yogurt. LB-Y showed the highest peptide concentration (54–67 µg/g; $p < 0.05$) among all other yogurts during the

storage, with the highest values found after one week of storage. CP-Y, IV-Y, and PG-Y showed a similar peptide concentration ($p > 0.05$) ranging from 27 to 38 µg/g during the 28 days.

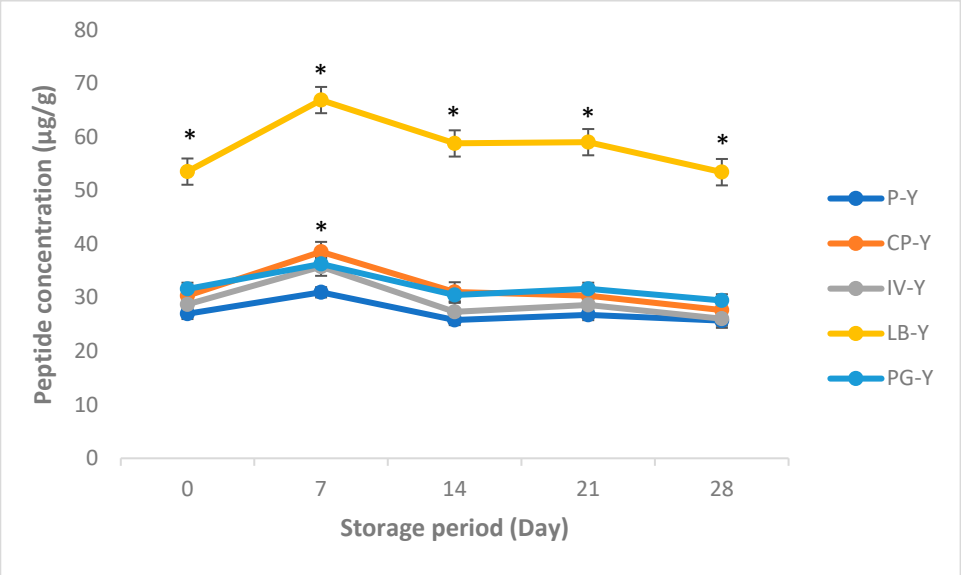

Figure 1. Changes in peptide concentration (µg/g) of yogurt (Y) enriched with water extract of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), or *Psidium guajava* during 28 days of refrigerated storage at 4 °C. Data are presented as mean ± SEM. * $p < 0.05$ as compared to control (P-Y).

Yogurt bacteria produce primary enzymes such as proteinase and peptidase to degrade milk proteins to bioactive peptides and amino acids during proteolytic activity [18,19]. Food-derived peptides that have a physiological impact on the body and have a nutritional value are referred to as biologically active or functional peptides. Milk proteins are potential sources of many biologically active peptides [20,21]. These bioactive peptides are inactive within the original protein, but once released, they act as regulatory compounds with a hormone-like activity that is based on the inherent amino acid composition and sequence [20,21]. Milk proteolysis from lactic acid bacteria (LAB) can produce a variety of bioactive peptides that may have therapeutic benefits, including antioxidant and anti-diabetic properties [19]. In the present study, the high peptide concentrations in the yogurt indicate a high rate of the proteolytic activity of LAB such as *Lactobacillus* spp. and *S. thermophilus* [22]. Further study is needed to investigate the viability of LAB in yogurt samples during refrigerated storage. LB-Y showed the highest peptide amount among all the yogurts during the 28 days of storage. This indicated that the addition of *L. barbarum* had the best effects in enhancing the proteolytic activity of the LAB in the yogurt. The overall result was in agreement with previous studies [23]. A previous study reported that the growth and viability of LAB in the presence of some medicinal plants such as coriander, cumin seeds, neem, garlic, cinnamon, nutmeg, and black and white pepper enhanced the proteolytic activity of LAB in yogurt [3,5,13,24,25]. In addition, the presence of phytomix containing a mixture of *Lycium barbarum*, *Momordica grosvenori*, *Psidium guajava*, and *Garcinia mangostana* improved the peptide content in yogurt [26]. A consistent decrease in peptide amount after two weeks might be due to the decrease in the viability of LAB. This occurs because of the high waste products produced by LAB during their growth [27].

3.2. Total Phenolic Content (TPC) and Radical Scavenging Activity of Yogurt

Control yogurt showed TPC between 16.5 ± 0.2 and 25.28 ± 0.14 µg GAE/g during the 28 days (Figure 2). All four types of herbal yogurt showed higher ($p < 0.05$) TPC than the control during the 28 days of storage, except for the 28-day-old IV yogurt (Figure 2).

No significant differences ($p > 0.05$) in TPC between both CP-Y and IV-Y (22–30 µg GAE/g) during 28 days of storage. TPC in LB-Y increased from 37.31 ± 0.16 to 55.34 ± 0.10 µg GAE/g during 28 days which was the highest value ($p < 0.05$) among all herbal yogurts. The TPC of PG-Y did not significantly change during storage (31–33 g GAE/g).

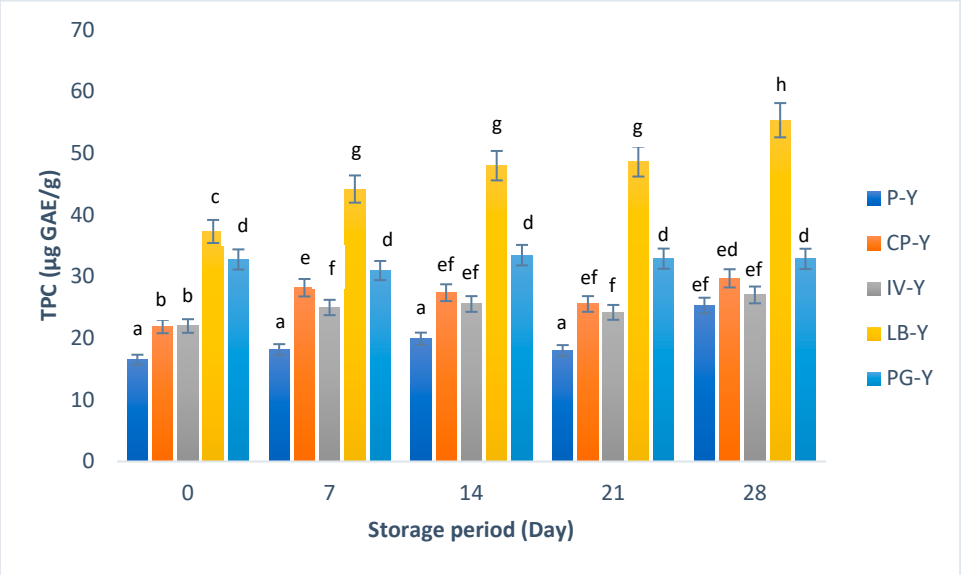

Figure 2. Changes in total phenolic content (TPC; µg GAE/g) of yogurt (Y) enriched with water extract of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), or *Psidium guajava* during 28 days of refrigerated storage at 4 °C. Data are presented as mean ± SEM. The abcdefgh means with different superscript letters indicate the level of significance at $p < 0.05$ compared to control (plain-yogurt; P-Y) and other treatments throughout the storage.

Radical scavenging activity of control yogurt ranged from 31% to 34% during the 28 days of storage (Figure 3). Yogurt treated with CP and PG decreased ($p < 0.05$) radical scavenging activity from 51.7 ± 1.7% and 57.23 ± 1.5% to 37.02 ± 1.4% and 41.7 ± 1.14%, respectively, during the 28 days of storage. IV-Y showed the highest ($p < 0.05$) radical scavenging activity (58−61%) among all herbal yogurts during the 4 weeks (Figure 3). LB-Y exhibited a significant increase in the radical scavenging activity (35%–41%) over a span of two weeks, followed by a decrease ($p < 0.05$) to 33% on day 28.

Phenolic phytochemicals are good dietary sources of natural antioxidants. These phenolic compounds that plants produce to safeguard themselves from biological and environmental challenges have positive impacts on human health [28]. Mustafa et al. [29] reported that phenolic compounds exhibit redox characteristics that enable them to function as reducing agents, hydrogen donators, and singlet oxygen quenchers. Therefore, determining the antioxidant activity of the plant is significantly influenced by the redox potential of phenolic compounds.

Antioxidant components are microconstituents found in food and play a role in scavenging free radicals and postponing or preventing lipid oxidation [30]. This is accomplished by preventing the beginning or propagation of oxidative chain reactions. Recently, antioxidant substances have become increasingly popular, particularly those that can prevent the deleterious effects of free radicals in the human body and prevent the degradation of fats and other nutrients in foods [31]. Antioxidants from natural sources are preferred over those from synthetic sources. Thus, there is a strong correlation between antioxidant potential and the nutrients found in foods, fruits, vegetables, and grains [32,33].

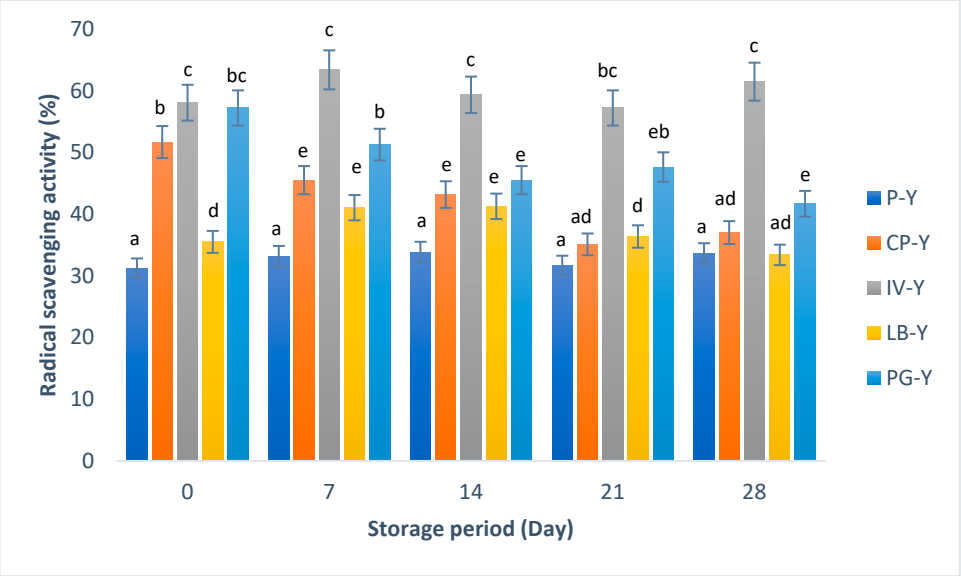

Figure 3. Changes in radical scavenging activity (%) of yogurt (Y) enriched with water extract of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), or *Psidium guajava* during 28 days of refrigerated storage at 4 °C. Data are presented as mean ± SEM. The abcde means with different superscript letters indicate the level of significance at $p < 0.05$ compared to control (plain-yogurt; P-Y) and other treatments throughout the storage.

In the present study, the elevation in TPC for all four types of herbal yogurt could be due to phytochemicals such as phenols, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids [6]. In addition, LB-Y had the highest TPC during the 28 days of storage. This might be due to the breakdown of phenolic compounds from the high-phytochemical ingredients present in the fruits of *L. barbarum*. In addition, the increase in TPC in LB-Y from 37.31 to 55.34 µg GAE/g during the 28 days could be attributed to the high degradation of phenols by LAB during storage. Several studies have proven that phenols are degraded by LAB, which leads to increased TPC [26]. *L. barbarum* is rich in antioxidants and phytochemical compounds such as beta-carotene, zeaxanthin, beta-cryptoxanthin, lutein, lycopene linoleic, palmitic, and oleic acids [33,34]. These compounds counter free radical damage to cells, which maybe prolong shelf life [23]. A strong correlation has been shown between dietary antioxidant consumption and glycaemic index and the prevention of diabetes [35]. In this study, IV-Y had the highest ($p < 0.05$) radical scavenging activity among all herbal yogurts during the 4 weeks. This could be due to the presence of phytochemicals such as kaempferol, cinnamaldehyde, and eugenol in *I. verum* [36]. Padmashree et al. [37] found that eugenol in *I. verum* is a compound with high antioxidant properties and is able to act as a reducing agent, hydrogen donor, and singlet oxygen quencher. The radical scavenging activity in IV-Y was higher than in the previously reported study on the antioxidant activity of phytomix yogurt containing a mixture of *L. barbarum*, *M. grosvenori*, *P. guajava*, and *G. mangostana* [26]. In addition, higher antioxidant activity in herbal yogurts might be due to their bioactive peptides and amino acids with antioxidant properties [3]. It was found that there was a weak correlation between the radical scavenging activity and the peptide concentrations of the four herbal yogurts (data not shown). However, only LB-Y showed a strong correlation ($R^2 = 0.63$; data not shown).

3.3. α-Amylase and α-Glucosidase Inhibitory Activities

All four herbal yogurts inhibited about 32–35% of the α-amylase activity on day 0 of storage, compared to only 25% for the control yogurt (Table 1). The α-amylase inhibitory activity increased ($p < 0.05$) to ~56% for CP-Y, LB-Y, and PG-Y compared to the control (46%) on day 7 of storage. Furthermore, LB-Y and PG-Y showed the highest α-amylase

inhibitory activity (58.94 ± 2.30% and 58.09 ± 2.67%, respectively; $p < 0.05$) on day 14 of storage. Refrigerated storage for up to 21 days negatively affected α-amylase inhibitory activity in all yogurts samples except CP-Y (Table 1). The lowest α-amylase inhibitory activity was shown on day 28 for the control (24.16 ± 1.74%) and herbal yogurts (26%–30%). The beneficial effect of the four herbal yogurts toward α-amylase inhibitory activity (IC_{50}) was higher than that of the control yogurt throughout the storage period. In addition, this effect was more pronounced on days 7 and 14 of storage (Table 1).

Table 1. Changes in α-amylase inhibitory activity (%) and IC_{50} (mg/g) of yogurt (Y) enriched with water extract of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), or *Psidium guajava* during 28 days of refrigerated storage at 4 °C.

Yogurt Samples / Storage Periods	0 Day	7 Day	14 Day	21 Day	28 Day
	α-Amylase Inhibitory Activity (%)				
P-Y	25.54 ± 1.28	46.53 ± 2.27	44.64 ± 2.96	24.92 ± 1.92	24.16 ± 1.74
CP-Y	34.36 ± 2.20 *	56.05 ± 2.19 *	48.83 ± 2.18	46.58 ± 2.94 *	26.4 ± 2.17
IV-Y	31.55 ± 1.01 *	51.21 ± 1.28	50.65 ± 1.32 *	34.76 ± 1.13 *	27.06 ± 1.58
LB-Y	34.52 ± 2.19 *	54.64 ± 2.52 *	58.94 ± 2.30 *	32.54 ± 1.85 *	27.47 ± 0.92 *
PG-Y	35.28 ± 1.72 *	55.88 ± 2.57 *	58.09 ± 2.67 *	35.84 ± 1.42 *	29.87 ± 1.45 *
	IC_{50} (mg/g)				
P-Y	4.77 ± 1.98	1.46 ± 1.79	1.38 ± 1.84	4.04 ± 1.82	3.67 ± 1.28
CP-Y	2.24 ± 1.72 *	0.72 ± 2.61 *	0.74 ± 1.24 *	1.88 ± 1.77 *	2.54 ± 2.08 *
IV-Y	1.95 ± 2.63 *	0.72 ± 2.92 *	0.86 ± 2.07 *	1.74 ± 1.47 *	2.39 ± 1.68 *
LB-Y	2.25 ± 0.51 *	0.73 ± 1.72 *	0.93 ± 2.27 *	1.84 ± 1.45 *	2.43 ± 1.57 *
PG-Y	2.19 ± 2.09 *	0.79 ± 1.49 *	0.75 ± 1.11 *	1.69 ± 1.18 *	2.54 ± 1.23 *

Data are presented as mean ± SEM. P = plain. * $p < 0.05$ as compared to control (P-Y).

Both control and herbal yogurt showed a similar trend to inhibit α- glucosidase activity, which significantly increased during the first two weeks and reached the highest level on day 14, followed by a significant reduction during the last two weeks (Table 2). All herbal yogurts showed higher α- glucosidase inhibitory activity ($p < 0.05$) than the control throughout the storage periods. The α-glucosidase inhibitory activity of herbal yogurt ranged from 10% to 12% compared to the control (3%) on day 0 of storage. Refrigerated storage for two weeks caused a significant increased ($p < 0.05$) in α-glucosidase inhibitory activity and was two times higher for CP-Y and IV-Y (20.45 ± 1.94% and 15.09 ± 1.90%, respectively), whereas the control yogurt increased about three-fold (10.26 ± 1.11%). CP-Y and LB-Y showed the highest ($p < 0.05$) α-glucosidase inhibitory activity among all herbal yogurts during two weeks of storage (Table 2). At the end of storage, the levels of α-glucosidase inhibitory activity in the control yogurt, IV-Y, and PG-Y were almost comparable to those at the beginning (Table 2).

The effectiveness of herbal yogurt in inhibiting 50% of the α-glucosidase activity was higher than that of the control yogurt throughout the storage periods (Table 2). CP-Y and LB-Y showed an almost four times higher α-glucosidase inhibitory activity (IC_{50}) than the control at 0 days of storage. The best α- glucosidase inhibitory activity (IC_{50}) for all the herbal yogurts was on day 14 of storage, with the lowest values seen for CP-Y and LB-Y (Table 2). In addition, CP-Y showed the highest α-glucosidase inhibitory activity (IC_{50}) among all samples at 21 and 28 days of storage.

Table 2. Changes in α-glucosidase inhibitory activity (%) and IC_{50} (mg/g) of yogurt (Y) enriched with water extract of *Codonopsis pilosula* (CP), *Illicium verum* (IV), *Lycium barbarum* (LB), or *Psidium guajava* during 28 days of refrigerated storage at 4 °C.

Yogurt Samples / Storage Periods	α-Glucosidase Inhibitory Activity (%)				
	0 Day	**7 Day**	**14 Day**	**21 Day**	**28 Day**
P-Y	2.66 ± 0.23	6.27 ± 0.59	10.26 ± 1.11	3.62 ± 1.25	3.35 ± 1.74
CP-Y	9.76 ± 1.40 *	18.2 ± 2.61 *	20.45 ± 1.94 *	13.07 ± 1.95 *	12.24 ± 1.66 *
IV-Y	7.73 ± 1.47 *	11.99 ± 2.76 *	15.09 ± 1.90 *	7.79 ± 1.13 *	6.71 ± 1.86 *
LB-Y	12.28 ± 0.43 *	17.37 ± 2.02 *	19.63 ± 1.24 *	11.43 ± 2.42 *	10.27 ± 0.93 *
PG-Y	9.71 ± 0.16 *	14.81 ± 2.99 *	16.98 ± 1.22 *	10.45 ± 2.44 *	9.44 ± 0.82 *
	IC_{50} (mg/g)				
P-Y	9.09 ± 2.67	3.57 ± 1.66	2.04 ± 1.28	6.39 ± 1.34	7.36 ± 1.61
CP-Y	2.41 ± 0.58 *	1.28 ± 1.58 *	1.14 ± 1.90 *	1.95 ± 0.54 *	2.09 ± 1.15 *
IV-Y	3.47 ± 1.31 *	2.19 ± 1.69 *	1.48 ± 1.91 *	3.17 ± 1.11 *	3.67 ± 1.64 *
LB-Y	2.05 ± 1.67 *	1.48 ± 1.04 *	1.20 ± 2.27 *	2.35 ± 2.31 *	2.64 ± 1.02 *
PG-Y	2.39 ± 1.52 *	1.76 ± 0.93 *	1.37 ± 1.98 *	2.22 ± 2.18 *	2.81 ± 1.14 *

Data are presented as mean ± SEM. P = plain. * $p < 0.05$ as compared to control (P-Y).

The enzyme α-amylase is a limiting enzyme that acts by catalysing the hydrolysis of the α-1,4-glucosidic linkages of starch, glycogen, and other oligosaccharides [4,5]. The inhibition of this enzyme in the humans digestive tract is considered to be effective in controlling diabetes since it reduces glucose absorption from starch [4,5]. The enzyme α-glucosidase, on the other hand, is a regulatory enzyme that breaks down disaccharides into simpler sugars that are readily absorbed by the intestines. Thus, inhibiting α-glucosidase can prolong the carbohydrate metabolism time, slows down glucose entry into the blood vessel through the intestine, and thus prevents postprandial hyperglycaemia [4].

The inhibitory activity of α-amylase and α-glucosidase might be due to the presence of miscellaneous peptides from the hydrolysis of milk protein [5]. An increased inhibition of α-amylase activity was correlated with OPA peptide concentrations for only CP-Y and LB-Y (R^2= 0.6 and 0. 5, respectively; data not shown). Similarly, the correlation between α-glucosidase inhibitory activity and OPA peptide concentrations was only present for CP-Y and LB-Y (R^2= ~ 0.4, respectively; data not shown). Further research needs to be carried out for the screening of such bioactive peptides with α-amylase and α-glucosidase inhibitory activity.

The low glycaemic index (GI) of yogurt along with its ability to inhibit α-amylase activity [38] can now be further increased using *I. verum*, *P. guajava*, *C. pilosula*, or *L. barbarum*. The highest α-amylase inhibitory activity of LB-Y and PG-Y on day 14 of storage could be attributed to constituents present in these plants, such as phenols and flavonoids. *L. barbarum* showed an anti-diabetic effect by reducing oxidation in patients with retinopathy [39,40]. Furthermore, the polysaccharides in *L. barbarum* were found to reduce the absorption of glucose in the intestine and facilitate insulin secretion [34,41]. The protein of *L. barbarum* also showed an insulin-like action that effectively reduces blood sugar [42]. Similarly, several studies investigated that guava leaves could be used as a potential anti-diabetic [43,44]. These effects can be attributed to the presence of tannins, flavonoids, pentacyclic triterpenoids, arjunolic acid, ursolic acid, glucuronic acid, oleanolic acid, and other compounds in guava leaves [45]. In addition, CP-Y and LB-Y demonstrated the greatest α-glucosidase inhibitory activity after 7 and 14 days of storage. The protein content and some of the polyphenols, flavonoids, and antioxidant compounds present in the plant may contain some α-glucosidase inhibitors [5]. *C. pilosula* contains 17 kinds of amino acids, fructose, and inulin, in addition to polyphenols and flavonoids such as 8β-hydroxyasterolid, pyrrolysine, α-spinasterol, phytosterols and triterpenes, polysaccharides, saponins, syringing,

and tangshennoside I [22]. According to the findings of this study, the alteration of yogurt fermentation by the addition of *I. verum*, *P. guajava*, *C. pilosula*, or *L. barbarum* could significantly increase the anti-diabetic effect after up to two weeks of storage. However, the further decreased α-amylase and α-glucosidase inhibitory activities in all herbal yogurt samples during the last two weeks of storage could be associated with the further degradation of milk proteins into inactive peptides and amino acids during the proteolytic activity of the yogurt bacteria. The balance between the formation of bioactive peptides and the subsequent breakdown into inactive peptides and amino acids plays a decisive role in the inhibition of α-amylase and α-glucosidase activities [5,24,25]. In addition, prolonged refrigerated storage could cause further degradation of phenolic compounds with anti-α-amylase and α-glucosidase activities [24,25].

4. Conclusions

The presence of LB in yogurt significantly improved peptide concentration. All herbal yogurts showed an increase in TPC during refrigerated storage, which has been associated with higher antioxidant activity. LB-Y and IV-Y showed the highest TPC and antioxidant activity, respectively, among other yogurts during the 28 days of storage. All herbal yogurts had the ability to inhibit the α-amylase and α-glucosidase activities over the two weeks of storage. The beneficial effect of the four herbal yogurts toward α-amylase and α-glucosidase inhibitory activities (IC_{50}) was higher than that of the control yogurt throughout the storage period, with the optimal consumption being within two weeks of storage. The maintained enhanced inhibition of α-amylase and α-glucosidase activity as well as that of the antioxidants during the storage period suggests that the inclusion of *C. pilosula*, *I. verum*, *L. barbarum*, and *P. guajava* into yogurt should be considered in further studies as a potential functional food to help reduce post-prandial hyperglycaemia. Further study is needed to identify the chemical compositions of all four herbal water extracts and their amylase and α-glucosidase inhibitory activities.

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