

## Effect of whey protein concentrate on probiotic viability and antioxidant properties of yogurt during storage and simulated gastrointestinal transit

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### Abstract

The present work investigated the effects of whey protein concentrate (WPC) on probiotic yogurt. Five different concentrations of WPC (0 - 10%) were evaluated. The results showed positive effects of WPC on yogurt's properties under simulated gastrointestinal (GI) transit and long-term storage. *In vitro* digestion of WPC-fortified yogurt during GI transit markedly promoted the antioxidant activities in a concentration-dependent manner. WPC supplementation was also shown to significantly enhance the viability of probiotics under GI transit and during refrigerated storage, to the recommended level for health benefits on daily intake. The optimal concentration for retention of physicochemical properties (water holding capacity and texture profile) of the yogurt during refrigerated storage for 28 days was 5% (w/w), while the addition of 10% (w/w) WPC yielded the highest radical-scavenging activity ( $15.3 \pm 0.1$  mg Trolox Eq./g), reducing power ( $575.3 \pm 2.3$   $\mu$ g Trolox Eq./g), and Fe<sup>2+</sup>-chelating ability ( $13.5 \pm 0.02$  mg EDTA Eq./g) under both gastric and pancreatic digestion conditions. The results obtained suggest that WPC-fortification promoted the overall quality of probiotic yogurt by improving its antioxidant activities and probiotic viability, as well as extending its shelf-life.

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### Keywords

antioxidant activity,  
whey protein concentrate,  
probiotic,  
yogurt

## Introduction

Yogurt is an important fermented dairy product, and of high nutritional value. The health benefits associated with eating yogurt are numerous such as promoting healthy digestion, lowering the risk of type 2 diabetes, protecting against colorectal and stomach cancers, preventing osteoporosis, promoting weight loss, improving the immune system, and reducing high blood pressure and plasma levels of LDL cholesterol. Hence, industrially-produced high-protein yogurt has achieved ongoing popularity and taken a considerable share of the global market. In Thailand, it has been reported that yogurt consumption is approximately USD 143 - 145 million (THB 4300 - 4500 million) per annum (Marketeer, 2018).

Research and development in dairy factories have been continually producing various forms of fortified yogurt by adding health-promoting ingredients such as essential amino acids, vitamin D, and probiotic bacteria. Whey protein is an ingredient that has received much attention in dairy manufacturing industries due to its high nutrient value (Tamime and Robinson, 2007). Whey protein has been fortified in

yogurt to reduce whey separation and to increase the firmness of the yogurt. The interaction of casein micelles and denatured whey proteins via intermolecular disulphide bonds helps to increase network connectivity and water retention (Guyomarc'h *et al.*, 2003; Mahomud *et al.*, 2017). Whey protein is a natural by-product of cheese production, which remains in solution after curdling of milk with rennet or acid treatment. The main components of bovine whey protein are  $\beta$ -lactoglobulin ( $\beta$ -LG; 35 - 65%),  $\alpha$ -lactalbumin ( $\alpha$ -LA; 12 - 25%), and some minor proteins including immunoglobulins (8%), bovine serum albumin (BSA; 6%), lactoferrin (LF; < 3%), and lactoperoxidase (0.3%) (Ramos *et al.*, 2015). Whey protein and its derivatives are rich in branched-chain amino acids (leucine, isoleucine, and valine) and essential amino acids (cysteine) (Tamime and Robinson, 2007) that help to maintain the body's nitrogen balance, as is particularly required by both endurance and power athletes (Hoffman and Falvo, 2004). In addition, within its amino acid sequence, whey protein has bioactive peptides that have been reported to possess high antioxidant activities (Peng *et al.*, 2010; Corrochano *et al.*, 2019). Whey protein can

be broken down by proteolysis, thus generating peptides that possess various antioxidant properties such as metal ion chelation, inhibition of lipid peroxidation, radical scavenging, ferric ion reduction, and oxygen radical absorbance. The antioxidant mechanisms of whey protein hydrolysates are diverse, depending on the sizes and amino acid sequences of the peptides released by the proteases, which have different specific activities (Hernández-Ledesma *et al.*, 2005; Pihlanto, 2006; Lin *et al.*, 2012).

Probiotics are live microorganisms that beneficially affect human health by mechanisms such as inhibition of the growth of foodborne pathogens, boosting of the immune system, and prevention of diarrhoea and cancers (Kailasapathy and Rybka, 1997; Li *et al.*, 2011). Probiotic microorganisms generally belong to the genera *Bifidobacterium* and *Lactobacillus*. The US Food and Drug Administration (FDA) recommends that a daily intake of  $10^8$  -  $10^9$  live cells is essential for maintaining healthy digestion (Knorr, 1998). Once ingested, probiotics can become established in the lower gastrointestinal (GI) tract, and persist under conditions of high bile salt concentration that allow them to be active within the human gut (Gerez *et al.*, 2012; Vargas *et al.*, 2015). Although the effects of whey protein concentrate (WPC) on probiotic cultures have been investigated, their effects in fortified yogurt on the viability of probiotics in the GI digestive tract and during a refrigerated storage have not been reported. Thus, the present work aimed to examine the cover effects of WPC on probiotic yogurt including: (1) the effects of WPC on the antioxidant activity and viability of probiotics under the simulated GI digestion; and (2) the effects of WPC on viability of probiotic and physical properties throughout a typical shelf life of commercial yogurt.

## Materials and methods

### Materials

Commercial freeze-dried yogurt starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*) and probiotic bacteria *L. acidophilus* (SACCO, Cadorago, Italy) were used for inoculation. Whey protein concentrate (WPC) containing 87.8% protein was purchased from a local supermarket (Power Corporation Co., Ltd., Bangkok, Thailand). Porcine pepsin, porcine pancreatin, 2,2'-azino-bis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), trinitrobenzenesulfonic acid (TNBS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Probiotic yogurt manufacture

To prepare the yogurt starter, low-fat bovine milk (1.0% fat and 8.5% solids non-fat) was heated at 75°C for 15 min, then immediately cooled, and maintained at a constant temperature of 42°C. The milk was inoculated with 0.005% (w/v) of the lyophilised yogurt starter and probiotics. Fermentation was stopped when the pH reached 4.6, and the yogurt was immediately stored at 4°C. Five sets of yogurt trials were made, using final WPC concentrations of 0, 2.5, 5, 7.5, and 10% (w/w). Low-fat milk was heated to 50°C, and WPC was added to each trial concentration. The milk-WPC mixture was then heated to 75°C for 15 min with continuous stirring. Afterwards, the sample was cooled to 42°C, and inoculated with 5% (w/w) yogurt starter probiotic culture. Each mixture was transferred to a 100-mL plastic cup, and further fermented at a constant temperature of 42°C until pH reached 4.6, and immediately stored at 4°C for 24 h prior to chemical analysis.

### *In vitro* pepsin and pancreatin GI digestions of yogurt

Probiotic yogurt, with and without WPC, were subjected to *in vitro* simulated GI digestions as described by Helal and Tagliacuzzi (2018) with slight modifications. Initially, samples were homogenised in 0.5% (w/v) NaCl solution. For the gastric-phase trial, the pH of each sample was adjusted to 2.5 with 0.5 M HCl. Pepsin (2000 U/mL) was added to the yogurt sample, which was then incubated at 37°C for 2 h. The sample was then subjected to the intestinal-phase trial. The pH was adjusted to 7.5 with 20% (w/v) Na<sub>2</sub>CO<sub>3</sub>, and then pancreatin and bile salts were added to a final concentration of 0.8 g/L and 10 mM, respectively. The reaction was incubated at 37°C for 3 h. Aliquots of the sample were collected before and after each simulated peptic and pancreatic digestion for the determination of antioxidant activities and cell viability.

### Determination of $\alpha$ -amino acid content

*In vitro* digestibility of WPC-fortified yogurt in the GI tract was determined by measuring the  $\alpha$ -amino acid content by the TNBS method, as described by Adler-Nissen (1979). Briefly, 50  $\mu$ L of each sample was mixed with 0.5 mL of 0.2 M sodium phosphate buffer, pH 8.2, and 0.5 mL of 0.005% TNBS reagent. The reaction mixture was incubated at 50°C for 1 h, then 1 mL of 0.1 M HCl was added, and the terminated reaction mixture was left standing at 25°C for 30 min. The absorbance of the sample was then measured at 420 nm. The results were

expressed as milligram leucine equivalent per gram of sample.

#### *Determination of antioxidant activities*

##### *ABTS<sup>•+</sup> radical-scavenging activity assay*

The ABTS<sup>•+</sup> radical-scavenging activities of the aliquot samples were determined as described by Wiriyaphan *et al.* (2012). Briefly, 20  $\mu$ L of samples were added to 2 mL of diluted ABTS<sup>•+</sup> solution. The mixture was then shaken for 30 s, left in the dark for 5 min, and the absorbance was measured at 734 nm. The degree of ABTS<sup>•+</sup> radical-scavenging activity of sample was estimated based on the Trolox standard curve, and was expressed as milligram Trolox equivalent per gram of sample.

##### *Ferric reducing antioxidant power (FRAP) assay*

The ferric reducing antioxidant power (FRAP) assay was performed according to the method reported by Wiriyaphan *et al.* (2012). Briefly, 1 mL of FRAP reagent (10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM FeCl<sub>3</sub>, and 0.3 M acetate buffer (pH 3.6) at a ratio of 1:1:10 (v:v:v) was added to 0.1 mL of aliquot sample, and immediately mixed. After standing at 37°C for 20 min, the absorbance of sample was measured at 593 nm. The ferric reducing activity of the tested sample was expressed as microgram Trolox equivalent per gram of sample.

##### *Metal-chelating capacity assay*

The ferrous-chelating capacity of the aliquot samples was determined by the method of Conway *et al.* (2013). Briefly, 50  $\mu$ L of 2 mM FeCl<sub>2</sub> was added to 100  $\mu$ L of the sample suspended in 2.4 mL of distilled water. The reaction was initiated by adding 100  $\mu$ L of 5 mM ferrozine solution. The reaction mixture was shaken, and left to stand at 25°C for 20 min prior to measuring the absorbance at 562 nm. EDTA standard solution, in different concentrations, were used to construct calibration curves. The chelation of ferrous ions of the tested sample was expressed as milligram of EDTA equivalent per gram of sample.

##### *Cell viability*

The viability of probiotic *L. acidophilus* in the prepared yogurt during *in vitro* simulated GI digestion and refrigerated storage of 1 - 28 days was tested. Cell counting was performed by serial dilutions with 0.9% (w/v) sterile saline solution. Aliquots of 1.0 mL of the diluted cells were plated in de Man Rogosa and Sharpe (MRS) agar (HiMedia, Mumbai, India) supplemented with 1% (w/v) bile

salts (Lima *et al.*, 2009). The culture plate was incubated at 37°C for 72 h under anaerobic conditions, and colony counts were recorded and expressed as log CFU/g sample.

##### *Effects of WPC supplement on physical properties of probiotic yogurt*

Water-holding capacity (WHC) and texture profile analysis were evaluated during refrigerated storage of 1 - 28 days. Water-holding capacity was determined as described by Akalin *et al.* (2012). Briefly, 20 g of probiotic yogurt (Y) was centrifuged at 5000 g at 20°C for 10 min, and then the whey supernatant (W) was collected and weighed. The WHC was defined as:

$$\text{WHC (\%)} = (Y - W) / Y$$

The texture profile analysis of sample was performed using a TA-CT3 texture analyser (Brookfield Texture Analyzer, Lorch, Germany), equipped with a 3.8-cm acrylic cylinder probe. The test speed was fixed at 1 mm s<sup>-1</sup> with two penetration cycles. The force exerted on the sample was recorded, and the parameters of hardness (g), adhesiveness (mJ), cohesiveness, springiness (mm), and chewiness (mJ) were evaluated. The penetration depth through the sample was 20 mm. The test was replicated three times.

##### *Statistical analysis*

All analyses obtained from experiments were carried out at least in triplicate. Statistical data were expressed as mean values with standard deviations. The data were statistically analysed by one-way ANOVA and Duncan's multiple range test at a 95% confidence level. Statistical significance of data was set at  $p < 0.05$ .

#### Results and discussion

##### *Changes in $\alpha$ -amino acid content during two-phase digestion*

The  $\alpha$ -amino acid content of yogurt significantly increased with increasing WPC concentrations, following sequential simulated GI digestive phases (Figure 1). An increase in  $\alpha$ -amino acid content indicated the action of pepsin and pancreatin in WPC proteolysis, yielding oligopeptides, and/or free amino acids in the WPC-fortified samples (Adler-Nissen, 1979). Comparing all WPC concentrations, the  $\alpha$ -amino acid content increased when the content of WPC increased from 0 to 2.5, 5, 7.5, and 10% (w/w) WPC in fortified yogurt. Proteolysis was significantly

greater in the pancreatic phase than in the gastric phase. The free  $\alpha$ -amino acid content of the undigested yogurt sample with each WPC content under GI digestion that was used as a negative control exhibited some peptide content, but this content was far less than that of the hydrolysed samples under any simulated GI digestion phase. This suggested that the peptide bonds of WPC in the fortified yogurt were initially hydrolysed by pepsin in the gastric phase, generating peptide fragments that were then hydrolysed by pancreatin to smaller peptides and free amino acids (Corrochano *et al.*, 2019). These results correlated with those of Conway *et al.* (2013), who showed that the degree of hydrolysis of the denatured WPC increased after peptic and tryptic digestion.

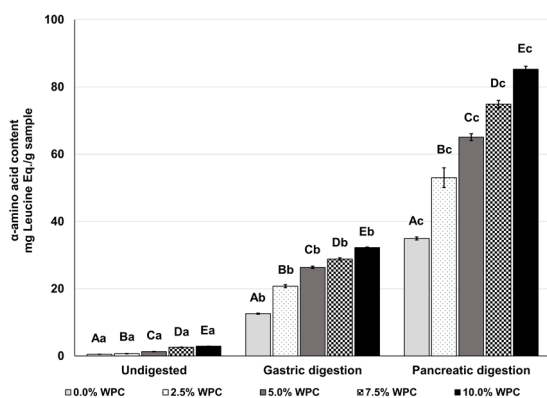


Figure 1. Changes in  $\alpha$ -amino acid content during simulated *in vitro* digestion by pepsin-pancreatin. Data are mean  $\pm$  standard deviation ( $n = 3$ ). Different uppercase superscripts denote significant differences between trials for the same sampling period of the *in vitro* assay ( $p < 0.05$ ). Different lowercase superscripts denote significant differences between different sampling periods of the *in vitro* assay for the same trial ( $p < 0.05$ ).

#### Effects of WPC on antioxidant activities of probiotic yogurt under two simulated GI conditions

##### ABTS<sup>•+</sup> radical-scavenging activity

The ABTS<sup>•+</sup> radical-scavenging activity of probiotic yogurt significantly increased with increasing WPC concentrations in both gastric and pancreatic digestions (Figure 2a). The highest ABTS<sup>•+</sup> radical-scavenging activity was observed in 10% (w/w) WPC fortified yogurt in all phases of digestion, with the value of  $1.2 \pm 0.1$  (undigested),  $9.6 \pm 0.1$  (gastric digestion), and  $15.3 \pm 0.1$  (pancreatic digestion) mg Trolox Eq./g sample. The values decreased when the content of WPC was reduced to 7.5, 5, 2.5, and 0% (w/w), respectively. The changing trend of the radical scavenging activity correlated with the elevated  $\alpha$ -amino acid content, indicating that bioactive peptides produced during proteolytic GI digestions contributed to higher

ABTS<sup>•+</sup> radical-scavenging activity. Peng *et al.* (2010) also reported that the WPC hydrolysate yielded higher ABTS<sup>•+</sup> scavenging capacity when compared with non-hydrolysed WPC, and that the scavenging capacity increased as the whey protein concentration increased. Another study also showed that WPC-fortified beverages increased radical scavenging during GI transit (Arranz *et al.*, 2019). Their results were similar to those of Corrochano *et al.* (2019), who showed that the production of ABTS<sup>•+</sup> radicals was inhibited by whey protein hydrolysates (WPI) and the main whey proteins ( $\alpha$ -LA,  $\beta$ -LG, BSA, and LF). It has been suggested that the reduction of ABTS<sup>•+</sup> radicals in the

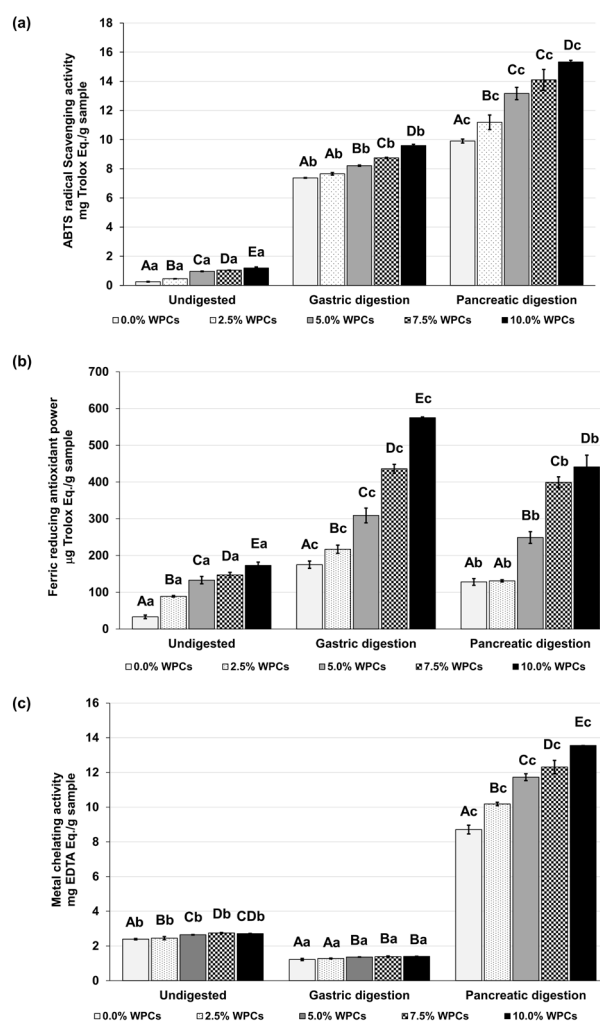


Figure 2. Antioxidant activity of yogurt fortified with and without WPC changes in (a) ABTS radical-scavenging activity, (b) ferric reducing antioxidant power, and (c) metal-chelating activity of yogurt during sequential *in vitro* gastric and intestinal conditions. Data are mean  $\pm$  standard deviation ( $n = 3$ ). Different uppercase superscripts denote significant differences between trials for the same sampling period of the *in vitro* assay ( $P < 0.05$ ). Different lowercase superscripts denote significant differences between different sampling periods of the *in vitro* assay for the same trial ( $p < 0.05$ ).



simulated GI digestion was positively correlated with the proteolytic digestion of WPC and the release of the antioxidant amino acid tryptophan.

#### *Ferric reducing antioxidant power (FRAP)*

The effects of WPC on the reducing antioxidant power of probiotic yogurt were tested. The results (Figure 2b) also showed that during the pepsin digestion stage, the reducing antioxidant power gradually increased in a concentration-dependent manner, but declined after the pancreatin digestion stage. The greatest reducing power was observed at 10% (w/w) WPC-fortified yogurt during *in vitro* pepsin digestion with values of  $173.3 \pm 9.1$  (undigested),  $575.3 \pm 2.3$  (gastric digestion), and  $440.7 \pm 32.0$  (pancreatic digestion) g Trolox Eq./g sample, respectively. The strong reducing power observed in WPC hydrolysates under gastric digestion has been suggested to be caused by reactive peptides generated during peptide hydrolysis, which could potentially react with free radicals, thus terminating the radical chain reaction (Kong and Xiong, 2006); while the decreasing reducing power after pancreatic digestion suggested that the whey proteins were more completely hydrolysed. In addition, some of the peptides generated may be less reactive with free radicals (Elias *et al.*, 2008). These results correlated with those of Iskandar *et al.* (2015), who reported that whey hydrolysates obtained from pepsin and trypsin digestion enhanced FRAP capacities. Peng *et al.* (2010) showed that FRAP values significantly increased with increasing WPI concentrations from 1.0 to 8.0% (w/w). Corrochano *et al.* (2019) also reported that the hydrolysis time and degree of hydrolysis enhanced the FRAP values of whey protein isolate after simulating tryptic GI digestion compared with intact WPI.

#### *Metal chelating capacity*

The results shown in Figure 2c clearly indicate that the  $\text{Fe}^{2+}$ -chelation capacity slightly decreased during the gastric phase, and then markedly increased with an increase in WPC concentration in the pancreatic phase. The positive effects of WPC hydrolysates on metal-chelating capacity suggest that proteolysis helped to increase peptide solubility, exposing free  $\alpha$ -carboxyl and  $\alpha$ -amino groups. In addition, a net anionic charge is established on a protein surface at high pH values; thus, at low pH (around pH 2.5 in the gastric phase), lower  $\text{Fe}^{2+}$ -chelating capacity was observed, as compared to the pancreatin phase (pH 7.5). These results are similar to those of Gad (2011), who showed that the degree of inhibition of WPC to the

ferrozine- $\text{Fe}^{2+}$  complex increased with increasing WPC concentrations. In addition, Conway *et al.* (2013) showed that the metal-chelating capacity increased with increasing WPC concentrations after peptic and tryptic digestion. Peng *et al.* (2010) also reported that an increase in enzymatic hydrolysis of WPI significantly enhanced antioxidant activities, which can act as a hydrogen donor, a metal ion chelator, and a radical stabiliser to inhibit lipid oxidation. Their data indicated that yogurt fortified with WPC can be used as a functional food to neutralise reactive oxidative species (ROS).

#### *Survival of *L. acidophilus* in WPC-fortified yogurt*

The survival rate of *L. acidophilus* probiotic bacteria, obtained from the *in vitro* viability assay, was found to markedly decrease after treatment under conditions of gastric digestion, then a further slight decrease following intestinal digestion (Figure 3). The initial population of *L. acidophilus* in yogurt was set at log 10.6 - 11.1 CFU/g. After gastric digestion, the counts of the viable *L. acidophilus* significantly increased with increasing WPC concentrations at  $p < 0.05$ . The highest viability was observed in the yogurt fortified with WPC of 7.5 and 10% (w/w) (*i.e.*, a reduction of  $\sim$  log 2.2 and 2.6 CFU/g, respectively). Conversely, a high susceptibility of *L. acidophilus* under conditions of low pH (pH 2.5) and pepsin was observed in the unfortified yogurt. These results suggest that the acid tolerance of *L. acidophilus* was influenced by the addition of WPC, and under intestinal digestion conditions, they exhibited the same trend as in the gastric phase. The fortified yogurt showed higher viable cell counts as compared to the unfortified samples, throughout 3 h of exposure to bile. These results suggest that the fortification of probiotic yogurt with whey protein enhanced cell viability under the simulated GI transit. This may be because WPC prevented or slowed down the damage to the bacterial cell proteins, facilitating protein repair, and mitigating the effects of acidic environments (Begley *et al.*, 2005; Vargas *et al.*, 2015). The survival of probiotic cells in the WPC-fortified yogurt was above log 6 CFU/g, a value in agreement with the standard requirement for products containing probiotics (Knorr, 1998). In previous reports, the addition of WPC and other milk proteins in yogurt resulted in an increase in counts of bifidobacteria when compared to no WPC supplement (Akalin *et al.*, 2007; Marafon *et al.*, 2011). The protective influence of whey protein on the viability of *L. acidophilus* and *L. casei* strains was previously observed in yogurt products supplemented with whey protein (Madureira *et al.*,

2011). The addition of whey protein hydrolysates up to 4% (w/w) improved the growth of *L. acidophilus* by three log cycles (Lucas *et al.*, 2004). These results indicated that WPC-enriched probiotic yogurt promoted the survival of probiotics throughout the two phases of GI digestion to the standard level required for products containing probiotics (Knorr, 1998).

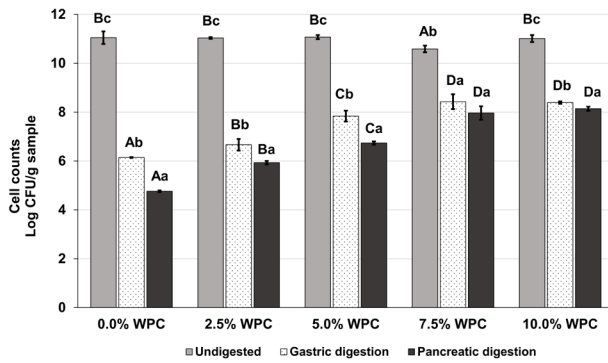


Figure 3. Survival of *L. acidophilus* in yogurt with different WPC concentrations during GI digestion process. Data are mean  $\pm$  standard deviation ( $n = 3$ ). Different uppercase superscripts denote significant differences between trials for the same sampling period of the *in vitro* assay ( $p < 0.05$ ). Different lowercase superscripts denote significant differences between different sampling periods of the *in vitro* assay for the same trial ( $p < 0.05$ ).

#### Evaluation of physicochemical properties during on-shelf storage

In this set of experiments, the effects of WPC on water-holding capacity (WHC), texture quality, and probiotic viability until the end of the shelf-life of the product were investigated.

When WHC was measured (Table 1), the value significantly increased with increasing content of WPC up to 5%, while a decrease in WHC was observed in yogurt fortified with 7.5 and 10% (w/w) WPC. It was seen that yogurt with 5% (w/w) WPC had a significantly higher WHC, and was more stable than others, with a WHC value of 97.4%. This might

be caused by the interactions of denatured whey proteins and  $\kappa$ -caseins, which improved the protein networks, and formed a homogeneous porous structure, in which a large amount of water was immobilised and entrapped, with a consequent increase in WHC (Lee and Lucey, 2010; Akalin *et al.*, 2012; Mahomud *et al.*, 2017). Similar studies also reported that the addition of WPC helped to maintain the stability of yogurt throughout its shelf life (Bierzuńska *et al.*, 2019; Rashid *et al.*, 2019). Other studies (Sodini *et al.*, 2005; Akalin, *et al.*, 2012; Mahomud *et al.*, 2017) also found that the increase of whey protein in yogurt promoted the WHC. However, a whey protein content of more than 5% (w/w) had effects on the physical properties that resulted in instability of the texture of the yogurt which exhibited undesirable characteristics including lumpiness, graininess, and a yellowish colour. These results might be due to a high content of solids (Rashid *et al.*, 2019). These results are consistent with those of Tamime and Robinson (2007) and Lee and Lucey (2010), who recommend that the whey protein concentration used to fortify yogurt mixes should be 0.6 - 4.0% (w/w).

Texture is one of the most essential indices of yogurt quality because a fine texture is usually the consumer's preference. Hardness, adhesiveness, cohesiveness, springiness, and chewiness of unfortified and WPC-fortified yogurts during refrigerated storage for 28 days are shown in Table 2. Considering day 1 of storage time, increasing concentrations of WPC significantly increased hardness, adhesiveness, and chewiness values as compared to the control samples. On the other hand, the addition of WPC did not significantly affect the cohesiveness or springiness of yogurt. Throughout the storage period of up to 28 days, WPC maintained the stability of yogurt by minimally decreasing its hardness, adhesiveness, and chewiness, suggesting that the addition of WPC produced consistency in

Table 1. Effects of WPC on the water-holding capacity of probiotic yogurts during 28 days of storage at 4°C.

Storage (day)	Water holding capacity (%)				
	Trial (% WPC)				
	0	2.5	5.0	7.5	10
1	65.4 $\pm$ 2.8 <sup>Aab</sup>	89.5 $\pm$ 3.0 <sup>Db</sup>	97.4 $\pm$ 4.9 <sup>Ea</sup>	79.8 $\pm$ 2.1 <sup>Ba</sup>	84.6 $\pm$ 1.6 <sup>Ca</sup>
7	72.6 $\pm$ 1.4 <sup>Ac</sup>	87.8 $\pm$ 1.8 <sup>Bab</sup>	96.8 $\pm$ 1.4 <sup>Ca</sup>	99.2 $\pm$ 0.5 <sup>Dd</sup>	98.7 $\pm$ 1.3 <sup>CDd</sup>
14	61.6 $\pm$ 3.0 <sup>Aa</sup>	89.0 $\pm$ 2.0 <sup>Bb</sup>	98.3 $\pm$ 0.5 <sup>Ca</sup>	87.5 $\pm$ 3.4 <sup>Bb</sup>	87.9 $\pm$ 1.5 <sup>Bb</sup>
21	67.5 $\pm$ 3.1 <sup>Ab</sup>	84.9 $\pm$ 1.8 <sup>Ba</sup>	95.6 $\pm$ 2.1 <sup>Ca</sup>	94.8 $\pm$ 1.8 <sup>Cc</sup>	94.6 $\pm$ 1.6 <sup>Cc</sup>
28	74.3 $\pm$ 2.9 <sup>Ac</sup>	88.3 $\pm$ 1.9 <sup>Bb</sup>	95.8 $\pm$ 1.4 <sup>Ca</sup>	89.4 $\pm$ 2.1 <sup>Bb</sup>	93.0 $\pm$ 1.7 <sup>Cc</sup>

Values are mean  $\pm$  standard deviation ( $n = 6$ ). Different uppercase superscripts within a row denote significant differences of WHC between different trials of yogurt ( $p < 0.05$ ). Different lowercase superscripts within a column denote significant differences of WHC during the storage periods ( $p < 0.05$ ).

textural characteristics. However, the addition of WPC > 5% (w/w) resulted in a dramatic decrease in all these physicochemical indices after storage for seven days. The cohesiveness and springiness values of unfortified and fortified yogurts were slightly different. These results indicate that suitable WPC-enrichment of yogurt contributed to the enhancement of its textural properties, except for cohesiveness and springiness. This is in agreement with results presented by many groups (Damin *et al.*, 2009; Akalin *et al.*, 2012; Fang and Guo, 2019), who reported that yogurt containing whey protein showed

higher firmness and adhesiveness than the control. This may be explained by the formation of the soluble protein complexes (disulphide-linked  $\beta$ -lactoglobulin and  $\kappa$ -casein) after adding WPC, which increases network connectivity and water retention in the yoghurt gel, thus increasing its firmness (Guyomarc'h *et al.*, 2003; Mahomud *et al.*, 2017). Also, the physical and sensory properties of yogurt gels are greatly influenced by the total solid content of the milk, especially the protein content (Lee and Lucey, 2010). Tamime and Robinson (2007) recommend that according to the total solid

Table 2. Texture profile analysis of probiotic yogurts with different concentrations of WPC during 28 days of cold storage.

Texture	Storage (day)	Trial (% WPC)				
		0	2.5	5.0	7.5	10
Hardness (g)	1	262 ± 15.9 <sup>Ac</sup>	317 ± 15.1 <sup>Bc</sup>	538 ± 10.5 <sup>Cd</sup>	545 ± 50.7 <sup>CDb</sup>	495 ± 13.0 <sup>Dc</sup>
	7	168 ± 16.8 <sup>Aa</sup>	282 ± 7.8 <sup>Cb</sup>	351 ± 10.3 <sup>Dc</sup>	199 ± 1.7 <sup>Ba</sup>	167 ± 3.6 <sup>Aa</sup>
	14	161 ± 6.4 <sup>Aa</sup>	283 ± 3.5 <sup>Cb</sup>	309 ± 25.4 <sup>Db</sup>	201 ± 12.3 <sup>Ba</sup>	174 ± 7.6 <sup>ABab</sup>
	21	192 ± 9.5 <sup>Ab</sup>	266 ± 16.7 <sup>Bb</sup>	333 ± 9.5 <sup>Cb</sup>	213 ± 9.3 <sup>Aa</sup>	191 ± 19.5 <sup>Ab</sup>
	28	166 ± 10.4 <sup>Aa</sup>	195 ± 14.7 <sup>Ba</sup>	217 ± 14.5 <sup>Da</sup>	209 ± 3.6 <sup>CDa</sup>	186 ± 4.0 <sup>Bab</sup>
Adhesiveness (mJ)	1	14.7 ± 0.3 <sup>Ad</sup>	16.1 ± 1.7 <sup>Aa</sup>	24.9 ± 3.8 <sup>Bb</sup>	25.7 ± 2.6 <sup>Bb</sup>	33.6 ± 3.4 <sup>Cb</sup>
	7	11.0 ± 1.5 <sup>Bbc</sup>	21.6 ± 1.7 <sup>Cb</sup>	21.7 ± 2.1 <sup>Cb</sup>	7.4 ± 0.7 <sup>Aa</sup>	5.0 ± 0.5 <sup>Aa</sup>
	14	10.0 ± 0.8 <sup>Bb</sup>	19.6 ± 2.0 <sup>Cab</sup>	22.5 ± 1.6 <sup>Db</sup>	6.9 ± 0.4 <sup>Aa</sup>	4.7 ± 0.2 <sup>Aa</sup>
	21	12.1 ± 1.0 <sup>Cc</sup>	21.1 ± 2.6 <sup>Db</sup>	21.5 ± 2.8 <sup>Db</sup>	8.6 ± 0.9 <sup>Ba</sup>	4.8 ± 0.2 <sup>Aa</sup>
	28	3.5 ± 0.3 <sup>Aa</sup>	16.5 ± 1.6 <sup>Ca</sup>	15.9 ± 1.2 <sup>Ca</sup>	7.8 ± 0.6 <sup>Ba</sup>	5.0 ± 0.6 <sup>Aa</sup>
Cohesiveness	1	0.57 ± 0.03 <sup>Aa</sup>	0.50 ± 0.02 <sup>Ab</sup>	0.45 ± 0.01 <sup>Aa</sup>	0.47 ± 0.01 <sup>Aa</sup>	0.50 ± 0.06 <sup>Aa</sup>
	7	0.49 ± 0.03 <sup>BCbc</sup>	0.41 ± 0.03 <sup>Aa</sup>	0.46 ± 0.01 <sup>Ba</sup>	0.52 ± 0.01 <sup>Cc</sup>	0.49 ± 0.01 <sup>BCa</sup>
	14	0.51 ± 0.02 <sup>Cbc</sup>	0.42 ± 0.02 <sup>Aa</sup>	0.48 ± 0.02 <sup>BCab</sup>	0.47 ± 0.02 <sup>BCab</sup>	0.47 ± 0.02 <sup>Ba</sup>
	21	0.49 ± 0.02 <sup>Cbc</sup>	0.44 ± 0.01 <sup>Aa</sup>	0.46 ± 0.04 <sup>ABa</sup>	0.49 ± 0.01 <sup>ABb</sup>	0.45 ± 0.02 <sup>ABa</sup>
	28	0.54 ± 0.05 <sup>Bc</sup>	0.56 ± 0.03 <sup>Bc</sup>	0.52 ± 0.02 <sup>Bb</sup>	0.46 ± 0.02 <sup>Aa</sup>	0.44 ± 0.01 <sup>Aa</sup>
Springiness (mm)	1	29.7 ± 1.6 <sup>Aab</sup>	30.0 ± 0.2 <sup>Aa</sup>	29.6 ± 0.7 <sup>Aa</sup>	30.6 ± 2.5 <sup>Aa</sup>	31.7 ± 0.7 <sup>Ab</sup>
	7	28.8 ± 1.5 <sup>ABab</sup>	28.3 ± 0.2 <sup>Aa</sup>	31.6 ± 1.1 <sup>Cb</sup>	30.9 ± 0.3 <sup>Ca</sup>	30.2 ± 0.2 <sup>BCa</sup>
	14	30.1 ± 0.7 <sup>Bab</sup>	28.3 ± 0.9 <sup>Aa</sup>	30.8 ± 0.5 <sup>Bab</sup>	30.5 ± 0.6 <sup>Ba</sup>	30.6 ± 0.2 <sup>Ba</sup>
	21	30.6 ± 0.1 <sup>Ac</sup>	30.2 ± 0.3 <sup>Aa</sup>	30.0 ± 1.3 <sup>Aab</sup>	30.9 ± 0.2 <sup>Aa</sup>	30.5 ± 0.3 <sup>Aa</sup>
	28	27.9 ± 1.9 <sup>Aa</sup>	30.4 ± 2.2 <sup>Ba</sup>	30.2 ± 0.2 <sup>Bab</sup>	30.5 ± 0.1 <sup>Ba</sup>	30.4 ± 0.1 <sup>Ba</sup>
Chewiness (mJ)	1	31.6 ± 2.0 <sup>Aa</sup>	46.8 ± 2.2 <sup>Bc</sup>	67.9 ± 4.4 <sup>Cd</sup>	72.8 ± 8.6 <sup>Cb</sup>	73.2 ± 3.9 <sup>Cb</sup>
	7	21.7 ± 3.8 <sup>Ad</sup>	32.2 ± 3.3 <sup>Bb</sup>	50.3 ± 3.0 <sup>Cbc</sup>	31.2 ± 0.4 <sup>Ba</sup>	23.9 ± 0.4 <sup>Aa</sup>
	14	24.0 ± 2.0 <sup>Ab</sup>	33.5 ± 2.3 <sup>Cb</sup>	45.0 ± 3.1 <sup>Dc</sup>	29.3 ± 2.4 <sup>Ba</sup>	24.5 ± 0.7 <sup>Aa</sup>
	21	28.3 ± 2.1 <sup>Abc</sup>	34.7 ± 2.6 <sup>Cb</sup>	41.7 ± 2.7 <sup>Db</sup>	31.4 ± 1.7 <sup>BCa</sup>	25.8 ± 2.8 <sup>Aa</sup>
	28	11.7 ± 1.8 <sup>Aa</sup>	19.0 ± 1.5 <sup>Ba</sup>	33.0 ± 0.8 <sup>Ea</sup>	28.6 ± 0.5 <sup>Ea</sup>	24.2 ± 0.2 <sup>Ca</sup>

Values are mean ± standard deviation ( $n = 3$ ). Different uppercase superscripts within a row denote significant differences of textural characteristics between different trials of yogurt ( $p < 0.05$ ). Different lowercase superscripts within a column denote significant differences of textural characteristics during the storage periods ( $p < 0.05$ ).

level, the greatest change in consistency was observed with 12 - 14 g/100 g in yogurt.

#### Survival of *L. acidophilus* during on-shelf storage

The cell counts of *L. acidophilus* in yogurts with and without WPC during refrigerated storage for up to 28 days are shown in Figure 4. The average initial cell count for each of the yogurts was  $\sim \log 10$  CFU/g. During the 28 days of storage, yogurt without WPC or fortified with 2.5% (w/w) WPC showed that the viability of *L. acidophilus* significantly ( $p < 0.05$ ) decreased, and that the cell count dropped below  $\log 6$  CFU/g after 21 days. On the other hand, supplementation of yogurt with  $> 5.0\%$  (w/w) WPC yielded significantly higher cell counts than in the WPC-free yogurt, and the cell viability remained above  $\log 8$  CFU/g (ranging from  $\log 8.8$  to  $8.9$  CFU/g) throughout the 28 days of storage. This protective effect on probiotic viability in refrigerated yogurt during storage probably came from the supply of peptides and amino acids in WPC, thus providing readily available nutritious sources necessary for the probiotic growth and reducing the effect of acidic environments (Shah, 2000; Akalin *et al.*, 2007; Marafon *et al.*, 2011). As previously reported, the viability of the probiotic during storage was improved by the addition of WPC and milk protein hydrolysates with the probiotic level greater than  $\log 6$  CFU/mL (Sodini *et al.*, 2002; Lucas *et al.*, 2004; Akalin *et al.*, 2007).

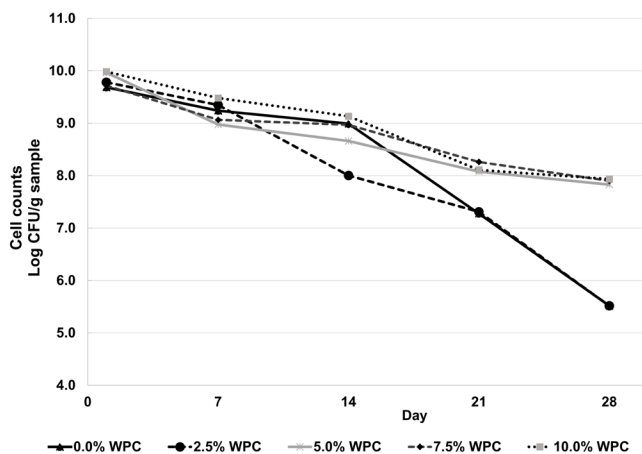


Figure 4. Survival of *L. acidophilus* in yogurt during refrigerated storage for 1, 7, 14, 21, and 28 days. Data are mean  $\pm$  standard deviation ( $n = 3$ ).

#### Conclusion

The present work showed that the addition of WPC to yogurt results in positive effects on its antioxidant properties and probiotic viability under GI transit that significantly increased with increasing

content of WPC. The highest value of the antioxidant activity was observed in 10% (w/w) WPC-fortified yogurt. These results suggest that the hydrolysis of whey proteins during GI digestion generated bioactive antioxidant peptides, thus resulting in an enhanced antioxidant activity. WPC-fortified yogurt enhanced the probiotic viability during simulated GI transit and refrigerated storage to the recommended level of health benefit for daily intake. In addition, yogurt fortified with WPC had significantly improved physical properties when compared with unfortified yogurt, where the supplementation with 5% (w/w) WPC was the most suitable for yogurt manufacture and provided an acceptable product.

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