



Effect of mulberry leaf extract fortification and probiotic fermentation on the bioactivities of cottage cheese

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Abstract

This study aims to enhance the bioactive property of cottage cheese by incorporating mulberry leaf extract (MLE) and applying probiotic fermentation. The MLE was produced via ultrasound-assisted water extraction (UW), pectinase-pretreated ultrasound-assisted water extraction (PUW) and cellulase-pretreated ultrasound-assisted water extraction. The MLE with the highest DPPH, FRAP, α -amylase inhibition (AI) and albumin denaturation inhibition (ADI) activities was selected to incorporate into the cheese. Lastly, the cheese was fermented with *Lactobacillus plantarum* TAR4. Results obtained reveal that UW and PUW produced MLE with the best bioactivities. Total phenolics, flavonoids, DPPH, FRAP, AI and ADI activities of the cottage cheeses were significantly enhanced with the fortification of MLE. Fermentation with *L. plantarum* TAR4 further enhanced the DPPH, FRAP, AI and ADI activities of the cheeses. Results of in-vitro digestion proved that the bioactivities exhibited by the cheeses did not diminished after digestion. Among the cheeses, fermented cheese fortified with MLE produced via UW (UW-LAB) showed the best bioactivity. After digestion, UW-LAB cheese exhibited 2.09 ± 0.12 mg g⁻¹ of FRAP, $74.77 \pm 1.90\%$ of DPPH, $64.91 \pm 1.16\%$ of AI and $38.83 \pm 2.50\%$ of ADI activities. This study recommends that combined MLE fortification with *L. plantarum* TAR4 fermentation was the best approach to improve nutrition value of cottage cheese.

Keywords Mulberry leaves · Lactic acid bacteria · Cottage cheese · Antioxidant activity · Anti-inflammation activity · Hypoglycemic activity

Introduction

Cheese is a famous dairy product. It is produced from milk in an extensive range of flavors and textures via different manufacturing setups. All types of cheeses made up of three common main ingredients, namely casein, fat, and brine. These components play important functions in the structural and rheological properties of cheese [1]. Cheese consumption is exponentially increased throughout the world, especially in the western countries such as Greece, Sweden, and Netherland for several decades. As a comparison with western countries, the rate of cheese consumption in Asian countries is relatively low [2]. Nonetheless, the abundance of health promoting factors in cheese such as essential

amino acids, minerals, vitamins, and probiotic bacteria have attracted interest of the health-conscious consumers [3]. According to few reports [4–6], fortifying cheese with asparagus powder, ginseng extract and *Rosmarinus officinalis* L. leaves had simultaneously increased the phenolic content and antioxidant capacity of cheese. According to Hasneen et al. [7], fortification of dairy products such as cheese and yogurt with herbal aqueous extract leads to the innovation of more variety of functional dairy foods. Therefore, this research was conducted to explore the potential approaches to improve the bioactivity of cottage cheese by using mulberry leaf extract (MLE).

Mulberry is a multi-functional plant and has been categorized as a functional food due to its prominent bioactivities [8]. Mulberry tree is widely cultivated in the Asia, particularly China, India, Korea, Africa, and Japan. Mulberry (*Morus* sp.) belongs to the Moraceae plant family and comprises several species. The three main species are *Morus nigra* (black mulberry), *Morus rubra* (red mulberry) and *Morus alba* (white mulberry) [9]. Different parts of the plant

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have their own uses, for instance, the mulberry fruit is usually eaten fresh or used to make jam, jelly and juice, whereas the mulberry leaves have been long served as the feed for silkworm in the silk industry and used to make herbal tea for revitalizing purposes. The bark and root are traditionally used as remedies for medicinal purposes [10]. In addition, mulberry leaves have also been widely used as medicine to relieve fever, improve eyesight, and lower blood sugar level in traditional Chinese medicine practice for several decades [11]. At present, mulberry leaves have attracted attention of modern healthcare practitioners due to its renowned anti-diabetic activity [12]. Besides, it has also been proven to exhibit antioxidant, anti-hyperlipidemia, anti-cancer, and anti-inflammation activities [13–16]. Thus, the inclusion of MLE was hypothesized to be able to improve the nutrition value of cottage cheese by exhibiting the nutraceutical properties of mulberry leaves.

Probiotic is the live microorganisms which are beneficial to health upon consumption [17]. *Lactobacillus* sp. such as *L. casei*, *L. acidophilus*, and *L. plantarum* and *Bifidobacterium* sp. such as *B. longum*, and *B. lactis* are the common probiotic strains used to enrich the nutritional benefits of foods [18]. Probiotics are proven to boost the immune system, improve inflammatory bowel condition, reducing the risk of certain cancers and maintaining body health [19]. Besides, probiotic bacteria fermentation could also produce a broad spectrum of bio-functional lipid (conjugated linoleic acid from linoleic acid), bioactive peptides and polyamines which have positive health effects. Furthermore, it also transforms certain phenolic and flavonoid compounds into biologically active metabolites and produce bacteriocin which exert anti-microbial property [20, 21]. These advantages contribute to the extensive use of lactic acid bacteria (LAB) as a popular commercial starter culture in food industry for functional food production [22].

In this study, combined effect of MLE fortification and probiotic fermentation on the antioxidant, anti-inflammation and hypoglycemic activities of cottage cheese was investigated. MLE was produced via ultrasound-assisted water extraction (UW), combined pectinase pretreatment ultrasound-assisted water extraction (PUW) and combined cellulase pretreatment ultrasound-assisted water extraction (CUW). The extraction technique that produced MLE with the highest antioxidant, anti-inflammation and hypoglycemic activities was selected to produce MLE for cottage cheese fortification. The effect of probiotic fermentation on the antioxidant, anti-inflammation and hypoglycemic activities were determined by fermenting the MLE-fortified cheese with *Lactobacillus plantarum* TAR4. The probiotic strain used in this project was isolated from fermented *Tapai* which is a popular food within this region. This warrants the source of origin of the probiotics and has the potential to target on the HALAL consumption market and functional food as aligned to the Sustainable Development

Goals (SDG3) which aspires to ensure healthy lives and promote well-being for all ages.

The combined effect of MLE fortification and probiotic fermentation was determined based on the changes of total phenolics, total flavonoids, DPPH scavenging activity, ferric reducing antioxidant power (FRAP), α -amylase inhibition activity (AI) and albumin denaturation inhibition activity (ADI) in the cottage cheese. The effect of in-vitro gastrointestinal digestion on the bioactivities of cheeses was also determined to evaluate the stability of the bioactive compounds in the cheese matrix upon digestion. This is a preliminary study to develop an integrated innovative approach to produce functional cheese via fortification and fermentation. As far as we know, study that investigate the interaction effect of nutraceutical fortification and fermentation on the bioactivities changes of cheese is limited in the field. Findings of this research provides a basic insight into the advantages of the use of integrated processing technology in boosting nutritional characteristics of functional food.

Materials and methods

Materials

Fresh mulberry leaves (*Morus alba*) were harvested from mulberry trees in the garden of Tunku Abdul Rahman University College. The collected mulberry leaves were washed, and vacuum dried at 45 °C and 15 kPa overnight (~ 16 h). The dried leaves were crushed into small pieces (< 50 mm) and kept in air-tight container until further usage. The food grade cellulase (~ 100 KU g⁻¹) and pectinase (~ 50 KU g⁻¹) enzymes used were purchased from Henan Wan Bang Industrial Co. Ltd., China. The enzymes were stored in an air-tight container at 4 °C. All chemicals used include gallic acid, Folin–Ciocalteu reagent, sodium carbonate, methanol, quercetin, aluminium chloride, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-S-triazine, hydrochloric acid, ferric chloride, α -amylase, starch, 3,5-dinitrosalicylic acid, albumin, pepsin, trypsin, bile salt, etc. were analytical grade with the brand name of Sigma, Merck and Chemsoln.

LAB used in this experiment was an isolated strain *Lactobacillus plantarum* TAR4 (NCBI GenBank database under the accession numbers MH012173). The LAB was grown in MRS broth (Oxoid, England) and the live cells were harvested after 48 h through centrifugation at 6373 × g for 5 min.

Preparation of mulberry leaf extract

Enzyme pretreatment

Prior to enzyme pretreatment, about 8% w/v of dried mulberry leaves was completely soaked in distilled water. For

pretreatment using pectinase enzyme, pH of the mixture was adjusted to 4.5. For pretreatment using cellulase enzyme, pH of the mixture was adjusted to 4.8. Next, about 0.5% w/v of cellulase and pectinase was added into the mixture, separately. The mixtures were mixed well, then incubated at 50 °C for 2 h in an incubator shaker at 150 rpm. An experimental control was carried out without the addition of enzyme.

Ultrasonic extraction

After pretreatment, the mixtures were placed in an ultrasonic water bath at 40 kHz and 250 W for 1 h. The temperature of ultrasonic extraction was maintained at 50 °C. The mulberry leaves residues were separated from the extract through centrifugation at $12,745 \times g$ for 10 min after the extraction was completed [23]. The supernatant (MLE) was collected and used as the additive to fortify the cottage cheese prepared in the subsequent study.

Preparation of cottage cheese

Cottage cheese was prepared according to the manufacturer instruction of food grade vegetarian rennet (MRDZ Malaysia, Kuala Lumpur, Malaysia). Briefly, about 1 L of pasteurized milk (Farm Fresh, Malaysia) was heated to 30–32 °C, then 3 g of food grade citric acid was added. The acidified milk was continuously stirred for 1 min to ensure complete dissolution of citric acid and left undisturbed for 15 min. Next, about 0.2 g of rennet was added. The mixture was stirred thoroughly for 1 min and left undisturbed for 20 min to allow cheese curd to form. The cheese curd was cut into cubes with knife in whey. The cheese cubes were left in the whey for another 15 min, then heated up the whey to 45 °C. The cheese was then separated from the whey by using cheesecloth. Next, about 12 g of salt was added into the whey and heated up to 82 °C. The cheese was placed in the hot whey for 15 s, then removed. The soft cheese curd was kneaded and folded for approximately 1 min. Next, the cheese was placed back into the hot whey for another 15 s. These steps were repeated for 15–20 min until the cheese is soft when cooled to room temperature. The cheese curd was then divided into small pieces with 20 g each.

To prepare cottage cheese containing probiotic *L. plantarum* TAR4, approximately 10^{10} cfu of cells were inoculated into 20 g of cheese and kneaded to mix well. Then, the cheese was incubated at 37 °C for 4 h. For cottage cheese fortified with MLE, about 1 g of MLE was added into the 20 g of cheese and kneaded to mix well. For cottage cheese containing MLE and probiotic, 1 g of MLE and 10^{10} cfu of *L. plantarum* TAR4 cells were added into the 20 g of cheese and kneaded to mix well. The cheese was then incubated at

37 °C for 4 h. Blank cheese was the plain cottage cheese without MLE and probiotic fermentation.

Simulated in-vitro gastrointestinal digestion

The method used was referring to Minekus et al. [24] with slight modification. Prior to digestion, simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) stock solution were prepared. Briefly, about 7.55 mL of 37.3 g L^{-1} potassium chloride, 1.85 mL of 68 g L^{-1} potassium hydrogen phosphate, 3.4 mL of 84 g L^{-1} sodium hydrogen carbonate, 0.25 mL of 30.5 g L^{-1} magnesium chloride hexahydrate and 0.03 mL of 48 g L^{-1} ammonium carbonate were mixed to prepare SSF electrolyte stock solution. To prepare SGF electrolyte stock solution, 3.45 mL of 37.3 g L^{-1} potassium chloride, 0.45 mL of 68 g L^{-1} potassium hydrogen phosphate, 6.25 mL of 84 g L^{-1} sodium hydrogen carbonate, 5.9 mL of 117 g L^{-1} sodium chloride, 0.2 mL of 30.5 g L^{-1} magnesium chloride hexahydrate and 0.25 mL of 48 g L^{-1} ammonium carbonate were mixed. To prepare SIF electrolyte stock solution, 3.4 mL of 37.3 g L^{-1} potassium chloride, 0.4 mL of 68 g L^{-1} potassium hydrogen phosphate, 21.25 mL of 84 g L^{-1} sodium hydrogen carbonate, 4.8 mL of 117 g L^{-1} sodium chloride and 0.55 mL of 30.5 g L^{-1} magnesium chloride hexahydrate were mixed. The SSF, SGF and SIF electrolyte stock solutions were made up to volume of 250 mL with distilled water, and then the pH was adjusted to 7, 3 and 7, respectively using 6 M hydrochloric acid. These electrolyte solutions were ready to be used for the subsequent digestion.

Firstly, about 5 mL of SSF electrolyte stock solution was added into 10 g of smashed cheese. Then, about 0.5 mL of α -amylase (1500 U mL^{-1}) was added into the mixture. Lastly, 1 mL of 75 mM calcium chloride dihydrate solution was added. Oral digestion was conducted at 37 °C and 200 rpm for 2 min. After oral digestion, the oral bolus was transferred into 7.5 mL of SGF electrolyte stock solution and about 1.6 mL of porcine pepsin (25 KU mL^{-1}) was added. The pH of the mixture was adjusted to 3 using 6 M hydrochloric acid. Prior to gastric digestion, about 5 μL of 0.3 M calcium chloride dihydrate was added. The mixture was incubated at 37 °C and 200 rpm for 2 h. After gastric digestion, approximately 10 mL of sample was withdrawn for analysis and the remaining gastric chyme was transferred into 5.5 mL of SIF electrolyte stock solution. Then, about 2.5 mL of pancreatin solution (800 U mL^{-1}) and 1.25 mL of 160 mM bile were added. pH of the mixture was adjusted to 7 using 1 M sodium hydroxide solution, followed by the addition of 20 μL 0.3 M calcium chloride dihydrate. The mixture was digested at 37 °C and 200 rpm for 2 h.

Total phenolics (TPC), flavonoids (TFC), ferric reducing antioxidant power (FRAP), DPPH activity, α -amylase inhibition activity (AI) and albumin denaturation

inhibition activity (ADI) of the samples obtained from the gastric and intestinal digestion were determined.

To determine TPC, TFC, FRAP, DPPH, AI and ADI of cheese before digestion, 10 mL of distilled water was added into 2 g of cheese sample, then subjected to ultrasonic water extraction (40 kHz, 250 W) at 50 °C for 1 h. The mixture was then centrifuged at $12,745 \times g$ for 5 min and the supernatant collected for analysis.

Chemical analysis

Total phenolics content (TPC)

TPC of MLEs, cheeses and digestion fluids after cheese digestion was determined according to protocol Baba and Malik [25]. Briefly, about 2 mL of Folin–Ciocalteu reagent was added into 400 μ L of sample/gallic acid standard solution and incubated at room temperature for 5 min. Then, about 1.6 mL of 7.5% sodium carbonate was added. The mixture was vortexed to mix well and incubated at room temperature in dark for 1 h. A series concentration of gallic acid (0.01–0.1 mg mL⁻¹) was used as the calibration standard. Absorbance of the mixture was measured at a wavelength of 765 nm by using a UV–Vis spectrophotometer (Merck, Germany) after 1-h incubation. The concentration of TPC in MLEs, cheeses and digestion fluids was expressed as mg gallic acid equivalents (GAE) per gram of dried leaves/cheese.

Total flavonoids content (TFC)

TFC of MLEs, cheeses and digestion fluids after cheese digestion was determined with aluminium chloride colorimetric method proposed by Baba and Malik [25] with slight modifications. Briefly, about 1 mL of sample/quercetin standard solution was added into 4 mL of distilled water. Then, about 0.3 mL of 5% sodium nitrite solution was added, then incubated at room temperature for 5 min. Next, about 0.3 mL of 10% aluminium chloride was added. After 5 min of incubation, about 2 mL of 1 M sodium hydroxide was added. The mixture was brought up to volume of 10 mL with distilled water. A series concentration of quercetin (0.01–0.5 mg mL⁻¹) was used as the calibration standard. The mixture was stood at room temperature (25 °C) for 15 min. The absorbance of the mixture was measured at a wavelength of 510 nm by using a UV–Vis spectrophotometer (Merck, Germany). TFC of MLEs, cheeses and digestion fluids was expressed as mg quercetin equivalents (QE) per gram of dried leaves/cheese.

In-vitro bioactivity assay

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to procedure proposed by Baba and Malik [25] with slight modifications. Initially, FRAP reagent was prepared by mixing 0.3 M acetate buffer, 0.01 M tripyridyltriazine (TPTZ) and 0.04 M hydrochloric acid at a ratio of 10:1:1. Then, about 0.2 mL of sample/ferrous sulphate (FeSO₄) standard solution was mixed with 3.8 mL of FRAP reagent. Next, the mixture was incubated at 37 °C for 30 min. Absorbance of the mixture was measured at a wavelength of 593 nm by using UV–Vis spectrophotometer (Merck, Germany). FRAP of the sample was expressed as ferrous sulphate equivalent per gram of leaves/cheese.

2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay

To determine DPPH activity of the sample, 1 mL of sample/ascorbic acid standard was added into 3.9 mL of 60 μ M DPPH solution. The mixture was then incubated in dark at room temperature for 30 min. The absorbance of the mixture was measured at a wavelength of 515 nm by using UV–Vis spectrophotometer. DPPH radical scavenging activity (%) was calculated by using formula: $[(\text{Absorbance}_{\text{blank}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{blank}}] \times 100\%$. Water was used as the experiment blank [25].

α -amylase inhibition activity assay

α -amylase inhibition activity (AI) of samples was determined according to protocol Mir et al. [26]. Briefly, about 0.25 mL of 0.5 mg mL⁻¹ α -amylase (30 U mg⁻¹) in 0.02 M phosphate buffer (pH 6.9) was added into 0.25 mL of sample. Then, about 0.25 mL of 1% starch solution was added, followed with incubation at 25 °C for 10 min. Upon completion, the enzyme activity was stopped by adding 0.5 mL of 1% dinitrosalicylic (DNS) reagent. The mixture was heated in boiling water bath for 5 min, then 5 mL of distilled water was added. Absorbance of the mixture was determined at wavelength of 540 nm by using an UV–Vis spectrophotometer. Experiment blank was conducted by replacing sample with distilled water, whereas sample blank was carried out by replacing 1% starch solution with distilled water. Acarbose (4 mg mL⁻¹) was used as positive control of the experiment. α -amylase inhibition activity of sample was determined by using formula: $[(\text{Absorbance}_{\text{blank}} - (\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{sample blank}})) / \text{Absorbance}_{\text{blank}}] \times 100\%$.

Albumin denaturation inhibition assay

Albumin denaturation inhibition activity (ADI) of sample was determined using protocols described by Kumari et al. [27]. Briefly, about 2 mL of sample was added into 2.8 mL of 0.02 M phosphate buffer 9pH 6.4) containing 0.2 mL of 5% albumin. The mixture was incubated at 37 °C for 15 min. Next, the mixture was heated to 70 °C and maintained for 5 min. The mixture was then allowed to cool down to room temperature. The absorbance of mixture was measured at wavelength 660 nm by using an UV–Vis spectrophotometer. Experiment blank was carried out by replacing sample with distilled water, whereas sample blank was conducted by replacing albumin with distilled water. Diclofenac sodium (4 mg mL⁻¹) was used as the positive control in this experiment.

Statistical analysis

All experiments were performed in triplicates and results were expressed as mean ± standard deviation. Statistical difference ($p < 0.05$) between samples was determined via one-way ANOVA and LSD's post hoc test. All the statistical analysis was conducted by using IBM SPSS statistics software version 20.

Results and discussion

Effect of different extraction methods on the chemical and bioactive properties of mulberry leaf extract (MLE)

Table 1 presents the total phenolics (TPC), total flavonoids (TFC), ferric reducing antioxidant power (FRAP), DPPH scavenging activity, α -amylase inhibition activity (AI) and albumin denaturation inhibition activity (ADI) of MLE produced via ultrasound-assisted water extraction (UW), combined pectinase pretreatment ultrasound-assisted

water extraction (PUW) and combined cellulase pretreatment ultrasound-assisted water extraction (CUW). Based on Table 1, TPC and TFC of MLE produced via PUW and CUW were significantly higher ($p < 0.05$) than MLE produced via UW. TPC of MLE produced via CUW (15.74 ± 0.52 mg g⁻¹) was the highest, followed by PUW (11.97 ± 1.78 mg g⁻¹). Cellulase pretreatment double up the TPC of MLE whereas pectinase pretreatment improved the TPC yield by only about 52%. TFC of MLE was increased by almost two-fold after the mulberry leaves were pretreated with pectinase and cellulase, respectively. TFC of MLE was increased from 11.80 ± 2.32 mg g⁻¹ to 21.23 ± 2.21 mg g⁻¹ and 20.68 ± 1.18 mg g⁻¹ after pectinase and cellulase pretreatment, respectively. This finding suggests that enzyme pretreatment is an effective technique to enhance the recovery of phenolics and flavonoids from mulberry leaves. The present finding corresponds to reports by Wang et al. on guava leaves [28], Rakariyatham et al. on longan peel [29] and Kapsakalidis et al. on blackcurrant pomace [30]. In their study, enzymatic treatment was proved to increase the phenolic and flavonoid recovery, subsequently enhanced the antioxidant activity of the extracts. The action of cellulase enzyme in the breakdown of cellulose and pectinase enzyme to hydrolyze the pectin have disrupted the structural integrity of plant cell wall, and thus facilitate the release of phenolics and flavonoids [31].

In this study, antioxidant potential of MLEs was evaluated using FRAP and DPPH assays. Results in Table 1 indicate that enzyme pretreatment exerted different effects on the FRAP and DPPH of MLE. FRAP of MLE was increased by about 7.5-fold when enzyme pretreatment was employed prior to ultrasound-assisted water extraction. The FRAP of MLE was increased from 8.93 ± 0.02 mg Fe²⁺ g⁻¹ to 68.05 ± 0.24 mg Fe²⁺ g⁻¹ (CUW) and 67.73 ± 0.28 mg Fe²⁺ g⁻¹ (PUW). However, DPPH of MLE was reduced by about 38–50% when enzyme pretreatment was employed. DPPH activity of MLE was reduced from $46.22 \pm 0.40\%$ to $28.22 \pm 0.09\%$ and $22.73 \pm 0.09\%$ respectively after PUW and CUW.

Table 1 Effect of different extraction methods on the total phenolics (TPC), total flavonoids (TFC), ferric reducing antioxidant power (FRAP), DPPH scavenging activity, α -amylase inhibition activity

(AI) and albumin denaturation inhibition activity (ADI) of mulberry leaf extract (MLE) (n=3)

| Samples | TPC (mg g ⁻¹) | TFC (mg g ⁻¹) | FRAP (mg FeSO ₄ g ⁻¹) | DPPH (%) | AI (%) | ADI (%) |
|---------|---------------------------|---------------------------|--|--------------------|--------------------|--------------------|
| UW | 7.87 ± 1.55^c | 11.80 ± 2.32^b | 8.93 ± 0.02^b | 46.22 ± 0.40^a | 22.72 ± 0.96^b | 45.35 ± 0.24^a |
| PUW | 11.97 ± 1.78^b | 21.23 ± 2.21^a | 67.73 ± 0.28^a | 28.22 ± 0.09^b | 26.11 ± 1.92^a | 43.73 ± 0.05^b |
| CUW | 15.74 ± 0.52^a | 20.68 ± 1.18^a | 68.05 ± 0.24^a | 22.73 ± 0.09^c | 28.33 ± 1.67^a | 34.83 ± 0.31^c |

DPPH, AI and ADI activities shown were activity exhibited by dilute MLE at a ratio of dilution 1:250

UW ultrasound-assisted water extraction, PUW combined pectinase pretreatment ultrasound-assisted water extraction, CUW combined cellulase pretreatment ultrasound-assisted water extraction

^{a-c}Different alphabets within the same column indicate there is significant difference ($p < 0.05$) between the samples in an analysis

Results of Pearson correlation analysis unveils that FRAP of MLEs was strongly correlated with its high TPC ($r=0.831$ at $p<0.01$) and TFC ($r=0.937$ at $p<0.01$). On the contrary, DPPH of MLEs was negatively correlated with TPC ($r=-0.907$ at $p<0.01$), TFC ($r=-0.903$ at $p<0.01$) and FRAP ($r=-0.977$ at $p<0.01$). This finding suggests that enzyme action probably exert a pronounced effect on the structural characteristic of phenolics and flavonoids released from the mulberry leaves. A broad spectrum of phytochemicals is found in mulberry leaves, include flavonoids (astragalins, kaempferol, quercetin, rutin, etc.), benzofurans (albufuran A, moracin, wittifuran E, etc.), phenolic acids (caffeic acid, ferulic acid, sinapic acid, etc.), alkaloids (1-deoxynojirimycin, auranthamide acetate, fagomine, pipercolic acid, etc.), coumarins (7-hydroxycoumarin, moralsin, scopolin, etc.), calcones and stilbenes [32]. These compounds are well known with their redox characteristic and always correlated with antioxidant activity. Besides, flavonoids contain active hydrogen, which can terminate the chain reaction of oxygen radicals, scavenge free radicals, and eliminate the toxic effect of the radicals [33].

Different compounds possess different free radicals scavenging capacity and reducing power. The number and position of functional groups in an antioxidant such as methoxy group, phenolic hydroxyl group and carboxylic acid group affect its antioxidant potential [34]. Both pectinase and cellulase are carbohydrases which are widely used to breakdown the plant cell wall by hydrolyzing the pectin and cellulose. These enzymes could also cleave the glycosidic linkages of the glycosides of polyphenols and flavonoids, subsequently release its aglycons which exhibit stronger antioxidant potential [35]. However, enzyme treatment does not always relate with the increase of antioxidant activity. In the study by Abbas et al. [36], total antioxidant capacity of red date syrup was reduced when pectinase-cellulase enzyme cocktail was used in the extraction. The extractability of different phenolics and flavonoids will not be the same under different extraction conditions because the chemical linkages cleavage in the cell wall structure was not the same. Therefore, phenolics and flavonoids profile of MLE produced from UW, PUW and CUW were expected to be different, hence contribute to different antioxidant potential. In addition, the reaction system will also interfere the antioxidant activity of a compound. In the FRAP assay, antioxidant reduces the colorless Fe^{3+} -TPTZ complex to blue-colored Fe^{2+} -TPTZ complex in aqueous system via sequential proton loss electron transfer mechanism. In the DPPH assay, the antioxidant scavenged DPPH radicals in methanol system via hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET-PT) and sequential proton loss electron transfer (SPLET) mechanisms. Therefore, an extract may not always display the same antioxidant potential in both

DPPH and FRAP assays [34]. This explained the differences in FRAP and DPPH activity of MLEs produced via different methods were different in this study.

α -amylase inhibition activity (AI) was determined to evaluate hypoglycemic activity of MLEs. α -amylase is a digestive enzyme that acts to degrade dietary carbohydrates into disaccharides. According to Kim et al. [37] and Anno et al. [38], MLE which is rich in quercetin, 1-deoxynojirimycin (DNJ), chlorogenic acid and soluble dietary fiber play a crucial role in hypoglycemic effect. These compounds are vital in the inhibition of α -amylase activity. In the study by Riche et al. [39], post-prandial blood glucose level was significantly decreased after 3 months of MLE-treatment in type-2 diabetic patients. MLE was found to effectively lower the blood glucose level and increase the insulin secretion [40]. Based on Table 1, AI of CUW-MLE ($28.33 \pm 1.67\%$) and PUW-MLE ($26.11 \pm 1.92\%$) was higher than UW-MLE ($22.71 \pm 0.96\%$). Although both pectinase and enzyme treatments had increased AI of MLE, the effect of pretreatment by both enzymes was no significant difference ($p>0.05$). Results of Pearson correlation analysis reveals that AI was positively correlated with TPC ($r=0.837$ at $p<0.01$), TFC ($r=0.671$ at $p<0.05$) and FRAP ($r=0.834$ at $p<0.01$). These results suggest that phenolics and flavonoids with antioxidant activity were also play an important role in the AI of MLE. In the study by Luo et al. [41], phenolics and flavonoids were found to have no significant difference in inhibiting α -amylase enzyme, but both were a better inhibitor than acarbose. In this study, 4 mg mL^{-1} of acarbose was proven to exhibit $91.67 \pm 1.36\%$ of AI. AI of all MLEs was threefold–fourfold lower than acarbose because these are crude extract. The flexibility of side chains and relative disposition of the OH groups of phenolic and flavonoid facilitates its binding via hydrogen bonds and hydrophobic interactions with amino acid residues near the active site of enzyme, hence changing the secondary structure of the enzyme's protein and effectively inhibit the enzyme activity. Different compounds have different inhibition potential because of its unique molecular arrangement [41].

Nevertheless, results in Table 1 show that enzyme pretreatment had cause significant reduction in albumin denaturation inhibition activity (ADI) of MLE. ADI of MLEs was found lower than diclofenac sodium ($93.38 \pm 0.06\%$) at 4 mg mL^{-1} . ADI of MLE ($45.35 \pm 0.24\%$) was reduced about 3.6% to $43.73 \pm 0.05\%$ after pectinase pretreatment and about 23.2% to $34.83 \pm 0.31\%$ after cellulase pretreatment. This finding proposes that CUW is not recommended to be used to produce MLE with the best bioactive characteristics. Although CUW-MLE contains the highest TPC, its TFC, FRAP and AI had no significance ($p>0.05$) with PUW-MLE, while its DPPH and ADI were the lowest among the samples. Results of Pearson correlation analysis unveils that ADI was positively correlated with DPPH activity of MLE

at $r=0.775$ at $p<0.05$. These findings postulate that anti-inflammation activity of MLE in term of its albumin denaturation inhibition capacity is contributed by the activity of free radical scavengers in the extract. Protein denaturation is one of the mechanisms of inflammation in human body [42]. Upon protein denaturation, the protein structure loses its functionality and precipitated. The macrophages will accumulate at the areas of accumulation of denatured protein. Antioxidant with free radicals scavenging activity acts to scavenge reactive oxygen species, so that the damage of cell membrane by reactive oxygen species can be avoided, hence reduce inflammation [43]. Therefore, low DPPH activity in MLE likely contributes to low ADI.

Based on results obtained in this section, MLEs produced via UW and PUW were selected to fortify cottage cheese in the next study due to their superior bioactive quality. MLE produced via UW presented the highest DPPH and ADI activity, whereas MLE produced via PUW exhibited the highest FRAP and AI activity. This finding elicits that both extraction methods have its pros and cons. In the following section, experiment of cottage cheese fortification was conducted to further determine the type of MLEs with the best characteristics for improving bioactivity of cottage cheese.

Effect of mulberry leaf extract (MLE) fortification and probiotic fermentation on the chemical and bioactive properties of cheese

In this section, effect of MLE fortification and probiotic fermentation on cottage cheese was investigated. Figure 1 shows the changes of total phenolics (TPC) and flavonoids (TFC) of different cottage cheeses (MLE fortified, probiotic fermented, and their combinations) after simulated in-vitro digestion. Results obtained indicate that MLE fortification and probiotic fermentation significantly ($p<0.05$) improved TPC and TFC of cottage cheeses. Probiotic fermentation had increased the TPC of cottage cheese from 0.13 ± 0.01 mg GAE g^{-1} to 0.34 ± 0.01 mg GAE g^{-1} and TFC from 0.07 ± 0.01 mg QE g^{-1} to 0.37 ± 0.01 mg QE g^{-1} . Besides, fortification of cottage cheese with PUW-MLE had increased its TPC and TFC to 0.37 ± 0.03 mg GAE g^{-1} and 1.27 ± 0.02 mg QE g^{-1} , respectively. TPC and TFC of cottage cheese fortified with UW were significantly lower ($p<0.05$) than PUW-fortified cheese. However, probiotic fermentation had increased both TPC and TFC of UW-fortified cheese but caused substantial reduction in PUW-fortified cheese. TPC and TFC of UW-LAB cheese became significantly higher ($p<0.05$) than PUW-LAB cheese after probiotic fermentation. This finding proposes that *L. plantarum* TAR4 fermentation is an effective process to enhance nutritional value of cottage cheese. Furthermore, MLE fortification has also been proven to substantially increase phytochemicals in the cheese. This result is in accordance with

study by Kobus-Cisowska et al. [44], whereby the addition of mulberry increased the phenolic acids level and improved the stability of other existing polyphenols in the bread. Furthermore, the study of Tomczyk et al. [45] showed that the addition of 1% mulberry leaves into creamed honey significantly increased its polyphenol compound and exerted pro-health properties. According to Katina et al. [46], probiotic fermentation enhances bioavailability of nutrients in fermented foods by breaking down the complexity of nutrient in food matrix. Fermentation technology is now widely used to promote the production of health-beneficial bioactive compounds for various functional foods production [47]. Through fermentation, the conjugated phenolic compounds are released into free form and the proteins degrade into medium- and short-chain peptides, which contribute to various biological functions [48]. However, based on results in Fig. 1, TPC and TFC in all cheeses were low (below 1 mg g^{-1}). This is likely due to the strong binding of polyphenols and flavonoids via hydrogen bonds and hydrophobic interactions to casein in the cheese matrix [49]. Thus, it restricted the extractability of phenolics and flavonoids for quantification.

Besides, Fig. 1a also shows that TPC of all cheeses was increased significantly after gastric and intestinal digestion. This result postulates that harsh gastric digestion environment at acidic pH did not cause phenolic degradation. On the contrary, acidic pH and pepsin enzyme action facilitate the breakdown of casein and other macromolecules in the cheese, hence promotes the release of bound phenolics [50]. Furthermore, the TPC level was continued to increase when the digestion was progressed to intestinal phase. This result elicits that phenolics in cheeses were quite stable upon digestion. The entrapment of phenolics in the solid cheese matrix and their interaction with the amino acid residues of casein delay the rate of release of phenolics, hence reduce their time of exposure to the harsh digestion conditions. Eventually, the rate of phenolics degradation was reduced while the bio-accessibility of the phenolics was enhanced [50]. Although the TPC of PUW- and UW-LAB-fortified cheeses was the highest among the tested samples before digestion, TPC of all MLE-fortified and probiotic fermented cottage cheeses had no significant difference ($p>0.05$) after digestion. Nonetheless, TPC retained after digestion of all MLE fortified and LAB fermented cheeses was significantly higher than the blank. This finding reveals that MLE fortification and probiotic fermentation could effectively improve phenolics availability in cheese intake.

However, Fig. 1b shows that TFC in cottage cheeses was reduced significantly ($p<0.05$) after in-vitro gastrointestinal digestion, except blank, UW-fortified and PUW-LAB cheeses. TFC of blank and PUW-LAB cheeses after intestinal digestion were higher than before digestion, whereas TFC of UW-fortified cheese had no significant difference

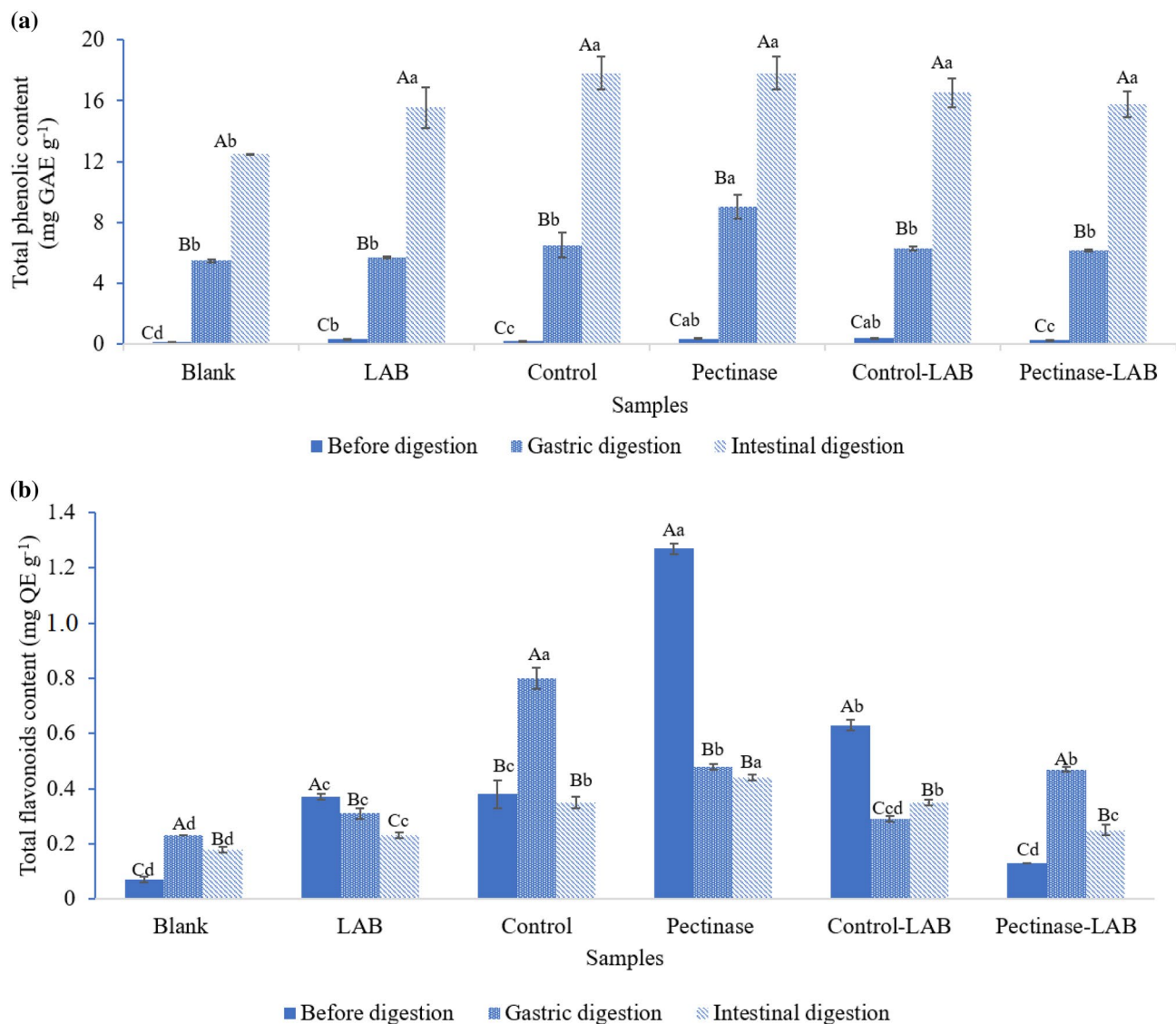


Fig. 1 Effect of in-vitro gastrointestinal digestion on the **a** total phenolics (TPC) and **b** flavonoids (TFC) changes in different types of cheese (n=3). (1) Blank indicates cheese without MLE fortification and LAB fermentation, LAB indicates LAB-fermented cheese without MLE fortification, UW indicates cheese fortified with UW-MLE, PUW indicates cheese fortified with PUW-MLE, UW-LAB indicates

LAB-fermented cheese fortified with UW-MLW and PUW-LAB indicates LAB-fermented cheese fortified with PUW-MLE. (2) A–C: Different alphabets indicate there is significant difference ($p < 0.05$) between different stages of digestion in a sample. (3) a–d: Different alphabets indicate there is significant difference ($p < 0.05$) between samples at the same stage of digestion

compared to before digestion. Although gastric digestion caused an increase of TFC of these three cheeses, intestinal digestion caused substantial TFC reduction. According to Bouayed et al. [51], free flavonoids may degrade due to long exposure to the harsh digestion environment. Furthermore, binding of flavonoids to some digestive enzymes such as α -amylase might also led to its low bio-accessibility [52]. Therefore, the cheeses with high free flavonoids before digestion tend to have low TFC after digestion. Phenolics and flavonoids which entrapped and strongly bound to casein will be more resistant to degradation upon digestion [50].

This hypothesis can be clearly seen in TFC change of PUW-fortified cheese during digestion. A drastic TFC reduction (~62%) was occurred in PUW-fortified cheese that contains the highest TFC before digestion. This finding suggests that flavonoids in PUW-MLE might just weakly bound to the casein in the cheese matrix, and thus prone to degradation upon gastric digestion under harsh condition. This outcome is supported by Jiao et al. [53] study. In their study, the bio-accessibility of flavonoid was significantly reduced after gastrointestinal digestion, whereby the recovery was only 15.68% on the dialyzed portion. According to Sharma et al.

[54], phenolic compounds are more resist to degradation than flavonoids under extreme conditions. Functional groups of flavonoids are more vulnerable to hydroxylation and degradation whereas covalently bound phenolic compounds are more stable [55]. This explains why most cheeses experience significant TFC reduction after intestinal digestion, except PUW- and UW-LAB cheeses. There was no significant TFC change ($p > 0.05$) observed in PUW-fortified cheese, while TFC of UW-LAB-cheese was slightly increased after intestinal digestion. Yet, all fortified and fermented cheeses still presented higher TFC than the blank after in-vitro

gastrointestinal digestion. Among them, the highest TFC was successfully preserved in PUW-fortified cheese after digestion.

Figure 2 presents the change of antioxidant potential of different cheeses in terms of its ferric reducing antioxidant power (FRAP) and DPPH scavenging activity after in-vitro gastrointestinal digestion. According to Fig. 2a, PUW-fortified cheese contains the highest FRAP ($0.81 \pm 0.08 \text{ mg g}^{-1}$), which was about double of the rest before digestion. FRAP of all the other cheeses ($0.44\text{--}0.52 \text{ mg g}^{-1}$) had no significant difference ($p > 0.05$) with the blank ($0.42 \pm 0.01 \text{ mg g}^{-1}$)

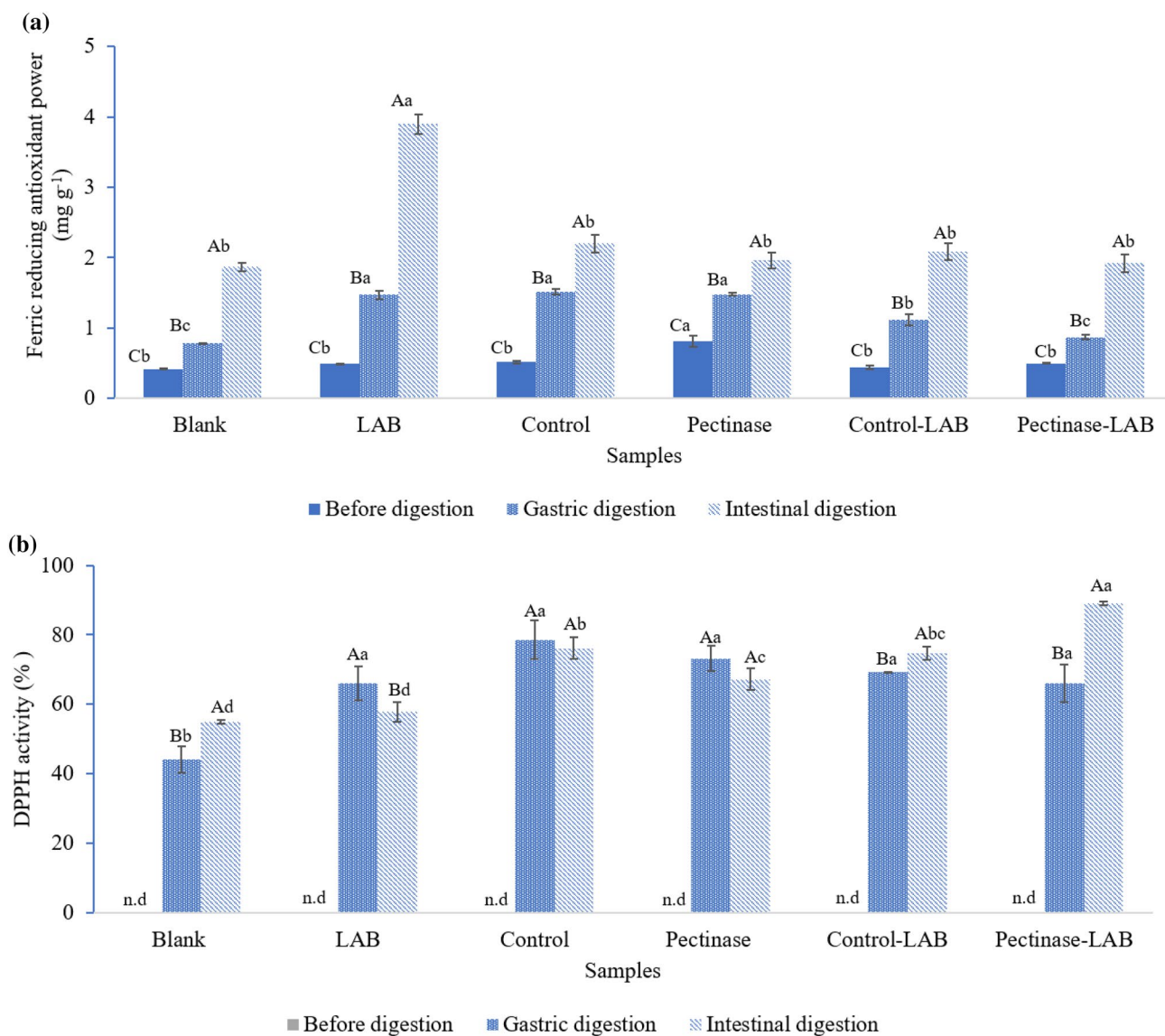


Fig. 2 Effect of in-vitro gastrointestinal digestion on the **a** ferric reducing antioxidant power (FRAP) and **b** DPPH scavenging activity changes in different types of cheese ($n=3$). (1) Blank indicates cheese without MLE fortification and LAB fermentation, LAB indicates LAB-fermented cheese without MLE fortification, UW indicates cheese fortified with UW-MLE, PUW indicates cheese fortified

with PUW-MLE, UW-LAB indicates LAB-fermented cheese fortified with UW-MLE. (2) A–C: Different alphabets indicate there is significant difference ($p < 0.05$) between different stages of digestion in a sample. (3) a–c: Different alphabets indicate there is significant difference ($p < 0.05$) between samples at a same stage of digestion

before digestion. Besides, DPPH scavenging activity was also not detected in all cheeses before digestion. Results of Pearson correlation analysis unveils that FRAP was strongly correlated with TFC of the cheeses ($r=0.833$ at $p<0.01$). This finding suggests that interactions between the phytochemicals with casein greatly reduced the antioxidant activity of the compounds. The binding of polyphenols with protein restricts the involvement of the compounds in the redox reactions and radical scavenging mechanisms [56]. Moreover, MLEs that were produced are believed to contain more water-soluble phytochemicals because water was used as the extraction solvent. Thus, DPPH assay in methanol system was believed to have a higher limit of detection for water-soluble antioxidants [57]. This explains why FRAP can be detected in cheeses before digestion, but not for DPPH activity.

However, both FRAP and DPPH activity had increased substantially after in-vitro gastrointestinal digestion in all cheeses. The disruption of cheese matrix and polyphenols-casein linkages during digestion upsurges the bioaccessibility of the bioactive constituents [58]. All MLE-fortified cheeses display higher DPPH activity than the blank. However, only LAB-fermented cheese exhibited higher FRAP than the blank. After both phases of digestion, LAB-fermented cheese showed the highest FRAP ($3.90 \pm 0.14 \text{ mg g}^{-1}$) whereas PUW-LAB cheese showed the highest DPPH activity ($88.97 \pm 0.59\%$). DPPH activity of LAB-fermented cheese was not significant different with the blank ($54.90 \pm 0.52\%$). According to the results of Pearson correlation analysis, FRAP shown after digestion

of cheeses was positively correlated with its TPC ($r=0.695$ at $p<0.01$). However, the DPPH activity did not correlate with both TPC and TFC. This finding proposes that PUW-MLE probably promotes the secretion of metabolites with free radical scavenging activity by *L. plantarum* TAR4 during fermentation. In the study by Renata et al. [59], DPPH activity of herb-fortified cottage cheeses with rosemary, pepper, and parsley respectively was higher than the blank. The snack fortified with 10% mulberry leaves powder was proven to exhibit high free radicals scavenging activity compared to the blank [57]. Moreover, cheddar cheese added with *Lactobacillus rhamnosus* was also found to contain higher antioxidant activity than the cheese without probiotic after digestion [60].

Figure 3 demonstrates the change of α -amylase inhibition activity (AI) of different types of cheese after in-vitro gastrointestinal digestion. AI of all cheeses, except UW-LAB cheese was enhanced after MLE fortification and probiotic fermentation. AI of cheese was increased about 37–39% from $50.61 \pm 0.57\%$ (blank) to $87.16 \pm 3.21\%$ and $89.76 \pm 1.81\%$ respectively after fermented with *L. plantarum* TAR4 and fortified with UW-MLE. However, combined UW-MLE-fortification and probiotic fermentation had reduced the AI by 27% to $23.81 \pm 1.59\%$, but not for the fortification with PUW-MLE. Combined PUW-MLE-fortification and probiotic fermentation enhanced AI to $78.92 \pm 1.81\%$. This finding was in line with the result of DPPH activity, whereby combined PUW-MLE-fortification and probiotic fermentation produced cottage cheese with the highest DPPH activity after digestion. According to

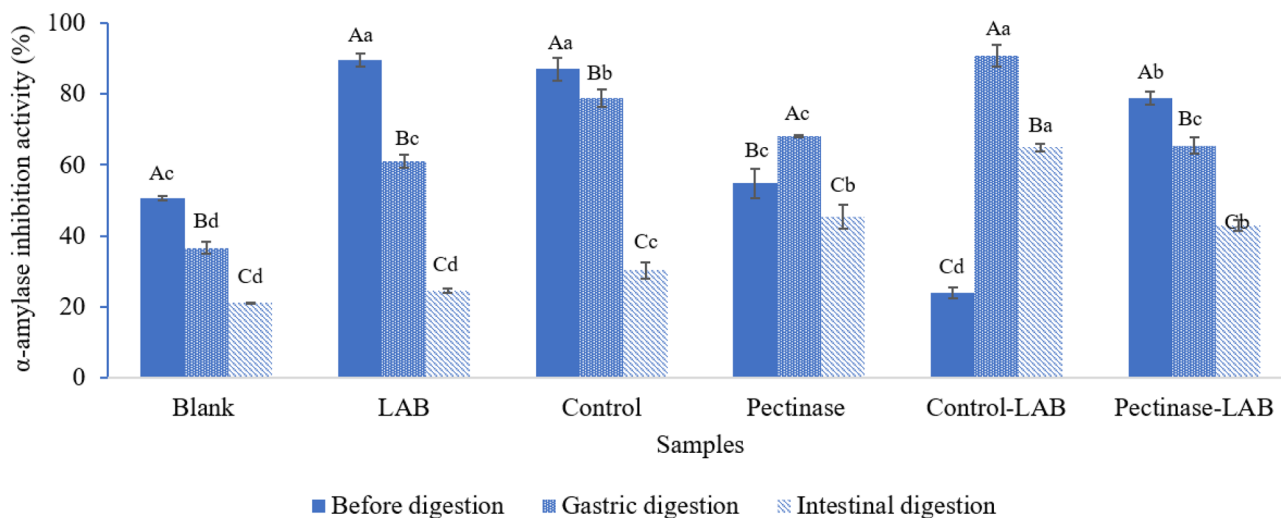


Fig. 3 Effect of in-vitro gastrointestinal digestion on the α -amylase inhibition activity (AI) change in different types of cheese ($n=3$). (1) Blank indicates cheese without MLE fortification and LAB fermentation, LAB indicates LAB-fermented cheese without MLE fortification, UW indicates cheese fortified with UW-MLE, PUW indicates cheese fortified with PUW-MLE, UW-LAB indicates LAB-

fermented cheese fortified with UW-MLW and PUW-LAB indicates LAB-fermented cheese fortified with PUW-MLE. (2) A–C: Different alphabets indicate there is significant difference ($p<0.05$) between different stages of digestion in a sample. (3) a–c: Different alphabets indicate there is significant difference ($p<0.05$) between samples at a same stage of digestion

Ujiroghene et al. [61], probiotic fermented products can be used as a complementary or adjuvant therapy in diabetes treatment. In their study, the sprouted quinoa yoghurt drink fermented with LAB showed prominent α -amylase inhibition activity of 86.11% at a concentration of 400 μ l. Furthermore, MLE is well-known to contain a broad spectrum of phytochemicals, such as kaempferol, rutin, myricetin, quercetin, etc. that possess α -amylase inhibition activity [62]. Therefore, the increase of AI of cottage cheese following MLE fortification was expected. During cheese production, whey served as the valuable protein source of bioactive peptides. LAB fermentation hydrolyzes the protein molecules into free amino acids and small peptides [63]. According to Siow et al. [64], aromatic amino acids of the peptides could inhibit α -amylase enzyme activity by forming hydrogen bonds, electrostatic interaction and van der Waals interaction with the amino acid residues of the active site of the enzyme, hence inducing enzyme conformation changes which subsequently lose its activity. This explained the relatively high AI showed in LAB-fermented cheese.

However, AI of LAB-fermented cheese unable to be retained after in-vitro gastrointestinal digestion. AI of LAB-fermented cheese was dropped by 3.7-fold to $24.39 \pm 0.62\%$ after digestion. Its AI was no significant different with the blank. Surprisingly, UW-LAB cheese which had the lowest AI before digestion exhibits the highest activity ($64.91 \pm 1.16\%$) after digestion. This finding suggests that LAB-fermentation likely modified the cheese protein matrix, and thus provide protection to the phytochemicals from degradation under harsh digestion condition. The fermentation

process improved the protein quality by altering the structure of protein, where it significantly reduced the α -helices as more random coil components were detected [65]. Protein degradation and aggregation is vital in physicochemical properties of a food [66]. The protein-rich solid dairy matrix entrapped phytochemicals and limit the degradation possibly due to the complex formed in the matrix act as protection layer where the bioactive compounds are sterically shielded and protected from oxidizing agents [58].

Figure 4 shows the change of albumin denaturation inhibition activity (ADI) of different types of cheese after in-vitro gastrointestinal digestion. Results in Fig. 4 clearly evidence that ADI was improved markedly with the MLE fortification and LAB fermentation. ADI of cottage cheese was increased from $7.99 \pm 0.71\%$ to $28.43 \pm 0.54\%$ after probiotic fermentation. Furthermore, combined UW-MLE fortification and probiotic fermentation did not improve the ADI of cheese ($26.52 \pm 1.01\%$) significantly ($p > 0.05$). These results further proved the important role of *L. plantarum* TAR4 in improving bioactive quality of cottage cheese. *L. plantarum* TAR4 fermentation not only improved the ADI of cottage cheese, but also increase the AI, DPPH and FRAP of the cheese. According to Santiago-López et al. [67], LAB fermentation in cheese curd tends to produce short chain peptides, γ -aminobutyric acid, conjugated linoleic acid, etc. which exhibited anti-inflammatory activity. In addition, the results also showed an increase of ADI in all cheeses upon digestion. ADI of blank cheese was increased by about threefold to $22.28 \pm 0.32\%$, whereas ADI of the fortified and fermented cheeses was improved by about 1.5-fold

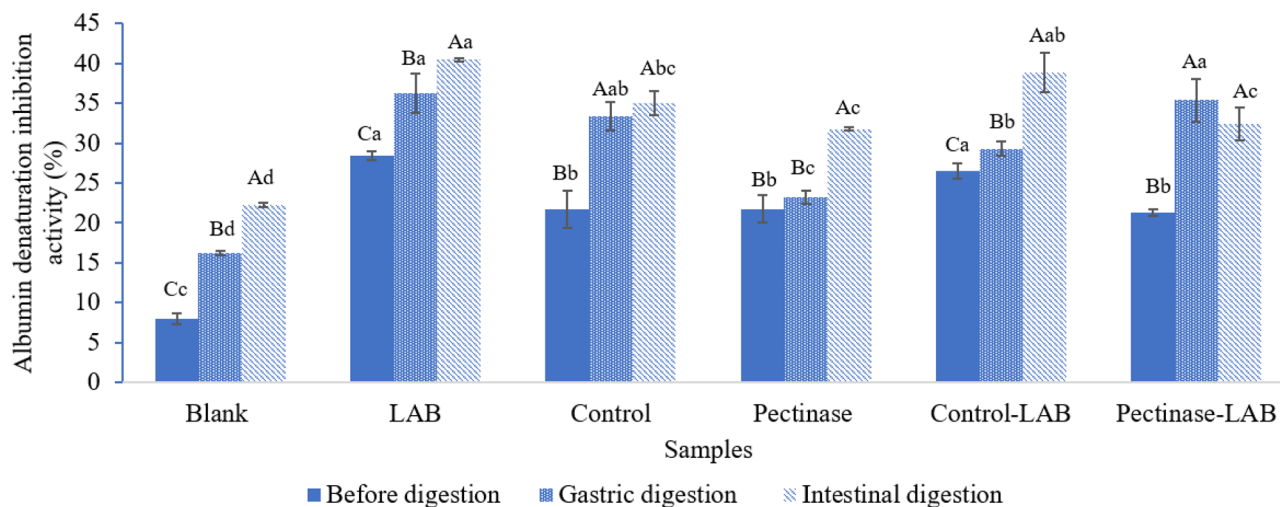


Fig. 4 Effect of in-vitro gastrointestinal digestion on the albumin denaturation inhibition activity (ADI) change in different types of cheese ($n=3$). (1) Blank indicates cheese without MLE fortification and LAB fermentation, LAB indicates LAB-fermented cheese without MLE fortification, UW indicates cheese fortified with UW-MLE, PUW indicates cheese fortified with PUW-MLE, UW-LAB indicates

LAB-fermented cheese fortified with UW-MLW and PUW-LAB indicates LAB-fermented cheese fortified with PUW-MLE. (2) A–C: Different alphabets indicate there is significant difference ($p < 0.05$) between different stages of digestion in a sample. (3) a–d: Different alphabets indicate there is significant difference ($p < 0.05$) between samples at a same stage of digestion

after gastrointestinal digestion. Among the cheeses, LAB-fermented cheese ($40.45 \pm 0.25\%$) and UW-LAB-fermented cheese ($38.83 \pm 2.50\%$) showed the highest ADI after digestion. This result suggests that digestion increased the ADI of cheese significantly. During gastrointestinal digestion, protein macromolecules were broken down. Therefore, more bioactive short chain peptides, fermentation metabolites and phytochemicals of MLE were released from the cheese curd, thus ADI was increased [68].

By comparing the FRAP, DPPH, AI, and ADI activities of all types of cheese, UW-LAB cottage cheese was claimed as the best functional cheese in this study. Result obtained indicates that UW-LAB cottage cheese had the highest AI and ADI activities after digestion. Although PUW-LAB cottage cheese exhibited the highest DPPH activity after digestion, its AI and ADI were approximately 22% and 7% lower than UW-LAB cottage cheese, respectively. The DPPH activity of UW-LAB cottage cheese was only about 14% lower than PUW-LAB cottage cheese. FRAP of both types of cheese had no significant difference ($p > 0.05$). In view of better nutrition benefits of UW-LAB cottage cheese, applying *L. plantarum* TAR4 fermentation on cottage cheese fortified with MLE produced via ultrasound-assisted water extraction was recommended as the best strategy.

Conclusion

In this study, ultrasound-assisted water extraction was proved to be the best method to produce MLE with the highest DPPH scavenging activity and ADI activity, whereas combined pectinase pretreatment ultrasound-assisted water extraction was the best to produce MLE with the highest FRAP and AI activity. Combined MLE fortification and *L. plantarum* TAR4 fermentation synergistically improved antioxidant, anti-inflammation, and hypoglycemic activities of cottage cheese. Moreover, the study also proved that in-vitro gastrointestinal digestion did not exert significant detrimental effects on the bioactive properties of the cottage cheese. From this study, *L. plantarum* TAR4 fermented cottage cheese fortified with MLE produced via ultrasound-assisted water extraction was proven as the best functional cheese with superior nutraceutical properties.

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Author contributions Study conception and design were contributed by PLT. Material preparation, data collection and analysis were performed by XYT. The *Lactobacillus plantarum* TAR4 strain was isolated by HYT. The first draft of the manuscript was written by HCC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data of this study are available from the corresponding author upon request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

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