Impacts of supplementation of probiotics on the prevalence of grape marc derived polyphenols in colonic digesta using in vitro digestion model

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Abstract. Grape marc is a by-product of winemaking that contains numerous polyphenols, the bioavailability of polyphenol is largely dependent on their metabolism by probiotics or gut microbiome. The two-way phenolic-microbiota interaction has been postulated as the microorganisms and specific polyphenols could affect each other and exert beneficial effects on the host health. However, the oral taken probiotics showed a low survival rate in the gastrointestinal tract, while using encapsulation technique can provide them with a protective microenvironment. The objective of this study was to investigate the bioaccessibility of polyphenols originated from whole grape marc, consisting of seeds, skins and stems, combined with encapsulated oral taken probiotics, using in vitro digestion model and analytical chemistry techniques. 23 phenolic compounds were evaluated in this study, which included phenolic acids, anthoxanthins and stilbenes. This study showed that the encapsulated probiotics were protected by microcapsules until being liberated in the large intestine, and the metabolism of some polyphenols derived from selected grape marc samples was promoted by probiotics. These results indicate the potential of combining grape marc polyphenols with probiotics to develop a new health product. Furthermore, this provides the wine industry with an opportunity to make a profit by using by-products.

1. Introduction

10 million tons of grape marc, which consisted of seeds, skins and stems, are produced annually worldwide by the wine industry after the fermentation of grapes [1]. Conventionally, they were used as compost and animal feed, or ingredients to produce a liquor called ‘Pomace Brandy’ [2,3]. Previous studies have found that part of the phenolic compounds, with several health-promoting potentials, such as antioxidant, anti-inflammatory, anti-carcinogenic and neuroprotective ability, is retained in the grape marc [4-7]. However, these benefits can only be achieved if the polyphenols are digested and absorbed by human. Direct absorption of dietary polyphenols by the human body in the small intestine is only 5 to 10 percent of total consumption, while the remaining 90 to 95 percent of unabsorbed polyphenols are delivered to the large intestine and potentially utilized by the gut microbiota [8]. Studies suggested that the poor bioavailability of dietary polyphenols might be due to its polymerized structure where dimers and polymers were difficult to be absorbed in the small intestine. Besides, glycosylation of polyphenols also compromised their bioavailability for the absorption in small intestine [9]. Research found that the
bioaccessibility of the polyphenols in the human body could be improved through their reaction with microorganisms, including gut microbiota and probiotics [10]. Gut microbiota could metabolise polyphenols by cleaving the glycosidic linkages in the phenolic compounds and breaking down the heterocyclic backbone in the phenolic structure, while the oral taken probiotics could enhance these effects if they can reach the gastrointestinal tract and modulate the gut microflora [11-13]. Lactobacillus plantarum is implemented as the model probiotics in this study because it is easy to produce, has a human origin, and has relatively higher tolerance to the low pH environment and bile salts exposure as compared to the other commonly used probiotics [14-15]. However, their oral delivery shows a low efficiency due to the low survival rate of free bacterial cells during digestion [16]. To protect the probiotics from being damaged, encapsulation techniques have been developed. Encapsulation consisted of the entrapment of target substance with a carrier agent, which could provide target substance with a micro-environment and therefore enhance its stability [17-18]. With the use of encapsulation, probiotics will not be released until they reach their desired destination [10].

In this study, alginate was chosen as the encapsulation material, and extrusion as the encapsulation technique. Alginate is a type of carbohydrate polymers extracted from algae [19]. Alginate contains mannuronic and guluronic acid; with the presence of calcium ions, it can lead to complex formation of compact and strong gels [20]. The effects of alginate have been demonstrated by some previous studies, for example, the non-encapsulated Bifidobacterium longum BIOMA 5920 was not able to survive in simulated gastric fluid, whereas the alginate encapsulated B. longum survived with a four log cycles reduction after two hours of gastric fluid treatment [21]. Similar results have been reported by Annan et al., as the survival rate of the free bacteria showed two log units lower compared with alginate encapsulated Bifidobacterium adolescentis 15703T [22]. For the extrusion technique, it has the characteristics of simple operation, cost-effective equipment, and no deleterious materials. This gentle operation process costs minimal damage to the cells with high cell viability achieved, under both aerobic and anaerobic conditions. However, the main drawback of this technique is that the slow formation of microcapsules, which makes it unsuitable to be adopted in large scale production [23]. Besides, the formation of high-diameter particles (>1mm) using extrusion method limits its applications in food industry, where mouthfeel is a crucial factor [24].

The interactions between phenolic compounds and probiotics, particularly the probiotic effect on the release of polyphenols derived from grape marc, is important factor for the polyphenols to exert beneficial health effects. Besides, the understanding of the metabolism and bioavailability of grape marc polyphenols requires further in-depth investigation. There are insufficient studies up to date to illustrate these issues. Therefore, in order to explore these research gaps, this study aims to investigate the bioaccessibility of polyphenols originated from grape marc combined with oral taken probiotic L. plantarum, using in vitro digestion model and analytical chemistry techniques. In details, the main objectives focus on the aspects as the following: i) to select one grape marc sample from red and white grape respectively collected from different wineries, by determining their total phenolic content and antioxidant activity; ii) to encapsulate probiotics using extrusion technique and investigate their effect; iii) to investigate the effects of probiotics on the release of polyphenols in the gastrointestinal tract using in vitro digestion model; iv) to determine the phenolic compounds of grape marc and the digesta of the combination of grape marc and probiotics with HPLC technique.

2. Materials and methods

2.1. Materials and chemicals

The following reagents and chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): MRS broth, calcium chloride, sodium alginate, sodium chloride, methanol, hydrogen chloride, 2.0N FCR, sodium carbonate solution, gallic acid, glacial acetic acid, sodium hydroxide, 2,4,6-Tris(2-pyridyl)-s-triazine, iron (III) chloride anhydrous, iron (II) sulfate heptahydrate, 2,2-Diphenyl-1-picrylhydrazyl, trolox, potassium chloride, monopotassium phosphate, sodium bicarbonate, magnesium chloride, ammonium carbonate, salivary α-amylase, porcine pepsin, pancreatin, sodium phosphate
dibasic, sodium phosphate monobasic, bile salts, peptone, yeast extract, casein, L-Cysteine HCl, Tween-80, guar, soluble starch, tryptone, pectin, mucin, sodium bicarbonate, magnesium sulfate, dipotassium phosphate, acetic acid 50% solution, 5-(hydroxymethyl)furfural, protocatechuic acid, p-hydroxybenzoic acid, caftaric acid, caffeic acid, p-coumaric acid, syringic acid, ferulic acid, trans-sinapinic acid, procyanidin B1, procyanidin B2, resveratrol, epicatechin, catechin, epicatechin gallate, polydatin, quercetin, quercetin 3-O-glucoside, quercetin 3-O-galactoside, quercetin 3-O-glucuronide, quercetin 3-O-rhamnoside, kaempferol and kaempferol 3-O-glucoside.

LAMVAB plates were purchased from media preparation unit of the department of microbiology & immunology, The University of Melbourne (VIC, Australia). All water used was prepared by Milli-Q Gradient water purification system (Millipore Australia Pty. Ltd., North Ryde, NSW, Australia). HPLC grade Acetonitrile was obtained from Merek Pty. Ltd. (Bayswater, VIC, Australia).

Eight grape marc samples, which consisted of seeds, skins and stems, were collected from 3 vineyards located in Victoria, Australia. 6 different red grape marc samples were collected from Mount Langi Ghiran winery in Grampians wine region. Middle Old Blocks marc (OM) and West Old Blocks marc (OW) were collected in 2016 vintage, the OM grapes were collected by hand picking while OW grapes were collected by machine. The grape marc of cabernet sauvignon (CS), shiraz marc from Contour Block (TR), shiraz marc from South Block (SS) and shiraz marc from the House Block (HB) were collected in 2018 from the same winery. White grape marc used were from Chardonnay grape collected in 2018 vintage from the Bowen Ridge Winery (BR) and Leura Park winery (LP) in Geelong wine region, Victoria, Australia.

2.2. Grape marc selection

The harvested grape marc samples were freeze-dried, blended into powder, extracted by methanol, centrifuged and filtered before further analyses [25]. One red and one white grape marc sample with the highest antioxidant and scavenging capacity were identified from the red and white grape marc collection for the further in vitro digestion experiment.

2.2.1. Determination of total phenolic content (TPC). The spectrophotometric detection of chromogens was the basis of TPC’s quantification. When the functional hydroxyl groups of phenols reacted with the reagent containing phosphomolybdic/ phosphotungstic acid complexes, the chromogens formed [26]. The absorbance of the reacted mixture was measured against the blank sample at the wavelength of 765nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland). The TPC of samples were expressed as gallic acid equivalents (GAE) [mg GAE/g sample dry mass (DM)]. All the determinations were triplicated.

2.2.2. Determination of ferric reducing antioxidant power (FRAP). The ability of antioxidants as reductants to reduce the colourless ferric tripyridyltriazine complex to blue ferrous-(2,4,6-tripyridyl-s-triazine)2 in the oxidation-reduction reaction was the basis of FRAP test. The absorbance of the reacted mixture was measured at the wavelength of 593nm [27]. The FRAP value of samples was expressed as Fe2+ equivalents (mM Fe2+ eq./g sample DM). All the determinations were triplicated.

2.2.3. Determination of DPPH radical scavenging capacity. The mechanism of donation of hydrogen to the DPPH radical by antioxidants, which acted as radical scavengers was the basis of DPPH assay. The absorbance of the reacted mixture was measured at the wavelength of 515nm [28-29]. The DPPH radical scavenging capacity of samples was expressed as Trolox equivalents (mg Trolox eq./g sample DM). All the determinations were triplicated.

2.3. In vitro digestion

2.3.1. Experimental design. Group A was the treatment group with faeces added in the in vitro digestion, which comprised of 2 types of grape marc powder, namely the white grape marc powder (WC) and red
grape marc powder (RC); and 4 treatment groups with the incorporation of probiotics, namely the white grape marc powder plus probiotics powder (WP), red grape marc powder plus probiotics powder (RP), white grape marc powder plus encapsulated probiotics (WPE), and red grape marc powder plus encapsulated probiotics (RPE). Group B was the treatment control group with no faeces added in the in vitro digestion, while other conditions were similar with group A, and named as WCn, RCn, WPn, RPn, WPe, RPe.

2.3.2. Probiotic preparation. The probiotics were sub-cultured and incubated in MRS-broth. After centrifugation, wash, resuspension and freeze-dry, the probiotics were processed into powder [30-31].

2.3.3. Encapsulation. 0.25 g freeze-dried probiotic powder was dissolved into 600 μl sterile saline solution, and then suspended in 800 μl 2% sodium alginate solution, which acted as hydrocolloid solution. 7% calcium chloride solution which acted as hardening solution was prepared and sterilised prior to the extrusion process. For each treatment, 25 mL of 7% calcium chloride solution was used to harden the droplets, and it was used only once for each treatment to ensure the same effect among treatments. Via a syringe, the viscous bacteria suspension was passed through an 18-gauge needle and allowed to free-fall onto a culture dish with 25 mL calcium chloride solution to generate droplets. The droplets were allowed to gel for 1 minute to form the encapsulated microcapsules. The hardening solution together with the microcapsules was then filtered by a sterilised cell tissue strainer and the microcapsules were transferred into the 50mL centrifuge tubes and ready for in vitro digestion [12,16].

2.3.4. In vitro digestion. The in vitro digestion experiment was carried out following two previous studies [32-33], with modifications to better fit the research objectives. There were 4 stages in this experiment, namely oral, gastric, small intestinal and large intestinal phases. The pig faeces, which collected freshly from 12-weeks old pigs in the animal house at the Dookie campus of the University of Melbourne, were used to simulate the microbial environment of the large intestine.

2.3.5. Microbial count. Drop plate method based on a published protocol was adopted for the quantification of lactobacilli, with modifications to better fit the research objectives [34].

2.3.6. Characterisation of polyphenols by HPLC analysis. The HPLC analysis of polyphenols was performed by the Waters 2690 Alliance HPLC separation module (Waters, Rydalmere, NSW, Australia) collocated with Waters 2998 Photodiode Array (PDA) detector, following a published protocol. The external standard method and standard addition HPLC calibration method were applied to identify and quantify the phenolic compounds in the sample extracts.

The preparation of samples for high-performance liquid chromatography (HPLC) analysis was conducted in three methods depending on the sample. For the grape marc samples, 5 g freeze-dried grape marc powder together with 50 mL 1% HCl v/v methanol were mixed and extracted for 24 h at 20 °C by the shaking incubator at 150 rpm, then the mixture was centrifuged at 6500 rpm for 10 minutes at 20 °C, and the supernatant was collected. The supernatants were filtered by 30mm × 0.45μm nylon syringe filter (Thermo Fisher Scientific, VIC, Australia) prior to being evaporated by a Hei-Vap rotary evaporator and vacuum pump (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). The residue was redissolved in 10 mL of HPLC grade methanol, then filtered by 30mm × 0.45μm nylon syringe filter and transferred to amber vials [35]. For the small intestinal digesta supernatant, they were 2-fold diluted by HPLC grade methanol, then filtered by 30mm × 0.45μm nylon syringe filter and transferred to amber vials. For the colonic fermentation digesta supernatant, 1mL of each sample were mixed thoroughly with 3mL of acetone: methanol (1:1, v/v) by a vortex for 30 s, and were then centrifuged at 5400g for 5 minutes at 20 °C. the resulted supernatant was collected in a 10mL-glass bottle and dried under nitrogen gas flow at room temperature. The residue was redissolved in 1.5 mL of HPLC grade methanol, then filtered by 30mm × 0.45μm nylon syringe filter and transferred to amber vials [36].
Two systems were performed separately to identify the phenolic acids (System I) and anthoxanthins and stilbenes (System II) in the sample extracts. For both systems, the mobile phases were the same, namely acetic acid in Milli-Q water (2:98, v/v) as solvent A, and 0.5% (v/v) acetic acid in water and acetonitrile (50:50, v/v) as solvent B. All the mobile phases were degassed using an ultrasonic degasser. In System I, the total run time was 60 minutes, all samples were injected with a volume of 10 µL, and the synchronous detection was conducted for hydroxybenzoic acids at 280nm and hydroxycinnamic acids at 320nm. In the elution process, the flow rate was kept constant at 1.0 mL/min, and a gradient program was adopted as, in the first 10 minutes, the proportion of solvent A decreased from 90 to 85%, followed by an isocratic period at 85% for 3 minutes. After that, the proportion of solvent A decreased from 85% to 75% for 7 minutes, and then further declined from 75% to 45% for 30 min. In the following 1 minute, its proportion decreased from 45 to 0% and kept at 0% for the next 5 minutes for column washing purpose. Finally, the proportion of solvent A was increased from 0% to 90% in last 4 minutes for equilibration. In System II, the total run time was 72 minutes, all samples were injected with a volume of 10 µL, and the synchronous detection was conducted for flavanols at 280nm, stilbenes at 320nm, and flavonols at 370nm. In the elution process, the flow rate was kept constant at 1.0 mL/min, and a gradient program was adopted as, from 90 to 76% solvent A (20 min), from 76 to 70% solvent A (20 min), from 70 to 45% solvent A (20 min), from 45 to 0% solvent A (5 min), 0% solvent A isocratic period (5 min) for column wash, and finally, from 0 to 90% solvent A in the last 2 minutes for equilibration [25].

2.3.7. Statistical analysis. Statistical analysis was conducted using Minitab 17 software (Minitab, Inc., NSW Australia). The significance of differences among the generated data was performed by doing the one-way analysis of variance (ANOVA). Results were demonstrated as mean ± standard deviation and noted with different letters when significant differences were found in the mean at the confidence level of p < 0.05.

3. Results and discussion

3.1. Total phenolic content and antioxidant activities of grape marc

The analyses of TPC, FRAP, DPPH (Table 1) showed that, among the red grape marc samples, sample CS showed a higher TPC and FRAP values compared to the other five samples, with the TPC of 30.9 ± 0.70 mg GAE/g sample DM, and FRAP value of 385 ± 17.6 mM Fe 2+ eq/g sample DM (DM, dry mass of the freeze-dried grape marc sample). Its DPPH radical scavenging capacity showed no difference with SS, TR and HB, but had the highest value of 27.4 ± 0.44 mg Trolox eq/g sample DM. In comparison with the reported value from the study by Rockenbach and his team, sample CS exhibited lower TPC and DPPH radical scavenging capacity than the Cabernet Sauvignon grape pomace with a TPC of 74.7 ± 2.22 mg GAE/g sample DM and 505 ± 4.62 μmol Trolox eq/g sample DM, respectively. Besides, TPC and DPPH activity of the grape pomaces from other varieties such as Merlot, Bordeaux, and Isabel were also higher than the sample CS [37]. However, as compared to another study, DPPH activity of sample CS of 109 ± 1.75 μmol Trolox eq/g sample DM (equivalent to 27.4 ± 0.44 mg Trolox eq/g sample DM) is higher than the grape pomaces from varieties such as Sangiovese (81.4 ± 1.14 μmol Trolox eq/g sample DM), Primitivo (77.9 ± 0.91 μmol Trolox eq/g sample DM), and Negro Amaro (26.9 ± 0.32 μmol Trolox eq/g sample DM) [38].

Between white grape marc samples, the sample BR had a higher TPC, FRAP and DPPH values compared with sample LP. Consequently, sample CS and sample BR were selected as the model grape marc in the further experiment.
Table 1. Total phenolic content and antioxidant activities of red and white grape marc samples.

| Sample Name | TPC (mg GAE/g sample DM) | FRAP (mM Fe2+ eq/g sample DM) | DPPH (mg trolox eq/g sample DM) |
|-------------|--------------------------|-------------------------------|--------------------------------|
| Red Grape   |                          |                               |                                |
| CS          | 30.9 ± 0.70 a            | 385 ± 17.6 a                  | 27.4 ± 0.44 a                  |
| SS          | 23.3 ± 1.67 b            | 260 ± 17.6 b                  | 27.3 ± 0.17 a                  |
| TR          | 22.0 ± 0.04 b            | 256 ± 18.4 b                  | 27.1 ± 0.23 a                  |
| HB          | 19.9 ± 1.39 c            | 210 ± 15.8 c                  | 26.3 ± 0.56 a                  |
| OM          | 13.2 ± 0.89 d            | 122 ± 8.54 d                  | 17.8 ± 3.46 b                  |
| OW          | 10.7 ± 0.48 e            | 100 ± 13.7 d                  | 16.7 ± 3.25 b                  |
| White Grape |                          |                               |                                |
| BR          | 17.8 ± 0.52 a            | 161 ± 3.52 a                  | 27.1 ± 0.16 a                  |
| LP          | 14.1 ± 1.81 b            | 123 ± 18.2 b                  | 24.6 ± 2.11 b                  |

Different letters within each row indicate statistically significant differences between treatments by one-way ANOVA (P < 0.05).

3.2. Microbial content of digesta

Table 2 showed the microbial counts of small and large intestinal digesta of different treatments, with the data being subtracted against the microbial counts of the blank group. According to the small intestinal digesta results, treatments with un-encapsulated probiotics showed a significantly higher value than those with encapsulated probiotics, which indicated that encapsulation could protect probiotics from releasing in the small intestine. Some previous research showed that encapsulated probiotics can better survive in simulated gastric and intestinal environment with bile salt added compared with free cells [39,40]. It is postulated that encapsulation technique can improve the low pH tolerance of probiotics passing through the in vitro gastric digestion which is acidic. Encapsulation also provides a protective barrier for the probiotics during the in vitro small intestinal digestion where the presence of bile salt that can cause damage to the probiotics [41]. Regarding large intestinal digesta results, treatments with encapsulated probiotics showed a significantly higher value than those without encapsulation, which indicated that encapsulation could effectively deliver probiotics to the large intestine, and the probiotics were released after colonic fermentation.

Table 2. Microbial counts of small and large intestinal digesta.

|            | Small intestinal digesta (CFU/g) | Large intestinal digesta (CFU/g) |
|------------|----------------------------------|----------------------------------|
| RM         | 0.00 b                           | 1.29×10^7 ± 7.27×10^6 b          |
| RP         | 2.50×10^7 ± 6.56×10^6 a           | 9.80×10^7 ± 4.91×10^7 b          |
| RPE        | 4.23×10^4 ± 3.28×10^4 b           | 1.60×10^9 ± 1.04×10^9 a          |
| RMn        | 0.00 b                           | 6.00×10^5 ± 3.61×10^5 b          |
| RPe        | 2.30×10^7 ± 2.65×10^6 a           | 7.67×10^7 ± 5.51×10^7 b          |
| RPEn       | 4.03×10^4 ± 4.08×10^4 b           | 6.83×10^8 ± 4.54×10^8 a          |
| WM         | 0.00 b                           | 3.53×10^8 ± 1.17×10^8 a          |
| WP         | 6.73×10^7 ± 3.06×10^6 a           | 9.50×10^8 ± 5.22×10^8 a          |
| WPE        | 7.47×10^5 ± 1.12×10^5 b           | 6.07×10^9 ± 6.87×10^9 a          |
| WMn        | 0.00 b                           | 7.00×10^7 ± 4.36×10^7 b          |
| WPN        | 6.97×10^7 ± 5.51×10^6 a           | 4.70×10^8 ± 4.59×10^8 b          |
WPEn $7.03 \times 10^4 \pm 6.51 \times 10^4 \text{b}$ $1.60 \times 10^9 \pm 7.00 \times 10^8 \text{a}$

Different letters within each row indicate statistically significant differences between treatments by one-way ANOVA ($P < 0.05$).

3.3. Polyphenol changes after in vitro digestion

Table 3 showed the 10 identified polyphenols out of 23 standard compounds, other polyphenols that were not identified from the grape marc samples and the supernatant of intestinal digested samples are: gallic acid, 5-(hydroxymethyl) furfural, caftaric acid, caffeic acid, sinapic acid, polydatin, quercetin 3-O-galatoside, quercetin 3-O-glucuronide, quercetin 3-O-glucoside, kaempferol 3-O-glucoside, quercetin 3-O-rhamnoside, epicatechin gallate and quercetin. Due to the detection limit for the methodology and equipment used, most of the target compounds were not identified. In the following relevant projects and further research, LC-MS analysis is recommended as an efficient alternative to evaluate the digestion of phenolic compounds.

3.3.1. Small Intestinal digesta. According to the results obtained in the HPLC analysis of small intestinal digesta, three major changes were observed. Firstly, the polyphenols that found in the grape marc did not exist in the digesta. This phenomenon was hypothesized as the parental polyphenols found in the grape marc were bio-transformed by the probiotics used into other phenolic metabolites after digestion, where those metabolites were not included in the standard compounds in this experiment. For example, L. plantarum IFPL.935 was shown to metabolize the flavan-3-ol enriched grape seed extract with the action of galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase that led to the formation of gallic acid, pyrogallol and catechol, respectively [42]. Secondly, the metabolites of polyphenols, such as protocatechuic acid was not found in the grape marc sample, but in the digesta. This is in agreement with some previous research, which showed that protocatechuic acid was a metabolite of catechins, protocatechuyl ester or cyanidin-glucosides 1–3. As protocatechuyl ester can be degraded by the esterase of L. plantarum to protocatechuic acid, the protocatechuic acid detected in small intestine digesta might be due to the degradation of protocatechuyl ester [43-45]. In addition, the third observation was that the polyphenols’ concentrations increased after digestion, this may be due to they were metabolites originated from other polyphenols. To support this, p-coumaric acid is reported to be attached to the cell wall covalently, and it is necessary to break down the ester linkages between polymers to release p-coumaric acid from the cell wall. Feruloyl esterases are reported to be the important enzymes in this reaction [46,47].

Comparing the effect of probiotics in the treatments, concentrations of polyphenols demonstrated a higher value among treatments without probiotics added than those with probiotics added. For example, by comparing the concentration of p-coumaric acid in RM, RP and RPE, it was found that p-coumaric acid in RM was significantly higher. According to a previous study, in controlled fermentation, the presence of lactobacillus could lead to the conversion of simple phenolic and the depolymerization of high-molecular-weight phenolic compounds [48]. Therefore, RP, which had the highest lactobacillus amount, had the lowest concentration of p-coumaric acid, which indicated that probiotics could promote the conversion of polyphenols into other forms that were easier to be absorbed.

Comparing the treatments with and without encapsulated probiotics, the concentrations of most of the polyphenols among treatments with encapsulation technique applied was lower than those without encapsulation. Therefore, the probiotic mainly plays a role in the treatments without encapsulation in the small intestine.

3.3.2. Colonic fermentation digesta. According to the results obtained in the HPLC analysis of the colonic fermentation digesta, syringic acid, and protocatechuic acid in red grape marc were detected in the large intestine. These polyphenols might be the metabolites originated from other phenolic compounds. A previous study showed that cyanidin-3-glucoside could undergo a significant degradation during digestion [49]. One of its major phenolic acid degradation products is protocatechuic acid.
Although cyanidin-3-glucoside was not analysed in this experiment, they have been found in grapes as an anthocyanin pigment [50]. Therefore, it is assumed that protocatechuic acid and ferulic acid were derived from cyanidin-3-glucoside in grape.

Comparing the treatments with and without probiotics, in majority treatments without probiotics added showed a significantly lower value than those with probiotics added. For example, the concentration of syringic acid among RMn, RPn and RPEn were $4.59 \pm 0.01$, $5.07 \pm 1.02$ and $7.59 \pm 1.93$ µg/g sample DM respectively. This might because of the release of encapsulated probiotics in the large intestine and therefore increased the production of syringic acid, as syringic acid is a product of microbial metabolism of anthocyanins and other polyphenols [51].

### 3.3.3. Small Intestinal digesta VS Colonic fermentation digesta

Comparing the difference of polyphenols’ concentrations between small intestinal digesta and colonic fermentation digesta, most of the polyphenols existed in colonic fermentation digesta exhibited increased concentrations. Take protocatechuic acid as an example, it showed an increase in RP and RPE in the large intestine, while it was not identified in RM after colonic fermentation. This phenomenon could be explained by the metabolism of protocatechuic acid from catechins, protocatechuoyl ester or cyanidin-glucosides by the action of lactobacillus. According to the results shown in Table 3, the concentration of protocatechuic acid increased in the large intestine, while catechin existed in grape marc samples but could not be identified in digesta. Therefore, catechin is suggested to be metabolized under the effect of L. plantarum with gut microbiota to produce protocatechuic acid.

**Table 3.** Polyphenols found in the grape marc, small and large intestinal digesta.

| Polyphenols          | Red grape (µg/g sample DM) | White grape (µg/g sample DM) |
|----------------------|-----------------------------|------------------------------|
|                      | RM | RP | RPE | RMn | RPn | RPEn | WM | WP | WPE | WMn | WPn | WPEn |
| p-hydroxybenzoic acid| ±  | —  | —   | 0.53| —   | —    | ±  | —  | —   | —   | —   | —     |
| Syringic acid        | ±  | 5.32| ±   | 53.99 ± 49.01 ± 45.58 | 2.06 ± 7.92 ± 0.64 | ±    | 15.38| 13.82 ± 12.98 ± 11.39 | ± 0.18| 1.33 ± | 0.67 ±1.03 |
| Ferulic acid         | —  | —  | —   | —   | —   | —    | —  | —  | —   | —   | —   | —     |
| Protocatechuic acid  | 23.10 ± 16.65 ± 3.58 | — | — | — | 19.23 | 0.11 ± 3.65 ± 3.58 | ± | — | — | — | — | — |
| p-coumaric acid      | 4.92 ± 3.93 ± 0.40 | — | — | — | 4.30 | 0.34 ± 0.52 ± 0.40 | ± | — | — | — | — | — |
4. Conclusion

According to the microbiial count results and the physical situation of the digested samples, this study demonstrated that extrusion technique using sodium alginate and chloride solution could encapsulate freeze-dried *L. plantarum* powder in small microcapsules and assisted in the delivery through digestive tract until their release in the large intestine. The HPLC analysis showed that 10 out of 23 common polyphenols compounds were abundant in the grape marc samples and the supernatant of intestinal digested samples. With the existence of *L. plantarum*, some complex phenolic compounds were metabolised to the simpler structure, and this effect was enhanced among the treatments with encapsulation technique applied during the colonic fermentation stage. Last but not least, the winery by-product grape marc showed a pharmaceutical potential due to its content of anti-oxidative phenolic compounds, which could benefit the winery industry in regard to waste utilisation.

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