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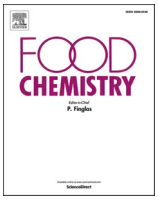

Alginate-inulin-chitosan based microspheres alter metabolic fate of encapsulated quercetin, promote short chain fatty acid production, and modulate pig gut microbiota

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ABSTRACT

Quercetin loaded alginate microspheres, fabricated with the inclusion of inulin as a prebiotic source and chitosan as protective coating (ALINCH-Q), were subjected to *in vitro* colonic fermentation using pig fecal microbiota, with empty microspheres ALINCH-E, unencapsulated quercetin UQ and media only Blank as parallel studies. ALINCH-Q altered quercetin biotransformation towards higher production of 3-hydroxyphenylpropionic acid and 3-hydroxyphenylacetic acid, and further metabolism of 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid compared to UQ. In addition, ALINCH-Q but not ALINCH-E or UQ significantly promoted SCFAs production compared to Blank. Furthermore, the ALINCH-Q microspheres altered the microbial compositions, increased the relative abundance of *Lactobacillus, Turicibacter, Eubacterium*, and *Clostridium*, while decreased that of the potentially pathogenic *Enterococcus*. The results suggest an interplay between the dietary fiber matrix and quercetin in producing these effects, and that ALINCH-Q could serve as a potential targeted delivery vehicle for quercetin to exert beneficial biological effects in the colon.

1. Introduction

Targeted delivery of bioactive to the colon presents a potential management strategy for colonic diseases such as Crohn's disease, ulcerative colitis, inflammatory bowel disease (IBD), and colorectal cancer for maximum efficacy (Liu et al., 2022b). Quercetin, a naturally occurring flavonol in our diets that is abundantly found in onions, tea, berries, apples and cherries, could be a potential food bioactive candidate (Kandemir et al., 2022). Studies have supported its pharmacological effects against colonic diseases through its strong antioxidant radical scavenging, reducing and metal chelating activity, and its bioactivity in regulating multiple cells signalling pathways and expression of inflammatory cytokines (Lin et al., 2019). Additionally, quercetin can also modulate the gut microbiota leaning to alleviating the microbiota dysbiosis linked to the pathogenesis of colonic diseases (Etxeberria et al., 2015; Lin et al., 2019; Tan et al., 2018). It is reported that the dietary intake of quercetin at a dose of 30 mg/kg in a colitis mice model could significantly ameliorated colitis, suppress the pro-inflammatory cytokines and enhance the growth of *Lactobacillus*, *Clostridia* and *Bifidobacterium,* which are shown to be effects beneficial to gut health (Lin et al., 2019).

However, quercetin exhibits low water solubility and is prone to undergo auto-oxidation and degradation in the upper gastrointestinal tract (GIT), significantly diminishing the amount and also the bioactivity of quercetin that reaches colonic site (Kandemir et al., 2022). Thus, there are increasing interests in encapsulating quercetin in various carriers for colon targeted delivery, most notably using natural biopolymers such as dietary fibers because they are not digestible in upper GIT but can be degraded by colonic microbial enzymes triggering release at the site (Liu et al., 2022b). Additionally, microbial metabolites derived from the fermentation of fibers such as short chain fatty acids (SCFAs) can provide health benefit to the gut, such as protective effects against intestinal inflammation by maintaining an intestinal barrier, inhibitory effects against carcinogenesis via their various immunomodulatory properties (Dalile et al., 2019). Furthermore, recent studies have supported the interplay between dietary fibers and phenolics in the gut, suggesting a synergistic effect on gut microbiota modulation (Loo et al., 2020; Loo et al., 2023).

There are number of dietary fibers with demonstrated capability as wall materials for encapsulating bioactive. Alginate is a dietary fiber commonly used for encapsulation due to its gel forming capability mediated by cross-linking with divalent cations under room

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temperature, making it favourable for entrapping bioactive (Agüero et al., 2017). Inulin is a known strong prebiotic that can be readily degraded by gut microbiota into SCFAs and support the growth of saccharolytic metabolizing bacteria, making it a promising blending material for enhanced therapeutic effects in gut (Le Bastard et al., 2020). In addition, to reduce the premature release of the core material, chitosan, another widely used poly-*N*-acetyl-D-glucosamine polysaccharide for encapsulation, can be coated outside the alginate gel *via* polyelectrolyte complex formation (Liu et al., 2022a). Despite various carriers fabricated with alginate and chitosan, our previous work has developed alginate-inulin microspheres with chitosan coating (ALINCH) as a viable colon targeted delivery system for quercetin. The ALINCH-Q microspheres fabricated in this study improved encapsulation efficiency (53.2%) compared to alginate-only microspheres (39.4%) by the combination of inulin filling and chitosan coating, while the retarded release of quercetin during *in vitro* gastrointestinal digestion was enhanced by the non-conventional external gelation method generating a more robust gel network. The ALINCH-Q microspheres could resist the gastrointestinal digestion and deliver > 80% of the loaded quercetin to the colon without observable burst release, and that the quercetin was released reaching the highest concentration at 3 h and completely degraded by 24 h during *in vitro* colonic fermentation with pig fecal microbiota (Liu et al., 2022a).

In addition, although many of the drug carriers targeting colonic site are test against their *in vitro* release kinetics, physical characterizations and proved to be viable for colon-targeted delivery, there are only a few studies examining their metabolic fate after reaching colon specifically regarding the (a) metabolic fate of the carriers, (b) metabolic fate of the core material, (c) effect of the carriers on short chain fatty acids (SCFAs) production arising from bacterial fermentation, and (d) modulatory effects of the carriers and core materials on gut microbiota (Aguirre-Calvo et al., 2020; Li et al., 2021; Liu et al., 2023; Wang et al., 2021; Wu et al., 2020). Thus, in this study, we further investigated the therapeutic potentials of ALINCH-Q microspheres by profiling the fiber and quercetin metabolites and their effects on the microbial compositions during batch colonic fermentation. We hypothesized that the quercetin-loaded ALINCH-Q microspheres could affect the metabolic fate of quercetin, enhance SCFAs production, and modulate the microbiota composition in an *in vitro* fermentation model using pig fecal microbiota. The results would potentially help with filling the gap of how microspheres might take effect by modulating the microbiota and revealing the possible metabolic fate of the microspheres after reaching colon.

2. Material and methods

2.1. Materials and reagents

The following chemicals and reagents were acquired from Sigma-Aldrich (Castle Hill, NSW, Australia): quercetin (≥95%, CAS number 117-39-5), 4-hydroxyphenylacetic acid (analytical standard, CAS number: 156-38-7), 3,4-dihydroxyphenylacetic acid (analytical standard, CAS number: 102-32-9), 3-(3-hydroxyphenyl)propionic acid (analytical standard, CAS number: 621-54-5), 3-hydroxyphenylacetic acid (≥99%, CAS number: 621-37-4), acetic, butyric, propionic, isobutyric, isovaleric and valeric acid analytical standards, alginic acid sodium salt from brown algae (low viscosity, CAS number 9005-38-3), inulin (from chicory, CAS number 9005-80-5), chitosan (low viscosity, from shrimp shells, 75%–85% deacetylated, CAS number 9012-76-4), calcium chloride dihydrate, TWEEN 80, bile salts, calcium chloride, sodium chloride, potassium chloride, sodium bicarbonate, magnesium chloride, ammonium carbonate, monopotassium phosphate, sodium phosphate dibasic, sodium phosphate monobasic, peptone, yeast extract, tryptone, casein from bovine, L-Cysteine, guar, soluble starch from potato, pectin, mucin, sodium bicarbonate, dipotassium phosphate, pepsin from porcine gastric mucosa (≥2500 units/mg).

The following chemicals and reagents were purchased from Merck (Australia): Ascorbic acid (L-), acetonitrile (LCMS grade), formic acid (>98%), methanol (LCMS grade), and dimethyl sulfoxide (DMSO). Pancreatin (procine Pancreas, USBiological life sciences, CAS: 8049-47-6) was purchased from Jomar Life Research (Mulgrave, Australia).

The sunflower oil was purchased from a local market and deionised water was generated from Milli Q system (Merck, Milli-Q® Direct).

2.2 Fabrication of microspheres

The fabrication of microspheres using emulsion-templated external gelation method was described in our previous study (Liu et al., 2022a). Briefly, $CaCl_2$ nanocrystals were freshly prepared as follows: 1 mL of 1 M $CaCl_2$ in ethanol was emulsified with 19 mL sunflower oil with 6% (w:w) TWEEN 80 using a probe sonicator (QSonica Sonicator, John Morries Scientific, Victoria, Australia) with 50 amplitude (50 μm) for 1 min to produce $CaCl_2$ nanocrystals dispersed in oil phase. The dietary fiber mixture was prepared by dissolving 2 g alginate, 1.5 g inulin in 96.5 g water. 20 g of the solution was then added with 4 mL of 20 mg/mL quercetin in DMSO for loaded or 4 mL of DMSO for empty mixture under constant stirring (1000 rpm) for 10 min. Loaded or empty mixture was emulsified with 50 mL sunflower oil containing 2% (w/w) TWEEN 80 using Ultra Turrax disperser at 5000 rpm for 5 min to form a W/O emulsion. Emulsions were gelled by adding of 6 mL of the above-mentioned $CaCl_2$ nanocrystals under constant stirring (1200 rpm) for 30 min. The gelled microspheres were harvested by adding 40 mL washing media (0.5% w:w $CaCl_2$ + 0.1% w:w TWEEN 80 in water) into the mixture and collected by centrifugation at 3000 rpm for 5 min. The microspheres were coated by immersing in the coating solution (1% w:w chitosan in 2% w/w ascorbic acid, pH = 4.75 to favor the electrostatic interactions between alginate and chitosan) for 30 min with stirring at 1000 rpm, washed 3 times using distilled water and each time collected by centrifugation, then freeze-dried and stored at −20 °C in the dark until use.

2.3 In vitro colonic fermentation using pig fecal material

Four treatment groups were set up for fermentation: Blank (fecal inoculum + basal media); ALINCH-E (100 mg empty ALINCH-E microspheres + fecal inoculum + basal media); ALINCH-Q (100 mg ALINCH-Q microspheres containing 5.46 mg of loaded quercetin + fecal inoculum + basal media); and UQ (5.46 mg unencapsulated quercetin equivalent to the quercetin content in 100 mg ALINCH-Q + fecal inoculum + basal media).

The colonic fermentation, fecal inoculum, buffer and basal media preparation were performed following the method of Loo et al. (2022). The fecal inoculum was prepared by homogenizing 40 g of freshly collected pig feces (pooled from 4 healthy female pigs) in 160 mL sterilized 0.1 M phosphate buffer and filtered through sterile cheese cloth. Fermentation was done by adding the samples into 5.0 mL of sterilized basal media (5 g soluble starch, 5 g peptone, 5 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 2 g pectin, 4 g mucin, 3 g casein, 1.5 g $NaHCO_3$, 0.8 g L-Cysteine HCl, 1.23 g $MgSO_4$ $7H_2O$, 1.0 g guar, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts, 0.11 g $CaCl_2$ and 1 mL Tween-80 and make up to 1 L in distilled water, final pH = 7.0), then added with 5.0 mL of the fecal inoculum. The mixture was vortexed, flushed with nitrogen, and incubated in a shaking incubator anaerobically for 24 h at 37 °C. Samples were collected at 0, 3, 6, 12, 24 h, snap frozen in liquid nitrogen and kept under −80 °C for further analysis.

2.4 Quantification of quercetin metabolites

The thawed fermentation fluids were extracted with 80% methanol, by mixing fermentation fluid with methanol at a ratio of 2:8 (v:v), vortexed for 1 min, shaking incubated overnight at 4 ℃, and centrifuged at 10,000 rpm for 5 min at 4 °C, and the supernatant of the extract was filtered through 0.45 μm syringe filter and 10 μL of the filtrate was

injected into a HPLC-DAD-ESI-MS system (Agilent 1260 Infinity II LC MSD, Australia) equipped with Phenomenex Gemini C18 reverse phase column (California, United States). The acquisition wavelengths of the DAD were set at 220 nm, 280 nm, 300 nm, and 370 nm. ESI chamber was set up with gas temperature of 325 °C, drying gas flow of 7.0 L/min, and capillary voltage of 3500 V to produce $[M-H]^-$ adducts. Mass spectrometer detector (MS) was set at mass range of 100.00 – 2000.00 with negative mode scanning. The LC mobile phases used were A (0.1% v/v formic acid in water) and B (0.1 %v/v formic acid in 95% v/v acetonitrile in water) with gradient as follow: 5% B (0–5 min), 10% B (5–10 min), 20% B (10–20 min), 30% B (20–30 min), 35 %B (30–35 min), 40% B (35–40 min), 50% B (40–45 min), 70% B (45–50 min), 100% B (50–70 min), 5% B (71–80 min) at a flow rate of 0.6 mL/min. Metabolites standards were run through the LCMS with same conditions to obtain standard mass/signal calibration curves for quantification.

2.5 SCFA analysis in fermentation fluid

The SCFA analysis was performed following the method of Gu et al (2019). The fermentation fluid was centrifuged at 8,000 rpm, 10 min at 4 °C, then the pH of the supernatant was adjusted to 2–3 with a few drops of 6 M HCl. Then, the supernatant was mixed with acid (1% formic acid with 1% orthophosphoric acid) at ratio of 3:7 (v:v). The mixture was vortexed and centrifuged at 1,000 rpm for 5 min, then the supernatant was filtered through 0.45 μm syringe filter. Two μL of the filtrate was injected into a gas chromatography system equipped with a flame ionization detector (GC-FID) (7890B Agilent, CA, USA) fitted with a capillary column (SGE BP21, 12 × 0.53 mm internal diameter with 0.5 μm film thickness (SGE International, Ringwood, VIC, Australia, P/N 054473). Helium carrier gas flow rate was set at 14.4 cc/min. The oven temperature gradient for the column was as follows: initial temperature was set at 100 °C for over the next 30 s, then increases to 180 °C at 6 °C/min, held for 1 min, and then increased at 20 °C/min for 5 min reaching 200 °C and held for 10 min. The air flow was 300 cc/min for injection port at 200 °C, while the hydrogen flow was 30 cc/min for FID detector (240 °C). 4-methyl-valeric is added as an internal standard with the injection sample at 1.59 mmol/L. Acetic, butyric, propionic, isobutyric, isovaleric and valeric acid analytical standards were run through GC-FID under the same conditions, to obtain standard plots for quantitation of these SCFAs in fermentation samples.

2.6 16 s rDNA extraction and sequencing

The preparation of the samples for DNA sequencing followed the method of Loo et al. (2022). Briefly, the fermented samples collected from colonic fermentation described in section 2.3 were thawed, centrifuged at 10,000 × g for 10 min and the supernatants were discarded. The sediments were washed with 2% polyvinylpolypyrrolidone in phosphate buffered saline for three times. Then 0.4 g of the pellets are homogenized with 1 mL of DNA shield solution (Zymo Research, California, U.S.A.) and kept under −20 °C. Bacterial DNA extraction and 16 s rDNA sequencing was performed by the Australian Genome Research Facility Ltd (Australia) as follows: bacterial DNA was extracted using the DNeasy® PowerSoil® Pro Kit (QIAGEN GmbH, Hilden, Germany), the 16 s rRNA gene from V1 to V3 regions were amplified by PCR using the 27F-519R primers, and sequencing was conducted on an Illumina MiSeq (San Diego, CA, USA) with a V3, 600 cycle kit.

2.7 Statistical analysis

Experiment results (n = 3) were expressed as mean ± SD, and the SD reported to 2 significant figures and decimal placing of the mean according to EURACHEM guidelines (Ellison and Williams 2012). Differences between treatment groups were analysed with one-way analysis of variance (ANOVA) and Tukey's comparison in Minitab® (Minitab Inc., State College, PA, USA). Origin Lab (OriginLab Corporation, Northampton, MA, USA) and R (R Core Team 2021) with "ggplot2" package were used for graph generation unless specified alternatively.

Microbiome bioinformatics were analysed with QIIME 2 2020.11 (Bolyen et al., 2019). Raw data were demultiplexed and quality filtered using q2-demux plugin and denoising with DADA2 (Loo et al., 2022). Alpha diversity was calculated using Shannon's diversity index and Pielou's evenness index. Factorial Kruskal-Wallis sum-rank test ($\alpha = 0.05$) was used to examine statistically significant differences within each treatment group. Non-parametric microbial interdependence test (NMIT) was performed using QIIME 2 environment for determination of longitudinal sample differences. Significant taxonomic differences after 24 h fermentation were analysed using linear discriminant analysis (LDA) effect size (LEfSe) (*https://huttenhower.sph.harvard.edu/galaxy/*) and graph was plotted with LDA score > = 2.0 (Segata et al., 2011). Pearson's correlation coefficient was calculated using R to examine the association between phenolic metabolites or SCFAs and bacterial genera, and heatmaps were plotted using R with "corrplot" package.

3. Results and discussion

3.1 Biotransformation of quercetin is affected by the alginate-inulin-chitosan fiber matrix comprising the microspheres.

Colonic microbiota has been shown to transform quercetin into metabolites with beneficial biological activities (Carregosa et al., 2022). We have previously shown that ALINCH-Q microspheres could remain stable under gastrointestinal digestion and deliver > 80% encapsulated quercetin to colon, and that the quercetin was released during the first 3 h and completely degraded by 24 h under the effect of pig fecal microbiota (S. Liu et al., 2022a). It is essential to further examine how the quercetin was metabolized by gut microbials, and whether the presence of dietary fiber matrix has any effect on its biotransformation.

We firstly monitored the biotransformation of released quercetin and identified four major quercetin metabolites produced during the colonic fermentation. These include 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3-hydroxyphenylpropionic acid. They were identified based on their retention times and molecular ion adducts generated comparing with reference standards in LC-ESI-MS (Table 1). These four metabolites have been reported as the main metabolites for quercetin under batch fecal fermentation before (Di Pede et al., 2020; Havlik et al., 2020). The concentration of the metabolites over 24 h of fermentation in the fermentation fluid is shown in Fig. 1.

4-hydroxyphenylacetic acid was detected at 3 h, and the level peaked at 6 h in all treatments (Fig. 1a). The production in Blank and ALINCH-E could arise from phenylacetic acids as the microbial metabolites of aromatic amino acids such as phenylalanine and tryptophan originated from the basal media, in addition from fermentation of quercetin (Dodd et al., 2017). At 12 h, it was no longer detected in Blank and ALINCH-E, indicating that 4-hydroxyphenylacetic acid from protein had been further transformed to simpler smaller molecules. But for the UQ and ALINCH-Q treatments at 12 h, significant level of 4-hydroxyphenylacetic acid remained indicating quercetin contributing to the build-up of this metabolite in addition to proteins. At 12 h the level in the UQ treatment was significantly ($p < 0.05$) higher than that in ALINCH-Q. Then it was further metabolised up till 24 h when 4-hydroxyphenylacetic acid remained at substantial amount for UQ but not for the ALINCH-Q treatment. This result inferred the tendency for further metabolism of 4-hydroxyphenylacetic acid in ALINCH-Q than in unencapsulated quercetin.

3,4-Dihydroxyphenylacetic acid, on the other hand, was not detected in the Blank and ALINCH-E treatments over the entire 24 h of fermentation, confirming its origination from quercetin (Fig. 1b). It was produced, instead, at 6 h in both UQ and ALINCH-Q treatments. The level in UQ treatment continued to increase into 12 h before decreasing by 24 h. On the contrary, the level in ALINCH-Q treatment reached a peak at 6 h

Table 1
Metabolites identified in fermentation fluid.

Compound	Formula	Retention time (min)	Molecular weight	Adduct ion	Theoretical ion product	*m/z* of molecular ion [M−H], adduct [2 M−H]
3,4-diHydroxyphenyl acetic acid	$C_8H_8O_4$	15.343	168.15	H-	167.15	167, 335
4-Hydroxyphenyl acetic acid	$C_8H_8O_3$	18.252	152.15	H-	151.15	151, 303
3-Hydroxyphenyl acetic acid	$C_8H_8O_3$	19.662	152.15	H-	151.15	151, 303
3- Hydroxyphenyl propionic acid	$C_9H_{10}O_3$	23.454	166.17	H-	165.17	165, 331

and then gradually decreased eventually not detected at 24 h. These effects implied that ALINCH-Q shifts the pattern of bacterial metabolism of quercetin towards further metabolism of 3,4-dihydroxyphenylacetic acid within the 24 h of fermentation time.

The production of 3-hydroxyphenylpropionic acid from UQ and ALINCH-Q treatments started later which was detected at 12 h and continued into 24 h (Fig. 1c). Significantly ($p < 0.05$) higher level of 3-hydroxyphenylpropionic acid were observed in ALINCH-Q treatment compared to that in UQ treatment at 12 h. This pattern was similar for 3-hydroxyphenylacetic acid, the level of which was approximately 3.8 folds ($p < 0.05$) higher in ALINCH-Q than in UQ at 24 h (Fig. 1d). This indicated that the ALINCH-Q shifted the biotransformation of quercetin to higher production of these two metabolites.

Overall, these results clearly indicate that ALINCH-Q microspheres can alter the metabolic pathway of encapsulated quercetin compared to unencapsulated quercetin in UQ. According to the literature, the quercetin could be metabolized through the postulated pathways outline in Fig. 1e. It has been proposed that quercetin is metabolized sequentially by gut microbiomes and undergoes C-ring fission leading to the production of the intermediate metabolite 3,4-dihydroxyphenylpropionic acid, which then undergoes degradation into 3,4-dihydroxyphenylacetic acid, or dehydroxylation into 3- or 4- hydroxyphenylpropionic acid. 3,4-Dihydroxylphenylacetic acid could also be further metabolized *via* dehydroxylation to 3- or 4- hydroxyphenylacetic acid. These two metabolites can be further transformed to phenylacetic acid or isomerized into 2-hydroxyphenylacetic acid (Appeldoorn et al., 2009; Dias et al., 2022; Najmanová et al., 2016; Serra et al., 2012). These metabolic pathways for quercetin help to explain the divergence of quercetin degradation pathways between ALINCH-Q and UQ treatments. The metabolite showing up earliest during fermentation was 3,4-dihydroxyphenylacetic acid, which could be the metabolic product of the ring fission intermediate 3,4-dihydroxyphenylpropionic acid. The intermediate was not detected in quantifiable level in this study probably due to its fast degradation during the first 3 h of fermentation. After this stage, ALINCH-Q was presumably altering the metabolic fate of 3,4-dihydroxyphenylacetic acid. While 3,4-dihydroxyphenylacetic acid from quercetin in UQ treatment was dehydroxylated into 4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid, in ALINCH-Q treatment it was more quickly depleted and dehydroxylated mainly into 3-hydroxyphenylacetic acid. This implies that ALINCH-Q microspheres could facilitate 4′-dehydroxylation of 3,4-dihydroxyphenylacetic acid. It has been reported that 3-hydroxyphenylacetic acid is significantly upregulated in unencapsulated quercetin compared to quercetin encapsulated in phytosome formulated with sunflower lecithin (Di Pede et al., 2020), which is the opposite of our study. The observed differences point to the encapsulating materials promoting the activities of different groups of bacteria differently, thus resulting in shifts of the metabolic pathways of quercetin.

Another metabolic pathway modulation can be inferred from the degradation of the ring-fission intermediate 3,4-dihydroxyphenylpropionic acid derived from quercetin. UQ treatment had higher tendency to produce 3,4-dihydroxyphenylacetic acid rather than 3-hydroxyphenylpropionic acid, but with ALINCH-Q treatment the latter was more substantially produced. This would imply dehydroxylation of the ring fission intermediate occurred instead of degradation which could again be attributed to the dietary fiber mix in the carrier altering bacterial activities. Dietary fiber modulating polyphenol biotransformation have been reported before. Inulin boosted the production of 3,4-dihydroxyphenylacetic acid from rutin as the parent compound after 24 h *in vitro* fermentation (Havlik et al., 2020), and chitosan and Konjac glucomannan promoted the production of 3-phenylpropionic acid from batch fermentation of grapefruit peel flavanone (Tang et al., 2022). Another study found that the fermentation of raftiline and pectin reduced the total production of phenolic acids from rutin by 78–85% (B. Mansoorian et al., 2015). There are a few possible mechanisms explaining this modulatory effect. Firstly, the fermentation of fiber produced SCFAs and reduced the pH of the fermentation fluid, which in turn could inhibit some acid-sensitive bacteria, reshaping the bacteria enzyme activities; secondly, the fibers could be favourably used as substrate by specific groups of bacteria, which in turn alter the metabolism of polyphenols; and thirdly, the fibers could delay or alter the velocity at which the bacteria degrade polyphenols, changing the metabolites productions (Havlik et al., 2020; Mansoorian et al., 2019). We hence speculate that these results indicate the interactive effects between dietary fibre and phenolic compounds on microbial catabolic pathways.

In conclusion, the alginate-inulin-chitosan fiber mix in the ALINCH-Q was shown to shift the metabolic pathway of quercetin towards further metabolism of 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, and significantly higher production of 3-hydroxyphenylpropionic acid and 3-hydroxyphenylacetic acid compared to UQ. This could arise from the effects of fiber matrix on modulating bacterial activities thus altering the enzymatic reactions governing the degradation and dehydroxylation of these metabolites.

3.2 Production of SCFA is enhanced by quercetin-loaded microspheres

Total (Fig. 2a) and specific SCFAs (Fig. 2b–g) were quantified after 0, 3, 6, 12, 24 h of fermentation to investigate the influence of ALINCH-Q on their productions. Production of total SCFAs increased with fermentation time from all treatments over the entire 24 h period. At 24 h, the mean total SCFAs concentrations were 109.8 ± 2.4, 110.3 ± 4.3, 116.1 ± 5.5 and 127.4 ± 2.5 mmol/L for Blank, UQ, ALINCH-E, and ALINCH-Q respectively. The concentrations of the total SCFA were within similar range as reported by previous study that fermented sugarcane fiber with phenolic extracts (Loo et al., 2022). At the end of the fermentation at 24 h, total SCFAs were the highest in quercetin-loaded microspheres (ALINCH-Q), significantly ($p < 0.05$) higher than that in Blank and UQ (Fig. 2a). The total SCFAs in empty ALINCH-E was in the middle between Blank and ALINCH-Q. These results point to loaded quercetin up regulating the production of total SCFAs by the fecal microbiota from the fiber matrix.

ALINCH-Q microspheres also promoted the production of individual SCFAs. Acetic acid was the most abundant SCFA, followed by propionic acid, butyric acid, isobutyric acid, and with valeric and isovaleric the least abundant. At 24 h, the productions of all SCFAs excluding isovaleric acid were significantly increased by ALINCH-Q ($p < 0.05$) compared to that in the Blank. Apart from 24 h, the enhanced SCFA production by ALINCH-Q was observed in earlier time points too. ALINCH-Q promoted valeric acid production at 12 h ($p < 0.05$), and

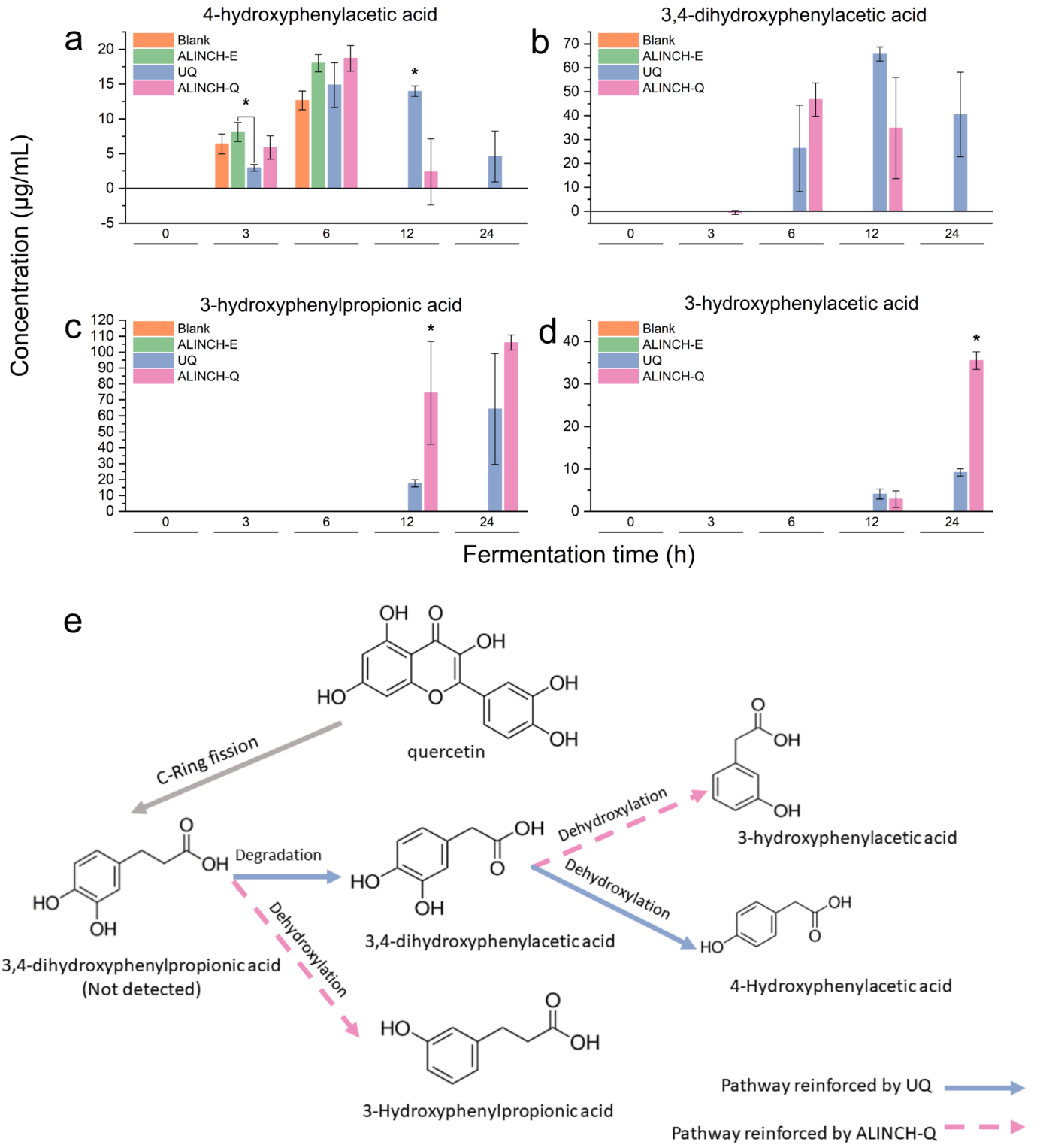

Fig. 1. Quercetin metabolites and postulated metabolic pathways. (a-d) Quercetin metabolites production during 24 h of *in vitro* colonic fermentation using pig fecal microbiota. (e) Postulated alteration of metabolic pathways of quercetin by microsphere encapsulation. Blank (10 mL fermentation fluid only), UQ (5.46 mg quercetin/10 mL fermentation fluid), ALINCH-E (100 mg empty alginate-inulin-chitosan microspheres/10 mL fermentation fluid), and ALINCH-Q (100 mg quercetin-loaded alginate-inulin-chitosan microspheres containing 5.46 mg quercetin /10 mL fermentation fluid). * Represents statistically significant difference ($p < 0.05$) compared to the other treatment group within each time point (The disappearance of the parent quercetin compound (UQ) has been reported in our previous paper (S. Liu et al., 2022a)).

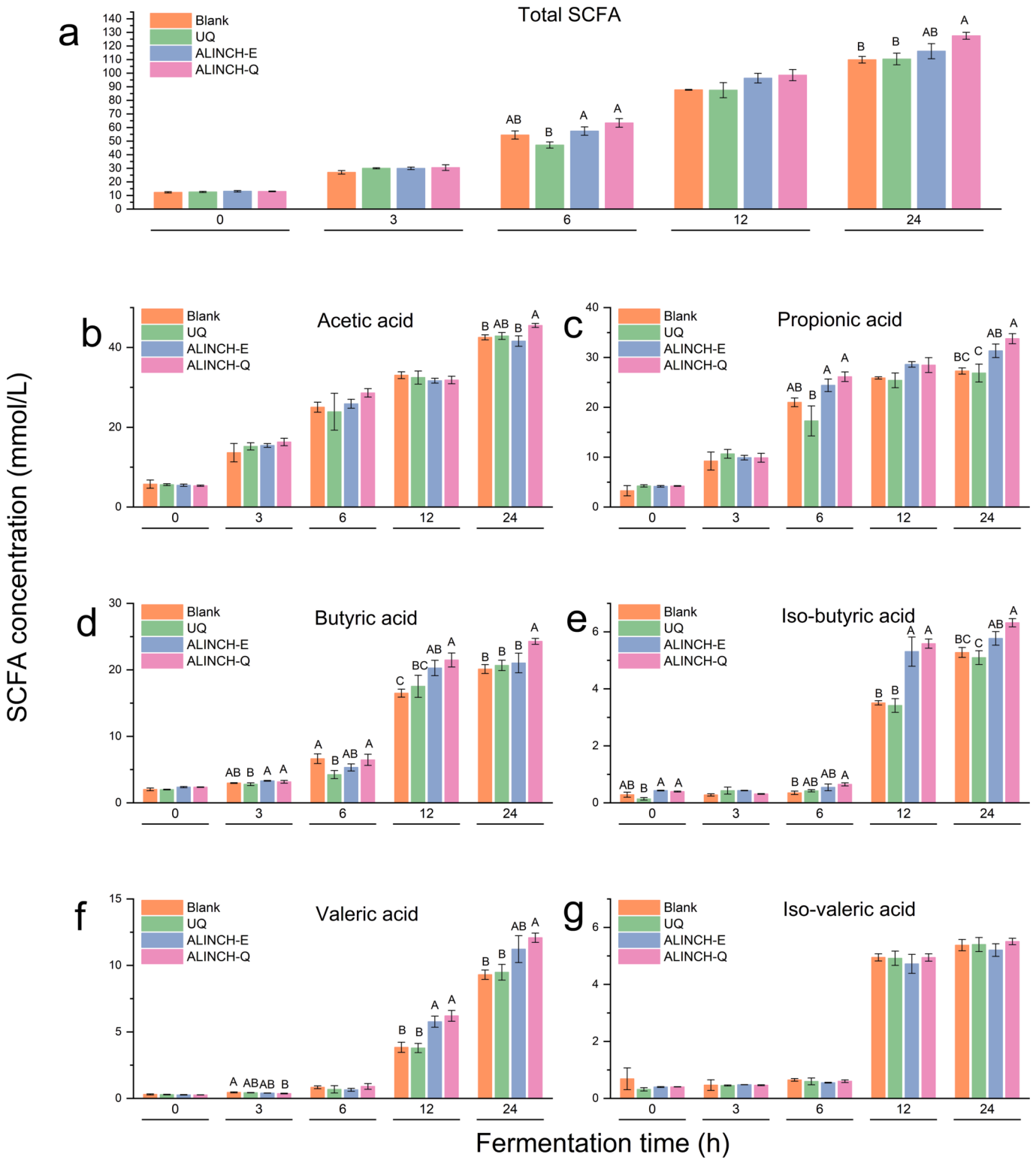

Fig. 2. SCFA concentration in mmol/L in fermentation fluid after 0, 3, 6, 12, 24 h of *in vitro* colonic fermentation. Total SCFA concentration in mmol/L (a), and concentration in mmol/L for acetic acid (b), propionic acid (c), butyric acid (d), isobutyric acid (e), valeric acid (f), and isovaleric acid (g). Blank, UQ, ALINCH-E and ALINCH-Q fermentation conditions as in Fig. 1. Means that do not share a letter are significantly different within each time point at $p < 0.05$.

isobutyric acid from 6 h and onwards ($p < 0.05$) compared to Blank. Interestingly, the production of butyric acid was enhanced by ALINCH-Q treatment from 12 h and was remarkably ($p < 0.05$) higher than not only that in the Blank but also those in ALINCH-E and UQ treatments at 24 h, suggesting much higher effect when fiber mixture and quercetin were combined (Fig. 2d).

The effect of ALINCH-E on SCFAs production was not as significant as ALINCH-Q. ALINCH-E increased production of butyric acid, isobutyric acid and valeric acid ($p < 0.05$) only at 12 h, but did not persist to 24 h, suggesting fiber mixture itself cannot provide continuous promotive effects on SCFA production. UQ did not exhibit any effect on production of all SCFAs compared to Blank. By contrast, the production pattern of

isovaleric acid, while increased with fermentation time, were similar for all the treatments. This could be due to the fact that unlike the non-branched SCFAs, branched SCFAs including isovaleric acids are produced from branched amino acids such as valine, leucine and isoleucine by microbial fermentation of proteins present in the media (Blakeney et al., 2019).

These results point to possible interplay between the dietary fiber carrier and quercetin in the production of SCFAs, where the combination of the dietary fiber matrix and quercetin has more prominent effect than either of them alone. SCFAs production was substantially enhanced only with the loading of quercetin into the fiber matrix in ALINCH-Q, presumably by supporting the growth of SCFA-producing bacteria. The interplay between dietary fibers matrix and encapsulated polyphenols has also been reported in the study of Wu et al. (2020), who found no effect on SCFAs production from gum Arabic or maltodextrin alone, but significant elevated production of acetic, propionic, and butyric acids by the encapsulated anthocyanins with dietary fiber matrix compared to blank control during batch colonic fermentation. Another study by Havlik et al. (2020) also found similar effects with rutin added to the batch fermentation of inulin, and that the production of propionic, butyric and isovaleric acids were particularly facilitated compared to that without rutin. In our study, quercetin itself did not contribute to SCFA production confirming the earlier study by Mansoorian et al. (2019) in batch fermentation experiment with fibers using human fecal microbiota.

Therefore, our result implicates the potential synergism between the alginate/inulin/chitosan fiber carrier and quercetin, where the promotive effects on SCFA production is greatest when the carrier is loaded with quercetin. This could be due their combine effects on SCFA-producing bacteria activities.

3.3 Effect of microspheres on modulation of pig gut microbiota

Microbial composition plays an important role in the biotransformation of phytochemicals and residual macronutrients in the gut; hence we collected the bacteria pellet after 0, 3, 6, 12, 24 h of fermentation and sequenced their 16 s rDNA to reveal the changes in microbiota community.

The venn diagram (Fig. 3a) showed that there were 246 unique operational taxonomic units (OTUs) present in the ALINCH-Q treatment, while 221, 210 and 133 unique OTUs in UQ, Blank and ALINCH-E respectively, meaning that the ALINCH-Q treatment had the most diverse microbiota compositions among four treatments. There were 148 OTUs shared among the four treatments, indicating that these OTUs were the core bacteria taxa. Alpha diversity indices were computed against each treatment group along different time points to find out how the microbial composition change within each group along time (Fig. 3c). Shannon's diversity index increased in all treatment groups significantly (Kruskal-Wallis pairwise, $p < 0.05$) from 6 h except for UQ, in which the significant increment was from 12 h. This indicates that the bacterial richness was enhanced during fermentation, but UQ delayed the process probably due to the inhibitory effect of quercetin on some bacteria groups. The change in Pielou's evenness reflected the distribution of the bacterial community. Significant increment of evenness was observed from 6 h in Blank and UQ to 24 h, whereas this trend started earlier at 3 h in ALINCH-E and ALINCH-Q (Fig. 3c). The addition of fiber material from microspheres promoted growth of variety of bacterial groups at early stage of fermentation by acting as carbohydrate substrates, resulting in a higher evenness index observed at earlier fermentation time points.

Nonparametric microbial interdependence test (NMIT) examines how the interdependencies of features of the microbial community between different treatment groups might differ from each other along time at a longitudinal level. PCoA plot was generated to visualize the longitudinal microbial interdependences (Fig. 3b). It showed that points in ALINCH-Q treatment were clustered at the bottom left of the graph and were well separated from other 3 treatments, indicating that the ALINCH-Q treatment led to a distinctive microbial pattern along 24 h fermentation compared to others, likely due to the presence of both quercetin and dietary fiber matrix. Additionally, ALINCH-Q and ALINCH-E clustered near to each other while Blank and UQ distributed sparsely on the right side of the plot. This pointed to ALINCH-E and ALINCH-Q exhibited different modulatory effect in the composition of microbial community along time from Blank and UQ, due to the presence of dietary fiber matrix. It has been previously shown that sugarcane fibers and sugarcane polyphenols together could also change the alpha diversity, alter longitudinal microbial pattern, and showed remarkable differences in NMIT analysis (Loo et al., 2022).

LefSe analysis was performed to examine the most differentially abundant taxa after 24 h of fermentation in all treatment groups (Kruskal-Wallis sum-rank test, $\alpha < 0.05$), to identify the potential biomarker taxa of each treatment using community-wide responses (S. Wang et al., 2021). It showed that the 4 treatment groups had distinct characteristic bacterial taxa (Fig. 3d). ALINCH-Q was characterised by the high abundance of *Lactobacillales*, and especially the genus *Lactobacillus*, in which many species have probiotic effects and are beneficial to gut health (Altermann et al., 2005). It also displayed high abundance of another 14 genera belonging to 7 distinct families. The high variety of biomarker taxa in ALINCH-Q showed that the addition of ALINCH-Q was associated with the occurrence of diverse bacterial groups. Interestingly, there was much fewer characteristic genera in ALINCH-E, with only *Enterococcus*, *Streptococcus* and *Allisonella* found prominently associated with ALINCH-E. UQ was found substantially associated with high abundance of Ruminococcaceae, in which there were 9 biomarker genera in this family. Also, UQ exhibited high abundance of *Escherichia-Shigella*. Instead, Blank showed quite different characteristic bacteria, mostly of Bacteroidales, and it was worth noting that Blank and UQ had overlapping taxa especially under Bacteroidales.

The modulation of most abundant bacterial genera (>0.1%) was analysed, and the relative abundance of specific taxa was computed across all samples at each time point (Fig. 4). One-way ANOVA was used to determine significant differences across different treatment groups at each time point for each bacterial genus (Supplementary Table 1).

Streptococcus was gradually decreased in all 4 treatments, and from 3 h UQ and Blank had higher abundance of *Streptococcus* ($p < 0.05$). But, from 6 to 24 h, ALINCH-Q and ALINCH-E slowed down the reduction in *Streptococcus* abundance compared to Blank and UQ, and *Streptococcus* abundance was significantly higher than Blank and UQ ($p < 0.05$) at 24 h. Dietary fibers may provide more energy source for *Streptococcus* thus slowing down the reduction in abundance. This is in accordance with the result of Uerlings et al. (2021), who found that batch fermentation of inulin and chicory roots (natural source of inulin) in piglet feces significantly increased abundance of *Streptococcus*. *Streptococcus* is naturally present in gut microbial community, and species like *S. thermophilus*, a lactic acid producing bacteria, have shown probiotic effect associated with reduction of the pro-inflammatory activity induced by uremic toxins (Vitetta et al., 2019).

Lactobacillus was found significantly higher in ALINCH-Q ($p < 0.05$) compared to Blank at 3 h and 6 h, however, neither ALINCH-E nor UQ alone showed significant effect. This indicates the potential synergistic effect between dietary fiber matrix and quercetin in affecting *Lactobacillus* growth, a genus in which many species possess probiotic effects and are beneficial to management of intestinal diseases. For example, *L. plantarum* is reported to have anti-inflammatory effects in a ulcerative colitis mouse model (Y. Wang et al., 2018). The dietary supplementation of another species *L. fermentum* improves the anti-oxidative system by enhancing superoxide dismutase and glutathione activities in piglets (A. N. Wang et al., 2013). Interestingly, this significant improvement on *Lactobacillus* by ALINCH-Q did not persist to 24 h, probably due to the depletion of dietary fiber substrate in the experiment such that the microbiota community slowly recovered to the original composition. To address this limitation, longer-term supplementation of ALINCH-Q on

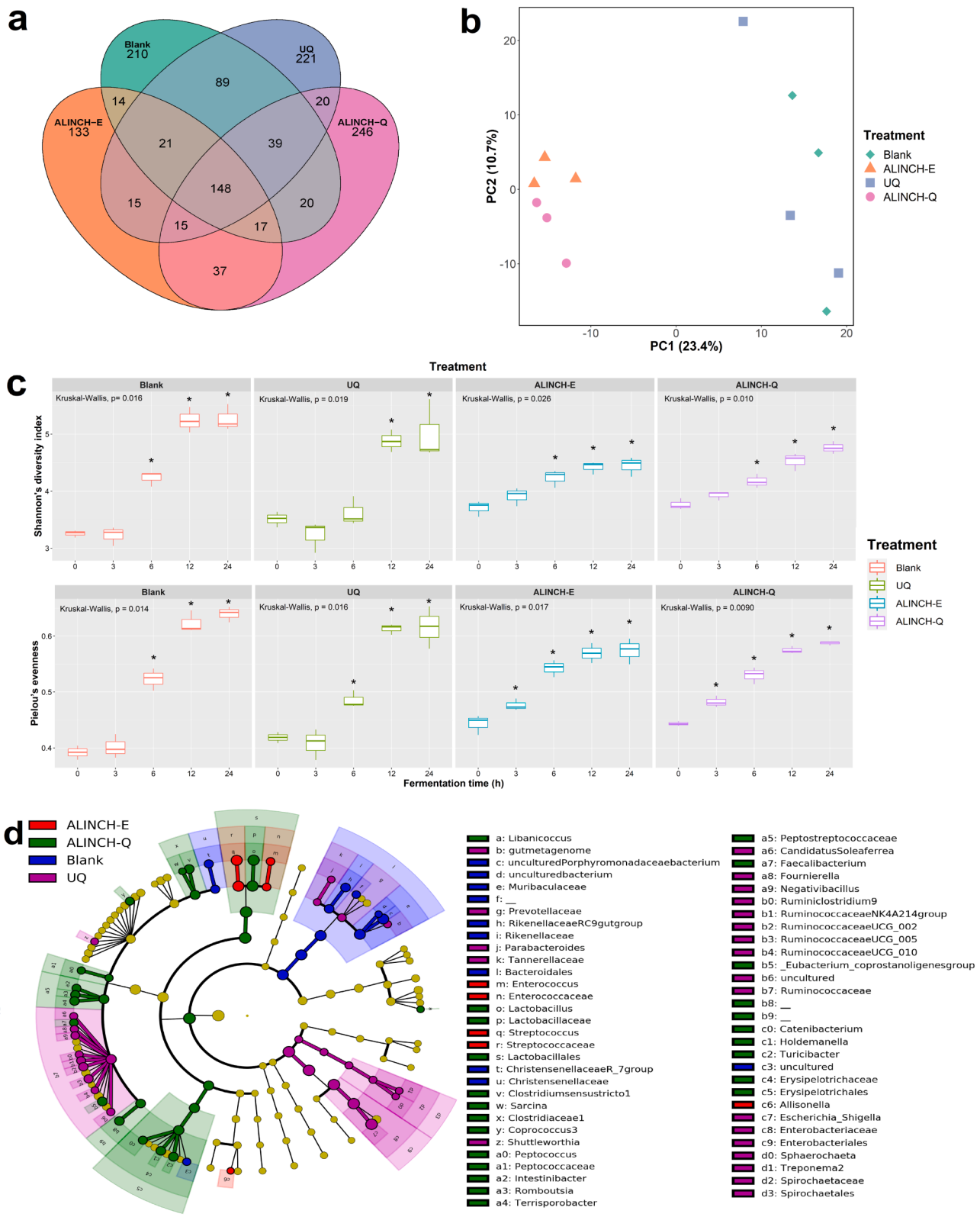

Fig. 3. Modulation of fecal microbiota by four treatments. (a) Venn diagram of OTUs counts (>97% nucleotide sequence identity) showing the shared and unique OTUs among four treatment groups (b) PCoA plot computed based on the nonparametric microbial interdependence test (NMIT) at genus level taxa for determination of between-group differences on longitudinal level. (c) Alpha diversity indices of fecal microbiota during 0, 3, 6, 12, 24 h of *in vitro* batch colonic fermentation. Statistical significance between timepoints and 0 h within each treatment group was analysed using Kruskal-Wallis test and denoted as significant with one asterisk (* = $p < 0.05$). (d) Linear discriminant analysis effect size (LEfSe) taxonomic cladogram (Kruskal–Wallis sum rank, test $\alpha < 0.05$; LDA score > 2.00) to identify statistically significantly discriminant bacterial taxa in different treatments. Colored circles refer to bacterial taxa that were significantly abundant in specific treatment group, while the yellow circles indicate insignificant taxa. Blank, UQ, ALINCH-E and ALINCH-Q fermentation conditions as in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

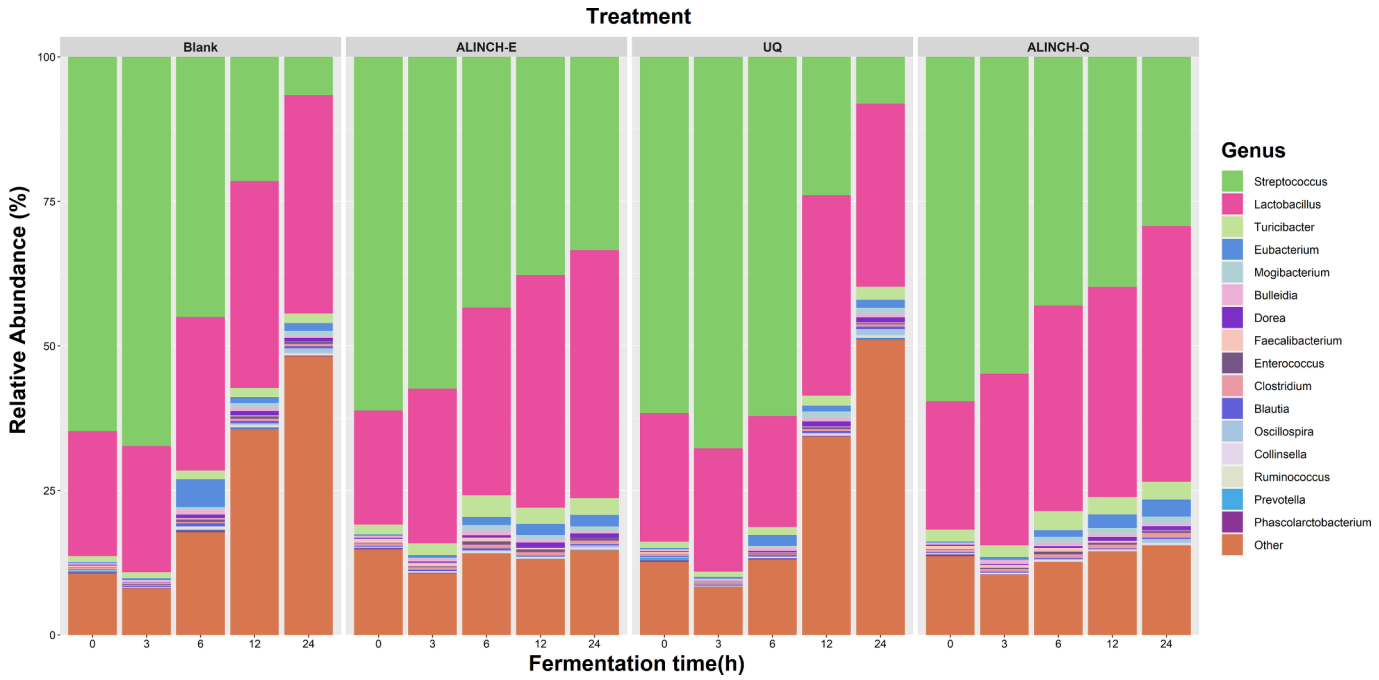

Fig. 4. Relative abundance of most abundant bacterial genera (>0.1%) in four treatment groups throughout 24 h fermentation and their correlation with bacterial metabolites. Alterations in relative abundance of fecal microbiota at genus level in treatments throughout 24 h. Relative abundance of most abundant genera (>0.1%) were plotted as stack bar plot for Blank, ALINCH-E, UQ and ALINCH-Q at 0, 3, 6, 12, 24 h of fermentation. Blank, UQ, ALINCH-E and ALINCH-Q fermentation conditions as in Fig. 1.

the microbiota modulatory effect should be investigated.

Turicibacter was found to be significantly ($p < 0.05$) higher in ALINCH-Q and ALINCH-E from 3 to 24 h compared to Blank, indicating that the dietary fiber promoted the growth of *Turicibacter*. The reduction of *Turicibacter* is reported to be substantially correlated with gut dysbiosis. It was found that dogs with acute hemorrhagic diarrhea also show significant decreases in *Turicibacter* spp. compared to healthy dogs (Honneffer et al., 2014). This points to up-regulation of *Turicibacter* might be related to alleviation of colon inflammation.

Eubacterium growth was significantly ($p < 0.05$) promoted by ALINCH-Q but not by ALINCH-E or UQ alone at 12 and 24 h, again suggesting potential synergistic effect between dietary fiber mix and quercetin. *Eubacterium* has been supported by recent studies as a beneficial bacterial genus in gut. Decreased abundance of *Eubacterium* is associated with higher occurrence of IBD in case control studies which are reviewed by Mukherjee et al. (2020). Species such as *E. rectale* and *E. hallii* are reported to be the major butyrate producers utilizing various complex carbohydrates (Louis & Flint, 2009), which is consistent with this study reporting an enhanced butyric acid production in ALINCH-Q. In addition, *E. ramulus* is another acknowledged species that can degrade quercetin by cleaving of the C ring, producing major metabolite 3,4-dihydroxyphenylacetic acid (Ulbrich et al., 2015), and this result is in accordance with this study that 3,4-dyhydroxyphenylacetic acid is one of the main metabolite product of quercetin. However, UQ did not significantly promote *Eubacterium* growth probably because of the quicker depletion of quercetin in UQ than in ALINCH-Q. This result supports the potential roles of ALINCH-Q microspheres rather than quercetin itself in ameliorating gut dysbiosis by fortifying growth of *Eubacterium* and the resulting production of butyric acid.

Clostridium was also significantly ($p < 0.05$) promoted by ALINCH-Q at 24 h compared to all other three treatments, showing synergism between fiber matrix and quercetin. The surge of *Clostridium* growth was observed from 6 h by both ALINCH-E and ALINCH-Q compared to Blank and UQ. But when progressed to 12 h, only ALINCH-Q showed significant elevation of *Clostridium* abundance compared to Blank. It was found that the oral administration of *trans*-resveratrol and quercetin positively affected growth of *C. clariflavum* and *C. methylpentosum* in a rat model (Etxeberria et al., 2015). In addition, *C. butyricum*, a major butyrate producer in human intestine, is reported to positively associated with impediment of intestinal tumor growth in mice model (Chen et al., 2020).

Enterococcus, which contains pathogenic species, were found significantly ($p < 0.05$) inhibited by UQ compared to Blank and inhibited by ALINCH-Q compared to ALINCH-E ($p < 0.05$) at a later fermentation time at 24 h. This means that quercetin can inhibit the growth of *Enterococcus* spp. that is linked to intestinal infections. Lin, Piao, and Song (2019) reported that quercetin oral supplementation decreased *Enterococcus* in a colitis mouse model and alleviated production of pro-inflammatory cytokines in the colon.

In summary, ALINCH-Q increased the alpha diversity indices and showed distinctive microbial community pattern along 24 h fermentation compared to other treatment groups. It also contained large variety of biomarker bacterial taxa, suggesting its role in shaping a diverse gut microbial community and potentials in combating gut dysbiosis. It specifically promoted the growth of beneficial bacterial genera such as *Lactobacillus*, *Eubacterium*, and inhibited pathogenic genera such as *Enterococcus*, which further implied its promising therapeutic effects in gut.

3.4 Correlation between SCFA production, generation of phenolic metabolites and pig gut microbiota profile

Our results show that the addition of ALINCH-Q microspheres can alter quercetin biotransformation (Fig. 1), changes production pattern of SCFAs (Fig. 2) and modulate pig fecal microbiota compositions (Figs. 3 and 4). To further investigate the association between bacterial genera and each metabolic substance (phenolic metabolites or SCFAs), correlation analysis was performed and were plotted as heatmaps shown in Fig. 5.

Streptococcus and *Faecalibaterium* were positively associated with quercetin, but negatively correlated with production of 3-hydroxyphenylpropionic acid and/or 3-hydroxyphenylacetic acid (Fig. 5a),

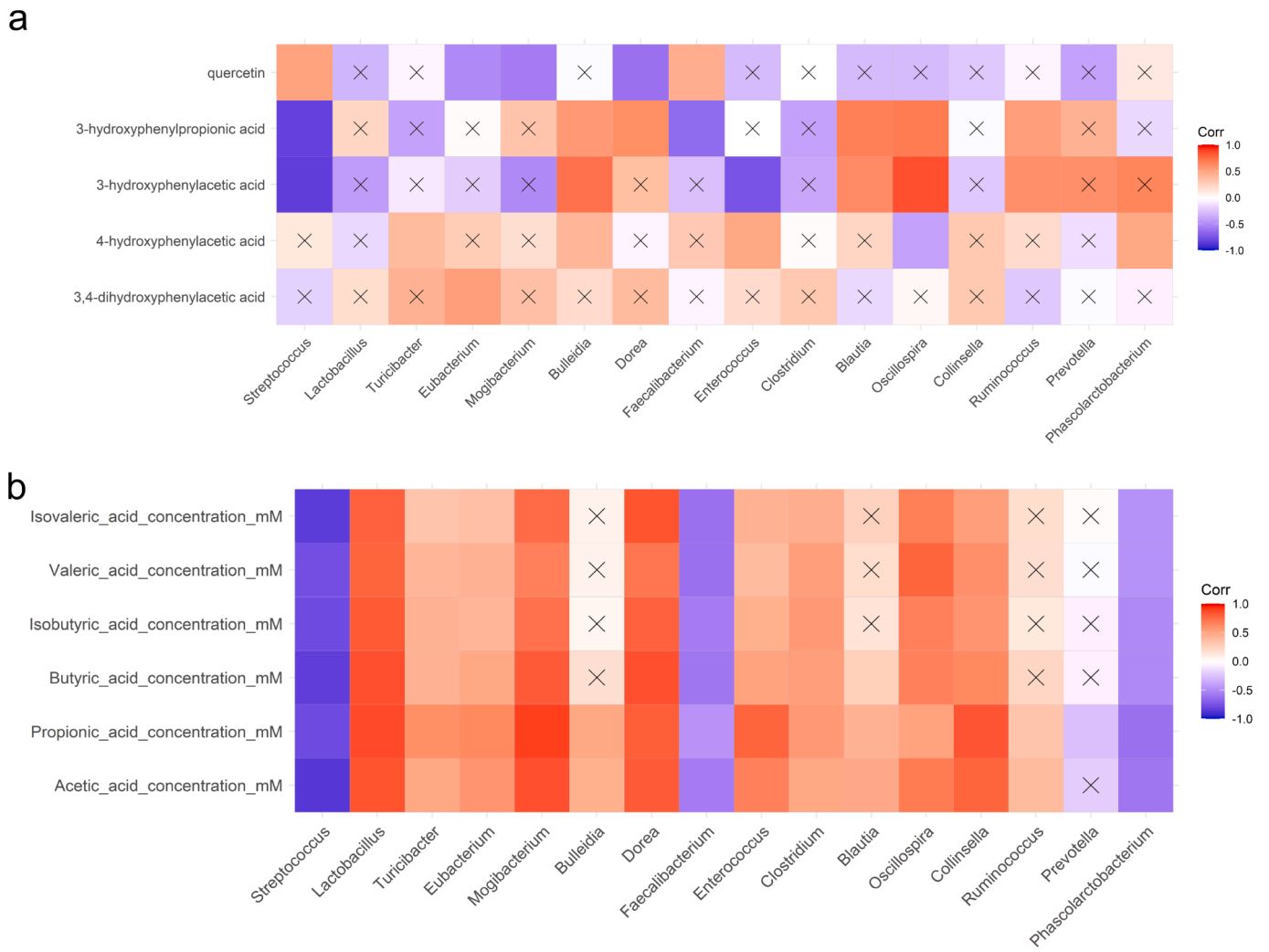

Fig. 5. Correlation of relative abundance of most abundant bacterial genera (>0.1%) with bacterial metabolites. Pearson's correlation (n = 60) heatmap between (a) quercetin and its metabolites and relative abundance of bacterial genera, (b) SCFA concentrations and relative abundance of bacterial genera (relative abundance > 0.1%), during 24 h *in vitro* fermentation. Red squares (positive correlation coefficient) indicate positive correlation while blue squares (negative correlation coefficient) indicate negative correlation. Squares without × are statistically significant at $p < 0.05$. Blank, UQ, ALINCH-E and ALINCH-Q fermentation conditions as in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

possibly because of their inferior capability of degrading quercetin to produce these metabolites. On the contrary, *Eubacterium, Mogibacterium* and *Dorea* were found to be negatively associated with quercetin, especially *Eubacterium* which was highly positively correlated with 3,4-dihydroxyphenylacetic acid (Fig. 5a). These correlations means that these bacteria genera are potentially quercetin degraders, particularly *Eubacterium* which is known to be capable of producing 3,4-dihydroxyphenylacetic acid as metabolite from quercetin (Ulbrich et al., 2015).

Furthermore, *Lactobacillus, Mogibacterium*, and *Dorea* had strong and positive correlations with all SCFAs production (Fig. 5b). *Turicibacter, Eubacterium, Enterococcus, Clostridium, Oscillospira,* and *Collinsella* were positively associated with SCFAs, but to a lesser extent, which possibly indicated their promotive roles in SCFA production. On the other hand, *Streptococcus, Faecalibaterium* and *Phascolarctobacterium* were negatively associated with all SCFAs (Fig. 5b). This insinuates that these three genera might negatively impact the production of SCFAs, or the other way around, the SCFAs have impeded the growth of these bacteria genera.

The result from correlation analysis helps to explain the modulation effect of ALINCH-Q on metabolites production and microbiota composition. ALINCH-Q produced the highest level of SCFAs and displayed high abundance of *Lactobacillus, Clostridium, Turicibacter* and *Eubacterium* which are positively associated with SCFAs production. With special regard to *Eubacterium*, the group contains species that are well-known butyrate producers and are also quercetin degraders.

4. Conclusion

During the *in vitro* batch colonic fermentation, the alginate-inulin-chitosan quercetin loaded microspheres (ALINCH-Q) shifted the metabolic pathway of quercetin to significantly higher production of 3-hydroxyphenylpropionic acid and 3-hydroxyphenylacetic acid compared to UQ, while promoted further metabolism of 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid. This indicates the effects of the fiber matrix on modulating bacterial activities altering the enzymatic reactions governing the degradation and dehydroxylation of these metabolites. In addition, ALINCH-Q significantly increased all SCFAs production, except for isovaleric acid, in a synergistic manner possibly because quercetin boosted the growth of SCFA-producing bacteria. Furthermore, by examining the microbiota composition, it was found that ALINCH-Q microspheres shifted the alpha diversity, showed distinct feature in NMIT analysis, and were characterized by the higher varieties of bacteria taxa in LEfSe analysis, suggesting its potential role in alleviating gut dysbiosis by increasing the richness and

diversity of the bacterial community. Differences in relative abundance of most abundant bacteria genera throughout fermentation showed that the microspheres enhanced the growth of *Streptococcus, Lactobacillus, Turicibacter, Eubacterium* and *Clostridium,* while inhibiting the growth of *Enterococcus*. *Lactobacillus*, *Eubacterium* were further found positively correlated with higher production of SCFAs and/or quercetin metabolites, further explaining the modulating effects of ALINCH-Q on the microbiota and their metabolites production. These results indicate that ALINCH-Q could be served as a functional delivery tool for quercetin that can exert health benefits in the gut.

CRediT authorship contribution statement

Siyao Liu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Yit Tao Loo:** Data curation, Formal analysis. **Zhenzhao Li:** Data curation, Formal analysis. **Ken Ng:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135802.

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