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Proceeding Paper

Influence of Probiotic Fermentation on the Bioactive Compounds, Glucosinolates Content and Antioxidant Properties of *Brassica oleracea* †

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Abstract: Symbiotic include the combination of prebiotics and probiotics, which promote good gut health and maintain balance of biological activity in the body. Brassica vegetable enriched with various health promoting compounds especially high level isothiocyanate that have been reported with good anticancer activities in cancer diseases and immunity enhancement. In this study, the use of cabbage as prebiotic coupling with probiotics strains had been investigate under fermentation process for optimal yield of total flavonoid (TFC), phenolic (TPC), glucosinolates (TGLs) and antioxidant activity. The power of single or combination strains of probiotic bacteria in the production of probiotic fermented cabbage (PFC) that yielding high bioactive compounds had been investigated. The addition of probiotic showed significant improvement of the bioactive compounds such as flavonoid, phenolic, glucosinolates contents and antioxidant activity compared to raw and fermented cabbage without probiotic bacteria. The PFC that consisting of 8 probiotics showed highest TFC (818.6 mg QE/100 g) and TGLs content (25.097 mg/100 g). The fermentation of single probiotic strain of *Lactobacills fermentus SK324* showed highest TFC (642.08 mg QE/100 g) and TPC (389.743 mg GA/100 g). However, there is no significant different on antioxidant activity among single or combination probiotic in the cabbage fermentation. The symbiotic PFC could offer high nutritional value and bioactive compounds that benefit for health. As such simple, cheap and easy to apply functional food could be developed to reduce the occurrence of cancer, in addition holds great promise for the future of medicine.

Keywords: functional food; symbiotic; health; prebiotic; glucosinolates

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1. Introduction

Cabbage is a vegetable from the Brassicaceae family that is grown and consumed globally. According to research by Shawon, et al. [1], consuming Brassica vegetables can help prevent various types of cancers such as breast, colon, and stomach. Cabbage contains phytochemicals that are beneficial to human health, such as antioxidants and antibacterial agents [2]. These active compounds help enhance the body's antioxidant and detoxification systems, which eliminate cancer-causing toxins.

The anticancer properties of Brassicaceae plants come from their glucosinolates, which can be hydrolysed into bioactive compounds such as isothiocyanates in the presence of myrosinase enzyme [3]. Cabbage has high levels of glucosinolates, which can be converted into anticancer ITCs [4]. However, the cooking process usually destroys natural

GSLs. his study aims to enhance the production of ITCs using selected probiotics to ferment cabbage extract. The fermented cabbage extract produced by the bacterial consortium could help discover anticancer bioactive compounds.

Probiotics are live microorganisms that help maintain a healthy and balanced gut microflora in the host's body [5]. Additionally, probiotics can produce enzymes that are not naturally present in the human digestive system. These probiotic enzymes have the ability to break down peptides and generate antioxidant and anti-inflammatory compounds [6]. Synbiotics refer to a combination of probiotics and prebiotics that work together to enhance the survival and integration of live microbial supplements in the digestive system. In this study, the use of cabbage as prebiotic coupling with different probiotics strains either single or combination strains had been investigate under fermentation process for optimal yield of total flavonoid (TFC), phenolic (TPC), glucosinolates (TGLs) and antioxidant activity. The symbiotic probiotic fermented cabbage (PFC) could offer high nutritional value and bioactive compounds that benefit for health.

2. Materials and Methods

2.1. Probiotics Sources

Probiotic bacteria sources had been obtained in freeze dried powder form from Dr Vijitra Luang-In of Mahasarakham University, Thailand. The types of probiotic bacteria used in this study including *Enterobacter xiangfangensis* 4A-2A3.1 (P1), *Lactococcus hircilactis* WS16 (P2), *Lactococcus lactis* WS18 (P3), *Lactococcus lactis* subsp. lactis TBRC 375 (P4), *Lactobacillus plantarum* SK321 (P5), *Lactobacillus fermentum* SK324 (P6), *Lactobacillus brevis* TRBC 3003 (P7) and *Bifidobacterium adolescentis* TBRC 7154 (P8).

2.2. Probiotic Activation and Cell Countings

The activation of probiotic strain was beginning with the incubation of the mixture comprising 2 mL of LB or MB and 10 mg freeze dried powder of bacteria strains at 37 °C with constant shaking at 150 rpm in orbital shaker up to 2–3 days until the culture grows and turn the culture solvents turbid. The probiotic concentration was counted with haemocytometer. About 6 μL of the mixture of cell suspension and trypan blue (1:1 ratio) loaded between the haemocytometer and cover glass by using micropipette (Eppendof, 10–100 μL). The goal is to have roughly 100–200 cells/square. Due to the bacterial cells are extremely small and abundance, the total number of cell count were counted from the centre box of haemocytometer and adopted the equation below:

$$A \times 5 \times 10^4 \times DF = \text{the number of cells/mL of suspension.} \quad (1)$$

where A = the mean number of cells per square, DF = dilution factor.

2.3. Cabbage Fermentation

White round cabbage (*Brassica oleracea*) from Cameron grower had been purchased from, Kai Ying Enterprise, Terengganu, Malaysia. After removing the core and outer layers, 6 kg of cabbage heads from the same batch were separated into leaf pieces. There are 8 single strains group (label as M1–M8), 4 combination strains (M9–M12) and 2 control (raw cabbage and fermentation cabbage without addition of probiotics). About 150 g of cut cabbage will put into a glass container and immerse in 150 mL fermented rice water at 7% salt concentration. The containers were capped tightly and kept at 25 °C for 3 days without shaking. The fermented cabbage and its pH data were collected at 48 h and 72 h fermentation period. Triplicate measurements were performed throughout the study.

2.4. Total Flavonoid Contents

The total flavonoid content (TFC) of fermented cabbage was analysed using the aluminium chloride ($AlCl_2$) colorimetric method (Chandra et al., 2014). About 500 μL of diluted standard quercetin solution and sample methanolic extract were mixed with 500 μL

of 2% $AlCl_2$ and loading in the cuvette. The mixtures were then incubated at room temperature for 60 min. The absorbance of the mixtures was measured and recorded spectrophotometrically at 415 nm using the UV-Vis spectrophotometer (UV-1800, Shimadzu Corp, Japan). Standard quercetin (QE) at 20, 40, 60, 80, 100 ppm were used as the standard to generate standard curve for measurement of the flavonoid's activity present in the samples. The TFC was obtained from the standard calibration curve and expressed as mg QE/100 g of samples.

2.5. Total Phenolic Contents

Total phenolic content (TPC) was measured using Folin–Ciocalteau's method (Chandra et al., 2014). A serial dilution of gallic acid (GA) was used as standard. About 100 µL of sample extract/GA was mixed with 500 µL of distilled water and 100 µL of Folin–Ciocalteu's (FC) phenol reagent and incubated at room temperature for 6 min. 1 mL of 7% (*w*/*v*) sodium carbonate solution and 500 µL of distilled water were then added to the mixture and kept in the dark for 30 min at room temperature. All the samples/standard was determined at the absorbance of 765 nm in UV-Vis spectrometer against a reagent blank. TPC was calculated based on the standard calibration curve of gallic acid and the values were expressed as mg GA/100 g.

2.6. Total Glucosinolate Contents

The spectrophotometric analysis was performed based on protocol with slight modifications as mentioned in Mawlong, et al. [7] and Ishida, et al. [8]. About 100 µL of sample extract was mixed with 3 mL of 2 mM sodium tetrachloropalladate and 0.3 mL of double distilled water. The stock of 2 mM sodium tetrachloropalladate (Na_2PdCl_4) can be prepared from mixing 58.8 mg Na_2PdCl_4 with 170 µL concentrated HCl and 100 mL double distilled water. After incubation at room temperature for 1 h, the absorbance was measured at 425 nm using UV-Vis spectrophotometer (Labomed UV-VIS Double beam UVD-3500). A blank was set following the same procedure without the extract. Glucosinolates reference standard (GRS) powder obtained from rapeseed colza (European Reference Material, BC367) was used as standard into the predicted formula and the value expressed as mg GRS/100 g.

2.7. Antioxidant Properties

The antioxidant properties of PFC were conducted from DPPH radical scavenging assay as described by Rahman, et al. [9]. The hydrogen atom donating ability of PFC extractives was determined by the decolorization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produces violet/purple colour in methanol solution and fades to shades of yellow colour in the presence of antioxidants. Serial dilution of sample extract was prepared at different concentration (3.125–200 µg/mL). About 100 µL of extracts are mixed with 150 µL of 0.1 mM DPPH solution, vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid (AA) was used as reference. Percentage DPPH radical scavenging activity was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(V0-V1)}{Vo} \times 100 \quad (2)$$

where V_0 is the absorbance of the DPPH control, and V_1 is the absorbance of the extractives/standard. Then growth inhibition activity was plotted against concentration, and from the graph of EC_{50} was calculated. EC_{50} obtained from DPPH scavenging activity represent the efficient concentration of sample to remove 50% active radicals, which also reduce the total reactive oxygen species.

2.8. Statistical Analysis

All the data obtained in triplicate were process with Statistical Package for Social Science (SPSS) software (SPSS Version 23, IMB Worldwide, USA) for the initial statistical analysis. One-way analysis of variance (ANOVA) were used for discrimination analysis. In the ANOVA analysis, the homogeneity variance test and Post Hoc Test using equal variances assumed Duncan, where n = 3 for each parameter were conducted. The treatments that fall in the different homogenous subset group and Duncan Post Hoc Test with $p < 0.05$ denoted for significantly difference among the substrate treatment.

3. Results and Discussion

3.1. Comparison of Single Probiotic Strain

From findings on the effect of single probiotic strains, PFC with probiotic *Lactobacillus fermentum SK324* (P6) at fermentation period 72 h showed optimal yield of TFC, TPC, TGLs and antioxidant activities following by *Bifidobacterium adolescentis TBRC 7154 (P8)*. Several studies come in agreement of the improvement yield with fermentation of *Lactobacillus fermentum* in grain sorghum [10,11], vinegar [12], rice [13]and juice [14]. The fermentation period at 72 h showed an overall higher yield of bioactive compounds than fermentation period at 48 h. During fermentation, the microorganisms present break down various compounds in cabbage, such as phenolics, into metabolites that are often more bioactive than the original compounds [15]. This conversion process can lead to an increase in the content of flavonoids and phenolic compounds, which are known to have antioxidant properties [16]. It may explained why 72 h fermentation promotes higher conversion of large compounds, resulting in the production of more bioactive metabolites that exhibit enhanced antioxidant properties [15].

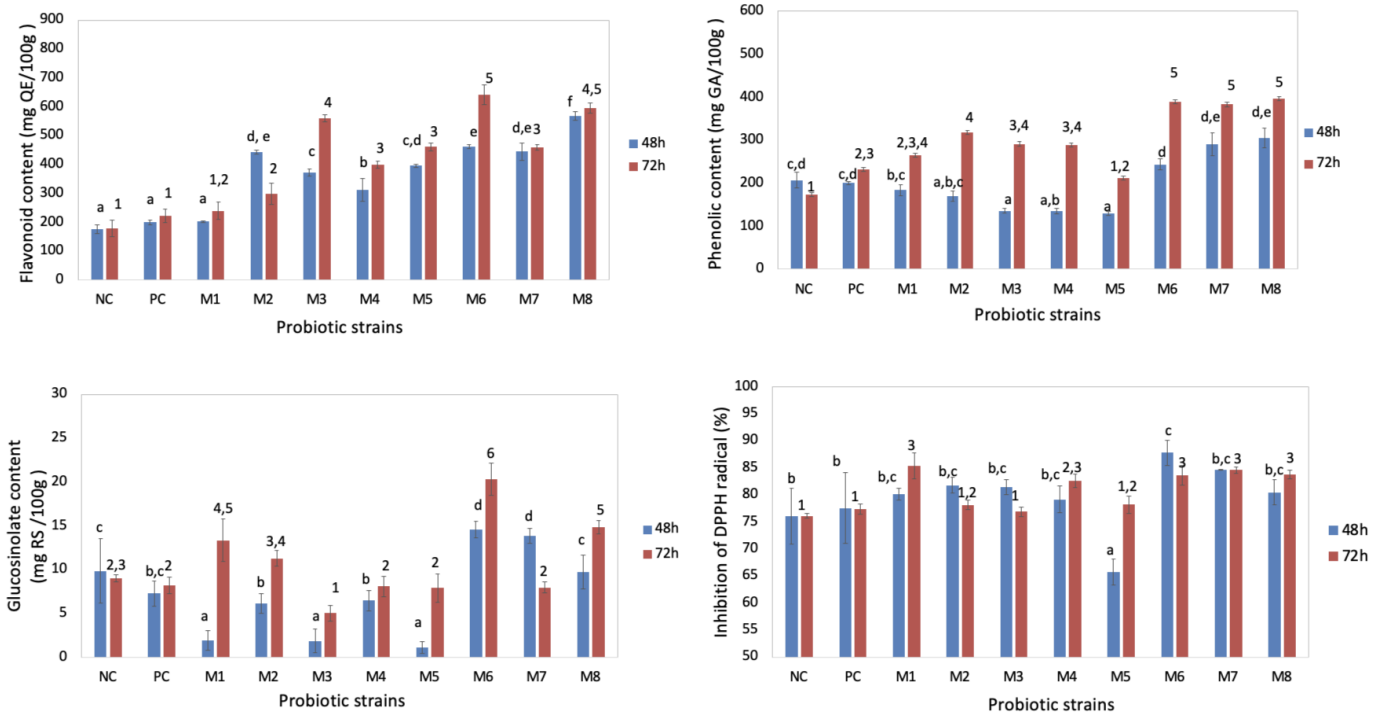

Figure 1. Bioactive compounds and antioxidant activities of PFC from single probiotic strain. (a) TFC, (b) TPC, (c) TGLs and (d) antioxidant activities with inhibition of DPPH radical. Notes: NC = raw cabbage, PC = fermented cabbage without no probiotic, M1–M8 fermentation group represented the single strains of probiotic added in respective order from P1–P8. Standard reference label QE = Quercetin, GA = Gallic acids, RS = Rapeseed and DPPH = 2,2-diphenyl-1-picrylhydrazyl**.**

3.2. Comparison of Combination of Probiotic Strains

Combination of multi-strain of probiotics showed significant improved in the total of bioactive compounds of PFC compared with single probiotic strains. PFC comprising 8 probiotic strains (M9) showed the highest yield of TFC (810.6 mg QE/100 g), TPC (494.62 mg GA/100 g) and TGLs (25.097 mg/100 g). Probiotic combined from different genus (M9 & M10) showed improved yield of bioactive compound than combination of probiotic from single genus (M11 & M12). Research suggests that a higher probiotic concentration added to the fermentation of cabbage can lead to a higher yield of bioactive compounds and increased antioxidant activity [17,18]. For instance, combination strains proven to be more efficient than single in the bioactive compound profile in yogurt [19]. Probiotics can contribute to the breakdown of cabbage components, such as phenolic compounds, into more bioactive forms [17]. This enzymatic activity by probiotics results in the production of metabolites with enhanced antioxidant properties [18]. More strains imply more chances of success; it can mean a broader spectrum of efficacy [20]. Different strains may target specific aspects of health, such as immune function, digestion, or gut health, allowing for a more comprehensive approach to addressing various health concerns [20]. This diverse range of strains can work synergistically to provide a more robust and versatile functionality in supporting overall health and well-being [21]. This synergistic cooperation can lead to amplified health benefits and increased effectiveness in functional food products [21].

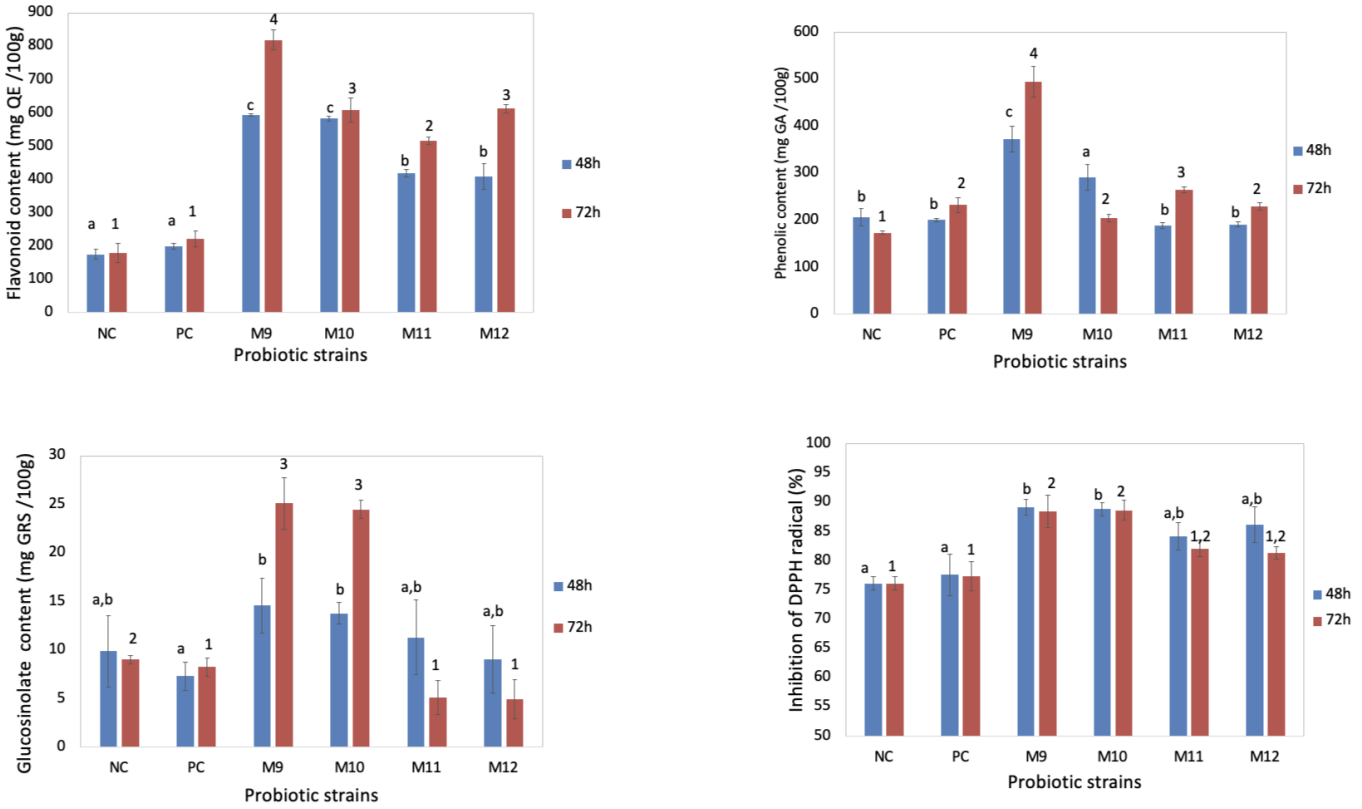

Figure 2. Bioactive compounds and antioxidant activities of PFC from combination of probiotic strain. (a) TFC, (b) TPC, (c) TGLs and (d) antioxidant activities with inhibition of DPPH radical. Notes: NC = raw cabbage, PC = fermented cabbage without no probiotic, M9 = addition of all 8 combined strains, M10 = addition of 10^6 CFU/mL 4 probiotic strains (P1, P3, P6, P8), M11 = addition of 10^6 CFU/mL 3 probiotic strains (P2, P3, P4), M12= 10^6 CFU/mL 3 probiotic strains (P5, P6, P7). Standard reference label QE = Quercetin, GA = Gallic acids, RS = Rapeseed and DPPH = 2,2-diphenyl-1-picrylhydrazyl.

4. Conclusions

Combination of multi-strains of probiotic fermented cabbage significantly improved the overall bioactive compounds level and higher antioxidant activities. Probiotics, whether in single-strain or multi-strain formulations, have gained significant importance and contribute to the field of functional food. Each strain has its own unique properties and mechanisms of action, and employing multiple strains expands the potential for success in delivering specific health benefits. Hence, cheap and easy to apply functional food such as cabbage could be developed to reduce the occurrence of disease and cancer, in addition holds great promise for the future of medicine.

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