รายงานวิจัยฉบับสมบูรณ์

The influence of microbes isolated from Thai fermented foods and beverages on the metabolic fate of glucosinolates

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สัญญาเลขที่ TRG5880096

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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Abstract

Project Code : TRG5880096

Project Title : The influence of microbes isolated from Thai fermented foods and beverages on the metabolic fate of glucosinolates

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Cancer has become the main cause of death in Thailand. Consumption of Cruciferous vegetables containing glucosinolate has been shown to reduce the cancer risks due to chemopreventive products namely isothiocyanate (ITC) generated by myrosinase-positive microbes in fermented foods. The aims of this work were to isolate and identify bacteria from eight Thai local fermented foods and beverages in Thailand with the capacity to metabolize glucosinolate using HPLC analysis and determine ITC using GC-MS analysis. The majority of ITC producers were *Enterobacter* and *Enterococcus* and the two highest ITC producers included *Enterobacter xiangfangensis* 4A2 (EX) from fermented fish and *Enterococcus casseliflavus* SB2 (EC) from fermented cabbage from 100% sinigrin degradation within 24 h. EC and EX were used to ferment Thai cabbage (*Brassica oleracea* L. var. capitata) containing glucoraphanin, glucoiberin and 4-hydroxy glucobrassicin for 3 days at 25°C. Sulforaphane and iberin produced by EX peaked at 294.1 and 117.4 μmol/100 g DW, respectively at day 1. Those produced by EC peaked at 242.6 and 51.7 μmol/100 g DW, respectively at day 2. These induced cabbage fermentations produced statistically higher chemopreventive ITCs than spontaneous cabbage fermentation over 3 days (p<0.01). Sulforaphane detected in induced cabbage fermentation with liquid portion were significantly (p<0.01) higher than fermented solid cabbage portion by almost three folds. However, extract of fermented cabbage (50 μg/mL) by EX at day 1 showed no antibacterial activity against *Enterococcus faecium, Pseudomonas aeruginosa* (PAO1), *Klebsiella pneumonia, Escherichia coli* and *Candida albicans*. Interestingly, the same extract showed 20.86% cytotoxicity towards lung cancer cell line NCI-H187. In addition, the same extract at 25 mg/mL exhibited 62% DPPH scavenging activity (DPPH assay), 6.51 mg $FeSO_4$/g dry weight (FRAP assay) and 5.21 mg VEAC/g dry weight (ABTS assay) which were higher than those in the extracts of cabbage by spontaneous fermentation. To conclude, Thai cabbage fermented by the selected bacterial culture for 1-2 days showed the potential as a functional food on the same par as kimchi and sauerkraut. One should consume a liquid portion of the fermented cabbage due to higher ITC level along with a solid portion to obtain the best health-promoting benefits from this functional food.

Keywords : Bacteria, Chemoprevention, Glucosinolate, Isothiocyanate, Myrosinase

บทคัดย่อ

Project Code : TRG5880096

Project Title : อิทธิพลของจุลินทรีย์ที่แยกได้จากอาหารและเครื่องดื่มหมักดองของไทยต่อการเมทาบอลิซึมของสารกลูโคซิโนเลต

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มะเร็งถือเป็นสาเหตุหลักในการคร่าชีวิตของประชากรในประเทศไทย การบริโภคผักตระกูลกะหล่ำที่อุดมไปด้วยสารกลูโคซิโนเลทซึ่งมีฤทธิ์ต้านมะเร็งจากผลิตภัณฑ์เมแทบอไลท์ที่ชื่อว่า ไอโซไธโอไซยาเนท ที่ถูกสร้างขึ้นโดยจุลินทรีย์ที่มีเอนไซม์ไมโรซิเนส จุดประสงค์ของงานวิจัยนี้ คือ การคัดแยกและจำแนกจุลินทรีย์ที่มีเอนไซม์ไมโรซิเนสในอาหารและเครื่องดื่มหมักดองในประเทศไทย โดยใช้เครื่อง HPLC เพื่อจำแนกเชื้อที่ย่อยสลายกลูโคซิโนเลทได้ และใช้เครื่อง GC-MS เพื่อศึกษาไอโซไธโอไซยาเนท จากผลการทดลอง พบว่า โดยส่วนใหญ่ จุลินทรีย์ที่ผลิตไอโซไธโอไซยาเนทได้ คือ *Enterobacter* และ *Enterococcus* และเชื้อที่ผลิตไอโซไธโอไซยาเนทได้สูงที่สุด คือ *Enterobacter xiangfangensis* 4A2 (EX) จากปลาส้ม และรองลงมา คือ *Enterococcus casseliflavus* SB2 (EC) จากผักกะหล่ำดอง หลังจากการบ่ม 24 ชั่วโมงกับซินิกริน ดังนั้น EX และ EC จึงถูกนำมาหมักผักกะหล่ำดอง (*Brassica oleracea* L. var. capitata) ซึ่งอุดมด้วยสารกลูโคไอเบอริน กลูโคราฟานินและ 4-ไฮดรอกซี่ กลูโคบราสซิซิน ที่อุณหภูมิ 25 องศาเซลเซียส เป็นเวลา 3 วัน พบว่าซัลโฟราเฟนและไอเบอรินถูกผลิตขึ้นโดย EX มีค่าสูงสุดที่ 294.1 และ 117.4 ไมโครโมล ต่อ 100 กรัมน้ำหนักผักแห้ง ตามลำดับ หลังการหมัก 1 วัน แต่ทว่าในกะหล่ำดองที่หมักด้วย EC พบผลิตภัณฑ์สองอย่างนี้ถูกผลิตขึ้นสูงที่สุดที่ 242.6 และ 51.7 ไมโครโมล ต่อ 100 กรัมน้ำหนักผักแห้ง ตามลำดับ ในวันที่ 2 การหมักผักกะหล่ำด้วยเชื้อที่คัดแยกมานี้ทำให้ได้ซัลโฟราเฟนและไอเบอรินที่สูงกว่าการหมักแบบธรรมชาติ ($p < 0.01$) ในเวลาหมักทั้ง 3 วัน สิ่งที่น่าสนใจ คือ ในน้ำผักดองจะมีซัลโฟราเฟนและไอเบอรินที่สูงกว่าในเนื้อผักดอง เกือบ 3 เท่า ($p < 0.01$) เมื่อนำสารสกัดจากผักดองที่หมักด้วย EX เป็นเวลา 1 วันไปทดสอบกิจกรรมการต้านเชื้อก่อโรค พบว่าสารสกัดที่ความเข้มข้น 50 ไมโครกรัมต่อมิลลิลิตร ไม่สามารถยับยั้งเชื้อ *Enterococcus faecium, Pseudomonas aeruginosa* (PAO1), *Klebsiella pneumonia, Escherichia coli* และ *Candida albicans* ได้ ส่วนผลการต้านเซลล์มะเร็ง พบว่าสารสกัดจากผักดองที่ความเข้มข้น 50 ไมโครกรัมต่อมิลลิลิตร แสดง 20.86% cytotoxicity ต่อเซลล์มะเร็งปอด (NCI-H187) แต่ก็ยังไม่ถึง 50% จึงยังไม่ถือว่าเป็นพิษต่อเซลล์มะเร็งปอด และไม่แสดงความเป็นพิษต่อเซลล์มะเร็งเต้านมและเซลล์ไตของลิง (Vero) อย่างไรก็ตาม สารสกัดผักกะหล่ำดองโดย EX เป็นเวลา 1 วัน ที่ความเข้มข้น 25 มิลลิกรัมต่อมิลลิลิตร แสดงกิจกรรมต้านอนุมูลอิสระที่ 62% DPPH scavenging activity, 6.51 มิลลิกรัม $FeSO_4$ ต่อกรัมน้ำหนักแห้ง และ 5.21 มิลลิกรัม VEAC ต่อกรัมน้ำหนักแห้ง ซึ่งโดยรวม มีค่าสูงกว่ากิจกรรมต้านอนุมูลอิสระของกะหล่ำดองที่หมักโดยธรรมชาติ โดยสรุป ผักกะหล่ำดองของประเทศไทยที่ถูกหมักโดยเชื้อที่แยกมาได้นี้มีศักยภาพที่จะเป็นอาหารฟังค์ชันเทียบเท่ากิมจิของประเทศเกาหลี และซาวเออร์เคราท์ของทวีปยุโรปได้ โดยควรทำการหมักเป็นเวลา 1-2 วัน และควรบริโภคน้ำผักดองด้วย เพราะว่ามีซัลโฟราเฟนและไอเบอรินในปริมาณที่สูง เพื่อยังผลให้เกิดประโยชน์ต่อสุขภาพของผู้บริโภคอย่างสูงสุด

คำสำคัญ : แบคทีเรีย, การต้านมะเร็ง, กลูโคซิโนเลต, ไอโซไธโอไซยาเนต, ไมโรซิเนส

The influence of microbes isolated from Thai fermented foods and beverages on the metabolic fate of glucosinolates

Objectives

1. To identify which microbe isolated from Thai fermented foods and beverages is able to degrade GSL.
2. To determine the degradation of GSLs in each Cruciferous plant extract by metabolism of microbes in isolation and combination i.e. microbial consortia.
3. To determine and optimize the production of the degradation products of GSLs in each Cruciferous plant extract by metabolism of microbes in isolation and combination.
4. To determine the antioxidant, antibacterial and anticancer activity of the fermented Cruciferous vegetables from metabolism of microbes in isolation and in combination.

Materials and Methods

1. Sample collection

Eight samples of fermented foods and beverages at the end of fermentative stage were purchased mainly from local markets in Mahasarakham, Thailand except for samples no. 7 and 8 for isolation of myrosinase-positive microbes. Samples (Fig. S1) included (1) Fermented cabbage (2) Picked onions (3) Fermented fish (4) Fermented pork (5) Fermented herbal drink (6) Fermented star fruit juice, (7) Water kefir from Nakhon Ratchasima and (8) Milk kefir from Kamphaeng Phet. Samples were stored at 4°C and analyzed within 24 h.

2. Isolation of GSL-metabolizing microbes

Solid food materials and liquid (5 g each, 10 g in total) or liquid beverage (10 ml) were weighed and mixed with 90 ml of sterile 0.85% NaCl solution. The mixture was homogenized in a sterile mortar and pestle for 5 min and mixed by vortexing for 5 min. The mixture was centrifuged at 4000g for 15 min and clear supernatant was obtained. Enrichment culture technique was used by inoculating 100 µl bacterial suspension into 900 µl LB broth containing 1 mM sinigrin for 2 days in anaerobic incubator and this step was repeated at day 4, 6 and 8 in fresh Luria-Bertani (LB) media (10 g Tryptone; 10 g NaCl; 5 g Yeast extract in 1 l). At day 10, 100 µl bacterial suspension was spread onto the selective minimal media M9 agar (1 M $MgSO_4$; 1 M $CaCl_2$; 50% Glucose; 1% Thiamine; 64 g Na_2HPO_4-$7H_2O$; 15 g KH_2PO_4; 2.5 g NaCl; 5.0 g NH_4Cl; 15 g agar in 1 l) containing 1 mM sinigrin and 2.5 mM barium acetate and incubated at 37°C for 72 h in the anaerobic incubator. Growth and opaque zone formation was an indicator of sinigrin degradation as seen from white precipitates of barium sulfate. This was a result of a release of sulfate group of GSL and thus GSL-metabolizing/myrosinase-positive isolates were selected from each food sample. Positive isolates were stored in 20% glycerol stocks in LB media at -80°C. All microbial isolates were deposited in the Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand (WDCM 1160).

3. *In vitro* sinigrin incubation

Sinigrin (1 mM) was incubated with each selected bacterial culture (100 µl, OD_{600nm} = 0.5) from the previous step in 100 µl LB media at 37°C without shaking in anaerobic incubator for 24 h. Bacterial culture was centrifuged at 16000g for 5 min and then 100 µl clear supernatant to be used for HPLC analysis and the other 900 µl for GC-MS analysis were kept at -20°C until use.

4. Genomic DNA isolation and 16S rDNA gene analysis

Selected isolates with the confirmed positive results of GSL degradation from HPLC analysis were cultured overnight for gram-staining, genomic DNA extraction and PCR-based 16S rRNA gene analysis using universal primers following the previous report (Luang-In et al., 2014).

5. Phylogenetic tree construction

The nucleotide sequences of isolated bacterial 16S rDNA, from this study and previous findings were compared with entries in the GenBank database. Similarly, putative myrosinases of bacterial relatives in the GenBank database were compared with the characterized myrosinases. The sequences were aligned using MUSCLE (Edgar, 2004). Phylogenetic trees were constructed using the maximum-likelihood method and assessing the reliability the constructed phylogenetic tree with 1000 bootstrap replicates, implemented in the Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software package (Kumar et al., 2015). The constructed phylogenetic trees of 16S rRNAs and proteins were drawn by FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) [http://tree.bio.ed.ac.uk/software/figtree/].

6. Starter culture preparation

Each of the two selected bacteria was grown in 10 ml LB media overnight and centrifuged at 8,000 g for 15 min at 4°C. The cells were washed twice with overnight fermented sticky rice water (initial pH 6.0). The inoculum concentration of 10^6 CFU/ml was inoculated in 3% (v/v) into the prepared cabbage-rice water jar (200 ml) to be fermented as mentioned below. Induced fermentations were defined by inoculation of either *Enterobacter xiangfangensis* 4A-2A3.1 (EX) or *Enterococcus casseliflavus* SB2X2 (EC) culture and spontaneous fermentations (N = Non-induced) were those without inoculation of either culture.

7. Cabbage fermentations

Thai white cabbage heads (*Brassica oleracea* L. var. capitata) were purchased from Pran Fresh Co. Ltd, Khon Kaen, Thailand. After removing core and outer layers, 3 kg of the cabbage heads from the same batch were separated into leaf pieces manually according to Thai local cabbage fermentation procedure. The spontaneous fermentation was performed by mixing torn plant materials well with 7% salt (w/v), washed with distilled water, and only 200 g solid plant materials were transferred into each replicate fermentation pot (200 ml glass container with lid already containing 200 mL fermented rice water pH 6.0 mixed with 7% salt). The salted cabbage materials were tightly pressed into the jars that were closed and kept at 25°C for 3 days without shaking. Triplicates were carried out throughout the study. The control

were those 200 g fresh cabbage heads separated into leaf pieces manually without fermentation at 0 h as the starting materials and they were determined for initial GSLs and initial ITC products to be compared with spontaneously fermented cabbage samples (N) and each of the cabbage fermentation induced by EX or EC.

8. Sampling and extraction of fermented cabbage

The fermentation experiment was carried out in parallel in 36 jars with triplicate in each of the three treatments (N, EC, and EX) from 0 to 3 days. Sampling was done at day 0, 1, 2 and 3, pH was measured immediately after opening the fermentation jars. For extraction of GSLs and ITC products, the whole samples from each jar collected as mentioned above were frozen at -80°C, dried in a freeze dryer, and processed accordingly for GSL and ITC analyses as mentioned below. For EC samples at day 2, half of cabbage leafy material and half of fermented liquid were taken separately for GSL and ITC determination to evaluate which part contained higher contents. Dried samples were ground to small pieces using a sterile mortar and pestle and weighed for extraction by 95% ethanol at concentration of 25 mg/ml at 25°C for 24 h. The mixture was centrifuged at 16000g for 5 min and clear supernatant was collected for antioxidant activity analyses.

9. Sample preparation and HPLC analysis to detect GSLs

The GSL extraction method was modified from the previous report (VicaŞ et al., 2011). Freeze dried samples (5 g) were ground and mixed with 5 ml of 70% methanol by shaking at 37°C for 5 min and the supernatant was collected after centrifugation at 8000g for 15 min. The remained solid sample was extracted again and the supernatant was mixed with the first extraction. The mixture was dried at 70°C in the oven and the dried residues were dissolved in 1 ml deionized water using vortex. One ml sample was processed in DEAE-25A anion exchange resin as previously described (Luang-In et al., 2014). HPLC–DAD system (Shimadzu, Japan) fitted with a Synergi 4u Hydro-RP 80A, 150 x 2 mm, 4.6 micron (Phenomenex Inc., Torrance, CA) protected with security guard column AQ C18 (4 x 3 mm), comprising of Shimadzu LC-20AC pumps, a SPD-M20A diode array detector and were used for GSL analysis using the following gradient: Water (Solvent A)–ACN (Solvent B) gradient 2% B (15 min), 2–25% B (2 min), 25–70% B (2 min), 70% B (2 min hold), 70–2% B (2 min), and 2% B (15 min) at a flow rate of 0.2 ml/min at 35ºC. Eluent was monitored at A229 nm. Quantification of desulfo-glucosinolate (DS-GSL) was achieved using known response factors for each GSL relative to an external standard (sinigrin). Pure sinigrin (Sigma-Aldrich, Singapore), glucoraphanin, glucoiberin (Youchemicals Ltd., China) and 4-hydroxy glucobrassicin (Clearsynth, India) were purchased as standards.

10. Sample preparation and GC-MS analysis to detect degradation products

Freeze-dried samples (500 mg) were mixed with 3 mL dichloromethane (DCM) in test tubes with tight lids for 24 h at 250 rpm at room temperature. The mixture was centrifuged at 16100g for 5 min and the supernatant (1 ml) was added with 0.5 g magnesium, mixed and then was centrifuged at 16100g for 20 min. The clear supernatants were transferred into vials and kept at -20 °C till GC-MS analysis. A Shimadzu

QP2010 system and a capillary column, Agilent HP-5MS (5% Phenylmethylsiloxane, 30 m × 0.25 mm i.d.; film thickness, 0.25 μm) were used for ITC analysis. GC-MS analytic conditions were performed as previously reported (Luang-In et al., 2014). The temperature was kept at 50°C for 5 min and ramped to 150°C at 5°C/min for 25 min, and then ramped to 250°C at 5°C/min for 15 min. The total 40 min run was carried out with a flow rate of 1 ml/min, average velocity of 36 cm/s, pressure of 7.56 psi and injection volume of 1 μl. Mass spectra were obtained by electron ionization (EI) over a range of 50−550 atomic mass units. Ion source temperature was 230°C, and the electron multiplier voltage was 70.1 eV. Authentic standards of allyl isothiocyanate and sulforaphane were purchased from Sigma-Aldrich Co. (Singapore). Identification was based on retention time and fragment ions (Table S1). Quantification of degradation products was calculated using an external standard curve of sulforaphane.

11. Antioxidant activity of the fermented cabbage

This was evaluated through the free radical scavenging effect on 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical as previously reported (Akowuah et al., 2005), FRAP assay (Benzie & Strain, 1996) and ABTS scavenging assay (Seeram et al., 2006) using 25 mg/ml freeze-dried extract dissolved in 95% ethanol as a starting solution.

12. Antimicrobial activity of the fermented cabbage extracts

The MIC assay were performed at Bioassay laboratory service (BIOTEC, Thailand) as follows. The reference pathogenic microbes (*Enterococcus faecium, Pseudomonas aeruginosa* (PAO1), *Klebsiella pneumonia, Escherichia coli* and *Candida albicans*) were grown in tryptic soy agar (TSA) at 37 °C for overnight. A single colony is inoculated in Mueller Hinton Broth (MHB) and incubated in a rotary shaker 200 rpm at 37°C for 30 min. Cells at a logarithmic growth were harvested and diluted to $2.5x10^5$ CFU/ml in MHB prior to assay. This assay is performed in 384-well plate in triplicate. Each well is added with 5 μl of 50 μg/ml of sample (or positive or negative control agents) and 45 μl of cell suspension. The positive control was amikacin or ofloxacin and the negative control was 0.5% dimethylsulfoxide (DMSO). Blank wells were added with 5 μl of 5% DMSO and 45 μl media. Plates are then incubated at 37 °C for 14 hours. Bacterial growth was observed by OD_{600nm} measurement using microplate reader. The OD units of test wells are subtracted with mean OD units of blank wells before calculation. Percent of bacterial inhibition is calculated by the following equation:

$$\% \text{ Inhibition} = [1- (ODT/ ODC)] x100$$

The results of antimicrobial activity from Bioassay laboratory service (BIOTEC, Thailand) appeared negative, and thus not included for discussion.

13. Anticancer activity test of the fermented cabbage extracts

Resazurin microplate assay (REMA) was used carried out at Bioassay laboratory service (BIOTEC, Thailand). Human small-cell lung (NCI-H187), human breast adenocarcinoma (MCF-7) and human colon adenocarcinoma Caco2 cell line (ATCC HTB-37) were grown and maintained in a complete medium (Minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 0.1 IU/ml Insulin-Transferrin-Selenium-X, 1.5 g/L sodium bicarbonate, 100 unit/ml penicillin and 100 µg/ml streptomycin) and incubated at 37 °C humidified incubator with 5% CO_2. Cells at a logarithmic growth are harvested and diluted to $2x10^4$ cells /ml in complete medium prior to assay. The positive control was ellipticine and the negative control was 1% DMSO. This assay was performed in 96-well plate in four replicate wells. First, plates were seeded with 200 µl of cell suspension or blank medium into well, and incubated at 37°C humidified incubator with 5% CO_2 for 48 h. Subsequently, culture medium was replaced with 200 µl of fresh medium containing test-compounds or 1% DMSO, and plates were further incubated for 24 h. After incubation period, the plates was added with 50 µl of 125 µg/ml resazurin solution and incubated at 37°C humidified incubator with 5% CO_2 for 4 h. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths by using the bottom-reading mode of fluorometer. The signal is subtracted with blank before calculation. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ Cytotoxicity} = [1-(FUT/ FUC)] \times 100$$

The results of anticancer activity from Bioassay laboratory service (BIOTEC, Thailand) appeared negative, and thus not included for discussion.

14. Statistical analyses

Triplicates were used for each treatment. Results are expressed as means $\pm$ standard deviation (SD). The significant differences between means are calculated by a one-way analysis of variance (ANOVA) and Duncan's multiple range test at $p < 0.01$ using SPSS package version 19.

Results and Discussion

1. GSL-metabolizing bacteria from Thai fermented foods and drinks

Twenty-one bacterial isolates from 8 sources of Thai local fermented foods and beverages were identified as GSL-metabolizing bacteria using selective M9 agar containing sinigrin substrate and identified at subspecies level using 16S rRNA gene analysis. The results show that the greatest number of newly identified GSL-metabolizing bacterial species came from Thai fermented cabbage followed by Thai fermented fish resulting in isolation of 8 and 3 bacterial species, respectively (Table 1). When sinigrin was metabolized by bacterial myrosinase, the degradation product i.e. isothiocyanate namely allyl isothiocyanate (AITC) was expected. It was observed that most GSL-metabolizing bacteria were able to produce AITC from sinigrin metabolism (Table 1) using GC-MS and HPLC analyses, respectively. The majority of ITC-producing bacteria belong to the genera *Enterobacter* and *Enterococcus* with the two highest ITC producers named as *Ent. xiangfangensis* 4A-2A3.1 (EX) from fermented fish and *Ec. casseliflavus* SB2X2 (EC) from fermented cabbage producing 65 and 61 nmol AITC, respectively from

100% sinigrin degradation within 24 h (Table 1). Therefore, these two isolates were chosen as a starter culture to ferment cabbage in further experiment. Although bacteria belong to the same genus e.g. Enterobacter, they exhibited different GSL-metabolizing capacity therefore possibly different myrosinase activity (Table 1). AITC was unstable in the culture media (Luang-In & Rossiter, 2015) and thus AITC product formation never reached 100% product formation. The highest % product formation was found in EX with 65% product formation. However, *Lactococcus hircilactis* WS16, *L. lactis* WS18 and *Bacillus* sp. KW3 did not produce AITC from 77-80% sinigrin degradation suggesting they may have different GSL metabolic enzymes or mechanisms from other bacteria in metabolizing GSL, but not producing ITC. Two bacterial species were found in more than one sample. *Enterobacter* sp. 1A-1A with 92% identity to *Enterobacter* sp. Md1-53 was found in fermented cabbage, pickled onions and fermented juices. *Enterobacter faecalis* 5A-2B with 99% identity to *Enterobacter faecalis* NW A20 was present in both fermented cabbage and fermented herbal drink. Thus, there were 17 bacterial strains from 21 isolates from 8 fermented food/drink samples. All these 17 bacterial strains at subspecies level have not been reported as GSL metabolizer and/or ITC producers before, yet they shared the same genus or the same species as those identified previously. Previous findings showed a variety of GSL-metabolizing bacterial strains such as *Bacillus thuringiensis* (El-Shora et al., 2016), Actinomycetes isolated from cotton soil (Madhuri & Anuradha, 2015), *E. coli* VL8, *Enterococcus casseliflavus* CP1 isolated from human faeces (Luang-In et al., 2014), *Lactobacillus plantarum* KW30, *Lactococcus lactis* subsp. lactis KF147, *Escherichia coli* Nissle 1917 isolated from foods (Mullaney et al., 2013), *Bifidobacterium pseudocatenulatum, B. adolescentis, B. longum* (Cheng et al., 2004), *Lb. agilis* R16 (Palop-llanos et al., 1995), and known myrosinase-producer *Ent. cloacae* isolated from soil (Tani et al., 1974). In this work, all the identified 17 bacteria belong to the above reported genus including Bacillus, Lactococcus, Escherichia, Enterobacter and Enterococcus. Contrary to popular belief, *Lc. hircilactis* WS16 (100% identity to *Lc. hircilactis* DSM 28960) and *Lc. lactis* WS18 (98% identity to *Lc. lactis* RCB787) in this study did not produce ITC from GSL metabolism (Table 1). This result indicated that not all LAB are able to produce ITC as previously thought. Similarly, Mullaney et al. (2013) found that *Lb. plantarum* KW30 and *Lc. lactis* subsp.lactis KF147 did not produce any ITC from glucoraphanin, glucoerucin and glucoiberverin. Instead they generated sulforaphane nitrile as well as erucin nitrile and iberverin nitrile. Most isolated bacteria have the highest % identity to the closest relative bacteria originated from food sources and plants followed by human/animal guts and environments located in mostly Asia including China, India, Korea, Pakistan, Thailand and also other parts of the world including Italy, Belgium, Brazil and South Africa (Table 1). Although probiotic reports of both *Ent. xiangfangensis* and *Ec. casseliflavus* are scarce, the genome of *Ent. xiangfangensis* isolated from Chinese traditional sourdough was recently published (Gu et al., 2014). In addition, *Ec. faecium*-group and *Ec. faecalis*-group were also isolated at the early stages of fermentation of cauliflower fermentation (Paramithiotis et al., 2010) indicating that Enterococcus and Enterobacter were commonly found in fermented foods.

Table 1 Twenty bacterial isolates with glucosinolate-metabolizing capacity isolated from Thai local fermented foods and drinks

No.	Accession no.[a]	Species	Closet relative species[b] (% identity) /Accession no.[c]/Origin of isolate[d]	Sinigrin degradation (nmol) [e]	AITC product (nmol) [e]	% product formation [f]
1. Fermented cabbage pH 3.87						
1	LC342980.1	*Enterobacter* sp. 1A-1A	*Enterobacter* sp. Md1-53 (92%) MF581459.1 Paeonia ostii root, China	73 ± 8	30 ± 5	41 ± 4
2	LC342981.1	*Enterobacter faecalis* 5A-2B	*Enterococcus faecalis* NW A20 (99%) MG543833.1 Raw meat, South Africa	78 ± 7	39 ± 11	50 ± 14
3	LC342982.1	*Enterobacter asburiae* 1B-1	*Enterobacter asburiae* voucher ST56 KT287073.1 100% Rumen China	75 ± 11	33 ± 8	44 ± 6
4	LC342983.1	*Enterobacter* sp. 1B-2	*Enterobacter* sp. NU33 (96%) MG459258.1 Plant growth-promoting bacteria in sugarcane, Brazil	79 ± 5	41 ± 7	52 ± 8
5	LC342984.1	*Enterobacter ludwigii* S1E9	*Enterobacter ludwigii* HTP04 (100%) KX024731.1 Isolated from shrimp gut, India	90 ± 8	50 ± 9	56 ± 7
6	LC342985.1	*Enterococcus casseliflavus* SB2X2	*Enterococcus casseliflavus* HMF4406 (98%) KT984002.1 Jeotgal (salted fermented food), Korea	100 ± 0	61 ± 4	61 ± 6
7	LC342986.1	*Bacillus* sp. SA8	*Bacillus* sp. SK123 (97%) KU060226.1 Honey bee apiary, Thailand	79 ± 8	39 ± 7	49 ± 6
8	LC342987.1	*Bacillus* sp. 1.1	*Bacillus* sp. BDU13 (96%) JX847614.1 Microbial diversity from fermented fish, India	87 ± 10	42 ± 11	48 ± 10
2. Pickled onion pH 4.81						
9	LC342980.1	*Enterobacter* sp. 1A-1A	*Enterobacter* sp. Md1-53 (92%) MF581459.1 Paeonia ostii root, China	73 ± 5	40 ± 5	55 ± 4
10	LC342988.1	*Enterobacter* sp. 2B-1B	*Enterobacter* sp. SR19 (100%) KF896099.1 Seawater sediment, Belgium	71 ± 0	39 ± 3	55 ± 6
3. Fermented fish pH 4.60						
11	LC342989.1	*Enterobacter xiangfangensis* 4A-2A3.1	*Enterobacter xiangfangensis* W31 (100%) KP813789.1 Storm water bacteria in two urban lakes China	100 ± 0	65 ± 3	65 ± 4
12	LC342990.1	*Bacillus* sp. 4A-1	*Bacillus* sp. S42 (100%) JX293317.1 Crystal tuff, China	71 ± 6	40 ± 4	56 ± 1
13	LC342991.1	*Bacillus* sp. 4B1	*Bacillus* sp. SO5.17 (97%) KC867296.1 Mine drainage, Brazil	73 ± 4	42 ± 9	58 ± 13
4. Fermented pork pH 4.73						
14	LC342992.1	*Enterococcus casseliflavus* 3.10A1	*Enterococcus casseliflavus* JFL12 (100%) KT343156.1 Fiber-degrading bacteria in rumen of Tibetan yak, China	74 ± 13	35 ± 8	47 ± 4
5. Fermented herbal drink pH 2.80						
15	LC342981.1	*Enterobacter faecalis* 5A-2B	*Enterococcus faecalis* NW A20 (99%) MG543833.1 Raw meat, South Africa	78 ± 7	35 ± 6	45 ± 5
6. Fermented juice pH 2.93						
16	LC342993.1	*Enterobacter* sp. 10-B1	*Enterobacter* sp. DBM3 (97%) KT957440.1 *Plutella xylostella* larval gut, China	77 ± 5	34 ± 11	42 ± 16

17	LC342980.1	*Enterobacter* sp. 1A-1A	*Enterobacter* sp. Md1-53 (92%) MF581459.1 Paeonia ostii root, China	73 ± 10	34 ± 8	47 ± 7
7. Water kefir from Nakhon Ratchasima pH 5.94						
18	LC336444.1	*Lactococcus hircilactis* WS16	*Lactococcus hircilactis* DSM 28960 (100%) KJ201026.1 Goat milk, Italy	77 ± 4	nd	na
19	LC336446.1	*Lactococcus lactis* WS18	*Lactococcus lactis* RCB787 (98%) KT260999.1 Bat guano, India	78 ± 3	nd	na
8. Milk kefir from Kamphaeng Phet pH 5.23						
20	LC342994.1	*Bacillus subtilis* KW3	*Bacillus subtilis* MA-48 (93%) KX426648.1 Rhizospheric soil in desert, Pakistan	80 ± 9	nd	na

[a] GenBank accession no. of our strains on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)

[b] Closet relative species and identity (%) from BLAST search on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)

[c] GenBank accession no. of closest relatives on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)

[d] Origins of closet relative species i.e. where each bacterium was isolated from

[e] Each isolate from fermented samples was cultured in LB medium containing 1 mM sinigrin for 24 h. After that sinigrin degradation and AITC production were determined by HPLC and GC-MS, respectively.

[f] % product formation = [AITC product (nmol)/Sinigrin degradation (nmol)] x 100%

nd = not detected; na = not available

2. Phylogenetic tree of GSL-metabolizing bacteria

Phylogenetic tree shows 17 GSL-metabolizing bacteria isolated from this work and 9 reference bacteria with GSL-metabolizing capacity from the previous reports were categorized into 3 main groups (Fig. 1).

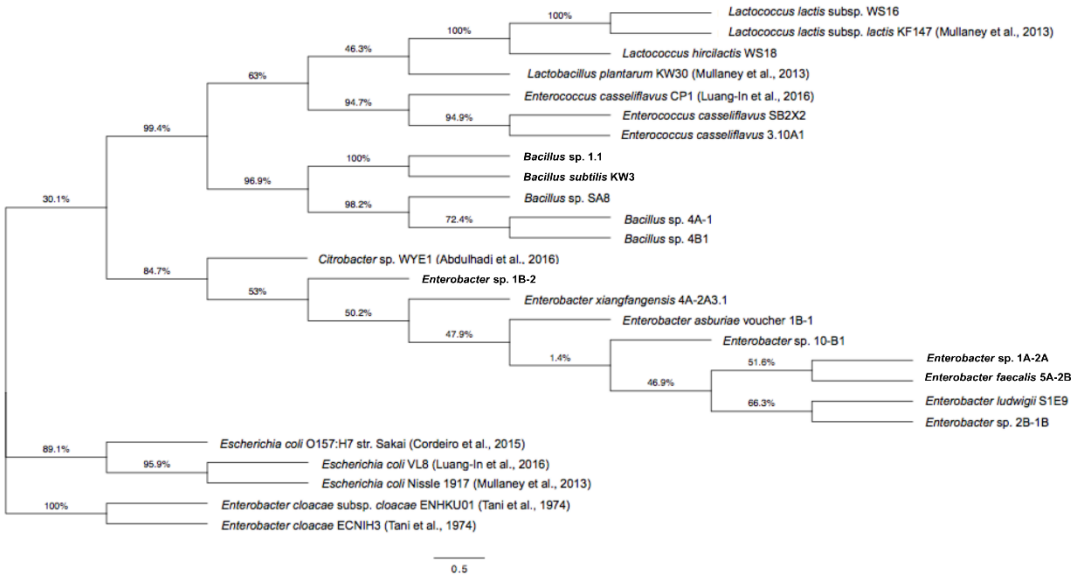

Fig. 1 Phylogenetic tree of GSL-metabolizing bacteria isolated from this work and the previous reports. It was inferred from 26 partial16S rRNA sequences from different bacteria by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The horizontal bar represents a distance of 0.5 substitutions per site. Evolutionary analyses were conducted in MEGA7 and the phylogenetic tree was drawn by using FigTree.

The first and biggest group (30.1 % node) comprised of all bacteria from this work including LAB, Enterococcus and Bacillus along with the reference LAB (Mullaney et al., 2013) and Enterococcus (Luang-In et al., 2014) from the previous findings. The subgroup consisted of Enterobacter as well as reference Citrobacter (Albaser et al., 2016). The second group (89.1% node) included only 3 reference *E. coli* bacteria (Mullaney et al., 2013; Luang-In et al., 2014; Cordeiro et al., 2015). Similarly, the third group (100% node) included only 2 reference Enterobacter bacteria (Tani et al., 1974). From this result, it seems *Enterobacter* spp. isolated from this work were evolutionarily closely related to *Citrobacter* sp. WYE1 (Albaser et al., 2016) than *Enterobacter cloacae* (Tani et al., 1974). This is the first report of identifying *Ent. xiangfangensis, Ent. ludwigii, Ent. asburiae* and several new *Bacillus* spp. as ITC producers. Phylogenetic tree of putative myrosinase enzymes was also constructed (Fig. 2). Phylogenetic tree shows 9 putative myrosinase from the closet relative bacteria found in NCBI database and 4 characterized myrosinases from *Brassica juncea* plant (Accession no. AAG54074.1), *Citrobacter* sp. WYE1 (Accession no. ALM58466.1) (Albaser et al., 2016), *Ent. cloacae* EcWSU1 (Accession no. AEW75128) (Cordeiro et al., 2015) and *Escherichia coli* O157:H7 str. TW14359 6 (Accession no. ACT73612.1) (Cordeiro et al., 2015) were categorized into 3 main groups (Fig. 2).

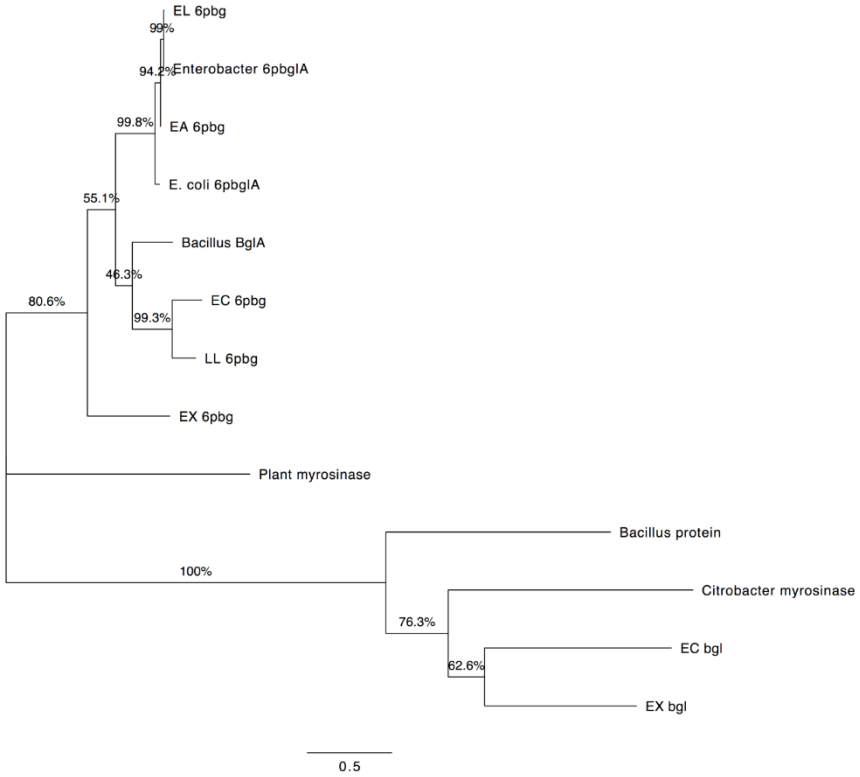

Fig. 2 Phylogenetic tree of putative myrosinases from the bacteria in this work and the previous reports. It was inferred from 13 amino acid sequences of putative myrosinases from different bacteria by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The horizontal bar represents a distance of 0.5 substitutions per site. Evolutionary analyses were conducted in MEGA7 and the phylogenetic tree was drawn by using FigTree.

The first bacterial myrosinase group categorized into Glycosyl Hydrolase 1 (GH1) family consists of 6-phospho-beta-glucosidases (6pbg) from the 2 reference myrosinases from *Ent. cloacae* EcWSU1 and *E. coli* O157:H7 str. TW14359 and those from the closet relative bacteria found in this work including *Ent. ludwigii, Ent. xiangfangensis, Ent. asburiae, Ec. casseliflavus, Bacillus* and *Lc. lactis.* On the other hand, the second bacterial myrosinase group categorized into GH3 family consists of beta-glucosidase (bgl) from the reference myrosinase from *Citrobacter* sp. WYE1 and those from the closet relative bacteria found in this work including *Ent. xiangfangensis, Ec. casseliflavus* and *Bacillus*. Clearly, plant myrosinase (GH1) was evolutionarily distinct from the other 2 bacterial myrosinase groups indicating differences in myrosinase evolution between plants and bacteria. *Citrobacter* sp. WYE1 myrosinase was active *in vitro* or in cell-free extract (Albaser et al., 2016); however those from *Lb. agilis* R16 (Llanos-Palop et al., 1995), *E. coli* O157:H7 (Cordeiro et al., 2015) and *Ec. casseliflavus* CP1 (Luang-In et al., 2014) were inactive *in vitro*, but only active *in vivo* or in intact cells. The closely evolved enzymes to *Citrobacter* sp. WYE1 myrosinase were Bacillus protein and 2 Bgl enzymes from *Ent. xiangfangensis* and *Ec. casseliflavus* (Fig. 2). These may be as active *in vitro* as *Citrobacter* sp. WYE1 myrosinase which will facilitate the study of myrosinase enzyme activity *in vitro* and hence decoding their amino acid sequences more easily than those only active *in vivo.*

3. Chemopreventive ITC products from fermented cabbage

Ent. xiangfangensis (EX) and *Ec. casseliflavus* (EC) were used to ferment Thai cabbage containing three GSLs namely glucoiberin (GIB), glucoraphanin (GRP) and 4-hydroxy glucobrassicin (GBS) at 430.5, 615.1 and 108.5 μmol/100 g dry weight, respectively (Table 2) for 3 day at 25°C. In comparison with Chinese cabbage (in kimchi) that contained five types of GLSs of approximately 8.3 μmol/g dry weight including glucoalyssin, gluconapin, glucobrassicanapin, glucobrassicin, and 4-methoxyglucobrassicin (Kim et al., 2017), it was clear that our GSLs were present in higher amounts indicating that cabbages in various countries contain various kinds of GSLs (Table S3) in various amounts depending on environmental factors such as geographical location, temperature, solar radiation, humidity and climatic conditions (Bohinc & Trdan, 2012). Degradation products of the three GSLs namely Iberin nitrile (IBN), iberin (IBR), Sulforaphane (SFN) and indole 3-acetonitrile (IAN), respectively were already detected in fresh cabbage; however in less amounts than those in fermented samples (Table 2). The product generation was due to intrinsic plant myrosinase in cabbage coming into contact with GSL upon tissue damage during handling cabbage process and possibly GSL hydrolysis occurred. Table 2 shows gradual GSL degradations in all treatments; however the spontaneous cabbage fermentation produced less IBR and SFN at days 2-3 suggesting that bacterial culture present may be able to degrade GSLs, but not capable of producing ITCs. However, IBN was found in less amounts at days 2-3 in the induced fermentation of cabbage than the spontaneous one, and that was possibly due to presence of bacterial enzymes transforming nitrile products to other metabolites. GRP was degraded more rapidly in bacterial induced cabbage fermentations than the spontaneous one indicating the presence of specific GRP-metabolizing bacteria in the samples (Table 2). GBS totally disappeared at the end of day 1 due to its initial low content in cabbage (Fig. 3A; Table 2). The degradation products included those mentioned above as in fresh cabbage along with palmitic acid and

linoleic acid (Fig. 3B). The IAN production in all treatments were similar at each day and gradually declined over time. The production of both chemopreventive IBR and SFN in EX-induced cabbage fermentation peaked at day 2 at 117.4 and 294.1 μmol/100 g dry weight, respectively which was significantly higher than IBR at 51.7 μmol/100 g dry weight, but not significantly higher than SFN at 242.6 μmol/100 g dry weight in EC-induced fermented cabbage at day 2. Overall degradation products in all treatments declined over 3 days and never reach 100% product formation (Table S2). This was possibly due to the unstable nature of ITCs in fermentation matrices (Luang-In & Rossiter, 2015). In addition, SFN content detected in the liquid portion of EC-induced cabbage fermentation at day 2 was significantly higher (p<0.01) than that found in the fermented cabbage solid portion by almost three folds (Table 2). This indicated that one should consume the liquid portion of the fermented cabbage along with the more commonly consumed fermented cabbage solid portion in Thailand to obtain the best health benefits from SFN. The pH values of the fermented cabbages at day 3 were 3.55 (N), 3.25 (EX) and 3.45 (EC) with no statistical difference (Fig. S2) and are similar to pH values of 3.27 – 3.67 of sauerkraut from Spanish cabbage over 7-day fermentation at 25°C by *Lactobacillus plantarum* (CECT 748) and *Leuconostoc mesenteroides* (CECT 219) (Table S3). Similarly, the ITC products including SFN at 39-49 μmol/100g dry weight, IBR, and IBR NIT were detected from metabolism of glucoraphanin and glucoiberin in sauerkraut from Spanish cabbage like in our product but in less amounts, except for allyl isothiocyanate (AITC) and allyl nitrile (ANIT) that were only found in their sauerkraut; however these products were not detected in their raw cabbage (Peñas et al., 2012) suggesting that fermentation was responsible for ITC and NIT productions. Similarly, sauerkraut made from cabbage grown in Finland produced SFN, AITC, ANIT and indole 3-carbinol (I3C) from glucoiberin, sinigrin and glucobrassicin, respectively (Tolonen et al., 2002). In contrast, sauerkraut made from cabbage grown in Germany only produced ascorbigen and I3C from glucoiberin, sinigirin, glucobrassicin, glucoraphanin and 4-methoxy glucobrassicin (Palani et al., 2016). SFN was also found in Korean kimchi, however in a less amount than in fresh cabbage (Kim et al., 1999), and sometimes not found at all (Hong & Kim, 2013). Different ITC production from different fermented cabbage products in various countries may be the result of different cultivars of cabbages used and bacterial species present in the fermentation. To ascertain functionality of Thai fermented cabbage in this work and for future commercial purposes, anticancer activity and antimicrobial activity of metabolites produced during induced cabbage fermentation as well as probiotic properties of both *Ent. xiangfangensis* and *Ec. casseliflavus* will be determined in the next project. In addition to ITCs as potential anticarcinogens, palmitic acid (16:0) and linoleic acid (18:2) as potential anticarcinogens or antimutagens (Nadathur et al., 1996; Bocca et al., 2010) were also present in fresh cabbage. After induced cabbage fermentation with either EC or EX for 3 days, palmitic acid and linoelic acid contents were increased by almost 5 folds and 10 folds, respectively (data not shown) suggesting induced fermentation added more nutritional value to cabbage than non-induced fermentation. Our finding was in accordance with the previous work showing that milk fermented with kefir had higher contents of healthy fatty acids when compared to unfermented milk (Guzel-Seydim et al., 2006). That was due to bacterial capacity to produce different fatty acids (Dykes et al., 1995) which employed the disassociated fatty acid synthesis II pathway, thus multiple discrete enzymes synthesize the fatty acid chain (White et al., 2005).

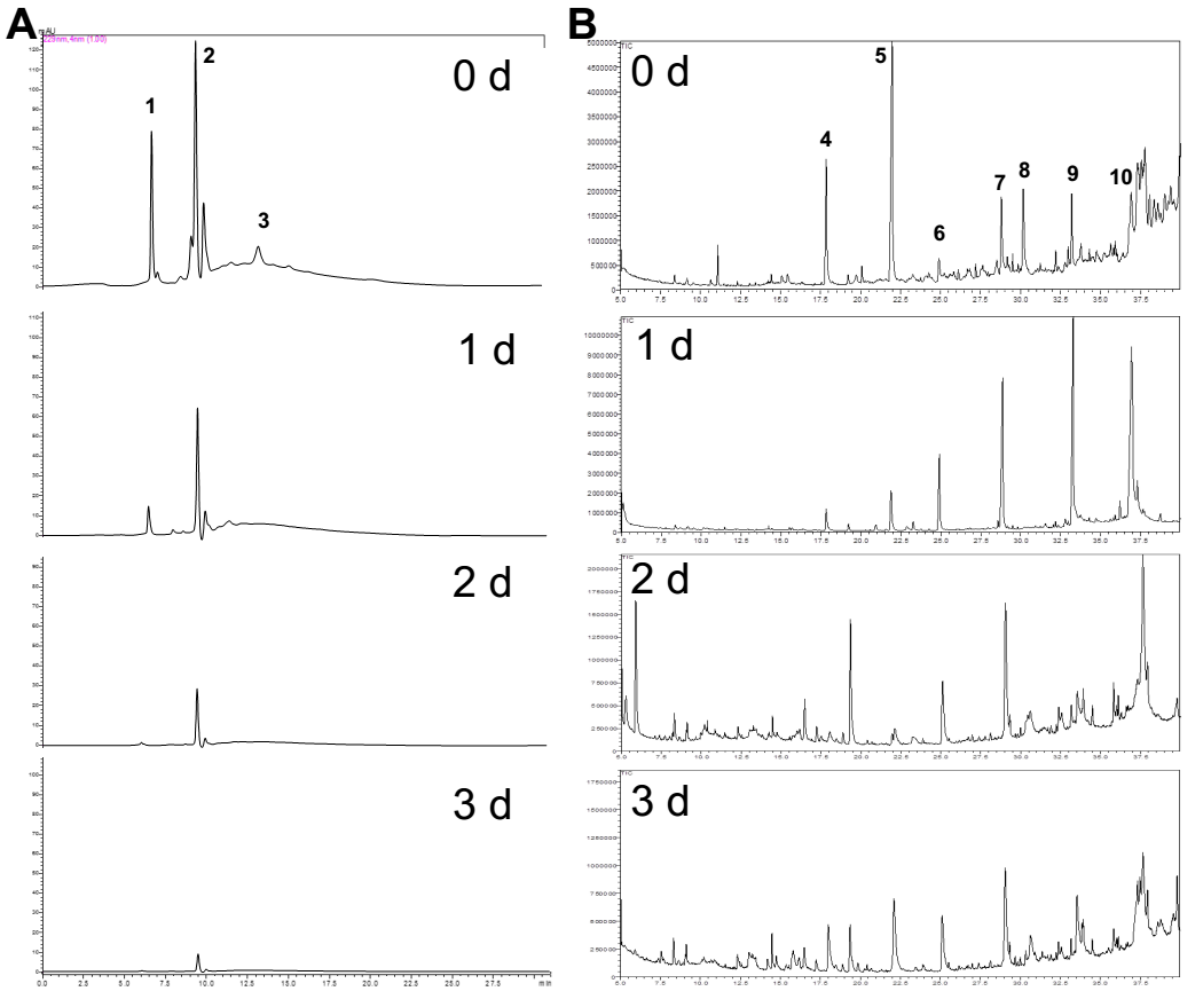

Fig. 3 (A) HPLC chromatograms of GSL profiles of fermented cabbage by EX from 0 – 3 days. (B) GC-MS chromatograms of metabolic profiles of fermented cabbage by EX from 0 – 3 days. HPLC peaks of GSLs; **1** Glucoiberin at 6.30 min, **2** Glucoraphanin at 9.44 min and **3** 4-Hydroxy glucobrassicin at 12.5 min. GC-MS peaks of products, **4** iberin nitrile at 17.9 min, **5** Pent-1,4-diene-3-one at 21.9 min, **6** iberin at 24.9 min, **7** sulforaphane at 28.9 min, **8** *indol-3-acetonitrile* at 30.2 min, **9** palmitic acid at 33.3 min and **10** linoleic acid at 36.9 min.

Table 2 Metabolism of glucosinoates in fermented cabbage with/without bacterial induction over 3 days.

Samples	Remaining GSL (µmol/ 100 g dry weight)			Products (µmol/ 100 g dry weight)			
	GIB	GRP	GBS	IBN	IBR	SFN	IAN
Cabbage	430.5±34.1aB	615.1±30.0aA	108.5±19.1aC	75.1±26.4aC	13.3±10.0bD	39.5±16.3dC	49.1±29.8abC
N0	273.8±15.8bB	534.4±30.4abA	30.2±4.4bC	61.82±28.4abC	11.2±6.0bD	56.2±26.0dC	52.4±19.1abC
N1	103.8±19.5cB	419.0±26.5cA	0.0+0.0cE	43.3±23.2abC	32.1±3.9bD	135.2±42.0abB	22.9±4.7abC
N2	20.9±12.0dB	217.5±14.8deA	0.0+0.0cC	35.2±15.4abB	11.57±5.8bD	127.9±60.0abA	15.8±2.0bB
N3	2.9±1.1dC	146.3±27.7deA	0.0+0.0cD	21.6±11.0bcB	8.6±3.9bB	112.2±52.0abA	10.7±3.1bB
EC0	305.7±29.8bB	514.2±42.6bA	27.4±6.9bD	64.3±23.3abC	15.4±5.9bD	45.6±22.0dC	60.5±21.8aC
EC1	91.8±17.3cC	359.9±31.8cdA	0.0+0.0cE	25.6±12.6abD	35.2±20.0bD	177.8±42.0abB	22.5±14.6abD
EC2	21.0±9.3dB	211.0±27.7deA	0.0+0.0cD	6.4±2.1cC	51.7±35.9abB	242.6±40.0aA	14.7±11.9abB
EC3	3.1±0.7dD	111±20.2fB	0.0+0.0cE	2.9±0.8cD	33.6±22.0bC	222.4±42.0aA	16.4±7.2abC
EX0	305.2±13.0bB	536.6±29.1abA	31.6±7.8aC	65.2±20.2abC	12.5±4.0bD	48.6±30.0dC	55.3±20.2abC
EX1	74.3±14.6cC	275.9±19.deA	0.0+0.0cF	26.6±12.3abD	117.4±41.9aB	294.1±44.0aA	7.7±3.7bE
EX2	11.1±6.4dD	160.8±29.0eB	0.0+0.0cE	6.5±3.8cD	78.2±38.0abC	252.6±46.0aA	16.3±7.4abD
EX3	2.4±0.6dD	85.3±24.7fB	0.0+0.0cE	13.8±3.1bcC	70.5±39.9abB	244.7±70.0aA	20.9±8.8abC
EC2 solid	5.2±1.6dC	93.3±23.6fA	0.0+0.0cD	3.6±2.3cC	19.2±10.0bB	69.7±34.0cA	5.9±1.2bB
EC2 liquid	16.3±7.5dC	117.7±34.4fB	0.0+0.0cF	3.5±0.7cE	30.8±22.0bC	173.3±48.0abA	10.4±1.3bD

Cabbage = fresh cabbage; N = Non-induced with EX for 0-3 days (N0, N1, N2, N3); EC = induced with EC for 0-3 days (E0, E1, E2, E3);

EX = induced with EX for 0-3 days (E0, E1, E2, E3); EC2 solid = Solid portion of fermented cabbage of EC at day 2; EC2 liquid = Liquid portion of fermented cabbage of EC at day 2. Different small letters indicate significantly statistical differences in values in the column ($p<0.01$) and capital letters indicate significantly statistical differences in values in the row ($p<0.01$)

4. Antioxidant activity of fermented cabbage

Since EX-induced cabbage fermentations yielded the highest ITCs, they were used for antioxidant activity evaluation in comparison with the spontaneous ones. The results showed that EX-induced cabbage fermentations exhibited significantly higher %DPPH scavenging activity, FRAP activity and ABTS activity than those found in the spontaneous fermentations over 3 days (Fig. 4). In addition, antioxidant activities of fresh cabbage at day 0 were similar to those in both spontaneous and induced fermentations and increased over time indicating bacterial metabolism was responsible for increasing antioxidants in the fermented cabbage as well as the increase of ITCs (Table 2). Lactic-fermented cabbage in China, prepared by a dry-salt method and extracted with methanol, showed antioxidant activity of DPPH radical scavenging effect at 60% (Sun et al., 2009). This was similar to DPPH radical scavenging effect at 62.1% at day 2 by EX-induced fermentation (Fig. 4). In addition, Lactic-fermented red cabbage sprouts gave significantly higher antioxidant functionalities than those of their unfermented/control counterparts. Fermented red cabbage sprouts inoculated with *Lb. plantarum* had the highest antioxidant activities (DPPH scavenging: 70.92%; TEAC: 1.94 mM Trolox equivalent), which was almost two-fold higher than those of unfermented treatments. These results indicated that Lactobacillus fermentation and our Enterobacter fermentation could be applied as a method to improve the potent antioxidant activities of vegetables (Hunaefi et al., 2013).

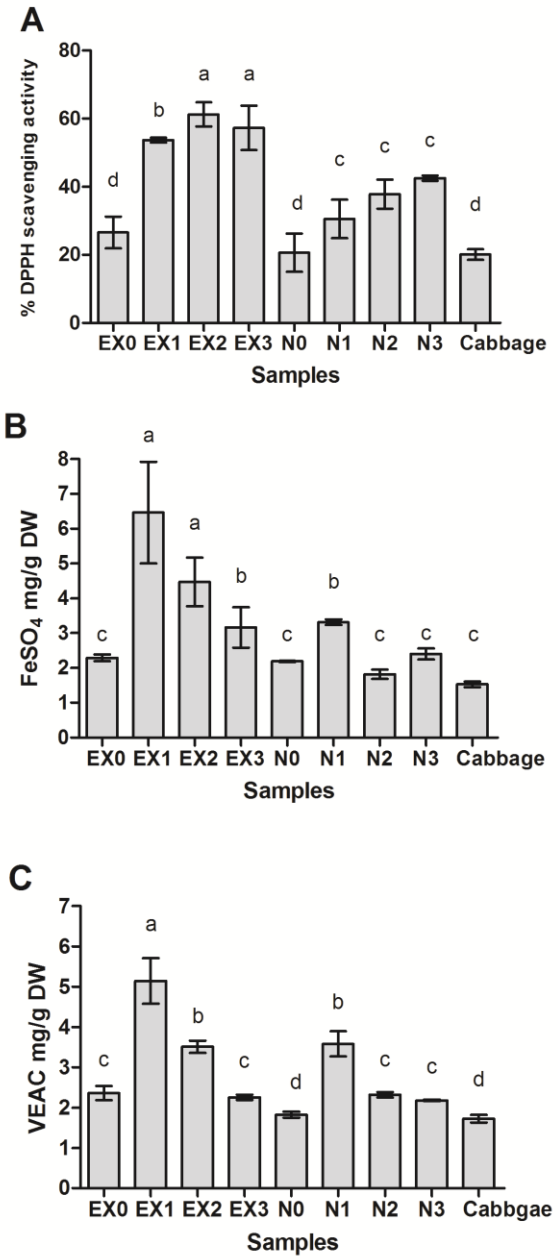

Fig. 4 Antioxidant activities from fermented cabbage with/without bacterial induction over 3 days. EX = induced with EX for 0-3 days (E0, E1, E2, E3); N = Non-induced with EX for 0-3 days (N0, N1, N2, N3). Different small letters indicate significantly statistical differences in values (p<0.01).

Conclusions

Induced cabbage fermentation by *Ent. xiangfangensis* or *Ec. casseliflavus* produced higher contents of chemopreventive SFN and IBR than those from the spontaneous fermentation and fresh cabbage. Fermented liquid and solid portions from fermented cabbage should be combined for consumption at day 1 or 2 for the highest ITCs and no nitrile product providing the best health-promoting benefits. GSL-metabolizing bacteria isolated from Thai fermented foods can be used as a starter culture for a traditional

Thai fermented cabbage. This can then be promoted as a functional food with chemopreventive properties from ITC products like the fermented cabbage product counterparts from different countries e.g. sauerkraut and kimchi.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

อยู่ระหว่างการพิจารณาโดยวารสาร Food Chemistry ซึ่งอยู่ในฐานข้อมูล ISI (Impact factor 2016 = 4.529)
Luang-In, V., Saengha, W., Udomwong, P., Gregory, M & Deeseenthum, S. (201X). Chemopreventive sulforaphane and iberin products in Thai cabbage fermented by myrosinase-positive bacterium.

2. การนำผลงานวิจัยไปใช้ประโยชน์

√ **ด้านสาธารณะ** **โดย** นิสิต และญาติของนิสิต
นำผลการทดลองที่ว่า เราควรบริโภคน้ำผักดองด้วย เพราะว่ามีซัลโฟราเฟนและไอเบอรินในปริมาณที่สูงกว่าชิ้นผักดองเสียอีก ไปเผยแพร่ให้แก่บุคคลรอบข้าง ทำให้ญาติของนิสิตหันมาบริโภคน้ำผักดองด้วย แทนที่จะทิ้งไปเหมือนแต่ก่อน

√ **ด้านวิชาการ** **โดย** ผู้สนใจด้านวิชาการ นิสิต อาจารย์และบุคคลทั่วไป
.ผู้เข้าร่วมประชุมดิ้งค์ความรู้ใหม่จากการนำเสนอเผยแพร่ผลงานวิจัยทางโพสเตอร์และในเล่มรวมบทคัดย่อของการประชุม The 4th International Postgraduate Symposium on Food, Agriculture & Biotechnology in ASEAN 2017 (IPSFAB2017), 30-31 August, 2017. Faculty of Technology, Mahasarakham University โดยมีผู้เข้าร่วมประชุมจากประเทศไทยและประเทศเพื่อนบ้านในอาเซียนกว่า 80 คน

โดย อาจารย์ และประเทศชาติ
ได้สร้างนักวิจัยรุ่นใหม่ในระดับปริญญาตรี 1 คน คือ นายวรโชติ แสงหา และขณะนี้นิสิตได้ศึกษาต่อในระดับปริญญาโท เพื่อทำงานวิจัยเป็นกำลังสำคัญของชาติต่อไป

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

Luang-In, V.*, Saengha, W., Udomwong, P., Deeseenthum, S. & Rossiter, J.T. Glucosinolate-metabolizing bacteria isolated from Thai fermented foods as a starter culture for a traditional Thai fermented cabbage to produce chemopreventive sulforaphane and iberin. The 4th International Postgraduate Symposium on Food, Agriculture & Biotechnology in ASEAN 2017 (IPSFAB2017), 30-31 August, 2017. Faculty of Technology, Mahasarakham University, Thailand (Poster presentation)

Luang-In, V.*, Saengha, W., Udomwong, P., Deeseenthum, S. & Rossiter, J.T. Glucosinolate-metabolizing bacteria isolated from Thai fermented foods as a starter culture for a traditional Thai fermented cabbage to produce chemopreventive sulforaphane and iberin. TRF-OHEC Annual Congress 2017, 11-13 January, 2017. Petchaburi Province, Thailand (Poster presentation)

Appendix

Glucosinolate-metabolizing bacteria isolated from Thai fermented foods as a starter culture for a traditional Thai fermented cabbage to produce chemopreventive sulforaphane and iberin

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INTRODUCTION

Recently, cancer has become the main cause of death in Thailand and consumption of Cruciferous vegetables has been shown to reduce the cancer risks due to chemopreventive products namely isothiocyanate (ITC) generated by plant myrosinase in Cruciferous vegetables or myrosinase-positive microbes in human gut during glucosinolate (GSL) metabolism (Luang-In et al., 2014) **(Fig. 1)**. Sulforaphane (SFN) has been the most studied and most effective chemopreventive agent exhibiting also other bioactivities. It is produced from glucoraphanin abundantly present in broccoli and cabbage. Hence we aimed to increase SFN products in Thai fermented cabbage for enhanced health benefits.

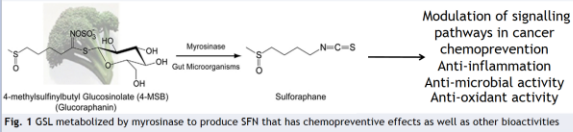

Fig. 1 GSL metabolized by myrosinase to produce SFN that has chemopreventive effects as well as other bioactivities

OBJECTIVES

1. To isolate and identify GSL-metabolizing bacteria from eight local fermented foods and beverages in Thailand.
2. To use GSL-metabolizing bacteria as a starter culture for a traditional Thai fermented cabbage to increase production of chemopreventive SFN and iberin (IBR).
3. To promote Thai fermented cabbage with increased ITC products as a functional food.

METHODOLOGY

1. Isolation of GSL-metabolizing bacteria

Eight fermented foods in Thailand were collected **(Fig.2)** for isolating GSL-metabolizing bacteria on M9 agar containing 1 mM sinigrin and 2.5 mM barium acetate. Colonies with white precipitate were selected.

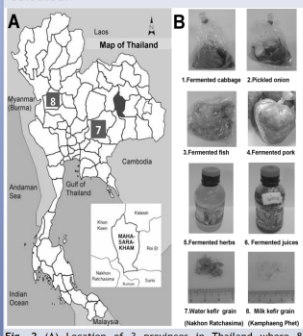

Fig. 2 (A) Location of 3 provinces in Thailand where 8 fermented samples were collected. (B) List of Thai local fermented foods and drinks used in this study.

3. Bacterial identification

Bacterial genomic DNA was extracted (Vivantis, Malaysia), analyzed by PCR-based 16S rRNA. Product was purified (Vivantis, Malaysia) and sequenced (1st BASE, Malaysia). Phylogenetic tree was constructed using MEGA 7.0 **(Fig. 4)**.

Fig. 4 Steps of bacterial identification

5. Metabolite analysis

ITC products in samples were detected by GC-MS **(Fig. 6)**. GSLs were detected by HPLC.

2. GSL degradation by HPLC analysis

Each of 21 positive isolates was cultured in LB + 1 mM sinigrin for 24 h to confirm their capacity to metabolize sinigrin by HPLC and to produce allyl isothiocyanate (AITC) by GC-MS **(Fig. 3)**.

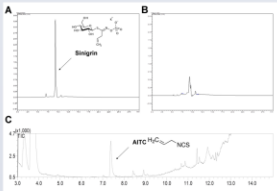

Fig. 3 Bacterial metabolism of sinigrin (A) HPLC chromatogram of sinigrin at 9.30 min at 0 h. (B) Sinigrin remained at 24 h. (C) GC-MS chromatogram of AITC product at 7.4 min at 24 h.

4. Fermentation of cabbage

E. xiangfangensis 4A2 (EX) from fermented fish and *E. casseliflavus* SB2 (EC) from fermented cabbage were chosen to induce cabbage fermentation vs spontaneous fermentation for 3 day at 25°C **(Fig. 5)**. Cabbage (*Brassica oleracea* L. var. capitata) was purchased from Pran Fresh Co. Ltd, Khon Kaen.

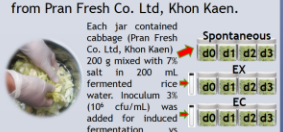

Fig. 5 Induced cabbage fermentation by EX, EC-induced vs spontaneous fermentation for 3 day at 25°C. Samples were collected each day for freeze-drying process.

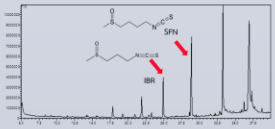

Fig. 6 GC-MS chromatogram of SFN at 29 min and IBR at 26 min.

RESULTS & DISCUSSION

Seventeen bacterial species from 8 food samples were identified as GSL-metabolizing bacteria. A majority of ITC-producing bacteria were *Enterobacter* and *Enterococcus* and the two highest ITC producers included *E. xiangfangensis* 4A-2A3.1 (EX) from fermented fish and *E. casseliflavus* SB2X2 (EC) from fermented cabbage producing 62 and 60 nmol AITC from 100% sinigrin degradation within 24 h **(Fig. 7)**. AITC may be unstable in culture media and thus % product formation never reached 100%. However, *Lactococcus hircilactis*, *L. lactis* and *Bacillus* sp. KW3 did not produce ITC from 77-80% sinigrin degradation suggesting they may have different GSL metabolic enzymes or mechanisms to other bacteria in metabolizing GSL, but not producing ITC.

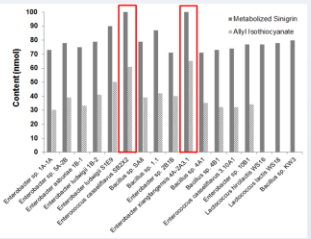

Fig. 7 GSL degradation and AITC production by selected bacteria

Phylogenetic tree shows these GSL-metabolizing bacteria were categorized into 3 main groups **(Fig. 8)**. Some were in the same groups as previously reported GSL-metabolizing bacteria. This is the first report of identifying *E. xiangfangensis*, *E. ludwigii*, *E. asburiae* and several new *Bacillus* spp. as ITC producers. EC and EX were used to ferment cabbage containing glucoiberin (GIB), glucoraphanin (GRP) and 4-hydroxy glucobrassicin (GBS) for 3 day at 25°C. EX produced more sulforaphane (SFN) and iberin (IBR) than EC and spontaneous cabbage fermentation over 3 days **(Table 1)**.

SFN peaked at 294.1 µmol/100 g DW and Iberin (IBR) at 117.4 µmol/100 g DW at day 1 by EX **(Table 1)**. SFN peaked at 242.6 µmol/100 g DW and IBR at 51.7 µmol/100 g DW at day 2 by EC **(Table 1)**. SFN detected in EC-induced cabbage fermentation with liquid portion was significantly ($p < 0.01$) higher than that in fermented solid cabbage portion by almost three folds. SFN and IBR were found in fresh cabbage as well, however in less content than fermented samples, due to endogenous myrosinase in cabbage getting into contact with GSL upon tissue damage during cabbage preparation and GSL hydrolysis occurred hence ITC products.

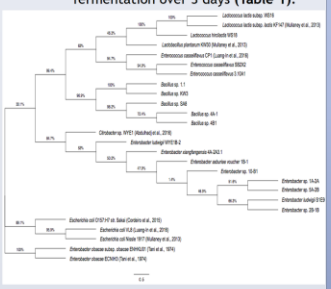

Fig. 8 Phylogenetic tree of selected and reference bacteria

Table 1 Metabolism of glucosinoates in fermented cabbage with/without bacterial induction over 3 days.

Samples	Remaining GSL (µmol/ 100 g dry weight)			Products (µmol/ 100 g dry weight)			
	GIB	GRP	GBS	IBN	IBR	SFN	IAN
Cabbage	430.5±34.1aB	615.1±30.0aA	108.5±19.1aC	75.1±26.4aC	13.3±10.0bD	39.5±16.3dC	49.1±29.8abC
N0	273.8±15.8bB	534.4±30.4abA	30.2±4.4bC	61.82±28.4aBC	11.2±6.0bD	56.2±26.0dC	52.4±19.1abC
N1	103.8±19.5cB	419.0±26.5cA	0.0+0.0cE	43.3±23.2abC	32.1±3.9bD	135.2±42.0abB	22.9±4.7abC
N2	20.9±12.0dB	217.5±14.8deA	0.0+0.0cC	35.2±15.4abB	11.57±5.8bD	127.9±60.0abA	15.8±2.0bB
N3	2.9±1.1dC	146.3±27.7deA	0.0+0.0cD	21.6±11.0bcB	8.6±3.9bB	112.2±52.0abA	10.7±3.1bB
EC0	305.7±29.8bB	514.2±42.6bA	27.4±6.9bD	64.3±23.3abC	15.4±5.9bD	45.6±22.0dC	60.5±21.8aC
EC1	91.8±17.3cC	359.9±31.8cdA	0.0+0.0cE	25.6±12.6abD	35.2±20.0bD	177.8±42.0abB	22.5±14.6abD
EC2	21.0±9.3dB	211.0±27.7deA	0.0+0.0cD	6.4±2.1cC	51.7±35.9abB	242.6±40.0aA	14.7±11.9abB
EC3	3.1±0.7dD	111±20.2fB	0.0+0.0cE	2.9±0.8cD	33.6±22.0bC	222.4±42.0aA	16.4±7.2abC
EX0	305.2±13.0bB	536.6±29.1abA	31.6±7.8aC	65.2±20.2abC	12.5±4.0bD	48.6±30.0dC	55.3±20.2abC
EX1	74.3±14.6cC	275.9±19.deA	0.0+0.0cF	26.6±12.3abD	117.4±41.9aB	294.1±44.0aA	7.7±3.7bE
EX2	11.1±6.4dD	160.8±29.0eB	0.0+0.0cE	6.5±3.8cD	78.2±38.0abC	252.6±46.0aA	16.3±7.4abD
EX3	2.4±0.6dD	85.3±24.7fB	0.0+0.0cE	13.8±3.1bcC	70.5±39.9abB	244.7±70.0aA	20.9±8.8abC
EC2 solid	5.2±1.6dC	93.3±23.6fA	0.0+0.0cD	3.6±2.3cC	19.2±10.0bB	69.7±34.0cA	5.9±1.2bB
EC2 liquid	16.3±7.5dC	117.7±34.4fB	0.0+0.0cF	3.5±0.7cE	30.8±22.0bC	173.3±48.0abA	10.4±1.3bD

Cabbage = fresh cabbage; N = Non-induced with EX for 0-3 days (N0, N1, N2, N3); EC = induced with EC for 0-3 days (E0, E1, E2, E3); EX = induced with EX for 0-3 days (E0, E1, E2, E3); EC2 solid = Solid portion of fermented cabbage of EC at day 2; EC2 liquid = Liquid portion of fermented cabbage of EC at day 2. GIB = Glucoiberin; GRP = Glucoraphanin; GBS; 4-hydroxyl glucobrassicin; IBN = Iberin nitrile; IBR = Iberin; SFN = Sulforaphane; IAN = Indole 3-acetonitrile
Different small letters indicate significantly statistical differences in values in the column ($p<0.01$) and capital letters indicate significantly statistical differences in values in the row ($p<0.01$)

CONCLUSION

Induced cabbage fermentation by EC or EX produced higher contents of chemopreventive SFN and IBR than those from spontaneous fermentation. Fermented liquid portion should be consumed alongside fermented solid cabbage portion from fermented cabbage collected at day 1 or 2 for the highest ITCs and best health-promoting benefits.

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Chemopreventive sulforaphane and iberin products from Thai cabbage fermented by myrosinase-positive bacterium

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Abstract

The aims of this work were to isolate bacteria from Thai local fermented foods and beverages in Thailand with the capacity to metabolize glucosinolate and produce chemopreventive isothiocyanates (ITCs) and used the selected strains for cabbage fermentation. *Enterobacter xiangfangensis* 4A-2A3.1 (EX) from fermented fish and *Enterococcus casseliflavus* SB2X2 (EC) from fermented cabbage were the two highest ITC producers. Consequently, EC or EX was used to ferment Thai cabbage (*Brassica oleracea* L. var. capitata) containing glucoiberin, glucoraphanin and 4-hydroxy glucobrassicin for 3 days at 25°C. Different amounts of iberin nitrile, iberin, sulforaphane and indole 3-acetonitrile were produced by spontaneous cabbage fermentation, EX- and EC-induced fermentation. However, statistically higher ITCs were detected in the latter two (p<0.01) with high antioxidant activities. One should consume a liquid portion of the fermented cabbage due to higher ITC level along with a solid portion to obtain the best health-promoting benefits from this functional food.

Abbreviations

ABTS, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; EX, *Enterobacter xiangfangensis*; EC, *Enterococcus casseliflavus*; FRAP, Ferric ion reducing antioxidant power; GBS, 4-hydroxy glucobrassicin; GIB, Glucoiberin; GRP, Glucoraphanin; GSL, Glucosinolate; IAN, Indole 3-acetonitrile; IBN, Iberin nitrile; IBR, Iberin; ITC, Isothiocyanate; SFN, Sulforaphane; VEAC, Vitamin C equivalent antioxidant capacity.

Keywords

Antioxidant; Enterococcus; Enterobacter; Fermented foods, Isothiocyanate, Myrosinase

1. Introduction

Today, cancer has led to 60,000 deaths a year among the Thai population and has been continuously the first common cause of death since 2000 and is set to continue on. The first five types of common cancers found in Thai population include liver and bladder, lung, colon, breast and cervical cancers. It is predicted that in the next 21 years there will be 24-million patients diagnosed with cancer in Thailand (Khuhaprema et al., 2012). The change of Thai dietary habits to a more Western style is likely to be attributed to health problems including cancers. Chemopreventive scheme should be promoted and implemented among Thai population to fight against cancer. At present, the putative role on cancer chemoprevention of cruciferous vegetables is attributed to the bioactivity of glucosinolates (GSLs) degradation products, mainly isothiocyanates (ITCs) (Traka & Mithen, 2009) and also other classes of antioxidant bioactive phytochemicals including phenolic compounds and flavonoids (Cartea et al., 2011). ITCs have been shown to protect against the most common cancer types, such as breast, lung, colon and prostate cancers in both *in vivo* and *in vitro* studies (Hayes et al., 2008). Problems can arise when plant myrosinases are rapidly denatured by cooking, and thus GSL hydrolysis and the production of ITCs as health promoters becomes dependent on the activity of the human gut microflora with GSL-degrading ability (Shapiro et al., 2001). Each individual has a unique microflora composition (The Human Microbiome Project, 2012) that would give rise to substantial differences in the capacity of the microflora to confer antioxidant and/or anticancer effect from metabolizing GSLs into ITCs. Since microbial consortia in the human gut are mainly determined by the food we eat, it is believed that we can manipulate the gut microbiota for the sake of our own health (Li et al., 2009). Accumulating evidence suggests that certain bacterial strains of human origin including *Bacteroides, Bifidobacterium, Lactobacilli* can metabolize GSLs to ITCs and/or nitriles (Cheng et al., 2004; Luang-In et al., 2016). To date, bacterial myrosinase genes/enzymes have

been sought after. The 6-Phospho-*β*-glucosidase encoded by the genes *bglA* and *ascB* (family GH1), and *chbF* (family GH4) in *Escherichia coli* O157:H7 were found to be responsible for sinigrin metabolism (Cordeiro et al., 2015). Soil bacterial myrosinase in the family GH3 was extracted from cell-free extracts of *Citrobacter* strain (WYE1) and its amino acid sequence was identified (Albaser et al., 2016). These findings show that bacterial myrosinases from different GSL-metabolizing bacterial strains are diverse. In this work, we aimed to identify GSL-metabolizing microbes from Thai local fermented foods and beverages and used the highest ITC bacterial producers as a starter culture to ferment Thai cabbage containing GSLs to produce chemopreventive ITC products. This will help upgrade Thai fermented cabbage to be a functional food. The isolated microbes will provide the potential use as starter cultures of Thailand origin to produce health-beneficial vegetables fermented foods or drinks or used as probiotics supplements. This is certainly a way to obtain quality food products at low cost to ensure enhanced health-beneficial effects for the Thai population.

2. Materials and Methods

2.1. Sample collection

Eight samples of fermented foods and beverages at the end of fermentative stage were purchased mainly from local markets in Mahasarakham, Thailand except for samples no. 7 and 8 for isolation of myrosinase-positive microbes. Samples (Fig. S1) included (1) Fermented cabbage (2) Picked onions (3) Fermented fish (4) Fermented pork (5) Fermented herbal drink (6) Fermented star fruit juice, (7) Water kefir from Nakhon Ratchasima and (8) Milk kefir from Kamphaeng Phet. Samples were stored at 4°C and analyzed within 24 h.

99 **2.2. Isolation of GSL-metabolizing microbes**

100 Solid food materials and liquid (5 g each, 10 g in total) or liquid beverage (10 ml) were
101 weighed and mixed with 90 ml of sterile 0.85% NaCl solution. The mixture was homogenized
102 in a sterile mortar and pestle for 5 min and mixed by vortexing for 5 min. The mixture was
103 centrifuged at 4000g for 15 min and clear supernatant was obtained. Enrichment culture
104 technique was used by inoculating 100 µl bacterial suspension into 900 µl LB broth
105 containing 1 mM sinigrin for 2 days in anaerobic incubator and this step was repeated at day
106 4, 6 and 8 in fresh Luria-Bertani (LB) media (10 g Tryptone; 10 g NaCl; 5 g Yeast extract in 1
107 l). At day 10, 100 µl bacterial suspension was spread onto the selective minimal media M9
108 agar (1 M $MgSO_4$; 1 M $CaCl_2$; 50% Glucose; 1% Thiamine; 64 g Na_2HPO_4-$7H_2O$; 15 g
109 KH_2PO_4; 2.5 g NaCl; 5.0 g NH_4Cl; 15 g agar in 1 l) containing 1 mM sinigrin and 2.5 mM
110 barium acetate and incubated at 37°C for 72 h in the anaerobic incubator. Growth and opaque
111 zone formation was an indicator of sinigrin degradation as seen from white precipitates of
112 barium sulfate. This was a result of a release of sulfate group of GSL and thus GSL-
113 metabolizing/myrosinase-positive isolates were selected from each food sample. Positive
114 isolates were stored in 20% glycerol stocks in LB media at -80°C. All microbial isolates were
115 deposited in the Natural Antioxidant Innovation Research Unit, Department of
116 Biotechnology, Faculty of Technology, Mahasarakham University, Thailand (WDCM 1160).
117

118 **2.3. *In vitro* sinigrin incubation**

119 Sinigrin (1 mM) was incubated with each selected bacterial culture (100 µl, OD_{600nm} = 0.5)
120 from the previous step in 100 µl LB media at 37°C without shaking in anaerobic incubator for
121 24 h. Bacterial culture was centrifuged at 16000g for 5 min and then 100 µl clear supernatant
122 to be used for HPLC analysis and the other 900 µl for GC-MS analysis were kept at -20°C
123 until use.

124

125 **2.4. Genomic DNA isolation and 16S rDNA gene analysis**

126 Selected isolates with the confirmed positive results of GSL degradation from HPLC analysis
127 were cultured overnight for gram-staining, genomic DNA extraction and PCR-based 16S
128 rRNA gene analysis using universal primers following the previous report (Luang-In et al.,
129 2014).

130

131 **2.5. Phylogenetic tree construction**

132 The nucleotide sequences of isolated bacterial 16S rDNA, from this study and previous
133 findings were compared with entries in the GenBank database. Similarly, putative
134 myrosinases of bacterial relatives in the GenBank database were compared with the
135 characterized myrosinases. The sequences were aligned using MUSCLE (Edgar, 2004).
136 Phylogenetic trees were constructed using the maximum-likelihood method and assessing the
137 reliability the constructed phylogenetic tree with 1000 bootstrap replicates, implemented in
138 the Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software package (Kumar et al.,
139 2015). The constructed phylogenetic trees of 16S rRNAs and proteins were drawn by FigTree
140 software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh,
141 Scotland, UK) [http://tree.bio.ed.ac.uk/software/figtree/].

142

143 **2.6. Starter culture preparation**

144 Each of the two selected bacteria was grown in 10 ml LB media overnight and centrifuged at
145 8,000 g for 15 min at 4°C. The cells were washed twice with overnight fermented sticky rice
146 water (initial pH 6.0). The inoculum concentration of 10^6 CFU/ml was inoculated in 3% (v/v)
147 into the prepared cabbage-rice water jar (200 ml) to be fermented as mentioned below.
148 Induced fermentations were defined by inoculation of either *Enterobacter xiangfangensis* 4A-

149 2A3.1 (EX) or *Enterococcus casseliflavus* SB2X2 (EC) culture and spontaneous
150 fermentations (N = Non-induced) were those without inoculation of either culture.
151
152 **2.7. Cabbage fermentations**
153 Thai white cabbage heads (*Brassica oleracea* L. var. capitata) were purchased from Pran
154 Fresh Co. Ltd, Khon Kaen, Thailand. After removing core and outer layers, 3 kg of the
155 cabbage heads from the same batch were separated into leaf pieces manually according to
156 Thai local cabbage fermentation procedure. The spontaneous fermentation was performed by
157 mixing torn plant materials well with 7% salt (w/v), washed with distilled water, and only 200
158 g solid plant materials were transferred into each replicate fermentation pot (200 ml glass
159 container with lid already containing 200 mL fermented rice water pH 6.0 mixed with 7%
160 salt). The salted cabbage materials were tightly pressed into the jars that were closed and kept
161 at 25°C for 3 days without shaking. Triplicates were carried out throughout the study. The
162 control were those 200 g fresh cabbage heads separated into leaf pieces manually without
163 fermentation at 0 h as the starting materials and they were determined for initial GSLs and
164 initial ITC products to be compared with spontaneously fermented cabbage samples (N) and
165 each of the cabbage fermentation induced by EX or EC.
166
167 **2.8. Sampling and extraction of fermented cabbage**
168 The fermentation experiment was carried out in parallel in 36 jars with triplicate in each of the
169 three treatments (N, EC, and EX) from 0 to 3 days. Sampling was done at day 0, 1, 2 and 3,
170 pH was measured immediately after opening the fermentation jars. For extraction of GSLs
171 and ITC products, the whole samples from each jar collected as mentioned above were frozen
172 at -80°C, dried in a freeze dryer, and processed accordingly for GSL and ITC analyses as
173 mentioned below. For EC samples at day 2, half of cabbage leafy material and half of

fermented liquid were taken separately for GSL and ITC determination to evaluate which part contained higher contents. Dried samples were ground to small pieces using a sterile mortar and pestle and weighed for extraction by 95% ethanol at concentration of 25 mg/ml at 25°C for 24 h. The mixture was centrifuged at 16000g for 5 min and clear supernatant was collected for antioxidant activity analyses.

2.9. Sample preparation and HPLC analysis to detect GSLs

The GSL extraction method was modified from the previous report (Vicaş et al., 2011). Freeze dried samples (5 g) were ground and mixed with 5 ml of 70% methanol by shaking at 37°C for 5 min and the supernatant was collected after centrifugation at 8000g for 15 min. The remained solid sample was extracted again and the supernatant was mixed with the first extraction. The mixture was dried at 70°C in the oven and the dried residues were dissolved in 1 ml deionized water using vortex. One ml sample was processed in DEAE-25A anion exchange resin as previously described (Luang-In et al., 2014). HPLC–DAD system (Shimadzu, Japan) fitted with a Synergi 4u Hydro-RP 80A, 150 x 2 mm, 4.6 micron (Phenomenex Inc., Torrance, CA) protected with security guard column AQ C18 (4 x 3 mm), comprising of Shimadzu LC-20AC pumps, a SPD-M20A diode array detector and were used for GSL analysis using the following gradient: Water (Solvent A)–ACN (Solvent B) gradient 2% B (15 min), 2–25% B (2 min), 25–70% B (2 min), 70% B (2 min hold), 70–2% B (2 min), and 2% B (15 min) at a flow rate of 0.2 ml/min at 35ºC. Eluent was monitored at A229 nm. Quantification of desulfo-glucosinolate (DS-GSL) was achieved using known response factors for each GSL relative to an external standard (sinigrin). Pure sinigrin (Sigma-Aldrich, Singapore), glucoraphanin, glucoiberin (Youchemicals Ltd., China) and 4-hydroxy glucobrassicin (Clearsynth, India) were purchased as standards.

199 **2.10. Sample preparation and GC-MS analysis to detect degradation products**

200 Freeze-dried samples (500 mg) were mixed with 3 mL dichloromethane (DCM) in test tubes
201 with tight lids for 24 h at 250 rpm at room temperature. The mixture was centrifuged at
202 16100g for 5 min and the supernatant (1 ml) was added with 0.5 g magnesium, mixed and
203 then was centrifuged at 16100g for 20 min. The clear supernatants were transferred into vials
204 and kept at -20 °C till GC-MS analysis. A Shimadzu QP2010 system and a capillary column,
205 Agilent HP-5MS (5% Phenylmethylsiloxane, 30 m × 0.25 mm i.d.; film thickness, 0.25 µm)
206 were used for ITC analysis. GC-MS analytic conditions were performed as previously
207 reported (Luang-In et al., 2014). The temperature was kept at 50°C for 5 min and ramped to
208 150°C at 5°C/min for 25 min, and then ramped to 250°C at 5°C/min for 15 min. The total 40
209 min run was carried out with a flow rate of 1 ml/min, average velocity of 36 cm/s, pressure of
210 7.56 psi and injection volume of 1 µl. Mass spectra were obtained by electron ionization (EI)
211 over a range of 50−550 atomic mass units. Ion source temperature was 230°C, and the
212 electron multiplier voltage was 70.1 eV. Authentic standards of allyl isothiocyanate and
213 sulforaphane were purchased from Sigma-Aldrich Co. (Singapore). Identification was based
214 on retention time and fragment ions (Table S1). Quantification of degradation products was
215 calculated using an external standard curve of sulforaphane.
216
217 **2.11. Antioxidant activity of the fermented cabbage**
218 This was evaluated through the free radical scavenging effect on 2,2′-diphenyl-1-
219 picrylhydrazyl (DPPH) radical as previously reported (Akowuah et al., 2005), FRAP assay
220 (Benzie & Strain, 1996) and ABTS scavenging assay (Seeram et al., 2006) using 25 mg/ml
221 freeze-dried extract dissolved in 95% ethanol as a starting solution.
222
223 **2.12. Statistical analyses**

224 Triplicates were used for each treatment. Results are expressed as means ± standard deviation
225 (SD). The significant differences between means are calculated by a one-way analysis of
226 variance (ANOVA) and Duncan's multiple range test at $p < 0.01$ using SPSS package version
227 19.
228
229 **3. Results and Discussion**
230 **3.1. GSL-metabolizing bacteria from Thai fermented foods and drinks**
231 Twenty-one bacterial isolates from 8 sources of Thai local fermented foods and beverages
232 were identified as GSL-metabolizing bacteria using selective M9 agar containing sinigrin
233 substrate and identified at subspecies level using 16S rRNA gene analysis. The results show
234 that the greatest number of newly identified GSL-metabolizing bacterial species came from
235 Thai fermented cabbage followed by Thai fermented fish resulting in isolation of 8 and 3
236 bacterial species, respectively (Table 1). When sinigrin was metabolized by bacterial
237 myrosinase, the degradation product i.e. isothiocyanate namely allyl isothiocyanate (AITC)
238 was expected. It was observed that most GSL-metabolizing bacteria were able to produce
239 AITC from sinigrin metabolism (Table 1) using GC-MS and HPLC analyses, respectively.
240 The majority of ITC-producing bacteria belong to the genera *Enterobacter* and *Enterococcus*
241 with the two highest ITC producers named as *Ent. xiangfangensis* 4A-2A3.1 (EX) from
242 fermented fish and *Ec. casseliflavus* SB2X2 (EC) from fermented cabbage producing 65 and
243 61 nmol AITC, respectively from 100% sinigrin degradation within 24 h (Table 1). Therefore,
244 these two isolates were chosen as a starter culture to ferment cabbage in further experiment.
245 Although bacteria belong to the same genus e.g. Enterobacter, they exhibited different GSL-
246 metabolizing capacity therefore possibly different myrosinase activity (Table 1). AITC was
247 unstable in the culture media (Luang-In & Rossiter, 2015) and thus AITC product formation
248 never reached 100% product formation. The highest % product formation was found in EX

249 with 65% product formation. However, *Lactococcus hircilactis* WS16, *L. lactis* WS18 and
250 *Bacillus* sp. KW3 did not produce AITC from 77-80% sinigrin degradation suggesting they
251 may have different GSL metabolic enzymes or mechanisms from other bacteria in
252 metabolizing GSL, but not producing ITC. Two bacterial species were found in more than
253 one sample. *Enterobacter* sp. 1A-1A with 92% identity to *Enterobacter* sp. Md1-53 was
254 found in fermented cabbage, pickled onions and fermented juices. *Enterobacter faecalis* 5A-
255 2B with 99% identity to *Enterobacter faecalis* NW A20 was present in both fermented
256 cabbage and fermented herbal drink. Thus, there were 17 bacterial strains from 21 isolates
257 from 8 fermented food/drink samples. All these 17 bacterial strains at subspecies level have
258 not been reported as GSL metabolizer and/or ITC producers before, yet they shared the same
259 genus or the same species as those identified previously. Previous findings showed a variety
260 of GSL-metabolizing bacterial strains such as *Bacillus thuringiensis* (El-Shora et al., 2016),
261 Actinomycetes isolated from cotton soil (Madhuri & Anuradha, 2015), *E. coli* VL8,
262 *Enterococcus casseliflavus* CP1 isolated from human faeces (Luang-In et al., 2014),
263 *Lactobacillus plantarum* KW30, *Lactococcus lactis* subsp. lactis KF147, *Escherichia coli*
264 Nissle 1917 isolated from foods (Mullaney et al., 2013), *Bifidobacterium pseudocatenulatum,*
265 *B. adolescentis, B. longum* (Cheng et al., 2004), *Lb. agilis* R16 (Palop-llanos et al., 1995), and
266 known myrosinase-producer *Ent. cloacae* isolated from soil (Tani et al., 1974). In this work,
267 all the identified 17 bacteria belong to the above reported genus including Bacillus,
268 Lactococcus, Escherichia, Enterobacter and Enterococcus. Contrary to popular belief, *Lc.*
269 *hircilactis* WS16 (100% identity to *Lc. hircilactis* DSM 28960) and *Lc. lactis* WS18 (98%
270 identity to *Lc. lactis* RCB787) in this study did not produce ITC from GSL metabolism (Table
271 1). This result indicated that not all LAB are able to produce ITC as previously thought.
272 Similarly, Mullaney et al. (2013) found that *Lb. plantarum* KW30 and *Lc. lactis* subsp.lactis
273 KF147 did not produce any ITC from glucoraphanin, glucoerucin and glucoiberverin. Instead

274 they generated sulforaphane nitrile as well as erucin nitrile and iberverin nitrile. Most isolated
275 bacteria have the highest % identity to the closest relative bacteria originated from food
276 sources and plants followed by human/animal guts and environments located in mostly Asia
277 including China, India, Korea, Pakistan, Thailand and also other parts of the world including
278 Italy, Belgium, Brazil and South Africa (Table 1). Although probiotic reports of both *Ent.*
279 *xiangfangensis* and *Ec. casseliflavus* are scarce, the genome of *Ent. xiangfangensis* isolated
280 from Chinese traditional sourdough was recently published (Gu et al., 2014). In addition, *Ec.*
281 *faecium*-group and *Ec. faecalis*-group were also isolated at the early stages of fermentation of
282 cauliflower fermentation (Paramithiotis et al., 2010) indicating that Enterococcus and
283 Enterobacter were commonly found in fermented foods.
284
285 **3.2. Phylogenetic tree of GSL-metabolizing bacteria**
286 Phylogenetic tree shows 17 GSL-metabolizing bacteria isolated from this work and 9
287 reference bacteria with GSL-metabolizing capacity from the previous reports were
288 categorized into 3 main groups (Fig. 1). The first and biggest group (30.1 % node) comprised
289 of all bacteria from this work including LAB, Enterococcus and Bacillus along with the
290 reference LAB (Mullaney et al., 2013) and Enterococcus (Luang-In et al., 2014) from the
291 previous findings. The subgroup consisted of Enterobacter as well as reference Citrobacter
292 (Albaser et al., 2016). The second group (89.1% node) included only 3 reference *E. coli*
293 bacteria (Mullaney et al., 2013; Luang-In et al., 2014; Cordeiro et al., 2015). Similarly, the
294 third group (100% node) included only 2 reference Enterobacter bacteria (Tani et al., 1974).
295 From this result, it seems *Enterobacter* spp. isolated from this work were evolutionarily
296 closely related to *Citrobacter* sp. WYE1 (Albaser et al., 2016) than *Enterobacter cloacae*
297 (Tani et al., 1974). This is the first report of identifying *Ent. xiangfangensis, Ent. ludwigii,*
298 *Ent. asburiae* and several new *Bacillus* spp. as ITC producers. Phylogenetic tree of putative

299 myrosinase enzymes was also constructed (Fig. 2). Phylogenetic tree shows 9 putative
300 myrosinase from the closet relative bacteria found in NCBI database and 4 characterized
301 myrosinases from *Brassica juncea* plant (Accession no. AAG54074.1), *Citrobacter* sp.
302 WYE1 (Accession no. ALM58466.1) (Albaser et al., 2016), *Ent. cloacae* EcWSU1
303 (Accession no. AEW75128) (Cordeiro et al., 2015) and *Escherichia coli* O157:H7 str.
304 TW14359 6 (Accession no. ACT73612.1) (Cordeiro et al., 2015) were categorized into 3
305 main groups (Fig. 2). The first bacterial myrosinase group categorized into Glycosyl
306 Hydrolase 1 (GH1) family consists of 6-phospho-beta-glucosidases (6pbg) from the 2
307 reference myrosinases from *Ent. cloacae* EcWSU1 and *E. coli* O157:H7 str. TW14359 and
308 those from the closet relative bacteria found in this work including *Ent. ludwigii, Ent.*
309 *xiangfangensis, Ent. asburiae, Ec. casseliflavus, Bacillus* and *Lc. lactis*. On the other hand,
310 the second bacterial myrosinase group categorized into GH3 family consists of beta-
311 glucosidase (bgl) from the reference myrosinase from *Citrobacter* sp. WYE1 and those from
312 the closet relative bacteria found in this work including *Ent. xiangfangensis, Ec. casseliflavus*
313 and *Bacillus*. Clearly, plant myrosinase (GH1) was evolutionarily distinct from the other 2
314 bacterial myrosinase groups indicating differences in myrosinase evolution between plants
315 and bacteria. *Citrobacter* sp. WYE1 myrosinase was active *in vitro* or in cell-free extract
316 (Albaser et al., 2016); however those from *Lb. agilis* R16 (Llanos-Palop et al., 1995), *E. coli*
317 O157:H7 (Cordeiro et al., 2015) and *Ec. casseliflavus* CP1 (Luang-In et al., 2014) were
318 inactive *in vitro*, but only active *in vivo* or in intact cells. The closely evolved enzymes to
319 *Citrobacter* sp. WYE1 myrosinase were Bacillus protein and 2 Bgl enzymes from *Ent.*
320 *xiangfangensis* and *Ec. casseliflavus* (Fig. 2). These may be as active *in vitro* as *Citrobacter*
321 sp. WYE1 myrosinase which will facilitate the study of myrosinase enzyme activity *in vitro*
322 and hence decoding their amino acid sequences more easily than those only active *in vivo*.
323

3.3. Chemopreventive ITC products from fermented cabbage

Ent. xiangfangensis (EX) and *Ec. casseliflavus* (EC) were used to ferment Thai cabbage containing three GSLs namely glucoiberin (GIB), glucoraphanin (GRP) and 4-hydroxy glucobrassicin (GBS) at 430.5, 615.1 and 108.5 µmol/100 g dry weight, respectively (Table 2) for 3 day at 25°C. In comparison with Chinese cabbage (in kimchi) that contained five types of GLSs of approximately 8.3 µmol/g dry weight including glucoalyssin, gluconapin, glucobrassicanapin, glucobrassicin, and 4-methoxyglucobrassicin (Kim et al., 2017), it was clear that our GSLs were present in higher amounts indicating that cabbages in various countries contain various kinds of GSLs (Table S3) in various amounts depending on environmental factors such as geographical location, temperature, solar radiation, humidity and climatic conditions (Bohinc & Trdan, 2012). Degradation products of the three GSLs namely Iberin nitrile (IBN), iberin (IBR), Sulforaphane (SFN) and indole 3-acetonitrile (IAN), respectively were already detected in fresh cabbage; however in less amounts than those in fermented samples (Table 2). The product generation was due to intrinsic plant myrosinase in cabbage coming into contact with GSL upon tissue damage during handling cabbage process and possibly GSL hydrolysis occurred. Table 2 shows gradual GSL degradations in all treatments; however the spontaneous cabbage fermentation produced less IBR and SFN at days 2-3 suggesting that bacterial culture present may be able to degrade GSLs, but not capable of producing ITCs. However, IBN was found in less amounts at days 2-3 in the induced fermentation of cabbage than the spontaneous one, and that was possibly due to presence of bacterial enzymes transforming nitrile products to other metabolites. GRP was degraded more rapidly in bacterial induced cabbage fermentations than the spontaneous one indicating the presence of specific GRP-metabolizing bacteria in the samples (Table 2). GBS totally disappeared at the end of day 1 due to its initial low content in cabbage (Fig. 3A; Table 2). The degradation products included those mentioned above as in fresh cabbage along

with palmitic acid and linoleic acid (Fig. 3B). The IAN production in all treatments were similar at each day and gradually declined over time. The production of both chemopreventive IBR and SFN in EX-induced cabbage fermentation peaked at day 2 at 117.4 and 294.1 µmol/100 g dry weight, respectively which was significantly higher than IBR at 51.7 µmol/100 g dry weight, but not significantly higher than SFN at 242.6 µmol/100 g dry weight in EC-induced fermented cabbage at day 2. Overall degradation products in all treatments declined over 3 days and never reach 100% product formation (Table S2). This was possibly due to the unstable nature of ITCs in fermentation matrices (Luang-In & Rossiter, 2015). In addition, SFN content detected in the liquid portion of EC-induced cabbage fermentation at day 2 was significantly higher (p<0.01) than that found in the fermented cabbage solid portion by almost three folds (Table 2). This indicated that one should consume the liquid portion of the fermented cabbage along with the more commonly consumed fermented cabbage solid portion in Thailand to obtain the best health benefits from SFN. The pH values of the fermented cabbages at day 3 were 3.55 (N), 3.25 (EX) and 3.45 (EC) with no statistical difference (Fig. S2) and are similar to pH values of 3.27 – 3.67 of sauerkraut from Spanish cabbage over 7-day fermentation at 25°C by *Lactobacillus plantarum* (CECT 748) and *Leuconostoc mesenteroides* (CECT 219) (Table S3). Similarly, the ITC products including SFN at 39-49 µmol/100g dry weight, IBR, and IBR NIT were detected from metabolism of glucoraphanin and glucoiberin in sauerkraut from Spanish cabbage like in our product but in less amounts, except for allyl isothiocyanate (AITC) and allyl nitrile (ANIT) that were only found in their sauerkraut; however these products were not detected in their raw cabbage (Peñas et al., 2012) suggesting that fermentation was responsible for ITC and NIT productions. Similarly, sauerkraut made from cabbage grown in Finland produced SFN, AITC, ANIT and indole 3-carbinol (I3C) from glucoiberin, sinigrin and glucobrassicin, respectively (Tolonen et al., 2002). In contrast, sauerkraut made from

374 cabbage grown in Germany only produced ascorbigen and I3C from glucoiberin, sinigirin,
375 glucobrassicin, glucoraphanin and 4-methoxy glucobrassicin (Palani et al., 2016). SFN was
376 also found in Korean kimchi, however in a less amount than in fresh cabbage (Kim et al.,
377 1999), and sometimes not found at all (Hong & Kim, 2013). Different ITC production from
378 different fermented cabbage products in various countries may be the result of different
379 cultivars of cabbages used and bacterial species present in the fermentation. To ascertain
380 functionality of Thai fermented cabbage in this work and for future commercial purposes,
381 anticancer activity and antimicrobial activity of metabolites produced during induced cabbage
382 fermentation as well as probiotic properties of both *Ent. xiangfangensis* and *Ec. casseliflavus*
383 will be determined in the next project. In addition to ITCs as potential anticarcinogens,
384 palmitic acid (16:0) and linoleic acid (18:2) as potential anticarcinogens or antimutagens
385 (Nadathur et al., 1996; Bocca et al., 2010) were also present in fresh cabbage. After induced
386 cabbage fermentation with either EC or EX for 3 days, palmitic acid and linoelic acid contents
387 were increased by almost 5 folds and 10 folds, respectively (data not shown) suggesting
388 induced fermentation added more nutritional value to cabbage than non-induced fermentation.
389 Our finding was in accordance with the previous work showing that milk fermented with kefir
390 had higher contents of healthy fatty acids when compared to unfermented milk (Guzel-
391 Seydim et al., 2006). That was due to bacterial capacity to produce different fatty acids
392 (Dykes et al., 1995) which employed the disassociated fatty acid synthesis II pathway, thus
393 multiple discrete enzymes synthesize the fatty acid chain (White et al., 2005).
394
395 **3.4. Antioxidant activity of fermented cabbage**
396 Since EX-induced cabbage fermentations yielded the highest ITCs, they were used for
397 antioxidant activity evaluation in comparison with the spontaneous ones. The results showed
398 that EX-induced cabbage fermentations exhibited significantly higher %DPPH scavenging

399 activity, FRAP activity and ABTS activity than those found in the spontaneous fermentations
400 over 3 days (Fig. 4). In addition, antioxidant activities of fresh cabbage at day 0 were similar
401 to those in both spontaneous and induced fermentations and increased over time indicating
402 bacterial metabolism was responsible for increasing antioxidants in the fermented cabbage as
403 well as the increase of ITCs (Table 2). Lactic-fermented cabbage in China, prepared by a dry-
404 salt method and extracted with methanol, showed antioxidant activity of DPPH radical
405 scavenging effect at 60% (Sun et al., 2009). This was similar to DPPH radical scavenging
406 effect at 62.1% at day 2 by EX-induced fermentation (Fig. 4). In addition, Lactic-fermented
407 red cabbage sprouts gave significantly higher antioxidant functionalities than those of their
408 unfermented/control counterparts. Fermented red cabbage sprouts inoculated with *Lb.*
409 *plantarum* had the highest antioxidant activities (DPPH scavenging: 70.92%; TEAC: 1.94
410 mM Trolox equivalent), which was almost two-fold higher than those of unfermented
411 treatments. These results indicated that Lactobacillus fermentation and our Enterobacter
412 fermentation could be applied as a method to improve the potent antioxidant activities of
413 vegetables (Hunaefi et al., 2013)
414
415 **4. Conclusions**
416 Induced cabbage fermentation by *Ent. xiangfangensis* or *Ec. casseliflavus* produced higher
417 contents of chemopreventive SFN and IBR than those from the spontaneous
418 fermentation and fresh cabbage. Fermented liquid and solid portions from fermented cabbage
419 should be combined for consumption at day 1 or 2 for the highest ITCs and no nitrile product
420 providing the best health-promoting benefits. GSL-metabolizing bacteria isolated from Thai
421 fermented foods can be used as a starter culture for a traditional Thai fermented cabbage. This
422 can then be promoted as a functional food with chemopreventive properties from ITC

423 products like the fermented cabbage product counterparts from different countries e.g.
424 sauerkraut and kimchi.

425

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433

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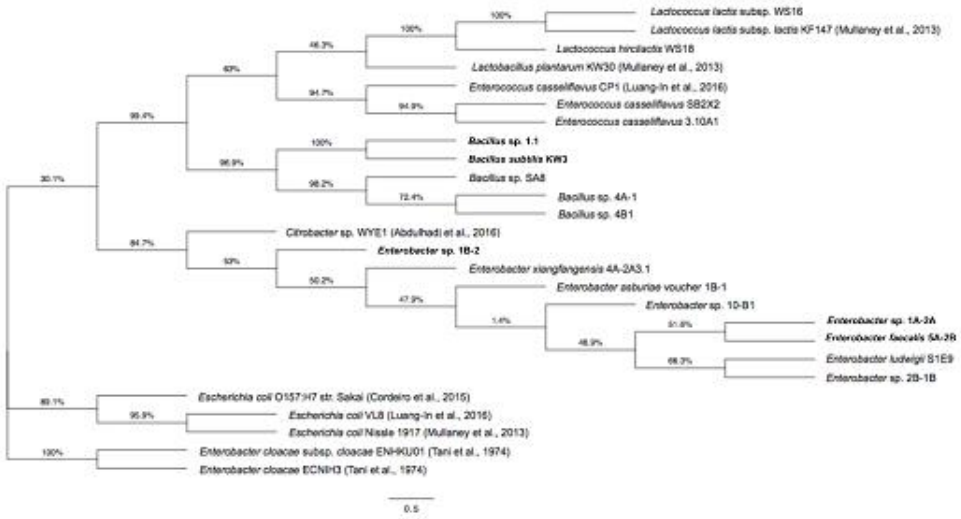
549

554 **FIGURES**



555

556 **Fig. 1** Phylogenetic tree of GSL-metabolizing bacteria isolated from this work and the
557 previous reports. It was inferred from 26 partial16S rRNA sequences from different bacteria
558 by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The
559 percentage of replicate trees in which the associated taxa clustered together in the bootstrap
560 test (1,000 replicates) is shown next to the branches. The horizontal bar represents a distance
561 of 0.5 substitutions per site. Evolutionary analyses were conducted in MEGA7 and the
562 phylogenetic tree was drawn by using FigTree.

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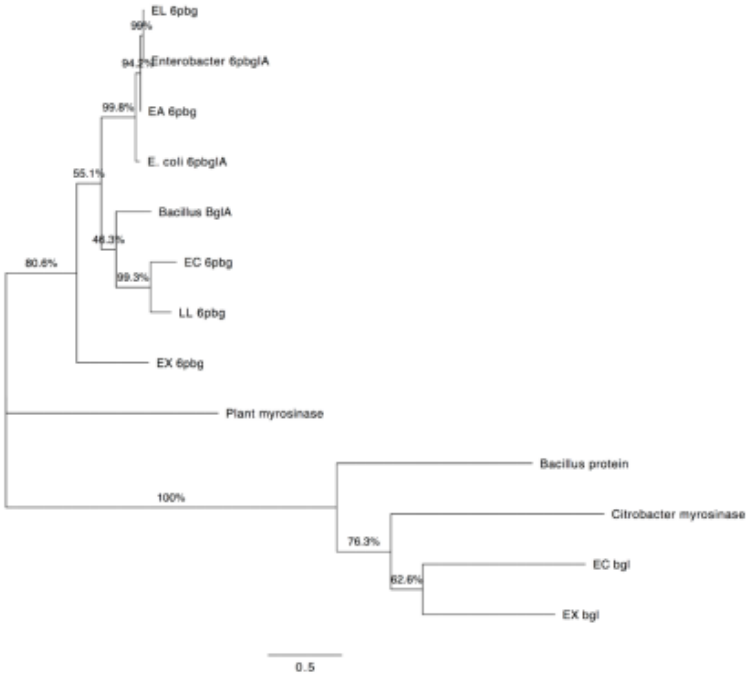
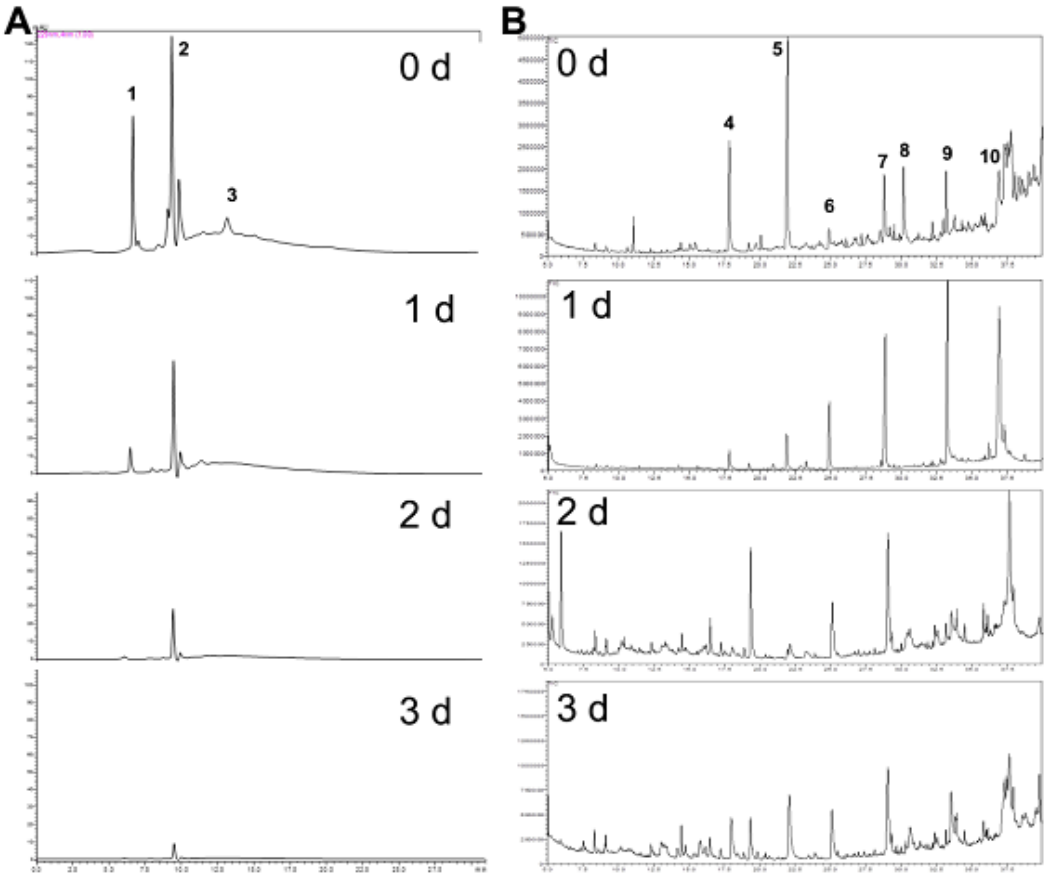


572

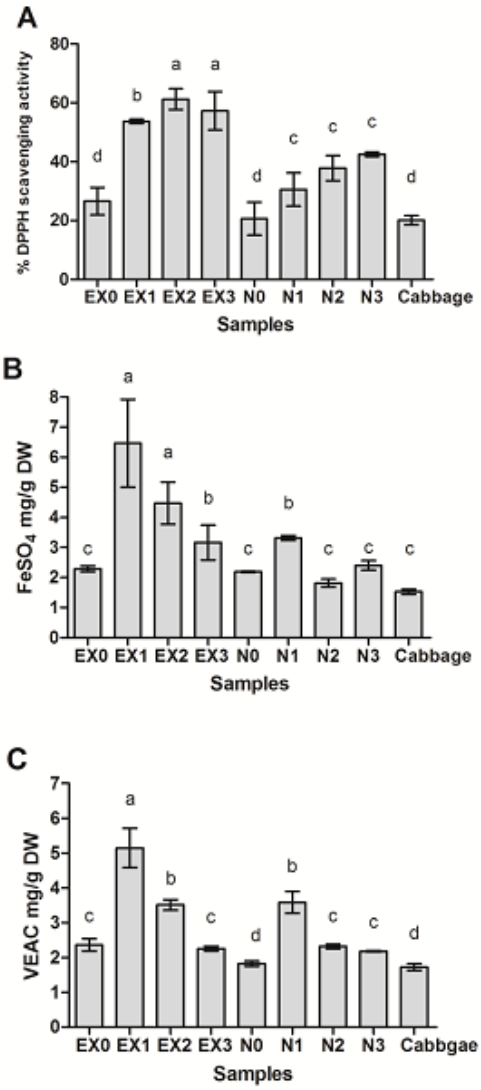
573 **Fig. 2** Phylogenetic tree of putative myrosinases from the bacteria in this work and the
574 previous reports. It was inferred from 13 amino acid sequences of putative myrosinases from
575 different bacteria by using the Maximum Likelihood method based on the Le_Gascuel_2008
576 model. The percentage of replicate trees in which the associated taxa clustered together in the
577 bootstrap test (1,000 replicates) is shown next to the branches. The horizontal bar represents a
578 distance of 0.5 substitutions per site. Evolutionary analyses were conducted in MEGA7 and
579 the phylogenetic tree was drawn by using FigTree.

580



581

582 **Fig. 3** (A) HPLC chromatograms of GSL profiles of fermented cabbage by EX from 0 – 3
583 days. (B) GC-MS chromatograms of metabolic profiles of fermented cabbage by EX from 0 –
584 3 days. HPLC peaks of GSLs; **1** Glucoiberin at 6.30 min, **2** Glucoraphanin at 9.44 min and **3**
585 4-Hydroxy glucobrassicin at 12.5 min. GC-MS peaks of products, **4** iberin nitrile at 17.9 min,
586 **5** Pent-1,4-diene-3-one at 21.9 min, **6** iberin at 24.9 min, **7** sulforaphane at 28.9 min, **8** indol-
587 3-acetonitrile at 30.2 min, **9** palmitic acid at 33.3 min and **10** linoleic acid at 36.9 min.



588

589 **Fig. 4** Antioxidant activities from fermented cabbage with/without bacterial induction over 3

590 days. EX = induced with EX for 0-3 days (E0, E1, E2, E3); N = Non-induced with EX for 0-3

591 days (N0, N1, N2, N3). Different small letters indicate significantly statistical differences in

592 values (p<0.01).

593

594

595

Table 1 Twenty bacterial isolates with glucosinolate-metabolizing capacity isolated from Thai local fermented foods and drinks

No.	Accession no.[a]	Species	Closet relative species[b] (% identity) /Accession no.[c]/Origin of isolate[d]	Sinigrin degradation (nmol) [e]	AITC product (nmol) [e]	% product formation [f]
1. Fermented cabbage pH 3.87						
1	LC342980.1	*Enterobacter* sp. 1A-1A	*Enterobacter* sp. Md1-53 (92%) MF581459.1 Paeonia ostii root, China	73 ± 8	30 ± 5	41 ± 4
2	LC342981.1	*Enterobacter faecalis* 5A-2B	*Enterococcus faecalis* NW A20 (99%) MG543833.1 Raw meat, South Africa	78 ± 7	39 ± 11	50 ± 14
3	LC342982.1	*Enterobacter asburiae* 1B-1	*Enterobacter asburiae* voucher ST56 KT287073.1 100% Rumen China	75 ± 11	33 ± 8	44 ± 6
4	LC342983.1	*Enterobacter* sp. 1B-2	*Enterobacter* sp. NU33 (96%) MG459258.1 Plant growth-promoting bacteria in sugarcane, Brazil	79 ± 5	41 ± 7	52 ± 8
5	LC342984.1	*Enterobacter ludwigii* S1E9	*Enterobacter ludwigii* HTP04 (100%) KX024731.1 Isolated from shrimp gut, India	90 ± 8	50 ± 9	56 ± 7
6	LC342985.1	*Enterococcus casseliflavus* SB2X2	*Enterococcus casseliflavus* HMF4406 (98%) KT984002.1 Jeotgal (salted fermented food), Korea	100 ± 0	61 ± 4	61 ± 6
7	LC342986.1	*Bacillus* sp. SA8	*Bacillus* sp. SK123 (97%) KU060226.1 Honey bee apiary, Thailand	79 ± 8	39 ± 7	49 ± 6
8	LC342987.1	*Bacillus* sp. 1.1	*Bacillus* sp. BDU13 (96%) JX847614.1 Microbial diversity from fermented fish, India	87 ± 10	42 ± 11	48 ± 10
2. Pickled onion pH 4.81						
9	LC342980.1	*Enterobacter* sp. 1A-1A	*Enterobacter* sp. Md1-53 (92%) MF581459.1 Paeonia ostii root, China	73 ± 5	40 ± 5	55 ± 4
10	LC342988.1	*Enterobacter* sp. 2B-1B	*Enterobacter* sp. SR19 (100%) KF896099.1 Seawater sediment, Belgium	71 ± 0	39 ± 3	55 ± 6
3. Fermented fish pH 4.60						
11	LC342989.1	*Enterobacter xiangfangensis* 4A-2A3.1	*Enterobacter xiangfangensis* W31 (100%) KP813789.1 Storm water bacteria in two urban lakes China	100 ± 0	65 ± 3	65 ± 4
12	LC342990.1	*Bacillus* sp. 4A-1	*Bacillus* sp. S42 (100%) JX293317.1 Crystal tuff, China	71 ± 6	40 ± 4	56 ± 1
13	LC342991.1	*Bacillus* sp. 4B1	*Bacillus* sp. SO5.17 (97%) KC867296.1 Mine drainage, Brazil	73 ± 4	42 ± 9	58 ± 13
4. Fermented pork pH 4.73						
14	LC342992.1	*Enterococcus casseliflavus* 3.10A1	*Enterococcus casseliflavus* JFL12 (100%) KT343156.1 Fiber-degrading bacteria in rumen of Tibetan yak, China	74 ± 13	35 ± 8	47 ± 4
5. Fermented herbal drink pH 2.80						
15	LC342981.1	*Enterobacter faecalis* 5A-2B	*Enterococcus faecalis* NW A20 (99%) MG543833.1 Raw meat, South Africa	78 ± 7	35 ± 6	45 ± 5
6. Fermented juice pH 2.93						
16	LC342993.1	*Enterobacter* sp. 10-B1	*Enterobacter* sp. DBM3 (97%) KT957440.1 *Plutella xylostella* larval gut, China	77 ± 5	34 ± 11	42 ± 16

17	LC342980.1	*Enterobacter* sp. 1A-1A	*Enterobacter* sp. Md1-53 (92%) MF581459.1 Paeonia ostii root, China	73 ± 10	34 ± 8	47 ± 7
7. Water kefir from Nakhon Ratchasima pH 5.94						
18	LC336444.1	*Lactococcus hircilactis* WS16	*Lactococcus hircilactis* DSM 28960 (100%) KJ201026.1 Goat milk, Italy	77 ± 4	nd	na
19	LC336446.1	*Lactococcus lactis* WS18	*Lactococcus lactis* RCB787 (98%) KT260999.1 Bat guano, India	78 ± 3	nd	na
8. Milk kefir from Kamphaeng Phet pH 5.23						
20	LC342994.1	*Bacillus subtilis* KW3	*Bacillus subtilis* MA-48 (93%) KX426648.1 Rhizospheric soil in desert, Pakistan	80 ± 9	nd	na

[a] GenBank accession no. of our strains on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)
[b] Closet relative species and identity (%) from BLAST search on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)
[c] GenBank accession no. of closest relatives on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)
[d] Origins of closet relative species i.e. where each bacterium was isolated from
[e] Each isolate from fermented samples was cultured in LB medium containing 1 mM sinigrin for 24 h. After that sinigrin degradation and AITC production were determined by HPLC and GC-MS, respectively.
[f] % product formation = [AITC product (nmol)/Sinigrin degradation (nmol)] x 100%
nd = not detected; na = not available

Table 2 Metabolism of glucosinoates in fermented cabbage with/without bacterial induction over 3 days.

Samples	Remaining GSL (µmol/ 100 g dry weight)			Products (µmol/ 100 g dry weight)			
	GIB	GRP	GBS	IBN	IBR	SFN	IAN
Cabbage	430.5±34.1aB	615.1±30.0aA	108.5±19.1aC	75.1±26.4aC	13.3±10.0bD	39.5±16.3dC	49.1±29.8abC
N0	273.8±15.8bB	534.4±30.4abA	30.2±4.4bC	61.82±28.4abC	11.2±6.0bD	56.2±26.0dC	52.4±19.1abC
N1	103.8±19.5cB	419.0±26.5cA	0.0+0.0cE	43.3±23.2abC	32.1±3.9bD	135.2±42.0abB	22.9±4.7abC
N2	20.9±12.0dB	217.5±14.8deA	0.0+0.0cC	35.2±15.4abB	11.57±5.8bD	127.9±60.0abA	15.8±2.0bB
N3	2.9±1.1dC	146.3±27.7deA	0.0+0.0cD	21.6±11.0bcB	8.6±3.9bB	112.2±52.0abA	10.7±3.1bB
EC0	305.7±29.8bB	514.2±42.6bA	27.4±6.9bD	64.3±23.3abC	15.4±5.9bD	45.6±22.0dC	60.5±21.8aC
EC1	91.8±17.3cC	359.9±31.8cdA	0.0+0.0cE	25.6±12.6abD	35.2±20.0bD	177.8±42.0abB	22.5±14.6abD
EC2	21.0±9.3dB	211.0±27.7deA	0.0+0.0cD	6.4±2.1cC	51.7±35.9abB	242.6±40.0aA	14.7±11.9abB
EC3	3.1±0.7dD	111±20.2fB	0.0+0.0cE	2.9±0.8cD	33.6±22.0bC	222.4±42.0aA	16.4±7.2abC
EX0	305.2±13.0bB	536.6±29.1abA	31.6±7.8aC	65.2±20.2abC	12.5±4.0bD	48.6±30.0dC	55.3±20.2abC
EX1	74.3±14.6cC	275.9±19.deA	0.0+0.0cF	26.6±12.3abD	117.4±41.9aB	294.1±44.0aA	7.7±3.7bE
EX2	11.1±6.4dD	160.8±29.0eB	0.0+0.0cE	6.5±3.8cD	78.2±38.0abC	252.6±46.0aA	16.3±7.4abD
EX3	2.4±0.6dD	85.3±24.7fB	0.0+0.0cE	13.8±3.1bcC	70.5±39.9abB	244.7±70.0aA	20.9±8.8abC
EC2 solid	5.2±1.6dC	93.3±23.6fA	0.0+0.0cD	3.6±2.3cC	19.2±10.0bB	69.7±34.0cA	5.9±1.2bB
EC2 liquid	16.3±7.5dC	117.7±34.4fB	0.0+0.0cF	3.5±0.7cE	30.8±22.0bC	173.3±48.0abA	10.4±1.3bD

Cabbage = fresh cabbage; N = Non-induced with EX for 0-3 days (N0, N1, N2, N3); EC = induced with EC for 0-3 days (E0, E1, E2, E3); EX = induced with EX for 0-3 days (E0, E1, E2, E3); EC2 solid = Solid portion of fermented cabbage of EC at day 2; EC2 liquid = Liquid portion of fermented cabbage of EC at day 2. Different small letters indicate significantly statistical differences in values in the column ($p<0.01$) and capital letters indicate significantly statistical differences in values in the row ($p<0.01$)

Supplementary data

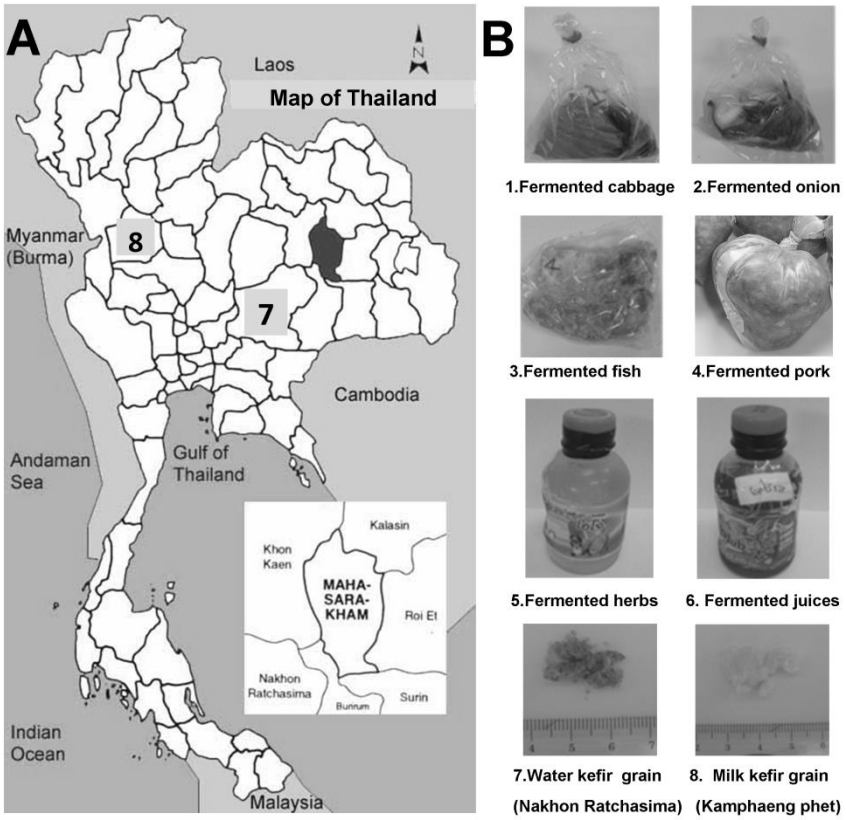


Fig. S1 (A) Location of Mahasarakham Province where most fermented samples were collected and Nakhon Ratchasima and Kamphaeng phet. (B) Thai local fermented foods and drinks used in this study.

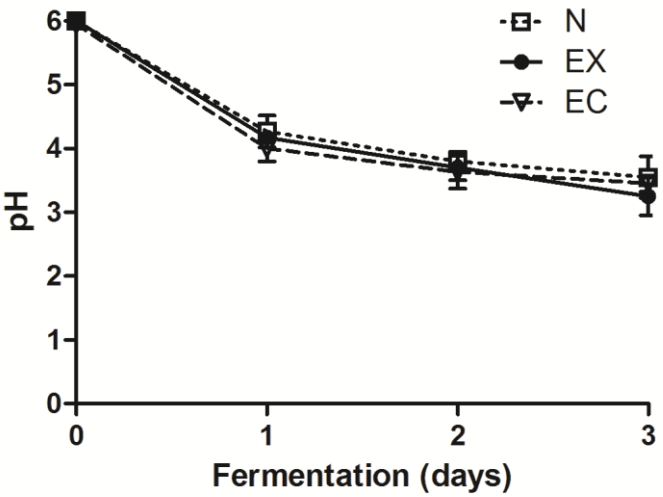


Fig. S2 The pH values of fermented cabbage with/without bacterial induction over 3 days.

Table S1 Mass spectral (MS) data of metabolites detected in this work

Substrate	Degradation products[a]	MS spectra data m/z	T_R (min)*
Sinigrin (SNG)	Allyl-ITC (AITC)	99 (M^+), 72, 65	6.9
	Allyl-NIT (ANIT)	67 (M^+), 52	n.d.
Glucoiberin (GIB)	Iberin (IBR)	163 (M^+), 130, 116, 100, 72	24.9
	Iberin nitrile (IBR NIT)	131 (M^+), 115, 69, 61	17.9
NA	Pent-1,4-diene-3-one	82 (M^+), 64, 55	21.9
Glucoraphanin (GRP)	Sulforaphane (SFN)	177 (M^+), 160, 115, 72	28.9
	Sulforaphane nitrile (SFN NIT)	145(M^+), 128, 82, 55	ND
4-Hydroxy Glucobrassicin (GBS)	Indole-3-acetonitrile (IAN)	155 (M^+), 130, 101, 77, 51	30.2
NA	Palmitic acid	256 (M^+), 213, 129, 73, 60	33.3
NA	Linoleic acid	280 (M^+), 193, 151, 69, 55	36.9

*Retention time at which degradation product was eluted as detected by GC-MS analysis.

**These compounds were not available as authentic standards however they were detected in the reactions during bacterial fermentations. Their identifications were made according to previously reported retention time and fingerprint profiles (Vaughn *et al*., 2005). NA, not available; n.d., not determined; ND, not detected.

Table S2 Percentage (%) production of degradation products upon glucosinolate metabolism during cabbage fermentation with/without induced bacterial culture over 3 days

Samples	% production		
	IBN + IBR	SFN	IAN
N0	46.6	69.6	92.5
N1	17.0	79.2	30.3
N2	11.4	43.0	23.8
N3	7.1	34.1	19.1
EC0	63.9	45.2	74.6
EC1	18.0	69.7	39.2
EC2	14.2	82.7	32.0
EC3	8.5	73.1	15.1
EX0	62.0	61.9	71.9
EX1	40.4	86.7	7.1
EX2	20.2	71.3	15.0
EX3	19.7	52.1	19.3
EC2 solid	5.4	14.2	11.0
EC2 liquid	8.3	54.6	18.8

Table S3 ITC products from different fermented cabbage products from different countries

Sample	GSL profiles	ITC products	pH at the end of fermentation	Fermentation method
Thai picked cabbage (white cabbage from Khon Kaen, Thailand)	Glucoiberin Glucoraphanin 4-hydroxy glucobrassicin	Iberin nitrile (IBN) Iberin (IBR) Sulforaphane (SFN) Indole-3-acetonitrile (IAN)	3.25-3.55 (3 days at 25 °C)	Spontaneous and induced fermentation by *Ec. casseliflavus* or *Ent. xiangfangensis* (This work)
Korean kimchi (white cabbage from Korea)	NA	2.2 ppm SFN (lower than fresh cabbage) 3-butenyl ITC 4-methylthiobutyl	4.53 (3 days at 20 °C)	Spontaneous (Kim et al., 1999)
Korean cabbage (*Brassica campestris* L. ssp. *peckinensis*, cultivar; 'Winter pride')	Gluconasturtiin Glucobrassicanapin 4-methyl-pentyl GSL Gluconapin	2-phenylethyl ITC 4-pentenyl ITC 4-methylpentyl ITC 3-Butenyl ITC	No fermentation	Fresh cabbage (Hong & Kim, 2013)
Sauerkraut (white cabbage cultivars Bronco and Megaton from Spain)	Sinigrin Glucoiberin Glucoraphanin	SFN (39-49 µmol/100g DW) IBR IBN allyl nitrile (ANIT) allyl isothiocyanate (AITC)	3.27 - 3.67 (7 days at 25 °C)	*Lactobacillus plantarum* (CECT 748) and *Leuconostoc mesenteroides* (CECT 219) by the Spanish Type Culture Collection (CECT, Valencia, Spain) (Peñas et al., 2012)
Sauerkraut (*Brassica oleracea* L. var. capitata cv. Lennox from Finland)	Sinigrin Glucoiberin Glucobrassicin	AITC ANIT SFN I3C	3.9 (3 days at 20 °C)	Spontaneous vs *Leuconostoc mesenteroides* and *Pediococcus dextrinicus* (1:1) (Tolonen et al., 2002)
Sauerkraut (*Brassica oleracea* L. var. capitata cv. Storema RZ and cv. Lennox from Germany)	Glucoiberin Sinigirin Glucobrassicin Glucoraphanin 4-Methoxy glucobrassicin	Ascorbigen (13.0 µmol/100 g FW) I3C (4.52 µmol/100g FW)	4 (9 days at 20 °C)	Spontaneous (Palani et al., 2016)