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Full Length Article

Probiotic Attributes, GABA-Producing Capacity and Cytotoxic Effects of Microbes Isolated from Thai Fermented Foods

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Abstract

Mental illnesses and cancer deaths have been alarmingly increased in Thailand over the past decade. There is a search for an alternative remedy from natural sources. This work revealed the probiotic attributes of eighteen microbes from Thai fermented food sources, their neurotransmitter-producing capacities and cytotoxic effects. The probiotic attributes were comprehensively assessed by gelatin hydrolysis, antibiotic susceptibility, auto-aggregation, virulence gene detection, hemolytic activity, hydrophobicity determination, bile and acid tolerance. Neurotransmitters were produced from precursors by microbes over 3 days and analyzed by high-throughput LC-MS/MS. Cytotoxic effects of the selected microbial extracts on MCF-7, HepG2, and HeLa were determined using MTT assay. The results showed that only seven strains; *Enterococcus casseliflavus* 3.10A1, *Enterobacter xiangfangensis* 4A-2A3.1, *Saccharomyces cerevisiae* TC6, *Pedicoccus pentosaceus* WS11, *Lactococcus lactis* subspp. *lactis* TBRC 375, *Lactobacillus brevis* TBRC 3003 and *Bifidobacterium adolescentis* TBRC 7154 exhibited promising probiotic potential in every probiotic assessment. Only *E. xiangfangensis* 4A-2A3.1 and *Bacillus* spp. PS15 were capable of producing gamma-aminobutyric acid (GABA) from monosodium glutamate (20 mg/mL). GABA production of 4.60 μg/mL over 2 days was found in *E. xiangfangensis* 4A-2A3.1; however, *Bacillus* spp. PS15 produced the highest GABA at 5.57 μg/mL on day 1. Moreover, *B. adolescentis* TBRC 7154 extract showed the lowest IC_{50} values against MCF-7 (750.02 μg/mL), HepG2 (681.08 μg/mL), and HeLa (425.50 μg/mL) followed by *E. xiangfangensis* 4A-2A3.1, *E. casseliflavus* 3.10A1 and *P. pentosaceus* WS11. These microbes can be used as probiotic supplement with neurodegeneration preventive or chemopreventive potential.

Keywords: Anticancer; Neurotransmitter; Microbes; Probiotics; Thai fermented foods

Introduction

Although, modern drugs and chemotherapy are the routine remedies of mental disorders and cancers, they produce adverse effects in patients and have become ineffective after a certain time of usage (Weinstein *et al.* 2016). Thus, natural product-derived treatment has been sought after. Scientific evidence in the past decade have demonstrated that human gut microbiota play a vital role in human health, general well-being and brain function through the gut-brain axis (Claesson *et al*. 2012; Davari *et al*. 2013; Hsiao *et al*. 2013). The beneficial microbes in the human gut are defined as 'probiotics'. Probiotics can be assessed using the following tests: gelatin hydrolysis, antibiotic susceptibility, autoaggregation, PCR detection of virulence gene, hemolytic activity, bile and acid tolerance, hydrophobicity etc. for the necessary survival in human gastrointestinal tract.

The novel subclass of probiotics called 'psychobiotics' has been emerged since 2013. These psychobiotics were first defined as probiotics that, when ingested in appropriate quantities, yield positive psychiatric effects in psychopathology (Dinan *et al*. 2013). They were shown to be able to produce neurotransmitters and also exert psychotropic effects in animal model or patients. For example, *Bifidobacterium* and *Lactobacillus* were reported to produce gamma-aminobutyric acid (GABA) (Dinan *et al*. 2013). *Bacillus, Escherichia* and *Saccharomyces* produced norepinephrine. *Streptococcus, Candida, Enterococcus* and

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Escherichia produced serotonin. *Serratia* and *Bacillus* produced dopamine, and *Lactobacillus* produced acetylcholine (Dinan *et al*. 2013). Interestingly, endophytic *Bacillus amyloquefaciens* SB-9 isolated from grape wine produced melatonin, 5-hydroxytryptophan, serotonin and N-acetylserotonin (Jiao *et al*. 2016).

In addition to neurotransmitter-producing capacity, certain microbes from fermented foods were shown to exhibit anticancer properties. The previous report showed that six lactic acid bacteria: *Lactococcus lactis* subspp. *lactis*, *L. lactis* subspp. *cremoris*, *L. lactis* subspp. *lactis biovar diacetylactis*, *L. plantarum*, *L. meseuteroides* subspp. *cremoris* and *L. casei* from the Japanese homemade kefir enhanced the cytotoxicity of human natural killer KHYG-1 cells to human chronic myelogenous leukemia K562 cells and colorectal tumor HCT116 cells (Yamane *et al*. 2018). In addition, *L. plantarum* isolated from kimchi was reported to strengthen phagocytosis, and exhibit cancer suppression in aseites carcinoma due to the polysaccharide chains of muramic acid in its cell well (Kim *et al*. 2011). Therefore, it is not surprised that probiotic bacteria have emerged as alternative treatment to human ailments and are extensively explored as biotherapeutics.

The bacterial genera mentioned above have been isolated from Thai fermented foods (water kefir, milk kefir, and fermented foods in Thailand) in the previous works (Luang-In and Deseenthum 2016; Luang-In *et al*. 2018a, b). Fermentation seems to be the lowest cost process to obtain probiotics-enriched functional foods. However, little is known about their probiotic attributes, neurotransmitter-producing potentials and cytotoxic effects on cancer cells.

Thus, the aims of this work were to determine which bacteria isolated from Thai fermented foods possessed probiotic attributes, neurotransmitter-producing capacity and cytotoxic effects on MCF-7, HepG2, and HeLa cells. The results provided the possibility to develop the novel probiotic/psychobiotic-rich functional foods or pills or cocktails at low cost to exert neurodegeneration preventive or chemopreventive effects.

Materials and Methods

Microbial sources

Eighteen microbial species were isolated from various Thai fermented foods (Luang-In and Deseenthum 2016), Thai milk kefir from Kamphaeng Phet Province, Thailand (Luang-In *et al*. 2018a) and Thai water kefir from Nakhon Ratchasima Province, Thailand (Luang-In *et al*. 2018b). All microbes were stored in 20% glycerol stocks at -80°C at Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Mahasarakham University, Thailand. Three bacterial strains including *Lactococcus lactis* subspp. lactis TBRC 375, *Lactobacillus brevis* TBRC 3003 isolated from pickled cabbage (*Brassica* spp.) and *Bifidobacterium adolescentis* TBRC 7154 isolated from human intestine were purchased from Thailand Bioresource Research Center (TBRC), Pathum Thani, Thailand and used as probiotic references.

Culture of microbial strains

Isolated acetic acid bacteria (AAB) strains, *Enterobacter* spp. and *Enterococcus* spp. were cultivated in Luria-Bertani broth (LB) pH 6.8 (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract). Lactic acid bacteria (LAB) strains were cultured in de Man, Rogosa and Sharpe (MRS) broth pH 6.8 (Difco, Detroit, MI, USA). *Bacillus* spp. was cultured in Tryptic Soy Broth (TSB) pH 6.8 (17 g/L tryptone, 3 g/L phytone, 5 g/L NaCl, 2.5 g/L glucose). Yeasts were grown in Yeast Peptone Dextrose (YPD) agar pH 7.0 (20 g/L glucose, 10 g/L yeast and 20 g/L peptone). All microbial cultures were anaerobically cultured for 24 h at 37°C, except yeasts and AAB that were aerobically cultured 30°C and 37°C, respectively. Standard cultures were prepared by inoculation of 10 mL corresponding broth with 10 μL of bacterial culture in a frozen stock (-80°C) and incubated at 37°C for 24 h. The cultures were then subsequently sub-cultured in 10 mL corresponding broth for 24 h at 37°C prior to inoculation into the test tubes for further assessments.

Assessments of probiotic properties of microbes

Gelatin hydrolysis: Gelatin hydrolysis due to the presence of gelatinase was assessed by spotting 1 μL of the 24 h microbial cultures onto the surface of Luria Bertani agar slants (BD, Franklin Lakes, NJ, USA) containing 3% (w/v) gelatin (BD, Franklin Lakes, NJ, USA) and incubated for 48 h at 37°C. Afterwards, the slants were maintained at 4°C for 4 h. Gelatin hydrolysis was recorded as positive when gelatin became liquid, and negative when gelatin was still solid (Zommiti *et al*. 2018).

Antibiotic susceptibility

Antibiotic susceptibility tests were conducted as per the National Committee for Clinical Laboratory Standards using the disk diffusion method (Wayne 2002). Six antibiotics, tetracycline, chloramphenicol, erythromycin, vancomycin, penicillin and streptomycin, were used in this study. All strains were sub-cultured two times from the frozen stock into 10 mL of corresponding broth and were incubated at 37°C for 24 h. These cultures were then spread on plates containing 20 mL of corresponding agar and then antibiotic disks (HiMedia, India) were placed on the surface of agar plates and then were incubated at 37°C for 24 h. Inhibition zones in diameters were recorded in triplicate and compared to score strains as resistant (R: $\leq$ 15 mm), moderate susceptibility (M: 16–20 mm) and susceptible (S: $\geq$ 21 mm) (Lapsiri *et al*. 2011), while streptomycin was reported as Minimal Inhibitory Concentration (MIC).

Autoaggregation

Auto-aggregation of the microbial strains was tested as in the previous study (Collado *et al*. 2008). Overnight cultures were centrifuged at 8,000 g for 10 min. The cell pellets were washed twice with Phosphate Buffer Saline (PBS) pH 7.4, and resuspended into PBS (10^8 CFU/mL). Cell suspensions (4 mL) were vortex-mixed for 20 s and the absorbance was measured at 600 nm (A_{600}) after incubation at 0 h and 24 h.

$$\text{Autoaggregation (\%)} = [1 - (A_{24\,h}/A_0) \times 100]$$

Where $A_{24\,h}$ refers to the absorbance of the suspension at 24 h and A_0 refers to the absorbance at time 0.

Hemolytic activity

Microbial cultures were streaked on blood agar plates supplemented with 5% (w/v) of sheep blood (EnvioMed, Thailand) in triplicate and incubated at 37°C for 48 h. Afterwards, the hemolytic activity was as scored as partial hydrolysis of the red blood cells by the appearance of a green zone (α-hemolysis), the total hydrolysis of red blood cells by clear zone appearance (β-hemolysis) or no reaction (γ-hemolysis).

Detection of virulence genes by PCR

Six virulence genes from all *Enterococcus* strains were determined by PCR screening. These genes included: (1) *agg* (Aggregation substance) for adhesion, Fwd primer: 5'AAGAAAAAGAA GTAGACCAAC 3', Rev primer: 5'AAACGGCAAGAC AAGTAAATA3', 1553 bp, annealing temp. 55°C. (2) *VanA* (Vancomycin resistance), Fwd primer: 5' CGGGGAAGATGGCAGTAT 3', Rev primer: 5' CGCAGGGACGGTGATTTT 3', 732 bp, annealing temp. 55°C. (3) *gelE* (Gelatinase) for translocation, Fwd primer: 5' ACCCCGTATCATTGGTTT 3', Rev primer: 5' ACGCATTGCTTTT CCATC 3', 419 bp, annealing temp. 54°C. (4) *Ent* (Enterocins) for antilisteria, Fwd primer: 5' AAATATTATGGAAATGGAGTGTAT 3', Rev primer: 5' CTCGTTAAGGTCCCTTCACG 3', 475 bp, annealing temp. 53°C. (5) *CylA* (Cytolysin) for cell lysis, Fwd primer: 5' ACTCGGGGATTGATAGGC 3', Rev primer: 5' GCTGCTAAAGCTGCGCTT 3', 688 bp, annealing temp. 55°C. (6) *Hyl* (Hyaluronidase) for translocation, Fwd primer: 5' GAGTAGAGGAATATCTTAGC 3', Rev primer: 5' AGGCTCCAATTCTGT 3', 661 bp, annealing temp. 54°C. The PCR reaction cocktail (25 μL) was as follows: gDNA template (0.02–5 μg, 2 μL), forward primer (0.1 – 1 μM, 1 μL), reverse primer (0.1 – 1 μM, 1 μL), One PCR mixture buffer (GeneDirex, Bio-Helix Co. Ltd., Taiwan) (1×, 12.5 μL) and nuclease-free water (8.5 μL). The PCR conditions in PCR thermocycler (Hybaid P×2 Thermo Scientific, USA) were as follows: (1) initial denaturation at 94°C for 1 min, (2) 32 cycles of denaturation at 94°C for 1 min, annealing at 53–55°C for 1 min and extension at 72°C for 1 min (3) final extension at 72°C for 10 min. DNA bands were analyzed on 0.8% agarose gel electrophoresis using Gel Documentation (Thermo Scientific, USA).

Cell surface hydrophobicity

Cell surface hydrophobicity relates to the microbial adherence to hydrocarbons. The higher cell surface hydrophobicity suggests the better probiotic attribute (Grajek *et al.* 2016). Overnight cultures were centrifuged at 4,000 g for 10 min. After centrifugation, the cell pellet was resuspended in PBS (pH 7.4). This procedure was repeated one more time. The cell suspension was then diluted with the PBS buffer to an absorbance at value of 0.5 in 3 mL, when measured at 600 nm. N-hexadecane (1 mL) was added to cell suspension culture, vortex-mixed for 2 min and incubated at 37°C until phase separation was observed. Subsequently, the absorbance of bacterial cells in the collected aqueous phases was determined at the 600 nm wavelength. The percentage of cell surface hydrophobicity was calculated from the formula:

$$H\% = (A_0\text{-}A) \times 100\%/A_0$$

Where A0 – is the absorbance of microbial cultures prior to the addition of n hexadecane and A is the absorbance after the addition of *n*-hexadecane measured at the aqueous phase

Tolerance to gastric acid

This was carried out according to Guo *et al*. (2015). Overnight cultures were harvested (12,000 g, 10 min, 4°C). The cell pellets were washed twice with sterile saline buffer (0.85%), and subsequently were resuspended in corresponding broth adjusted by HCl to pH 3.0. The cultures were anaerobically incubated at 37°C for 1.30 h. Viable cell counting was conducted using the plate count method. Each sample (1 mL) was collected at 0 h and 1.30 h under sterile conditions, and made in 10-fold serial dilutions with sterile saline buffer (0.85%). The dilutions were plated on corresponding agars and anaerobically incubated at 37°C for 24 h before calculation in $\log_{10}$ CFU/mL and survival rate (%) was calculated from (cell number at final time/cell number at initial time) × 100%.

Tolerance to bile salts

Tolerance to bile salts was conducted as in the previous method (Guo *et al*. 2015). Overnight cultures were harvested (12,000 g, 10 min). Cell pellets were washed twice with sterile saline buffer (0.85% NaCl), and subsequently resuspended in corresponding broths

supplemented with 0.3% (w/v) oxgall (Amresco, USA) at 37°C under anaerobic conditions. Each sample (1 mL) was collected at 0 h and 3 h and made in 10-fold serial dilutions with sterile saline buffer (0.85%). The dilutions were plated on corresponding agars and anaerobically incubated at 37°C for 24 h before calculation in $\log_{10}$ CFU/mL and the survival rate (%) was calculated from (cell number at final time/cell number at initial time) × 100%.

Screening for neurotransmitter-producing microbes by LC-MS/MS

Microbial cultures were cultured in 1 mL LB broths containing 20 mg/mL monosodium glutamate (MSG) (HiMedia, India), 200 mg/L L-tryptophan (HiMedia, India), 200 mg/L L-tyrosine (HiMedia, India) and 0.15 m*M* pyridoxal-5 phosphate as co-factor for GABA-synthesizing enzyme (TCI, Japan) at 37°C for 24, 48, 72 h. Culture broths were centrifuged at 16,000 g for 5 min and supernatant was filtered through a 0.22-μm filter and the filtrates were used directly for LC-MS/MS analysis. The operating conditions used for LC-MS/MS and specific parameters were as follows: Shidmazu SIL-20AC model with Insertsil ODS-3 C18 column (150 mm × 2.1 mm) was used, the flow rate was 0.2 mL/min, the injection volume was 2 μL, mobile phase A was acetonitrile and mobile phase B was 0.45% formic acid, the run time was 10 min, oven model was CTO-20AC at 38°C, acquisition mode was MRM, CID gas was 230 kV and interface volt was 4.59 kV. GABA (Mw = 103.12) was eluted at the retention time 1.65 min, the parent ion was 104 (m/z) and the daughter ion was 87 (m/z).

Cancer cell lines

The human cervical adenocarcinoma (HeLa), breast adenocarcinoma (MCF-7), and hepatocellular carcinoma (HepG2) cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% of fetal bovine serum and 100 U/mL of penicillin, 100 μg/mL of streptomycin, then incubated at 37°C under 5% CO_2. DMEM media for cell lines cultures were renewed every 2–3 days until 80% confluency was reached. Cultured cell lines were washed with PBS, pH 7.2 before trypsinization with 0.25% Trypsin-EDTA. DMEM media were added to cell lines and the cell colonies were counted using inverted microscope (NIB-9000, Xenon, China).

Crude microbial extraction for cytotoxicity assay

Overnight cultures (1% v/v) were inoculated in corresponding broths (100 mL) in 500 mL flasks at 37°C at 200 rpm for 2 days. Negative controls were broths without microbial inoculations. The crude microbial extracts were obtained from whole cultures; consisting of microbial cells and broths. After that, 100 mL ethyl acetate (ETAC) was added to microbial cultures for crude microbial extraction at 37°C at 200 rpm for 6 h and the ETAC layer was separated and dried using a rotatory evaporator, dissolved in 95% ethanol and stored at -20°C till further analysis.

Cytotoxicity assay

Cytotoxicity was measured using 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetra zolium bromide (MTT) assay (Sigma, USA) following the previous method (Siddiqui *et al*. 2017). MCF-7, HeLa and HepG2 cells (5×10^3 cells/mL) were pipetted into 96-well plates and incubated at 37°C under 5% CO_2 for 72 h. Crude microbial extracts (0, 400, 600, 800 and 1,000 μg/mL) were added to wells and incubated for 72 h. MTT (5 mg/mL) dissolved in PBS buffer (pH 7.2) was added to the wells and incubated at 37°C under 5% CO_2 for 4 h. MTT was removed and 200 μL DMSO was added to dissolve the formazan and the purple color appeared if cells were alive. Doxorubicin was used as a positive control. The absorbance was measured at 590 nm using microplate reader (M965+, Mastertech, Taiwan). Cytotoxicity of crude microbial extracts against cancer cells was measured as IC_{50} value. When % cytotoxicity was ≤ 50%, it represented non-cytotoxic effect and when % cytotoxicity was >50%, it represented cytotoxic effect. Cell morphology was also observed using an inverted microscope (NIB-100, Xenon, China).

Statistical analysis of data

Data were collected in triplicate and results were reported as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan multiple range test by the software S.P.S.S. (demo version) was used to separate the means at $p < 0.05$.

Results

Probiotics attributes of microbes

The result showed that half of all microbes exhibited gelatin hydrolysis or gelatinase activity. Those included all *Bacillus* spp., *L. casei* WS13, WS15, *A. pasteurianus* WS3 and WS4. However, *Enterococcus* spp., *Enterobacter* spp., *M. guilliermondii* TC15, *S. cerevisiae* TC6, *P. pentosaceus* and certain *A. pasteurianus* showed negative results *i.e.*, no gelatinase (Table 1). The percentage of auto-aggregation at 5 h was ranged from 13.57 to 84.53% and at 24 h from 23.92 to 97.38%. In all strains, as time passed by from 5 h to 24 h, the percentage of auto-aggregation increased. Only two strains; *E. xiangfangensis* 4A-2A3.1 and *Enterobacter* spp. 1B-2 had less than 50% percentage of auto-aggregation at

Table 1: Gelatin hydrolysis, auto-aggregation and hemolysis properties of microbes

No.	Microbial strains	Source	Gelatin hydrolysis	Auto-aggregation (%) at 5 h	Auto-aggregation (%) at 24 h	Hemolysis level
1	*Enterococcus casseliflavus* SB2x2	PC	-	80.41 ± 0.53 ^{c}	95.29 ± 0.00 ^{b}	β
2	*Enterobacter ludwigii* S1E9	PC	-	69.09 ± 0.14 ^{h}	72.86 ± 0.16 ^{i}	γ
3	*E. xiangfangensis* 4A-2A3.1	PC	-	27.77 ± 0.11 ^{m}	42.14 ± 0.11 ^{m}	γ
4	*E. casseliflavus* 3.10A1	PC	-	45.33 ± 0.00 ^{k}	67.125 ± 0.49 ^{j}	γ
5	*Enterobacter* spp.1B-2	PC	-	6.13 ± 0.25 ^{o}	23.92 ± 0.25 ^{n}	γ
6	*Bacillus siamensis* PS23	MK	+	78.42 ± 0.08 ^{d}	61.42 ± 0.65 ^{k}	α
7	*B. subtilis* KW5	MK	+	70.44 ± 0.90 ^{g}	97.38 ± 0.00 ^{a}	α
8	*B. tequilensis* PS21	MK	+	74.37 ± 0.00 ^{e}	84.10 ± 0.13 ^{g}	β
9	*Bacillus* spp. PS15	MK	+	82.54 ± 0.26 ^{b}	86.43 ± 0.40 ^{f}	γ
10	*Lactobacillus casei* WS13	WK	+	71.79 ± 0.13 ^{f}	92.82 ± 1.89 ^{c}	α
11	*Meyerozyma guilliermondii* TC15	WK	-	13.57 ± 1.52 ^{n}	54.00 ± 0.13 ^{l}	γ
12	*Acetobacter pasteurianus* WS4	WK	+	57.68 ± 0.57 ^{j}	81.43 ± 1.07 ^{h}	α
13	*L. casei* WS15	WK	+	57.21 ± 1.03 ^{j}	92.06 ± 0.10 ^{cd}	β
14	*Saccharomyces cerevisiae* TC6	WK	-	64.88 ± 0.38 ^{i}	81.30 ± 0.23 ^{h}	γ
15	*Pedicoccus pentosaceus* WS12	WK	-	81.50 ± 0.14 ^{bc}	91.11 ± 0.25 ^{de}	γ
16	*Pedicoccus pentosaceus* WS11	WK	-	84.53 ± 0.10 ^{a}	90.36 ± 0.21 ^{e}	γ
17	*A. pasteurianus* WS3		+	77.59 ± 1.31 ^{d}	91.99 ± 0.14 ^{cd}	α
18	*A. pasteurianus* WS7	WK	-	33.63 ± 0.18 ^{l}	85.93 ± 0.11 ^{f}	α
19	*Lactococcus lactis* subspp. *lactis* TBRC 375	PC	-	77.77 ± 0.21 ^{m}	92.14 ± 0.17 ^{m}	γ
20	*L. brevis* TBRC 3003	PC	-	56.00 ± 0.11 ^{m}	75.00 ± 1.41 ^{m}	γ
21	*Bifidobacterium adolescentis* TBRC 7154	intestine	-	69.00 ± 1.41 ^{m}	82.00 ± 0.11 ^{m}	γ

Means with same lower-case letter did not differ significantly from each other according to Duncan Multiple range's test at $P < 0.05$
PC = Pickled cabbage; MK = Milk kefir; WK = Water kefir

both 5 h and 24 h. Four microbial strains including *E. casseliflavus* 3.10A1, *P. pentosaceus* WS12, *E. casseliflavus* SB2x2 and *P. pentosaceus* WS11 exhibited > 80% auto-aggregation at both 5 and 24 h (Table 1) suggesting they are most likely to attach to colonocytes in the human gut. The highest percentage of autoaggregation at 24 h was found in *B. subtilis* KW5 (97.38%) and at 5 h was found in *P. pentosaceus* WS11 (84.53%). The results showed that only 3 microbial strains including *E. casseliflavus* SB2x2, *B. tequilensis* PS21 and *L. casei* WS15 exhibited β-hemolysis which may present harm to human health since it may cause hemolysis. The remaining strains showed γ-hemolysis and α-hemolysis indicating safety for use as probiotic in humans (Table 1).

The results showed that only four bacterial strains (Table 2) namely *E. casseliflavus* 3.10A1, *B. siamensis* PS23, *A. pasteurianus* WS4, and *A. pasteurianus* WS3 were susceptible to all six antibiotics; tetracycline, chloramphenicol, erythromycin, vancomycin, penicillin and streptomycin. In addition, most of the tested microbial strains appeared to be moderate susceptible or susceptible to most of the six antibiotics used in this work. However, *E. ludwigii* S1E9, *Enterobacter* spp. 1B-2 and *Bacillus* spp. PS15 were found resistant to three or four antibiotics.

Virulence in enterococci is linked to several factors, such as *ace*, *agg*, *ccf*, *cpd*, *cob*, *cyl*L, *esp*, *gel*E, and *efa*A and formation of biofilm (Chuang-Smith *et al*. 2010). Here, the presence of 6 virulence genes including (1) *agg* (Aggregation substance) for adhesion, (2) *VanA* (Vancomycin resistance), (3) *gelE* (Gelatinase) for translocation, (4) *Ent* (Enterocins) for antilisteria, (5) *CylA* (Cytolysin) for cell lysis, and (6) *Hyl* (Hyaluronidase) for translocation was evaluated. This is the first report of investigating the occurrence of *agg, VanA, gelE, Ent, CylA* and *Hyl* genes in *E. casseliflavus* isolated from Thai fermented foods. The results showed no virulence gene products with designated sizes in *E. casseliflavus* SB2x2 or *E. casseliflavus* 3.10A1 (Fig. 1). However, the product band of 300 bp for *CylA* from *E. casseliflavus* SB2x2 may confirm β-hemolysis result of *E. casseliflavus* SB2x2 suggesting that it may express *CylA* (Cytolysin) for red blood cell autolysis. The PCR products of 400 and 150 bp of *VanA* in *E. casseliflavus* SB2x2 may be results of non-specific annealing of primers that did not correspond to vancomycin susceptibility of this strain. Thus, *E. casseliflavus* 3.10A1 with better safety (*i.e*., γ-hemolysis and no virulence genes) was chosen over *E. casseliflavus* SB2x2.

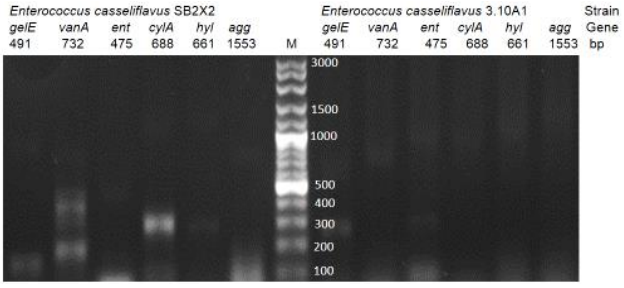

Fig. 1: PCR products of virulence genes in *E. casseliflavus* SB2x2 and *E. casseliflavus* 3.10A1on agarose gel electrophoresis

Twelve out of twenty-one microbes from the previous assessments were selected for the next experiment based on promising results from each evaluation. The results showed that eight out of twelve microbial strains including *E. casseliflavus* 3.10A1, *E. xiangfangensiss* 4A-2A3.1, *S. cerevisiae* TC6, *M. guilliermondii* TC15, *P. pentosaceus* WS11, *L. lactis* subspp. *lactis* TBRC 375, *L. brevis* TBRC 3003 and *B. adolescentis* TBRC 7154 were classified as low

Table 2: Antibiotics susceptibility of microbes

Microbial strains	Clear zone (mm)					MIC
	Tetracycline(30 μg)	Penicillin (10 IU)	Chloramphenicol (30 μg)	Erythromycin (15 μg)	Vancomycin (30 μg)	Streptomycin (μg)
Enterococcus casseliflavus SB2x2	28	22	29	25	22.5	1
	S	S	S	S	S	S
E. ludwigii S1E9	17	0	22	0	0	0
	M	R	S	R	R	R
E. xiangfangensis 4A-2A3.1	10	22	24.5	19.5	18	5
	R	S	S	M	M	S
E. casseliflavus 3.10A1	28	16	25	18.5	18.5	3
	S	M	S	M	M	S
Enterobacter spp.1B-2	17	0	22	0	0	5
	M	R	S	R	R	S
Bacillus siamensis PS23	19.5	21	24.5	25.5	16.5	3
	M	S	S	S	M	S
B. subtilis Y31.1 KW5	20	20	28.5	22	18	0
	S	S	S	S	M	R
B. tequilensis PS21	27	27	25	31	27	5
	S	S	S	S	S	S
Bacillus spp. PS15	22	0	22	0	0	0.1
	S	R	S	R	R	S
Lactobacillus casei WS13	23	18	29	22	20	15
	S	M	S	S	S	M
Meyerozyma guilliermondii TC15	25	18	27.5	22	19	0
	S	M	S	S	M	R
Acetobacter pasteurianus WS4	25	21	28	23.5	21	3
	S	S	S	S	S	S
Lactobacillus casei WS15	25	13.5	28	21	14.5	7.5
	S	R	S	S	R	M
Saccharomyces cerevisiae TC6	26	26	31	26	20	10
	S	S	S	S	S	M
Pedicoccus pentosaceus WS12	20	22.5	26	25	0	1
	S	S	S	S	R	S
P. pentosaceus WS11	23	17	30	12.5	0	5
	S	M	S	R	R	S
A. pasteurianus WS3	26.5	27.5	26.5	24.5	23	3
	S	S	S	S	S	S
A. pasteurianus WS7	20.5	24.5	27.5	0	20.5	0.1
	S	S	S	R	S	S
Lactococcus lactis subsp. *lactis* TBRC 375	23	21	22	23	22	0.1
	S	S	S	S	S	S
L. brevis TBRC 3003	24	21	23	24	22	0.1
	S	S	S	S	S	S
Bifidobacterium adolescentis TBRC 7154	25	22.5	25	23.5	23	0.1
	S	S	S	S	S	S

MIC= Minimal inhibitory concentration; R= Resistant (diameter of clear zone ≤ 15 mm); M= Moderate susceptible (diameter of clear zone 16-20 mm); S= Susceptible (diameter of clear zone ≥ 21 mm)

% hydrophobicity ranging from 1.89 to 16.46%. *E. xiangfangensis* 4A-2A3.1 was found to have the highest % hydrophobicity of 13.57% among microbes tested whereas *E. ludwigii* S1E9, *E. ludwigii* 1B-2, *Bacillus* spp. PS15, and *P. pentosaceus* WS12 showed no hydrophobicity property at all (Table 3).

Seven out of twelve microbes were selected for the next test. Tolerance of microbes to acidic condition in human stomach and bile in the intestine is important to enable the strain survival in acid rich-foods for longer time without a decrease in microbial population and persistence in intestinal habitat. Only four strains from the previous test were examined for acid and bile tolerance. The findings showed that all four selected isolates and three reference strains showed similarly high survival rate (90.45–99.78%) in simulated gastric juice pH 3.0 for 1.30 h, which indicated their resistance towards the acidic pH (Table 4). Similarly, high survival rate of 95.66–100.00% in 0.3% bile salts for 3 h was also detected among all the strains indicating that they were tolerant towards intestinal habitat (Table 4).

Neurotransmitter-producing capacity of microbes

Results of this study disclosed that only two bacteria including *E. xiangfangensis* 4A-2A3.1 and *Bacillus* spp. PS15 were found to produce GABA following 20 mg/mL MSG treatment (Table 5; Fig. 2). GABA production remained rather constant at 4.60 μg/mL over 2 days for *E. xiangfangensis* 4A-2A3.1; however, *Bacillus* spp. PS15 produced the highest GABA at 5.57 μg/mL on day 1 and afterwards decreased rapidly to 1.42 and 1.01 μg/mL on day 2 and 3, respectively. This may be a result of GABA

Table 3: Percentage of hydrophobicity of microbes

No.	Microbial strains	Hydrophobicity (%)
1	*Enterobacter ludwigii* S1E9	0.00 ± 0.00 [e]
2	*Enterobacter xiangfangensis* 4A-2A3.1	13.57 ± 1.85 [ab]
3	*Enterococcus casseliflavus* 3.10A1	1.89 ± 0.15 [d]
4	*Enterobacter ludwigii* 1B-2	0.00 ± 0.00 [e]
5	*Bacillus* spp. PS15	0.00 ± 0.00 [e]
6	*Meyerozyma guilliermondii* TC15	5.75 ± 1.63 [c]
7	*Saccharomyces cerevisiae* TC6	5.73 ± 1.61 [c]
8	*Pedicoccus pentosaceus* WS12	0.00 ± 0.00 [e]
9	*Pedicoccus pentosaceus* WS11	12.28 ± 5.20 [ab]
10	*Lactococcus lactis* subspp. *lactis* TBRC 375	16.46 ± 5.45 [a]
11	*Lactobacillus brevis* TBRC 3003	13.66 ± 0.93 [ab]
12	*Bifidobacterium adolescentis* TBRC 7154	14.38 ± 1.39 [ab]

Means with same lower-case letter did not differ significantly from each other according to Duncan Multiple range's test at $P < 0.05$

Table 4: Acid and bile tolerance of microbes

Microbial strains	Gastric juice pH 3.0			0.3% bile salts		
	Cells (log_{10} CFU/mL)			Cells (log_{10} CFU/mL)		
	0 h	1.30 h	Survival rate (%)	0 h	3 h	Survival rate (%)
E. casseliflavus 3.10A1	11.00 ± 0.23 [a]	10.36 ± 0.69 [a]	94.18	8.94 ± 0.69 [a]	8.55 ± 0.25 [a]	95.66
E. xiangfangensis 4A-2A3.1	9.25 ± 0.01 [a]	9.23 ± 0.09 [a]	99.78	9.27 ± 0.45 [a]	9.06 ± 0.16 [a]	97.75
S. cerevisiae TC6	10.08 ± 0.21 [a]	10.01 ± 0.11 [a]	99.30	8.84 ± 0.06 [a]	8.61 ± 0.17 [a]	97.41
P. pentosaceus WS11	10.89 ± 0.16 [a]	10.84 ± 0.01 [a]	99.54	8.97 ± 0.01 [a]	8.84 ± 0.03 [a]	98.58
L. lactis subspp. *lactis* TBRC 375	11.00 ± 0.05 [a]	9.95 ± 1.48 [b]	90.45	8.74 ± 1.48 [b]	8.66 ± 0.33 [a]	99.00
L. brevis TBRC 3003	10.85 ± 0.09 [a]	10.81 ± 1.04 [b]	99.68	9.00 ± 0.00 [a]	9.00 ± 0.00 [a]	100.00
B. adolescentis TBRC 7154	10.84 ± 0.09 [a]	10.72 ± 0.08 [b]	98.94	8.99 ± 0.15 [a]	8.88 ± 0.35 [a]	98.77

Means with same lower-case letter did not differ significantly from each other according to Duncan Multiple range's test at $P < 0.05$

Table 5: Microbial production of GABA over 72 h

Microbial strains	GABA production (μg/mL) at each incubation time		
	24 h	48 h	72 h
E. xiangfangensis 4A-2A3.1	4.60 ± 0.04 [b,A]	4.59 ± 0.28 [a,A]	1.21 ± 0.12 [a,B]
Bacillus spp. PS15	5.57 ± 1.09 [a,A]	1.42 ± 0.22 [b,B]	1.01 ± 0.03 [b,C]

Means with same lower-case letter and upper case letter within the same columns and rows, respectively did not differ significantly from each other according to Duncan Multiple range's test at $P < 0.05$

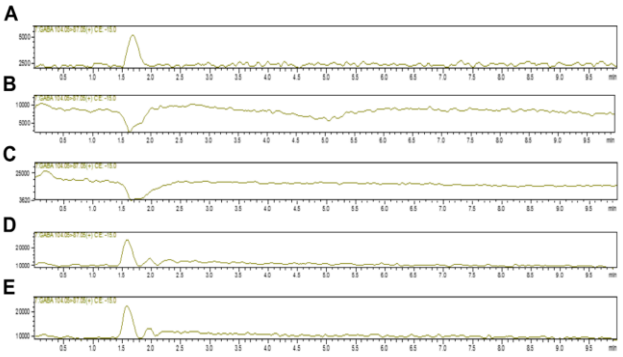

Fig. 2: LC-MS/MS chromatograms of GABA standard and GABA detection in bacterial cultures. **A**: GABA standard (1 ppm). **B**: Control (media supplemented with substrates and co-factor without bacterial culture). **C**: Control (microbes not producing GABA). **D**: GABA detection in *Bacillus* spp. PS15. **E**: GABA detection in *E. xiangfangensis* 4A-2A3.1

degradation or conjugated to other microbial products over time. Unfortunately, none of microbes studied in this work produce any other neurotransmitters such as serotonin, dopamine or melatonin (data not shown).

Cytotoxicity of microbial extracts against cancer cells

Microbial products have been known as good candidates for anticancer agents due to a plethora of bioactive molecules such as antioxidant enzymes, secondary metabolites and bioactive peptides. The results showed that all five microbial strains inhibited HepG2, MCF-7 and HeLa cells in a dose-dependent manner (Fig. 3). HepG2 cells were most susceptible to microbial extracts as observed by the highest cytotoxicity (%) in most concentrations, while MCF-7 cells were most resistant as observed by the lowest cytotoxicity (%) in most concentrations (Fig. 3).

The lowest half maximal inhibitory concentration (IC_{50}) values of 681.08 μg/mL on HepG2, 750.02 μg/mL on MCF-7 and 425.50 μg/mL on HeLa, respectively were found from *B. adolescentis* TBRC 7154 extracts (Table 6). This strain was most cytotoxic towards three cancer cells followed by *E. xiangfangensis* 4A-2A3.1, *E. casseliflavus* 3.10A1 and *P. pentosaceus* WS11 as seen by lower IC_{50} values. Doxorubicin, one of the most extensively used commercial anticancer drug, was used as a positive control. It showed the lowest IC_{50} values against all cancer cell lines

Table 6: IC_{50} (µg/mL) of crude microbial extracts for cytotoxic activity against cancer cells

Microbial strains	HepG2	MCF-7	HeLa
	IC_{50} (μg/mL)	IC_{50} (μg/mL)	IC_{50} (μg/mL)
E. xiangfangensis 4A-2A3.1	684.48 ± 1.62b,B	806.25 ± 0.53f,C	468.16 ± 0.87d,A
E. casseliflavus 3.10A1	710.81 ± 0.34d,B	752.07 ± 0.69b,C	549.54 ± 0.57f,A
P. pentosaceus WS11	707.54 ± 0.49c,B	772.08 ± 1.31c,C	437.07 ± 1.48c,A
L. lactis subspp. *lactis* TBRC 375	704.20 ± 1.26c,B	777.36 ± 1.00d,C	524.45 ± 0.16e,A
B. adolescentis TBRC 7154	681.08 ± 1.31c,B	750.02 ± 1.00d,C	425.50 ± 0.26e,A
Doxorubicin	9.10 ± 0.22	7.04 ± 0.80	0.61 ± 0.04

Means with same lower-case letter and upper case letter within the same columns and rows, respectively did not differ significantly from each other according to Duncan Multiple range's test at $P < 0.05$

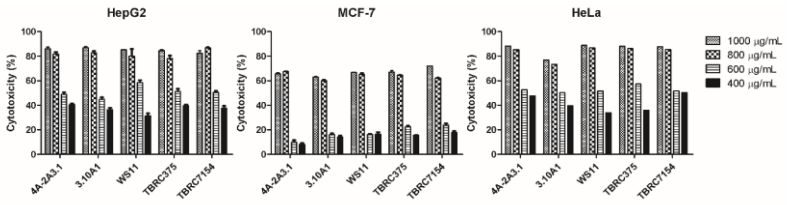

Fig. 3: Cytotoxicity of five microbial extracts in different concentrations against HepG2, MCF-7 and HeLa cells

at 0.6–9.1 μg/mL. A negative control (broth extract without microbes) showed no cytotoxicity (data not shown).

Discussion

In this work, both strains of *E. casseliflavus* showed no gelatin hydrolysis. Similarly, the results of no gelatin hydrolysis of *Enterobacter* spp., *P. pentosaceus*, certain *A. pasteurianus*, *M. guilliermondii* TC15, and *S. cerevisiae* TC6 in this work were in accordance with the previous reports showing the same species with no gelatinase activity (Fakruddin *et al*. 2017; Chi *et al*. 2018; Zajc *et al*. 2019). This indicated greater potential use as probiotics since these strains are unlikely to express gelatinase, a metalloendopeptidase, which is able to degrade hemoglobin, insulin, collagen, casein, fibrinogen and gelatin (Kanemitsu *et al*. 2001) and thus may harm the hosts. It is known that the species of *Enterococcus* showed their expressions of gelatinase depending on the niche of isolation (Araújo and Ferreira 2013).

The auto-aggregation ability can be used as one of the indicator for probiotic attribute. This is linked to the adherence capacity to the human intestinal lining, protection of the gastrointestinal tract of the host from pathogen colonization (García-Cayuela *et al*. 2014) and immunomodulatory effects (Xu *et al*. 2010). Thus, probiotics are expected to exert high auto-aggregation. However, percentage of auto-aggregation is not directly translated to *in vivo* adhesion due to involvement of host factors such as normal microflora, defense mechanisms, and peristaltic rhythms that modulate the microbial attachment (Caggia *et al*. 2015). Interestingly, *S. cerevisiae* TC6 in this work showed similar percentage of auto-aggregation (64.88%) to that of *S. cerevisiae* IFST062013 (61.34%), a potential probiotic, isolated from fruit in Bangladesh (Fakruddin *et al*. 2017).

The previous findings showed that *Enterococcus* spp. exhibited β-hemolysis (Igbinosa and Beshiru 2019). In contrast to the result from this work, others have found that *B. tequilensis* YC5-2 (Luis-Villaseñor *et al*. 2011) and *B. tequilensis* FR9 (Rani *et al*. 2016) exhibited γ-hemolytic activity causing no health hazard. The differences of hemolytic activity may lie in specific strain genetic makeup. Contrary to popular belief, hemolytic activity was found in *Bacillus*, but it is used as commercial human probiotics (Hoa *et al*. 2001). Although the *in vitro* hemolysis does not always result in any negative effect to fish and pigs (Trapecar *et al*. 2011), EFSA guidelines do not recommend hemolysin-producing microbes as feed additives (European Food Safety Authority 2011).

In this work, most of the tested microbial strains appeared to be moderate susceptible or susceptible to most of the six antibiotics suggesting they may have probiotic potential since they are not likely to have transferrable antibiotics resistance genes to pathogens. However, *E. ludwigii* S1E9, *Enterobacter* spp. 1B-2 and *Bacillus* spp. PS15 were found resistant to three or four antibiotics indicating that they may be used for patients undertaking long-term antibiotic remedy.

The previous study showed that certain *Bacillus clausii* with specific antibiotic resistance mechanisms has been used as probiotics in humans for the treatment of infectious bacterial diarrhea (Bozdogan *et al*. 2014).

In contrast to this finding, the *hyl* gene has also been found in *E. casseliflavus* recovered from food (Trivedi *et al*. 2011). The conflicting results from this work and of other report concerning the presence of virulence genes among microbial isolates might be resulted from differences in the reservoir of the various countries or the ecological origin of strains (Gulhan *et al*. 2015).

The findings in this work were supported by Sica *et al*. (2012) showing that *P. pentosaceus* S17 and S19 had very

low hydrophobicity to n-hexadecane at 0.01% and *E. mundtii* S21 at 0.05%. *S. cerevisiae* VIT-ASN03 was reported to exhibit only 4% hydrophobicity (Suvarna *et al*. 2012) which was similar to *S. cerevisiae* TC6 in this work. In contrast, the previous study showed a much higher hydrophobicity value of 52.4% for *L. lactis* DF04Mi (Furtado *et al*. 2014) when compared to 16.46% from *L. lactis* subs. lactis TBRC 375 in this work. It is known that hydrophobicity variation can occur among related species and same species (Schar-Zammaretti and Ubbink 2003). Although, certain strains had a high cell surface hydrophobicity, they were not able to properly attach to HT-29 and Caco-2 cells (Todorov *et al*. 2008). However, a relatively low hydrophobicity (38%) strain, *L. pentosus* ST712BZ, attached to HT-29 cells at 63%. Hydrophobicity may partly contribute to adhesion, but it is not a requirement for strong adherence to human intestinal cells. In addition, the physiology of the cell, availability of nutrient and pH of the microbial cultivation medium also influence physiochemical properties of the microbial cell surface (Hamadi *et al*. 2004).

Similarly, certain yeast strains *S. cerevisiae* from various sources have been documented as acid- and bile-tolerant (Agarwal *et al*. 2001; Helmy *et al*. 2019; Kim *et al*. 2019). Other authors found similarly high survival ability of *P. pentosaceus* (Ilavenil *et al*. 2016; Zommiti *et al*. 2018; Ladha and Jeevaratnam 2018). In contrast to this result, the other report showed that *E. casseliflavus* WECA01 exhibited weak bile resistance at 30.50% survival rate (Zhang *et al*. 2016).

This is the first report of *E. xiangfangensis* as GABA producer, which may express glutamate decarboxylase (GAD), the enzyme that catalyzes the biosynthesis of GABA from decarboxylating glutamate to GABA (Dhakal *et al*. 2012). However, its GABA production was much lower in this work compared to the commonly found GABA-producing LAB strains.

Previously, *L. brevis* DPC6108 isolated from human intestine was found to produce GABA at 20.47 mg/mL from 20 mg/mL MSG over 2 days (Barrett *et al*. 2012). In literature, very few findings have reported the GABA-producing capacity from *Bacillus* spp. In this work, *Bacillus* spp. PS15 was found to produce very low GABA amount over 3 days, compared to the previously reported *Bacillus subtilis* ATCC 6051 with the highest GABA production at 19.74 mg/mL for 120 h (Wang *et al*. 2018). It is thought that the optimum conditions differ from one microbe to the next due to the different characteristics of the GADs (Dhakal *et al*. 2012) and thus effects of temperature, pH, media additives, and cultivation time should be investigated to achieve the highest GABA production. GABA is one of the major inhibitory neurotransmitter in the mammalian central nervous system. GABA helps increase the growth hormones, plasma concentration and the synthesis of protein in the brain (Cho *et al*. 2007). The GABA-producing strains are of great interest to be used to manufacture GABA-enrich food products (Coda *et al*. 2010).

When compared to the previous finding (Phonnok *et al*. 2010), most of the microbes in this work still had much lower cytotoxicity against cancer cells as observed by high IC_{50} values. The differences in cytotoxicity may lie in genetics of each strain, different genes responsible for producing bioactive compounds such as immune toxins, antioxidant enzymes, secondary metabolites, exopolysaccharides, and bioactive peptides that account for anticancer effects.

Conclusion

Only seven microbial strains confer most promising probiotic potential with safety for use in humans; *E. casseliflavus* 3.10A1, *E. xiangfangensis* 4A-2A3.1, *S. cerevisiae* TC6, *P. pentosaceus* WS11, *L. lactis* subspp. *lactis* TBRC 375, *L. brevis* TBRC 3003 and *B. adolescentis* TBRC 7154. In addition, *E. xiangfangensis* 4A-2A3.1 and *Bacillus* spp. PS15 were found to be GABA producers which may be used as ingredients in cocktails or pills or GABA-enrich food products to prevent GABA-deficient mental disorders. Moreover, *B. adolescentis* TBRC 7154, *E. xiangfangensis* 4A-2A3.1, *E. casseliflavus* 3.10A1, *P. pentosaceus* WS11 and *L. lactis* subspp. *lactis* TBRC 375 displayed cytotoxic effects against HepG2, MCF-7 and HeLa. These microbes may be used as potential biotherapeutic reagents or food supplements to prevent cancer.

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Author Contributions

VL designed, conducted the experiments, analyzed data and wrote the manuscript. WS and TK conducted the experiments. BB and SD designed the experiments. SNT, NLM and AN edited the manuscript draft. All authors listed have read and approved the manuscript for publication.

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