Agr. Nat. Resour. 58 (2024) 643–652

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Prebiotic effect of spices, herbs and Thai curry under *in-vitro* digestion on attachment of probiotic bacteria on large intestine Caco-2 cells

Jutamat Klinsoda*, Kanokwan Yodin, Khemmapas Treesuwan

Institute of Food Research and Product Development, University of Kasetsart, Bangkok 10900, Thailand

Article Info

Article history:
Received 23 April 2024
Revised 6 October 2024
Accepted 7 October 2024
Available online 31 October 2024

Keywords:
Caco-2,
Prebiotic,
Probiotics,
Spice/Herb extract,
Thai curry

Abstract

Importance of the work: Little information is available on the prebiotic properties of spices or herbs in Thai curries after digestion and their interaction with probiotic microbiota.

Objectives: To investigate the *in-vitro* viability and adherence to Caco-2 cells of selected probiotics presenting in human and commercial products (*Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium bifidum*) after inoculation in 14 spice or herb extracts and 8 Thai curries and passing through an *in-vitro* digestion model.

Materials and Methods: The selected probiotics were passed in the *in-vitro* digestion model and further evaluated for large intestine Caco2-cell adhesion.

Results: In total, 14 spice or herb extracts (chili, garlic, galangal, turmeric, shallot, lemon grass, basil, red cotton tree flowers, cloves, coriander root, cumin, shrimp paste, cinnamon and finger root) and Thai curries had no inhibitory effects on the selected probiotics. *L. casei* inoculated in Nam Ngeaw curry had the greatest Caco-2 cell adhesion (2.37%), whereas *L. casei* was lower after exposure to turmeric extracts (0.27%; $p < 0.05$). The adhesion of *L. acidophilus* in Gaeng Aom and Nam Ngeaw curries was similar to the control, whereby *L. acidophilus* after exposure to other spice/herb extracts and curries lost adherence ($p < 0.05$). There were no significant differences in adherence to Caco-2 cells of *B. bifidum* incubated in the Thai curries, with the adhesion of *B. bifidum* being lower in garlic extract compared to coriander root extract ($p < 0.05$).

Main finding: The screening results of the spice or herb extracts and mixed spices/herbs in Thai curries was able to reveal their prebiotic properties to support Caco-2 cell adhesion of probiotics after ingestion.

* Corresponding author.
E-mail address: ifrjmk@ku.ac.th (J. Klinsoda)

https://doi.org/10.34044/j.anres.2024.58.5.11

Introduction

During the COVID-19 pandemic, a new food trend developed involving herb and spice extracts as an immune booster (Prakasita et al., 2019). Several commercial spices, herbs and probiotic products have been prominently launched on markets worldwide and in Thailand (Trevanich, 2021). Since Thai curry consists of various spices and herbs, they may be a source of many bioactive compounds and might offer a prebiotic effect. Spices and herbs contain a number of phenolic and flavonoid compounds that can contribute to significant antioxidant activities (Duenas et al., 2015: Milutinovic´ et al., 2021). Fresh spices contain bioactive ingredients in higher concentrations than dried ones (Krisch et al., 2010). Many studies have been reported to have antimicrobial properties and can be used as food additives against a variety of Gram-positive and Gram-negative bacteria, including the lactic acid bacteria (LAB) group (Lu et al., 2017). On the other hand, many spices, fruits and vegetables in Southern Thailand have been reported to be natural candidate prebiotics such as asparagus, onion, cereals, and garlic (Wichienchot et al., 2011). Prebiotics are polysaccharides and oligosaccharides that can withstand digestion and absorption in the small intestine; therefore, they can be selectively fermented by probiotic bacteria native to the large intestine (Davani-Davari et al., 2019). Some evidence from *in-vitro* and *in-vivo* studies has indicated that polyphenols in prebiotics modulate the intestinal microbiota composition (Duenas et al., 2015: Milutinovic´ et al., 2021). However, there has been little research into the antimicrobial and prebiotic properties of spices or herbs on probiotic microbiota. Furthermore, there is emerging concern that some specific LAB, considered to be probiotic bacteria, may be affected by spices that may halt any health benefit on the host (Cocetta et al., 2019). The prebiotic properties are unknown of many spice/herb extracts after ingestion, including the mixed spices/herbs in Thai curries. Therefore, it is important to determine whether the growth stimulation of probiotics and their adherence capacity of probiotics are influenced by the consumption of herbs and spices.

Probiotic products, which at nearly 33% account for the largest component of the functional food market, have been popular in various food and feed industries because of their scientifically claimed benefits to health, establishing balanced levels of intestinal microbiota, developing the immune system, and preventing colonization by pathogens (Ouwehand and Salminen, 2003; Han et al., 2021). Probiotic microbiota, such as bifidobacteria and lactobacilli, have been known as normal inhabitants and immune modulators to produce cytokines for maintaining the gut and immune systems when attached to intestinal cells (Bahrami et al., 2011; Javanshir et al., 2021). However, before probiotics can be beneficial to human health, they must survive in sufficient numbers through the manufacturing processes and subsequently survive in the gastrointestinal (GI) tract, as well as retain their properties and functionality to be effective in the host (de Oliveira et al., 2017). Several factors have been reportedly involved in the functionality of probiotics such as the number of probiotic survivals, acid resistance, bile resistance and intestinal surface adhesion (Han et al., 2021). Furthermore, external factors, such as food composition and food structure, can affect their functionality (Farnworth and Champagne, 2010).

These criteria for screening LAB strains and assessing their functional features before using them as probiotics are crucial, especially for colonizing epithelial adhesion sites (de Oliveira et al., 2017; Han et al., 2021). Because of the dynamics associated with human microbiota, adhesion to the intestinal mucosa is a necessary criterion to allow a probiotic to exert its effects (Jiang et al., 2021). Due to the difficulty of *in vivo* study, bacterial adherence has been performed using intestinal cell lines of human origin (Fedi et al., 2021). The Caco-2 cell line derived from human colon adenocarcinoma has been used in pre-clinical investigations (Duary et al., 2011). The Caco-2 cell is considered the most common *in-vitro* model to screen new chemical entities because of the good prediction of its solubility, bioavailability and the possibility of drug-drug or herb-drug interactions in the gut lumen (Awortwe et al., 2014; Fedi et al., 2021). This developed cell line has been widely used as a model for probiotic adherence and also as a pathogen (Fedi et al., 2021). Bacterial adhesion depends to differing degrees on the compounds, strains and intestinal cells. The hydrophobic nature of the outermost surface of microorganisms facilitates the adhesion of bacteria to the host epithelium and the adhesiveness of LAB may involve several forces such as passive forces, electrostatic interactions, hydrophobic steric forces, lipoteichoic acids, and lectins (de Oliveira et al., 2017; Javanshir et al., 2021). However, different bacterial strains of the same genus and species may exert completely different effects on the host (Javanshir et al., 2021). Therefore, the specific properties of individual strains should be demonstrated in a case-by-case manner.

The effect of the food matrix on the survival and persistence of probiotics has been reported (Morelli and Pellegrino, 2021). The boiling of herbs causes a loss of soluble compounds

(soluble fiber, ash and phenolic compounds) and antimicrobial properties (Salamatullah et al., 2021; Sikora et al., 2008). Zhou et al. (2012) showed that the modification of soluble-to-insoluble dietary fiber ratio using thermal treatment resulted in no significant change in the fiber contents. Plant consumption is one of the important factors in the gut modulation. The phenolic compounds in the plant (garlic, cumin and cloves) have been reported to influence bacterial adhesion because of the presence of hydroxyl groups, which enable the adhesion of bacteria to epithelial cells (de Souza et al., 2018). The consumption of foods consisting of herbs and spices is linked to the abundant and complex microbiota in humans and there is strong evidence that phenolic compounds can control the balance of microbiota (de Souza et al., 2018). Currently, there has been little scientific data reported about the prebiotic effect of spices and herbs post ingestion on probiotic bacteria. The interactions of probiotics with the food matrix, such as Thai curries, may influence probiotic colonization. To gain insight into how spices or herbs in Thai curries change the adhesion properties of probiotic microbiota to intestinal epithelial cells, the present study aimed to investigate the Caco-2 cell adhesion of three probiotic strains (lactobacilli and bifidobacteria) after inoculation in 14 selected spice/herb extracts and 8 Thai curries based on *in-vitro* digestion. Evaluation of the prebiotic properties of the 14 spice/herb extracts and 8 Thai curries were also highlighted to reveal their inhibitory effects on probiotic microbiota.

Materials and Methods

Preparation of spice/herb extracts

In total, 14 spices and herbs [chili (*Capsicum frutescens* L.); garlic (*Allium sativum* L.); galangal (*Alpinia galanga* (L.) Willd.); turmeric (*Curcuma longa* L.); shallot (*Allium ascalonicum* L.); lemon grass (*Cymbopogon citrates* (DC.) Stapf; basil (*Ocimum basilicum* Linn.); red cotton tree flowers (*Bombax ceiba* L.); cloves (*Syzygium aromaticum* (L.) Merr. & Perry); coriander root (*Coriandrum sativum* L.); cumin (*Cuminum cyminum* L.); cinnamon (*Cinnamomum* spp.); finger root (*Boesenbergia pandurata* Holtt.); and shrimp paste] were purchased from local supermarkets in Bangkok, Thailand (Table 1). All spices/herbs were rinsed with water and dried in a hot-air oven at 80°C for 24 hr. Then, 100 g of dry weight samples were separately cut into small pieces (2 cm × 2 cm) and extracted using hot water in a spice-to-water ratio of 1:6 at 100°C for 10 min. Each extract was passed through filter paper (pore size: 10 µm; Whatman; Kent, UK) according to the method of Chan et al. (2011) with the modification of the ratio and stored for further investigation in sterile glass bottles at 4°C.

Preparation of Thai curry solution

In total, eight Thai curry pastes (Hang-lay curry, Nam Ngeaw curry, Gaeng Aom curry, Red curry, Green curry, Massaman curry, Yellow curry, and Som curry) were purchased from local supermarkets in Bangkok, Thailand (Table 1). According to Thai traditional cooking directions of use on the commercial label, the pastes (Hang-lay curry, Nam Ngeaw curry, Red curry, Green curry, Massaman curry, and Yellow curry) were weighed to produce samples with a paste-to-coconut milk ratio of 1:4, boiled on a hot plate, and cooled at room temperature, whereas the other pastes (Gaeng Aom curry and Som curry) were weighed to produce samples with a paste-to-water ratio of 1:4, boiled on a hot plate, and cooled at room temperature. Each curry was stored in sterile glass bottles at 4°C until further investigation.

Table 1 Characterization of Thai curry pastes and their spice components

Thai region	Type of curry	Spice components
Northern	Hang-lay curry	shallot 25%, chili 15%, garlic 12%, lemon grass 12%, galangal 4%, mixed spices (coriander root, cloves, cinnamon) 10%
	Nam Ngeaw curry	chili 25%, shallot 20%, garlic 17%, shrimp paste 10%, mixed spices (coriander root, cloves, cinnamon) 5%, red cotton tree flowers 2%
North Eastern	Gaeng Aom	chili 22%, shallot 20%, garlic 18%, lemon grass 15%, shrimp paste 10%, galangal 8%
	Red curry	chili 36%, garlic 15%, lemon grass 14%, shallot 13%, galangal 6%, shrimp paste 1.5%, kaffir lime 1%
Central	Green curry	green chili 31%, lemon grass 21%, garlic 18%, galangal 8.5%, shrimp paste 4%, kaffir lime 2%, coriander root 1%, cloves 0.5%, cumin 0.5%
	Massaman curry	chili 22%, lemon grass 20%, garlic 18%, shallot 14%, galangal 7%, mixed spices (coriander root, cloves, cinnamon) 5%, shrimp paste 4%
Southern	Yellow curry	yellow chili 30%, shallot 22%, garlic 21%, shrimp paste 12%, cumin 1%, curry powder 1%
	Som curry	shallot 34%, chili 27%, shrimp paste 12%, finger root 3%

Preparation of bacterial suspensions

The probiotic strains were obtained from the Thailand Institute of Scientific and Technological Research (TISTR). Both *Lactobacillus* species (*Lactobacillus acidophilus* TISTR 2365 and *Lactobacillus casei* TISTR 1463) were grown aerobically at 37°C in DeMan, Rogasa and Sharpe (MRS; Oxoid; Basingstoke; UK) broth, whereas *Bifidobacterium bifidum* TISTR 2129 was grown anaerobically at 37°C in MRS supplemented with 0.05% L-cysteine hydrochloride (Merck; Darmstadt, Germany). All cultures were maintained on 40% (volume per volume) sterile glycerol (Himedia; Maharashtra, India) and stored at -80°C until further investigation.

Agar well diffusion assay

Agar well diffusion assay was used to test the inhibition effect of the spice/herb extracts and Thai curries on the three probiotic bacteria. The fresh culture of each probiotic bacterium was obtained from an overnight-grown MRS culture for *Lactobacillus* species and an overnight-grown MRS supplemented with 0.05% L-cysteine hydrochloride for *B. bifidum*. About 100 mL of the fresh cells (1×10^7 colony forming units (CFU)/mL) from each culture were pipetted into MRS agar. Thereafter, the MRS agar was poured onto the plate and dried. A hole (6 mm in diameter) was punched using a sterile cork-borer aseptically. Separate wells were filled with a volume of 50 µL of one of the spice/herb extracts or Thai curries and then incubated at 37°C for 24 hr before measuring the zone of inhibition. The size of the clear zone was used to measure the growth inhibition by each probiotic bacterium by the individual extracts or curries. The spice/herb extracts and curries without a clear zone were selected to test the simulated gastrointestinal conditions.

Species survival under simulated gastrointestinal conditions

The fresh cells of *Lactobacillus* species (approximately 1×10^7 CFU/mL) were prepared from an overnight-grown MRS culture using centrifugation at 5,000×*g* for 5 min; then, the bacterial suspension was resuspended twice in phosphate buffer saline (PBS, pH 7.2; Himedia; Maharashtra, India) solution (Gagnon et al., 2004). After centrifugation, the supernatant was discarded. The residual pellet was resuspended in 500 µL of a 0.85% NaCl solution (Merck; Darmstadt, Germany). The preparation of *B. bifidum* was carried out in the same manner as the lactobacilli but with the growth media being MRS supplemented with 0.05% L-cysteine hydrochloride. Each fresh cell suspension was added to 500 µL of each of the spice/herb extracts and Thai curries. Subsequently, three *in-vitro* enzyme treatments were used to simulate digestive stresses: amylase (1 mg/mL; purified water pH 8) for 5 min, followed by pepsin (3 mg/mL; PBS solution, pH 2) incubated at 37°C for 3 hr and pancreatin (1 mg/mL; PBS solution, pH 8) incubated at 37°C for 24 hr, respectively (Maragkoudakis et al., 2006; Both et al., 2010). The incubation under aerobic or anaerobic conditions depended on the test microorganism, as previously described. The viable colony counts after exposing the spice/herb extracts and Thai curries to the simulated gastro-intestinal conditions were determined after incubation at 37°C for 24 hr. The numbers of probiotic cultures after exposure to the extracts, Thai curries and digestive stress were quantified based on 10-fold serial dilution before the adhesive assay, which was designated 'Pre-attachment'.

Preparation of Caco-2 cell cultures

Caco-2 cells were placed in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma-Aldrich; MA; USA), supplemented with 10% fetal bovine serum (Gibco; CA; USA) and grown under standard conditions (37°C, 5% CO_2, 95% relative humidity). The Caco-2 cells were placed in a tissue culture flask with a 75 cm^2 growth surface. Before the adhesion assay, the Caco-2 cell monolayers were washed once with PBS at pH 7.2 and the cells were detached using 0.1% trypsin- ethylenediaminetetraacetic acid (EDTA) solution for 15 min. After observation of detachment under the microscope, the DMEM medium was added to stop the trypsin reaction by pipetting in the medium to disperse the Caco-2 cells. The cell density was counted and seeded at a ratio of 2×10^5 cells/mL in 24-well tissue culture plates. To reach the cell density of 2×10^4 cells/cm^2, the cells were replaced in a new culture medium every 3 d under standard conditions (37°C, 5% CO_2, 95% relative humidity).

Adhesive properties of selected probiotic strains on Caco-2 cells

The adhesive property analysis was performed according to the method of Gagnon et al. (2004) and Maragkoudakis et al. (2006) (Fig. S1). The obtained Caco-2 cells in 24-well tissue culture plates were washed twice with PBS at pH 7.2; then, 0.5 mL of bacterial cell density corresponding to about

1×10^7 CFU/mL from each probiotic culture ('Pre-attachment') was pipetted into each well and incubated at 37°C for 2 hr. To remove the non-attached bacteria, the tissue culture plates were washed five times with PBS at pH 7.2. To collect the adhered bacteria, the probiotic cells were detached using 0.25 mL of 0.05% trypsin-EDTA solution per hole for 15 min. The 0.25 mL PBS was added to stop the trypsin reaction in each well and then mixed by pipetting in the medium to separate the bacterial cells from the Caco-2 cells. After incubation, the suspensions were counted for the number of probiotic bacteria released from the Caco-2 cells in colony forming units per plate count. The number of adhered bacteria was quantified based on 10-fold serial dilution after adhesive assay and designated 'Post-attachment'.

The adhesive capacity was expressed as the percentage of adhesion with respect to the number of bacteria added (% CFU Post-attachment / CFU Pre-attachment) as represented in Equation 1:

$$\%\text{Adhesion} = \frac{\text{CFU count from Post-attachment} \times 100}{\text{CFU count from Pre-attachment}} \quad (1)$$

To obtain an image of the Caco-2 cell adherence under an inverted microscope, the Caco-2 cells were seeded in 6-well tissue culture plates with a glass coverslip producing a cell density of 1.9×10^5 cells per well in 3mL of the medium after 15 d of incubation, as previously described. A sample (1 mL) of bacterial culture was added to each well and incubated under standard conditions (37°C, 5% CO_2, 95% relative humidity). The cells were washed five times with PBS at pH 7.2. The bacterial cells were fixed to the cells using 4% methanol in PBS and then colored using Giemsa stain for 20 min. Finally, the stain color was rinsed and the dried sample was imaged under an inverted microscope, according to Duary et al. (2011).

Statistical analysis

The results were presented as %Adhesion, derived from three replications. The different datasets of probiotics (*L. acidophilus*, *L. casei* and *B. bifidum*) incubated in the different Thai curries or spice/herb extracts were subjected to analysis of variance with Completely Randomized Design (CRD) model using the 'emmeans' package in RStudio (version 2023.03.1+446; https://rstudio.com/products/rstudio/download/), an integrated development environment for R, a programming language for statistical computing (R Core Team, 2023). Significant differences ($p < 0.05$) were tested based on Tukey's honestly significant difference using the TukeyHSD function in R. Data were expressed as mean ± SD values.

Results

Pre-evaluation of prebiotic properties of spice/herb extracts based on agar well diffusion assay

The commercial Thai curries and their spice and herb components were used to evaluate prebiotic properties. Based on the results after boiling, the 14 spices and herbs (chili, garlic, galangal, turmeric, shallot, lemon grass, basil, red cotton tree flowers, cloves, coriander root, cumin, shrimp paste, cinnamon and finger root) and 8 Thai curries (Hang-lay curry, Nam Ngeaw curry, Gaeng Aom, Red curry, Green curry, Massaman curry, Yellow curry and Som curry) had no inhibitory effects on any of the selected probiotic strains based on agar well diffusion assay (Table S1). Accordingly, all Thai curries and spice/herb extracts were selected for the next experiment on the simulated gastrointestinal conditions.

Survival of three probiotic microbiota under simulated gastrointestinal conditions

The survival of the three types of probiotic cells was evaluated after incubating each one in the various spice/herb extracts and Thai curries and passing through the *in-vitro* enzyme treatments under simulated gastrointestinal conditions (Tables 2–3), with the viable cells of the three probiotic microbiota being more than approximately 1×10^8 CFU/mL after digestive stress. In the control group, the numbers of *L. acidophilus, L. casei* and *B. bifidum* were 7.64×10^8 CFU/mL, 4.37×10^8 CFU/mL, and 8.45×10^7 CFU/mL. The three probiotics incubated in the garlic extract had more than 1×10^9 CFU/mL of survival cells after digestive stress. *L. acidophilus* had the highest survival in the garlic extract (1.34×0^9 CFU/mL), while the lowest survival was in the clove extract (6.65×10^7 CFU/mL). Survival of *L. casei* was greater than 1×10^9 CFU/mL in the six extracts (garlic, shallot, lemon grass, basil, cumin and shrimp paste). The growth of viable *B. bifidum* was the highest in the garlic extract (1.44×10^9 CFU/mL) and the lowest in the clove extract (6.3×10^7 CFU/mL).

Table 2 Number of viable probiotic cells in Thai curries after passing simulated gastro-intestinal conditions (designated as Pre-attachment)

Type of extract/curry	Growth of viable cells (CFU/mL)		
	L. acidophilus	*L. casei*	*B. bifidum*
Control [1]	7.64×10^8	4.37×10^8	8.45×10^7
Hang-lay curry	1.92×10^8	3.63×10^8	2.85×10^8
Nam Ngeaw	5.35×10^6	1.38×10^7	8.2×10^7
Gaeng Aom	1.01×10^7	1.73×10^7	7.6×10^7
Red curry	1.58×10^8	5.7×10^8	3.12×10^8
Green curry	1.47×10^8	5.10×10^8	2.57×10^8
Massaman curry	3.41×10^8	4.80×10^8	3.66×10^8
Yellow curry	2.66×10^8	7.00×10^8	3.10×10^8
Som curry	3.35×10^6	7.00×10^6	2.07×10^7

CFU = colony forming units.

[1] Control treatment = probiotics without inoculation with Thai curries after passing simulated gastrointestinal conditions.

Table 3 Number of viable probiotic cells in spice/herb extracts after passing simulated gastro-intestinal conditions (designated as Pre-attachment)

Type of extract/curry	Growth of viable cells (CFU/mL)		
	L. acidophilus	*L. casei*	*B. bifidum*
Control [1]	7.64×10^8	4.37×10^8	8.45×10^7
Chili	5.17×10^8	2.31×10^8	9.30×10^8
Galangal	9.46×10^8	7.8×10^8	6.4×10^8
Garlic	1.34×10^9	2.7×10^9	1.44×10^9
Turmeric	1.55×10^8	3.15×10^8	4.3×10^8
Shallot	9.30×10^8	2.27×10^9	1.14×10^8
Lemon grass	7.43×10^8	1.69×10^9	5.9×10^8
Basil	5.33×10^8	1.25×10^9	1.21×10^9
Red cotton tree flowers	5.61×10^8	7.00×10^8	1.38×10^8
Cloves	6.65×10^7	6.10×10^6	6.3×10^7
Coriander root	1.30×10^9	3.50×10^7	4.80×10^8
Cumin	1.04×10^9	1.37×10^9	1.58×10^9
Shrimp paste	9.27×10^8	1.18×10^9	8.9×10^7
Cinnamon	1.06×10^8	1.21×10^8	8.6×10^7
Finger Root	2.35×10^8	3.40×10^8	3.92×10^8

CFU = colony forming units.

[1] Control treatment = probiotics without inoculation with spice/herb extracts after passing simulated gastrointestinal conditions.

Adhesive properties of three probiotic microbiota on Caco-2 cells

To evaluate the prebiotic efficiency of the Thai curries and spice/herb extracts, the percentages of Caco-2 cell adhesion were calculated (Tables 4–5) and compared to the control group (probiotics without inoculation in Thai curries and spice or herb extracts), as shown in Tables S2–S3. Adhesion of the probiotic strains on Caco-2 cells as viewed under the microscope (32× magnification) showed that the probiotic strains could adhere to Caco-2 cells (Fig. 1). After the simulated gastrointestinal stress, there were significant differences in the numbers of viable cells of the three probiotics with Caco-2 cell adhesion according to the strain and different spice/herb extracts and Thai curries. However, the %Adhesion level largely varied among the triple replicates in some spices/herbs extracts and Thai curries. The crude extracts and curries coating the probiotic cells may have disturbed Caco-2 cell adhesion. This resulted in large SD values and problems with the statistical analysis.

Among the Thai curries, the percentages of Caco-2 cell adhesion of *L. acidophilus* for the Gaeng Aom (1.41%), control sample (1.13%), and Nam Ngeaw curry (0.87%) were significantly higher than for the other curries (Table 4). Only the Gaeng Aom curry increased the adherence to Caco-2 cells by 0.28% compared to the control (Table S2). There was a significant reduction in the %Adhesion represented in *L. acidophilus* inoculated with nine of the spice or herb extracts (garlic, turmeric, shallot, lemon grass, basil, red cotton tree flowers, cumin, shrimp paste, and cinnamon) compared to the control sample (Table 5; Table S3).

Lactobacillus casei in the Thai curries and spice/herb extracts had greater adhesive properties on Caco-2 cells after passing through the simulated gastrointestinal conditions. The adhesion of *L. casei* in the Nam Ngeaw curry (2.37%) was significantly higher than the adhesion level of the other curries (Table 4). The Nam Ngeaw curry increased the adhesion level of *L. casei* by 1.95% compared to the control sample (Table S2). The adherence to Caco-2 cells of *L. casei* was the highest in the coriander root extract (1.34%; Table 5). In contrast, the %Adhesion of *L. casei* after exposure to turmeric extracts (0.27%) was significantly lower than for the coriander root extract. There was no significant difference in adhesion of *L. casei* between the other spice/herb extracts and the control.

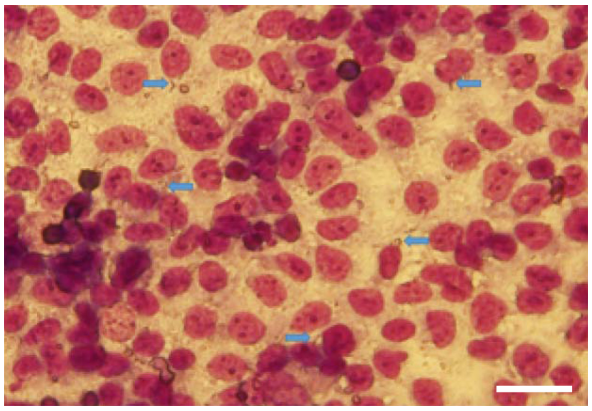

Fig. 1 Example of probiotic strain adherence on Caco-2 cells (32× magnification, scale bar = 100 µm), where blue arrows indicate probiotics attached to Caco-2 cells using Giemsa stain

Table 4 Adhesion level (%) of probiotics incubated in Thai curries on Caco-2 cells

Type of curry	Mean of %Adhesion (± SD)[1]		
	L. acidophilus	*L. casei*	*B. bifidum*
Control[2]	1.13±0.21[a]	0.42±0.28[b]	0.47±0.29
Hang-lay curry	0.06±0.02[b]	0.22±0.11[b]	0.16±0.07
Nam Ngeaw curry	0.87±0.21[ab]	2.37±1.16[a]	0.77±0.31
Gaeng Aom	1.41±0.42[a]	1.13±0.3[b]	0.34±0.06
Red curry	0.13±0.09[b]	0.12±0.02[b]	0.11±0.07
Green curry	0.12±0.07[b]	0.24±0.16[b]	0.22±0.19
Massaman curry	0.06±0.03[b]	0.15±0.08[b]	0.04±0.01
Yellow curry	0.05±0.03[b]	0.19±0.01[b]	0.07±0.02
Som curry	0.41±0.04[b]	0.77±0.25[b]	1.09±0.02

Data shown as mean ± SD, derived from triple replications.

[1] Means in a column with different lowercase superscripts are significantly ($p < 0.05$) different based on analysis of variance and Tukey's honestly significant different test.

[2] Control treatment = probiotics without inoculation with Thai curries or spice/herb extracts after passing simulated gastrointestinal conditions.

Table 5 Adhesion level of probiotics incubated in spice/herb extracts on Caco-2 cells

Type of extract	Mean of %Adhesion (± SD)[1]		
	L. acidophilus	*L. casei*	*B. bifidum*
Control[2]	1.13 ± 0.21[a]	0.42 ± 0.28[ab]	0.47 ± 0.29[ab]
Chili	0.51 ± 0.08[ab]	1.17 ± 0.39[ab]	0.11 ± 0.09[ab]
Garlic	0.17 ± 0.06[b]	0.25 ± 0.13[ab]	0.06 ± 0.04[b]
Galangal	0.64 ± 0.18[ab]	0.6 ± 0.33[ab]	0.68 ± 0.39[ab]
Turmeric	0.28 ± 0.23[b]	0.27 ± 0.05[b]	0.21 ± 0.04[ab]
Shallot	0.19 ± 0.01[b]	0.29 ± 0.16[ab]	0.13 ± 0.07[ab]
Lemon grass	0.40 ± 0.1[b]	0.34 ± 0.23[ab]	0.20 ± 0.06[ab]
Basil	0.40 ± 0.1[b]	0.31 ± 0.13[ab]	0.27 ± 0.04[ab]
Red cotton tree flowers	0.42 ± 0.09[b]	0.41 ± 0.18[ab]	0.42 ± 0.21[ab]
Cloves	0.57 ± 0.35[ab]	0.91 ± 0.68[ab]	0.82 ± 0.43[ab]
Coriander root	0.68 ± 0.59[ab]	1.34 ± 0.85[a]	1.15 ± 1.07[a]
Cumin	0.44 ± 0.01[b]	0.53 ± 0.31[ab]	0.33 ± 0.12[ab]
Shrimp paste	0.48 ± 0.01[b]	0.80 ± 0.35[ab]	0.34 ± 0.16[ab]
Cinnamon	0.11 ± 0.09[b]	0.45 ± 0.27[ab]	0.28 ± 0.19[ab]
Finger Root	0.59 ± 0.05[ab]	0.46 ± 0.11[ab]	0.35 ± 0.14[ab]

Data shown as mean ± SD, derived from triple replications.

[1] Means in a column with different lowercase superscripts are significantly ($p < 0.05$) different based on analysis of variance and Tukey's honestly significant different test.

[2] Control treatment = probiotics without inoculation with Thai curries or spice/herb extracts after passing simulated gastrointestinal conditions.

The *B. bifidum* inoculated in Thai curries passing through the digestion model had no significant differences in adhesive properties among curries and the control sample (Table 4). *B. bifidum* in Som curry (1.09%) and Nam Ngeaw curry (0.77%) attached to the Caco-2 cells (Table 4) in similar numbers to the control treatment (0.47%), as shown in Table S2. The adherence to Caco-2 cells of *B. bifidum* was the highest in the coriander root extract (1.15%), as shown in Table 5. In contrast, the adhesion of *B. bifidum* was significantly lower in the garlic extract compared to the coriander root extract Table 5), with the %Adhesion levels of *B. bifidum* inoculated in the other spice/herb extracts and the control sample not being significantly different.

Discussion

Analysis of the results of the current study provided new insight into the beneficial effects of individual spices, herbs and Thai curries after *in-vitro* ingestion on prebiotic properties. The evaluation of adhesive properties among the three probiotic strains (*L. acidophilus*, *L. casei* and *B. bifidum*) in this study has provided a first impression of the persistence of probiotic strains on Caco-2 cell adhesion when these probiotic cells were added to spice or herb extracts or Thai curries, followed by incubation using a simulated gastrointestinal model. The mixture of spices or herbs in Thai curries and the individual effect of each extract similarly influenced the survival of the three probiotics. The % Adhesion levels on the Caco-2 cell differed according to the type of spice/herb extract or Thai curry. Based on the current findings, *L. casei* with inoculation in the Nam Ngeaw curry significantly enhanced Caco-2 cell attachment compared to the probiotics not incubated in the Thai curries. This could be explained by the individual spice or herb extracts containing phenolic compounds that influenced the survival of the probiotic bacteria and consequently maintained the adhesive property of the probiotics (de Souza et al., 2018). The synergistic effect of the combined or mixed herbs in the Thai curries could be used to develop representative food products to support the growth of probiotic microbiota. However, the digestive stress affected the adhesion property of *L. acidophilus* incubated in some of the spice/herb extracts.

The antimicrobial activity of the spice or herb extracts in the current study was not investigated; however, garlic and ginger have been reported to not lose their antimicrobial ability, even up to 100°C (Cocetta et al., 2019), which was consistent with the current study. Based on the results for the Thai curries that included individual spice or herb extracts (especially the garlic extract), boiling had no inhibitory effects on the three-probiotic microbiota (*L. acidophilus*, *L. casei* and *B. bifidum*). This suggested that the inhibitory effect of the spice or herb extracts has more influence on pathogenic bacteria than on probiotic bacteria. A similar finding was reported by Lu et al. (2017), who showed the prebiotic-like activity of herbs or spices, such as turmeric, on the promotion of

the growth of beneficial bacteria (*Bifidobacterium* spp. and *Lactobacillus* spp.), whereas cinnamon, rosemary and turmeric suppressed the growth of pathogenic bacteria (*Clostridium spp*. and *Fusobacterium*). Based on *in-vitro*, *in-vivo* experiments and human studies, Duenas et al. (2015) confirmed that plant diets containing polyphenol-rich dietary sources could reduce potential pathogens and certain Gram-negative *Bacteroides spp*. while enhancing bifidobacteria and lactobacilli.

Several studies (Takahashi et al., 2005; Guan et al., 2021) have tested the food matrix factor of insoluble dietary fiber (such as lignin and complex carbohydrates) in plants on gut microbiota; however, it is very hard to draw conclusions. In the present study, the spice/herb extracts and Thai curries did not affect the survival and prebiotic properties of the probiotics after gastro-intestinal digestion. According to the characterization of Thai curry paste, Chili, Garlic, Shallot Lemon grass, Shrimp paste and Galangal were the main ingredients (Table 1). The amount of these spices or herbs varied according to the type of curry. There were similar prebiotic effects of the spice/herb extracts and Thai curries on the survival of the three probiotic microbiota, with garlic supporting the growth of all three probiotic microbiota. The three probiotic bacteria survived in the tested Thai curries and in the spice/herb extracts, especially *L. casei*, which had higher viable cells after digestive stress compared to the other two probiotics tested, which might indicate that the survival of probiotic microbiota (such as *L. casei*) after incubation in Thai curries or spice/herb extracts and then passing through the simulated gastrointestinal conditions reflected their resistance along the gastrointestinal tract. One possible explanation is that the spice or herb extracts contain complex carbohydrates (Milutinovic´ et al., 2021), which can act as a carbon source to support the growth of the three probiotic microbiota. In addition, most herbs and curries contain complex carbohydrates, which are not digested by human enzymes; therefore, they can pass through the upper gut to enter the large intestine (Li, 2010). The current results were consistent with this reported finding, providing insight into the effects of the spice-specific strain in the spice or herb extracts and Thai curries on the probiotic microbiota under simulated gastrointestinal conditions.

The present results indicated that the spices or herbs and Thai curries supported the probiotic microbiota (*L. acidophilus*, *L. casei* and *B. bifidum)* to differing degrees regarding Caco-2 cell attachment due to the spice-specific strain (Duenas et al., 2015). Among the Thai curries after gastrointestinal digestion, the highest growth-stimulating effect of *L. casei* on Caco-2 cells was with Nam Ngeaw curry (a water-based curry), reflecting that the consumption of Nam Ngeaw curry may enhance probiotic ability on Caco-2 cell adhesion. The combined spices and herbs may have enhanced the adhesiveness of the two potential probiotic strains to epithelial cells based on a synergistic effect (Duenas et al., 2015; Milutinovic´ et al., 2021). The survival of probiotics was similarly reported by de Souza et al. (2018), who showed that phenolic compounds could improve the adhesion capacity and survival of probiotics during their exposure to the gastrointestinal tract. Furthermore, the coriander root extract had the highest (1.34%) adherence to Caco-2 cells of *L. casei* and *B. bifidum*, with the other spice/herb extracts not having significant differences in adhesion level compared to the control sample. This was due to the higher viable cell numbers of the two probiotics in the coriander root extract after passing through the digestive enzymes and thereby having a greater number of the probiotic cells for Caco-2 cell attachment. These findings were in accordance with Volstatova et al. (2017), who reported that apple pulp extracts enhanced the adhesiveness of probiotic strains on epithelial cells. In the present work, Thai curries with a coconut base containing high levels of fat and nutrients may have obstructed Caco-2 cell adhesion of the probiotic bacteria. Similar findings reported that hot Thai curries affected the adhesion of probiotic microbiota to intestinal cells (Settharaksa et al., 2012). Based on the current results, *L. acidophilus* lost its Caco-2 cell adhesion property after exposure to the spice/herb extracts (garlic, turmeric, shallot, lemon grass, basil, red cotton tree flowers, cumin, shrimp paste and cinnamon), although some spice/herb extracts supported the growth of probiotics in the current study. The finding on the loss of properties was similar to Bustos et al. (2012) who confirmed that all flavan-3-ols significantly inhibited *L. acidophilus* LA-5 and *L. plantarum* IFPL379 adhesion except epigallocatechin gallate, which enhanced *L. acidophilus* LA-5 adhesion to Caco-2. Compared with this other study, the ability of Caco-2 cell adhesion had similar adhesion rates with *L. casei* Shirota ACA-DC 6002, which had 2.4 + 0.4% (Maragkoudakis et al., 2006. *B. bifidum* inoculated in Thai curries or spice/herb extracts passing through the digestion model were not significantly different in their adherence levels to Caco-2 cells compared to the control treatment. Based on the current results, *B. bifidum* resisted all stress conditions and attached the Caco-2 cells. Charnchai et al. (2016) reported that *B. animalis* BF052 had considerable probiotic properties that were stable, including resistance under simulated gastrointestinal conditions and adherence ability to Caco-2 cells. Charnchai et al. (2016) reported several factors

that affected the viability of bifidobacteria, such as the presence of oxygen, the temperature and pH and osmotic changes. Based on the current results, there were differences in the adhesion ability of the probiotic microbiota because the viability and stability of the probiotics were challenged after passing through the GI tract and its associated stresses until their adherence to the intestinal epithelium (Javanshir et al., 2021).

In this regard, the results of the screening of the herb extracts and Thai curries in the current study have provided new insight into the beneficial effects on the prebiotic properties of individual herbs and Thai curries after ingestion. This information could strengthen the evidence confirming that natural spices or herbs or Thai curry products can maintain differing levels of the probiotic properties of probiotic microbiota (specifically *L. acidophilus*, *L. casei* and *B. bifidum)*. Several factors of the extraction could influence prebiotic activity. The tested probiotic bacteria (*L. casei*) is a potential probiotic candidate for further food development involving products containing herbs or spices and may also have potential as a food immunity enhancer. The viability of the probiotic microbiota (*L. casei*) may indicate their resistance and adaptability to the gastrointestinal tract and their subsequent adherence to the intestinal epithelium. Intestinal permeability can be preserved by lactobacilli since *Lactobacillus spp*. can be a modulator of the immune response in the intestine (Javanshir et al., 2021) and has anti-inflammatory effects in Caco-2 cells (Han et al., 2021). In addition, these probiotic strains could be highly stable probiotic starters with the stable functionality that is needed for commercial food applications.

Conclusion

The current study provided insight into screening the prebiotic properties of three probiotic microbiota (*L. acidophilus*, *L. casei* and *B. bifidum)* to support probiotic Caco-2 cell adhesion after ingestion. Based on the results, the 14 spice/herb extracts and 8 Thai curries had no inhibitory effects on the three probiotic microbiota. The prebiotic properties of *L. casei* persisted after its inoculation in the spice or herb extracts or Thai curries and passing digestive stress. The adhesion of *L. casei* on Caco-2 cells was the highest in the Nam Ngeaw curry, with some herb/spice extracts causing a reduction in *L. acidophilus* adhesion. Further study on different concentrations of the herb extracts used in Thai curries should investigate any dose-dependent effect to guarantee any functional claim of health-promoting effects. The current study highlighted the gap in risk assessment of commercial herb-probiotic interactions. The risk assessment should be evaluated regarding herbal resistance to ingestion as well as their modulatory effects on the gut microbiome and the immune system among Thai consumers.

Acknowledgments

This work was supported by the Biodiversity-based Economy Development Office (Public Organization)-National Research Council of Thailand. Mr. Wanchai Pantavee provided important advice during the experiment.

Conflict of Interest

The authors declare that there are no conflicts of interest.

References

Awortwe, C., Fasinu, P.S., Rosenkranz, B. 2014. Application of Caco-2 cell line in herb-drug interaction studies: Current approaches and challenges. J. Pharm. Pharm. Sci. 17: 1–19. doi.org/10.18433/J30K63

Bahrami, B., Child, M.W., Macfarlane, S., Macfarlane, G.T. 2011. Adherence and cytokine induction in Caco-2 cells by bacterial populations from a three-stage continuous-culture model of the large intestine. Appl. Environ. Microbiol. 77: 2934–2942. doi.org/10.1128/AEM.02244-10

Bustos, I., García-Cayuela, T., Hernández-Ledesma, B., Peláez, C., Requena, T., Martínez-Cuesta, M.C. 2012. Effect of flavan-3-ols on the adhesion of potential probiotic lactobacilli to intestinal cells. J. Agric. Food. Chem. 12: 60: 9082–9088. doi.org/10.1021/jf301133g

Chan, K.W., Iqbal, S., Khong, N.M.H., Babji, A.S. 2011. Preparation of deodorized antioxidant rich extracts from 15 selected spices through optimized aqueous extraction. JMPR. 5: 6067–6075. doi: 10.5897/JMPR.9001043

Charnchai, P., Jantama, S.S., Prasitpuriprecha, C., Kanchanatawee, S., Jantama, K. 2016. Effects of the food manufacturing chain on the viability and functionality of *Bifidobacterium animalis* through simulated gastrointestinal conditions. PLoS One 11: e0157958. doi.org/10.1371/journal.pone.0157958

Cocetta, V., Catanzaro, D., Borgonetti, V., et al. 2019. A fixed combination of probiotics and herbal extracts attenuates intestinal barrier dysfunction from inflammatory stress in an *in vitro* model using caco-2 cells. Recent Pat. Food Nutr. Agric. 10: 62–69. doi.org/10.2174/2212798410666180808121328

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S.J., Berenjian, A., Ghasemi, Y. 2019. Prebiotics: Definition, types, sources, mechanisms, and clinical applications. Foods 8: 92. doi.org/10.3390/foods8030092

de Oliveira, G.L.V., Leite, A.Z., Higuchi, B.S., Gonzaga, M.I., Mariano, V.S. 2017. Intestinal dysbiosis and probiotic applications in autoimmune diseases. Immunology 152: 1–12. doi.org/10.1111/imm.12765

de Souza, E.L., de Albuquerque, T.M.R., dos Santos, A.S., Massa, N.M.L., de Brito Alves, J.L. 2018. Potential interactions among phenolic compounds and probiotics for mutual boosting of their health-promoting properties and food functionalities–A review. Crit. Rev. Food Sci. Nutr. 59: 1645–1659. doi.org/10.1080/10408398.2018.1425285

Duary, R.K., Rajput, Y.S., Batish, V.K., Grover, S. 2011. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. Indian J. Med. Res. 134: 664–671. doi: 10.4103/0971-5916.90992

Duenas, M., Munoz-Gonzalez, I., Cueva, C., Jimenez-Giron, A., Sanchez-Patan, F., Santos-Buelga, C., Moreno-Arribas, M.V., Bartolome, B. 2015. A survey of modulation of gut microbiota by dietary polyphenols. Biomed Res. Int. 2015: 850902. doi.org/10.1155/2015/850902

Farnworth, E.R., Champagne, C.P. 2010. Production of probiotic cultures and their incorporation into foods. In: Watson, R.R., Preedy, V.R. (Eds.). Bioactive Foods in Promoting Health: Probiotics and Prebiotics. Academic Press. Amsterdam, the Netherlands, pp. 3–17.

Fedi, A., Vitale, C., Ponschin, G., Ayehunie, S., Fato, M., Scaglione, S. 2021. *In vitro* models replicating the human intestinal epithelium for absorption and metabolism studies: A systematic review. J. Control. Release. 335: 247–268. doi.org/10.1016/j.jconrel.2021.05.028

Gagnon, M., Kheadr, E.E., Blay, G.L., Fliss, I. 2004. *In vitro* inhibition of *Escherichia coli* O157:H7 by Bifidobacterial strains of human origin. Int. J. Food Microbiol. 92: 69–78. doi.org/10.1016/j.ijfoodmicro.2003.07.010

Guan, Z.W., Yu, E.Z., Feng, Q. 2021. Soluble dietary fiber, one of the most important nutrients for the gut microbiota. Molecules 26: 6802. doi.org/ 10.3390/molecules26226802.

Han, S., Lu, Y., Xie, J., et al. 2021. Probiotic gastrointestinal transit and colonization after oral administration: A long journey. Front. Cell. Infect. Microbiol. 11: 609722. doi.org/10.3389/fcimb.2021.609722

Javanshir, N., Hosseini, G.N.G., Sadeghi, M., Esmaeili, R., Satarikia, F., Ahmadian, G., Allahyari, N. 2021. Evaluation of the function of probiotics, emphasizing the role of their binding to the intestinal epithelium in the stability and their effects on the immune system. Biol. Proced. Online 23: 23. doi.org/10.1186/s12575-021-00160-w

Jiang, Q., Xu, N., Kong, L., Wang, M., Lei, H. 2021. Promoting effects of 6-Gingerol on probiotic adhesion to colonic epithelial cells. Food Sci. Technol. 41: 678–686. doi.org/10.1590/fst.17420

Krisch, J., Pardi, Z., Tserennadmid, R., Papp, T., Vágvölgyi, C. 2010. Antimicrobial effects of commercial herbs, spices and essential oils in minced pork. Acta Biol. Szeged. 54: 131–134.

Li, L. 2010. Assessing prebiotic effects of resistant starch on modulating gut microbiota with an *in vivo* animal model and an *in vitro* semi-continuous fermentation model. M.Sc. thesis, College of Agriculture and Life Sciences, Iowa State University. Iowa, IA, USA.

Lu, Q.Y., Summanen, P.H., Lee, R.P., Huang, J., Henning, S.M., Heber, D., Finegold, S.M., Li, Z. 2017. Prebiotic potential and chemical composition of seven culinary spice extracts. J. Food Sci. 82: 1807–1813. doi.org/10.1111/1750-3841.13792

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., Tsakalidou, E. 2006. Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int. Dairy J. 16: 189–199. doi.org/10.1016/j.idairyj.2005.02.009

Milutinovic´, M., Dimitrijevic´-Brankovic´, S., Rajilic´-Stojanovic´, M. 2021. Plant extracts rich in polyphenols as potent modulators in the growth of probiotic and pathogenic intestinal microorganisms. Front. Nutr. 8: 688843. doi: 10.3389/fnut.2021.688843

Morelli, L., Pellegrino, P. 2021. A critical evaluation of the factors affecting the survival and persistence of beneficial bacteria in healthy adults. Benef. Microbes 12: 321–331. doi: 10.3920/BM2021.0017

Ouwehand, A.C., Salminen, S. 2003. *In vitro* adhesion assays for probiotics and their *in vivo* relevance: A review. Microb. Ecol. Health Dis.15: 175–184. doi.org/10.1080/08910600310019886

Prakasita, V.C., Asmara, W., Widyarini, S., Wahyuni, A.E.T.H. 2019. Combinations of herbs and probiotics as an alternative growth promoter: An *in vitro* study. Vet. World 12: 614–620. doi: 10.14202/vetworld.2019.614-620

R Core Team. 2023. RStudio: Integrated development for R. Boston, MA, USA. http://www.rstudio.com/

Salamatullah, A.M., Hayat, K., Arzoo, S., et al. 2021. Boiling technique-based food processing effects on the bioactive and antimicrobial properties of basil and rosemary. Molecules 26: 7373. doi.org/10.3390/molecules26237373

Settharaksa, S., Jongjareonrak, A., Hmadhlu, P., Chansuwan, W., Siripongvutikorn, S. 2012. Flavonoid, phenolic contents and antioxidant properties of Thai hot curry paste extract and its ingredients as affected of pH, solvent types and high temperature. Int. Food Res. J. 19: 1581–1587.

Sikora, E., Cieslik, E., Leszczynska, T., Filipiak-Florkiewicz, F., Pisulewski, P.M. 2008. The antioxidant activity of selected cruciferous vegetables subjected to aquathermal processing. Food Chem. 107: 55–59. doi.org/10.1016/j.foodchem.2007.07.023

Takahashi, T., Karita, S., Ogawa, N., Goto, M. 2005. Crystalline cellulose reduces plasma glucose concentrations and stimulates water absorption by increasing the digesta viscosity in rats. J. Nutr. 135: 2405–2410. doi.org/10.1093/jn/135.10.2405

Trevanich, S. 2021. Probiotic beverages in Thailand: Health attributes and future trends. In: Panda, S.K., Kellershohn, J., Russell, I. (Eds.). Probiotic Beverages. Academic Press. Cambridge, MA, USA, pp. 81–97.

Volstatova, T., Marsik, P., Rada, V., Geigerova, M., Havlik, J. 2017. Effect of apple extracts and selective polyphenols on the adhesion of potential probiotic strains of *Lactobacillus gasseri R* and *Lactobacillus casei FMP*. J. Funct. Foods 35: 391–397. doi.org/10.1016/j.jff.2017.06.005

Wichienchot, S., Thammarutwasik, P., Jongjareonrak, A., Chansuwan, W., Hmadhlu, P., Hongpattarakere, T., Itharat, A., Ooraikul, B. 2011. Extraction and analysis of prebiotics from selected plants from southern Thailand. Songklanakarin J. Sci. Technol. 33: 517–523.

Zhou, X., Qian, Y., Zhou, Y., Zhang, R. 2012. Effect of enzymatic extraction treatment on physicochemical properties, microstructure and nutrient composition of tartary buckwheat bran: A new source of antioxidant dietary fiber. Adv Mat Res. 396–398: 2052–2059.