

***In vitro* EVALUATION OF HERBAL EXTRACT POWDER AND PROBIOTIC PROPERTIES FOR COORDINATING USAGE**

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Abstract

Determination of the *in vitro* properties of 6 herbal extracts and 4 probiotic bacteria was performed to examine their potential for being used as a co-functional product. The anti-oxidant and anti-inflammatory activities of the re-soluble extracts of *Cordyceps sinensis*, *Ganoderma lucidum*, *Tagetes erecta*, *Terminalia chebula* Retz., *T. bellirica* Roxb., and *Phyllanthus emblica* were investigated. *P. emblica* and *T. chebula* possessed the top 2 highest DPPH radical scavenging activities of 94.07% and 88.75%, respectively. For anti-inflammation, the highest percentage was found in *T. bellirica* (12.11%). There was no antibacterial effect of those herbal solutions on 4 probiotic bacteria, i.e. *Bifidobacterium longum* BCC 51139, *Pediococcus acidilactici* TISTR 2309, *Lactobacillus acidophilus* TISTR 2365, and *Lactobacillus casei* TISTR 1463. For *in vitro* cholesterol reduction, *B. longum* and *L. casei* were the strains with the most potential (87.68% and 85.71% decline, respectively). Model capsules of a mixture of herbal powder (Triphala herbs including *T. chebula*, *T. bellirica*, and *P. emblica*) and each freeze-dried probiotic were prepared. The acid tolerance test showed that the cell numbers of all 4 bacteria in the model capsules decreased only 0.5 log CFU/g at pH 2.0 when compared to those at pH 6.4, whilst *L. acidophilus* was sensitive to bile salt (> 5 log reduction). The decimal reduction time was exploited to evaluate the storage life of the capsules at room temperature. The D-values of *L. acidophilus* and *P. acidilactici* were 46 and 25 days, respectively, whereas *L. casei* and *B. longum* had 15 days of D-value.

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Introduction

Herbal products are currently in high demand due to increasing health concern issues. The global market value of traditional medicine products was US\$60 billion in 2010 and was predicted to reach US\$5 trillion within the next 4 decades (Nirmal *et al.*, 2013). Recently, nutraceutical products derived from Cordyceps, Ganoderma, and Triphala have been of growing interest in Thailand. *Ganoderma lucidum* contains secondary metabolites (triterpenoids and ganodermin) which contribute to the suppression of *Botrytis cinerea* and *Fusarium oxysporum* (Erjavec *et al.*, 2012). Furthermore, findings of anti-cancer activity (Yuen and Gohel, 2005), and inhibition of oxidation reaction, cholesterol reduction, and immunity improvement (Sanodiya *et al.*, 2009) have been published. There is evidence that *Cordyceps* has been exploited in traditional Chinese medicine for more than 13 centuries. Its bioactive constituents and therapeutic applications were extensively reviewed by Holliday and Cleaver (2008). Research has indicated that *C. sinensis* possesses many bioactive constituents, such as polysaccharide, adenosine, cordycepin, cordycepic acid, and ergosterol. The polysaccharide component in *C. sinensis* plays a role in antioxidant, immunomodulatory, and anti-fibrotic activities (Wang *et al.*, 2015). The components of Triphala, an ayurvedic formulation, include *Terminalia chebula* Retz., *Terminalia bellerica* Roxb., and *Phyllanthus emblica* Linn. Several bioactive compounds are constituents of Triphala, such as tannin, quinones, flavonoids, gallic acid, and vitamins (Zaveri *et al.*, 2016). Anti-inflammation (Sabina and Rasool, 2008), antitumor activity (Sandhya *et al.*, 2006a), antioxidant activity (Sandhya *et al.*, 2006b), anti-mutagenic activity (Kaur *et al.*, 2002) and reduction of blood sugar (Rajan and Antony, 2008) associated with Triphala have been examined by board investigations.

Probiotics have been widely recognized as beneficial microorganisms that need to be administered in an appropriate amount in order to provide a health benefit to the host (FAO/WHO, 2002). The scientific proof of health advantages relevant to the probiotic bacteria available in the food industry has been extensively reviewed (Kechagia *et al.*, 2013). Although these microorganisms can be found in the gastrointestinal tracts of humans and other animals, the number and sort of population may depend on individual consumption, stress, age, and the antibiotic used. Many probiotic strains that are available commercially and are considered as functional supplements in several food products were first established in the dairy industry (Socol *et al.*, 2010). It is foreseen that the use of herbal products with probiotic microorganisms will be a new alternative supplement beside fermented dairy products. *In vitro* assessment has revealed a primary selection of the possible target materials, and the potential capabilities of herbal extracts have been evaluated in the literature. An example of the antioxidant efficiency of Triphala was studied by Naik *et al.* (2005), and the anti-inflammatory activity of such a herbal formula was also studied by Rasool and Sabina (2007). A similar investigation was conducted into ethanol extracts of the marigold flower and sensitive plant (Kaur *et al.*, 2016). Likewise, the probiotic properties were also screened using *in vitro* protocols, i.e. of fermented vegetables (Sayedboworn *et al.*, 2014), of silage (Ohmomo *et al.*, 1999), and of cheese (Zago *et al.*, 2011). This research studied some beneficial competency of the commercial powder forms of herbal extracts and some properties of potential probiotic bacteria. The ultimate aim of this study was to examine the potential of use as a cofunctional product between plant extracts and live probiotics by *in vitro* experiments. Additionally,

the model capsules of the coordinating mixture were prepared and evaluated for their shelf-life using the decimal reduction time of each type of probiotic bacterium in the storage conditions.

Materials and Methods

Microorganisms and Culturation

The potential probiotic strains were *Lactobacillus casei* TISTR 1463, *Pediococcus acidilactici* TISTR 2309, and *Lactobacillus acidophilus* TISTR 2365 and they were purchased from the Microbial Center, Thailand Institute of Scientific and Technological Research. *Bifidobacterium longum* BCC 51139 was bought from the National Center for Genetic Engineering and Biotechnology. All strains were from fermented food or human origin. *Lactobacillus* and *Pediococcus* were cultured on MRS agar (Difco) at 37°C in a 5% CO₂ incubator for 48 h. For *Bifidobacterium*, the strain was grown on MRS agar supplemented with 0.05% (w/v) L-cysteine HCl and was anaerobically incubated at 37°C for 48 h.

Test bacteria including *Escherichia coli* TISTR 073, *Staphylococcus aureus* TISTR 029, *Bacillus cereus* TISTR 037, and *Salmonella* sp. (isolated from chicken intestine) were in the laboratory stocks. These bacteria were sub-cultured in nutrient agar (NA) and were incubated at 37°C for 24 h prior to use.

Effect of Herbal Extract Solution on the Growth of Probiotic Bacteria

The powder extracts of *Terminalia chebula* Retz., *Terminalia bellerica* Roxb., *Phyllanthus emblica* Linn., *Cordyceps sinensis*, *Ganoderma lucidum*, and *Tagetes erecta* were provided by Healthy Home Trepala Ltd (Bangkok, Thailand). Each dried powder was dissolved in distilled water to a concentration of 1 mg/ml. Afterwards, the solution was filtered through a 0.2 µm sterile membrane (Millipore).

Four potential probiotic strains, grown as previously mentioned, were suspended in a normal saline solution. The bacteria were adjusted to an initial concentration of 10⁵-10⁶ CFU/ml before plating on MRS agar. The solidified agar was aseptically punched with a cork borer (0.6 mm in diameter) and 30 µl of prepared herbal solution was placed into the well. All plates were incubated at 37°C in a 5% CO₂ incubator for 48 h. The inhibition zone of growth around the well was observed.

Antioxidant Activity by DPPH Radical Scavenging Method

The DPPH radical scavenging activity was determined according to the method of Manzocco *et al.* (1998). The dried herbal extracts were dissolved in methanol (0.1 mg/ml). One ml of herbal solution was mixed with 2 ml of 0.5 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. The solution absorbance at a wavelength of 517 nm was measured at 0 min and 30 min. The percentage of the DPPH radical scavenging was calculated as follows:

$$\% \text{ inhibition of DPPH radical} = \frac{[Ab - Aa]}{Ab} \times 100 \quad (1)$$

where Ab and Aa were the absorbance at 0 min and 30 min, respectively.

In vitro Anti-inflammatory Activity

The anti-inflammatory percentage was carried out using the egg albumin method (modified from Kaur *et al.*, 2016). The herbal samples were liquefied in distilled water to the testing concentration of 10 mg/ml. Thereafter, 2 ml of a sample was added into test tubes containing 200 µl of fresh egg white and 2.8 ml of phosphate buffered saline (PBS, pH 6.4). The tubes were incubated at 37°C for 15 min and the reaction was stopped by heating at 70°C for 5 min. The tubes were then cooled in ice and the sample absorbance was measured at 660 nm. The control sample was the same volume of egg white and PBS mixed with 2 ml of distilled water. The following equation was

calculated in order to obtain the suppression percentage of the protein denature:

$$\% \text{ Inhibition} = 100 \times (V_t / V_c - 1) \quad (2)$$

where V_t and V_c were the absorbance of the sample and control, respectively. The anti-inflammatory effect of the herbal samples was compared to the standard curve of the reaction by diclofenac sodium in the range of 0.2-1.0 mg/ml.

Dissolving Percentage of the Herbal Powder

The percentage of dissolution was adapted from water-soluble index analysis by Anderson *et al.* (1969). Three grams of the herbal powder was dissolved with 30 ml of distilled water. The samples were vortexed and incubated in a water bath at 90°C, or 37°C, for 30 min. Afterwards, the samples were centrifuged at 5,000 g for 10 min. The supernatant was dried at 105°C for 12 h before weighing. The water dissolving percentage was calculated as follows:

$$\% \text{ Dissolving} = 100 \times (W_d / W_s) \quad (3)$$

where W_d was the weight of the dried supernatant and W_s was the initial weight of the dried sample.

Antibacterial Determination of Probiotic Bacteria

This method was modified from bacteriocin screening by Ohmomo *et al.* (1999). To examine the function of the anti-foodborne bacteria by probiotics, the probiotic bacteria were streaked on MRS agar (containing 2% dextrose) and tryptic soy agar (TSA, no glucose). Only half of the Petri dish was streaked while the other half was left as a blank. All plates were anaerobically incubated at 37°C for 2-3 days until the colony appeared along the streaked line. Thereafter, the test bacterial suspension was prepared to obtain 10^5 - 10^6 CFU/ml of initial concentration. One ml of suspended cells was transferred into a 10 ml of molten NA before overlaying on top of the grown probiotic plates. The plates were further incubated at 37°C for 24 h. The

inhibition zone around the streak area was observed.

Coaggregation Analyze

The probiotic and test bacteria, cultured as mentioned above, were suspended in PBS to obtain approximately 10^8 CFU/ml of cell concentration. An equal volume of the dual culture of each probiotic strain and test strain were mixed by vortex for 10 s. After incubation at an ambient temperature for 5 h, the absorbance at 600 nm was measured. The control tubes were also prepared in the same manner using only an individual bacterial strain. The percentage of coaggregation was calculated using the following formula (Handley *et al.*, 1987):

$$\begin{aligned} \% \text{ Coaggregation} \\ = \frac{([A_x + A_y] / 2) - A_{xy}}{([A_x + A_y] / 2)} \times 100 \quad (4) \end{aligned}$$

where A_x and A_y represent the absorbance of the probiotic and test bacteria, respectively, and A_{xy} is the OD_{600} of the mixture of the probiotic and test bacteria.

In vitro Assay of Cholesterol Reduction

Probiotic strains grown in the suitable medium as previously stated were inoculated (1%) in MRSOCH broth, MRS broth and 3 g/l oxgall supplemented with 1% (v/v) filtered sterile Lipid cholesterol-rich (Sigma, L-4646), before use and were further incubated at 37°C for 16 h. The samples were then centrifuged at 8,000 g for 5 min. After transferring 0.5 ml of supernatant to a new tube, 3 ml of 95% ethanol and 2 ml of 50% (w/v) potassium hydroxide were sequentially added. The tubes were placed in a water bath at 60°C for 10 min and were cooled to room temperature. The volume of 5 ml of hexane was dispensed and vortexed for 20 s and then 3 ml of distilled water was added and mixed well. The tubes were left at room temperature for 15 min. The upper part of 2.5 ml (hexane phase) was transferred to a new tube and dried by evaporation at 60°C. Thereafter, the tube was mixed with 2 ml of o-phthalaldehyde reagent (Sigma-Aldrich, St. Louis, MO, USA) and was left at room temperature for 10 min

before the addition of 1 ml concentrated sulfuric acid. After mixing well and leaving at room temperature for 10 min, the absorbance (A_{550}) was measured and compared to the standard curve of 1-10 $\mu\text{l/ml}$ of cholesterol (Font de Valdez and Taranto, 2001).

Preparation of Model Capsule

Prior to making the model capsules, the probiotic bacteria were freeze-dried using 10% (w/v) mannitol as a cryoprotectant (Savini *et al.*, 2010). Subsequently, each probiotic bacterium was mixed with Triphala extract powder (in the ratio of 1:4) and then was packed in a 500 mg capsule.

Acid Tolerance Assay

PBS (pH 6.4) was prepared and adjusted to pH 2.0 and 3.0 with 1M HCl. The solution was dispensed (9 ml) into a test tube before sterilizing at 121°C for 15 min. One gram of the model capsule was inserted into each PBS tube and then was incubated at 37°C for 3 h. After that, each tube was diluted and the cell numbers were determined by the drop plate (20 μl) technique on a suitable medium (adapted from Ren *et al.*, 2014).

Resistance to Bile Salt Assay

A sample of 1 g was added into 9 ml of the MRS broth tubes containing 0% (control), 0.3%, 0.5%, and 1% (w/v) of bile salts (HiMedia, Mumbai, India). After incubation at 37°C for 24 h under anaerobic conditions, the samples were taken for enumeration by the drop plate (20 μl) method on a suitable medium (adapted from Ren *et al.*, 2014).

Estimation of Cell Reduction Using D Value

Survival of the probiotic bacteria in the model capsule was examined every 20 days after storage at room temperature. The samples were taken to make a serial dilution with saline peptone water and the cell numbers were counted using the drop plate (20 μl) method on MRS agar incubated at 37°C for 48 h in 5% CO_2 . The cell numbers were plotted in a semi-log scale against time in order to obtain the time required for decrease of the bacteria in 1 log cycle (D-value).

Results and Discussion

In vitro Properties of Herbal Extract Powder

The *in vitro* properties of the herbs that are available commercially in the form of extract powder are shown in Table 1. It has long been shown from many research works that the extracts from herbs exhibit various functional properties which benefit health. For example, Triphala, including *Terminalia chebula*, *T. Bellirica*, and Indian gooseberry, has an anti-oxidant effect (Naik *et al.*, 2005) and anti-inflammatory activity (Rasool *et al.*, 2007). However, laboratory extraction substances were used for these tests. The commercial forms that are usually available in dried powder form have not been tested for those properties. Thus, our work examined the *in vitro* properties using the commercial herbs. The results indicated that the *T. bellirica* extract powder (10 mg) exhibited the highest anti-inflammatory value of 12.11% and the

Table 1. Anti-inflammatory, antioxidant, and dissolving properties of the herbal extract powder

Extract	Anti-inflammation (%)	DPPH (%)	Water Dissolving (%)
<i>Cordycep sinensis</i>	11.12 $\pm$ 0.06 ^d	44.75 $\pm$ 0.35 ^e	94.94 $\pm$ 0.89 ^b
<i>Garnoderma lucidum</i>	4.75 $\pm$ 0.08 ^c	55.62 $\pm$ 0.17 ^d	97.13 $\pm$ 1.01 ^{cd}
<i>Tagetes erecta</i>	3.49 $\pm$ 0.06 ^b	15.20 $\pm$ 0.35 ^f	79.80 $\pm$ 0.30 ^a
<i>Terminalia chebula</i>	11.33 $\pm$ 0.05 ^e	88.75 $\pm$ 1.66 ^b	97.07 $\pm$ 0.14 ^{cd}
<i>Terminalis bellirica</i>	12.11 $\pm$ 0.08 ^f	73.67 $\pm$ 0.44 ^c	96.16 $\pm$ 0.10 ^c
<i>Phyllanthus emblica</i>	1.32 $\pm$ 0.00 ^a	94.07 $\pm$ 0.09 ^a	98.01 $\pm$ 1.25 ^d

Data indicate mean $\pm$ standard deviation, n= 3, ^{a-f}: different alphabets in the same column are significantly different ($P \leq 0.05$).

lowest value was obtained from *P. emblica* which was only 1.32%. When compared with 1 mg diclofenac sodium (of 78.17% anti-inflammatory value), it can be deduced that 10 mg of *T. bellirica* had an equivalent anti-inflammatory value to 0.15 mg of diclofenac sodium. For Cordyceps and *T. chebula*, the anti-inflammation effects were equal to 0.14 mg of diclofenac sodium. In contrast, the results of the antioxidant ability (% inhibition of DPPH radical) showed that the highest value was found in *P. emblica* (94%). For the water-soluble capacities, all of the extracts dissolved at 90°C were more than 95% except *Tagetes erecta* (marigold flower extract) of which an insoluble yellowish froth was left on the surface. The dissolving temperature (90°C) mimicked the household electric kettle temperature that is used to liquefy an instant herbal drink. When dissolved at 37°C (body temperature), the percentages of water solubility were 1-3% lower than those dissolved at 90°C for all herbal powders, except the marigold flower (<60%). Three herbal powders used in this experiment, i.e. *T. bellirica*, *T. Chebula*, and *P. emblica*, were extracted with deionized water. The analysis information provided that the active ingredient was Gallic acid for both

species of *Terminalia*, whereas vitamin C was the active substance of *P. emblica*. The marigold flower was extracted with water and ethanol, and 10% lutein was the active compound. Therefore, this might be the reason why *T. erecta* had a lower solubility. Unfortunately, the extraction solvents for *C. sinensis* and *G. lucidum* were not provided by the trading company.

Antibacterial Properties of Probiotic Bacteria

Potential probiotic bacteria of 4 Thai culture collection strains were tested to obtain the antibacterial ability against some foodborne bacteria, as shown in Figure 1. All the probiotic strains that were grown on MRS agar could suppress the growth of 4 tested bacteria, whereas only *B. longum* and *L. casei* which were propagated on TSA inhibited the colony of the 4 test strains. These results disclosed that the antibacterial function of the probiotics was mainly acid production because the MRS containing glucose (2%) and the final pH (after 2 days of incubation) dropped to around 4.1-4.3. In contrast, for the TSA (without sugar), where the final pH was higher than 6, the role of inhibition was caused by other produced

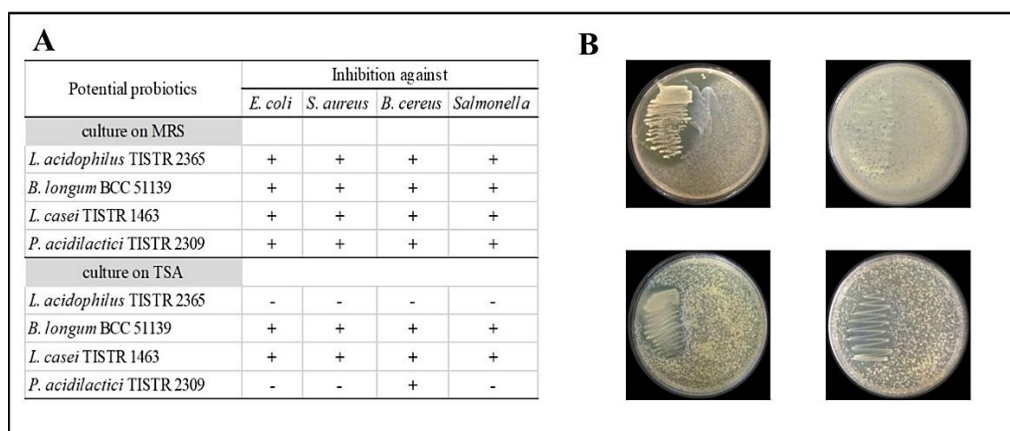


Figure 1. Antagonistic activity of probiotic bacteria on some foodborne diseases. A) Positive suppression when probiotic strains were grown on MRS and TSA. B) The pictures illustrate as follows: top left, *E. coli* overlaid on *P. acidilactici* streaked MRS; top right, on TSA for half a plate; bottom left, *S. aureus* overlaid on *B. longum* streaked on half a plate of MRS; and bottom right, on TSA for half a plate

substances such as bacteriocins. The results of the coaggregation percentages are illustrated in Figure 2. The coaggregation exhibits the possibility of the probiotic strains adhering to the pathogenic strains. Consequently, this phenomenon prevents the pathogenic bacteria from colonizing on the intestinal mucous (Reid *et al.*, 1988). Osmanagaoglu *et al.* (2010) reported that *P. pentosaceus* OZF had a coaggregation reaction with *E. coli* LMG 3038 and *S. Typhimurium* SL 1344 of 12.99% and 6.26%, respectively. Other literature showed that the coaggregation ability between *L. acidophilus* M92 with *E. coli* 3014 and *S. Typhimurium* were 15.11% and 15.70%, respectively (Kos *et al.*, 2003). For this present work, the results revealed that the coaggregation percentage of the probiotic strains with the test bacteria were mostly higher than 30%.

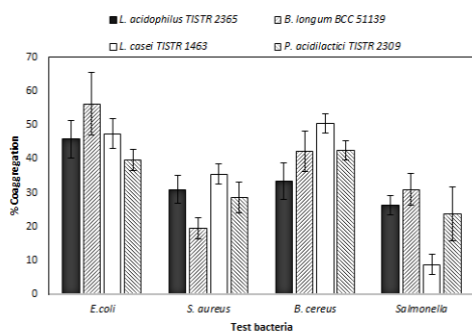


Figure 2. Coaggregation reaction between each potential probiotic strain and some foodborne bacteria. The error bars were the standard deviation of triplicate data

Cholesterol Reduction

The results of cholesterol reduction (Figure 3) exhibited that the 4 strains of probiotics used cholesterol in the culture broth differently. When starting with 6.0 $\mu\text{l/ml}$ of lipid cholesterol, the amount was reduced by *L. casei*, *P. acidilactici*, *L. acidophilus*, and *B. longum* to 0.87, 4.94, 5.88, and 0.75 $\mu\text{l/ml}$, respectively. The highest efficiency was found in *B. longum* which was able to decrease 87% cholesterol in the tube. The lowest percentage

was obtained from *L. acidophilus* which reduced approximately 3.4% of cholesterol. Cholesterol is a major cause of cardiovascular, cerebrovascular, and many other diseases (Ren *et al.*, 2014). Klaver and Meer (1993) confirmed that ingestion of fermented food containing *Lactobacillus* and *Bifidobacterium* spp. reduced the level of cholesterol in human blood. The ability of probiotic microorganisms to reduce cholesterol is due to the mechanisms involving the bile salt hydrolysis and the ability of the metabolites of cholesterol to construct cell membrane for cell growth (Argyri *et al.*, 2013; Kumar, *et al.*, 2012).

Antagonistic Assessment of Herbal Extract and Probiotics

Studies of herbal extracts on the antimicrobial effect have mostly focused on the pathogenic strain. For instance, the crude extract of *P. emblica* conquered the growths of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sp., *Klebsiella* sp., *Salmonella* sp., *Bacillus subtilis*, and *Bacillus Cereus* (Nahor and Ahmed, 2012). However, the antagonistic effect on probiotic bacteria has not been studied. The results in Table 2 disclosed that 6 herbal solutions (1 mg/ml) had no bactericidal effect against the 4 probiotic strains tested. Although *P. emblica* showed a few inhibition zones on *B. longum* and *P. acidilactici*, the suppression could be due to the acidic pH of this herb which was very low (pH 4). Meanwhile, each of the other aqueous herbal solutions had a higher pH (>pH 7). Therefore, these herbal

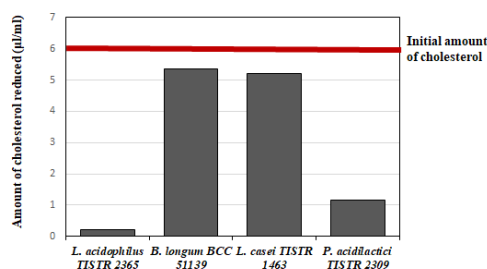


Figure 3. Amount of cholesterol decreased by probiotic bacteria. (The bold line indicates the initial amount of cholesterol)

Table 2. Determination of the herbal extract solution on suppression of probiotic bacteria

Extract	Inhibition zone (mm)			
	<i>B. longum</i> BCC 51139	<i>P. acidilactici</i> TISTR2309	<i>L. acidophilus</i> TISTR 2365	<i>L. casei</i> TISTR1463
<i>Cordyceps sinensis</i>	0	0	0	0
<i>Ganoderma lucidum</i>	0	0	0	0
<i>Tagetes erecta</i>	0	0	0	0
<i>Terminalia chebula</i>	0	0	0	0
<i>Terminalia bellirica</i>	0	0	0	0
<i>Phyllanthus emblica</i>	9.70 ±0.57	10.00±0.23	0	0

Mean ± SD, n = 3

Table 3. Survival numbers of probiotic bacteria after incubating the capsules in various acidic and bile salt conditions for 3 and 24 h, respectively

Freeze-dried bacteria	Number of cell survivals (Log CFU/g)					
	pH 6.4	pH 3	pH 2	0% Bile salt	0.3% Bile salt	0.5% Bile salt
<i>L. acidophilus</i> TISTR 2365	7.49±0.04	7.34±0.31	7.00±0.13	9.18±0.16	< 2.7	< 2.7
<i>B. longum</i> BCC 51139	6.87±0.07	6.54±0.47	6.37±0.19	9.13±0.06	6.16±0.15	4.96±0.24
<i>L. casei</i> TISTR 1463	7.03±0.07	6.86±0.08	6.47±0.07	9.76±0.10	6.97±0.07	5.16±0.41
<i>P. acidilactici</i> TISTR 2309	7.15±0.06	6.87±0.05	6.68±0.07	10.09±0.04	7.79±0.08	6.95±0.05

Data show mean ± SD, n = 3.

extracts might be consumed at the same time as probiotic bacteria without destroying them.

Properties of Probiotic Bacteria and Herbal Formula in Model Capsule

From the previous results, a model capsule of the herbal extract plus the probiotics strains was developed. The Triphala formula was selected to be the herbal component in the capsule because of the high percentage of both anti-inflammation and radical scavenging properties, and also its recognition in traditional medicine. The 500 mg capsule was composed of the Triphala formula (62.5% *T. chebula*, 25% *T. bellirica*, and 12.5% *P. emblica*) and each freeze-dried probiotic strain (4:1 ratio). The acid and bile salts tolerance of the probiotics in the model capsules were determined (Table 3). The pH 2 and pH 3, and the 0.3% and 0.5% bile salts are similar to those found in the stomach and small intestine, respectively. The results revealed that all probiotics were resistant to acidic conditions because the cell numbers dropped approximately 0.5 log CFU/g. Nonetheless, *L. acidophilus* TISTR 2365 was the most tolerant to bile salt. It has been

reported that the acid could interfere with the peptidoglycan of gram-positive bacteria and changed the membrane composition which culminated in the cell structural alteration (Sanhueza *et al.*, 2015). Bile salt also affected the lipid on the cell membrane and it affected the expression of the genes that maintained the properties of the cell wall and cell membrane (Muganga *et al.*, 2015).

The anti-inflammation and antioxidant activity of the Triphala in the model capsule appeared to be stable for a long period. The % DPPH of the capsule formula after 8 months' storage remained high at 95.76 %. The anti-inflammation efficiency was 19.62% when tested with 10 mg of Triphala. Therefore, the 400 mg of Triphala in the capsule would be equal to 45 mg of diclofenac sodium.

The decimal reduction time (D-value) of the viable and culturable probiotics was carried out in order to predict the shelf-life of the model capsule. The D-value is the time it takes for the microorganism to decrease 10% (or 1 log) under a certain condition. Figure 4 shows that *L. acidophilus* TISTR 2365 had the longest survival time in the capsules kept at

room temperature. Meanwhile, the culturability of *B. longum* BCC 51139 and *L. casei* TISTR 1463 dropped 10 times after 15 days of the storage period at room temperature. Therefore, the initial number of probiotic microorganisms in the product is important and must be correlated to the expected shelf-life of the capsule in accordance with the required regulations. As stated by the announcement of the Ministry of Public Health in Thailand, the number of probiotics in a food supplement at the time of consumption must be $>6 \log$ CFU/g. In addition, there are also other factors that could affect the storage life of a product, such as packaging and storage temperature (Pimentel *et al.*, 2015).

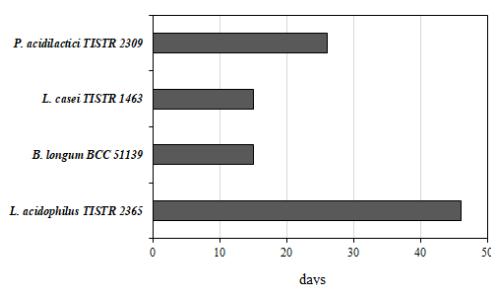


Figure 4. Decimal reduction time of probiotic bacteria packed in model capsules

Conclusions

The commercial powders of 6 herbal extracts were examined for the existence of some properties. The anti-inflammatory and antioxidant functions were subsistent in different percentages. Some probiotic properties of 4 potential strains were also determined. The *B. longum* BCC 51139 and *L. casei* TISTR 14634 seemed to be the appropriate strains because of their highly effective cholesterol reduction and their anti-foodborne bacterial effect. The solution of the herbal extract in this work was proven to be able to reconcile with the probiotic bacteria. Hence, the model capsules of a food supplement containing the mixture of some herbal extract powders and probiotics were developed. The shelf-life was estimated using

the D-value of the probiotic bacteria. The bioactive availability of Triphala in the capsule remained high for a long storage period. Although the survival cells of the *B. longum* and *L. casei* in the acid and bile salts simulating the stomach and intestine conditions were high, the viability of the cells after storage at room temperature was lower than those of *P. acidilactici* and *L. acidophilus*. This current research revealed an efficient approach to develop the new food supplement with the use of decimal reduction time to aid the prediction of the storage life of the probiotic bacteria.

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References

- Anderson, R.A., Conway, H.F., Pfeifer, V.F., and Griffin, E.L., Jr. (1969). Gelatinization of corn grits by roll-and extrusion-cooking. *Cereal Sci. Today*, 14(1): 4-12.
- Argyri, A.A., Zoumpopoulou, G., Karatzas, K.A., Tsakalidou, E., Nychas, G.J., Panagou, E.Z., and Tassou, C.C. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.*, 33:282-291.
- Erjavec, J., Kos, J., Ravnikar, M., Dreo, T., and Sabotič, J. (2012). Proteins of higher fungi - from forest to application. *Trends Biotechnol.*, 30(5):259-273.
- FAO/WHO. (2002). Guidelines for the Evaluation of Probiotics in Food. Joint Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report, London, ON, Canada, p. 1-11.
- Font de Valdez, G. and Taranto, M. (2001). Cholesterol reduction and bile salt hydrolase activity. In: *Food Microbiology Protocols*. Spencer, J.F.T. and Ragout de Spencer, A.L. (eds). Humana Press Inc., Totowa, NJ, USA, p. 171-181.
- Handley, P.S., Harty, D.W.S., Wyatt, J.E., Brown, C.R., Doran, J.P., and Gibbs, A.C.C. (1987). A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate

- strains of *Streptococcus salivarius*. J. Gen. Microbiol., 133:3,207-3,217.
- Holliday, J. and Cleaver, M. (2008). Medicinal value of the caterpillar fungi species of the genus *Cordyceps* (Fr.) link (Ascomycetes): A review. Int. J. Med. Mushrooms, 10(3):219-234.
- Kaur, S., Arora, S., Kaur, K., and Kumar, S. (2002). The *in vitro* antimutagenic activity of Triphala - an Indian herbal drug. Food Chem. Toxicol., 40:527-534.
- Kaur, J., Shabir, S., and Khan, M.U. (2016). *In vitro* antioxidant and antiinflammatory activity of *Calendula officinalis* and *Mimosa pudica*. World J. Pharmacol. Pharm. Sci., 5 (2):1,388-1,391.
- Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., and Fakiri, E.M. (2013). Health benefits of probiotics: A review. International Scholarly Research Notices, 481651. doi: 10.5402/2013/481651,
- Klaver, F.A.M. and Meer, R. (1993). The assumed assimilation of cholesterol by *Lactobacilli* and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. Appl. Environ. Microb., 59(4):1,120-1,124.
- Kos, B., Susković, J., Vuković, S., Simpraga, M., Frece, J., and Matosić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J. Appl. Microbiol., 94(6):981-987.
- Kumar, M., Nagpal, R., Kumar, R., Hemalatha, R., Verma, V., Kumar, A., Chakraborty, C., Singh, B., Marotta, F., Jain, S., and Yadav, H. (2012). Cholesterol-lowering probiotics as potential biotherapeutics for metabolic diseases. Exp. Diabetes Res., 902917. 14p. doi: 10.1155/2012/902917902917,
- Manzocco, L., Anese, M., and Nicoli, M.C. (1998). Antioxidant properties of tea extracts as affected by processing. LWT-Food Sci. Technol., 31:694-698.
- Muganga, L., Liu, X., Tian, F., Zhao, J., Zhang, H., and Chen, W. (2015). Screening for lactic acid bacteria based on antihyperglycaemic and probiotic potential and application in synbiotic set yoghurt. J. Funct. Foods, 16:125-136.
- Nahor, U. and Ahmad, Z. (2012). Antimicrobial activity of *Phyllanthus Emblica* and *Allium Sativum*: Comparative analysis of antimicrobial action of crude and ethanolic extract of these natural plant products. J. Pharm. Biol. Sci., 4(3):21-26.
- Naik, G.H., Priyadarsini, K.I., Bhagirathi, R.G., Mishra, B., Mishra, K.P., Banavalikar, M.M., and Mohan, H. (2005). *In vitro* antioxidant studies and free radical reactions of triphala an ayurvedic formulation and its constituents. Phytother. Res., 19(7):582-586.
- Nimal, S.A., Pal, S.C., Otimenyin, S.O., Aye, T., Elachouri, M., Kundu, S.K., Thandavarayan, R.A., and Mandal, S.C. (2013). Contribution of herbal products in global market. The Pharma Review. Nov- Dec, p. 95-104.
- Ohmomo, S., Kobayashi, M., Yajima, M., Suyanandana, P., Sudka, P., and Bomchai, P. (1999). Screening of thermophilic lactic acid bacteria producing bacteriocins in the tropics. Japan Agricultural Research Quarterly, 33:125-131.
- Osmanagaoglu, O., Kiran, F., and Ataoglu, H. (2010). Evaluation of *in vitro* probiotic potential of *Pediococcus pentosaceus* OZF isolated from human breast milk. Probiotics Antimicro., 2(3):162-174.
- Pimentel, T.C., Madrona, G.S., Garcia, S., and Prudencio, S.H. (2015). Probiotic viability, physicochemical characteristics and acceptability during refrigerated storage of clarified apple juice supplemented with *Lactobacillus paracasei* ssp. *paracasei* and oligofructose in different package type. LWT-Food Sci. Technol., 63:415-422.
- Rajan, S.S. and Antony, S. (2008). Hypoglycemic effect of triphala on selected non insulin dependent diabetes mellitus subjects. Ancient Science of Life, 27(3):45-49.
- Rasool, M. and Sabina E.P. (2007). Antiinflammatory effect of the Indian ayurvedic herbal formulation Triphala on adjuvant-induced arthritis in mice. Phytother. Res., 21(9):889-894.
- Reid, G., McGroarty, J.A., Angotti, R., and Cook, R.L. (1988). *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Can. J. Microbiol., 34(3):344-351.
- Ren, D., Li, C., Qin, Y., Yin, R., Du, S., and Ye, F. (2014). *In vitro* evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. Anaerobe, 30:1-10.
- Sabina, E.P. and Rasool, M. (2008). An *in vivo* and *in vitro* potential of Indian ayurvedic herbal formulation Triphala on experimental gouty arthritis in mice. Vasc. Pharmacol., 48:14-20.
- Sandhya, T., Lathika, K.M., Pandey, B.N., and Mishra, K.P. (2006a). Potential of traditional ayurvedic formulation, Triphala, as a novel anticancer drug. Cancer Lett., 231:206-214.
- Sandhya, T., Lathika, K.M., Pandey, B.N., Bhilwade, H.N., Chaubey, R.C., Priyadarsini, K.I., and Mishra, K.P. (2006b). Protection against radiation oxidative damage in mice by Triphala. Mutat. Res-Gen. Tox. En., 609:17-25.
- Sanhueza, E., Paredes-Osses, E., González, C.L., and García, A. (2015). Effect of pH in the survival of *Lactobacillus salivarius* strain UCO_979C wild type and the pH acid acclimated variant. Electron. J. Biotechnol., 18(5):343-346.
- Sanodiya, B.S., Thakur, G.S., Baghel, R.K., Prasad, G.B., and Bisen, P.S. (2009). *Ganoderma lucidum*: a potent pharmacological macrofungus. Curr. Pharm. Biotech., 10(8):717-742.
- Savedboworn, W., Riansa-ngawong, W., Sinlapacharoen, W., Pajakang, S., Patcharajarukit, B., and Tipkanon, S. (2014). Assessment of probiotic properties in lactic acid bacteria isolated from fermented vegetables. Appl. Sci. Eng. Prog., 7(4):53-65.
- Savini, M., Cecchini, C., Verdenelli, M.C., Silvi, S., Orpianesi, C., and Cresci, A. (2010). Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. Nutrients, 2(3): 330-339.
- Socol, C.R., de Souza Vandenbergh, L.P., Porto, L., Spier, M.R., Medeiros, A.B.P., Yamagishi, C.T., De Dea Lindner, J., Pandey, A., and Thomaz-

- Soccol, V. (2010). The potential of probiotics: a review. *Food Technol. Biotech.*, 48(4):413-443.
- Wang, J., Kan, L., Nie, S., Chen, H., Cui, S.W., Phillips, A.O., Phillips, G. O., Li, Y., and Xie, M. (2015). A comparison of chemical composition, bioactive components and antioxidant activity of natural and cultured *Cordyceps sinensis*. *LWT-Food Sci. Technol.*, 63: 2-7.
- Yuen, J.W. and Gohel, M.D. (2005). Anticancer effects of *Ganoderma lucidum*: a review of scientific evidence. *Nutrition and Cancer*, 53(1):11-17.
- Zago, M., Fomasari, M.E., Carminati, D., Burns, P., Suárez, V., Vinderola, G., Reinheimer, J., and Giraffa, G. (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiol.*, 28:1,033-1,040.
- Zaveri, H., Rathva, V., Sant, A., and Dave, D. (2016). Triphala: an alternative therapy in periodontics – a critical review. *Journal of Clinical Periodontology and Implant Dentistry*, 1(1):5-11.