Stability of spray-dried synbiotics containing *Lactobacillus plantarum* DSM 2648 and exopolysaccharide from *Pediococcus acidilactici* TISTR 2612 and its vivo effectiveness

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**ABSTRACT**

Spray drying is an economical process for dehydrating microorganisms into a powder form that preserves viability for use at the industrial scale. It is especially applicable for probiotics and other temperature sensitive functional foods. In this study, the survival and functionality of synbiotics were evaluated after spray drying. A mixture of stationary phase probiotic culture (*Lactobacillus plantarum* DSM 2648) and 2 % (w/v) of exopolysaccharide (EPS) produced from *Pediococcus acidilactici* TISTR2612 was spray-dried with outlet air temperature of 70 ±1°C. Results showed a probiotic survival rate decrease from 96.74 % (log 10.04 CFU/g) after spray-drying to 96.01, 96.00, 93.18 and 92.17 % during room temperature storage in a desiccator for 1, 2, 3 and 4 weeks, respectively. In simulated gastric juice (pH 2) and in 2 % of bile salts solution, probiotics survival rates were 97.94 and 91.56 % and EPS hydrolysis reached 3.26 and 7.23 %, respectively, after incubation at 37 °C for 12 h. In vivo testing of the impact of synbiotics on reducing colorectal cancer risk showed a significant decrease of β-glucuronidase bacterial enzyme activity, with 36.56 and 50.55 % reduction in male and female rats, respectively. Finally, the acute oral toxicity (LD50) testing was performed by feeding rats with the maximum dose of synbiotics (5 g per kg body weight). No mortality or organ abnormalities (from necropsy findings) were observed in any of the rats after oral administration for 15 days. These results suggest that synbiotics could potentially be a safe functional food for aiding disease prevention in the future.

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INTRODUCTION

Functional foods are well known for their abilities to improve functions in the body by reducing inflammation, fighting off disease, supporting good gut health, and improving nutrient absorption, among other benefits. Several food components, including carotenoids, dietary fiber, fatty acids, flavonoids, specific minerals, prebiotics and probiotics have been linked to functional benefits (FAO, 2007). Probiotics and prebiotics are two food components that can be used together for greater effect. Probiotics are living microorganisms that are beneficial to the digestive system, and prebiotics are nondigestible food ingredients that help stimulate probiotic growth and activity. The consumption of probiotics may help reduce fecal bacteria, intestinal inflammation, and incidence of colorectal cancer (CRC) (O’Mahony et al., 2001; Biliboni et al., 2005; Gaudier et al., 2005). To confer these and other health benefits, probiotics should be ingested in adequate amounts, usually at 10^10–10^12 CFU/g of product (FAO/WHO, 2002; FAO/WHO, 2006). By improving the viability of probiotics, such as bifidobacteria and lactobacilli in the colon, and inhibiting the growth of pathogenic bacteria, probiotics can further improve gut microbial balance (Fuller, 1989; Gibson and Roberfroid, 1994). When a mixture of probiotics and prebiotics is used in synergy, the result is a symbiotic formulation. Symbiotics ensure greater probiotic proliferation in the gut and more efficient implantation in the colonic microbiota by using complimentary prebiotics to improve the survival of probiotic bacteria during passage through the upper intestinal tract (Roberfroid, 1998; Goorbyere et al., 2011). Saulnier et al. (2008) reported that symbiotics were more effective than probiotics or prebiotics alone in modulating the gut microflora by increasing the beneficial bacteria. In CRC biomarker investigation, the combination of Bifidobacterium longum and inulin was more efficient at decreasing β-glucuronidase activity on colonic aberrant crypt fo1d (ACF) than B. longum or inulin alone (Rowland et al., 1998). Moreover, dietary symbiotics significantly reduced colorectal proliferation, DNA damage and the capacity of fecal water to induce necrosis in polyp-pectomized patients and favorably altered the colonic bacteria ecosystem in polypl and colon cancer patients (Rafter et al., 2007). Thus, symbiotics are gaining interest for dietary supplementation to reduce multiple factors associated with colon cancer risk in humans.

CRC is the third most commonly diagnosed cancer among men and women in the United States and the fourth leading cause of cancer-related deaths in the world (Ahnold et al., 2016; Rebecca et al., 2017). In Thailand, CRC cases are increasing with average annual change of 4.1% and 3.3% in men and women, respectively (Virani et al., 2017). The factors with “Westernization” such as obesity, smoking, alcohol consumption, low fruit-vegetables intake and physical inactivity are associated with this considerable increasing rate (Center et al., 2009). Therefore, the decrease incidence of CRC is likely driven by changes in lifestyle and dietary patterns (Ahnold et al., 2016) and development of functional food ingredients is an alternative of interest.

Spray drying is a dehydration process that can be used to produce viable probiotic powders for incorporation into food systems (Fu and Chen, 2011). This process offers high production rates and low operating costs (Corcoran et al., 2004). Process parameters of spray drying, including inlet and outlet air temperatures, feed flow rate, residence time at drying chamber, thermodynamic process, species and strains type, adaptation of the bacteria to heat or osmotic stress condition, growth state are associated with the survival of probiotics (Gardier et al., 2000; Ananta et al., 2005; Lapsiri et al., 2012; Behboudi-Johobedh et al., 2013; Bustos and Borquez, 2013). In addition, stationary phase cultures of bacteria are suitable for the spray drying process (Corcoran et al., 2004).

In a previous study, the combination of Lactobacillus plantarum DSM 2648 and exopolysaccharide (EPS) produced from Pediococcus acidilactici TISTR 2612 showed the highest potential for β-glucuronidase reduction (Chaiongkarn, et al., 2017). β-glucuronidase is associated with the conversion of procarcinogens to carcinogens in colon cancer (Goldin and Gorbach, 1976; Goldin and Gorbach, 1980). In the present study, spray drying with outlet air temperature of 70 ±1°C was used for incorporating the combination of L. plantarum and EPS as a symbiotic ingredient for further use in functional food or dietary supplements. The aims of this study were to examine the survival and stability of L. plantarum DSM 2648 after spray drying and during powder storage in the presence of 2 % w/v EPS produced by P. acidilactici TISTR 2612 as a probiotics carrier and to investigate the effectiveness of this symbiotic product for reducing the risk of CRC via in vivo assessment of fecal bacteria enzyme investigation (β-glucuronidase).

MATERIALS AND METHODS

Preparation of EPS from P. acidilactici TISTR 2612

A single colony of P. acidilactici TISTR 2612 was grown in 5 mL of MRS broth (Himedia, India) at 37°C under anaerobic condition for 16-18 h. Fresh overnight culture was scaled up to 100 and finally to 1,000 mL (at the rate 5% v/v) in MRS broth containing 1% sucrose for EPS inducing at 37°C under anaerobic condition for 48 h (Dathong et al., 2016). The cells were removed after centrifugation at 8,000 rpm for 10 min. The supernatant was then collected and concentrated by rotary evaporator to one third of its volume. EPS was precipitated with two volumes of cold 95% ethanol. The mixture was stored at 0°C for 48 h and centrifuged at 8,000 rpm for 10 min. The supernatant was discarded and the EPS was resuspended in 10-50 mL of distilled water and then freeze-dried. A 2% EPS solution was prepared by dissolving EPS powder in 500 mL of distilled water, followed by autoclaving at 121°C for 15 min.

Preparation of probiotic culture for spray drying

In an initial experiment that defined the growth of L. plantarum DSM 2648 under broth culture conditions, the stationary phase was reached after 12-24 h of fermentation (Chaiongkarn et al., 2017). A single colony of L. plantarum DSM 2648 (DSMZ Bacteria collection, Germany) was grown in 5 mL of MRS broth (Himedia, India) under anaerobic condition at 37°C for 16-18 h. One percent of the starter cell solution (with OD600 of 0.5) was added to 50 mL MRS broth and incubated at 37°C for 16-18 h. A 50 mL of the cell suspension was scaled up to 500 mL in MRS broth (at the rate 10% v/v) and placed at 37°C for 24 h. Stationary phase L. plantarum DSM 2648 was harvested after centrifugation at 8,000 rpm for 10 min and resuspended in 200 mL of 0.85% of sterile NaCl. The cell suspension was centrifuged and washed repeatedly at least 2 times. Finally, probiotics cell pellet was resuspended with 500 mL EPS solution (2% w/v). To validate the survival rate of probiotics during the drying process, cell counts were observed before and after spray drying.
A 0.5 mL cell suspension was collected for serial dilution in 4.5 mL of normal saline while 0.5 g of spray-dried sample was rehydrated in 5 mL of normal saline and was serially diluted and plated on MRS agar+0.5% CaCO3 under anaerobic conditions at 37°C for 48 h. The growth of the probiotics was enumerated as CFU/g (of dry matter) and cell counts were expressed as mean of duplicate measurement.

Spray-drying process and storage

A laboratory scale spray dryer (Buchi mini spray dryer, model B290, Flawil, Switzerland) was used to process synbiotic microparticles at an air inlet and outlet temperature of 135-140°C and 70±1°C, respectively. The feed solution was atomized into droplets with 0.7 mm i.d. nozzle, at aspirator pressure of 100 %, atomizer pressure of 650 Nl/h and flow rate of 12 mL/min. The percentage of bacteria survival was calculated as following: % survival = (N/N0) x 100, where N was the number of bacteria in the spray-dried powder (CFU/g) and N0 represented the number of bacteria in the cell solution before drying (described above). Aliquot powders of synbiotics were placed in sterile bottles sealed with parafilm and stored in a desiccator at room temperature. Viability of probiotics was determined at 1, 2, 3 and 4 weeks. For long-term investigation, synbiotics powders were kept in vacuum sealed aluminum foil bags and stored at 4°C and room temperature for 8 month.

Simulated gastrointestinal and bile salts solution tolerance test

Simulated gastric juice (SG) was prepared as described by Lian et al. (2003). Briefly, 0.3% of pepsin was dissolved in 0.5% (w/v) NaCl solution, adjusted to pH 2.0 with 1 M HCl and 1 M NaOH and sterilized through a membrane with 0.2 μm (Life Sciences, Ann Arbor, MI, USA). The solution should be used immediately and could be kept in the fridge (not longer than 24 hours). Bile salt (Himedia, India) was dissolved in distilled water to a concentration of 2 % (w/v) and the solution was sterilized at 121°C for 15 minutes. One gram of spray dried synbiotic microparticles was separately dissolved in 10.0 mL of SG (pH 2) and 2% of bile salts solutions. The suspensions were taken at 0 h and 12 h after incubation at 37°C to determine viability of *L. plantarum* DSM 2648. EPS hydrolysis was determined following the methods by Nelson N, (1944), Dubois et al. (1956), Krasse and Carlsson (1970) and Wichienchot et al. (2010). The hydrolysis degrees of samples were calculated according to the formula as following, where reducing sugar released is the difference between reducing sugar content at specified time and initial reducing sugar content.

\[
\text{Hydrolysis degree } (%) = \frac{\text{Reducing sugar released}}{\text{Total sugar-initial reducing sugar}} \times 100
\]

**Animals**

This study was approved by the TISTR Animal Ethical Committee no.T58001. Wistar rats (7 weeks old) weighting 267-304 g and 181-210 g (male and female) were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom. The rats were allowed to acclimatize to the housing condition with standardized conditions according to animal experiment guidelines at 24 ± 2°C, 50 ± 10% humidity, 12 h light/dark cycle. The rats were housed in cages bedded with dried corn cob and dried water hyacinth, and fed standard laboratory animal food pellets and RO drinking water *ad libitum.*

**Treatments and β-glucuronidase assay**

Rats were randomly divided into 2 groups of each gender to experiment and control group (n=5/each group). In treatment group; rats were orally administered with UHT milk containing 2% synbiotic products (5 mL/kg body weight) with containing log 9 to log 10 CFU/g of probiotics. For the control group; the rats were fed UHT milk only. The rats were fed via oral gavage daily for 4 weeks. After dosing, all animals were observed once per day and their body weights were recorded weekly and at the end of the test. The mean body weight gain of the test group animals was calculated and compared to that of the control group rats using ANOVA (p-value ≤ 0.05). At the end of the test, all survivors were homogenized by CO2 asphyxiation. Likewise, the fresh feces of each rat in the treatment group was taken from the large intestinal part of the caecum after death and placed in sterile aluminum foil and then mixed until homogenization. The homogenized-fecal samples (1.5 g) were dissolved in 30 mL phosphate buffer at pH 7.0 and sonicated for 2 min (4 cycles, 30 sec, 0°C). The suspension of feces was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was collected to a sterile tube and kept at -80°C until it was carried out for the assessment of β-glucuronidase. The β-glucuronidase assay was conducted as described by Goldin and Gorbach (1976). The protocol for enzymatic assay of β-glucuronidase (EC 3.2.1.31, Sigma Chemical Co., St. Louis, Mo.) was based on the determination of phenolphthalein released from phenolphthalein β-D-glucuronide and the results were displayed as units/g solid ±SD.

**Acute oral toxicity testing**

This experiment was approved by TISTR Animal Ethical Committee no.50001. The acute oral toxicity determination (LD50) of synbiotics was carried out with slight modification to the Test Guideline No. 423: Acute oral toxicity-Acute toxic class method of the OECD Guidelines for Testing of Chemicals (2001). Rats were randomly divided into 3 groups of each gender (n=5/each group). All animals were fasted for 16 h with free access to water. After fasting, the rats were administered synbiotics orally at dose 2 g/kg and 5 g/kg. Distilled water was given to the control group. After administration of the test samples, mortality and clinical signs which included changes in skin, fur, eyes and mucous membranes were noted at the first 4 h subsequently for 72 h and thereafter for 15 days. The body weight was recorded at day-1 (after fasting), day-8 and day-15. At the end of the test (day-15), all survivors were weighed and homogenized by CO2 asphyxiation (AVMA Guidelines for the Euthanasia of Animals, 2013) and any gross pathological changes were recorded for each rat. The mean body weight gains of the treatment groups were calculated in comparison to the control group.

**Scanning electron microscopy (SEM)**

EPS and synbiotics microparticles were sent to National Metal and Materials Technology Center (MTEC) for morphology analysis. The samples were sputter coated with a thin layer of gold. Scanning electron microscopy (SEM) micrographs were acquired using a scanning electron microscope (HITACHI, S-3400N, Japan) at an accelerating voltage of 20 kV.

**Statistical analysis**

The results of three replicates were displayed as mean ±SD. Statistical analysis was performed by SPSS (IBM SPSS Statistics Software version 18, Co., Ltd., Thailand). The difference between the means was analyzed by Turkey’s method at p-value < 0.05.
RESULTS AND DISCUSSION

Survival and stability of probiotics after spray drying process

After spray drying, the yields of synbiotic microparticles were between 65 and 80 % with probiotics survival at 96.74% as shown in Figure 1(a) N0 = log 10.38±0.08 and N=log 10.04±0.11 CFU/g. Results indicated that EPS was suitable for probiotics encapsulation with high survival rate during spray drying process. Jantzen et al. (2013) found that survival rate of L. reuteri decreased by log 2 cycles after drying and log 1 cycle during 4 weeks of storage when bacteria was encapsulated with whey from slurry fermentation. Carrier media can improve survival rates after the spray drying process and enhance survival in simulated gastrointestinal conditions (Hamsupo et al., 2005; Paez et al., 2012). To investigate product stability during storage at RT, the viable numbers of L. plantarum DSM 2648 significantly decreased after a storage period for 4 weeks (p-value < 0.05), according for the survival of 96.01, 96.00, 93.18 and 92.17 %, respectively. Although the survival of probiotics tends to decrease during storage at RT, the viability of probiotics did not decrease from the beginning of storage at log 10.04 CFU/g when the synbiotic microparticle was stored under vacuum seal at RT and 4°C for 8 month (showing 110.25 and 110.57 % survival, respectively). The results suggested that for prolonged shelf-life probiotic products should be kept at vacuum seal due to oxygen content as an important factor affecting probiotic survival and growth during the storage period (Tripathi et al. 2014).

For the morphological analysis of spray-dried microparticles, EPS had smooth spherical shape Figure 2 (a) while L. plantarum DSM 2648-encapsulated with EPS displayed bacteria cells covered with EPS Figure 2 (b). Thus, the high survival of probiotics during the spray drying was probably due to thermal protection of probiotics cells with EPS (Fritzen-Freire et al., 2012).

Figure 1. Probiotics survival after spray drying (a) and during storage at RT for 4 weeks (b)

Figure 2. SEM of EPS without probiotics (a) and powder of synbiotics (b) using high vacuum technique (scale bar 100 μm). The arrow indicated the L. plantarum DSM 2648 was covered with EPS.
Tolerance to simulated gastrointestinal (SGI) and bile salts solution

The survivals of microencapsulated cells of \(L.\) \textit{plantarum} DSM 2648 were 97.94\% and 91.56\% after 12 h exposure to SGI pH 2 and 2\% bile salts solution, respectively, while the EPS was hydrolyzed by 3.26\% and 7.23\%, respectively (Table 1). The results suggested that \(L.\) \textit{plantarum} DSM 2648 and EPS produced by \(P.\) \textit{acidilactici} TISTR 2612 were more susceptible to 2\% bile salts than SGI (pH 2). More than 80\% of viable probiotics and more than 90\% of EPS were resistant to SGI (pH2) and 2\% bile salts solution.

The obtained results indicated that EPS produced from \(P.\) \textit{acidilactici} TISTR 2612 could protect \(L.\) \textit{plantarum} DSM 2648 during the spray drying process, SGI pH 2 and 2\% of bile salts solution.

Table 1. Survival rate of \(L.\) \textit{plantarum} DSM 2648 and EPS hydrolysis after 12 h exposure to SGI pH 2 and 2\% bile salts solution

<table>
<thead>
<tr>
<th>% Survival of (L.) \textit{plantarum} DSM 2648</th>
<th>% Hydrolysis of EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGI (pH 2)</td>
<td>97.94</td>
</tr>
<tr>
<td>2% bile salts</td>
<td>91.56</td>
</tr>
</tbody>
</table>

The effectiveness on \(\beta\)-glucuronidase reduction

The effectiveness of probiotic microorganisms on reducing the risk of CRC \textit{in-vivo} was investigated via \(\beta\)-glucuronidase enzyme activity. \Five grams of synbiotics per kg of rat body weight per day was used in all periods of experimentation. After a period of 4 weeks, there were no significant differences in final body weight between dietary group in the same gender, showing 406.4 ± 10.8 and 404.0 ± 9.0 in male rats and 250.6 ± 11.5 and 246.60 ± 6.1 in female rats of the control and treatment group, respectively. On the other hand, the \(\beta\)-glucuronidase enzyme activity of rats treated with synbiotics was significantly lower than the control (p-value =0.000) showing 36.56 % and 50.55 % reduction in male and female rats, respectively.

Fecal bacteria enzymes such as \(\beta\)-glucuronidase, nitroreductase and azoreductase commonly produced by \(Bacteroides\), \(Clostridium\), and \(Enterobacteriaceae\) are associated with the risk of colon cancer (Raman \textit{et al.}, 2013), as their activities generate toxic and carcinogenic substances. Fecal enzyme activity can be influenced by diet (Goldin and Gorbach, 1976). Hylia \textit{et al.} (1998) found that the consumption of resistant starch significantly decreased total concentrations of fecal bile acids in healthy volunteers that may be relevant for cancer prevention. Results from the present study suggested that probiotics microorganisms have potentially important effects on decreasing the risk of CRC \textit{in-vivo} experiments. In the previous study, oligofructose-based prebiotics such as fructooligosaccharides and inulin were emphasized for colon cancer protection. In DMH-induced CRC in rats study, Hughes and Rowland (2001) found that oligofructose and long chain inulin had protective effects at an early stage in the onset of cancer. An administration of \(L.\) \textit{rhamnosus} GG and \(L.\) \textit{acidophilus} to DMH-treated Sprague Dawley rats was more efficient at decreasing aberrant crypt foci (ACF) counts and \(\beta\)-glucuronidase activity (Verma and Shukla, 2012). In this experiment, we found a new source of prebiotics (EPS produced from \(P.\) \textit{acidilactici} TISTR 2612) that had a potential effect to reduce the risk of CRC.

Figure 3. \(\beta\)-glucuronidase enzyme activity of investigated rats after 4 weeks of feeding with synbiotics for 4 weeks. ** p-value < 0.05

Table 2. Summary data from acute oral toxicity (LD50) in rats

<table>
<thead>
<tr>
<th>Gender</th>
<th>Groups</th>
<th>Body weight increasing (g)</th>
<th>Gross pathology after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days 8</td>
<td>Days 15</td>
</tr>
<tr>
<td>Male rats</td>
<td>Control</td>
<td>9.60 ± 0.60</td>
<td>61.00 ± 3.08</td>
</tr>
<tr>
<td></td>
<td>2 g/kg body weight</td>
<td>9.60 ± 0.81</td>
<td>71.20 ± 2.80*</td>
</tr>
<tr>
<td></td>
<td>5 g/kg body weight</td>
<td>9.40 ± 0.68</td>
<td>58.20 ± 3.42</td>
</tr>
<tr>
<td>Female rats</td>
<td>Control</td>
<td>13.40 ± 2.15</td>
<td>33.40 ± 2.63</td>
</tr>
<tr>
<td></td>
<td>2 g/kg body weight</td>
<td>14.80 ± 2.41</td>
<td>34.40 ± 2.01</td>
</tr>
<tr>
<td></td>
<td>5 g/kg body weight</td>
<td>10.20 ± 0.58</td>
<td>24.40 ± 1.47</td>
</tr>
</tbody>
</table>

*p-value increasing (mean SE), mean comparison of final body weight using ANOVA (* indicate p-value < 0.05)

Acute oral toxicity (LD50)

Acute toxicity determination is a method for assessing acute oral toxicity that involves a recognition of a dose level that causes mortality (Subhal and Geetha, 2017). The mortality and clinical signs which included changes in skin, fur, eyes and mucous membranes, weight were noted after rats were fed with synbiotics for the first 4 h, 8 days and thereafter for 15 days, respectively. The results showed that synbiotics did not significantly affect female rats body weight (p-value=0.687 at days 15) while male rats fed with 2 g of synbiotics had significant body weight increase from control and 5 g feeding rats at day 15. The results of acute oral toxicity test showed no mortality or abnormal organs from necropsy findings in any male or female rats (Table 2).
CONCLUSIONS

The spray drying process was suitable for production and preservation of the symbiotic products. The survival of stationary phase probiotics after spray drying at outlet air temperature of 70 ± 1°C in the presence of 2% (w/v) of EPS was high (96.74%). So, EPS produced by P. acidilactici had the capacity to protect L. plantarum DSM 2648 during spray drying process and storage. The probiotic survival rates of 96.01%, 96.00%, 93.18% and 92.17% were achieved after 1, 2, 3 and 4 weeks of storage in a dessicator, respectively. The survival rate of probiotics in stimulated gastrointestinal pH 2 and 2% of bile salts at 37°C for 12 h were 97.94% and 91.56% while the hydrolysis of EPS was reached 3.26% and 7.23%, respectively. The effectiveness of symbiotics for reducing the risk of colorectal cancer in-vivo suggested that the activity of β-glucuronidase significantly decreased in rats orally administered with 5 g of symbiotics per kg of body weight/day for 4 weeks. Further study of symbiotics indicated that no acute toxicity effects were observed in the group of rats fed with symbiotics in the maximum dose after 15 days. From these results, symbiotic microparticles could contribute to functional food ingredient development in the future.

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