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Original Article

Study of prebiotic properties from edible mushroom extraction

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ABSTRACT

The prebiotic properties were investigated of seven edible mushrooms: *Auricularia auricula-judae*, *Lentinus edodoes*, *Pleurotus citrinopileatus*, *Pleurotus djamor*, *Pleurotus ostreatus*, *Pleurotus ostreatus* (Jacq.Fr.) Kummer and *Pleurotus pulmonarius*. All mushrooms were extracted using distilled water and ethanol at a ratio of 1:4 vol per volume, respectively, at 80 °C and shaken at 150 revolutions per minute for 1–4 hr before the total carbohydrates and total reducing sugar were determined. After 3 hr of extraction, *P. ostreatus* had the maximum yield of total carbohydrates (6.7325 ± 0.0261 mg/mL) and total reducing sugar (2.6737 ± 0.0027 mg/mL). Based on high performance liquid chromatography analysis, *A. auricula-judae* had the highest levels of galactose and maltrotriose (928.26 mM and 112.59 mM, respectively), while *L. edodoes* had a high lactulose level (229.64 mM). Each mushroom extract was supplemented in Man Rogosa Sharpe broth for cultivation of probiotic strains of *L. acidophilus* and *L. plantarum*. Next, the prebiotic properties were determined based on probiotic growth stimulation, pathogenic inhibition (against *Bacillus cereus*, *Escherichia coli*, *Samonella Paratyphi* and *Stapphylococcus aureus*) and gastrointestinal tolerance (in amylase, bile extract and HCl). High probiotic growth stimulation resulted for *L. acidophilus* cultured with *L. edodoes* extract (1.9779 ± 0.0032), and for *L. plantarum* cultured with *P. pulmonarius* extract (1.9702 ± 0.0072). The widest inhibition zone of *S. Paratyphi* in the culture of *L. acidophilus* was 1.1500 ± 0.0707 cm with *P. ostreatus* extract. The highest survival percentage for gastrointestinal tolerance of probiotics after incubation for 2 hr with HCl was 13.64% for *P. djamor* extract cultured in *L. acidophilus*.

Introduction

Mushrooms provide rich bio-active compounds with immunomodulatory (Guggenheim et al., 2014), cardiovascular (Guillamon et al., 2010), anti-inflammatory (Elsayed et al., 2014), anti-diabetic (Ganeshpurkar et al., 2014), anti-oxidant and anti-microbial (Kosanić et al., 2012) benefits. Scientists have been analyzing the active compositions in mushrooms for many purposes, such as diet food (Akindahunsi and Oyetayo, 2006; Rao et al., 2009) and pharmaceutical (Siqian and Shah, 2015; Valverde et al., 2015) and cosmeceutical (Taofiq et al., 2016) applications.

Edible mushrooms contain high bioactive polysaccharide which is interesting as a functional food as well as a safe food, especially as they are a good source of prebiotic substances which contain short chain sugars such as glucose, galactose, fructose and N-acetylglucosamine (Patel and Goya, 2011) which are non-digestible carbohydrates that stimulate the growth of beneficial microorganisms. These microorganisms function as probiotics because of their potential to inhibit pathogenic microorganisms in the gastrointestinal tract. In addition, prebiotic compounds are gastrointestinal tolerant in the presence of salivary amylase, gastric juice or bile extract, maintaining properties to activate the beneficial microbes for host health (Chowdhury et al., 2015).

Many studies have reported the use of prebiotic substances from mushroom extraction as non-digestible food ingredients to demonstrate probiotic growth stimulation and pathogenic microbial inhibition. For example, medicinal mushrooms were extracted and non-digestible carbohydrate was separated to be used for growth stimulation of beneficial bacteria in the gastrointestinal tract (Gao, 2009). Extracts from some edible mushrooms such as *Lentinus edodes* and *Pleurotus eryngii* demonstrated high potential anti-microbial effects against some pathogens, for example *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium* (Tinrat, 2015). Many edible mushrooms have health-promoting qualities, including anti-cancer, anti-obesity, anti-diabetes and anti-biotic properties (Zhu et al., 2015; Friedman, 2016).

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As already mentioned, edible mushrooms have become attractive as functional food and pharmaceutical products because of their safety and availability; and are, moreover, an inexpensive and natural food. The aims of this study were to examine the prebiotic properties (probiotic growth stimulation, pathogenic inhibition and gastrointestinal tolerance) of the seven edible mushrooms; *Auricularia auricula-judae*, *Lentinus edodoes*, *Pleurotus citrinopileatus*, *Pleurotus djamor*, *Pleurotus ostreatus*, *Pleurotus ostreatus* (Jacq.Fr.) Kummer and *Pleurotus pulmonarius* were extracted by a mixture of distilled water and ethanol. Total carbohydrates and total reducing sugar were determined by spectrophotometer. Selected mushroom extracts were analyzed and quantified for their components using high performance liquid chromatography (HPLC).

Materials and methods

Materials

Seven kinds of three-month-old edible mushrooms were used in this experiment:, *Auricularia auricula-judae*, *Lentinus edodoes*, *Pleurotus citrinopileatus*, *Pleurotus djamor*, *Pleurotus ostreatus*, *Pleurotus ostreatus* (Jacq.Fr.) Kummer and *Pleurotus pulmonarius,* which were kindly provided by the Thai Traditional Medicine College, Rajamangala University of Technology Pathum Thani province, Thailand. All other chemicals were analytical grade from Sigma-Aldrich (Saint Louis, USA) and Fluka (Darmstadt, Germany).

Microorganism

Probiotic *Lactobacillus* strains (*L. acidophilus* TISTR 1338 and *L. plantarum* TISTR 1465) and pathogenic bacteria (*B. cereus, E. coli, S. Paratyphi* and *S. aureus*) were cultivated in an appropriate medium, Man Rogosa Sharpe medium (MRS), Nutrient agar (NA) or Nutrient broth (NB) from Hi-media (Bangkok, Thailand).

Mushroom extraction

All edible mushrooms were cleaned and dried at 105 °C overnight, then blended thoroughly for 5 min using a blender. Three grams of each sample were transferred into separate test tubes. All samples were extracted by adding 5 mL of a mixture of distilled water and ethanol (95% volume per volume, v/v) at a ratio of 1:4 v/v, respectively. The extracted mushrooms were incubated in a shaker at 80°Cand 150 revolutions per minute for 4 hr. Then, 1 mL of each sample was taken every 30 min and centrifuged at 10,000×g for 15 min at room temperature. After that, all supernatants were kept at 4 °C for total carbohydrate and total reducing sugar analysis. The mushroom extracts at 3 hr were collected and evaporated at 60 °C for further investigation.

Total carbohydrate determination

The total carbohydrate content of the mushroom extracts were determined using the phenol sulfuric acid method (Dubois et al., 1956). In brief, 0.25 mL of an appropriate diluted sample was prepared in a test tube and 1.25 mL concentrated sulfuric acid (95% v/v) and 0.25 mL phenol (5% v/v) were immediately added. The mixture was heated at 100 °C for 5 min, then cooled to room temperature. The total carbohydrate content of the mushroom sample was determined using colorimetry at 490 nm absorbance. Standard and blank samples were prepared and analyzed in the same way, except for adding 0.25 mL of glucose to the standard sample and 0.25 mL of distilled water to the blank sample.

Total reducing sugar determination

All mushroom extracts were determined for total reducing sugar using 3,5-dinitrosalicylic acid (DNS) assay according to (Miller, 1959). After the extraction process, each extracted mushroom sample was centrifuged at 10,000×g at room temperature for 15 min. A supernatant aliquot of 1 mL was mixed with 1 mL DNS reagent and incubated at 100 °C for 5 min. After cooling the mixture to room temperature, the reducing sugar was measured using spectrophotometry at 540 nm absorbance. The standard and blank samples were prepared and analyzed in the same way, except for adding 1 mL of glucose to the standard sample and 1 mL of distilled water to the blank sample.

High performance liquid chromatography determination

The extracted mushrooms were analyzed and the components quantified using HPLC with an Inertsil® NH2 column at 30 °C. Acetronitrile (75% v/v) in distilled water was applied as a mobile phase at a constant flow rate of 1.4 mL/min in CarboPac™ (4.6 × 250 mm), then analyzed using differential refractometry (modified method from Hernandez et al., 1998). The standard compounds (galactose, lactulose, maltotriose, maltotetraose) were used for peak identification.

Prebiotic properties

Probiotic growth stimulation

L. acidophilus and *L. plantarum* were cultured at 37 °C for 48 hr under anaerobic conditions in MRS broth (used as the control), compared with culture medium supplemented with 10 mg/mL of each mushroom extract and commercial prebiotic compounds (fructooligosaccharides (FOS) and inulin). After incubation, the cultures were quantified by measuring the optical cell density using spectrophotometry at 620 nm (Siragusa et al., 2009).

Pathogenic growth inhibition

The cultivations of *L. acidophilus* and *L. plantarum* at 37 °C for 48 hr in MRS broth complemented with 10 mg/mL of each mushroom extract were centrifuged at 10,000×g and 4 °C for 15 min. Supernatant samples were collected separately from the cell pellets for inhibition testing and tolerance determination. Pathogenic bacteria (*B. cereus, E. coli, S. Paratyphi, S. aureus*) were cultured in NB at 37 °C for 24 hr. A 50 μl aliquot sample of each pathogenic bacteria cultivation was inoculated in NA using a spread plate technique and dried. Supernatant from each probiotic culture was dropped onto the sterilized filter paper and placed onto the pathogenic bacteria plates. After incubation at 37 °C for 24 hr, inhibition efficiency was measured by comparing the diameter of the clear zone from the plate containing probiotic supernatant with that of the media without mushroom extract (control) and the culture with commercial prebiotic compounds (Rousseau et al., 2005).

Gastrointestinal tolerance

The pelleted cells of *L. acidophilus* and *L. plantarum* were washed twice with 1 mL of phosphate-buffered saline buffer at pH 7.0 and centrifuged at 8000×g and 4 °C for 15 min. Then, they were incubated in 1 mL of each of the gastrointestinal conditions (100 units/mL alpha-amylase, 0.3% w/v bile extract and 0.1 M HCl) at 37 °C for 0.5 hr, 1.0 hr, 2.0 hr and 3.0 hr. Gastrointestinal tolerance was determined by the percentage of colony forming units per milliliter of *Lactobacillus* surviving the spread plate technique in NA and incubation at 37 °C for 48 hr (Kondepudi et al., 2012).

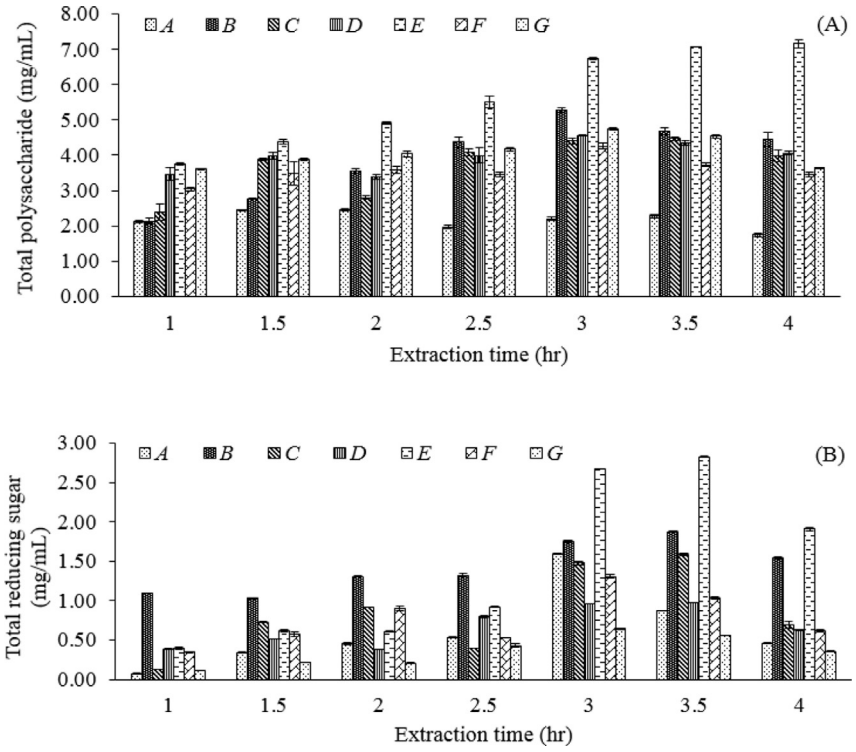

Fig. 1. Total carbohydrate (A) and total reducing sugar (B) from mushroom extraction using distilled water and ethanol at 80 °C and 150 revolutions per minute for 1–4 hr, where A = *Auricularia auricula-judae*, B = *Lentinus edodoes*, C = *Pleurotus citrinopileatus*, D = *Pleurotus djamor*, E = *Pleurotus ostreatus*, F = *Pleurotus ostreatus* (Jacq.Fr.) Kummer and G = *Pleurotus pulmonarius*, and error bars indicate ± SD.

Results and discussion

Mushroom extraction and total carbohydrate and reducing sugar determination

After mushroom extraction using a mixture of distilled water and ethanol, all mushroom extracts collected at different times were analyzed for total carbohydrate and total reducing sugar count using the phenol sulfuric acid and DNS methods. The mushroom extracts after 1 hr and 2 hr contained lower carbohydrate levels and reducing sugar than the extract after 3 hr and 4 hr (data not shown). However, both determinants had not increased compared with the samples after 3 hr of extraction. The experiment showed that the sample extracted after 3 hr had higher total carbohydrate and total reducing sugar levels than the samples extracted after the other periods. Azmi et al. (2012) reported the extraction of carbohydrates from plants, carried out at high temperature (but not more than 80 °C) and over longer extraction periods (not more than 4 hr), was caused by hydrolysis of carbohydrate and this decreased the extraction yield. For this reason, the mushroom extracts at 80 °C after 3 hr of extraction had high carbohydrate levels (Fig. 1), especially for *P. ostreatus*, which produced high concentrations of total carbohydrate and total reducing sugar of 6.7325 ± 0.0261 and 2.6737 ± 0.0027 mg/mL respectively, and for *L. edodoes* at 5.2764 ± 0.0520 and 1.7457 ± 0.0165 mg/mL, respectively. Interestingly, *A. auricula-judae* produced a high level of total reducing sugar (1.5909 ± 0.0078 mg/mL) but a low total carbohydrate value (2.1994 ± 0.0563 mg/mL), while *P. pulmonarius* produced a high level of total carbohydrate (4.7472 ± 0.0474 mg/mL) but a low level of total reducing sugar (0.6440 ± 0.0066 mg/mL).

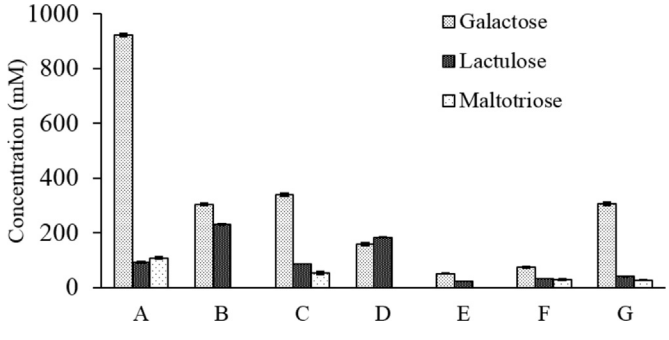

Fig. 2. Composition of galactose, lactulose and maltotriose from mushroom extract after 3 hr using high performance liquid chromatography analysis, where A = *Auricularia auricula-judae*, B = *Lentinus edodoes*, C = *Pleurotus citrinopileatus*, D = *Pleurotus djamor*, E = *Pleurotus ostreatus*, F = *Pleurotus ostreatus* (Jacq.Fr.) Kummer and G = *Pleurotus pulmonarius*, and error bars indicate ± SD.

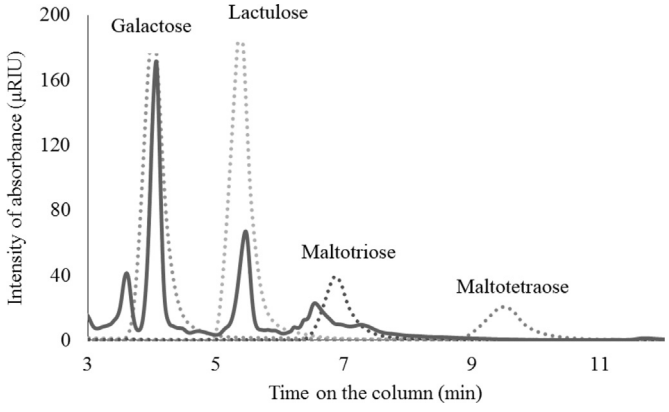

Fig. 3. High performance liquid chromatography chromatogram of *Auricularia auricula-judae* extracted after 3 hr (solid line) compared with standard compounds (dotted line).

Table 1
Growth of *L. acidophilus and L. plantarum* cultivation in media with mushroom extract and commercial prebiotic supplements at 37 °C for 48 hr determined using the optical density at 620 nm (OD620) where values = mean ± SD (n = 3).

Medium culture supplement	OD620	
	L. acidophilus	*L. plantarum*
Control	1.8515 ± 0.0425	1.8295 ± 0.0123
FOS	1.9226 ± 0.0057	1.8321 ± 0.0300
Inulin	1.9032 ± 0.0146	1.8460 ± 0.0058
A	1.9093 ± 0.0049	1.9402 ± 0.0145
B	1.9779 ± 0.0032	1.9326 ± 0.0180
C	1.9747 ± 0.0084	1.9378 ± 0.0088
D	1.9735 ± 0.0067	1.9378 ± 0.0155
E	1.9131 ± 0.0041	1.9348 ± 0.0116
F	1.9180 ± 0.0074	1.9247 ± 0.0081
G	1.9374 ± 0.0077	1.9702 ± 0.0072
G		

FOS = fructooligosaccharides; A = *Auricularia auricula-judae*; B = *Lentinus edodoes*; C = *Pleurotus citrinopileatus*; D = *Pleurotus djamor*; E = *Pleurotus ostreatus*; F = *Pleurotus ostreatus* (Jacq.Fr.) Kummer; G = *Pleurotus pulmonarius*.

These results indicated that the composition of carbohydrate in the mushroom extracts included both reducing sugar and non-reducing sugar, which are expected to be prebiotic substances. Therefore, the carbohydrate content required determination of both the total carbohydrate and total reducing sugar. More explanation is provided in an article review (Wang et al., 2017) which discusses the extraction of several saccharides and polysaccharides from a variety of mushrooms, focusing on their relationships with bioactive compounds.

High performance liquid chromatography determination

The 3 hr mushroom extracts were selected for HPLC analysis. In order to compare the sugar quantities of extracted mushrooms by peak identification, galactose, maltotriose, maltotetraose and lactulose were used as standards, as they have been identified in previous studies as major saccharide constituents in various kinds of mushrooms (Aida et al., 2009). The composition of the mushroom extracts analyzed using HPLC are presented in Fig. 2. As illustrated in Fig. 3, the chromatogram of *A. auricula-judae* had the

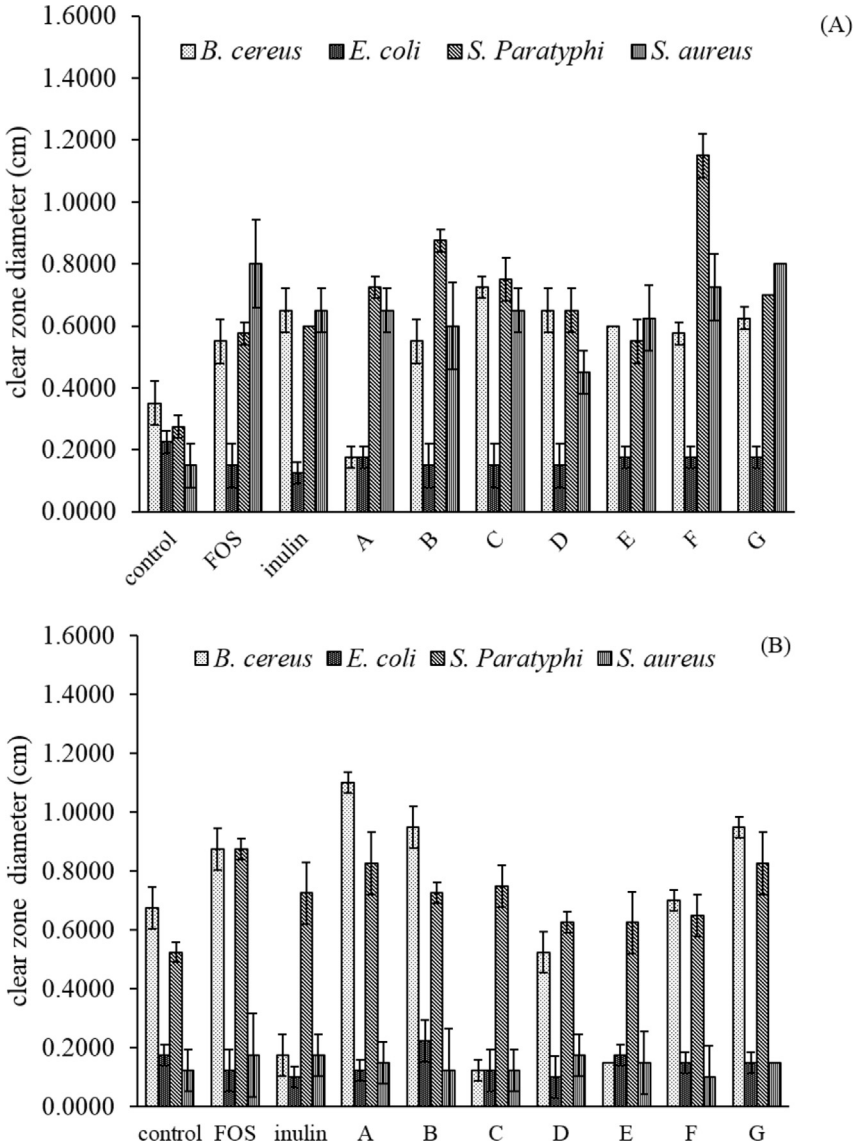

Fig. 4. Clear zone diameter of pathogenic inhibition; *B. cereus*, *E. coli*, *S. Paratyphi* and *S. aureus* from: (A) *L. acidophilus*; (B) *L. plantarum* cultivation in media with mushroom extract and commercial prebiotic supplements, where FOS = fructooligosaccharides, A = *Auricularia auricula-judae*, B = *Lentinus edodoes*, C = *Pleurotus citrinopileatus*, D = *Pleurotus djamor*, E = *Pleurotus ostreatus*, F = *Pleurotus ostreatus* (Jacq.Fr.) Kummer and G = *Pleurotus pulmonarius*, and error bars indicate ± SD.

highest galactose and maltrotriose levels (928.26 mM and 112.59 mM), while high lactulose was found in *L. edodoes (*229.64 mM). However, the polysaccharide compositions of the mushroom extracts used in this study were considered based on the sugars that influence probiotic growth stimulation (lactulose and maltotriose) previously reported (Sekhar et al., 2013).

Prebiotic properties

Probiotic growth stimulation

After both probiotics (*L. acidophilus* and *L. plantarum*) were cultivated in MRS with and without the mushroom extracts, they were compared with the commercial prebiotics (FOS and inulin). The results summarized in Table 1 show that all cultivations were not probiotic-growth inhibited, measured at an optical density of 620 nm (significant differences at $p < 0.05$). Notably, the cultivation with L. *edodoes* and *P. pulmonarius* extracts had the highest growth of *L. acidophilus* (1.9779 ± 0.0032) and *L. plantarum* (1.9702 ± 0.0072), respectively. This could be explained more by the study of Nowak et al. (2017) which investigated the ability of polysaccharides from 53 mushroom species, extracted using ethanol and distilled water, to promote the metabolism of beneficial microorganisms such as *Lactobacillus* strains. In that study, the mushroom polysaccharides stimulated stronger growth of probiotics than commercial prebiotics like FOS and inulin.

Pathogenic growth inhibition

The pathogenic inhibition efficiency of the probiotic cultivations is shown in Fig. 4. All culture media containing the mushroom extracts and the commercial prebiotic compounds had better pathogenic inhibition ability (*B. cereus, E. coli, S. Paratyphi* and *S. aureus*) than the control sample. The culture of *L. acidophilus* with extracts of *L. edodoes*, *P. citrinopileatus* and *P. ostreatus* (Jacq. Fr.) Kummer produced wider clear zones of *S. Paratyphi* inhibition (0.8750 ± 0.0354 cm, 0.7500 ± 0.0707 cm and 1.1500 ± 0.0707 cm, respectively) as shown in Fig. 4A, while, *L. plantarum* culture with extracts of *A. auricula-judae* and *L. edodoes* had high efficiency regarding *B. cereus* inhibition with diameters of the clear zone of 1.1000 ± 0.1414 cm and 0.9500 ± 0.0707 cm, respectively (Fig. 4B). Alves et al. (2012) reported some bioactive compounds such as peptides (plectasin), polysaccharides (beta-glucan), organic acid (benzoic acid) and phenolic compounds (catechin) from mushroom extraction. In particular, *Lentinus edodoes* has potential for broad anti-microbial action not only against mainly *E. coli*, because it also inhibited *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Moreover, Ishikawa et al. (2001) evaluated the antimicrobial ability of *Lentinula edodes* extract and found that it had potential to inhibit foodborne pathogens (*B. cereus, B. subtilis, Staphylococcus aureus, Staphylococcus epidermidis*) based on it producing the largest inhibition zone.

Gastrointestinal tolerance

The tolerance to gastrointestinal conditions (alpha-amylase, bile extract, HCl) was demonstrated by the survival of probiotic strains (*L. acidophilus* and *L. plantarum*) after incubation in the gastrointestinal conditions for 0.5–3 hr and the results for each mushroom extract were then compared with those of commercial prebiotics. Fig. 5 shows the survival percentage of *L. acidophilus* (Fig. 5A) and *L. plantarum* (Fig. 5B) cultured under the gastrointestinal

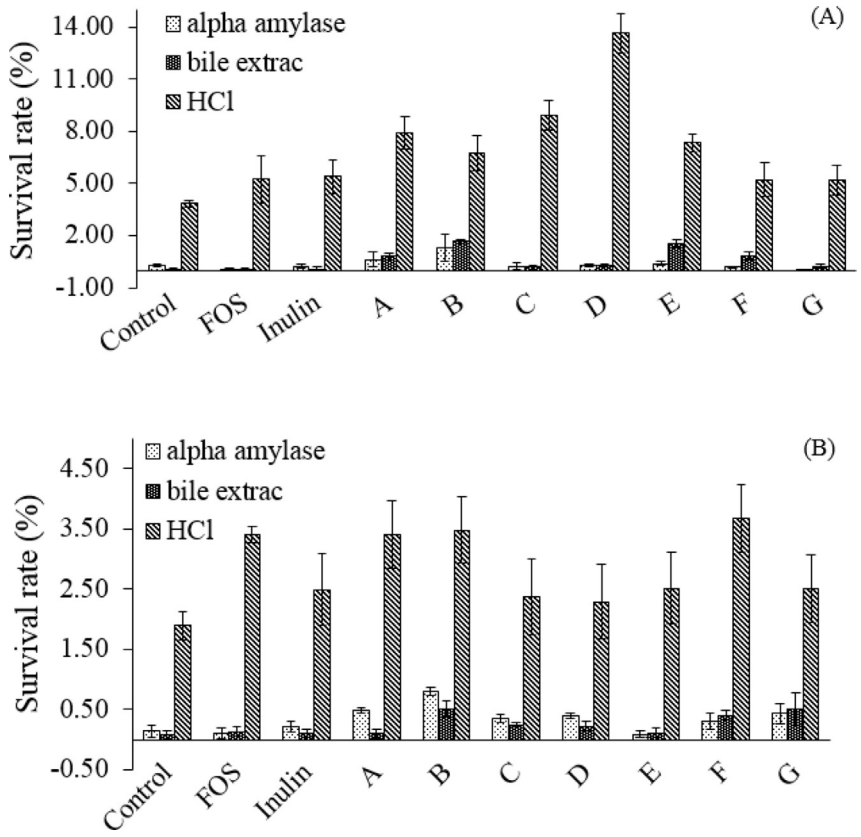

Fig. 5. Survival percentage of: (A) *L. acidophilus*; (B) *L. plantarum* after incubation under gastrointestinal conditions (alpha-amylase, bile extract and HCl for 2 hr) in culture with extracted mushrooms and commercial prebiotics supplements, where FOS = fructooligosaccharides, A = *Auricularia auricula-judae*, B = *Lentinus edodoes*, C = *Pleurotus citrinopileatus*, D = *Pleurotus djamor*, E = *Pleurotus ostreatus*, F = *Pleurotus ostreatus* (Jacq.Fr.) Kummer and G = *Pleurotus pulmonarius*, and error bars indicate ± SD.

conditions. After 2 hr of incubation, the probiotics could survive in the culture with or without mushroom extract or commercial prebiotic. Interestingly, both probiotic strains could tolerate HCl and showed higher survival percentage rates than under the other conditions; in particular *L. acidophilus,* when cultivated in the medium containing *P. djamor* extract, had the highest survival rate (13.64%). Noticeably, the culture containing *L. edodoes* extract had higher probiotic survival of both strains. Moreover, this mushroom extract had a greater capacity to tolerate all gastrointestinal conditions. The high lactulose level in the *L. edodoes* extract may be explained by the polysaccharide, as heteropolysaccharide, that could stabilize the *Lactobacillus* strain under the gastrointestinal conditions, as previously described in Bhakta and Kumar (2013).

From this study, seven edible mushrooms were extracted using a mixture of distilled water and ethanol and then the total carbohydrate and total reducing sugar amounts were determined. After 3 hr of extraction, *P. ostreatus* had the highest total carbohydrate and total reducing sugar which indicated the prebiotic compound yield. After HPLC analysis, the chromatograms showed galactose and maltotriose in the *A. auricula-judae* extract and high lactulose using the *L. edodoes* extract. The prebiotic properties of mushroom extracts were further investigated for probiotic growth stimulation. *L. edodoes* and *P. pulmonarius* had better growth stimulation in the *L. acidophilus* and *L. plantarum* cultures than in the control samples. The cultivation of *L. acidophilus* using *P. ostreatus* (Jacq.Fr.) Kummer extract produced a higher inhibition effect on *S. Paratyphi* than with the other pathogens, while *A. auricula-judae* in *L. plantarum* culture inhibited *B. cereus* more efficiently. Noticeably, *L. acidophilus* cultured using *P. djamor* extract had the highest survival percentage in HCl after 2 hr of incubation. From this study, the edible mushrooms extracts displayed highly efficient prebiotic properties compared with FOS and inulin. Therefore, edible mushrooms have rightly become more attractive as a functional food ingredient, which can add value to agricultural products of Thailand.

Conflict of interest

The authors declare that there are no conflicts of interest.

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