A B S T R A C T

The prebiotic properties were investigated of seven edible mushrooms: Auricularia auricula-judae, Lentinus edodes, Pleurotus citrinopileatus, Pleurotus djamor, Pleurotus ostreatus, 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. auricularia-judae had the highest levels of galactose and maltotriose (928.26 mM and 112.59 mM, respectively), while L. edodes 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, Salmonella Paratyphi and Staphylococcus aureus) and gastrointestinal tolerance (in amylase, bile extract and HCl). High probiotic growth stimulation resulted for L. acidophilus cultured with L. edodes 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.

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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 edodes_, _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).

**Materials and methods**

**Materials**

Seven kinds of three-month-old edible mushrooms were used in this experiment:; _Auricularia auricula-judae_, _Lentinus edodes_, _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 °C and 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. Acetonitrile (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_ and _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 plates 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-amyolase, 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).
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. edodes 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).
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

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**Table 1**

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

FOS – fructooligosaccharides; A – *Auricularia auricula-judae*; B – *Lentinus edodes*; 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
highest galactose and maltotriose levels (928.26 mM and 112.59 mM), while high lactulose was found in *L. edodes* (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. edodes* and *P. pulmonarius* extracts had the highest growth of *L. acidophilus* ($1.9779 \pm 0.0032$) and *L. plantarum* ($1.9702 \pm 0.0072$), 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. edodes*, *P. citrinopileatus* and *P. ostreatus* (Jacq. Fr.) Kummer produced wider clear zones of *S. Paratyphi* inhibition ($0.8750 \pm 0.0354$ cm, $0.7500 \pm 0.0707$ cm and $1.1500 \pm 0.0707$ cm, respectively) as shown in Fig. 4A, while, *L. plantarum* culture with extracts of *A. auricula-judae* and *L. edodes* had high efficiency regarding *B. cereus* inhibition with diameters of the clear zone of $1.1000 \pm 0.1414$ cm and $0.9500 \pm 0.0707$ 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 edodes* 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](image-url)
conditions. After 2 hr of incubation, the probiotics could survive in the culture with or without mushroom extract or commercial probiotic. 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. edodes 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. edodes 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 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 B. cereus 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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References


