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EFFECT OF MUSHROOM EXTRACT ON THE GROWTH OF ISOLATED LACTIC ACID BACTERIA AND IMMOBILIZATION AS BIO−BEADS GEL

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ABSTRACT

Bacteria membered in lactic acid producing group containing many dominant properties can be applied in diverse fields. This research was aimed to select effective lactic acid bacterial strains and enhanced their growth with mushroom extracts. Fermented vegetable samples were used as a source to achieve lactic acid bacteria by isolating on MRS medium. Fifteen isolates were lactic producing bacteria that exhibited clear zone around their colony on medium. Bacterial isolates FV2, FV4, and FV14 have produced the highest lactic acid content in quantitative investigation. In addition, FV2 and FV4 have been found the ability to suppress growth of pathogenic bacteria *Salmonella* Typhimurium and *Staphylococcus aureus*, with the highest inhibitory activity at 200 AU/mL. Isolates FV2 and FV4 have been classified as *Lactiplantibacillus plantarum* FV2 and *Pediococcus pentosaceus* FV4 based on their 16S rRNA gene sequences. Mushroom extracts of *Pleurotus pulmonarius* and *Lentinus squarrosulus* (Mont.) have been discovered oligosaccharide, and antioxidants activity, including the property to support the growth and lactic acid production of *L. plantarum* FV2 and *P. pentosaceus* FV4. High survival of lactic acid bacteria within bio−bead gel structure have been found more than naked cell under strong acid condition, including storage at 4 °C up to 28 days. Bio−bead gels containing lactic acid bacteria and mushroom extracts could be applied as the starter culture to produce fermented milk that they promoted coagulation of milk protein, high lactic acid bacteria, and antioxidant activity.

Keywords: Lactic acid bacteria; *Lactiplantibacillus plantarum*; *Pediococcus pentosaceus*; Milk fermentation; *Pleurotus pulmonarius*; *Lentinus squarrosulus* Mont.

INTRODUCTION

Preservation of foods are the important process to extend shelf life and destroy many food spoilage microorganisms without preservatives application, including increase good flavor. Several processes of preservation based on different types of food products such as moist−heat, dry−heat, pH, and water activity (AW) have been utilized. The spontaneous fermentation process occurred with several beneficial microorganisms are used to against many food poisoning factors such as spoilage microorganisms, and foodborne pathogen contamination. Various raw materials such as vegetables, fruits, milk, and fish can be used to manufacture many kinds of fermented food. Especially, vegetables and fruits have been found numerous acid producing microorganisms on their surface such as lactic− and acetic−acid bacteria, mold, and yeast. During fermentation, organic acids, hydrogen peroxide, bacteriocin, diacetyl, ethanol, fatty acids, and CO_2 are generated that most of them destroy non−beneficial microorganisms and occur a good flavor of foods (**Angmo *et al.*, 2016**).

Fermented foods contain many acid producing bacteria that can produce bacteriocin and bacteriocin−like substances with broad− and narrow−antimicrobial properties (**Yang *et al.*, 2014**). These microorganisms are mostly lactic acid producing bacteria, which are considered as probiotic agents due to their non−pathogenic nature and health benefits. Certain dominant strains of bacteria in this group such as *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp., and *Bifidobacterium* sp., are known to produce bioactive compounds that inhibit growth of pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella* sp., *Bacillus* sp., *Escherichia coli* along with the ability to ferment several raw material types occurring homo−or hetero−products. These beneficial bacteria are utilized in the development of cost effective fermentation processes that promote human health and flavor of foods (**Koponen *et al.*, 2012; Wu *et al.*, 2009; Zhai *et al.*, 2014**). To increase the efficiency of probiotics bacteria, they must survive under stress conditions, especially acidic gastric environment of stomach and small intestine (**Corcoran *et al.*, 2005**). The acid tolerance of lactic acid bacteria is the important property of another attribute that used as the criteria to select potentially probiotic strains.

Several natural sources of substances as prebiotic properties to enhance the growth of beneficial endogenous strains from the gastrointestinal tract or probiotic including lactic acid bacteria can be obtained from many raw materials of food such as vegetables, fruits, and edible mushrooms. Many mushroom species considered as nutritious macro−fungi containing fruiting bodies and mycelia, are the interesting sources of many value substances as major polysaccharides and water present in mushrooms, proteins, and amino acids (10%–40%), and fats (2%–8%), vitamins and antioxidants, residual salts, and metals (**Cerletti *et al.*, 2012**). Many substances in carbohydrate group have been found in mushrooms such as chitin, hemicellulose, β− and α−glucans, mannans, xylans, and galactans, which can be applied as carbon sources including minerals for probiotic bacteria growth (**Cerletti *et al.*, 2012**). These mushrooms not only support bacterial growth as prebiotics, but also contain medicinal properties such as antitumor, antimicrobial, antioxidant, antiviral, and immunomodulating (**Singdevsachan *et al.*, 2016**). The most economic mushroom species cultivated worldwide are *Agaricus bisporus* (button mushroom), *Lentinus edodes* (shiitake), *Pleurotus spp* (oyster mushrooms), *Auricula 1 uricular* (wood ear mushroom), *Flamulina velutipes* (winter mushroom), *Volvariella volvacea* (straw mushroom), and *Lentinus squarrosulus* (log white fungi) (**Ayimbila *et al.*, 2022**).

Immobilization of bacterial cell, this technique exhibit many advantages more than free cells. Several outstanding attributes comprise the maintenance of stable and active biocatalysts, high volumetric productivity, improved process control, protection of cells against damage and reduced susceptibility to contamination (**Fijałkowski *et al.*, 2015**). The development of bacterial cell with immobilization technique has to consider as follow the effective and safety strains, benefits for health, stability during storage, processing step, and the effect on the food (**Burgain *et al.*, 2011; Fijałkowski *et al.*, 2015**). Selection either natural or synthetic carrier matrix for the immobilization should be nonreactive, non−toxic, inexpensive, available in a large quantity, easy to handle, highly stable and high capacity with effective yield and preservation. (**Fijałkowski *et al.*, 2015**). In addition, many carrier matrix types used to support for the immobilization are alginate, carrageenan, agar, glass beads, polyvinyl alcohol, and polyacrylamide (**Fijałkowski *et al.*, 2015; Liu *et al.*, 2016**)

Thus, this research was aimed to select effective lactic acid bacterial strains, and investigated their abilities. Moreover, extract of mushrooms were applied to promote the growth of lactic acid bacteria, and developed as bio−bead gel.

MATERIAL AND METHODS

Selection of lactic acid bacteria

Isolation of bacteria

Fermented Wild Spider Flower (*Gynandropsis gynandra* Linn.) sample was weighed as 25.0 g into plastic bags. The sample bag was filled with 225.0 mL of 0.85% (w/v) sodium chloride (NaCl) solution and finely crushed by using a stomacher (IUL INSTRUMENTS, BARCELONA, SPAIN) for 10 minutes. A suspension was ten−fold serially diluted with 0.85% (w/v) sodium chloride solution, and 0.1 mL aliquot was spread on De Man, Rogosa and Sharpe (MRS) agar (HI−MEDIA, INDIA) containing 1% (w/v) calcium carbonate ($CaCO_3$). Lactic acid bacteria on MRS agar were grown by incubating at 30 ˚C for 24−48 hours. Bacterial colony presenting either a clear zone or halo zone on medium were selected and purified to a single colony on MRS agar with incubation at 30 ˚C for 24−48 hours.

Measurement of lactic acid production

All bacterial isolates were prepared by culturing with MRS broth and incubation at 30 ˚C for 24−48 hours. Culture broths were collected the supernatant by using a centrifuge (DYNAMICA, HONG KONG) at 8,500 rpm for 10 minutes, and then determined pH values with a digital pH meter (DYNAMICA, HONG KONG), and titratable acidity with 0.02 M sodium hydroxide (NaOH) to end point of reaction.

Inhibitory activity to pathogenic bacteria

All lactic acid bacterial isolates were determined inhibitory activity to *S.* Typhimurium and *S. aureus*. The inoculums of each lactic acid bacteria were prepared by culturing with MRS broth and incubation at 120 rpm, 30 ˚C for 24−48 hours. Bacterial cell were collected by centrifuging at 8500 rpm for 10 minutes, washed and re−suspended with distilled water. The turbidity of cell concentration were measured by using a spectrophotometer (THERMO FISHER SCIENTIFIC, GENESYS 10 UV SCANNING) with an absorbance at 600 nm to 0.05 (10^8 cfu/mL). The prepared bacterial inoculums were inoculated to MRS broth with incubation at 120 rpm, 30 ˚C for 24−48 hours, and then collected the samples every 2 hours. Culture broth samples were centrifuged at 8500 rpm for 10 minutes, and then supernatants were filtered with a 0.2 µm membrane filter. The inhibitory activity was qualitatively investigated by using agar well diffusion on Muller Hinton agar (HI−MEDIA, INDIA). Pathogenic bacteria were prepared by using Muller Hinton broth and adjusted the turbidity of cell concentration to McFarland scale of 0.5. Prepared cell−free supernatant of lactic acid bacteria 50 µL aliquot were inoculated into the well of Muller Hinton agar swabbed with each pathogenic bacterium. The inhibitory activity were observed the halo zone on medium after incubating at 37 ˚C for 24 hours. The highest inhibitory activity of pathogenic bacteria were selected for quantitative estimation. The culture filtrates collected every 2 hours were diluted to the final concentrations as 1x, 2x, 4x, 6x, 8x, and 10x for investigation the inhibitory activity with agar well diffusion and expressed the results as Arbitrary Unit (AU) /mL. One arbitrary unit was defined as the reciprocal of the highest dilution showing a clear inhibition zone around the well (**Shehata *et al.*, 2019**).

Identification of lactic acid bacteria with 16S rRNA gene

Lactic acid bacteria were prepared by culturing with MRS agar and incubation at 30 ˚C for 24−48 hours. Genomic DNA mini kit (Blood/culture cell) (GENEAID BIOTECH LTD., TAIWAN) was utilized to extract bacterial genome. Two primers as 20F (5'−GAGTTTGATCCTGGCTCAG−3') and 1500R (5'−GTTACCTTGTTACGACTT−3') were applied to amplify the full−length 16S rRNA gene (**Brosius *et al.*, 1986**). A reaction mixture 100 µl was consisted of 15−20 ng of DNA template, 2.0 µmoles each of the two primers, 2.5 units of Taq polymerase, 2.0 mM magnesium chloride ($MgCl_2$), 0.2 mM dNTP and 10 µl of 10xTaq buffer (750 mM Tris−HCl, 200 mM ammonium sulfate ($(NH_4)_2SO_4$) and 0.1% Tween 20, pH 8.8). Polymerase chain reaction (PCR) was programmed by following the initial step at 94 ˚C for 3 minutes, 25 cycles at 94 ˚C for 1 minute, at 50 ˚C for 1 minute, at 72 ˚C for 2 minutes, and at 72 ˚C for 3 minutes (DNA Engine Dyad® Thermal Cycler, Bio−Rad Laboratories). The PCR products were obtained on 0.8% (w/v) agarose gel electrophoresis and purified with a GenepHlowTM Gel/PCR Kit (GENEAID BIOTECH LTD., TAIWAN). The purified PCR products were stored at -20 ˚C for further step. Then, purified PCR products were investigated the sequences with an ABI Prism® 3730XL DNA Sequence (APPLIED BIOSYSTEMS, FOSTER CITY, CALIFORNIA, USA). The bacterial nucleotide informations were submitted to National Center for Biotechnology Information (NCBI), and compared the similarity data by BLAST tool. The other closely related bacterial 16S rRNA sequences were obtained from GenBank database. Phylogenetic tree diagram was constructed by using neighbor−joining method with bootstrap analysis (1,000 replications).

Analysis of mushroom extracts

Mushroom extracts preparation

Stalk and fruiting bodies of mushroom species *P. pulmonarius* and *L. squarrosulus* Mont., were prepared by washing with water and air dried. Prepared samples were finely crushed with blender to obtain a crude extract. The sediment of mushrooms after crushing were suspended by using distilled water (1:2). Suspension of crude extracts were pasteurized by boiling at 75 ˚C for 20 minutes, and cooling. Then, pasteurized suspensions were filtered by using sterilized muslin cloth and filter paper Whatman No.1. Sterilization of mushroom extract suspensions were done by using 0.2 µm syringe filter.

Measurement of mushroom inulin−oligosaccharide

Crude mushrooms extracts were determined an inulin−oligosaccharide by using thin layer chromatography (TLC). Prepared mushrooms extracts and standard inulin−oligosaccharide suspension were dropped on the TLC aluminum sheets (Silica gel 60 F_{254}) and air dried. Acetone and methanol were prepared as the saturated solution in a ratio 1:2 (v/v) for using as mobile phase. Spot of substances on the TLC sheets were detected by spraying a reagent containing sulfuric acid (H_2SO_4) and 95% ethanol in a ratio 1:1 (v/v) and then heated at 110 ˚C for 5 minutes. The distance of substances movement on TLC plates would be presented after heating. Rate of Flow (R_f) values were obtained for calculating and comparing the data. Moreover, reducing sugar of mushroom extracts were quantitatively estimated by using dinitrosalicylic acid (DNS) method (**Miller, 1959**) along with standard inulin−oligosaccharide at the concentration of 0−80.0 mg/L.

Total phenolic compound assay

A 0.2 mL aliquot of mushroom extracts suspension was mixed to 0.2 mL of 10% (v/v) Folin−Ciocalteu reagent followed by 2.0 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) and 2.5 mL of distilled water. The reaction was activated by incubating at room temperature for 90 minutes under dark condition. Total phenolic contents were estimated on the level of blue color and measured by using UV−visible spectrophotometer (THERMO FISHER SCIENTIFIC, GENESYS 10 UV SCANNING) with an absorbance at 765 nm, compared to standard gallic acid with concentration of 0−1.6 mg/mL presenting result as mg GAE/g extract (**Yingngam *et al.*, 2014**).

DPPH (2,2 diphenyl−1−picrylhydrazyl) radical scavenging assay

Prepared solution of DPPH 6×10^{-3} M in ethanol was diluted to a final concentration as 6×10^{-5} M. An aliquot of 1.0 mL of mushroom extract suspension was transferred to DPPH solution 3.0 mL and incubated under dark at room temperature for 40 minutes. The antioxidant activity of mushroom extract was done by observing the reducing purple color to yellow and measured by UV−visible spectrophotometer with an absorbance at 517 nm. The percentage of radical scavenging activity was calculated by using a formula as % DPPH scavenging = [(absorbance of control - absorbance of the sample)/absorbance of control)] x 100 (**Brand−Williams *et al.*, 1995; Majhenič *et al.*, 2007**).

ABTS˙ [2,2'−azinobis−(3−ethyl−benzothiazoline−6−sulfonic acid)] assay

Oxidation of ABTS with potassium persulfate ($K_2S_2O_8$) were reacted to generate ABTS˙. ABTS salt 0.0768 g was added to 0.0132 g of potassium persulfate in 20 mL of distilled water to prepare an ABTS stock solution that was diluted with ethanol and adjusted to 0.7 at OD 734 nm before utilization. An aliquot of mushroom extract suspension 0.25 mL was mixed to 2.5 mL of ABTS solution and incubated at room temperature for 5 minutes under dark condition. The reaction of the mixture was measured by using UV−visible spectrophotometer with an absorbance at 734 nm, and the percentage of radical scavenging activity was calculated by using a formula of % ABTS scavenging = [(absorbance of control - absorbance of the sample) /absorbance of control] x 100 (**Mayur *et al.*, 2010; Re *et al.*, 1999**).

Effect of mushroom extracts on bacterial growth

Bacterial isolates exhibiting the highest lactic acid production were selected. The inoculums bacteria were prepared by culturing with MRS broth and incubation at 120 rpm, 30 ˚C for 24−48 hours. Each starter were inoculated to MRS broth supplemented with sterilized suspension of mushroom extracts 0.5%, 1.0%, and 2.0% (v/v), and incubation at 120 rpm, 30 ˚C for 24−48 hours. The growth of bacteria were investigated by using plate count technique on MRS agar with incubation at 30 ˚C for 24−48 hours, and expressed the results as log cfu/mL.

Immobilization of lactic acid bacteria

Development of bio-beads gel

Bacterial isolates exhibiting the highest lactic acid production and growth with mushroom extracts were selected to develop bio−beads gel. Bacteria was prepared the inoculums by culturing with MRS broth and incubation at 120 rpm, 30 ˚C for 24−48 hours. Bacterial cell were collected by using a centrifuge at 8500 rpm, 10 minutes and discard a supernatant. Cell pellets were twice washed and re−suspended with sterilized distilled water. Turbidity of cell concentration was measured by using a spectrophotometer with an absorbance at 600 nm to 0.05 (10^8 cfu/mL). Bacterial cell were prepared into a single and mixed inoculums. Bacterial inoculums (10 % v/v) and mushroom extract were thoroughly mixed with 4% (w/v) sodium alginate into a homogeneous solution on a magnetic stirrer for 5 minutes. Subsequently, the mixture was reacted with a 4% (w/v) calcium chloride ($CaCl_2$) solution on a magnetic stirrer for 5 minutes. The immobilized cells (bio−beads gel) as a complete spherical shape were washed with a 4% (w/v) calcium chloride solution for 2 minutes. Changing bacterial number of bio−beads gel were estimated during storage at 4 ˚C for 0−28 days by sampling every 7 days, compared to naked cells. Plate count technique was used to investigate the bacterial number on MRS agar by incubating at 30 ˚C for 24−48 hours. Changing size of bio−beads gel were measured by Vernier caliper.

Effect of artificial gastric juice

To determine the endurance of bacteria under strong acid conditions, bio−bead gels and naked cells were investigated. Gastric juice solution was prepared that consisted of sodium chloride 2.05 g/L, potassium dihydrogen phosphate (KH_2PO_4) 0.60 g/L, calcium chloride 0.11 g/L, and potassium chloride (KCl) 0.37 g/L, and pH was adjusted to 2.0 with 1 M hydrochloric acid (HCl). This solution was sterilized by autoclaving at 121 ˚C for 15 minutes, and then pepsin 0.0133 g/L and lysozyme 0.01 g/L were added to a sterilized solution when low temperature. In addition, the synthetic pancreatic juice was prepared that comprised of dibasic sodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) 50.81 g/L, sodium chloride 8.50 g/L and bile salts 3.00 g/L, and the pH was adjusted to 8.0. Sterilization of solution was autoclaved at 121 ˚C for 15 minutes, and then pancreatin 0.10 g/L was added after low temperature (**Mesquit *et al.*, 2011**). The bio−bead gels and naked cells of bacteria were examine the endurance to gastric juice solution by incubating at 37 ˚C for 2 hours. Subsequently, they were investigated the resistance to pancreatic juice by incubating at 37 ˚C for 1 hour. The survival of bacteria under stress conditions were investigated by using a plate count technique on MRS agar and incubation at 37 ˚C for 24−48 hours.

Production of fermented milk

Bio−bead gel formulas containing the highest lactic acid bacteria after investigation the endurance to either artificial gastric and pancreatic juice were selected to apply as starter culture of fermented milk. Raw milk from farm was prepared by heat−treating at 95 ˚C for 3 minutes and then immediately cooled in ice box. Prepared milk in containers were inoculated with either bio−beads gel or naked cell inoculum with 10% (v/v) and incubation at 30 ˚C for 6−12 hours to observe milk curdling. Subsequently, the samples were collected to estimate lactic acid value titrated with sodium hydroxide, and pH with pH meter. In addition, bacterial numbers were measured by using plate count technique on MRS agar and incubation at 30 ˚C for 24−48 hours.

RESULTS

Selection of lactic acid bacteria

A total 15 lactic acid bacteria has been obtained from fermented vegetable sample that exhibited the halo zone around their colony on MRS agar after incubation. Almost bacterial colony morphology on MRS agar displayed the characteristics of white, convex, and raised forms with entire edges. Investigation of bacterial cell staining were Gram−positive with the shape of bacillus and coccus (Table 1). All lactic acid bacteria have exhibited the accumulation of acid in MRS broth from quantitative investigation and reduced the pH values to acid ranges. Particularly, bacterial cultivation for 48 hours displayed higher lactic acid amount increasing in culture broth than incubation for 24 hours. In addition, bacterial isolates FV2, FV4, and FV14 demonstrated the highest values of lactic acid accumulation along with a lower pH than 4.0 (Figure 1).

Table 1 Colony and cell morphology of lactic acid bacteria on MRS agar

No.	Isolates	Characteristics	
		Colony*	Gram and cell
1	FV1	Small, White, Circular, Convex, Entire, Smooth	Positive−Lactobacillus
2	FV2	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
3	FV3	Small, White, Circular, Convex, Entire, Smooth	Positive−Lactococcus
4	FV4	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
5	FV5	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
6	FV6	Small, White, Circular, Convex, Entire, Smooth	Positive−Lactobacillus
7	FV7	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
8	FV8	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
9	FV9	Small, White, Circular, Convex, Entire, Smooth	Positive−Lactobacillus
10	FV10	Small, White, Circular, Convex, Entire, Smooth	Positive−Lactobacillus
11	FV11	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
12	FV12	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
13	FV13	Small, White, Circular, Convex, Entire, Smooth	Positive−Lactobacillus
14	FV14	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus
15	FV15	Small, White, Circular, Convex, Entire, Smooth	Positive−Staphylococcus

* Colony morphology consisted of size, color, shape, elevation, margin, and texture.

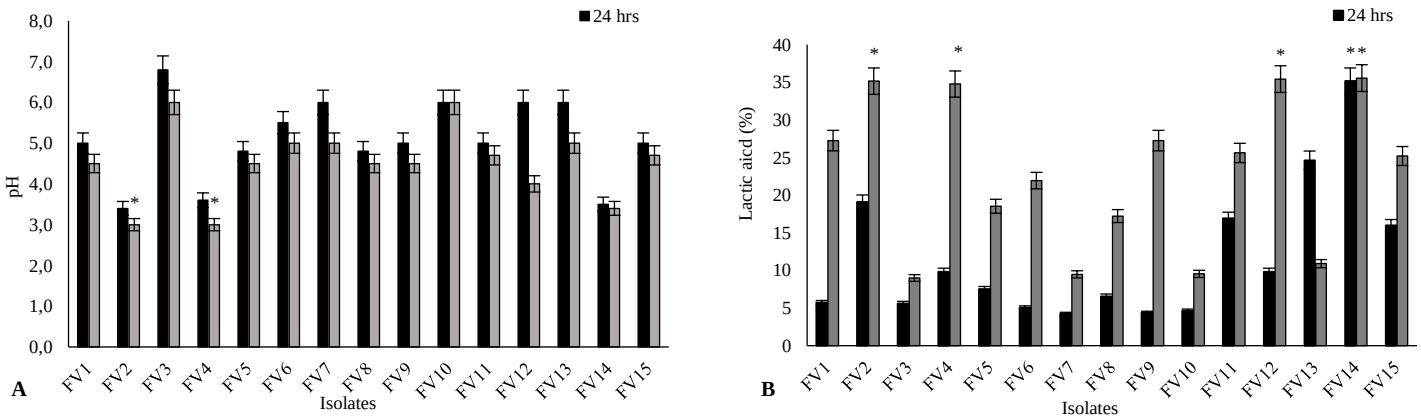


Figure 1 Lactic acid content (a) and pH values (b) from lactic acid bacteria grown with MRS broth incubated at 30 ˚C for 24−48 hours. *$p < 0.05$ significant (Compare mean with One−Way ANOVA, Duncan test)

Inhibitory activity to pathogenic bacteria

All 15 lactic acid bacteria with the capability to produce lactic acid were investigated the inhibitory activity against pathogenic bacteria. Six hours after incubation, the culture broth collected from isolates FV1−FV4, FV9, and FV14 exhibited the growth of indicator bacteria by presenting an inhibitory zone on Muller Hinton agar (Table 2). The bacterial isolates were more effective in suppressing the growth of Gram−negative *S.* Typhimurium compared to Gram−positive *S. aureus*. Lactic acid bacteria FV2 and FV4 limited the spread of pathogenic strains swabbed on the medium by exhibiting wide diameters of halo zones around the inoculated well. Thus, bacterial isolates FV2 and FV4 were selected to quantitatively determine the inhibitory activity against the growth of pathogenic bacteria. The initial inhibitory activity against *S. aureus* has been found as 40 AU/mL at 2 hours. The inhibitory activity progressively increased during 8−18 hours (*S. aureus*) and 8−24 hours (*S.* Typhimurium) and displayed the highest value of 200 AU/mL. The inhibitory activity of isolates FV2 and FV4 against *S. aureus* diminished after 20 hours of incubation. Therefore, the optimum time of the inhibitory activity from bacterial isolates FV2 and FV4 against the growth of *S. aureus* and *S.* Typhimurium was observed to be 6−18 hours and 8−24 hours, respectively (Figure 2).

Table 2 Screening the inhibitory activity against pathogenic bacteria

Isolates	Inhibitory levels	
	***S.* Typhimurium**	***S. aureus***
FV1	+	-
FV2	++	+
FV3	+	-
FV4	++	++
FV5	-	-
FV6	-	-
FV7	-	+
FV8	-	-
FV9	+	-
FV10	-	-
FV11	-	-
FV12	-	-
FV13	-	-
FV14	++	-
FV15	-	-

++: Maximum (16.0−20.0 mm); +: Minimum (10.0−15.0 mm); -: Not detected

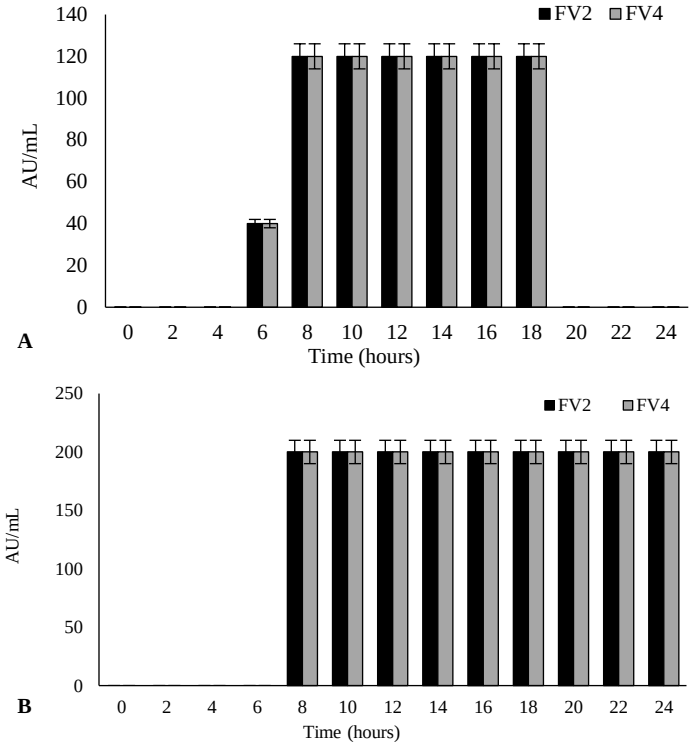


Figure 2 The inhibitory activity of lactic acid bacteria FV2 and FV4 against pathogenic bacteria *S. aureus* (a) and *S.* Typhimurium (b) after incubation at 37 ˚C for 24 hours.

Identification of lactic acid bacteria

The species of bacterial strains FV2 and FV4 have been identified with the analysis of 16S rRNA sequences by obtaining the PCR product size as 1,434 and 1,507 bp, respectively. Base on the data of NCBI GenBank, lactic acid bacteria FV2 exhibited the similarity value as 100% to genus *Lactiplantibacillus* with the various species of *L. pentosus*, *L. plantarum*, *L. argentoratensis*, including *Lactobacillus plantarum*. While, FV4 has closely related to genus *Pediococcus* and *Lactobacillus* as 99.87% and 99.93%, respectively, including the species of *P. pentosaceus* as 100%. The sequence of 16S rRNA genes from isolates FV2 and FV4 have been deposited in the NCBI GenBank database with the accession number OQ880489 and OQ880498, respectively. The phylogenetic tree diagram of bacterial FV2 and FV4 displayed close relationships to the *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* (Figure 3).

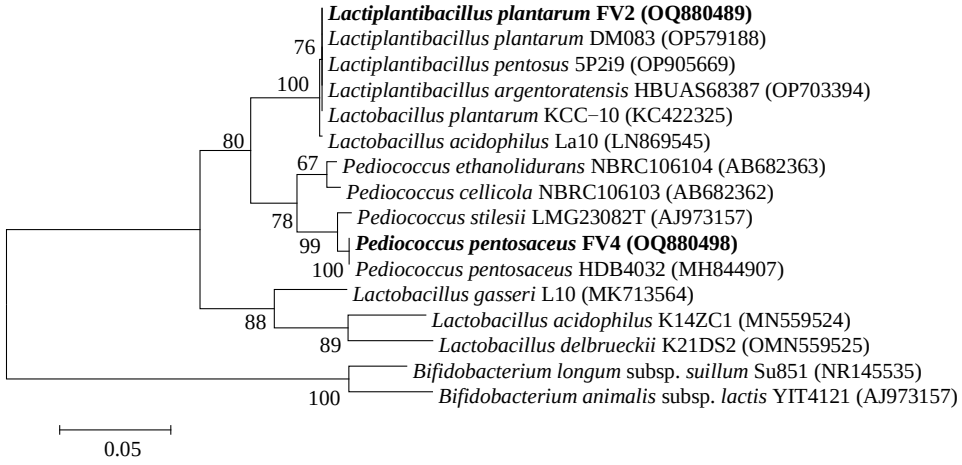
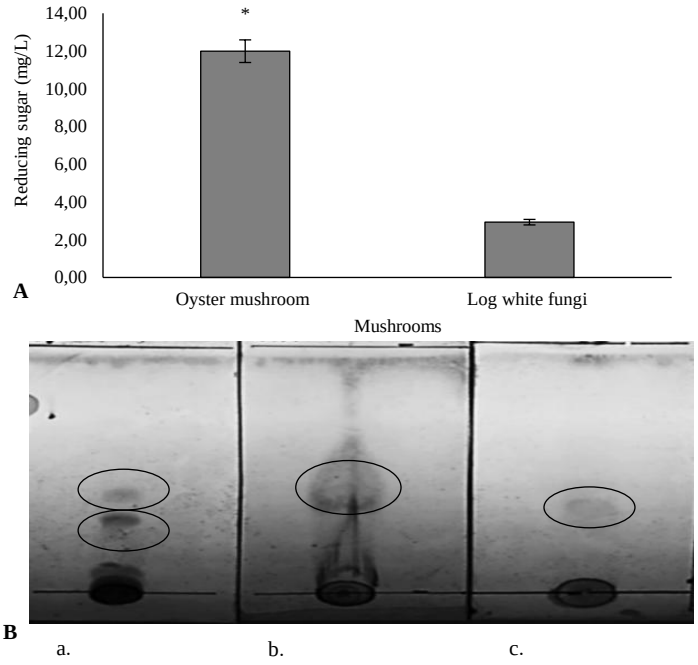


Figure 3 Phylogenetic relationships of lactic acid bacteria based on 16S rRNA gene sequences. *L. plantarum* FV2 with accession no. OQ880489 and *P. pentosaceus* FV4 with accession no. OQ880498 constructed by neighbor−joining method on *p*−distance model. Bootstrap values based on 1000 replicates at branch points. GenBank accession numbers are shown in parentheses.

Analysis of mushroom extracts

The investigation of mushrooms extracts (*P. pulmonarius* and *L. squarrosulus*) on TLC plate showed a single band of substance with R_f value of 0.412. While standard inulin−oligosaccharide exhibited double bands on the TLC plate containing two ranges of R_f values as 0.329 and 0.423. In addition, reducing sugar obtained from mushrooms *P. pulmonarius* has been discovered higher the amount than *L. squarrosulus* (Figure 4). Additionally, the antioxidant activity of mushroom *L. squarrosulus* showed a high percentage of radical scavenging activity as measured by DPPH and ABTS assay, including total phenolic compound, compared to *P. pulmonarius* (Table 3).



*p < 0.05 significant (Compare mean with One−Way ANOVA, Duncan test)

Figure 4 Analysis of inulin−oligosaccharide on TLC plate (a) Oyster mushroom (*P. pulmonarius*) (b) Log white fungi (*L. squarrosulus* Mont.) (c) (A) and reducing sugar from mushrooms (B).

Table 3 Antioxidant activity of the mushroom extracts

Samples	Total phenolic (mgGAE/g extract)	% Radical scavenging activity (at 7.5 mg/mL)	
		DPPH	ABTS
P. pulmonarius	34.40 ± 0.50	27.42 ± 4.69	98.61 ± 2.12
L. squarrosulus Mont.	60.50 ± 0.70*	66.24 ± 4.21*	99.91 ± 0.08

*p < 0.05 significant (Compare mean with One−Way ANOVA, Duncan test)

Effect of mushroom extracts on bacterial growth

Utilization either inulin−oligosaccharide or mushroom extracts as the sole carbon source of culture medium significantly enhanced the growth of *L. plantarum* FV2 and *P. pentosaceus* FV4. Commercial inulin−oligosaccharide as prebiotic in MRS broth has supported the growth of both bacterial strains higher than crude of mushroom extracts. However, the potentiality of mushroom extracts have contributed bacterial amount increasing based on the concentration levels of extracts. Utilization of 2.0% (v/v) mushroom extracts clearly demonstrated a higher number of bacteria than 0.5 and 1.0% (v/v). Lactic acid has been produced in MRS broth supplemented with various carbon source indicated that the ability of inulin−oligosaccharide and mushroom extracts could support the growth of bacteria. Incubation times were another influential factor of increasing bacterial amounts and lactic contents. Utilization either inulin−oligosaccharide or mushroom extracts with different concentrations with incubation for 48 hours significantly enhanced the growth and lactic acid values of *L. plantarum* FV2 and *P. pentosaceus* FV4, compared to 24 hours of incubation (Figure 5).

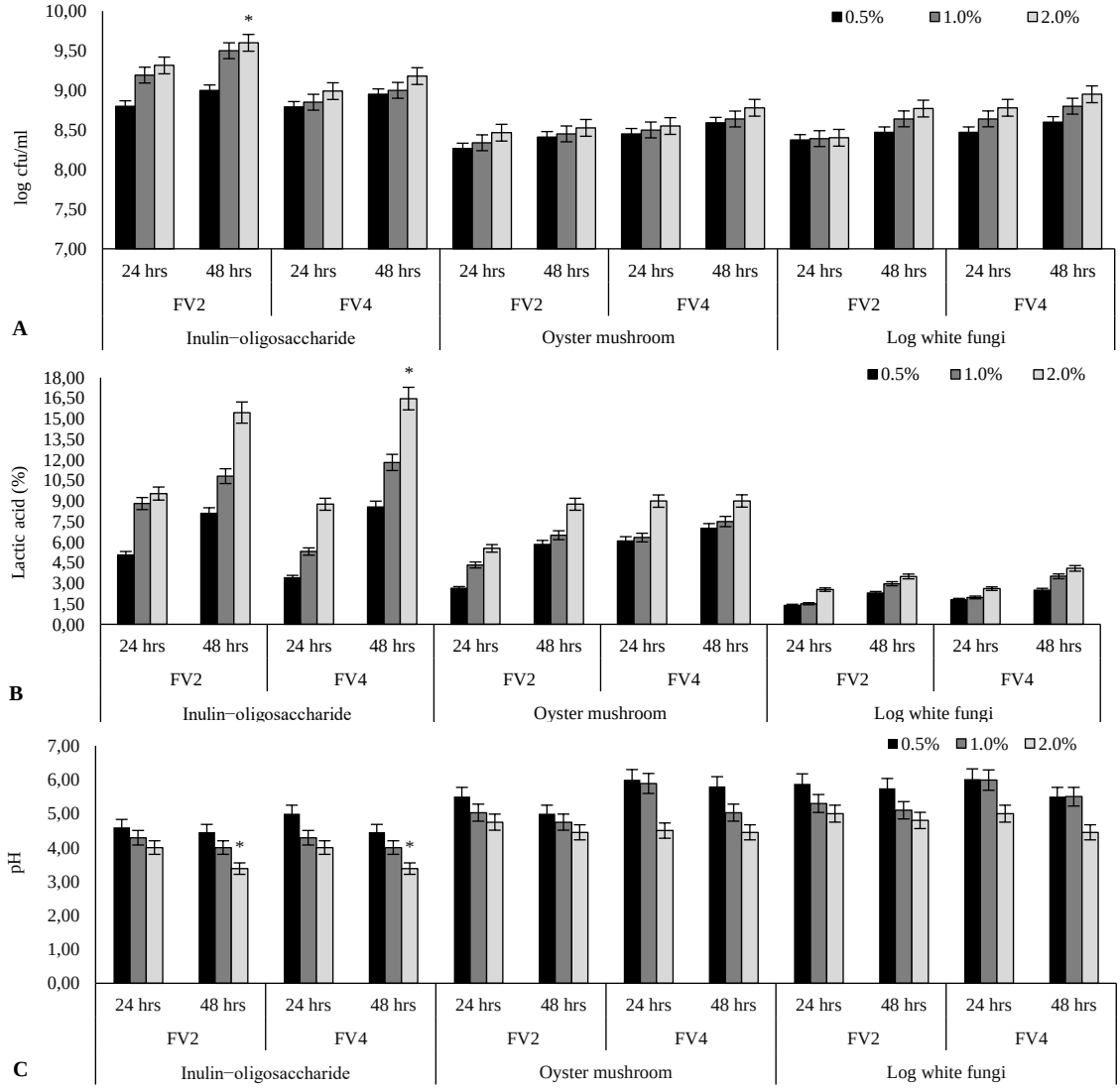


Figure 5 The growth (a), lactic accumulation (b), and pH value (c) of *L. plantarum* FV2 and *P. pentosaceus* FV4 cultured by using various carbon sources as follows Inulin−oligosaccharide; Oyster mushroom (*P. pulmonarius*); Log white fungi (*L. squarrosulus* Mont.). *p < 0.05 significant (Compare mean with One−Way ANOVA, Duncan test)

Immobilization of bacteria

Naked cells of *L. plantarum* FV2 and *P. pentosaceus* FV4 demonstrated a greater reduction in bacterial amount than immobilized cells as bio−bead gel during 1−2 hours of incubation under acid condition. Presence of inulin−oligosaccharide or mushroom extracts including its physical structure of bio−beads gel evidently enhanced the endurance of mixed or single lactic acid bacteria. Especially, *L. plantarum* FV2 displayed a little reduction of bacterial amount with the changing value as 0.083 log cfu/mL since 0 until 2 hours, including *P. pentosaceus* FV4 and mixed culture as 1.03−1.14 log cfu/mL (Table 4). Whereas, naked cell of mixed bacteria F2 and F4 without inulin−oligosaccharide or mushroom extracts have been extremely reduced the bacterial amount since 0 until 2 hours as 2.64 log cfu/mL, including single naked cell as 2.53−2.64 log cfu/mL.

Every formulas of bio−beads gel exhibited a transparent colorless texture and a spherical shape with average diameter as 4.31 mm (at 0 day). Preservation of bio−beads gel to prolong the shelf life at 4 ˚C for 0−28 days did not change the size of bio−beads gel and stable on their physical appearance during storage. Particularly, at 4 ˚C remained the stable size of bio−beads gel displaying average diameter as 4.30 mm since 0 until 28 days of storage (Table 5).

To prolong the shelf−life of bacterial cells, they have been stored at 4 ˚C to delay the metabolic process of the cells. The population of lactic acid bacteria whether in the form of naked cells or bio−beads gel contained a high number of living cells, and was stable during 0 until 7 days with average 7.69−8.59 log cfu/mL, respectively. Increasing the storage time since 7 up to 14 days could reduce viability the naked cells of single culture, whereas mixture of naked cells significantly retained the number of viable cells similar to bio−bead gel. The number of viable cells gradually decreased when stored since 21 till 28 days, compared to the initial bacterial population at 0 days. On the other hand, the structure of the bio−bead gel protected cells damage, and contained nutrients of prebiotics or mushroom extracts, including both of supplements have maintained the number of living cells average as 7.36 log cfu/mL at 28 days of storage, compared to naked cell as 6.00 log cfu/mL (Figure 6).

L. plantarum FV2 and *P. pentosaceus* FV4 in the form of bio−bead gel and naked cell have been utilized as starter culture to produce fermented milk. These bacterial starters were able to coagulate milk protein forming curd, which displayed semi−solid layer separated by whey firmness, including contain fermented odor after incubation at 37 °C for 6 hours. Especially, the application of bio−beads gel containing mixed bacteria and mushroom extracts as starter culture have been discovered the highest number of lactic acid bacteria in fermented milk as 8.03 log cfu/mL at the end of incubation, compared to other formulas (Figure 7). The result indicated that the persistence of lactic acid bacteria in products have supported the success of benefit bacterial strains onto target of applications. Fermented milk made by bio−beads gel containing *L. plantarum* FV2 and mushroom extracts as starter culture has increased the accumulation of lactic acid value by 28.13% and reduce pH of product to acid (pH 4.0), including enhanced the coagulation and curd of milk. Consequently, fermented milk products not only has contained a high amount of beneficial lactic acid bacteria but also they had prebiotics and antioxidant of mushroom extracts.

Table 4 Survival of *L. plantarum* FV2 and *P. pentosaceus* FV4 under artificial gastric juice condition

Treatments	Log CFU/mL		
	0 hour	1 hour*	2 hour**
FV2 (Naked cell)	8.34 ± 0.00^{ab}	7.05 ± 0.03^{b} (15.4)	5.70 ± 0.00^{e} (17.0)
FV4 (Naked cell)	8.31 ± 0.00^{b}	6.96 ± 0.01^{b} (16.2)	5.78 ± 0.00^{e} (15.0)
FV2 + FV4 (Naked cell)	8.36 ± 0.01^{a}	6.96 ± 0.01^{b} (16.7)	5.64 ± 0.00^{e} (16.8)
FV2 (Bio− bead inulin−oligosaccharide)	8.21 ± 0.01^{de}	7.45 ± 0.02^{b} (9.1)	6.65 ± 0.06^{c} (10.2)
FV4 (Bio−bead inulin−oligosaccharide)	8.25 ± 0.01^{c}	7.60 ± 0.03^{a} (7.9)	6.90 ± 0.06^{bc} (8.9)
FV2 + FV4 (Bio−bead inulin−oligosaccharide)	8.22 ± 0.01^{cd}	7.74 ± 0.19^{a} (5.8)	7.22 ± 0.06^{ab} (6.6)
FV2 (Bio−bead mushroom extracts)	8.23 ± 0.01^{cd}	7.87 ± 0.05^{a} (4.3)	7.40 ± 0.06^{d} (6.0)
FV4 (Bio−bead mushroom extracts)	8.18 ± 0.01^{e}	7.53 ± 0.23^{a} (7.8)	7.15 ± 0.12^{ab} (4.8)
FV2 + FV4 (Bio−bead mushroom extracts)	8.14 ± 0.03^{f}	7.56 ± 0.12^{a} (6.9)	7.00 ± 0.12^{a} (7.1)

Means with the same letters are not significantly different ($p < 0.05$). Compare mean with One−Way ANOVA, Duncan test.
* Value in bracket showed the reducing percentage compared to 0 hour.
** Value in bracket showed the reducing percentage compared to 1 hour.

Table 5 Diameter of bio−beads *L. plantarum* FV2 and *P. pentosaceus* FV4 during storage at 4 ˚C

Treatments	Diameter (mm)				
	0 day	7 day*	14 day**	21 day***	28 day****
FV2 (Bio−bead inulin−oligosaccharide)	4.49 ± 0.07^{a}	4.4 ± 0.08^{a} (0.7)	4.45 ± 0.07^{a} (0.4)	4.44 ± 0.07^{a} (0.1)	4.44 ± 0.07^{a} (0.0)
FV4 (Bio−bead inulin−oligosaccharide)	4.27 ± 0.02^{b}	4.28 ± 0.04^{b} (0.1)	4.26 ± 0.03^{b} (0.2)	4.27 ± 0.02^{b} (0.0)	4.27 ± 0.02^{b} (0.0)
FV2 + FV4 (Bio−bead inulin−oligosaccharide)	4.43 ± 0.02^{a}	4.43 ± 0.02^{a} (0.1)	4.42 ± 0.02^{a} (0.2)	4.43 ± 0.02^{a} (0.1)	4.43 ± 0.02^{a} (0.0)
FV2 (Bio−bead mushroom extracts)	4.18 ± 0.02^{c}	4.17 ± 0.02^{c} (0.1)	4.17 ± 0.02^{c} (0.0)	4.18 ± 0.02^{c} (0.0)	4.18 ± 0.02^{c} (0.0)
FV4 (Bio−bead mushroom extracts)	4.22 ± 0.06^{bc}	4.21 ± 0.05^{bc} (0.1)	4.22 ± 0.06^{bc} (0.1)	4.22 ± 0.06^{bc} 0.0)	4.22 ± 0.06^{bc} (0.0)
FV2 + FV4 (Bio−bead mushroom extracts)	4.27 ± 0.02^{b}	4.26 ± 0.03^{b} (0.1)	4.27 ± 0.02^{b} (0.1)	4.27 ± 0.02^{b} (0.0)	4.27 ± 0.02^{b} (0.0)

Means with the same letters are not significantly different ($p < 0.05$). Compare mean with One−Way ANOVA, Duncan test. * Value in bracket showed the change in diameter (%) compared to 0 days. ** Value in bracket showed the change in diameter (%) compared to 7 days. *** Value in bracket showed the change in diameter (%) compared to 14 days. **** Value in bracket showed the change in diameter (%) compared to 21 days.

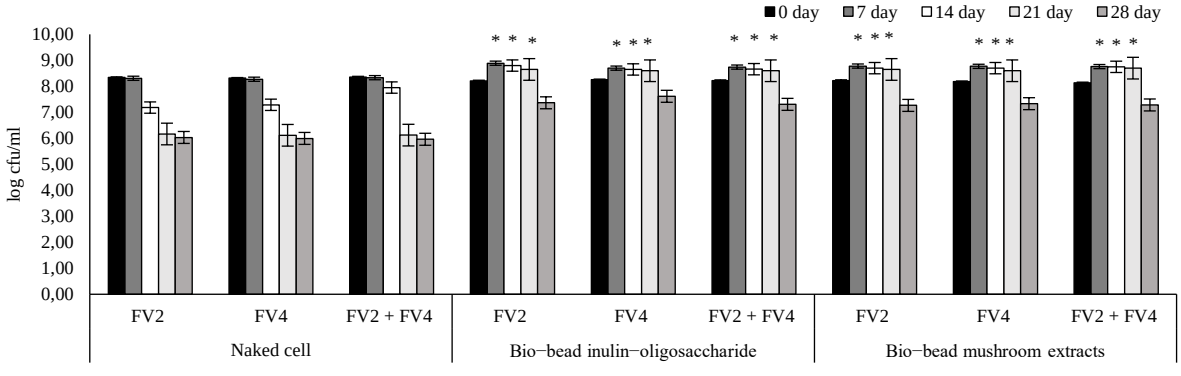


Figure 6 Survival of *L. plantarum* FV2 and *P. pentosaceus* FV4 in bio−beads and naked cells during storage at 4 ˚C for 28 days. $p < 0.05$ significant (Compare mean with One−Way ANOVA, Duncan test)

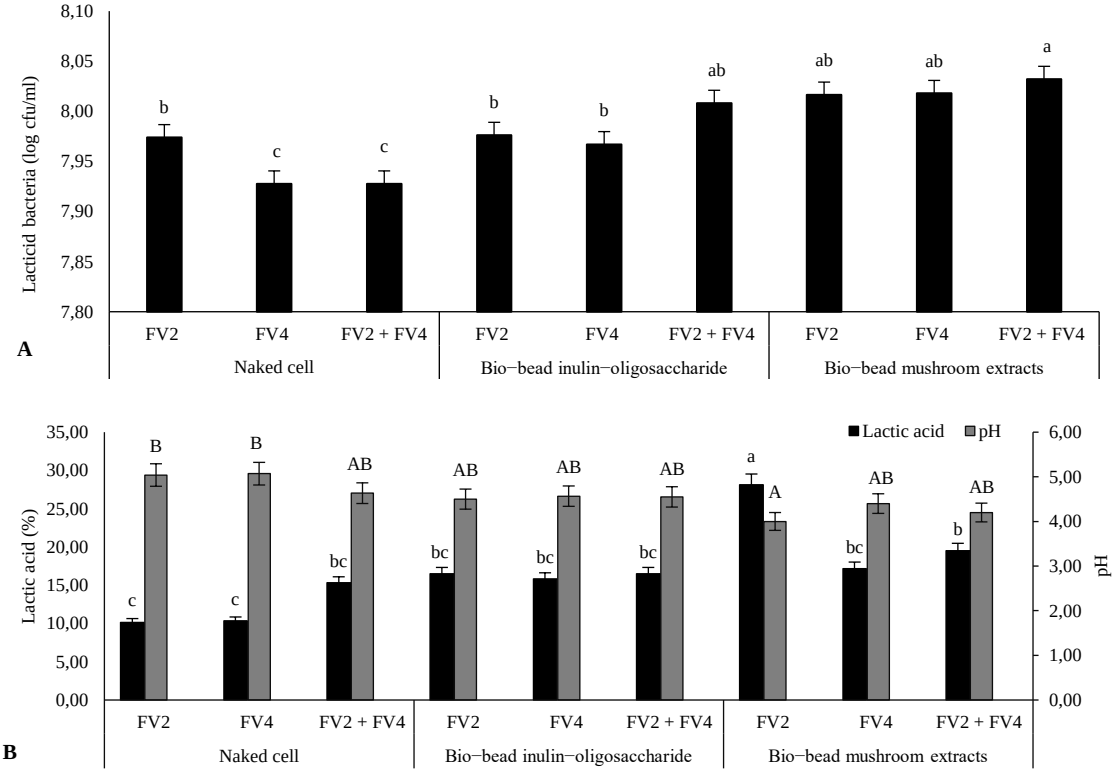


Figure 7 Lactic acid bacteria (a) and lactic acid content (b) of fermented milk produced by *L. plantarum* FV2 and *P. pentosaceus* FV4. Values represent the mean ± SD. Means with the same letters are not significantly different ($p < 0.05$). Compare mean with One−Way ANOVA, Duncan test.

DISCUSSION

Dominance of acid producing microorganisms have played an important role in the preservation and production of fermented foods that occurred spontaneously or through natural fermentation. Many lactic acid bacteria have been commonly found in fermented foods and produced lactic acid as a major organic acid from homo− or hetero−fermentative processes. These bacteria also synthesized bioactive compounds as secondary metabolites that accumulated in fermented foods (**Abedi and Hashemi, 2020**). Bacteriocins or bacteriocin−like substances with the characteristics as follow small molecules, cationic, heat stable, amphiphilic and membrane permeabilizing peptides have been accumulated in several fermented foods as antimicrobial peptides or proteins synthesized from ribosome consisting of the ability to inhibit other related (narrow spectrum) or non−related (broad spectrum) microorganisms (**Yang *et al.*, 2014; Zacharof and Lovitt, 2012**). Plantaricins and pediocins as the bacteriocin substances have been synthesized by bacterial strains *L. plantarum*, and *P. pentosaceus*, respectively, and had the effect to reduce the colonization and growth of pathogenic bacteria (**Porto *et al.*, 2017**). Moreover, bacterial strains *L. plantarum* and *L. paraplantarum* isolated from fermented foods demonstrated the antioxidant activity by DPPH assay and were capable endurance the damage from hydrogen peroxide by exhibiting efficient free radical scavenging activity (**Arasu *et al.*, 2013; Devi *et al.*, 2016**). Likewise, *P. pentosaceus* obtained from fermented food also contained the ability to scavenge hydrogen peroxide (**Watanabe *et al.*, 2016; Yamamoto *et al.*, 2019**).

Mushrooms contained many beneficial substances in different parts of them as follow fruiting bodies, and mycelium, including culture medium. Glucan, a homopolysaccharide made up of d−glucose playing an important cell wall component of mushrooms. It could be served as the dietary fiber and natural polysaccharide with the prebiotic properties that enhance the growth of beneficial gut microbiota. Edible mushrooms also contained true heteroglycans with arabinose, mannose, fructose, galactose, xylose, glucose, and glucuronic acids (**Cerletti *et al.*, 2012; Ruthes *et al.*, 2021**). The hydroxyl groups of monosaccharide units from mushrooms genus *Pleurotus* have played an essential role in the ABTS and DPPH radical scavenging activity, and total phenolic compounds with high volume when extracted with water (**Boonsong *et al.*, 2016; Fasoranti *et al.*, 2019**). Additionally, *L. squarrosulus* extract has been detected the activity of radical scavenging (**Ayimbila *et al.*, 2021; Okoro, 2012**).

Acidogenic and aciduric bacteria consisted the mechanisms to prevent their cell damage in acidic environments such as human gastric and fermentative foods. These mechanisms as follows proton pumping by F1−F0−ATPase, glutamate decarboxylase system, formation of a protective cloud of ammonia, high cytoplasmic urease activity, repair or protection of macromolecules, and biofilm formation (**Liu *et al.*, 2015**). The digestive system of human had many steps, with the initial stage being breaking down food to 2.0 mm or smaller size through the chewing process and degrading carbohydrates by amylase. Then, the small particles of digested food have been transferred to the gastric phase with very low pH (pH 1−2) of hydrochloric acid for 0.5−2 hours depending on various kinds of nutrients (**Alegría *et al.*, 2015; Mackie and Rigby, 2015**). This acidic condition destroyed and limited the amount of contaminated bacteria by mechanisms of active protease enzymes such as trypsin, chymotrypsin, and intestinal peptidases (**Brenneman *et al.*, 2013**).

Artificial gastric juice, which had a strong acid with pH 3.0, could destroy and degrade microbial cells. *L. plantarum* FV2 and *P. pentosaceus* FV4 could be protected the damage of their cells with the structure of edible alginate compound. A gel made from alginate, a polysaccharide that cross−links with calcium chloride ions, have been used to entrap bacterial cells supplemented with beneficial mushroom extracts. This gel property had a slow rate of degradation, especially under acidic stress, due to its structure of (l−4)−linked β−D−mannuronate and α−L−guluronate (**Gacesa, 1992**). Alginate as the carrier matrix could withstand to different factors of environment as follows pH, temperature, time, and ionic strength, which affected on the appearance and size of its particles (**Liu *et al.*, 2016**). Pores structure of alginate allowing the exchange of substances between the interior and exterior of the bio−beads gel could transport nutrients to bacterial cells (**Sergeeva *et al.*, 2019**). Lactic acid bacteria could utilize nutrients of milk, especially lactose for their growth and conversion to bacterial byproducts such as lactic acid, acetic acid, ethanol, and CO_2. Effective strains of acid−tolerant bacteria, *P. pentosaceus* and *L. plantarum*, obtained from fermented food with the ability of antimicrobial substances synthesis could be used as the starter inoculum to produce fermented milk and other foods in the future.

CONCLUSION

Lactic acid bacteria *L. plantarum* FV2 and *P. pentosaceus* FV4 have highly produced lactic acid, and exhibited the inhibitory activity against pathogenic bacteria. Application of edible mushroom extracts, especially 2% concentration, extremely enhanced the growth of bacteria and lactic acid production. *L. plantarum* FV2 and *P. pentosaceus* FV4 survived within the structure of bio−beads gel under strong acid condition of artificial gastric juice, including the extension of the shelf life at 4 ˚C for 28 days. Moreover, bio−beads gel could be applied as the starter culture to produce fermented milk that they contained high amount of lactic acid bacteria and antioxidant activity of mushroom extracts.

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