

Effect of *Ganoderma lucidum* polysaccharides on the growth of *Bifidobacterium* spp. as assessed using Real-time PCR

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Abstract: The use of component from *Ganoderma lucidum* as prebiotic source is interesting as the *G. lucidum* itself was known for more than a decade in the traditional Chinese medicine. In this work, *Ganoderma lucidum* crude polysaccharides (GLCP) and Polysaccharide-fraction number 2 (PF-2) were used as carbon sources in the fermentation with *Bifidobacterium* sp. The results showed the potential of prebiotic effect of the *G. lucidum* extract in batch-culture fermentation based on increment in the growth of bacteria used (0.4 – 1.5 log₁₀ CFU/mL) after 18h fermentation. Fermentation was further done using faecal materials as bacterial inocula and bacterial growth changes were examined using real-time PCR. The results showed the ability of GLCP and PF-2 to support the growth of *Bifidobacterium* genus with 0.3 and 0.7 log₁₀ cells/ml increased, respectively. Interestingly, *Lactobacillus* which is known as beneficial bacterial genus also showed growth increment with 0.7 and 1 log₁₀ cells/ml increased. The competition for carbon sources thus inhibits the growth of potentially harmful genus, *Salmonella* (0.3 and 0.5 log₁₀ cells/ml) in comparison to the control.

Keywords: *Ganoderma lucidum* crude polysaccharides, polysaccharide-fraction number 2, *Bifidobacterium* spp., prebiotic

Introduction

Over the centuries, food for keeping a healthy gastrointestinal track (GIT) has evolved and risen into a very important issue among the public and researchers, in particular. Many people have gone beyond looking for food simply to maintain normal health and are seeking to optimise performance and wellness in addition to reducing the risk of some diseases such as cardiovascular disease, cancer and osteoporosis. This has set the scene for increased demand and development of functional foods (Delphine *et al.*, 2009).

One of the functional foods that is increasing in demand is prebiotic, which has been proven through scientific research for its beneficial effects. Prebiotics are defined as “non-digestible dietary food ingredients that, when passing through the colon will benefit the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria *in situ*” (Gibson and Roberfroid, 1999). Prebiotics provide food for the growth of

lactobacilli and bifidobacteria. These non-fermentable carbohydrates can be found naturally not only in cereals such as wheat and oat, but even in garlic, chicory and artichokes. However, their quantity is present in small amounts. Fruit and vegetables are among the most common, available and easily obtained prebiotic in the diet. Although it is preferable to obtain prebiotics from natural sources, this may not always be practical. Prebiotics can be incorporated into the diet through foods such as cereals and dairy products. Direct consumption of prebiotics is already available in the market in the capsule form.

New research is being proposed to find and establish new prebiotics synchronised with the demands of the market and people. The potential of polysaccharides from mushroom is a growing interest among researchers. Polysaccharides from *Pleurotus ostreatus* and *Pleurotus eryngii* were reported to be able to support the growth of *Lactobacillus* sp. (Andriy *et al.*, 2009). Thus, interest was increased on the potential of *Ganoderma lucidum*, the well-known medical fungi as prebiotic. The *G. lucidum* mushroom

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contains pharmacologically active variables and the constituents of *G. lucidum* include polysaccharides (including beta-D-glucans, heteropolysaccharides and glycoproteins), triterpenes, germanium, essential and non-essential amino acids, sterols, lipids, antioxidants, vitamins B1, B2, B6, iron, calcium, and zinc (Huie, 2004; McKenna, 2002). This mushroom has been reported to be effective in the treatment of chronic hepatopathy, hypertension, hyperglycaemia and neophasia (Franz, 1989; Furusawa *et al.*, 1992; Shiao *et al.*, 1994). The polysaccharides were also reported to have anti-tumour properties (Miyazaki and Nishijima, 1981; Wang *et al.*, 1997) and hypoglycaemic activities (Hikino *et al.*, 1985; Tomoda *et al.*, 1986).

Thus, this study was conducted in order to determine the ability of oligosaccharides from *Ganoderma lucidum* crude polysaccharide (GLCP) extract and second fraction of polysaccharide fractions (PF-2) to selectively promote the growth of *Bifidobacterium* strains and reveal the effect of GLCP and PF-2 as prebiotic towards the bacterial microflora in human faeces.

Materials and Methods

Ganoderma lucidum extract preparation

The dried fruit bodies of *G. lucidum* were purchased from Malimas Healthcare Sdn. Bhd. (Selangor, Malaysia). The polysaccharides from fruiting bodies were extracted and fractionated according to Bao *et al.* (2002) with some modifications. Briefly, the fruiting bodies were boiled and fractionated with DEAE-cellulose column, eluted with H₂O and followed by 0.005, 0.2, 0.5 M NaCl solutions at a flow rate of 1.0 mL/min. The fractions were collected and separated based on the NaCl solutions concentration to have four fractions namely; PF-1, PF-2, PF-3 and PF-4. Fractionation was performed for several times and fractions with the same elution times were pooled together. PF-2 was the only fraction used to further the tests because of its potential in supporting bifidobacterial growth (Mohd Hamim, 2009). PF-2 was kept in 4°C. Fructooligosaccharides (FOS) was used as prebiotic control obtained from Orafit (Teinen, Belgium).

Proximate analysis of GLCP

Proximate analysis of GLCP was carried out according to AOAC method (1995). Moisture, protein, ash and crude fiber contents were analysed. Total carbohydrate contents were determined in percent by the formula, [100% - (%protein + %fat + %ash + %moisture)].

Determination of growth rates and survival of bacteria in GLCP and PF-2

The growth of *B. pseudocatenulatum* G4, *B. longum* BB536 and *B. breve* ATCC 15700 on glucose, GLCP and PF-2 was examined using batch culture fermentation. The bacteria were grown in the 1L bioreactor (B. Braun, France) for 24h in 37°C. Sampling was done for every 6h. Growth rates and the ability of bacteria to grow in GLCP and PF-2 were observed. Glucose was used as the control.

Determination of prebiotic ability of GLCP and PF-2 in faecal culture

Fresh voided faecal samples were collected in sterile plastic bags, and kept cool in insulated boxes until they were transferred to the laboratory where they were immediately analysed. Faecal slurry inoculum was prepared 1h prior to fermentation. Fresh faecal samples were collected from three healthy people who practiced normal Malaysian diet and did not take any probiotic supplements or any antibiotics for 3 months before sampling. Faecal samples (10 g) from each person were mixed and homogenised in 300 mL of 0.1 M phosphate-buffered saline (PBS), pH 7.0 (10% w/v). Then, the faecal slurry was filtered using metal sieve to discard food and solid particles from inoculum suspension. All the procedures were done in laminar flow to prevent any contamination from undesirable outside microorganisms. Four types of carbon sources namely glucose, FOS, GLCP and PF-2 were added to anaerobic faecal batch cultures separately and ran for 24h fermentation. The effects upon major groups of the microbiota namely *Bifidobacterium*, *Lactobacillus*, *Bacteroides* and *Salmonella* genus were evaluated using real-time PCR. The temperature of bioreactor was maintained for 37°C along the fermentation process to simulate human body temperature for optimum bacterial growth.

Quantitative real-time PCR

Cell concentration in fermentation samples was determined using molecular method. Duplication samples from fermentation bottles (2 mL each) were taken after 12 h and 24 h of fermentation and were stored at -20°C. After the extraction of DNA, 4 genera and species of predominant bacteria in human GIT namely *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Salmonella* were detected and quantified using quantitative real-time PCR method. The cell concentration in the samples was determined using standard curves generated from known concentration of plasmid DNA that carried the targeted gene for each genus and species. Quantification of four genera of bacteria in fermentation products was

achieved by using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each set of primers (Fite *et al.*, 2004). The real-time PCR machine generated standard curve automatically by using the standard plasmid DNA and specific primers for each bacterial genus and species. Standard curves for quantification of number of cells in samples were generated using known concentration of plasmid DNA containing target gene. Plasmid DNA that was previously extracted from competent cells was measured for its concentration (g/ μ L) using spectrophotometry method at the absorbance of 260 nm. Then, the plasmid DNA was serially diluted (10-fold) until five times before 5 μ L from each dilution were mixed with PCR mixture. The target genes were amplified by real-time PCR using the same primers in triplicate. The concentration of plasmid DNA was converted from g/ μ L in stock solution to copies/ μ L in PCR mixture. PCR amplification were performed using primers Forward 5'-CTCCTGGAAACGGGTGG-3' and Reverse 5'-GGTGTCTTCCCGATATCTACA-3' for *Bifidobacterium* genus (Matsuki *et al.*, 2003), Forward 5'-TGGAAACAGGTGCTAATACCG-3' and Reverse 5'-CCATTGTGGAAGATTCCC-3' for *Lactobacillus* genus (McOrist *et al.*, 2002), Forward 5'-TCCACCTGGGGAGTACGCCG-3' and Reverse 5'-TATGGCACTTAAGCCGACACC-3' for *Bacteriodes* genus (McOrist *et al.*, 2002) and Forward 5'-ACAGCAAATGCGGATGCTTT-3' and Reverse 5'-GAGCGCTCAGTGTAGGACTC-3' for *Salmonella* genus (Carlson *et al.*, 1999), to amplify 16S rRNA genes in this selected bacteria. Determination of specificity for each primer set and the optimum annealing temperature was done using a BiometraTM T Gradient thermocycler (Biometra, Goettingen, Germany). The primers were synthesised commercially by First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia). Real-time PCR amplification for the detection and enumeration of bacterial DNA was performed in a computer-operated Rotorgene 3000 real-time PCR system from Corbett Research, Australia. SYBR green master mix (Qiagen, USA) was used as a reporter dye in real-time PCR assay. The DNA template that had already been extracted from fermentation sample was mixed with other real-time PCR components in one 0.2 mL PCR tube before analyzed using real-time PCR machine.

Organic acid analysis

Profiles of SCFA were analysed by high-performance liquid chromatography (HPLC) (Shimadzu LC-10AS Liquid Chromatography, Japan) with a Shimadzu SPD-10AV UV-VIS detector. An

organic column packed with 9 μ m of polystyrene divinylbenzene ion exchange resin (Aminex HPX-87H; 300 mm \times 7.8 mm, Bio-Rad Laboratories, USA) and maintained at 65°C was used. The UV detector was set at 220 nm and the mobile phase was 0.009 N sulphuric acid with a flow rate of 0.7 ml/min.

Statistical analysis

Data analysis was done using MINITAB version 14 (Minitab Inc., PA, United States). Differences between growth rate constants were checked by ANOVA for pure culture fermentation. Differences between bacterial counts at 0, 18 and 24 h of fermentation for each batch culture, treatments, and concentration of organic acids were checked for significance by paired t-test, assuming equal variances and considering both sides of distribution. A probability of $P < 0.05$ was used as criterion for statistical significance.

Result and Discussion

The composition analysis of the GLCP

The yield of hot water extracts for *G. lucidum* shows that the carbohydrates (polysaccharides) was the highest (82.2%), while ash was the lowest (1.5%). The yield of protein, fat and moisture were 3.6, 4.3 and 8.4 percent, respectively. The fractionation process produced four types of fractions known as polysaccharides fraction one (PF-1), polysaccharides fraction two (PF-2), polysaccharides fraction three (PF-3) and polysaccharides fraction four (PF-4) as shown in Figure 1. However, based on the previous experiment, PF-2 was the only fraction selected for further experiment because of its ability to maintain and support the growth of *Bifidobacterium* sp. in the previous studies (Mohd Hamim, 2009).

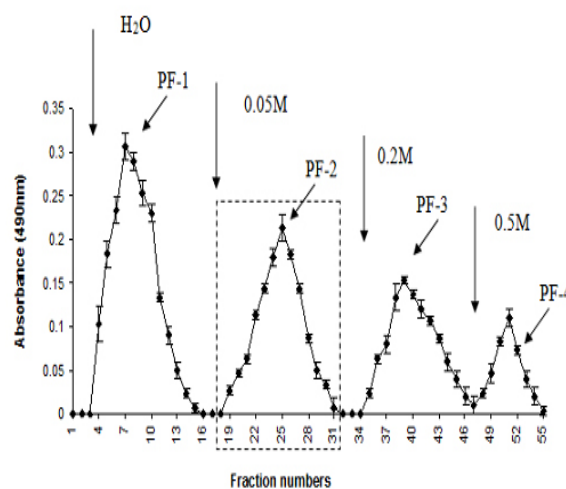


Figure 1. DEAE-cellulose elution profile of the polysaccharides present in the aqueous extract of *Ganoderma lucidum* fruit bodies. The column was eluted stepwise with H₂O, 0.05, 0.1, 0.2 and 0.5 M NaCl solutions. PF-2 was used in the further fermentations (marked with the box).

Fermentation of GLCP and PF-2 in pure culture

Polysaccharides from mushroom were found to be able to induce the growth of probiotic bacteria. Andriy *et al.* (2009) reported that polysaccharides from *Pleurotus ostreatus* and *Pleurotus eryngii* supported the growth of *Lactobacillus* strains. The research has proven the ability of polysaccharides from mushroom to act as prebiotics. In this study, polysaccharides from *Ganoderma lucidum* extract were able to increase the growth of three selected *Bifidobacterium* strains. GLCP are first polysaccharides produced through extraction and purification. Based on Fig. 2, bacterial were shown of optimum growth at 18h and slightly reduced after 24h. At 18h, GLCP was shown slight increase in the bacterial growth for *B. pseudocatenulatum* G4, *B. longum* BB536, *B. breve* ATCC 15700 with 0.69, 0.59 and 0.53 log₁₀ CFU/ml, respectively when compared to initial inoculums. The increase was because of the present of simple sugar such as glucose, in the crude extract (GLCP) itself. The present of simple sugar was also found in crude extract from several previous researches such as Saidou *et al.* (2011) with 22-36 percent of glucose from local African legumes. In the algal researches by Attachai and Anong (2010), glucose ranged within 0.09 to 0.45 percent was detected. The natural quantity after being extracted in the crude extract was the cause for the differences in glucose composition. As for PF-2, the sugar was produced through fractionation process. Fermentation of PF-2 was shown increase in the growth of *Bifidobacterium* strains (1.42, 1.61 and 1.73 log₁₀ CFU/ml) compared to the initial inoculums. In comparison to FOS, the difference in bacterial growth for PF-2 at 18h was between 0.9 – 1.6 log₁₀ CFU/ml for those three types of *Bifidobacterium* strains.

As for commercial prebiotic, FOS seems to have the ability to increase the growth of *Bifidobacterium* strains higher than GLCP and PF-2 with maximum growth at 18 h with range 2.6 to 3 log₁₀ fold bacterial count. As for *B. longum* BB536, when the fermentation reached 18 h, the growth of this bacterium seemed to increase and managed to reach 9.94 log₁₀ CFU/ml in FOS, respectively at the end of 24 h fermentation. Extended fermentation might be necessary to observe carbon source depletion point for this particular bacterium. Based on the growth profiles of *Bifidobacterium* strains, all strains were able to consume and degrade the component of crude GLCP and PF-2. As for *G. lucidum*, polysaccharides that are beneficial to human are also beneficial to *Bifidobacterium* strains from the perspective of this research.

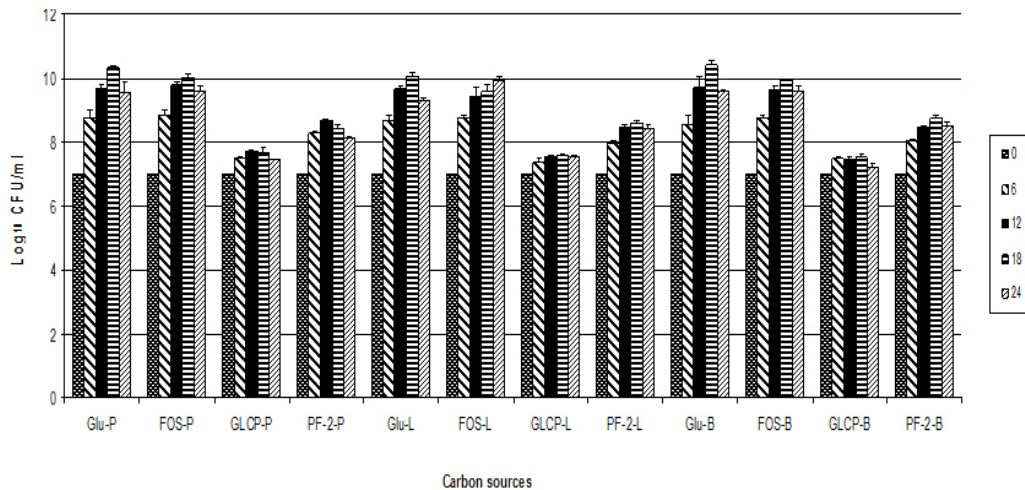
From the Figure 2, all of the *Bifidobacterium*

strains used possessed a different growth rate in the fermentation process with glucose, FOS, GLCP or PF-2 as their carbon sources. The growth rates of *Bifidobacterium* strains were determined by the ability to ferment each carbon sources and degree of polymerisation (DP) structure as *Bifidobacterium* sp. preferred to use low-DP carbohydrates (Cummings *et al.*, 2001). From the three strains assessed, the best growth rates belonged to *B. pseudocatenulatum* G4 in FOS. FOS has the lowest DP structure among these four carbon sources. Even though the *Bifidobacterium* strains gave different log numbers of bacteria when grown in different types of prebiotics and carbon sources, the growth patterns were almost the same as they consumed the carbon sources on the same biochemical pathway.

Fermentation of GLCP and PF-2 in human faecal culture

Fermentation of polysaccharides in faecal cultures was conducted to show the prebiotic capabilities on all *Bifidobacterium* strains. The changes in cell number of bacteria were monitored using real-time PCR analysis. The fermentations were performed in the bioreactor with faecal slurry as the bacterial inoculum. Initial numbers of bacteria were different from each other with the highest being *Bacteriodes* with 7.4 log₁₀ cells/ml, followed by *Bifidobacterium*, *Lactobacillus* and *Salmonella* with concentrations at 7.2, 6.5, and 2.8 log₁₀ cells/ml, respectively. In these fermentations, the ability of bacteria to use carbon sources was shown with differences in the increment of bacterial cell numbers. Using real-time PCR analysis, the numbers of bacteria will be shown merely increment in the results as the machine will calculate not only alive but also dead bacteria.

In the human gut, bacterial composition changes according to daily diet taken by the host. FOS was proven to be able to increase *Bifidobacterium* genus (Bouhnik *et al.*, 1996) thus giving a better gut health to the host. Based on Table 1, all selected carbon sources were able to increase the number of *Bifidobacterium* genus with the range between 0.3 log₁₀ cells/ml to 1.1 log₁₀ cells/ml. In the prebiotic treatment, the increase in the growth of the bacteria tested could be seen with significant numbers ranging from 0.6 log₁₀ cell/ml to 1.8 log₁₀ cell/ml after 24 h of fermentation. GLCP and PF-2 that were chosen to be prebiotics candidates were able to increase the growth of total *Bifidobacterium* genus by 0.3 log₁₀ cell/ml and 0.7 log₁₀ cells/ml, respectively. The increase of *Bifidobacterium* genus showed significant difference ($P \leq 0.05$) in PF-2 at 24 h when compared to the initial



Glu-P, FOS-P, GLCP-P, PF-2-P = Carbon sources used for fermentation of *B. pseudocatenulatum* G4; Glu-L, FOS-L, GLCP-L, PF-2-L = Carbon sources used for fermentation of *B. longum* BB536; Glu-B, FOS-B, GLCP-B, PF-2-B = Carbon sources used for fermentation of *B. breve* ATCC 15700. Data are expressed as mean±standard error (N=3).

Figure 2. Growth of *Bifidobacterium* spp. in different types of carbon sources in faecal batch culture fermentation

count. However, no significant difference ($P>0.05$) could be seen for GLCP and PF-2 when compared to the control. On the other hand, the increase of cell number for *Lactobacillus* genus was interestingly higher compared to *Bifidobacterium* genus with 0.7 log₁₀ cells/ml for GLCP and 1 log₁₀ cells/ml for PF-2. *Lactobacillus* is known for its beneficial effects and most of the probiotics come from this genus. The increase of *Lactobacillus* was significantly different ($P\leq 0.05$) when compared to the initial count of cell numbers with the highest growth at 1.8 log₁₀ cells/ml in FOS. The increase was even higher as compared to *Bifidobacterium* genus thus showing their ability to be better at fermenting polysaccharides. *Bacterioides* also showed an increase in FOS, GLCP and PF-2. However, the increase was far too low when compared to the control. Thus, it could be concluded that *Bacterioides* does not prefer to use a FOS, GLCP and PF-2 as a carbon source. The inhibition of *Salmonella* was clearly shown in the FOS and PF-2 treatment. The inhibition of potential pathogenic bacteria fulfilled one of the prebiotic criteria. As the increase of *Bifidobacterium* and *Lactobacillus* in GLCP and PF-2 media was not as significantly ($P>0.05$) high as in FOS, the ability of GLCP and PF-2 to support the growth of those bacteria increases the expectation for a new prebiotic source.

Organic acids production

Acetic acid and lactic acid are two types of main organic acids produced by *Bifidobacterium* genus in ratio 3 to 2. In this research, the ability of *Bifidobacterium* genus in fermenting GLCP and PF-2 were shown by quantity of organic acid produced along their fermentation process (Table 2). The production of organic acids was in line with the

Table 1. Changes in bacterial population (log₁₀ cells/ml) in batch cultures after 12 h and 24 h of incubation using real-time PCR based on total number of cells (viable and dead).

Group	Time (h)	Glucose (control)	FOS (prebiotic control)	GLCP	PF-2
<i>Bifidobacterium</i>	N ₀	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.4
	12	7.6 ± 0.4 ^a	8.2 ± 0.3 ^{aA}	7.4 ± 0.3	7.5 ± 0.3
	24	8.0 ± 0.4 ^a	8.3 ± 0.3 ^{aA}	7.5 ± 0.2 ^B	7.9 ± 0.1 ^a
<i>Lactobacillus</i>	N ₀	6.5 ± 0.8	6.5 ± 0.8	6.5 ± 0.8	6.5 ± 0.8
	12	7.3 ± 0.6 ^a	8.0 ± 0.7 ^{aA}	6.9 ± 0.7 ^a	7.1 ± 0.7 ^a
	24	7.9 ± 0.5 ^a	8.3 ± 0.6 ^{aA}	7.2 ± 0.6 ^{aB}	7.5 ± 0.7 ^a
<i>Bacterioides</i>	N ₀	7.4 ± 0.3	7.4 ± 0.3	7.4 ± 0.3	7.4 ± 0.3
	12	7.9 ± 0.4 ^a	7.7 ± 0.3 ^a	7.5 ± 0.3	7.6 ± 0.3 ^a
	24	8.1 ± 0.4 ^a	7.9 ± 0.4 ^a	7.6 ± 0.2 ^{aB}	7.7 ± 0.3 ^{aB}
<i>Salmonella</i>	N ₀	2.8 ± 0.8	2.8 ± 0.8	2.8 ± 0.8	2.8 ± 0.8
	12	3.1 ± 0.6	2.8 ± 0.9	2.9 ± 0.8	2.9 ± 0.9
	24	3.4 ± 0.5	2.9 ± 0.9	3.1 ± 0.8	2.9 ± 0.9

N0 = Initial inoculum
a Significantly different from initial count ($P\leq 0.05$) at 0h
A Significantly different from glucose ($P\leq 0.05$) with higher value
B Significantly different from glucose ($P\leq 0.05$) with lower value
Values are mean log₁₀ cells/ml culture ± SD for three replicates.

Table 2. Production of lactic acid and acetic acid in batch cultures fermentation of human faecal microbiota and significant difference between GLCP and PF-2 with glucose (control) and FOS (prebiotic control).

Carbon sources	Hours	Lactic acid (µmol/ml)	Acetic acid (µmol/ml)
Glucose (control)	0	3.0 ± 0.8	5.3 ± 0.4
	12	20.2 ± 1.4	26.7 ± 0.7
	24	29.3 ± 0.9	38.5 ± 1.2
FOS (prebiotic control)	0	3.0 ± 0.8	5.3 ± 0.4
	12	28.5 ± 2.1 ^a	36.2 ± 1.1 ^a
	24	39.6 ± 0.7 ^a	46.8 ± 1.4 ^a
GLCP	0	3.0 ± 0.8	5.3 ± 0.4
	12	6.9 ± 1.6 ^{aA}	10.1 ± 0.9 ^{aA}
	24	8.2 ± 1.3 ^{aA}	13.3 ± 1.2 ^{aA}
PF-2	0	3.0 ± 0.8	5.3 ± 0.4
	12	11.4 ± 0.7 ^{aA}	15.7 ± 1.3 ^{aA}
	24	15.2 ± 1.1 ^{aA}	21.1 ± 0.6 ^{aA}

^a Significantly different from Control ($P\leq 0.05$) with higher value
^b Significantly different from Control ($P\leq 0.05$) with lower value
^A Significantly different from FOS ($P\leq 0.05$) with lower value

growth of *Bifidobacterium* genus which shown the high numbers of this bacteria in faecal batch fermentation. Organic acid also one of the factors identified in inhibiting pathogenic bacteria in several researches. In the research by Midolo *et al.* (2005), the high concentration of organic acid was reported to be able to inhibit the growth of pathogenic bacteria in the gut such as *Helicobacter pylori*. In this study, the primers used for quantification using real-time PCR assay were counting both the dead and alive bacteria thus only increases of bacteria could be seen. Therefore, the inhibition of *Salmonella* genus only could be seen by comparing the growth of *Salmonella* genus in glucose medium with the other carbon sources containing media. From Table 1, the cell number of *Salmonella* genus was slightly increased or in same numbers as initial inoculums. When compared to the glucose (control), the difference was between 0 to 0.1 log₁₀ cells/ml which resulted from the inability of *Salmonella* genus to grow in GLCP and PF-2 and thus can be concluded that the *Salmonella* growth was inhibited. The inhibition of *Salmonella* sp. by *Bifidobacterium* sp. was also shown by Araya-Kojima *et al.* (1995) by administration of *B. longum* BB536.

Conclusion

Fermentation of *Ganoderma lucidum* extracts have shown prebiotic ability of polysaccharides in increasing the number of bifidobacteria. The increase in the number of *Lactobacillus*, which is also well-known for its probiotic capability, should be considered as part of success in this study. The production of organic acid during prebiotic treatments showed domination of *Bifidobacterium* species throughout the fermentation process. The pattern of its production and inhibition of potential pathogenic bacteria suggest that the organic acids might directly confer antimicrobial action.

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