

# Fermentation of *Dioscorea hispida* oligosaccharides as prebiotic potential by lactic acid bacteria and bifidobacterium

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**Abstract.** *Dioscorea hispida* oligosaccharides (DHOS) were intended to be used as a synbiotic product. Fermentability of DHOS by lactic acid bacteria and Bifidobacterium was studied. *In vitro* DHOS fermentations by *L. acidophilus* FTDC 2131, *L. casei* ATCC 393, *L. casei* FTCC 0442, *L. plantarum* FTCC 0350 and *B. bifidum* 12 were at 37°C for 24 h. DHOS was the most preferred substrate by *L. acidophilus* FTDC 2131, *L. plantarum* FTCC 0350 and *B. bifidum* 12, whilst, fructooligosaccharides (FOS) and inulin was the best substrate for the growth of *L. casei* ATCC 393 and *L. casei* FTCC 0442, respectively. The pH reduction in DHOS fermentation medium was comparable to the commercial FOS suggesting that DHOS was able to enhance the selected bacterial growth and produced organic acids. Fermentation of DHOS by *B. bifidum* 12 had exhibited the highest prebiotic activity score (+0.713) and produced 30.42 mM lactic, 22.63 mM acetic and 0.71 mM propionic acids. The incorporation of DHOS in the medium culture of the selected bacteria was able to enhance the bacterial growth, reduce the pH medium, produce substantial amount of lactic, acetic and propionic acids, and exhibit positive prebiotic activity score.

## 1. Introduction

*Dioscorea hispida* belongs to Dioscoreaceae family which has about 1137 species with only 600 species are edible [1]. *D. hispida* is a monocotyledonous perennial climbing plant that produces tubers. However, the tubers contain water soluble toxic alkaloid dioscorine. Consequently, *D. hispida* tubers have low economic value, although a plant of *D. hispida* can generally produce 15 kg of mature tubers. However, sometimes the plant can produce up to 25-30 kg of tubers depending on the climate. Each 100 g of tubers could produce up to 20 g of starch [2]. *D. hispida* tuber is synonymous with poor people daily food as well as an important food during famine [3]. *D. hispida* is widely found as a wild plant in tropical and subtropical regions of the world. Besides the tubers, *D. hispida* plant has significant benefit in ethnopharmaceutical practice as the plant extract has been traditionally used in Bangladesh to treat arthritis, ulcer and skin infections [4]. It was also revealed that the bioactive extracts of *D. hispida* plant had antioxidant, anti-microbial, thrombolytic and membrane stabilization activities [4].

Tubers and roots which generally do not contain gluten that causes incidence of celiac disease or allergic reactions are important sources of carbohydrates in tropical and subtropical countries as staple foods [5]. Therefore, attempts are being made by the Malaysian government to breed and cultivate the *D. hispida* plant for its tubers in large scale to fulfil the government food security agenda [6].



Properties of *D. hispida* starch are unique which can be used as a new source of starch for various applications [7]. Conventionally, *D. hispida* tubers are submerged in free flowing water such as river stream or submerged in large volume of contained water for 3-5 days with daily change of water to remove the dioscorine. Usually, the tubers are consumed together with grated coconut or dipping sauce after boiling, steaming or frying the tubers [2, 3].

Starch of *D. hispida* tuber could be value added by hydrolysing the starch into oligosaccharides. Oligosaccharides are a group of carbohydrate polymers with degree of polymerisation (DP) of 2 to 12. The oligosaccharides are regarded as prebiotic if they are resistant to digestion and absorption in the human small intestine but fermented into short chain fatty acids by beneficial intestinal microflora in the large intestine [8]. The US National Centre for Complementary and Integrative Health refers prebiotic as dietary substance that favours the growth of beneficial microbes over the harmful ones [9]. The Centre also refers synbiotic as a product that contains both prebiotic and probiotic. The US Food and Drug Agency only recognizes inulin, fructooligosaccharides, galactosyloligosaccharides and xylooligosaccharides from various sources as prebiotics although lactulose, mannitol, maltodextrin, raffinose and sorbitol are also commercially used as prebiotics [10]. In this research, starch obtained from *D. hispida* tuber was further processed via enzymatic hydrolysis to oligosaccharides. Experiments were conducted to evaluate the prebiotic activity of *D. hispida* oligosaccharides by selected lactic acid bacteria (*L. acidophilus* FTDC2131, *L. casei* ATCC39, *L. casei* FTCC0442, *L. plantarum* FTCC0350) and *B. bifidum* 12 against gut pathogens (*E. coli* FTDC201, *C. coli* c519, *C. perfringens* c438).

## 2. Materials and methods

### 2.1. Materials

*D. hispida* tubers were obtained from Marang, Terengganu, Malaysia. Commercial prebiotics FOS (Raftilose, Orafti® P95, BENEIO-Orafti) and inulin (Raftiline, Orafti® GR, BENEIO-Orafti) were used as positive controls were provided by DPO (M) Pvt. Ltd. (Kuala Lumpur, Malaysia).

### 2.2. Bacterial strains

Pure cultures of lactic acid bacteria were obtained from Bioprocess Technology Division (School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia) and Food Technology Research Centre (Malaysian Agricultural Research and Development Institute, Selangor, Malaysia). *Bifidobacterium bifidum* 12 was provided by Chr-Hansen A/S (Malaysia Regional Office, Selangor, Malaysia) and pathogens (*Escherichia coli* FTDC 201, *Campylobacter coli* c519 and *Clostridium perfringens* c438) were obtained from the Institute for Medical Research, Kuala Lumpur, Malaysia. Lactic acid bacteria and bifidobacterium cultures were maintained on de Man, Rogosa, Sharpe agar (Hi-Media, India). *E. coli* and *C. coli* were maintained on the tryptic soy agar (Hi-Media, India), while *C. perfringens* was on Wilkins Chalgren anaerobic agar (Hi-Media, India).

### 2.3. Preparation of *D. hispida* tuber flour and starch samples

*D. hispida* tubers (total weight of 15 kg) were peeled, washed and cut into slices before submerged in excessive water for 4 days with daily constant change of water. Then, the slices were dried in an oven (Mettler UL40) at 50°C until a constant weight was obtained. The dried tuber slices were grinded and sieved to obtain flour with particle size of less than 180 µm. Starch was extracted from *D. hispida* tuber flour according to Hoover and Senanayake [11]. The starch was ground to particle size of 180 µm and stored in an airtight container until used.

### 2.4. *Dioscorea hispida* oligosaccharides (DHOS)

DHOS was prepared by the enzymatic hydrolysis of *D. hispida* starch. Initially, the starch slurry (10%, w/v) in 0.05 M citrate phosphate buffer, pH 5 was gelatinized at 100°C for 30 min [12]. Then, 5 mL of

50 ppm  $\text{CaCl}_2$  was added, followed by 0.15 mL enzyme solution which consist of 25 GNU  $\alpha$ -amylase and 125 NPUN pullulanase (Novozyme, Bagsvaerd, Denmark) and the sample mixture was incubated in a shaking water bath of 150 rpm for 12 h. The enzymatic hydrolysis was deactivated by boiling the samples for 10 min. The samples were centrifuged at  $2500\times g$  for 15 min and supernatant was transferred into clean tubes and analysed for reducing sugars (dinitrosalicylic acid method) and oligosaccharides concentration (HPLC). Maltooligosaccharides standard (Supelco 4-7265, USA) was used to estimate the concentration of *D. hispida* oligosaccharides.

### 2.5. *In vitro* fermentation and determination of cell viability

For lactic acid bacteria and bifidobacterium, fermentation medium was de Man, Rogosa, Sharpe broth except that the dextrose was replaced with 1% (w/v) of *D. hispida* oligosaccharides, FOS or inulin. Tryptic soy broth was for both *E. coli* and *C. coli* whereas Wilkin Chalgren anaerobic broth was for *C. perfringens*. Fermentation process was carried out at  $37^\circ\text{C}$  (in aerobic or anaerobic condition according to the required oxygen of the bacteria) for 24-72 h after inoculating with 1 mL bacterial inoculum ( $10^6$  colony-forming unit, CFU) in 20 mL medium. All preparation, incubation of pre-culture and fermentation, sampling, serial dilution and agar plating for *B. bifidum* and *C. perfringens* (obligate anaerobic cultures) were carried out in an anaerobic chamber supplied with an atmospheric system of 85%  $\text{N}_2$ , 10%  $\text{CO}_2$  and 5%  $\text{H}_2$ . The viable counts were determined by standard spread plate method incubated for 24-48 h and referred as  $\log_{10}$  CFU/mL.

### 2.6. Prebiotics activity score

The prebiotic activity score (PAS) was calculated according to Huebner et al [13]:

$$\text{PAS} = \left( \frac{\text{Cell density changes on prebiotic}}{\text{Cell density changes on glucose}} \right) - \left( \frac{\text{Cell density changes of pathogen on prebiotic}}{\text{Cell density changes of pathogen on glucose}} \right) \quad (1)$$

The changes in cell density were the differences between the viable count at 0 h and 24 h (in  $\log_{10}$  CFU/mL).

### 2.7. Determination of lactic and short chain fatty acids

Cell free supernatants (20  $\mu\text{L}$ ) were filtered and injected into a high performance liquid chromatographer (Shimadzu, Kyoto, Japan), which was attached with IC pak-ion exclusion column, 300 mm  $\times$  7.8 mm (Waters, USA) and UV-Vis detector (Shimadzu Model SPD-20A, Kyoto, Japan) at 210 nm. The elution took place at  $50^\circ\text{C}$  with 5 mM degassed  $\text{H}_2\text{SO}_4$  at a flow rate of 0.6 mL/min. Concentrations of organic acids in the samples were referred to standard calibration curves of lactic, acetic and propionic acids (HPLC grade, Fluka, Steinheim, Germany).

### 2.8. Statistical analysis

The triplicate data was subjected to a one-way analysis of variance (ANOVA), and the significance of the differences between means was determined by the Duncan test, where  $p < 0.05$  was considered statistically significant. The Statistical Package for Social Science, version 14.0 (SPSS Inc., U.S.A) was used for the analysis.

## 3. Results and discussion

### 3.1. *Dioscorea hispida* oligosaccharides

Figure 1 shows the HPLC chromatogram of *D. hispida* oligosaccharides. It contains a mixture of carbohydrate polymers of maltose (DP 2) to maltooligosaccharides with DP 10. The mixture also contained glucose but the amount was insignificant compared to the amount of oligosaccharides.

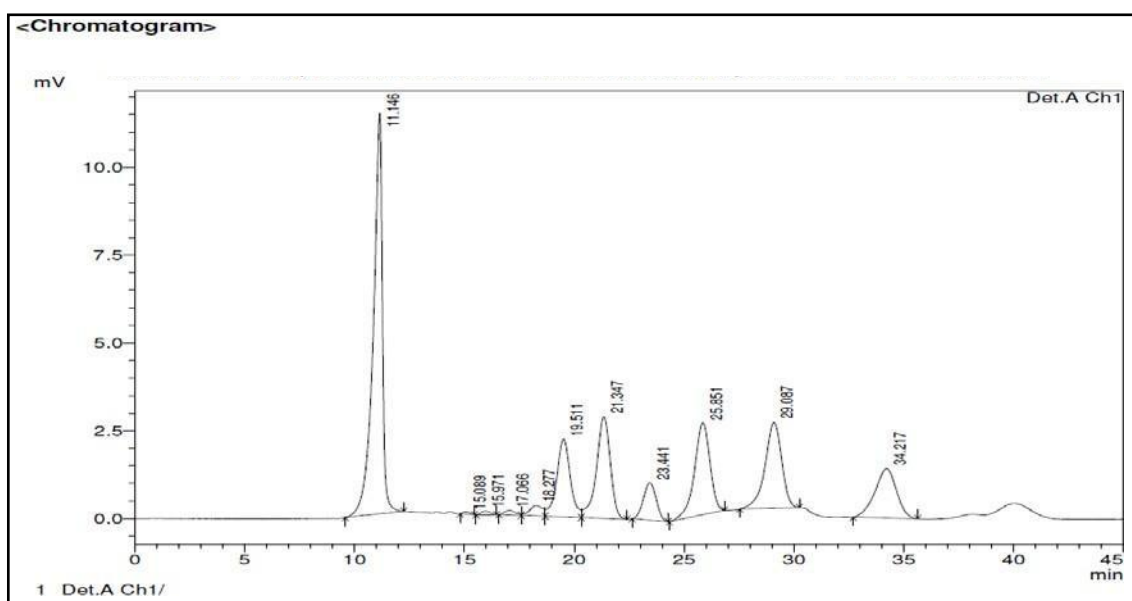


Figure 1. HPLC chromatogram of *D. hispida* oligosaccharides (DHOS).

### 3.2. Growth of lactic acid bacteria and bifidobacterium on *D. hispida* oligosaccharides, inulin and FOS

The increase in viable count of the microbes in different substrates after 24 h fermentation was recorded in Table 1. It shows that all microbes can grow in the substrates. In fact, *D. hispida* oligosaccharides and commercial prebiotics also supported the growth of pathogens. Thus, increase in viable counts of beneficial bacteria alone cannot be used as indicator to prebiotic characteristic of a substrate.

Table 1. Increase in the cell density of selected lactobacilli, bifidobacterium and pathogens in media containing glucose, *D. hispida* oligosaccharides (DHOS), FOS and inulin after 24 h of fermentation.

Bacterial cultures	Cell density ( $\log_{10}$ CFU/mL)*			
	Glucose	DHOS	FOS	Inulin
<i>L. acidophilus</i> FTDC 2131	$2.55 \pm 0.13^c$	$2.72 \pm 0.14^a$	$2.69 \pm 0.10^b$	$2.63 \pm 0.04^b$
<i>L. casei</i> FTCC 0442	$1.75 \pm 0.01^d$	$2.24 \pm 0.01^c$	$2.65 \pm 0.04^b$	$2.90 \pm 0.12^a$
<i>L. casei</i> ATCC 393	$3.17 \pm 0.90^a$	$3.15 \pm 0.06^a$	$2.96 \pm 0.04^c$	$3.07 \pm 0.10^b$
<i>L. plantarum</i> FTCC 0350	$2.46 \pm 0.12^b$	$2.52 \pm 0.07^a$	$2.47 \pm 0.03^b$	$2.10 \pm 0.08^c$
<i>Bifidobacterium bifidum</i> 12	$2.33 \pm 0.01^d$	$3.53 \pm 0.03^a$	$3.25 \pm 0.03^b$	$3.10 \pm 0.09^c$
<i>Escherichia coli</i> FTDC 201	$2.14 \pm 0.11^c$	$1.73 \pm 0.01^d$	$2.34 \pm 0.02^b$	$2.82 \pm 0.08^a$
<i>Campylobacter coli</i> c159	$2.24 \pm 0.10^a$	$2.14 \pm 0.02^b$	$2.02 \pm 0.01^c$	$2.16 \pm 0.11^b$
<i>Clostridium perfringens</i> c438	$2.03 \pm 0.08^a$	$1.62 \pm 0.01^c$	$1.82 \pm 0.10^b$	$1.63 \pm 0.03^c$

\* Increase in cell density was the differences between the viable count at 24 h and 0 h (in  $\log_{10}$  CFU/mL).

<sup>abcd</sup> Different superscript indicate significant different within a row for different bacteria ( $p < 0.05$ ).

Therefore, Huebner et al. [13] had introduced a formulae to calculate prebiotic activity score, taking into account that a substrate, be it prebiotic or not a prebiotic, could support growth of both beneficial microbes and pathogens.

Substrate with prebiotic characteristic should selectively support the growth of beneficial microbes more than the pathogens. Prebiotic activity score is calculated by the difference of increase in viable count of beneficial microbes in potential prebiotic over glucose and increase in viable count of pathogens in potential prebiotic over glucose. Positive and big score indicates a good prebiotic substrate. Many researchers interested in studying oligosaccharides from various sources to be claimed as prebiotics such as oligosaccharides from dragon fruit flesh [14], pectic oligosaccharides from orange peel wastes [15], oligosaccharides from coffee mannan [16] and refined arabinoxylooligosaccharides from wheat bran [17]. All of these oligosaccharides have supported the growth of beneficial microorganisms.

### 3.3. Prebiotic Activity Score

Figures 2(a-c) show the prebiotic activity scores (PAS) of *D. hispida* oligosaccharides and commercial prebiotics for five beneficial microbes; *L. acidophilus* FTDC 2131, *L. casei* FTCC 0442, *L. casei* ATCC 393, *L. plantarum* FTCC 0350 and *B. bifidum* 12 against three detrimental microbes; *E. coli*, *C. coli* and *C. perfringens*. Higher and positive prebiotic score indicates better substrate. Generally, PAS of *D. hispida* oligosaccharides and commercial prebiotics (FOS and inulin) were positive and significantly higher than that of blank medium (medium with glucose). *D. hispida* oligosaccharides had higher PAS than inulin for all the beneficial microbes ( $p < 0.05$ ) except *L. plantarum* against *E. coli*, *L. casei* FTCC 0442 against *C. perfringens* and *C. coli*. *D. hispida* oligosaccharides exhibited high PAS with *L. casei* FTCC 0442 (+0.471) and *B. bifidum* 12 (+0.470) against *E. coli*, with *B. bifidum* 12 against *C. coli* (+0.613) and *C. perfringens* (+0.713). Commercial FOS scored positive PAS for all beneficial microbes against *E. coli* (+0.04 to +0.171), *C. coli* (+0.034 to +0.500) and *C. perfringens* (+0.037 to +0.501). Commercial prebiotic inulin had positive PAS for most beneficial microbes but inulin PAS were negative for *L. acidophilus* FTDC 2131 (-0.287) and *L. casei* ATCC 393 (-0.392) against *E. coli*, and for *L. plantarum* FTCC 0350 against *C. coli* (-0.151). Thus, it was assumed that the prebiotic activity score of each substrate is largely depending on the substrate itself and type of bacterial strains. PAS of longan fruit hydrolysates was +1.69 with *L. acidophilus* La5 and PAS of the same substrate with *B. lactis* was +1.44 [18].

Nevertheless, pairing *D. hispida* oligosaccharides with *B. bifidum* 12 scored the highest PAS and thus the fermentation media of different substrates by *B. bifidum* 12 were further analysed for lactic, acetic and propionic acids.

### 3.4. Concentration of lactic, acetic and propionic acids

Table 2 displays the concentration of lactic, acetic and propionic acids after 24 h fermentation of substrates by *B. bifidum* 12. Fermentation of *D. hispida* oligosaccharides produced significantly the highest concentration of acetic and propionic acids. The highest lactic acid produced by synergistic of *D. hispida* oligosaccharides and *B. bifidum* 12 was 30.42 mM, significantly lower than FOS but higher than inulin. Ability to produce lactic and short chain fatty acids is one of 8 criteria for a probiotic [19]. Lactic and short fatty acids reduce the intraluminal pH which favours the growth of bifidogenic and other lactic acid bacteria. Short chain fatty acids also stimulate water and electrolyte absorption in the intestine and hence reduce the risk of diarrhoea and dehydration. They also increase colonocyte proliferation and metabolic energy production [20].

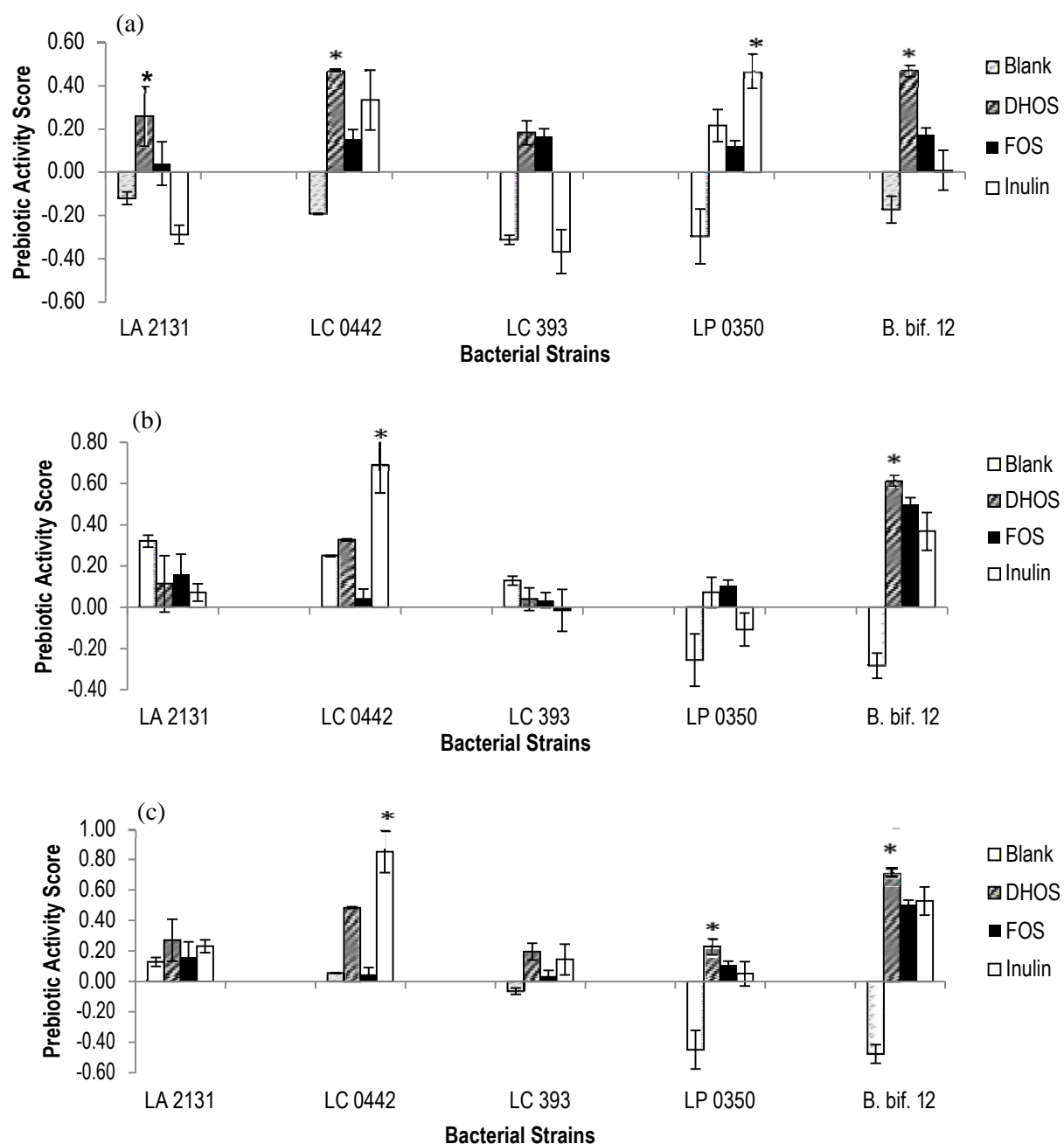


Figure 2. Prebiotic activity scores for of bacterial cultures supplemented with 1% substrates (DHOS, FOS and inulin) by lactobacilli and bifidobacterium against (a) *Escherichia coli*, (b) *Campylobacter coli* and (c) *Clostridium perfringens*. Data were expressed as mean  $\pm$  SD from calculation of three independent experiments.\*Bar indicates the highest score within the same bacterial strains ( $p < 0.05$ ). LA2131, *L. acidophilus* FTDC2131; LC0442, *L. casei* FTCC0442; LC393, *L. casei* ATCC393; LP0350, *L. plantarum* FTCC0350; B. bif 12, *B. bifidum* 12.

Table 2. Concentration of lactic, acetic and propionic acids after 24 h fermentation of different substrates by *B. bifidum* 12.

Substrate	Lactic acid (mM)	Acetic acid (mM)	Propionic acid (mM)
Glucose	53.95 ± 1.72 <sup>a</sup>	19.45 ± 0.01 <sup>b</sup>	0.55 ± 0.01 <sup>c</sup>
DHOS	30.42 ± 0.33 <sup>b</sup>	22.63 ± 0.14 <sup>a</sup>	0.71 ± 0.06 <sup>a</sup>
FOS	53.92 ± 0.09 <sup>a</sup>	13.31 ± 0.11 <sup>c</sup>	0.69 ± 0.21 <sup>b</sup>
Inulin	15.47 ± 0.88 <sup>c</sup>	19.00 ± 0.09 <sup>b</sup>	0.63 ± 0.05 <sup>b</sup>

Data were expressed as mean ± SD from three independent experiments.

<sup>abc</sup> Different superscript alphabets indicate significant different concentrations of a particular acid among different substrates ( $p < 0.05$ ).

#### 4. Conclusion

*D. hispida* oligosaccharides were able to selectively enhance the growth of lactobacilli and bifidobacterium strains better than the growth of pathogens. *D. hispida* oligosaccharides was the most preferred substrate by *L. acidophilus* FTDC 2131, *L. plantarum* FTCC 0350 and *B. bifidum* 12 as compared to inulin and FOS. Fermentation of DHOS by *B. bifidum* 12 had significantly exhibited the highest PAS against the pathogens and produced substantial lactic (30.42 mM), acetic (22.63 mM) and propionic (0.71 mM) acids.

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