Nondigestible Carbohydrates Isolated from Medicinal Mushroom Sclerotia as Novel Prebiotics

*Shane Gao, Connie K. M. Lai, & Peter C. K. Cheung**

Department of Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

* Address all correspondence to Peter C. K. Cheung, University Science Centre, Department of Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; Tel.: +852 26096144; Fax: +852 26035745; petercheung@cuhk.edu.hk

ABSTRACT: Nondigestible carbohydrates (NDCs) extracted from two medicinal mushroom sclerotia, including *Poria cocos* (PC) and *Polyporus rhinocerus* (PR), were 98.4% and 100.0% carbohydrate by weight, respectively. These sclerotial NDCs were incubated separately with *Bifidobacterium longum, Lactobacillus brevis,* and *Clostridium celatum* for *in vitro* fermentation, with lactulose as the positive control. The sclerotial NDCs stimulated the growth of *B. longum* and *L. brevis* more preferentially than *C. celatum* after 24 hours of fermentation. The short-chain fatty acids (SCFAs) profile of sclerotial NDCs was dominated by acetate (98.4% of total SCFAs). The utilization of the sclerotial NDCs in terms of organic matter disappearance (OMD) by the bacteria during fermentation was consistent with the bacterial growth. The potential application of NDCs from mushroom sclerotia as novel prebiotics for gastrointestinal health was discussed.

KEY WORDS: mushroom sclerotia, medicinal mushrooms, prebiotics, nondigestible carbohydrates (NDCs), short-chain fatty acids (SCFAs)

I. INTRODUCTION

Functional foods have enjoyed great strides, increasing at a rate of 15%–20% per year.[1] Prebiotics, probiotics, and synbiotics as functional food ingredients have recently been in research spotlights for their positive influence on constipation, hyperlipidemia, diabetes, obesity, diverticular diseases, colon cancer, and other digestive-related diseases.[2] Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health.[3] Most of the currently available prebiotics are extracted from natural sources such as (2→1)-β-fructans, but prebiotic potential has also been shown for other nondigestible carbohydrates (NDCs) such as galacto-oligosaccharides, xylo-oligosaccharides, soybean oligosaccharides, polyols and polydextrose.[4] Synbiotics, which is one of the most popularly developed ideas in recent years, strengthen the matching possibilities between prebiotics and probiotics, because the digestion of prebiotics by probiotics largely depends on the compatibility between probiotic enzymes and the chemical and structural characteristics of prebiotics. However, because investigating the fermentability of novel prebiotics in humans is time consuming, expensive

ABBREVIATIONS

NDC: nondigestible carbohydrate; **PC:** *Poria cocos*; **PR:** *Polyporus rhinocerus*; **SCFA:** short-chain fatty acids; **OMD:** organic matter disappearance; **RCB:** Reinforced Clostridial Broth; **GC:** gas chromatography.

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and difficult, current preliminary assessments of the efficacy of novel prebiotics have always turned to *in vitro* fermentation with human fecal microflora or human-derived pure cultures by continuous or static-batch fermentation.[5]

Mushroom sclerotia are rich in NDCs such as β-glucans, which are different from the currently well-developed fructan-based prebiotics. These mushroom NDCs are mainly polysaccharides with (1→3)-β-glucan as the main chain and (1→6)-β glucosyl branches. Their fermentability by human-derived bacteria as prebiotics has rarely been studied. In this study, we compared the prebiotic potential of two novel NDCs extracted from two mushroom sclerotia—*Poria cocos* F.A. Wolf (PC) and *Polyporus rhinocerus* Cooke (PR). Parameters, including the pH of fermentation broth, SCFA outputs, and effects on bacterial proliferation, as well as the organic matter disappearance (OMD), were all analyzed during a 72-hour *in vitro* batch fermentation by three human-derived pure cultures of *Bifidobacterium longum, Lactobacillus brevis,* and *Clostridium celatum.* The possible relationship between the mushroom NDC structures and their fermentation characteristics are also discussed.

II. MATERIALS AND METHODS

A. Sample Preparation and Characterization

Mushroom sclerotia of *Poria cocos* (PC) and *Polyporus rhinocerus* (PR) were cultivated and identified by the Sanming Mycological Institute in the Fujian province of the Chinese Mainland. NDCs (β-glucan rich) were isolated from these two mushroom sclerotia by the current protocols developed in our laboratory.[6,7] In short, individual sclerotial powders (particle size between 0.5 and 1.0 mm) were separately subjected to the following extraction at 1:25 (w/v): ultrasonic extraction (alkaline swelling in 1.0 M NaOH for 48 hours followed by ultrasonication [Sonics VCX600]) for 30 minutes in an ice-bath prior to dialysis using ultrapure water (tubing with molecular weight cut-off 6000–8000). The NDCs extracted were lyophilized and their carbohydrate content was determined by the phenol-sulfuric acid test method.[8] The protein content of the NDCs was determined by the Lowry method, as previously described.[9]

B. Bacterial Strains, Culture Media, and Fermentation Conditions

Three pure cultures—*Bifidobacterium longum* (JCM 1217), *Lactobacillus brevis* (JCM 1059), and *Clostridium celatum* (JCM 1394)—were bought from the Japan Collection of Microorganism (JCM) and stored at –80°C. To activate the bacteria, 2% Reinforced Clostridial Broth (RCB, Oxoid, Hampshire, UK) was used for *B. longum* and *C. celatum* while MRS Broth (Oxoid, Hampshire, UK) was used for *L. brevis* under at 37°C anaerobic conditions. Basal medium (2%) (Oxoid, Hampshire, UK) was used for *in vitro* fermentation with or without supplementation by NDC samples. The activated bacteria were inoculated at 0.8% (v/v) in a basal medium with 1% of the mushroom NDCs supplemented with 0.8% (v/v) oxyrase (Oxyrase, Mansfield, USA) to generate an anaerobic environment. The positive control was 1.0% (w/v) lactulose (Wako Pure Chemical Industries, Osaka, Japan) added to the basal medium, and the blank control contained only basal medium. Fermentation were performed by gently shaking (100 rpm) at 37°C. Whenever the fermentation bottles were opened, argon was blown to remove the air in them.

C. Growth Monitoring by pH and Bacterial Enumeration

During the 72-hour fermentation, the pH value of the fermentation broth was monitored at 24–hour intervals with a pH meter (Φ240 pH/Temp Meter, Beckman). At the same time, an aliquot of 200 μL was immediately transferred into sterile Eppendorfs and refrigerated at –20°C for subsequent bacterial proliferation analysis by trypan blue assay using a hemacytometer (AO Scientific Instruments, New York, USA). Another aliquot measuring 600 μL was poured into Eppendorfs filled with 8.0 μL of copper sulfate (10 g/L, w/v) for terminating the fermentation, and the mixture was stored at –20°C

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for SCFA analysis by gas chromatography (GC). All of the above operations were carried out in a Class I safety cabinet (NuAire201-430, NUNR) to avoid contamination.

D. SCFA Extraction and Quantification by GC

Short-chain fatty acids (SCFAs) produced in the fermentation broth were extracted according to the protocols described by Wong et al.[5] In brief, the stored aliquot of 600 μL was thawed and centrifuged (4800 rpm) at 4°C for 30 minutes; then an aliquot of 350 μL of the upper supernatant was transferred into a new Eppendorf, followed by 82.5 μL of metaphosphoric acid (25%, w/v) to acidify the SCFAs and 67.5 μL of methyl pentanoic acid as the internal standard at 4.0 mg/mL (catalog no. 6220601, Alltech, USA). The SCFAs in the mixture were extracted twice with 500 μL of diethyl ether, and the upper layers of ethyl ether fractions were pooled and dehydrated with anhydrous sodium sulfate before being filtered through a 0.45-μm membrane into GC vials. GC protocols followed those described by Wong et al.[5] Individual SCFAs expressed on an organic-matter basis were calculated according to the equation described by Wong et al.[5]

E. Organic Matter Disappearance Analysis

To evaluate the efficiency of the bacterial conversion of organic matter in NDCs into the SCFAs, the fermentation residues were first dried at 120°C in a vacuum oven (Sheldon Manufacturing Inc., Oregon, USA) and weighed before ashing in a 600°C muffle furnace (Cole-Parmer, Illinois, USA) overnight. The OMD percentage is expressed as follows:

$$\text{OMD}\ (\%) = \{[\text{OM}_{\text{original}} - (\text{OM}_S - \text{OM}_B)]/\text{OM}_{\text{original}}\} \times 100\%$$

in which $\text{OM}_{\text{original}}$ is the mean weight of organic matter (difference between weight of dry matter and weight of ash) of the substrate before fermentation, OM_S is the mean weight of organic matter of the nonfermented residue with NDCs added after fermentation, and OM_B is the mean weight of organic matter in the blank control after fermentation.

F. Statistical Analysis

All experiments were conducted in triplicate. Results were expressed as mean ± standard deviation (SD). Data collected were analyzed by ANOVA, and differences between means were compared by the Tukey's multiple comparison test using SPSS (13.0 version for Windows). A value $P < 0.05$ was considered as statistically significant.

III. RESULTS AND DISCUSSION

A. Composition Analysis of Three NDCs

The yield of NDCs obtained from PC and PR was found to be 94.8% and 82.3%, respectively. The carbohydrate (mainly β-glucan) content of the NDCs from PC and PR was 98.4% and 100.0% by weight, respectively (Table 1). Glucose was the predominant sugar in the NDCs (Table 1). Our previous studies on the linkage analysis of the two NDCs suggested that they are mainly high molecular weight glucans with β-(1→3) main chain and β-(1→6) branches.[5,6] These mushroom sclerotial β-glucans are structurally very different from cereal β-glucans with β-(1→3) and β-(1→4) linkages that are commonly found in barley and oats, which have also rarely been studied for their prebiotic characteristics.[10]

B. Bacterial Proliferation and pH Changes

According to the growth curve of the three bacteria in basal medium, the exponential stage was found within the first 24-hour fermentation, whereas the stationary stage occurred at 72 hours (data not shown). Therefore, proliferation of the three bacteria in different substrates was examined after 24 hours of fermentation, whereas those samples used for SCFA and OMD analysis were sampled after 72 hours of fermentation. There was no significant

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TABLE 1
Chemical Characterization of *Poria cocos* (PC) and *Polyporus rhinocerus* (PR) Sclerotial Nondigestible Carbohydrates (NDCs)[a]

		Chemical components			Sugar composition		
NDCs	Yield (%)	Carbohydrate	Protein	Uronic acids	Glc[b]	Glc-NAc[c]	Gal[d]
PC	94.8	98.4	n.d.[e]	0.30	98.9	n.d.	0.30
PR	82.3	100.0	n.d.	n.d.	99.0	1.02	n.d.

[a] Data are presented as mean % dry weight.
[b] Glucose.
[c] N-acetyl glucosamine.
[d] Galactose.
[e] Not detected.

difference ($P > 0.05$) in the initial number of the three bacteria at the beginning of the fermentation between the NDCs and the control (Table 2). After a 24-hour fermentation, NDCs from PR could significantly ($P < 0.05$) stimulate the number of *Bifidobacterium longum* and *Lactobacillus brevis* compared with both lactulose and NDCs from PC (Table 2). The percentage of increase for *L. brevis*

TABLE 2
Changes in Bacterial Population After 24 hours of *In Vitro* Fermentation with *Poria cocos* (PC) and *Polyporus rhinocerus* (PR) Sclerotial Nondigestible Carbohydrates (NDCs) and Lactulose (Lac)

Bacteria	Substrates	No. ($\times 10^7$) (t = 0 h)[a]	No. ($\times 10^7$) (t = 24 h)[a]	Percentage of increase[b] ($\times$100%)
B. longum	PC	7.36 ± 2.37[cx]	49.6 ± 4.23[x]	6.74
	PR	5.04 ± 1.06[x]	72.4 ± 7.30[y]	14.3
	Lac	6.00 ± 2.10[x]	54.9 ± 1.62[x]	9.16
L. brevis	PC	5.41 ± 0.56[x]	64.3 ± 3.70[y]	11.9
	PR	5.05 ± 1.54[x]	95.7 ± 5.68[z]	18.9
	Lac	2.69 ± 1.15[x]	51.1 ± 2.72[x]	19.0
C. celatum	PC	5.23 ± 1.76[x]	34.3 ± 20.0[x]	6.56
	PR	3.39 ± 1.07[x]	8.80 ± 1.06[y]	2.60
	Lac	2.95 ± 1.79[x]	44.8 ± 4.18[x]	15.2

[a] Data are values of mean ± SD (n = 3) of bacterial number expressed as $\times 10^7$/mL of the fermentation broth.
[b] Ratio of number of bacterial populations at 24 hours compared to that at 0 hours.
[c] Different letters (x–z) indicate significant differences compared with lactulose for each bacterial species (one-way ANOVA, Tukey's multiple comparison, $P < 0.05$).

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and *B. longum* between NDCs from PR and lactulose was comparable (Table 2). For *Clostridium celatum*, an important pathogen in the human colon, the situation was different. NDC from PR showed significantly stronger inhibition ($P < 0.05$) than both lactulose and NDC from PC during the 24-hour fermentation (Table 2).

The pH variation at the 24-hour interval during the 72-hour fermentation (ranging from 6.69 ± 0.01 to 5.87 ± 0.05) was not apparent in this batch of fermentation, which may be due to the buffering effects of the medium used.

In the limited amount of literature that is available, partially hydrolyzed β-glucans from oats and barley appear to stimulate lactobacilli more than bifidobacteria.[11] Support of the growth of *Bifidobacterium animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* and *Lactobacillus casei* by hydrolysates of barley β-glucans has also been recently demonstrated in mono-culture fermentation.[12] Inhibition of the growth of pathogenic bacteria such as *Clostridium celatum* has not been reported.

C. SCFA Production

In general, SCFA production (expressed as mmol/g of organic matter) in different combination systems of bacteria and NDCs increased in a time-dependent manner during the 72 hours of fermentation (Fig. 1). When comparing the SCFA products produced by the same bacteria incubated with different substrates, NDC from PR gave a significantly ($P < 0.05$) higher output of acetate (2.47 ± 0.232 mmol/g) than that from PC (1.68 ± 0.195) for *Bifidobacterium longum* (Fig. 1a), which was consistent with the bacterial proliferation results. For *Lactobacillus brevis* and *Clostridium celatum*, NDCs from PR and PC yielded a similar ($P > 0.05$) acetate output after the 72-hour fermentation (Figs. 1b–1c). As for the SCFA composition, acetate was the predominant component (acetate > 98.4%), followed by minor amounts of propionate and butyrate (less than 1.60%), which was consistent with the facts that *Bifidobacterium* and *Lactobacillus* mainly produce mainly acetate and lactate rather than butyrate.[13] However, lactic acid, which is supposed to be produced by both *Bifidobacterium* and *Lactobacillus*, was not observed. It might be possible that lactic acid produced as an intermediate was utilized preferentially because of the limited carbon source available in the closed fermentation system.[14] *In vitro* fermentation using human fecal inoculum on purified oat β-glucan with β-(1→3) and β-(1→4) mixed linkages gave a ratio of acetate:propionate:butyrate at 39:27:34, which was in contrast with the present result of sclerotial NDCs.[15] As for *Clostridium celatum*, which was commonly reported as a butyrate producer, the amount of butyrate and propionate detected was also very small (less than 1.68%) during the above fermentation process. The above observations might be due to the fact that the rate, relative ratios, and extent of SCFA production depend on the composition of the colonic microflora (monoculture vs. fecal inoculum) as well as the chemical composition (monosaccharide profile) and structural arrangement (linkage and degree of branching) of the constituents of NDCs available for fermentation.[16–18]

D. OMD Percentage

The percentage of organic matter in lactulose and the NDC extracts from PC and PR was very close (94.2%, 99.2%, and 98.4% respectively). The OMD percentage of the sclerotial NDCs and lactulose after 72-hour *in vitro* fermentation using three pure cultures was presented in Table 3. The two NDCs of PC and PR exhibited very high OMD (especially by *Lactobacillus brevis*) compared with other plant-food materials (OMD by fecal bacterial fermentation for oat, corn bran, and soy were 12.2, 11.2, and 57.7%, respectively), as previously reported.[19–22] These observed differences might be partly due to the fact that pure bacterial cultures rather than human fecal bacteria and a longer incubation time (72 hours instead of 24 hours) were used in the present study, and *L. brevis* seemed to utilize the two sclerotial NDCs more efficiently than the other two bacteria (Table 3). For *Bifidobacterium longum*, the OMDs of NDCs from both PC and lactulose did not differ significantly ($P > 0.05$). Utilization of all the substrates by *Clostridium celatum* in terms of the OMD was the least among the three bacteria (Table 3). In general, the trend of the OMD was

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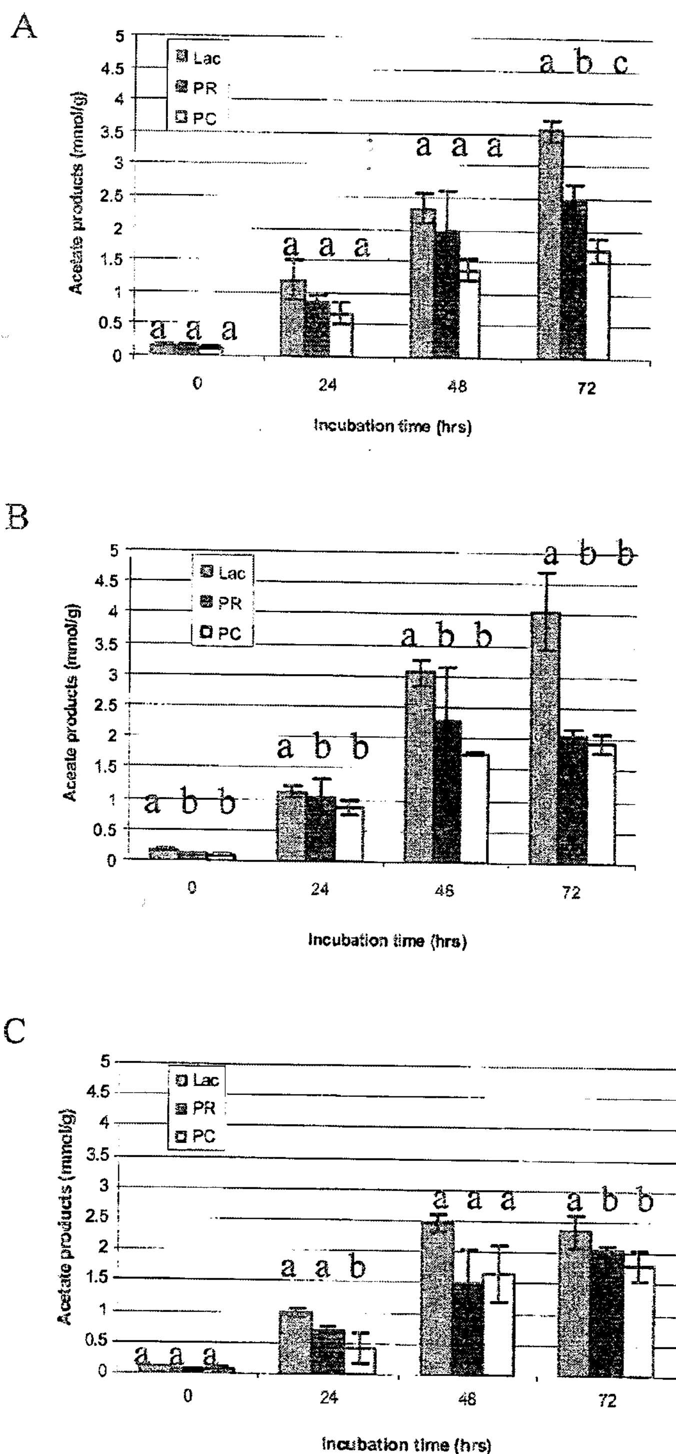

FIGURE 1. Acetate production by bacteria. (A) *Bifidobacterium longum*, (B) *Lactobacillus brevis*, and (C) *Clostridium celatum* expressed as mmol/g of organic matter (lac, lactulose). Different letters indicate significant differences in the different substrates within the same fermentation time point (one-way ANOVA, Tukey's multiple comparison, $P < 0.05$).

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TABLE 3
Organic Matter Disappearance (OMD) After 72 Hours of *In Vitro* Fermentation with *Poria cocos* (PC) and *Polyporus rhinocerus* (PR) Sclerotial Nondigestible Carbohydrates (NDCs) and Lactulose (Lac) Using Three Pure Bacterial Cultures[a]

Bacteria/Substrates	*Bifidobacterium longum*	*Lactobacillus brevis*	*Clostridium celatum*
PC	$42.8 \pm 3.68^{a,x}$	$94.5 \pm 23.5^{b,x}$	$23.7 \pm 11.4^{a,x}$
PR	$29.8 \pm 5.37^{a,y}$	$59.9 \pm 36.3^{a,x}$	$15.5 \pm 12.7^{a,x}$
Lac	$38.1 \pm 3.87^{a,x}$	$96.2 \pm 21.4^{b,x}$	$33.1 \pm 24.3^{a,x}$

Note: Data are means ± SD (n = 3). Different letters (a,b,c) indicate significant differences among OMD of different bacteria incubated with each substrate (one-way ANOVA, Tukey's multiple comparison, $P < 0.05$). Different letters (x,y) indicate significant differences among OMD of each bacteria incubated with different substrates (one-way ANOVA, Tukey's multiple comparison, $P < 0.05$).

consistent with the corresponding bacterial growth (Table 2).

IV. CONCLUSIONS

The two sclerotial NDCs used in this study are β-glucan-type polysaccharides that differ greatly in their chemical structure from the current commercial prebiotics, including fructans and nonfructose oligosaccharides. Furthermore, on the basis of their stimulation of the growth of beneficial bacteria and inhibition of pathogenic ones together with their SCFA production (mainly acetate), the two sclerotial NDCs from *Poria cocos* and *Polyporus rhinocerus* are potential novel prebiotics. Further *in vivo* studies using animal models and humans are necessary to find out the prebiotic effects of these mushroom NDCs. More detailed structural elucidation is required to delineate the structure–function relationship of these sclerotial β-glucans in terms of the contribution of glycosidic linkages and molecular weights toward the selectivity of fermentation by probiotic bacteria. These results would provide important insights into the synbiotic effect between mushroom prebiotics and probiotics in the prevention and treatment of colon cancer in humans.

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