Food Chemistry 152 (2014) 251–260

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In vitro evaluation method for screening of candidate prebiotic foods ☆

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ARTICLE INFO

Article history:
Received 23 June 2013
Received in revised form 18 November 2013
Accepted 22 November 2013
Available online 1 December 2013

Keywords:
NMR
Multivariate statistical analysis
Metabonomics
Microbiota
Welsh onion

ABSTRACT

The aim of this work was to develop a simple and rapid *in vitro* evaluation method for screening and discovery of uncharacterised and untapped prebiotic foods. Using a NMR-based metabolomic approach coupled with multivariate statistical analysis, the metabolic profiles generated by intestinal microbiota after *in vitro* incubation with feces were examined. The viscous substances of Japanese bunching onion (JBO_{VS}) were identified as one of the candidate prebiotic foods by this *in vitro* screening method. The JBO_{VS} were primarily composed of sugar components, especially fructose-based carbohydrates. Our results suggested that ingestion of JBO_{VS} contributed to lactate and acetate production by the intestinal microbiota, and were accompanied by an increase in the *Lactobacillus murinus* and *Bacteroidetes* sp. populations in the intestine and fluctuation of the host-microbial co-metabolic process. Therefore, our approach should be useful as a rapid and simple screening tool for potential prebiotic foods.

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1. Introduction

The co-evolution of mammalian-microbial symbiosis is accompanied by extensive interactive modulations of metabolism and physiology, facilitated by the crosstalk between the host and symbiotic community. Microbial symbionts often provide traits that their hosts have not evolved on their own, and may synthesise essential amino acids and vitamins or process otherwise indigestible components in the diet, such as plant polysaccharides (Flint, Bayer, Rincon, Lamed, & White, 2008; Turnbaugh et al., 2007). The composition of intestinal microbial communities is highly variable (Turnbaugh et al., 2007), and can be significantly affected by alterations in diet (Flint et al., 2008). Interactive modulations of individuals with variations in their microbial symbionts are likely to affect human health and disease (Turnbaugh et al., 2007).

The interactive modulations affecting human health are considerably engaged by beneficial microbial symbionts such as *Lactobacilli* and *Bifidobacteria*, which are currently the most marketed probiotic bacteria worldwide (Saulnier, Spinler, Gibson, & Versalovic, 2009). The beneficial microbial symbionts are responsible for preventing infection, enhancing the immune system, and providing increased nutritional value to food (Fukuda et al., 2011; Saulnier et al., 2009; Ventura et al., 2009). The growth and activity of these beneficial microbial symbionts is enhanced by prebiotic foods, such as fructo-oligosaccharide (FOS) and galacto-oligosaccharides, in the human gastrointestinal tract (Saulnier et al., 2009). Therefore, evaluation of the effects of prebiotic foods on the dietary interactive modulations of the host and the beneficial microbial symbionts are important for human health.

Some foods and their components are customarily considered to play an important role in human health. For example, Japanese bunching onion (JBO) (synonym for welsh onion; *Allium fistulosum* L.), an edible perennial plant, is considered to be beneficial for human health in Japan. The edible portions of the JBO are the green stalk and the white bulb, which are used as ingredients in Asian cuisine, especially in East and Southeast Asia. The edible portion of JBO has been reported to show hypolipidemic effects (Yamamoto, Aoyama, Hamaguchi, & Rhi, 2005), antioxidant effects in rats fed

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http://dx.doi.org/10.1016/j.foodchem.2013.11.126

a high-fat and high-sucrose diet (Yamamoto & Yasuoka, 2010), and hypoglycemic effects in an animal model of diabetes mellitus (Kang et al., 2010). In addition, the JBO is customarily considered a preventative food against the common cold and influenza in Japan. In support of this, a recent study reported that the macromolecular component obtained by aqueous solvent extraction of JBO had anti-influenza activity (Lee et al., 2012). However, viscous substances (VS), a macromolecular component extractable by aqueous solvent and produced in the cavity of JBO (JBO_{VS}), are not well-characterized with regard to their chemical and mineral compositions. Moreover, limited biological information about the effects of the JBO_{VS} on host-microbial symbiotic systems in the intestines is available. Thus, a large number of foods and their components derived from plants such as JBO_{VS} may have undiscovered human health benefits.

Candidate functional and prebiotic foods are usually evaluated using *in vitro* cell assays and/or animal experiments in mice and rats. Animal experiments, however, are generally time-consuming and present several ethical issues. With this in mind, it was envisaged that a simple and rapid *in vitro* method involving the use of *in vitro* cell assays would be a much more suitable method for the screening of functional and prebiotic foods. Therefore the objective of this study was to develop a simple and rapid *in vitro* evaluation method for screening and discovery of uncharacterised and untapped prebiotic foods. To accomplish this objective, a metabolomic approach was employed, which is a powerful tool well suited to provide metabolic profiles that contain information pertaining to the ecosystem and community response. Multivariate metabolic profiling offers a practical approach for measuring the metabolic endpoints that are directly linked to whole system activity (Nicholson, Holmes, & Wilson, 2005). In addition to this, some approaches, including our developed methods, have been successfully applied to characterising the metabolic consequences of nutritional intervention, monitoring the metabolic dynamics in microbial ecosystems, and linking the relationships between microbial communities and their metabolic information (Date et al., 2010; Li et al., 2008; Rezzi, Ramadan, Fay, & Kochhar, 2007).

Herein we describe an *in vitro* evaluation method for a rapid and simple screening of candidate prebiotic foods and their components. The JBO_{VS} and other foods and their components were evaluated by an *in vitro* screening method based on the metabolic dynamics of microbial communities obtained by nuclear magnetic resonance (NMR) spectroscopy and denaturing gradient gel electrophoresis (DGGE) fingerprinting. In addition, we characterised the chemical components in the JBO_{VS} by NMR spectroscopy and inductively coupled plasma optical emission spectrometry (ICP-OES)/mass spectrometry (ICP-MS) analysis. Furthermore, the effects of the JBO_{VS} observed by *in vitro* screening were evaluated on the host-microbial symbiotic system in the intestines by an approach based on metabolic and microbial community profiles obtained by NMR spectroscopy and DGGE fingerprinting, respectively.

2. Materials and methods

2.1. Materials

The materials used in a screening method were as follows: FOS from chicory, raffinose, stachyose, pectin from apple, wheat-bran, carrageenan (Sigma-Aldrich Japan Co., Ltd., Tokyo, Japan), chlorella (Chlorella Industry Co., Ltd., Tokyo, Japan), starch from wheat, glucan, agar (Wako Pure Chemical Industries, Ltd., Osaka, Japan), onion, kelp, Japanese mustard spinach, arrowroot, starch from arrowroot (purchased from a market), JBO, and JBO_{VS}. The variety of JBO used in this study was Fuyuougi 2. The JBOs themselves were obtained from the Kanagawa Agricultural Technology Center (Kanagawa, Japan). The JBOs were planted in soil taken from the farm at the Center for about 3 months at the mature stage, and the resulting JBO_{VS} were collected from the JBO cavity when the JBO was harvested.

2.2. In vitro incubation

Male 10-week-old BALB/cA mice (CLEA Japan, Inc., Tokyo, Japan) were housed at 23–25 °C and 50–60% relative humidity with a 12 h light-dark cycle. The mice were fed CLEA Rodent Diet CA-1 (CLEA Japan, Inc., Tokyo, Japan). Fresh fecal sources were collected from the mice. The collected fecal sources (1%) were suspended anaerobically in phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4{\cdot}12H_2O$, and 0.2 g KH_2PO_4 per litre distilled water, pH 7.0) with 0.5% (w/v) of each substrate. The PBS solutions containing 5 mg/L resazurin and 1 mg/L cysteine hydrochloride were used to provide an indication of the amount of oxygen in the medium and act as an oxygen scavenger, respectively. These solutions were pretreated with pure CO_2 (>99.9%) gas and autoclaved before being mixed with the fecal sources. The substrates examined were from the above-mentioned list of materials, as well as a control (no addition of substrate). All substrates were freeze-dried and crushed to a powder by using an Automill machine (Tokken, Inc., Chiba, Japan). The PBS solutions including fecal source and the substrate were purged with N_2 gas to allow for any residual oxygen to be displaced from the headspace. The suspensions were incubated at 37 °C under anaerobic conditions in an incubator without shaking. Resazurin remains colourless under anaerobic conditions and turns red in the presence of oxygen, and great care was taken to ensure that the experiments were conducted under anaerobic conditions to prevent the solution turning red. The suspensions were collected after 12 h incubation and then centrifuged. The supernatants of the centrifuged samples were used as samples for NMR measurements. The experiments (i.e., *in vitro* incubation) were performed 3–5 times for each substrate and the control.

2.3. Animal experiments

All animal experiments were approved by the Animal Research Committees of RIKEN Yokohama Research Institute and Yokohama City University. Animals were kept in environmentally controlled animal facilities at the Yokohama City University. All efforts were made to minimise suffering with minimal use of mice. Male 10-week-old BALB/cA mice (CLEA Japan, Inc., Tokyo, Japan) were housed at 23–25 °C and 50–60% relative humidity with a 12 h light-dark cycle. The mice were fed a CLEA Rodent Diet CA-1 (CLEA Japan, Inc., Tokyo, Japan) for 1 week before commencement of experiments. The experimental diet consisted of 5% JBO_{VS} mixed with CLEA Rodent Diet CA-1 (control diet) excluding fibre contents. The mice were fed the experimental diet for a week after a week of the control diet intakes. Thirty-two fecal pellets were collected from the mice. The pellets were lyophilized and then stored at −80 °C.

2.4. NMR spectroscopy

The supernatants of the collected samples from the *in vitro* experiments were suspended in 10% (v/v) deuterium oxide (D_2O) and 1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. JBO_{VS} and 32 fecal samples from the *in vivo* experiments were freeze-dried and 50 mg of JBO_{VS} and 5 mg of the freeze-dried fecal samples were extracted with 600 μl of a phosphate buffer solution (0.1 M K_2HPO_4/KH_2PO_4, pH 7.0), containing 90% D_2O and 1 mM DSS at 50 °C for 5 min. After

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