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Isolation and assessment of acidic and neutral oligosaccharides from goat milk and bovine colostrum for use as ingredients of infant formulae

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ABSTRACT

Acidic and neutral oligosaccharides (OSs) from goat milk or bovine colostrum were assessed for the potential uses as ingredients of infant formulae or functional foods. For that purpose, neutral and acidic OS fractions were isolated by a modified charcoal column method. The elution protocol of the charcoal column was improved to decrease the column size to approximately 1/9 of the traditional process. Then the inhibitory potencies of the neutral and acidic OSs isolated from the two mammals were compared at 2.5 and 5.0 mg mL^{-1} using OSs from human breast milk as a reference. These OS fractions inhibited the adhesion of *Salmonella enterica* to Caco-2 cells, although some differences were not statistically significant. Finally, goat milk OSs were determined as more potential ingredients of infant formulae or functional foods in the aspect of the potency of adherence inhibition, safety, and the availability of the starting material.

1. Introduction

Milk oligosaccharides (OSs) have received great attention for their anti-infective properties and as a result, the structures of more than 100 human milk OSs have been reported (Boehm & Stahl, 2007; Urashima, Messer, & Oftedal, 2017). In particular, human milk OSs have been reported to have high anti-infective activity (Kunz, Kuntz, & Rudloff, 2014; Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005; Perrer et al., 2005; Yang, Chuang, & Chen, 2012), anti-inflammatory activity (Daddaoua et al., 2006), and regulatory activity of gut microbiome diversity and composition (Gomez-Gallego, Garcia-Mantrana, Salminen, & Collado, 2016; Thum, McNabb, Young, Cookson, & Roy, 2016; Thum et al., 2017; Urashima et al., 2012). Of these properties, the anti-infective potency of OSs is remarkable in protecting infants from various pathogenic bacteria (Morrow et al., 2005; Perrer et al., 2005) or viruses (Yang et al., 2012).

Other than OSs, various materials in human breast milk such as lactoferrin, immunoglobulins, lysozyme, and cellular components including macrophages and lymphocytes have been recognised as effective against infection by pathogenic bacteria or viruses (Hassiotou et al., 2013; Orlando, 1995). However, these materials are less functional for such purposes, in terms of process and storage stability. The aim of the present study is to specifically investigate OSs for potential uses as functional food ingredients.

The mechanism underlying the protective effect by OSs has been proposed to be mediation of the binding of pathogens to intestinal cells. Interestingly, the glycan moieties of the intestinal mucosal cell surface act as receptors for pathogens, and the structures of human milk OSs have high homology to the partial structures of these glycans (Newburg, 1999). As a result, human milk OSs presumably act as competitive inhibitors by binding to the pathogens (Bode & Jantscher-Krenn, 2012). For example, human milk OSs containing α-1,2-linked fucosyl residues were reported to inhibit the binding of a heat-stable toxin of *Escherichia coli* to colonic T84 cells in vitro (Crane, Azar, Stam, & Newburg, 1994). L-Fucosylated OSs from human milk have also been reported to inhibit the binding of *Campylobacter jejuni* to carcinoma-derived human epithelial cells in vitro and the *campylobacter* colonisation of mice in vivo

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(Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). In addition, 2′-fucosyllactose (2′-FL) and 3-fucosyllactose (3-FL) were reported to inhibit the adhesion of *Pseudomonas aeruginosa* and enteric pathogens to human intestinal and respiratory cell lines (Weichert et al., 2013). Furthermore, human and avian influenza viruses have been shown to utilize α-2,6-L-sialylated OSs and α-2,3-L-sialylated OSs, respectively, as their receptors (Sato, Hanagata, Kiso, Hasegawa, & Suzuki, 1998). Finally, Coppa et al. (2006) separated human milk OSs into acidic OSs fraction, neutral high-molecular weight OSs fraction, and neutral low-molecular weight OSs fraction. When they examined the potency of each of the three fractions to inhibit the adhesion of *Escherichia coli (E.coli)* serotype O119, *Vibrio cholerae*, or *Salmonella fyris* to Caco-2 human epithelial colorectal cells, and they concluded that OSs present in human breast milk were one of the important defensive factors against acute diarrhoeal infections in breast-fed infants, consistent with previous studies (Newburg, 2003; Zopf & Roth, 1996).

As human breast milk is not a practical source of OSs for the ingredients of infant formulae, goat milk and bovine colostrum are considered as a candidate of alternative source of OSs. Human breast milk contains 5–15 g L^{-1} OSs in addition to D-lactose (Boehm & Stahl, 2007; Coppa et al., 1999; Daddaoua et al., 2006; Kunz et al., 2014; Urashima et al., 2012), while goat milk contains 0.25–0.30 g L^{-1} OSs (Daddaoua et al., 2006; Gopal & Gill, 2000; Martinez-Ferez, Guadix, & Guadix, 2006a; Martinez-Ferez et al., 2006b; Oliviera, Wilbey, Grandison, & Roseiro, 2015). In addition, although bovine mature milk contains very low concentration of OSs (30–60 mg L^{-1}), bovine colostrum is reported to contain a far larger amount of complex OSs (approximately 1 g L^{-1}; Urashima, Taufik, Fukuda, & Asakuma, 2013). Furthermore, 2′-FL and α-2,6-L-sialyllactose (6′-SL) are found in goat milk (Albrecht et al., 2014), though the concentration is relatively lower than that in human milk.

The object of this study is to search a new candidate source of OSs that would serve as ingredients of infant formulae instead of human breast milk OSs to protect infants against pathogens such as bacteria or viruses. The same OSs would be applicable not only to the infant formulae, but also to functional foods or supplements for aged persons. Therefore, the aim of the present study is to determine which of the goat milk OSs or the bovine colostrum OSs are more preferable source as the ingredients of infant formulae or functional foods.

Usually, the isolation of OSs from mammalian milk sources has been performed by gel chromatography (Kobata, Yamashita, & Takasaki, 1987), adhesion chromatography using charcoal columns (Johnson & Srisuthep, 1975; Ward, 2009), or ultrafiltration–nano-filtration technology (Martinez-Ferez et al., 2006b). In the present study, a charcoal column method was selected for the isolation of Oss, applying several modifications that could permit adaptation to industrial processing in the future. Then, the neutral and acidic OSs isolated from goat milk and bovine colostrum were compared for their potency to inhibit the adhesion of *Salmonella enterica* IID604 to Caco-2 cells using human breast milk OSs as a positive control.

2. Materials and methods

2.1. Materials

Goat milk was a kind gift from Fastfarm Co., Ltd. (Niigata, Japan). Bovine colostrum produced from day 0 to day 3 after parturition from three Holstein cows was kindly supplied by Niigata Prefecture Agricultural Junior College (Niigata, Japan). Twenty millilitres each of day 0–3 post-parturition colostrum from 3 cows were mixed and frozen until use. Human breast milk, which was a mixture of breast milk from more than 20 Japanese women of various blood types, was donated by Meiji Milk Products Co. Ltd. (present name, Meiji Co. Ltd., Tokyo, Japan) and was frozen until use. Charcoal for the column chromatography was purchased from Wako Pure Chemical Industries Ltd. ("Charcoal, Activated", average particle size of 300 μm; Catalog No. 031-02135; Wako Pure Chemical Industries Ltd., Osaka, Japan). The reagents for analytical standards or reference samples for high pH anion exchange chromatography (HPAEC) and nuclear magnetic resonance (NMR) analyses, D-lactose as a negative control in inhibition experiments, propidium iodide for the staining the bacteria and Coco-2 cell nuclei were purchased from Sigma–Aldrich Japan (Tokyo, Japan).

2.2. Isolation of OSs fractions from natural milk sources

Each of the raw goat milk, bovine colostrum (a mixture of 4 days × 3 cows), and human breast milk samples was diluted with the same volume of distilled water and centrifuged at 4 °C for 30 min at 4000 × *g*. The aqueous layer was treated with 4 vol of 2:1 (v/v) chloroform/methanol to remove lipids using separating funnel, and the chloroform layer containing lipids was discarded. The emulsion during the separating funnel treatment was centrifuged at 4000 × *g* for 30 min at 4 °C, and the aqueous layer containing OSs was mixed with the aqueous layer of the separating funnel treatment. The pH of the mixed aqueous solution was adjusted to 4.5 with 100 mM HCl, and the solution was stored at 4 °C for 2 h. The precipitated proteins (mainly casein) were removed by centrifugation at 4 °C for 30 min at 4000 × *g*. The pH of the supernatant was then adjusted to 6.5 with 100 mM NaOH solution and centrifuged at 10,000 × *g* for 30 min at 4 °C to obtain a clear solution containing the OSs. Finally, the solution was concentrated and lyophilised.

The following procedure is described for the preparation of goat milk OSs, but the isolation from the other sources was also performed in a mostly analogous manner. A column (5 × 50 cm) packed with 50 g of activated charcoal was washed with 60% (v/v) ethanol containing 0.1% (v/v) trifluoroacetic acid (TFA) (2 L), then with distilled water (2 L), and finally equilibrated with 4% (v/v) ethanol (3 L). The lyophilised OSs powder (9.11 g) obtained from 200 mL goat milk was dissolved in 4% (v/v) ethanol (4 L), and the solution was loaded onto the charcoal column. The column was washed with 4% (v/v) ethanol (4 L) to wash away D-lactose. The neutral OSs were first eluted with 50% (v/v) ethanol (4 L), and the eluent was concentrated with rotary evaporator. The obtained syrup was dissolved in water and lyophilised to obtain 17.1 mg of neutral OSs. The column was then washed with 8% (v/v) ethanol containing 0.1% (v/v) TFA to wash away a contaminating citric acid and other organic acids. Ten mL aliquots of the eluent were collected at appropriate intervals and concentrated to confirm the absence of citric acid using NMR. Finally, the acidic OSs were eluted with 50% (v/v) ethanol containing 0.1% (v/v) TFA (4 L), concentrated, and lyophilised to obtain 34.5 mg of pure acidic OSs.

2.3. Analyses

^{1}H NMR spectra were measured using an Avance III 400 MHz NMR spectrometer (Bruker Co., Billerica, MA, USA) in D_2O at room temperature. HDO signal (4.80 ppm) was used as a reference of chemical shifts, since HDO signal resonates at δ 4.80 ppm when internal reference of 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (DSS, δ 0.00 ppm) was used. HPAEC analyses were performed on a Dionex-300 Bio-LC system (Sunnyvale, CA, USA) equipped with pulsed amperometric detector, CarboPac PA-100 column (4 × 250 mm), and its guard column (5 × 50 mm), at a flow rate of 1 mL min^{-1}. Neutral OSs were eluted using a gradient of