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In vitro fermentation properties of pectins and enzymatic-modified pectins obtained from different renewable bioresources

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ABSTRACT

The suitability of artichoke and sunflower by-products as renewable sources of pectic compounds with prebiotic potential was evaluated by studying their ability to modulate the human faecal microbiota *in vitro*. Bacterial populations and short-chain fatty acid (SCFA) production were measured. Reduction of the molecular weight of artichoke pectin resulted in greater stimulation of the growth of *Bifidobacterium*, *Lactobacillus* and *Bacteroides/Prevotella*, whilst this effect was observed only in *Bacteroides/Prevotella* for sunflower samples. In contrast, the degree of methoxylation did not have any impact on fermentability properties or SCFA production, regardless of the origin of pectic compounds. Although further *in vivo* studies should be conducted, either pectin or enzymatically-modified pectin from sunflower and artichoke by-products might be considered as prebiotic candidates for human consumption showing similar ability to promote the *in vitro* growth of beneficial gut bacteria as compared to well-recognized prebiotics such as inulin or fructo-oligosaccharides.

1. Introduction

One of the most complex polysaccharides that exist in the cell wall of all higher plants is pectin (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). Pectin is not a single structure and comprises of a family of plant cell wall polysaccharides that contain galacturonic acid (GalA) linked at α-1,4 positions. It mainly consists of a GalA-rich backbone, known as homogalacturonan (HG ≈ 65%) which is partially methyl-esterified in C-6 and O-acethyl-esterified in positions 2 and 3 (Mohnen, 2008). Rhamnose residues interrupt the HG structure to form rhamnogalacturonan I (RG-I ≈ 20–35%) which is based on a backbone consisting of a repeating disaccharide of GalA and rhamnose residues. In addition, some rhamnose residues may contain sidechains consisting of α-L-arabinose and/or β-D-galactose (arabinans, galactans and arabinogalactans). RG-II constitutes ≈ 2–10% of pectin and is the most complex, but is also believed to be the most conserved part of pectin molecules. RG-II has a HG backbone and is branched with rhamnose and other minor sugars such as fucose, glucuronic acid and methyl-esterified glucuronic acid among other rare carbohydrates such as apiose, 2-*O*-methylxylose, and 2-*O*-methylfucose (Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014; Noreen et al., 2017).

The biological effects of pectins have been mainly studied on *in vitro* assays and they are highly fermentable dietary fibres. Furthermore, pectic-oligosaccharides (POS) have been proposed as a new class of prebiotics capable of exerting a number of health-promoting effects (Olano-Martin, Gibson, & Rastall, 2002). These benefits include a desirable fermentation profile in the gut (Gómez, Gullón, Yáñez, Schols, & Alonso, 2016), potential *in vitro* anti-cancer properties (Maxwell et al., 2015), potential for cardiovascular protection (Samuelsson et al., 2016), as well as antibacterial, anti-inflammatory and antioxidant properties, among others (Míguez, Gómez, Gullón, Gullón, & Alonso, 2016). Nevertheless, the details of the underlying mechanisms are still largely unknown and additional studies are needed on the structure-function interrelationship, as well as on the claimed effects caused by POS in humans (Gullón et al., 2013).

Apart from POS, whose degree of polymerization range from 3 to 10, during the past few years there has been a flourishing interest towards pectin derivatives, especially the so-called “modified pectins” (MP), a term standing for pectin-derived, water-soluble polysaccharide of lower molecular weight (Mw) than the original pectin and, normally, produced from citrus peel and pulp (Holck et al., 2014). These compounds can be obtained from pectins in their native form using chemical and enzymatic treatments, which produce lower Mw HG and fragments enriched in RG (Morris, Belshaw, Waldron, & Maxwell,

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2013). The break-down of pectins not only leads to modification of their physico-chemical and gelling properties (Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015), but also modulation of their bioactivity (Morris et al., 2013).

There are several *in vitro* and *in vivo* studies on the ability of MP to inhibit tumour growth and metastasis (Morris et al., 2013; Nangia-Makker et al., 2002; Park et al., 2017). Citrus MP inhibits *in vitro* and *in vivo* angiogenesis in different types of cancer by blocking the association of galectin-3 to its receptors (Zhang, Xu, & Zhang, 2015). Other beneficial health properties might include the reduction of atherosclerotic lesions (Lu et al., 2017), anti-inflammatory and antioxidant properties (Popov & Ovodov, 2013; Ramachandran, Wilk, Melnick, & Eliaz, 2017) or immunostimulatory properties (Vogt et al., 2016). However, most of these studies were performed using cell cultures or in mice and extrapolation of the results to human or clinical investigations should be considered with caution.

Nonetheless, only a few recent studies have addressed the prebiotic potential of MP in terms of the fermentation properties. A slight or no increase was observed in the faecal lactobacilli count during an *in vivo* study with rats fed with citrus MP (Odun-Ayo, Mellem, & Reddy, 2017). Di et al. (2017) compared five structurally different citrus pectic samples (3 of them were POS and 2 were MP) and found that two POS and one MP exhibited bifidogenic effects with similar fermentabilities in human faecal cultures. These authors concluded that Mw and degree of methoxylation did not affect their bifidogenic properties; however, structural diversity in pectic compounds is possible as long as significant arabino- and galacto-oligosaccharide content is present. Fanaro et al. (2005) investigated the effect of acidic oligosaccharides from pectin on intestinal flora and stool characteristics in infants, showing that they were well tolerated as ingredient in infant formulae but did not affect intestinal microecology.

To the best of our knowledge, the fermentation and prebiotic properties of pectin derived from artichoke (Sabater, Corzo, Olano, & Montilla, 2018) and sunflower (Muñoz-Almagro, Rico-Rodriguez, Wilde, Montilla, & Villamiel, 2018) by-products have not been explored. In the case of artichoke, only one previous study showed a selective growth of two specific strains, *i.e. Lactobacillus plantarum* 8114 and *Bifidobacterium bifidum* ATCC 11,863 which was ascribed to the combination of its high inulin and low methoxylated pectin contents (Fissore, Santo Domingo, Gerschenson, & Giannuzzi, 2015). Also, Costabile et al. (2010) reported, in a double-blind, cross-over study carried out in healthy adults, a pronounced prebiotic effect (*i.e.*, increasing of bidifobacteria and lactobacilli) of a very-long-chain inulin derived from artichoke on the human faecal microbiota composition. The lack of knowledge of potential alternative sources of active pectic compounds for human consumption is surprising as previous studies reported that structure and composition can make a significant difference to the fermentation properties (Onumpai, Kolida, Bonnin, & Rastall, 2011). Thus, bifidogenic properties seem to highly depend on the composition and structure of pectins, with neutral sugar content and GalA:Rha ratio being critical factors (Di et al., 2017).

In this context, considering the structural diversity of pectins dependent on their origin, the aim of this study was to evaluate the effect of a variety of pectins and enzymatic-modified pectins from different sources (in particular, citrus, sunflower and artichoke) on the profile changes in human faecal microbiota population and fermentation metabolites, *i.e.* short-chain fatty acids.

2. Materials and methods

2.1. Raw material

Sunflower by-products based on heads and leftover stalks and artichoke by-products derived from external bracts, leaves and stems, were supplied by Syngenta AG and Riberebro S.L. (Spain), respectively. Prior to experiments, raw material was ground with a knife mill to particle size < 500 μm. Commercial citrus pectin (trade name Ceampectin®, ESS-4400) was kindly provided by CEAMSA (Porriño, Pontevedra, Spain).

2.2. Pectin extraction and modification

Sunflower pectin was extracted from 1 kg of dried substrate by suspending in 20 L of sodium citrate (0.7%) at 52 °C, pH 3.2 for 184 min under agitation and the residue was precipitated with ethanol and then freeze-dried (Muñoz-Almagro, Rico-Rodriguez, Wilde et al., 2018). Artichoke pectin was extracted using a cellulase from *Trichoderma reesei* (Celluclast® 1.5 L, Novozymes, Bagsvaerd, Denmark) in an orbital shaker at 50 °C, pH 5 with constant shaking (200 rpm) following the method described by Sabater et al. (2018). After hydrolysis, samples were centrifuged (1300 x *g* for 10 min at 4 °C) and supernatants were filtered through cellulose paper. Residues were washed and precipitated in 70% ethanol, centrifuged (1200 x *g*, 20 min) and then freeze-dried. Extraction yield of pectin (expressed as percentage) represents the amount of pectin extracted from 100 *g* of initial dried raw material, being 10.0% and 22.1% the obtained values for sunflower and artichoke pectin, respectively.

The extracted sunflower and artichoke pectins, as well as the commercial citrus pectin were then subjected to an enzymatic treatment using a commercial cellulase from *Aspergillus niger* (Sigma Aldrich, Steinheim, Germany) with pectinolytic activity to reduce their Mw. Then, the resulting material was transferred to a continuous membrane reactor to separate the modified pectin from oligosaccharides and free sugars formed (Olano-Martin, Mountzouris, Girbson, & Rastall, 2001). The reactor consisted of an ultrafiltration dead-end stirred cell (model 8000, Amicon, Watford, U.K.) where the substrate was added and then pushed from a pressurized feed tank filled with water at a rate matching the permeate flow rate. All filtrations were carried out with an Ultracel® ultrafiltration disk membrane, with a Mw cut-off (MWCO) of 3 kDa and a diameter of 76 mm as determined by the manufacturers. Checking of absence of low Mw carbohydrates in the ultrafiltered samples was accomplished by the analysis of the resulting retentates and permeates by SEC-ELSD following the method described in Section 2.3.2. All pectin and MP samples were free from monosaccharides, as well as oligosaccharides below 10 kDa (Fig. 1).

2.3. Characterisation of pectin and enzymatic-modified pectin samples

2.3.1. Monosaccharide analysis

Monosaccharide analysis was performed after the acid hydrolysis of samples with 2 M trifluoroacetic acid (TFA) at 110 °C for 4 h. After that, released monosaccharides were analysed by gas chromatography (GC) in an Agilent Technologies gas chromatograph (7890 A) equipped with a flame ionisation detector (FID). Prior to GC analysis, trimethylsilyl oximes (TMSO) of monosaccharides were formed (Cardelle-Cobas, Martínez-Villaluenga, Sanz, & Montilla, 2009). 500 μL of hydrolysed samples were evaporated to remove the acid and then 400 μL of phenyl-β-glucoside (0.5 mg/mL) used as internal standard (IS) were added. Afterward, the mixture was dried at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 250 μL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisylazane (250 μL) and TFA (25 μL) at 50 °C for 30 min. Derivatisation mixtures were centrifuged at 6700 x *g* for 2 min and supernatants were injected in the GC-FID.

Analyses were carried out using a DB-5HT capillary column (15 m x 0.32 mm x 0.10 μm, J&W Scientific, Folson, California, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 °C, respectively. The oven temperature was programmed from 150 to 380 °C at a heating rate of 1 °C/min until 165 °C and then up to 300 °C at a heating rate of 10 °C/min. Injections were made in the split mode (1:5).