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Characterisation and *in vitro* fermentation of resistant maltodextrins using human faecal inoculum and analysis of bacterial enzymes present

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ABSTRACT

A commercially available resistant maltodextrin (RMD), which is made out of corn starch by chemically and enzymatically modification, consists of atypical starch linkages and a low average molecular mass of 2 kDa. These characteristics of RMD make it rather complicated to identify with any chromatographic or masspectrometric method at this moment.

A batch fermentation with human inocula over 48 h under anaerobic conditions showed that the degradation of the indigestible RMD is slow in comparison to dietary fibres like FOS. The RMD is only fermentable for ca. 60% and no specific oligosaccharide within the RMD population seems to be preferentially utilised by the microbiota present.

Short chain fatty acid (SCFA) production, with acetic acid as the main SCFA, started after *ca.* 5 h of fermentation, while the increase of SCFA at 11 h was concurrent with the major degradation of the oligosaccharides of RMD. The microbiota composition as analysed by HITchip, revealed that RMD slightly stimulated the growth of bifidobacteria in comparison to the blank.

The activity of carbohydrate degrading enzymes, produced by the microbiota during the *in vitro* fermentation, revealed that potential capacity to degrade typical starch linkages was high, but the RMD was only slowly and partly degradable during the incubation of 20 h.

1. Introduction

Resistant starch (RS) belongs to the group of dietary fibre and can be categorised into four types. RS type 1 is physically unavailable starch, RS 2 is raw starch and RS 3 is retrograded starch (Cummings, Edmond, & Magee, 2004; Topping, Fukushima, & Bird, 2003). Fibersol-2® used in this study belongs to RS 4, chemically modified starches. It is a thermally and enzymatically treated starch, which has resulted in a wide range of resistant maltodextrins (RMD) (Ohkuma & Wakabayashi, 2001). To obtain such a resistant maltodextrin, starch is heated with small amounts of hydrochloric acid under low-moisture conditions and at the same time hydrolysed by transglucosidases. Afterwards, the solution is further hydrolysed by an amylase and spray dried. This leads to RMDs, which are soluble, heat stable, non-digestible dietary fibre that consist of α(1-4), α(1-6), (1-2) and (1-3) glycosidic linkages (Ohkuma & Wakabayashi, 2001).

RMDs were demonstrated to have a stool volume increasing- and laxative beneficial health effect (Flickinger et al., 2000), attenuate the postprandial blood glucose level and positively affect the fat metabolism (Okuma & Hashizume, 2009). RMD's indigestibility in the upper gastrointestinal tract and partially fermentability has been determined in rat models and it was shown that 38% of the RMD investigated is excreted unfermented in faeces (Tsuji & Gordon, 1998). However, little is known about the fermentability of RMD with human faecal inoculum and the resulting fermentation metabolites. Since this type of dietary fibre is applied in many food products, its fermentation characteristics are of interest.

It has been demonstrated in an *in vivo* rat fermentation study, that substrates present specifically induce the expression of enzymes in the bacteria being active on these substrates (Sonnenburg et al., 2005). The enzymes produced determine the degradation process of the substrate and can be secreted by the

Abbreviations: RMD, resistant maltodextrin; FOS, Fructooligosaccharides; PNP, *p*-nitrophenyl; CMC, carboxymethyl cellulose; RAX, rye arabinoxylan; WAX, wheat arabinoxylan; SSP, soluble soy polysaccharide; LBG, locust bean gum; LMP, low methylated pectin; HMP, high methylated pectin

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bacteria in the lumen or are bound to the bacteria´s cell wall. It is known that amylase activity towards starch is primarily provided by cell wall bound bacterial enzymes (Macfarlane & Englyst, 1986). Thus, the enzyme activity levels of extracellular and cell wall bound enzymes towards many polysaccharides can be illustrative for the fermentation potential of the microbiota.

The objective of this study is to characterise the structure of an commercially available RMD and investigate its *in vitro* fermentation behaviour by means of human faecal inoculum. In addition, the microbiota composition was analysed as well as the activities of the microbial enzymes produced during the *in vitro* fermentation.

2. Materials and methods

2.1. Substrate RMD

The commercial name of the RMD used is Fibersol-2® and was obtained from ADM/Matsutani LLC (Hyogo, Japan).

2.2. Culture medium for in vitro fermentation

The culture medium was based on the standard ileal efflux medium (SIEM) and modified as described elsewhere (Ramasamy, Venema, Schols, & Gruppen, 2014). All medium components were obtained from Tritium Microbiology (Veldhoven, The Netherlands). The medium included a 1 M MES buffer pH 5.8 (Ramasamy et al., 2014).

2.3. Human faecal inoculum

Standardized, pooled inoculum from 8 healthy European adults (25–45 years old) was obtained from TNO (Zeist, The Netherlands), prepared and validated as described elsewhere (Minekus et al., 1999). The subjects were not treated with antibiotics 2 months before faecal donation. The inoculum was activated in SIEM culture medium for 16 h and diluted 10 times before use.

2.4. In vitro fermentation

For each time point two times a 20 mL fermentation bottle was filled with 9 mL culture medium containing 9 mg/mL substrate and 1 mL of the activated inoculum under anaerobic conditions. Hence, the inocula were in total 100 times diluted at the start of the fermentation experiment. The bottles were immediately closed with rubber stoppers, sealed with an aluminium cap and incubated at 37 °C during shaking at 130 rpm for maximum 48 h. Blanks with either no substrate or no inoculum were prepared. Time points were 0, 5, 6, 10, 11, 24 and 48 h. After each fermentation time, 5.5 mL of the solution was heated for 5 min at 100 °C. The remaining volume was directly frozen in liquid nitrogen, stored at −80 °C and used for microbiota composition and enzyme activity analysis.

2.5. Chemical and enzymatic analyses

2.5.1. Determination of starch content

Total starch and resistant starch contents were analysed according to AOAC method 996.11 and 2002.02 with enzyme kits from Megazyme (Bray, Ireland), respectively, including extensive α-amylase and amyloglucosidase digestion.

2.5.2. Constituent monosaccharide composition

The constituent monosaccharide composition was determined using a pre-hydrolysis step with 72% (w/w) sulphuric acid at 30 °C for 1 h, followed by hydrolysis with 1 M sulphuric acid at 100 °C for 3 h. The monosaccharides released were derivatized to alditol acetates and analysed by gas chromatography using inositol as an internal standard (Englyst & Cummings, 1984). The absence of uronic acid (UA) in the samples was substantiated by using the colorimetric m-hydroxydiphenyl assay (Ahmed & Labavitch, 1978) automated on a skalar autoanalyser (Skalar, Breda, The Netherlands) as described elsewhere (Thibault, 1979).

2.5.3. Glycosidic linkage composition

The monosaccharide linkage composition was determined using the permethylation with methyl iodide as described elsewhere (Pettolino, Walsh, Fincher, & Bacic, 2012) using 4 times higher substrate and end reagent quantities. The hydrolysis, acetylation and analysis with GC–MS was performed as described elsewhere (Pustjens et al., 2014).

2.5.4. Size exclusion chromatography

The RMD (2 mg/mL) as such or the RMD fermentation liquids were centrifuged (10 min, RT, 18,000*g*) and the supernatant was analysed for molecular weight distribution with high performance size exclusion chromatography (HPSEC) on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Shodex RI-101 refractive index detector (Showa Denko, Tokyo, Japan). Three TSK-Gel columns connected in series (4000-3000-2500 SuperAW; 150×6 mm^2) were used for the analysis. These columns were preceded by a TSK Super AW-L guard column (35×4.6 mm^2). All columns were from Tosoh Bioscience (Tokyo, Japan) and covered a molecular mass range from 0.2–250 kDa. Samples (20 μL) were injected and eluted with 0.2 M $NaNO_3$, at 40 °C with a flow rate of 0.6 mL/min. Pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA) were used for calibration.

For semi-preparative size exclusion chromatography (SEC) 100 mg RMD or maltodextrin (Avebe, Veendam, The Netherlands) was solubilised in 4 mL water and manually injected on an Äkta purifier (GE Healthcare, Uppsala, Sweden) equipped with three Superdex 30 Hiload 26/60 prep grade columns in series (molecular mass range of approximately 0.1–10 kDa). A flow rate of 1.7 mL/min, water with 0.5% (v/v) ethanol as eluent at a column temperature of 35 °C was used. Fractions (7 mL) collected were analysed with MALDI-TOF-MS to determine the molecular weight of dextrins present.

2.5.5. Oligosaccharide profiling with HPAEC and HILIC–MS

After centrifugation of the RMD as such or the fermentation digest (10 min, RT, 18,000*g*), the supernatant was diluted twice and analysed for its oligosaccharide profile using high performance anion exchange chromatography (HPAEC) on a ICS5000 system (Dionex), with a CarboPac PA-1 column (2×250 mm^2) in combination with a Carbopac PA-1 guard column (2×50 mm^2). The flow rate was 0.3 mL/min with an eluent profile starting with 0.02 M NaOH until 13 min, then increasing to 0.1 M NaOH till 15 min, followed by a linear gradient of 0–500 mM NaOAc in 1 M NaOH till 45 min, followed by a gradient to 1 M NaOAc in 0.1 M NaOH in 1 min and 7 min at 1 M NaOAc in 0.1 M NaOH. After this, the column was equilibrated with 0.1 M NaOH for 3 min and 0.02 M NaOH for 20 min. An ICS5000 ED (Dionex) pulsed amperometric detector and Chromeleon software version 7 were used.

Hydrophilic interaction chromatography with mass spectrometry detection (HILIC–MS) was used to characterise the RMD as such. An Acquity UPLC BEH Amide column (1.7 μm, 2.1 mm × 150 mm; Waters, Milford, MA, USA) in combination with an Acquity UPLC BEH Amide VanGuard precolumn (1.7 μm 2.1 mm × 5 mm; Waters) were used. The mobile phase was used as described previously (Leijdekkers et al., 2014). MS detection was