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Unsaturated oligogalacturonic acids are generated by in vitro treatment of pectin with human faecal flora

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Abstract

Pectins with a degree of esterification (DE) of 95, 66, 34 and 0%, respectively, were incubated in vitro with human faecal flora (pH 7.8). The concentration and composition of oligogalacturonic acids (oligoGalA) generated were determined using high-performance thin-layer chromatography (HPTLC) with UV and colorimetric detection. In the first period of the anaerobic degradation, the pectin macromolecules were fragmented into unsaturated oligoGalA as intermediate products by the action of bacterial pectate lyases. Depending on the incubation time and the DE of pectin, the amount of unsaturated oligoGalA having different degrees of polymerization changed continuously. These oligoGalA were present in the cultures for some hours. Mixtures of unsaturated di-, tri- and tetraGalA were the end products of a pectate lyase action. Later, the oligoGalA disappear as a result of their further fermentation by the gastrointestinal microflora under formation of short-chain fatty acids (SCFA). Low-esterified pectins were depolymerized and fermented faster than the highly esterified by the human faecal flora in vitro. Furthermore, a mixture of unsaturated oligoGalA prepared from pectic acid by the action of pectate lyase from *Erwinia carotovora* was completely fermented by human faecal flora.

Keywords: Pectin fermentation; Oligogalacturonic acids; Short-chain fatty acids; Pectate lyase; Human faecal flora

1. Introduction

Pectin is not depolymerized by human intestinal enzymes, however, it is fermented practically almost in the colon by the gastrointestinal microflora [1–4]. In most studies, only the concentration and composition of the short-chain fatty acids (SCFA)—the physiologically important end-products of the fermentation of pectin—were estimated [3,5,6]. The backbone of pectin is composed of 1→4-linked α-D-galactosyluronic acid residues, some of which are methyl-esterified [7]. Oligogalacturonic acids (oligoGalA) are the intermediate- or end-products of pectin degradation following the action of microbial or plant *endo*-polygalacturonases, *endo*-pectin lyases or *endo*-pectate lyases [8]. However, it is not known whether oligoGalA are present or accumulate in the colon or whether they are metabolised immediately by the microflora.

Pectin and its enzymatic degradation products have been shown to influence the absorption, incorporation, and the renal excretion of lead [9]. A precondition for these effects is the resorption of oligoGalA from the colon and it has been shown that oligoGalA injected intravenously or injected directly in the caecum of rats appeared in the urine [10].

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Table 1
Composition of the pectin preparations

Pectin preparation	Degree of esterification (%)	Galacturonan (%)	Intrinsic viscosity [η] (mL/g galacturonan)
Pectic acid	0	59.3	420
Low-esterified pectin	34.4	74.4	395
High-esterified pectin	66.0	71.5	920
Very highly esterified pectin	94.7	72.7	271

Besides HPAEC [11,12], thin-layer chromatography (TLC) with different modifications was used by several authors for characterisation of oligoGalA [13–16].

The aim of this study was to investigate the microbial breakdown of the homogalacturonan part of pectins with different degrees of esterification (DE) during its incubation with human faecal flora and the separation and determination of oligogalacturonic acids using high-performance TLC (HPTLC).

2. Experimental

Pectins.—High- and low-esterified citrus pectins without additives were obtained from Copenhagen Pectin A/S, Lille Skensved, Denmark. The DE with methanol of these preparations were 66 and 34%, respectively (Table 1). For the preparation of a very highly esterified pectin, 100 g pectin with a DE of 66% was further esterified using initially 800 mL MeOH/40 mL concd H_2SO_4 (6 d) and then 1000 mL MeOH/37.5 mL concd H_2SO_4 (6 d) at 4 °C. Finally, this pectin was washed with 80% EtOH. The DE was increased to 95% by this procedure.

For the preparation of pectic acid, 25 g high-esterified citrus pectin was fully de-esterified in 400 mL 50% EtOH containing 5 g NaOH and 5 g KOH (30 min at 20 °C). After acidification, the pectic acid was isolated from EtOH [17].

Characterisation of pectins.—The galacturonan content of the pectin and oligoGalA preparations was determined by the *meta*-hydroxydiphenyl (MHDP) method [18]. Methyl ester groups were analysed by the chromotropic acid method [19] and the DE was also characterised using titrimetric analysis [20]. The intrinsic viscosity [η] was determined in 0.155 M NaCl (high-esterified pectin) or in 0.05 M NaCl/0.005 M Na–oxalate (low-esterified pectin) at 25.0 °C and pH 6.0 using an Ubbelohde viscosimeter. The intrinsic viscosity is related empirically to the molecular weight by the Mark–Houwink relation [21].

The pectin preparations used are characterised in Table 1.

Preparation of oligogalacturonic acids.—A pectate lyase (EC 4.2.2.2.) culture concentrate (220 lyase U/mL) from *Erwinia carotovora* (Institute of Potato Research, Groß Lüsewitz, Germany) was purified by dialysis (24 h at 4 °C) against water for removal of sugars and other low-molecular substances. The enzyme was isolated by precipitation and centrifugation in aq 50% EtOH at 4 °C and freeze-drying. In the lyophilised preparation, the pectate lyase activity [22] was 5400 U/g.

For the preparation of mixtures of unsaturated oligoGalA, 100 mL pectic acid solution (1% galacturonan) was incubated at 30 °C and pH 8.5 with pectate lyase (40 U) in the presence of 2 mM $CaCl_2$. The degradation was stopped by adding 1 M HCl up to pH 2.0 and 100 mL aq 96% EtOH. After stirring, the mixture was centrifugated for 30 min (3000*g*) at 4 °C. The supernatant which contained the oligoGalA fraction was heated for 10 min at 80 °C to inactivate the enzyme and then centrifuged for a second time and dried in vacuum. The content of galacturonan in the oligoGalA preparation was approximately 50%.

In vitro incubation.—The pectin media were prepared by treating 0.75 g galacturonan with 5 mL aq 70% EtOH at 20 °C for 20 min (decrease of possible microbial contamination of the pectin preparations) and then solubilizing in 150 mL 0.067 M phosphate buffer (pH 7.8) under shaking whilst gassing with CO_2.

Fresh human faeces (5 g) were collected from a healthy 40-year-old female who had not taken antibiotics for at least 4 months prior to the experiments and ate a normal Western diet. The faecal sample was transferred immediately into the sterile pectin medium under anaerobic conditions in closed vessels at 37 °C.

Samples (7 mL) were removed under sterile conditions from the incubation suspension immediately before and after inoculation and then after different periods (up to 24 h).

Determination of short-chain fatty acids.—Isobutyrate (internal standard), perchloric acid and NaOH solution were added to 1 mL of the incubation suspensions, and these mixtures were freeze-dried. The dried materials were homogenised in a mixture of 100 μL 5 M HCOOH and 400 μL acetone. A 1 μL sample of the organic phase was assayed on a 25 × 0.32 (i.d.) Carbowax M (20 μm) column attached to a Hewlett–Packard 5890 A gas chromatograph with flame-ionisation detector and split injector. Helium was used as carrier gas at a column flow of 1.0 mL/min. The auxiliary helium gas flow was maintained isothermally at 125 °C, whereas the injector port and the detector temperature were 200 °C.

Determination of the macromolecular pectin fraction and oligoGalA.—For the determination of the macromolecular pectin and oligoGalA, 4 mL of the incubation suspensions were mixed immediately with 1 mL 0.2 M HCl and 5 mL aq 96% EtOH and then centrifuged for 30 min (6000*g*) at 4 °C. The macromolecular pectin was estimated colorimetrically [18] in the residues after its extraction with 0.5 M EDTA (pH 6.0) followed by coagulation in aq 50% EtOH.

The oligoGalA were determined in the supernatant using HPTLC. For inactivation of the bacterial enzymes, the supernatant was heated at 85 °C for 15 min.

High-performance thin-layer chromatography.—The oligoGalA-containing solutions (1.5–2.5 μL) were applied 8 mm from the bottom of 10 × 10 cm HPTLC Silica Gel 60 F_{254} plates (E. Merck, Darmstadt, Germany) by spraying (6 mm streaks) with the automatic TLC sampler III (Camag, Muttenz, Switzerland). The chromatograms were developed several times with 1-propanol–water mixtures (between 7:4.50 and 7:2.75) in an automatic developing chamber (ADC; Camag); run distance 40–80 mm, drying time 10 min, heating time 1.5 min, and precondition time 5 min [16].

The double bonds of unsaturated oligoGalA were measured at 235 nm using a TLC scanner II with CATS software (Camag). The plates were than dipped twice for 3 s in a mixture of 20 mL concd sulfuric acid, 20 mL water and 360 mL acetone using a chromatogram immersion device III (Camag) and heated for 5 min at 80 °C. After cooling, the plates were dipped twice for 3 s in a 0.5% solution of MHDP (Eastman Kodak, Rochester, NY) in acetone and then heated again for 10 min at 100 °C. The red–brown spots on a pale blue ground were scanned at 525 nm [16].

The HPTLC was calibrated using a mixture of oligoGalA with a degree of polymerization (DP) of between 2 and > 7 prepared from pectic acid by the action of pectate lyase from *Erwinia carotovora* [16].

3. Results and discussion

Complete human faecal flora from a fresh faecal sample was tested in vitro for its ability to degrade pectin. Macromolecular pectins with intrinsic viscosities of > 270 mL/g galacturonan and with different degrees of esterification were used as substrates for the microbial degradation studies (Table 1). Formation and disappearance of the oligoGalA were investigated in batch-cultures at several times during an incubation time up to 24 h. The composition and concentration of the oligoGalA were estimated chromatographically in the fraction soluble in aq 50% ethanol.

In the starting phase of incubation, the galacturonan macromolecules were enzymatically depolymerized under formation of relatively high-molecular degradation products (results not shown). The concentration of macromolecular pectin decreased continuously in the batch cultures of whole faecal flora throughout the incubation period. Pectin with

a DE of 95% (Fig. 1(B)) was degraded more slowly than the low-esterified substrate with a DE of 34% (Fig. 1(A)). The amount of the low-molecular galacturonan fraction containing the oligoGalA increased during the first period of the incubation and reached a maximum after approximately 8 h (pectin with DE 34%) or 12 h (pectin with DE 95%) under the incubation conditions used. The sum of high- and low-molecular galacturonans decreased continuously throughout the incubation period. The amount of the total galacturonans (sum of macromolecular pectin and oligoGalA) was diminished to 50% after approximately 10 h for the low-esterified substrate and after approximately 12 h if the high-esterified pectin was used. After 24 h, galacturonans disappeared almost completely when low-esterified pectins were added as substrate

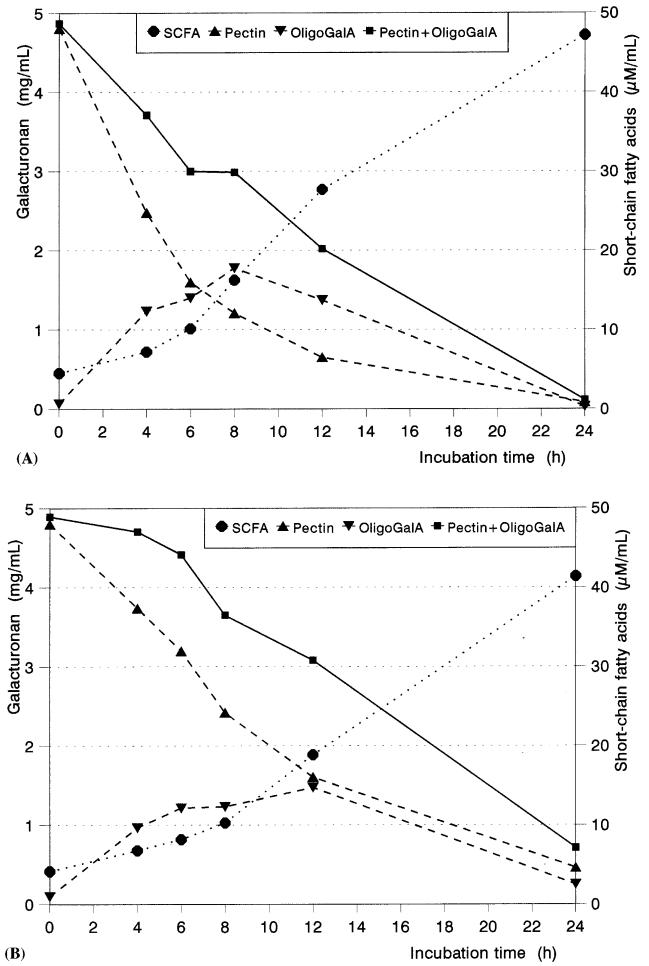

Fig. 1. Variation in macromolecular pectin and oligoGalA fractions as well as formation of SCFA during in vitro fermentation of pectin: (A) pectin with a DE of 34%; (B) pectin with a DE of 95%.

(Fig. 1(A)). In contrast, both galacturonan fractions were still present after an incubation time of 24 h using pectin with a DE of 95% as substrate (Fig. 1(B)).

OligoGalA are not the end-products of the degradation of pectin by the gastrointestinal microflora. The monomeric degradation product formed from the polysaccharide molecules is not stable. It is quickly rearranged to 4-deoxy-5-ketouronic acid and fermented further to SCFA, which were estimated by GLC. As shown in Fig. 1, the concentration of SCFA increased continuously in incubations with both high- and low-esterified pectins but reached higher concentrations with low-esterified pectins as substrate. The molar proportion of the SCFA was 78–90% acetate, 5–11% propionate and 8–15% butyrate. Valerate and *iso*-valerate were found in very low concentrations.

Typical chromatograms of the oligoGalA formed in the culture using HPTLC are shown in Fig. 2. After 8 h incubation of the pectin with a DE of 34%, a spectrum of oligomers with a DP between 2 and 7 was found in the fraction soluble in aq 50% ethanol. The spots were visualised initially by measuring the absorption at 235 nm which is closely related to the formation of delta-4,5-double bonds as a result of the enzymatic splitting of glycosidic linkages between the GalA units of pectin and the formation of unsaturated oligoGalA (Fig. 2(A)).

The peak between the unsaturated oligomers with DP of 2 and 3 indicated not an oligoGalA. This peak was also present when the plates were measured at 300 or 400 nm, whereas under these conditions the unsaturated oligoGalA gave no peaks (not shown).

The peak areas in Fig. 2(A) are only related to the number of double bonds independent of the length of the saccharide chains or the DP. Therefore, they are a measure for the amount of molecules with a double bond.

Subsequently, the plates were developed by dipping in the MHDP reagent [16], which is a sensitive colorimetric method for determination of pectin [18] (Fig. 2(B)). Using this second detection method, the appearing peak areas are related to the concentration of galacturonic acids present in the spots. Earlier it

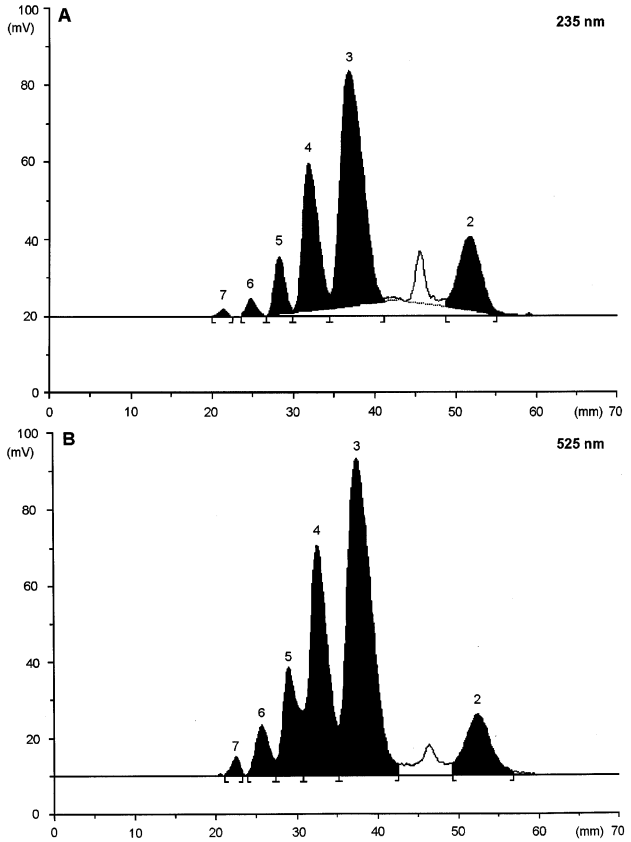

Fig. 2. Determination of unsaturated oligoGalA after 8 h incubation of pectin (DE 34%) with human faecal flora: (A) detection at 235 nm; (B) detection at 525 nm after treatment with the *m*-hydroxydiphenyl reagent (peaks 2–7: DP 2–7).

was shown that saturated and unsaturated oligoGalA have different R_f values in the applied HPTLC technique [16]. Because no additional peaks were found in the second detection method, it can be concluded that no saturated oligoGalA were produced beside the unsaturated by the action of the human microflora on the pectins under the used conditions.

Permanent changes in the composition of the unsaturated oligoGalA were measured during the incubation period (Fig. 3). Under the experimental conditions, the quantity of oligoGalA formed increased up to an incubation time of approximately 6–8 h. Subsequently, a continuous decrease in oligoGalA was measured during the in vitro fermentation of pectin. After 24 h incubation, these oligomers disappeared completely. During 2–6 h of incubation, predominantly unsaturated oligomers with a chain length from 2 to 7 appeared, whereas later only di-, tri- and tetramers were identified in the supernatant after addition of ethanol to the medium. The DE of the pectins influenced both quality and quantity of oligomers formed during the action of human faecal flora. Generally, oligoGalA with a higher DP were mostly found in higher concentrations during fermentation of low-esterified pectins.

The results of this study show that unsaturated oligogalacturonic acids were present in the cultures as intermediate products in variable concentration and composition for some hours depending on the fermentation conditions and the DE of pectin. These in vitro results suggest that the resorption of the oligoGalA in the colon may be possible due to the relatively long-term existence of these metabolites.

Under the conditions applied with buffered medium, the pH values were relatively stable during the whole incubation period. In the absence of buffer, the pH would decrease as a result of the formation of SCFA. In such a case, the activity of pectate lyases and naturally the degradation of pectin substrates would be reduced [8].

Finally, the effect of human faecal flora on a mixture of unsaturated oligoGalA prepared by action of pectate lyase from *E. carotovora* on pectic acid was investigested. This mixture consisted of oligoGalA with a DP between 2 and 8 (Table 2). The galacturonan concentration in this experiment was 0.84%. Although the step of depolymerization of the macromolecules is missing, the further enzymatic depolymerization to the di-, tri- and tetramers lasted for some hours. After 24 h incubation, oligoGalA disappeared from the medium. The permanent changes in concentration and composition of the oligomers are summarised in Table 2.

4. Conclusions

Pectin as a dietary fibre passes through the small intestine as a macromolecule. Because of its properties (e.g., viscosity, ion-exchange), pectin is able to interact with steroids [23] or metal ions [24] in the upper part of the gastrointestinal tract. By the action of microflora

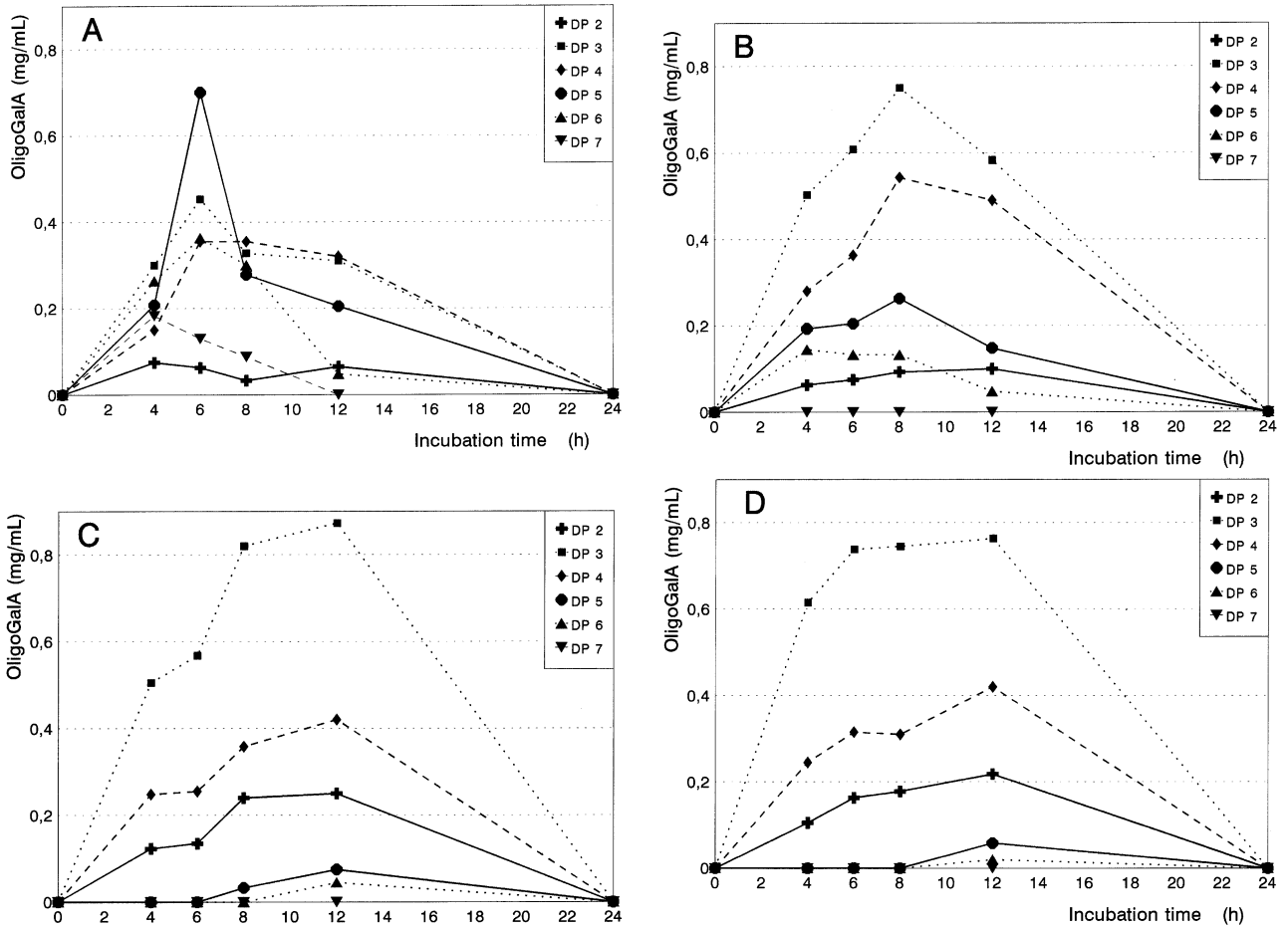

Fig. 3. Qualitative and quantitative composition of oligoGalA during incubation of pectins with human faecal flora in vitro: (A) pectic acid with DE 0%; (B) pectin with DE 34%; (C) pectin with DE 66%; (D) pectin with DE 95%.

in the large bowel, pectin is extensively degraded [25]. In general, intact pectin does not appear in faeces, however, in some studies it was shown that galacturonans are not completely fermented in vitro and in vivo [26,27].

In this study, it is shown that during the in vitro fermentation of pectin substrates, unsaturated oligoGalA were present as intermediate metabolites of pectin degradation for some hours. They could potentially be absorbed by the host if these molecules were produced and retained in the large bowel in vivo. Therefore, they can be involved in different physiological processes, for example in resorption of lead ions from the colon and in its transport [9,10].

Because of the faster fermentation rate of low-esterified pectins and the appearance of unsaturated oligoGalA as the sole products produced in this fermentation, it is suggested that pectate lyases of the endo-type are the key enzymes in pectin degradation by human—and additionally by rat [9]—gastrointestinal microflora.

These findings are supported by data from the literature: Jensen and Canale-Parola [28] described two *Bacteroides* species as pectin-degrading microbes in the human intestinal flora (*B. pectinophilus* and *B. galacturonicus*). Matsuura [29] detected a pectate lyase of the endo-type in human faecal extracts which splits pectic acid into unsaturated oligoGalA. Furthermore, pure strains of human gut bacteria (*Bacteroides* and *Eubacterium* species) were able to degrade pectin [26]. Cell-associated pectin-degrading enzymes were also found in *B. thetaiotaomicron* grown in media with pectic acid as sole carbon source [30]. Likewise, *Clostridium butyricum–C. beijerinckii* isolated from human faeces also splits pectin [31].

Table 2
Degradation of an oligogalacturonic acid mixture (prepared from pectic acid by action of pectate lyase from *Erwinia caratovora*) by human faeces flora

Incubation time (h)	pH	Galacturonan (%)	Analysis method[a]	Composition of oligoGalA in the culture[b] (%)						
				Di	Tri	Tetra	Penta	Hexa	Hepta	Octa
0	7.82	0.835	A	9.3	28.1	16.8	22.0	19.8	3.7	0.3
			B	10.2	29.4	15.9	19.1	20.2	4.8	0.4
1	7.72	0.830	A	14.0	39.4	21.4	14.4	8.5	2.3	0
			B	14.6	40.2	20.1	14.1	9.0	2.0	0
2	7.64	0.835	A	17.2	45.9	22.7	9.2	4.0	1.0	0
			B	17.9	46.7	23.3	7.3	3.6	0.8	0
4	7.35	0.635	A	22.8	53.7	23.5	0	0	0	0
			B	24.3	54.6	21.1	0	0	0	0
8	7.30	0.470	A	20.3	53.6	26.1	0	0	0	0
			B	22.2	53.0	24.6	0	0	0	0
24	7.30	(0.012)	A	0	0	0	0	0	0	0
			A	0	0	0	0	0	0	0

[a] HPTLC with UV detection (A) and with colorimetric detection (B).
[b] Similar results were obtained with other chromatographic techniques (HPAEC).

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