**Behaviour of citrus pectin during its gastrointestinal digestion and
fermentation in a dynamic simulator (simgi®)**

Alvaro Ferreira-Lazarte, F. Javier Moreno, Carolina Cueva, Irene Gil-Sánchez and Mar
Villamiel*

Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM).

C/ Nicolás Cabrera, 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid,
Spain

*Author to whom correspondence should be addressed:

Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM),

C/ Nicolás Cabrera 9, Campus de la Universidad Autónoma de Madrid,

E-28049 Madrid (Spain).

Tel: +34 910017951

E-mail: m.villamiel@csic.es

Abstract

During the last years, there is an increasing interest toward the potential prebiotic properties of pectin. However, most of the studies have been carried out in batch *in vitro* assays without a previous gastrointestinal digestion. *In vivo* investigations provide the best models for studying the influence of these compounds on the activity of microbiota. However, due to the difficulty of this type of assays, *in vitro* multistage dynamic models are presented as a good alternative. Thus, the behaviour during digestion and potential prebiotic properties of a commercial citrus pectin was examined using a Dynamic Gastrointestinal Simulator (simgi®) model for the human gut, which allows to study the behaviour of compounds in five different compartments (stomach, small intestine, ascending, transverse and descending colon). A remarkable non-digestibility of citrus pectin in the upper gastrointestinal tract was observed following its monitoring by HPLC-ELSD which determined that ~88% of fed citrus pectin remained intact during its transit through the stomach and small intestine. Fermentation of pectin stimulated the growth of beneficial bacteria such as *Bifidobacterium* spp, *Bacteroides* spp and *Faecalobacterium prausnitzii*. A high increase of short-chain fatty acids (SCFA) was observed, in concordance with the bacterial growth, especially in acetate and butyrate, which was produced due to direct fermentation of pectin or by cross-feeding interaction between bacteria. As a consequence of the pH decrease, the levels of ammonia were also decreased. This is the first study on the digestibility and fermentation of pectin carried out in a complex dynamic gastrointestinal simulator, being of special relevance the results obtained for *F. prausnitzii*.

Keywords: non-digestible carbohydrate; pectin; fermentation; prebiotic; *in vitro* gastrointestinal model; intestinal digestibility; dynamic *in vitro* model

Introduction

Pectins are a family of plant cell wall polysaccharides with glycan domains that contain galacturonic acid (GalA) units with α -1,4 linkages. It mainly consists of a GalA-rich backbone, known as homogalacturonan (HG $\approx$ 65%), of which a number of residues are methyl esterified at the C-6 position, thereby conferring a specific degree of methoxyl esterification (DM) to the polymer. This degree of esterification and its distribution pattern define the charge distribution over the polymer playing a major role in the dimerization of pectin chains through the formation of junction zones, either via cooperative Ca^{2+} complexation or at reduced water activity as well as pH, thus defining the gelation properties of pectin (Dongowski, Lorenz, & Proll, 2002; Fraeye et al., 2010). Furthermore, rhamnose residues interrupt the HG structure to form rhamnogalacturonan I (RG-I $\approx$ 20-35%) which is based on a backbone consisting of a repeating disaccharide of $[\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1}\rightarrow 4)\text{-}\alpha\text{-D-GalAp-(1}\rightarrow]$ residues. RG-I has a number of side chains attached to its backbone and the length of these chains can vary from single neutral glycosyl to polymeric side chains of different types (1 $\rightarrow$ 5)- α -L-arabinans, (1 $\rightarrow$ 4)- β -D-galactans, arabinogalactans-I, arabinogalactans-II (Buffetto et al., 2015). RG-II constitutes $\approx$ 2-10% of pectin and is the most complex part of pectin, it has a HG backbone branched with rhamnose and other minor sugars such as fucose, glucuronic acid, methyl-esterified glucuronic acid, apiose, 2-O-methylxylose, and 2-O-methylfucose (Holek, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014; Lara-Espinoza, Carvajal-Millán, Balandrán-Quintana, López-Franco, & Rascón-Chu, 2018; Noreen et al., 2017). The suitability of pectin for specific applications is governed by the structural features, including molecular weight (Mw), neutral sugar content, proportion of HG:RG-I regions or the degree of esterification (Ferreira-Lazarte, Kachrimanidou, Villamiel, Rastall, & Moreno, 2018; Sila, Van Buggenhout, Duvetter, Van Loey, &

Hendrickx, 2009). These factors can affect its applicability as thickeners or as gelling and stabilizing agents (Gullón et al., 2013).

Pectins, as other dietary fibers, are believed to be resilient to digestion reaching the hindgut where they are fermented by the colonic microbiota (Lunn & Buttriss, 2007). However, before they reach the colon, these heteropolysaccharides are subjected to the singular luminal environment of the upper digestive tract that can contribute to chemical and physicochemical changes affecting the rate and extent of the fermentation in the colon (Hoebler, Guillon, Fardet, Cherbut, & Barry, 1998). The intestinal degradation of pectin has been studied with substantially dissimilar results. In studies involving human subjects, Chinda et al. (2003) and Saito et al. (2005) as well as Holloway (1983) observed that around 90% and 60-85% of apple and citrus pectin, respectively, reached the terminal ileum but the procedures to evaluate total pectin were not robust enough to identify the possible structural and physical changes that take place.

However, the capability of pectins to be fermented by the intestinal microbiota it is well known, being the arabino- and galacto-oligosaccharides content one of the most important factors, even more relevant than Mw (Di et al., 2017; Onumpai, Kolida, Bonnin, & Rastall, 2011). In this sense, there are some investigations that report a better bifidogenic effect, which means a growth of *Bifidobacteria* population, of pectins and pectic-polysaccharides with higher arabino- and galacto-oligosaccharides content, over modified pectin and pectic-oligosaccharides (POS) with lower Mw (Di et al., 2017; Ferreira-Lazarte et al., 2018).

Most of these studies are *in vitro* and often restricted to faecal samples, since *in vivo* investigations with animals and human trials have various drawbacks, such as high costs, ethical constraints, inter-individual variations and limitations in sampling from the small and large bowel (Venema & Van Den Abbeele, 2013; Verhoeckx, K., Cotter,

P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, 2015). Nonetheless, even if they have limitations based on the absence of a physiological host environment, *in vitro* models are reproducible, since they allow better control of the experimental variables than animal or human studies. In general, they are rapid and simple methods and, therefore, relatively inexpensive and cost-effective. Furthermore, they allow a reduction of the samples size when this is a limiting factor (Verhoeckx, et al., 2015).

Therefore, several *in vitro* models have been developed to simulate the multistage processes of human gastrointestinal digestion (Alminger et al., 2014; Cascone et al., 2016; Hur, Lim, Decker, & McClements, 2011; Marzorati et al., 2011; Verhoeckx, et al., 2015). Among all these models, complex multi-compartmental continuous systems overcome the limitations present on static models, which do not reproduce the dynamic environment of the GIT (e.g. pH changes, peristaltic movements, gastric emptying, continuous changes, and secretion flow rates) (Ouweland, Vaughan, 2006). Nowadays, dynamic gastrointestinal digestion simulators are still limited. The SIMulator Gastro-Intestinal (simgi®, Madrid, Spain) (Barroso, Cueva, Peláez, Martínez-Cuesta, & Requena, 2015) comprise five different compartments system, which simulates the different regions of the GIT such as, stomach (ST), small intestine (SI) and three compartments simulating the ascending (AC), transverse (TC) and descending (DC) regions of the human colon. The simgi® represents a fully computer controlled multi-compartmental system, which allows joint or separated simulation of the gastric and colonic fermentative processes. Thus, this is a flexible modulating system that combines a gastric compartment that simulates peristaltic mixing movements, a reactor simulating the small intestine and three-stage continuous reactors for reproducing the colon region-specific microbiota and its metabolism (Barroso et al., 2015).

Therefore, the aim of the present study was to examine the *in vitro* gastrointestinal digestion of a commercial citrus pectin using the Dynamic Gastrointestinal Simulator (simgi®), and its impact on the subsequent fermentation by the colonic microbiota.

2. Materials and methods

2.1. Samples of pectin

Commercial citrus pectin (trade name Ceampectin®, ESS-4400) was kindly provided by CEAMSA (Porriño, Pontevedra, Spain). Galacturonic acid (GalA) content, degree of methoxylation (DM), molecular weight (Mw) and neutral sugar content of the tested pectin were described in previous studies in our laboratory (Muñoz-Labrador, Moreno, Villamiel, & Montilla, 2018).

2.2. simgi® model assays digestion

The dynamic gastrointestinal simulator simgi® was used in the operating mode to work with the five units simulating the stomach (ST), small intestine (SI) and the ascending (AC), transverse (TC) and descending colon (DC) regions (Barroso et al., 2015). Figure 1 shows the experimental protocol of the simgi® trial. The operation of the dynamic model was validated and optimized in previous studies (Barroso et al., 2016, 2015; Cueva et al., 2015). Faecal slurry was obtained from a healthy volunteer who had no received any antibiotic treatment in the previous 3 months of the experiment. Then, faecal samples were diluted (20% w/w) in sterilised phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.0, Oxoid, Basingstoke, UK) containing 1 g/L sodium thioglycolate as reduced agent. The nutritive medium was adapted from the studies mentioned before and it was constituted by potato starch (Difco™, BD) (7 g/L), glucose (Difco™, BD) (0.4 g/L), yeast extract (Oxoid, ThermoFisher Scientific) (3 g/L),

Figure 1. Schematic representation of the experimental protocol of the simgi® trial developed to assess the gastrointestinal digestion and fermentation properties of citrus pectin (30 g/L in nutritive medium)

special peptone (Oxoid, ThermoFisher Scientific) (1 g/L), mucin from porcine stomach (Sigma-Aldrich, Merk) (4 g/L) and L-cysteine (Panreac AppliChem) (0.5 g/L). All compounds were dissolved in 1 L of distilled water and sterilized at 121 °C for 21 min with a final pH of 6.0.

The ascending, transverse and descending colon compartments were filled and pre-conditioned with the nutritive medium that feed the system during the stabilization period; 250 mL (AC), 400 mL (TC) and 300 mL (DC) of nutritive medium were added and later inoculated with 20 mL of fresh faecal slurry (20% w/v).

A stabilisation period of 12 days was applied to allow the intestinal microbiota to adapt to environmental conditions present in the colon compartments and to form a stable microbial community (Barroso et al., 2015). This stabilisation was approached by feeding the small intestine with nutritive medium (75 mL, pH 2) mixed with pancreatic juice (40 mL of a solution of 12 g/L NaHCO₃ (VWR Chemicals), 6 g/L oxgall dehydrated fresh bile (Difco™, BD) and 0.9 g/L porcine pancreatin (Sigma-aldrich)) three times a day during 12 days (Van Den Abbeele et al., 2010). After stabilisation period of the colonic microbiota, the simgi® was subjected to a 2-week experiment, which consisted of adding 240 mL of the commercial citrus pectin per day (3 doses of 80 mL) dissolved in the feeding nutritive medium (30 g/L, pH 3.1). This sample was added directly to the stomach during 14 days, where it was mixed with gastric electrolytes and pepsin by the simulated peristaltic moves, controlling the decrease of pH by adding 0.5 M HCl. After stomach digestion, stomach content was automatically transferred to the small intestine vessel where digestion was performed during 2 h at 37 °C (pH = 7.0). Then, this content was transferred to the following compartment (AC) at a flow rate of 5 mL/min, which simultaneously activated the transit of colonic content between the AC, TC and DC compartments at the same flow rate. The temperature (37

°C), continuous flushing of nitrogen and pH were continuously controlled by the system. pH in the colonic units was controlled by addition of 0.5 M NaOH and 0.5 M HCl to keep values of 5.6 ± 0.2 in the AC, 6.3 ± 0.2 in the TC and 6.8 ± 0.2 in the DC. Finally, a 1-week washout period was included at the end of the experiment by feeding the simgi® daily with nutritive medium. During the whole study, samples were collected every day at regular time points from the three colon vessels: During stabilisation period (< Day 13, and immediately prior to pectin feeding (Day 13*), during pectin feeding period, samples were also taken in stomach and small intestine compartments (Day 13-27) and after the beginning of washout period (Day 27- 34). Finally, all collected samples were immediately centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were aliquoted and stored at – 20 °C until being analysed for short-chain fatty acids (SCFA), ammonium and molecular weight estimation of the tested pectin. Pellets were stored at – 80 °C until further analysis of total bacteria and main bacterial groups by qPCR. Microbial plate count analyses were performed at the time of the sample collection.

2.3. *Estimation of the molecular weight (Mw) of pectin*

The estimation and monitoring of Mw of pectin samples during the gastrointestinal digestion with the simgi® was carried out by Size Exclusion Chromatography (SEC), according to the method described by [Muñoz-Almagro, Rico-Rodriguez, Villamiel, & Montilla, \(2018\)](#). Analysis was carried out on a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Boeblingen, Germain), equipped with two consecutive TSK-GEL columns (G5000 PW_{XL}, 7.8 x 300 mm, particle size 10 µm, G2500 PW_{XL}, 7.8 x 300 mm, particle size 6 µm; Tosoh Bioscience, Stuttgart, Germany). Centrifuged samples from the different compartments were first diluted before HPLC

analysis: 1/10, 1/4 and 1/2 in HPLC water for ST, SI and AC, TC, DC compartments, respectively. Diluted samples were filtered and eluted (20 µL) with 0.1 M $\text{NH}_4\text{CH}_3\text{CO}_2$ at a flow rate of 0.5 mL/min for 80 min at 30 °C. The eluent was monitored with an Evaporative Light Scattering Detector (ELSD) (Boeblingen, Germain) at 30 °C. Pullulans of Mw 805, 200, 10, 3 and 0.3 kDa were used as standards to calibration. All the Mw values specified were weight-average.

2.4. Short-chain fatty acids (SCFA) analysis

SCFA analysis was performed by liquid chromatography using a UV-975 detector following the method described by Sanz et al., (2005). Briefly, samples from the different colon compartments (AC, TC and DC) were filtered and injected on a HPLC system (Agilent Technologies, Germany) equipped with a UV-975 detector and automatic injector. SCFA were separated using a Rezex ROA Organic Acids column (300 x 7.8 mm) (Phenomenex, Macclesfield, UK) thermostated at 50 °C. Mobile phase was sulphuric acid 0.005 mM in HPLC grade water at a flow rate of 0.5 mL/min under isocratic elution. The elution profile was monitored at 210 nm and peaks were compared to standards to be identified. Data acquisition and integration were done using Agilent ChemStation software (Wilmington, DE, USA). Calibration curves of all SCFA were obtained from the analysis of standard solutions of lactic, formic, acetic, propionic, butyric, valeric and isovaleric acid, ranging the concentrations of 1-100 mM.

2.5. Ammonium determination

Ammonium levels were determined using the Ammonium test (Spectroquant Ammonium Test, Merck), following the manufacturer's instructions. Briefly, serial dilutions of an ammonium standard solution (10 g/L) were used to prepare calibration

curves. Simgi® samples were diluted with deionized water (1:10). Just prior to performing the measurement at 25 °C, 5 mL of reactive NH₄-1 and reactive NH₄-2 were added to the diluted standards or samples. The mixture was shaken between each reagent addition. Then, the absorbance was quantified at 690 nm. Analyses were performed in duplicate. The results were expressed as mg of NH₄⁺ contained in each colon compartment.

2.6. Microbial analyses

2.6.1 Plate count

Collected samples from the different colon compartments were diluted (1/10) in a physiological solution (0.9 %) and were plated on eight types of genera and selective media as follows: Tryptic Soy Agar (TSA) (Becton and Dickson & Company, BD) for total aerobes; Wilkins-Chalgren agar (BD) for total anaerobes; MacConkey agar (BD) for *Enterobacteriaceae*; Enterococcus agar (BD) for *Enterococcus* spp.; MRS agar (Pronadisa) for lactic acid bacteria and Tryptose Sulfite Cycloserine agar (TSC) (Pronadisa) for *Clostridium* spp. Plates were incubated at 37 °C for 24-48 h in an anaerobic chamber (BACTRON Anaerobic/Environmental Chamber, SHELLAB, USA), except for TSA which was incubated in aerobic conditions (Nüve Incubator EN 120, NÜVE, Turkey).

2.6.2 Bacterial DNA extraction and quantitative polymerase chain reaction (qPCR)

Bacterial DNA extraction of pellets from AC, TC and DC compartments was performed using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommended protocol. Extracted DNA of all samples was stored at -80 °C until analysis.

The amplification and detection of bacterial DNA was carried out on a ViiA7 Real-Time PCR System (Applied Biosystems). Specific 16s rRNA-targeting primers were used in this study to determine total bacteria, *Bacteroides* spp, *Bifidobacterium* spp, *Enterobacteriaceae*, *Enterococcaceae*, *Lactobacillus* spp, *Faecalibacterium prausnitzii* and the *Clostridium cluster XIVa*. Reactions were done in triplicate in 384-well plates using SYBR® Green PCR Master Mix (Applied Biosystems). Final volume of each amplification reaction was 10 µL: 5 µL of SYBR® Green, 0.3 µL of each primer (10 µM), 3.4 µL of nuclease-free water purified for PCR (Sigma-Aldrich) and 1 µL of DNA template. Thermal cycling consisted of an initial cycle of 95 °C, 3 min, followed by 40 cycles of 95 °C, 15 s and 1 min at the appropriate primer-pair temperature (Gil-Sánchez et al., 2017). In order to quantify bacterial groups, DNA isolated from selected bacterial strains was used, *Bacteroides fragilis* for *Bacteroides*, *Bifidobacterium longum* for *Bifidobacterium*, *Blautia coccoides* for *Clostridia XIVa*, *F. prausnitzii* for *Faecalobacterium prausnitzii*, *Escherichia coli* for *Enterobacteriaceae*, *Enterococcus faecium* for *Enterococcaceae*, *Lactobacillus plantarum* for *Lactobacillus* and *B. fragilis* for *total bacteria*. Standard curves were generated by plotting threshold cycles (CT) vs. bacterial quantity expressed as colony-forming units (CFU)/mL.

2.7. Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 23.0. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to determine significant differences among the bacterial group populations obtained after the plate counting and qPCR analysis and organic acid concentrations to test the main effects of factors studied (time, pectin feeding, compartment). Differences were considered significant at $p < 0.05$ ($n = 3$).

3. Results

Characterisation results showed GalA as the main component with 66.5 ± 0.2 % of the total carbohydrates; galactose was the second main component with 20.2 ± 0.1 % and rhamnose and arabinose were present with 5.8 and 3.5 %, respectively. Glucose, mannose and xylose were also determined in minor values, 1.8, 1.4 and 0.9 %, respectively. **Interval of Mw** and methoxylation degree of pectin were determined as 100-800 kDa (**Average 350 ± 30 kDa**) and 70.7 %, respectively ([Ferreira-Lazarte et al., 2018](#)).

3.1. *Effect of digestion and fermentation on pectin molecular weight*

The behaviour of pectin during the chronic feeding period (**Figure 1**) was evaluated by monitoring the Mw during its passage through the different compartments (ST, SI, AC, TC and DC). **Table 1** shows the quantitative results obtained by SEC-ELSD determination. Analyses were carried out just before starting the feeding period at day 13 (representing the nutritive medium), and three random days during the chronic feeding (Day 15, 24 and 27) as well as the last day of the washout period (Day 34). Results showed a high average Mw for citrus pectin (350 ± 30 kDa) which represented almost 54 % of total content when mixed with nutritive medium, whereas the latter was mainly constituted of low Mw carbohydrates (<18 kDa). Values at Day 13* (before feeding with citrus pectin) showed almost no changes between all compartments. During the chronic feeding, pectin showed no changes in the stomach compartment when compared to the intact pectin (before feeding the system), whereas a slight decrease can be observed after the small intestine passage, showing a high resistance of citrus pectin to the upper gastrointestinal digestion. However, fermentation in the three

Table 1

Effect of the gastrointestinal digestion (simgi®) on the estimation and distribution of Mw (**Average Mw**) of the studied pectin (3%, w/v).

Sample/Day	Compartment	Concentration of carbohydrate fraction mg/L (%)		
		350 ± 30 kDa	40 ± 5 kDa	< 18 kDa
Medium	-	-	-	100.0 ± 0.0
Medium+Pectin	-	53.6 ± 1.2	-	46.4 ± 0.0
Day 13*	ST	5.7 ± 0.5	-	94.2 ± 0.1
	SI	6.9 ± 0.1	-	93.0 ± 0.3
	AC	5.0 ± 0.2	-	94.9 ± 0.2
	TC	4.2 ± 0.1	-	95.8 ± 0.1
	DC	4.3 ± 0.0	-	95.5 ± 0.0
Day 15	ST	56.6 ± 0.6	-	42.8 ± 0.9
	SI	52.9 ± 0.4	-	46.7 ± 0.5
	AC	8.5 ± 0.6	15.1 ± 0.8	75.8 ± 0.6
	TC	9.5 ± 0.2	4.8 ± 0.1	85.2 ± 0.3
	DC	9.8 ± 0.1	4.6 ± 0.1	85.1 ± 0.1
Day 24	ST	56.5 ± 1.5	-	42.0 ± 0.3
	SI	47.6 ± 0.3	-	55.9 ± 0.7
	AC	5.4 ± 0.1	17.2 ± 0.9	76.9 ± 0.1
	TC	4.8 ± 0.4	3.5 ± 0.0	91.4 ± 0.5
	DC	4.7 ± 0.2	2.2 ± 0.0	92.8 ± 0.3
Day 27	ST	55.8 ± 0.9	-	43.5 ± 0.2
	SI	47.7 ± 0.8	-	55.7 ± 0.6
	AC	1.9 ± 0.0	17.7 ± 0.6	79.7 ± 0.1
	TC	0.6 ± 0.0	3.7 ± 0.1	95.5 ± 0.1
	DC	0.5 ± 0.0	2.5 ± 0.0	96.8 ± 0.0
Day 34	ST	9.8 ± 0.0	-	89.7 ± 0.4
	SI	7.0 ± 0.1	-	92.8 ± 0.7
	AC	4.0 ± 0.0	0.0 ± 0.0	95.8 ± 0.6
	TC	3.9 ± 0.0	0.0 ± 0.0	96.0 ± 0.2
	DC	2.0 ± 0.0	0.0 ± 0.0	98.0 ± 0.0

Data are expressed as the mean ± SD (n=2).

*Sample taken before feeding with citrus pectin.

different sections of colon gave rise to a remarkable effect on pectin Mw. At this stage, it can be seen the presence of a new chromatographic peak of lower Mw (40 ± 5 kDa) than the peak corresponding to the intact pectin (350 ± 30 kDa), as well as an increase in the abundance of the peak including low Mw carbohydrates (<18 kDa), probably due to the fermentation of pectin (**Figure 2**). Lastly, washout period showed almost no presence of any carbohydrates since feeding with nutritive medium/pectin was substituted with only nutritive medium (**Table 1**).

3.2. Evolution of the microbial community

The computer-controlled multicompartmental dynamic gastrointestinal model used in this study, allowed us to monitoring the gastrointestinal digestion and fermentation in the different compartments/sections due to its capability to simulate *in vitro* the microbial conditions that characterize the different regions of the gut.

3.2.1. Plate counts

The composition of the microbial community and the bacterial counts reached during the last day of each period (stabilisation, feeding and washout period) in the AC, TC and DC was first evaluated by plate counts (**Table 2**). Results showed a significant increase in the counts of total bacteria, enterococcus and lactic bacteria families in the AC, after feeding with citrus pectin (Day 27). In the case of TC and DC all counted bacteria showed a decrease or no significant change ($p < 0.05$) after the feeding period, with the exception of *Enterococcus* in the DC. Counts after washout period (Day 34) showed a decrease in almost all bacteria and compartment which could be in correspondence with the absence of feeding with pectin in this period.

Figure 2. Qualitative evolution of citrus pectin (Mw distribution) in the five compartments (Stomach (dilution 1/10), Small Intestine (1/4), Ascending Colon (1/2), Transverse Colon (1/2) and Descending Colon (1/2)) during feeding of the dynamic simulator of the gastrointestinal tract (simgi®).

Table 2

Average plate count measurements ($n=3$) expressed in log CFU/mL, analysed in the ascending (AC), transverse (TC) and descending colon (DC) of the dynamic gastrointestinal model (simgi®) at the end of stabilisation period (day 13), chronic intake (day 27) and washout period (day 34) with citrus pectin.

Bacterial group		Sampling Day		
		Day 13 Stabilisation period	Day 27 Feeding period	Day 34 Washout period
Total aerobes	AC	7.56 (0.01) ^{b,1}	8.04 (0.12) ^{c,2}	7.26 (0.13) ^a
	TC	7.57 (0.11) ^{c,1}	7.52 (0.08) ^{b,1}	7.24 (0.12) ^a
	DC	7.37 (0.01) ^b	6.86 (0.03) ^a	6.93 (0.22) ^a
Total anaerobes	AC	8.26 (0.07) ^a	8.50 (0.02) ^{ab,2}	8.59 (0.08) ^{b,1}
	TC	8.38 (0.04) ^b	7.96 (0.07) ^{a,1}	8.10 (0.14) ^{ab}
	DC	8.30 (0.11) ^c	7.35 (0.02) ^a	7.95 (0.10) ^b
Lactic bacteria	AC	5.76 (0.10) ^b	6.42 (0.14) ^{c,1}	4.59 (0.02) ^a
	TC	6.30 (0.06) ^{b,3}	6.63 (0.12) ^{b,2}	4.57 (0.15) ^a
	DC	5.98 (0.23) ^{c,2}	5.81 (0.04) ^b	5.08 (0.05) ^{a,1}
Enterobacteria	AC	7.79 (0.05) ^{b,1}	7.90 (0.03) ^{b,2}	7.26 (0.23) ^{a,1}
	TC	7.67 (0.13) ^{b,1}	7.48 (0.12) ^{b,1}	7.22 (0.02) ^{a,1}
	DC	7.37 (0.28) ^b	6.88 (0.03) ^a	6.69 (0.02) ^a
Enterococcus	AC	5.90 (0.05) ^a	7.77 (0.07) ^{c,2}	6.91 (0.24) ^{b,1}
	TC	6.42 (0.12) ^{c,1}	6.29 (0.09) ^{bc}	5.37 (0.23) ^a
	DC	6.03 (0.03) ^a	7.50 (0.04) ^{c,1}	6.58 (0.04) ^{b,1}
<i>C. perfringens</i>	AC	7.55 (0.09) ^{a,1}	7.51 (0.01) ^{a,1}	7.45 (0.03) ^{a,2}
	TC	7.63 (0.07) ^{b,1}	7.75 (0.05) ^{b,2}	6.19 (0.06) ^a
	DC	7.22 (0.02) ^b	7.22 (0.09) ^b	6.52 (0.06) ^{a,1}
<i>Lactobacillus</i>	AC	6.14 (0.11) ^{c,1}	3.78 (0.07) ^b	2.97 (0.14) ^a
	TC	6.15 (0.07) ^{b,1}	4.67 (0.09) ^{b,1}	3.56 (0.08) ^{a,1}
	DC	5.59 (0.03) ^c	5.14 (0.05) ^{b,2}	3.95 (0.10) ^{a,2}

^{a,b,c} Different letters mean significant differences ($p < 0.05$) between the end of stabilisation (Day 13), feeding (Day 24-27) and washout period (Day 34) on the same compartment.

^{1,2,3} Different numbers mean significant differences ($p < 0.05$) between compartments on the same day of analysis.

Standard deviation values are in brackets.

3.2.2. qPCR analysis

Given that important modifications in the HPLC profiles of pectin were found together with the trends observed by the plate counts, a qPCR analysis was also done at the last day of each period in the AC, TC and DC compartments in order to better assess changes in the microbial population during the *in vitro* fermentation of citrus pectin (Table 3). In general, higher amounts of bacteria were obtained with qPCR as compared with plate counts, which is in consonance with the fact that only a small fraction of the range of gut bacterial groups found had been, up to now, cultured (Zoetendal, Vaughan, & De Vos, 2006). The rest of bacteria were often labelled as “unculturable” due to the generally fastidious anaerobic growth requirements (Allen-Vercoe, 2013). Counts of total bacteria group were about 9.5 log copy number/mL at the end of stabilisation period and increased significantly ($p < 0.05$) after citrus pectin feeding in AC and TC whereas in DC a lower and non-significant increase ($p > 0.05$) was determined. In addition, a decrease was observed in all compartments after washout period.

Among all bacterial groups that were studied, a huge and significant increase in counts of *Bifidobacterium* spp, *Bacteroides* spp, *F. prausnitzii* and *Enterobacteriaceae* was observed after the feeding with the citrus pectin in all colon sections with the exception of *Enterobacteriaceae* in TC. Furthermore, an overall and statistically significant decrease was found after the washout period, with some exceptions (i.e., *Bacteroides* spp in AC, or *Enterobacteriaceae* in all colon sections). In contrast, a decrease in *Lactobacillus* spp (in all colon sections) and *Enterococaceae* (in TC) was also observed after the feeding period with pectin. These values increased during the washout period for *Enterococaceae* whereas did not present any changes in *Lactobacillus* spp.

Table 3

Mean values ($n = 3$) of the qPCR data (log copy number/mL) for the microbial groups analysed in the ascending (AC), transverse (TC) and descending colon (DC) of the dynamic gastrointestinal model (simgi®) at the end of stabilization period (day 13), chronic intake (day 27) and washout period (day 34) with citrus pectin.

Bacteria group	Compartment	Stabilisation period Day 13*	Chronic intake period Day 27	Washout period Day 34
Total bacteria	AC	9.49 (0.05) ^a	9.82 (0.02) ^c	9.68 (0.04) ^b
	TC	9.50 (0.23) ^a	10.16 (0.01) ^b	9.48 (0.06) ^a
	DC	9.43 (0.01) ^a	9.59 (0.02) ^a	9.14 (0.33) ^a
Lactobacillus	AC	5.24 (0.13) ^b	≤ 4 ^a	≤ 4 ^a
	TC	5.79 (0.24) ^b	≤ 4 ^a	≤ 4 ^a
	DC	5.53 (0.18) ^b	4.39 (0.28) ^a	4.03 (0.48) ^a
Bifidobacterium	AC	5.44 (0.04) ^a	8.56 (0.06) ^c	6.43 (0.13) ^b
	TC	5.87 (0.14) ^a	8.02 (0.09) ^c	6.77 (0.05) ^b
	DC	5.50 (0.07) ^a	8.54 (0.02) ^c	6.98 (0.02) ^b
Bacteroides	AC	8.45 (0.08) ^a	9.24 (0.01) ^b	9.18 (0.02) ^b
	TC	8.85 (0.01) ^a	9.64 (0.03) ^b	8.79 (0.07) ^a
	DC	8.53 (0.05) ^a	8.93 (0.02) ^c	8.70 (0.02) ^b
Faecalobacterium prausnitzii	AC	≤ 5 ^a	7.43 (0.14) ^b	≤ 5 ^a
	TC	≤ 5 ^a	8.08 (0.18) ^b	≤ 5 ^a
	DC	≤ 5 ^a	7.17 (0.21) ^b	5.05 (0.10) ^a
Enterococaceae	AC	≤ 4 ^a	5.68 (0.06) ^b	≤ 4 ^a
	TC	6.83 (0.05) ^b	4.19 (0.28) ^a	6.40 (0.02) ^b
	DC	6.05 (0.04) ^a	5.50 (0.03) ^a	5.69 (0.45) ^a
Enterobacteriaceae	AC	6.98 (0.02) ^a	8.66 (0.03) ^b	8.55 (0.07) ^b
	TC	8.32 (0.08) ^{ab}	8.21 (0.19) ^a	8.60 (0.07) ^b
	DC	7.70 (0.24) ^a	8.23 (0.25) ^b	8.37 (0.02) ^b

^{a,b,c} Significant differences ($p < 0.05$) were noted with different letters according to the ANOVA analysis for the same bacterial group

*Sample taken before feeding with citrus pectin.

Standard deviation values are in brackets.

Finally, it should be pointed out that *Clostridia XIVa* group was also determined showing no changes in all compartments between stabilisation, chronic and washout period (data not shown).

3.3. Metabolic activity

The metabolic activity of the microbiota before, during and after feeding with pectin in the different colonic reactors of the simgi® was evaluated by monitoring the content of SCFA (fermentative metabolism) and of ammonium (proteolytic metabolism).

3.3.1. Ammonium determination

Evolution of ammonia during the simulation of the gastrointestinal digestion in the three colon compartments is shown in **Figure 3**. A slight but significant decrease ($p < 0.05$) in ammonium concentration is observed during the feeding with the citrus pectin, as compared with stabilisation and washout periods. Also, as it has been observed in previous studies with the same system (simgi®) (Barroso et al., 2016, 2015; Gil-Sánchez et al., 2017), ammonium concentration gradually increased from AC to the DC compartment because of the accumulation of products in the system, which lacks of any absorption steps between the different compartments. These values showed that proteolytic metabolism occurred through the entire colon compartments during the whole experiment, but it was substantially diminished during the chronic feeding period with citrus pectin.

Figure 3. Ammonium Evolution (mM) during gastrointestinal digestion of citrus pectin in the simgi®

3.3.2 Short-chain fatty acid (SCFA) analysis

The major end-products of indigestible carbohydrates metabolism by the colonic microbiota are SCFA. SCFA evolution (mM) during the stabilisation period, chronic feeding of citrus pectin and washout period is shown in **Figure 4**. SCFA concentrations presented no changes during the stabilisation period in all three compartments. During the chronic feeding with pectin these levels showed a significant increase in all major SCFA (acetate, propionate and butyrate) which decreased after elimination of nutritive medium/pectin administration. As expected, SCFA production consisted mainly of acetate, butyrate and propionate with small amounts of lactate and valerate in all compartments. Acetate was the most abundant SCFA, followed by butyric and propionic acid, which showed increases up to 297, 92 and 60 % after chronic feeding, as compared to the initial levels (Day 13), respectively. Afterwards, they showed a considerable decrease during washout period started. Overall, the total SCFA average molar production was compartment-dependent, being higher in the transverse and descending colon. Regarding minor SCFA, results also showed significant values of valerate in all three compartments (Khodaei, Fernandez, Fliss, & Karboune, 2016). Formic acid was detected in the AC compartment reaching values of 2.7-8.0 mM whereas TC and DC presented concentrations below 1.1 mM. Lactic acid production was only detected at fermentation days with the citrus pectin (1.3, 8.7 and 4.0 mM in ascending, transverse and descending colon, respectively). Although lactate is not a SCFA, it is usually considered in the metabolism of bacteria as a product of saccharolytic fermentation. Furthermore, valerate has been described as a primary end product of lactate fermentation (Almeida et al. 2017; Unger et al. 2016; Yoshikawa et al 2018).

Figure 4. Evolution in concentration of SCFA in the ascending (AC), transverse (TC) and descending colon (DC) of the simgi® during the stabilisation (D1-D13), feeding (D13-D27) and washout period (D27-34) with citrus pectin solution (3 g/L). ^{1,2,3} Numbers represent differences ($p < 0.05$) between the data at the end of each period

4. Discussion

Several studies have shown that oligosaccharides deriving from pectins exert bifidogenic activities. Furthermore, there are also studies that have demonstrated a significant growth of bacteria in intact pectins suggesting a potential role of this polysaccharide as a prebiotic (Ferreira-Lazarte et al., 2018; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016; Yang, Martínez, Walter, Keshavarzian, & Rose, 2013). In fact, in a static *in vitro* study, we have recently shown that either pectin or enzymatically-modified pectin from different by-products stimulates beneficial bacteria of colonic microbiota (Ferreira-Lazarte et al., 2018). However, no investigation has been carried out on the properties of pectin as a substrate for fermentation including a previous passage through the upper gastrointestinal tract. This study remarks, for the first time, the use of the simgi® to evaluate the effect of the upper gastrointestinal digestion on a commercial citrus pectin and its effect on colonic microbiota metabolism.

Pectin taken from the stomach compartment showed almost no changes when compared to the initial status, whereas samples taken from the small intestine revealed some loss of pectin, 6.5, 15.7 and 14.7 % for Day 15, Day 24 and Day 27, respectively. Holloway et al. (1983) observed a loss of pectin of 15-40 % in an *in vivo* study with ileostomy samples. In the same way, Saito et al. (2005) found that approximately 90 % of ingested pectin was recovered in the terminal ileum in an *in vivo* study collecting endoscopy retrograde samples. These studies attributed the loss of pectin to the possible degradation by bacteria within the digestive tract, especially the terminal ileum. However, given that in our prototype of digestion the presence of bacteria is confined to the colon compartments, changes observed in the Mw of pectin after its passage through

the SI could be related to other chemical effects due to the interaction with pancreatic fluids and bile salts (Miller, Buchanan, Eastwood, & Fry, 1995).

Regarding the effect of digested pectin on microbiota, higher amounts of microbial population were obtained with *q*PCR analysis when compared with plate counts. In general, results obtained showed that citrus pectin favourably impacts on microbiota composition and functionality in the three compartments (AC, TC and DC) of the simgi® model.

Pectin fermentation produced an increase in the counts of total bacteria, compared to the initial state, with significant increases in the proximal regions (ascending and transverse colon) probably due to the content of polysaccharide coming from the small intestine. Furthermore, high methoxyl pectins, as it is the case of citrus pectin here assayed, have showed a slower fermentation in the large intestine of rats, which allows the fermentation to take part in all three compartments (Dongowski, Lorenz, & Proll, 2002).

According to some authors, increments up to 0.5 - 1.0 log₁₀ in *Bifidobacterium* populations could be considered as a major shift in the gut microbiota towards a potentially healthier composition of intestinal microbiota (Kolida & Gibson, 2007). *Bifidobacterium* and lactobacilli have been traditionally considered as the major microbial targets for prebiotic action, due to their beneficial effects (Roberfroid et al., 2010). Similar values of *Lactobacillus* spp and *Bifidobacterium* spp populations at the end of stabilisation period were observed (Table 3). Increases up to 2.15 - 3.12 log₁₀ in *Bifidobacterium* group was determined in all compartments, being the highest increase of all bacteria determined. This could be attributed to the high galactose/arabinose content of the studied pectin (23.8 %) (Di et al, 2017; Onumpai et al., 2011). However, unlike *Bifidobacterium*, *Lactobacillus* group showed a significant decrease after feeding

with citrus pectin. In related studies, [Olano-Martin et al \(2002\)](#) reported that both POS and citrus pectin significantly increased the number of *Bifidobacteria*, whereas lactobacilli numbers only increased with POS although this increase was not statistically significant. Furthermore, [Chen et al. \(2013\)](#), showed an increase of *Bifidobacteria* during the *in vitro* fermentation of apple pectin (DM 70%) and POS, whereas *Lactobacillus* population presented no changes or even similar values for pectin compared to the negative control after 24 h of fermentation. [Li, Zhang, & Yang, \(2018\)](#) also showed a decrease of *Lactobacillus* when feeding rats with pectin extracted from citrus peels in an *in vivo* study.

Faecalobacterium prausnitzii values reported to be the second highest increase during the fermentation of pectin in the simulator ($2.17 - 3.03 \log_{10}$). *F. prausnitzii* is one of the most abundant commensal bacteria in the healthy large intestine and is one of the main **producers** of butyrate in the human colon ([P. Louis, Scott, Duncan, & Flint, 2007](#); [Petra Louis, Hold, & Flint, 2014](#)). Furthermore, low *F. prausnitzii* levels were correlated with the recurrence of inflammatory bowel disease and it has confirmed to have anti-inflammatory effects ([Onumpai et al., 2011](#); [Sokol et al., 2008](#)). Likewise, it has been suggested that this bacterium could be a good probiotic candidate to counterbalance dysbiosis in Crohn's disease patients ([Scott, Martin, Duncan, & Flint, 2014](#); [Sokol et al., 2009](#)). **Moreover**, previous studies have shown that this bacterium could have a major role in pectin utilization in comparison with other two abundant pectin-utilizing species, *Bacteroides thetaiotaomicron* and *Eubacterium eligens* ([Lopez-Siles et al., 2012](#)).

Bacteroides population showed also a high increase being the third highest increase of all bacteria determined with values of $0.4 - 0.8 \log_{10}$. *Bacteroides* are one of the enterotypes of the human microbiota, which are responsible for the major part of

polysaccharide digestion occurring in the human large intestine (Flint, Scott, Duncan, Louis, & Forano, 2012; Salazar et al., 2009). In fact, many strains from human faeces can produce various pectinolytic enzymes, including polygalacturonase, pectin methylesterase, extracellular and cell-associated pectate lyase (Dekker & Palmer, 1981; Jensen & Canale-parola, 1986). Hence, *Bacteroides* could be involved in cross-feeding with *Bifidobacteria* by releasing breakdown products of pectin which might be utilized by the latter.

Enterococaceae and *Enterobacteriaceae* groups presented different behaviour compared to the bacteria mentioned before. Significant increase were found in the AC for both bacteria during the feeding period with citrus pectin, whilst TC showed a decrease of *Enterococaceae* and no significant change for *Enterobacteriaceae*. Nevertheless, a significant increase in *Enterobacteriaceae* population was found in DC whereas stable levels were observed for *Enterococaceae* after feeding with citrus pectin.

Concerning the proteolytic and saccharolytic activity of microorganisms, SCFA concentrations increased during the chronic feeding with pectin. Lactic and formic acid were observed in low concentrations since produced lactic acid is considered to be an intermediate metabolite and can be further metabolized within the colon and turned into butyric and propionic acids through cross-feeding by gut bacteria (Duncan, Louis, & Flint, 2004; Reichardt et al., 2014). Similarly, formic acid is used by microorganisms that have a particularly important role in anaerobic metabolism, via interspecies cross-feeding interactions (Petra Louis et al., 2014). Results obtained showed an increase for valerate during the pectin fermentation. Khodaei et al (2016) also reported a small amount of valerate when testing a galactose/rhamnose rich polysaccharide with similar values compared with recognised prebiotic, such as FOS.

The major end-products of saccharolytic fermentation are acetate, propionate and butyrate, which have a combined concentration of 50-150 mM in the colon (Petra Louis et al., 2014). High levels of SCFA are desirable since, among other benefits, the corresponding decrease in the pH values can suppress the growing of pathogenic bacteria. **Figure 4** shows a significant high increase of these compounds, being acetate the major SCFA produced followed by butyric and propionic, respectively.

Given the complexity of the human microbiota, it is challenging to attribute a particular fermentation end-product to a specific bacterial group, however, acetate is typically generated via *bifidus* pathway, and more specifically it is a major end-product of *Bifidobacterium* fermentation (Sanz et al., 2005). Thus, the high production of acetate observed in our study can be ascribed to the growth of *Bifidobacterium* population in presence of pectin. The high increase of propionate concentrations after feeding the system with pectin is in good agreement with the increase in *Bacteroides* population, one of the main propionate-producing bacteria in the human colon. Propionate has also been shown to exert beneficial effects such as protective role against carcinogenesis through the decrease in human colon cancer cell growth (Hinnebusch, Meng, Wu, Archer, & Hodin, 2002; Jan et al., 2002). In addition, propionate and formate were reported to reduce the activity of *E. coli* and *Salmonella* at pH 5 (Beatriz Gullón, Gullón, Sanz, Alonso, & Parajó, 2011; Topping & Clifton, 2001). Significant increases in butyrate concentrations were also observed in all three compartments, with the second highest levels after acetate. *F. prausnitzii* might utilise apple, citrus and sugar beet pectin as a source of growth and butyrate formation as shown by using pure cultures and *in vitro* models (Lopez-Siles et al., 2012; Chung et al., 2017; Onumpai et al., 2011; Gómez et al., 2016). Thus, butyrate levels concur with the high increase of *F. prausnitzii* population observed. Furthermore, higher levels of

butyrate can also be explained due to cross-feeding between *Bifidobacteria* and *F. prausnitzii* since the latter is able to use the acetate produced by *B. adolescentis* thereby boosting butyrate formation (Rios-Covian, Gueimonde, Duncan, Flint, & De Los Reyes-Gavilan, 2015). Apart from these effects, butyrate is known to affect several components of the colonic defence barrier, resulting in enhanced protection against luminal antigens (Hamer et al., 2008; Havenaar, 2011).

Regarding ammonia concentration, Figure 3 showed a slight but significant decrease in ammonium concentration during the feeding with citrus pectin. It is noteworthy that lower proteolytic activities are usually associated with health-promoting effects (Ichikawa & Sakata, 1998), since it can be a potential carcinogenic agent at relatively low concentrations, as has been shown by the increase in mucosal damage and colonic adenocarcinoma in a rat model (Petra Louis et al., 2014; Windey, de Preter, & Verbeke, 2012). A significant positive correlation was observed between SCFA levels and ammonia excretion where more acidic conditions favour the excretion of ammonia due to the protonation and formation of poorly absorbed ammonium ion (Petra Louis et al., 2014).

5. Conclusions

The *in vitro* study of citrus pectin using the dynamic gastrointestinal simulator simgi® pointed out its high indigestibility, since a reduced hydrolysis of pectin (~12%) was detected in the upper gastrointestinal tract (ST and SI), mainly due to chemical interactions with pancreatic fluids and bile salts. Findings also highlight the important role played by pectin in stimulating beneficial bacteria such as *Bifidobacteria*, *F. prausnitzii* and *Bacteroides* (especially in the first two bacterial groups). A high increase in acetate, propionate and butyrate concentration was observed due to

fermentation of pectin by the microbiota but also to cross-feeding interactions between different bacteria. Increase in SCFA also produced a decrease in ammonia concentration, which is associated with health-promoting effects. This is the first study of gastrointestinal digestion and fermentation of pectin in a dynamic gastrointestinal simulator and, although further *in vivo* studies should be conducted, the data obtained confirmed the potential of pectin to be considered shortly as emergent prebiotics with a possible use for human consumption.

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Abbreviations used

GIT, GastroIntestinal Tract

POS, pectic-oligosaccharides

simgi®, SIMulator Gastro-Intestinal model

AC, Ascending colon

TC, Transverse colon

DC, Descending colon

Mw, Molecular weight

DM, Degree of methoxylation

SCFA, Short Chain Fatty Acid

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