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An evaluation of the prebiotic potential of microbial levans from *Erwinia* sp. 10119

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Abstract

Levan, a bacterial exopolysaccharide, has been suggested to have several biological activities, such as anti-tumour activity and lowering blood pressure. There is also interest in its potential prebiotic activity. This study investigated the fermentation profile of a levan fraction from *Erwinia* sp. 10119 (average DP =137) throughout a three-stage continuous gut model system, in which inulin HP (average DP = 40) was included as a comparison. Levan-type fructan was found to selectively stimulate the growth of *Bifidobacterium* and *Eubacterium rectale* - *Clostridium coccoides* group in all fermenter vessels, with significant ($p<0.05$) increases in the concentration of both acetate and butyrate. The increases in *Bifidobacterium* population were significantly ($p<0.05$) higher in the models treated with levan-type fructan (0.8 to1.24 log cell/mL) compared to the models treated with inulin HP (0.62-0.7 log cell/mL), indicating

Abbreviations: EPS, exopolysaccharide; DP, degree of polymerisation; inulin HP, high performance inulin; Mn, number average molecular weight; Mw, weight average molecular weight; PDI, polydispersity index; SCFA, short chain fatty acid; FOS, fructooligosaccharide; SOD, superoxide dismutase; CAT, catalase; FISH, fluorescent in situ hybridization; OD, optical density; NMR, nuclear magnetic resonance; BCA, bicinchoninic acid; BSA, bovine serum albumin; SS1, steady state 1; SS2, steady state 2; DAPI , 6-diamidino-2-phenylindole dihydrochloride; TSP, 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt; MW, molecular weight.

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a stronger bifidogenic effect of levan-type fructan and a prolonged persistence in the colon due to its higher DP.

Key words: exopolysaccharide (EPS), short chain fatty acid (SCFA), gut model, gut microbiota.

1. Introduction

A dietary prebiotic is "a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al 2010). Various potential beneficial effects of prebiotics have been studied including, control of intestinal transit time and bowel habit, and reduction of risk of atherosclerosis, osteoporosis, obesity, type-2 diabetes, cancer, infections and allergies (Laparra and Sanz, 2010). The establishment of a prebiotic effect requires appropriate nutritional feeding trials with defined health outcomes (Gibson et al., 2010). Short chain fatty acids (SCFAs), the principle end products of microbial metabolism, can activate G-coupled-receptors, inhibit histone deacetylases, and serve as energy sources, hence affect various physiological processes and may contribute to health and disease (Koh et al., 2016). Human studies, however, are not suitable for evaluating the impact on the metabolism of the microbiota as SCFAs are largely absorbed from the colon (Verbeke et al., 2015). To obtain data on faecal metabolism, *in vitro* gut models can be appropriate model systems.

Many common diseases associated with the human large intestine, such as colon cancer and ulcerative colitis, arise in the distal colon where proteolytic fermentation predominates and potentially toxic metabolites e.g. ammonia, hydrogen sulphide, and cresol are produced

(Andriamihaja, et al., 2015; De Preter et al., 2007; Hughes, et al., 2008; Gibson, 2004; McBurney et al., 1987; Ijssennagger, et al., 2016; Oliphant & Allen-Vercoe, 2019). Consequently, there is interest in designing novel prebiotics targeting the distal region of the colon in order to reduce the products of protein metabolism and to increase saccharolytic activity which results in short chain fatty acids. As suggested by a number of studies comparing the fermentation of FOS and inulin in continuous *in vitro* model systems (Rumessen et al., 1990; Van de Wiele et al., 2007) and *in vivo* animal or human trials (Costabile et al., 2010; Patterson et al., 2010; Tuohy et al., 2001), FOS (low DP) are rapidly fermented in the proximal colon, while inulin (high DP) appears to have a more sustainable fermentation through the gut and hence may provide more functional effects in the distal colon. It is clear that there is a positive relationship between DP and the persistence of prebiotics in the gut.

Inulin and levan are two main types of fructans. Inulin-type fructan consists of linear (2-1) linked β-D-fructosyl units attached to the fructosyl moiety of sucrose, with DP ranging from 2 to 60. Inulin has been widely used as a prebiotic, fat replacer, sugar replacer, and texture modifier due to its versatile physicochemical properties and beneficial role in gastric health (Shoaib et al., 2016). Industrial production of inulin has been achieved by extraction from inulin containing plants, such as Jerusalem artichoke and chicory, whereas inulin from microbial sources are less well studied (Ahmed and Rashid, 2017). Levan-type fructan is linked by β (2-6) linkages with occasional β (2-1) branching. Compared with inulin, only a small amount of levan is synthesised in plants by the action of sucrose:sucrose 6 fructosyltransferases (6-SST) and some other fructosyltransferases with relatively small chains (DP <10 to 100) (Öner et al., 2016). Bacterial levans on the contrary, are produced from sucrose by transfructosylation with levansucrase, and many are in the range of DP 5000

to 50000 depending on the bacteria and culture conditions (Ortiz-Soto et al., 2019). Levan has been marketed in both Korea and Japan, with several claimed health benefits, for example, inhibiting hyperglycaemia and oxidative stress induced by diabetes (Dahech et al., 2011), exhibiting anti-tumour activity against typical tumour cell lines (Abdel-Fattah et al., 2012; Calazans et al., 2000; Esawy et al., 2013; Yoon et al., 2004) and increasing superoxide dismutase (SOD) and catalase (CAT) in the heart (Abdel-Fattah et al., 2012; Dahech et al., 2013). However, these health claims need to be substantiated by more reliable *in vivo* studies. In addition to these potential activities, bacterial strains of several genera were shown to grow on levan or levan-derived FOS, i.e. *Bacteroides* (Adamberg et al., 2014; Sonnenburg et al., 2010), *Lactobacillus* (Martel et al., 2010; Yong et al., 2007), *Bifidobacterium* (Porras-Dominguez et al., 2014). Several levan degrading enzymes have also been identified in gut microbes. A fructofuranosidase from *Bifidobacterium longum* subsp. Infantis was found acting on structurally diverse fructans (Ávila-Fernández et al., 2016). An endo-levanase (BT1760) from *Bacteroides thetaiotaomicron,* an abundant commensal gut bacterium, has been biochemically studied (Mardo et al., 2017; Sonnenburg et al., 2010). More recently, the crystal structure of BT1760 was presented by Ernits et al. (2019). The prebiotic potential of levan-type fructans has been studied by some researchers mainly using *in vitro* model systems or animals (Adamberg et al., 2018; Bello et al., 2001; Hamdy et al., 2018; Kang et al., 2000; Marx et al., 2000). However, results have not been consistent due to the use of different model systems and the various origins of levan-type fructans used in these studies. Adamberg et al (2018) documented that the growth of *Collinsella* (Actinobacteria) was enhanced at cultivation of faecal inocula on levans. However, utilisation of levans by bacteria belong to this genus can be hardly evaluated as the majority of this genus are currently uncultured (Almedida et al., 2019). On the other hand, there could be a numerous population of uncultivated gut bacteria that may respond to presence of levan-type fructan. Therefore,

the shifts in gut microbe composition brought by supplementation of levan-type fructans remain to be fully revealed.

In order to evaluate the fermentation profile and gut persistence of long-chain levan-type fructan, this study was carried out using a pH controlled, three-stage continuous gut model mimicking the different regions of the human large intestine. The effects on growth and activity of gut microbiota were analysed by fluorescent *in situ* hybridization (FISH) and high performance liquid chromatography (HPLC).

2. Materials and Methods

2.1 Fermentation, fractionation and purification of levan

2.1.1 Fermentation

Media suitable for levan production were prepared by adding 23 % w/v sucrose into 100 mL Nutrient Broth No.2 (Oxoid) in baffled flasks (Sigma), followed by autoclaving at 121°C for 15 min. *Erwinia* sp.10119, isolated from cherry tree gum, was purchased from the National Collections of Industrial, Marine and Food Bacteria (NCIMB). A single colony of *Erwinia* sp. 10119 from a 48 h pre-inoculated agar plate was incubated in 30 mL Sterilin bottles containing 10 mL Nutrient Broth overnight. This pre-incubated cell suspension was then added into 100 mL levan producing media to achieve a starting optical density (OD at 600 nm) of 0.05. Flasks containing the inoculated media were incubated at 25°C on a shaking incubator at a speed of 125 rpm for 72 h.

After fermentation, the culture solution was diluted 1:3 with deionised water and centrifuged at low speed (2,991 *g*) to spin down most of the bacterial cells. After that, a probe sonicator (Soniprep 150, MSE) was used to apply ultrasound to the supernatant at 1-micron amplitude for up to 15 min to de-aggregate the mixture. This crude liquor was then centrifuged again to completely remove residual bacteria cells. The supernatant was collected and stored in 1L Duran bottles at 4°C for no more than 24 h before next treatment.

2.1.2 Levan fractionation by acid thermal hydrolysis

In order to obtain lower DP *Erwinia* levan as the final product, the crude levan-containing supernatant was subjected to acid-thermal hydrolysis. Prior to hydrolysis, the supernatant was rapidly heated to 70°C by a steamer to minimise uneven heating throughout the container. Acid-thermal hydrolysis was carried out in an oven at 70°C without agitation; 0.1% v/v acetic acid was added into a 1 L Duran bottle containing 900 mL of the crude levan solution for 50 min before being stopped by addition of 2M NaOH. The crude hydrolysis liquor was cooled to room temperature and stored at 4°C for no more than 2 days before it was passed through a membrane filtration process.

2.1.3 Levan purification by membrane filtration

Ultrafiltration was carried out using a high pressure test unit (Osmonic Desal, Le Mee sur Saine, France). The unit consisted of a feed tank of 4 L capacity, a piston pump and two stainless steel flow cells with a stainless porous sheet membrane support. These cross flow cells were connected in parallel. Two flat sheet asymmetric thin film composite membranes (GE, Osmonic Desal, Gilson Scientific, Luton, UK) with MWCO of 10 kDa were used.

Membranes were cut into circular forms with an area of 81cm^2. Each new membrane was immersed overnight in 2 L of deionised water to remove any preservatives prior to use.

Non-continuous diafiltration by volume reduction was employed, whereby the working feed volume of 3.0 L was concentrated to 1.5 L by removing the permeate, while recycling the retentate to the feet tank at 300 psi pressure. The feed was diluted back to its initial volume with deionised water and the diafiltration was repeated three times to remove more of the low molecular weight material. The purified liquor was then concentrated to 500 mL as further concentration results in insufficient circulation. The cumulative retentate was then precipitated with 3 volumes of ethanol in 250 mL Sterilin bottles and left to precipitate at 4°C overnight. After removal of ethanol, the precipitates were dried in a biosafety cabinet, reconstituted with deionised water, then store at – 80°C for 24 h and freeze dried at -55°C for 48 h with a VirTis Bench Top freeze dryer (VirTis Sentry 2.0, SP Scientific, Ipswich, UK), and stored in an airtight container at room temperature for further analysis.

2.2 Analysis of levan

NMR was used to confirm the structure of levan by comparing the ^{1}H NMR and ^{13}C NMR spectra with that of levan purchased from Sigma (extracted from *Erwinia herbicola*). NMR samples were prepared by dissolving freeze dried levan in D_2O (20 mg/mL m/v) and then analysed by Bruker Nanobay 400MHz NMR spectroscopy. One-dimensional ^{1}H NMR spectra were obtained by applying a zg30 pulse sequence at 400MHz with a 3.9584 sec acquisition time, 1 sec relaxation delay, 8278 Hz spectral width, and 296.2 K temperature. Internal solvent D_2O was used as chemical shift reference (δ = 4.79). One-dimensional ^{13}C NMR spectra were obtained by applying a zgpg30 pulse sequence with a 1.3665 sec acquisition time, 1.5 sec relaxation delay, 23980 Hz spectral width, and 295.2 K temperature.

160 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP) was used as chemical shift
161 reference ($\delta = 0.00$). Analysis of the protein content in the final levan product was carried out
162 by BCA (bicinchoninic acid) protein assay kit (Sigma). Bovine serum albumin (BSA)
163 solutions (50-500 μg/mL) were used as standards. The working reagent was prepared by
164 mixing 100 mL of reagent A (a solution containing bicinchoninic acid, sodium carbonate,
165 sodium tartrate and sodium bicarbonate in 0.1N NaOH, pH 11.25) with 2mL of Reagent B
166 (4% (w/v) $CuSO_4 \cdot 5H_2O$). 2 mL of the working reagent were added to each Eppendorf
167 containing 0.1 mL of BSA standards or freeze dried levan sample (10g/L). After that, these
168 mixed solutions were incubated at 37 ºC for 30 mins. After incubation, the absorbance was
169 read at 562 nm. The apparent molar mass was determined by HPLC-RI (Agilent 1100 series,
170 Winnersh, UK) using a PL aquagel-OH MIXED-H 8μm size exclusion column (Varian,
171 INC., England) before and after hydrolysis. The column temperature was 30℃ and HPLC
172 grade water was used as mobile phase at 0.4 mL/min. Sucrose，glucose，FOS，inulin ST
173 and Dextrans (50-1400kDa) were used as external standards. Size exclusion chromatography
174 was also used to determine retention of desired levan fraction after ultrafiltration.

175

176 2.3 Three-stage continuous culture system

177 A scaled-down version of the three-stage continuous culture simulation of the human colon
178 (Macfarlane et al., 1998) was used to investigate the effect of a hydrolysed levan fraction
179 from *Erwinia* sp. 10119, on the faecal microbiota. The scaled down system was run at the
180 same dilution rate (i.e., a rate at which fresh medium was added) to the conventional gut
181 model when operating at a retention time of 48 h (flow rate of 6.25ml/h). The gut model
182 system includes three glass fermenters simulating conditions in the proximal colon (Vessel 1,
183 80 mL, pH 5.5), transverse (Vessel 2, 100 mL, pH 6.2) and distal colon (Vessel 3, 120 mL

pH 6.8) and was fed with a complex medium through a peristaltic pump (Watson-Marlow, Cornwall, UK). The intervention dose used in this study (3 g/day) was selected based on previous human studies (Costabile et al., 2010; Tuohy et al., 2001), 8 g/day and 10 g/day respectively. An average of 9g/day (provides 18 kcal of energy per day) was selected, however, as a scaled-down model was used, containing one-third of the normal medium volume, a value of 3g/day was added to the system (Macfarlane et al., 1998). The entire system, including the medium reservoir, was constantly stirred and maintained in an anaerobic condition by continuously sparging with nitrogen. The pH of each vessel was automatically adjusted using pH controllers (Fermac 260; Electrolab, Tewkesbury, UK) by adding 0.5 N HCL and 0.5 N NaOH. The temperature of the culture was maintained at 37 °C by a circulating water bath. Sterile vessels were filled with pre-sterilized medium containing (per litre): 5 g starch, 5 g peptone water, 5 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 KCl, 4 g mucin, 3 g casein, 2 g xylan, 2 g arabinogalactan, 1.5 g $NaHCO_3$, 1.25 g $MgSO_4 \cdot 7H_2O$, 1 g guar gum, 1 g inulin (Orafti® ST, Beneo, Tienen, Belgium), 0.8 g cysteine-HCl, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts, 0.15 g $CaCl_2 \cdot 6H_2O$, 0.005 g $FeSO_4$, 0.05 g haemin, 1 mL Tween 80, 0.01 mL vitamin K and 4 mL resazurin solution (0.25 g/L) as a redox indicator.

Vessels were inoculated with freshly prepared faecal slurries (20% w/w in PBS). The faecal slurries were prepared in strainer stomacher bags (Seward, UK) to remove large particles and were homogenised in a stomacher (Stomacher 400; Seward, West Sussex, UK) for 2 min at medium speed. Faecal samples from three healthy adults were used, each of them was used to inoculate two gut models (one for levan and one for inulin HP). Volunteer selection was based on three criteria, i.e., generally healthy without any current medication; should not

have taken any antibiotics or pro/pre-biotic tablets over the last 6 months, should not be a frequent consumer of pro/prebiotic containing food or beverages.

For each donor, two gut models were inoculated in parallel, 28.6 mL (V1), 33.3 mL (V2), and 37.5 mL (V3) were inoculated into culture medium (51.4 mL (V1), 66.7 mL (V2), 82.5 mL (V3)). The systems were run as batch cultures for the first 24 h after inoculation to stabilise the bacterial populations. After this, all the vessels were connected and the medium flow was initiated until eight full volume turnovers (16 days) were completed (steady state 1, SS1). 5 mL of sample were taken from each vessel on 3 consecutive days for analysis of bacterial populations and SCFA accumulation. Once stable organic acid profiles were obtained, 3 g of hydrolysed levan or inulin HP (Orafti® HP, Beneo, Tienen, Belgium) were added into vessel 1 of the respective gut models on a daily basis for another eight turnovers (16 days), until stable organic acid profiles were observed from samples taken on 3 consecutive days (steady state 2, SS2).

2.4 Bacterial enumeration

Bacterial groups were enumerated using fluorescent *in situ* hybridization (FISH) with 16S rRNA oligonucleotide probes (Table 1). The probes were labelled with the fluorescent Cy3 dye as described by Sarbini et al. (2011). Samples (375 mL) were fixed for 4 h at 4° C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:4 (v/v), washed twice with filtered PBS (0.22 μm pore size), and resuspended in 300 mL of a PBS–ethanol mixture (1:1, v/v). Prior to hybridization, samples were diluted to appropriate concentration and 20 μl of each sample were pipetted onto Teflon- and poly-L-lysine-coated, six-well (10 mm diameter each) slides (Tekdon Inc., Myakka City, FL, USA). In order to make the cells permeable to

the hybridization buffer, the slides were dried in a bench top oven at 50°C for 15 min before being finally dehydrated in an ethanol series (50 %, 80 % and 96 % (v/v) ethanol, 3 min each). Fifty microliters of hybridization buffer (containing 5 ng probe /mL) were applied onto the surface of each well. Hybridizations were performed for 4 h in an ISO20 oven (Grant Boekel, Cambridge, UK). Hybridization temperatures for each probe are listed in Table 1. For the washing step, slides were placed in 50 mL of pre-warmed wash buffer containing 20 µl of 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 ng/mL; Sigma, St Louis, MO, USA) for 15 min. They were then washed (2–3 s) in ice-cold water and dried under a stream of compressed air. After that, 5 µl of antifade reagent (polyvinyl alcohol mounting medium with DABCOe antifade, Sigma) were added to each well and a coverslip was applied. Slides were stored in the dark at 4º C until cells were counted under a Nikon E400 Eclipse microscope (Nikon, Kingston upon Thames Surrey, UK). Slides were counted within a week, but could be kept for up to three months due to the use of antifading agents. DAPI slides were visualised with the aid of a DM 400 filter and probe slides with the aid of a DM 575 filter. Fifteen fields of view were geometrically picked and counted. The average counts were calculated and used for statistical analysis.

2.5 Short chain fatty acid (SCFA) analysis

A sample (1 mL) from the gut model vessels was dispensed into 1.5 mL Eppendorf tubes and centrifuged at 13 000 x g for 10 min to sediment bacteria and other solids. Supernatants were filtered using 0.2 µm polycarbonate syringe filters (Whatman International Ltd, Maidstone, Kent, UK) and injected with internal standard (diethylbutyric acid, Sigma) at a ratio of 4:1 into an HPLC system (Merck, Whitehouse Station, NJ, USA) equipped with refractive index (RI) detection. The column used was an ion-exclusion REZEX ROA organic acid column

(Phenomenex, Inc., Macclesfield, Cheshire, UK) maintained at 85°C. Sulfuric acid in HPLC-grade water (0.0025M) was used as the eluent and the flow rate was maintained at 0.5 mL/min. The carboxylic acids in the samples were quantified using calibration curves of acetic, propionic, butyric, valeric and formic acid, in concentrations ranging between 2.5 and 100 mM.

Table 1 Oligonucleotide probes and hybridisation conditions used in this study

Probe name	Sequence (5′ to 3′)	Bacterial groups enumerated	Hybridization pre-treatment	Formamide (%) in hybridization buffer	Temperature (°C)		Reference
					Hybridization	Washing	
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* group	None	0	50	50	Harmsen et al. (2000)
Bac303	CCAATGTGGGGGACCTT	*Bacteroides/ Prevotella*	None	0	46	48	Manz et al. (1996)
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	None	0	50	50	Langendijk et al. (1995)
Chis150	TTATGCGGTATTAATCTYCCTTT	*Clostridium histolyticum* group	None	0	50	50	Franks et al. (1998)
Prop853	ATTGCGTTAACTCCGGCAC	*Clostrium* cluster IX	None	0	50	50	Walker et al. (2005)
Erec482	GCTTCTTAGTCARGTACCG	*Eubacterium rectale-Clostridium coccoides* group	None	0	50	50	Franks et al. (1998)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus-Enterococcus* spp.	Lysozyme	0	50	50	Harmsen et al. (1999)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* spp.	None	0	50	50	Walker et al. (2005)
Frpau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* group	None	0	58	58	Hold et al. (2003)
EUB338‡	GCTGCCTCCCGTAGGAGT	Total bacteria	None	35	46	48	Daims et al. (1999)
EUB338II‡	GCAGCCACCCGTAGGTGT	(mixed EUB338 probes)	None	35	46	48	Daims et al. (1999)
EUB338III‡	GCTGCCACCCGTAGGTGT		None	35	46	48	Daims et al. (1999)

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2.6 Statistical analysis

Statistical analysis was performed using SPSS for Windows, Version 18.0. A paired independent t-test was used to determine significant changes for each bacterial group and SCFA concentration between steady state 1 (SS1) of faecal fermentation without adding test substrate and steady state 2 (SS2) after adding the test substrate. One-way analysis of variance (ANOVA) and Tukey's posthoc test were used to determine significant differences in bacterial group populations and SCFA concentrations among the different volunteers. Differences were considered to be significant when $p<0.05$.

3. Results and Discussion

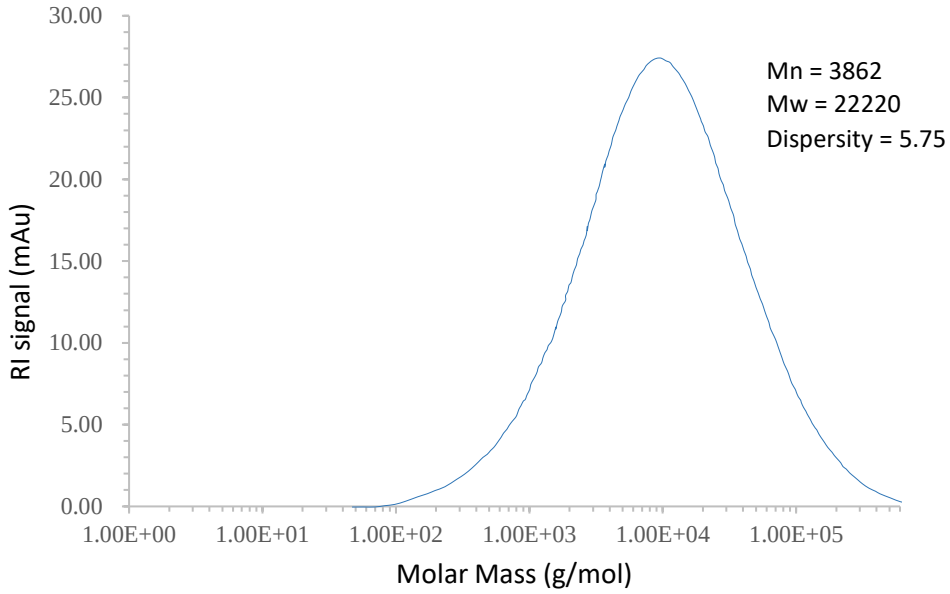
3.1 Levan characterization

Comparison of the chemical shifts in ^{13}C NMR and ^{1}H NMR spectrum of levan produced in this experiment with that for Sigma levan reveals the [→6)-β-D-Fru*f*-(2→]n main chain structure of produced levan. Chemical shifts were shown in Table 2. Residual protein was non-detectable in the final product. The result of size exclusion chromatography showed that the molecular mass of natural levan produced by *Erwinia* sp. 10119 was about DP 7719 (1.3 x 10^6 Daltons), which is comparable to the previous reported molecular range weight of levan produced from Erwinia herbicola (1.1 x 10^6-1.6 x $l0^6$ Daltons) (Keith et al., 1991). After acid-thermal hydrolysis, the molecular mass of resulting levan fraction was examined again by size exclusion chromatography. The molecular weight distribution of obtained levan fraction was illustrated in Fig.1. The hydrolysed levan fraction had a weight average molecular weight (Mw) of approximately 22220 g mol^{-1} (DP = 137) and a dispersity of 5.75. Membrane processing resulted in a moderate DP levan fraction with 15 % (w/w) of mono- and oligo-saccharides. The results are expressed as mass percentages of the compounds left after

ultrafiltration based on GPC area measurements. Overall, the type of linkage, DP and purity of levan fractions produced by the present used method, consisting of centrifugation, sonication, acid-thermal hydrolysis and ethanol precipitation were found satisfactory, and these factions could be subjected to the three-stage gut model for further evaluation of their prebiotic potential and gut persistency.

Table 2 Comparison of ^{1}H and ^{13}C NMR data of lavean produced in this study with Sigma Levan (*Erwinia herbicola*) and chemical shifts data published for levan from *Bacillus* sp. 3B6 and *Zymomonas mobilis* (Angeli et al., 2009, Matulová et al., 2011)

Compound		^{1}H and/or ^{13}C chemical shifts /δ						
		1, 1'	2	3	4	5	6, 6'	
Levan (*Erwinia* sp. 10119)	H	3.644	3.570	-	4.066	3.982	3.836	3.775 3.437
	C	59.79	103.79	76.19	75.11	80.24	63.33	
Levan (Sigma-*Erwinia herbicola*)	H	3.645	3.571	-	4.067	3.984	3.838	3.775 3.440
	C	59.81	103.78	76.21	75.13	80.24	63.33	
Levan (*Bacillus* sp. 3B6*)*	H	3.77	3.670	-	4.178	4.096	3.958	3.898 3.549
	C	60.75	105	77.15	76	81.07	64.2	
Levan (*Zymomonas mobilis*)	H	3.8	3.7	-	4.3	4.2	4.0	3.85 3.55
	C	61.4	105.1	77.5	76.6	81.3	64.6	



296

297 **Fig.1. Molecular weight distribution of hydrolysed levan analysed by Size exclusion**
298 **chromatography (n=3).** Mn, number average molecular weight; Mw, weight average molecular weight;
299 Dispersity= Mw/Mn.

300

301 3.2 Changes in microbiota composition upon inulin and levan supplementation

302 The work presented here is the first to evaluate the fermentation selectivity of levan-type
303 fructan in a continuous gut model system. Fig. 2 shows the bacterial concentrations before
304 (SS1) and after (SS2) the addition of test substrates. The mean values for each probe are
305 averages of three volunteers for each substrate (inulin HP and levan-type fructan) in each
306 vessel (V1, V2 and V3) representing different regions of the colon, i.e. proximal, transverse
307 and distal colon. Significant stimulation ($p<0.05$) of *Bifidobacterium* was observed for both
308 test substrates in all vessels, which is in line with results from Tuohy et al. (2001), who
309 reported a stimulation of *Bifidobacterium* after an *in vivo* inulin HP intervention (8g/ day). In
310 comparison, using a five-stage *in vitro* continuous Simulator of the Human Intestinal
311 Microbial Ecosystem (SHIME), Van de Wiele et al. (2004) only observed an increase of
312 *Bifidobacterium* in vessel 1 (proximal colon) when natural chicory inulin (average DP <10)

313 was used as a supplement at a dose equal to 5 g/day. Furthermore, the population of
314 *Bifidobacterium* was significantly higher in all three vessels in the models treated with levan
315 than with inulin HP, exhibiting a stronger bifidogenic effect of levan. This is supported by
316 Ávila-Fernández et al. (2016), who fund that a β-fructofuranosidase from *Bifidobacterium*
317 *longum* subsp. *infantis* ATCC 15697 has higher affinity for levan-type than inulin-type FOS.
318 No significant changes were observed in population of *Lactobacillus-Enterococcus* for both
319 substrates. This is in line with the results of Tuohy et al. (2002) and Harmsen et al. (2002), in
320 which this groups of bacteria were unaffected by supplementation with inulin HP.

321

322 A decrease in *Bacteroides - Prevotella* (ranging from 0.1 to 0.86 log cells/mL) was observed
323 when inulin HP was added, while there were no significant changes in the levan models.
324 Harmsen et al., (2002) has reported similar results in a human study on inulin in which 10
325 healthy volunteers were given inulin HP for 14 days at a dose of 9 g/day. Inulin HP did not
326 have any significant stimulation effect on the *Bacteroides – Prevotella* group, and a decrease
327 of 0.6 log cells/mL has been observed during the treatment. The decrease in number of
328 *Bacteroides* was also reported by two other studies evaluating the prebiotic potential of high
329 molecular weight inulin by human studies (Costabile et al. 2010; Tuohy et al. 2002).
330 Moreover, Sonnenburg et al. (2010) showed that many studied *Bacteroides* species (for
331 example *B. thetaiotaomicron* and *B. vulgatus*) did not grow on inulin. (Sonnenburg et al.,
332 2010).

333

334 Varied responses to inulin and levan were found for different group of Clostridium, including
335 commensal (*Eubacterium rectale- Clostridium coccoides* groups, *Clostridium* cluster IX,
336 *Roseburia spp.* and *Faecalibacterium prausnitzii)* and pathogenic species (*Clostridium*

337 *histolyticum*). Significant stimulation ($p<0.05$) of *Eubacterium rectale- Clostridium*
338 *coccoides* groups was observed for both test substrates in all vessels, and populations of this
339 group of bacteria were significantly higher in all three vessels in the models treated with
340 levan than with inulin HP. Significant increase in population of *Roseburia* was observed in
341 all vessels treated with both substrates. A different result has been reported by Ramirez-
342 Farias et al. (2008) that no significant increase was found in number of *Roseburia* after inulin
343 supplementation as a mean of all volunteers. However, they also documented a strong
344 increase upon inulin ingestion of two volunteers and suggested a possible variance between
345 volunteers in which different strain/species of *Roseburia* were present (Ramirez-Farias et al.,
346 2008). This assumption was then supported by Sheridan et al. (2016), who found that all
347 tested *R. inulinivorans* strains were capable of utilizing inulin, whereas *R. intestinalis*, *R.*
348 *hominis* and *R. faecis* strains did not grow on inulin as the sole carbohydrate source (Sheridan
349 et al., 2016). *Clostridium* cluster IX also showed a significant decrease (ranging from 0.34 to
350 0.73 log cells/mL) in all vessels treated with both substrates. A similar finding has been
351 reported by Van de Wiele et al. (2006), in which the numbers of clostridia decreased after
352 intervention with inulin HP using SHIME model at a dose of 2.5 g/day. The population of
353 *Faecalibacterium prausnitzii*, significantly decreased in the models with added inulin HP in
354 all three vessels (0.57 log cells/mL decrease in V1, 0.44 log cells/mL decrease in V2 and 0.3
355 log cells/mL decrease in V3), while the addition of levan-type fructan led to an increase in
356 vessel 2 (0.25 log cells/mL), after a decrease in vessel 1 (0.61 log cells/mL), also suggesting a
357 transit-dependent effect. In contrast to the present study, many researchers have documented
358 stimulative effects of inulin on the population of *F. prausnitzii* (Lopez-Siles et al., 2017;
359 Ramirez-Farias et al., 2008). Besiders, Kleessen et al. (2007) reported that no significant
360 change was observed in *F. prausnitzii* counts in volunteers consumed snack bars containing
361 inulin (7.7 g/d) after a 7-d run-in period. However, Moens and De Vuyst (2017) studied the

362 utilisation of inulin by *F. prausnitzii* in pure cultures and suggested that *F. prausnitzii* could
363 degrade both FOS and inulin. Moreover, Moen et al. (2016) documented that a cross-feeding
364 interactions between bifidobacteria and acetate-depending, butyrate-producing *F. prausnitzii*
365 was observed and can be either a beneficial relationship or dominated by competition,
366 depending on the inulin degradation capacities of the bifidobacterial strains involved.
367 Therefore, decrease in number of *F. prausnitzii* seen in the present study could be possibly
368 explained by the competition between *F. prausnitzii* and other dominating gut microbes.
369 However, this will have to be confirmed in other intervention studies. In addition, no
370 significant changes were observed for *Clostridium histolyticum.* This is in line with the
371 results of Tuohy et al. (2002) and Harmsen et al. (2002), in which this groups of bacteria
372 were unaffected by supplementation with inulin HP.

373

374 For the *Atopobium* cluster, with addition of inulin HP, no significant increase was observed in
375 the first two vessels, but there was a 0.31 log cells/mL increase in vessel 3, suggesting a
376 stimulation effect in the distal colon. This cluster of bacteria is a dominant member of the
377 faecal microbiota of healthy humans, which making up around 8 % of the microbiota
378 (Thorasin et al., 2015). The result found in the present study is in good agreement with
379 Costabile et al. (2010), who also reported a significant increase of *Atopobium* after 14 days
380 supplementation with inulin. However, no significant difference was obtained with addition
381 of levan-type fructan across all three vessels.

382

383 Briefly, both inulin and levan contributed to beneficial shifts towards the gut microbial
384 composition, indicated by significant increase in population of *Bifidobacterium* spp. Such
385 bifidogenic effect is widely accepted as beneficial to the host health due to the carbohydrate-

386 fermenting pattern of bacteria strains belong to the genus *Bifidobacterium* (Meyer and Stasse-
387 Wolthuis, 2009). Also, a stronger bifidogenic effect of levan-type fructan has been observed.
388 Various groups of commensal bacteria were enumerated and some of them showed different
389 responses between the two types of fructans. The main differences were among the changes
390 in the populations of *the Bacteroides-Prevotella* group, *Atopobium* cluster *and F prausnitzii*,
391 due to their capacities of using different fructans in the complex gut ecosystem. In addition,
392 no stimulation of pathogenic clostridia (*Clostridium histolyticum*) was observed for both
393 substrates, indicating that no adverse effect on the host health was induced by inulin and
394 levan supplementation in terms of their effects on *Clostridium histolyticum*.

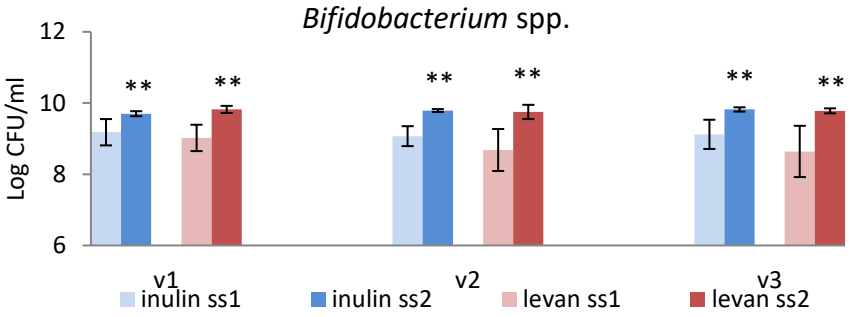
395

396 Compared with inulin, the effect of levan-type fructans on the human gut microbiota has
397 hitherto only been studied using batch cultures. Marx et al. (2000) tested the abilities of
398 various bacterial genera to ferment long chain levan and levan oligosaccharides in pure
399 cultures, including several strains of *Bifidobacterium* and found that only levan
400 oligosaccharides demonstrated an enrichment effect on the tested strains (*B. adolescentis, B.*
401 *breve, B. longum* and *B. pseudocatenulatum*). In another study, the *in vitro* fermentation
402 properties of a commercial levan from Sigma (originating from *Erwinia herbicola*) and two
403 self-isolated levan-type exopolysaccharides (originating from *Lactobacillus sanfranciscensis*)
404 were studied using human faeces as an inoculum (Bello et al., 2001). An enrichment of
405 *Bifidobacterium* species was found with the levan type exopolysaccharides produced by
406 *Lactobacillus sanfranciscensis*, but not for levan from *Erwinia herbicola*. Levan synthesized
407 using levansucrase from *Pseudomonas syringae was* found to act as an easily degradable
408 substrate for *Bacteroides thetaiotaomicron* when tested with pure culture (Adamberg et al.,
409 2014). Mardo et al. (2017) reported that the endo-levanase from *B. thetaiotaomicron* can
410 degrade various β-2, 6-linked polyfructan levans. They also suggested that the long chain

levan molecules were degraded into FOS with a cell surface bound endo-levanase BT1760, and the FOS was then consumed by *B. thetaiotaomicron* and other gut bacteria, including health-promoting bifidobacteria and lactobacilli (Mardo et al., 2017). Such cross-feeding effect was previously documented by Rakoff-Nahoum et al. (2014), who reported that fructose and oligosaccharides liberated from levan metabolized by *B. thetaiotaomicron* could support the growth of Bacteroidales species without levan utilising ability. Crystal structure of the BT1760 supported this assumption once again (Ernits et al., 2019). Hamdy et al. (2018) investigated the prebiotic activity of *Bacillus* levan in rat feeding trails. They found that levans produced by two strains of *Bacillus subtilis* (HMNig-2 and MENO2) both have the ability of lowing coliform count and increasing lactobacillus count in gut especially when used together which the probiotic strain *B. subtilis* HMNig-2 and MENO2. More recently, both natural levan from *Erwinia herbicola*, and a low molecular weight (8 kDa), highly branched levan from *Bacillus amyloliquefaciens* JN4 were shown to exhibit antiadhesive activity against enterotoxigenic *Escherichia coli*, whereas such activity was not found with chicory inulin (Cai et al., 2019) The fermentation of levanheptaose on some components of the intestinal microbiota was studied by Kang et al (2000) using pure cultures. Growth of *Bifidobacterium adolescentis*, *Lactobacillus acidophilus* and *Eubacterium limosum* were stimulated, whereas numbers of *Clostridium perfringens*, *E. coli* and *Staphylococcus aureus* remain unchanged compared with control cultures. Kang et al (2000) also studied the effect of levanheptaose on the gut microbiota of rats. The ingestion of levanheptaose resulted in a 1 log cell/ mL increase in the faecal counts of endogenous bifidobacteria, without affecting *Lactobacillus* sp. The amount of butyrate as well as β-fructosidase activity were increased, whereas pH was reduced in rats fed levanheptaose diets as compared to those on the control diet (Kang et al., 2000).

436 The molecular weight of a fructan has an effect on the fermentation behaviour (Biedrzycka
437 and Bielecka, 2004). The results of *in vitro* studies using pure cultures indicate the specificity
438 for *Bifidobacterium* spp., with the exception of *B. bifidum*, when utilising short chain
439 fructooligosaccharides and inulins of average DP over 9, but not highly polymerized inulins
440 (average DP = 40). The results of subsequent *in vivo* studies on rats also suggested the
441 selectivity of fructooligosaccharides and low DP inulins, for *Bifidobacterium* spp., while the
442 effects of highly polymerized inulin were more diverse and related to the presence and ability
443 of other bacteria to initiate degradation (Biedrzycka and Bielecka, 2004). On the other hand,
444 degree of branching also has effects on the fermentation property of levan. Although this
445 effect has not been fully understood, Yoon et al. (2004) found that anti-tumour activity of
446 levan towards SNU-1 and HepG2 tumour cell lines decreased rapidly as the degree of
447 branching reduced. Also，Benigar et al. (2014) found that levan from three different origins
448 (i.e. *Bacillus subtilis, Zymomonas mobilis*, and *Erwinia herbicola*) differed in their structural
449 and dynamic properties in aqueous solutions. Their Small-Angle X-ray Scattering result
450 indicated that, in aqueous solution, *Bacillus subtilis* levan was the least entangled with the
451 most flexible structure while *Erwinia herbicola* levan was the most entangled, reflected in
452 higher solution turbidity at the same concentration (Beinigar et al., 2014). Factors affecting
453 the particle-forming property were mainly MW and branching of levan molecules.
454 Furthermore, as reported by Bello et al. (2001), a bifidogenic effect was not found with
455 natural *Erwinia herbicola* levan compared with levan from other sources, we hypothesised
456 that this particle-forming property could possibly limit the contact between digestion
457 enzymes of gut bacteria and natural levan molecules and hence restrict their bioactivity. In
458 the current study, after acid hydrolysis, turbidity of levan solution was reduced to a great
459 extent and the hydrolysed levan (DP =137) showed enhanced prebiotic potential comparing
460 with inulin HP as well as a gut persistence given by large molecular weight.

461


Bifidobacterium spp.
Log CFU/ml
12
10
8
6
**
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**
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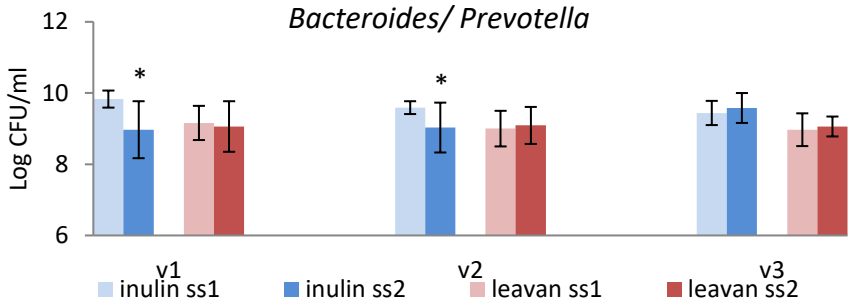
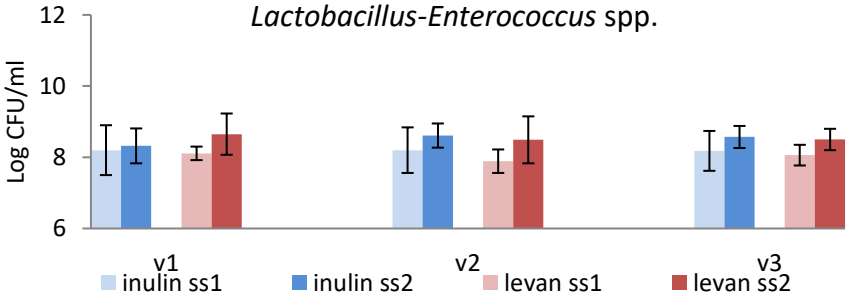
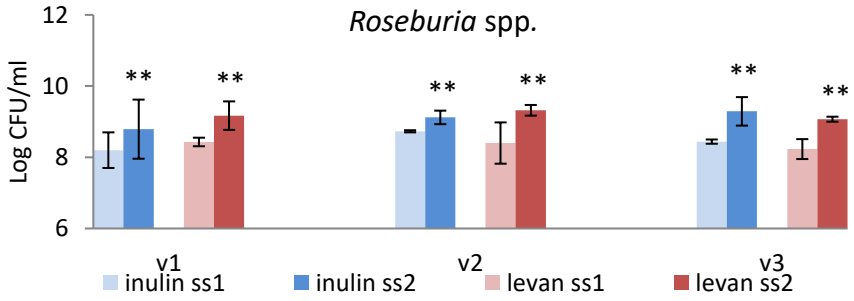
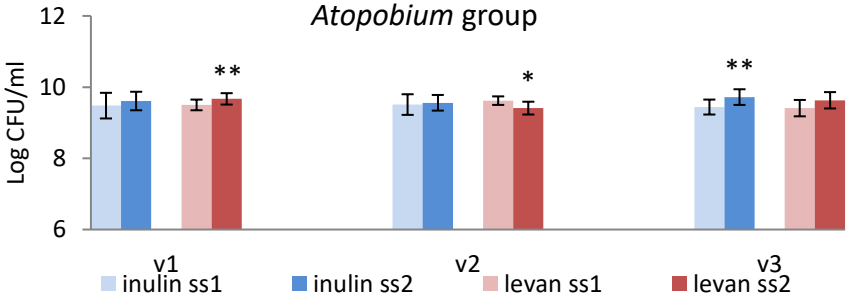
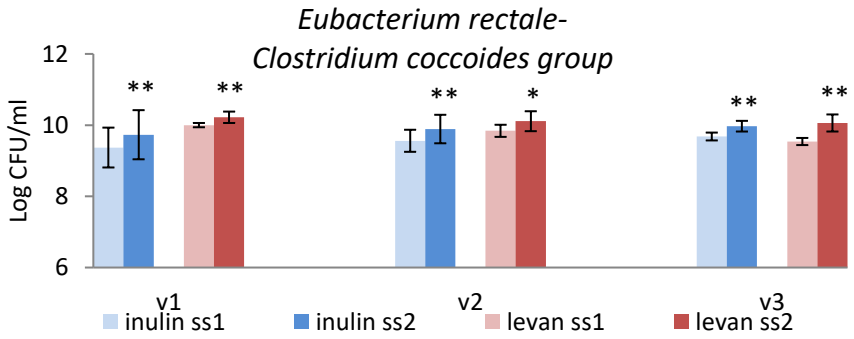
v1
v2
v3
inulin ss1
inulin ss2
levan ss1
levan ss2


Bacteroides/ Prevotella
Log CFU/ml
12
10
8
6
*
*
v1
v2
v3
inulin ss1
inulin ss2
leavan ss1
leavan ss2


Lactobacillus-Enterococcus spp.
Log CFU/ml
12
10
8
6
v1
v2
v3
inulin ss1
inulin ss2
levan ss1
levan ss2


Roseburia spp.
Log CFU/ml
12
10
8
6
**
**
**
**
**
**
v1
v2
v3
inulin ss1
inulin ss2
levan ss1
levan ss2


Atopobium group
Log CFU/ml
12
10
8
6
**
*
**
v1
v2
v3
inulin ss1
inulin ss2
levan ss1
levan ss2


Eubacterium rectale-
Clostridium coccoides group
Log CFU/ml
12
10
8
6
**
**
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*
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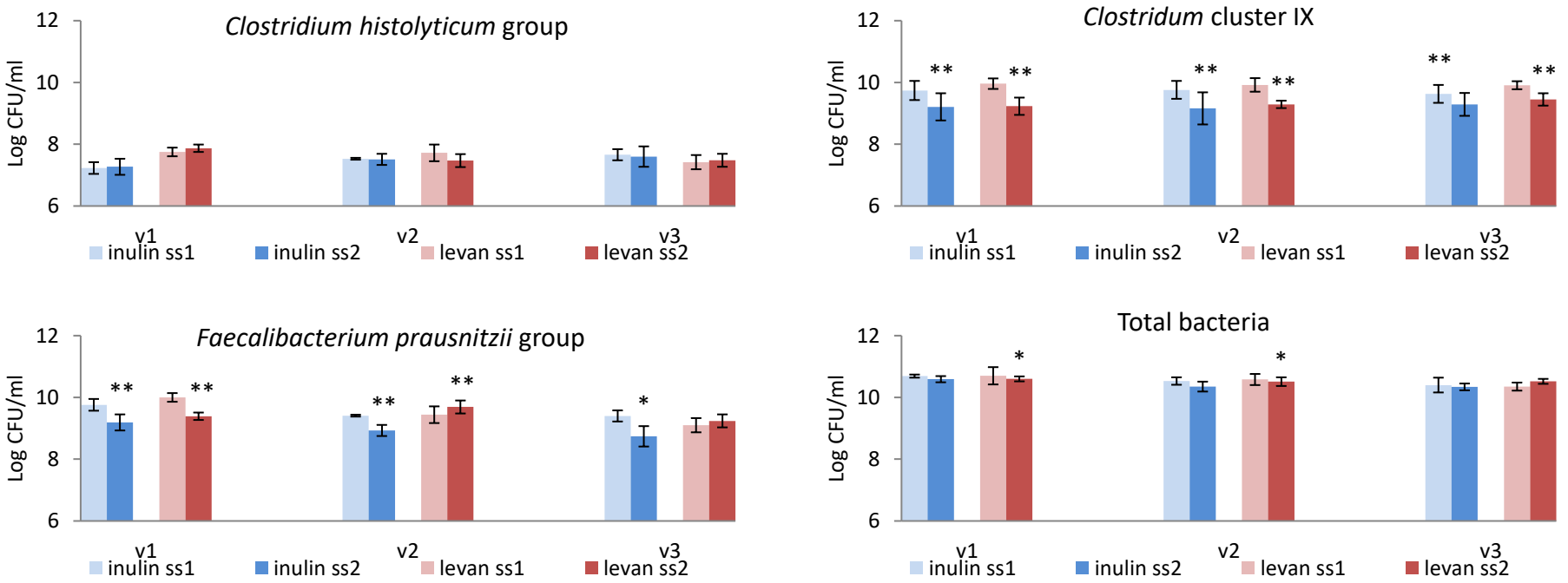
**
v1
v2
v3
inulin ss1
inulin ss2
levan ss1
levan ss2



462

463

464 **Fig. 2. Mean bacterial populations (log10 cell per mL) in each vessel of the three-stage continuous system (vessel 1, 2 and 3) at steady state 1 and steady state**
465 **2.** Standard deviation is shown in parentheses with n=3. *denoted a significant change in bacterial number in SS2 compared to SS1 at $P < 0.05$; ** denoted a
466 significant change in bacterial number in SS2 compared to SS1 at $P < 0.01$. SS1: Steady state 1, fermentation without adding test substrate; SS2: steady state 2, after
467 adding the test substrate.

3.3 Short chain fatty acid production

Changes in SCFA concentrations are shown in Fig. 3. In the gut model system, SCFA accumulate across all three vessels, which is not physiological so only changes in vessel will be discussed here. Acetate, butyrate, and propionate were the three main SCFAs accumulating during fermentation. The concentration of acetate significantly ($p<0.05$) increased in all three vessels for both test substrates ranging from 14.16 mM to 30.37 mM, while both inulin HP and levan-type fructan led to a large increase in vessel 1 (30.37mM and 29.74 mM, respectively), with no significant differences between them. This is consistent with the changes in number of *Bifidobacterium* spp., which is known as a lactate and acetate producer. Lactate was not detected in any vessel due to consumption by butyrate producing bacteria such as *Clostridium* cluster IV (e.g. *Faecalibacterium prausnitzii* group and *Eubacterium rectale- Clostridium coccoides* group) and XIVa (e.g. *Roseburia spp*.). Accordingly, the butyrate concentration also increased significantly after the intervention, both substrates giving rise to significant increases ranging from 26.03 mM to 65.41mM, in line with the increase in number of *Clostridium* cluster IV and XIVa. Enrichment in acetate and butyrate production has been reported by many inulin intervention studies (Alexander et al., 2018; Thøgersen et al.,2018; van der Beek et al., 2018). Moreover, similar result has been documented regarding levan-type fructan. Adamberg et al. (2018) found that administration of levan corrected the metabolic pattern of overweight children faecal consortium by increasing the production of butyrate and acetate. Besides, as the concentration of acetate and butyrate increased, the accumulation of propionate significantly decreased after addition of inulin HP while treatment with levan-type fructan did not affect the propionate concentration significantly. This is the main difference between SCFAs production induced by supplementation of two tested substrates and is consistent with the changes in number of *Bacteroides – Prevotella*, which is known as an acetate, lactate, and propionate producer.

493 Total organic acids also significantly increased in all vessels. There was no significant
494 difference between the test substrates.

495

496 Overall, a possible route of levan metabolism was proposed based on the experimental data
497 of the present study and information from the literature. Levans are firstly degraded by
498 *Bifidobacterium, Bacteroides,* and *Lactobacillus* to produce fructose, FOS and organic acids,
499 especially lactate and acetate. The hydrolysis products and SCFAs are then utilized by
500 butyrate-producing bacteria (such as *Eubacterium, Faealibacterium* and *Roseburia*) and other
501 nondegraders of levans with the production of butyrate and other metabolic products.

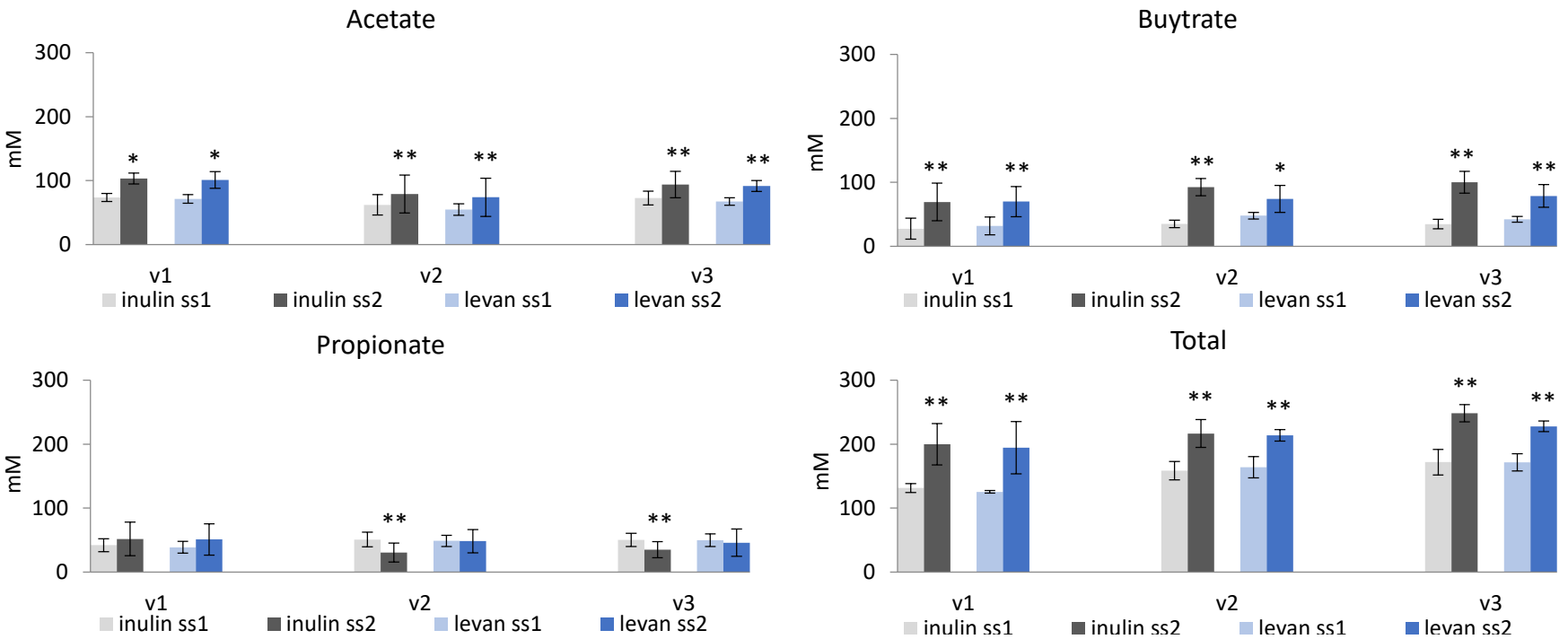


Fig. 3 Mean short chain fatty acid concentration (mM) in each vessel of the three-stage continuous system (vessel 1, 2 and 3) at steady state 1 and steady state 2, Standard deviation is shown in parentheses with n=3. *denoted a significant change in bacterial number in SS2 compared to SS1 at $P < 0.05$; ** denoted a significant change in bacterial number in SS2 compared to SS1 at $P < 0.01$. SS1: Steady state 1, fermentation without adding test substrate; SS2: steady state 2, after adding the test substrate.

4. Conclusion

The *in vitro* fermentation of a hydrolysis product of levan (average DP =137), produced from *Erwinia* sp. 10119 was investigated in pH controlled three-stage compound continuous gut model systems compared with inulin HP (average DP = 40). *Bifidobacterium* and *Eubacterium rectale - Clostridium coccoides* groups significantly increased in all vessels including the 3rd vessel simulating the distal colon. The increases were significantly higher in the models treated with levan-type fructan (0.8 to1.24 log cell/mL) compared to the inulin HP models (0.62-0.7 log cell/mL), indicating a stronger bifidogenic effect of levan-type fructan and prolonged persistence in the colon due its higher DP. Both acetate and butyrate significantly increased in all the vessels of the system, although no significant difference was observed between them. The structural differences among levan from different microorganisms have not been determined in this study. However, it has been reported by various researchers that the molecular weight and degree of branching varies among levans from different producers, which most likely results in differences in their fermentation properties. Thus, it would be valuable to evaluate these relationships in well designed *in vitro* and *in vivo* studies with the aim to develop novel prebiotics based on these polymers.

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