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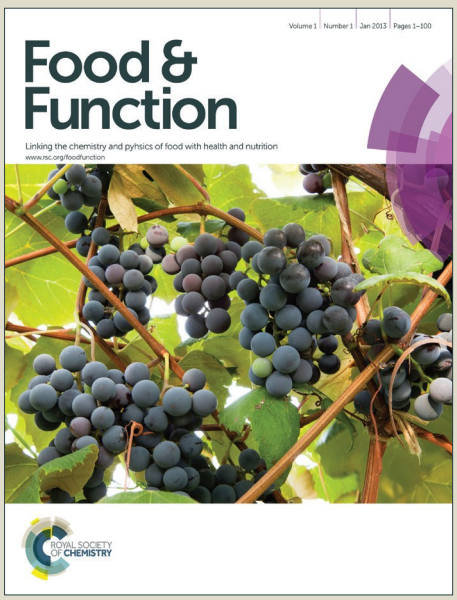

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The fate of ^{13}C-labelled and non-labelled inulin predisposed to large bowel fermentation in rats

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Abstract

The fate of stable-isotope ^{13}C labelled and non-labelled inulin catabolism by the gut microbiota was assessed in a healthy rat model. Sprague-Dawley male rats were randomly assigned to diets containing either cellulose or inulin, and were fed these diets for 3 days. On day (d) 4, rats allocated to the inulin diet received ^{13}C-labelled inulin. The rats were then fed the respective non-labelled diets (cellulose or inulin) until sampling (d4, d5, d6, d7, d10 and d11). Post feeding of ^{13}C-labelled substrate, breath analysis showed that ^{13}C-inulin cleared from the host within a period of 36 hours. Faecal ^{13}C demonstrated the clearance of inulin from gut with a ^{13}C excess reaching maximum at 24 hours (d5) and then declining gradually. There were greater variations in caecal organic acid concentrations from d4 to d6, with higher concentrations of acetic, butyric and propionic acids observed in the rats fed inulin compared to those fed cellulose. Inulin influenced caecal microbial glycosidase activity, increased colon crypt depth, and decreased the faecal output and polysaccharide content compared to the cellulose diet. In summary, the presence of inulin in the diet positively influenced large bowel microbial fermentation.

Keywords: ^{13}C-labelled inulin; non-digestible carbohydrates; microbial fermentation; short-chain fatty acids

1. Introduction

The positive association between dietary fibre consumption and human health reinforces the need to include adequate amounts of dietary fibre in our daily diet to maintain optimal health. Dietary fibre consists of carbohydrates that are resistant to digestion in the upper gastrointestinal (GI) tract and are subsequently available for fermentation by microorganisms in the lower GI tract. Prebiotics are defined as "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''.[1] The positive functional attributes of prebiotics have encouraged the food industry to explore using them as an ingredient in food products. Fructo-oligosaccharides, galacto-oligosaccharides, inulin and resistant starch have been shown to exhibit prebiotic characteristics.[2, 3]

Depending on the type and level of supplementation into diets, dietary fibre can alter several host functions including digestive processes, GI motility and resident microbiota composition.[4] In the lower GI tract, the type and availability of dietary fibres as fermentable substrates for microbiota alters their population balance, and the composition and quantity of fermentation end-products. Early studies have emphasised the biological relevance of organic acids mainly short-chain fatty acids (SCFAs) to health,[5] and have been utilising SCFAs in the large bowel as a biomarker to demonstrate the functional benefits of whole foods (fruit and vegetables) and food components.

In our previous study, we measured the rate and extent of digesta transit through the GI tract using titanium dioxide (TiO_2) as an indigestible marker, and found that the TiO_2 ratios rapidly increased within 24 h and reached a maximum level within 48 h in the caecum.[6] Recently, the utilisation of carbon from a ^{13}C-labelled inulin by the microbiota in caecum of rats was evaluated over a 24 h period by RNA-stable-isotope probing (RNA-SIP), to identify the members of microbiota that utilised the ^{13}C-labelled substrate.[7] In the current study, rats

were fed diet containing inulin or cellulose to evaluate whether the microbiota continues to adapt to fermentable carbohydrates over time, thereby engendering distinctions in microbial fermentation products and changes in colon morphology. Rats allocated to the inulin diet received ^{13}C-labelled inulin at a single time point to evaluate the transit time in the host GI tract and predisposition of inulin to large bowel microbial fermentation.

2. Materials and methods

2.1. Animals

Three week old male Sprague-Dawley rats were obtained from a conventional out-bred colony (Food Evaluation Unit, Plant & Food Research, Palmerston North). They were housed in individual metabolic cages in a temperature controlled room (22 ± 1°C, humidity 60 ± 5 %) with a 12 h light/dark cycle. The rats were randomly assigned (n = 6) to the experimental diets (cellulose or inulin) and sampling times (d4, d5, d6, d7, d10 and d11). The experiment was carried out in 3 blocks over time with treatments allocated evenly across and within the blocks. All the animal procedures were approved by AgResearch Grasslands Animal Ethics Committee, Palmerston North, New Zealand (application number: 11816) according to the Animal Welfare Act 1999, New Zealand. All the rats were given *ad libitum* access to diets and water throughout the study. Diets were supplemented with microcrystalline cellulose (Ceolus PH-102), a non-fermentable polysaccharide, and inulin (Fibruline XL, degree of polymerisation >20), a readily fermented carbohydrate. Rats were fed cellulose or inulin diet for three days. On day (d) 4, rats assigned to the inulin diet received ^{13}C-inulin. The ^{13}C-inulin was purchased from Iso*Life* (Wageningen, The Netherlands). The ^{13}C-inulin had a uniform isotopic enrichment of 97 atom % ^{13}C and comprised 97% fructans (degree of polymerisation ≥ 3). From d5 onwards, the rats were fed the respective non-labelled diets (cellulose or inulin) until sampling (Figure 1). The ingredient compositions of the

experimental diets are shown in Table 1. Rat weights, food and water intake were recorded daily. Faeces were collected every 12 hours from d4 to d11. An accumulated breath sample was collected from each rat receiving the ^{13}C-inulin every 12 hours from d4 to d6, thereafter on d7, d10 and d11 and immediately prior to euthanasia. To take this breath sample, each rat was placed in a 300 mL sealed polyethylene container for 5 minutes followed by sampling of the container's air. At the allocated sampling time, the rats were euthanased by CO_2 asphyxiation and the caecum contents were collected, snap frozen in liquid nitrogen and then stored at -80°C until analysis. Colon tissues were excised and stored in 10% formalin for histology.

2.2. Analysis of breath ^{13}C-CO_2

The ^{13}C isotope ratios for the CO_2 present in breath samples were determined by isotope ratio mass spectrometry (IRMS). The breath samples (10 μL) were injected on to a Thermo Trace gas chromatograph (Thermo Fisher Scientific, Auckland, New Zealand) equipped with a porapakQ column (30 m × 1.5 mm × 25 um) (Agilent Technologies, Santa Clara, CA, USA) operating isothermally at 65°C using Helium carrier gas, 1.5 cm^3/sec flow rate, interfaced with a Conflo-III continuous flow inlet and a Delta-V plus IRMS (Thermo-Finnigan, Bremen, Germany) acquiring data for masses 44, 45 and 46 m/z. The ^{13}C ratio for the breath CO_2 was determined relative to a calibrated CO_2 reference gas ratio running concurrent to sample analysis. Data was processed using Isodat software. The ^{13}C breath CO_2 atom percent excess (APE) for enriched samples was calculated relative to the ^{13}C ratios measured for breath CO_2 samples (d4) collected from the rats prior to receiving the ^{13}C-inulin.

2.3. Analysis of caecal organic acids

Acetic, butyric, formic, lactic, propionic and succinic acids concentrations in the caecal contents of each rat were quantified by gas chromatography as described by Richardson et al.[8] Briefly, the samples were analysed using a Shimadzu gas chromatograph system (GC-17A, Kyoto, Japan) with flame ionization detector and an HP-1 column (10 m × 0.53 mm × 2.65 μm) (Agilent Technologies).

For rats receiving ^{13}C-inulin, the resulting samples from the organic acid analysis were also analysed for the incorporation of ^{13}C into various organic acids by a Shimadzu gas chromatograph system (GC-17A) equipped with a Shimadzu mass selective detector (QP-5050a) and a ZB-5MS column (30 m × 0.25 mm × 0.25 μm) (Phenomenex, Auckland, New Zealand). The mass selective detector was operated using selected ion acquisition targeting the *t*-butyl-dimethyl-silyl organic acid derivative, m/z M-57, fragment ion mass for the complete distribution of ^{12}C and ^{13}C isobaric species. The chromatograms were acquired and peaks areas integrated using GC solution software (Shimadzu). The ^{13}C APE for the organic acids and ^{13}C mole percent excess (MPE) for the enriched isobaric species were calculated relative to the natural abundance and distribution of ^{12}C and ^{13}C isobaric species present for the d4 samples collected prior to the onset of feeding ^{13}C-inulin.

2.4. Analysis of caecal microbial enzymes

The model substrates used in the current study have host-derived glycosidic linkages and plant cell wall linkages representative of most of the endogenous and dietary polysaccharides to which the resident microbiota would be exposed to in the GI tract. The glycosidase model substrates were all 4-nitrophenyl-1-linked sugars as follows: α-arabinofuranoside, α-arabinopyranoside, α-galactopyranoside, α-glucopyranoside, α-fucopyranoside, α-N-acetylgalactosaminide, α-mannopyranoside, α-rhamnopyranoside, β-galactopyranoside, β-glucopyranoside, β-N-acetylglucosaminide, β-xylopyranoside, β-galacturonide and β-

glucuronide. The N-acetyl-glycosaminides represent some mucin linkages, the glucuronide represents some host connective tissue linkages, and the remainder correspond to plant glycosidic linkages, including the arabinopyranoside, found in a class of ginsenosides from ginseng (*Panax* spp.). The fucopyranoside and galactopyranosides can be found in both host and plant tissue, with the former commonly occurring as terminal residues.

Caecal samples from two animals in the same treatment were pooled and suspended in an equal volume of sterile deionised water. The pooled slurry was then diluted with four parts of 25 mM Na succinate buffer (pH 6.0) and divided into aliquots. An aliquot was mixed with protease inhibitor cocktail (Sigma-Aldrich), resulting in a 10-fold diluted caecal slurry in a final concentration of 20 mM succinate buffer (pH 6.0). The resulting caecal extracts and enzymes were placed on to 384-well assay plates containing 0.833 mM 4-nitrophenyl-sugar substrates in 25 mM Na succinate buffer (15.0 μL). The caecal extracts (5.0 μL) were loaded in triplicate on to the plates (40-fold diluted caecal fractions in 0.625 mM substrate in 25 mM Na succinate buffer), immediately covered to minimise evaporative losses, and incubated for 90 min at 37°C. The enzyme reactions were terminated and 4-nitrophenol colour developed by the addition of 0.5 M Na glycine buffer pH 9.6 (20.0 μL). The absorbance was measured at 405 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Colon histology

Tissues from the colon were excised, thoroughly rinsed with phosphate buffered saline and stored in 10% formalin until embedded in paraffin. Transverse tissue sections of 5 μm thick (3 sections per rat) were generated from each rat. The sections were stained with Alcian blue (pH 2.5) and haematoxylin and eosin for microscopic examination.[9] A bright field microscope (Model Axiophot; Carl Zeiss Inc., Goettingen, Germany) fitted with a cellSens

Dimension software, version 1.5 (Olympus Corporation, Tokyo, Japan) was used to measure the crypt depth from randomly selected intact colon crypts through visualising at 20 × magnification. The same image was used to count goblet cells per crypt from randomly selected intact colon crypts.

2.6. Faecal non-digested polysaccharides

Finely ground faecal samples (100 mg) from each rat were used to measure the total faecal dietary fibre, previously described by Paturi et al.[6] Briefly, the faecal samples were extracted twice with 5 ml 80% ethanol, with the residue collected by centrifugation between washes. The residues were washed with 2 mL acetone, air-dried at 60°C, and finely crumbled. The residues were then subjected to acid hydrolysis (1 mL 12M H_2SO_4, 35°C for 1h), followed by the addition of distilled water (7 mL) and heating (100°C for 1 h). The total polysaccharide in the hydrolysates was measured as reducing sugars using a glucose reference.

2.7. Faecal ^{13}C analysis

The total carbon and ^{13}C ratio for the 12 hourly collected faecal samples of rats was analysed by an elemental analyser coupled to an isotope ratio mass spectrometer (IRMS). Samples were homogenised by grinding the faecal pellets in a mortar and pestle after which a weighed subsample (1 mg) was transferred into a 3 × 5 mm tin foil cup (IVA Analysentechnik eK, Meerbusch, Germany). The encapsulated samples were then combusted by a Flash HT2000 elemental analyser (Thermo Fisher Scientific) on a single $CrO_3/Cu/AgCo_2O_4$ oxidation-reduction reactor tube operated at 1020°C interfaced onto the IRMS by a Conflo-IV continuous flow interface (Thermo Finnigan, Bremen, Germany). The IRMS acquired data for mass 44, 45 and 46 m/z. The ^{13}C ratio for the resulting CO_2 peak was determined relative to the concurrent analysis of a calibrated CO_2 reference gas with the elemental analysers TCD

signal being integrated to determine the samples total carbon content using a calibrated response. The ^{13}C faecal total carbon APE for enriched samples was calculated relative to the ^{13}C ratios measured for faecal samples (d4), collected from the rats prior to receiving ^{13}C-inulin.

2.8. Statistical analysis

The data were analysed using analysis of variance (ANOVA), with diet and sampling time as block factors (random effects). The treatment effects were analysed two ways; once using the conventional factorial arrangement of diet, sampling time, diet × sampling time, and block × diet × sampling time; the other with diet and sampling time effect for each diet as treatment factors, and block (1, 2 or 3) and block × diet × sampling time as block factors. The organic acid data was log transformed to stabilise the variance prior to ANOVA. All analyses were carried out using GenStat 14th edition (VSN International, Hemel Hempstead, UK).

3. Results and discussion

3.1. Rat body weight and food intake

The initial body weight, daily weight gain and food intake of rats fed the experimental diets was similar ($P > 0.05$) (Supplementary Table S1).

3.2. ^{13}C breath measurements

The ^{13}C breath CO_2 measurements of rats fed inulin are presented in Figure 2. With breath sampling only being performed every 12 h, insufficient data was obtained to characterise the profile of expired ^{13}C excesses: the observed excess maximums are therefore not indicative of the true maximums and likewise the decay profile could not be modelled to explore differences between rates of fermentable fibre metabolism and clearance. The low ^{13}C excess

for 36 h breath CO_2 samples and subsequent samples suggests that most of the ingested ^{13}C-inulin was metabolised and cleared from the rats within this 36 h period.

3.3. Caecal organic acids

The fermentation of inulin led to a significant increase in caecal organic acid concentrations compared with cellulose (Table 2). Acetic, butyric and propionic acid concentrations were higher in rats fed the inulin diet ($P < 0.001$ for the diet effect). Significant diet × day interaction effects were observed in butyric ($P = 0.007$) and lactic ($P = 0.008$) acids. One of the important attributes of dietary fibres is their availability as a rich substrate for microbial fermentation in the large bowel, thereby altering the microbiota metabolism and releasing metabolites that have importance to host health. For example SCFAs, such as acetic, butyric and propionic acids are known to have a varied range of health benefits.[10] In the present study, greater differences in caecal fermentation profiles were observed between the cellulose and inulin treatments from d4 to d6 compared to the later days of the study (d7-d11), suggesting that dietary fibre changes can swiftly alter the composition and metabolism of resident microbiota.[11]

The labelling of fermentable carbohydrates with stable isotopes assists in obtaining specific insights into fermentation profiles of the resident microbiota in the large bowel.[7, 12, 13] The mean ^{13}C APE and MPE for labelled isotopomers for major organic acids found in the caecal digesta collected at 24 h (d5) from the onset of feeding ^{13}C-inulin are presented in Table 3. Comparing the acetic acid ^{13}C APE for rats fed labelled inulin relative to the calculated ingested inulin ^{13}C APE suggests that 70% of their caecal acetic acid originated from the fermentation of inulin. The origin of the remaining 30% is unclear; it is possible that cellulose fermentation took place or the acetate was derived from mucosal or dietary protein bypassing foregut metabolism. The majority of ^{13}C incorporation was associated with organic

acid isotopomers containing two enriched carbons. The distribution of observed isotopomer enrichments was consistent with the conservation of enriched carbons derived through the Embden-Meyerhof pathway converting fructose to organic acids. The appearance of the mono labelled acetate isotopomer species suggests the occurrence of cross feeding of labelled and unlabelled fructose and its metabolism via the bifidobacteria[14]. Alternatively, *de novo* synthesis of caecal organic acids occurred from H_2 and the partially labelled fermentation CO_2. The caecal organic acid ^{13}C excesses for samples collected on d6 were less than 0.35% and less than 0.1% for the remaining samples collected on d7, d10 and d11 indicating the rapid clearance of ^{13}C-inulin through the caecum (data not shown). In this study, labelling of inulin with a stable isotope (^{13}C) allowed us to monitor its utilisation by resident microbiota and the subsequent release of organic acid metabolites.[15, 16]

3.4. Caecal microbial enzymes

The ability of caecal microbiota to catabolise complex carbohydrates to monosaccharides was investigated using model substrates selected from the NCBI database panel of (exo)-glycosidases possessed by the highly metabolically flexible carbohydrate degrading gut bacteria, *Bacteroides thetaiotaomicron* VPI-5482 (http://www.ncbi.nlm.nih.gov/bioproject/399).[17] There was a significant effect of diet on the caecal microbial glycosidase activities of rats (Table 4). Rats fed the inulin diet had increased caecal α-arabinofuranosidase ($P = 0.004$), α-arabinopyranosidase ($P = 0.012$), α-fucosidase ($P = 0.034$); α-galactosidase ($P < 0.001$), α-glucosidase ($P = 0.001$), β-glucosidase ($P < 0.001$) and β-glucuronidase ($P = 0.003$) compared to rats fed the cellulose diet. Despite this, activities against substrates representative of mucin glycosidic linkages (fucosidase, N-acetyl-glucosidase, and N-acetyl-galactosidase) and plant glycosidic linkages (rhamnosidase, galacturonidase, and xylosidase) were similar between the diet groups. The unchanged mucin

linkage activities may reflect that there were no changes in mucin degradation throughout the duration of the rat trial, whilst the unchanged plant linkage activities are consistent with the absence of material containing these linkages in the diets.

Increases in glucosidases, galactosidases and glucuronidases are unsurprising. Galactosidases are ubiquitous amongst microorganisms, whilst the primary glycosidases produced by the intestinal microbiota are β-glucosidase and β-glucuronidase; the ability of prebiotics and probiotics in reducing these bacterial enzymes has been reported earlier.[18-20] The increased β-glucosidase and β-glucuronidase enzymes in rats fed inulin could be due to enhanced proliferation of certain members of the microbiota. A recent study has associated the abundance of various genera in the human faecal microbiota with the microbial enzymes, β-glucosidase and β-glucuronidase.[21] Similarly, an increase in arabinopyranosidase could be representative of increased abundance of bacteria possessing this activity, as this arabinopyranoside substrate captures the activity of a class of ginsenosidases constitutively expressed by some members of the microbiota, such as bifidobacteria species.[22, 23]

Collectively, changes in caecal microbial glycosidase activities against model substrates were consistent with changes in the fermentation metabolic end-products, organic acids. This suggests that ongoing metabolic changes within the microbiota were occurring, in other words, various members of the microbiota throughout the trophic chain were changing in abundance. In a previous study, RNA-SIP showed that members of the family *Bifidobacteriaceae, Bacteroidaceae, and Lachnospiraceae* are the primary users of ^{13}C-inulin.[7] These data are consistent with the elevated glycosidase enzyme activities observed in the current study. However, interpretation of these results must be undertaken with caution as the enzyme activities are against model colorimetric substrates, and expressed on a concentration basis (Table 4), which means they are influenced by faecal output (Table 5),

such that significant differences between treatments may reflect the effects of intracaecal dilution or concentration rather than actual amounts of enzyme produced.

3.5. Colon morphology

In the colon, anaerobic fermentation of dietary fibres by microbiota releases organic acids, particularly SCFAs that can induce the proliferation of mucin producing goblet cells.[24] In the present study, rats fed inulin diet showed a significant increase in colon crypt depth (P = 0.047) compared to cellulose. However, there was no significant diet × day interaction effect on colon crypt depth (P = 0.726) and goblet cell per crypt (P = 0.708) (Figure 3). In our previous study, we observed a similar short-term feeding effect of inulin on increasing the colon crypt depth in rats.[25]

3.6. Faecal output and non-digested polysaccharides

Faecal output (Table 5) and faecal polysaccharide contents (Table 6) were lower in rats fed inulin compared to cellulose, irrespective of the day of collection, demonstrating the utilisation of these digestion resistant fibres by microbiota in the GI tract. If these differences in faecal output reflect differences in caecal loading, this affects the organic acids and microbial enzyme activities reported in Tables 2 and 4 as these are presented as concentrations rather than as amounts per caecum. The importance of allowing for bulking effects in interpreting results from the gut samples has been highlighted previously,[26] and is relevant to all of the analytical results of caecal contents expressed on a concentration basis. Polysaccharide content, as a percentage of the faeces, from the rats fed inulin was lower than those fed cellulose (Table 6). Faecal output (Table 5) multiplied by polysaccharide content gave values for polysaccharide output of 0.86 g for cellulose and 0.47 g for inulin, indicating

that inulin was more fully utilised than cellulose as fermentable substrates by the microbiota in the GI tract.

3.7. Faecal ^{13}C

The mean excess quantity of ^{13}C expressed in faecal samples for rats receiving the labelled dietary carbohydrate compared to the excess quantity of ^{13}C ingested for the 60 h period are presented in Table 7. The ^{13}C excess ratio profile reached a maximum at 24 h (d5), and then declined during the following 24 h period to a plateau at 60 h. A small quantity of ingested ^{13}C was excreted in the faeces of rats fed inulin within 60 h and is estimated to represent 23% of ingested ^{13}C component.

4. Conclusions

The ingestion of ^{13}C-labelled and non-labelled inulin showed the fermentability by resident microbiota of the laboratory rat, a model of mammalian digestion. Incorporation of dietary ^{13}C into caecal organic acids and its expulsion in the breath CO_2 demonstrated the utilisation of inulin by the resident microbiota. Low faecal output and polysaccharide content were observed in rats fed inulin compared to cellulose. The caecal organic acids showed differences between the predisposition of dietary inulin and cellulose to microbial fermentation; inulin being more fermentable than cellulose. Ingestion of inulin diet altered the caecal microbial glycosidase activity and colon crypt depth suggesting changes in resident microbiota composition and metabolism and colon morphology in rats.

Conflict of interest

The authors declare that they have no conflict of interest.

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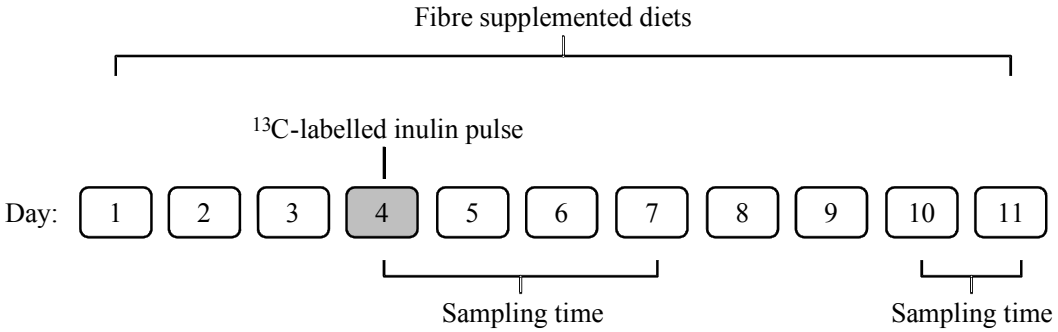

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403 **Figure 1.** Schematic view of experimental design.

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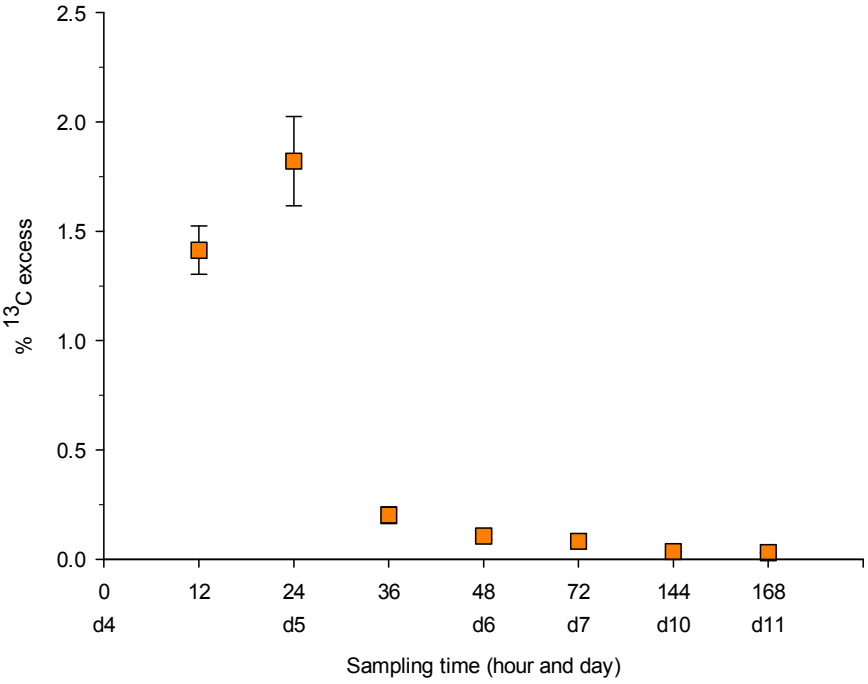
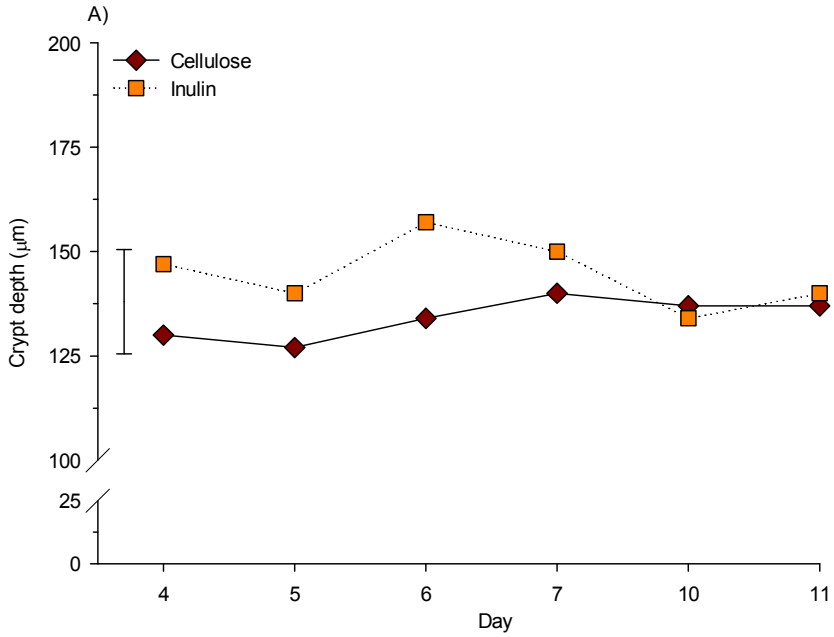


406

407 **Figure 2.** Mean ^{13}C atom percent excess for breath CO_2 samples of rats fed inulin. Error bars
408 represent standard error of the mean. Excess is relative to $^{13}C/^{12}C$ ratio for time 0 h breath
409 CO_2 samples.

410



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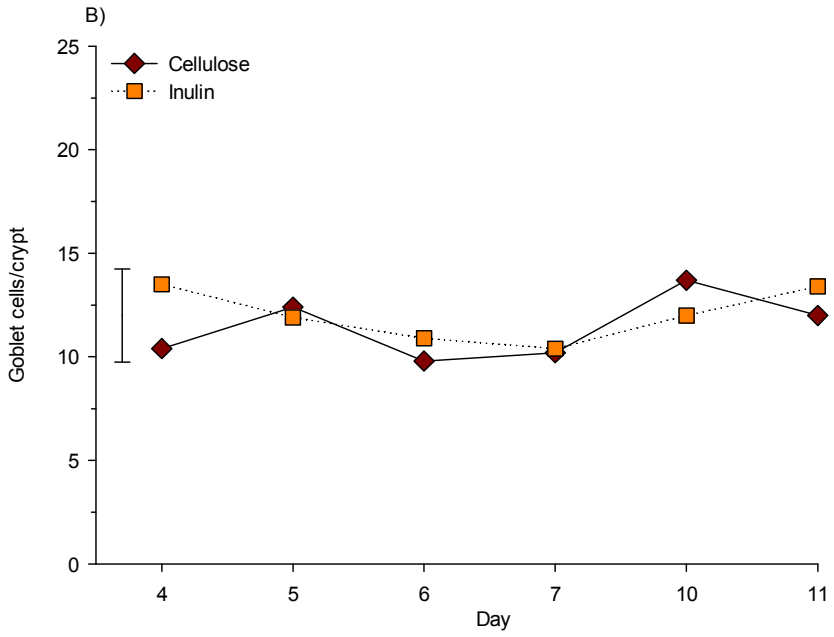

413

414 **Figure 3.** Influence of diets containing cellulose or inulin on colon morphology A) crypt
415 depth and B) goblet cells/crypt in rats. The error bars are least significant difference between
416 two mean at the 5% level (22 degrees of freedom).

417

418 **Table 1.** Ingredient compositions (g/kg) of experimental diets.

Ingredient	Cellulose	Inulin	
Lactic casein[a]	120	120	120
Vitamin mixture[b]	50	50	50
Mineral mixture[c]	50	50	50
Corn oil[d]	120	120	120
Starch[e]	540	540	540
Sucrose[f]	45	45	45
Cellulose[g]	75	25	25
Inulin[h]		50	30.3
^{13}C-Inulin[i]			19.7

419 [a]Alacid 80 mesh, New Zealand Milk Products, Wellington, New Zealand.
[b]Mixture contains the following components: (mg/kg diet) Retinol acetate 5.0, DL-α-tocopheryl acetate 100.0, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500.0; (μg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.
[c]Mixture contains the following components: (g/kg diet) Ca 6.29, Cl 7.79, Mg 1.06, P 4.86, K 5.24, Na 1.97; (mg/kg diet) Cr 1.97, Cu 10.7, Fe 424.0, Mn 78.0, Zn 48.2; (μg/kg diet) Co 29.0, I 151.0, Mo 152.0, Se 151.0.
420 [d]Davis Trading Company, Palmerston North, New Zealand.
421 [e]Wheaten corn flour, Starch Australasia, Goodman Fielder Group, Tamworth, NSW,
422 Australia.
423 [f]Chelsea Sugar Refinery, Auckland, New Zealand.
[g] Ceolus PH-102, Asahi Kasei chemicals corporation (Tokyo, Japan).
424 [h]Fibruline XL, Cosucra, Warcoing, Belgium.
425 [i]Isolife, Wageningen, The Netherlands.
426

427 **Table 2.** Caecal organic acids in rats fed experimental diets.

Diet	Day	Acetic	Butyric	Formic	Lactic	Propionic	Succinic
Cellulose	4	24.0	1.9	0.9	0.2	5.3	1.9
Cellulose	5	21.9	1.8	0.2	0.3	5.6	2.4
Cellulose	6	24.5	2.0	5.6	0.3	6.6	3.3
Cellulose	7	25.7	2.2	0.2	0.3	6.8	1.9
Cellulose	10	24.5	4.0	0.3	0.2	6.0	0.7
Cellulose	11	16.2	1.9	0.4	0.1	4.7	0.4
Inulin	4	50.1	14.5	19.6	40.0	15.4	13.4
Inulin	5	43.7	10.0	2.5	42.8	9.3	19.9
Inulin	6	40.7	10.2	8.6	2.0	16.6	5.5
Inulin	7	22.4	3.5	2.7	0.6	8.9	2.3
Inulin	10	30.2	4.7	7.2	1.3	12.5	2.4
Inulin	11	21.4	2.8	13.2	0.7	8.5	1.9
LSR % (22 df)[a]		165	225	2767	713	217	272
P values							
Diet (1 df)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Day (5 df)		0.015	0.050	0.306	0.005	0.517	<0.001
Diet × Day (5 df)		0.149	0.007	0.734	0.008	0.684	0.056

428 The organic acids are expressed as μmol/g of caecum content.

429 [a]Least significant ratio (LSR) is equivalent of the least significant difference for data which
430 was log-transformed before ANOVA; two means are significantly different if the ratio of the
431 higher to the lower is more than the LSR. df – Degrees of freedom.

432

433 **Table 3.** Labelled organic acids isotopomer mole percent excess and ^{13}C atom percent excess
434 for rat caecal digesta samples collected on d5.

		Inulin	
		Mean	SEM
Acetic	$^{13}C_1$ MPE	8.8	1.0
	$^{13}C_2$ MPE	22.9	0.8
	^{13}C APE	27.3	0.8
Butyric	$^{13}C_1$ MPE	10.2	1.2
	$^{13}C_2$ MPE	30.9	0.5
	$^{13}C_3$ MPE	7.6	0.6
	$^{13}C_4$ MPE	7.9	0.4
	^{13}C APE	31.5	0.9
Propionic	$^{13}C_1$ MPE	6.7	0.6
	$^{13}C_2$ MPE	17.6	2.4
	$^{13}C_3$ MPE	9.2	0.5
	^{13}C APE	23.1	1.6

435 $^{13}C_1$ MPE: mole percent excess of labelled isotopomer species containing 1 ^{13}C labelled
436 carbon, position of labelled C not determined. Mole percent excess (MPE) relative to
437 distribution natural abundance of ^{13}C labelled isotopomers determined for time 0 h samples.
438 ^{13}C atom percent excess (APE) relative to $^{13}C/^{12}C$ ratio for time 0 h samples. SEM – Standard
439 error of the mean.
440

441

Table 4. Microbial enzyme profiles in the caecum of rats fed experimental diets.

Diet	Day	α-araF	α-araP	α-fuc	α-gal	α-galNAc	α-glc	α-man	α-rha	β-gal	β-galU	β-glc	β-glcNAc	β-glcU	β-xyl
Cellulose	4	0.4	0.2	0.4	1.0	0.4	1.1	0.1	0.2	1.5	0.4	0.3	1.8	1.1	0.6
Cellulose	5	0.3	0.2	0.2	0.8	0.5	0.8	0.3	0.2	1.8	0.1	0.3	1.7	0.7	0.3
Cellulose	6	0.2	0.0	0.3	0.8	0.4	0.8	0.2	0.1	0.9	0.1	0.1	1.1	0.6	0.2
Cellulose	7	0.3	0.0	0.2	1.0	0.5	1.0	0.0	0.1	1.2	0.3	0.2	1.9	0.9	0.3
Cellulose	10	0.4	0.1	0.3	1.0	0.6	0.8	0.0	0.1	1.4	0.2	0.4	1.7	0.9	0.5
Cellulose	11	0.2	0.1	0.1	0.6	0.2	0.7	0.0	0.1	0.9	0.2	0.2	1.2	0.6	0.1
Inulin	4	0.7	0.2	0.4	2.1	0.3	2.0	0.2	0.3	1.9	0.3	0.6	1.9	1.8	0.4
Inulin	5	0.8	0.3	0.4	1.8	0.3	1.9	0.1	0.1	1.7	0.2	0.6	1.6	0.9	0.5
Inulin	6	0.4	0.1	0.3	1.6	0.3	1.4	0.1	0.1	0.9	0.2	0.5	1.3	1.1	0.2
Inulin	7	0.5	0.3	0.3	1.9	0.3	1.1	0.1	0.2	0.9	0.4	0.7	1.5	1.5	0.4
Inulin	10	0.6	0.2	0.6	2.0	0.6	1.4	0.1	0.2	1.7	0.2	0.6	1.7	1.0	0.5
Inulin	11	0.6	0.2	0.6	2.6	0.4	1.6	0.1	0.3	1.6	0.4	0.9	2.1	2.1	0.2
LSD (21 df)[a]		0.4	0.2	0.4	1.0	0.5	1.0	0.2	0.2	1.2	0.2	0.5	1.1	0.9	0.4
P values															
Diet (1 df)		0.004	0.012	0.034	<0.001	0.835	0.001	0.904	0.061	0.540	0.188	<0.001	0.609	0.003	0.574
Day (5 df)		0.429	0.336	0.629	0.848	0.731	0.668	0.022	0.338	0.287	0.038	0.815	0.594	0.200	0.134
Diet × Day (5 df)		0.865	0.542	0.535	0.491	0.803	0.735	0.206	0.353	0.799	0.512	0.731	0.617	0.318	0.851

The data are expressed as Units/mL of caecum content, where 1 Unit equals 1 nmol/min.

[a]LSD – Least significant difference between two means at the 5% level; df – Degrees of freedom.

Microbial enzymes: α-araF – α-arabinofuranosidase; α-araP – α-arabinopyranosidase; α-fuc – α-fucosidase; α-gal – α-galactosidase; α-galNAc – α -N-acetylgalactosaminidase; α-glc – α-glucosidase; α-man – α-mannosidase; α-rha – α-rhamnosidase; β-gal – β-galactosidase; β-galU – β-galacturonidase; β-glc – β-glucosidase; β-glcNAc – β -N-acetylglucosaminidase; β-glcU – β-glucuronidase; β-xyl – β-xylosidase.

Table 5. Faecal output of rats fed experimental diets.

Diet	Day	Faeces output (g)
Cellulose	4	–
Cellulose	5	1.7
Cellulose	6	1.3
Cellulose	7	1.3
Cellulose	10	1.6
Cellulose	11	1.5
Inulin	4	–
Inulin	5	1.2
Inulin	6	1.3
Inulin	7	1.1
Inulin	10	1.2
Inulin	11	1.2
LSD (18 df)[a]		0.3
P values		
Diet (1 df)		<0.001
Day (4 df)		0.083
Diet × Day (4 df)		0.125

[a]LSD – Least significant difference between two means at the 5% level; df – Degrees of freedom.

Table 6. Faecal polysaccharides of rats fed experimental diets.

Diet	Day	Faecal polysaccharides (% dry weight)
Cellulose	4	60.8
Cellulose	5	61.5
Cellulose	6	56.5
Cellulose	7	60.3
Cellulose	10	57.5
Cellulose	11	60.6
Inulin	4	47.0
Inulin	5	37.1
Inulin	6	35.8
Inulin	7	38.0
Inulin	10	41.6
Inulin	11	40.2
LSD (22 df)[a]		12.1
P values		
Diet (1 df)		<0.001
Day (5 df)		0.612
Diet × Day (5 df)		0.798

[a]LSD – Least significant difference between two means at the 5% level; df – Degrees of freedom.

Table 7: Faecal ^{13}C excess to ingested ^{13}C excess ratio for rats fed labelled inulin diet.

Sampling time (h)	Inulin
12	0.02
24 (day 5)	0.19
36	0.11
48 (day 6)	0.12
60	0.03
LSR % (10 df)[a]	355
P value	
Time (4 df)	0.008

Excesses calculated relative to mean time 0 h faecal ^{13}C content and quantity of ^{13}C labelled and unlabelled NDC ingested.

[a]Least significant ratio (LSR) is equivalent of the least significant difference for data which was log-transformed before ANOVA; two means are significantly different if the ratio of the higher to the lower is more than the LSR. df – Degrees of freedom.