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ORIGINAL ARTICLE

# Digestive-resistant carbohydrates affect lipid metabolism in rats

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**Abstract**
*Introduction* Digestion resistant carbohydrates (DRC) are complex carbohydrates that resist digestion and absorption in the small bowel. Diets high in DRC can have wide ranging impacts on the health of the host, which include changes to immunity and allergy, incidence of cardiovascular disease, and obesity.
*Objectives* The aim of this study was to characterise the effects of DRC (inulin, konjac or resistant starch) on large intestinal short-chain fatty acid (SCFA) concentrations and serum metabolite and lipid profiles.
*Methods* A rat model was used to compare the effects of feeding a basal diet or the basal diet containing 5 % inulin, konjac or resistant starch for 14 days.
*Results* Of the three DRC, inulin had the greatest effect; ten serum phospholipids differed significantly in abundance between inulin-treated and control rats. In particular phosphatidylcholines and lysophosphatidylcholines containing fatty acyl chains 22:5, 22:4, 20:4, 18:0 and 16:0 were increased in the inulin-fed group, whereas phosphocholines containing fatty acyls 20:5 and 22:6 were decreased.
*Conclusion* These results indicated an impact on both *n*-3 and *n*-6 fatty acid metabolism as a result of inulin dietary intake. Increased intestinal concentrations of SCFA were detected in rats fed DRC, but only inulin caused appreciable changes to serum lipid profiles.

**Keywords** Metabolomics · SCFA · Serum lipids · Resistant starch · Konjac · Inulin · Rat

## 1 Introduction

Digestion resistant carbohydrates (DRC) are complex carbohydrates that resist digestion and absorption in the small bowel. After ingestion, DRC can reach the large bowel and become substrates for hydrolysis and fermentation by some members of the microbial community, and consequently alter the microbiota composition and function. Fermentation of DRC by the microbiota produces short-chain fatty acids (SCFA), which in turn can be utilised by other members of the microbial community and by the host itself as a source of energy (Kripke et al. 1989).

Diets high in DRC have been suggested to have wide-ranging impacts on the health of the host, which include immunity and allergy (Rodriguez-Cabezas et al. 2003; Trompette et al. 2014), cardiovascular disease (Liu et al. 1999), and obesity (Everard et al. 2014; Reinhardt et al. 2009). Consumption of DRC has also been shown to reduce blood cholesterol and triglyceride levels in rats fed high fat diets (Fak et al. 2015), and decrease fat deposition

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(Yamada et al. 2003). SCFA produced by bacterial fermentation can alter lipid metabolism and deposition through a number of mechanisms (dos Reis et al. 2015), including signalling via the SCFA receptor, G Protein-coupled receptor 43 (Kimura et al. 2013; Zaibi et al. 2010), and 5′ AMP-activated protein kinase activity in liver and muscle (Gao et al. 2009). It is recognised that the benefits of DRC are mediated by more than just their total amount in the diet and that the specific biological effects of DRC depend on the source and structure, which in turn can affect their fermentability. The structure and composition of each DRC might determine which mechanism of action is modulated and consequently which biological effect a specific DRC has on body function.

Konjac (KJ), produced from the corm of the *Amorphophallus konjac* plant, is typically incorporated as a thickening agent into Asian foods. It commonly contains up to 75 % KJ glucomannan, a complex carbohydrate consisting of D-glucose and D-mannose units joined by β-1,4 glycosidic bond linkages. KJ glucomannan resists digestion in the small bowel (Li et al. 2005) and has been shown to alter the faecal microbiota composition in rodents and humans (Young et al. 2013; Chen et al. 2006), in particular on the relative abundance of *Bifidobacterium* (Young et al. 2013). In addition KJ supplementation for 28 days altered serum metabolite profiles in rats, particularly in relation to lipids (Young et al. 2013).

Maize-derived resistant starch (RS) and inulin (IN) are other complex polysaccharides that escape absorption in the small bowel and can serve as sources of fermentable substrate for the large bowel microbiota (Kleessen et al. 1997; Loh et al. 2006). RS, like digestible starch, is composed of glucose subunits. However, the compact secondary structure of RS prevents access of mammalian digestive hydrolases and accounts for its resistant nature. Inulin is a heterogeneous polymer of fructose units linked by β(2,1) bonds and is found in many plants (Swennen et al. 2006). RS caused a seven-fold bloom in *Bifidobacterium* in newly weaned rats along with an increase in host expression of *Gsta2* and *Ela1* genes and also altered the serum metabolite profiles (Young et al. 2012). Feeding IN to young rats also increased *Bifidobacterium* abundance in the caecum, along with the *Lachnospiraceae*, and *Bacteroidaceae* families (Tannock et al. 2014). Consumption of both RS and IN has been shown to prevent body-weight and waist circumference gain (Du et al. 2010), normalise expression levels of transcription factors involved in lipogenesis and cholesterol metabolism (Polakof et al. 2013), and reduce serum cholesterol and triglyceride levels (Han et al. 2013). Inulin supplementation has been shown to decrease blood triglycerides in rats and hamsters (Delzenne et al. 2002) while in humans, contradictory results have been reported (Williams and Jackson 2002).

To better understand the effects of DRC on lipid metabolism throughout the body, we mapped the effects of DRC on serum polar metabolites, lipid profiles and SCFA production in the large bowel. A weanling rat's model was used to compare the effects of feeding diets containing 5 % of IN, KJ or RS for 14 days on SCFA concentrations in the ileum, caecum and colon, and serum metabolite and lipid profiles. In addition to the traditional analytical chemistry methods, non-targeted metabolite analysis (metabolomics) was applied to further define the relationship between dietary DRC consumption and concentrations of SCFAs in the large bowel. In particular, lipidomics was included to increase the understanding of the link between DRC and lipid metabolism.

## 2 Materials and methods

### 2.1 Rat experiment

Newly weaned 21 days old male Sprague–Dawley rats individually housed in hanging wire mesh cages were fed a lactic casein based basal diet (BD) or the basal diet supplemented with 5 % DRC in the form of RS, IN or KJ for 14 days ($n = 10$). The compositions of the diets are described in Table 1. RS was the high amylose maize RS2 type resistant starch Hi-maize 1043 (National Starch and Chemical Company, Bridgewater, NJ, USA). KJ was produced from freeze-dried *Amorphophallus konjac* corms grown at Plant and Food Research, Pukekohe, New Zealand. Macronutrient composition of KJ was analysed by AsureQuality Ltd (Auckland, New Zealand), with total dietary fiber calculated using AOAC method 985.29, and insoluble fiber determined using AOAC method 991.42. IN was Fibruline XL (Cosucra, Warcoing, Belgium), a long chain inulin (average degree of polymerisation >20).

The experiment was conducted with the approval of the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand) under the oversight of the Crown Research Institute Animal Ethics Committee according to the New Zealand Animal Welfare Act 1999. The rats were kept under strict 12 h light cycles. Food and water was provided ad libitum and consumption of food was monitored daily.

The rats were weaned from mothers that had been fed standard rodent chow (LabDiet, St. Louis, MO 63144, USA). After receiving the appropriate diet for 14 days, rats were euthanised by carbon dioxide overdose and blood was collected by cardiac puncture without an anticoagulant. The blood samples were left to stand at room temperature for 2 h, after which the serum was separated from red blood cells by centrifugation for 10 min at 1500 RCF. Serum samples were then snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Samples of

**Table 1** Diet composition

| | Basal diet (BD) | Inulin diet (IN) | Resistant starch diet (RS) | Konjac diet (KJ) |
|---|---|---|---|---|
| Lactic casein | 120 | 120 | 120 | 120 |
| Vitamin mix[a] | 50 | 50 | 50 | 50 |
| Mineral mix[b] | 50 | 50 | 50 | 50 |
| Corn oil | 65 | 65 | 65 | 65 |
| Corn starch | 650 | 637.5 | 625 | 600 |
| Sucrose | 40 | 40 | 40 | 40 |
| Cellulose | 25 | 25 | 25 | 25 |
| KJ[c] | – | – | – | 50 |
| RS[d] | – | – | 50 | – |
| IN[e] | – | 50 | – | – |

Composition of experimental diets (g/kg)

[a] Vitamin 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

[b] Mineral 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

[c] Konjac (Plant and Food Research, Pukekohe, New Zealand)—protein (9 %), fat (0.9 %), moisture (10.5 %), ash (5.3 %), total fiber [52.3 % (insoluble fiber, 35.1 %; soluble fiber, 17.2 %)], total sugar and digestible carbohydrates (22 %), energy 9.79 kJ/g

[d] Resistant starch (Hi-maize 1043, National Starch and Chemical Company, Bridgewater, NJ, USA)

[e] Inulin (Fibruline XL, Cosucra, Warcoing, Belgium)

ileal, caecal and colon contents were also snap frozen in liquid nitrogen and stored at −80 °C.

## 2.2 Chemicals

L-tyrosine-(phenyl-3,5-$d_2$) and ammonium formate were purchased from Sigma-Aldrich Chemical Co (St Louis, MO). Trigonelline and β-alanine were purchased from Fluka Analytical (Hannover, Germany). Internal standard for SCFA analysis (2-ethyl butyric acid) was purchased from Sigma. Ultrapure water was obtained from a Milli-Q® system (Millipore, Bedford, MA). Acetonitrile, methanol and isopropyl alcohol (IPA) were purchased from Thermo Fisher Scientific (Auckland, New Zealand) and were of Optimal LC–MS grade. Acetone was purchased from BDH Laboratory Supplies (Poole, England). β-Alanine betaine was synthesised by methylation of β-alanine according to the method of Li et al. (C. Li et al. 2010).

## 2.3 Short-chain fatty acid analysis

Acetic, propionic, *iso*-butyric, butyric, lactic, and succinic acids in ileal, caecal, and colonic digesta were derivatised with *N*-methyl-*N*-E-butyldimethylsilytrifluoracetamide and measured using the capillary gas chromatography (GC) method described by Jensen et al. (1995), with the following modifications: digesta was homogenised by vortexing with 0.1 g of glass beads (0.3 mm diameter) in 8 mL of homogenization medium (0.9 % NaCl w/v, 0.1 % Tween 20 v/v) and 1 mL internal standard (100 mM 2-ethyl butyric acid) per gram of digesta. GC separation and detection of acids was carried out using a Shimadzu GC-17A chromatograph equipped with an Agilent HP-1 methyl silicone gum column (10 m × 0.53 mm × 2.65 μm) and a flame ionization detector (both from Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas at 10 kPa for the first 6 min, and was then increased to 15 kPa at 5 kPa per minute for the final 3 min. The column temperature at time of injection was 70 °C, which was then increased to 80 °C at 10 °C per minute, followed by an increase to 260 °C at 20 °C per minute. The detector flame was maintained with a hydrogen/air mixture with both gases supplied at 50 psi. Detector and injector temperatures were both set to 260 °C. Samples were assayed in duplicate. Areas of acid peaks were normalised against area of the internal standard peaks. Concentrations of acids were calculated against a standard curve using a linear curve fit. Differences between means were analysed by one-way ANOVA with DRC type as the independent variable.

## 2.4 Sample preparation for metabolomics

Serum samples were randomised and defrosted on ice for 1 h. Two aliquots were taken from each serum sample and

prepared for hydrophilic interaction chromatography liquid chromatography mass spectrometry (HILIC LC–MS) and lipid analysis by reverse-phase liquid chromatography mass spectrometry (RP LC–MS), respectively, immediately to avoid repeated freezing/thawing of the samples. For each of the two batches (HILIC and lipidomics) two blank samples (containing no serum) and one quality control (QC) sample were prepared. The QC sample consisted of 10 µL of serum taken from ten randomly selected samples.

Samples for HILIC LC–MS analysis were prepared by transferring 100 µL of serum to 1.5 mL centrifuge tubes cooled on ice. 300 µL of a chilled mixture of acetone:methanol:acetonitrile (1:1:1 v/v/v) was added to each tube followed by vortex mixing for 10 s. Samples were left on ice for 20 min and were then centrifuged at 13,000 RCF at 4 °C for 10 min. 250 µL of the supernatant was transferred to LC vials fitted with 250 µL inserts and the solvent was evaporated overnight using a Savant SpeedVac (Thermo Scientific). Samples were reconstituted in 200 µL MilliQ water-acetonitrile (1:1, v/v) containing 10 mg/L of L-tyrosine-(phenyl-3,5-$d_2$) (internal standard for HILIC positive mode).

Samples for lipid analysis were prepared by modification of a previously published method (Sarafian et al. 2014). In brief, 100 µL of each serum sample was transferred to 1.5 mL centrifuge tubes cooled on ice. 300 µL ice-cold *iso*-propanol (IPA) was added to each tube followed by vortex mixing for 10 s. Samples were left at −20 °C for 22 h and were then centrifuged at 14,000 RCF at 4 °C for 10 min. 250 µL of the supernatant was transferred to LC vials fitted with 250 µL inserts. Samples were stored at −20 °C for 24 h until LC–MS analysis.

### 2.5 LC–MS conditions for HILIC and lipid analyses

Polar and lipid extracts were analysed on a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisting of an Accela 1250 quaternary UHPLC pump, a Thermo-PAL auto-sampler fitted with a 15,000 psi injection valve and a 2 µL injection loop connected to a Q Exactive Orbitrap mass spectrometer with electrospray ionisation. Samples were cooled in the auto-sampler at 4 °C and the injection volume was 2 µL.

Polar metabolites were separated on a ZIC®-pHILIC polymeric bead based column (Merck, 100 mm × 2.1 mm, 5 µm i.d.) with a ZIC®-pHILIC metal-free guard column at 25 °C using a gradient elution program at a flow rate of 250 µL/min. The mobile phase consisted of acetonitrile-formic acid (99.9:0.1, v/v; solvent A) and water-ammonium formate (16 mM, pH 6.3; solvent B) using the following elution program: 97 % A (0–1 min), 97–70 % A (1–12 min), 70–10 % A (12–14.5 min), 10 % A (14.5–17 min), 10–97 % A (17–18.5 min), 97 % A (18.5–24 min). Eluent from the first 1.5 min and last 5 min of the chromatographic run was diverted to waste.

Mass spectrometric data of polar metabolites was collected in profile data acquisition mode covering a mass range of m/z 55–1,100 with a mass resolution setting of 35,000 and a maximum trap fill time of 100 ms using the Xcalibur software package (provided by the manufacturer). Samples were run in both positive and negative ionisation mode. Parameters in positive ion mode: spray voltage, 3.5 kV; capillary temperature, 320 °C; S-lens 55 V. Parameters in negative ion mode: spray voltage, −3.6 kV; capillary temperature, 320 °C; S-lens 55 V. The settings for the nitrogen source gas were identical for both modes: sheath gas, 50; auxiliary gas, 10; sweep gas, 5 (arbitrary units).

Lipids were separated on an Acquity CSH $C_{18}$ column (100 mm × 2.1 mm, 1.7 µm i.d.; Waters Corp., Milford, Massachusetts, US) at 65 °C using a gradient elution program at a flow rate of 600 µL/min. The mobile phase consisted of acetonitrile–water-formic acid (59.95:39.95:0.1 v/v + 10 mM ammonium formate; solvent A) and isopropyl alcohol-acetonitrile-formic acid (99.95:9.95:0.1 v/v + 10 mM ammonium formate; solvent B) using the following elution program: 85–70 % A (0–2 min), 70–52 % A (2–2.5 min), 52–18 % A (2.5–11 min), 18–1 % A (11–11.5 min), 1 % A (11.5–12 min), 1–85 % A (12–12.1 min), 85 % A (12.1–15 min). Eluent from the last min of the chromatographic run was diverted to waste.

Mass spectrometric data of lipids was collected in profile data acquisition mode covering a mass range of m/z 200-2,000 with a mass resolution setting of 35,000 and a maximum trap fill time of 250 ms using the Excalibur software package (provided by the manufacturer). Samples were run in both positive and negative ionisation mode. Parameters in positive ion mode: spray voltage, 4 kV; capillary temperature, 275 °C; S-lens 50 V. Parameters in negative ion mode: spray voltage, −4.0 kV; capillary temperature, 275 °C; S-lens −100 V. The settings for the nitrogen source gas were identical for both modes: sheath gas 40; auxiliary gas 10; sweep gas 5 (arbitrary units). Data dependent fragmentation spectra were collected for every lipidomics sample using the same mass range and mass resolution settings as in full scan mode, with an isolation window of 1.5 m/z and normalised collision energy of 30.

### 2.6 Data processing and analysis

Metabolites eluting between 3 and 18 min for the HILIC analysis and between 1 and 11 min for the lipidomics analysis were extracted from the LC–MS data and aligned using PhenoAnalyzer (SpectrometricWorks Ltd, Manchester, UK). The main peak detection settings for HILIC

data were area threshold, 100,000; minimum and maximum peak width threshold of 0.1 and 1.4 min respectively and m/z peak detection window, 5 ppm. For the lipidomics data, the main peak detection settings were area threshold, 500,000; minimum and maximum peak width threshold of 0.09 and 1.0 min respectively; m/z peak detection window, 10 ppm. The resulting data matrices of mass spectrometric features and peak areas were cleaned up by removing all features containing detected peak areas (peak area >0) in less than 30 % of the samples.

Data was further processed and analysed using the online tool MetaboAnalyst, versions 2.0 and 3.0 (Xia et al. 2015, 2012). Data from the four analytical streams were analysed separately. As a first step, data was filtered using relative standard deviation filtering, where the features with the largest absolute value of the coefficient of variation (expressed as a percentage) were removed from the dataset (Xia et al. 2012). HILIC positive data was normalised to the internal standard (tyrosine-$d_2$) and HILIC negative, lipidomics positive and negative data were normalised to the total sum of intensities of all mass spectrometric features to account for changes in instrument response over time. Different data transformation methods (log and cube root transformation) and scaling methods (no scaling, autoscaling, Pareto scaling and range scaling) were used for each dataset in order to obtain a data distribution as close as possible to normal. Log transformation and Pareto scaling resulted in the most normally distributed data for lipidomics positive; no transformation and autoscaling for lipidomics negative; cube root transformation and autoscaling for HILIC positive; and log transformation and Pareto scaling for HILIC negative.

Principal component analysis (PCA) was used to obtain an overview of each of the four datasets and find potential outliers. Next, pairwise comparisons of each of the treated groups (IN, KJ or RS) to the BD group in each dataset were made using Partial least squares-discriminant analysis (PLS-DA) and a *t* test. Both methods yielded very similar results. False discovery rate correction was used with the t-test to reduce the risk of Type I errors (false positives). Mass spectrometric features with FDR <0.05 were considered to differ significantly between treatment groups. The peak areas of these significant mass spectrometric features were re-extracted from the raw chromatogram using Xcalibur Quan-browser with a 5 ppm window for the target mass, and peaks with very low intensities were discarded.

### 2.7 Metabolite identification

$MS^2$ data was automatically collected for most ions with intensity $>2 \times 10^5$ in the lipidomics streams. $MS^2$ methods were set up for ions that were found to differ significantly in abundance between BD and treated in the HILIC streams (normalised collision energies of 70–80) and for ions in the lipid stream where $MS^2$ data had not been collected automatically (normalised collision energy of 30). Accurate mass in combination with the $MS^2$ data was used to putatively annotate polar and lipid metabolites by matching them to information in online databases: HMDB (http://www.hmdb.ca/) and LIPID MAPS (http://www.lipidmaps.org/) and METLIN (http://metlin.scripps.edu/). The identities of some of the polar metabolites were then confirmed by comparing accurate mass, retention time and $MS^2$ spectrum of the metabolite with that of the authentic reference compound analysed under identical experimental conditions, followed by spiking of the serum with authentic compound and repeating the analysis. Thus, lipids were identified to level 2 ('Putatively annotated compounds') and some polar metabolites to level 1 ('Identified compounds') (Sumner et al. 2007).

Identified lipids were named using the nomenclature used in the Human Metabolome Database. For example, lysophosphatidylcholine with a 16:0 fatty acid chain was named LPC(16:0) and phosphatidylcholine with one 22:4 and one 18:0 fatty acid chain was named PC(22:4/18:0). Neither the positions of the fatty acyl chains in PC nor the positions of the double bonds were determined in this study.

## 3 Results and discussion

### 3.1 Feed intake and growth performance

No significant differences in body weight gain ($P = 0.39$) or food intake ($P = 0.44$) were observed after 14 days of feeding BD or BD supplemented with 5 % RS, IN or KJ. All animals remained healthy throughout the experiment.

### 3.2 Short chain fatty acid analysis

All DRC affected SCFA acid concentrations in the caecum and colon compared with the BD-fed group (Table 2). The difference in total SCFA concentrations in the ileum of KJ and BD rats was a trend ($P = 0.07$). Mean caecal concentrations of propionic acid also tended to be higher ($P = 0.07$) in DRC-fed rats compared to BD-fed rats. The total concentration of propionic acid across the ileum, caecum, and colon of each rat, showed a significantly higher ($P < 0.05$) concentration of propionic acid in DRC-fed rats compared with BD-fed rats (BD 10.41 ± 0.52; IN 18.40 ± 3.68; KJ 17.97 ± 2.90; RS 20.58 ± 2.19 μmol/g ± SEM). Propionic acid is an important mediator of lipid metabolism by inhibiting hepatic lipid biosynthesis (Nishina and Freedland 1990; Demigne et al. 1995).

**Table 2** Mean SCFA concentrations (±standard error) in digesta from the ileum, caecum and colon of rats fed BD, IN, KJ or RS

| Tissue | Diet | Acetic acid (μmol/g) | Propionic acid (μmol/g) | Isobutyric acid (μmol/g) | Butyric acid (μmol/g) | Lactic acid (μmol/g) | Total SCFA (μmol/g) |
|---|---|---|---|---|---|---|---|
| Ileum | BD | 17.76 ± 3.48 | 0.34 ± 0.10 | 0.27 ± 0.070 | 0.82 ± 0.15 | 10.91 ± 1.00 | 36.4 ± 4.04 |
| Ileum | IN | 25.49 ± 3.36 | 0.60 ± 0.15 | 0.39 ± 0.10 | 1.31 ± 0.34 | 6.79 ± 1.00 | 44.1 ± 4.74 |
| Ileum | KJ | 27.44 ± 5.32 | 0.47 ± 0.11 | 0.27 ± 0.03 | 6.52 ± 3.17 | 12.92 ± 3.92 | 56.0 ± 7.61 |
| Ileum | RS | 22.30 ± 3.28 | 0.40 ± 0.10 | 0.28 ± 0.05 | 2.35 ± 1.35 | 9.90 ± 1.10 | 40.45 ± 3.78 |
| Caecum | BD | 32.39 ± 2.46 | 8.81 ± 0.51 | 1.39 ± 0.06 | 3.95 ± 0.45 | 6.10 ± 0.98 | 56.51 ± 2.90 |
| Caecum | IN | 35.84 ± 6.38 | 12.94 ± 2.06 | 0.84 ± 0.11 | 6.52 ± 1.48*** | 4.52 ± 0.72 | 67.26 ± 8.89 |
| Caecum | KJ | 37.50 ± 3.80 | 12.57 ± 1.20 | 1.16 ± 0.11 | 13.99 ± 2.26*** | 6.05 ± 0.36 | 76.51 ± 5.67 |
| Caecum | RS | 36.36 ± 2.81 | 14.08 ± 1.41 | 1.13 ± 0.07 | 8.97 ± 1.02*** | 4.50 ± 0.56 | 68.74 ± 4.26 |
| Colon | BD | 21.60 ± 2.77 | 1.27 ± 0.48 | 0.74 ± 0.16 | 2.09 ± 0.41 | 3.58 ± 0.45 | 35.61 ± 3.95 |
| Colon | IN | 29.38 ± 2.23 | 4.90 ± 1.77 | 0.54 ± 0.10 | 5.85 ± 1.24*** | 4.12 ± 0.34 | 52.71 ± 5.39** |
| Colon | KJ | 32.66 ± 2.71* | 3.57 ± 1.47 | 0.76 ± 0.12 | 9.47 ± 1.22*** | 5.59 ± 0.56 | 62.20 ± 4.14** |
| Colon | RS | 37.08 ± 4.58* | 5.20 ± 1.31 | 0.46 ± 0.07 | 5.61 ± 0.60*** | 17.11 ± 9.08 | 75.04 ± 10.54** |

Asterisks are indicating a significant difference in SCFA from BD (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$)

Furthermore, propionate has also been shown to lower blood glucoses and alter high-density lipoprotein and triglyceride concentrations in human subjects (Todesco et al. 1991). Given the altered serum lipid profiles in IN fed rats (see Sect. 3.4), it seems likely that these effects are mediated, at least in part, by the elevated levels of propionic acid found in the small and large bowel DRC fed rats.

In the caecum, butyric acid concentrations were significantly higher in all DRC groups ($P < 0.001$) compared to BD (Table 2). Similarly, colon concentrations of butyric acid were also significantly higher ($P < 0.001$) in DRC fed rats compared to BD (Table 2). In addition, colon concentrations of acetic acid were higher ($P < 0.05$ in RS and KJ fed rats compared to BD (Table 2). Like propionate, acetate also plays a role in lipid metabolism. In mice, acetic acid is able to suppress fat deposition by up regulating liver expression of fatty acid oxidation enzymes (Kondo et al. 2009) and through activation of the free fatty acid receptor GPR43 (Kimura et al. 2013). Acetic acid activation of GPR43 can also stimulate the release of leptin from mouse mesenteric adipocytes (Zaibi et al. 2010), leading to inhibition of hunger.

### 3.3 LC–MS peak detection and data analysis

Peak picking of mass/retention time pairs and chromatographic alignment resulted in data matrices of mass spectrometric features and peak areas for each of the four analytical streams. The number of mass spectrometric features detected was: HILIC positive mode, 1459; HILIC negative mode, 1252; lipidomics positive mode, 2288; and lipidomics negative mode, 1130. These numbers were reduced to 875, 751, 1372 and 678 mass spectrometric features respectively, following relative standard deviation filtering.

Principal component analysis of data from each of the four analytical streams showed that there were no major differences between treatment groups; the only trend was that the IN group seemed different from the other three treatment groups in lipidomics positive, and that the KJ group seemed slightly different from the other three treatment groups in HILIC positive (Fig. S1).

### 3.4 Lipid analysis

The addition of DRC into the diet affected serum lipid profiles in all three treatment groups (compared with BD), however the effect was most markedly in the IN group.

Univariate analysis (*t* test, FDR) of the lipid data showed that 55 mass spectrometric features differed significantly between the IN and BD groups in positive mode and 14 in negative mode. After removal of isotope peaks the list of significant features was reduced to 34 in positive mode and 7 in negative mode. Peak areas for these peaks were assessed in the raw chromatograms, and very small peaks and unresolved peaks were removed. Most of the lipids were putatively characterised from accurate mass and $MS^2$ fragmentation data, and the majority of the lipids were either *lyso*-phosphatidyl cholines (LPC) or phosphatidyl cholines (PC). 11 out of 14 lipids were more abundant in the IN-treated group, and 3 were more abundant in the BD group (metabolites 1–14, Table 3; Fig. S2).

LPCs and PCs were characterised based on their accurate mass and their characteristic $MS^2$ fragmentation pattern (Hsu and Turk 2009; Murphy and Axelsen 2011). LPCs were detected as [M+H] in positive mode, and as

formate adducts $[M+HCOO]^-$ in negative mode. The base peak in the positive mode $MS^2$ spectrum was m/z 184.0728 which corresponds to phosphocholine. In some cases, a small fragment at [M+H-18] was observed, corresponding to loss of water from the parent ion. The negative mode $MS^2$ spectra confirmed the identity of the fatty acyl chain of the LPC. In total, 4 LPCs were identified: LPC(22:4) and LPC(22:5) were more abundant in the IN-treated group, whereas LPC(20:5) and LPC(22:6) were more abundant in the BD group (metabolites 1–3 and 5, Table 3).

PCs were detected as $[M+H]^+$, and in some cases also as $[M+Na]^+$, in positive mode. Just like the LPCs, PCs were also detected as the formate adduct $[M+HCOO]^-$ in negative mode. The base peak in the positive mode $MS^2$ spectra of $[M+H]^+$ was m/z 184.0728 corresponding to phosphocholine. In most cases this was the only peak found in the positive mode $MS^2$ spectrum, and unlike LPCs no fragment arising from loss of water from the molecular ion was found. The sodium adducts $[M+Na]^+$ fragmented quite differently from the $[M+H]^+$ ions (Hsu and Turk 2009). The two fatty acyl chains in the PC were identified from the negative mode $MS^2$ spectra (Table 3). In total, 5 phosphocholines were identified, all of which were more abundant in the IN-treated group: PC(22:5/16:0), PC(22:4/16:0), PC(22:5/18:0), PC(22:4/18:0) and PC(22:5/20:4) (Table 2).

The increase in serum LPC(22:4), LPC(22:5), PC(22:5/16:0), PC(22:4/16:0), PC(22:5/18:0), PC(22:4/18:0) and PC(22:5/20:4) and the decrease in LPC(20:5) and LPC(22:6) following IN intake for 14 days points to effects on both the *n*-6 and *n*-3 polyunsaturated fatty acid (PUFA) metabolism pathways. The results suggest that the *n*-6 pathway, in which linoleic acid is converted in several steps to arachidonic acid (20:4) then to adrenic acid (22:4) and finally to docosapentaenoic acid (DPA, 22:5), is upregulated whereas the *n*-3 pathway, in which α-linolenic acid is converted in several steps to eicosapentaenoic acid (EPA, 20:5) then to docosapentaenoic acid (DPA, 22:5) and finally to docosahexaenoic acid (DHA, 22:6), is downregulated (Fig. 1). However, DPA is also part of the *n-3* pathway. The lipid analysis method used in this study is unable to distinguish between DPA (*n-3*) and DPA (*n-6*) resulting in uncertainty around which DPA isomer is the more abundant and thus which pathway is affected.

Previous studies in humans have shown that a decrease in EPA and DHA concentrations in serum might indicate a lower omega-3 index (concentration of EPA + DHA as a percentage of total fatty acids), which has been associated with increased risk of cardiovascular disease in some studies (von Schacky 2014). However, results from other studies indicate that the relationship between cardiovascular disease and lipids is more complex (Rasmiena et al. 2013; Bellis et al. 2014). EPA and DHA are also associated with reducing inflammation (Weldon et al. 2007; Knoch et al. 2009) and therefore decreases in these fatty acids would seem to be undesirable. In addition, *n-3* PUFA, such as EPA and DHA, regulate hepatic lipid metabolism by activating fatty acid oxidation and inhibiting fatty acid synthesis with changes in lipid composition throughout the body as a result (Jump et al. 2006). Such changes in lipid composition can in turn contribute to the onset of atherosclerosis, diabetes and obesity (Jump 2011). While IN is regarded as a beneficial food for bowel and overall health in humans (Slavin 2013), the results of the present rat study show that the effects of foods are complex and consequently predictions of their functions are equally complex. Although IN appears to have unfavourable effects on lipid metabolism by decreasing serum levels of EPA and DHA no weight gain was observed in the IN-fed rats compared with BD-fed rats. Although rat models come with inherent limitations with regard to their suitability as a model for human lipid metabolism, they can provide important mechanistic information for informing further studies (Iannaccone and Jacob 2009). These studies may include liver lipid measurements, long term feeding studies, and human clinical studies.

The remaining five lipids that were significantly different between the IN and BD groups could not be identified to level 2 due to inconclusive $MS^2$ spectra and/or limited success with database searches. Further univariate analysis (*t* test, FDR) of the lipid data showed that two mass spectrometric features differed significantly between KJ and BD groups in positive mode (metabolites 15 and 16, Table 3; Fig. S2). Unfortunately, neither of these lipids could be identified from their accurate mass, $MS^2$ spectra or database searches.

The effects of dietary supplementation with RS on serum lipids were minimal; only two mass spectrometric features were found to be significantly more abundant in the RS-treated group compared with the BD group (metabolites 17–18, Table 3; Fig. S2). Neither of these lipids could be identified from their accurate mass, $MS^2$ spectra or database searches, but their fragmentation pattern indicates that they are likely related. Both fragment by neutral loss of $CO_2$ (loss of 43.9890 Da), indicating the presence of a carboxyl group, and neutral loss of $C_3H_2O_3$ (loss of 86.0010 Da). The mass difference between these two lipids is 28.0318 mass units. This could indicate that the two lipids are homologues differing by on $C_2H_4$ unit. Neither metabolite was detected in positive mode. Both lipids elute early indicating quite polar structures. The accurate mass indicates the molecular formula $C_{18}H_{29}O_4$ for the ion at m/z 309.2079 and $C_{20}H_{33}O_4$ for m/z 337.2397. These suggested molecular formulae seem plausible given the fragmentation pattern which indicates the presence of at least 3 oxygens and also supports the

**Table 3** Mass spectrometric features from the lipid analysis differing significantly (FDR > 0.05) between rats fed BD and rats fed BD supplemented with IN, KJ or RS

| Metabolite no | m/z (for parent ion) | Mode | r.t. (min) | Ion detected | Identification | Identification level[a] | FDR | Level | Important $MS^2$ fragments |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 542.3245 | Pos | 1.36 | $[M+H]^+$ | LPC(20:5) | 3 | 0.028 | BD > IN | 524.3226, |
| | 586.3149 | Neg | | $[M+HCOO]^-$ | | | | | 184.0729, |
| | | | | | | | | | 104.1070* |
| | | | | | | | | | 301.2182 |
| 2 | 568.3404 | Pos | 1.53 | $[M+H]^+$ | LPC(22:6) | 3 | 0.043 | BD > IN | 184.0731 |
| | 612.3307 | Neg | | $[M+HCOO]^-$ | | | | | 327.2330 |
| 3 | 570.3543 | Pos | 2.20 | $[M+H]^+$ | LPC(22:5) | 3 | 0.0015 | IN > BD | 184.0732, |
| | 614.3463 | Neg | | $[M+HCOO]^-$ | | | | | 104.1072 |
| | | | | | | | | | 329.2487, |
| | | | | | | | | | 285.2588 |
| 4 | 572.3611 | Pos | 2.15 | $[M+H]^+$? | Unknown lipid | 3 | 0.0014 | IN > BD | – |
| | 616.3515 | Neg | | $[M+HCOO]^-$ | | | | | – |
| 5 | 572.3713 | Pos | 2.34 | $[M+H]^+$ | LPC(22:4) | 3 | 0.017 | IN > BD | 554.3592, |
| | 616.3617 | Neg | | $[M+HCOO]^-$ | | | | | 184.0729, |
| | | | | | | | | | 104.1070 |
| | | | | | | | | | 331.2647 |
| 6 | 803.6103 | Pos | 6.00 | $[M+H]^+$ | Unknown lipid | 3 | $9.0 \cdot 10^{-4}$ | IN > BD | 184.0728 |
| | 847.6007 | Neg | | $[M+HCOO]^-$ | | | | | – |
| 7 | 807.6372 | Pos | 7.01 | $[M+H]^+$ | Unknown lipid | 3 | 0.049 | IN > BD | 184.0728 |
| | 851.6276 | Neg | | $[M+HCOO]^-$ | | | | | – |
| 8 | 808.5845 | Pos | 6.89 | $[M+H]^+$ | PC(22:5/16:0) | 3 | 0.0014 | IN > BD | 184.0734 |
| | 830.5669 | Pos | | $[M+Na]^+$ | | | | | 647.5015, |
| | 852.5833 | Neg | | $[M+HCOO]^-$ | | | | | 146.9819, |
| | | | | | | | | | 86.0969 |
| | | | | | | | | | 329.2487, |
| | | | | | | | | | 255.2328 |
| 9 | 823.6034 | Pos | 7.13 | Unknown | Unknown lipid | 3 | 0.025 | IN > BD | – |
| 10 | 810.6002 | Pos | 7.13 | $[M+H]^+$ | PC(22:4/16:0) | 3 | 0.0073 | IN > BD | 184.0734 |
| | 854.5917 | Neg | | $[M+HCOO]^-$ | | | | | 331.2646, |
| | | | | | | | | | 255.2329 |
| 11 | 836.6164 | Pos | 7.62 | $[M+H]^+$ | PC(22:5/18:0) | 3 | 0.0015 | IN > BD | 184.0735 |
| | 858.5989 | Pos | | $[M+Na]^+$ | | | | | 675.5330, |
| | 880.6073 | Neg | | $[M+HCOO]^-$ | | | | | 146.9819, |
| | | | | | | | | | 86.0969 |
| | | | | | | | | | 329.2488, |
| | | | | | | | | | 283.2644 |
| 12 | 838.6323 | Pos | 7.88 | $[M+H]^+$ | PC(22:4/18:0) | 3 | 0.010 | IN > BD | 184.0735 |
| | 860.6140 | Pos | | $[M+Na]^+$ | | | | | 677.5481, |
| | 882.6230 | Neg | | $[M+HCOO]^-$ | | | | | 146.9819, |
| | | | | | | | | | 86.0969 |
| | | | | | | | | | 331.2644, |
| | | | | | | | | | 283.2643 |
| 13 | 856.5859 | Pos | 6.21 | $[M+H]^+$ | PC(22:5/20:4) | 3 | 0.0014 | IN > BD | 184.0728 |
| | 900.5760 | Neg | | $[M+HCOO]^-$ | | | | | 329.2496, |
| | | | | | | | | | 303.2332 |
| 14 | 880.5850 | Pos | 5.76 | $[M+H]^+$ | Unknown lipid | 3 | 0.0051 | BD > IN | – |
| | 924.5754 | Neg | | $[M+HCOO]^-$ | | | | | – |

**Table 3** continued

| Metabolite no | m/z (for parent ion) | Mode | r.t. (min) | Ion detected | Identification | Identification level[a] | FDR | Level | Important $MS^2$ fragments |
|---|---|---|---|---|---|---|---|---|---|
| 15 | 586.5410 | Pos | 9.84 | Unknown | Unknown lipid | 3 | 0.040 | BD > KJ | – |
| 16 | 720.5540 | Pos | 6.83 | $[M+H]^+$ | Unknown lipid | 3 | 0.017 | KJ > BD | – |
| | 764.5443 | Neg | | $[M+HCOO]^-$ | | | | | 255.2334, 241.2176 |
| 17 | 309.2079 | Neg | 1.16 | Unknown | Unknown | 4 | 0.029 | RS > BD | 265.2179, 223.2068 |
| 18 | 337.2397 | Neg | 1.75 | Unknown | Unknown | 4 | $2.37 \cdot 10^{-4}$ | RS > BD | 293.2498, 251.2387 |

[a] Levels of metabolite identification (1–4) according to (Sumner et al. 2007): *level 1* identified, *level 2* putatively annotated, *level 3* putatively characterised, *level 4* unknown

theory that the difference between the two lipids is a $C_2H_4$ unit. Since it is unclear whether the detected ions are $[M-H]^-$ or $[M+HCOO]^-$ database searches (HMDB, LIPIDMAPS) were conducted for both options, but results were inconclusive pointing towards either eicosanoids, sterol lipids or oxidised lipids.

### 3.5 HILIC LC–MS analysis

Univariate analysis (*T* test, FDR) of the HILIC data showed that there were clear differences between the KJ-treated group and the BD group: 3 mass spectrometric features in positive mode and 4 in negative mode differed significantly between the two groups, and all of them were more abundant in the KJ-treated group (Table 4, Fig. S2).

Of these significant mass spectrometric features, the three that were found in HILIC positive mode were significant with FDR of $1.54 \times 10^{-8}$ to $4.97 \times 10^{-12}$ (metabolites 1–3, Table 4). Two of these metabolites were identified to level 1, and were found to be trigonelline (CHEBI:18123) and β-alanine betaine (CHEBI:28825). Both of these metabolites were detected in HILIC positive mode only, which is expected due to their permanently positively charged nitrogen. β-Alanine betaine (metabolite 1, Table 4) fragments by losing either trimethylamine ($N(CH_3)_3$), $HOCOCH{=}CH_2$ or $HOCOCH_2CH_3$ to give rise to fragments m/z 73.0282, 60.0808 and 58.0652, respectively, which is consistent with previous reports (Naresh Chary et al. 2012) (Figs. S3 and S4). Trigonelline (metabolite 2, Table 4) fragments by expelling CO and $CO_2$, forming fragment ions m/z 110.0593 and 94.0646 (Figs. S5 and S6). The metabolite that differed most between KJ and BD (metabolite 3, Table 4) could not be identified from its accurate mass, $MS^2$ spectrum and database searches. However, since its fragmentation pattern is similar to that of β-alanine betaine and the mass difference between the two is 18.0104 Da, metabolite 3 could potentially be a hydroxylated β-alanine betaine, with the hydroxyl group likely being either in the 2- or 3-position. This structure would explain the fragmentation pattern, i.e. loss of formic acid, $HOCOCH{=}CH_2$ or $HOCOCH_2CH_3$ to give rise to fragments m/z 102.0907, 60.0807 and 58.0652 (Fig. S7). Unlike β-alanine betaine, metabolite 3 does not fragment by expelling $N(CH_3)_3$. Instead, it loses formic acid to form a resonance-stabilised cation. The proposed structure of metabolite 3 is also supported by its retention time of 9.90 min (compared with β-alanine betaine at 9.05 min) indicating a more polar compound.

Trigonelline is an alkaloid and an osmolyte found in many plants, seeds and vegetables and is thus obtained from dietary sources. For instance, it has been suggested as a biomarker of coffee intake in humans (Lang et al. 2011). However, trigonelline is also a product of niacin metabolism: niacin is methylated to form trigonelline, which is excreted via the urine. Niacin is either obtained from the diet or synthesised from tryptophan by the large bowel microbiota.

β-Alanine betaine is also an osmolyte which is found in plants (Duhazé et al. 2003; Tipirdamaz et al. 2006), soil bacteria (Mohamed Ahmed et al. 2009, 2010) and in some corals (Hill et al. 2010). It can act as an osmolyte and an osmoprotectant in plants (Maimaiti et al. 2014; Murakeözy et al. 2003; Murata et al. 2012; Raman and Rathinasabapathi 2003). Since there is no evidence in the literature that this metabolite can be produced by humans or bowel microbiota, this metabolite might originate from the KJ corm itself. In fact, it seems likely that metabolites 1–3 (Table 4) all originate from the KJ and are not a result of changes in rat metabolism induced by KJ. If that is indeed the case, these three metabolites could potentially be used as biomarkers of KJ intake. Such biomarkers are valuable in nutritional studies for assessing dietary intake.

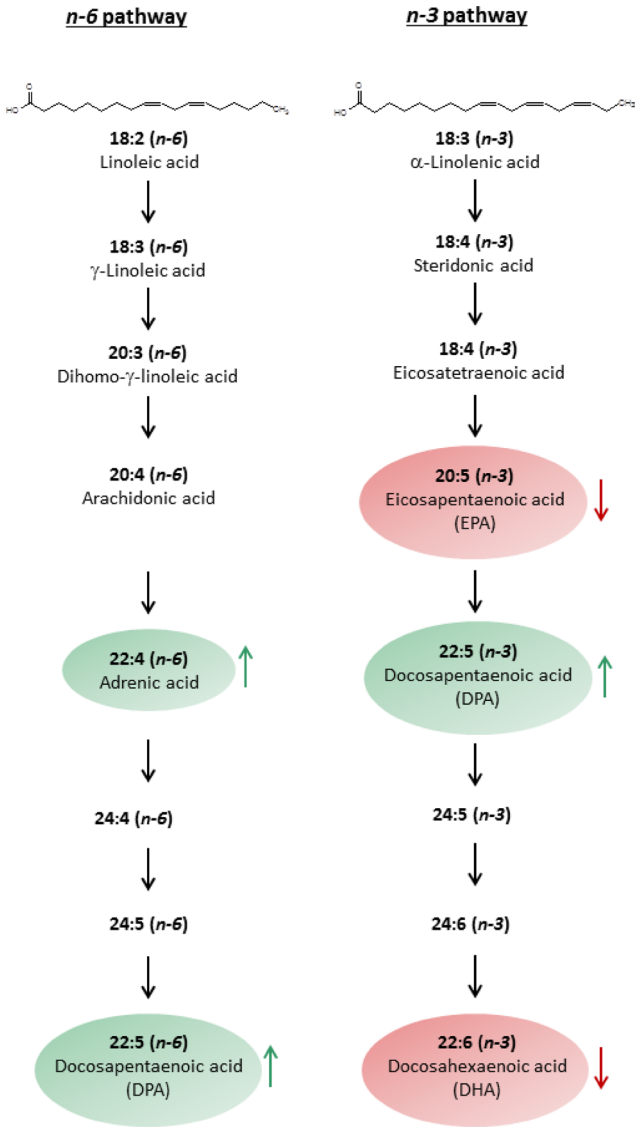


**Fig. 1** Pathways for synthesis of *n-6* and *n-3* PUFAs in mammals. DPA is synthesised from linolenic acid by the *n-6* pathway and DHA from α-linolenic acid by the *n-3* pathway. Fatty acids shaded in *green* were more abundant in the IN-fed group compared with the BD-fed group. Fatty acids shaded in *red* were less abundant in the IN-fed group compared with the BD-fed group (Color figure online)

The other polar metabolites found to be more abundant in the KJ-treated group, ions m/z 373.6087, 375.6064 and 377.6042, were likely related, with a retention time of 11.64 min. The mass spectra of these three ions show groups of peaks, indicating a polymeric structure.

IN or RS treatment did not affect the abundance of polar metabolites in blood serum.

### 3.6 Effects of different DRC on lipid metabolism

SCFA produced by the bowel microbiota are known to affect lipid metabolism of the host (dos Reis et al. 2015). In the current study IN, KJ and RS all caused increased SCFA production in the intestine. However, only IN had an impact on the serum lipid profile compared to KJ or RS supplementation. These results indicate that the changes in lipid metabolism caused by IN are not only derived from the SCFA. A further possible mechanism for IN induced changes on lipid metabolism may relate to microbiota-mediated changes in lipid metabolism through the activity of bile salt hydrolases (BSH). These enzymes are produced by many members of the microbiota, including *Bifidobacterium* (Jarocki et al. 2014) and *Lactobacillus* (Altermann et al. 2005; McAuliffe et al. 2005), which are increased by diets containing IN (Tannock et al. 2014; Meyer and Stasse-Wolthuis 2009; Tarini and Wolever 2010). They transform bile salts which play a major role in absorption and metabolism of lipids. Bile salts are produced from cholesterol in the liver and modification of bile acids by BSH enzymes reduces reabsorption rates of the conjugated bile acids, therefore increasing the rate of faecal excretion (Öner et al. 2014; Tsai et al. 2014; Lye et al. 2010; Gérard 2013). As a result, more cholesterol is drawn from the circulation in order to synthesise more bile acids. Through this pathway, the microbiota can influence lipid metabolism by modulating the circulation and recycling of LDL cholesterol (Joyce et al. 2014). In addition, BSH activity by the microbiota can lead to increased hydrogel-forming properties of some bile salts, further decreasing the reabsorption of bile salts in the intestine (Joyce et al. 2014).

The physiochemical structure of DRC also plays an important role in regulating lipid metabolism. Dietary fibre is able to bind bile salts, phospholipids and cholesterol (van Bennekum et al. 2005; Vahouny et al. 1980), which may change their bioavailability or activity. However, the binding capacity of each DRC is affected by factors such water holding capacity, solubility and viscosity.

## 4 Concluding remarks

DRC are a chemically diverse group of complex polysaccharides, and their equally diverse biological effects are evident from this study. Although all three tested DRC (IN, KJ and RS) increased the production of SCFA in the intestine they had very different effects on serum metabolites, in particular serum lipids. Whereas KJ and RS had little effect, IN clearly impacts on phosphatidylcholine and *lyso*-phosphatidylcholine levels in serum: The decrease in EPA and DHA-containing phospholipids and the increase in adrenic acid and likely DPA-containing phospholipids indicates changes in both *n-3* and *n-6* fatty acid metabolism following IN intake which may lead to altered hepatic lipid metabolism.

**Table 4** Mass spectrometric features from the HILIC/MS analysis differing significantly (FDR > 0.05) between rats fed BD and rats fed BD supplemented with IN, KJ or RS

| Metabolite no | m/z (for parent ion) | Mode | r.t. (min) | Ion detected | Identification | Identification level[a] | FDR | Level | Important $MS^2$ fragments |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 132.1020 | Pos | 9.05 | $[M+H]^+$ | β-Alanine betaine | 1 | $5.05 \times 10^{-8}$ | KJ ≫ BD | 73.0282, 60.0808, 59.0729, 58.0652 |
| 2 | 138.0549 | Pos | 9.53 | $[M+H]^+$ | Trigonelline | 1 | $1.54 \times 10^{-8}$ | KJ ≫ BD | 110.0593, 94.9891 |
| 3 | 148.0968 | Pos | 9.90 | $[M+H]^+$ | Hydroxylated β-alanine betaine? | 3 | $4.97 \times 10^{-12}$ | KJ ≫ BD | 102.0907, 60.0807, 59.0729, 58.0651 |
| 4 | 373.6087 | Neg | 11.64 | Unknown | Polymer? | 4 | 0.014 | KJ > BD | – |
| 5 | 377.6042 | Neg | 11.64 | Unknown | Polymer? | 4 | 0.014 | KJ > BD | – |
| 6 | 375.6064 | Neg | 11.64 | Unknown | Polymer? | 4 | 0.014 | KJ > BD | – |
| 7 | 473.7461 | Neg | 11.66 | Unknown | Unknown | 4 | 0.049 | KJ > BD | – |

[a] Levels of metabolite identification (1-4) according to (Sumner et al. 2007)

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**Compliance with ethical standards**

**Conflict of interest** All authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving animals were in accordance with the ethical standards of the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand) according to the New Zealand Animal Welfare Act 1999.

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