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## Title page

### Systemic Availability and Metabolism of Colonic-derived Short-chain Fatty Acids in Healthy Subjects – a stable isotope study

#### Short-chain Fatty Acid Systemic Availability and Metabolism in humans

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### Key Point Summary

- SCFAs are bacterial metabolites produced during colonic fermentation of undigested carbohydrates, such as dietary fibre and prebiotics, and could mediate the interaction between diet, the microbiota and the host.
- We quantified the fraction of colonic administered SCFA that could be recovered in the systemic circulation, the fraction that was excreted via breath and urine and the fraction that was used as a precursor for glucose, cholesterol and fatty acids.
- This information is essential to understand the molecular mechanisms by which SCFA beneficially affect physiological functions such as glucose and lipid metabolism and immune function.

### Abstract

The short-chain fatty acids (SCFAs), acetate, propionate and butyrate are bacterial metabolites that mediate the interaction between diet, the microbiota and the host. In this study, the systemic availability of SCFAs and their incorporation into biologically relevant molecules was quantified. Known amounts of  $^{13}\text{C}$ -labelled acetate, propionate and butyrate were introduced in the colon of 12 healthy subjects using colon delivery capsules and plasma levels of  $^{13}\text{C}$ -SCFAs and of  $^{13}\text{C}$ -glucose,  $^{13}\text{C}$ -cholesterol and  $^{13}\text{C}$ -fatty acids were measured. The butyrate producing capacity of the intestinal microbiota was quantified as well. Systemic availability of colonic-administered acetate, propionate and butyrate was 36%, 9% and 2%, respectively. Conversion of acetate into butyrate (24%) was the most prevalent interconversion by the colonic microbiota and was not related to the butyrate-producing capacity in the faecal samples. Less than 1% of administered acetate was incorporated into cholesterol and <15% in fatty acids. On average, 6% of colonic propionate was incorporated into glucose. The SCFAs were mainly excreted via the lungs after oxidation to  $^{13}\text{CO}_2$  whereas less than 0.05% of the SCFAs were excreted into urine. These results will allow future evaluation and quantification of SCFAs production from  $^{13}\text{C}$ -labelled fibres in the human colon by measuring  $^{13}\text{C}$ -labelled SCFA concentrations in blood.

### Abbreviation list

AUC, area under the curve; CDC: colonic delivery capsule; Cl, clearance;  $C_{ss}$ , steady state concentration; cumPDR, cumulative percentage of administered dose; D, administered dose; FFAR, free fatty acid receptor; GC-C-IRMS, gas chromatography combustion isotope ratio mass spectrometry; GC-MS, gas chromatography mass spectrometry; GPR, G-protein coupled receptor; I, infusion rate; MCT, monocarboxylate transporter; OCTT, orocecal transit time; RT-PCR, real time polymerase chain reaction; SA, systemic availability; SCFA, Short-chain fatty Acid; TCA, tricarboxylic acid;  $T_{20\%}$ , time of release of the CDC

### Body of paper

#### Introduction

Short chain fatty acids, mainly comprising acetate, propionate and butyrate are produced in the large intestine by microbial degradation of undigested carbohydrates and to a lesser extent proteins. They are increasingly recognised as signalling molecules that mediate the interaction between the diet, the gut microbiota and the host. Locally in the gut, SCFA are crucial for intestinal health because they serve as the major energy substrates for the colonocytes and because of their anti-inflammatory and anti-carcinogenic properties (Hamer *et al.*, 2008). In addition, they affect gut and host metabolism by activating the G-protein coupled cell surface receptors GPR-41 and GPR-43 (Brown *et al.*, 2003), later renamed as free fatty acid receptors (FFAR)-3 and FFAR-2, that are involved in the regulation of glucose and lipid metabolism (den Besten *et al.*, 2013b). Activation of those receptors on the enteroendocrine L-cells in the colon results in the secretion of the gut-derived satiety hormones glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), providing a mechanism for the beneficial effect of SCFA on energy intake and satiety (Canfora *et al.*, 2015). Finally, a fraction of the colonic produced SCFA reaches the systemic circulation and directly affects the function and metabolism of peripheral organs and tissues such as the liver, the pancreas, adipocytes, immune cells and skeletal muscle tissue. As a consequence, SCFA have been involved in maintaining glucose and lipid metabolism and may provide an important target to tackle disorders that are associated with disturbances of glucose and lipid metabolism such as obesity, metabolic syndrome or type 2 diabetes.

To further understand how SCFAs provide a mechanistic link between the diet, the microbiota and health benefits, it is absolutely necessary to quantify the extent to which fermentation-derived SCFAs reach the systemic circulation. Quantitative information on the assimilation of SCFAs into biologically relevant molecules in humans may also contribute to unravelling the mechanism via which SCFAs elicit systemic effects. Currently, SCFAs are most often measured in faeces or in fasting blood samples. However, because of the rapid absorption of SCFAs by the colonocytes, only an estimated 5% of produced SCFAs is excreted in faeces (McNeil *et al.*, 1978) rendering faecal measurements not representative for colonic SCFA production. In addition, the extensive metabolism in colonocytes and the liver results in low plasma levels of SCFAs.

In this work, stable isotope technology was applied to determine the systemic availability of each SCFA after colonic administration of  $^{13}\text{C}$ -labelled SCFAs. The systemic availability is the fraction of an administered dose of unchanged substrate that reaches the systemic circulation. In addition, the occurrence of interconversions was evaluated and the incorporation of the colonic-derived  $^{13}\text{C}$ -SCFAs into glucose, free fatty acids and cholesterol was quantified. Stable isotope technology is a very sensitive analytical technique that allows measuring selectively in plasma those SCFAs that originate from the colon.

## Materials & Methods

### Ethical Approval

The study procedure was approved by the Ethics Committee of the Leuven University Hospital, Leuven, Belgium. Written, informed consent was obtained from all subjects and the study conformed to the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (NCT01757379).

### Colonic Delivery of SCFAs

Known amounts of the individual  $^{13}\text{C}$ -labelled SCFAs ( $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate or  $^{13}\text{C}$ -butyrate) were delivered into the colon of healthy subjects using colon delivery capsules (CDCs). Hard gelatine capsules (size 0) were filled with either approximately 200 mg of  $^{13}\text{C}$ -labelled sodium acetate ([1- $^{13}\text{C}$ ]acetate), 170 mg of  $^{13}\text{C}$ -labelled sodium propionate ([1- $^{13}\text{C}$ ]propionate) or 495 mg of  $^{13}\text{C}$ -labelled sodium butyrate ([1- $^{13}\text{C}$ ]butyrate) (Euriso-top,

St Aubin, Cédex, France) together with 9.0% (w/w) citric acid (Sigma-Aldrich, Bornem, Belgium). The addition of citric acid was necessary to keep the pH of the capsule contents below pH 6.5 and to prevent in this way dissolution of the coating from the inside-out due to water intrusion into the capsules. Before and after filling, the capsules were weighed to determine their exact content. The capsules were coated manually with a pH-dependent film made up of Eudragit S100 (Evonik, Darmstadt, Germany) with diethyl phthalate (Omega Pharma, Nazareth, Belgium) (25% w/w with respect to polymer) as plasticizer. Eudragit S100 only dissolves at a pH > 7.0 (Chourasia *et al.* 2003). The coating thickness was estimated from the increase in weight after coating and expressed in mg/cm<sup>2</sup> capsule surface. Coating thickness was varied between 13.5 mg/cm<sup>2</sup> and 23.3 mg/cm<sup>2</sup>.

Similar capsules containing 5.2 mg (in acetate capsules) or 4.0 mg (in propionate and butyrate capsules) methylene blue were prepared to assess the pH resistance of the polymer coating. The release of methylene blue from the coated capsules was monitored during *in vitro* dissolution tests in sodium phosphate buffers (0.1 M) of pH 6.3, 6.8, 7.0 and 7.2 according to the USP XXV paddle method (150 rpm) at 37 °C. The concentration of the marker was quantified colorimetrically at 664 nm. All measurements were performed in duplicate.

### Study Design

Twelve healthy subjects (7 F/ 5 M, aged 26 ± 6 years, BMI 22 ± 3 kg/m<sup>2</sup>) participated in a randomized cross-over study. Exclusion criteria were previous abdominal surgery (except from appendectomy), history of chronic gastro-intestinal conditions such as inflammatory bowel diseases, irritable bowel syndrome and celiac disease, or being on a low calorie or vegetarian diet. Female subjects were excluded if pregnant or lactating. All subjects were free of medication influencing the gut transit or intestinal microbiota for 14 days and of antibiotics for 1 month. Intake of pre- and probiotics was prohibited during the study period. Subjects were also excluded if they had donated blood during the last three months, suffered from low blood haemoglobin levels or had participated in experiments involving ionizing radiation during the last year. Each subject performed three test days (one type of SCFA per test day) with one week in between. During the three days prior to a test day, the subjects consumed a low-fibre diet and avoided alcohol intake. On the evening prior to the test day, a non-fermentable standard meal (lasagne) was offered to avoid changes in colonic fermentation during the different test days. In the morning of the test, the subjects received

two coated capsules containing either  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate or  $^{13}\text{C}$ -butyrate with a standard breakfast (pancake, 250 kcal) labelled with inulin- $^{14}\text{C}$ -carboxylic acid (74 kBq, American Radiolabelled Chemicals, St. Louis, MO, USA) as a marker for orocecal transit time (OCTT) (Verbeke *et al.*, 2005). During each test day, a primed continuous intravenous infusion of deuterated SCFAs was applied ( $[^2\text{H}_3]$ -acetate:  $10 \mu\text{mol.kg}^{-1} + 20 \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ ;  $[^2\text{H}_5]$ -propionate:  $1 \mu\text{mol.kg}^{-1} + 2 \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ ;  $[^2\text{H}_7]$ -butyrate:  $0.5 \mu\text{mol.kg}^{-1} + 1 \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ ) to quantify the clearance of each SCFA. Before the breakfast and at regular time points during the day, breath, blood and urine samples were collected. Breath samples were collected every 20 min up to 10 h after breakfast. Blood samples were collected every hour during the first 4 h, every 20 min from 4 to 9 h and every 40 min from 9 h to 12 h. A basal urine sample and 0-4 h, 4-8 h, 8-12 h and 12-24 h urine fractions were collected in recipients containing neomycin to prevent bacterial growth. After 4 h and 8 h, the subjects received a standard non-fermentable meal (white bread with ham or cheese). Finally, all subjects delivered a faecal sample that within 10 h after collection was frozen at  $-80^\circ\text{C}$  until analysis.

### **Analytical Procedures**

***Analysis of Breath Samples.*** Measurements of  $^{13}\text{CO}_2$ ,  $\text{H}_2$  and  $^{14}\text{CO}_2$  in breath samples were performed as described previously (Verbeke *et al.*, 2005). The results for  $^{13}\text{CO}_2$  were expressed as cumulative percentages of administered dose (cumPDR) (Braden *et al.*, 2007). The time of release from the capsules ( $T_{20\%}$ ) was defined as the time at which 20% of the cumPDR was recovered in breath. The cumPDR values after 12 h were corrected for  $^{13}\text{CO}_2$  that is produced via oxidation but is not excreted using a correction factor of 0.55 as suggested by Maurer *et al.* (Maurer *et al.*, 2013). The OCTT was defined as the time at which a significant increase in  $^{14}\text{C}$  from background was seen in breath, *i.e.* 2.5 times the standard deviation of all previous points above the running average of all previous points (Verbeke *et al.*, 2005).

***Analysis of Total SCFA Concentrations in Plasma and Urine Samples.*** Total SCFA concentrations in plasma were measured using gas chromatography (GC) after purification and concentration of the samples with hollow fibre supported liquid membrane extraction as described by Zhao *et al.* (Zhao *et al.*, 2007). To measure total SCFA concentrations in urine, samples (3.0 mL) were spiked with 150  $\mu\text{L}$  internal standard mix (83  $\text{mg.L}^{-1}$  2-ethyl-butyric acid and 200  $\text{mg.L}^{-1}$  3-methyl-valeric acid) and 500  $\mu\text{L}$  of 1.0 M sodium hydroxide and concentrated to dryness. After addition of 200  $\mu\text{L}$  37.0% HCl to the residue, SCFAs were

extracted in 1.0 mL diethyl ether. The ether extracts were injected (0.5  $\mu$ L) in pulsed splitless mode into the injector at 200 °C. Chromatographic analysis was carried out on a fused-silica capillary column with Free Fatty Acid Phase (DB-FFAP 125–3237, J&W Scientific, Agilent Technologies, Santa Clara, California, USA) of 30 m  $\times$  0.53 mm i.d. coated with 0.50  $\mu$ m film thickness using an Agilent 6890N GC system equipped with a flame ionization detector (Agilent Technologies). Column temperature was started at 100 °C (3 min) and increased to 140 °C (5 min) at a rate of 4 °C/min followed by an increase to 235 °C (5 min) at a rate of 40 °C/min. Helium was used as carrier gas in a constant flow mode of 4.2 mL/min and the detector temperature was set at 240 °C. The flow rates of the detector gases hydrogen, air and nitrogen as makeup gas were 30, 300 and 20 mL/min, respectively. Data handling was carried out with a HP ChemStation Plus software (B.04.03, Agilent Technologies).

**Analysis of  $^{13}\text{C}$ - and  $^2\text{H}$ -enrichments of SCFA in Plasma and Urine Samples.** Plasma samples were deproteinised and extracted according to Morrison *et al.* (Morrison *et al.*, 2004) whereas urine samples were only dried and extracted. The extracted samples for analysis of  $^{13}\text{C}$ -enrichment of SCFAs were injected (4  $\mu$ L) in splitless mode at 240 °C into a Delta-XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Trace gas chromatograph and a combustion interface type 3 (GC-C-IRMS). An AT-Aquawax-DA column (30 m  $\times$  0.53 mm i.d. and 1.00  $\mu$ m film thickness; Grace, Lokeren, Belgium) was used. The initial oven temperature was 80 °C (3 min) and ramped to 140 °C at a rate of 4 °C/min followed by an increase to 240 °C (10 min) at a rate of 16 °C/min. Helium was used as carrier gas in a constant flow mode of 2.5 mL/min. After elution from the GC column, the compounds were oxidized into  $\text{CO}_2$  in the combustion reactor after which the enrichment was measured in the IRMS detector. To calculate the enrichment of the SCFAs from the measured  $^{13}\text{CO}_2$  enrichment, the number of labelled carbon atoms per molecule and the enrichment of the administered substrate ( $^{13}\text{C}$ -SCFAs) were taken into account. Data were processed using Isodat NT (version 2.0, Finnigan<sup>TM</sup>, Thermo Fisher Scientific).

For the analysis of the  $^2\text{H}$ -enrichments of SCFAs, 1  $\mu$ L of the extracted samples was injected at 240 °C in splitless mode into the injector of a gas chromatograph equipped with a quadrupole system (Trace GC Ultra and DSQ II, Thermo Electron Corporation, Waltham, MA, USA) with an AT-Aquawax DA column (30 m  $\times$  0.25 mm i.d. and 0.25  $\mu$ m film thickness; Grace). The initial oven temperature was 40 °C (3 min) and ramped to 140 °C at a rate of 4 °C/min followed by an increase to 240 °C (6 min) at a rate of 16 °C/min. Helium was used as carrier gas in a constant flow mode of 1 mL/min. The transfer line was

maintained at 240 °C. The mass spectrometer was operated in full scan monitoring mode ( $m/z$  33-650) and the source temperature was set at 250 °C. Xcalibur™ software (Thermo Fisher Scientific) was used for the automatization of the GC-MS system and for data acquisition.

**Analysis of  $^{13}\text{C}$ -glucose.** Total plasma glucose concentrations and  $^{13}\text{C}$ -glucose enrichments were measured using GC-C-IRMS after derivatization of glucose to its aldonitrile penta acetate derivative according to Schierbeek *et al.* (Schierbeek *et al.*, 2009). The derivatized samples were injected (1  $\mu\text{L}$ ) in splitless mode into the GC-C-IRMS instrument equipped with an AT-5ms capillary column (30 m x 0.32 mm i.d. and 1.0  $\mu\text{m}$  film thickness; Grace). The injector temperature was set at 250 °C and the initial oven temperature amounted to 95 °C (30 s) and was increased to 300 °C (15 min) at 20 °C/min. The helium carrier flowed at a constant rate of 1.5 mL/min. To calculate the enrichment of  $^{13}\text{C}$ -glucose from the measured enrichment of  $^{13}\text{CO}_2$ , it was assumed that one propionate molecule was incorporated per glucose molecule.

**Analysis of  $^{13}\text{C}$ -free fatty acids.** The concentrations and  $^{13}\text{C}$ -enrichment in plasma of palmitic (C16:0), stearic (C18:0) and oleic (C18:1, n-9) acid were measured using GC-C-IRMS after prior conversion of the fatty acids into their corresponding methyl esters as described by Wang *et al.* (Wang *et al.*, 2012). Samples were injected (1  $\mu\text{L}$ ) at 250 °C in a splitless mode into an AT-aquawax-DA column (30 m x 0.53 mm i.d. and 1.0  $\mu\text{m}$  film thickness; Grace). The initial oven temperature was 50 °C (2 min) and ramped to 200 °C (10 min) at a rate of 10 °C/min followed by an increase to 220 °C (15 min) at a rate of 10 °C/min. Helium was used as carrier gas in a constant flow mode of 2.5 mL/min. To calculate the enrichment of  $^{13}\text{C}$ -fatty acids from the measured enrichment of  $^{13}\text{CO}_2$ , we assumed that one acetate molecule was incorporated per fatty acid molecule.

**Analysis of  $^{13}\text{C}$ -cholesterol.** Plasma samples for  $^{13}\text{C}$ -enrichment measurements of cholesterol were saponified and extracted in sequence with hexane and diethyl ether according to Paik *et al.* (Paik *et al.*, 2008). The supernatant layer was silylated using N,O-bis(trimethylsilyl)trifluoroacetamide. The  $^{13}\text{C}$ -enrichment of cholesterol in plasma samples was measured using GC-C-IRMS analysis with an Rxi-5ms capillary column (30 m x 0.25 mm i.d. and 0.5  $\mu\text{m}$  film thickness; Interscience, Breda, The Netherlands). Samples were injected (1  $\mu\text{L}$ ) in splitless mode at 250 °C into the column. The initial oven temperature was 240 °C (1 min) and was increased to 300 °C (15 min) at 20 °C/min. Helium gas was used as carrier gas and flowed at a constant rate of 2.5 mL/min. To calculate the enrichment of  $^{13}\text{C}$ -cholesterol from the measured enrichment of  $^{13}\text{CO}_2$ , it was assumed that one acetate or

propionate molecule was incorporated per cholesterol molecule. Total cholesterol concentrations were determined using standard laboratory techniques.

**Analysis of Butyrate-producing Capacity in Faecal Samples.** Real-time PCR was used to quantify *Clostridium* cluster IV, *Clostridium* cluster XIV, butyryl-CoA:acetate-CoA transferase and butyrate kinase genes in faecal samples as described previously (Boets *et al.*, 2015).

### Calculations

**Systemic availabilities of colonic acetate, propionate and butyrate.** The systemic availability of each SCFA was determined by measuring  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate plasma concentrations up to 12 h after colonic delivery of  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate, respectively, on three separate test days. At each time point, total (labelled + unlabelled) SCFA concentrations and  $^{13}\text{C}$ -SCFA enrichments were measured. The plasma concentration of  $^{13}\text{C}$ -SCFAs consists of the concentration of  $^{13}\text{C}$ -SCFAs naturally present in the plasma and the concentration originating from the colon. The concentration of  $^{13}\text{C}$ -SCFAs originating from the colon at each time point ( $n_{\text{colon}}$ ) was calculated according to equation 1 and allowed constructing  $^{13}\text{C}$ -SCFA concentration *versus* time graphs:

$$n_{\text{colon}} = n_{\text{tot}} \times \frac{AP_{\text{tot}} - AP_{\text{plasma } t_0}}{AP_{\text{colon}} - AP_{\text{plasma } t_0}} \quad (\text{equation 1})$$

with  $n_{\text{tot}}$ : total concentration of SCFAs (labelled + unlabelled),  $AP_{\text{tot}}$ :  $^{13}\text{C}$ -enrichment of the SCFAs measured in plasma at time point t,  $AP_{\text{plasma } t_0}$ :  $^{13}\text{C}$ -enrichment of the SCFAs measured in plasma at time 0, and  $AP_{\text{colon}}$ :  $^{13}\text{C}$ -enrichment of the administered substrate.

To allow calculating the clearance of the SCFAs, a primed constant infusion with  $^2\text{H}$ -SCFAs was applied during each test day. The  $^2\text{H}$ -enrichments of the SCFAs were multiplied with the SCFA plasma concentrations to calculate the  $^2\text{H}$ -SCFA concentrations. Subsequently, the clearance ( $\text{Cl}$ ,  $\text{L}\cdot\text{h}^{-1}$ ) was calculated from the  $^2\text{H}$ -SCFA infusion rate ( $I_{^2\text{H-SCFA}}$ ,  $\mu\text{mol}\cdot\text{h}^{-1}$ ) and the steady state  $^2\text{H}$ -SCFA plasma concentration ( $C_{\text{ss}}$ ,  $\mu\text{mol}\cdot\text{L}^{-1}$ ) according to equation 2:

$$\text{Cl} = \frac{I_{^2\text{H-SCFA}}}{C_{\text{ss}}} \quad (\text{equation 2})$$

$^{13}\text{C}$ -SCFA concentration *versus* time graphs were used to calculate the exposure to  $^{13}\text{C}$ -SCFAs in plasma using the trapezoidal rule. Finally, knowing the administered amount of

each  $^{13}\text{C}$ -SCFA, the systemic availability of  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate was calculated according to equation 3:

$$SA_{SCFA} = \frac{AUC \times Cl_{SCFA}}{D_{capsule}} \times 100 \quad (\text{equation 3})$$

with SA: systemic availability (%), AUC: area under the curve of the  $^{13}\text{C}$ -SCFA concentration *versus* time graph ( $\mu\text{mol}\cdot\text{h}\cdot\text{L}^{-1}$ ), Cl: clearance of the respective SCFA ( $\text{L}\cdot\text{h}^{-1}$ ), and  $D_{capsule}$ : administered dose of the respective SCFA ( $\mu\text{mol}$ ).

**Assimilation of SCFAs into biologically relevant molecules.** The fraction of colonic-derived propionate used for glucose production was quantified from the cumulative amount of  $^{13}\text{C}$ -glucose appearing in the plasma after colonic administration of  $^{13}\text{C}$ -propionate according to equation 4.

$$F_{^{13}\text{C}\text{-label recovered as glucose}} = \frac{AUC \times Cl_{glucose}}{D_{^{13}\text{C}\text{-propionate}}} \times 100 \quad (\text{equation 4})$$

with  $F_{^{13}\text{C}\text{-label recovered as glucose}}$ : the fraction of administered  $^{13}\text{C}$  recovered in glucose (%), AUC: the area under the curve of the  $^{13}\text{C}$ -glucose *versus* time graph ( $\mu\text{mol}\cdot\text{h}\cdot\text{L}^{-1}$ ),  $Cl_{glucose}$ : the clearance ( $\text{L}\cdot\text{h}^{-1}$ ) of glucose that was calculated for each individual according to Jani *et al.* (Jani *et al.*, 2008) and amounted to  $8.9 \pm 1.2 \text{ L}\cdot\text{h}^{-1}$ , and  $D_{^{13}\text{C}\text{-propionate}}$ : the dose of colonic administered  $^{13}\text{C}$ -propionate ( $\mu\text{mol}$ ).

Similarly, the fraction of  $^{13}\text{C}$ -acetate incorporated into the most abundant fatty acids (palmitic, stearic and oleic acid) was calculated according to equation 4 using the respective AUCs of the  $^{13}\text{C}$ -fatty acids *versus* time graphs, the clearance of palmitic, stearic and oleic acid, respectively, and the administered dose of  $^{13}\text{C}$ -acetate. The clearance of the fatty acids was obtained from literature and amounted to 48, 30 and 45  $\text{L}\cdot\text{h}^{-1}$  for palmitic, stearic and oleic acid, respectively.

Finally, the extent to which intestinal acetate and propionate were used for *de novo* synthesis of cholesterol was determined. The fractional incorporation of  $^{13}\text{C}$ -acetate and  $^{13}\text{C}$ -propionate into cholesterol was calculated according to equation 4 using the AUC of the  $^{13}\text{C}$ -cholesterol *versus* time graph, the clearance of cholesterol and the administered dose of  $^{13}\text{C}$ -acetate and  $^{13}\text{C}$ -propionate, respectively. The clearance of cholesterol was calculated for each individual based on his/her body weight and cholesterol levels as described by Turner *et al.* (Turner *et al.*, 2012) and amounted to  $0.15 \pm 0.04 \text{ L}\cdot\text{h}^{-1}$ .

## Statistical analysis

Statistical analyses were performed using SPSS, version 22.0 (IBM Corp, New York, USA). All results are presented as means  $\pm$  standard deviations (SDs). Assumptions of normality were checked with a Shapiro-Wilk test. The OCTT and time of  $^{13}\text{CO}_2$  in breath samples were compared with a paired samples T-test. Bivariate correlations were performed with Spearman's rho. Significance was accepted at the 5% level.

## Results

### Delivery of SCFAs into the colon of healthy subjects

*In vitro* dissolution tests indicated an appropriate release profile of the marker methylene blue from the CDCs (release after 45 min at pH = 7.0) with a coating thickness of 18.8 mg/cm<sup>2</sup> capsule surface (Figure 1 A).

To evaluate the *in vivo* performance of the capsules, the time at which  $^{13}\text{CO}_2$ , resulting from oxidation of released  $^{13}\text{C}$ -SCFAs, appeared in breath was compared with the OCTT which was estimated from an increase of  $^{14}\text{CO}_2$  in breath (Verbeke *et al.*, 2005). A simultaneous increase in breath of  $^{13}\text{CO}_2$  and  $^{14}\text{CO}_2$  indicates a correct release of the CDCs content in the proximal colon (Figure 1 B). No significant differences were observed between the  $^{13}\text{CO}_2$  excretion time ( $383 \pm 105$  min) and the OCTT ( $399 \pm 84$  min) in any of the test days ( $p = 0.202$ ) (Figure 1 C).

### Systemic availabilities of colonic acetate, propionate and butyrate

$^{13}\text{C}$ -SCFA concentration *versus* time graphs were constructed for the three test days of each subject and a representative example is shown Figure 2 A. By calculating the area under these  $^{13}\text{C}$ -SCFA concentrations *versus* time graphs an exposure of  $1.92 \pm 1.13$ ,  $0.48 \pm 0.26$  and  $0.21 \pm 0.20$   $\mu\text{mol.h.L}^{-1}$  was obtained for  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate, respectively. Total body clearance of acetate, propionate and butyrate amounted to  $938 \pm 287$ ,  $647 \pm 176$  and  $1237 \pm 592$  L.h<sup>-1</sup>, respectively. On average, the fraction of colonic-derived acetate, propionate and butyrate appearing in plasma amounted to  $36 \pm 21\%$ ,  $9.2 \pm 5.9\%$  and  $2.4 \pm 1.9\%$ , respectively (Figure 2 B).

### SCFAs are interconverted by gut microbiota

The individual  $^{13}\text{C}$ -SCFAs were administered on separate days to allow evaluating the extent of interconversion between the three SCFAs. After administration of  $^{13}\text{C}$ -acetate, the extent of its conversion into  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate was determined by quantifying the appearance of  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate in plasma. Interconversion was quantified in similar ways after administration of  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate. The incorporation of  $^{13}\text{C}$ -acetate into butyrate (Figure 3 A) was quantitatively the most significant interconversion but all interconversions were detected. To confirm that conversion of acetate into butyrate was due to bacterial and not to human metabolism, uncoated  $^{13}\text{C}$ -acetate capsules were administered. As these released  $^{13}\text{C}$ -acetate already in the proximal gastrointestinal tract,  $^{13}\text{C}$ -acetate was absorbed before it had been in contact with bacteria and appeared in plasma already after 120 min. No  $^{13}\text{C}$ -butyrate in plasma was observed, confirming that contact with bacteria is essential for the interconversion to occur (Figure 3 B and 3 C).

### **Quantification of the butyrate-producing capacity of the intestinal microbiota**

To relate the interconversion of acetate into butyrate to the intestinal microbiota composition of the subjects, the butyrate-producing capacity in a faecal sample was quantified. RT-PCR was used to quantify genes coding for butyrate kinase and butyryl-CoA:acetate CoA-transferase which are the dominant terminal enzymes in the production of butyrate (Louis & Flint, 2007; Vital *et al.*, 2013). In addition, the most abundant clusters of butyrate producing bacteria, *i.e.* *Clostridium* clusters IV and XIVa, were quantified (Figure 4 A). However, the extent of acetate into butyrate conversion was not significantly related to any of these parameters of butyrate-producing capacity (Figure 4 B-E).

### **Utilization of colonic-derived propionate in gluconeogenesis**

Plasma  $^{13}\text{C}$ -glucose concentrations increased simultaneously with  $^{13}\text{C}$ -propionate concentrations (Figure 5 A). The cumulative amount of  $^{13}\text{C}$ -glucose was  $23 \pm 20.5 \mu\text{mol.h.L}^{-1}$ . We calculated that  $5.9 \pm 4.7\%$  of the colonic administered propionate was used for gluconeogenesis (Figure 5 B).

### **Incorporation of acetate into fatty acids**

The  $^{13}\text{C}$ -enrichment and total concentrations of the most abundant plasma fatty acids, palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1, n-9) were quantified after  $^{13}\text{C}$ -acetate administration. Enrichment was mainly observed in palmitic acid and to a lesser extent in stearic and oleic acid (Figure 5 C). Up to  $12.0 \pm 8.7\%$ ,  $1.0 \pm 0.9\%$  and  $1.0 \pm 1.0\%$  of

administered acetate was assimilated into palmitic, stearic and oleic acid, respectively (Figure 5 D).

### **Incorporation of colonic-derived acetate and propionate into cholesterol**

After administration of  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -cholesterol readily appeared in plasma whereas  $^{13}\text{C}$ -cholesterol was hardly detectable after administration of  $^{13}\text{C}$ -propionate (Figure 5 E). Only  $0.101 \pm 0.076\%$  and  $0.007 \pm 0.005\%$  of administered  $^{13}\text{C}$ -acetate and  $^{13}\text{C}$ -propionate were assimilated into cholesterol, respectively (Figure 5 F).

### **Conversion of short-chain fatty acids into carbon dioxide**

The extent to which  $^{13}\text{C}$ -substrates are converted to  $^{13}\text{CO}_2$  at the whole-body level is most often estimated from the excretion of  $^{13}\text{CO}_2$  in breath. After 12 h, the cumulative percentage of  $^{13}\text{C}$ -label recovered as  $^{13}\text{CO}_2$  in breath after administration of  $^{13}\text{C}$ -propionate amounted to  $47 \pm 13\%$ , whereas  $33 \pm 10\%$  of administered  $^{13}\text{C}$ -acetate and  $18 \pm 3\%$  of administered  $^{13}\text{C}$ -butyrate was recovered (Figure 6). Application of the correction factor for the  $^{13}\text{CO}_2$  that is retained in the body indicated that up to  $86 \pm 24\%$  of administered  $^{13}\text{C}$ -propionate,  $60 \pm 18\%$  of administered  $^{13}\text{C}$ -acetate, and only  $33 \pm 6\%$  of administered  $^{13}\text{C}$ -butyrate was oxidized into  $^{13}\text{CO}_2$  within 12 h.

### **Urinary excretion**

Subjects collected urine for 24 h in different fractions (0-4 h, 4-8 h, 8-12 h, 12-24 h) to allow quantification of the urinary excretion of  $^{13}\text{C}$ -SCFAs. After 24 h, only up to  $0.031 \pm 0.020\%$  of  $^{13}\text{C}$ -acetate,  $0.008 \pm 0.002\%$  of  $^{13}\text{C}$ -propionate and  $0.0006 \pm 0.0003\%$  of  $^{13}\text{C}$ -butyrate were retrieved in urine.

## **Discussion**

By applying a direct, stable isotope based approach, the present study enabled to provide quantitative information on the uptake and elimination of SCFAs in different body compartments and their assimilation into biologically relevant molecules in humans. This information may contribute to deciphering the mechanistic link between fibre consumption and its health benefits.

### **Colonic delivery of SCFA**

Targeting of orally administered compounds to the colon can be achieved using various strategies including the use of coatings with a pH-sensitive polymer, formulation of time-released systems, use of carriers that are specifically degraded by colonic bacteria, covalent linkage of the compound with a carrier, bioadhesive systems and osmotic controlled drug delivery systems (for a review, see Charousia *et al.*, 2003). These formulations have been mainly developed to target drugs to the colon either for treatment of local diseases such as Crohn's disease or ulcerative colitis but also because of the potential to deliver therapeutic peptides or proteins. Due to absence of peptidases in the colon, peptide drug might be absorbed unchanged after peroral administration. Targeted delivery of SCFA to the colon has previously been achieved using carriers such as starch (Annison *et al.* 2003) or inulin (Chambers *et al.*, 2014). Upon arrival in the colon, the carriers are fermented by the resident bacteria and release the SCFA that are attached to them. We preferred to use gelatine capsules with a pH-dependent coating as a convenient vehicle to deliver well known amounts of SCFA to the colon.

#### **Systemic availability of SCFAs**

Estimates of the extraction of the SCFAs during passage through the splanchnic bed have been reported and are based either on differences in SCFA concentrations in peripheral blood compared to their concentrations in portal blood obtained during abdominal surgery (Peters *et al.*, 1992; Bloemen *et al.*, 2009) or on isotope dilution experiments (Pouteau *et al.*, 1996). Estimates for splanchnic extraction of acetate vary from 40-75% (Pouteau *et al.*, 1996; Vogt *et al.*, 2004) whereas an extraction value of 90% for propionate has been mentioned (Vogt *et al.*, 2004). As butyrate is the preferred energy source for colonocytes, the majority of absorbed butyrate may already be consumed by the colonic mucosa. According to Cook and Sellin (1998), 70-90% of butyrate is metabolized in the colonocytes (Cook & Sellin, 1998). Bloemen *et al.* showed that the release of butyrate from the liver is not significantly different from zero, suggesting a splanchnic extraction of almost 100% (Bloemen *et al.*, 2009). Of note, all studies mention a wide interindividual variation in SCFA levels, both in peripheral and in portal blood (Peters *et al.*, 1992; Bloemen *et al.*, 2009).

We combined colonic administration of  $^{13}\text{C}$ -labelled SCFA with intravenous administration of  $^2\text{H}$ -labelled SCFA to determine the SCFA systemic availability, assuming a similar behaviour of both types of labelled SCFA. Although simultaneous infusion of  $^{13}\text{C}$ -acetate and  $^2\text{H}$ -acetate in the gut of pigs resulted in higher  $^{13}\text{C}$ -acetate than  $^2\text{H}$ -acetate enrichment in

plasma, suggesting *in vivo* isotope exchange of the deuterium hydrogen atoms in acetate and thus underestimation of the deuterium enrichment (Kien *et al.*, 1996), we did not find evidence for such isotope exchange in our study. Indeed, the ion masses used to quantify acetate and  $^2\text{H}_3$ -acetate were  $m/z$  60 and 63, respectively, with no quantifiable ions at mass  $m/z$  61 nor 62. Similarly, we did not find evidence for isotope exchange of  $^2\text{H}_7$ -butyrate which was quantified using a fragment ion with  $m/z$  of 63, rather than the parent ion. Therefore, even if isotope exchange occurred in the remaining part of the molecule, which we cannot exclude, this will not have affected our results.  $^2\text{H}_5$ -propionate was quantified using the parent ion at  $m/z$  79. Although smaller peaks at  $m/z$  78 and 77 were visible suggesting isotope exchange, the same ratios were observed in the infusion solution as in the plasma samples indicating that the exchange likely occurred in the ion source of the mass spectrometer rather than *in vivo*.

The values for systemic availability of acetate (36%) and propionate (9%) obtained in this study correspond to a splanchnic extraction of 64% and 91%, respectively, which is in nice agreement with reported values. Also in the present study, a large interindividual variation was observed. In addition, the direct stable isotope approach enabled detecting  $^{13}\text{C}$ -butyrate in plasma after colonic administration of  $^{13}\text{C}$ -butyrate and to quantify its systemic availability (2%) or splanchnic extraction (98%).

### Metabolism of SCFAs

Within 12 h after colonic administration, the vast majority of propionate (86%) was converted into  $\text{CO}_2$  of which 47% was recovered in breath. The remainder was retained in the body bicarbonate pool. Except from being a substrate for gluconeogenesis, no other pathways for incorporation of propionate into other metabolites are known and the fate of propionate seems to be a rapid and almost complete conversion into  $\text{CO}_2$ , which proceeds most likely in the liver. These results do not allow differentiating between the fraction of propionate that is directly converted into  $\text{CO}_2$  and that used for gluconeogenesis followed by oxidation of the resulting glucose into  $\text{CO}_2$ . After administration of  $^{13}\text{C}$ -acetate, recovery in breath was lower compared to that of  $^{13}\text{C}$ -propionate. This may be explained by labelled C that does not appear as  $^{13}\text{CO}_2$  but is exchanged in other metabolites. These are most likely metabolites of the tricarboxylic acid (TCA) cycle (van Hall, 1999), as acetate enters the TCA cycle after conversion into acetyl-CoA. This exchange is only temporary since the resulting metabolites re-enter the oxidative pathways at a later time. This also explains the observation that breath

$^{13}\text{CO}_2$  recoveries after intravenous  $^{13}\text{C}$ -acetate infusion increase with the duration of the infusion and only reach a plateau after 12 h of infusion (Mittendorfer *et al.*, 1998). Breath  $^{13}\text{CO}_2$  recoveries after colonic administration of  $^{13}\text{C}$ -acetate have not been reported in literature. Reported  $^{13}\text{CO}_2$  recoveries after intravenous  $^{13}\text{C}$ -acetate administration vary from 40% to 81% (Wolfe & Jahoor, 1990; Pouteau *et al.*, 1996; Mittendorfer *et al.*, 1998). The low recovery of  $^{13}\text{CO}_2$  after administration of  $^{13}\text{C}$ -butyrate (33%) is probably in part due to exchange of the label into TCA metabolites. After absorption in the colonocytes, butyrate is taken up in the mitochondria of the cells where it undergoes the  $\beta$ -oxidation pathway, which involves a series of five enzymes, to form acetyl-CoA that subsequently enters the TCA cycle (De Preter *et al.*, 2012). A few other studies that compared the rate of colonic butyrate oxidation in patients with ulcerative colitis and healthy controls, report on  $^{13/14}\text{CO}_2$  recoveries in breath after colonic administration of  $^{13}\text{C}$ - or  $^{14}\text{C}$ -labelled butyrate. Six hours after rectal instillation of  $^{14}\text{C}$ -butyrate enemas, 52.9 (44.4-61.5)% of administered label was retrieved in breath (Den Hond *et al.*, 1998). In another study, about one fourth of rectally instilled  $^{13}\text{C}$ -butyrate was recovered as  $^{13}\text{CO}_2$  after 4 hours (Simpson *et al.*, 2000). After intravenous infusion of  $^{13}\text{C}$ -butyrate in five healthy subjects, the 6-h cumulative excretion of  $^{13}\text{CO}_2$  amounted to  $49.9 \pm 2.2\%$  (Den Hond *et al.*, 1998). For all  $^{13}\text{C}$ -SCFA, higher fractions of administered dose are excreted in breath as  $^{13}\text{CO}_2$  than appearing in plasma indicating that they are metabolized prior to arrival in the systemic circulation, i.e. presumably in the colonocytes and liver.

In the human kidney, monocarboxylate transporters (MCTs) are responsible for the reabsorption of SCFAs and ketone bodies (Halestrap & Meredith, 2004). Although lactate is quantitatively the most important substrate for these transporters, they have been shown to also reabsorb SCFAs. As a consequence, urinary excretion of SCFAs is low. After continuous infusion of sodium butyrate for 10 days in patients with acute leukaemia, only  $0.21 \pm 0.04\%$  of the administered dose was retrieved in urine (Miller *et al.*, 1987). In healthy subjects, urinary levels of SCFA have been reported in a range of 0.8-130  $\mu\text{M}$  for acetate and below 3  $\mu\text{M}$  for propionate and butyrate (Perry *et al.*, 1970).

### ***In vivo* cross-feeding**

Cross-feeding involves the supply of breakdown products of carbohydrates after partial hydrolysis by primary degraders as secondary substrates to other bacteria (De Vuyst & Leroy, 2011). In particular, acetate is known to function as an intermediate in cross-feeding

interactions between colon bacteria, and plays a key role in colonic butyrate production (Morrison *et al.*, 2006; Riviere *et al.*, 2015). Morrison *et al.* incubated human faecal slurries *in vitro* with  $^2\text{H}$ -labelled SCFAs and quantified the interconversion between the SCFAs (Morrison *et al.*, 2006). After 6 h, 0.28 mol/mol labelled acetate was incorporated into butyrate. In the present study, conversion of  $^{13}\text{C}$ -acetate into  $^{13}\text{C}$ -butyrate was estimated to be about 24%. Although the contact time of  $^{13}\text{C}$ -acetate with the intestinal microbiota was shorter *in vivo* than *in vitro* as  $^{13}\text{C}$ -acetate was rapidly absorbed into the plasma after release from the CDCs, the value is only slightly lower than the Morrison data, suggesting that this interconversion proceeds quite rapidly. Whereas no evidence for incorporation of  $^2\text{H}$ -acetate into propionate or  $^2\text{H}$ -propionate into butyrate was found in the *in vitro* study, all possible interconversions could be detected, albeit to a lower extent than the acetate-into-butyrate interconversion. This discrepancy is likely due to the different analytical techniques used in both studies. Morrison *et al.* applied GC-MS analysis which allowed differentiating between different isotopomers of the SCFAs, but is a less sensitive technique than the isotope ratio mass spectrometry applied in the present study. In a recent study in mice, enrichments of cecal SCFAs were quantified after a 6-h cecal infusion of  $^{13}\text{C}$ -labelled SCFA. A high conversion was observed from acetate into butyrate, a low conversion from butyrate into acetate and between butyrate and propionate and very little interconversion between acetate and propionate (den Besten *et al.*, 2013a).

### **Propionate as a substrate for gluconeogenesis**

Studies in lactating cows report that up to 61% of glucose in blood is derived from gluconeogenesis with propionate as the substrate (Wiltout & Satter, 1972). In sheep, corresponding values between 42% and 59% have been reported (Leng *et al.*, 1967). On the other hand, up to 32% of colonic-derived propionate is incorporated into glucose in sheep and up to 62% in mice (Leng *et al.*, 1967; den Besten *et al.*, 2013a). Compared to those values, the extent of incorporation of colonic-derived propionate into glucose (5.9%) is limited in humans, as shown by the present study. Indeed, propionate is the major substrate for gluconeogenesis in ruminants (Bergman, 1990) whereas in humans, lactate and pyruvate are the major gluconeogenic substrates with minor contributions of alanine and glycerol (Garber *et al.*, 1974). Several methods based on stable isotope technology have quantified *in vivo* the contribution of gluconeogenesis to the glucose production in humans (Previs & Brunengraber, 1998). However, contributions of all gluconeogenic substrates are included in

these estimates and no human data on the contribution of propionate to gluconeogenesis are available.

More recently, it has been shown in rats that propionate is incorporated into glucose at the level of the intestine (De Vadder *et al.*, 2014). The resulting glucose is sensed in the walls of the portal vein and induces a nervous signal to the brain that influences food intake and glucose homeostasis. These results may provide a mechanistic role for propionate in the beneficial effects of dietary fibre. Unfortunately, due to inability to obtain portal blood from healthy subjects, our results do not allow determining whether the observed incorporation of  $^{13}\text{C}$ -propionate into glucose occurs in the intestine or in the liver.

### **Incorporation of acetate into fatty acids**

Indirect methods to estimate *de novo* lipogenesis such as indirect whole-body calorimetry as well as direct methods such as  $^2\text{H}_2\text{O}$  infusion and mass isotopomer distribution analysis (MIDA) confirmed that *de novo* lipogenesis (DNL) is a quantitatively minor pathway under normal conditions of typical high-fat diets (Hellerstein *et al.*, 1991; Hellerstein *et al.*, 1996; Bjorntorp and Sjostrom, 1978). Absolute DNL was estimated at less than 1 g synthesized per day which is low compared to a dietary fat intake of typically 100 g per day (Hellerstein *et al.*, 1996). Studies using MIDA intravenously infuse  $^{13}\text{C}$ -acetate to enrich the acetyl-CoA-precursor pool and express the fractional hepatic DNL as fraction of newly synthesised lipid compared to the lipid already present and not as fraction of administered acetate incorporated in the lipid. As a consequence, it is not possible to compare the results obtained in the present study to those results reported in literature. Almost 15% of colonic-administered acetate was incorporated into fatty acids in the present study. However, this only corresponds to an absolute amount of  $0.229 \pm 0.175$  g of newly synthesized fatty acids which is still far below the 1 g threshold. It needs to be taken into account that only 5 mmol of  $^{13}\text{C}$ -labelled acetate was administered in the colon delivery capsules whereas the amount of acetate produced in the colon on a western diet (20 g of dietary fibre) has been estimated at about 150 mmol per day (Wolever *et al.*, 1995).

### **Incorporation of acetate and propionate into cholesterol**

It has been calculated that the rate of *de novo* synthesis of cholesterol amounts to  $10 \pm 6$  mg.kg $^{-1}$ .day $^{-1}$  in healthy adults (Renfurm *et al.*, 2004). As the biosynthesis of cholesterol starts from the two-carbon acetate group of acetyl-CoA, it has been suggested that dietary fibres that are fermented into a high proportion of acetate might increase serum

cholesterol levels. However, unlike propionate and butyrate, the majority of plasma acetate originates from endogenous metabolism and the contribution of colonic-derived acetate to cholesterol synthesis may well be less important. Indeed, the present study showed that less than 0.1% of colonic-administered acetate was incorporated into cholesterol. This value is of the same order of magnitude as the value of 0.7% reported by Hellman *et al.* after oral administration of  $^{14}\text{C}$ -acetate (Hellman *et al.*, 1954). In mice, the fractional synthesis of cholesterol from acetate was  $0.7 \pm 0.1\%$  (den Besten *et al.*, 2013a). Propionate did not contribute to cholesterol synthesis neither in this study nor in the study in mice mentioned before (den Besten *et al.*, 2013a).

### **Limitations of the present study**

For the calculation of the systemic availability, it was assumed that the absorption of the  $^{13}\text{C}$ -SCFAs from the colonic lumen was quantitative. The major part of SCFA is transported in dissociated form across the apical membrane of the colonocytes by the monocarboxylate transporter 1 (MCT-1), the electrogenic sodium dependent monocarboxylate transporter 1 (SMCT-1) or an SCFA- $\text{HCO}_3^-$  exchanger of unknown identity (den Besten *et al.*, 2013). It is indeed generally believed that the colon has a large capacity to absorb SCFAs with estimates of 6.1-12.6  $\mu\text{mol}/\text{cm}^2\cdot\text{h}$  (He *et al.*, 2006). As the human colon has a mucosal surface area of about 20000  $\text{cm}^2$  (Helander & Fandriks, 2014), up to 120-250 mmol of SCFAs could be absorbed per hour. To confirm a quantitative SCFA absorption, a complete stool collection for 3-5 days after each test day would have been required for quantifying residual  $^{13}\text{C}$ -SCFAs. This would have placed an additional burden on the protocol which was already onerous for the participants and was therefore not performed. Only one stool sample was collected for the analysis of the microbial butyrate-producing capacity.

In this study, the metabolic fate of the SCFA was evaluated in standardized feeding conditions (i.e. after administration of a non-fermentable standard breakfast and lunch). Whether the oxidation and/or incorporation of SCFA in biomolecules differs in fasting conditions remains to be investigated.

### **Conclusions**

The setup and methodology used in the present study allowed quantifying the percentages of acetate, propionate and butyrate originating from the colon that reached the systemic circulation. In addition, the metabolism and assimilation of acetate, propionate and butyrate into biologically relevant molecules were measured.

In the future, these results will allow evaluating and quantifying SCFA production of varying  $^{13}\text{C}$ -labelled fibres in the human colon by measuring  $^{13}\text{C}$ -labelled SCFA concentrations in blood and multiplying the amounts of SCFAs that reached the plasma with the systemic availability index calculated in this study. In addition, the availability of plasma clearance values for each SCFA will allow calculating fluxes under different conditions when combined with plasma SCFA measurements.

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## **Disclosures**

None of the authors has a conflict of interest to declare.

## **Author contribution**

The work was performed at the Translational Research Center for Gastrointestinal Disorders (Laboratory of Digestion and Absorption) of the KU Leuven, Leuven, Belgium.

E.B., H.H. and K.A.V. were responsible for the study concept and design. E.B., L.D., H.H. and K.V. executed the intervention study and acquired the data. E.B., S.V.G., L.D., K.V., T.P., V.D., G.V., L.D.V., C.M.C., P.A., J.A.D. and K.A.V. were responsible for data analysis and interpretation. T.P., K.V., G.V. and P.A. provided technical and material support. E.B., S.V.G., L.D., T.P., K.V., V.D.P., H.H., G.V.M., L.D.V., C.M.C., P.A., J.D., K.A.V. contributed to the writing and critically revising of the manuscript.

All authors approved the final version of the manuscript.

All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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## Figure legends

Figure 1: In vitro and in vivo evaluation of the performance of the colon delivery capsules. (A) In vitro dissolution profile of colon delivery capsules at different pH,  $n = 2$ . (B) A representative example of the appearance of  $^{14}\text{CO}_2$  and  $^{13}\text{CO}_2$  in breath after administration of  $^{13}\text{C}$ -acetate colon delivery capsules. The simultaneous rise in breath of  $^{14}\text{CO}_2$  and  $^{13}\text{CO}_2$  indicates a correct delivery of  $^{13}\text{C}$ -acetate in the colon. (C) Comparison of the time of release of the  $^{13}\text{C}$ -SCFAs from the capsules (indicated by the time point at which 20 % of the cumulative amount recovered was obtained,  $t_{20\%}$ ) and arrival of the test meal in the colon (indicated by the orocecal transit time, OCTT),  $n = 36$  except for OCTT  $n = 30$ . Results are expressed as means and standard deviations.

Figure 2: Calculation of the systemic availability of colonic-administered  $^{13}\text{C}$ -SCFAs. Typical graph depicting (A)  $^{13}\text{C}$ -acetate concentrations in plasma versus time. (B) Systemic availability results of acetate, propionate and butyrate for all 12 subjects. Results are expressed as means and standard deviations.

Figure 3: Cross-feeding of SCFAs. (A) Overview and quantitative indication of the interconversions between acetate, propionate and butyrate,  $n = 12$ . (B) Appearance of  $^{13}\text{C}$ -acetate in plasma after administration of a coated and uncoated capsule filled with  $^{13}\text{C}$ -acetate. Without coating,  $^{13}\text{C}$ -acetate was released in the proximal intestine and appears at an earlier time in plasma compared to a coated capsule. (C) Appearance of  $^{13}\text{C}$ -butyrate in plasma after administration of a coated and uncoated capsule filled with  $^{13}\text{C}$ -acetate.  $^{13}\text{C}$ -butyrate is only formed when  $^{13}\text{C}$ -acetate is properly released in the colon.

Figure 4: Butyrate-producing capacity. (A) Gene copy numbers of butyrate-producing colon bacteria and butyrate-producing colon enzymes in faecal samples,  $n = 11$ . (B-C) Correlation between acetate-into-butyrate conversion and enzymes involved in butyrate synthesis,  $n = 11$ . (D-E) Correlation between acetate-into-butyrate conversion and the most abundant butyrate-producing bacteria,  $n = 11$ .

Figure 5: Assimilation of  $^{13}\text{C}$ -SCFAs in biologically relevant molecules. (A) Typical example that shows the appearance of  $^{13}\text{C}$ -propionate followed by  $^{13}\text{C}$ -glucose in plasma after colonic administration of  $^{13}\text{C}$ -propionate. (B) Fraction of administered  $^{13}\text{C}$ -propionate recovered in glucose,  $n = 12$ . (C) Typical example that shows the appearance of  $^{13}\text{C}$ -acetate followed by  $^{13}\text{C}$ -palmitate,  $^{13}\text{C}$ -stearate and  $^{13}\text{C}$ -oleate in plasma after colonic administration of  $^{13}\text{C}$ -acetate. (D) Fraction of administered  $^{13}\text{C}$ -acetate recovered in palmitate (C16), stearate (C18) and oleate (C18:1),  $n = 12$ . (E) Typical examples that show the appearance of  $^{13}\text{C}$ -acetate and  $^{13}\text{C}$ -propionate followed by  $^{13}\text{C}$ -cholesterol in plasma after colonic administration of  $^{13}\text{C}$ -acetate. (F) Fraction of administered  $^{13}\text{C}$ -acetate and  $^{13}\text{C}$ -propionate recovered in cholesterol,  $n = 12$ . Results are expressed as means and standard deviations.

Figure 6: Recovery of  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate in breath as  $^{13}\text{CO}_2$ . Results are expressed as means and standard deviations,  $n = 12$ .

Figure 1

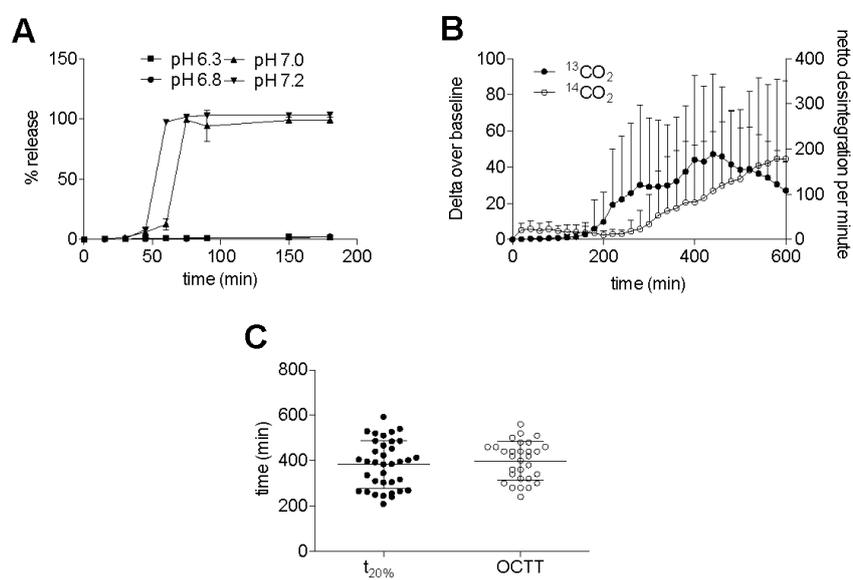


Figure 2

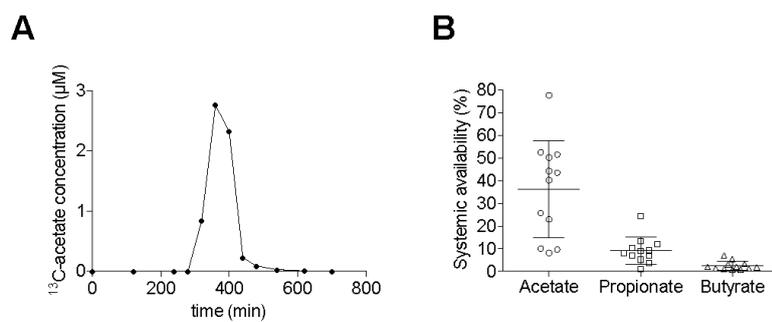


Figure 3

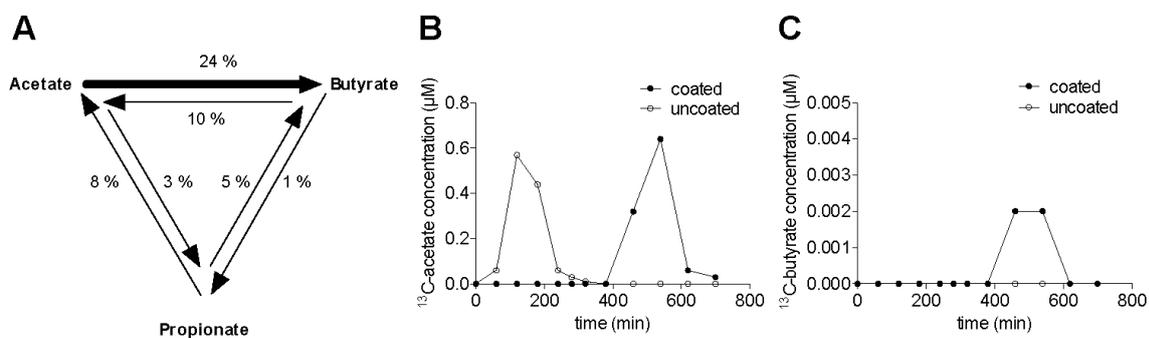


Figure 4

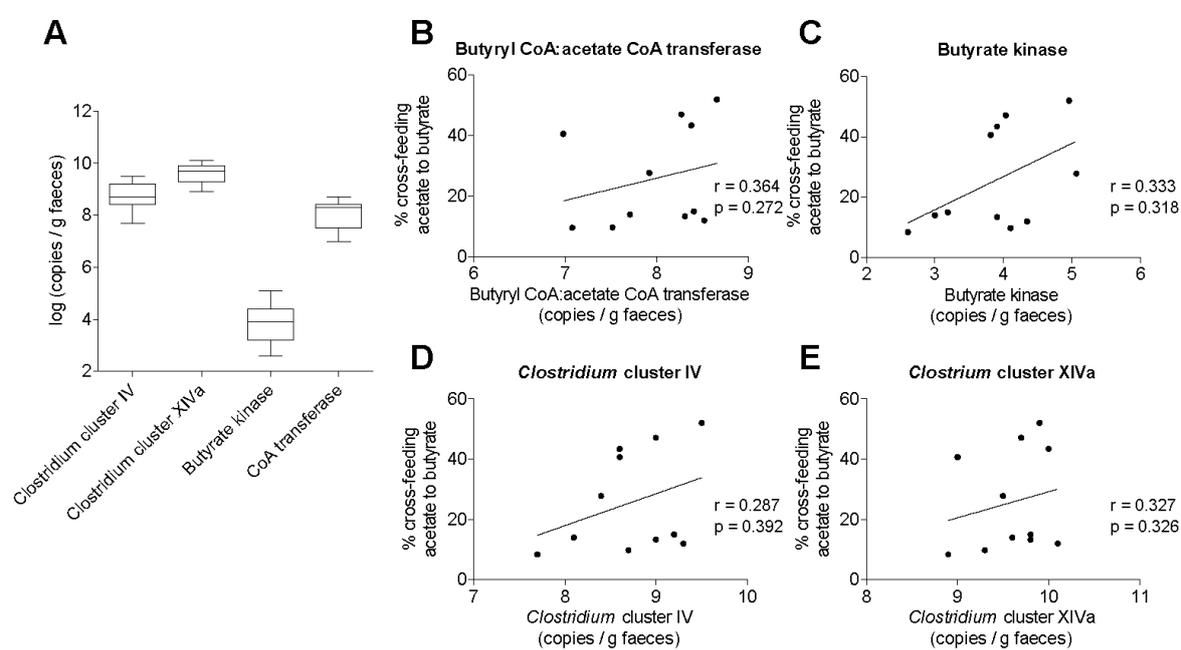


Figure 5

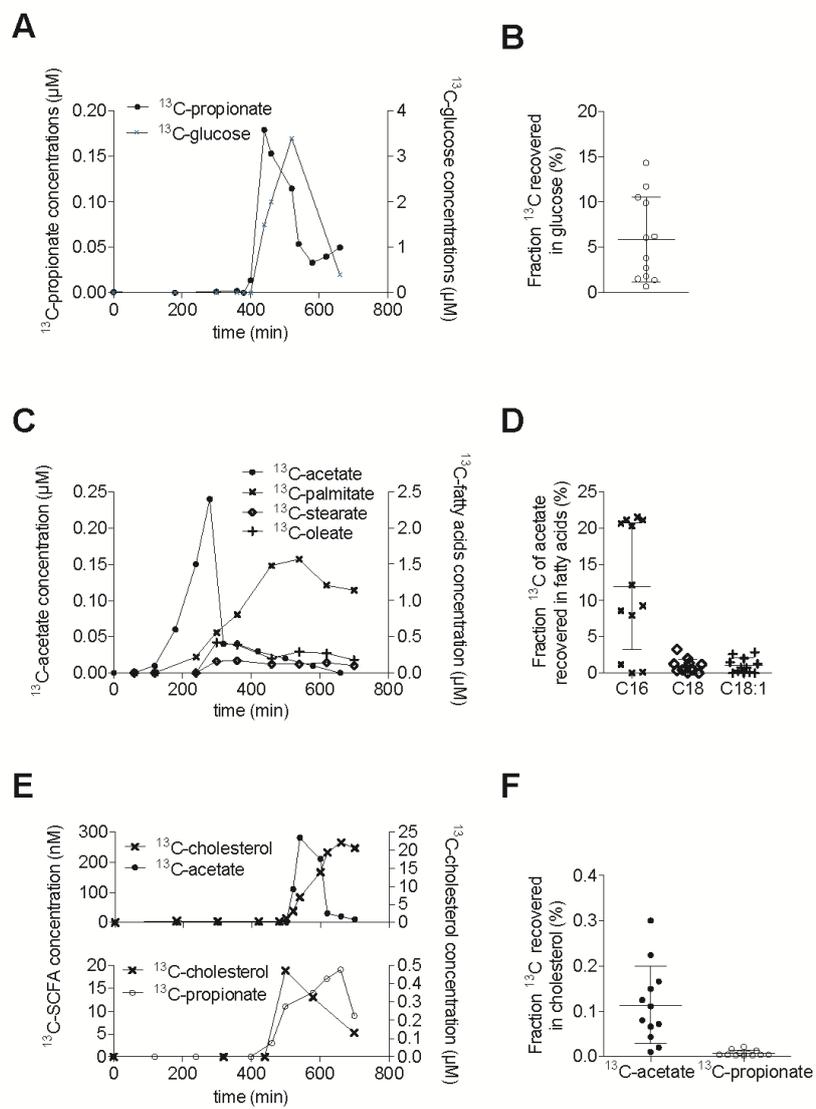


Figure 6

