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Production rates and metabolism of short-chain fatty acids in the colon and whole body using stable isotopes

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Short-chain fatty acids (SCFA; mainly acetate, propionate and butyrate) are largely produced in non-ruminants during the colonic bacterial fermentation of non-digestible carbohydrates. These intestinal exogenous SCFA pass in part through the splanchnic bed and reach the peripheral bloodstream, mixing with the endogenous circulating SCFA. The whole-body turnover of SCFA is thus composed of an endogenous peripheral turnover and an exogenous production that depends on dietary intake of non-digestible carbohydrates. In the present work methods were developed for determining the SCFA turnover in animals and in human subjects using stable isotopes. The original studies performed to determine endogenous and exogenous metabolism of SCFA in animals and in human subjects are summarised. Using intravenous infusion of ^{13}C-labelled SCFA the whole-body turnover of acetate, propionate and butyrate was assessed in rats in a fasted state. The endogenous turnover of acetate and its oxidation were determined in healthy human subjects in the post-absorptive state, using intravenous infusion of [1-^{13}C]acetate. Intragastric tracer infusions were performed to evaluate the splanchnic first-pass retention of acetate in adults. Finally, an original model was developed in healthy human subjects using intravenous infusion of [1-^{13}C]acetate to determine *in vivo* the true colonic acetate production after ingestion of a non-digestible disaccharide. These present studies using stable isotopes provide the basis for a novel strategy to evaluate *in vivo*, in human subjects, the production of SCFA in the large intestine.

Short-chain fatty acids: Whole-body and colonic production: Stable isotopes

Short-chain fatty acids (SCFA) are largely produced in non-ruminants from colonic or caecal bacterial fermentation of non-digestible carbohydrates, with acetate, propionate and butyrate representing ≤70, 15 and 15% respectively (Bergman, 1990). Since SCFA are major participants in gut maintenance and may also be beneficial active contributors to the peripheral metabolism in human subjects (Bergman, 1990; Macfarlane & Cummings, 1991; McIntyre *et al*. 1993; Veldman *et al*. 1999), numerous investigations have been undertaken to develop a method for accurately determining SCFA production.

The production of SCFA has been assessed using *in vitro* models (Barry *et al*. 1995) and *in vivo* in pigs, dogs and rats (Bergman, 1990; Bleiberg *et al*. 1992; Rémésy *et al*. 1995) and also in human subjects (Cummings *et al*. 1986). However, no approach has been satisfactory in determining the true colonic production of SCFA *in situ* in healthy human subjects. Several authors have determined SCFA production in human faeces (Bergman, 1990), which actually represents the state of the rectum and not that of the caeco-colon. Some authors have measured SCFA concentrations in the peripheral blood circulation where the majority of SCFA had already been catabolised (Pomare *et al*. 1985; Rumessen *et al*. 1992). The production rate of SCFA has been inadequately described, especially that of the colon in healthy human subjects. No clear agreement on acetate turnover emerges from the literature. Using radioactive tracers, Skutches *et al*. (1979) and Seufert *et al*. (1984) determined the whole-body acetate turnover to be about 6–8 μmol/kg per min in human subjects and 1 μmol/kg per min in an obese patient in the post-absorptive state. Rocchiccioli *et al*. (1989) and Kien *et al*. (1992) found an acetate turnover of 54 and 64 μmol/kg per min in an adult and in preterm infants respectively using stable-isotope-labelled tracers. Recently, Mittendorfer *et al*. (1998) evaluated the acetate turnover to be 3·4 μmol/kg per min in healthy adults in the post-absorptive state using a [1,2-^{13}C]acetate tracer. For propionate Sbaï *et al*. (1994) reported a turnover of

Abbreviations: SCFA, short-chain fatty acids; MPE, mole percent excess.
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0·64 μmol/kg per min in patients with propionic acidaemia using a [1-^{13}C]propionate tracer. No data are available for butyrate whole-body turnover in human subjects. The production and utilisation rates of SCFA in the whole body have not been established either in human subjects or in non-ruminants. The *in vivo* colonic SCFA production rate is still inaccessible, with good reason, and needs to be verified in healthy adults.

In the following work major aspects of the turnover of SCFA in the whole body are demonstrated using a stable-isotope approach. The objective was to determine precisely in animals and in human subjects those SCFA that originate from the endogenous metabolism and those that originate from the exogenous and colonic supply. The endogenous and exogenous production rates of SCFA constitute the two major components of the whole-body turnover of peripheral SCFA. Endogenous turnover of SCFA in the peripheral circulation was evaluated in rats, dogs and human subjects. From the determination of the endogenous production of SCFA using the isotope-dilution technique, a model was developed in human subjects to evaluate the exogenous flux of SCFA originating from the intestinal fermentation of non-digested carbohydrate. The final study quantified the colonic production of acetate in healthy human adults who had received an oral dose of non-digestible disaccharide.

Principle of the isotope-dilution technique to assess turnover

The isotope-dilution technique used in the following work was based on a closed volume of distribution of a metabolite (e.g. acetate in the peripheral blood volume) in which the production and elimination rates were at a steady-state. In that volume at equilibrium the labelled tracer (e.g. [1-^{13}C]acetate) was infused at a constant and known rate until a new steady-state had been reached. Samples of blood (representative of the volume of distribution) were then collected at regular intervals, and the amount of label (isotopic enrichment) was determined using MS. From the isotopic enrichment at a steady-state the production or elimination rates of the metabolite of interest could be calculated. The level of isotopic enrichment was inversely proportional to the production and elimination rates; the lower the enrichment, the higher the production rate, with high production reducing the isotopic enrichment. The production (rate of appearance) and utilisation (rate of disappearance) rates represent the turnover at steady-state.

Isotopic enrichments and concentrations of short-chain fatty acids in plasma

Analyses of SCFA concentration in portal-vein plasma (or in faeces) have been performed in a number of laboratories and there have been no major analytical difficulties (Dankert *et al*. 1981; Cummings *et al*. 1986; Rémésy *et al*. 1995). However, simultaneous analysis of SCFA concentrations, and particularly isotopic enrichments, in human arterial or venous plasma are more challenging (Simoneau *et al*. 1994; Powers *et al*. 1995, Pouteau *et al*. 2001), as these measurements, especially that of acetate, are subject to substantial contamination because acetate is a ubiquitous molecule. As a result, particular attention needs to be paid to avoiding (or minimising) contamination in all reagents and in all preparation steps, and to reporting consistent and reproducible measurements. Our procedures for the analysis of plasma SCFA concentrations and isotopic enrichments have been described previously (Simoneau *et al*. 1994; Pouteau *et al*. 2001). Briefly, plasma was deproteinised, the supernatant fraction acidified with HCl and the organic acids isolated using a diethyl ether extraction procedure. The organic acids were then converted to tert-butyl-dimethyl-silyl-SCFA derivatives. The final solution was injected into a GC connected to a quadrupole MS. Chemical ionisation and selected ion monitoring were used to enhance sensitivity. [^{2}H]acetate was used as an internal standard for simultaneous measurement of concentrations and isotopic enrichments of [1-^{13}C]acetate, [1-^{13}C]propionate and [1-^{13}C]butyrate. Plasma concentrations were expressed in μmol/l and isotopic enrichments in mole percent excess (MPE).

Calculation of whole-body turnover

Rate of appearance of endogenous short-chain fatty acids

The endogenous rates of appearance (μmol/kg per min) of acetate, propionate or butyrate in the whole-body circulation were calculated according to the equation for steady-state during the last hour of kinetic studies on individuals in the fasted or post-absorptive (12 h overnight-fasted) state. From the determination of isotopic enrichment of each SCFA in plasma (MPE) and knowing the infusion rate (μmol/kg per min), the rate of appearance (whole-body or endogenous turnover; Wolfe, 1992; Pouteau *et al*. 1996) was calculated. Endogenous turnover was assessed in subjects in the fasted or post-absorptive steady-state with no (or minimal) colonic SCFA production.

The oxidation rate (μmol/kg per min) of acetate was then calculated from the ^{13}C-isotopic enrichments of expired CO_2:plasma acetate according to the calculation of Wolfe (1992), taking into account the CO_2 flux variable measured by indirect calorimetry.

Splanchnic first-pass retention

Splanchnic first-pass retention (intestine and liver first-pass retention) was determined as the difference between the rate of appearance of acetate during intravenous tracer infusion and the apparent rate of appearance during intragastric infusion of [1-^{13}C]acetate (Pouteau *et al*. 1996).

Exogenous and colonic acetate production

Although volunteers were not in the steady-state after ingestion of a non-digestible carbohydrate, the whole-body acetate turnover (μmol/kg per min) was calculated using the equation for steady-state according to Miles *et al*. (1987) and Jensen *et al*. (1990). The equivalence of steady- and non-steady-state equations was tested by intravenously infusing healthy volunteers with labelled acetate and an increasing amount of unlabelled acetate. The instantaneous whole-body acetate turnover was calculated using the steady-state equation at each sampling point during the

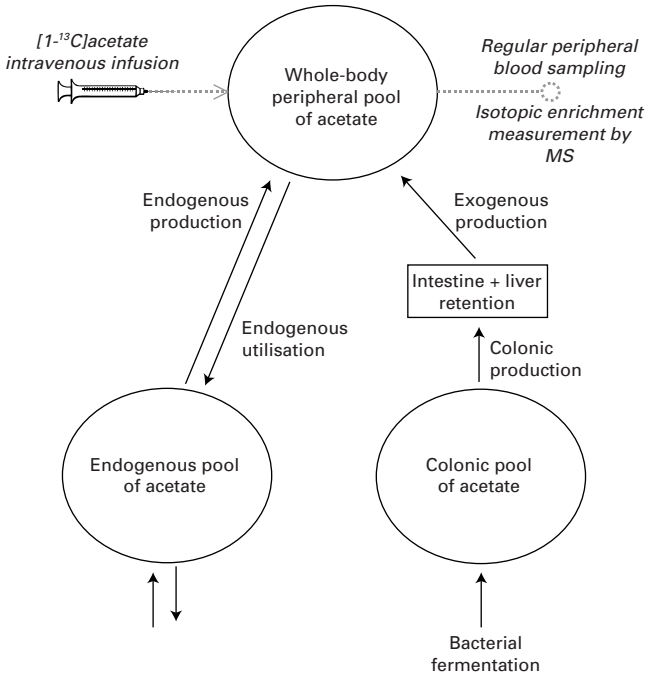

Fig. 1. Model of the whole-body peripheral pool of acetate in healthy adult human subjects. The whole-body peripheral turnover of acetate is determined by the rates of endogenous turnover and the exogenous production. Whole-body turnover is assessed from isotopic enrichment measurements on blood samples following an intravenous continuous infusion of [1-^{13}C]acetate tracer in healthy volunteers. Exogenous production is defined as colonic production after accounting for splanchnic retention. The colonic production of acetate following intake of non-digestible carbohydrate is obtained after determination of the endogenous turnover and the splanchnic retention.

kinetic study. This whole-body acetate turnover represented the endogenous turnover and the exogenous production according to our model (Fig. 1).

Exogenous acetate production (μmol/kg per min) was obtained following intake of non-digestible carbohydrate, from the difference between whole-body acetate turnover and the endogenous production measured before fibre intake (Pouteau *et al*. 1998*b*). Endogenous acetate turnover and instantaneous whole-body turnover were estimated before and after lactulose ingestion respectively. The rate of appearance of exogenous acetate in the peripheral circulation beyond the hepatic filter was calculated, and finally the colonic production was derived (Fig. 1). For development of the model it was confirmed that the endogenous production was unchanged for that range of acetate concentrations (< 550 μmol/l) and with a low physiological exogenous supply (< 11 μmol/kg per min). Exogenous acetate production was the quantity of acetate (mol) per unit time measured in the systemic blood circulation that had been recovered from colonic fermentation and the splanchnic retention. The recovery (mmol) during 6 h observation was measured from the area under the curve of exogenous acetate production, using a trapezoidal method. Colonic acetate production (mmol) was calculated from the exogenous acetate entering the systemic circulation, taking into account the splanchnic first-pass retention of acetate.

In vivo studies of short-chain fatty acid endogenous metabolism

The endogenous metabolism of SCFA originates from body cells producing and utilising SCFA; cells having the necessary enzymes. This information is available, particularly for acetate. Knowles *et al*. (1974) have demonstrated the activities of acetyl-CoA hydrolase and synthetase that respectively release acetate into, and take up acetate from, the peripheral circulation. In a comparison with ruminant species Annison & White (1962) described in sheep an endogenous turnover of acetate additional to the exogenous rumen turnover. In non-ruminant species the endogenous production of SCFA should probably account for a major part of the whole-body peripheral production of SCFA. To evaluate the endogenous metabolism of SCFA, subjects were studied in the post-absorptive or the fasted state. Under such physiological conditions the intestinal fermentation process was minimised, as well as intestinal SCFA production. A series of determinations of whole-body acetate turnover were carried out in healthy human volunteers in the post-absorptive state (Pouteau *et al*. 1996). A primed constant infusion of [1-^{13}C]acetate was administered

Table 1. Endogenous turnover of short-chain fatty acids: turnover (rate of appearance) of acetate, propionate and butyrate determined using the same stable-isotope-labelled tracer approach in rats, dogs and human subjects (from Pouteau *et al*. 1996, 1998*a*) and oxidation of acetate and its contribution to resting energy expenditure in human subjects (from Pouteau *et al*. 1996)*
(Values are means with their standard errors)

	Turnover (μmol/kg per min)		Oxidation (μmol/kg per min)		Concentration (μmol/l)	
	Mean	SE	Mean	SE	Mean	SE
Human subjects in post-absorptive state						
Acetate	8·3	0·6	5·7 (6·5% of energy expenditure)	0·5	138	1
Dogs in 24 h fasted state						
Acetate	24·4	2·4			144	17
Rats in 22 h fasted state						
Acetate	29·2	3·8			356	23
Propionate	4·2	1·2			6·1	3·5
Butyrate	0·31	0·03			0·07	0·2

*Since all subjects were either in a fasted or post-absorptive state, the turnover was considered to be the endogenous turnover.

intravenously, the bicarbonate pool being primed with $NaH^{13}CO_3$. Arterial blood and expired air samples were then collected at regular intervals during the study. Endogenous turnover, oxidation rate and concentration of acetate were calculated from isotopic measurements (Table 1). About 70 % of the endogenous acetate turnover was readily oxidised in these volunteers, who were in the post-absorptive state. Further details have been reported, in particular that 7 % of the energy expenditure was attributable to acetate (Pouteau *et al*. 1996).

Furthermore, the endogenous acetate turnover was investigated in Beagle dogs. A whole-body turnover of acetate, equivalent to the endogenous turnover, of about 24 μmol/kg per min was observed in the 24 h fasted state (Pouteau *et al*. 1998*a*).

There have been fewer reports on endogenous production of propionate and butyrate in human subjects than there have been for acetate metabolism. The occurrence of endogenous propionate and butyrate metabolism in non-ruminants has been demonstrated by Breves *et al*. (1993), Sbaï *et al*. (1994) and Kien *et al*. (2000). Before undertaking a clinical trial for the evaluation of endogenous propionate and butyrate whole-body turnover in human subjects, a rat model was developed in which [1-^{13}C]acetate, [1-^{13}C]propionate or [1-^{13}C]butyrate was infused intravenously at a rate of 1 μmol/kg per min, and arterial and portal blood was collected. Isotopic enrichment and concentration were measured and the endogenous turnover rates for acetate, propionate and butyrate were calculated. The ^{13}C-isotopic enrichments of acetate, propionate and butyrate increased throughout the study period and reached a plateau during the last 0·5 h of the infusion. The isotopic enrichments of acetate, propionate and butyrate in the plasma of rats were 3·5 (SE 0·6), 21·9 (SE 3·7) and 76·6 (SE 1·7) MPE respectively. The concentrations and the peripheral endogenous turnover rates are shown in Table 1.

In summary, under these conditions, in animals and in human subjects in a fasted-state or in the post-absorptive state, the whole-body peripheral turnover of SCFA is essentially the endogenous production and utilisation.

There have been many determinations of SCFA turnover in non-ruminants and large discrepancies have been reported, probably due to analytical problems and physiological variations between individuals (Rocchiccioli *et al*. 1989; Kien *et al*. 1990; Bleiberg *et al*. 1992; Sbaï *et al*. 1994; Simoneau *et al*. 1994; Mittendorfer *et al*. 1998). Previous studies have shown that peripheral plasma acetate concentration increases in proportion to lactulose oral intake in healthy human subjects (Pomare *et al*. 1985). However, the concentration variable should be considered as only one factor in production and utilisation rates. The use of tracer techniques allows the dynamic situation to be determined by assessing the rates of production and utilisation (the turnover). Determination of the turnover adds another dimension to the accuracy of monitoring SCFA metabolism. Furthermore, the non-invasive aspect of using stable isotopes allows direct determination of SCFA turnover in the peripheral whole body in human subjects and in animals.

In the present work we determined the basal endogenous turnover of acetate in rats, dogs and human subjects using the same stable-isotope-tracer technique and analytical methods (Pouteau *et al*. 1996, 1998*a*). The endogenous acetate turnover increased with decreasing size of the species studied. This observation, not surprisingly, was consistent with the blood flow of the various species. As our determination of acetate turnover was of the first in human subjects, we compared present values with those of authors who had used radio-labelled tracers. The use of radioactive-tracer techniques (Skutches *et al*. 1979) gave identical results for turnover of acetate in human subjects to our stable-isotope approach, 6–8 μmol/kg per min under similar physiological conditions. The similarity of the findings validated our acetate turnover determination using the stable-isotope infusion technique.

There are few data available on propionate and butyrate turnover in non-ruminants (Breves *et al*. 1993; Kien *et al*. 2000). The level of peripheral propionate production in rats was not insignificant (about 4 μmol/kg per min), and probably originates from amino acid metabolism in the liver or the whole-body muscle mass. A slow, but not insignificant, turnover of butyrate was also found in the peripheral circulation of rats in the fasted state. Furthermore, the presence of endogenous butyrate in piglets (in the postprandial state) has been reported by Kien *et al*. (2000).

As a result of reported studies and the present work the endogenous turnover of acetate has been accurately assessed in animals and in healthy human subjects. Furthermore, the existence of a peripheral turnover of propionate and butyrate has been confirmed in rats. Determination of propionate and butyrate endogenous production rates in healthy human subjects would be an interesting question to address in future investigations.

Clinical studies on exogenous and colonic production of acetate

The exogenous production of SCFA, in particular that of acetate, was defined earlier as the measurable amount per time unit that is additional to the endogenous turnover, originating from the intestine and from splanchnic retention. Following determination of the whole-body turnover of SCFA (endogenous and exogenous rates), the splanchnic retention was taken into account in the calculation of the true *in vivo* production in the large intestine (the colonic production). The focus was on acetate, since acetate is the main SCFA reaching the peripheral circulation. A protocol was established in healthy volunteers to assess the splanchnic first-pass retention of acetate (Pouteau *et al*. 1996). The first part of the study consisted of an intragastric infusion of [1-^{13}C]acetate for 3 h through a naso-gastric feeding tube without any priming injection, followed by an intravenous infusion of the same tracer for 3 h at an identical rate. It was shown that isotopic enrichment of the plasma acetate during the intragastric infusion of [1-^{13}C]acetate was about twofold lower than that during the intravenous infusion. The apparent acetate turnover determined from the intragastric infusion was two to three times higher than when using the intravenous infusion (21·3 (SE 3·2) and 8·0 (SE 1·0) μmol/kg per min respectively; $P < 0·01$, paired *t* test). The splanchnic first-pass retention of acetate was calculated to be about 60 (SE 7) % in human subjects.

Knowing the endogenous turnover and the splanchnic first-pass retention of acetate, it was possible to finalise a model to determine the colonic production of acetate in human subjects *in vivo* following ingestion of a non-digestible carbohydrate (Fig. 1). A clinical study was done that aimed to quantify the amount of acetate produced in the large intestine of volunteers who had received an oral dose of 20 g lactulose. The endogenous turnover was assessed before lactulose intake and then the change in whole-body acetate turnover throughout the kinetic study was determined. From this change the exogenous contribution was calculated and thereafter the colonic production (Pouteau *et al*. 1998*b*). A 7 h primed constant infusion of [1-^{13}C]acetate was administered intravenously to healthy male adults before and after ingestion of 20 g lactulose. Before lactulose intake the plasma acetate concentration and H_2 level were found to be low, and the endogenous turnover of acetate was similar to previous values (6·0 (SE 0·7) μmol/kg per min). After lactulose intake the mean breath H_2 and the plasma acetate concentration increased and then returned to approximately initial values within 6 h. Compared with the basal endogenous rate, whole-body acetate turnover increased, reached a plateau (9·2 (SE 0·9) μmol/kg per min) and then decreased in a manner similar to that of the H_2 and acetate levels (Fig. 2). The calculated exogenous acetate production increased (3·2 (SE 0·4) μmol/kg per min) and then decreased similarly, the residual exogenous acetate production being about 0·5 μmol/kg per min (Fig. 2). The amount of exogenous acetate recovered in the peripheral blood was 56·1 (SE 4·3) mmol over 6 h. Using our model it was determined that 20 g lactulose yields 140·4 (SE 11·7) mmol colonic acetate from bacterial fermentation (Pouteau *et al*. 1998*b*).

In summary, while determining instantaneous whole-body peripheral turnover of acetate in human subjects it was possible to evaluate, by comparison with the initial post-absorptive state, the appearance of exogenous production of acetate after an oral intake of non-digestible lactulose. Furthermore, when the splanchnic first-pass retention is accounted for the true intra-lumen production of acetate in the large intestine during bacterial fermentation could be calculated.

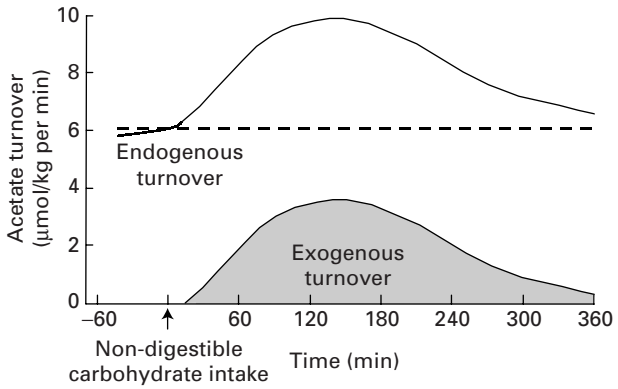

Fig. 2. Production of colonic acetate after oral intake of lactulose by healthy volunteers. Plot of the instantaneous whole-body turnover of acetate (μmol/kg per min; —) throughout the kinetic study. From –60 min to time zero (↑) the whole-body turnover represented the mean endogenous turnover (----). After ingestion of 20 g lactulose at time zero, the mean exogenous turnover of acetate (▩) increased. (From Pouteau *et al*. 1998*b*.)

Numerous authors have attempted to evaluate the colonic fermentation process for complex carbohydrate in human subjects (Cummings, 1994) using stable isotopes (Kien *et al*. 1990, 1992, 1996), but there have been no reports on the determination of exogenous and colonic acetate production rates in healthy adults. One study reported a high acetate whole-body turnover (64 μmol/kg per min) in preterm infants during lactose feeding and intra-gastric [1-^{13}C]acetate infusion (Kien *et al*. 1992). Although preterm infants cannot be compared with adult subjects, the Kien *et al*. (1992) study probably overestimated acetate turnover because of splanchnic retention of the tracer. Interestingly, the same authors evaluated lactose fermentation in preterm infants using an oro-gastric infusion of [1-^{13}C]glucose and D-[1-^{13}C]lactose (Kien *et al*. 1992, 1996), but the change in labelled glucose enrichment was assessed without direct measurement of acetate, propionate and butyrate production.

In the present work the colonic acetate originating from the bacterial fermentation process was determined *in vivo*. When compared with Miller & Wolin's (1979) stoichiometric equation for carbohydrate fermentation, 86 % of the acetate was recovered within the 6 h measurement period after lactulose ingestion. Some of the colonic acetate production might have been lost in faeces or utilised by bacteria. Nevertheless, the apparent loss could also be explained by residual exogenous acetate production at the end of the study, suggesting that the lactulose was not completely fermented, as suggested by the finding that the levels of H_2 and acetate did not return exactly to initial levels. Cummings *et al*. (1989) estimated total SCFA production to be 9·4 mmol/d per g available fermentable substrate from the gastrointestinal tract of patients undergoing surgery. In the present work, if it is considered that acetate represents 60–75 % of the total SCFA from carbohydrate fermentation (Bergman, 1990), about 185–205 mmol total colonic SCFA was thus produced from 20 g lactulose (9·25–10·25 mmol/d per g fermentable substrate). Our present model shows a very similar level of colonic SCFA production from non-digestible carbohydrate in healthy volunteers when compared with the findings of Cummings *et al*. (1989) and Cummings (1994) in patients undergoing surgery (i.e. conditions not applicable to normal healthy human subjects).

Conclusion

SCFA (acetate, propionate and butyrate) are produced mainly in the hindgut of non-ruminants, as well as in the whole-body peripheral circulation. The overall whole-body production of SCFA originates from an exogenous intestinal supply as well as from endogenous metabolism. In order to show the presence of the exogenous and endogenous components of SCFA production, a series of kinetic studies were carried out in animals and in human volunteers. To determine the endogenous SCFA turnover subjects were in the basal fasted or post-absorptive state. Exogenous production in human subjects following ingestion of lactulose was then investigated and the colonic production

of acetate calculated. Similarly, whole-body peripheral production rates of propionate and butyrate could be determined in human subjects.

The originality of our work was the strategy and kinetics used to evaluate SCFA production from colonic bacterial fermentation in human subjects *in vivo*, requiring state-of-the-art analyses of peripheral plasma SCFA. Distinguishing the endogenous turnover from the whole-body peripheral turnover was the key to determining the exogenous turnover *in vivo* in human subjects, in order to evaluate production of SCFA in the large intestine. This unique approach could be further extended to the investigation of other non-digestible carbohydrates in healthy adults as well as in patients with diseases related to bacterial fermentation in the large intestine.

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