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**Influence of the type of indigestible carbohydrate on plasma and urine short chain fatty acid profiles in healthy human volunteers<sup>1</sup>**

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Running title: SCFA profiles in healthy volunteers

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## **Abstract**

**Background:** Health effects of whole grain foods are becoming more evident. In this study, we analyzed the SCFA profiles in urine and serum derived from the colonic fermentation process of  $^{13}\text{C}$  barley meals, prepared from barley grown under  $^{13}\text{CO}_2$ -atmosphere.

**Methods:** In a crossover study 5 volunteers ingested intact barley kernels (high content of non-starch polysaccharides (NSP) and resistant starch) and barley porridge (high content of NSP only). Using a newly developed stable isotope technology we monitored 14 h and 24 h postprandially  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate in plasma and urine, respectively. The oro-cecal transit time of the meals was measured with the hydrogen breath test.

**Results:** The oro-cecal transit time was 6 h and did not differ between the two test meals. An increase of  $^{13}\text{C}$ -acetate was observed already early after ingestion of the meals ( $< 6$  h) and was attributed to early fermentation of the test meal. A rise in plasma  $^{13}\text{C}$ -propionate in the fermentation phase could only be detected after the porridge and not after the kernel meal. An increase in  $^{13}\text{C}$ -butyrate was only found in the fermentation phase and was higher after the barley kernels. Urine  $^{13}\text{C}$ -short chain fatty acids data were consistent with these observations.

**Conclusion:** The difference in the profiles of  $^{13}\text{C}$ -acetate,  $^{13}\text{C}$ -propionate and  $^{13}\text{C}$ -butyrate indicates that NSP combined with resistant starch results in an altered fermentation profile than dietary fiber alone.

**key-words:** fatty acids, volatile; stable isotope; barley; fermentation

## Introduction

Whole grain foods have been associated in epidemiological studies with the prevention of cardiovascular disease, obesity and type 2 diabetes (Anderson *et al.*, 2000; Koh-Banerjee *et al.*, 2003; McKeown *et al.*, 2002). One of the characteristics of whole grain foods is their relatively high content of resistant starch (RS), if unprocessed (Livesey *et al.*, 1995), and of non-starch polysaccharides (NSP). These components were related in intervention studies to improved insulin sensitivity (Ito *et al.*, 2006; Robertson *et al.*, 2003; Robertson *et al.*, 2005; Weickert *et al.*, 2006) and glucose tolerance (Brighenti *et al.*, 2006; Nilsson *et al.*, 2006). Furthermore, effects on lipid metabolism in adipocytes (Higgins *et al.*, 2006; Kabir *et al.*, 1998) and postprandial lipid oxidation (Higgins *et al.*, 2004) are reported. It is likely that those effects of NSP and RS are mediated through fermentation of RS and NSP in the colon which results in the production of the short-chain fatty acids (SCFA) acetate, propionate and butyrate. There is emerging evidence that SCFA not only exert effects in the colonic epithelial cells but also enter the circulation and can influence metabolic processes in other tissues and organs. Effects of acetate and propionate on glucose and lipid metabolism in the liver have been reported (Anderson *et al.*, 1984; Fushimi *et al.*, 2006; Kok *et al.*, 1996; Nishina *et al.*, 1990; Venter *et al.*, 1990; Wolever *et al.*, 1989). Acetate is used in the liver as substrate for the synthesis of cholesterol and fatty acids whereas it seems that propionate inhibits these processes (Nishina *et al.*, 1990). Effects of SCFA on adipose tissue metabolism are likely as SCFA have been identified to be ligands for the orphan G protein-coupled receptor GPR41 which is primarily expressed in adipose tissue (Brown *et al.*, 2003) and stimulation of leptin production in adipocytes by propionate (not acetate) has been described (Xiong *et al.*, 2004). Since adipose tissue is known to secrete various signalling peptides influencing among others insulin sensitivity, feeding behaviour and inflammation (Gimeno *et al.*, 2005) it could constitute an important link between SCFA production and peripheral metabolic effects. To investigate the interaction between colonic SCFA production and peripheral tissue it is necessary to obtain information about the extent to which fermentation derived SCFA reach systemic circulation.

Furthermore, as different SCFA can exert different and even opposing effects, information is needed as to how different fermentable substrates affect these SCFA patterns in the circulation.

Recently, we developed new analytical methods to determine low-level enrichment of  $^{13}\text{C}$ -SCFA in human plasma and urine (Ferchaud-Roucher *et al.*, 2006; Morrison *et al.*, 2004). These methods were applied to test the hypothesis that ingestion of a test meal with a high content of NSP results in a different  $^{13}\text{C}$ -SCFA plasma profile than a test meal that in addition contains RS.  $^{13}\text{C}$ -enriched barley was obtained by culturing plants under  $^{13}\text{CO}_2$  enriched atmosphere for the test meals. The test meals administered were intact cooked barley kernels (high RS and NSP) and barley porridge (high NSP).

## **Materials and methods**

### ***Subjects***

Five healthy volunteers with a mean age of  $25 \pm 7$  y and BMI of  $22.4 \pm 2.4$  participated in the study. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (except for appendectomy). The subjects were free of antibiotics or any other medical treatment influencing gut transit or intestinal microbiota for at least 3 months. The study protocol complied with the Helsinki Declaration and was approved by the Ethics Committee of the University of Leuven. All subjects gave written informed consent. Each of the subjects performed two tests, respectively with barley porridge and barley kernels as test meal.

### ***Study design***

After an overnight fast, the volunteers presented at the laboratory where they provided a basal urine sample and two breath samples for measurement of respectively  $H_2$  and  $^{13}CO_2$ . A catheter was introduced in an antecubital vein and a basal blood sample was withdrawn. Consequently, the volunteers received a test meal prepared from  $^{13}C$ -labelled barley as well as a glass of water. The test meal was always consumed within 20 min. Blood samples were collected every hour during the first 5 h, every 20 min from 5 to 8 h and every 40 min from 8 up to 14 h. Breath samples were collected every 20 min up to 6 h for analysis of  $^{13}CO_2$  and up to 10 h for analysis of  $H_2$ . Three volunteers collected all urine for 24 h in different fractions using specified receptacles to which neomycin was added for prevention of bacterial growth. After 6 h, 9 h and 12 h, a standard, light and non-fermentable meal was offered to the volunteers (sandwich with ham or cheese). Unlimited access to water was allowed from 6 h after the start of the test.

### ***Test meals***

#### ***Production of labelled barley***

Two batches of  $^{13}\text{C}$ -labelled barley were produced. Experimental details are mentioned in supplementary information. The first batch weighed 165 g and its  $^{13}\text{C}$  enrichment was 1.852 atom %  $^{13}\text{C}$  excess. The second batch weighed 171 g and its  $^{13}\text{C}$  enrichment was 2.985 atom %  $^{13}\text{C}$  excess. The average carbon content was 39.6 %.

#### *Preparation of test meals*

A barley pearling machine was used to remove the outer hull of the barley kernels. For the porridge, pearl barley kernels were milled in an electric household grain mill (SAMAP Elsässer grain mill F 100) on the finest grade. Barley porridge was prepared by addition of about 86 g of flour to 450 mL water along with 1 g of NaCl after which this portion was cooked in a microwave oven for 5 min at 600 W. For the barley kernel test meal about 86 g of pearl barley kernels were boiled at low temperature in 320 mL of water until all water was absorbed by the kernels.

#### *Analytical procedures*

##### *Analysis of breath samples*

Breath was collected in Exetainers (PDZ, Cheshire, UK). The  $^{13}\text{C}$ -breath content was determined by isotope ratio mass spectrometry (ABCA, PDZ, Cheshire, UK). We assumed  $\text{CO}_2$ -production to be 300 mmol/m<sup>2</sup> body surface area per hour (Ghoos et al., 1993). The body surface area was calculated by the weight-height formula of Haycock et al (Haycock *et al.*, 1978). The results for digestion of barley were expressed as the cumulative percentage of administered dose of  $^{13}\text{C}$  excreted over 6 h, the maximal excretion rate of  $^{13}\text{C}$  and the time to maximal excretion rate of  $^{13}\text{C}$ . The amount of administered dose of  $^{13}\text{C}$  was calculated taking into account the known enrichment of the labelled barley and the fact that an 86-g portion of barley contains 56 g of total starch.

Hydrogen was measured in an  $\text{H}_2$ -monitor (MEC, Brussels, Belgium) and expressed in parts per million (ppm).



Oro-cecal transit time (OCTT, i.e. the time that elapses between intake of a meal and arrival of the meal in the colon) was calculated on the basis of H<sub>2</sub>-excretion as described previously (Geypens et al., 1999).

#### *Analysis of SCFA concentrations in plasma samples*

Concentrations of SCFA were determined on a gas chromatography - mass spectrometry (GC-MS) (Hewlett Packard, Palo Alto, CA, USA) using electron impact ionisation as previously described (Ferchaud-Roucher *et al.*, 2005).

#### *Analysis of <sup>13</sup>C-enrichment in plasma samples*

After addition of 200 µL of NaCl (4M) (≥ 99.8% purity, Sigma-Aldrich) to 1 mL of plasma, proteins were removed by centrifugation at 4575 g for 2 h 40 min using Amicon centrifugal filter devices YM-30 (Millipore, Bedford, MA, USA). Plasma ultrafiltrates were concentrated to 200 µL under a nitrogen stream and acidified to pH 2.5-3 by adding 10 µL of HCl (2 M).

SPME fiber (CAR/PDMS from Supelco (Bellefonte, PA, USA)) was exposed to the analytes by immersion in the sample at 40 °C for 45 min. The SCFA adsorbed on the SPME fiber were injected by direct desorption into a gas chromatography (HP 5890, Hewlett Packard, Palo Alto, CA, USA). The <sup>13</sup>C-SCFA analysis was performed using a gas chromatography - combustion - isotope ratio mass spectrometry (GC-C-IRMS) (Delta S, Finnigan MAT, Bremen, Germany) (Ferchaud-Roucher *et al.*, 2006).

#### *Calculation of the enrichment of <sup>13</sup>C-SCFA*

The data were obtained using the formula:

$$\delta^{13}\text{C}(\text{‰}) = \left[ \left( \frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}}}{^{13}\text{C}/^{12}\text{C}_{\text{VPDB}}} - \frac{^{13}\text{C}/^{12}\text{C}_{\text{VPDB}}}{^{13}\text{C}/^{12}\text{C}_{\text{VPDB}}} \right) \right] * 1000$$

(where  $^{13}\text{C}/^{12}\text{C}_{\text{VPDB}} = 0.0112372$ ),

The observed abundance of <sup>13</sup>C in a sample, given in Atom Percent (AP) is defined as:

$$AP = \frac{100}{1 + \frac{1}{(1 + 0.001 * \delta^{13}C_{sample}) * {}^{13}C/{}^{12}C_{VPDB}}}$$

The isotopic enrichment in a sample as compared to the basal value is given in Atom Percent Excess (APE):

$$APE = AP_{sample} - AP_{basal\ value}$$

Finally, plasma  $^{13}C$ -SCFA concentrations were calculated by multiplying the APE of the respective SCFA with its plasma concentration.

#### *Analysis of urine samples*

SCFA concentration and  $^{13}C$  abundance was measured in urine following the method of Morrison and co-workers (Morrison *et al.*, 2004).

#### *Statistical analysis*

Results are expressed as mean values and standard deviations. Because of the small number of subjects non-parametric statistical evaluation (Wilcoxon test and Mann-Witney test) was performed using SPSS software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, USA). The level for statistical significance was set at  $P < 0.05$ . Differences for which  $P$ -values were  $<0.1$  were considered as tendencies.

## Results

### *OCTT*

OCTT was calculated on the basis of an increase in H<sub>2</sub>-excretion. No statistically significant differences were observed in OCTT values of barley porridge (360 ± 47 min) as compared to barley kernels (372 ± 18 min) ( $P = 0.705$ ).

### *Digestion of administered <sup>13</sup>C-barley*

The cumulative <sup>13</sup>C-excretion (33.9 ± 19.5 vs 26.7 ± 15.1 % of administered dose) as well as the peak excretion (7.9 ± 4.9 vs. 6.3 ± 3.6 % of administered dose/h) were not significantly different for <sup>13</sup>C-barley porridge and for <sup>13</sup>C-barley kernels, suggesting no difference in the ultimate quantity of tracer oxidised. The time to maximal <sup>13</sup>C-excretion was significantly shorter for the porridge meal (200 ± 32 min) indicating a faster digestion than that of the kernel meal (300 ± 42 min) ( $P = 0.043$ ).

### *<sup>13</sup>C-SCFA concentrations in plasma*

The plasma concentrations of <sup>13</sup>C-SCFA are presented in Figure 1.

After administration of both test meals, an increase in <sup>13</sup>C-acetate was already observed in the early phase (< 360 min) (Figure 1, A). A second peak in <sup>13</sup>C-acetate concentration was observed in the later phase (> 360 min) and was higher after the barley kernel test meal than after the barley porridge test meal. The cumulative plasma concentration tended to be higher after barley kernels ( $P=0.064$ ). A rise in <sup>13</sup>C-propionate concentration was detected after the barley porridge meal for only 3 volunteers but never in the plasma after the barley kernels meal (Figure 1, B). An increase of <sup>13</sup>C-butyrate concentration was only found in the later phase (> 360 min) and was comparable after the barley kernels and barley porridge meal (Figure 1, C). Consequently, cumulative <sup>13</sup>C-butyrate plasma concentrations were not different for both meals ( $P=0.65$ ).

The proportion of  $^{13}\text{C}$ -acetate of total  $^{13}\text{C}$ -SCFA in plasma was 99% after barley kernels and 98.3% after barley porridge.

#### *Excretion of $^{13}\text{C}$ -SCFA in urine*

Figure 2 (A-C) shows the cumulative urinary excretion of the respective  $^{13}\text{C}$ -SCFA. Cumulative  $^{13}\text{C}$ -acetate excretion after 24 h was similar after both meals ( $P=0.57$ ). However, after barley porridge,  $^{13}\text{C}$ -acetate excretion proceeded somewhat faster than after barley kernels which is in agreement with the higher  $^{13}\text{C}$ -acetate plasma concentrations in the early phase. Unlike in plasma,  $^{13}\text{C}$ -propionate was retrieved in urine after both meals although in lower concentrations after barley kernels. A tendency towards a higher cumulative excretion after barley porridge than after barley kernels was observed ( $P=0.098$ ). The urinary excretion of  $^{13}\text{C}$ -butyrate was borderline significantly higher after barley kernels ( $P=0.057$ ).

Also in urine,  $^{13}\text{C}$ -acetate was by far the most predominant  $^{13}\text{C}$ -SCFA (97.4% of total  $^{13}\text{C}$ -SCFA after barley kernels and 93.5% after barley porridge).

## Discussion

In vitro fermentation studies using human (Pylkas *et al.*, 2005; Velazquez *et al.*, 2000; Weaver *et al.*, 1992) or swine inocula (Smiricky-Tjardes *et al.*, 2003) have shown that the amounts and the pattern of SCFA production vary among different types of fermentable carbohydrate. Similarly, the type of indigestible carbohydrate consumed was found to influence the distribution of SCFA in the hindgut of rats (Henningsson *et al.*, 2002) and pigs (Bird *et al.*, 2004).

Due to the inaccessibility of the colon in healthy, free-living humans it is difficult to estimate the colonic production profile of SCFA and their bioavailability to the host. Stable isotope tracer techniques have been suggested as a way to estimate the colonic acetate production (Pouteau *et al.*, 1998) and the whole body acetate flux (Morrison *et al.*, 2004). Ahmed *et al.* studied colonic SCFA formation in patients with left-sided colectomy and found a higher SCFA production and in particular butyrate production after a high-RS diet as compared to a low-RS diet (Ahmed *et al.*, 2000). Pomare *et al.* measured venous blood SCFA in humans after administration of beverages containing SCFA or fermentable carbohydrates but only found a rise in acetate concentrations (Pomare *et al.*, 1985).

In the present study, we measured  $^{13}\text{C}$ -labelled SCFA after administration of labelled barley porridge and barley kernels. The use of isotopes allowed to selectively detect SCFA derived from colonic fermentation with high sensitivity.

Barley porridge is rich in NSP whereas barley kernels contain RS in addition to NSP. The  $^{13}\text{CO}_2$ -excretion data showed that the porridge test meal was digested faster as compared with the kernel test meal (shorter time to peak excretion) which is in agreement with the higher amount of rapidly available glucose in the porridge meal (supplemental information). In contrast, the extent of digestion was relatively similar for both test meals (similar cumulative  $^{13}\text{C}$ -excretion). The amount of substrate reaching the colon may have been similar in both occasions, however, the profile of enriched SCFA in plasma differed depending on the test meal administered. Based on several studies suggesting that RS is a good source for butyrate production (Brouns *et al.*, 2002; Casterline *et al.*, 1997; De Schrijver *et al.*, 1999; Weaver *et al.*, 1992), we assumed a higher colonic  $^{13}\text{C}$ -butyrate production after the barley

kernel meal than after the porridge meal. Nevertheless, plasma  $^{13}\text{C}$ -butyrate concentrations were rather similar after both meals. A similar pattern was observed in urinary  $^{13}\text{C}$ -butyrate excretion up to 12 h. However, urinary  $^{13}\text{C}$ -butyrate excretion from 12 to 24h tended to be higher after barley kernels than after barley porridge ( $P=0.074$ ), suggesting that butyrate production in the colon proceeds slower than acetate or propionate production. This might be explained by the fact that butyrate is not produced directly from the fermented carbohydrate but through conversion of acetate (so-called mechanism of cross-feeding)(Falony et al., 2006; Morrison et al., 2006).

In vitro studies have shown that fermentation of barley derived  $\beta$ -glucans by human faecal microbiota yields SCFA in a molar ratio of acetate:propionate:butyrate of 51:32:17 (Hughes et al., 2008), which is considered propionate-rich. However, the  $^{13}\text{C}$ -SCFA were retrieved in plasma as well as in urine in a molar ratio of acetate:propionate:butyrate of 98:1:1, reflecting the considerable differential absorption and metabolism in colonic epithelial cells and liver.

Data on  $^{13}\text{C}$ -enrichment of SCFA in urine confirmed the observed difference in SCFA profile after the two test meals. Interestingly,  $^{13}\text{C}$ -enriched propionate was also observed in urine after the barley kernel test meal although to a lower extent than after the porridge meal. The measurement of low  $^{13}\text{C}$  enrichment involves subtraction of basal  $^{13}\text{C}$  abundance from each analysis. At low SCFA concentrations (as occurs with propionate and butyrate) this results in larger errors in estimating enrichment. However, the generally good agreement between plasma and urinary data, which were analysed in different laboratories, suggests that analysis of urinary SCFA enrichment profiles provides an adequate and non-invasive way to monitor the availability of SCFA to the host. This observation confirms previous data in which a similar whole body acetate flux was obtained from measurement of urine or plasma tracer enrichments (Morrison et al., 2004).

The OCTT of both test meals was measured to allow correlation of changes in plasma  $^{13}\text{C}$ -SCFA concentrations to the digestion phase (before OCTT) or the fermentation phase (after OCTT). OCTT values were similar for both test meals and amounted to approximately 6h.

When looking at the kinetics of appearance of  $^{13}\text{C}$ -labelled SCFA in the circulation, it was found that plasma concentration of  $^{13}\text{C}$ -acetate was already increased as early as after 3h, i.e. during the early phase when digestion would be thought to dominate (<6h).  $^{13}\text{C}$ -acetate could be derived from early

bacterial degradation of the test meal, a hypothesis which is favoured by the concomitant appearance of labelled propionate and butyrate, albeit to a lesser extent. In addition, all labelled SCFA were found in the 0-6h urine fraction as well. It is not clear whether this early fermentation should be due to an easily fermentable compound of the test meal, to rapid transit of the head of the meal, to rapid transit of a soluble component of the (soluble fibre rich) meal or to fermentation in the small intestine where, in the more distal parts, the density of bacterial populations lies between  $10^4$  to  $10^8$  cells/ml (as compared to  $10^{12}$  cells/ml in the colon) (Tannock *et al.*, 1995).

The methodology used in the present study allowed quantitative description of the SCFA reaching the circulation after consumption of a test meal rich in indigestible carbohydrates. To be able to relate colonic SCFA production and SCFA availability to the effects of SCFA on peripheral tissues, it will be necessary to further adapt the methodology, probably by in vivo application of known quantities of isotope-labelled SCFA.

In conclusion, the method described in this study allows to evaluate SCFA profiles obtained after administration of different indigestible carbohydrates in healthy humans. The results of the present study have clearly shown that meals containing dietary fiber combined with RS result in altered SCFA profiles as compared to meals containing dietary fiber alone.

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## Figure legends

Figure 1: Mean plasma concentrations  $^{13}\text{C}$ -acetate (A),  $^{13}\text{C}$ -propionate (B) and  $^{13}\text{C}$ -butyrate (C) after a barley kernels (BK) (—◆—) and barley porridge (BP) (—■—) test meal (n=5 subjects; mean  $\pm$  sd). Left panel: instantaneous concentrations, right panel: cumulative concentrations.

Figure 2: Mean cumulative urinary concentrations of  $^{13}\text{C}$ -acetate (A),  $^{13}\text{C}$ -propionate (B) and  $^{13}\text{C}$ -butyrate (C) after a barley kernels (BK) and barley porridge (BP) test meal (n=3 subjects; mean  $\pm$  sd)



Supplementary information is available at <http://www.nature.com/ejcn/index.html>

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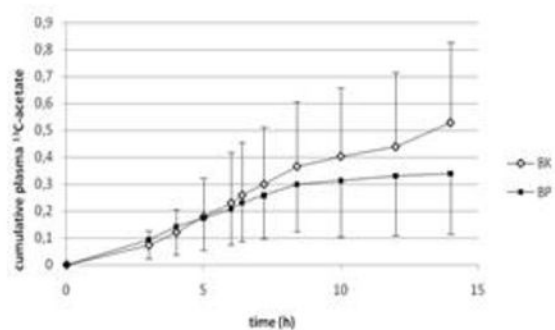
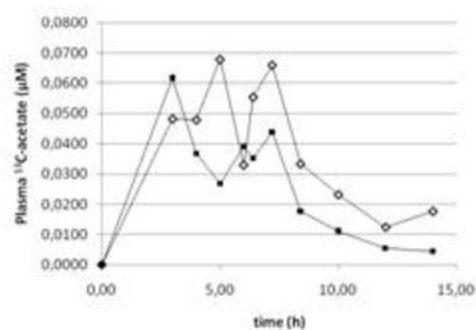
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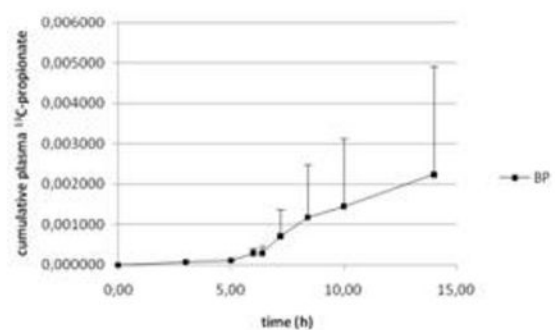
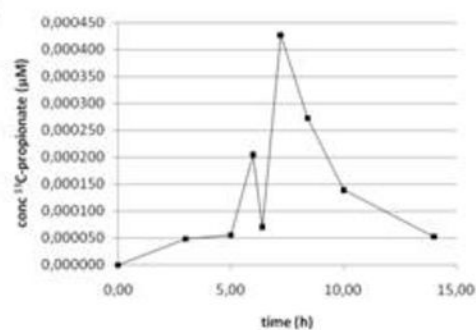
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Fig. 1

A



B



C

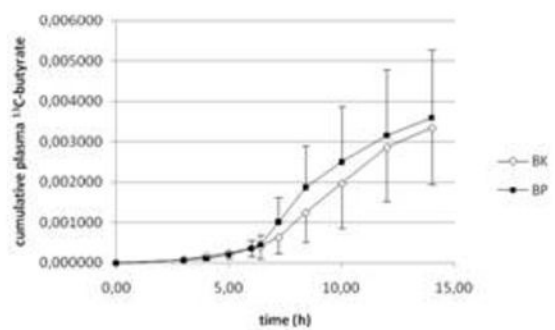
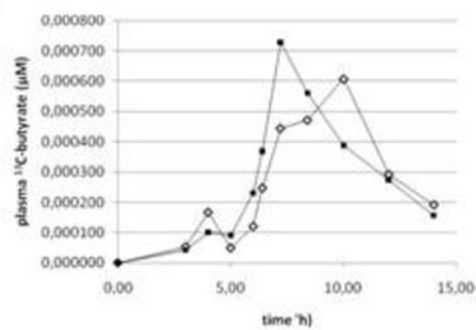




Fig. 2

