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ORIGINAL CONTRIBUTION

Metabolic signature of ^{13}C-labeled wheat bran consumption related to gut fermentation in humans: a pilot study

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Abstract

Purpose The aim of this pilot study was to analyze concomitantly the kinetics of production of ^{13}C-labeled gut-derived metabolites from ^{13}C-labeled wheat bran in three biological matrices (breath, plasma, stools), in order to assess differential fermentation profiles among subjects.

Methods Six healthy women consumed a controlled breakfast containing ^{13}C-labeled wheat bran biscuits. H_2, CH_4 and $^{13}CO_2$, $^{13}CH_4$ 24 h-concentrations in breath were measured, respectively, by gas chromatography (GC) and GC-isotope ratio mass spectrometry (GC-IRMS). Plasma and fecal concentrations of ^{13}C-short-chain fatty acids (linear SCFAs: acetate, propionate, butyrate, valerate; branched SCFAs: isobutyrate, isovalerate) were quantified using GC-combustion-IRMS. Gut microbiota composition was assessed by16S rRNA gene sequencing analysis.

Results H_2 and CH_4 24 h-kinetics distinguished two groups in terms of fermentation-related gas excretion: high-CH_4 producers vs low-CH_4 producers (fasting concentrations: 45.3 ± 13.6 ppm vs 6.5 ± 3.6 ppm). Expired $^{13}CH_4$ was enhanced and prolonged in high-CH_4 producers compared to low-CH_4 producers. The proportion of plasma and stool ^{13}C-butyrate tended to be higher in low-CH_4 producers, and inversely for ^{13}C-acetate. Plasma branched SCFAs revealed different kinetics of apparition compared to linear SCFAs.

Conclusion This pilot study allowed to consider novel procedures for the development of biomarkers revealing dietary fiber-gut microbiota interactions. The non-invasive assessment of exhaled gas following ^{13}C-labeled fibers ingestion enabled to decipher distinct fermentation profiles: high-CH_4 producers vs low-CH_4 producers. The isotope labeling permits a specific in vivo characterisation of the dietary fiber impact consumption on microbiota metabolite production.

Clinical trial registration The study has been registered under the number NCT03717311 at ClinicalTrials.gov on October 24, 2018.

Keywords Short-chain fatty acids · Stable isotopes · Gut microbiota · ^{13}C-labeled dietary fiber · Methane · Branched short-chain fatty acids

Laure Meiller and Valérie Sauvinet have contributed equally to this work.

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Introduction

Dietary fibers represent a huge interest for the scientific and medical community, but also for the agri-food industry, through their interaction with host microbiota and metabolism. Dietary fibers are known to be beneficial for human health and their consumption at a sufficient level (most countries recommend a daily intake of 25–35 g for adults) is associated to adequate transit time and, reduced blood glucose and cholesterol concentrations, and reduced risk of developing some types of cancers and cardiovascular diseases [1, 2]. However, because of the broad diversity of dietary fibers (resistant starches, non-starch polysaccharides, oligosaccharides, lignin and analogous polysaccharides), their specific mechanisms of action related to human health remain to be elucidated. Dietary fibers are generally classified according to their water solubility and viscosity, obtained via in vitro methods but these characteristics do not always predict their distinct physiological effects [3, 4]. Moreover, metabolic responses to dietary intervention including fibers also vary among individuals, highlighting the need for precise and personalized characterization of food impact on health. When assessing the impact of dietary fiber consumption on human health, the metabolites produced by bacterial communities during fermentation such as hydrogen (H_2), methane (CH_4) and short-chain fatty acids (SCFAs) could be of major interest. Once absorbed into the bloodstream, these gases diffuse into the pulmonary alveoli and ~ 10% end up in the exhaled air [5]. Their measurement in the exhaled air could be a potential biomarker of gut microbiota-diet interaction and fermentation profiles in response to dietary fiber ingestion. Hydrogen originates exclusively from the gut microbiota and plays a key role in many microbial metabolic pathways [6]. Methane is a final product of the fiber degradation and is produced by methanogen microorganisms from hydrogen and CO_2 produced by the gut microbiota [7]. It was observed that the coproduction of methane and hydrogen, as measured in breath excretion test, was associated with greater body mass index and body fat [8–10]. In addition to dietary sources, SCFAs are produced in the colon during bacterial fermentation of undigested carbohydrates and to a lesser extent of proteins. They constitute the main products of saccharolytic fermentation of non-digestible carbohydrate in the large intestine [11, 12]. Their numerous roles in human physiology are now well documented and make them of greatest interest as well for health [13].

In order to precisely characterize the metabolic fate of dietary fibers and impact on microbiota-host interaction, the use of stable isotope labeled dietary fiber has appeared as an interesting tool. Using an indirect strategy based on stable isotope dilution method, Boets et al. infused ^{13}C-SCFA to individuals to quantify the in vivo colonic production of SCFAs after consumption of inulin, a readily fermentable carbohydrate [11]. Deroover et al. used uniformly stable isotope labeled inulin to study the impact of wheat bran consumption on SCFA production [14, 15]. We followed a more direct strategy consisting in labelling the fiber of interest itself [16, 17].

In this exploratory study, ^{13}C-labeled wheat bran biscuits have been provided to 6 healthy female subjects at breakfast. Expired H_2 and CH_4 concentrations were measured for 24 h to identify the fermentation profile of the subjects. The first objective was to follow concomitantly the kinetics of labelling appearance in microbiota fermentation metabolites in 3 different biological matrices (plasma, stools, breath) following the labeled wheat bran biscuits ingestion. The second objective was to investigate whether differences in the individuals' fermentation profile alter the production of labeled metabolite within the 3 matrices. Moreover, we also evaluated specifically the concentrations of branched short-chain fatty acids (BCFAs) concentrations in plasma and stools, regarding their growing interest in the gut environment.

Materials and methods

Study population

Six healthy women, 29.7 ± 1.7 years, were recruited to participate in this study. Mean body mass index (BMI) was 23.2 ± 0.9 kg/m^2 and all subjects had a regular diet defined as three meals per day. Exclusion criteria were the use of antibiotics, prebiotics and probiotics, during the month before the investigation. Their usual fiber consumption was of 23 ± 1 g/d. The clinical study has been registered under the number NCT03717311 at ClinicalTrials.gov on October 24, 2018.

Wheat bran biscuits

Wheat bran was obtained from soft wheat (*Triticum aestivum,* Scipion) grown in an atmosphere enriched in ^{13}C ($^{13}CO_2/CO_2$ ~ 11%). After grinding the wheat grains, the wheat bran was separated from the wheat flour and underwent a micronization, after being mixed with a standard wheat bran obtained from soft wheat (*T. aestivum*, Crousty) grown in open fields [18]. The final ^{13}C-enrichment of wheat bran was obtained by mixing ^{13}C-enriched wheat bran with an enrichment of 5.56392 Atom% to the standard wheat bran with an enrichment of 1.08106 Atom% to obtain a final enrichment of 3 Atom% of wheat bran. Biscuits were then made from this wheat bran using the rotary moulding technology. The main ingredients, white flour, butter, sugar and water were mixed altogether to obtain a dough. Manually, the biscuits were formed using a metal cercle to obtain

Table 1 Nutritional composition of the ^{13}C-naturally labeled wheat bran biscuits

	Ingested quantity	Lipids	Proteins	Sugars	Starch	Dietary fibers
Biscuits (g)	136	21.1	15.5	24.8	40.4	19.2
Of which labeled wheat bran (g)	40	1.8	6.1	1.8	9.4	16.7
Energy (kcal)	547	190	62	99	162	35
Energy %	–	34.7	11.3	18.1	29.5	6.4

The nutritional parameters were measured analytically by UPSCIENCE (Saint-Nolff, France) with internal methods adapted from international reference methods (fiber: AOAC 985–29; total starch: enzymatic method as described in the French standard V18-121; fat and moisture: methods described in the French Decree of 8 September 1977; protein: Kjeldahl method). The ingredients in descending order of proportion were: ^{13}C-labeled wheat bran (AP = 3.0%), flour, sugar, egg, butter, water, sodium bicarbonate, salt and pyrophosphate

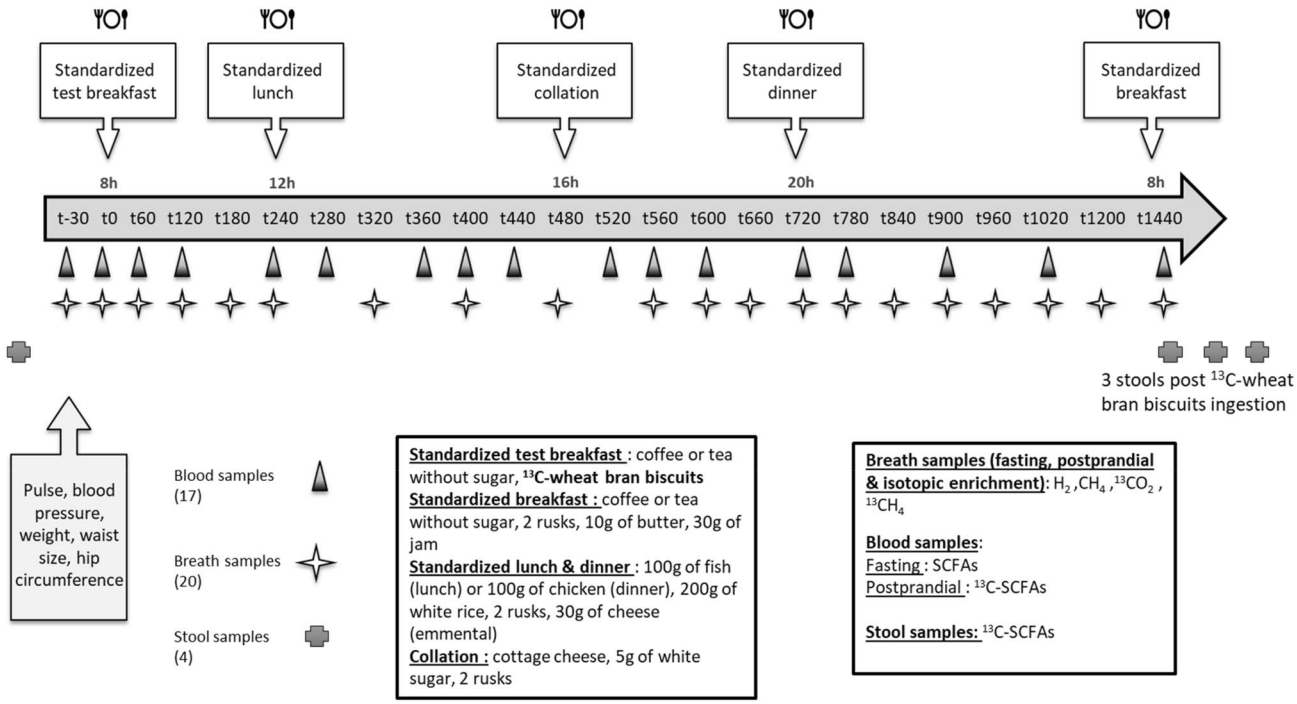

Fig. 1 Metabolic test design. A standardized test breakfast including ^{13}C-labeled wheat bran biscuits, and several standardized meals or collations were administered throughout the 24 h-exploration day. Breath samples were collected for the measurement of H_2, CH_4, $^{13}CO_2$ and $^{13}CH_4$; blood samples were collected for the analysis of ^{13}C-SCFA concentrations; stools were collected for the analysis of.^{13}C-SCFA concentrations up to 4 days post ingestion, depending on the subjects (3 stools collected post ingestion for all)

individual 17 g biscuits after cooking. The cooking process was done in a rotative oven at 160 °C during 30 min. (see Table 1 for composition). Only the wheat bran part of the biscuits was labeled with carbon 13.

Study design

Each subject was considered for a 24 h-period including a test day at the Centre de Recherche en Nutrition Humaine Rhône-Alpes (Fig. 1). During the seven days before the test, they were asked to avoid rich-^{13}C-food items (maize based, sugar cane, exotic fruits, etc.). During three days prior to the test day, subjects were instructed to consume a low fiber diet, consisting of white bread, and no more than 100 g of cooked vegetables per day among a selection which has been provided to them, raw fruits and vegetables being totally excluded. They were also asked to avoid alcohol consumption. In the evening prior to the test day, the subjects consumed a fully-digestible and non-fermentable standardized meal. To ensure that they followed the instructions they were given, the subjects had to fill dietary records. After an overnight fast, the subjects presented themselves at the Centre

de Recherche en Nutrition Humaine Rhône-Alpes and two fasting breath samples for the measurement of exhaled metabolites, and one basal stool were collected. A catheter (Introscan Safety®, B. Braun, Melsungen, Germany) was placed into an antecubital vein in the forearm to collect all blood samples during the test day. After collection of a basal blood sample, a standardized test breakfast was administered to the subjects (8:00 am). The breakfast consisted of 8 × 17 g of labeled biscuits (containing ^{13}C-wheat bran) accompanied by a 300 mL drink (water, unsweetened tea or coffee, according to their choice). This represented 40 g of labeled wheat bran and 19.2 g of dietary fiber. The detailed course of the test day is displayed in Fig. 1. Briefly, plasma ($n = 17$) and breath ($n = 20$) samples were collected on various time points for 24 h, as well as a baseline stool sample and the three first stools following the ^{13}C-wheat bran ingestion. Standardized -lunch (12:00 am), -collation (4:00 pm), and -dinner (8:00 pm) were served to the subjects.

Analysis of breath samples

H_2 and CH_4 concentrations were analysed through the Breath Tracker analyser Quintron using solid-state sensors and patented breath collection systems (Quintron, Quintron Instruments, Milwaukee, WI). CO_2 was simultaneously measured to assess the alveolar air quality in samples.

The ^{13}C-labeling gases apparition from the ingested biscuits was measured in exhaled CO_2 and CH_4 collected into 12 mL glass tubes (Exetainer®, Labco, Ltd, UK). $^{13}CO_2$ to $^{12}CO_2$ ratios were measured on a continuous-flow inlet system connected to an Isoprime isotope ratio mass spectrometer (Elementar UK Ltd, Cheadle Hulme, UK), the abundance of $^{13}CH_4$ was measured using a greenhouse gas system interfaced to the Precision Isotope ratio Mass Spectrometry (Elementar UK Ltd, Cheadle Hulme, UK).

SCFA analysis of plasma samples

Blood samples were centrifuged at 3600 g and + 4 °C for 10 min. EDTA-Plasma was stored at − 80 °C until extraction. SCFA concentrations and isotopic enrichments were determined after deproteinisation and acidification. First, 15 µL of an internal standard (2-ethylbutyric acid, 1 mM) was added to 1 mL of plasma in a centrifugal filter device (Vivaspin 2 CTA 20 KD, Sartorius), gently vortexed and then centrifuged at 4000 g and + 4 °C for 120 min to remove proteins and high molecular weight compounds. Plasma filtrate was then acidified to pH 2–3 by adding 18 µl of HCl 2 M before analysis.

Plasma ^{13}C-SCFA enrichment and concentration were measured combining SPME (solid phase microextraction) autosampler (MPS robotics, Gerstel) with GC-Combustion-IRMS (Isoprime, GV Instruments, Manchester, UK). SCFAs were adsorbed by liquid immersion SPME onto the coated fiber (Carboxen/polydimethylsiloxane; 75 µm film thickness; automatic from Supelco, Bellefonte, PA, USA) at 50 °C for 50 min, and then were thermally desorbed in the GC injector at 250 °C for 1 min. The injector is fitted with a pre-drilled septum (Agilent) and a narrow bore SPME injection liner (0.75 mm i.d.; Supelco). Analyses were performed in splitless mode at 250 °C on a fused-silica column (DB-FFAP 30 m × 0,25 mm × 0,25 µm film thickness; Agilent), with a splitless time of 1 min. Helium was used as the carrier gas. The SCFAs were separated at constant flow (1.2 mL.min^{-1}) with the following oven program: (a) 80 °C for 1 min; (b) increase at a rate of 20 °C.min^{-1} to 120 °C; (c) increase at a rate of 10 °C.min^{-1} to 205 °C; (d) hold at 205 °C for 2.5 min. A post run of 2 min at 240 °C was applied between each injection. The GC effluent was diverted to a flame ionisation detector until the elution of the target peaks before being switched into a copper oxide furnace maintained at 850 °C which produced CO_2 and water from the sample. Water and CO_2 passed through Nafion tubing, where water was removed, while CO_2 was transferred to the IRMS instrument via an open-split interface. Before and after the CO_2 peaks arising from sample combustion, a reference CO_2 gas calibrated against PDB was sequentially injected in the IRMS instrument for 30 s. The $^{13}CO_2/^{12}CO_2$ ratios of samples were expressed as δ^{13}C ‰ relative to PDB where PDB is the Pee Dee Belemnite international standard ($(^{13}C/^{12}C)_{PDB} = 0.0112372$).

SCFA analysis of stool samples

All subjects were provided with sterile containers and instructed on how to collect the stool samples at home and how to send back the samples to the CRNH Rhône-Alpes. The stool samples (about one gram) were frozen immediately at − 20 °C after collection at subjects' home and stored at − 80 °C when received at the CRNH Rhône-Alpes until analysis.

Sample was thawed and homogenized using a spatula. They were then maintained at + 4 °C. Approximately 200 mg (exactly weighted) were used for the determination of the dry matter and the same quantity was needed for the SCFA analysis. Briefly, 200 mg of stools were suspended in 1 mL of HCl 120 mM so that the pH of the suspension be between 2 and 3 (< SCFAs pKa). 2-ethylbutyric acid was used as the internal standard (IS) and was spiked to obtain 88 µg of IS / 200 mg of thawed stools. The suspension was vigourously vortexed and then centrifuged at 4000 g and + 4 °C for 30 min. The supernatant was filtered (Chromafil Xtra CA-45/25- cellulose acetate, Macherey Nagel, Duren, Germany) before analysis.

Stool ^{13}C-SCFA enrichment and concentration were measured by GC-Combustion-IRMS (Delta V Advantage,

Thermo Scientific, Bremen, Germany). Analyses were performed with the same parameters as the plasma SCFAs except for the following parameters: injection of 1 µL with a split 1:10 ratio at 270 °C, and combustion at 1000 °C (Isolink, Thermo Scientific).

SCFA Standard curves

Calibration curves were used to quantify the SCFAs from plasma and stools.

For plasma samples, two calibration curves were prepared. For the first one, a pool of plasma was spiked with increasing amounts of standard solution VFA (Volatil Free Acid mix, 10 mM, Supelco) from 0 to 15 µM and for the other, with acetic acid only (Sigma) from 0 to 150 µM.

For stools samples, the matrix was too heterogenous to make adequate standard curve, so calibration curve was prepared with standard solution VFA only (10 mM) from 0 to 1,5 mM in HCl 3,5 mM.

VFA standard (10 µM) and acid acetic (100 µM) solutions were used as quality control.

Gut Microbiota composition analysis

The fecal bacteria DNA extraction, sequencing and analysis was performed as previously reported [19]. Bacterial DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the 'Protocol Q' that was described by Costea and colleagues [20] with a slight modification: a reduction in time of bead beating step. Cells are mechanically lysed by running the FASTPREP Instrument for 2 min at max speed (beating 1 min and resting 5 min).

Library construction and the Illumina sequencing protocol has been previously described in detail [21]. In brief, the V5-V6 regions of the 16S rRNA gene were targeted for PCR amplification using primers 784 F [5′-RGGATTAGATAC CC-3′] and 1064 R [5′- CGACRRCCATGCANCACCT-3′]. 16S rRNA gene amplicons were sequenced by the MiSeq platform (300 bp paired-end length) at the University of Minnesota Genomics Center. All samples of this study were sequenced in the same run. Paired-end reads were merged, demultiplexed and conducted quality control implementation (length = 281 bp, mean sequence quality score ≥ 30) using QIIME2 pipeline with DADA2 [22, 23], which we refer to herein as amplicon sequences variants (ASV). The average of the sequence in all samples ($n = 60$) was 19,884. An even depth of 8,290 sequences per sample was used to conduct microbiome diversity. We assigned the sequences to taxonomic categories including kingdom, phylum, class, order, family and genus levels using a pre-trained Naive Bayes classifier based on Silva 132 99% OTUs database [24].

Calculations and statistical analysis

The objective of this pilot study was to determine the metabolic signature of ^{13}C-enriched wheat bran fermentation. Due to the lack of available published data on this specific objective, a standard power calculation to obtain the number of subjects needed for the study was not possible. Here, the choice of the sample size relayed on data from two studies. The first study, carried out in 10 subjects showed that a moderate dose of carbohydrates, 3 times/day was able to increase the production of SCFA [14]. The second study which aimed at determining the production of SCFA in 2 healthy subjects using different isotopic tracers allowed to validate a new method of detection of SCFA through the measurement of their isotopic enrichment in ^{2}H and ^{13}C [25].

^{13}C-SCFA concentrations were calculated as described elsewhere [14]. Briefly, the ^{13}C-SCFA plasma and stool concentrations were calculated according to Equation below:

$$\left[^{13}\text{C SCFA}\right] = \frac{[SCFA] \text{ X } APE}{AP_{Fiber} - AP_{basal}}$$

Where [^{13}C-SCFA] is the ^{13}C-concentration of each SCFA at time point; [SCFA] is the total concentration of each SCFA at time point; APE is the Atom Percent Excess of each SCFA at time point; AP_{fiber} is the atom percent of the ^{13}C-wheat bran of the ingested biscuits; AP_{basal} is the atom percent of each SCFA at time point t0.

The cumulative concentrations of ^{13}C-SCFAs in plasma and stools, of $^{13}CO_2$ in breath were calculated using the linear trapezoidal rule (AUC) and expressed in µmol/L·h, mg/g.d and Atom Percent Excess.h, respectively. We measured 8h40- and 24 h-cumulative ^{13}C-SCFAs concentrations in plasma, 24 h-cumulative $^{13}CO_2$ in breath. For stools, we cumulated the results of the three stools following the ^{13}C-wheat bran ingestion. Data are presented as means ± SEMs. Due to the exploratory nature of the study (n = 6), descriptive analyses were performed and simple statistical comparisons between high-CH_4 and low-CH_4 producer groups using the non-parametric two-tailed Student's t-test were carried out. Differences between means were considered significant when p value < 0.05.

Results

CH_4 and H_2 excretion profiles in breath samples at baseline and following ^{13}C-labeled biscuit ingestion

As shown in Fig. 2, at baseline, the analysis of H_2 and CH_4 kinetics in breath samples allowed to distinguish two groups in terms of fermentation-related gas excretion: a high-CH_4

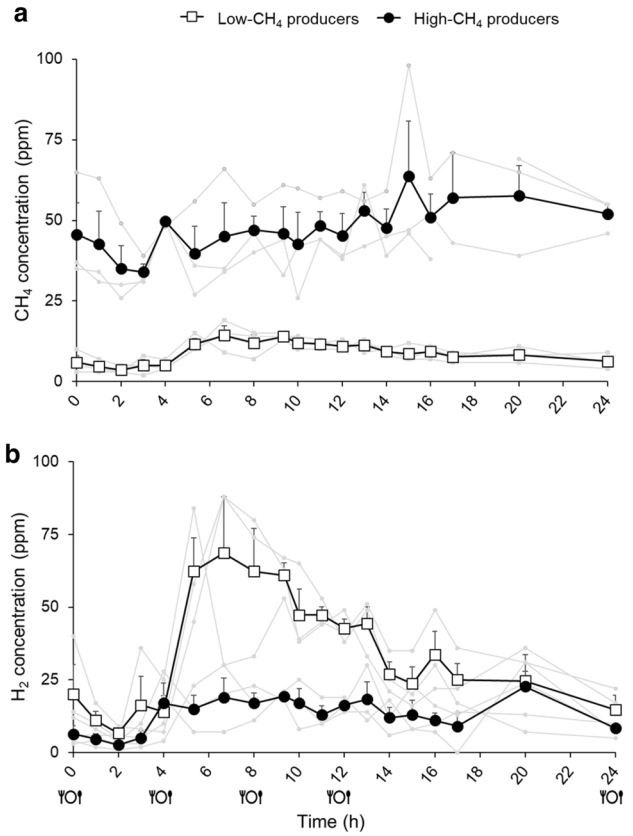

Fig. 2 CH_4 and H_2 excretion profiles in breath samples. Concentrations in CH_4 (**a**) and H_2 (**b**) of breath samples collected at baseline and following the 24 h after the standardized test breakfast ingestion. Individuals kinetics in grayed out ($n=6$). Mean ± SEM for the three low-methane producers (-□-) versus the mean ± SEM for the three high-methane producers (-●-)

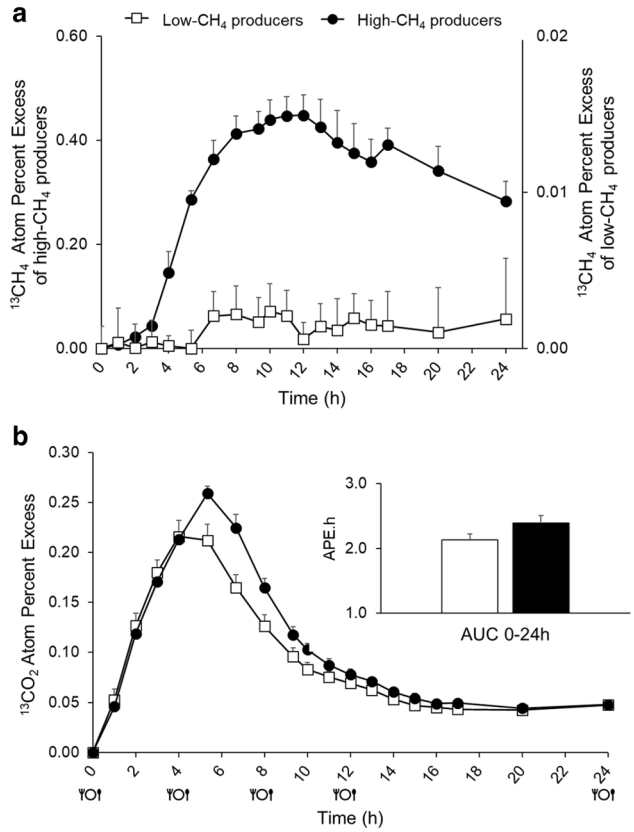

Fig. 3 $^{13}CH_4$ and $^{13}CO_2$ excretion profiles in breath samples. Atom Percent Excess (APE) of $^{13}CH_4$ (**a**) and $^{13}CO_2$ (**b**) of breath samples collected at baseline and following 24 h after meal ingestion for the three low- or high-methane producer subjects. Data are represented as mean ± SEM. Insert corresponding to 24 h-AUC for.$^{13}CO_2$ (**b**)

producer group ($n=3$) with high baseline CH_4 concentration (45.3 ± 13.6 ppm) and low baseline H_2 concentration (7.3 ± 5.8 ppm) *vs* a low-CH_4 producer group ($n=3$) with low baseline CH_4 concentration (6.5 ± 3.6 ppm) and high baseline H_2 concentration (20.8 ± 16.0 ppm). Difference between high and low baseline CH_4 concentration was significant ($p<0.05$). Following the ^{13}C-wheat bran biscuits' ingestion, the differences in exhaled H_2 and CH_4 concentrations between the two groups were maintained throughout the test. Between 3 and 5 h after the ingestion of the ^{13}C-wheat bran biscuits and after lunch, a substantial increase in breath H_2 concentrations was observed in both groups with a higher extent in the low-CH_4 producers; this was also observed for CH_4 concentrations in the low-CH_4 producers but was not visible in the high-CH_4 producers due to the important relative basal concentrations.

$^{13}CH_4$- and $^{13}CO_2$- excretion profiles in breath samples

Figure 3 shows the $^{13}CH_4$- and $^{13}CO_2$- excretion profiles in breath samples, respectively. A difference between the high-CH_4 and low-CH_4 producer groups in $^{13}CH_4$- and $^{13}CO_2$-kinetic appearance was observed, and was much more pronounced for $^{13}CH_4$.

In high-CH_4 producer group, $^{13}CH_4$ appearance in breath samples started from the second hour following the ^{13}C-wheat bran biscuits' ingestion and was massive and prolonged until the end of the exploration with no back to the baseline after 24 h. The maximal ^{13}C-enrichment compared to the baseline was 0.4487 ± 0.0380 Atom % Excess. In the low-CH_4 producer group, a weak $^{13}CH_4$ appearance in breath was measured from the 6th hour post-ingestion until the 17th hour, with a maximal enrichment compared to baseline at 0.0024 ± 0.0004 Atom % Excess. (Fig. 3A).

For both groups, the $^{13}CO_2$ excretion (Fig. 3B) rapidly increased after ingestion of ^{13}C-wheat bran biscuits and reached a maximum between approximately 4 and 5 h

post-ingestion which was, respectively, 0.2590 ± 0.0075 and 0.2218 ± 0.0120 Atom % Excess in high-CH_4 and low-CH_4 producer groups. The $^{13}CO_2$ excretion declined to almost baseline after 24 h. Nevertheless, the ^{13}C-enrichment was more important in the high-CH_4 producer group especially over the period from 4 and 10 h post-test meal ingestion and the cumulative ^{13}C-enrichment over 24 h tended to be more important for high-CH_4 producer group compared to low-CH_4 producer group (AUC Fig. 3B).

^{13}C-SCFA plasma concentrations

Mean fasting plasma SCFA concentrations were 77 ± 11 µM for acetate, 1.8 ± 0.3 µM for propionate, 0.9 ± 0.2 µM for butyrate, 1.7 ± 0.4 µM for isobutyrate, and 5.0 ± 0.6 µM for isovalerate. Figure 4 shows concentrations of plasma ^{13}C-acetate, ^{13}C-propionate, ^{13}C-butyrate, ^{13}C-isobutyrate and ^{13}C-isovalerate measured for 24 h following the ingestion of the ^{13}C-wheat bran biscuits**.** Valerate was not detectable in the plasma samples. The results showed a quick emergence of the labelling in the plasma SCFAs from the first or second hour after ingestion of the ^{13}C-labeled wheat bran biscuits. Two different patterns of the labeled SCFAs appearance were observed. On the one hand, linear SCFAs ^{13}C-acetate, ^{13}C-propionate and ^{13}C-butyrate (Fig. 4a–c) exhibited continuous appearance kinetics throughout the day of exploration, with no return to baseline after 24 h. On the other hand, branched SCFAs (BCFAs): ^{13}C-isobutyrate and ^{13}C-isovalerate (Fig. 4d,e), produced a rapid and important rise in concentrations with a plateau between the 2^{d} and the 5th hour post-ingestion for ^{13}C-isobutyrate concentrations and between the 1st and the 5th hour post-ingestion for ^{13}C-isovalerate concentrations. The return to basal values operated from more than 8 h post-ingestion. The difference between the two patterns was even clearer when cumulative concentrations from the 8h40-period were reported to the cumulative concentrations measured during all the exploration day: 75 ± 3% of the branched ^{13}C-SCFAs appeared in plasma during this first period against 47 ± 9% for the linear chain ^{13}C-SCFAs (data non shown, $p < 0.05$).

Figure 5 presents plasma ^{13}C-SCFA concentrations within the two groups, low- and high-CH_4 producers. The sum of ^{13}C-cumulative plasma concentrations of the five SCFAs was not different between groups (Fig. 5a). Individually, no difference in ^{13}C-isobutyrate- and ^{13}C-isovalerate-plasma apparition was observed between low- and high-CH_4 producer groups. However, the ^{13}C-acetate-, ^{13}C-propionate- and ^{13}C-butyrate- kinetics seemed to vary according to the group (Fig. 5b–d). Plasma concentrations of ^{13}C-acetate and ^{13}C-propionate actually tended to be higher in the high-CH_4 producers compared to the low-CH_4 producers. The corresponding cumulative plasma concentrations over 8h40 min also tended to be more important for high-CH_4 producer group compared to low-CH_4 producer group for acetate and propionate, respectively (69.5 ± 8.6 vs 44.7 ± 11.4 and 0.7 ± 0.2 vs 0.4 ± 0.1 µmol.h/L). Inversely, plasma concentrations of ^{13}C-butyrate tended to be more important in the low-CH_4 producers compared to the high-CH_4 producers with corresponding cumulative plasma concentrations over 8h40min at 1.1 ± 0.5 vs 0.6 ± 0.2 µmol.h/L.

Figure 5e shows the percentage of individual ^{13}C-SCFA plasma concentration for each group. ^{13}C-acetate represented more than 93% of the plasma ^{13}C-SCFAs whatever the group. ^{13}C-butyrate proportion seemed to be higher in low- than in high-CH_4 producer group (respectively 3.27 ± 0.66% vs 1.19 ± 0.40%) and it was the opposite tendency for ^{13}C-acetate (respectively 92.9 ± 1.7 vs 95.8 ± 0.6%). However, these trends did not reach significance. No proportion variation was noted for the other SCFAs between both groups.

^{13}C-SCFA stool concentrations

In stool samples, basal SCFA concentrations from the six subjects were 6.93 ± 0.61 mg/g dry matter for acetate, 2.32 ± 0.57 mg/g dry matter for propionate, 2.87 ± 1.04 mg/g dry matter for butyrate, 0.58 ± 0.10 mg/g dry matter for valerate, 0.53 ± 0.09 mg/g dry matter for isobutyrate, and 0.85 ± 0.16 mg/g dry matter for isovalerate. Enrichment and concentration were sufficient to evaluate the kinetics of excretion of six ^{13}C-SCFAs: acetate, propionate, butyrate, isobutyrate, isovalerate and valerate presented in Fig. 6 as a function of time for each subject. First, the kinetic of the six ^{13}C-SCFA stool concentration were quite similar for one given subject. However, a high inter-individual variability was observed. Among the 6 subjects, 4 subjects continued to excrete the ^{13}C-SCFAs in the third stool collected after the ingestion of the ^{13}C-wheat bran biscuits, while 1 subject reached his maximal ^{13}C-SCFA concentrations in the second stool collected post ingestion. 1 subject did not exhibit any increase in ^{13}C-SCFAs concentrations. The maximal ^{13}C-SCFA concentrations measured in the six subjects after the ^{13}C-wheat bran ingestion were 792 µg/g dry matter for acetate, 292 µg/g dry matter for propionate, 24 µg/g dry matter for butyrate, 58 µg/g dry matter for valerate, 61 µg/g dry matter for isobutyrate, and 74 µg/g dry matter for isovalerate. If making the assumption that one carbon 13 atom from the fiber is transferred on the produced SCFAs, that is equivalent to a ^{13}C-enrichment expressed in mole percent excess of 0.23 for acetate, 0.38 for propionate, 0.47 for butyrate, 0.48 for valerate, and 0.40 for isobutyrate and isovalerate.

Proportions of each ^{13}C-SCFA reported to the total ^{13}C-SCFAs measured in the stools are shown in Fig. 7, according to the fermentation profile of low- or high-CH_4 producers. Differences between groups were observed for

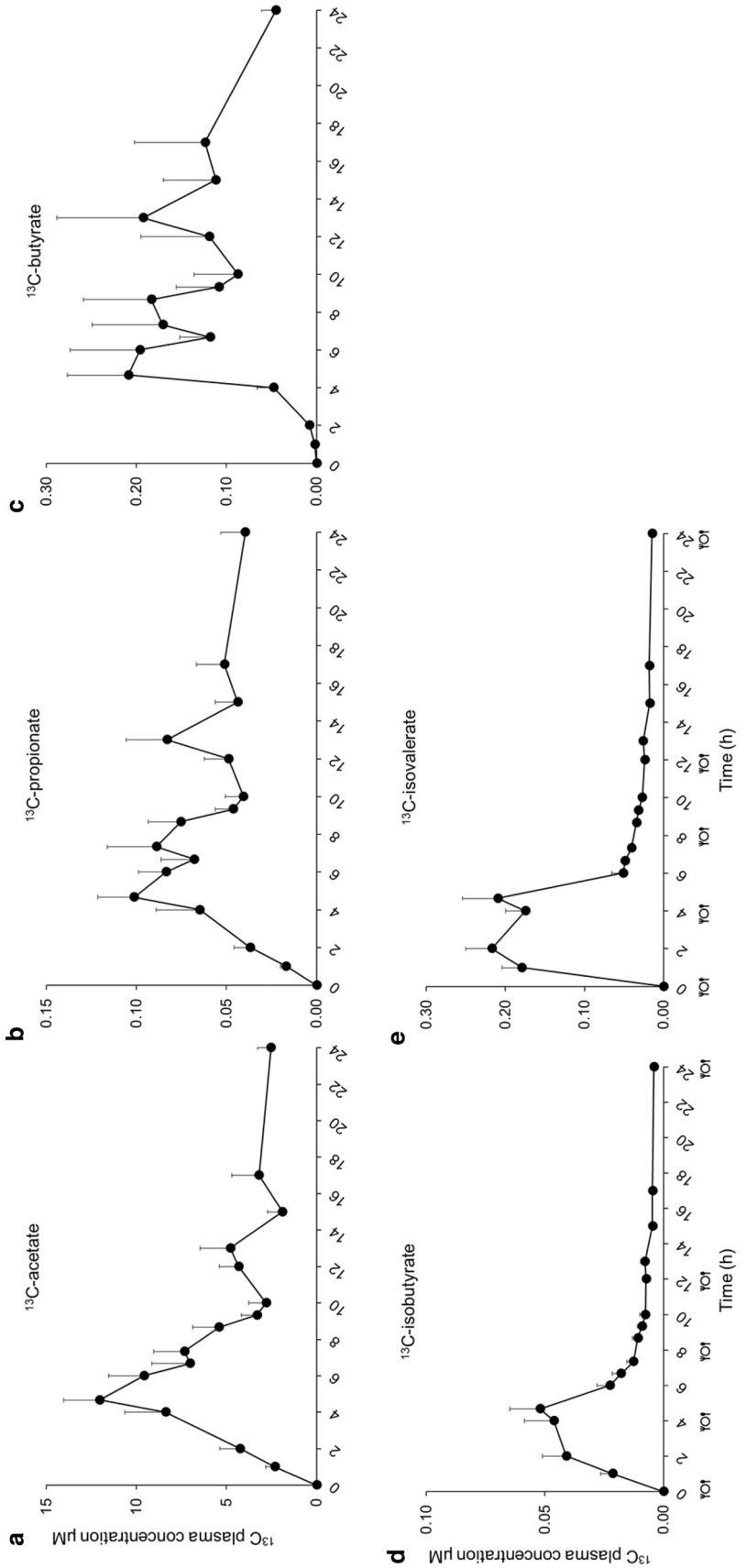

Fig. 4 Plasma concentrations kinetics of ^{13}C-acetate (**a**), ^{13}C-propionate (**b**), ^{13}C-butyrate (**c**), ^{13}C-isobutyrate (**d**), ^{13}C-isovalerate (**e**), as a function of time ($n = 6$). Data are represented as mean ± SEM

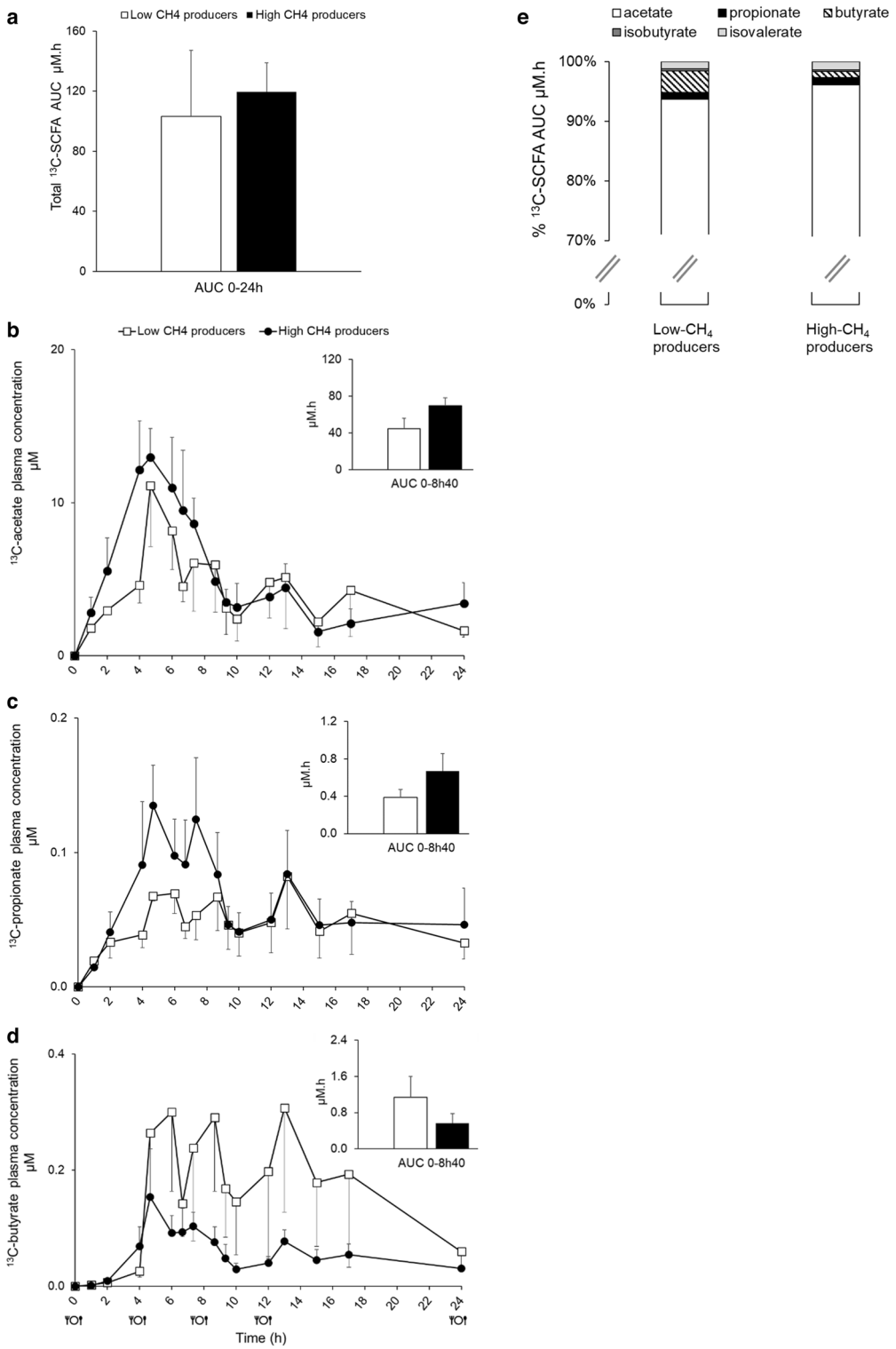

Fig. 5 ^{13}C-SCFA plasma concentrations according to the fermentation profile of the subjects. For the three low- or high-methane producer subjects: AUC of total ^{13}C-SCFA plasma concentrations (**a**); ^{13}C-acetate (**b**), ^{13}C-propionate (**c**) and ^{13}C-butyrate (**d**) concentrations with the corresponding AUC (0-8h40min); ^{13}C-SCFA proportions recovered in plasma over 24 h (**e**)

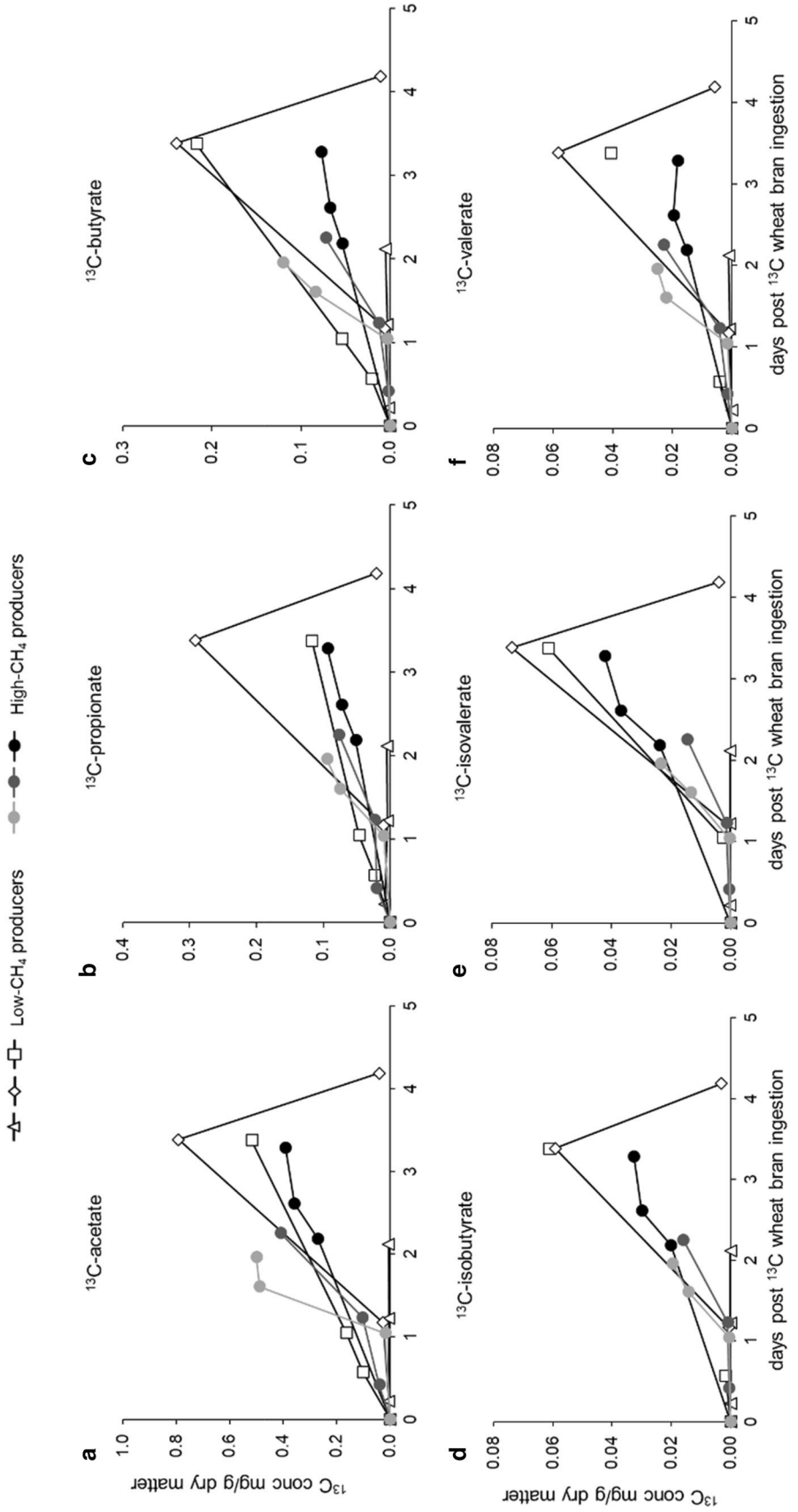

Fig. 6 Time course for the excretion of ^{13}C-acetate (**a**), ^{13}C-propionate (**b**), ^{13}C-butyrate (**c**), ^{13}C-isobutyrate (**d**), ^{13}C-isovalerate (**e**), ^{13}C-valerate (**f**) in the 3 stools collected post ^{13}C-wheat bran ingestion for each subject ($n = 6$). Stool concentrations are expressed in mg/g dry matter

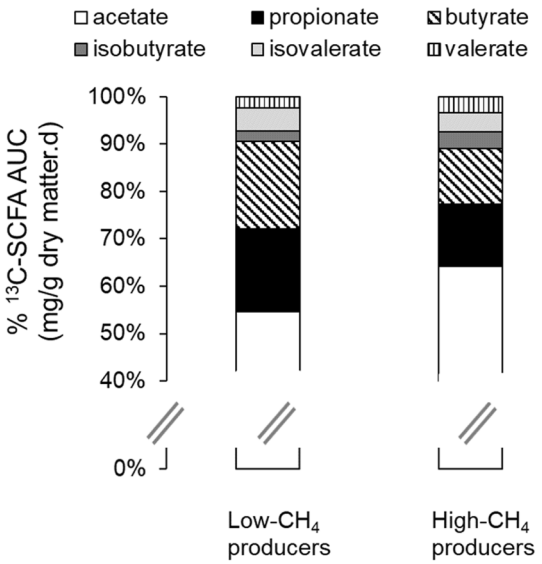

Fig. 7 Proportions of cumulative ^{13}C-SCFAs excreted in stools in the three low- or high-methane producer subjects

^{13}C-acetate, ^{13}C-propionate, ^{13}C-butyrate and ^{13}C-isobutyrate. Notably, ^{13}C-propionate and ^{13}C-butyrate proportions seemed to be more important in the low- than in the high-CH_4 producer group (^{13}C-propionate: $23.5 \pm 6.9\%$ vs $13.2 \pm 0.9\%$; ^{13}C-butyrate: $20.1 \pm 2.2\%$ vs $11.7 \pm 0.8\%$, respectively) whereas ^{13}C-acetate and ^{13}C-isobutyrate proportions looked less important in the low- than in the high-CH_4 producer group (^{13}C-acetate: $48.6 \pm 7.1\%$ vs $65.5 \pm 2.0\%$; ^{13}C-isobutyrate: $1.3 \pm 1.3\%$ vs $8.1 \pm 3.0\%$, respectively). No proportion variation was noted for ^{13}C-isovalerate and ^{13}C-valerate between both groups.

Gut microbiota analysis

Overall gut microbiota diversity (observed OTUs, Shannon and Pielou index) was not significantly different between the two groups. When assessing the relative abundance of bacteria at different taxonomy levels, we did not show significant differences within the present small group of subjects. However, we observed that an ASV belonging to Methanobrevibacter genus was lacking within the low-CH_4 group compared to the high-CH_4 group.

Discussion

In this pilot study, we followed the metabolic fate of a ^{13}C-labeled dietary fiber, specifically ^{13}C-wheat bran formulated into biscuits. We investigated for the first time whether fermentation profile differs among subjects and if so, whether labeled gut microbiota metabolites production in 3 biological matrices was altered accordingly ($^{13}CH_4$ in breath, ^{13}C-SCFAs in plasma and stools). We distinguished two distinct fermentation profiles: low- or high-CH_4 producers by analyzing breath excretion gases and we observed that differences in plasma SCFAs kinetics also may exist according to this profiling.

After ^{13}C-wheat bran ingestion, higher plasma and stool ^{13}C-acetate and plasma ^{13}C-propionate concentrations were observed in the high-CH_4 producer group, whereas the proportion of plasma and stool ^{13}C-butyrate seemed to be higher in low-CH_4 producers. To the best of our knowledge, this is the first study that revealed differences in plasma- and stool-SCFA production in response to fiber ingestion according to individuals' breath excretion profile in relation to gut fermentation. The results of this study also revealed differences in plasma SCFA kinetics depending if considering linear or branched fatty acids.

First, when analyzing exhaled H_2 and CH_4 24 h-kinetics following the labeled wheat bran ingestion, we distinguished two fermentation profiles: the high-CH_4 producers and the low-CH_4 producers, which were, respectively, low-H_2 producers and high-H_2 producers. Consistently, the kinetics of appearance of $^{13}CH_4$ in exhaled air were extremely different between the two groups with a much higher $^{13}CH_4$ in the high-CH_4 producers group. These findings corroborate the results of several works reviewed by de Lacy Costello et al. in 2013 [7]. The measurement of breath volatile metabolites has recently been linked to different fermentation profile and to the composition of the microbiota in another study by our group, following the ingestion of chitin-glucan [26]. The production of H_2 and/or CH_4 by gut fermentation will partly depend also on the composition of gut microbiota. Interestingly, CH_4 is a by-product of the anaerobic metabolism of methanogens, which involves the use of H_2 and CO_2. Since H_2 is used for CH_4 production, the fact that high-CH_4 producers exhibit low H_2 production is coherent. In the literature, 35 to 50% of the adult population have been reported to be CH_4-producer [27]. Similarly, 50% of the 6 subjects included in our study were high-CH_4 producers. This breath CH_4 production is generally largely associated to the presence of methanogens that belong to the Archaea domain and are located in the distal part of the colon [28]. In the pilot study, the microbiota of high-CH_4 producer group was characterized by the presence of methane-producing bacteria, an ASV belonging to Methanobrevibacter genus whereas it was absent within the low-CH_4 producer group. As the small size of our study could not allow further conclusions, it would be of great interest to combine such comprehensive multi-matrices metabolites profiling with microbiota analysis in a future larger scale clinical study to establish direct links between labeled metabolites and implicated species. It would be of high interest to go even further, using a more enriched dietary fiber and RNA stable isotope probing, to potentially

show carbon utilization from the labeled fiber by specific bacterial populations in gut microbiota [29]. Indeed, during the fiber fermentation process, the methanogens use as a substrate the hydrogen co-produced with SCFAs by the primary fermenting bacteria [30]. By removing this metabolic product, methanogens stimulate the production of SCFAs and enhance the availability of calories to the host [9]. Concerning the exhaled $^{13}CO_2$, slight differences observed on the kinetics between the two fermentation profile groups were not significant, and should be considered with caution. Indeed $^{13}CO_2$ excretion is not exclusively related to microbiota metabolism but to both the ^{13}C-metabolite oxidation resulting from the fermentation by the intestinal microbiota and the oxidation of the digestible part of the bran. If we refer to Table 1, it appears clearly that not only dietary fibers are ^{13}C-labeled, but sugars, starch, lipids, and proteins too. These macronutrients are digestible and represent a mass ratio of 1,1: 1 compared to the non-digestible dietary fiber part. In comparison to bran, inulin, which was tested in other works, is entirely non digestible and reach the colon intact [17].

The major end-products of microbial fermentation, acetate, butyrate and propionate have a recognized and well documented health interest [13]. Regarding SCFAs, up to now, the majority of studies have relied on measurements of fecal SCFA concentrations. In recent years, due to the development of analytical techniques adapted to small concentrations of these analytes, SCFA measurement in serum or plasma are more frequent. On note, the physiological significance of SCFA measurement may vary according to the chosen matrix. Indeed, fecal SCFA concentrations result from three parameters, namely colonic SCFA production, their use by the colonocytes to fulfill their energetic needs (80% of the absorbed SCFAs [31]) and their absorption into the systemic circulation. It was shown in Humans post-mortem [32] that SCFA concentrations were the highest in the ascending colon and decreased progressively until the rectal area which was found similar to stools in term of SCFA concentrations. However, a large majority of SCFAs produced by microbial fermentation are absorbed by colonocytes [33], meaning that fecal SCFAs represent only about 5% of the initial colonic production. As for plasma concentrations, based on data in the same study [32], and approximating that 1 kg of luminal matter is equivalent to 1 L [13], it appeared that acetate, propionate and butyrate measured in peripheral blood represented, respectively, only 0.1%, 0.02%, and 0.02% of the ascending colon concentration, that means an extremely minor part of SCFAs produced following the fermentation process. Indeed, if 80% of the absorbed SCFAs are used by colonocytes, the 20% remaining are transported first in the portal vein, and then to the liver, where a non-negligible part is uptaken [34]. The systemic availability of ^{13}C-labeled acetate, propionate and butyrate in human was previously evaluated, respectively, at 36, 9 and 2% of the dose administered directly in the colon [12]. SCFAs can be produced by other routes than microbial fermentation. Acetate is produced by fat oxidation, propionate is produced by branched-chain amino acid metabolism [35], and butyrate can be synthetized de novo from acetyl Co A, even if this hepatic pathway is sparingly used under normal circumstances in humans [36]. All these pathways interfere with the appearance kinetics of SCFAs in both matrices and limit the interpretation of these data. This further underlines the advantage to use a ^{13}C-labeled dietary fiber to specifically target the metabolites produced by the fermentation of the ingested fiber. Of note, fasting plasma [37–40] and basal fecal [41–43] SCFA concentrations observed during this study were in accordance with those published in the same type of population.

Concerning plasma, labeled ^{13}C-SCFAs were calculated according to Deroover et al., by multiplying the SCFA concentration and its ^{13}C-abundance [14]. We showed a transitional increase in plasma for each ^{13}C-SCFA measured, with two different apparition profiles depending on the type of SCFA's carbon tail. The linear SCFAs (acetate, propionate, butyrate), had a constant but of medium intensity labeled appearance kinetic throughout the exploration day, similar to those presented by Verbeke et al. [16], whereas the branched short-chain fatty acids, BCFAs, (isobutyrate and isovalerate), had a sharp rise of labeling appearance in the first 5 h only. To the best of our knowledge, it is the first time that BCFAs plasma kinetics are investigated in such context, demonstrating the critical difference with linear SCFAs profile. Historically, the attention has been mainly focused on the major SCFAs produced by microbial fermentation in the gut (acetate, propionate and butyrate); still little is known about the role that BCFAs play. They are produced, in more confidential quantities, through the microbial fermentation of branched-chain amino acids [44]. In our case, they probably come from the fermentation of the ^{13}C-wheat bran protein part. Nowadays, there is a growing interest to better understand the health impact of BCFAs and which role they can play in the gut environment [45]. Indeed, some authors have reported that an increased production of BCFAs has been associated with preventing irritable bowel syndrome [46] or improving insulin sensitivity on population with disturbed metabolism [47]. On the other side, BCFAs seem to be involved in the development of benign prostatic hyperplasia [48], have been related to a worse lipid profile in hypercholesterolaemia patients [49] and isovalerate may be one of the contributors to depressive disorders [50]. Thus, their role in human health still need to be clarified.

Concerning ^{13}C-SCFAs stool concentrations, a huge inter-subject variability was observed. This inter-variability may be partly related to the intestinal transit time and the variable excretion profiles, inherent to the subjects. The

number of three stools collected post ^{13}C-wheat-bran ingestion was clearly insufficient in our pilot study. Van der Beek et al. collected one single stool the first day following the investigation day that means around 24-48 h post ingestion of ^{13}C-inulin (3 At% as in our study) and observed ^{13}C-labeling between 0.3 and 0.4 Mol Percent Excess (MPE) for acetate, propionate and butyrate [17]. The maximal MPE values measured in our subjects between 24-48 h post ingestion were similar. For future studies, if the oro-fecal transit time can not be evaluated as performed by Deroover et al. [14, 15], we recommend that five stools should be collected post ^{13}C-labeled dietary fiber ingestion.

The present strategy, consisting in labelling the fibers of interest using stable isotope, has already been performed in the past using barley [16] or inulin [17]. However, to our knowledge, this is the first study using isotopic labeling which follows gut microbiota metabolites simultaneously in 3 different biological matrices. Compared to some previous studies [11, 14, 15], providing the ^{13}C-labeled wheat bran incorporated into biscuits was selected as a more physiological and direct strategy. In vitro dietary fiber fermentation experiments using fresh fecal inocula showed that SCFA profiles were different according to the dietary fiber used as a substrate [51]. Recently, our team showed that increasing the diversity of dietary fiber intake in a population of subjects at cardiometabolic risk improved lipid and insulin sensitivity parameters, and modified gut microbiota, promoting bacteria families involved in their degradation [52]. This underlines the importance to characterize in vivo the impact of dietary fiber consumption on gut microbiota metabolite production to propose the best mix of dietary fibers in the aim to improve metabolic health related to the gut microbiota.

Conclusion

This innovative pilot study allows to consider novel procedures, especially non-invasive monitoring of breath metabolites, for the development of biomarkers of fiber-gut microbiota interactions. We explored the possibility to follow specifically the metabolic fate of a dietary fiber using stable isotope methodology permitting a specific in vivo characterization of the dietary fiber impact consumption on microbiota metabolite production. Breath excretion profiling could be of high interest to distinguish specific response to dietary intervention and to carve personalized dietary interventions.

Larger scale clinical studies are now needed to confirm the observed associations between distinct breath excretion, gut microbiota composition and metabolites production.

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Author contributions LM, VS, AEB, AM, SCB, JW, AMN, ML, NMD, SV and JAN contributed to the study design. AEB contributed to data collection. LM, VS, CM and AM performed the analysis. LM, VS, HR and JAN wrote the manuscript. All authors reviewed the manuscript.

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Data availability Data will be available from the corresponding author on reasonable request.

Declarations

Conflict of interest A Meynier and S Vinoy are Mondelez International employees. No potential conflict of interest was reported by the other author(s).

Informed consent The study protocol was in line with the Declaration of Helsinki and was approved by the Ethics Committee (Comité de Protection des Personnes Nord-Ouest IV, France, Registration Number: 2018 A00949 46). All participants signed written informed consent.

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