Faecal microbial metabolites of proteolytic and saccharolytic fermentation in relation to degree of insulin resistance in adult individuals

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Abstract

The gut microbiota may affect host metabolic health through microbial metabolites. The balance between the production of microbial metabolites by saccharolytic and proteolytic fermentation may be an important determinant of metabolic health. Amongst the best-studied saccharolytic microbial metabolites are the short-chain fatty acids acetate, propionate and butyrate. However, human data on the role of other microbial fermentation by-products in metabolic health are greatly lacking. Therefore, we compared in a cross-sectional study the faecal microbial metabolites (caproate, lactate, valerate, succinate, and the branched-chain fatty acids (BCFA) (isobutyrate, isovalerate)) between insulin sensitive (homeostatic model assessment of insulin resistance (HOMA-IR), HOMA-IR<1.85, IS) and insulin resistant (HOMA-IR>1.85, IR) individuals. Additionally, we assessed the relationships between faecal metabolites and markers of metabolic health including fasting glucose, insulin, free fatty acids, insulin resistance (HOMA-IR) and fasting substrate oxidation in 86 individuals with a wide range of body mass index. Faecal metabolite concentrations did not significantly differ between IS and IR. Furthermore, there were no associations between microbial metabolites and metabolic health markers, except for a slight positive association of isovalerate with carbohydrate oxidation (E%, *std* β 0.194, $P=0.011$) and fat oxidation (E%, *std* β -0.075, $P=0.047$), also after adjustment for age, sex and BMI. In summary, faecal caproate, lactate, valerate, succinate, and BCFA (isobutyrate, isovalerate) were not different between IR and IS individuals, nor was there any association between these faecal metabolites and parameters of metabolic health. Further human intervention studies are warranted to investigate the role of these microbially-derived fermentation products and their kinetics in metabolic health and insulin sensitivity.

Keywords: gut-derived metabolites, insulin sensitivity, excessive body weight

1. Introduction

Metabolic health is impacted by gut health maintenance which is intricately modulated through various mechanisms, including the preservation of a balanced microbial composition and functionality (Canfora *et al.*, 2015). In addition, a high microbial diversity has been linked to gut health as well as to an improved insulin sensitivity and metabolic health in various animal and human studies (Castaner *et al.*, 2018; Koliada *et al.*, 2017; Ley *et al.*, 2005; Mariat *et al.*, 2009; Schwiertz *et al.*, 2010; Turnbaugh *et al.*, 2006). In contrast, the presence of gut dysbiosis has been linked to the development of obesity and its complications (Shen *et al.*, 2013).

Metabolites derived from the microbial fermentation of indigestible foods play an important role in the microbes-host metabolic crosstalk. In the proximal colon, microbial fermentation of mainly indigestible carbohydrates (saccharolytic fermentation) produces short chain fatty acids (SCFA, acetate, propionate and butyrate) as well as other metabolites (e.g. lactate, succinate, valerate, caproate) and gases (e.g. methane, carbon dioxide and hydrogen) (Canfora *et al.*, 2015; Koh *et al.*, 2016). The distal portion of the colon specialises more in proteolytic fermentation (residual peptides and proteins) and produces a wide variety of metabolites including phenolic compounds, indoles, hydrogen sulphide as well as branched chain fatty acids (BCFA), such as isovalerate and isobutyrate (Canfora *et*

al., 2019; Mika *et al.*, 2016; Ran-Ressler *et al.*, 2014). In general, the majority of microbial metabolites derived from proteolytic fermentation are associated with adverse gut and metabolic health effects. An increased production of proteolytic fermentation by-products elicited by high protein diets (HPD) or low-fibre diets may lead to altered production of microbial derived metabolites (e.g. increase faecal BCFA and decrease faecal SCFA), alterations in gut microbiota, colonic epithelium as well as impaired detoxification capacity of gut mucosa and increased inflammation (Blachier *et al.*, 2019).

Furthermore, in human small-scale observational studies, faecal BCFA concentrations have been reported to be higher in individuals with obesity (Tiihonen *et al.*, 2010) and hypercholesterolemia (Granado-Serrano *et al.*, 2019) as well as associated to non-alcoholic fatty liver disease (NAFLD) progression (Da Silva *et al.*, 2018). Interestingly, the gut microbiome from obese individuals has shown a higher biosynthetic capacity for BCFA precursors, branched chain amino acids (Ridaura *et al.*, 2013), which circulating concentrations have been related to tissue specific insulin resistance in obese individuals (Vogelzangs *et al.*, 2020) as well as with type 2 Diabetes mellitus (T2DM) (Gannon *et al.*, 2018; Newgard *et al.*, 2009). Currently, there is a lack of studies that investigate the role of microbial fermentation by-products besides the major SCFA acetate, propionate and butyrate. The balance between saccharolytic fermentation products with positive effects and proteolytic fermentation products with overall adverse effects on metabolic health may be important in determining the net effect on energy and substrate metabolism, insulin sensitivity and metabolic health, as recently reviewed (Canfora *et al.*, 2019). Therefore, we hypothesised that faecal microbial metabolite concentrations (e.g. BCFA) are different between more pronounced insulin resistant and more pronounced insulin sensitive phenotypes. In the present cross-sectional study, we investigated whether faecal microbial metabolites (caproate, lactate, valerate, succinate, and the BCFA (isobutyrate, isovalerate)) varied based on HOMA-IR by comparing values in a more insulin sensitive and more insulin resistant group, as determined by the median HOMA-IR value (1.85) of our population. Secondly, we assessed the associations of these faecal metabolites with fasting glucose, insulin, free fatty acids (FFA), insulin resistance ((homeostatic model assessment of insulin resistance (HOMA-IR)) and fasting substrate oxidation in a continuous analysis the whole cohort.

2. Materials and methods

Study participants

After meeting the inclusion criteria, a cohort of 86 Caucasian men and women aged 20-69 years with a BMI between 19.2 and 35.2 kg/m² from the vicinity of Maastricht was divided based on the HOMA-IR median (1.85) in insulin sensitive (IS, HOMA-IR<1.85) and insulin resistant individuals (IR, HOMA-IR>1.85). Additionally, in the total cohort, associations between faecal metabolites and parameters of metabolic health were assessed. Eligibility of the participants was assessed via a general health questionnaire, medical history and anthropometry during an initial screening visit. Exclusion criteria were as follows: use of antibiotics, prebiotics, or probiotics 3 months before or during the study, diagnosis of T2DM, gastrointestinal or cardiovascular diseases, abdominal surgery, participants with life expectancy shorter than 5 years, alcohol or drug abuse and participants following a hypocaloric diet. Participants did not use β -blockers, lipid- or glucose-lowering drugs, antioxidants, or chronic corticosteroids. All protocols were reviewed and approved by the local Medical Ethical Committee (MUMC+) and conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul, South Korea). Written informed consent was obtained from all participants.

Study design

This cross-sectional analysis included metabolic parameters as well as faecal metabolites (caproate, lactate, valerate, succinate, BCFA) of previously performed interventions (Canfora and Blaak, 2018; Müller *et al.*, 2020; Van der Beek *et al.*, 2018). The drop-out in these intervention studies was on average 10-15%. In the present study, we collated and analysed study data at baseline and thus prior to the respective interventions. In all studies, sample collection and measurements were conducted according to the same standard operating procedures. Two days prior to the baseline investigation day, participants were asked to refrain from intense physical activity and alcohol consumption. On the evening before the investigation day, subjects were provided with a standardised meal composed of 65 E% carbohydrates with a low fibre content, 20 E% fat and 15 E% protein to ensure a standard meal with a low amount of dietary fibre the evening prior to the baseline measurements.

Used data sets

For this analysis we included baseline data from the following intervention human *in vivo* studies. The data set includes an intervention study with arabinoxylan-oligosaccharide (AXOS) in healthy participants with slow gastrointestinal transit (Clinical trial No. NCT02491125) (Müller *et al.*, 2020), an intervention study with prebiotic inulin in overweight to obese individuals on substrate metabolism (Clinical trial No. NCT03711383) (Van der Beek *et al.*, 2018) and an intervention study with beta glucan in lean-overweight individuals (Clinical trial No. NCT03714646) (Canfora and Blaak, 2018).

Baseline investigation day

After an overnight fast (>10 h), participants came to the laboratory by car or public transport. Anthropometry was measured including height, weight and waist to hip ratio. After inserting a cannula into the antecubital vein, blood samples were taken to measure plasma metabolites in the fasted state. After the blood sampling, participants were in a resting, half-supine position and fasting substrate oxidation was measured for 30 min using an open circuit ventilated hood system (Omnicol, MUMC+, Maastricht, the Netherlands). Fat and carbohydrate oxidation were calculated according to the equations of De Weir and Frayn (Frayn, 1983; De Weir, 1949), assuming that protein oxidation accounted for 15% of total energy expenditure. Fat and carbohydrate oxidation are expressed as percentage of energy expenditure.

Analysis of faecal microbial metabolites

Faecal samples were collected at home and stored in the subjects' freezer at -20 °C maximum of two days before the baseline investigation day, transported on dry ice, and stored on arrival at the university at -80 °C. BCFA, lactate, succinate, caproate and valerate were measured using gas chromatography-mass spectrometry as described previously (Garcia-Villalba *et al.*, 2012).

Blood collection and biochemical analysis

Blood was collected in pre-chilled EDTA tubes (0.2 mol/l EDTA; Sigma, Dorset, UK) for insulin, glucose, FFA, analyses during fasting conditions. Plasma glucose and FFA were determined using commercially available reagent kits for glucose (Glucose HK CP; Horiba ABX Pentra, Montpellier, France) and FFA (NEFA-HR (2) assay, Wako, Sopachem BV, Ochten, the Netherlands). Plasma insulin was determined with a commercially

available radioimmunoassay (RIA) kit (HI-14K Human Insulin specific RIA HI-14K; Millipore Merck, Darmstadt, Germany) according to the manufacture's protocol. HOMA-IR was calculated as the product of (glucose (mmol/l) × insulin (μU/ml)/22.5), using fasting values (Matthews *et al.*, 1985).

Statistical analysis

First, we divided the total cohort into two groups based on the HOMA-IR median (1.85), insulin sensitive (IS, HOMA-IR<1.85) and insulin resistant individuals (IR, HOMA-IR>1.85) to compare the microbial metabolites on group level. The normality of data was assessed with the Gaussian distribution and Kolmogorov-Smirnov procedure. Variables that were not normally distributed were ln-transformed. In case of missing data, the participant was excluded from the analysis. Differences between IS and IR characteristics were analysed using a Student's independent samples t-test. Next, we used simple/multiple linear regression to investigate the associations between faecal metabolites (as independent variables) and metabolic parameters (as dependent variables) in the whole cohort. Multiple linear regression models were adjusted for the covariates age, sex and BMI. All data were analysed using SPSS 22.0 (IBM, Armonk, NY, USA) with significance set at $P<0.05$.

3. Results

The mean age of the participants in our cohort was 45.8 ± 15 years and 60.5% of participants were male. To study differences between the aforementioned faecal metabolites including proteolytic fermentation by-products (BCFA) in relation to insulin sensitivity on group level, we divided the total cohort into two groups based on the HOMA-IR median (1.85), insulin sensitive (IS, HOMA-IR<1.85) and insulin resistant individuals (IR, HOMA-IR>1.85). While there were differences between groups in body weight, BMI, fasting insulin, fasting glucose, 2-h glucose and HOMA-IR, there were no differences in faecal metabolites between IS and IR groups (Table 1). We found a weak association of faecal isovalerate concentrations with carbohydrate oxidation E% ($std \beta$ 0.194, $P=0.011$) and fat oxidation E% ($std \beta$ -0.075, $P=0.047$) in the whole cohort (Table 2), which was maintained after adjustment for age, sex and BMI. However, we did not find further associations between the faecal metabolites valerate, caproate, succinate, lactate and BCFA concentrations with fasting metabolites (glucose, insulin, FFA), HOMA-IR and fasting substrate oxidation in the whole cohort.

Table 1. Characteristics of insulin sensitive (HOMA-IR<1.85) and insulin resistant (HOMA-IR>1.85) individuals.¹

Parameter	Insulin sensitive (n=43)		Insulin resistant (n=43)		P-value
	Mean ± SD	Range	Mean ± SD	Range	
Sex (F/M)	18/25		16/27		
Age (y)	46±15	20-66	46±16	20-69	0.850
Body weight (kg)	73.7±11.3	52-103	83.8±15.2	57-144	0.000
BMI (kg/m ²)	24.0±2.2	19-28	27.1±3.6	19-35	0.000
Waist:hip ratio	0.85±0.11	0.64-1.07	0.89±0.12	0.70-1.07	0.123
HOMA-IR	1.3±0.3	0.6-1.9	3.0±1.4	1.9-9.0	0.000
Fasting plasma insulin (mU/l)	5.8±1.5	2-9	12.3±5.8	7-39	0.000
Fasting plasma glucose (mmol/l)	4.9±0.4	4-6.3	5.5±0.8	4-7.4	0.000
2h glucose (mmol/l)	4.7 ±1.8	2.1-11.0	6.2±2.3	2-10.8	0.002
Fasting plasma free fatty acids (μmol/l)	517±214	117-949	468±146	96-759	0.222
Faecal isobutyrate (μmol/g)	1.8±0.9	0.6-4.3	2.0±1.3	0.1-5.4	0.586
Faecal valerate (μmol/g)	1.7±1.5	0.1-8.4	1.8±1.5	0.1-5.3	0.857
Faecal isovalerate (μmol/g)	2.7±1.6	0.1-6.3	2.7±2.6	0.1-12.8	0.988
Faecal caproate (μmol/g)	1.0±0.8	0.1-3.1	1.3±1.5	0.1-7.1	0.314
Faecal succinate (μmol/g)	1.3±1.0	0-3.6	1.8±2.6	0.1-15.3	0.289
Faecal lactate (μmol/g)	1.0±1.1	0.1-4.7	0.9±0.9	0.1-3.3	0.706
Energy intake (kcal/d)	1,924±503	1,065-3,600	1,957±471	1,265-3,165	0.786
Carbohydrate intake, E%	46±7	32-63	46±6	34-60	0.905
Fat intake, E%	34±7	16-50	35±6	23-49	0.455
Protein intake, E%	17±4	10-27	17±4	9-25	0.610
Dietary fibre (g/MJ)	2.4±0.8	1.1-4.1	2.4±0.6	1.4-4.3	0.948

¹ BMI = body mass index; HOMA-IR = homeostasis model assessment of insulin resistance; SD = standard deviation.

4. Discussion

In this cross-sectional analysis, we showed that the faecal concentrations of BCFA and other faecal metabolites (lactate, succinate, caproate and valerate) were not different between IS and IR individuals. In addition, we could not find a relationship between these microbiota-derived metabolites and insulin resistance and metabolic health parameters in a continuous analysis in the total group.

Our findings are in line with a study that did not find differences in faecal BCFA concentrations between overweight/obese and healthy individuals (Fernandes *et al.*, 2014). In contrast, another cross-sectional study showed increased total faecal BCFA (isovalerate, isobutyrate and 2 methylbutyrate) and valerate concentrations in obese (30-37 BMI kg/m²) hyperinsulinaemic individuals as compared to non-obese normo-insulinemic individuals (Tiihonen *et al.*, 2010). Furthermore, increased faecal isobutyrate concentrations have been previously related to NAFLD progression in both rodents (Le Roy *et al.*, 2013) and humans (Da Silva *et al.*, 2018) and higher faecal BCFA have been associated with hypercholesterolemia (Granado-Serrano *et al.*, 2019).

In view of the above inconsistent data, it is important to consider that faecal concentrations depend on various factors including microbial community (Feng *et al.*, 2018), intestinal transit time (Müller *et al.*, 2018), diet (Den Besten *et al.*, 2013; Tang *et al.*, 2019), age (Nagpal *et al.*, 2018) and lifestyle (Zhong *et al.*, 2019). Additionally, gut-derived circulating metabolite concentrations may relate more strongly with changes in whole body metabolic markers than faecal concentrations as we have previously suggested for SCFA (Müller *et al.*, 2019). Of note, obese individuals have been reported to have lower circulating isobutyrate and higher isovalerate concentrations as compared to healthy individuals (Aragonès *et al.*, 2020). In addition, an *in vitro* study using rat and human adipocytes showed that both BCFA (isobutyrate and isovalerate) inhibited cytosolic (intracellular) lipolysis and lipogenesis using supraphysiological concentrations (10 mmol/l) (Heimann *et al.*, 2016).

Thus, although our study did not show differences in faecal BCFA between metabolic phenotypes, other studies (38,39) may suggest alteration in circulating BCFA in metabolically compromised individuals and possible tissue-specific

Table 2. Relationship between faecal metabolites with metabolic markers.¹

Metabolite	Faecal isobutyrate				Faecal valerate			
	std β	P-value	std β adj	P-value	std β	P-value	std β adj	P-value
FFA, $\mu\text{mol/l}$	-0.079 $\pm$ 0.061	0.196	-0.070 $\pm$ 0.056	0.211	-0.008 $\pm$ 0.045	0.858	0.015 $\pm$ 0.042	0.713
Insulin, mU/l	-0.007 $\pm$ 0.057	0.917	0.023 $\pm$ 0.056	0.686	-0.003 $\pm$ 0.051	0.950	-0.005 $\pm$ 0.044	0.911
HOMA-IR	-0.013 $\pm$ 0.071	0.855	0.026 $\pm$ 0.057	0.646	0.004 $\pm$ 0.055	0.939	-0.004 $\pm$ 0.046	0.931
Glucose, mmol/l	-0.008 $\pm$ 0.015	0.596	0.002 $\pm$ 0.013	0.865	0.006 $\pm$ 0.011	0.611	0.000 $\pm$ 0.009	0.976
Fat, E%	-0.078 $\pm$ 0.042	0.068	-0.073 $\pm$ 0.042	0.086	-0.008 $\pm$ 0.032	0.813	-0.002 $\pm$ 0.032	0.957
CHO, E%	0.123 $\pm$ 0.082	0.140	0.117 $\pm$ 0.083	0.167	0.045 $\pm$ 0.062	0.468	0.039 $\pm$ 0.063	0.539
	Faecal isovalerate				Faecal caproate			
	std β	P-value	std β adj	P-value	std β	P-value	std β adj	P-value
FFA, $\mu\text{mol/l}$	-0.018 $\pm$ 0.053	0.742	-0.029 $\pm$ 0.049	0.556	-0.016 $\pm$ 0.046	0.731	-0.007 $\pm$ 0.043	0.866
Insulin, mU/l	-0.030 $\pm$ 0.061	0.625	-0.014 $\pm$ 0.052	0.782	0.092 $\pm$ 0.046	0.051	0.073 $\pm$ 0.040	0.075
HOMA-IR	-0.028 $\pm$ 0.066	0.672	0.001 $\pm$ 0.054	0.979	0.094 $\pm$ 0.048	0.057	0.070 $\pm$ 0.040	0.084
Glucose, mmol/l	0.000 $\pm$ 0.014	0.982	0.015 $\pm$ 0.012	0.216	0.000 $\pm$ 0.010	0.981	-0.003 $\pm$ 0.008	0.691
Fat, E%	-0.073 $\pm$ 0.037	0.047	-0.075 $\pm$ 0.037	0.047	-0.011 $\pm$ 0.032	0.738	-0.011 $\pm$ 0.031	0.725
CHO, E%	0.187 $\pm$ 0.074	0.012	0.194 $\pm$ 0.075	0.011	0.003 $\pm$ 0.067	0.960	0.009 $\pm$ 0.057	0.892
	Faecal succinate				Faecal lactate			
	std β	P-value	std β adj	P-value	std β	P-value	std β adj	P-value
FFA, $\mu\text{mol/l}$	0.003 $\pm$ 0.049	0.951	-0.052 $\pm$ 0.045	0.257	0.040 $\pm$ 0.050	0.419	-0.032 $\pm$ 0.048	0.507
Insulin, mU/l	0.009 $\pm$ 0.052	0.867	0.001 $\pm$ 0.045	0.981	0.017 $\pm$ 0.052	0.745	-0.015 $\pm$ 0.048	0.747
HOMA-IR	0.001 $\pm$ 0.055	0.979	0.004 $\pm$ 0.045	0.929	-0.005 $\pm$ 0.055	0.925	-0.030 $\pm$ 0.541	0.541
Glucose, mmol/l	-0.008 $\pm$ 0.012	0.508	0.004 $\pm$ 0.010	0.719	-0.023 $\pm$ 0.011	0.031	-0.015 $\pm$ 0.010	0.133
Fat, E%	-0.015 $\pm$ 0.035	0.675	-0.031 $\pm$ 0.036	0.390	0.019 $\pm$ 0.035	0.584	-0.006 $\pm$ 0.037	0.872
CHO, E%	-0.044 $\pm$ 0.070	0.535	-0.033 $\pm$ 0.073	0.652	-0.032 $\pm$ 0.072	0.658	0.002 $\pm$ 0.078	0.976

¹ Std β (adj) = standardised β coefficient + standard error of coefficient of faecal branched chain fatty acids (isobutyrate, isovalerate), valerate, caproate, succinate and lactate as dependent variable in a simple regression and multiple regression analysis adjusted for age, sex and body mass index. E% = percentage of energy expenditure; FFA = free fatty acids, HOMA-IR = homeostatic model assessment of insulin resistance; CHO = carbohydrate oxidation.

metabolic effects. More research investigating BCFA dynamics may shed light on whether BCFA handling is disturbed in different metabolic phenotypes and/or whether BCFA need to enter the circulation to exert metabolic effects.

Of note, various human studies reported that a HPD could increase faecal BCFA/SCFA ratio through a higher proteolytic fermentation, possibly inducing adverse health consequences in the long-term (Blachier *et al.*, 2019; Russell *et al.*, 2011). Additionally, a human study reported that a high fibre diet (resistant starch type 2 and arabinoxylan fibres) increased saccharolytic/proteolytic fermentation ratio while a Western diet did not (Hald *et*

al., 2016). Collectively, current literature shows that the capacity of dietary interventions to modulate saccharolytic/proteolytic fermentation may have an impact on gut and metabolic health. A further interesting aspect to consider in future research is the microbial phenotype. For example, a study showed that differences in saccharolytic/proteolytic fermentation ratio may depend on microbial enterotype (microbial community stratification in *Bacteroides*, *Prevotella*, *Ruminococcus*) (Arumugam *et al.*, 2011). For instance, the *Bacteroides* enterotype has been linked to higher consumption of protein and saturated fats, whereas *Prevotella* enterotype has been associated to a lower protein intake (Wu *et al.*, 2011).

5. Conclusions

In conclusion, the present data do not show any alteration in proteolytic fermentation metabolites (faecal BCFA) and the saccharolytic metabolites lactate, succinate, valerate and caproate with degree of insulin resistance. Future nutritional interventions with the aim to modulate saccharolytic/proteolytic fermentation should measure the kinetics of these metabolites as well as plasma concentrations taking into account the metabolic (Bouter *et al.*, 2018; Hansen *et al.*, 2020; Lim *et al.*, 2016) and microbial phenotypes (Arumugam *et al.*, 2011; Holscher, 2017; Kovatcheva-Datchary *et al.*, 2015).

Conflict of interest

The authors declare no conflict of interest.

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