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RESEARCH PAPER

Short-chain fatty acids and bile acids in human faeces are associated with the intestinal cholesterol conversion status

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Background and Purpose: The analysis of human faecal metabolites can provide an insight into metabolic interactions between gut microbiota and the host organism. The creation of metabolic profiles in faeces has received little attention until now, and reference values, especially in the context of dietary and therapeutic interventions, are missing. Exposure to xenobiotics significantly affects the physiology of the microbiome, and microbiota manipulation and short-chain fatty acid administration have been proposed as treatment targets for several diseases. The aim of the present study is to give concomitant concentration ranges of faecal sterol species, bile acids and short-chain fatty acids, based on a large cohort.

Experimental Approach: Sterol species, bile acids and short-chain fatty acids in human faeces from 165 study participants were quantified by LC–MS/MS. For standardization, we refer all values to dry weight of faeces. Based on the individual intestinal sterol conversion, we classified participants into low and high converters according to their coprostanol/cholesterol ratio.

Abbreviations: BMI, body mass index; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DFH, diluted faeces homogenate; LCA, lithocholic acid; LOQ, limit of quantification; SCFAs, short-chain fatty acids; UDCA, ursodeoxycholic acid.

Key Results: Low converters excrete more straight-chain fatty acids and bile acids than high converters; 5th and 95th percentile and median of bile acids and short-chain fatty acids were calculated for both groups.

Conclusion and Implications: We give concentration ranges for 16 faecal metabolites that can serve as reference values. Patient stratification into high or low sterol converter groups is associated with significant differences in faecal metabolites with biological activities. Such stratification should then allow better assessment of faecal metabolites before therapeutic interventions.

KEYWORDS
bile acids, faecal short chain fatty acids, sterols

1 | INTRODUCTION

Metabolites in human faeces can be seen as attractive surrogate markers to track changes of the metabolic activity of the microbiota induced by diet or disease. Of particular interest, faeces are easily accessible and provide a non-invasive window to study the outcome of the gut microbiota–host interaction through the analysis of metabolic end products. It is particularly relevant that exposure to xenobiotics markedly affects the physiology, structure and gene expression of the active microbiome (Maurice et al., 2013). However, not only the effects of antibiotics (Vrieze et al., 2014) have been studied in recent years, as anti-diabetic drugs such as **metformin** (Napolitano et al., 2014) or **GLP-1 receptor** agonists and **dipeptidyl peptidase-4 (DPP-4)** inhibitors (Smits et al., 2016) have complex effects on intestinal bile acid metabolism due to gut-based pharmacology. Moreover, microbiota manipulation and short-chain fatty acid (SCFA) administration have been proposed as treatment targets for several diseases including gastrointestinal and neurological diseases (Bliss & Whiteside, 2018; Gill et al., 2018; Russo et al., 2018; Soldavini & Kaunitz, 2013; Stilling et al., 2016). Treatment with **butyrate**, by a possible interaction with bile acids, even for hepatic inflammation, has recently been suggested, based on a mouse model (Sheng et al., 2017). Nonetheless, there is an enormous number of studies dealing with effects of microbiota-targeted interventions such as prebiotics, probiotics and diet on intestinally produced signalling molecules (see Dalile et al. (2019), Koh et al. (2016), Verbeke et al., 2015). Even in oncology, faecal SCFAs have gained interest and are associated with the effects of **programmed cell death-1 (PD-1, CD279)** inhibitors (Nomura et al., 2020).

Until now, reference values in faecal samples are lacking, especially in various physiological and physiopathological contexts of health and disease. Because faecal samples contain a complex mixture of metabolites, there is a need for establishing clear guidelines for faecal sample collection, preparation and analysis. The first aim of this study was to provide an overview of concentration ranges of sterol species including **cholesterol** and bile acids and SCFAs in human faeces.

What is already known

- Cholesterol is converted to coprostanol by the gut microbiota.

What does this study add

- Low converters excrete more straight short-chain fatty acids and bile acids than high converters.
- Concentration ranges of faecal short-chain fatty acids, bile acids and sterols based on dry weight.

What is the clinical significance

- Patients should be stratified into high or low converter groups to assess therapeutic interventions correctly.
- Concentration ranges provided here may be used as reference values.

The second aim of this study was to investigate the relation between cholesterol–coprostanol conversion status and faecal excretion of metabolites (SCFAs and bile acids). Studies from as early as the 1930s have indicated that the microbiome mediates the conversion of sterols to stanols (Schoenheimer, 1931). Several studies and reviews have shown that the distribution of microbial cholesterol-to-coprostanol conversion in human populations is bimodal, with a majority of high converters (almost complete cholesterol conversion) and a minority of low converters (Benno et al., 2005; Eneroth et al., 1964; Korpela & Adlercreutz, 1985; Lichtenstein, 1990; Wilkins & Hackman, 1974).

There are numerous studies and reviews indicating that SCFAs play an important role in the maintenance of gut and metabolic

health (Blaak et al., 2020). SCFAs are mainly produced in the large intestine from indigestible carbohydrates and to a minor extent from several amino acids (Blachier et al., 2017; Koh et al., 2016). However, the major limitation concerning SCFAs is the inability to measure SCFA production in vivo. Assessment of in vivo production and absorption kinetics of SCFAs is challenging due to the inaccessibility of the colon and rapid absorption by the colonocytes. To complicate matters, the SCFAs are rapidly metabolized in the liver, and small proportions enter the peripheral circulation resulting in large variations in plasma with very low concentrations. Nevertheless, in many studies, faecal SCFAs were used as a proxy of gut-derived SCFA production. Keeping this in mind, interpretation of faecal concentrations is difficult, but most authors also agree that more information on actual SCFA fluxes and kinetic studies on SCFA metabolism are urgently needed (Blaak et al., 2020; Canfora et al., 2019; Gill et al., 2018; McOrist et al., 2011; Müller et al., 2019; Sakata, 2019). Intervention studies are quite often difficult to interpret implying that there may be responders and non-responders to interventions, depending on the initial metabolic profile (Blaak et al., 2020). Our laboratory has wide experience with the analysis and interpretation of sterol species (Matysik et al., 2011, 2012; Matysik & Schmitz, 2013; Pataj et al., 2016; Schott et al., 2018), and it is well-accepted that classification as a sterol converter or a non-converter depends on the abundance of cholesterol-converting bacteria in the intestine. The question is, can the SCFA values be classified and thus allow better interpretation, if they are correlated with the cholesterol conversion status, which is a very individual parameter.

Second, there seems to be a link between bile acids and SCFAs in faeces caused by the group of microbes that remove the major metabolic end product, hydrogen. Bile acids are amphipathic end products of cholesterol metabolism with multiple physiological functions. In the colon, 7α-dehydroxylation of primary bile acids, leading to deoxycholic acid (DCA) and lithocholic acid (LCA), is the most quantitatively important and the most physiologically significant conversion of bile acids in humans (Hamilton et al., 2007). These secondary bile acids can exert deleterious cytotoxic effects on the intestinal epithelium when present in excess (Ajouz et al., 2014; Duboc et al., 2013). There is published evidence that, during high-fibre diets, there is an increase of butyrogenesis and suppression of secondary bile acid synthesis within a few days and switching to a high-fat, low-fibre diet reversed these changes. One explanation of these effects is the level of removal of hydrogen. If hydrogen is allowed to accumulate, it inhibits the fermentation process and thus butyrogenesis. The level of hydrogen in the gut depends on the level of those microbes that remove hydrogen, namely the methanogens, sulfate reducers and acetogens (Katsidzira et al., 2019; O'Keefe et al., 2015). This raises the question of a potential crosstalk between bile acids and SCFAs that may depend on the composition of individual colonic microbiomes.

In that context, our study proposes concentrations of metabolites that can be used as reference values under special consideration of the cholesterol conversion.

2 | METHODS

2.1 | Subjects

Human faeces samples were obtained from participants of four different European studies under the EU project MyNewGut. Here, only data from time point 0 were analysed, before any intervention or therapy was started. Due to study design, the participants did not define their diets at study entry, before any intervention started. For each subgroup, there was a different approach (AXOS, PUFAs, protein, maltodextrin, etc.) so that we could not collect enough meaningful data to draw conclusions based on special diet intervention.

- Thirty overweight and obese individuals (body mass index [BMI] of 25 to 40 $kg \cdot m^{-2}$) with markers of the metabolic syndrome were recruited in a Danish study, conducted at the University of Copenhagen, Department of Nutrition, Exercise and Sports (Denmark) from August 2014 to June 2015. Study details are described elsewhere (Kjolbaek et al., 2020). Complete data set was obtained for 24 participants.
- Forty-two healthy male and female participants were recruited from September 2014 to May 2015 at Avicenne Hospital (Bobigny, France). Inclusion criteria were age 18–45 years and overweight (25 < BMI < 30) (Beaumont et al., 2017). Complete data set was obtained for 36 participants.
- Twenty-four subjects with metabolic syndrome from a Dutch study (age 51–70, BMI 30–41) received faecal microbiota transplantation (data submitted but not published until yet). Samples were taken before transplantation.
- Forty obese women with diagnosed low food addiction, 40 obese women with diagnosed high food addiction and 40 healthy normal weight women were recruited at the Endocrinology Unit of the S. Orsola-Malpighi University Hospital of Bologna, Italy (Guzzardi et al., 2018). Complete data set was obtained for 81 participants.

The local ethics committees (see references above) approved all the studies.

2.2 | Sample collection

Stool samples were mostly collected at participants' homes and kept in portable freezers until they were delivered to the study centres. There was a standard operating procedure for collection and transport of faeces in the whole project. For reasons of comparability and safe transport, we decided from the beginning of the study to relate all values to dry weight (dw). It was not possible for the study centres to determine an exact wet weight of the stool sample. Study centres were asked to prepare a raw faeces homogenate by adding 500-µl methanol to stop residual enzymic activity to approximately 500-mg fresh faeces and store this homogenate at −70°C or lower. These samples were transported on dry ice to our laboratory.

We added 2.5 ml of 70% isopropanol and homogenized in a gentleMACS dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Between preparation steps, samples were kept on ice. The dry weight of this raw faeces homogenate was determined by overnight drying of exactly 1.0 ml of this mixture in a vacuum centrifuge. For further analysis, the raw faeces homogenate was diluted to a final concentration of 2.0-mg dry weight·ml^{-1} (diluted faeces homogenate [DFH]). Sufficient homogenization was evaluated by repeated determination of dry weight in a former report (Schott et al., 2018). The DFH was used for extraction of sterol and bile acid analysis. For analysis of SCFAs, an aliquot of the DFH was centrifuged, and 50 µl of the clear supernatant was subjected to derivatization.

2.3 | Biochemical analysis

The SCFAs - acetate, propionate, butyrate and isobutyrate - were measured by LC–MS/MS after derivatization to 3-nitrophenylhydrazones (Liebisch et al., 2019). Sterols and stanols (coprostanol, 5α-cholestanol, sitosterol, 5α-sitostanol, 5β-sitostanol, campesterol and 5α-campestanol) were quantified by LC–high-resolution MS (LC–MS/HRMS) after derivatization to *N*,*N*-dimethylglycine esters (Schott et al., 2018). Faecal bile acids were quantified by LC–MS/MS using stable isotope dilution analysis with a modified method for serum (Krautbauer et al., 2016; Scherer et al., 2009). We quantified the free bile acids **ursodeoxycholic acid** (UDCA), chenodeoxycholic acid (CDCA), cholic acid (CA), **deoxycholic acid** (DCA) and lithocholic acid (LCA), as well as their glycine (G) and taurine (T) conjugated species. The study centres provided the anthropometric data including sex, age and BMI.

2.4 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). Data were analysed using SPSS v. 25. The Mann–Whitney *U*-test and Kruskal–Wallis *H* test were used for non-normally distributed variables. The level of significance was set to $P < .05$.

2.5 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro et al., 2019).

3 | RESULTS

Full data sets of bile acids, sterols and SCFAs were obtained for 165 participants. Table 1 reflects the distribution regarding sex, age and BMI. The relatively small number of participants with BMI < 25 and the predominance of women are due to the original study designs. All studies were designed to reveal effects of dietary interventions or any weight loss strategies with respect to overweight and obese people. We show in Table S1 the basic characteristics of the four single cohorts.

3.1 | SCFAs

Table 2 shows the range (5th and 95th percentile) of faecal acetate, propionate, butyrate and isobutyrate. There were no significant differences in terms of sex, age or BMI (data not shown).

3.2 | Bile acids

Table 3 shows the range of the most abundant faecal bile acids UDCA, CDCA, DCA and LCA, whereas CA and glycine- and taurine-conjugated bile acids were mostly below the limit of quantification (LOQ). There is no significant difference of all free bile acids with regard to sex and age. However, a positive association was observed between faecal bile acids and BMI. Figure 1 shows box and whisker plots for total faecal bile acids and the major bile acid species, DCA. Study participants with BMI > 30 have significantly higher

TABLE 2 Concentration ranges of SCFA in faeces (µmol·g^{-1})

SCFA	Range (median)
Acetate	53.2–518 (194)
Propionate	19.1–200 (75)
Butyrate	13.5–199 (58)
Isobutyrate	3.6–20.0 (9.6)

Abbreviation: SCFA, short-chain fatty acid. Ranges shown are the 5th–95th percentiles with (medians).

TABLE 1 Characteristics of the study participants

	Sex		Age (years)					BMI			
n	Female	Male	<20	21–35	36–50	51–65	>65	<25	25–30	30.1–35	>35
165	133	32	1	74	55	28	7	34	64	48	19

Abbreviation: BMI, body mass index.

TABLE 3 Concentration ranges of major bile acids in faeces (nmol·g^{-1})

Faecal bile acid	Range (median)
UDCA	<LOQ—241 (25)
CDCA	4.9–853 (27)
DCA	97.4–3190 (756)
LCA	132–1311 (425)

Abbreviations: CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; LOQ, limit of quantitation; UDCA, ursodeoxycholic acid. Ranges shown are the 5th–95th percentiles with (median); the LOQ for UDCA was 10 nmol·g^{-1}.

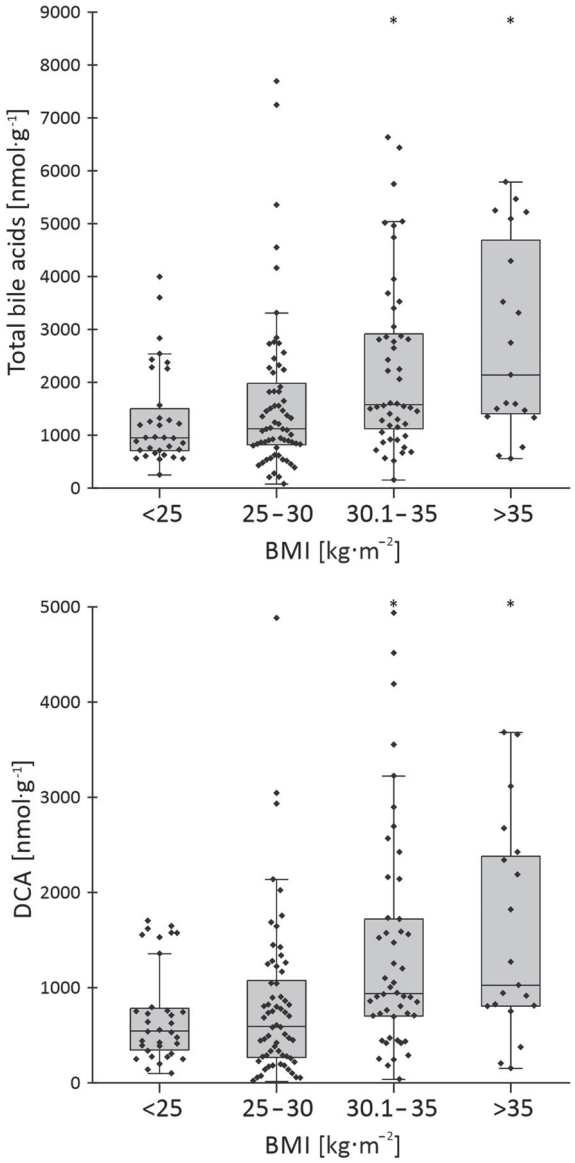

FIGURE 1 Total faecal bile acids and the major bile acid species deoxycholic acid (DCA), related to levels of body mass index (BMI). Data are shown as box (25th and 75th percentiles) and whisker (5th and 95th percentiles) plots, with the median shown as a bar within the box. *P < .05, significantly different from BMI<25

excretion of total bile acids, compared with those with BMI < 30 and BMI < 25.

3.3 | Sterols and stanols

It has been established that cholesterol is mainly converted into coprostanol, and this conversion occurs in a part of the human population only. Following previous studies, we divided the study participants in high and low converters based on the intestinal cholesterol conversion. Therefore, we calculated the ratio of coprostanol to the sum of all cholesterol species, that is, cholesterol, coprostanol and 5α-cholestanol. As shown in Table 4. The following statistical analysis was applied only to study participants with a coprostanol/sum cholesterol ratio <40%, classed as low converters and to study participants with a coprostanol/sum cholesterol ratio >60% classed as high converters. Table 5 shows the range (5th and 95th percentile) of faecal cholesterol, coprostanol, 5α-cholestanol, sitosterol, 5α-sitostanol, 5β-sitostanol, campesterol and 5α-campestanol for low and high converters. There were no differences in BMI and sex,

TABLE 4 Distribution in low and high converters of cholesterol

Coprostanol/sum cholesterol species (%)	*n*
<20	40
20–40	11
41–60	14
61–80	42
>80	58

TABLE 5 Concentration ranges of sterol and stanol species in faeces (μmol·g^{-1})

	Low converter	High converter
	Coprostanol/sum cholesterol <40%	Coprostanol/sum cholesterol >60%
Compound	*n* = 51	*n* = 100
Cholesterol	27.6–96.3 (49.5)	1.7–18.2 (5.9)
Coprostanol	<LOQ—28.4 (0.5)	17.2–78.8 (39.6)
5α-Cholestanol	0.43–1.40 (0.67)	0.45–1.70 (0.98)
Sitosterol	6.9–16.6 (10.8)	0.6–5.3 (1.9)
5β-Sitostanol	<LOQ—9.7 (0.48)	4.8–28.9 (13.7)
5α-Sitostanol	0.28–1.60 (0.75)	0.32–1.63 (0.77)
Campesterol	1.4–5.5 (2.57)	0.13–1.79 (0.48)
5α-Campestanol	0.2–1.21 (0.64)	0.2–1.24 (0.54)

Abbreviation: LOQ, limit of quantitation. Data shown are the 5th–95th percentiles with (medians); the LOQ for coprostanol was 0.3 μmol·g^{-1}; LOQ for 5β-sitostanol was 0.11 μmol·g^{-1}.
[b]Limit of quantitation (LOQ) coprostanol 0.3 μmol·g^{-1}; LOQ 5β-sitostanol 0.11 μmol·g^{-1}.

regarding these groups. However, the low converters were significantly younger.

3.4 | SCFAs and bile acids in faeces of high and low converters

There is a significant difference in the faecal SCFAs found in samples from high and low converters with higher values of acetate, propionate and butyrate in faeces of low converters, than those in faeces from high converters (Figure 2). In addition, we show in Figure 2 those participants with cholesterol conversion <20% and those with cholesterol conversion >80%. The differences of SCFAs are even more marked. In other words, the lower the cholesterol conversion, the higher the SCFA production. In Table 6, we present the 5th and 95th percentile of SCFAs and bile acids for low and high converters. Free bile acid excretion shows the same relationship (see Figure 2), whereas the levels of LCA do not show this association (Table 6). A statistical analysis was not carried out for CA species due to many missing values, that is, values below the LOQ. Nevertheless, it is noteworthy that those participants with very high CDCA values also have high CA values. These 5–8 participants belong to different studies and have at first view nothing in common.

4 | DISCUSSION

One aim of the report here was to give an overview of the range of faecal values from an adequately high number of individuals when consuming their normal habitual diet, that is, without special dietary

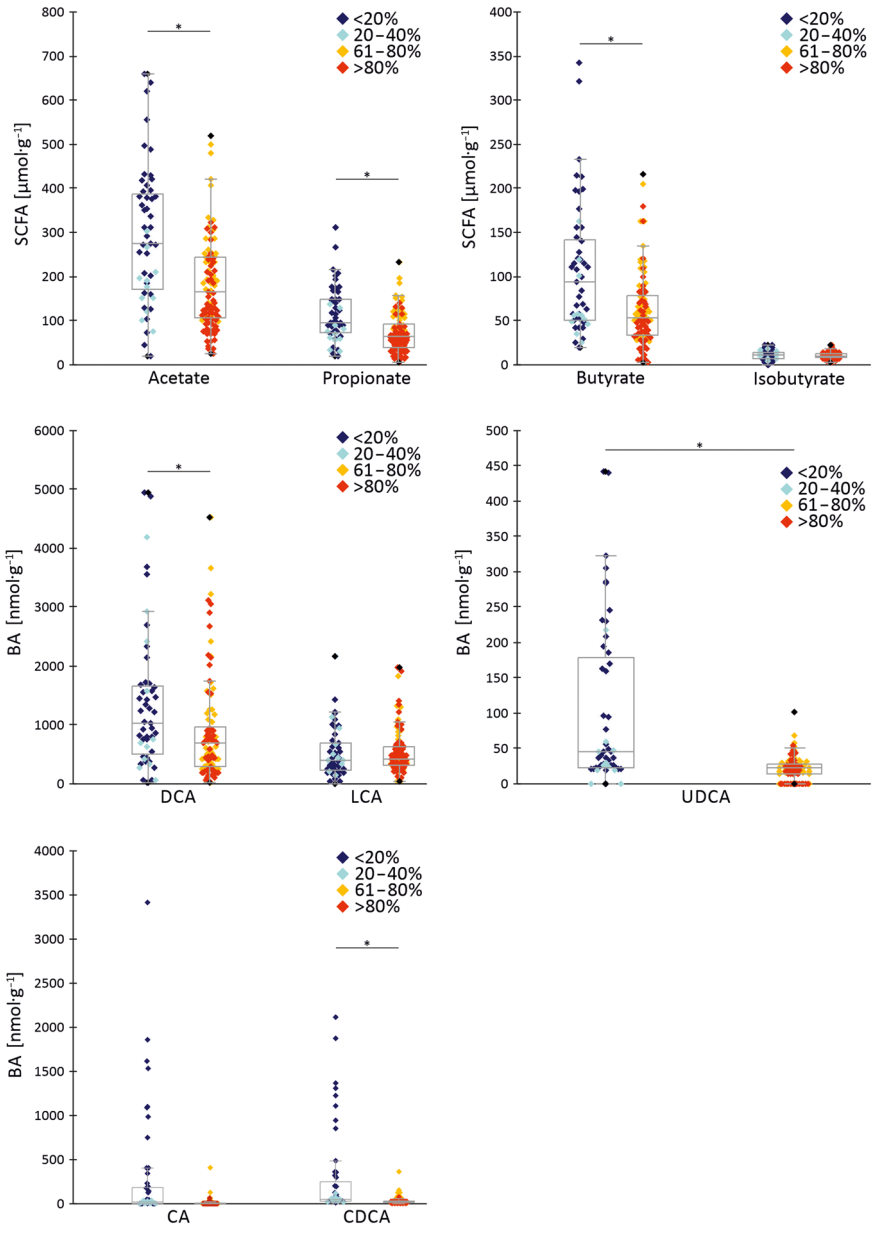

FIGURE 2 Faecal short-chain fatty acids (SCFAs) (acetate, propionate, butyrate and isobutyrate) and bile acids (BAs) (ursodeoxycholic acid [UDCA], chenodeoxycholic acid [CDCA], cholic acid [CA], deoxycholic acid [DCA] and lithocholic acid [LCA]), related to levels of intestinal cholesterol conversion; four groups of conversion - <20%, <40%, >60% and >80% - were used. *P < .05, significantly different as indicated

TABLE 6 Concentration ranges of faecal SCFA (μmol·g^{-1}) and bile acids (nmol·g^{-1}) of low and high cholesterol converters

Compound	Low converter ($n = 51$)	High converter ($n = 100$)	*P* value
Acetate	61–628 (274)	53–404 (166)	1.36×10^{-5}
Propionate	27–237 (96)	17–153 (63)	1.42×10^{-5}
Butyrate	25–269 (94)	6.4–162 (53)	7.74×10^{-5}
Isobutyrate	2.6–21 (11.2)	3.9–18 (9.4)	0.22
UDCA	<LOQ—370 (45)	<LOQ—53 (22)	2.19×10^{-8}
CDCA	17–1568 (49)	<LOQ—90 (21)	1.70×10^{-10}
DCA	52–4467 (1025)	101–3036 (687)	0.004
LCA	39–1302 (397)	190–1336 (427)	0.40

Abbreviations: CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; LOQ, limit of quantitation, SCFA, short-chain fatty acid; UDCA, ursodeoxycholic acid. Data shown are the 5th–95th percentiles with (medians); the LOQ for UDCA was 10 nmol·g^{-1}; the LOQ for CDCA was 4.0 nmol·g^{-1}.

intervention. The samples result from four different European studies. All values were measured in our laboratory at the same time to avoid batch effects. For this report, only values at study entry were analysed. To enable comparability among our and other studies, we calculated all values relative to the dry weight of stool. The results confirmed that there is a wide range of faecal SCFAs, cholesterol, plant sterols and their 5α/β-derivative species as well as bile acids, with an up to 10-fold or 100-fold difference for SCFAs and bile acids, respectively, between the highest and lowest levels. We confirmed the typical ratio of faecal SCFA concentrations to be roughly 3:1:1 for acetate to propionate to butyrate, which fits with the SCFA level detected in the large intestine (Cummings et al., 1987).

Our data indicate that BMI and the classification into low and high sterol converters are significant contributors to this inter-individual variation. Consistent with published data, we found one third of our samples were low converters and the remainder (two thirds) were high sterol converters (Gerard, 2013). This distribution is also reflected in the 5β-sitosterol fraction. Due to missing standards, 5β-campesterol could, however, not be quantified.

An important result is that low converters have much higher amounts of straight SCFAs in faeces than high converters (Table 6). Branched SCFAs , such as isobutyrate, that are produced by the microbiota from amino acids (Blachier et al., 2007) do not show this distribution. In contrast to straight SCFAs, which are generally considered as beneficial for the colon and rectum mucosa notably for their role of energy substrate in absorptive colonic epithelial cells (Wong et al., 2006), these branched-chain short-chain fatty acids are associated with both beneficial effects (Boudry et al., 2013) and harmful effects on colonic mucosa and metabolic health (Canfora et al., 2019). Thus, the relevance of these branched-chain SCFAs to the health of the host has not yet been determined.

Although cholesterol metabolism by the gut microbiota has been known for many years, the genes and enzymes involved in the conversion of cholesterol to coprostanol are still largely unknown. Only a few coprostanoligenic bacteria have been isolated so far, and very few cholesterol-metabolizing strains are available. Most of the cholesterol-reducing bacteria isolated and characterized are members of the genus *Eubacterium*, except for *Bacteroides* sp. strain D8 (Gérard et al., 2007). Strains of *Bifidobacterium*, *Lactobacillus* and *Peptostreptococcus* were also reported to reduce cholesterol to coprostanol (Lye et al., 2010). Recently, new bacterial phylotypes belonging to the Lachnospiraceae and Ruminococcaceae families have been associated with high cholesterol conversion (Antharam et al., 2016). Whether or not those strains are involved in SCFA production at all is still unclear. Our data suggest an inverse association is more likely. Basically, the human intestine absorbs coprostanol poorly, in contrast to its absorption of cholesterol. There is one report about an inverse relationship between plasma cholesterol levels and the ratio of cholesterol-to-coprostanol conversion in the faeces (Sekimoto et al., 1983). In addition, in animal models, a high-efficiency conversion of cholesterol to coprostanol was linked to lower serum cholesterol levels (Li et al., 1995, 1998). Therefore, one hypothesis of a relationship between low cholesterol conversion and higher faecal bile acids could be that in low converters, the higher level of plasma cholesterol is leading to a higher level of total bile acids. In our studies, this is speculative because due to the study design, we did not have plasma values for all participants.

Another hypothesis is that a higher cholesterol concentration in the colon might be exploited for additional functionality by pathogenic microbes (Antharam et al., 2016). Whether the entrapment and incorporation of free cholesterol into bacterial membranes plays a role remains speculative as mechanisms are still unknown (Kriaa et al., 2019). However, distinct strains of both *Lactobacillus* and *Bifidobacterium* were suggested to perform such activity in vitro, and several bacterial genera, including *Clostridium*, *Bifidobacterium* and *Lactobacillus*, exert bile salt hydrolase (BSH) activity (Ridlon et al., 2006) producing higher amounts of free bile acids.

An explanation about the causal relationship of the inverse correlation between cholesterol conversion and SCFAs is not straightforward. For that reason, we show in Table S2 the distribution into three groups (non-converters [<5%] and low [5–40%] and high [>40%] converters), as proposed by Benno et al., (2005). Here, the differences of higher SCFA and bile acid values of non-converters/low converters compared with high converters are also highly significant. Furthermore, we demonstrated that our findings are also valid in two of the four single cohorts (Table S3). For the other two cohorts, there was an imbalance of too few low converters ($n = 1$ and $n = 2$, respectively), and statistics was therefore not possible.

Assuming that faecal SCFAs might reflect the net result of production and absorption, a first hypothesis could be that the colonic microbiome composition for non-converters and low converters favours a higher abundance of phylogenetic groups of bacteria responsible for SCFA production. Unfortunately, we have no data from microbiological investigations in all these studies.

A second hypothesis could be that the elevated bile acids in non-converters and low converters inhibit an uptake of SCFAs as shown for butyrate uptake in tumour and non-tumour intestinal epithelial cells with an inhibitory effect of CDCA (Gonçalves et al., 2012). In other words, in the presence of high levels of bile acids, lower amounts of SCFAs are absorbed and more are excreted.

On the other hand, increased SCFA levels could lower pH through direct acidification (McOrist et al., 2011) when fermentation is promoted. A more acid environment may stabilize the protonated form of the bile acid. This is as more pronounced as higher the acidity of the molecule - CA > CDCA > DCA. CA and CDCA are more acidic and thus exhibit more extreme values as seen in Figure 2. LCA is, however, an exception that does not follow this general trend. LCA has only one hydroxyl group and is the most hydrophobic species among the bile acids. The effects of pH on LCA might not be as marked as on the others.

The subdivision into high and low sterol converters enables a new assessment of faecal SCFA values. We know that the microbial function of cholesterol converting once established is essentially stable over time. This is supported by our studies (Kunz & Matysik, 2019) and by those of others (Benno et al., 2005; Midtvedt & Midtvedt, 1993; Wilkins & Hackman, 1974). Thus, the status of a low or high converter is not dependent on diet. An exception is during antibiotic treatment (Midtvedt et al., 1990), which was an exclusion criterion in our studies. That non-converters and low converters excrete significantly more SCFAs seems therefore to not depend on a special diet or lifestyle. A limitation is that we have no data about intestinal transit times. There is, however, also no published evidence that cholesterol conversion is dependent on transit time.

The level of bile acids, in contrast to SCFAs, was in our study positively associated with BMI. Study participants with high BMI had a significantly higher excretion of bile acids. This is an important finding because high levels of DCA in blood, bile and in faeces have been correlated with an increased risk of cholesterol gallstone disease and colon cancer, two relatively frequent diseases in Western societies.

We did not test for significant differences between the different four countries/study sites because of the inhomogeneity of the participants. As mentioned in section 2, each study centre had different study designs; for example, one study was focused on women only and another one to people with very high BMI.

4.1 | Concordance with previous studies

Our SCFA data agree well with published data on SCFAs in faeces either for values based on dry weight or for values based on wet weight assuming faecal moisture from 61% to 85% (Han et al., 2015; McOrist et al., 2008; Verbeke et al., 2015). The concentrations of sterols reported here are in concordance with results described in our studies and those of others (Batta et al., 2002; Keller & Jahreis, 2004; Korpela & Adlercreutz, 1985; Reddy et al., 1977; Schott et al., 2018; Weststrate et al., 1999; Wilkins & Hackman, 1974). The advantage here is the high number of samples ($n = 165$), which enables us to classify the participants into low and high converter groups without losing statistical power due to too small sample size. For a correct quantification of faecal bile acids, we decided to modify our LC–MS/MS method (Scherer et al., 2009) in such a way that we had a linear calibration for DCA and LCA, at the expense of minor species like CA and CDCA. DCA and LCA are the dominant species in faeces, and a correct quantification is necessary for any meta-analysis. Therefore, primary bile acids are sometimes below LOQ. At the time of measurement, we could not quantify oxo-bile acids representing up to 35% of the faecal pool (Franco et al., 2019). This might be a drawback of our study. As conjugated bile acids must first be de-conjugated by bacterial BSHs to be metabolized into secondary bile acids (Long et al., 2017; Thomas et al., 2008), almost no conjugated secondary bile acids were present in the intestinal content. Consequently, conjugated compounds are mostly below the LOQ. There are a few studies reporting glycine conjugated bile acids in faeces (Breuninger et al., 2019; Mitry et al., 2019). But these were metabolomic approaches, and bile acid species were not confirmed by authentic standards and not quantified.

It is interesting to note that there were no associations between faecal SCFAs and BMI, although there are reports on the relationship between faecal SCFAs and obesity. Some reports recorded positive associations of SCFAs and BMI including a recently published meta-analysis of seven different studies (de la Cuesta-Zuluaga et al., 2018; Kim et al., 2019; McOrist et al., 2011). In contrast, for example, the analysis of a large cohort of 160 participants with a wide range of BMI values did not reveal significant associations to faecal SCFAs, but interestingly, there was an inverse relation to circulating SCFAs (Müller et al., 2019). One explanation could be that we had no data of waist-to-hip ratio, which is known to be a better predictor of obesity or metabolic syndrome than BMI (Björntorp, 1991; Kissebah, 1996).

4.2 | Limitations of our study

The major limitation of our study is the imbalance between women and men, with the predominance of women. Another is that, due to the study design, we had many overweight and obese participants. However, our results are still applicable to a significant proportion of the global population as the number of overweight individuals continues to increase (WHO, 2016). For example, nationally representative data for Germany collected in 2008–2011 show that 67.1% of men and 53.0% of women aged 18–79 years were overweight (based on measured height and weight). The proportion of men and women that were obese was 23.3% and 23.9%, respectively (Mensink et al., 2013).

Another limitation is the fact that participants collected a faecal sample only once at each time point. Hence, we have no data about individual variations. Moreover, we have no information about water content of faeces, intestinal transit times, pH values, because we decided from the beginning of the study to relate all values to the dry weight of the faecal samples.

In conclusion, based on a large number of study participants (165 with complete data), we have been able to give a general quantitative overview of several metabolites in human faeces that can used as reference values. The intestinal cholesterol conversion is a distinctive feature to evaluate SCFA and bile acid concentrations. A stratification into low and high converters could then allow assessing faecal metabolites more appropriately, before therapeutic or dietary interventions. A subdivision based on <20 and >80% cholesterol conversion results in a wider difference of SCFAs and bile acids. We did not present, however, values according to such grading because of a limited number of participants in those groups. The strength of our calculation is that our database on (i) a large cohort; (ii) an uncontrolled diet, which should reflect the behaviour of the normal population; and (iii) a comprehensive data set from various countries in Europe.

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AUTHOR CONTRIBUTIONS

S.K., G.L., H-F.S. and S.M. performed the analytical measurements. S.K. and S.M. performed the statistical analysis. L.K., A.A., F.B., M.B., M.N., A.H., S.R., U.P. and P.I. collected the data. S.M. wrote the manuscript. S.K., L.K., F.B., M.B. and S.R. edited the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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