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# Temporal stability of fecal metabolomic profiles in irritable bowel syndrome

Cristina Iribarren<sup>1</sup>  | Otto Savolainen<sup>2,3</sup> | Maria Sapnara<sup>1</sup> | Hans Törnblom<sup>4</sup>  |  
Magnus Simrén<sup>4,5</sup>  | Maria K. Magnusson<sup>1</sup>  | Lena Öhman<sup>1</sup> 

<sup>1</sup>Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

<sup>2</sup>Chalmers Mass Spectrometry Infrastructure, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden

<sup>3</sup>Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio, Finland

<sup>4</sup>Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

<sup>5</sup>Center for Functional Gastrointestinal and Motility Disorders, University of North Carolina, Chapel Hill, North Carolina, USA

## Correspondence

Lena Öhman, Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, Box 435, 405 30 Gothenburg, Sweden.  
Email: [lena.ohman@gu.se](mailto:lena.ohman@gu.se)

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## Abstract

**Background:** The potential of the fecal metabolome to serve as a biomarker for irritable bowel syndrome (IBS) depends on its stability over time. Therefore, this study aimed to determine the temporal dynamics of the fecal metabolome, and the potential relationship with stool consistency, in patients with IBS and healthy subjects.

**Methods:** Fecal samples were collected in two cohorts comprising patients with IBS and healthy subjects. For Cohort A, fecal samples collected during 5 consecutive days were analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS). For Cohort B, liquid chromatography-MS (LC-MS) was used to analyze fecal samples collected at week 0 (healthy and IBS) and at week 4 (patients only). Stool consistency was determined by the Bristol Stool Form scale.

**Key Results:** Fecal samples were collected from Cohort A (seven healthy subjects and eight IBS patients), and Cohort B (seven healthy subjects and 11 IBS patients). The fecal metabolome of IBS patients was stable short-term (Cohort A, 5 days and within the same day) and long-term (Cohort B, 4 weeks). A similar trend was observed over 5 days in the healthy subjects of Cohort A. The metabolome dissimilarity was larger between than within participants over time in both healthy subjects and IBS patients. Further analyses showed that patients had greater range of stool forms (types) than healthy subjects, with no apparent influence on metabolomic dynamics.

**Conclusion & Inferences:** The fecal metabolome is stable over time within IBS patients as well as healthy subjects. This supports the concept of a stable fecal metabolome in IBS despite fluctuations in stool consistency, and the use of single timepoint sampling to further explore how the fecal metabolome is related to IBS pathogenesis.

## KEYWORDS

fecal metabolites, irritable bowel syndrome, longitudinal, metabolomics, temporal dynamic

Maria K. Magnusson and Lena Öhman shared senior authorship.

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## 1 | INTRODUCTION

Despite extensive global research,<sup>1</sup> the complex and multifactorial nature of irritable bowel syndrome (IBS) challenge the identification of disease-specific biomarkers.<sup>2,3</sup> As a consequence, IBS is diagnosed by symptom-based criteria<sup>4,5</sup> and for management and research purposes, patients are often categorized into IBS subtypes based on the predominant bowel habit.<sup>6</sup>

The fecal microbiome and metabolome have been suggested as potential noninvasive diagnostic tools in IBS, but also in other gastrointestinal diseases.<sup>3,7-10</sup> We and others have shown that gut microbiota is highly stable over time in patients with ulcerative colitis<sup>7,11</sup> and Crohn's disease,<sup>8</sup> and may have diagnostic use.<sup>8</sup> Similarly, patients with colorectal cancer can be discriminated from healthy subjects based on the fecal metabolomic profile,<sup>9</sup> which can also distinguish different disease stages.<sup>12</sup> In IBS, cross-sectional studies demonstrate that subsets of patients present with an altered fecal microbiota composition<sup>13-20</sup> and/or unbalance of the fecal microbial-derived metabolome<sup>10,15,17,19</sup> which can be related to IBS subtypes,<sup>10,21</sup> clinical<sup>13,18</sup> and even psychological symptoms,<sup>19,20</sup> although not all reports fully agree.<sup>15,19,22</sup> However, most of these studies do not account for the dynamic disease course of IBS that can include changes in gut transit time,<sup>23</sup> fluctuation of stool consistency,<sup>24</sup> and gastrointestinal symptoms,<sup>25</sup> all potential confounders of the outcome of microbiota-related studies.<sup>10,15,17,21,23,26</sup> Overall, the fecal microbiota composition has been linked to stool consistency in both health and IBS.<sup>27,28</sup> Longitudinally, some studies demonstrate a stable day-to-day core microbiota regardless of bowel movement fluctuations,<sup>26,29,30</sup> which supports the potential of fecal microbiota as a biomarker<sup>3</sup> and the validity of single-point fecal sampling.<sup>28</sup> In IBS it still remains to be determined if the often large variation of bowel habits within an individual influences the fecal metabolome, and thereby the reliability of the single-point sampling. To our knowledge, there are no available studies assessing the stability of the fecal metabolome explicitly in IBS, although a few clinical trials report that at least specific unique metabolites seem to be quite stable after microbiota modulation.<sup>26,31,32</sup> Therefore, the aim of this study was to determine the temporal dynamics of the fecal metabolome of patients with IBS and healthy subjects in relation to the stool consistency of each bowel movement.

## 2 | MATERIALS AND METHODS

### 2.1 | Study populations

This study comprised two cohorts, both consisting of adult patients with IBS and healthy subjects (>18 years of age), that provided fecal samples for metabolomic analysis. Clinical data and samples were part of prospective and independent studies.

Cohort A encompassed patients diagnosed with IBS according to the Rome III criteria.<sup>4</sup> Fecal samples from all subtypes were collected between 2016 and 2018 as part of a larger study with another

### Key points

- The fecal metabolome has been suggested to have the potential to serve as a biomarker in irritable bowel syndrome (IBS) but its stability and the influence of stool consistency fluctuations over time have not previously been assessed.
- This study demonstrates that the fecal metabolome of IBS patients is subject-specific and stable, short- and long-term, despite fluctuations in stool consistency.
- Our findings support the concept of a subject-specific and stable fecal metabolome, as well as the validity of single timepoint measurements to further explore the fecal metabolome in IBS.

primary endpoint than presented in this manuscript (not published). Only samples from IBS patients with mixed bowel habits (IBS-M) according to the Bristol Stool Form (BSF) scale,<sup>33</sup> with at least four consecutive samples, were analyzed for fecal metabolites (see below). Healthy subjects with no current or prior history of gastrointestinal diseases were included as controls during the same period. The same exclusion criteria applied to both patients and healthy subjects, including presence of severe diseases (e.g., liver disease and pulmonary disease), neurological and/or psychiatric diseases, episode of infectious gastroenteritis during the last month, or ongoing upper respiratory tract infection. Besides, regular intake of NSAID (>1 pill per week) and known addiction to alcohol and drugs were reasons of exclusion. Other factors that could influence the outcome of the fecal metabolomic analysis, such as specific dietary habits, were not recorded in the original study. Subjects were not controlled for other factors that could influence the outcome of the present study, such as probiotic or medication intake. Furthermore, IBS patients were required to have undergone colonoscopy during the last 5 years and were excluded if the colonoscopy reported any significant findings such as inflammation or tumor (with benign and/or cancerous origin) >1 cm. All patients of Cohort A were recruited from primary healthcare centers in Södra Älvsborg (i.e., Gråbo, Lerum, Floda and Sandared), Sweden, as well as from the gastroenterology unit at Södra Älvsborgs Hospital, Borås, Sweden. Healthy subjects were recruited at Södra Älvsborgs Hospital through internal advertisement among hospital personnel, students, or healthy relatives of patients.

Cohort B encompassed patients with IBS diagnosed according to the Rome IV criteria<sup>5</sup> who provided fecal samples between 2017 and 2018. Only samples from patients with either predominant diarrhea (IBS-D) or mixed bowel habits (IBS-M) based on the BSF scale<sup>33</sup> were selected among the participants of the placebo group of a previously reported intervention study<sup>34,35</sup> and were analyzed for metabolites (see below). All patients were well-characterized and presented with moderate to severe symptoms (IBS Severity Scoring System (IBS-SSS)<sup>36</sup> ≥ 175).<sup>35</sup> Initially patients classified as IBS-D (predominantly presenting with watery stools (scores of 6 and 7)

according to the Bristol Stool Form (BSF) scale<sup>33</sup> were selected from a previous study to increase the group size.<sup>37</sup> However, the limited fecal sample availability of this IBS subtype in the placebo group resulted in the addition of a small group of IBS-M patients with high severity of symptoms, defined by IBS-SSS total score  $\geq 175$ . These patients were randomly selected among those reporting an IBS-D like profile at the time of sampling. As controls, healthy subjects with no history of gastrointestinal diseases were randomly selected from an earlier study conducted in 2017.<sup>38</sup> For patients in Cohort B, presence of other severe organic, psychiatric or neurological diseases, pregnancy, and breastfeeding were reasons of exclusion. Besides, patients were not allowed to take probiotics, antibiotics, or medication at least 1 month prior to sample collection. Patients were asked not to modify their dietary habits throughout the study and no violation of these recommendations were recorded.<sup>34,35</sup> IBS patients were recruited from the functional gastrointestinal disorders' outpatient clinic at the Sahlgrenska University Hospital (Gothenburg, Sweden) or through advertisements in the local newspaper, whereas healthy subjects were recruited through advertisements among hospital personnel, students, or healthy relatives of patients and received monetary reimbursement.

All subjects of both cohorts received oral and written information and gave written informed consent according to the declaration of Helsinki before any study procedures. If participants showed inability to understand Swedish, they were not included. All IBS patients included in this study experienced gastrointestinal symptoms throughout the respective study periods. The corresponding study protocols were approved by the Regional Ethical Review Board: Dnr 590-16 (7 September 2016) (Cohort A), Dnr 266-16 (18 April 2016), and Dnr 548-16 (4 July 2016) (Cohort B).

## 2.2 | Clinical questionnaires and sample collection

At inclusion, all subjects completed validated self-report symptom questionnaires assessing the overall severity of current gastrointestinal IBS symptoms (Gastrointestinal Symptom Rating Scale-IBS, GSRS-IBS)<sup>39</sup> and the presence of anxiety and depression (Hospital Anxiety and Depression scale, HAD) either on paper or electronically. Briefly, the GSRS-IBS questionnaire consists of five main domains (abdominal pain, bloating, constipation, diarrhea, and satiety) that evaluate the severity of 13 gastrointestinal symptoms. Each question is graded using a 7-point Likert scale (no discomfort at all = 1, very severe discomfort = 7), and adds up to a minimum total score of 13 and a maximum of 91 points.<sup>39</sup> The HAD questionnaire comprises 14 questions about the anxiety and depression dimensions, with a total score range between 0 and 21 points, respectively. For each domain, this questionnaire has a validated cut-off to classify patients according to the presence (score  $\geq 8$ ) or absence (score  $< 8$ ) of clinically significant psychological symptoms, where higher scores indicate greater severity.<sup>40</sup>

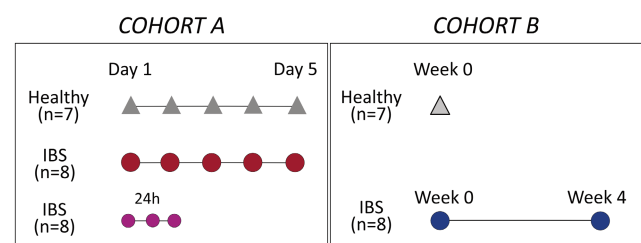
All subjects recorded their bowel movements during the study using a stool diary based on the BSF scale.<sup>33</sup> In short, this scale

classifies the stool form into seven categories, from separate hard lumps (type 1) to completely liquid stool (type 7).<sup>33</sup> All subjects registered the date of the bowel movements and the corresponding stool consistency in a stool diary by using the BSF scale. In Cohort A, the sample collection was related to the registered defecation timepoint and stool consistency. By contrast, Cohort B filled in a stool diary on the days (up to 7 days) before the corresponding visit to the clinic (week 0 and week 4).<sup>35</sup> This difference in sample collection and report of stool form was addressed by using the stool form (BSF) of the last collected fecal sample prior to visiting the clinic by each participant of Cohort B.

The overview of the sample collection is graphically represented in Figure 1. Subjects in Cohort A were asked to collect a sample from each defecation during 5 consecutive days. From these, the first fecal sample of each day was selected for analysis. Furthermore, from IBS patients, additional fecal samples up to 3 consecutive bowel movements during one of those days were selected. All subjects of Cohort B collected fecal samples a maximum of 4 days before the visit at the clinic, and only the IBS patients provided an additional fecal sample after 4 weeks.<sup>35</sup> In all cases, the study subjects collected a scoop of the whole specimen into a transport tube and kept them in the freezer (approximately at  $-18^{\circ}\text{C}$ ) at home. While Cohort A did not receive any specific instruction, both cohorts were provided with a toilet seat paper to help with sample collection. The time window of sample storing at home was similar in both cohorts, being no more than 7 days from the first sampling timepoint for Cohort A and a maximum of 3 days for Cohort B, until handing in to the corresponding center. All samples were then stored on site at  $-80^{\circ}\text{C}$  until further analysis.

## 2.3 | Fecal supernatants and metabolome analysis

Fecal supernatants generated from ultracentrifuged fecal samples were used for metabolome analysis. Briefly, feces were weighed (approximately 1 g) and dissolved in cold phosphate-buffered



**FIGURE 1** Graphical overview of the time intervals of fecal sample collection in the study cohorts. Fecal samples were collected from healthy subjects and patients with irritable bowel syndrome (IBS) in Cohort A and Cohort B at the timepoints indicated by the symbols. Each row represents one study group, the triangles correspond to healthy subjects and the circles to IBS patients. Note that the symbols of the 24 h-interval indicate the first three bowel movements that occurred during one of those 5 days.

saline (PBS) (2x w/v). The mixture underwent a two-step centrifugation process: centrifugation at 3000g for 10 min, followed by ultracentrifugation at 35000g for 2 h at 4°C. The resulting fecal supernatants were collected and stored at -80°C until analysis at Chalmers Mass Spectrometry Infrastructure (Gothenburg, Sweden).

Fecal supernatants from Cohort A were analyzed in one batch using gas chromatography-tandem mass spectrometry (GC-MS/MS) as described previously.<sup>41</sup> Metabolites were extracted from fecal supernatants with a water:methanol (1:9 v/v) solution containing ten stable isotope labeled internal standards, followed by evaporation to dryness and derivitization by oxymation and silylation. The derivitized extracts were then injected and analyzed using a GC-MS/MS system (Shimadzu GCMS TQ-8030 system, Shimadzu Europa GmbH, Duisberg, Germany). Full scan data was collected between 50 and 750 m/z and the data was processed by using an in-house Matlab (Mathworks, Natick, MA, USA) script.<sup>42</sup> The identification of spectral features was done using the retention index (RI), diagnostic ion and spectral matching, and confirmed by visually inspecting the peaks. Signal intensities (peaks) were normalized based on the internal standard peak intensities<sup>41</sup> and log<sub>10</sub>-transformed prior statistical analysis to reduce skewness of biological data. The final dataset comprised a total of 155 unique metabolites.

Fecal supernatants from Cohort B were analyzed in two batches using liquid chromatography-quadrupole time of flight mass spectrometry (UHPLC-qTOF) as described previously.<sup>43</sup> Metabolites were extracted from fecal supernatants with a water:methanol (1:9 v/v) solution before injection into a LC-MS system consisting of 1290 Infinity II UPLC coupled to a 6550 qTOF-MS (Agilent Technologies, CA, USA). Samples were injected and separated by using both a reversed phase (Waters Acquity UPLC HSS T3 column) and hydrophilic interaction chromatography (HILIC) (Waters Acquity UPLC NH2) columns. Briefly, the reversed mobile phases included (A) water and (B) methanol, both containing 0.04% formic acid, and had a flow of 0.4 mL/min with a linear gradient elution as follows: 0–6 min 5–100% B and 6–10.5 min 100% B. The HILIC mobile phases included (A) 10 mM ammonium formate in water and (B) 20 mM ammonium formate 90/10 (v/v) acetonitrile/water, and the following gradient: 0–1 min 100% B, 1–8 min 100–30% B and 8–8.1 min 70–100% B. Electrospray ionization was used both in positive and negative mode and data was collected between m/z 50–1600 in centroid mode with a threshold abundance of 200. Fragmentation data was collected by using data dependent acquisition in quality control samples.<sup>43</sup> The data was preprocessed using the *notame* analytical workflow<sup>44</sup> in RStudio (R version 4.2.1, Vienna, Austria). This script involved drift correction within- and between-batches, random forest-based imputation using the *missForest* package, and clustering of features to remove weak and repeated features.<sup>44</sup> Data was log<sub>10</sub>-transformed before between-batch correction to minimize plausible instrument-related batch effects, and to reduce skewness of biological data. The final dataset comprised a total of 6999 spectral features.

## 2.4 | Data and statistical analysis

No power calculation was performed since the current study design was exploratory, using already available cohorts and datasets. Demographic participant data were analyzed using  $\chi^2$  test and Mann-Whitney *U* test. Demographic analyses for each cohort were carried out in IBM SPSS Statistics for Windows, version 28.0.1 (IBM Corp. Armonk, NY, USA). In all statistical analyses,  $p < 0.05$  were considered statistically significant.

Multivariate analyses were carried out in R Studio (version 4.2.1). Patterns in the metabolome profiles (X-variables) between the study groups of cohort A and cohort B, respectively, were assessed in principal component analysis (PCA) plots using the *pca3d* package, *prcomp*-function, with z-score scaling. Furthermore, the evenness of the fecal metabolomic profiles over time was estimated using the Bray-Curtis dissimilarity of the *vegan* package, where dissimilarity index values of 0 indicate identical metabolome composition, and a nonidentical metabolome equals to 1.

Data and statistical analysis of fecal metabolome dissimilarities were performed in GraphPad Prism (version 9.4.1). Boxplots were used to show within- versus between-subject analyses at inclusion and over time of all dissimilarities between the samples for a unique participant (within) versus the dissimilarities for a unique participant to all non-related samples of that study group (between). Comparisons within each study group (within versus between-participant dissimilarities) were carried out with Mann-Whitney *U* test. The dissimilarity index for a unique participant between its own consecutive samples (within-participant analyses: 1 versus 2, 2 versus 3, etc.) and the mean dissimilarity index for a unique participant to all nonrelated samples of that study group at each timepoint were calculated and presented in dot plots to show the evolution of the metabolite profile over time. Finally, comparisons of the BSF values between the groups were performed using non-parametric Levene test for equal variances in IBM SPSS Statistics for Windows.

## 3 | RESULTS

### 3.1 | Demographic and clinical characteristics of study participants

The characteristics of both study cohorts (A and B) at inclusion are displayed in Table 1. Cohort A included seven healthy subjects and eight IBS patients with alternating diarrhea and constipation (IBS-M) fulfilling the inclusion criteria for this cohort. The distribution of age, gender, the stool type reported based on the Bristol stool form (BSF) scale, and the proportion of subjects with and without anxiety and/or depression were similar between the groups. As expected, patients reported more severe gastrointestinal symptoms (Gastrointestinal Symptom Rating Score for IBS, GSRS-IBS total score) ( $p < 0.001$ ), and higher Hospital Anxiety and Depression (HAD) total score ( $p < 0.01$ ) as compared to healthy subjects (Table 1). For Cohort B,

TABLE 1 Patient demographics at inclusion.

	Cohort A		Cohort B	
	Healthy (n = 7)	IBS (n = 8)	Healthy (n = 7)	IBS (n = 11)
Age <sup>a</sup>	42 [28–47]	42 [20–50]	22 [20–36]	54 [26–71]**
Sex (Female:Male) <sup>b</sup>	5:2	7:1	5:2	7:4
IBS subtype	–	IBS-M=8	–	IBS-D=8 IBS-M=3
Bristol stool scale, stool form (type) <sup>a,c</sup>	3 [2–6]	3 [1–6]	4 [3–5]	6 [2–7]*
GSRS-IBS total score <sup>d</sup>	1.23 (1.1–1.5)	<b>3.9 (2.8–4.5)***</b>	1.15 (1–1.2)	<b>3.6 (2.9–4.5)***</b>
HADS total score <sup>d</sup>	3.57 (3–5)	<b>10.50 (6–14.2)**</b>	10 (7–10)	10 (4–11)
No anxiety:anxiety <sup>b,e</sup>	7:0	5:3	6:1	8:3
No depression:depression <sup>b,e</sup>	7:0	7:1	7:0	11:0

Abbreviations: IBS, Irritable Bowel Syndrome; IBS-D, IBS with predominant diarrhea; IBS-M, IBS with mixed loose and hard stools; GSRS-IBS, Gastrointestinal Symptoms Rating Score for IBS; HADS, Hospital Anxiety and Depression Scale.

Note: Between-group differences within the same cohort are shown in bold and with a symbol (\*P < 0.05 vs. healthy; \*\*P < 0.01 vs. healthy; or \*\*\*P < 0.001 vs. healthy).

<sup>a</sup>Data shown as median and range.

<sup>b</sup>Number of subjects.

<sup>c</sup>Cohort A, Bristol stool scale corresponds to the stool form (type) of the collected fecal sample. Cohort B, Bristol stool scale corresponds to the last type reported before sample collection.

<sup>d</sup>Data shown as median (25th–75th percentile).

<sup>e</sup>Patients with anxiety and depression classified based on a validated cut-off level  $\geq 8$  (clinically relevant symptoms).

TABLE 2 Overview of fecal sample collection.

	Cohort A		Cohort B	
	Healthy (n = 7)	IBS (n = 8)	Healthy (n = 7)	IBS (n = 11)
Day 1/Week 0	7	8	7	11
Day 2	6	8	–	–
Day 3	7	8	–	–
Day 4	7	6	–	–
Day 5	2	7	–	–
Week 4	–	–	–	11
Timepoint 1	8	–	–	–
Timepoint 2	8	–	–	–
Timepoint 3	7	–	–	–

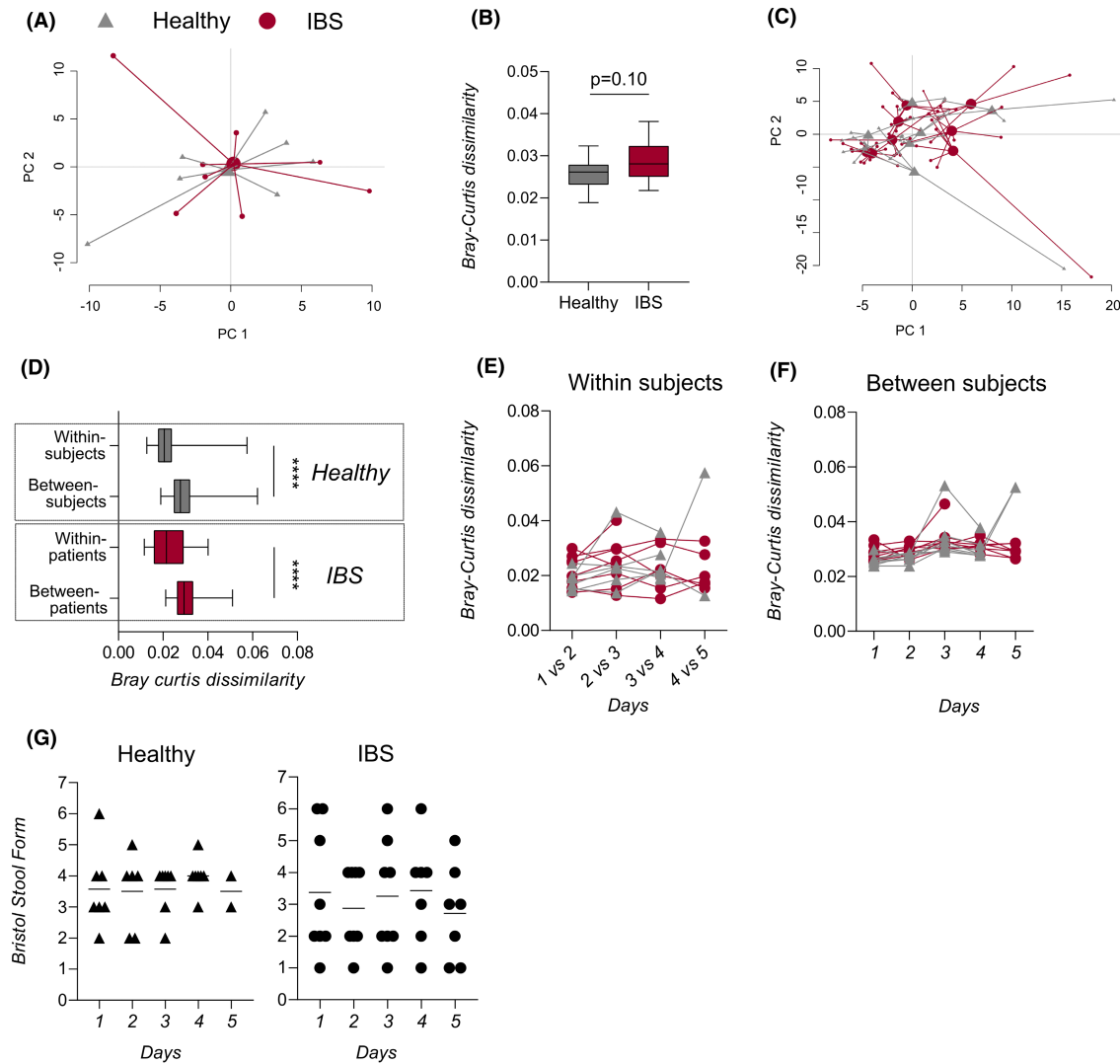
seven healthy subjects and 11 IBS patients (eight patients with predominant diarrhea (IBS-D) and three with IBS-M) fulfilling the corresponding inclusion criteria were included. In Cohort B, patients with IBS were older ( $p < 0.01$ ) than the healthy subjects. While patients presented with more severe gastrointestinal symptoms according to GSRS-IBS total score ( $p < 0.001$ ), both study groups in Cohort B had similar gender distribution and similar anxiety and depression scores (Table 1).

Study subjects provided fecal samples at the timepoints specified in Table 2. For Cohort A, subjects provided samples during 5 consecutive days (IBS=37 samples, healthy=29 samples). Five healthy subjects did not collect any fecal sample on day 5. In addition, a total of 23 fecal samples were collected by IBS patients

within the same day (Table 2). For Cohort B, seven fecal samples from healthy subjects were available at inclusion (week 0), whereas a total of 22 samples were collected at week 0 and at week 4 by IBS patients (Table 2).

### 3.2 | Dynamics of fecal metabolome over days in IBS patients and healthy subjects

In Cohort A, temporal dynamics of the metabolome over days were evaluated in fecal supernatants by GC-MS/MS. At day 1, a principal component analysis (PCA) of the fecal metabolomic profiles ( $n = 155$  spectral features) showed no clustering for healthy subjects and IBS patients (Figure 2) and was not influenced by age, sex, or stool form (Figure S1). The compositional variance between the samples based on the spectral feature intensity signals was assessed by Bray-Curtis dissimilarity. Healthy subjects and IBS patients had similar metabolomic composition (dissimilarity index close to 0), with a tendency towards a higher dissimilarity in the IBS patients ( $p = 0.10$ ) (Figure 2B). A PCA based on the fecal metabolomic profile, linking intraindividual samples provided over a period of 5 days to their centroid, revealed that fecal supernatants tended to localize close to those provided by the same individual, with no clear separation between study groups (Figure 2C). Furthermore, both healthy subjects and IBS patients showed higher dissimilarities in the metabolome between individuals than within individuals (Figure 2D). Overall, only minor fluctuations within- and between-subjects were detected over time (Figure 2E, F). Importantly, even though the mean stool form was

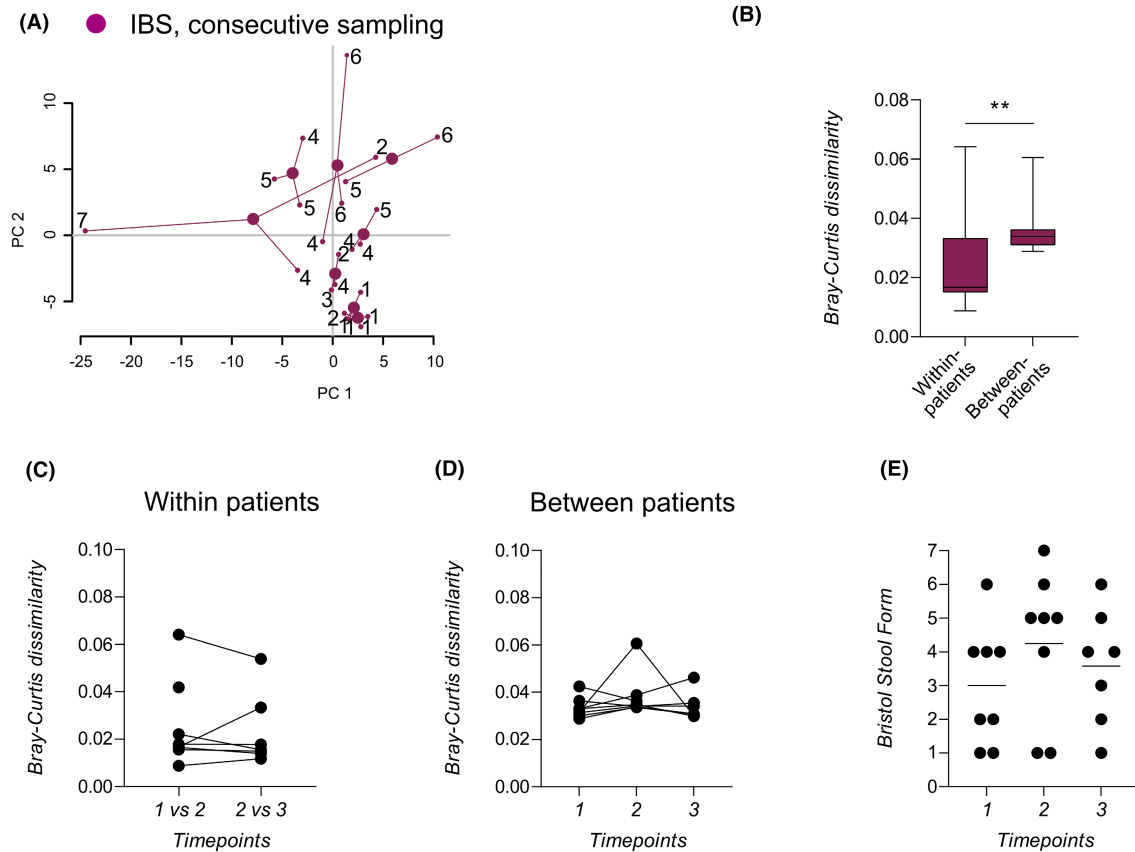


**FIGURE 2** Temporal dynamics of fecal metabolome over 5 days in healthy subjects and patients with IBS. Fecal samples of Cohort A were obtained from healthy subjects ( $n=7$ ) and IBS patients ( $n=8$ ) on 5 consecutive days. Fecal supernatants were analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS) resulting in 155 spectral features. (A) Principal component analysis (PCA) based on the fecal metabolomic profiles of healthy subjects (dark gray triangles) and IBS patients (merlot red circles) at day 1 of sampling. (B) Between-subject metabolome dissimilarities in healthy subjects and IBS patients at day 1 of sampling. (C) PCA showing the fecal metabolomic profiles of healthy subjects and IBS patients over 5 days. Samples originating from individual patients are linked to their centroids. (D) Within- and between-subject metabolome dissimilarity over 5 consecutive days in healthy subjects and IBS patients. (E) Within- and (F) between-subject metabolome dissimilarities for each healthy subject and IBS patient over 5 sampling days. (G) Stool forms reported over 5 days by the participants using the Bristol stool form (BSF) scale. (B, D–F) The dissimilarities were analyzed by Bray–Curtis dissimilarity index. (G) The BSF scale grades the stool form on a 7-point Likert scale, where type 1 corresponds to hard stools and type 7 to watery stools. (G) shows individual BSF values and the mean stool form reported at each sampling day. \*\*\*\* $p < 0.0001$ .

similar for IBS and healthy over time (Figure 2G), the IBS group reported a larger variance of stool types over time than the healthy group ( $p < 0.001$ , nonparametric Levene test for equal variances).

We then assessed the fecal metabolomic dynamics of consecutive samples taken the same day provided by IBS patients from Cohort A. A PCA plot based on the fecal metabolomic profile linking intraindividual samples provided during the same day to their centroid revealed that most samples from the same individual tended to cluster close together (Figure 3A). Also, consecutive samples

taken the same day had higher compositional variance between- than within-patients (Figure 3B). At individual level, within- and between-dissimilarities were quite stable over the three sampling points (Figure 3C, D). Only one individual had a metabolomic profile more unstable within the same day and reported extreme stool types (from 2 to 7) throughout the sampling points. Nevertheless, in general the metabolomic profiles remain stable despite a large variation in BSF scores (Figure 3E), gastrointestinal (intermediate to moderate), and psychological symptoms.



**FIGURE 3** Temporal dynamics of fecal metabolome over the same day in patients with IBS. Fecal samples of Cohort A were obtained from IBS patients ( $n=8$ ) during the first three bowel movements that occurred during the same day. Fecal supernatants were analyzed by GC-MS/MS resulting in 155 spectral features. (A) PCA based on the fecal metabolomic profiles of IBS patients from fecal samples collected during the same day. Samples originating from individual patients are linked to their centroids and the stool form reported at each specific timepoint using the BSF scale is indicated. (B) Within- and between- subjects metabolome dissimilarity in IBS patients over 3 consecutive sampling timepoints during the same day. (C) Within- and (D) between- subjects metabolome dissimilarities for each IBS patient over the same day. (E) Stool forms reported during the same day by the participants using the BSF scale. (B–D) The dissimilarities were analyzed by Bray–Curtis dissimilarity index. (E) The BSF scale grades the stool form on a 7-point Likert scale, where type 1 corresponds to hard stools and type 7 to watery stools. (E) shows individual BSF values and the mean BSF reported at each sampling timepoint.  $**p < 0.01$ .

### 3.3 | Dynamics of the fecal metabolome over 4 weeks in IBS patients

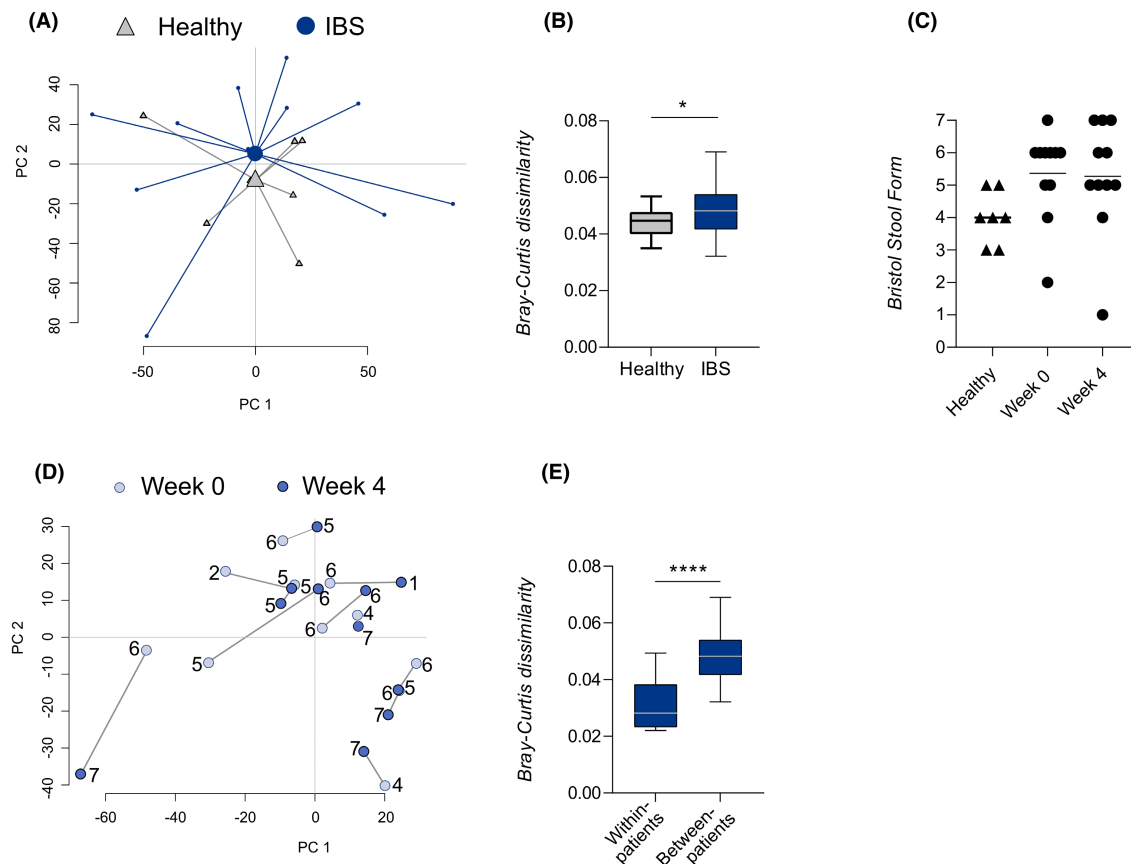
Next, in Cohort B, the temporal dynamics of the metabolome in fecal supernatants were analyzed by LC-MS metabolomics. At week 0, a PCA of the fecal metabolomic profiles ( $n=6999$  spectral features) showed large overlap between healthy subjects and IBS patients (Figure 4A). Again, age, sex, and stool form did not seem to influence the metabolome of IBS patients and healthy subjects (Figure S2A–C). With dissimilarity index close to 0, the metabolome profile differed more (higher dissimilarity) in IBS patients as compared to healthy subjects at week 0 ( $p < 0.05$ ) (Figure 4B) and IBS patients reported a larger range of stool types over time than the healthy group at week 0, which only reported normal stool consistencies (types 3–5) (Figure 4C). A PCA based on the fecal metabolomic profile linking intra-individual samples provided at week 0 and week 4 demonstrated that most of the samples provided by the same individual tended to localize close to each other, regardless of the consistency of the bowel movement (Figure 4D). At group level, patients had similar metabolome at week 0 and week 4, independently of the stool

form (Figure S2D). Finally, temporal stability over 4 weeks was evaluated by assessing the Bray-Curtis dissimilarities and, like Cohort A, results showed greater metabolome variation between- than within- IBS patients (Figure 4D).

## 4 | DISCUSSION

This study gives evidence of both short-term and long-term stability of the fecal metabolomic profile of patients with IBS as well as short-term fecal metabolomic profile stability in healthy subjects. The metabolome dissimilarity over time was higher between than within individuals of both IBS patients and healthy subjects. Further, the greater range of stool forms among IBS patients had no apparent influence on fecal metabolomic dynamics. Together these findings support the notion of a stable fecal metabolome in patients with IBS, despite fluctuations of stool consistency.

The potential of the fecal microbiome and metabolome to serve as biomarkers for diseases has attained increasing interest recently.<sup>3,7–10</sup> Yet, the validity of such fecal biomarkers for IBS, where



**FIGURE 4** Temporal dynamics of fecal metabolome throughout 4 weeks in healthy subjects and patients with IBS. Fecal samples of Cohort B were obtained from healthy subjects ( $n = 7$ ) at one timepoint and from IBS patients ( $n = 11$ ) at week 0 and after 4 weeks. Fecal supernatants were analyzed by liquid chromatography-mass spectrometry (LC-MS) resulting in 6999 spectral features. (A) PCA based on the fecal metabolomic profiles of healthy subjects (light gray triangles) and IBS patients (dark blue circles) at week 0. (B) Between-participant metabolite dissimilarities in healthy subjects and IBS patients at week 0. (C) The stool form of the last bowel movement before the first study visit by the healthy subjects and prior week 0 and week 4 by the IBS patients was recorded using the BSF scale. (D) PCA based on the fecal metabolome profiles of IBS patients at week 0 (light blue circles) and week 4 (dark blue circles). Samples originating from individual patients are linked and the stool form on the last bowel movement reported by the patients using the BSF scale, it is indicated. (E) Within- and between-IBS patient metabolome dissimilarities between week 0 and week 4. (B, E) The dissimilarities were analyzed by Bray-Curtis dissimilarity index and data are shown in boxplots as median (min-max). (C, D) The BSF scale grades the stool form on a 7-point Likert scale, where type 1 corresponds to hard stools and type 7 to watery stools. (C) shows individual BSF values and the mean of the last stool forms reported before the study visit. \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ .

variations in stool form, number of bowel movements, stool water content, and transit time are hallmarks of disease, has been unclear. Therefore, the current study, demonstrating that the fecal metabolome within an individual IBS patient is relatively constant over time, spanning over hours, days, and weeks, is of major importance. Bray-Curtis dissimilarity index close to zero in IBS patients and healthy subjects support similar overall fecal metabolite composition in both study groups throughout the study periods. Our results clearly display that the interindividual differences are larger than intraindividual differences in the fecal metabolome of IBS patients as well as of healthy subjects, and the metabolomic dissimilarities over time of both study groups show comparable fluctuation patterns. Thus, our findings support a stable longitudinal individual-specific fecal metabolite signature, suggesting that single timepoint sampling of individuals with symptoms compatible with IBS may be used as a biomarker for the disease.

To our knowledge, the number of studies aiming for longitudinal profiling of the intestinal microenvironment in IBS is limited<sup>21,30,45,46</sup> and predominantly focus on the fecal microbiota composition. In relation to metabolites, data extracted from the placebo group of clinical trials including IBS patients indicate that at least specific unique metabolites remain constant over time.<sup>26,31,32</sup> Another study, including only two IBS patients, demonstrated that the functional microbial profile measured at gene expression level was stable within subjects when having mild symptoms, but less stable when symptoms worsened.<sup>47</sup> While a recent study added important knowledge to the longitudinal aspects of both microbiota and metabolites in a larger cohort of IBS patients, they did not report the individual variation of the metabolite profiles over time.<sup>21</sup> Still, specific unique microbial derived metabolites, such as cholic acid and chenodeoxycholic acid were reported to be associated with a flare.<sup>21</sup> Due to the few studies on the topic, our study determining

the stability of fecal metabolome profile in IBS patients over time fills a so far unmet need, although further studies expanding and deepening the knowledge are warranted, including investigations of the variation of specific metabolites over time in relation to symptom fluctuations, including pain. In addition, while this study did not include patients with IBS-C, it would be of interest to assess the dynamics of fecal metabolites in samples from these patients bearing in mind the evident difficulties related to consecutive sample collection. Furthermore, future studies should consider controlling for menstrual cycle phase when considering week by week variation of metabolites in women of reproductive age.<sup>48</sup>

IBS symptoms, such as severity and predominant stool pattern, may fluctuate from 1 day to another,<sup>24,28</sup> and it has been suggested that the intestinal microenvironment vary with bowel habits. Interestingly, it was recently reported that between-subject but not within-subject variation in microbiota over time can partially be explained by variation in stool consistency of IBS patients.<sup>28</sup> Similarly, another study found no consistent association of fecal microbiota composition or short chain fatty acid signatures to IBS severity or stool pattern over time.<sup>26</sup> In our study, we followed patients over hours, days, and weeks and noted the stool consistency of the collected sample. In line with the literature,<sup>24</sup> healthy subjects had normal stools whereas the range of stool types over time was larger among IBS patients. Altogether, we could not identify any link between the metabolomic profile stability and the changes in stool consistency. This lack of association should however be interpreted with caution since we did not correct for fecal water content and the accuracy of subjective assessment of stool consistency to subtype IBS has recently been called into question.<sup>49,50</sup> Despite this, our data may imply that previous reports of differences in the fecal metabolome related to bowel habits in cross sectional studies most likely reflect individual differences in metabolome composition rather than bowel habits and disease activity.<sup>10,15,17,19</sup>

To our knowledge, this is the first longitudinal study that assessed the variation of the fecal metabolome in IBS patients and healthy subjects over hours, days, and weeks in relation to stool consistency. Although the findings presented may have major impact on the field, our study has several limitations. First, this study was designed to capture the longitudinal fluctuations of fecal metabolites making use of available metabolomic data from previous studies conceived for other purposes. The small sample size and heterogeneity of IBS pathophysiology limits the possibility of drawing firm conclusions on the differences in the metabolomic signatures between IBS and healthy subjects, or to identify specific unique metabolites linked to IBS or health. Therefore, study group specific metabolite panels are not presented. Furthermore, dietary habits, known to affect metabolites, were not considered but participants were asked to not change their diet throughout the study. While no major changes of dietary habits or medication were detected in Cohort B, this information was not available for Cohort A. Nevertheless, considering potential heterogeneity with regards to dietary registration, participants still displayed a stable fecal metabolome over time in both cohorts.

Because fecal samples may differ in water content, we asked the participants in cohort A to record the consistency after each bowel movement, but this task was not done in Cohort B. We therefore acknowledge that the link between fecal metabolome and stool consistency of Cohort B needs careful interpretation, even though patterns were similar in both cohorts. It may however be considered as a strength that the two IBS study groups were recruited at different type of healthcare centers and belonged to different IBS subtypes based on either Rome III or IV, respectively. Further, recruitment methods and the study timelines and timepoints for samples collection differed between cohorts, and thereby limited direct comparisons between the two, but allowed us to determine stability over short- as well as long-time periods. Other challenges related to fecal sampling<sup>51</sup> such as differences of sample collection and short-term storage were not controlled for. Nevertheless, all samples were long-term stored and prepared the same way. There is no indication that a limited numbers of freezing-and-thaw cycles will affect the overall metabolite profiles, confounding the present results.<sup>52</sup> The use of two different metabolomic methods did not allow for detailed comparisons between the two cohorts, but both methodological approaches show similar patterns, suggesting that metabolomic signatures based on different sizes of datasets (155 metabolites vs. 6999 spectral features) may be equally useful to determine fecal metabolome profiles, although at different depth.

In summary, by applying consecutive sampling and two different metabolomic analyses methods we have shown short- and long-term stability of the fecal metabolomic profile in IBS patients, regardless of the fluctuations of stool consistency. Similar patterns were seen in healthy subjects over short-time. While future larger studies are required, this study supports the concept of a subject-specific and stable metabolome as well as the use of single timepoint sampling to explore how the fecal metabolome is related to IBS pathogenesis.

## AUTHOR CONTRIBUTIONS

CI processed the biological data, performed analyses, and interpreted, prepared visualizations of the data and drafted the manuscript. MS (Sapnara) prepared the fecal supernatants. OS performed metabolomic analysis of samples and provided intellectual and scientific input on metabolomic methods and statistical analysis. MS (Simrén) and HT collected the subject materials and provided intellectual and scientific input on the manuscript. MKM processed the biological data, performed analyses, and interpreted, and drafted the manuscript. LÖ involved in conceptualization of the study, obtained funding, and drafted the manuscript. MKM and LÖ share senior authorship. All authors approved the final version of the article, including the authorship list.

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## CONFLICT OF INTEREST STATEMENT

CI, OS, MS (Sapnara), and HT do not have conflicts of interest to declare. MS (Simrén) has received unrestricted research grants from Danone, Ferring Pharmaceuticals and Glycom (now DSM); served as advisory board member for Ammirall, Allergan, Albireo, AstraZeneca, Danone, Nestlé, Glycom (now part of DSM), Menarini, and Shire, as well as a speaker for AlfaSigma, Allergan, Ammirall, Alimentary Health, Biocodex, Kyowa Kirin, Menarini, Tillotts, Takeda, and Shire. MKM has served as a speaker for Janssen-Cilag and Takeda. LÖ has received a financial support for research by Genetic Analysis AS, Biocodex, Danone Research and AstraZeneca and served as Consultant/Advisory Board member for Genetic Analysis AS, and as a speaker for Biocodex, Ferring Pharmaceuticals, Takeda, AbbVie, and Meda.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

Cristina Iribarren  <https://orcid.org/0000-0002-0632-7552>

Hans Törnblom  <https://orcid.org/0000-0003-2117-9874>

Magnus Simrén  <https://orcid.org/0000-0002-1155-1313>

Maria K. Magnusson  <https://orcid.org/0000-0002-8888-4968>

Lena Öhman  <https://orcid.org/0000-0001-8142-2106>

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## SUPPORTING INFORMATION

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

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