Microchemical Journal 200 (2024) 110255

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Short-chain fatty acids in plasma and feces: An optimized and validated LC-QqQ-MS method applied to study anorexia nervosa

Laura Mayo-Martínez [a], M. Paz Lorenzo [a], Gabriel Á. Martos-Moreno [b,c,d,e], Montserrat Graell [f,g], Coral Barbas [a], Francisco J. Rupérez [a], Jesús Argente [b,c,d,e,h], Antonia García [a,*]

[a] Centro de Metabolómica y Bioanálisis (CEMBIO), Facultad de Farmacia, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, Boadilla del Monte, Madrid 28660, Spain
[b] Departments of Pediatrics & Pediatric Endocrinology, Hospital Infantil Universitario Niño Jesús, Madrid 28009, Spain
[c] La Princesa Research Institute, Madrid 28006, Spain
[d] Department of Pediatrics, Universidad Autónoma de Madrid, Madrid 28029, Spain
[e] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid 28029, Spain
[f] Psiquiatry Department, Hospital Infantil Universitario Niño Jesús, Madrid 28009, Spain
[g] Centro de Investigación Biomédica en Red Salud Mental (CIBERSAM), Instituto de Salud Carlos III, Madrid 28029, Spain
[h] IMDEA Food Institute, CEI UAM & CSIC, Madrid 28049, Spain

A B S T R A C T

Short-chain fatty acids (SCFAs) are organic saturated monocarboxylic acids with fewer than six carbon atoms. Their recently described relevance in health and disease has brought to the table the need for a convenient method for their analysis. The current research presents an optimized and validated LC-QqQ-MS method for the absolute quantification of SCFAs in plasma and feces. This method has proven to be accurate and precise in a wide range of concentrations (0.2 – 200 μM). It has been applied to more than 500 samples showing good precision, reproducibility, and linearity results. The method was applied to study samples from anorexia nervosa (AN) patients and matched healthy controls (HC). The results showed significant differences in the concentration of plasmatic and fecal SCFAs between both groups whose implications in AN are yet to be determined. Nonetheless, these findings denote important alterations in the SCFAs homeostasis in AN which can be linked to the metabolic alterations and the intestinal dysbiosis already characterized in the disease. More research is still required to identify the function of these metabolites in the pathophysiology of AN, but the present work provides a reliable methodology and valuable information to continue delving into the potential role of the microbiota as a therapeutic target *via* the gut-brain axis.

1. Introduction

SCFAs: biosynthesis, transport, and relationship with health/disease.

The SCFAs are organic saturated monocarboxylic acids with less than six carbon atoms [1]. There is increasing evidence about the role of these compounds in health and disease. Whilst they can be endogenously produced in the liver, the main source of SCFAs is the anaerobic fermentation of indigestible polysaccharides by the microbiota in the large intestine [2].

Acetate, propionate, and butyrate are the most abundant and the main subproducts of bacterial fermentation of dietary fibers and resistant starch. While the two firsts are primarily absorbed, butyrate is a major energy source for colonocytes, and it can activate intestinal gluconeogenesis participating in energy homeostasis. SCFAs that are not metabolized in the colon reach portal circulation, and they serve as energy sources for hepatocytes, except for acetate which is not oxidized in the liver. The remaining SCFAs enter the systemic circulation and peripheral organs, exerting functions such as appetite regulation, immune modulation, brown tissue activation etc. [3,4]. SCFAs have proven effects on host health status and have been related to the pathogenesis of several diseases from allergies or asthma to cancer or autoimmune diseases [5]. In addition, they are thought to play a pivotal role in the microbiota-gut-brain axis, mediating different processes in the CNS [6]. All these three metabolites can cross the blood–brain barrier (BBB), and they seem to contribute to its development in early life stages and to the maintenance of its integrity. Moreover, they participate in neuromodulation mechanisms [4,7–9]. Besides, the branched short-chain fatty acids (BSCFAs) isobutyric and isovaleric acids can come from the microbial fermentation of dietary amino acids isoleucine and valine and have been proposed as markers of microbiota protein metabolism and

* Corresponding author at: Centro de Metabolómica y Bioanálisis (CEMBIO), Facultad de Farmacia, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, Boadilla del Monte, 28660, Spain.
E-mail address: antogar@ceu.es (A. García).

https://doi.org/10.1016/j.microc.2024.110255
Received 19 November 2023; Received in revised form 21 February 2024; Accepted 26 February 2024
Available online 3 March 2024
0026-265X/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

found them positively correlated with obesity, and metabolic diseases [10].

SCFAs can directly cross the intestinal epithelial barrier in the unionized form. However, the major part exists in an ionized state and requires specific transporters for the uptake. The active transport of SCFAs is mediated mainly by proton-dependent or sodium-coupled monocarboxylate transporters. In addition, SCFAs can interact with their receptors on enteroendocrine cells to promote indirect signaling to the brain via the systemic circulation or vagal pathways, inducing the secretion of anorectic gut hormones such as glucagon-like peptide 1 (GLP1) and peptide YY (PYY) [3–5]. In a mirror image of the recently developed drugs for obesity treatment based on gastrointestinal peptide analogs, SCFAs could turn into a plausible base for the development of therapeutic agents with an appetite-promoting effect [11].

As previously introduced, SCFAs have important effects on brain neurochemistry, and they are greatly involved in gut-brain axis regulation. Firstly, they reduce the BBB permeability improving its overall integrity and function by upregulating the expression of tight junctions [6]. SCFAs increase neurogenesis by modulating the levels of neurotrophic factors and improving neuronal homeostasis and function. Particularly, propionate and butyrate modulate the levels of serotonin and dopamine, noradrenaline, and adrenaline levels by regulating tryptophan 5-hydrolase 1 and tyrosine hydroxylase, respectively. Furthermore, acetate is known to modify the levels of glutamine, glutamate, and γ-aminobutyric acid (GABA) in the hypothalamus and increase anorexigenic neuropeptide expression [3,4,12]. Thus, the microbiota-gut-brain axis appears as a novel therapeutic target for neurological and psychiatric diseases, and supplementation with SCFAs seems to be a feasible therapy for improving eating behavior [13].

Analysis of SCFAs

Given the importance of SCFAs and their local effects on the gastrointestinal tract and the peripheral role in CNS or the immune system, they have become one of the targets in recent research. As a result, there is an evident need for their reliable quantitation in biological samples such as biofluids, and feces. However, this is not an easy task; the low molecular weight and high polarity and volatility, among others, hamper the instrumental analysis. The bioavailability and function of these metabolites are usually assessed in fecal samples, but the plasma levels of SCFAs are critical for the systemic effects of such compounds. The main trouble with blood samples especially in humans is the low concentration of SCFAs in this matrix, which is in the μM-nM range. Different analytical techniques have been used for the analysis of SCFAs in the different biological matrices, such as nuclear magnetic resonance (NMR), gas chromatography coupled to mass spectrometry (GC–MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) or capillary electrophoresis (CE) with different detectors. Among them, GC–MS is the technique of the most frequent choice owing to its affordability, sensitivity, and reproducibility. Moreover, it is a major analytical platform for the analysis of small polar compounds such as aldehydes, carboxylic acids, or ketones [10,14]. Nonetheless, complex sample pretreatment, including clean-up and derivatization (mostly alkylation), and long analytical runs, might produce important metabolite losses, poor analyte recovery, and low throughput.

Liquid chromatography coupled to mass spectrometry (LC-MS) has been proposed as a suitable alternative for the analysis of microbial metabolome due to its high sensitivity and short analytical runs [15–21]. Still, several aspects need to be considered. Firstly, carboxylic acids could be poorly detected by negative MS ionization mode due to their natural structure. However, the direct analysis of polar compounds such as SCFAs is hindered by low chromatographic performance and low ionization efficiency. In reverse phase chromatography, the most widely applied of all LC retention mechanisms, hydrophilic compounds present low retention and high matrix effect, resulting in poor quantitation performance and metabolite losses. Chemical derivatization with labeling reagents that could give bigger parent masses and less polar derivatives and enhance ionization efficacy is highly recommended. In this regard, some authors referred to 50 to 1500-fold increased detection sensitivities of the analytes after chemical derivatization [21]. Labeling agents include chemical compounds such as aniline [15], 3- nitrophenylhydrazine, O-benzyl hydroxylamine [10] or 4-acetoamido-7-mercapto-2,1,3-benzoxadiazole [22] among others. HILIC chromatography offers an alternative that improves the chromatographic performance for small polar metabolites, but the instability of carbonyl compounds in the ion source still burdens the detection [23]. Indeed, there are very few references concerning the direct analysis of SCFAs. Saha et al. described a method for the direct analysis of SCFAs in different biological matrices from mice and human feces by LC-QqQ-MS just after the dilution and extraction of metabolites in acid media. The chromatographic retention was by a graphitic carbon column and under extreme conditions by positive ESI ionization, although SCFAs do not present a moiety susceptible to protonation [10]. Van Eijk et al., in their work, proposed a method without sample derivatization for the analysis of SCFAs in human plasma. However, they required a neutralization step between the reverse phase chromatography and the MS analysis under negative ESI ionization. Samples were deproteinized in acidic conditions and measured with hydrochloric acid mobile phases and an ethanol gradient, which are harsh conditions that may limit the lifespan of the column [24].

To overcome all the previously described analytical challenges, chemical derivatization has been proposed to enhance detection sensitivity, ionization efficiencies, carbonyl compounds stability, and chromatographic retention and resolution. Importantly, with metabolites of low molecular weight, the chemical derivatization increases the mass, as well as the symmetry of the peaks and their retention time, leading to an overall improved chromatographic detection [23,25,26]. In addition, the positive ionization mode usually renders higher ionization efficiency than the negative mode in ESI. So, many derivatization strategies are based on the addition of a labeling agent with a protonated function group [23].

Recently, some LC-MS methods have been reported for the analysis of SCFAs after chemical derivatization in different biological matrices, although they are frequently determined in feces but their determination in serum is more challenging due to their low concentration [20]. Zhang et al. reported a method for the quantification of SCFAs in fecal samples following a dual derivatization strategy. The samples were labeled with a light derivatizing reagent, Dans-Hz or DMED, and a pool of commercial standards was labeled with the heavy mass structural analogs, either N, N-diethyldansulfonyl hydrazide (Dens-Hz) or N, N-diethyl-1,2-ethanediamine (DEEA), respectively [16]. Han et al. assessed the SCFAs in human feces after $^{13}C_6$-3NPH derivatization. They proposed a method in which standards and samples are derivatized separately and they are mixed before injection [27]. Similarly, some authors add the labeled internal standard/reagent to the human serum/plasma before the derivatization with 3-NPH, which allows following the entire process [18,20]. Parallelly, Zhao et al. used a similar approach with dual derivatization in urine samples. For the samples they used ^{12}C-DnsHz and as an internal standard they spiked ^{13}C-Dns-Hz labeled control, which was a pool of all the samples labeled with ^{13}C [17]. The main drawback of this method is that only allows the relative quantitation of the metabolites. Additionally, Hut et al. used light and heavy structural labeling with DP (2-hydrazinylpyrimidine) for standards and DMP (2-hydrazinyl4,6-dimethylpyrimidine) for rat plasma samples [19].

Here we present an accurate, sensitive, selective, and robust high throughput methodology for the analysis of eight SCFAs (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and 2-methylbutyric acids) by LC-MS/MS (MRM) in feces and plasma samples. Analytes are measured after derivatization with Dns-Hz based on internal standard (IS) calibration with their stable isotope-labeled standards. For our developed strategy Dns-Hz was selected as the derivatizing reagent since it provides fast and simple reactions under mild conditions, with high performance, and improved sensitivity in positive MS ionization mode.

The method was validated according to the European Medicines Agency (EMA) ICH guideline [28] for linearity, accuracy, precision, sensitivity, matrix effect, stability, carryover, selectivity, and specificity. Besides it was applied to samples (both feces and plasma) from healthy controls (HC) and anorexia nervosa (AN) patients to unveil the possible role of SCFAs in the neuropathophysiology of the disease.

2. Materials and methods

2.1. Reagents

Standards, chemicals, and reagents of analytical grade were used. Acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids, were purchased from Sigma-Aldrich (Steinheim, Germany) with purity over 99 %, as well as acetic acid-1-^{13}C, propionic acid-1-^{13}C, and butyric acid-1-^{13}C. The 2-methylbutyric acid standard, and the deuterated standards including acetic acid-2,2,2-d3, propionic acid-3,3,3-d3, butyric acid-4,4,4-d3, and valeric acid-2,2,3,3,4,4,5,5,5-d9 were purchased from Cymit Quimica (Barcelona, Spain).

Dilutions of standards, solutions, and aqueous mobile phases were prepared with ultrapure water obtained from a MilliQ® system (Millipore, Billerica, MA, USA), with acetonitrile (ACN) LC-MS quality or with MES buffer from Sigma-Aldrich (Steinheim, Germany). Formic acid (FA) LC-MS quality was used for mobile phases and was also from Sigma-Aldrich (Steinheim, Germany).

The derivatization reagents Dansyl Hydrazine (Dns-Hz), N-[3-(Dimethylamino)propyl]-N'-ethylcarbodiimide (EDT), and copper (II) chloride, were from Sigma-Aldrich, except for 1-Hydroxy-7-azabenzotriazole (HOAT) which was supplied by Cymit Quimica (Barcelona, Spain).

2.2. Working solutions and standards for method validation

Individual stock solutions of standards (labeled and non-labeled ones) were prepared in ultrapure water with a concentration of 1000 ppm (μg/mL), aliquoted, and kept in the freezer (- 20 °C). The working mixed solutions were prepared by dilution of the previous ones at 0.50 mM of labeled SCFAs and 0.25 mM of the non-labeled ones. For the distinct levels of the calibration curves, the working solutions were prepared by mixing the labeled and non-labeled standards accordingly. Blank samples were prepared for each batch and used along the analysis to ensure proper working conditions. All the stock solutions were prepared and kept under −20 °C for defrosting and derivatization for each batch analysis.

2.3. Samples

Sixty pubertal (Tanner stage II to V) female patients diagnosed with anorexia nervosa (AN) according to the DSM-V criteria, mean age 15.26 ± 1.90 years; mean BMI −1.68 ± 0.57 SDS, hospitalized in the Department of Psychiatry in a tertiary care specialized hospital were enrolled in the study. Their average time of disease evolution and weight loss prior to hospital admission were 10.19 ± 17.53 months and 9.03 ± 5.72 kg, respectively.

Thirty-two healthy females (mean age 19.28 ± 4.94 years; mean BMI + 0.03 ± 0.67 SDS) visited the Department of Endocrinology in the same hospital and were studied to rule out any underlying disease to be used as the control group.

Patients above the age of 12 years and their parents or guardians gave informed assent/consent as required by the local ethics committee, which had previously approved the study (CI: R-0017/19), in accordance with the "Ethical Principles for Medical Research Involving Human Subjects" adopted in the Declaration of Helsinki by the World Medical Association.

Stool and plasma samples from AN patients and controls were collected at the Department of Endocrinology following patient hospital admission before the onset of treatment (between 8 and 9 a.m. in the outpatient clinic for controls) after a minimum of 10 h of fasting and immediately stored at (-80 °C) under appropriate conditions until the day of the analysis. For validation purposes, a pool of plasma and stool samples were used, respectively.

2.4. Sample extraction protocol

Stool samples.

Stool samples from patients (n = 60) and controls (n = 21) were lyophilized (Labogene Scanvac CoolSafe) for 48 h. Once lyophilized, 10 mg of the lyophilized fecal samples were resuspended in 4 mL of ultrapure water. They were vigorously vortex-mixed for 1 min, and they were sonicated in a water bath sonicator for 15 min. Later, they were centrifuged for 10 min 16,000 g at 4 °C, and 100 μL from the supernatant were transferred to a new Eppendorf tube. 20 μL of the internal standard solution with labeled SCFAs in H_2O were added to the tube.

For standard solutions, 100 μL of the standard was mixed with 20 μL of the labeled internal standard mixture in a tube.

Finally, 50 μL either from the sample or standard solutions were taken for derivatization. The sample treatment is specified in Fig. 1.

Plasma samples.

Plasma samples from patients (n = 60) and controls (n = 32) were thawed in ice before the extraction and derivatization. After homogenization by vortexing, 100 μL of the samples were transferred to an Eppendorf tube.

At this point, 20 μL of the internal standard mixture with the labeled SCFAs (700:700:600, H_2O: IS working solution: ACN, v/v/v) were added to each sample. Subsequently, samples were gently vortexed for 1 min and transferred to Centrifree Millipore® (30 kDa) filters (Merck KGaA, Darmstadt, Germany) for deproteinization by centrifugation (2000 × g, 90 min, 4 °C), and 50 μL of the extract was directly transferred to a new tube for derivatization.

For standard solutions, 100 μL of the standard was mixed with 20 μL of the labeled SCFAs mix (700:700:600, H_2O: IS working solution: ACN, v/v/v) in a tube. Lastly, 50 μL of the standard solutions were taken for derivatization. The whole protocol for sample treatment is described in Fig. 1.

2.5. Derivatization procedure for samples and standards

Samples and standards were derivatized with Dns-Hz following a protocol based on Zhao et al., 2018 [17] for urine but optimized for fecal and plasma samples. For the derivatization 50 μL of either sample extract or standard mixture were taken to an Eppendorf tube and 20 μL of EDC were added and vortex-mixed. Then, 20 μL of the HOAT solution (10 mM, H_2O) were added to each tube and vortex-mixed again followed by the addition of 20 μL of Dns-Hz solution (10 mg/mL, ACN). After gently mixing the tubes, they were shaken for 60 min at 20 °C (room temperature). Then, 20 μL of the $CuCl_2$ solution (100 mM, H_2O) were added to quench the reaction, and the tubes were incubated for 30 min at 40 °C in an oven. Finally, the samples were centrifuged at 16,000 *g* at 4 °C for 10 min and the supernatant was taken for the analysis. The global scheme of sample treatment is detailed in Fig. 1.

2.6. LC-MS analysis

The LC system was constituted by a degasser (1260 Infinity series, Agilent), a binary pump, and a multisampler at 4 °C (1260 Infinity II series, Agilent). 20 μL and 5 μL of the derivatized plasma and stool samples were injected respectively into a reverse-phase column with a suitable precolumn (Acquity BEH C8 100 mm x 2.1 mm, 1.7 μm) from Waters (Mildford, MA, USA) thermostated in a UHPLC Column oven (1290 Infinity II Multicolumn Thermostat) at 60 °C. The mobile phase consists of eluent A (5 % ACN and 0.1 % FA in ultrapure water) and eluent B (0.1 % FA in ACN). They were pumped at a 0.350 mL/min flow

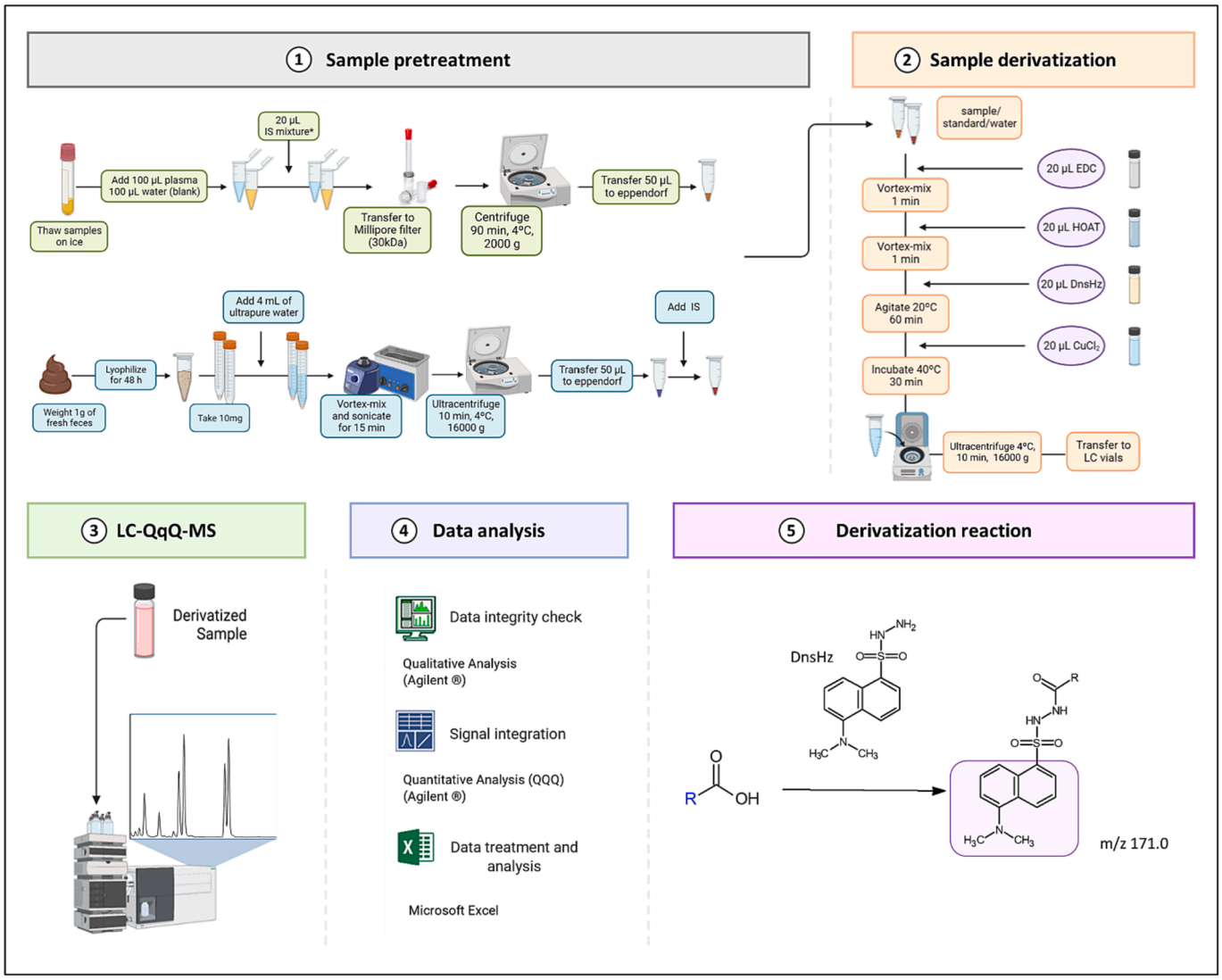

Fig. 1. Summary of the proposed method for the analysis of SCFAs in plasma and stool samples. From sample treatment to data analysis. **1**: Sample pretreatment and SCFAs extraction; **2**: Sample derivatization; **3**: Instrumental analysis; **4**: Data treatment; **5**: Scheme of the derivatization reaction with DnsHz, the highlighted moiety corresponds to the transition used for detection in the MRM mode. *IS mixture: (700:700:600, H_2O:IS working solution: ACN, v/v/v). Dns-Hz: Dansyl hydrazine.

rate with a total run time of 19.7 min. The method gradient started at 30 % of B and went up to 45 % in 8.5 min and it was held until 11.5 min. Then it went up to 90 % at 11.6 min and held until 13.6 min. Lastly, the initial conditions were restored in 0.1 min and the system was re-equilibrated for 6 min until the end of the analysis. Data was collected in dynamic multiple reaction monitoring (MRM), following previously studied transitions for these compounds, with an electrospray ionization source (ESI) in the positive mode in a triple quadrupole mass analyzer (G6470A, Agilent). The capillary voltage was set at 3500 V, the gas temperature was 200 °C, and the flow was 7 L/min. The nebulizer was fixed at 35 psi and the sheath gas temperature and flow rate were 250 °C and 7 L/min, respectively.

All the ions, the MRM-optimized transitions by the Mass Hunter Optimizer software (Agilent Technologies), and the retention times are included in Table 1. Quantification was based on the internal standard calibration with stable isotope standards *(Area Acid / Area labeled Acid)* considering the same counterpart acids, except for isobutyric, for which either D3-butyric acid or butyric acid-1- ^{13}C were used, and for 2-methylbutyric and isovaleric for which d9-valeric acid or butyric acid-1- ^{13}C were used as the reference internal standard according to the closest standard in retention time.

2.7. Method validation

According to the European Medicines Agency (EMA) ICH guideline M10 on bioanalytical method validation [28], the method was evaluated for selectivity, specificity, matrix effect, accuracy and precision, carry-over, stability, linearity, and sensitivity.

The method for the quantitation of SCFAs was validated by the internal standard calibration method with their relative peak area (Area SCFA/ Area SCFA*) using labeled standards of the SCFAs (SCFA*). The use of labeled IS avoided interferences with the analytes of interest and minimized the matrix effect. When the analogous IS was not available, the nearest IS in elution order was applied. Thus, for isobutyric acid, the butyric-d3 and butyric-1-^{13}C acids were used for feces and plasma, respectively. For 2-methylbutyric and isovaleric acids, the valeric acid-d9 was used for the fecal SCFAs analysis, and butyric acid-1-^{13}C was used as the reference standard in plasma including also the valeric acid. Linearity, precision, accuracy, recovery, and quantitation of samples were calculated according to the IS calibration method, considering the relative response area of the analyte and its corresponding labeled IS.

2.7.1. Linearity, accuracy, and precision

Linearity for SCFAs was assessed by testing in triplicate a mixture of

Table 1
MS parameters for each Dns-Hz derivatized SCFA and measured in positive polarity. Frag: Fragmentor; CE: Collision energy; Cell Acc: Cell accelerator voltage. RT: Retention time. (**F**): IS for feces. (**P**): IS for plasma.

Compound	MS/MS parameters					RT (min)
	Precursor ion (m/z)	Product ion (m/z)	Frag (V)	CE (V)	Cell Acc (V)	
Aceticacid-d_3 (F)	311.1	171	119	25	4	2.9
Acetic acid-1-^{13}C (P)	309.1	171	119	25	4	2.9
Acetic acid	308.1	171	119	25	4	2.9
Propionic acid-d3 (F)	325.1	171	129	25	4	4.8
Propionic acid-1-^{13}C (P)	323.1	171	129	25	4	4.8
Propionic acid	322.1	171	129	25	4	4.8
Butyric acid-d3 (F)	339.1	171	124	29	4	6.9
Butyric acid-1-^{13}C (P)	337.1	171	124	29	4	6.9
Isobutyric acid	336.1	171	124	25	4	6.6
Butyric acid	336.1	171	124	29	4	6.9
2-Methylbutyric acid	350.1	171	129	25	4	8.7
Isovaleric acid	350.1	171	129	25	4	9.1
Valeric acid-d9 (F)	359.1	171	124	29	4	9.2
Valeric acid	350.1	171	129	25	4	9.3

the SCFAs over a concentration range of 0.2 – 200 μM in seventeen levels of concentrations (n = 17) covering the range expected from previous trials for plasma and fecal samples.

To prove the reproducibility of the instrumental response to each analyte, the *instrumental precision* was determined with 10 injections of a mixture of SCFAs standards.

Intra and *inter-day precision* and *accuracy* (Equation 1) were tested with replicates of QCs at four concentrations across the assay range, including LLOQ, low, mid, and high QCs on the same day (n = 5 each concentration level) and on three different days respectively (n = 15 each level). For plasma samples, a pool of plasma was prepared by mixing plasma samples from the validation cohort to make a homogeneous pool for the whole validation. The pool was split into three parts that were used each day. On the day of the analysis, the same aliquots of plasma were independently enriched with the standards and IS and mixed to reach the desired concentration for the validation of the method. Then each QC was filtered, and the individual aliquots were taken to proceed with the derivatization process and the LC-MS analysis.

For feces, a pool of fecal samples was prepared to obtain a homogeneous mixture for validation purposes. From that mixture, on every validation day, 10 mg of lyophilized were weighed and resuspended in 4 mL of ultrapure water. From the supernatant obtained after the extraction of those 10 mg, all the QCs for that validation day were prepared, following a similar procedure to the one already described for plasma. The aliquots were divided into concentration levels and enriched with standards following 1–1 additions to achieve the desired concentration. Individual aliquots were taken for each analyzed QC for the derivatization and the instrumental analysis.

Precision was expressed as the CV (coefficient of variation), and accuracy was expressed as the relative error.

Equation 1: Accuracy (%) = 100 x Measured concentration of spiked sample/Nominal concentration

2.7.2. Sensitivity

The LOD was estimated as 3 the signal-to-noise ratio, respectively. The LLOQ was experimentally checked with the lowest concentration level of the calibration curve for precision and accuracy.

2.7.3. Matrix effect

Matrix effects on analyte quantification were calculated by comparing the slope of linear regression curves of the standards and the slope of the linearity with spiked samples from precision and accuracy according to Equation 2.

Equation 2: Matrix effect (%) = 100 x (1-Slope sample/Slope standards).

2.7.4. Stability

Based on previous experience with labeled standards, stock solutions were kept in the freezer (-20 °C) and defrosted on the day of the analysis. The stability of the stock solutions stored at −20 °C was evaluated by comparing the responses of a working mixture of standards one year after its preparation. Additionally, the stability of the derivatized samples was conducted by analyzing mid-range QCs for 0, 24, 48, and 72 h under 4 °C in the autosampler tray.

2.7.5. Carryover, selectivity, and specificity

Carryover was assessed by analyzing blank samples before the linearity and after the highest standard of the regression curve, comparing their obtained MRM profiles.

Selectivity and specificity were evaluated by comparing the MRM chromatograms of Dns-Hz derivatized i) SCFAs standards ii) SCFAs labeled IS iii) plasma and serum pools and iv) blank samples (either blanks with IS and no SCFAs, and blanks without SCFAs or IS).

2.8. Application to the study of anorexia nervosa

Plasma samples. The quantitation of SCFAs (μmol/L) in plasma samples was performed by interpolation of the relative response with respect to the IS in the obtained regression lines for each analyte.

Stool samples. For the concentration (μmol/g) in the stool samples, the relative signal for each analyte was interpolated in the regression line, and the result was later expressed as μmol of analyte in dry weight of feces (g of lyophilized feces).

2.9. Data treatment and statistics

Analytical control and data treatment were performed with Agilent Mass Hunter Qualitative and Agilent Mass Hunter QQQ Quantitative software programs, respectively (version 10.0).

Univariate statistical analyses (UVDA) were performed to compare the obtained results in patients and controls. For the UVDA, parametric t-tests were performed using Matlab R2022b with in-house scripts for assessing the statistically significant AAs between groups.

3. Results and discussion

The sample treatment and the instrumental parameters for the LC-MS/MS ESI positive mode were optimized for the extraction and derivatization of the SCFAs in plasma and stool samples, its detection, and quantification, including the resolution of the stereoisomers.

3.1. Sample extraction and derivatization

The method was based on Zhao et al. [17] with several modifications. The former method analyzed SCFAs in urine samples after derivatization with Dns-Hz. However, the analysis with plasma and feces was challenging due to the intrinsic properties of the matrices. Plasma samples contain higher protein content. Therefore, additional steps were optimized for removing proteins. Firstly, deproteinization by the addition of methanol or acetonitrile 1:3 ratio (v/v) was tried but the signal from the analytes was minimal due to sample dilution. To overcome such an issue, cutoff filtration, instead of dilution, was included after the addition of their labeled IS.

Concerning the analyte extraction, as SCFAs are polar, for stool samples, we decided to select ultrapure water as the extractant. We evaluated different extractant volumes to determine which was optimal for the analysis. Moreover, we extended the method to analyze and quantify all the SCFAs, starting from acetic, propionic, and, butyric and adding isobutyric, 2-methylbutyric, isovaleric, and valeric acids. In addition, we performed the complete validation of the optimized method to quantify them with reliability.

In the preliminary trials, we found acetic acid bias and contaminations during the optimization. As a result, to minimize those signals, the entire deproteinization and derivatization protocol was conducted under a fume hood and wearing a gas mask. This was critical to reduce the contamination of the samples by ambient acetic acid.

3.2. Chromatographic optimization

We aimed to design a methodology with high sensitivity and selectivity for the analysis of SCFAs. Four different columns were assessed, being the Acquity BEH-C8 the most suitable one giving the best-resolved peaks. The first approach consisted of a Zorbax Extend C18 (150 mm,

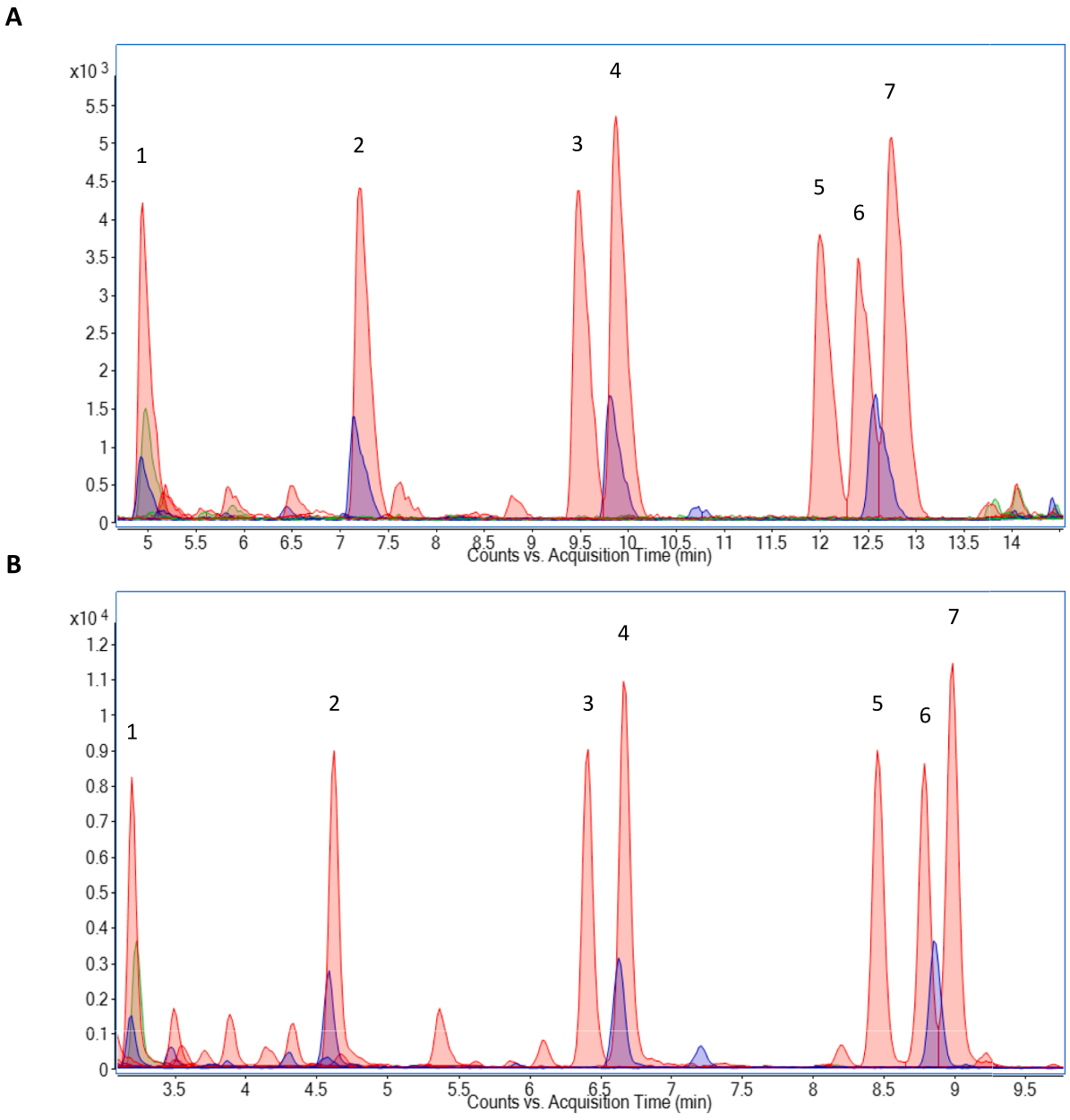

Fig. 2. Comparison of a standard mix analyzed using the different columns tested. Red peaks correspond to physiological standards and the blue ones are the labelled IS. **A**: Zorbax Extend C18 and **B**: Acquity BEH C8. Compounds are numbered from **1 to 7**. **1:** Acetic acid, **2**: Propionic acid, **3**: Isobutyric acid, **4**: Butyric acid, **5**: 2-Methylbutyric acid, **6**: Isovaleric acid, **7**: Valeric acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.1 mm, 1.8 μm) from Agilent (Santa Clara, California) previously selected by Zhao et al. [17]. This column showed good isomer resolution and narrow peaks (Fig. 2A). Nonetheless, to increase the separation between the isoforms of C-5 fatty acid the Acquity BEH columns from Waters (Mildford, MA, USA) were evaluated. Acquity BEH-C8 with the corresponding guard column was used, being the one that gave the highest performance regarding total run time and peak resolution (Fig. 2B). Additionally, this column presented higher sensitivity for the studied compounds as shown in Fig. 2, where the peak width is broader in chromatogram B, corresponding to Acquity BEH-C8. As can be shown, the Acquity BEH C8 column provides improved isomer separation, narrower peaks, higher sensitivity, and shorter retention times.

For the separation of the SCFAs in positive ionization mode, acidic mobile phases give satisfactory results. The selected eluents were described elsewhere [17]. The eluent A contained 5 % (v/v) of ACN and 0.1 % (v/v) of formic acid in ultrapure water, pH(app) = 2.71, and eluent B was 0.1 % (v/v) formic acid in ACN. The flow was adjusted to 0.350 mL/min so as not to overpass the HPLC pump pressure threshold of 600 bars. Then, the gradient was modified to improve the isomer separation and reduce the total run time, ensuring optimal chromatographic performance. The obtained resolution for each isomer was enough for the identification and quantification.

Next, the injection volume was optimized, by assessing the relative area obtained when injecting volumes from 5 to 25 μL in five different levels. The preferred volume related to the intensity of the signals compared to the background noise and the internal standard, was 20 μL for plasma samples. However, for stool samples, the injection volume was optimized at 5 μL since a good analyte response was obtained, and reducing the injection volume improves the column preservation and longevity. Thus, these volumes were fixed for the rest of the analysis. Figs. 3 and 4 represent the MRM chromatograms corresponding to the different SCFAs in the biological matrices studied compared to the 100 μM standard.

3.3. Method validation

3.3.1. Sensitivity, linearity, and selectivity

For the linearity by the IS calibration method, all the obtained correlation coefficients (r) were above 0.990. The correlation coefficients and the linear intervals are listed in Tables 2 and 3. Linearity was studied at 17 concentration levels 0–0.2–0.5–1.0–2.5–5.0–10.0–15.0–20.0–25.0–50.0–75.0–100.0–125.0–150.0–175.0–200.0 μM and it was confirmed for all the SCFAs with a broad linear range of three orders of magnitude for most of them, being acetic and propionic acids the only ones among all the studied analytes presenting bias. The LOD was estimated by SNR (signal-to-noise ratio) = 3. The LLOQ was confirmed experimentally, and accuracy and precision were evaluated at that concentration level. The obtained results are included in Tables 2 and 3.

Selectivity was evaluated by comparing the MRM profiles of the blank (no analyte, but with IS), double blank (no analyte, no IS), blank non-derivatized (no analyte, but with IS), and the spiked samples derivatized (analytes and IS) following Equation 3. In the study of selectivity, it must be demonstrated that the responses of interfering components at the retention times of the analyte or internal standard in the blank samples are not significant. For them to be not significant, these signals must not exceed 20 % of the analyte response to the LLOQ and 5 % of the IS response in the LLOQ sample for each matrix considered.

Equation 3: Selectivity (%) = (Signal in blank/Signal in sample) x 100.

In the blank samples without derivatization, good selectivity values were obtained for all the labeled and physiologic SCFAs, as shown in Table 4. The highest value was obtained for acetic acid, with 10.35 % for the physiological analyte which is still within the limit fixed by the EMA guidelines which permit up to a response detected and attributable to the interfering compounds up to a 20 % of the analyte at the LLOQ. Regarding the labeled SCFAs, all the analytes were within the range fixed in the guidelines [28].

3.3.1.1. Accuracy and precision. The inter- and intra-day *accuracy* and *precision* were assessed by the analysis of spiked QC samples at four different concentrations, ranging from the LLOQ to a high concentration level. The added concentrations in the feces validation assay were 0.2 μM, 0.4 μM, 75.0 μM, and 175.0 μM for each QC concentration level, respectively. In the plasma validation study, the spiked concentrations were 0.2 μM, 0.6 μM, 8.3 μM, and 20.8 μM to LLOQ, Low, Medium, and High QC levels, respectively. As it is shown in Tables 5 and 6, the RSD for all the LLOQs was below 20 % and the accuracy According to the EMA guidelines all of our SCFAs satisfied the recommendations since the

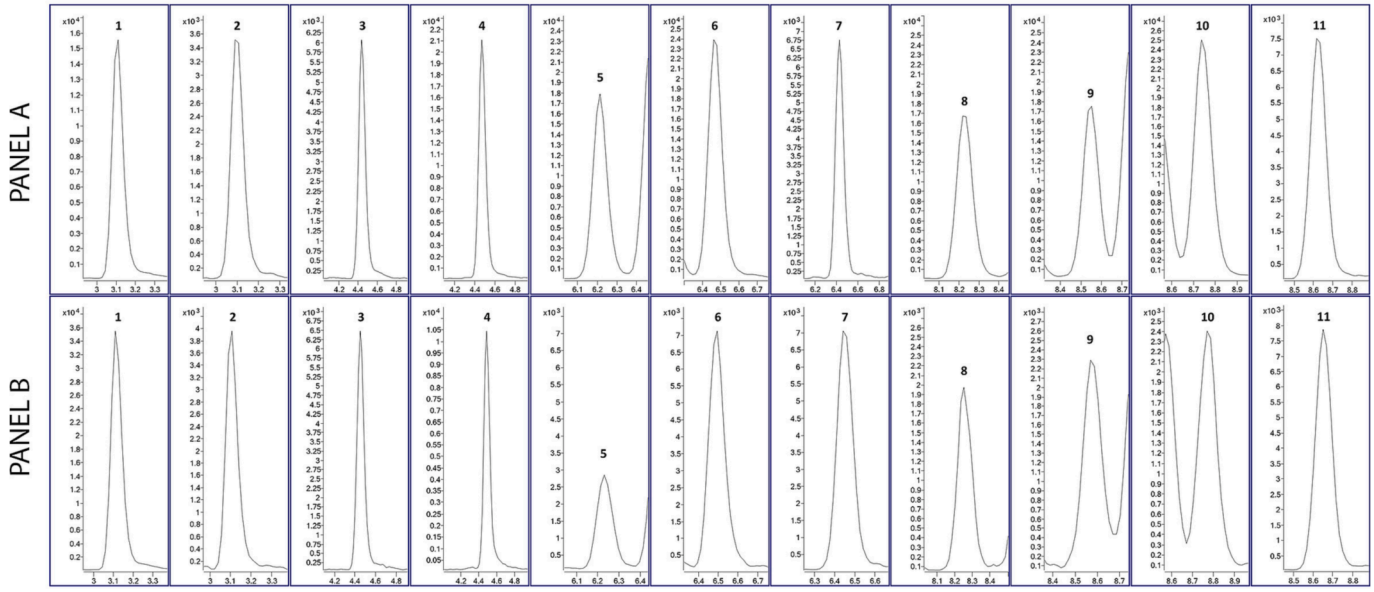

Fig. 3. MRM chromatograms of DnsHz derivatives of SCFAs obtained by HPLC-MS/MS. **Panel A** corresponds to 100 μM standards, **Panel B** corresponds to an Anorexia study sample from feces. Compounds are numbered from 1 to 11. **1**: Acetic acid, **2**: Aceticacid-d_3, **3**: Propionic acid, **4:** Propionic acid-d3, **5**: Isobutyric acid, **6**: Butyric acid, **7**: Butyric acid-d3, **8**: 2-Methylbutyric acid, **9**: Isovaleric acid, **10**: Valeric acid, **11**: Valeric acid-d9.

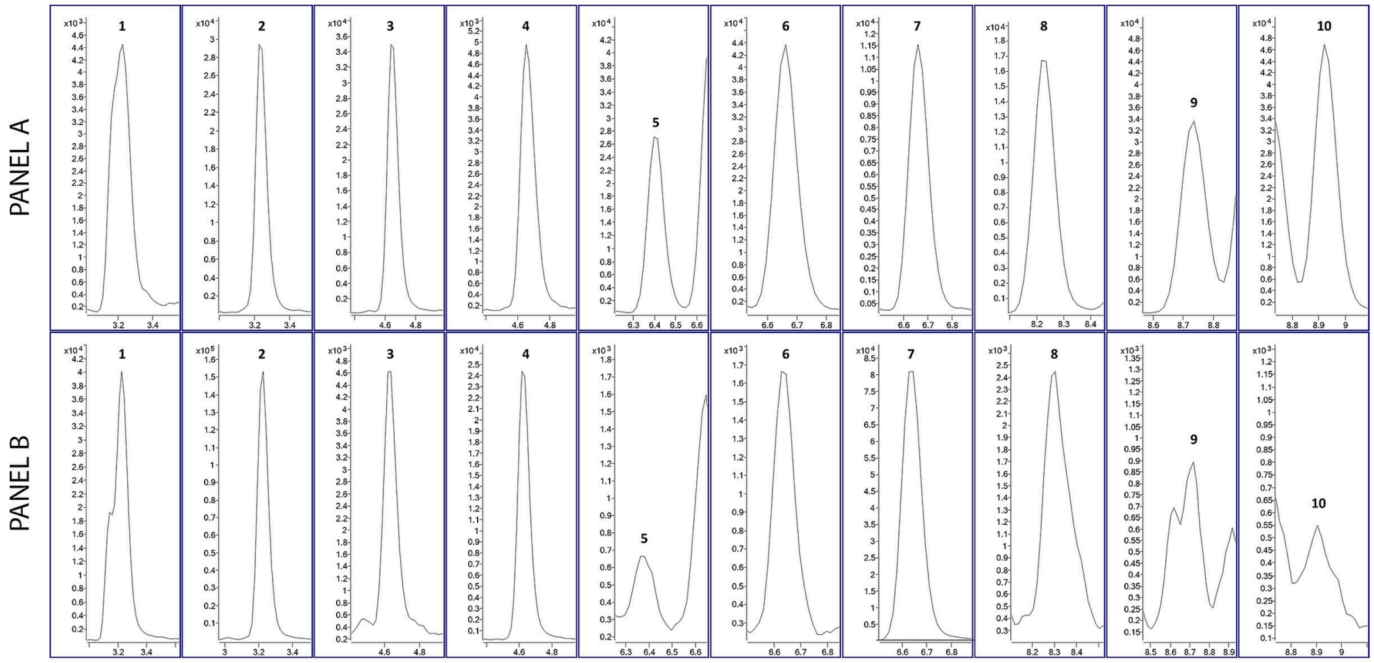

Fig. 4. MRM chromatograms of DnsHz derivatives of SCFAs obtained by HPLC-MS/MS. **Panel A** corresponds to 100 μM standards, **Panel B** corresponds to an Anorexia study sample from plasma. Compounds are numbered from 1 to 10. **1**: Acetic acid, **2**: Acetic acid-1-13C, **3**: Propionic acid, **4**: Propionic acid-1-13C, **5**: Isobutyric acid, **6**: Butyric acid, **7**: Butyric acid-1-13C, **8**: 2-Methylbutyric acid, **9**: Isovaleric acid, **10**: Valeric acid.

Table 2
Linearity study for plasmatic SCFAs, LOD (estimated), and LLOQ (experimentally determined). Linear ranges from 0.2 to 200 μM, except for acetic acid 5.0– 200 μM. C.I.: confidence interval.

SCFA	RT	Range (μM)	Slope ± C.I.	Intercept ± C.I.	r	LOD (nM) (S/N = 3)	LLOQ (nM)	IS
Acetic acid	3.2	5.0 – 200	$0.0350 \pm 5.6x10^{-3}$	$0.48 \pm 2.5x10^{-1}$	0.994	41.1	5000	Acetic acid 1-^{13}C
Propionic acid	4.6	0.20 – 200	$0.0559 \pm 3.8x10^{-3}$	$0.532 \pm 4.7x10^{-2}$	0.996	15.5	200	Propionic acid 1-^{13}C
Isobutyric acid	6.3	0.20 – 200	$0.0204 \pm 1.3x10^{-3}$	$0.01 \pm 1.2x10^{-1}$	0.995	44.1	200	Butyric acid 1-^{13}C
Butyric acid	6.9	0.20 – 200	$0.0318 \pm 7.3x10^{-3}$	$0.01 \pm 9.3x10^{-1}$	0.996	17.3	200	Butyric acid 1-^{13}C
Isovaleric acid	8.6	0.20 – 200	$0.0244 \pm 1.9x10^{-3}$	$0.00 \pm 1.6x10^{-1}$	0.995	122.4	200	Butyric acid 1-^{13}C
Valeric acid	8.9	0.20 – 200	$0.0355 \pm 1.8x10^{-3}$	$0.00 \pm 1.5x10^{-1}$	0.995	181.8	200	Butyric acid 1-^{13}C

Table 3
Linearity study for fecal SCFAs, LOD (estimated), and LLOQ (experimentally determined). Linear ranges from 0.2 to 200 μM, except for acetic acid 5.0– 200 μM. C.I.: confidence interval.

SCFA	RT	Range (μM)	Slope ± C.I.	Intercept ± C.I.	r	LOD (nM) (S/N = 3)	LLOQ (nM)	IS
Acetic acid	3.2	5.0 – 200	$0.0329 \pm 5.3x10^{-3}$	$1.00 \pm 3.5x10^{-1}$	0.998	41.1	5000	Acetic acid-2,2,2-d3
Propionic acid	4.6	0.20 – 200	$0.03459 \pm 3.3x10^{-4}$	$0.043 \pm 5.2x10^{-2}$	0.9990	15.5	200	Propionic acid-3,3,3-d3
Isobutyric acid	6.9	0.20 – 200	$0.02487 \pm 3.8x10^{-4}$	$-0.026 \pm 3.4x10^{-2}$	0.998	44.1	200	Butyric acid-4,4,4-d3
Butyric acid	6.3	0.20 – 200	$0.0360 \pm 1.1x10^{-3}$	$-0.06 \pm 1.4x10^{-1}$	0.9994	17.3	200	Butyric acid-4,4,4-d3
2-Methylbutyric acid	8.3	0.20 – 200	$0.02279 \pm 3.5x10^{-4}$	$-0.040 \pm 4.2x10^{-2}$	0.9993	22.2	200	Valeric acid-2,2,3,3,4,4,5,5,5-d9
Isovaleric acid	8.6	0.20 – 200	$0.02400 \pm 1.6x10^{-4}$	$0.001 \pm 1.8x10^{-2}$	0.9990	122.4	200	Valeric acid-2,2,3,3,4,4,5,5,5-d9
Valeric acid	8.9	5.0 – 200	$0.0332 \pm 3.1x10^{-3}$	$-0.06 \pm 3.3x10^{-1}$	0.9990	181.8	200	Valeric acid-2,2,3,3,4,4,5,5,5-d9

obtained values were in the range of **100 ± 15 %.** Concerning precision, the accepted variation is 20 % for the LLOQ and 15 % for the other concentration levels. So, as to this statement, the method is validated for precision in all ranges of concentration for all the acids (RSD < 15 %) inter and intraday.

Instrumental precision was evaluated by the RSD of 10 consecutive injections of the same solution corresponding to the medium QC. The obtained values of RSD range between 1.2 and 5.9 %. We can state from the consistency and reproducible values depicted in Tables 5 and 6 that all are acceptable.

3.3.1.2. Matrix effect. The *matrix effect* was calculated in percentage by comparing the slopes of the spiked QC samples at the four different concentration levels and those of the standard regression lines. The obtained values are included in Tables 5 and 6, and they range from − 4.5 to 2.3 % in fecal samples and from −10.8 to 13.0 % in plasma samples. Thus, the method fulfills the specifications for the matrix effect.

3.3.1.3. Carryover. The carry-over was assessed by injecting double blank samples (no analyte, no IS) after the highest value of the linear range. The peak areas of the analytes and the IS in the blank were less than 20 % and 5 % of the LLOQ, respectively.

Table 4
Selectivity values for the SCFAs.

SCFA	Selectivity (%) in blank samples without derivatization	Selectivity (%) in blank samples derivatized (no IS)
Acetic acid-1-^{13}C	0.13 %	4.9 %
Propionic acid-1-^{13}C	0.13 %	1.3 %
Butyric acid-1-^{13}C	0.07 %	0.22 %
Aceticacid-d_3	0.15 %	0.83 %
Propionic acid-d3	0.18 %	0.24 %
Butyric acid-d3	0.12 %	0.24 %
Valeric acid-d9	0.07 %	0.04 %
Acetic acid	0.05 %	10.3 %
Propionic acid	0.13 %	1.9 %
Isobutyric acid	0.48 %	1.3 %
Butyric acid	0.12 %	0.94 %
2-Methylbutyric acid	0.60 %	0.92 %
Isovaleric acid	0.77 %	0.75 %
Valeric acid	0.49 %	0.78 %

3.3.1.4. Stability. For stability assays samples were derivatized and kept in the tray autosampler at 4 °C until their analysis. Injection right after sample preparation was considered 100 %, and the subsequent stability tests were calculated as the percentage of recovery related to the initial conditions.

According to the results, after 24 h all the analytes were stable with little variations (<15 %) in both matrices. However, at 48 h the acetic acid Dns-Hz derivative in plasma was not stable. At 72 h the only stable SCFAs in plasma were propionic, isobutyric, butyric, isovaleric, and valeric acids. Thus, plasma samples must be injected and analyzed within the first 24 h. Nonetheless, all SCFAs in feces were stable for 72 h, except isovaleric acid which was stable up to 48 h.

The stability of the stored standard working solutions at −20 °C was evaluated one year after the preparation showing variation percentages below 15 % in all the cases. The variation of the signal for all the studied SCFAs was below +/- 15 %.

The results of the stability assays are included in Fig. 5.

Here we present a methodology for the analysis of eight SCFAs by LC-MS/MS in human plasma and stool samples in less than 10 min. After the step-by-step validation study of the proposed method according EMEA ICH validation guide, pieces of evidence were collected, data processed, and discussed. Consequently, our method has demonstrated to be reliable for the absolute quantitation of SCFAs in plasma and stool samples with high sensitivity, precision, and accuracy. Fully validated methods for SCFAs after derivatization are scarce. Low LOD and LOQ are reported and LLOQs were studied for accuracy and precision and the results fulfill the guide specifications.

Besides, a major concern of previously reported methods is the addition of the internal standard after derivatizing the sample. Thus, the derivatization efficiency of the individual samples is not monitored or assessed. Here, stable isotope-labeled SCFAs of the targeted analytes were added before the derivatization to control the entire sample preparation.

3.4. Application of the method to the study of anorexia nervosa

Proven the specificity, linearity, precision, accuracy, LLOQ, and stability of the method, it was applied to different study samples. Fecal and plasma samples of anorexia nervosa patients and controls were analyzed to quantify their SCFAs. Results obtained from control individuals were in agreement with previously reported results for feces and plasma samples especially when 2-methylbutyric was quantified and differentiated from isobutyric and butyric acids [14,29]. It has been previously reported a dysbiosis of the intestinal microbiome in AN patients under prologued restrictive diets [30–32]. Moreover, decreased fecal concentrations of the main SCFAs (acetic, propionic, and butyric acids) have been occasionally reported in AN [30,33–35]. However, as stated for plasma samples, sparse information is reported in general about plasmatic SCFAs in AN and their eventual role in this disease. Thus, the combined study of these metabolites in plasma and feces becomes of potential clinical and scientific significance.

The results obtained showed that acetic acid was the most abundant SCFA despite the biological matrix (Fig. 6). From all the studied SCFAs, isobutyric acid was quantified in stool samples but negligible concentrations were found in plasma samples from AN patients, falling below the LLOQ. Thus, it was not quantified although it was detected. It is important to highlight the simultaneous quantitation of SCFA including acetic and 2-methylbutyric acid for the first time in plasma samples from both control individuals and anorexic patients.

Significant differences were found in the SCFA profile between AN and the control group (Fig. 6). However, the abundance of SCFAs is related to the multiple mechanisms governing SCFAs' homeostasis.

A pairwise comparison (*t*-test) of the concentrations of SCFAs in plasma and feces in AN and controls was performed (Tables 7 and 8). Significant changes were found in all the quantified analytes in plasma. Increased concentrations of acetic, 2-methylbutyric, isovaleric, and valeric acids next to an important decrease in propionic and butyric acids were found in AN plasma samples compared to controls. For fecal SCFAs, there was a significant decrease in acetic, butyric, and valeric acids in patients (p-value < 0.05). However, there was no significant alteration in the concentration of propionic and isobutyric acids in feces. Later, a correlation analysis between plasma and fecal levels of SCFAs was performed. However, no significant correlations between plasma and fecal SCFAs concentrations were found in either AN patients or controls, as has been previously described [36–39].

The human acetic acid plasma pool has three major sources: the gut microbiota synthesis in the intestine, the endogenous acetogenesis from acetyl-CoA hydrolysis, and the exogenous intake of acetic acid from the diet. Importantly, the second route is activated in prolonged starvation. Acetic levels significantly increased after periods of fasting in mice [40,41]. During fasting, already described in AN as a core symptom of the disease, an acetyl-CoA hydrolase called acyl-CoA thioesterase 12 (ACOT12) becomes activated and generates free acetic acid from acetyl-CoA in the liver. Whereas most of the hepatic propionic and butyric acids are directly metabolized in the liver, the acetic acid flows to general circulation [42,43]. Thus, increased concentrations of plasma acetic acid could be expected in AN patients. Interestingly, acetic acid is an important modulator of body weight through different mechanisms. It has been described to participate in central appetite signaling via hypothalamic mechanisms, regulate the expression of gut-satiety neuropeptides, and improve lipid metabolism and energy expenditure. In short, acetic acid reduces appetite signaling and increases satiety and energy expenditure [44,45]. Thus, higher concentrations of acetic acid in plasma in AN patients might be contributing to the chronification of their reduced food intake. On the contrary, fecal acetic acid is reduced in AN patients. This finding can be related to a modified diet, intestinal dysbiosis, altered acetic acid absorption by the intestine cells, or a combination of them, yielding lower fecal acetic acid in patients. This finding reinforces our hypothesis that the main source of their higher plasma acetic acid is the endogenous production and not the microbiota or the diet (also supported by the lack of correlation between plasma and feces acetic acid concentration observed).

Propionic acid is mainly derived from microbial fermentation [46]. Whereas acetic acid can be endogenously produced under some circumstances, propionic acid is mainly absorbed after gut bacteria synthesis and metabolized by the liver as an energetic substrate [47]. So, under caloric restriction and low energy intake, it is plausible that the propionic acid concentration in plasma could decrease as a consequence of enhanced liver metabolism. Propionic acid has demonstrated lipid-lowering effects, lipogenesis reduction in the liver, and reduction of

Table 5
Precision, accuracy (inter and intra-day), recovery, and matrix effect in spiked derivatized QCs from feces samples.

			Inter-day (n = 15)	Intra-day (n = 5)				
SCFAs	**Concentration level**	**Spiked concentration (μM)**	**Accuracy (%)**	**Sample precision**				
RSD (%)	**Accuracy (%)**	**Sample precision**						
RSD (%)								
Instrumental precision (n = 10)								
RSD (%)	**Recovery**							
Matrix effect (%)								
Acetic acid	LLOQ	0.2	87.2	2.5	89.6	12.6	3.1	2.3
	Low QC	0.4	87.2	0.6	87.4	0.8		
	Med QC	75.0	94.1	3.7	93.5	2.1		
	High QC	175.0	95.8	2.9	96.3	2.0		
Propionic acid	LLOQ	0.2	89.6	8.3	91.7	6.9	2.1	0.3
	Low QC	0.4	91.0	2.1	92.6	12.6		
	Med QC	75.0	96.7	2.2	96.0	1.4		
	High QC	175.0	99.7	1.4	100.4	3.9		
Isobutyric acid	LLOQ	0.2	103.9	2.1	105.9	5.5	5.9	0.7
	Low QC	0.4	104.4	5.8	103.9	4.8		
	Med QC	75.0	98.9	3.3	96.7	1.8		
	High QC	175.0	99.9	2.1	100.8	2.5		
Butyric acid	LLOQ	0.2	106.8	7.2	103.7	6.7	2.1	2.0
	Low QC	0.4	104.3	3.8	103.9	5.2		
	Med QC	75.0	94.2	2.5	95.7	4.4		
	High QC	175.0	98.3	6.0	100.2	4.1		
2-Methylbutyric acid	LLOQ	0.2	109.6	4.8	118.7	7.0	5.0	−4.5
	Low QC	0.4	101.2	6.1	113.8	9.7		
	Med QC	75.0	101.7	4.2	99.7	4.3		
	High QC	175.0	105.0	5.7	104.8	4.1		
Isovaleric acid	LLOQ	0.2	100.2	3.9	106.0	6.1	4.4	−4.1
	Low QC	0.4	102.7	7.7	103.8	7.9		
	Med QC	75.0	100.9	4.4	98.8	4.1		
	High QC	175.0	104.4	6.0	104.5	3.8		
Valeric acid	LLOQ	0.2	97.6	1.3	95.4	6.5	3.1	0.5
	Low QC	0.4	98.2	4.2	93.0	6.8		
	Med QC	75.0	94.6	3.4	93.5	3.1		
	High QC	175.0	99.4	4.0	99.6	2.8		

cholesterol absorption [48]. Thus, lower propionate in plasma in AN might contribute to the well-known altered lipid metabolism and hypercholesterolemia usually observed in AN patients (mainly driven by a central downregulation of thyroid function). However, the impact of the propionic acid disturbances in AN is still to be determined.

Among the SCFAs, butyric acid has been the most widely studied. Fecal butyric acid has been described to participate in host responses of gastrointestinal health, regulate the immune and inflammatory responses, and modulate the intestinal barrier function. There is also growing evidence of the neuroprotective effects of butyric acid on the brain via the gut-brain axis [49,50]. Butyric acid has been deeply related to psychiatric disorders as an inductor of histone hyperacetylation and a reduction in depressive-like and anxiety behaviors [49]. Thus, reduced concentrations of fecal and plasmatic butyric acid in patients might be involved in their psychological symptomatology, including depressive symptoms and impairment of social functioning.

Regarding the branched SCFAs (BCFAs) (isobutyric, isovaleric, and 2-methylbutyric acids) little information is available. BCFAs are produced by the endogenous catabolism of branched amino acids (isoleucine, leucine, and valine) or by the bacterial fermentation of non-digestible proteins in the gut [51]. So, the observed increase in the BCFAs levels in AN could be related to modified microbiota composition, altered food intake, or distorted protein metabolism. As protein catabolism is induced in starvation, an increase of plasmatic BCFAs is expected, as it is shown with 2-methylbutyric acid. The obtained differences in fecal BCFAs could be related to dysbiosis and a modified diet that will produce alterations in the production and absorption of such metabolites.

Lastly, valeric acid, which is a minor product of the intestinal microbiota [50] was decreased in stool samples from AN patients whereas it was below the LLOQ in plasma. Although there is not much knowledge on its function, valeric acid can cross the intestinal barrier and function as a ligand for GPCRs producing different effects on metabolism, immunity, and blood pressure regulation [51]. Previous studies have also shown that the increase in valeric acid concentration is correlated with the levels of inflammation markers (C-reactive protein and white blood cell count) [51]. Nonetheless, the effects in AN pathophysiology are still unclear and need further research.

SCFAs are a necessary waste product of the intestinal microbiota produced after the fermentation of nondigestible carbohydrates and resistant starch. Thus, diet becomes a key factor in their concentration, which varies according to the daily intake and type of food [52]. Therefore, substantial modifications in the diet in AN would have an important effect on the concentration of SCFAs. Additionally, diet is one of the most important factors inducing intestinal dysbiosis, which has been previously described in AN [53–55]. This dysbiosis can also produce differences in fecal and plasmatic SCFAs according to two main facts. Firstly, an altered microbiota composition can modify the ratio of SCFAs producers leading to differences in the concentration of such metabolites. Secondly, the microbiota is a key element in the modulation of intestinal barrier integrity and permeability, so changes in the microbiome can directly affect colonic absorption, ending in overall

Table 6
Precision, accuracy (inter and intra-day), recovery, and matrix effect in spiked derivatized QCs from plasma samples.

SCFAs	Concentration level	Spiked concentration (μM)	Inter-day (n = 9) Accuracy (%)	Inter-day (n = 9) Sample precision RSD (%)	Intra-day (n = 3) Accuracy (%)	Intra-day (n = 3) Sample precision RSD (%)	Instrumental precision (n = 10) RSD (%)	Recovery Matrix effect (%)
Acetic acid	LLOQ	0.2	95.8	13.1	100.0	4.3	1.2	−10.8
	Low	0.6	110.7	1.1	111.7	1.2		
	Medium	8.3	107.0	5.3	106.4	3.3		
	High	20.8	97.3	5.3	94.9	4.1		
Propionic acid	LLOQ	0.2	99.6	4.8	100.0	5.2	1.8	5.2
	Low	0.6	103.3	5.5	106.1	5.6		
	Medium	8.3	88.2	3.1	88.7	3.0		
	High	20.8	93.2	4.1	92.9	3.1		
Isobutyric acid	LLOQ	0.2	100.0	17.4	104.3	12.5	3.8	−0.7
	Low	0.6	96.7	5.2	98.5	5.1		
	Medium	8.3	96.6	7.0	98.7	4.0		
	High	20.8	100.7	6.0	101.1	2.7		
Butyric acid	LLOQ	0.2	103.7	8.1	100.0	13.1	3.8	6.4
	Low	0.6	96.1	6.4	93.5	9.9		
	Medium	8.3	98.7	3.7	99.9	5.3		
	High	20.8	94.6	3.9	95.2	3.5		
Isovaleric acid	LLOQ	0.2	114.2	4.3	112.4	0.0	2.4	2.2
	Low	0.6	92.2	4.0	92.3	5.2		
	Medium	8.3	89.4	4.0	89.5	4.0		
	High	20.8	92.3	3.8	90.6	2.5		
Valeric acid	LLOQ	0.2	100.0	12.2	94.6	14.2	2.4	13.0
	Low	0.6	86.7	13.5	92.0	8.0		
	Medium	8.3	85.2	0.9	85.6	3.7		
	High	20.8	86.7	0.8	86.8	4.4		

modified SCFAs concentrations in feces and plasma [49]. These metabolites can be rapidly and efficiently absorbed in the colon where they can be metabolized as energy substrates, and less than 5 % is excreted in feces [1,56–58]. As a result, although fecal concentrations have been used to determine the production rate by intestinal microbiota, they are also a surrogate measure of their colonic absorption and metabolism. Lastly, SCFAs can cross the epithelial barrier and enter the circulation via the hepatic portal vein, which connects the gastrointestinal tract, spleen, and liver. In fact, in humans, it has been reported that portal vein concentrations of SCFAS are quite higher than those encountered in peripheral blood. Therefore, it can be inferred that the liver acts as a major sink for gut-produced SCFAs, where they can be metabolized via β-oxidation, used for the synthesis of ketone bodies, or converted to acetyl coenzyme A [49]. Thus, alterations in all these processes will produce significant variations in the SCFA profile in AN patients.

4. Conclusions

In this work, we propose a robust, optimized, and validated method for the absolute quantitation of SCFAs in plasma and fecal samples, that can be performed in less than 10 min. It is based on their simultaneous quantification by the stable isotope dilution method with labelled standards. The sample treatment including deproteinization and Dns-Hz derivatization has proven to be a suitable method for the extraction and quantification of SCFAs in both matrices. The time required for sample treatment (plasma and lyophilized feces) is 4 h per daily batch and up to 72 samples can be analyzed each day. Moreover, samples are completely stable in the autosampler vial for more than 24 h and more than 500 samples can be subsequently analyzed in a row without cleaning the equipment (source, injector, needle, etc.).

Once validated, the methodology was applied to a study with human plasma and feces from AN patients and controls. The Results suggested significant changes in plasma and fecal SCFAs profile in patients with AN compared to controls, whose consequences on disease pathogenesis largely remain to be determined. Acetic acid was found to be the most abundant SCFA in both matrices, and its plasmatic concentration was significantly higher in AN patients, potentially playing a role in modulating appetite and energy expenditure in the disease. The decrease in fecal acetic, propionic, butyric, and valeric acid concentrations in AN patients suggests alterations in diet, intestinal dysbiosis, or impaired absorption, contributing to lower SCFA levels in feces. Butyric acid, known for its beneficial effects on gastrointestinal health and its impact on the gut-brain axis, was found to be reduced in both fecal and plasma samples in AN patients. The branched SCFAs (isobutyric, isovaleric, and 2-methylbutyric acids) showed varied patterns, with 2-methylbutyric acid being significantly increased in plasma, likely linked to increased protein catabolism during starvation. However, further research is needed to fully understand the implications of altered BCFAs in AN. Valeric acid, although decreased in the feces and increased in the plasma of AN patients, lacks comprehensive knowledge about its functions, particularly in the context of AN pathophysiology.

It is important to consider that SCFA concentrations are influenced by multiple factors, including diet, microbiota composition, and host metabolism, which can lead to significant variations in SCFA profiles between individuals. Thus, while this study provides valuable insights into SCFAs in AN, further research is necessary to elucidate the precise role of SCFAs in the disease and their potential as biomarkers or therapeutic targets.

Rapid, reliable, sensitive, and cost-effective LC methods for biomarkers are required to quantify SCFA in feces and plasma samples. This method allows for the quantification of SCFAs in both biological matrices overcoming their low concentration in blood samples (nM-μM range). The proposed methodology may assist physicians in diagnosing and monitoring AN in clinics. Understanding the intricate interplay between SCFAs and AN pathophysiology could have considerable clinical and scientific implications for the management and treatment of this complex disorder.

CRediT authorship contribution statement

Laura Mayo-Martínez: Writing – original draft, Methodology, Formal analysis, Data curation. **M. Paz Lorenzo:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Gabriel Á. Martos-Moreno:** Writing – review & editing, Visualization,

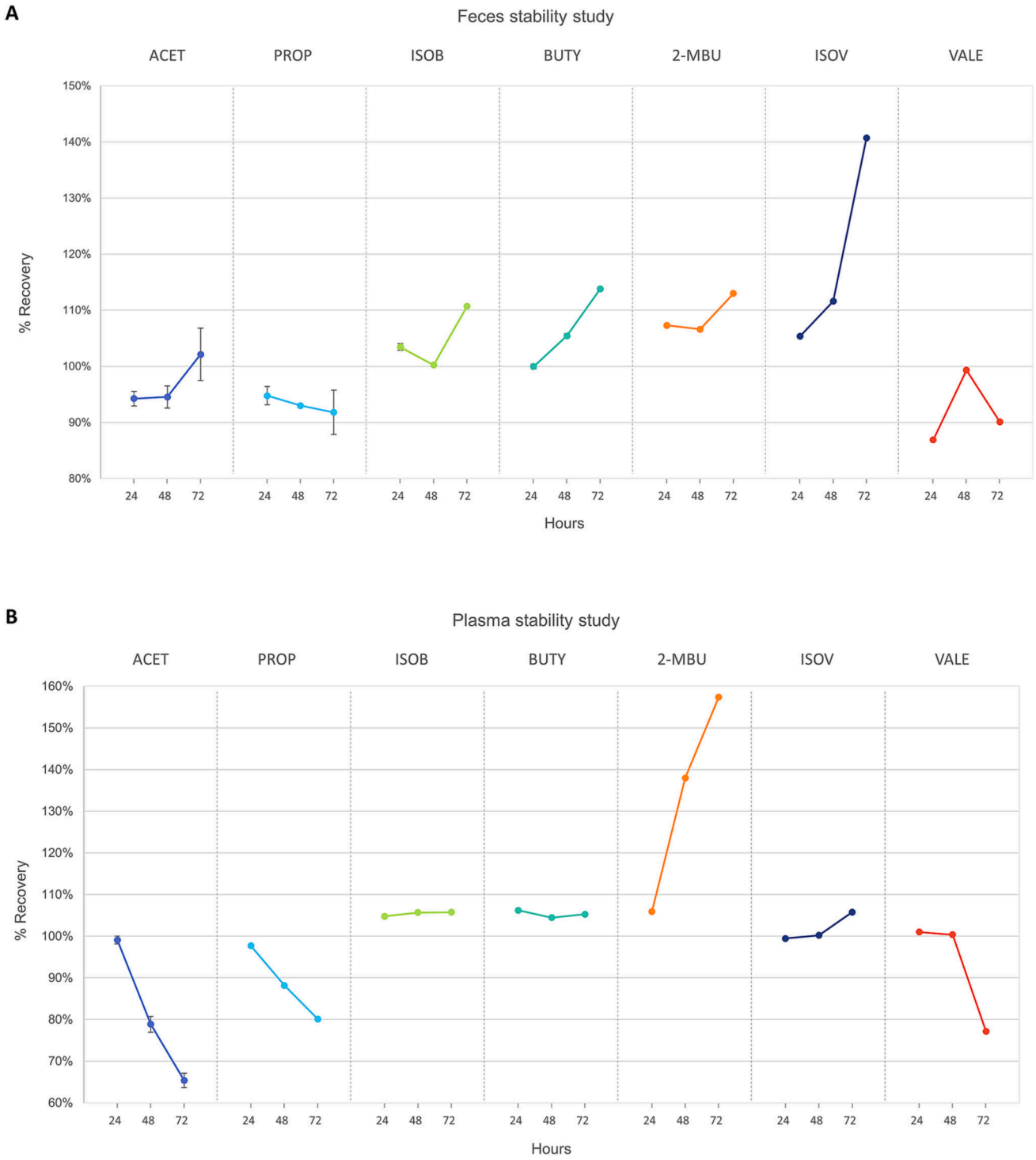

Fig. 5. Stability study of SCFAs derivatives in spiked QCs at the autosampler tray (4 °C). A: Stability is feces. B: Stability in plasma. ACET: Acetic acid, PROP: Propionic acid, ISOB: Isobutyric acid, BUTY: Butyric acid, 2-MBU: 2-Methylbutyric acid, ISOV: Isovaleric acid, VALE: Valeric acid. Error bar: SEM.

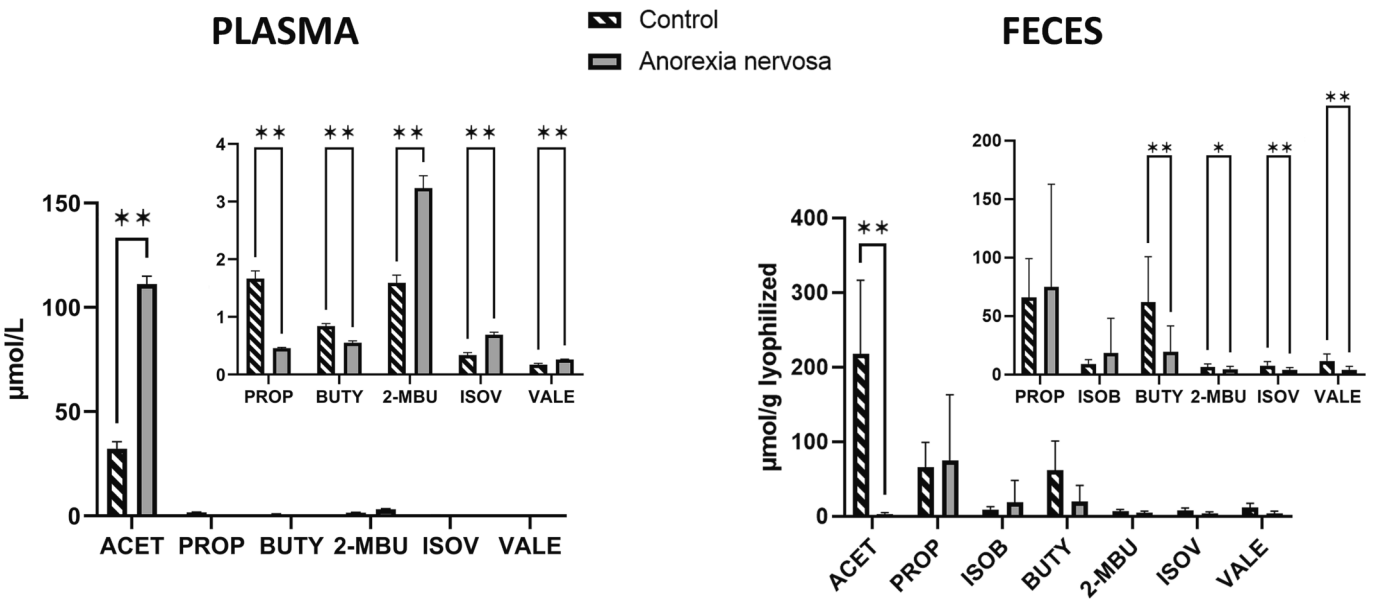

Fig. 6. SCFAs levels in AN patients and controls. NS = non-significant, **FDR-p < 0.01, *FDR-p < 0.05. Error bars: SEM.

Table 7
Pairwise comparison of SCFAs plasma profile in AN patients and controls (*t*-test). *p-value < 0.05, **p-value 0.01, ***p-value < 0.0001, ns: non-significant.

µmol/L	AN (Average ± SEM)	HC (Average ± SEM)	Log_2(FC)	*p*-value	*p*-Bonferroni
Acetic acid	111.17 ± 3.71	32.23 ± 3.34	1.79	***	***
Propionic acid	0.46 ± 0.01	1.67 ± 0.13	−1.86	***	***
Isobutyric acid	0.03 ± 0.01	0.49 ± 0.04	–	–	–
Butyric acid	0.59 ± 0.03	0.84 ± 0.04	−0.51	***	***
2-Methylbutyric acid	3.24 ± 0.21	1.59 ± 0.13	1.02	***	***
Isovaleric acid	0.69 ± 0.04	0.34 ± 0.12	1.03	***	***
Valeric acid	0.26 ± 0.01	0.17 ± 0.02	0.59	***	***

Table 8
Pairwise comparison of fecal SCFAs profile in AN patients and controls (*t*-test). *p-value < 0.05, **p-value 0.01, ***p-value < 0.0001, ns: non-significant.

µmol SCFA/g lyophilized feces	AN (Average ± SEM)	HC (Average ± SEM)	Log_2(FC)	*p*-value	*p*-Bonferroni
Acetic acid	3.20 ± 0.20	217.39 ± 21.65	−6.09	***	***
Propionic acid	74.98 ± 11.24	65.79 ± 7.25	0.19	ns	ns
Isobutyric acid	18.42 ± 2.84	9.11 ± 8.51	1.02	ns	ns
Butyric acid	19.46 ± 0.32	61.78 ± 0.58	−1.67	***	***
2-Methylbutyric acid	4.52 ± 0.25	6.34 ± 0.76	−0.49	***	*
Isovaleric acid	3.94 ± 0.38	7.63 ± 1.31	−0.95	***	***
Valeric acid	3.89 ± 3.79	11.59 ± 0.80	−1.57	***	***

Supervision, Resources, Investigation, Conceptualization. **Montserrat Graell:** Visualization, Investigation. **Coral Barbas:** Writing – review & editing, Visualization, Resources, Investigation, Conceptualization. **Francisco J. Rupérez:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization. **Jesús Argente:** Writing – review & editing, Visualization, Supervision, Resources, Formal analysis, Conceptualization. **Antonia García:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Funding

This research was funded by the Ministry of Science and Innovation of Spain (MCIN) MCIN/AEI/10.13039/501100011033, by ERDF A way of making Europe, grant number PID2021-122490NB-I00 (AGF, FJR), Ministry of Science and Innovation of Spain (FIS-PI19/00166 to JA) and by Centro de Investigación Biomédica en Red Fisiopatología de Obesidad y Nutrición, Instituto de Salud Carlos III (JA and GAMM). LMM is a recipient of the MCINN FPU grant (FPU19/05505).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

[1] N.I. McNeil, J.H. Cummings, W.P.T. James, Short chain fatty acid absorption by the human large intestine, Gut. 19 (1978) 819–822, https://doi.org/10.1136/GUT.19.9.819.
[2] A. Agus, K. Clément, H. Sokol, Gut microbiota-derived metabolites as central regulators in metabolic disorders, Gut. 70 (2021) 1174–1182, https://doi.org/10.1136/gutjnl-2020-323071.
[3] Y.P. Silva, A. Bernardi, R.L. Frozza, The role of short-chain fatty acids from gut microbiota in gut-brain communication, Front Endocrinol (lausanne). 11 (2020), https://doi.org/10.3389/fendo.2020.00025.

[4] B. Dalile, L. Van Oudenhove, B. Vervliet, K. Verbeke, The role of short-chain fatty acids in microbiota–gut–brain communication, Nat Rev Gastroenterol Hepatol. 16 (2019) 461–478, https://doi.org/10.1038/s41575-019-0157-3.

[5] J. Tan, C. McKenzie, M. Potamitis, A.N. Thorburn, C.R. Mackay, L. Macia, The Role of Short-Chain Fatty Acids in Health and Disease, in: Adv Immunol, Academic Press Inc., 2014: pp. 91–119. https://doi.org/10.1016/B978-0-12-800100-4.00003-9.

[6] Y. Dong, C. Cui, The role of short-chain fatty acids in central nervous system diseases, Mol Cell Biochem. 477 (2022) 2595–2607, https://doi.org/10.1007/S11010-022-04471-8.

[7] X. Qian, R. Xie, X. Liu, S. Chen, H. Tang, Mechanisms of short-chain fatty acids derived from gut microbiota in alzheimer's disease, Aging Dis. 13 (2022) 1252. https://doi.org/10.14336/AD.2021.1215.

[8] T.J. Wenzel, E.J. Gates, A.L. Ranger, A. Klegeris, Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells, Molecular and Cellular Neuroscience. 105 (2020), https://doi.org/10.1016/j.mcn.2020.103493.

[9] L. Michel, A. Prat, One more role for the gut: microbiota and blood brain barrier, Ann Transl Med. 4 (2016), https://doi.org/10.3978/j.issn.2305-5839.2015.10.16.

[10] S. Saha, P. Day-Walsh, E. Shehata, P.A. Kroon, Development and Validation of a LC-MS/MS Technique for the Analysis of Short Chain Fatty Acids in Tissues and Biological Fluids without Derivatisation Using Isotope Labelled Internal Standards, Molecules 2021, Vol. 26, Page 6444. 26 (2021) 6444. https://doi.org/10.3390/MOLECULES26216444.

[11] B. Pati, S. Sendh, B. Sahu, S. Pani, N. Jena, N.C. Bal, Recent advancements in pharmacological strategies to modulate energy balance for combating obesity, RSC Med Chem. 14 (2023) 1429–1445, https://doi.org/10.1039/D3MD00107E.

[12] C.S. Byrne, E.S. Chambers, D.J. Morrison, G. Frost, The role of short chain fatty acids in appetite regulation and energy homeostasis, International Journal of Obesity 2015 39:9. 39 (2015) 1331–1338. https://doi.org/10.1038/ijo.2015.84.

[13] E. Medawar, S.B. Haange, U. Rolle-Kampczyk, B. Engelmann, A. Dietrich, R. Thieleking, C. Wiegank, C. Fries, A. Horstmann, A. Villringer, M. von Bergen, W. Fenske, A. Veronica Witte, Gut microbiota link dietary fiber intake and short-chain fatty acid metabolism with eating behavior, Translational Psychiatry 2021 11:1. 11 (2021) 1–11. https://doi.org/10.1038/s41398-021-01620-3.

[14] M. Primec, D. Mičetić-Turk, T. Langerholc, Analysis of short-chain fatty acids in human feces: a scoping review, Anal Biochem. 526 (2017) 9–21, https://doi.org/10.1016/j.ab.2017.03.007.

[15] D.G. Bihan, T. Rydzak, M. Wyss, K. Pittman, K.D. McCoy, I.A. Lewis, Method for absolute quantification of short chain fatty acids via reverse phase chromatography mass spectrometry, PLoS One. 17 (2022) e0267093.

[16] J. Zhang, S. Yang, J. Wang, Y. Xu, H. Zhao, J. Lei, Y. Zhou, Y. Chen, L. Wu, M. Zhou, Y. Li, Y. Li, Integrated LC-MS metabolomics with dual derivatization for quantification of FFAs in fecal samples of hepatocellular carcinoma patients, J Lipid Res. 62 (2021) 100143, https://doi.org/10.1016/J.JLR.2021.100143.

[17] S. Zhao, L. Li, Dansylhydrazine isotope labeling LC-MS for comprehensive carboxylic acid submetabolome profiling, Anal Chem. 90 (2018) 13514–13522, https://doi.org/10.1021/acs.analchem.8b03435.

[18] H.Y. Liao, C.Y. Wang, C.H. Lee, H.L. Kao, W.K. Wu, C.H. Kuo, Development of an efficient and sensitive chemical derivatization-based LC-MS/MS method for quantifying gut microbiota-derived metabolites in human plasma and its application in studying cardiovascular disease, J Proteome Res. 20 (2021) 3508–3518, https://doi.org/10.1021/acs.jproteome.1c00147.

[19] T. Hu, Y. Sun, H. Li, P. Du, L. Liu, Z. An, Dual derivatization strategy for the comprehensive quantification and double bond location characterization of fatty acids by ultra-high performance liquid chromatography-tandem mass spectrometry, J Chromatogr a. 1639 (2021), https://doi.org/10.1016/j.chroma.2021.461939.

[20] A. Shafaei, V. Vamathevan, J. Pandohee, N.G. Lawler, D. Broadhurst, M.C. Boyce, Sensitive and quantitative determination of short-chain fatty acids in human serum using liquid chromatography mass spectrometry, Anal Bioanal Chem. 413 (2021) 6333, https://doi.org/10.1007/S00216-021-03589-W.

[21] R. Jiang, Y. Jiao, P. Zhang, Y. Liu, X. Wang, Y. Huang, Z. Zhang, F. Xu, Twin derivatization strategy for high-coverage quantification of free fatty acids by liquid chromatography-tandem mass spectrometry, Anal Chem. 89 (2017) 12223–12230, https://doi.org/10.1021/ACS.ANALCHEM.7B03020.

[22] H.E. Song, H.Y. Lee, S.J. Kim, S.H. Back, H.J. Yoo, A facile profiling method of short chain fatty acids using liquid chromatography-mass spectrometry, Metabolites. 9 (2019), https://doi.org/10.3390/METABO9090173.

[23] T.Y. Zhang, S. Li, Q.F. Zhu, Q. Wang, D. Hussain, Y.Q. Feng, Derivatization for liquid chromatography-electrospray ionization-mass spectrometry analysis of small-molecular weight compounds, TrAC - Trends in Analytical Chemistry. 119 (2019), https://doi.org/10.1016/j.trac.2019.07.019.

[24] H.M.H. van Eijk, J.G. Bloemen, C.H.C. Dejong, Application of liquid chromatography-mass spectrometry to measure short chain fatty acids in blood, J Chromatogr B Analyt Technol Biomed Life Sci. 877 (2009) 719–724, https://doi.org/10.1016/j.jchromb.2009.01.039.

[25] A. Gomez-Gomez, A. Soldevila, N. Pizarro, V. Andreu-Fernandez, O.J. Pozo, Improving liquid chromatography-tandem mass spectrometry determination of polycarboxylic acids in human urine by chemical derivatization. comparison of o-benzyl hydroxylamine and 2-picolyl amine, J Pharm Biomed Anal. 164 (2019) 382–394, https://doi.org/10.1016/j.jpba.2018.10.055.

[26] P. Deng, Y. Zhan, X. Chen, D. Zhong, Derivatization methods for quantitative bioanalysis by LC-MS/MS, Bioanalysis. 4 (2012) 49–69, https://doi.org/10.4155/bio.11.298.

[27] J. Han, K. Lin, C. Sequeira, C.H. Borchers, An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry, Anal Chim Acta. 854 (2015) 86–94, https://doi.org/10.1016/j.aca.2014.11.015.

[28] E. Medicines Agency, ICH guideline M10 on bioanalytical method validation 4 Step 2b 5, 2019. www.ema.europa.eu/contact.

[29] A. Jaochico, D. Sangaraju, S.K. Shahidi-Latham, A rapid derivatization based LC-MS/MS method for quantitation of short chain fatty acids in human plasma and urine, Bioanalysis. 11 (2019) 741–753, https://doi.org/10.4155/bio-2018-0241.

[30] C. Morita, H. Tsuji, T. Hata, M. Gondo, S. Takakura, K. Kawai, K. Yoshihara, K. Ogata, K. Nomoto, K. Miyazaki, N. Sudo, Gut dysbiosis in patients with anorexia nervosa, PLoS One. 10 (2015) e0145274.

[31] S. Trinh, B. Herpertz-Dahlmann, J. Seitz, Role of the microbiome and the gut-brain interaction in anorexia nervosa, Nervenheilkunde. 39 (2020) 67–73, https://doi.org/10.1055/a-1037-2119.

[32] A.M. Monteleone, J. Troisi, G. Serena, A. Fasano, R.D. Grave, G. Cascino, F. Marciello, S. Calugi, G. Scala, G. Corrivetti, P. Monteleone, The gut microbiome and metabolomics profiles of restricting and binge-purging type anorexia nervosa, Nutrients. 13 (2021) 1–13, https://doi.org/10.3390/nu13020507.

[33] F. Borgo, A. Riva, A. Benetti, M.C. Casiraghi, S. Bertelli, S. Garbossa, S. Anselmetti, S. Scarone, A.E. Pontiroli, G. Morace, E. Borghi, Microbiota in anorexia nervosa: the triangle between bacterial species, metabolites and psychological tests, PLoS One. 12 (2017), https://doi.org/10.1371/journal.pone.0179739.

[34] P. Prochazkova, R. Roubalova, J. Dvorak, J. Kreisinger, M. Hill, H. Tlaskalova-Hogenova, P. Tomasova, H. Pelantova, M. Cermakova, M. Kuzma, J. Bulant, M. Bilej, K. Smitka, A. Lambertova, P. Holanova, H. Papezova, The intestinal microbiota and metabolites in patients with anorexia nervosa, Gut Microbes. 13 (2021) 1–25, https://doi.org/10.1080/19490976.2021.1902771.

[35] A. Ghenciulescu, R.J. Park, P.W.J. Burnet, The gut microbiome in anorexia nervosa: friend or foe? Front Psychiatry. 11 (2021) 1463, https://doi.org/10.3389/FPSYT.2020.611677/BIBTEX.

[36] S.J. Chen, C.C. Chen, H.Y. Liao, Y.T. Lin, Y.W. Wu, J.M. Liou, M.S. Wu, C.H. Kuo, C. H. Lin, Association of Fecal and Plasma Levels of short-chain fatty acids with gut microbiota and clinical severity in patients with parkinson disease, Neurology. 98 (2022) e848.

[37] I. Nilsson, J. Xu, R. Landberg, C. Lavebratt, C. Bulik, M. Landén, Plasma concentrations of short-chain fatty acids in active and recovered anorexia nervosa, (2022). https://doi.org/10.21203/rs.3.rs-1744940/v1.

[38] L. Calderón-Pérez, M.J. Gosalbes, S. Yuste, R.M. Valls, A. Pedret, E. Llauradó, N. Jimenez-Hernandez, A. Artacho, L. Pla-Pagà, J. Companys, I. Ludwig, M. P. Romero, L. Rubió, R. Solà, Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study, Sci Rep. 10 (2020), https://doi.org/10.1038/s41598-020-63475-w.

[39] M. Müller, M.A.G. Hernández, G.H. Goossens, D. Reijnders, J.J. Holst, J.W. E. Jocken, H. van Eijk, E.E. Canfora, E.E. Blaak, Circulating but not faecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans, Sci Rep. 9 (2019), https://doi.org/10.1038/s41598-019-48775-0.

[40] N. Trivedi, H.E. Erickson, V. Bala, Y.S. Chhonker, D.J. Murry, A concise review of liquid chromatography-mass spectrometry-based quantification methods for short chain fatty acids as endogenous biomarkers, Int J Mol Sci. 23 (2022), https://doi.org/10.3390/ijms232113486.

[41] M. Zeng, H. Cao, Fast quantification of short chain fatty acids and ketone bodies by liquid chromatography-tandem mass spectrometry after facile derivatization coupled with liquid-liquid extraction, Journal of Chromatography b. 1083 (2018) 137–145, https://doi.org/10.1016/J.JCHROMB.2018.02.040.

[42] Z.T. Schug, J. Vande Voorde, E. Gottlieb, The metabolic fate of acetate in cancer, Nat Rev Cancer. 16 (2016) 708–717, https://doi.org/10.1038/nrc.2016.87.

[43] J.R. Moffett, N. Puthillathu, R. Vengilote, D.M. Jaworski, A.M. Namboodiri, Acetate revisited: a key biomolecule at the nexus of metabolism, epigenetics, and oncogenesis – part 2: acetate and ACSS2 in health and disease, Front Physiol. 11 (2020), https://doi.org/10.3389/fphys.2020.580171.

[44] M.A.G. Hernández, E.E. Canfora, J.W.E. Jocken, E.E. Blaak, The Short-Chain Fatty Acid Acetate in Body Weight Control and Insulin Sensitivity, Nutrients 2019, Vol. 11, Page 1943. 11 (2019) 1943. https://doi.org/10.3390/NU11081943.

[45] G. Frost, M.L. Sleeth, M. Sahuri-Arisoylu, B. Lizarbe, S. Cerdan, L. Brody, J. Anastasovska, S. Ghourab, M. Hankir, S. Zhang, D. Carling, J.R. Swann, G. Gibson, A. Viardot, D. Morrison, E.L. Thomas, J.D. Bell, The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism, Nature Communications 2014 5:1. 5 (2014) 1–11. https://doi.org/10.1038/ncomms4611.

[46] E. Hosseini, C. Grootaert, W. Verstraete, T. Van de Wiele, Propionate as a health-promoting microbial metabolite in the human gut, Nutr Rev. 69 (2011) 245–258, https://doi.org/10.1111/j.1753-4887.2011.00388.x.

[47] L. Hoyles, T. Snelling, U.-K. Umlai, J.K. Nicholson, S.R. Carding, R.C. Glen, S. McArthur, Microbiome–host systems interactions: protective effects of propionate upon the blood–brain barrier, Microbiome. 6 (2018) 55, https://doi.org/10.1186/S40168-018-0439-Y.

[48] A. Haghikia, F. Zimmermann, P. Schumann, A. Jasina, J. Roessler, D. Schmidt, P. Heinze, J. Kaisler, V. Nageswaran, A. Aigner, U. Ceglarek, R. Cineus, A. N. Hegazy, E.P.C. Van Der Vorst, Y. Doring, C.M. Strauch, I. Nemet, V. Tremaroli, C. Dwibedi, N. Krankel, D.M. Leistner, M.M. Heimesaat, S. Bereswill, G. Rauch, U. Seeland, O. Soehnlein, D.N. Muller, R. Gold, F. Backhed, S.L. Hazen, A. Haghikia, U. Landmesser, Propionate attenuates atherosclerosis by immune-dependent regulation of intestinal cholesterol metabolism, Eur Heart J. 43 (2022) 518–533, https://doi.org/10.1093/eurheartj/ehab644.

[49] R.M. Stilling, M. van de Wouw, G. Clarke, C. Stanton, T.G. Dinan, J.F. Cryan, The neuropharmacology of butyrate: the bread and butter of the microbiota-gut-brain

axis? Neurochem Int. 99 (2016) 110–132, https://doi.org/10.1016/j.neuint.2016.06.011.
[50] P. Markowiak-Kopeć, K. Śliżewska, The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome, Nutrients. 12 (2020), https://doi.org/10.3390/nu12041107.
[51] Y. Gao, H. Chen, J. Li, S. Ren, Z. Yang, Y. Zhou, R. Xuan, Alterations of gut microbiota-derived metabolites in gestational diabetes mellitus and clinical significance, J Clin Lab Anal. 36 (2022), https://doi.org/10.1002/JCLA.24333.
[52] G. Den Besten, K. Van Eunen, A.K. Groen, K. Venema, D.J. Reijngoud, B.M. Bakker, The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism, J Lipid Res. 54 (2013) 2325, https://doi.org/10.1194/JLR.R036012.
[53] C.R. Martin, V. Osadchiy, A. Kalani, E.A. Mayer, The brain-gut-microbiome Axis, cell Mol, Gastroenterol Hepatol. 6 (2018) 133, https://doi.org/10.1016/J.JCMGH.2018.04.003.
[54] T.G. Dinan, J.F. Cryan, The microbiome-gut-brain Axis in health and disease, Gastroenterol Clin North Am. 46 (2017) 77–89, https://doi.org/10.1016/j.gtc.2016.09.007.
[55] T.G. Dinan, J.F. Cryan, The impact of gut microbiota on brain and behaviour: implications for psychiatry, Curr Opin Clin Nutr Metab Care. 18 (2015) 552–558, https://doi.org/10.1097/MCO.0000000000000221.
[56] G. Rechkemmer, K. Rönnau, W.V. Engelhardt, Fermentation of polysaccharides and absorption of short chain fatty acids in the mammalian hindgut, Biochem. Physiol. 9 (4) (1998) 563–568, https://doi.org/10.1016/0300-9629(88)90668-8. PMID: 2902962.
[57] M. Müller, M.A.G. Hernández, G.H. Goossens, D. Reijnders, J.J. Holst, J.W. E. Jocken, H. van Eijk, E.E. Canfora, E.E. Blaak, Circulating but not faecal short-chain fatty acids are related to insulin sensitivity, lipolysis and GLP-1 concentrations in humans, Scientific Reports. 9 (1) (2019) 1–9, https://doi.org/10.1038/s41598-019-48775-0.
[58] D.L. Topping, P.M. Clifton, Short-chain fatty acids and human colonic function: roles of resistant starch and non starch polysaccharides, Physiological Reviews. 81 (3) (2001) 1031–1064, https://doi.org/10.1152/PHYSREV.2001.81.3.1031/ASSET/IMAGES/LARGE/9J0310145003.JPEG.