

## ORIGINAL RESEARCH

## Gut microbiota in very early systemic sclerosis: the first case-control taxonomic and functional characterisation highlighting an altered butyric acid profile

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## ABSTRACT

**Objectives** In systemic sclerosis (SSc), gastrointestinal involvement is one of the earliest events. We compared the gut microbiota (GM), its short-chain fatty acids (SCFAs) and host-derived free fatty acids (FFAs) in patients with very early diagnosis of SSc (VEDOSS) and definite SSc.

**Methods** Stool samples of 26 patients with SSc, 18 patients with VEDOSS and 20 healthy controls (HC) were collected. The GM was assessed through 16S rRNA sequencing, while SCFAs and FFAs were assessed by gas chromatography-mass spectrometry.

**Results** In patients with VEDOSS, an increase in Bacteroidales and Oscillospirales orders and a decrease in Bacilli class, *Blautia*, *Romboutsia*, *Streptococcus* and *Turicibacter* genera was detected in comparison with HC. In patients with SSc, an elevated number of Acidaminococcaceae and Sutterellaceae families, along with a decrease of the Peptostreptococcaceae family and *Anaerostipes*, *Blautia*, *Romboutsia* and *Turicibacter* genera was found in comparison with HC. Patients with SSc and VEDOSS had a significantly lower butyrate and higher acetate with respect to HC. In VEDOSS, an increase in Oscillospiraceae family and *Anaerostipes* genus, and a decrease in *Alphaproteobacteria* class, and Lactobacillales order was identified with respect to SSc. Moreover, patients with VEDOSS exhibited higher acetate and lower valerate compared with definite SSc.

**Conclusion** A GM dysbiosis with depletion of beneficial anti-inflammatory bacteria (especially butyrate-producing) and a significant decrease in faecal butyrate was identified in patients with VEDOSS. This early GM imbalance may foster the growth of inflammatory microbes, worsening intestinal dysbiosis and inflammation in early SSc stages. The potential butyrate administration in early disease phases might be considered as a novel therapeutic approach to mitigate gastrointestinal discomfort and progression preserving patient's quality of life.

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ We searched PubMed using the terms (“systemic sclerosis”) AND (“VEDOSS”) AND (“gut microbiome”) for studies that assessed gut microbiota (GM) composition and function in early systemic sclerosis (SSc), published in English between database's inception and 31 December 2023.
- ⇒ Most of the studies on GM were based on definite SSc compared with healthy controls (HC), and did not include early patients (ie, very early diagnosis of SSc (VEDOSS)).

## WHAT THIS STUDY ADDS

- ⇒ To our knowledge, this is the first time that GM composition and its metabolites, as well as host-derived free fatty acids, were evaluated in patients with VEDOSS in comparison with patients with definite SSc and HC.
- ⇒ The study explored the potential correlations between microbial elements and intestinal health parameters.
- ⇒ In three independent cohorts, a significant GM dysbiosis was identified in patients with VEDOSS, marked by the depletion of beneficial anti-inflammatory bacteria, especially those producing butyrate.
- ⇒ Accordingly, a significant decrease in faecal butyrate was seen.
- ⇒ In very early SSc, the GM imbalance may favour the growth of inflammatory microbes, worsening intestinal dysbiosis and inflammation.

## INTRODUCTION

Systemic sclerosis (SSc) is a complex and heterogeneous fibrosing autoimmune disease

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings suggest that butyrate administration in very early SSc might be an innovative therapeutic approach to slow gastrointestinal disease progression, control symptoms and maintain the quality of life.

affecting internal organs characterised by high mortality and morbidity.<sup>1</sup> In SSc, there is growing evidence that gut microbiota (GM) and its metabolites may contribute to disease onset and evolution such as the progression of gastrointestinal (GI) involvement.<sup>2</sup> It is well documented that the GI system is extensively affected in >90% of patients with SSc starting from the earliest disease phase.<sup>3</sup> However, studies on GM contribution to GI symptomatology are still scarce. To date, several studies suggested that in SSc the GM composition is altered when compared with healthy controls (HC).<sup>4</sup> The commensal bacteria genus *Lactobacillus* was found in greater abundance, while the genus *Faecalibacterium* and protolerogenic bacteria species were significantly decreased in SSc.<sup>5</sup> Furthermore, recent studies demonstrated a different GM composition in patients with SSc according to GI involvement.<sup>6</sup> Moreover, a dysbiosis characterised by the abundance of *Akkermansia muciniphila*,<sup>5</sup> *Fusobacterium* spp<sup>6</sup> and *Prevotella* spp was detected in patients with SSc with more severe GI symptoms.<sup>5</sup>

Although the mechanisms involved in SSc onset are still unclear, immune activation is a pivotal event leading to fibrosis.<sup>7</sup> It is well documented that GM and its metabolites can shape the host immune system, thus contributing to the maintenance of immune cell homeostatic mechanisms or promoting inflammation when the homeostasis of various T-cell subsets is disturbed. The short-chain fatty acids (SCFAs) are the primary GM metabolites exerting a remarkable immunomodulatory activity<sup>8</sup> and our recent data showed a different and interdependent salivary and faecal microbiota composition related to the presence of specific circulating autoantibodies in patients with SSc.<sup>9</sup>

It is well known that GI involvement in SSc is one of the earliest clinical features and, in the last decade, the clinical criteria to reach a very early diagnosis have been validated (ie, very early diagnosis of SSc (VEDOSS)). In this scenario, the present work aimed to evaluate, for the first time, the GM composition and its metabolites, especially SCFAs, as well as host-derived free fatty acids (FFAs), in patients with VEDOSS compared with patients with definite SSc and HC. Moreover, we explored the potential correlations between microbial elements and intestinal health parameters.

## METHODS

### Patients

Twenty-six patients with SSc classified according to the American College of Rheumatology/EULAR 2013 criteria (24 females, mean age 64.8±11.9 years, with

a disease duration (from RP onset) of 18.8±10.5), 18 patients with VEDOSS<sup>10</sup> (17 females, mean age 51.7±16.1 years, with a disease duration (from RP onset) of 11.2±9.2 years) and 20 HC matched for age and sex (14 females, mean age 48±11.8 years) were consecutively enrolled at the division of Rheumatology, Careggi, Hospital of Florence, between January 2020 and December 2022.

Exclusion criteria were: (i) age <18 years; (ii) use of antibiotics or non-steroidal anti-inflammatory drugs or any other prebiotic or probiotic supplement or any proton pump inhibitors in the previous 3 months (ms); (iii) recent diagnosis (<3 ms) of bacterial or parasitic infections of the GI tract; (iv) trip to exotic areas in the last 5 years; (v) GI pacemaker at baseline; (v) total parenteral nutrition or gastrostomy-jejunostomy tube at baseline or within the last 3 ms and (vi) inability to provide informed consent.

For each patient, demographic, and clinical, laboratory and instrumental data were collected. Clinical data included the history or presence of puffy fingers, digital ulcers, telangiectasia, smoking habits and the modified Rodnan skin score (mRSS). GI symptoms were assessed using the University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Tract Instrument (UCLA GIT 2.0) questionnaire. Laboratory data included antinuclear antibodies, SSc-specific antibodies (anticentromere antibodies (ACA), antitopoisomerase I antibodies and anti-RNA polymerase III antibodies). Instrumental data included nailfold videocapillaroscopic (NVC) pattern.

Clinical and laboratory features of enrolled patients are shown in [table 1](#), while pharmacological treatments are shown in online supplemental table S1.

Detailed methods and any associated references of the present case-control study are available in the online supplemental methods. The bash and R scripts through which the microbiota data have been processed, filtered and analysed are publicly available at [https://github.com/LeandroD94/Papers/tree/main/2023\\_SSc\\_vs\\_VEDOSS](https://github.com/LeandroD94/Papers/tree/main/2023_SSc_vs_VEDOSS)

## RESULTS

### Clinical features

Demographic and clinical features of patients with VEDOSS and definite SSc are detailed in [table 1](#), while past or ongoing treatment is reported in online supplemental table S1. All patients with SSc were ANA positive, 12 (46.2%) had ACA and 12 (46.2%) had antitopoisomerase I antibodies, while only two patients (7.6%) were anti-RNA polymerase III positive. Among patients with VEDOSS, eight were positive for ACA (44.4%), one (5.6%) was positive for antitopoisomerase I and one (5.6%) was positive for anti-RNA polymerase III. The mean±SD mRSS of patients with SSc was 3.8±4.2, while no skin involvement

**Table 1** Demographic and clinical data of patients with VEDOSS and patients with SSc and HC

	VEDOSS (n=18)	SSc (n=26)	HC (n=20)
Clinical and disease features			
Age (years), mean±SD	51.7±16.1	64.8±11.9	48.0±11.8
Female, n (%)	17 (94.4%)	24 (92.3%)	14 (70.0%)
Disease duration in years (from Raynaud phenomenon onset), mean±SD	11.2±9.2	18.8±10.5	NA
Disease subset			
lcSSc, n (%)	NA	13 (50%)	NA
dcSSc, n (%)	NA	13 (50%)	NA
Smoke			
History of smoking, n (%)	6 (33.3%)	8 (30.8%)	4 (20.0%)
Smokers, n (%)	2 (11.1%)	4 (15.4%)	3 (15.0%)
ANA positivity, n (%)	18 (100%)	26 (100%)	NA
Specific autoantibodies			
Antitopo I, n (%)	1 (5.6%)	12 (46.2%)	NA
ACA, n (%)	8 (44.4%)	12 (46.2%)	NA
Anti-RNA polymerase III, n (%)	1 (5.6%)	2 (7.6%)	NA
Other autoantibodies*	4 (22.2%)	3 (11.5%)	NA
NVC pattern			
Aspecific, n (%)	12 (70.6%)	2 (7.7%)	NA
Early, n (%)	4 (23.5%)	7 (26.9%)	NA
Active, n (%)	1 (5.9%)	9 (34.6%)	NA
Late, n (%)	0	8 (30.8%)	NA
Digital ulcers, n (%)	0	14 (53.8%)	0
Puffy fingers, n (%)	8 (44.4%)	7 (26.9%)	0
Telangiectasia, n (%)	2 (11.1%)	14 (53.8%)	0
Joint involvement			
Arthralgia, n (%)	10 (55.6%)	7 (26.9%)	0 (0.0%)
Arthritis, n (%)	1 (5.6%)	3 (11.5%)	0 (0.0%)
UCLA GIT 2.0 total score, mean±SD	0.314 (±0.266)	0.448 (±0.449)	NA
Reflux score, mean±SD	0.240 (±0.454)	0.545 (±0.523)	NA
Bloating score, mean±SD	0.714±0.684	0.519±0.714	NA
Faecal storage score, mean±SD	0.028±0.114	0.308±0.722	NA
Diarrhoea score, mean±SD	0.139±0.466	0.404±0.501	NA
Social function score, mean±SD	0.273±0.340	0.389±0.650	NA
Emotional well-being score, mean±SD	0.197±0.385	0.459±0.676	NA
Constipation, mean±SD	0.194±0.453	0.288±0.315	NA

\*Other autoantibodies in VEDOSS: one PM-Scl75, one NOR90, one anticardiolipin and one Ro52; other autoantibodies in patients with SSc: two antihistone, one Ro52 and one PM-Scl75.

ACA, anticentromere antibodies; ANA, antinuclear antibodies; antitopo I, antitopoisomerase I antibodies; dcSSc, diffuse cutaneous SSc; HC, healthy control; lcSSc, limited cutaneous SSc; NA, not available; NVC, nailfold videocapillaroscopy; SSc, systemic sclerosis; UCLA GIT 2.0, University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Tract Instrument questionnaire; VEDOSS, very early diagnosis of SSc.

was detectable in the VEDOSS group. All patients showed Raynaud's phenomenon, and eight (44.4%) of the patients with VEDOSS and seven (26.9%) of the patients with SSc presented with puffy fingers. No difference in the UCLA GIT 2.0 score was found between patients with VEDOSS and SSc.

### Faecal microbiota characterisation

#### Different microbial community among SSc, VEDOSS and HC groups

First, the faecal microbial communities were characterised in the enrolled patients with SSc and VEDOSS as well as in HC. The abundance of the five most represented microbial phyla and 10 most represented microbial genera in faecal samples is reported in online supplemental figure S1A,B. The five most represented phyla

were Firmicutes, Bacteroidota, Actinobacteriota, Verrucomicrobiota and Proteobacteria (online supplemental figure S1A), while the top 10 genera were *Ruminococcus*, *Akkermansia*, *Alistipes*, *Fecalibacterium*, *Subdoligranulum*, *Bifidobacterium*, *Prevotella*, *Blautia*, *Bacteroides* and *UCG-002* (online supplemental figure S1B).

The alpha diversity of faecal samples from the three investigated groups displayed significant differences for all the indices evaluated: observed richness ( $p=0.009$ ), the Shannon index ( $p=0.0002$ ) and evenness ( $p=2e-07$ ) across the groups (online supplemental figure S2). Furthermore, significant differences have resulted also in almost every pairwise comparison (see  $p$ -adjusted reported in online supplemental table S2), with the exclusion of the observed richness between HC and VEDOSS samples and the Shannon index between HC and SSc samples which were not significantly different.

### The faecal microbiota composition of VEDOSS is more similar to that of SSc than HC

Hierarchical clustering on amplicon sequence variants (ASVs) and Principal Coordinate Analysis on genera using Hellinger distance were performed to investigate the similarity in microbial abundance profiles across patients. In detail, distinct clusters were evidenced by both methods, with VEDOSS faecal samples appearing much more comparable to those of SSc than to HC (figure 1A,B). As shown in figure 1A and online supplemental table S3, every comparison, both general and pairwise, was statistically significant according to permutational multivariate analysis of variance (PERMANOVA). Furthermore, a significant difference in dispersion is featured in the comparison between SSc and HC ( $p$ -adjusted=0.0027) and VEDOSS and HC ( $p$ -adjusted=0.0375) but not between SSc and VEDOSS samples ( $p$ -adjusted=0.3392).

To assess the genera shared by the three groups, we used a Venn diagram considering only the genera with minimal abundance  $>0.1\%$  at least in 10% of the whole dataset (figure 1C). The data showed that 114 genera were common among SSc, VEDOSS and HC, while two genera (ie, *Megasphaera* and *Rikenellaceae RC9 gut group*) were found both in SSc and VEDOSS but not in HC. Moreover, a sparse partial least square discriminant analysis (sPLS-DA) was computed to further distinguish the microbiota of patients with SSc and VEDOSS and HC (figure 2A). The prediction accuracy of this classification model is equal to 100% when classifying the nine subjects casually selected and left out from the model building to serve as a test dataset. However, the resulting balanced error rate from cross-validation amounts to about 30%. Despite this, we used its estimations to further improve the confidence of our conclusions by matching the genera that have been selected by sPLS-DA with DESeq2 results (see the paragraph below). Accordingly, *Blautia* and *Turicibacter* genera were confirmed to be relevant changes in HC (figure 2B). Further details about this model are available in the R script (see online supplemental methods) and provided as supplemental files.

### VEDOSS and SSc display different taxonomic microbiota profile

Patients with VEDOSS reported a significant increase in members of the Oscillospiraceae family and the *Bacteroidales*, *Oscillospirales*, *UCG-002* and *UCG-005* genera, as well as a decrease in the Bacilli class, Eggerthellaceae families and *Blautia*, *Erysipelotrichaceae UCG-003*, *Romboutsia*, *Streptococcus* and *Tucibacter* genera when compared with HC. In patients with SSc, a significant increase in Acidaminococcaceae, *Eubacterium\_coprostanoligenes*, Sutterellaceae families and *Eubacterium\_coprostanoligenes* and *UCG-005* genera was detected, together with a significant decrease in Peptostreptococcaceae family and *Anaerostipes*, *Blautia*, *Erysipelotrichaceae UCG-003*, *Eubacterium\_halli*, *Romboutsia* and *Turicibacter* genera, compared with HC. Finally, patients with VEDOSS reported a significant increase in the Oscillospiraceae family, *Anaerostipes* and *UCG-002* genera, as well as a decrease in *Alphaproteobacteria* class, Lactobacillales order, Eggerthellaceae and Marinifilaceae families and *Erysipelatoclostridium* genus, when compared with patients with SSc. Figure 3 is a graphical representation of the differential analysis at all taxonomic ranks.

### Evaluation of the lipidomics profile

#### Different faecal SCFA, medium-chain fatty acid and long-chain fatty acid profiles in SSc, VEDOSS and HC groups

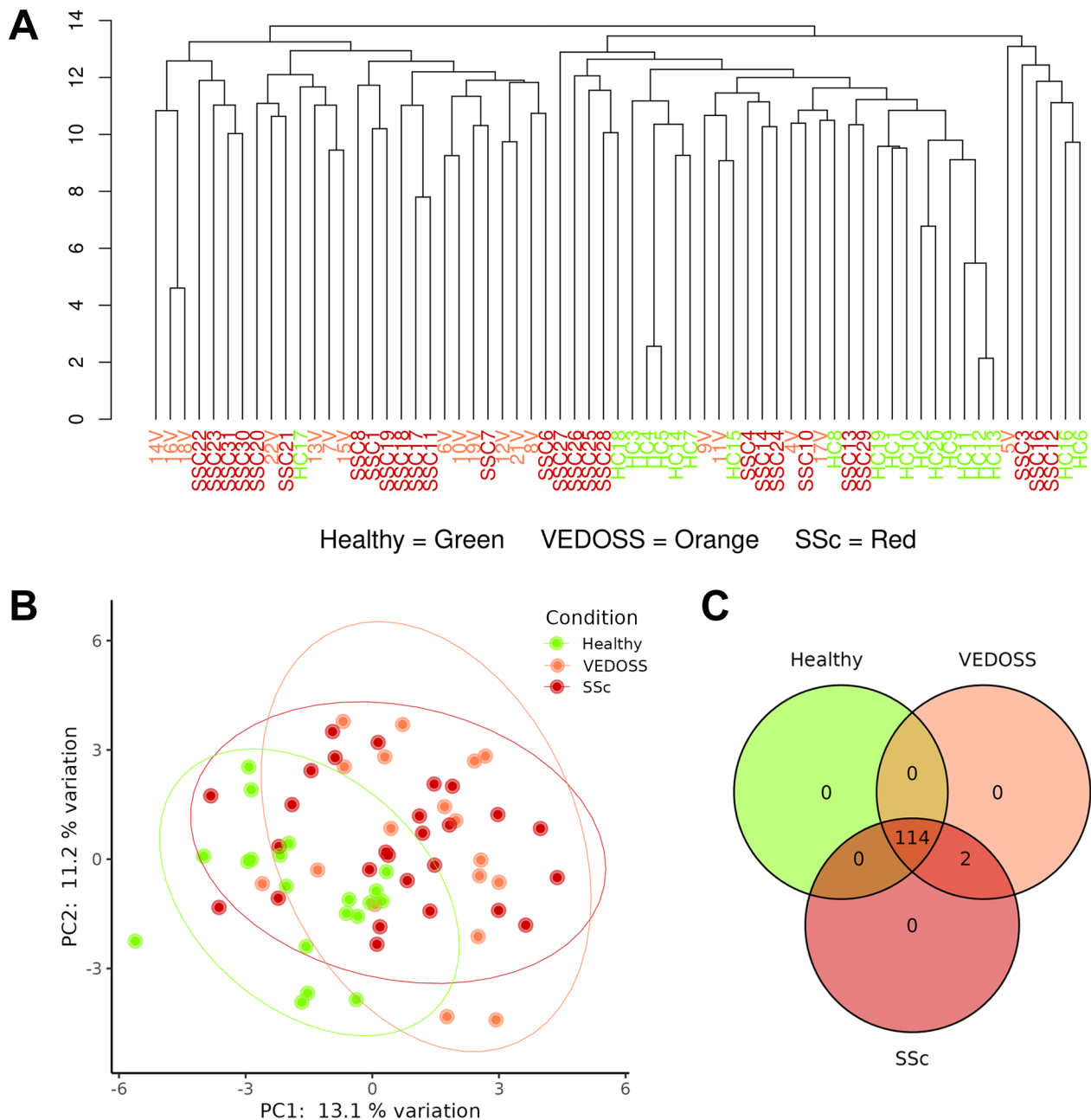
To investigate possible differences in the metabolic output among the three study groups, we analysed both microbial (linear and branched SCFAs) and host-derived FFAs in faecal samples (figure 4). Both patients with VEDOSS and SSc showed significantly higher levels of acetic and nonanoic acids but lower levels of butyric acid in comparison with HC. Patients with VEDOSS reported a higher abundance of tetradecanoic acid in comparison with HC. On the other hand, octanoic acid was increased only in patients with SSc with respect to HC. Notably, patients with VEDOSS reported significantly lower abundances of acetic, isohexanoic, heptanoic and octanoic acids, as well as higher abundances of valeric and tetradecanoic acids than patients with SSc (figure 4).

#### Significant differences in serum FFAs among SSc, VEDOSS and HC groups

In addition, we evaluated the circulating abundance of the same lipids as it provides information on the systemic distribution of FFA, which is well known to have a relevant impact on different host functions, especially immunity modulation. The analysis of serum FFA levels showed significant changes among HC, and patients with VEDOSS and SSc.

As shown in figure 5, patients with VEDOSS displayed higher serum levels of isobutyric, hexanoic, isohexanoic, octanoic, decanoic and dodecanoic acids but lower levels of acetic, propionic, butyric, 2-methylbutyric, valeric, isovaleric, 2-ethylhexanoic, hexadecanoic and octadecanoic acids than HC.

In patients with SSc, significantly higher serum levels of hexanoic and dodecanoic acids, but lower levels of acetic, propionic, butyric, 2-methylbutyric, isobutyric,



**Figure 1** (A) Hierarchical clustering analysis on ASVs and (B) Principal Coordinate Analysis on genera, both computed using Hellinger distance, showing that samples separate into groups. (C) Venn diagram of the three groups including only genera with minimal abundance >0.1% at least in seven samples (10% of the whole dataset). PC, principal component; SSc, systemic sclerosis; VEDOSS, very early diagnosis of systemic sclerosis.

valeric, isovaleric, isohexanoic, 2-ethylhexanoic, octanoic, hexadecanoic and octadecanoic acids were detected compared with HC (figure 5). In addition, patients with VEDOSS showed lower circulating levels of butyric, 2-methylbutyric and isovaleric acids but higher abundances of isobutyric, valeric, isohexanoic, 2-ethylhexanoic, octanoic, decanoic and octadecanoic acids compared with patients with SSc (figure 5).

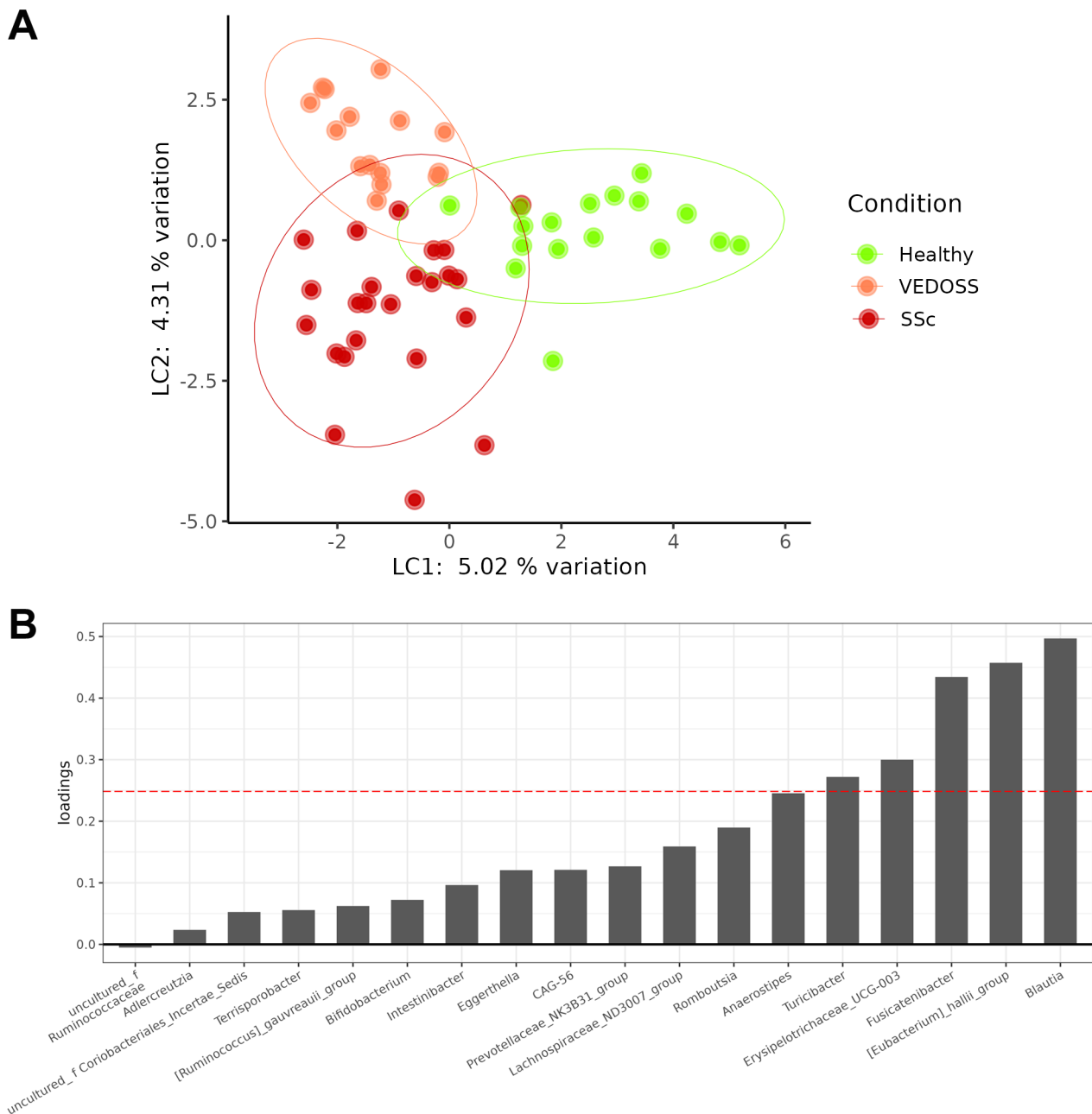
### Functional profiles of faecal microbiota in patients with SSc and VEDOSS

As we observed significant alterations in the faecal microbiota composition when comparing the three

groups, the functional metagenomics inferred using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) analysis was performed to evaluate differences in the bacterial functional profiles. Clearly, the pathway related to L-glutamate and L-glutamine biosynthesis was more abundant in HC than in patients with VEDOSS. The results are reported in online supplemental figure S3.

### Associations between the GM composition and GI symptoms

Since the GI tract is the most affected internal organ in SSc, we investigated the potential correlation of the altered microbiota taxa and FFAs composition

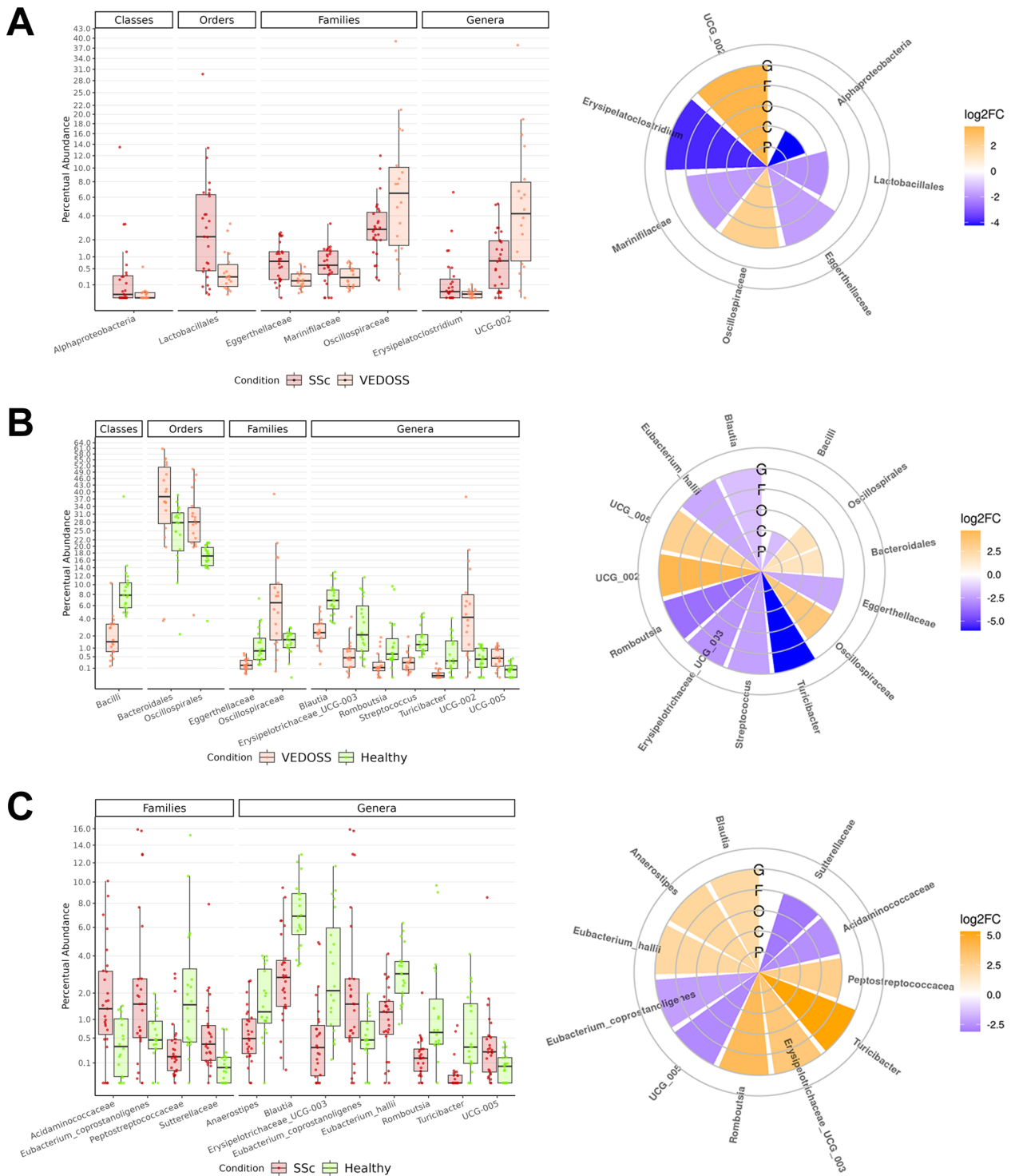


**Figure 2** (A) Representation of sample similarities according to their projections on a space defined by two sparse partial least square discriminant analysis (sPLS-DA) latent components. The ellipses display the 95% CI for each group centroid. (B) Loadings of the genera selected by sPLS-DA for latent component (LC1). A red dashed line is plotted at 50% of the maximum loading value to highlight the most discriminant genera. SSc, systemic sclerosis; VEDOSS, very early diagnosis of systemic sclerosis.

with intestinal health parameters (ie, UCLA GIT 2.0 and its single items as reflux, bloating, diarrhoea, social function, emotional well-being and constipation) in both patients with SSc and VEDOSS. Of note, only in patients with VEDOSS, we observed a positive correlation between faecal *Alphaproteobacteria* and reflux assessed by the UCLA GIT 2.0 questionnaire ( $\rho=0.69$ ,  $p=0.030$ ) (online supplemental figure S4). No correlation was detected for faecal SCFAs and medium-chain fatty acids (MCFAs) as well as for circulating FFAs with GI symptoms.

## DISCUSSION

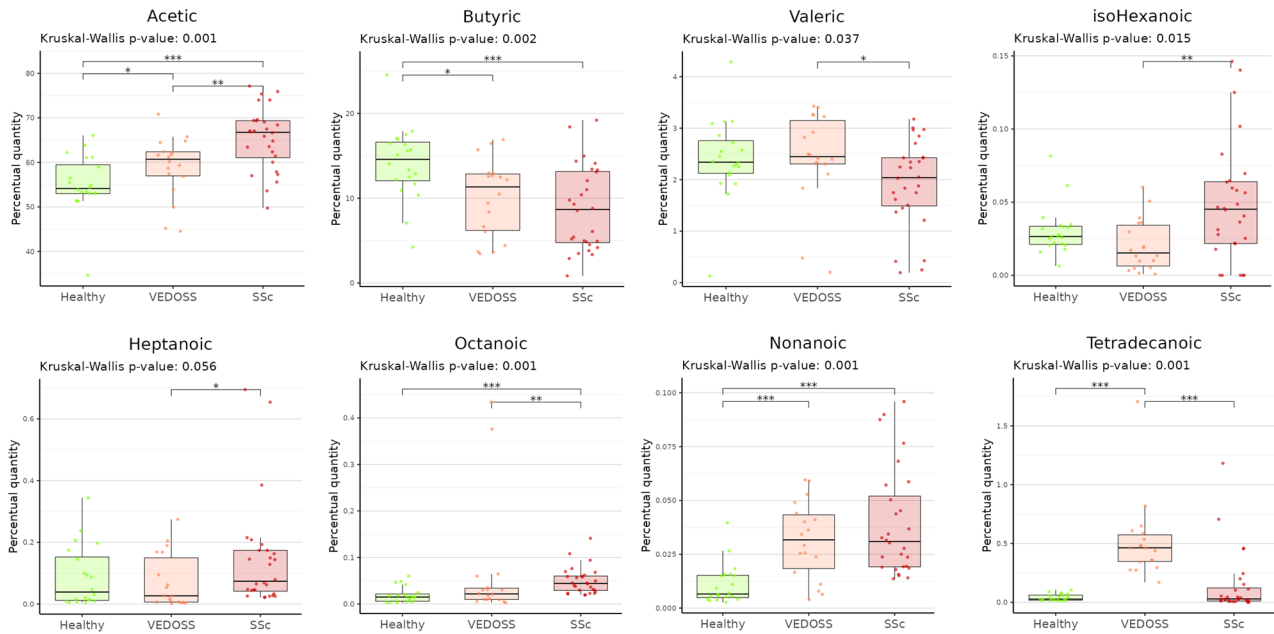
Today, research on patients with VEDOSS attracts a large interest to get insight into the main reasons why some patients progress rapidly while others exhibit stable VEDOSS features for several years before progression to definite SSc. In the last decade, significant efforts have been dedicated to unravelling the molecular features that could serve as early signatures of SSc. In this context, our data clearly show that the GM and its metabolites are significantly modified in patients with definite SSc and in patients with VEDOSS. In fact, we documented that



**Figure 3** Box plot (on the left) and CircoTax plot (on the right) showing the results of taxa-level differential abundance analysis and log<sub>2</sub> fold change (log<sub>2</sub>FC) between (A) patients with SSC and VEDOSS, (B) VEDOSS and HC and (C) SSC and HC in faecal samples. The colours in the box plots distinguish the three sample groups while the colour range in the CircoTax plot displays the FC intensity. Letters in the CircoTax plot indicate the taxonomic depth, in detail. C, class; F, family; G, genus; HC, healthy control; O, order; P, phylum; SSC, systemic sclerosis; VEDOSS, very early diagnosis of systemic sclerosis.

bacterial alpha diversity is significantly different among VEDOSS, SSC and HC groups. The PERMANOVA and the hierarchical clustering further showed a distinct microbial profile corresponding to each sample group and that a different dispersion (interindividual variability) is featured when comparing SSC or VEDOSS with

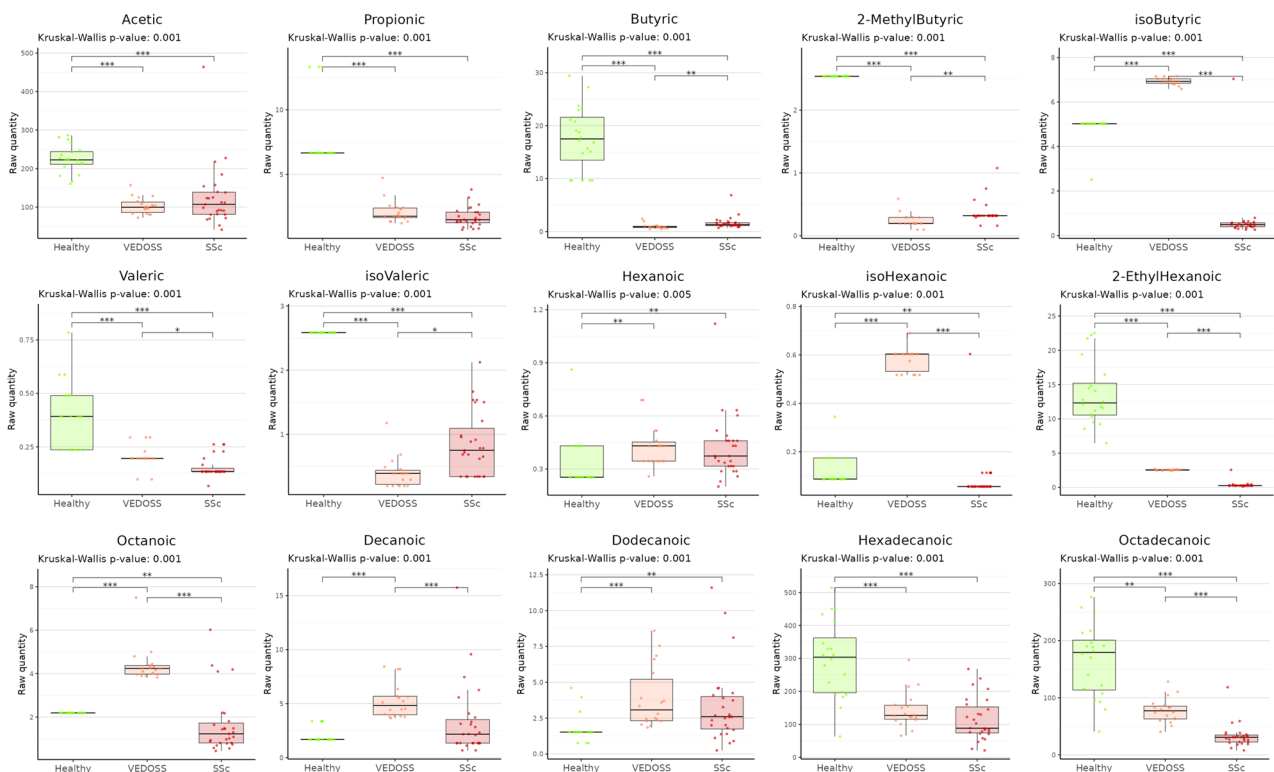
HC, even though the VEDOSS faecal samples appeared much more comparable to SSC than to HC. Although the differences in dispersions may be an informative result, such differences may also lead to biased results in PERMANOVA analysis if the sample groups are not perfectly balanced.<sup>11</sup>



**Figure 4** Bar plots showing the faecal short-chain fatty acid, medium-chain fatty acid and long-chain fatty acid abundances in patients with VEDOSS, patients with SSc and HC. Statistical significance was assessed using the Kruskal-Wallis test with the Dunn correction. Adjusted p values <0.05 were considered statistically significant. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . HC, healthy control; SSc, systemic sclerosis; VEDOSS, very early diagnosis of systemic sclerosis.

As far as we know, we report for the first time that differences in GM between patients with VEDOSS and definite SSc are moderate and that very significant modifications of the GM can be observed between VEDOSS/

SSc and HC. This result highlights a potential pathogenetic pathway that could be independent of the treatments used in the two groups of patients. In fact, while many of the patients with scleroderma were treated with



**Figure 5** Bar plots showing the free fatty acid (FFA) abundances in patients with VEDOSS, patients with SSc and HC. Statistically significant differences were assessed using the Kruskal-Wallis test with the Dunn correction. Adjusted p values <0.05 were considered statistically significant. \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . HC, healthy control; SSc, systemic sclerosis; VEDOSS, very early diagnosis of systemic sclerosis.



immunosuppressive drugs, none of the patients with VEDOSS were. It is well known that drugs can modify the microbiota composition; despite this, the differences in the microbiota between VEDOSS and SSc were moderate, while they were very significant when compared with healthy subjects. Our results suggest that there are many unknown aspects of the link between microbiota composition and disease pathogenesis.

Moreover, SSc and VEDOSS samples shared two genera, *Megasphaera* and *Rikenellaceae\_RC9\_gut\_group*, which were not represented in HC, but notably have been previously related to other rheumatic and autoimmune diseases.<sup>12,13</sup> In detail, *Megasphaera*, a genus belonging to the phylum Firmicutes, was previously reported positively linked with osteoarthritis<sup>12</sup> (and *Rikenellaceae\_RC9\_gut\_group*, belonging to the Rikenellaceae family), and was found enriched in faeces from mice with rheumatoid arthritis (RA).<sup>13</sup>

In line with our and other previous studies,<sup>9,14</sup> the GM analysis revealed that a large part of the sequences collected were classifiable into five phyla: Firmicutes, Bacteroidota, Proteobacteria, Actinobacteria and Verrucomicrobia.

Regarding the microbial composition of VEDOSS samples, we observed a significant decrease in several GM probiotic strains, such as Bacilli class and *Blautia* genus, both of which are known to have beneficial effects on intestinal homeostasis.<sup>15</sup> In particular, *Blautia* prevents inflammation by upregulating intestinal regulatory T cells (Treg) with the production of SCFAs, such as butyric and acetic acids.<sup>16</sup> Additionally, in VEDOSS we observed a reduction of *Turicibacter* that has been linked to an increased expression of proinflammatory tumour necrosis factor and nuclear factor- $\kappa$ B1, as well as to decreased intestinal butyrate abundance.<sup>17</sup> In line with our results, the *Turicibacter* spp decrease has also been reported in patients with SSc.<sup>6</sup> Furthermore, a decrease in butyrate-producing microflora has already been documented in patients with SSc,<sup>6</sup> and here we report for the first time that such a decline is also present in patients with VEDOSS. Of note, a significant reduction in butyrate levels was detected both in faecal and serum samples from patients with VEDOSS, suggesting a poor immunomodulatory effect also at a systemic level. Thus, our data might suggest that butyrate supplementation could be used already in patients with VEDOSS to control gut inflammation. This approach could improve GI symptoms, as suggested by promising results in SSc mouse models.<sup>18</sup> Additionally, we observed an increase in Oscillospiraceae family members in patients with VEDOSS compared with HC. Interestingly, members of the Oscillospiraceae family have been previously associated with various inflammatory diseases.<sup>19</sup>

For the first time, the comparison between patients with SSc and patients with VEDOSS documented an increase in some proinflammatory bacterial flora members, such as *Alphaproteobacteria* class and the Marinifilaceae family. In detail, *Alphaproteobacteria*, belonging to the phylum

Proteobacteria, represents a pathological ‘microbial signature’.<sup>20</sup> In agreement with our results, members of this phylum are over-represented in several diseases with an inflammatory phenotype.<sup>21</sup> In our patients with VEDOSS, a positive correlation between faecal *Alphaproteobacteria* and reflux assessed by the UCLA GIT 2.0 questionnaire was found. Strikingly, an alteration in the *Alphaproteobacteria* composition has been documented in the oesophageal microbiota and correlated with oesophageal adenocarcinoma risk in Barrett’s oesophagus, which is a consequence of longstanding gastro-oesophageal reflux.<sup>22</sup> Consistent with previous results in patients with SSc, we also observed an overabundance of the Lactobacillales order.<sup>23</sup> Such a result was unexpected as there is large evidence suggesting the ability of several Lactobacillales strains to improve gut inflammation.<sup>24</sup> However, in our patients, the increase in Lactobacillales order was mainly represented by the Streptococcaceae family, which was previously found to increase in the gut of patients with RA.<sup>25</sup>

Additionally, it should be remarked that, in patients with SSc, a reduction of GM members showing beneficial properties including the maintenance of gut homeostasis, immunosuppressive and anti-inflammatory functions and SCFA production has been documented.<sup>26</sup> Consistent with a previous study,<sup>6</sup> a significant decrease in faecal and serum butyrate abundances was observed in SSc, along with a rise in *Eubacterium coprostanoligenes*, previously documented in patients with RA,<sup>27</sup> as well as in Acidaminococcaceae and Sutterellaceae families that have proinflammatory properties.<sup>28</sup> In particular, Sutterellaceae were found to increase in active juvenile idiopathic arthritis.<sup>29</sup>

Our results suggest that patients with VEDOSS show early gut dysbiosis presumably due to the loss of a probiotic and protective anti-inflammatory bacterial flora. Such an early proinflammatory environment might result in dysregulated intestinal homeostasis, further triggering abnormal systemic inflammation and immune responses that in turn may favour the proliferation of some dangerous and proinflammatory gut microbes. This condition might exacerbate intestinal dysbiosis and inflammation in SSc. The decline of protective/anti-inflammatory bacteria, mostly butyrate-producing, linked to the simultaneous increase in proinflammatory GM members could boost SSc gut inflammation further evolving into fibrosis and GI dysmotility.

Recent studies highlighted the association between the microbiome and related metabolites, with alterations in lipid metabolism in patients with SSc.<sup>6</sup> As far as SCFAs are concerned, here we demonstrate that faecal butyrate gradually decreases from VEDOSS to SSc. Given the anti-fibrotic effects of butyrate recently reported, we might speculate that the progressive decrease in this SCFA from VEDOSS to SSc could correlate with, and potentially play a role in, disease progression.

Park *et al* in a single study showed how the administration of sodium butyrate in a bleomycin mouse with

dermal and lung fibrosis reduced the expression of the alpha-smooth muscle actin ( $\alpha$ -SMA) in the skin (a myofibroblast marker), reduced collagen deposits and skin thickness, suppressed macrophages proliferation, reduced the expression of profibrotic and proinflammatory genes in fibrotic skin and decreased  $\alpha$ -SMA protein in fibrotic lung tissue.<sup>18</sup>

Moreover, butyrate plays a crucial role in maintaining gut-brain axis integrity, supporting cognitive function, and potentially protecting against neurodegenerative diseases.<sup>30</sup>

Surprisingly, a significant gradual increase of acetate was found in faecal samples from VEDOSS to SSc, while both VEDOSS and SSc serum samples showed a significant decrease compared with HC. This is in line with the known context-specific effect of acetate. Indeed, at the systemic level acetate reduces inflammation in various mouse models, including arthritis, colitis and asthma,<sup>31</sup> while acetic acid infusion directly into the colon strongly induces inflammation as the ulcerative colitis experimental model.<sup>32</sup> In addition, we found a significant decrease in circulating propionic acid, known for its anti-inflammatory function, both in VEDOSS and SSc. Of note, propionate administration was reported to ameliorate inflammatory arthritis by dampening synovial fibroblast pathogenic phenotype.<sup>33</sup>

Among MCFAs, hexanoic (caproic) acid was significantly enriched at the systemic level both in VEDOSS and SSc compared with HC. In the gut, MCFAs were shown to support the differentiation of the T helper 1 (Th1) and Th17 cells and to suppress the Treg development.<sup>34</sup> In particular, hexanoic acid seems to be prototypically endowed with such proinflammatory properties through the activation of p38 MAPK signalling.<sup>35</sup>

Overall, patients with VEDOSS and SSc reported an increased quantity of proinflammatory FFAs, such as MCFAs and LCFAs, but reduced levels of anti-inflammatory SCFAs both in faecal and serum samples. Hence, our results may suggest that the identification of altered lipid metabolic pathways may be crucial to understanding the pathophysiology of GI involvement, particularly the rise in inflammation that then evolves into fibrotic remodelling and GI dysmotility.

Metabolic pathways and shared metabolites are fundamental in the crosstalk among microorganisms or between microorganisms and the host. Interestingly, the PICRUSt2 analysis estimated a decrease in the L-glutamate and L-glutamine biosynthesis bacterial pathway in patients with VEDOSS. It is known that alterations in glutamine metabolism may be involved in the pathogenesis of fibrotic disorders.<sup>36</sup> Indeed, the conversion of glutamine to glutamate is essential for the production of  $\alpha$ -ketoglutarate, a key precursor of type I collagen.<sup>37</sup> Interestingly, high glutamine levels were found in SSc circulation<sup>38</sup> and SSc fibroblasts showed increased expression of glutaminase, that is, the mitochondrial enzyme that catalyses the breakdown of glutamine to form glutamate, supporting the hypothesis that altered glutamine metabolism might

be involved in SSc pathogenesis.<sup>39</sup> Although emerging studies point towards the involvement of bacterial microflora in the regulation of collagen production,<sup>9,40</sup> further studies will be needed to investigate the potential link between microbiome composition/metabolism and the onset/progression of the SSc-related fibrotic process.

We are aware that our study has some limitations. First, considering the small sample size, our findings should be validated in larger cohorts of patients. Furthermore, we should consider that GM sampling has an intrinsic variability and may not reflect the continuous dynamic changes of microbial flora over time or in response to exogenous stimuli. We are also aware that drugs can affect the GM composition, so the different therapeutic approaches between VEDOSS and definite SSc could introduce a bias. Therefore, a larger number of clinically well-characterised samples from patients with VEDOSS and treatment-naïve definite SSc should be enrolled to confirm our preliminary findings.

However, the core novelty of our study lies in the exploration of the GM architecture and function in patients with VEDOSS. Overall, our data documented that the gut community of patients with VEDOSS and SSc is characterised by distinct shifts in GM composition and associated metabolites. The observed shift in microbial taxa at different taxonomic levels suggests that microbiome-based strategies might be useful for early characterisation, staging and treatment of SSc, which needs further extensive validation. Indeed, future investigations will improve our understanding of whether the observed GM alterations contribute to disease characterisation or merely reflect secondary changes caused by intestinal inflammation and/or other disease-associated conditions. In-depth longitudinal studies on microbiome changes and disease evolution will help clarify the role of microbial communities in SSc pathogenesis and its associated GI manifestations.

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