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ORIGINAL CONTRIBUTION

Gut microbiome–short-chain fatty acids interplay in the context of iron deficiency anaemia

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Abstract

Purpose Anaemia is a global health concern, with iron deficiency anaemia (IDA) causing approximately 50% of cases. Affecting mostly the elderly, pregnant and adult women and children, physiopathology of IDA in relation to the gut microbiome is poorly understood. Therefore, the objective of this study is to analyse, in an animal model, the effect of IDA on the gut microbiome along the gastrointestinal tract, as well as to relate intestinal dysbiosis to changes in microbial metabolites such as short chain fatty acids (SCFA).

Methods IDA was experimentally induced through an iron deficient diet for a period of 40 days, with twenty weaned male Wistar rats being randomly divided into control or anaemic groups. Blood samples were collected to control haematological parameters, and so were faecal and intestinal content samples to study gut microbial communities and SCFA, using 16S rRNA sequencing and HPLC–UV respectively.

Results An intestinal dysbiosis was observed as a consequence of IDA, especially towards the distal segments of the gastrointestinal tract and the colon. An increase in SCFA was also noticed during IDA, with the major difference appearing in the colon and correlating with changes in the composition of the gut microbiome. Clostridium_sensu_stricto_1 and Clostridium_sensu_stricto_4 showed the greatest correlation with variations in butyric and propionic concentrations in the colon of anaemic animals.

Conclusions Composition of intestinal microbial communities was affected by the generation of IDA. An enrichment in certain SCFA-producing genera and SCFA concentrations was found in the colon of anaemic animals, suggesting a trade-off mechanism against disease.

Keywords Iron deficiency anaemia · Gut microbiome · Short-chain fatty acids · Intestinal microbial community · Microbial metabolites

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Introduction

Anaemia is a multiethiological syndrome characterised by the reduction of haemoglobin levels below physiological concentrations. Global prevalence of anaemia reached 32.4% in the latest years, with an increased incidence in certain populations such as the elderly, pregnant and adult women or children [1]. Indeed, it has been related to cognitive impairment and dementia, maternal and perinatal mortality, and delayed mental and motor development, respectively.

Approximately, iron deficiency causes 50% of anaemia cases, being, therefore, considered the most prevalent micronutrient deficiency worldwide [2], especially in underdeveloped countries [1]. Moreover, iron deficiency anaemia (IDA) appears as a consequence of clinically relevant disorders, particularly malabsorption diseases (inflammatory bowel disease, celiac disease or *Helicobacter pylori* infections), which are becoming increasingly prevalent [1].

Current treatment consisting of iron supplements shows evident limitations, including side effects such as oxidative stress, or limited intestinal absorption, which cause diarrhoea, constipation or intestinal inflammation in around 30–50% of patients [3, 4]. IDA is often accompanied with inflammation [5], and presumably, cells with high division rates, namely enterocytes, will be affected by shortage of essential micronutrients such as iron. Excess of iron has also been reported to exert proinflammatory effects on the intestinal mucosa, as well as to impair intestinal barrier function [6].

The human intestine harbours a complex microbial community, composed of up to one thousand species [7] and trillions of microbes [8]. Commensal, symbiotic and pathogenic bacteria are natural members of the gut microbiome, playing a key role in host homeostasis. Indeed, essential functions related to pathogen protection, nutrition, metabolism, and immunity are mediated by the intestinal microbial community.

Over the last years, the relationship between the pathogenesis of IDA and the gut microbiome has remained elusive. Intestinal dysbiosis produced by IDA is poorly understood, and so is the influence of the gut microbiome on the generation and recovery of the disease. Iron metabolism involves different oxidation states and solubilities, which requires specific transport systems across the epithelium [9]. The acquisition of iron in the duodenum is regulated via hypoxia-inducible factor (HIF) 2α, a transcriptional factor which targets three key iron absorptive genes: divalent metal transporter 1, duodenal cytochrome b, and ferroportin [9]. Due to its complexity, iron metabolism can be easily disrupted and both endogenous and exogenous factors might interfere with intestinal absorption. Lately, a pivotal role of microbial metabolites has been described in the duodenum, with diaminopropane and reuterin impairing iron uptake in enterocytes through degradation of HIF2α [9]. Other microbial metabolites, such as butyrate, propionate and acetate, from here on short-chain fatty acids (SCFA), have been proposed as mediators of iron absorption [9–11]. Specifically, a decrease in the relative abundance of butyrate-producing genera, such as *Roseburia*, *Coprococcus* and *Butyricicoccus,* has been found in infants suffering IDA, with no changes in faecal butyrate levels [11]. In accordance with Dostal et al. [10], SCFA-producing bacteria, such as *Roseburia spp./Eubacterium rectale* group, were decreased in iron-deficient rats, being propionate and butyrate also diminished in the cecal contents. Lastly, propionate and butyrate have been shown both to increase HIF2α RNAm and to inhibit HIF2α activity at the protein level in vitro. Having not been confirmed these results in vivo, the underlying molecular mechanisms regarding SCFA-HIF2α interaction are yet to be elucidated [9]. Other functions, such as the implications of butyrate in mental development, have been linked to SCFA in the context of IDA [11].

Considering the lack of understanding as far as SCFA and IDA are concerned, and their potential as physiopathological mediators, more in-depth studies are still needed on this field. Comprehensive analysis regarding the effect of IDA on the microbiome along the gastrointestinal tract have not been performed to date, being the existent ones focused on specific segments, mainly duodenum and faeces [9–11]. Moreover, no clear relationships have been established between members of the gut microbiome and changes in SCFA during IDA. Hence, the objective of this study was to provide a holistic view of the impact of IDA on the microbial community in the small (duodenum, jejunum and ileum) and large (cecum and colon) intestine, as well as in faeces, using an animal model of iron deficiency. It, therefore, aimed to set the foundations for the study of SCFA as microbial metabolites with a potential role in the pathogenesis of IDA.

Methods

Animal model

Animal housing, care, handling procedures, and experimental protocols were approved by the Ethics Committee of the University of Granada and the local government Junta de Andalucía (ref 06/06/2019/100) in accordance with European guidelines (Declaration of Helsinki; Directive 2010/63/EU). Animal experiments were performed in the Animal Service of the University of Granada, with controlled sanitary and environmental parameters. Twenty

weaned male Wistar rats, purchased from Charles River Laboratories (France), were used for the study, being diets and deionized water available ad libitum. Animals were housed in groups, using ventilated, thermoregulated cages with controlled temperature (23 ± 2 °C), humidity (60 ± 5%), and a twelve-hour circadian rhythm.

IDA was experimentally induced through an iron-deficient diet for a period of 40 days [12]. Briefly, animals were randomly divided into the control (C, $n = 11$) or anaemic (A, $n = 9$) group, receiving the AIN93G diet (iron = 48.08 mg/kg ± 0.77) [13] or the iron-deficient counterpart (iron = 2.55 mg/kg ± 0.021), respectively. Faecal samples were collected at baseline and at days 20 and 40 using metabolic chambers, and so were blood samples from the caudal vein, using EDTA as anticoagulant to control haematological parameters. At day 40, animals were intraperitoneally anaesthetised using sodium pentobarbital (Richter Pharma AG, Austria) and bled out by cardiac puncture. The small and large intestine were then isolated, being divided into their respective segments: duodenum, jejunum and ileum, and cecum and colon, respectively. Intestinal contents from the small intestine were obtained after washing each segment with sterile saline solution (0.9% sodium chloride). As for the large intestine, solid contents were directly squeezed out. Samples were immediately frozen at − 80 ºC until analysis (Supplementary Fig. 1).

Haematological tests

Red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, leukocytes and platelets were measured using an automated haematology analyser Mythic 22CT (C2 Diagnostics, Grabels, France).

Sample preparation

For SCFA determination, liquid intestinal contents from each segment in the small intestine were centrifuged (6000 rcf, 10 min, 4 ºC), the supernatants recovered and the pellets frozen for subsequent DNA isolation procedures. Obtained supernatants were centrifuged again at maximum speed (10 min, 4 ºC) and filtered (0.22 µm) to eliminate suspended particles. As for the large intestine, 200 mg of intestinal content from each segment were weighted and homogenised in 1.8 mL of saline solution. Suspensions were subsequently centrifuged and filtered (0.22 µm), following the same procedure as in the small intestine. Once prepared, samples were transferred to a vial for HPLC analysis.

SCFA analysis by HPLC

SCFA analysis (acetic, propionic and butyric acids) was carried out by high-performance liquid chromatography (HPLC) using the Acquity UPLC-I Class System (Waters Corporation, USA) with an UV–vis detector set at 210 nm (TUV Detector). Dilutions of SCFA standards (Acetic acid: A6283, Sigma-Aldrich; Propionic acid: 81,910, Sigma-Aldrich; Butyric acid:108,111,000, Acros Organics) were prepared in saline solution at concentrations ranging from 87 to 0.087 mM for acetic acid, 67–0.067 mM for propionic acid and 54.5–0.0545 mM in the case of butyric acid.

A Waters CORTECS™ C18 column (2.1 × 100 mm, 1.6 µm) was used at room temperature, at a flow rate of 0.2 mL/min; water buffer (solvent A)/acetonitrile (solvent B) gradient elution was performed as follows: from 1 to 100% B and down to 1% B, 0–7.5 min. The injected sample volume was 10uL.

DNA isolation, high-throughput sequencing and bioinformatic analysis

DNA isolation was performed using QIAamp DNA Stool Mini Kit (19590, QIAGEN) using 250 mg of faeces or intestinal content, according to the manufacturer's instructions. DNA quality and amount were determined using a spectrophotometer (NanoDrop 2000 UV–Vis,ThermoFisher Scientific, Waltham, MA, USA).

PCR amplification products of the V1–V3 variable regions of the 16S rRNA gene [14] were obtained using fusion universal primers 27F (Illumina adaptors + 5'AGA GTTTGATCMTGGCTCAG3') and 533R (Illumina adaptors + 5'TTACCGCGGCKGCTGGCACG3'), as described by Soriano-Lerma et al. [15]. Amplicon multiplexing and sequencing was carried out with a dual indexing tag-tailed design using 8nt indexes from the Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA). Paired-end sequencing of 16S PCR amplicon libraries was performed using the Illumina MiSeq instrument with v3 kit chemistry (300 + 300 bp). Demultiplexing was performed by Illumina BaseSpace software with default settings. Bioinformatic analysis and quality-filtering were carried out using Mothur software (v 1.43.0, University of Michigan Medical School, Ann Arbor, MI, USA), following the standard Miseq SOP. Chimeric reads were identified and excluded using Chimera UCHIME. Redundant, non-chimeric FASTA files were taxonomically classified using Silva v132 database. Abundance was expressed as a percentage with respect to the total number of sequences in each sample. Genera with total abundance higher than 0.01% were considered for statistical analysis.

Microbial functional analysis was carried out using Phylogenetic Investigation of Communities by Reconstruction

of Unobserved States (PICRUSt) on high-throughput 16S rRNA gene sequencing data [16]. KEGG microbial pathways classified at level 3 were statistically analysed as described by Díaz-Faes et al. [17].

Statistical analysis

After assessing normality in the variables with Shapiro–Wilk test, non-parametric Mann–Whitney *U* test was carried out using SPSS v.20.0 (SPSS Inc., Chicago, IL, USA). Principal coordinate analysis (PCoA) based on Bray Curtis distances was implemented in PRIMERe Permanova + (PRIMER-E Ltd, Plymouth, UK). Heatmaps were performed using R software (R Foundation for Statistical Computing Vienna 2012), and so were Pearson correlations. Clustering procedures based on Bray Curtis distances were performed in Past 4.02 [18]; dendrograms were drawn using MEGA7 [19]. Linear discriminant analysis Effect size (LEfSe) was carried out using Python 3.7.6, with default parameters [20]. Correlation network diagrams were represented using Gephi 0.9.2, with a cut-off value of − 0.5 and 0.5 and Fruchterman Reingold algorithm. Multivariant correlation analysis was implemented via Statgraphics Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA).

For all statistical analyses, a p value < 0.05 was considered as significant.

Results

Determination of haematological parameters confirmed the induction of IDA

A decrease in the number of red blood cells, haemoglobin concentration, haematocrite and mean corpuscular volume by day 20 (d20) and day 40 (d40) confirmed that IDA had been correctly induced (Table 1).

IDA is associated with intestinal dysbiosis along the gastrointestinal tract, especially in the large intestine

Sequencing of 16S rRNA gene amplicons from intestinal content samples and faeces resulted in a total of 1.860.784 sequences after bioinformatic processing. Principal Coordinate Analysis (PCoA) considering bacterial relative abundances at the genus level in faeces collected at baseline, d20 and d40 revealed differences between control and anaemic animals over time. PCoA accounted for 57.2% of bacterial variation considering the first two principal coordinates (Supplementary Fig. 2). Samples were separated along the Y axis according to the duration of the deficiency (Supplementary Fig. 2a) and along the X axis according to their iron status (Supplementary Fig. 2b). Specifically, control and anaemic baseline samples grouped together, while d20 and d40 samples were segregated into anaemic and control groups. D20 and d40 samples clustered separately from baseline samples (Supplementary Fig. 2a and 2b).

Genera-based PCoA of intestinal contents showed a clear distinction between samples belonging to the small and large intestine along the X axis (Supplementary Fig. 3a), regardless of their iron status (Supplementary Fig. 3b) and explaining 50.8% of bacterial variation. These results were further confirmed by Bray Curtis distance-based clustering methods (Supplementary Fig. 4), where samples were also divided into two clusters according to the major anatomical region.

As microbial differences might be overlooked when analysing samples with great dissimilarity, genera-based PCoA was performed separately in the small and large intestine to analyse disease-driven dysbiosis. In the small intestine, duodenum, jejunum and ileum were separated along the X axis, explaining 47.3% of bacterial variation (Supplementary Fig. 5a). Duodenum showed an intermediate microbial composition, while jejunum and ileum samples clustered separately (Supplementary Fig. 5a). Anaemic and control

Table 1 Haematological parameters during the development of iron deficiency anaemia (day 20 and day 40)

	Control group		Anaemic group	
	d20	d40	d20	d40
Red blood cells (10^6/µL)	6.56 ± 0.56	8.12 ± 0.45	3.07* ± 0.65	2.15* ± 0.51
Haemoglobin (g/dL)	13.27 ± 3.06	16.05 ± 0.89	6.55* ± 1.17	4.16* ± 0.52
Haematocrit (%)	43.32 ± 4.65	47.89 ± 2.75	17.98* ± 2.71	21.34* ± 9.27
Mean corpuscular volume (fL)	65.97 ± 2.29	59.14 ± 1.54	59.15* ± 4.95	40.22* ± 0.65
Mean corpuscular haemoglobin (pg)	20.32 ± 4.84	19.82 ± 0.67	22.14 ± 5.22	20.60 ± 6.27
Mean corpuscular haemoglobin concentration(g/dL)	30.87 ± 7.58	33.49 ± 0.75	36.99* ± 6.73	25.95 ± 17.11
Leukocytes (10^3/µl)	10.47 ± 3.06	13.23 ± 2.25	13.61* ± 3.03	10.51* ± 1.48
Platelets (10^3/µL)	901.27 ± 407.42	889.64 ± 445.55	Over range	Over range

Means and standard deviations are shown for each group, time and parameter. (*) represents statistical differences ($p < 0.05$) between control and anaemic group at each respective time (d20 and d40)

samples also showed different microbial composition, as they were separated along the Y axis (17.5% of bacterial variation) (Supplementary Fig. 5b). Same results were obtained for the large intestine, with cecum and colon samples being separated along the Y axis (17.3% of bacterial variation) (Supplementary Fig. 6a), and anaemic and control animals along de X axis (37.5% of bacterial variation) (Supplementary Fig. 6b). It is worth highlighting that a higher percentage of microbial variation was explained by the segregation of intestinal content samples into anaemic and control groups in the large intestine compared to the small intestine (37.5 vs. 17.5%, see above).

Relative abundances considering the fifty most abundant genera were represented in a heatmap in each experimental group and intestinal segment (Fig. 1). Again, a clear distinction of samples belonging to the small and large intestine was shown. Differences between control and anaemic animals were less apparent in the small intestine considering the

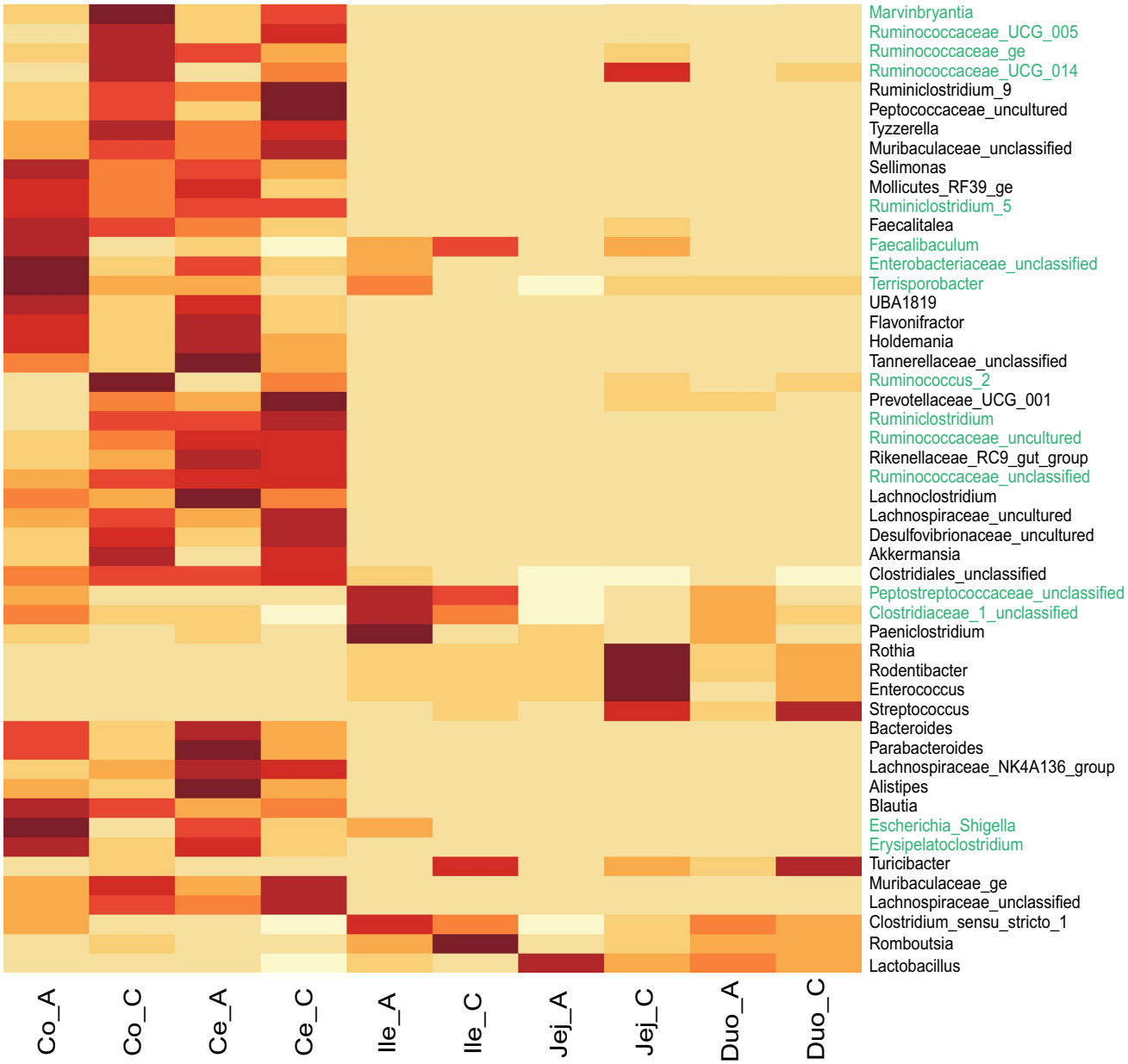

Fig. 1 Heatmap representing mean relative abundance of the fifty most abundant genera in each intestinal segment and experimental group. A yellow-to-red scale has been used to score abundances, with red colours corresponding to greater values. Values have been scaled in the row direction to illustrate differences in microbial genera between experimental groups. Highlighted in green are bacterial taxa with clear differences between colon and cecum, therefore emphasizing a greater dysbiosis in the colon. Co: Colon, Ce: Cecum, Ile: Ileum, Jej: jejunum, Duo: duodenum; _A and _C stand for anaemic and control group. R software was used to represent the plot

majority of microbial genera, with dysbiosis being greater in the distal part of the gastrointestinal tract. Particularly, the colon showed more drastic changes in the relative abundance of certain genera (Fig. 1, highlighted in green, see legend).

During IDA, an enrichment in SCFA-related metabolic pathways and certain SCFA-producing bacteria is noticed towards the distal part of the gastrointestinal tract

Linear discriminant analysis Effect size (LEfSe) was next applied to identify differentially distributed bacteria for control and anaemic animals in the small and large intestine. As stated before, a lower number of bacterial genera with statistical differences between experimental groups was found in the small intestine (Fig. 2) compared to the large intestine, showing a greater dysbiosis in the latter (Fig. 3).

The majority of microbial genera with statistical differences between the control and anaemic groups in the small intestine matched those altered in the large intestine (Figs. 2, 3). However, new enriched genera appeared in the large intestine of anaemic animals, such as *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_4*, *Flavonifractor*, *Intestinimonas* or *UBA1819* (Fig. 3). Members from the *Lachnospiraceae* and *Ruminococcaceae* family were especially abundant in control animals both in the

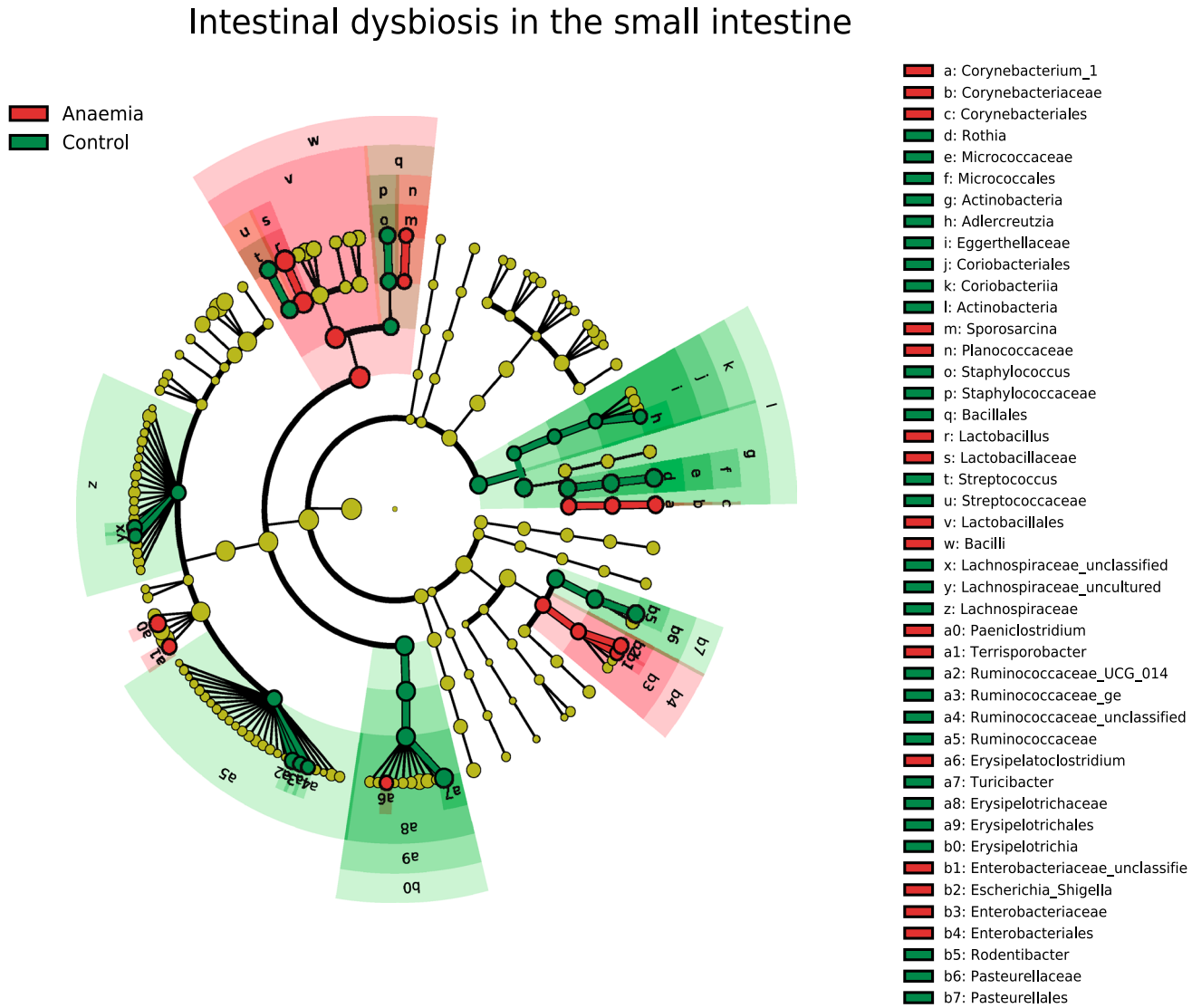

Fig. 2 Linear discriminant analysis Effect size (LEfSe): cladogram for differentially distributed taxa ($p < 0.05$, LDA > 2) between control and anaemic groups in the small intestine. Taxonomic features are represented in a hierarchical structure, with higher phylotypes oriented towards the inner part of the plot. Taxa showing significant differences are coloured according to their greatest abundance in either the control or anaemic group (red for anaemia, green for control, yellow for non-significant). Python 3.7.6 was used in the implementation of the statistical analysis

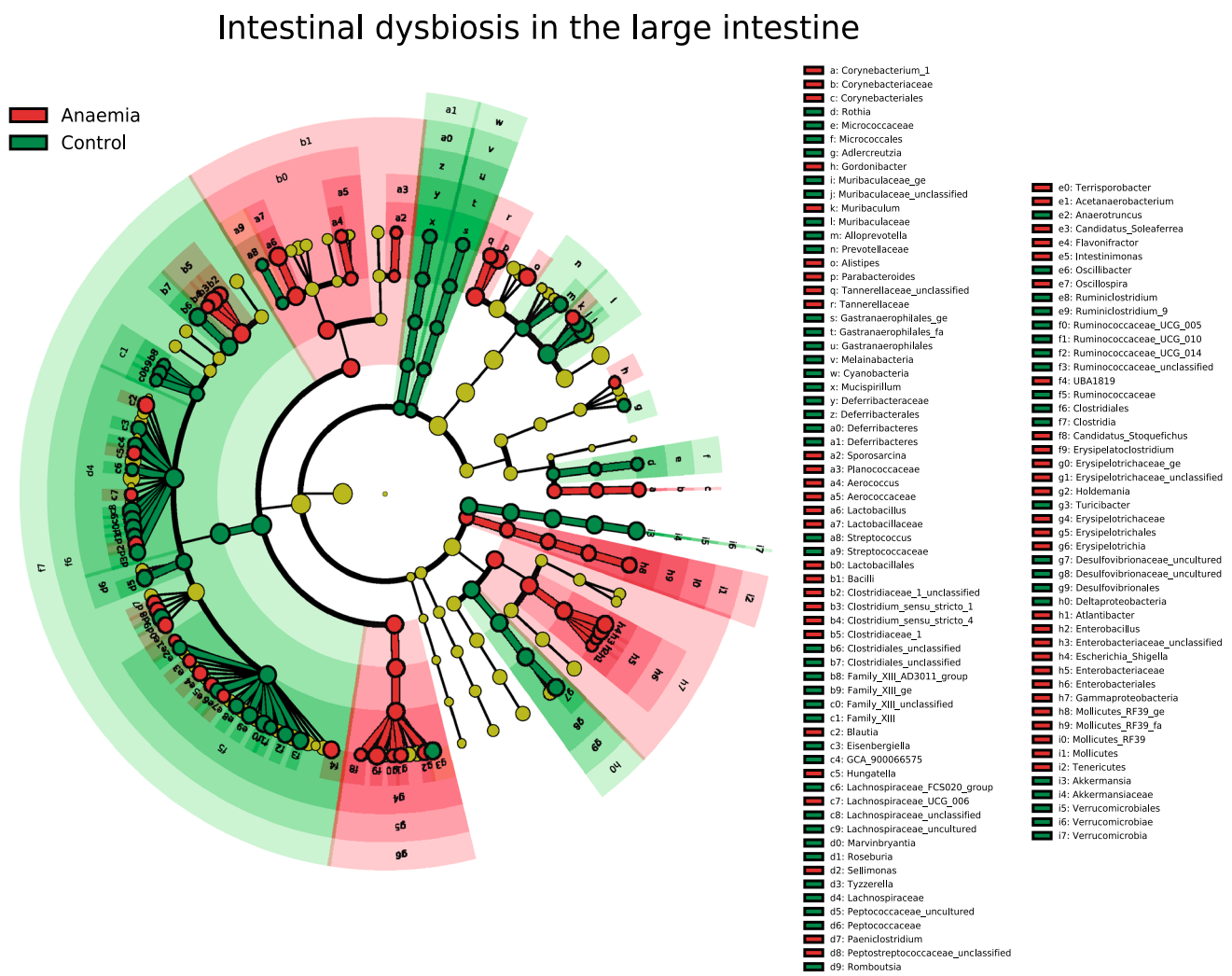

Fig. 3 Linear discriminant analysis Effect size (LEfSe): cladogram for differentially distributed taxa ($p < 0.05$, LDA>2) between control and anaemic groups in the large intestine. Taxonomic features are represented in a hierarchical structure, with higher phylotypes oriented towards the inner part of the plot. Taxa showing significant differences are coloured according to their greatest abundance in either the control or anaemic group (red for anaemia, green for control, yellow for non-significant). Python 3.7.6 was used in the implementation of the statistical analysis

small and large intestine, with specific genera differing between both sites (Figs. 2, 3). *Romboutsia* and *Roseburia* were, however, only enriched in the large intestine in control animals.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was employed to infer microbial functional traits. LEfSe was implemented on microbial functional data to identify which metabolic pathways were more prevalent during IDA along the gastrointestinal tract. In agreement with a less intense dysbiosis, no metabolic pathways were enriched in the small intestine of anaemic animals, while 15 pathways did in the case of the large intestine (Table 2). The top five (phosphotransferase system, butyrate metabolism, fatty acid metabolism, carbon fixation pathways and propionate metabolism), which showed the highest enrichment, were related to acetic, propionic, and butyric acid metabolism.

An increase in SCFA concentration parallels changes in the gut microbiome during IDA

SCFA were determined in intestinal contents from each segment along the gastrointestinal tract, showing an increase in anaemic animals in relation to control ones, especially in distal segments (Fig. 4). Specifically, propionic acid significantly increased in the jejunum of anaemic rats compared to control ones, while the colon showed significant differences in all three fatty acids (Fig. 4). However, faeces from control and anaemic rats showed no statistical differences for any fatty acid at d40 (Supplementary Fig. 7).

Table 2 KEGG microbial pathways differentially enriched in IDA

KEGG microbial pathways at level 3	LDA (log10)	*p* value
Bacterial phosphotransferase system	2.6403	0.0002
Butyrate metabolism	2.4731	0.0063
Fatty acid metabolism	2.4437	0.0021
Carbon fixation pathways	2.4308	0.0260
Propionate metabolism	2.4257	0.0007
Valine, leucine and isoleucine degradation	2.4242	0.0088
Replication, recombination and repair proteins	2.3643	0.0001
Benzoate degradation	2.3106	0.0008
Geraniol degradation	2.2296	0.0074
Lysine degradation	2.1943	0.0037
Beta-alanine metabolism	2.1381	0.0023
Tryptophan metabolism	2.1130	0.0194
Limonene and pinene degradation	2.0609	0.0048
Aminobenzoate degradation	2.0199	0.0209
Fatty acid biosynthesis	2.0039	0.0001

Effect size-related parameter LDA and *p* values are included in subsequent columns for each pathway

An increase in butyric, propionic and acetic acids was also noticed towards the distal parts compared to proximal ones, both in control and anaemic animals (d40) (Fig. 4). In control rats, the highest levels of butyric acid were found in the cecum; propionic acid significantly increased along the jejunum, ileum and cecum, whereas acetic acid showed its greatest concentration in the cecum, with a significant decrease in the jejunum and colon (Fig. 4). In anaemic rats, butyric acid progressively increased from the jejunum to the colon, where it reached the highest level. Propionic acid showed the lowest concentration in the jejunum and the highest in the colon, while acetic acid showed its greatest concentration in the cecum (Fig. 4).

Specific bacterial genera are correlated with SCFA concentrations in the colon of control and anaemic animals

Network diagrams illustrating positive and negative correlations between SCFA and bacterial genera were elaborated, considering the colonic region of anaemic and control animals (Figs. 5, 6). Correlations were drawn as edges, using cut-off values of − 0.5 and 0.5 and a red–blue scale according to their weight; positive and negative values were represented in red and blue, respectively. SCFA and bacterial genera were represented as nodes, which were also coloured according to the number of connections in a similar scale, with red colours corresponding to greater connectivity. Node and label size were adjusted so that highly connected nodes showed bigger size and labels. A greater number of nodes and edges were found in the colon of anaemic animals, with 61 nodes and 77 edges (Fig. 5), as opposed to 33 and 41 found in control rats (Fig. 6). Both in the case of anaemic and control animals, parallel correlations could be observed between butyric and propionic acids, with acetic acid showing the opposite tendency. Such is the case of *Ruminococcaceae_ge* in the colon of anaemic animals, which showed negative correlations with butyric and propionic acids while being positively correlated to acetic acid (Fig. 5). *Lachnospiraceae_NK4A136_group* and *Romboutsia* also followed the same pattern in the colon of control animals (Fig. 6).

Aiming to identify which bacterial genera mainly contributed to variations in butyric, propionic and acetic acids, multivariant regression analysis was next performed for each SCFA in the colon of both experimental groups. *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_4* explained 83.77% of butyric acid variation in anaemic animals, while *Clostridium_sensu_stricto_1* was responsible for 54.6% of changes in propionic acid. Lastly, contribution of *Akkermansia* and *Christensenellaceae_R-7_group* to acetic acid variation was worth 83.68% (Supplementary Fig. 8a). Different microbial genera were identified as main contributors to SCFA variation in the colonic region of control animals. Specifically, *Rhodospirillales_uncultured_ge*, *Paeniclostridium*, *Romboutsia*, *Ruminococcaceae_UCG_005* and *Pasteurellaceae_unclassified* explained 98.14% of butyric acid variation. Lastly, *Lachnospiraceae_NK4A136_group* and *Muribaculaceae_ge* represented 59.37% and 73.42% of changes in propionic and acetic acid, respectively (Supplementary Fig. 8b).

Discussion

Although required in small proportions, iron is an essential nutrient for most organisms. It contributes to a considerable number of biological processes including oxygen transport, DNA synthesis, energy production and cell proliferation [2]. A tightly regulated system operates to maintain iron levels within normal ranges, with intestinal absorption representing a key regulatory step [21].

A unique host–microbiome crosstalk takes place along the gastrointestinal tract through several mechanisms, including the production of microbial metabolites such as SCFA [22]. In this study, an intestinal dysbiosis was triggered in response to IDA, mainly towards the lower gastrointestinal tract and characterised by an enrichment in metabolic pathways related to SCFA and specific SCFA-producing bacteria.

First, genera-based Principal Coordinate Analysis (PCoA) of faeces collected at baseline, d20 and d40 showed a differentiation of anaemic and control animals at d20 and d40, but not at baseline (Supplementary Fig. 2). Therefore,

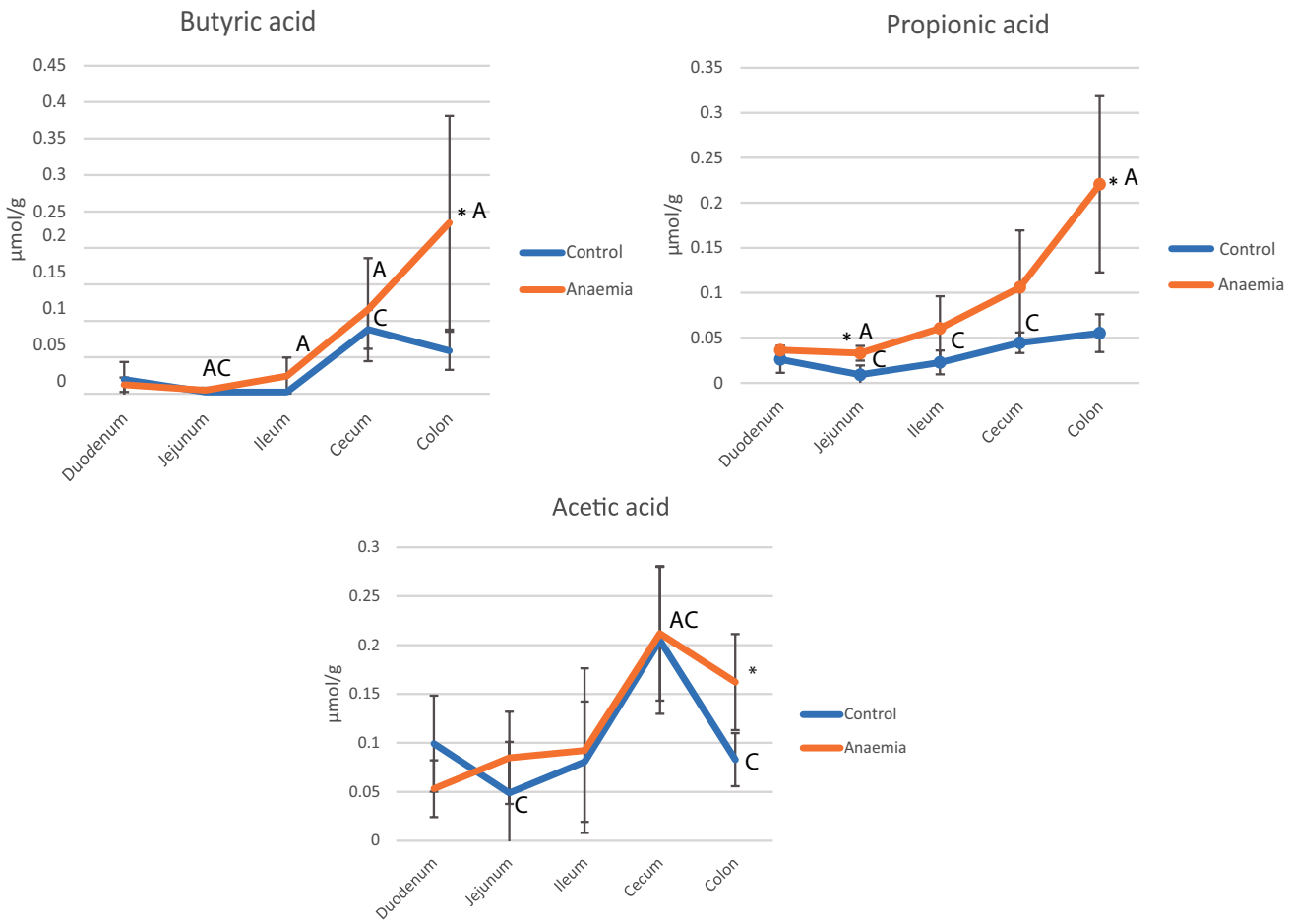

Fig. 4 Changes in butyric, propionic and acetic acids concentrations (µmol/g) in intestinal contents along the digestive tract. Means are shown for each experimental group (control and anaemic), and standard deviations are illustrated by error bars. (*) represents statistical differences ($p < 0.05$) between control and anaemic groups in that intestinal region. C and A represents statistical differences ($p < 0.05$) between the intestinal region in question and the one immediately before in control and anaemic groups respectively

intestinal dysbiosis at the genus level appeared at d20 and was maintained until d40. PCoA considering intestinal contents revealed that the main differentiating feature in the microbiome structure was the major anatomic site, since samples belonging to the small and large intestine were clearly divided irrespective of iron status (Supplementary Fig. 3a and 3b, Supplementary Fig. 4). Differences in the composition of gut microbial communities in the small intestine became more evident towards the most distal parts; duodenum showed an intermediate microbial composition while jejunum and ileum clustered differentially (Supplementary Fig. 5a). A greater percentage of bacterial variation is explained by segregation of samples into intestinal segments belonging to the small intestine (47.3%) compared to the separation observed by iron status (17.5%) (Supplementary Fig. 5b). In contrast, a sectional division could also be observed in the large intestine but contributed to a lesser extent to bacterial variation (17.3%) when compared to disease-mediated effects (37.5%) (Supplementary Fig. 6a and 6b). Therefore, changes in microbiome structure in the large intestine are mainly attributed to the influence of iron deficiency. Particularly, a greater dysbiosis was noticed in the colon (Fig. 1), where intestinal bacteria are more diverse and resilient [23, 24].

Specific bacterial genera showed differences in their relative abundance in response to iron deficiency, both in the small (Fig. 2) and large intestine (Fig. 3). Gut dysbiosis in the upper gastrointestinal tract resembled that of the lower tract, although a higher number of microbial genera was altered in the latter. In accordance with Dostal et al. [10] and Mcclorry et al. [11], *Roseburia* genus, a classic butyrate producer, was depleted in the large intestine of anaemic animals, while other SCFA-producing bacteria, such as *Clostridium* members [25], were increased (Fig. 3). Conversely, the small and large intestine in control rats were characterised by members of *Lachnospiraceae* and *Ruminococcaceae* families. *Lactobacillus* was also enriched in the small and large intestine in anaemic animals, as already

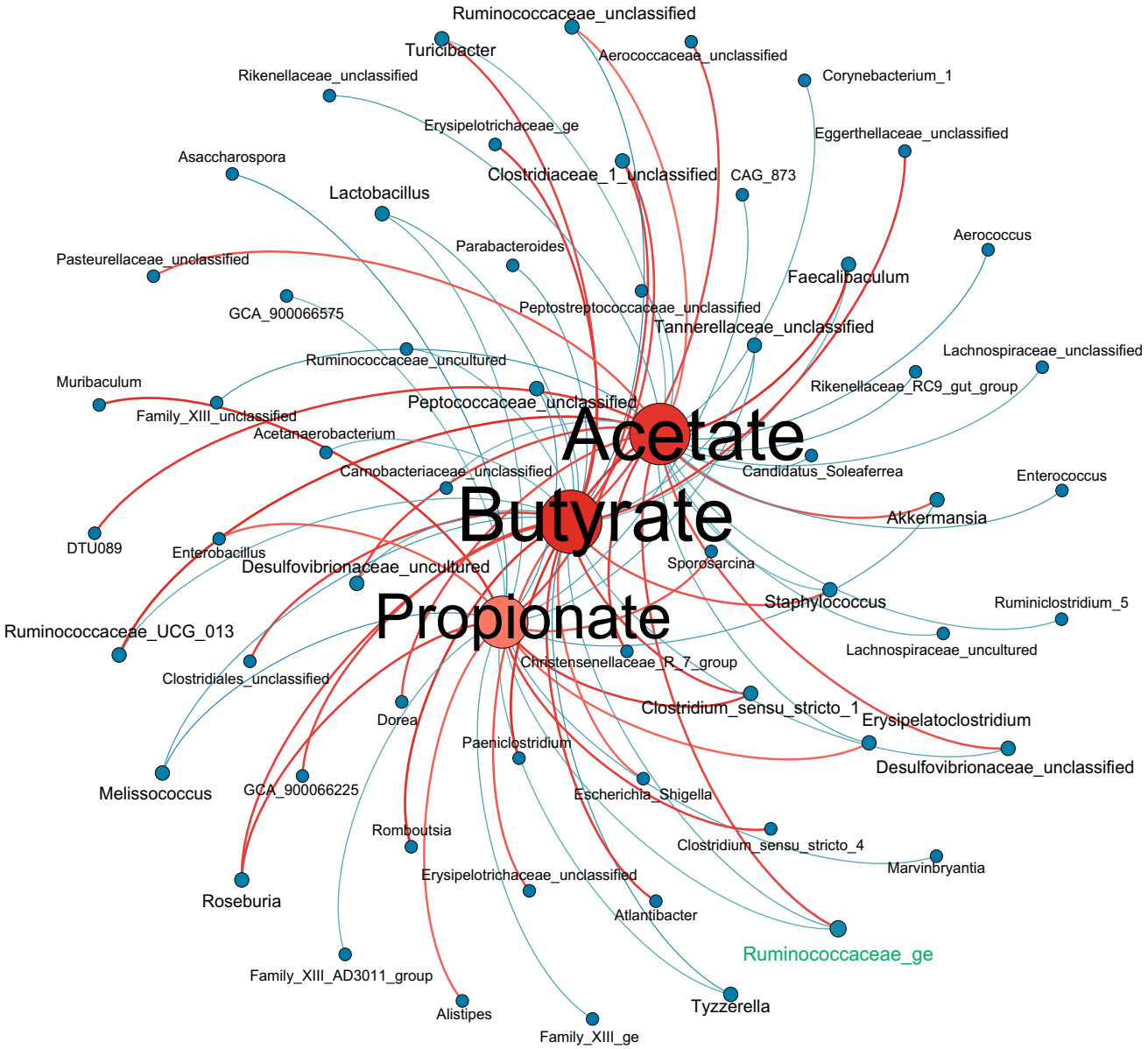

Fig. 5 Correlation network between short chain fatty acids (SCFA) and microbial community members at the genus level in the colon of the anaemic group. Correlations were drawn as edges, using cutoff values of –0.5 and 0.5 and a red-blue scale according to their weight; positive and negative values are represented in red and blue respectively. SCFA and bacterial genera are represented as nodes, using a similar colour scale according to their connectivity. Node and label size were adjusted so that highly connected nodes showed bigger size and labels. Highlighted in green are microbial genera mentioned in the text. Gephi 0.9.2 was used to represent the plot

described by Das et al. [9]; a pivotal role of reuterin, produced by *Lactobacillus reuterii*, was described in mice suffering IDA, impairing iron absorption. Iron-limiting conditions can affect the growth of certain bacterial genera both in the gut and other biological niches [26]. Certain genera, such as *Roseburia* or *Bacteroides*, heavily depend on iron as a cofactor or substrate for their metabolic reactions [10]. As a butyrate producer, *Roseburia genus* has been shown to affect colonic motility and to exert anti-inflammatory properties and immune regulatory effects [27]. On the other hand, *Bacteroides* has been associated with enhanced glucose metabolism and lipolysis, playing a preventive role on the development of obesity and diabetes [28]. A depletion in *Roseburia* genus was noticed in the large intestine of anaemic animals, while no changes in *Bacteroides* were found (Fig. 3).

Functional analysis of 16S rRNA sequencing data revealed that, during IDA, the five most enriched metabolic pathways in the large intestine were related to SCFA metabolism (Table 2). Bacterial phosphotransferase systems

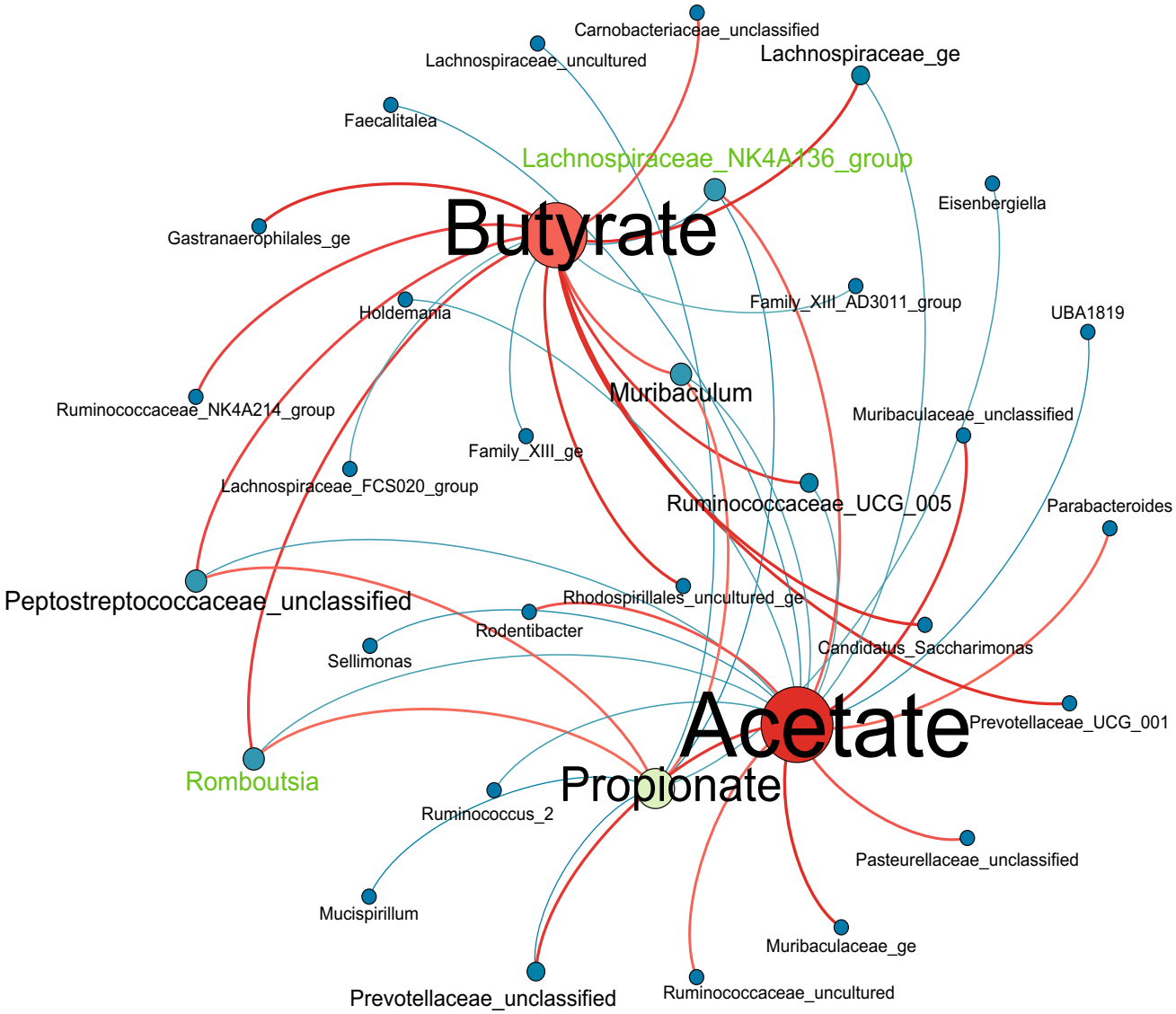

Fig. 6 Correlation network between short chain fatty acids (SCFA) and microbial community members at the genus level in the colon of the control group. Correlations were drawn as edges, using cutoff values of –0.5 and 0.5 and a red-blue scale according to their weight; positive and negative values are represented in red and blue respectively. SCFA and bacterial genera are represented as nodes, using a similar colour scale according to their connectivity. Node and label size were adjusted so that highly connected nodes showed bigger size and labels. Highlighted in green are microbial genera mentioned in the text. Gephi 0.9.2 was used to represent the plot

contribute to carbohydrate uptake [29], the main source for SCFA production [30]. Similarly, carbon fixation pathways are also related to acetate, propionate and butyrate synthesis [31, 32]. Consequently, variations in SCFA concentrations paralleled changes in the intestinal microbial community. SCFA concentration was higher in anaemic compared to control animals, especially towards the most distal segments of the digestive tract such as the colon, while no changes were observed in the upper gastrointestinal tract or faeces (Fig. 4, Supplementary Fig. 7). Although absorption or use of SCFA by enterocytes could be an influential factor affecting compartment variations in anaemia, the observed enrichment in SCFA-related metabolic pathways and certain SCFA-producing bacteria towards the lower gastrointestinal tract suggest that the main contributor to SCFA variations in these segments is an increased production of these metabolites by the gut microbiome during IDA. To the best of our knowledge, this is the first study showing an increase in SCFA in anaemia, as opposed to what had been previously reported [9–11]. No changes or a decrease in SCFA had been shown in similar studies, although iron deficiency had been induced for a shorter period of time and faecal samples were used instead. Volatility of this type of metabolites could belie slight changes if biological samples

are not rapidly gathered and processed. Unlike intestinal content samples, which were immediately gathered during the animal sacrifice, faeces stayed in the metabolic chambers since their emission until their collection, a few hours later. Therefore, volatilization could partly explain the absence of significance in faecal SCFA between the anaemic and control groups in our study and others [9, 11].

An increase in the concentration of butyric, propionic and acetic acids was also noticed both in control and anaemic animals from the most proximal to the most distal segments of the digestive tract (Fig. 4), as already described by Dongyao et al. [24]. The fermentative capacities in the upper and lower gastrointestinal tract yield different end products, lactate and butyrate being the major metabolites in each respective region.

Correlations between bacterial genera and SCFA in the colonic region of control and anaemic animals were illustrated using network diagrams. Considering the same cut-off values, a higher number of nodes and edges was found in the iron-deficient group (Figs. 5, 6). Therefore, contribution of microbial genera to variations in SCFA was greater in the case of anaemic compared to the control condition. Parallel correlations were observed in the case of butyric and propionic acids, whereas acetic acid showed an inverse tendency. Cross-feeding mechanisms involving acetate as a substrate for the production of butyrate could support this finding [30].

Statistically significant correlations were subsequently analysed by multivariant correlation analysis to discern which bacterial genera mostly contributed to variations in SCFA concentrations. Interestingly, in the colon of anaemic animals, *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_4* explained over 80% of changes in butyric acid, while the former represented over 50% of variations in propionic acid (Supplementary Fig. 8a). Different microbial genera were associated with the production of SCFA in the colon of control animals, such as members of *Lachnospiraceae* and *Ruminococcaceae* families.

SCFA are considered the major products derived from the gut microbiome fermentative activity in the large intestine [30]. Although acetic acid can partly be synthetized by eukaryotic cells, bacterial metabolic activity in the gut is mainly responsible for propionate and butyrate levels. Not only do SCFA serve beneficial purposes at the local and systemic level, but their depletion has also been linked to several alterations such as insulin resistance [33], colon cancer [34], inflammatory bowel disease [undefined] or metabolic syndrome [36]. Among their beneficial functions, it is worth highlighting their positive influence on intestinal health. Butyrate constitutes the main source of energy for colonocytes; it is locally consumed and barely reaches the bloodstream. It exerts protective effects on the intestinal barrier through the modulation of gene expression, including tight junction proteins, antimicrobial peptides or components of the mucus layer [37]. Intestinal anti-inflammatory effects have also been reported in the case of butyrate through the generation of T regulatory cells and secretion of IL-18 and IL-10 [30], allowing an increased immune tolerance towards commensal bacteria. In normal colonocytes, butyrate has also been shown to enhance self-renewal of stem cells, favouring intestinal health [30]. Unlike butyrate, propionic and acetic acids have a more systemic effect. They are mainly absorbed in the colon, reaching the liver through the portal vein; acetate shows the highest systemic availability, reaching peripheral tissues at considerable concentrations [38]. Propionate has been related to glucose homeostasis in the liver [38], while acetate has been associated with appetite regulation [30]. All things considered, such increase in SCFA during the development of IDA suggests that trade-off mechanisms are taking place to compensate physiological alterations during the disease, namely alterations in the intestinal tract, IDA-derived insulin resistance [39] or appetite reduction. Unlike what has been previously described [9], loss of iron-dependent bacterial genera, such as *Roseburia*, could also support this hypothesis, since reducing microbial iron competitors can enhance iron bioavailability for the host.

The implications of SCFA in the host–microbiome crosstalk during iron deficiency anaemia still remain to be investigated. In this study, an intestinal dysbiosis was produced as a result of IDA, especially in the most distal segments of the gastrointestinal tract. Such dysbiosis was characterised by an enrichment in metabolic pathways related to SCFA metabolism and certain SCFA-producing genera in the large intestine, including *Clostridium_sensu_stricto_1* and *Clostridium_sensu_stricto_4*. Being one of the major butyrate producers in the gut [25], these microbial genera, which showed the greatest contribution to the variation in butyric and propionic acid in the colon of anaemic animals, represent an interesting taxa to study during iron deficiency anaemia. More effort should be put into studying SCFA as chemical mediators in the context of IDA, confirming a beneficial role during the development of the disease in each type of tissue according to their biological gradient. The use of SCFA or SCFA-producing bacteria during iron deficiency anaemia could be of scientific interest to tackle local and systemic disease-derived alterations and to enhance disease management.

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Authors' contributions MS, JAGS and ILA developed the original idea, contributed to the design of the study and critically revised the manuscript. A.S.L, M.J.M.A and M.G.B participated in the in vivo model of iron deficiency. VPC, VSM, ALR and MOG did the laboratory analysis and produced the experimental data. A.S.L performed the bioinformatic and statistical analysis and wrote the original draft. MS, JAGS and ILA equally contributed and jointly supervised this work.

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Data availability All datasets supporting the conclusions of this article will be made available in the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) upon request. Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Experimental procedures described in this study have been performed in accordance with European guidelines (Declaration of Helsinki; Directive 2010/63/EU) and approved by the Ethics Committee of the University of Granada and the local government Junta de Andalucía (ref 06/06/2019/100).

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