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ABSTRACT

Background: Iron deficiency anaemia (IDA) is closely associated with the gut microbiome, as microbial composition influences iron bioavailability. Small intestinal bacterial overgrowth (SIBO), a form of dysbiosis, may interfere with anaemia treatment, yet it has not been investigated as a direct consequence of iron deficiency. This study aimed to characterize the small intestinal dysbiosis linked to IDA and to identify microbial patterns indicative of SIBO.

Methods: an animal model of IDA was employed to analyse the microbiome of the small intestine, focusing on community structure and functional properties. Anaemia was confirmed using haematological and biochemical markers. Microbiome profiling was conducted through 16S rRNA gene sequencing. In addition, bacterial load was quantified by 16S rRNA qPCR.

Results: qPCR confirmed a significantly elevated bacterial load across all three regions of the small intestine during anaemia, reaching levels compatible with SIBO. A progressive increase was observed in alpha diversity from the jejunum to the ileum during IDA. Taxonomic analysis revealed enrichment of fermentative and colonic-associated species, including *Clostridium, Escherichia-Shigella* and *Lactobacillus*. Lastly, functional predictions indicated increased activity in pathways related to carbohydrate fermentation and gas production—metabolic signatures typically linked to SIBO.

Conclusion: Iron deficiency was found to induce marked taxonomic and functional alterations in the small intestinal microbiome, especially in distal regions, accompanied by an increased bacterial load. These findings support the concept that iron deficiency promotes microbial shifts characteristic of SIBO, suggesting that iron deficiency may act as a predisposing factor in its pathogenesis.

Keywords: Iron deficiency anaemia, iron deficiency, Small intestine microbiome, SIBO, Microbial functionality

List of abbreviations

ASV	Amplicon sequence variant
GMM	Gut microbial modules
IDA	Iron deficiency anaemia
Hb	Haemoglobin
HCT	Hematocrit
KO	KEGG orthologs
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
PCoA	Principal coordinate analysis
PLT	Platelets
qPCR	quantitative PCR
RBC	Red blood cells
RDW	Red cell distribution width
rRNA	ribosomal RNA
SCFA	Short chain fatty acids
SIBO	Small intestine bacterial overgrowth
Sobs	Observed species
TIBC	Total iron binding capacity

WBC	White blood cell	

1. Background

The World Health Organisation has recognised iron deficiency anaemia (IDA) as the most common nutritional deficiency in the world, with 30% of the population being affected (1). Susceptible subjects for IDA include growing children, the elderly as well as pregnant and non-pregnant women due to its increased demand for iron or its lower intake. Anaemia affects negatively the quality of life, especially because of its frequent relapses, the lack of a therapy with high effectivity and few side effects (1).

Studies in patients and animal models have revealed that IDA is associated with low intestinal health in terms of the gut microbiome and the intestinal barrier. Studies in anaemic infants show that the gut microbiome of patients suffering IDA differs from healthy controls, with a predominance of *Proteobacteria* phyla and a decrease in short chain fatty acid (SCFA)-producing bacteria such as *Roseburia, Coprococcus* and *Butyricicoccus*. An alteration in the gut microbiome was also found in pre-menopausal women suffering IDA, with a decrease in family *Ruminococcaceae*, order *Clostridiales*, class *Clostridia* and genera *Faecalibacterium* (2). A decrease in *Faecalibacterium* genera was also observed in an independent study including women with IDA (3). Studies in animals have also confirmed this dysbiosis, with families such as *Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae*, *Prevotellaceae* or *Porphyromonadaceae* decreasing as a consequence of a nutritionally induced iron deficiency. An increase in species of the genera *Clostridia* along with an increased production of SCFA was also noticed in an animal model of IDA, along with impaired intestinal barrier functionality(4,5).

However, there is a lack of studies analysing the effect of iron deficiency on the small intestine microbiome, especially due to its difficult accessibility for sample collection. Das et al. described that specific microbial metabolites, namely 1,3-diaminopropane and reuterin,

were able to impair iron absorption *in vitro* and *in vivo* through the inhibition of HIF-2α, a master regulator of iron absorption in the small intestine (6). This study sets the foundation for the consideration of the small intestine microbiome as a mediator of iron absorption and opens up new venues to investigate microbiome-targeting therapies to treat or aid in the recovery of IDA.

In this sense, small intestinal bacterial overgrowth (SIBO) gains importance in the context of IDA as changes in the number or composition of the bacterial populations in the small intestine might interfere with iron absorption. SIBO is a newly defined clinical entity and it is the subject of active research in relation to its pathogenic mechanisms, therapeutic and diagnostic tools, as evidenced by the continuous growth in publications (7). Iron malabsorption has been well described as a consequence of SIBO due to host-microbial competition and the damaged intestinal absorption surface(8). However, SIBO has never been studied as a consequence of iron deficiency. In previous studies by our research group, we found an increase in the bacterial load and in gas production in the large intestine triggered by the development of IDA, along with a decreased intestinal motility in the colon and an impaired intestinal barrier function (4). According to these results, we hypothesize that SIBO might develop as a result of iron deficiency. This is of special relevance and needs to be considered in the clinical management of IDA, since the development of SIBO might impair iron absorption and IDA treatment.

Taking the afore-mentioned aspects into consideration, the objective of this study is to characterize small intestinal dysbiosis associated with iron deficiency and to identify microbial patterns indicative of SIBO.

2. Methods

2.1. Animal model, experimental design and diets

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 June 06, 2019/100). These methods were carried out in accordance with the Declaration of Helsinki (World Medical Association Declaration of Helsinki, 2014) (9) and ARRIVE guidelines. Animal experiments were conducted at the Animal Facility of the University of Granada under controlled sanitary and environmental conditions. Twenty weaned male Wistar albino rats with an initial weight of 55 ± 5 g, purchased from Charles River Laboratories (France), were used for the study, with diets and deionized water available *ad libitum*. Animals were housed in stainless-steel cages in groups, using ventilated, thermoregulated cages with controlled temperature (23 ± 2 °C), humidity (60 ± 5 %), and a circadian rhythm (12h light; 12h dark).

Animal experiments were performed as already described (4,5). Briefly, IDA was experimentally induced over a period of 40 days through an iron deficient diet for the anaemic group (n=9) while a standard diet (AIN-93G diet) was fed to the control group at the same time (n=11). Both the control and the iron-free diet were prepared at the Diet Facility of the University of Granada. The AIN-93G diet was formulated according to the protocol described by Reeves et al. (10), while the iron-deficient diet was obtained by using an iron-free mineral mix instead of the standard one in the AIN-93G formulation.

At the end of the experimental period, animals, previously fasted overnight, were anesthetized intraperitoneally with sodium pentobarbital (Sigma Diagnostics, St Louis), and euthanized via exsanguination by cardiac puncture. Samples collected for this study include blood samples obtained by cardiac puncture and small intestine content samples. One aliquot of blood was treated with EDTA to control haematological parameters, and the other one was centrifuged (1500× g, 4 □, 15 min) without anticoagulant to separate the serum for subsequent analysis of serum iron, total iron-binding capacity (TIBC), ferritin, and hepcidin. Small

intestine content samples were obtained washing each segment (duodenum, jejunum, ileum) with sterile saline solution (0.9% sodium chloride) and collecting the flow through (2 mL). Intestinal samples were immediately frozen at -80^{o}C until further analysis.

*2.2. Haematological t*ests

Key haematological parameters for anaemia estimation includes determination of red blood cell numbers (RBC), haemoglobin levels (Hb), haematocrit (Hct), white blood cell count (WBC), platelets count (PLT) and red cell indices such as mean cellular volume (MCV), mean cellular haemoglobin (MCH), mean cellular haemoglobin concentration (MCHC) and red cell distribution width (RDW). All parameters were analyzed using an automated haematology analyser Mythic 22CT (C2 Diagnostics, France) (4,5).

The following indicators of iron status were assessed: serum iron, total iron-binding capacity (TIBC), transferrin saturation, serum ferritin and hepcidin. Serum iron, total iron-binding capacity (TIBC), and transferrin saturation were determined using Sigma Diagnostics Iron and TIBC reagents (Sigma, St Louis, USA). The percentage of transferrin saturation was calculated by dividing the serum iron level by TIBC and multiplying by 100. Serum ferritin and hepcidin were measured by ELISA assay (BioVendor GmbH, Heidelberg, Germany and DRG Instruments GmbH, Marburg, Germany, respectivetly).

2.3. DNA isolation and high-throughput sequencing

Small intestinal content samples were pelleted by centrifugation at 6000 rcf for 10 min at 4^{o}C. The obtained pellet underwent DNA extraction using the QIAamp DNA Stool Mini Kit 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 were obtained as previously described (4,5). 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.

2.4. Quantitative PCR (qPCR)

To quantify the total bacterial load, 16S rRNA gene-targeted qPCR was performed using the QuantStudio 6 High-throughput Realtime PCR system. SYBR Green Master Mix (4309155, Thermo Scientific) was used in a total reaction mixture volume of 10 µL. The universal bacterial primers were F: 5′-AAACTCAAAKGAATTGACGGGG-3′ and R: 5′-GGGTTGCGCTCGTTRYGG-3′. Primers in a final concentration of 0.5 µM each and 1 µL of previously diluted DNA (20ng/µL) were added to the PCR master mix, in MicroAmp Fast 96-Well reaction plates. Cycling conditions included 95°C for 10 min and 40 cycles consisting of denaturalization at 95°C for 15 s and annealing-extension at 60°C for 1 min. Negative controls containing no template DNA were subjected to the same procedures. The specificity of the amplified products was determined by analysis of melting curves, which were obtained using QuantStudio 6 default parameters. The number of DNA copies per sample was obtained based on standard curves, for which known concentrations of Escherichia coli 16S gene were used. All samples and the standard curve were analysed in technical triplicates. Results were expressed as mean of Number of 16S copies per mL of intestinal content.

2.5. Bioinformatic analysis

Sequences were processed via Mothur software (v 1.48.3, 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. Amplicon sequence variants (ASV) were defined with the UNOISE3 algorithm implemented in the pre.cluster command of Mothur pipeline. Relative abundances for each ASV were calculated and lowly abundant ASVs (whose relative abundance was below 0.05%) were filtered out. Coverage was estimated yielding values ranging from 75 to 85%.

Microbial functional analysis was carried out using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States version 2 (PICRUSt2) on high-throughput 16S rRNA gene sequencing data (11). KEGG orthologs (KO) genes(12) obtained were used to calculate Gut Microbial Modules (GMMs)(13) using the omixer-rpmR package (14).

2.6. Statistical analysis

Statistical analysis of data was performed using R sofware (version 4.4.3) and GraphPad Prism (version 9.0c).

Student t-tests were employed to assess statistical differences in haematological and biochemical parameters between the experimental groups. Statistical differences between experimental groups considering alpha diversity indexes and bacterial load were assessed by the t-test or the Mann-Whitney test depending on the normality of the dataset. Differential abundant analysis for ASVs and GMMs were identified using Microbiome Multivariable Association with Linear Models (MaAsLin) 2 R package (15). Principal coordinate analysis (PcoA) was performed using the PRIMER-E software (PRIMER-E Ltd, Plymouth, United Kingdom).

Statistical significances were considered at $p < 0.05$. P-adjusted values to account for False Discovery Rate (FDR) were calculated via MaAsLin2 for ASVs and GMMs statistical analyses.

3. Results

3.1. Haematological parameters

A routine complete blood count is one of the most basic and commonly used diagnostic tests in clinical practice, primarily assessing the cellular components of blood. It is frequently employed in the diagnosis of IDA.

At the end of the 40-day experimental period, RBC, Hb concentration, HCT, MCV, MCH, and MCHC were significantly reduced in the IDA group compared to the normal control group. In contrast, RDW and PLT count were significantly increased in the anaemic group, and the white blood cell count remained unchanged (Supplementary Table1).

In relation to indicators of iron status, a significant decrease in serum iron, transferrin saturation, ferritin and hepcidin and an increase of TIBC were observed in the anaemic group compared with the control group. All these findings confirm the successful establishment of IDA in the animal model (Supplementary Table1).

3.2. Iron deficiency anaemia increases the bacterial load in the small intestine

First, to investigate whether there was a bacterial growth pattern consistent with SIBO, bacterial load was assessed across the three segments of the small intestine. Quantitative PCR (qPCR) targeting the 16S ribosomal RNA gene was performed to achieve an accurate quantification of the bacterial content. Results were expressed as the number of 16S rRNA gene copies per mL of intestinal content. An increased number of 16S gene copies across all

three segments of the small intestine during anaemia was observed, showing highly significant differences between the control and anemic groups (Figure 1).

3.3. Iron deficiency anaemia triggers taxonomic alterations in the small intestinal microbiome

To broadly assess the effect of iron deficiency on the structure of the small intestinal microbiome, four estimators of alpha diversity indexes were calculated. The number of observed species (sobs), InvSimpson, Shannon and Pielou indexes were estimated for the duodenum, jejunum and ileum and compared between the control and the anaemic group.

Changes in alpha diversity indexes were more notable towards the distal regions of the small intestine. No significant differences were found for any index in the duodenum (Figure 2A). Sobs and Shannon index also increased in the jejunum during iron deficiency (Figure 2B). All indexes increased in the anaemic group in the ileum, indicating a higher alpha diversity during iron deficiency (Figure 2C).

Subsequently, changes in beta diversity were examined using multivariate analyses in each intestinal segment during IDA. Again, changes in the bacterial community between the anaemic and control groups were more notable in the distal regions of the small intestine. Principal coordinate analyses (PCoA) at the ASV level revealed a marked clustering between the anaemic and the control groups in all the intestinal segments, which indicates that microbial communities in the small intestine are altered during iron deficiency. In the duodenal region, samples are segregated along the X axis into experimental groups, which explains 38% of the total sample variation (Figure 3A). A higher percentage of the total sample variation (43% and 52%) is explained with the segregation of samples along the X axis into the anaemic and control groups in the jejunal (Figure 3B) and ileal regions (Figure 3C), respectively, which indicates that the microbial dysbiosis derived from iron deficiency tends to increase towards the distal segments of the small intestine.

MaAslin 2 was used to identify individual ASVs differing between the control and anaemic group in each intestinal segment. A total of 6 ASVs differed in the duodenum (Supplementary Table 2), 6 in the jejunum (Supplementary Table 3) and 13 in the ileum (Supplementary Table 4). Again, a higher number of ASVs differed in distal segments. The top 10 most variable ASVs for each segment were plotted in a heatmap for a clearer visualization (Figure 4). FigureFigureFigure

In samples corresponding to the duodenal content (Figure 4A), an increase in the abundance of anaerobic and spore-forming species was observed, particularly those belonging to the genus *Clostridium*. Conversely, an increase in other relevant species due to their role as fermenters, such as *Lactobacillus sp*., was noticed in the jejunal segment during anaemia (Figure 4B). In this region, an increase of a single species from the genus *Clostridium* was identified within the anaemic group. Similarly, as observed in the duodenal content, there was a pronounced increase in species of the genus *Clostridium* and *Escherichia-Shigella* in the ileum, which are classically found in the colon and large intestine (Figure 4C). Collectively, the data obtained indicate that, across the different sections of the small intestine, the microbiome composition varies significantly between groups, reflecting an increased abundance of fermentative bacteria during iron deficiency.

3.4. Iron deficiency anaemia triggers functional alterations in the small intestinal microbiome

Due to the observed increased abundance of ASVs belonging to the genera *Clostridium*, *Lactobacillus* and *Escherichia-Shigella*, and the intestinal dysbiosis observed in most small intestinal regions, bacteria functionality was next studied. Functional analysis of the gut microbiome is essential to determine whether the observed dysbiosis exhibits a pattern consistent with SIBO, and whether it aligns with the associated clinical symptoms.

Functional analyses were carried out on the microbial communities of all intestinal regions by inferring functional profiles from taxonomic sequencing data. Gut microbial modules (GMMs) differing between the control and anaemic group in all segments were obtained by MaAslin 2. A total of 21, 41, and 27 significantly altered GMMs were identified in the duodenum (Supplementary Table 5), jejunum (Supplementary Table 6), and ileum, respectively (Supplementary Table 7). From these, the GMMs showing a coefficient higher than 1.5 (highly abundant in the anaemic group and likely associated with SIBO) with a maximum of 10, were selected for each segment and graphically represented in Figure 5 to facilitate intersegmental comparison.

In the duodenum (Figure 5), increased GMMs in the anaemic group included metabolic pathways related to propionate production, butyrate production, hydrogen metabolism, and catabolic routes of sugars and amino acids such as fucose and glutamate degradation. In the jejunal content (Figure 5), similar to the duodenal segment, GMMs related to sugar and amino acid metabolism were found to be more abundant during IDA. In the ileum (Figure 5), an increased abundance of gas producing pathways such as propionate was noticed, along with an increased metabolism of amino acids and certain carbohydrates, consistent with the patterns detected in other intestinal segments. Supplementary Table 7 also shows hydrogen metabolism is increased in the ileum during IDA.

4. Discussion

IDA is a global public health issue characterized by impaired iron absorption and utilization. Iron bioavailability and metabolism are closely influenced by the composition and function of the gut microbiome. SIBO, a form of dysbiosis, has been proposed as a potential contributing factor to iron deficiency. However, whether iron deficiency can trigger the development of SIBO has not been investigated to date. In the present study, we characterized

the small intestinal dysbiosis triggered as a consequence of iron deficiency. The results have shown differences in the small intestinal microbiome between anaemic and control groups, especially in the distal segments of the small intestine, and functional microbial patterns indicative of SIBO have been identified.

qPCR results confirmed an increased bacterial load in all the intestinal segments within the small intestine (Figure 1). Currently, there is no universally established threshold of 16S rRNA gene copies/mL for the definitive diagnosis of SIBO. However, several studies, including that by Leite et al. estimate that the physiological bacterial load in the jejunum is approximately 10□ copies/mL. (16). Considering this threshold, the bacterial load found during iron deficiency in this study would be considered pathological and compatible with SIBO. Possible mechanisms that favour a higher bacterial load in the upper gastrointestinal tract could include impaired gastrointestinal motility, which was observed as an intestinal barrier dysfunction during iron deficiency in the same animal model (4).

Alpha diversity in the small intestine is a hallmark of gut health (17,18). The analysis of duodenal content revealed no significant differences between the two groups (Figure 2A). The jejunum showed higher values for sobs and Shannon indexes during iron deficiency (Figure 2B), while all indexes increased in the anaemic group in the ileum (Figure 2C). Changes in alpha diversity are more pronounced towards distal segments, which may reflect abnormal colonization by multiple taxa from an ectopic region like the large intestine. However, recent studies in human subjects have reported opposite findings in patients with SIBO. Bamba et al. observed a significant reduction in alpha diversity, specifically a lower Shannon index (19). Similarly, other human studies, such as that by Shin et al., also reported reduced alpha diversity in individuals with SIBO (20). The present study yielded opposite results regarding differences in diversity indexes, suggesting that the relationship between

anaemia and gut dysbiosis may be more complex than initially assumed. The portion of the small intestine analyzed, underlying causes of SIBO, types of diet and medication or chronicity of the disorder in humans may also be important factors. Human studies often use low biomass mucosal samples while animal studies use high biomass luminal samples, which can impact diversity. Moreover, human studies of SIBO often includes different underlying, chronic causes of SIBO while SIBO in animal studies is acute and caused by only one causative agent, which can also bias alpha diversity trends.

Principal Coordinates Analysis (PCoA) revealed a progressive shift in microbiome composition along the regions of the small intestine in both control and anaemic groups, being the dysbiosis more intense towards distal segments (Figures 3A, 3B and 3C). In particular, an enrichment of anaerobic and spore-forming bacterial species, notably members of the genus *Clostridium*, was identified in the duodenum (Figure 4A). Similar studies have demonstrated that iron deficiency alters the intestinal microbiome in the colon, increasing the bacterial load and *Clostridium spp* (4). In the jejunum, an increase was also observed in species relevant for their fermentative role, such as *Lactobacillus* spp., during anaemia (Figure 4B). *Lactobacillus* is known for its ability to ferment undigested carbohydrates, producing primarily lactic acid, but also gases such as hydrogen, methane, and carbon dioxide. This fermentative activity can contribute to gastrointestinal symptoms, including bloating and abdominal distension. While *Lactobacillus spp*. are typically associated with the small and large intestine, their presence has been found significantly increased in the small intestine of patients suffering from SIBO (21). Notably, certain species of *Lactobacillus* genus have been found increased in the small intestine during iron deficiency, producing metabolites that impair iron absorption (6). *Lactobacillus* genus does not require iron to proliferate (22), which might be the reason underlying its increase in the small intestine during iron deficiency.

The ileum exhibited a marked increase in species of the genus *Clostridium, Lactobacillus* and *Escherichia-Shigella*, which are typically abundant in the colon and large intestine (Figure 4C). In general, species within the *Clostridium* genus play a beneficial role in maintaining intestinal health; however, certain species, such as *Clostridium perfringens*, have a detrimental impact in the context of SIBO. *C. perfringens* produces a wide range of toxins that compromise the intestinal barrier and can exacerbate symptoms such as abdominal distension, diarrhea, and malabsorption(23). Several studies have reported an overgrowth of *Clostridium* species in duodenal aspirates from patients with SIBO (20,24).

In studies such as the one conducted by Siddique et al. two distinct types of SIBO were identified, one of colonic origin and another of aerodigestive origin (24). In the present study, the anaemic group exhibited an increased abundance of bacterial genera such as *Lactobacillus, Escherichia-Shigella* and *Clostridium* throughout the small intestine. These genera were found increased in the large intestine of the same anaemic animals (4,5), which, along with the fact that the dysbiosis is more notable in the distal segments of the small intestine, suggests a possible colonic origin of the overgrowth.

The functional dysbiosis observed during anaemia also showed a SIBO pattern. In the duodenum (Figure 5), elevated GMMs in the anaemic group included metabolic pathways related to propionate and butyrate production, hydrogen metabolism, and catabolic routes of sugars and amino acids such as fucose and glutamate degradation, as previously described in the literature, along with traits characteristic of fermentative bacteria (25,26). In the jejunal content (Figure 5), as well as in the duodenal segment, metabolic pathways related to amino acid metabolism, carbohydrate metabolism and gas production were found to be more abundant during IDA. The ileum showed—similarly to other segments of the small intestine—an increased abundance of metabolic pathways related to amino acid and carbohydrate catabolism, along with gas producing pathways such as propionate or hydrogen (Figure 5, Supplementary

Table 7). Hydrogen production and sugar fermentation pathways are commonly associated with SIBO, as they are significantly increased in patients with this condition. Indeed, hydrogen breath tests are routinely used clinically to diagnose SIBO. These pathways partly contribute to the abdominal bloating characteristic of SIBO and are linked to increased severity of gastrointestinal symptoms (16).

Taken together, this study reveals taxonomic and functional alterations of the small intestine microbiome during IDA that suggest a SIBO pattern, with an increase in the abundance of colonic species such as *Escherichia-Shigella spp.*, *Clostridium spp* and fermentative species such as *Lactobacillus spp,* an increase in sugar and amino acid catabolic pathways as well as gas producing pathways (hydrogen and SCFA), and an increased bacterial load. Future perspectives in this field include an in-depth genomic and metabolomic characterization of SIBO during iron deficiency. The development of SIBO as a consequence of iron deficiency is of special relevance considering SIBO can impair iron absorption and IDA treatment. Therefore, the development of therapeutic strategies that consider SIBO as a component of the pathology are key in the clinical management of IDA.

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AUTHOR CONTRIBUTIONS

M.S. and A.S.L contributed to project design and conceptualisation. M.I.L.A, A.S.L, M.S and M.J.M.A performed the animal study and sample collection. A.S.L. carried out DNA extractions, sequencing, bioinformatic and statistical analyses. M.G.R. performed qPCR experiments. M.I.L.A and A.S.L drafted the manuscript. J.A.G.S and M.S. provided funding. J.S.S.S contributed to the reviewed version of the manuscript. A.S.L. J.A.G.S., M.S, M.I.L.A. revised the final version of the manuscript.

DATA AVAILABILITY

Raw data supporting the analyses presented in this study have been uploaded to the SRA (PRJNA1291205)

ADDITIONAL INFORMATION

Ethics approval

All experimental procedures were approved by the Ethics Committee of the University of Granada and the local government Junta de Andalucía (ref June 06, 2019/100) and conducted

following the ARRIVE guidelines and European guidelines (Declaration of Helsinki; Directive 2010/63/EU).

Competing interests

The authors declare that they have no competing interests

FIGURE LEGENDS

Figure 1. Quantification of the bacterial load in all segments of the small intestine in control and anaemic animals by qPCR.(A) Duodenal region (B) Jejunal region (C) Ileal region

Figure 2. Alpha diversity indexes: observed species (sobs), InvSimpson, Shannon and Pielou in the small intestinal segments of control and anaemic groups. (A) Duodenal region (B) Jejunal region (C) Ileal region

Figure 3. Principal coordinate analyses (PCoA) at ASV level based on Bray-Curtis distances. Plots for intestinal contents in each segment of the small intestine considering highly abundant bacterial ASVs. Samples are represented by coloured symbols according to the legend. (A) Duodenal region (B) Jejunal region (C) Ileal region

Figure 4. Heatmap illustrating the relative abundance of the top 10 most variable ASVs in the small intestinal segments from control and anaemic groups. Abundance levels are represented using a yellow-to-red gradient scale (see colour legend). Values have been scaled in the row direction to illustrate differences in microbial ASVs between experimental groups. (A) Duodenal region (B) Jejunal region (C) Ileal region

Figure 5. Highly increased gut microbial modules (GMMs) (MaAsLin coefficient > 1.5, maximum of 10) in each segment of the small intestine and both experimental groups, represented as $\log_2$FC.