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applied microbiology

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In Vitro Modelling of a Typical Dietary Intake in Restrictive Anorexia Nervosa Results in Changes to Gut Microbial Community and Metabolites

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Abstract: Anorexia nervosa (AN) is a psychiatric illness with harmful physical consequences. Studies have observed differences in the faecal microbiota of patients with AN compared to healthy controls. Diet has an impact on the gut microbiota, facilitating an altered community, such changes could impact the gut–brain axis. In this study, a three-stage gut model system that mimics the luminal microbiology of the large intestine was conducted to identify relationships between diet and gut microbiota. A microbial medium was developed to provide nutrients more appropriate to restricting subtype AN (R-AN). The model was inoculated with faeces and samples were taken to compare differences in the microbiota and end products following the fermentation of healthy control medium (HC) compared to R-AN medium. Then, 16S amplicon sequencing along with flow cytometry–fluorescence in situ hybridisation were used to ascertain changes in the microbiota. Gas chromatography (GC) was used to assess changes in microbial metabolites. There were reduced levels of SCFA following the fermentation of R-AN medium. The fermentation of R-AN media led to fewer total bacteria numbers, along with less bifidobacteria and *Rumincoccus* proximally, but more *Clostridium* and Enterobacteriaceae. Nutrient-deficient medium resulted in reduced neurotransmitter-producing bacteria, reduced butyrate-producing bacteria, and increased protein-utilising bacteria, all of which could be maintaining factors in AN. The model system provides a novel tool for exploring how extreme dietary changes impact the microbiota and could therefore could be useful for assessing appropriate gut–brain targeted treatments.

Keywords: anorexia nervosa; dysbiosis; restricted diet; gut–brain axis; 16S metagenomics analysis; malnutrition; micronutrients; macronutrients

Citation: Liu, L.; Poveda, C.; Jenkins, P.E.; Iddrisu, I.; Walton, G.E. In Vitro Modelling of a Typical Dietary Intake in Restrictive Anorexia Nervosa Results in Changes to Gut Microbial Community and Metabolites. *Appl. Microbiol.* **2024**, *4*, 1642–1660. https://doi.org/10.3390/applmicrobiol4040112

Academic Editor: Ian Connerton

Received: 25 November 2024
Revised: 3 December 2024
Accepted: 6 December 2024
Published: 10 December 2024

1. Introduction

Anorexia nervosa (AN) is a psychiatric illness with harmful physical consequences and pathophysiological mechanisms that are yet to be fully elucidated [1–3]. Some studies have highlighted gut microbial differences in people with AN compared to healthy controls. Such differences have led to suggestions that the microbiota may have a role to play in AN, particularly when considering that the microbiota could impact mental wellbeing through the gut–brain axis [4–7]. However, a lack of standardised approaches in current research makes it difficult to firmly establish the gut microbiota as a key component in AN [8–11]. The restricting subtype of AN (R-AN) is characterised by the restriction of energy intake and the absence of binge-eating or purging behaviour [12]. Diet has an impact on the gut microbiota composition and richness during both restrictive and refeeding phases [13,14]. Metagenomic studies on AN have provided insight into how diet and nutrition can alter the gut microbiome, making it possible to understand more about this modifiable factor

of the disease [15]. However, there is still much to learn about how the microbiota and associated metabolites could be implicated in the pathophysiology of R-AN. With growing interest in the gut–brain axis, this leads clear research questions as to whether changing this microbial community could help in supporting recovery.

Healthy human intestinal microbiota are dominated by bacteria from two phyla: Firmicutes (including the genera *Lactobacillus*, *Clostridium*, and *Enterococcus*) and Bacteroidetes (including *Bacteroides*), which together account for over 90% of the known genetic categories of the intestinal system [16]. Other bacteria, such as representatives from the phyla Actinobacteria (*Bifidobacterium*), Proteobacteria (*Escherichia coli*), Fusobacteria, Verrucomicrobia, and Cyanobacteria, are present to a lesser extent [17]. However, the proportion in their representation differs depending on many external factors. It has been noted that gut microbiota composition plays a crucial role in the development of several pathologies [18–20]. For example, the Firmicutes/Bacteroidetes (F/B) ratio is higher in obese people compared to lean people and tends to decrease with weight loss [21,22], with lower levels of Firmicutes also reported in AN [4,23]. Additionally, AN pathophysiology also involves an autoimmune component that may be related to gut bacterial antigens, such as Caseinolytic protease B (ClpB), an anorexigenic bacterial protein produced by Enterobacteriaceae [24]. CIpB may be able to stimulate Peptide YY (PYY) production, thus suppressing appetite by impacting on hormone secretion and satiety [25].

Within the gastrointestinal (GI) tract, there are differences in physiology, digestion, the retention time of food, substrate availability, host secretions, pH, and oxygen tension between different anatomical regions [26]. The large intestine is the most intensely inhabited area of the human microbiome [17]. It is characterised by a slow flow rate ranging from 0.3 to 20 mL/min, which can vary depending on whether a person is fasting or has eaten [27,28] and where pH ranges from acidic to neutral, and is dominated by specialised obligate anaerobes. Important differences in the intestinal environment occur between proximal, transverse, and distal regions and more locally between the intestine cavity and surface [26]. By considering these factors, it is possible to model this complex microbial community

A three-stage continuous culture system was validated by Macfarlane et al., 1998 [29]. This system was designed to mimic nutritional and physiochemical conditions of microbiota in the large intestine and was determined by measuring contents of the human large intestine obtained from sudden death victims [29]. The main substrates available to microorganisms growing in the large intestine were undigested foods, along with host-derived substrates [30,31]. Originally the constituents of culture medium were determined on the basis of large intestine contents [26]. By manipulating this medium, it might be possible to study how the microbial community might react in the presence of different nutrients. Studying this microbial community could be of great importance. For example, the GI microbiota can respond to changes in nutrients in the lumen and impact on neuron and hormone signals of the gastrointestinal tract, a process that influences appetite and food intake [32]. Therefore, in the following study, original, healthy gut model medium was altered to develop a medium based on the dietary intake of individuals with R-AN. This medium was used within a three-stage continuous culture system to determine the likely impact of these large nutritional changes on the gut microbial community and its end products, compared to the healthy control medium. Therefore, the aims of the current study were to (1) establish a three-stage continuous culture gut model system based on nutrient intake and gastrointestinal transit times appropriate for R-AN patients and (2) compare changes observed within this system to existing data of AN patients. Overall, this should result in the development of a tool to better study the dynamics of the gut microbial community associated with R-AN. The relevance of microbial changes can therefore be considered alongside the pathophysiology of the illness. This tool can then be used when considering how dietary modulations could impact the microbiota during treatment.

2. Materials and Methods

2.1. Modelling Dietary Intake R-AN and HCs

To establish an R-AN gut model system, a medium was developed based on dietary information reported in restrictive AN individuals [33] (restricted diet = 12, quartile 1) (Table 1), and mineral intake data were obtained from a study by Jauregui et al. (2009) [34]. These data were used to contrast the dietary intake of healthy adults obtained from British Nutrition Foundation (BNF) guidelines [35]. This information was used with the existing gut model medium recipe of Macfarlane et al. (1997) to determine the likely composition of nutrient components to reach the large intestine [36]. The diets were compared, and a new medium was developed based on the differences between the macronutrient and micronutrient content of the diets. A table detailing medium development compared to the healthy control can be found in a previous publication [37]. Details of the two media can be found in Table 2.

Table 1. Energy and nutrient intake reported by individuals with restrictive anorexia (R-AN) and comparison with British Nutrition Foundation (BNF) recommendations for estimated average requirements for adults.

Daily Dietary Composition		**Estimated Average Requirement for Adults (Normal Value)**	**R-AN Diet (n = 12) [33]**	**Sites of Absorption [38]**
Energy intake	Kcal	2175	736	-
Protein intake	g	50	33	Ileum and colon
Carbohydrate	g	260	110	-
Dietary fibre	g	30	12	Colon
Fat	g	70	17	Ileum
Potassium	mg	3500	2600	Ileum and colon
Chloride	mg	2500	1825	Duodenum and colon
Sodium	mg	1600	1168	Colon
Magnesium	mg	270	227	Duodenum
Phosphorus	mg	550	607	Jejunum
Calcium	mg	700	545	Jejunum
Iron	mg	14.8	7.9	Duodenum
Vitamin K	µg	-	-	Ileum and colon
Vitamin B12	µg	1.5	2.2	Ileum
Thiamin	mg	0.8	0.704	Jejunum
Riboflavin	mg	1.1	1.1	Jejunum
Niacin	mg	13	8.6	Duodenum
Folate	µg	200	234	Duodenum
Vitamin B6	mg	1.2	0.9	Jejunum
Vitamin A	mg	600	503	Ileum
Vitamin C	mg	40	70	Ileum
Vitamin E	mg	3	2.7	Ileum
Vitamin D	µg	10	1.7	Ileum
Selenium	µg	60	39	Duodenum
Zinc	mg	7	5.4	Jejunum

The UK set of Dietary Reference Values (DRVs) including estimated average requirements (EARs) and Reference Nutrient Intakes (RNIs). In this study, for the group of 18–60-year-old females, macronutrient requirements were based on EARs and micronutrient and protein requirements were based on RNIs.

Table 2. Healthy control (HC) and restrictive anorexia (R-AN) gut model media.

Medium Ingredient	HC Gut Model Medium (g/L) SS1 [29]	R-AN Medium (g/L) SS2 [37]
Starch	5	2.1
Peptone water	5	3.3
Tryptone	5	3.3
Yeast extract	4.5	2.97
Casein	3	1.98
Guar gum	1	0.4
Inulin	1	0.4
Pectin	2	0.8
Arabinogalactan	2	0.8
Xylan	2	0.8
KCI	4.5	3.28
NaCI	4.5	3.28
$NaHCO_3$	1.5	1.095
$MgSO_4 \cdot 7H_2O$	1.25	1.05
KH_2PO_4	0.5	0.55
K_2HPO_4	0.5	0.55
$CaCI_2 \cdot 6H_2O$	0.15	0.117
Hemin	0.5	0.0265
$FeSO_4 \cdot 7H_2O$	0.0005	0.000795
Vitamin K	10 µL	6.68 µL
L-cystiene HCl	0.8	0.8
Tween 80	1 mL	1 mL
Resazurin (0.25 g/L)	4 mL	4 mL
Mucin (porcine gastric type III)	4	4
Bile salts	0.4	0.4

2.2. Three-Stage Continuous Culture System

A three-stage continuous culture system was conducted in quadruplet, modelling the proximal (V1, 80 mL, pH = 5.5), transverse (V2, 100 mL, pH = 6.2), and distal colonic regions (V3, 120 mL, pH = 6.8), as a scaled-down version of the original gut model system [29]. The systems were held under anaerobic conditions by sparging with N_2 (15 mL/min) and held at a continuous temperature of 37 °C. A 20% faecal slurry was made in anaerobic phosphate-buffered saline (PBS solution: 0.1 mol/L; pH 7.4) using a faecal sample obtained from a healthy female donor who had not taken antibiotics within 6 months and was not a regular consumer of prebiotic or probiotic supplements. The systems were inoculated to give a final concentration of 6% faecal slurry. The faecal donors were four healthy females (aged 25–43 years). The experiment was conducted 4 times with a stool sample from a different faecal donor for each run. Initially, the system was allowed to stabilise for 24 h before starting the flow at a rate that enabled 300 mL to pass through the system in 64 h, as appropriate for AN [39]. The system was run with the HC medium until equilibrium was reached (steady state 1 (SS1)), determined by the stabilisation of SCFA over 3 days; this was after 512 h (8 full-volume turnovers). The medium was changed over to R-AN, and the model was continued until a second equilibrium was reached (steady state 2 (SS2)) after a further 512 h. At each steady state, samples were taken from the three vessels of the models to determine the microbial community and metabolites associated with the HC media or the R-AN media.

2.3. Total Bacteria and SCFA Analysis

For total bacterial community analysis, a 750 µL supernatant of gut model fluid was centrifuged at 13,000× *g* for 5 min. The pellet was then resuspended in 375 µL filtered 0.1 M PBS and fixed with 1125 µL filtered paraformaldehyde (PFA 4% *v*/*v*) for 4 to 8 h at 4 °C. The sample was washed twice with 1 mL PBS to remove PFA and resuspended in filtered 600 µL ethanol–PBS (1:1, *v*/*v*). The samples were kept at −20 °C prior to fluorescence in situ hybridisation–flow cytometry (FISH-FCM) analysis. For FISH-FCM, positive and negative control probes were used to establish a threshold of brightness so only hybridised cells containing the relevant probe were included in the quantification. The methods used to analyse FISH-FCM and SCFA/BCFA production are reported in detail elsewhere [37]. In SCFA/BCFA analysis, an internal standard was used within each sample to ensure appropriate quantification after sample derivitisation. Additionally, an external standard solution was used to obtain an accurate quantification of these metabolites.

2.4. DNA Extraction, Quantification, and Qualification

A 1 mL gut model sample was centrifuged at 13,000× *g* for 10 min, and the pellet was stored at −20 °C until DNA extraction. DNA extraction was conducted using a QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with some modifications. Cell pellets were defrosted on ice and washed with 0.5 mL PBS. Each pellet was resuspended in 1 mL of InhibitEX buffer, and the suspension was transferred to a new tube with acid-washed glass beads (≤100 µm) (Sigma Aldrich, Dorset, UK). Samples were subjected to a bead beater (FastPrep-24™ 5G) for 60 s and put on ice for 60 s. This step was repeated 3 times. The sample was then vortexed for 1 min and centrifuged at 13,000× *g* for 1 min, 0.6 mL of supernatant was transferred to a fresh tube with 25 µL of Proteinase K, and 0.6 mL of AL buffer was added before vortexing for 15 s. Samples were incubated at 70 °C for 10 min; 0.6 mL of ethanol (99.9%) was added to the lysate, and this was vortexed to mix, and 0.6 mL of the lysate was added to a QIAamp spin column. Samples were centrifuged at 13,000× *g* for 1 min, and the tubes containing the filtrate were discarded. The spin column was placed into a new 2 mL collection tube. The previous step was repeated until all of the lysate was loaded onto the spin column. The spin column for each tube was opened, and 0.5 mL of buffer AW1 was added before centrifuging at 13,000× *g* for 1 min. The filtrate was discarded. Spin columns were then placed in a new 2 mL collection tube, and 0.5 mL of AW2 buffer was added. The tubes were centrifuged for 3 min at 13,000× *g* and the collection tubes containing the filtrate were discarded. In new 2 mL collection tubes, the spin columns were placed and centrifuged for 3 min at 13,000× *g*. The QIAamp spin columns were then transferred into a new set of 1.5 mL microcentrifuge tubes, and 0.03 mL of distilled water was added. These tubes were incubated at room temperature for 1 min and then centrifuged at 13,000× *g* for 1 min. The microcentrifuge tubes with the filtrate were discarded, the spin column was placed into a new 1.5 mL microcentrifuge tube, and the previous step was repeated to elute the DNA. The DNA concentration of the final product was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). A 2 µL volume of sample was placed on the Nanodrop pedestal, and the DNA quantity was assessed. Each sample was diluted in ddH_2O to provide a concentration within the range of 10 to 50 ng/µL.

2.5. 16S rRNA Gene-Based Next-Generation Sequencing (NGS) and Bioinformatics

16S rRNA sequencing was carried out by Eurofins (Ebersberg, Germany; project NG-27480). Briefly, 16S rRNA gene sequences were amplified from extracted DNA samples, within the V3-V4 region (forward, 349F: 5′-GYGCASCAGKCGMGAAW-3′; reverse, 806R: 5′-GGACTACVSGGGTATCTAAT-3′) of the 16S rRNA gene by polymerase chain reaction (PCR), as previously described [40]. After sequencing, the de-multiplexing of the data based on the Illumina index reads was performed, and raw data were converted to FASTQ files. Illumina adapters were removed using the FASTP program, and error correction was

performed where two reads overlapped. Raw paired end reads were subjected to quality filtering using software before paired end read assembling with Fast Length Adjustment of short reads (FLASh) software (2.2.00 version). For precise Operational Taxonomic Unit (OTU) analysis, data containing sequence errors (i.e., merged sequences shorter than 458 bp, raw reads with ambiguous base cells, chimeric sequences) were removed. The remaining representative reads from non-chimeric clusters were clustered de novo into OTUs (97% similarity threshold) using the Cluster Database at High Identify with Tolerance (CD-HIT) software program Version 4.6 (University of California, San Diego, CA, USA) based on 99% similarity accounting for PCR and sequencing errors of less than 1%. After pre-clustering [41], the trimmed reads were also checked, and chimeric sequences were removed using an implementation of the UCHIME program (version 4.2.40).

2.6. Data Processing and Bioinformatic Analysis

The pooled libraries were paired-end read sequenced on a MiSeq System (Illumina Inc., San Diego, CA, USA). OTU clustering and taxonomic information was converted to FASTQ files and processed using CLC Genomics Workbench version 21.0.4 and the CLC Microbial Genomics Module (QIAGEN Bioinformatics) (Aarhus, Denmark). Sequences were first trimmed, merged, and then clustered into OTUs at a 99% sequence similarity level using the Amplicon-Based OTU clustering tool. The creation of new OTUs was allowed considering 99% taxonomic similarity. The most abundant sequences were selected as representative of each cluster and then assigned to a taxonomy level using CLC Microbial Genomics default values and the SILVA Database September 2016 release.

2.7. Statistical Analysis

2.7.1. 16S Metagenomic Sequencing and Analysis

The classification of 16S rRNA forward sequence reads was performed using CLC Genomics Workbench version 21.0.4. The OTU log-fold change between healthy and anorexic model conditions was statistically analysed using FDR correction and Kruskal–Wallis test. The false discovery rate (FDR) was determined to correct *p*-values. Fold change (FC) > 1.5 and FDR-adjusted *p*-value < 0.05 were considered for miRNAs with healthy conditions versus anorexic conditions.

2.7.2. 16S rRNA Gene-Based Next-Generation Sequencing (NGS) and Bioinformatics

Data from FISH-FCM and gas chromatography (GC) were analysed with SPSS version 27 (IBM Corp., Armonk, NY, USA). Changes in total bacteria and SCFA/BCFA production were assessed between the 2 steady states using one-way analysis of variance (ANOVA). Significant differences were assessed with a post hoc Tukey HSD (Honestly Significant Difference) test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Microbial Profiles in the Stimulation of Proximal, Transverse, and Distal Colon (HC Versus R-AN)

The faecal-derived microbial communities in the different gut model vessels following the fermentation of HC and R-AN media were profiled. The composition of the intestinal microbiota was significantly influenced by R-AN medium at every taxonomic level ($p < 0.05$) (Figure 1). At the phylum level, the predominant bacterial taxa were Firmicutes and Bacteroidetes followed by Actinobacteria, Proteobacteria, and Fusobacteria. When compared to the microbial phylum profile between the fermentation of HC and R-AN media, the relative abundance of Firmicutes and Actinobacteria showed an increasing trend following fermentation of HC medium. However, after R-AN fermentation, there was an increase in the relative abundance of Bacteroidetes, Proteobacteria, and Fusobacteria, although the former two did not reach significance. Significant differences in phylum levels between R-AN and HC are reported in Table 3.

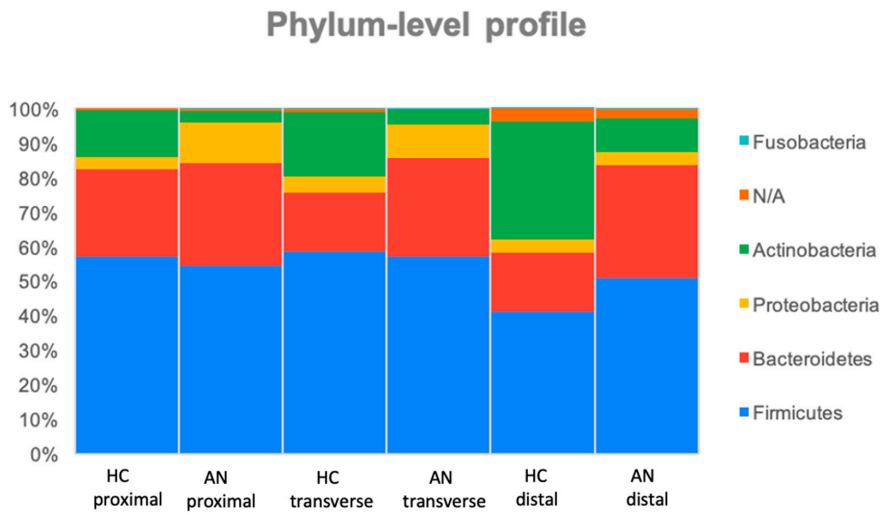

Figure 1. Phylum-level profile. Relative abundance of common microbial taxa. Bar chart showing the relative abundance of the most represented microbial taxa, defined as having a mean relative abundance of >1% in gut model of proximal, transverse, and distal colon following the fermentation of healthy control (HC) medium and then restrictive anorexia (R-AN) medium (n = 4). N/A: data not available.

Table 3. Taxa significantly increased (+) or decreased (-) in abundance following fermentation of healthy control (HC) and restrictive anorexia (R-AN) medium (data are expressed as means ± standard deviation). AN, R-AN medium; HC, healthy control medium. n = 4.

Taxonomic Level	Classification	Colon Site	HC Relative Abundance	AN Relative Abundance	AN	HC Versus AN *p*-Value
Phylum	*Proteobacteria*	Proximal	3.50 ± 3.80	11.70 ± 18.80	+	0.04
	Actinobacteria	Proximal	13.60 ± 7.80	3.40 ± 3.10	-	<0.001
		Transverse	18.60 ± 9.00	4.60 ± 4.80	-	0.01
		Distal	34.30 ± 25.90	9.60 ± 10.90	-	0.01
Family	*Eubacteriaceae*	Proximal	0.00 ± 0.00	0.14 ± 0.23	+	0.01
		Transverse	0.02 ± 0.04	0.42 ± 0.73	+	0.03
		Distal	0.00 ± 0.00	3.97 ± 4.93	+	0.001
	Tissierellaceae	Transverse	4.70 ± 11.50	1.40 ± 1.70	-	0.05
		Distal	0.91 ± 0.31	11.60 ± 7.10	+	<0.001
	Mogibacteriaceae	Transverse	0.70 ± 1.50	0.16 ± 0.30	-	0.05
		Distal	0.13 ± 0.11	0.94 ± 1.00	+	0.01
	Actinomycetaceae	Distal	0.01 ± 0.02	0.00	-	0.04
	Porphyromonadaceae	Proximal	0.09 ± 0.009	0.76 ± 0.76	+	<0.001
		Transverse	0.24 ± 0.19	1.40 ± 1.59	+	0.01
	Lactobacillaceae	Transverse	0.30 ± 0.30	0.13 ± 0.15	-	0.02
		Distal	0.28 ± 0.09	0.13 ± 0.03	-	0.01
	Desulfovibrionaceae	Proximal	0.00	0.16 ± 0.20	+	<0.001
		Transverse	0.11 ± 0.15	0.81 ± 0.83	+	0.03
	Campylobacteraceae	Transverse	0.03 ± 0.05	0.00	-	0.02
		Distal	0.13 ± 0.19	0.00	-	0.003
	Enterobacteriaceae	Proximal	0.70 ± 0.87	10.97 ± 17.91	+	0.05
		Transverse	13.19 ± 18.88	8.37 ± 12.74	-	0.03
	Bifidobacteriaceae	Transverse	15.10 ± 9.70	3.10 ± 4.10	-	0.001
	Coriobacteriaceae	Transverse	3.50 ± 2.40	1.50 ± 0.80	-	0.006
	Veillonellaceae	Proximal	6.62 ± 5.87	22.90 ± 18.75	+	0.002
	Methylobacteriaceae	Proximal	0.00	0.46 ± 0.82	+	0.004
		Transverse	0.00	0.13 ± 0.25	+	0.003
		Distal	0.00	0.00 ± 0.00	+	0.003
	Fusobacteriaceae	Proximal	0.00	0.26 ± 0.74	+	<0.001
	Alcaligenaceae	Proximal	2.74 ± 3.80	0.16 ± 0.16	-	0.00
	Dethiosulfovibrionaceae	Transverse	0.00	0.11 ± 0.14	+	0.03
		Distal	0.00	0.09 ± 0.05	+	0.04

At the family level (Figure 2), Actinomycetaceae was only observed following the fermentation of HC media in the distal colon, being undetectable following R-AN medium fermentation ($p < 0.05$). The family Campylobacteraceae was also only observed following HC medium fermentation (transverse and distal colon) ($p < 0.05$ and $p < 0.01$, respectively). In contrast, some families were undetectable in the HC medium and only significantly increased after R-AN medium fermentation. For example, the relative abundance of Methylobacteriaceae significantly increased in all vessels (proximal, $p < 0.005$; transverse, $p < 0.05$; and distal, $p < 0.05$) after the fermentation of R-AN medium. The abundance of the families of Fusobacteriaceae and Desulfovibrionaceae significantly increased in V1 (proximal, both $p < 0.001$) after the fermentation of R-AN medium. The families of Barnesiellaceae and Oxalobacteraceae significantly increased after the fermentation of R-AN medium in V2 (transverse, $p < 0.05$) and V3 (distal, $p < 0.05$). The abundance of Dethiosulfovibrionaceae significantly increased in V2 and V3 (transverse and distal colon, both $p < 0.05$) following the fermentation of R-AN medium.

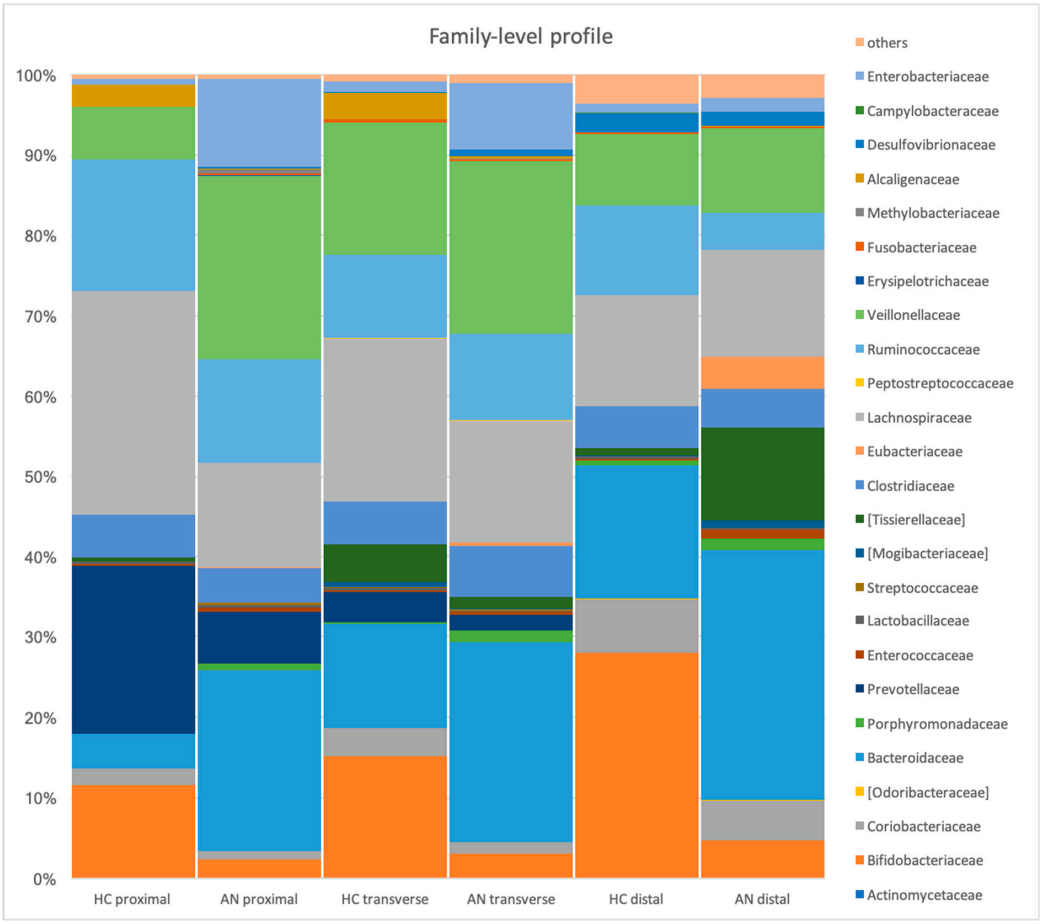

Figure 2. Family-level profiles. Relative abundance of common microbial families. Bar charts showing the relative abundance of the most represented microbial taxa, defined as having a mean relative abundance of >1% in gut model of proximal, transverse, and distal colon following the fermentation of healthy control (HC) medium and then restrictive anorexia (R-AN); (n = 4).

At the genus level (Figure 3), some genera were only observed in the HC model, whilst the R-AN medium was unable to support the growth of these. On the contrary, compared to the HC medium, there were some genera only detectable following the fermentation of R-AN medium. Significant differences at the phylum, family, and genus levels post the fermentation of AN and HC media are reported in Tables 3 and 4.

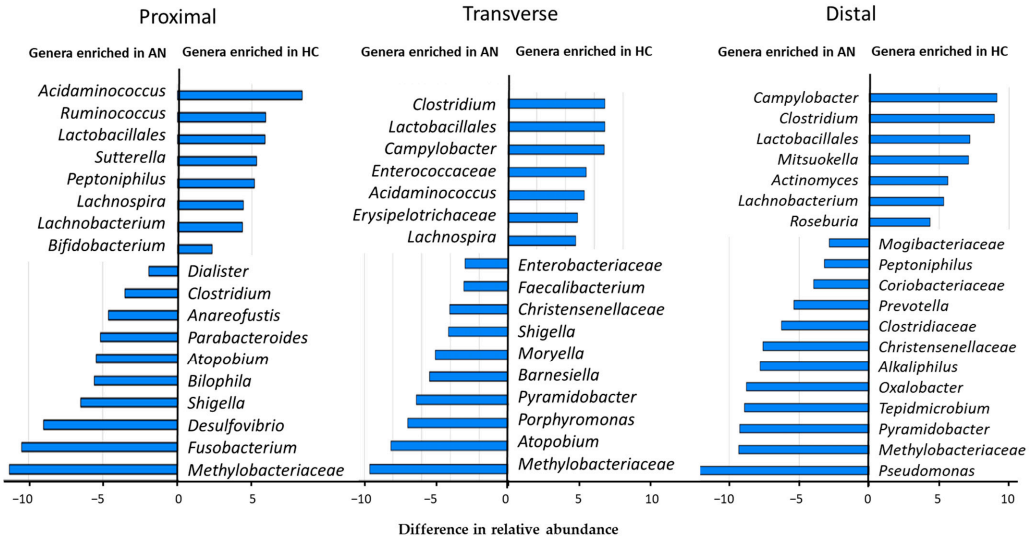

Figure 3. Taxonomic differences were detected between healthy control (HC) and restrictive anorexia (R-AN) using CLC workbench analysis (corrected *p* values). Only genera with significant differences in the relative abundance between HC controls and the R-AN condition are displayed.

Table 4. Genus-level profile from each vessel V1, V2, and V3, mimicking the proximal, transverse, and distal colon of in vitro colonic model. Differences between healthy control (HC) and restrictive anorexia (R-AN) medium microbiome (HC microbiome versus R-AN microbiome). Only genera with significant differences in the relative abundance between HC and R-AN are displayed. Genera significantly increased (+) or decreased (-) in abundance following the fermentation of R-AN medium and HC medium (data are expressed as means ± standard deviation).

Genus	Colon Site	HC Relative Abundance	R-AN Relative Abundance	R-AN	*p* Value
Lachnospira	Proximal	2.39 ± 1.95	0.12 ± 0.11	-	<0.001
	Transverse	1.86 ± 2.05	0.08 ± 0.08	-	<0.001
Methylobacteriaceae	Proximal	0.00	0.41 ± 0.82	+	<0.001
	Transverse	0.00	0.12 ± 0.24	+	<0.001
	Distal	0.00	0.00 ± 0.00	+	0.03
Sutterella	Proximal	2.45 ± 3.80	0.14 ± 0.16	-	0.001
Fusobacterium	Proximal	0.00	0.30 ± 0.74	+	0.002
Parabacteroides	Proximal	0.01 ± 0.01	0.64 ± 0.76	+	0.002
Desulfovibrio	Proximal	0.00	0.12 ± 0.20	+	0.002
Atopobium	Proximal	0.00 ± 0.01	0.21 ± 0.04	+	0.01
	Transverse	0.00	0.36 ± 0.71	+	<0.001
	Distal	0.00	0.91 ± 1.22	+	0.007
Ruminococcus	Proximal	13.09 ± 7.87	6.17 ± 4.71	-	0.02
Peptoniphilus	Proximal	0.53 ± 0.81	0.02 ± 0.03	-	0.03
	Distal	0.61 ± 0.56	5.03 ± 6.51	+	0.005
Clostridium	Proximal	0.12 ± 0.18	0.94 ± 1.23	+	0.04
	Transverse	4.92 ± 1.85	6.49 ± 6.18	+	0.01
	Distal	0.00	0.01 ± 0.01	+	0.02
Bifidobacterium	Proximal	12.18 ± 7.56	2.69 ± 3.09	-	0.04
Dialister	Proximal	5.16 ± 4.90	24.65 ± 18.75	+	0.04
Anaerofustis	Proximal	0.00	0.01 ± 0.00	+	0.04
Lachnobacterium	Proximal	0.45 ± 0.88	0.01 ± 0.02	-	0.05
	Distal	0.11 ± 0.12	0.00 ± 0.01	-	0.01
Lactobacillales	Proximal	0.01 ± 0.00	0.00	-	0.05
	Transverse	0.02 ± 0.04	0.00	-	0.01
	Distal	0.09 ± 0.13	0.00	-	0.006

Table 4. *Cont.*

Genus	Colon Site	HC Relative Abundance	R-AN Relative Abundance	R-AN	*p* Value
Porphyromonas	Transverse	0.00 ± 0.01	0.81 ± 1.61	+	0.01
Campylobacter	Transverse	0.02 ± 0.05	0.00	-	0.02
	Distal	0.13 ± 0.18	0.00	-	0.01
Enterobacteriaceae	Transverse	0.25 ± 0.35	1.83 ± 2.95	+	0.03
Faecalibacterium	Transverse	3.56 ± 6.43	5.45 ± 9.07	+	0.03
Pyramidobacter	Transverse	0.00	0.10 ± 0.10	+	0.03
Tepidimicrobium	Distal	0.01 ± 0.01	2.67 ± 3.77	+	0.003
Pseudoramibacter_ Eubacterium	Distal	0.00	3.46 ± 4.90	+	0.003
Mitsuokella	Distal	1.12 ± 1.57	0.02 ± 0.03	-	0.01
Alkaliphilus	Distal	0.00	0.18 ± 0.26	+	0.02
Coriobacteriaceae	Distal	0.04 ± 0.05	0.08 ± 0.08	+	0.02
Pyramidobacter	Distal	0.00	0.10 ± 0.14	+	0.03
Mogibacteriaceae	Distal	0.10 ± 0.07	0.83 ± 1.03	+	0.03
Oxalobacter	Distal	0.00	0.07 ± 0.09	+	0.03
Roseburia	Distal	0.11 ± 0.05	0.01 ± 0.02	-	0.04

3.2. Total Bacteria, SCFA, and BCFA Production

There were fewer total bacteria following the fermentation of R-AN media, along with lower levels of SCFA. Changes in total bacteria, SCFA, and BCFA concentrations are shown in Table 5.

Table 5. Total bacteria and short-chain fatty acid/branched-chain fatty acid (SCFA/BCFA) concentrations detected by fluorescence in situ hybridisation coupled with flow cytometry (FISH-FCM) (Log_{10} cells/mL) and gas chromatography (GC) (mM), respectively, from each vessel V1, V2, and V3, mimicking the proximal, transverse, and distal colon of within a pH-controlled in vitro faecal (6%) colonic model. Samples were collected at steady state 1 (SS1) (healthy control (HC) medium) and SS2 (restrictive anorexia (R-AN) medium). Data are expressed as means ± standard deviation. Not significant is referred to as n.s. Significant differences in each vessel between SS1 and SS2 are indicated. Total bacteria and SCFA/BCFA increased (+) or decreased (-) in amounts following the fermentation of R-AN medium compared to HC medium (data are expressed as means ± standard deviation).

	Colon Site	HC	R-AN	R-AN	*p* Value
Total bacteria (log_{10} cells/mL)	Proximal	8.50 ± 0.12	8.16 ± 0.05	-	0.0018
	Transverse	8.23 ± 0.23	7.73 ± 0.32	-	0.05
	Distal	8.00 ± 0.09	7.28 ± 0.40	-	0.01
Acetate (mM)	Proximal	30.02 ± 8.59	16.79 ± 1.96	-	0.023
	Transverse	42.64 ± 17.17	21.31 ± 9.98	-	n.s.
	Distal	46.83 ± 28.44	31.94 ± 13.40	-	n.s.
Butyrate (mM)	Proximal	28.80 ± 3.26	16.82 ± 5.98	-	0.012
	Transverse	37.37 ± 6.08	17.16 ± 9.57	-	0.011
	Distal	36.61 ± 12.20	20.29 ± 7.76	-	n.s.
Propionate (mM)	Proximal	17.54 ± 9.40	14.27 ± 13.78	-	n.s.
	Transverse	28.82 ± 8.11	15.03 ± 6.66	-	0.039
	Distal	27.44 ± 10.21	19.54 ± 9.97	-	n.s.
BCFA (Iso-butyrate + Isovalerate) (mM)	Proximal	2.91 ± 3.38	2.67 ± 3.61	-	n.s.
	Transverse	6.11 ± 1.64	3.08 ± 1.86	-	0.05
	Distal	5.70 ± 1.84	7.06 ± 4.16	+	n.s.

4. Discussion

An in vitro model system represents an innovative tool that allows the dynamic microbial communities of the gastrointestinal tract to be studied, permitting adjustments to a variety of parameters, including nutrients, temperature, pH, and retention time. Manipulating these factors can enable the modelling of physiologically relevant conditions, reducing the requirement for human intervention studies [29,42,43]. In this study, a three-stage gut model was developed to investigate the effect of a modelled R-AN diet on the microbial community. The drastic changes in diet, modelled as a change in nutrient medium, led to extensive microbial differences following fermentation of HC medium and R-AN medium. The current study provides experimental evidence to support what has been observed in patients with AN in vivo.

At the phylum level, when comparing the microbiota following fermentation of HC medium compared to AN medium, it was observed that there was an increased relative abundance of Proteobacteria in the proximal regions of the model following R-AN medium fermentation. Similarly, in 2017, Borgo et al. analysed stool samples from 15 AN patients compared to healthy controls and noted increased abundance of Proteobacteria in individuals with AN [4]. Whilst this observation has not been made in other AN studies, a high abundance of Proteobacteria has been related to dysbiosis in hosts with metabolic or inflammatory disorders with potentially detrimental effects on gut microbiota composition and immune function [44,45]. It is therefore likely that this phylum may have a similar effect in AN.

The F/B ratio of the proximal, transverse, and distal colon following the fermentation of R-AN medium was lower than following HC medium fermentation, although this change was not significant. The most abundant bacteria in human gut microbiota are Firmicutes and Bacteroidetes phylum members. However, across three previous studies, the average F/B ratio was lower in AN individuals than in healthy groups [6,46,47]. It is worth noting that the F/B ratio is frequently reported to be increased in obese people compared to lean people and decreases with weight loss [21,48], suggesting that these microbial groups are likely to be sensitive to dietary changes. As such, the microbial change in the F/B ratio observed here would make sense in the context of R-AN.

Actinobacteria are a phylum associated with the maintenance of gut homeostasis [49] and include *Bifidobacterium* as a key member. Mack et al. (2016) and Morita et al. (2015) reported the microbial profile of AN patients compared to healthy controls, observing that Actinobacteria were elevated in AN patients and remained this way following weight regain [5,23]. The increase in this phylum during AN is likely to relate to the increased fibre ratio relative to other macronutrients [50]. In contrast, within the current model system, a reduction in Actinobacteria was observed following R-AN medium fermentation. However, the reduced Actinobacteria in the current study was only observed in the proximal regions; thus, this change might not translate to faeces, although it might be reasonable to expect higher Actinobacteria levels more distally following the fermentation of R-AN media. Reasons for this discrepancy could be due to the reduced levels of FOS in the R-AN medium, reducing the available substrate to the AN microbiota; alteration in the FOS levels of the medium may help to better model this. It is also worth noting that an increase in Actinobacteria has been observed in faeces of individuals with obesity [51]. This is also likely to be a result of enhanced levels of carbohydrates reaching the large intestine. Carbohydrates commonly include dietary ingredients that promote the growth of the Actinobacteria phyla, specifically *Bifidobacterium* [52–54]. A difference in the expected result highlights the fact that the dietary intake of each person with AN will differ, resulting in a different microbial profile, but through modulating the medium, a model that more closely relates to the microbiota typically associated with AN may be reached.

The observation of significantly increased Enterobacteriaceae in the proximal and transverse regions following the fermentation of R-AN medium revealed similar differences to those observed by Borgo et al. (2017) and Fan et al. (2023) when comparing persons with AN with healthy controls [4,48]. This observation makes sense when considering several

characteristics of AN. The family Enterobacteriaceae are associated with gut inflammation, which favours bacterial translocation, promoting systemic inflammation [55]. A study has reported Enterobacteriaceae to play a role in eating disorders (EDs), where species of *Escherichia coli* are capable of producing small-protein sequence ClpB [56]. ClpB appears to interfere with a melanocyte-stimulating hormone (a-MSH) involved in anxiety and satiety signalling as an appetite regulator [57]. Higher levels of Enterobacteriaceae are associated with AN and also the gut–brain axis [56]. Furthermore, Borgo et al. (2017) also identified higher levels of Enterobacteriaceae in individuals with AN [4]. Increased Enterobacteriaceae have been associated with higher levels of lipopolysaccharides (LPSs) in blood, molecules linked to systemic inflammation that have been linked to symptoms of depression and anxiety [58]. Increased LPS levels imply a possible common inflammatory pathway shared by AN and mood disorders [56,57]. Consistent with this observation, an increase in this family may be associated with a higher production of neuropeptide ClpB, which may have a role to play in GBA communication in individuals with AN.

In the current study, the relative abundance of Fusobacteria was higher in all three colon segments following fermentation of AN medium compared to that of HC, particularly within the proximal region. Whilst this result has not been previously observed in AN, it is noteworthy because Fusobacteria are more frequently cultured from Kwashiorkor, an acute form of protein malnutrition [59]. In a study by Smith et al. (2013), faecal samples from children with kwashiorkor were transferred to germ-free mice, leading to drastic weight loss and metabolic abnormalities compared to the control group that received transplants from healthy children. This indicates that gut microbial community during starvation may play a role in body weight regulation [60]. Although malnutrition secondary to AN develops more chronically, it is plausible that microbial communities selected by a low-energy gut environment may perpetuate in individuals with AN and could have an impact on weight loss and behaviour [61].

An additional observation of the in vitro model was that Dethiosulfovibrionaceae significantly increased in the transverse and distal regions following AN medium fermentation, whereas it was undetectable after the fermentation of HC media. Whilst a link between AN and Dethiosulfovibrionaceae has not previously been observed, a study found that Dethiosulfovibrionaceae significantly increased in obese woman during a phase of rapid weight and body fat mass loss following bariatric surgery [62]. The study considered the potential of bacteria changes to be associated with changes in appetite, and it is therefore possible that this group of bacteria could have a role to play in the maintenance of AN and other eating problems. At the genus level, compared to the HC medium, the current study observed a significant decreased relative abundance of *Lactobacillales* in all three colon regions following R-AN medium fermentation, although the levels in the HC model were below 0.1% abundance. A study conducted by Armougoum et al. showed reduced *Lactobacillus* in AN compared to patients with obesity [63], suggesting that sufficient nutrients may not be available to support the growth of this genus in this mixed microbial community. *Lactobacillus* has been linked to a reduced anxiety response, the alleviation of depression, and increased resilience to stress [64–67]. Furthermore, γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter present throughout the central nervous system, has been shown to be secreted by a number of bacteria, including *Lactobacillus* and *Bifidobacterium* [68]. Therefore, it is possible that a particular probiotic strain intervention could significantly affect GABA modulation through neurotransmitter production.

Compared to in the HC medium, the relative abundance of *Clostridium* significantly increased in all three colon regions following the fermentation of R-AN medium, which is consistent with studies on AN patients when compared to HC groups [7,20,47], contrasting the findings of Borgo et al. (2017), which suggested a reduced relative abundance of *Clostridium* in AN faecal samples [4]. However, as *Clostridium* is a large genus of microbes, changes based on species may vary. For example, a study by Queipo-Ortuno et al. observed increased *Clostridium* group IV levels in the rat model of R-AN following exercise [69], and a study conducted by Mack et al. (2016) indicated higher levels of *Clostridium* cluster I and

cluster XI in faeces of individuals with AN when compared to healthy participants [23]. The use of 16S amplicon sequencing provides good resolution for genus distinction, but it is difficult to comment further on the species resolution of the data.

Coriobacteriaceae were significantly increased within the R-AN medium distal region, consistent with a study by Kleiman et al. (2015) in faecal samples of AN patients. It is worth noting that Mortia et al., 2015, did not observe any change in this microbial group, which could relate to the difference in the macronutrient intake within AN patients [70]. However, Morkl et al. (2017) observed *Coriobacteriaceae* as the only enriched phylotype in faeces of AN patients compared to in other microbial groups [71]. *Coriobacteriaceae* colonise the GI tract and are implicated in bile salt conversion and lipid metabolism [72]. As such, it is possible that this genus gains a competitive advantage with this state of limited nutrients and is therefore able to proliferate in the colons of those with AN.

The *Roseburia* genus identified by this study was significantly reduced following distal AN media fermentation; this reduction has also been observed in the faecal samples of individuals with AN [4,23,47,73]. This difference was well mirrored within the in vitro model system, and such a change is of interest because *Roseburia* are carbohydrate-degrading bacteria and key butyrate producers [29,74]. Decreased levels of *Roseburia* have also been found in patients with inflammatory bowel diseases [75]. Low-grade inflammation and altered gut permeability have been observed in an AN animal model [76,77], which could be in part driven by the reduced levels of butyrate, suggesting a putative role in AN's pathophysiology. As such, a future direction of this research could look for using novel *Roseburia* probiotics to determine if this can help to counter some of the negative associations mentioned.

Compared to the HC model, *Atopobium* was significantly increased in all three colon regions of the AN model. However, a study indicated that the level of the *Atopobium* genus in patients with AN is not significantly different to participants without AN [5]. However, Atopobium has been observed to be elevated in major depressive disorder (MDD) and bipolar disorder [78]. Given significant comorbidity with AN and both MDD and bipolar disorder [78–81], the in vitro findings relating to the *Atopobium* genus might be a link between shared mechanisms of eating and mood disorders. Similarly, the family Desulfovibrionaceae was significantly increased in the proximal and transverse colon of the AN model. Desulfovibrionaceae were elevated in an AN study [82]; this family produce hydrogen sulphide, a pro-inflammatory agent that may disrupt gut barrier function. Desulfovibrionaceae have also been linked to depression and may contribute to shared pathophysiological mechanisms between gut inflammation and mood disorders [83]. Using in vitro models to determine ways to counter these effects could help in finding ways to readdress the microbial balance, whilst determining possible links with the gut–brain axis.

Methylobacteriaceae were detected only following the fermentation of AN media including the proximal, transverse, and distal regions with an increasing trend when compared to the HC media. There are no previous observations regarding AN's faecal microbial status related to levels of *Methylobacteriaceae*, although this family may have a negative effect on immunocompromised hosts as they have been reported to cause infections post colonisation, including symptoms of fever, bloodstream infections, peritonitis, and pneumonia [80,84].

Another aspect worth considering is that in much AN research, the number of mucin-degrading bacteria is enhanced in AN samples [23]. In the current model, the mucin concentrations were the same in the HC and AN models, which may result in mucin becoming a key source of substance for the existing bacteria. In the current model, an increase in mucin utilisers in the AN model was not observed, suggesting that the concentration of mucin relative to the other nutrients needs consideration. Furthermore, the source of mucin of the in vitro model (being porcine) may not be optimal. Gastric III porcine mucin exhibits distinct glycosylation patterns compared to MUC2, the primary mucin in the large intestine. MUC2 contains complex O-glycans unique to the gut environment, which support the growth of specific mucin-degrading bacteria such as the *Akkermansia*

muciniphila and *Bacteroides* species. In contrast, the simpler glycan structures in PGM III reduce its ability to effectively support these bacterial populations [85,86]. Thus, through optimising the mucin in vitro, a better model may be obtained.

Within the current model, there were significantly decreased total bacteria in AN medium within the proximal, transverse, and distal regions, which is in line with findings of Morita et al. (2015), who found lower total bacteria in the faces of those with AN when compared to healthy subjects [Morita et al., 2015]. Previous studies that measured faecal samples in AN individuals reported decreased levels of SCFAs when compared to healthy subjects [4,5,23,87], likely as a result of reduced carbohydrate intake, thus reducing substrate available for conversion to SCFA [87]. Furthermore, a depletion in carbohydrate-fermenting *Roseburia* could result in decreased propionate production [74]. Studies by Bailey et al. (2011) and Borgo et al. (2017) observed that butyrate concentration was negatively correlated with depression and anxiety scores [4,88]. In agreement with the above findings, in the current model, the levels of total bacteria and SCFA were significantly reduced upon fermentation of AN media when compared to HC media.

The increased concentration of total BCFAs, particularly iso-butyrate and iso-valerate, has been observed in stool samples of patients with AN [23]. BCFAs are typically markers of protein fermentation. In the current study, the AN model resulted in decreased levels of BCFAs when compared to the HC model; there was, however, a tendency for more BCFA distally, but this did not reach significance. This result may be due to limited protein availability in the anorexic medium. Furthermore, as SCFA and BCFA are largely absorbed, the faecal concentrations do not accurately reflect production within the GI tract.

It is worth noting that there are some limitations associated with the AN medium formulation. In this study, the AN medium was designed based on data from a specific group of twelve individuals with restrictive-type anorexia (BMI: 17.3 $\pm$ 0.3; age: 22 $\pm$ 1 years), so this AN medium recipe was stratified according to subtype and quartiles of extreme lower dietary nutrient intake compared to the age-specific Dietary Reference Intake levels. However, a recent meta-analysis suggested that the average protein daily intake for AN was 53.7 g, which is similar to the recommended dietary intake [89]. Therefore, it is important to note that the model system designed here is specific for restrictive AN at the extreme lower end of intake, and other medium variations would be necessary if modelling different conditions. Within this study, there were many observations that made sense to be occurring in the AN microbiota but had not previously been observed. This could be because the current model was an extreme system, whilst the publications on AN microbiota have represented a range of individuals, not all at the same extreme end of low nutritional intake. Nevertheless, the links of the in vitro gut model findings with physiological observations of AN suggest that the data generated are of importance and the model did appropriately indicate microbial changes in an extremely low nutrient diet.

As such, this study highlights how dietary restrictions can impact the microbial community. The in vitro system also provides insight into possible differences between the HC and AN microbiome in the proximal, transverse, and distal colonic regions, i.e., further up the GI tract than would normally be studied. Concentrating only on faecal samples may neglect significant region-specific microbial changes that are impacted by restricted diet in AN patients. Several findings of this study are considered alongside with previous observations, including the gut microbiome in AN, MDD, and malnutrition, and further confirm that nutritional deficiency is associated with profound alterations in the bacterial community structure [5,23,63,90].

As such, it seems that the fermentation of the AN medium mimics core features of restricting dietary AN, resulting in several changes in the intestinal microbial ecosystem, some common and others that map well to changes that might be expected in AN. Whilst no two persons with AN will have the same dietary intake, an approximation based on reports allows typical intakes of persons with R-AN to be modelled as a useful approach for studying microbial aspects of this condition. AN is, however, a multifaceted and complex condition, but these models provide an innovative technological tool that is less restricted

by ethical concerns. As such, with these models, it may be possible to study likely effects of interventions on R-AN gut microbiota. Such approaches could be useful for optimising re-feeding approaches, when also considering the microbiota and the gut–brain axis.

5. Conclusions

Within this study, a medium was created to approximate the likely dietary nutrients available in the colon of those with R-AN. When compared to the gut model validated for healthy individuals, the restricted approach led to large differences in microbiota concentrations and SCFA concentrations. These included reduced microbial numbers and lower levels of health-associated bifidobacteria and SCFA-producing *Ruminococcus*. Conversely, there were higher levels of bacteria that include potentially pathogenic organisms; e.g., Enterobacteriacae and groups containing proteolytic genera, such as *Clostridium*. When considered alongside changes to the faecal microbiota reported in the faeces of persons with AN, similar increases in proteolytic microorganisms and lower levels of SCFAs were observed. As such, the R-AN medium within gut model systems could be a useful tool for studying AN. The model was based on R-AN patients within the lower quartile for nutritional intake, so the results may not translate perfectly to all AN situations. Nevertheless, useful links associated with reduced nutrient intake were identified. When considering therapies that implicate the microbiota, the gut model system enables a clear way to model the microbiota and their end products, minimising the need to test in patients. Such a system is likely to be of great value when considered alongside the GBA for developing dietary interventions to support recovery.

Author Contributions: Conceptualization, L.L., P.E.J. and G.E.W.; methodology, L.L., G.E.W. and C.P.; software, L.L.; validation, L.L. and G.E.W.; formal analysis, L.L.; investigation, L.L. and C.P.; resources, L.L.; data curation, L.L., G.E.W., C.P. and I.I.; writing—original draft preparation, L.L. and G.E.W.; writing—review and editing, L.L., G.E.W., C.P., P.E.J. and I.I.; visualisation, P.E.J. and G.E.W.; supervision, G.E.W., C.P. and P.E.J.; project administration, L.L.; funding acquisition, L.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data generated is presented in the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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