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Modulation and adaptation of gut microbial metabolic functions under probiotic and postbiotic treatment using a novel *in vitro* anaerobic pseudo-colon system

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Abstract

Probiotics and postbiotics compounds found in food influence gut microbiota to attenuate chronic metabolic diseases; however, the underlying mechanisms are not yet fully understood. This study employed a customized *in vitro* anaerobic pseudo-colon system (AMMR) to evaluate the impacts of *Lactiplantibacillus plantarum* (probiotic) and butyrate (postbiotic) on gut microbial composition and functionality, using human fecal samples. Metagenomic (16S rRNA) profiling and untargeted metabolomic (GC-MS) analysis were conducted after 48-h treatments. The results showed that butyrate supplementation markedly enhanced microbial diversity, inhibited opportunistic pathobionts (e.g., *Enterococcus*, *Klebsiella*), and selectively enriched butyrate producers (e.g., *Lachnoclostridium*), while diminishing the Firmicutes:Bacteroidetes ratio. It increased indole levels metabolically and redirected pathways towards amino acid synthesis and energy metabolism, while suppressing fatty acid formation. Contrarily, *L. plantarum* exhibited modest alterations in microbial diversity while enhancing *Bacteroides* and *Klebsiella*, and preserving elevated *Enterococcus* levels. It elevated saturated fatty acids (octanoic/capric acid) and enhanced amino acid catabolic pathways (valine/leucine) and redox regulators (taurine metabolism). Correlation analysis revealed that butyrate was associated with fiber-degrading microbes, whereas *L. plantarum* was associated with lactic acid bacteria, suggesting distinct ecological niches and interaction patterns. These findings collectively indicate that butyrate and *L. plantarum* elicit complementary microbial alterations, i.e., butyrate directly transforms microbial structure and metabolism towards an anti-inflammatory phenotype, while *L. plantarum* largely influences via metabolic byproducts and niche adjustment. The complementary actions highlight the therapeutic potential of integrated probiotic-postbiotic approaches for the enhancement of gut health.

Keywords:
Butyrate; *Lactiplantibacillus*; Postbiotic; Probiotic; Microbiome; Metabolism.

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Introduction

Emerging gut microbiota-centric approaches, comprising probiotics (beneficial live microorganisms), prebiotics (microbe-feeding), postbiotics (microbe-derived metabolites), and synbiotics (synergistic combinations), are transforming translational research by facilitating a favourable modulation of the host-microbiome axis to prevent or address metabolic, immune, and neuropsychiatric disorders. By defining mechanistic pathways, such as the improvement of gut barrier integrity, modulation of systemic inflammation, and production of bioactive compounds, these interventions expedite the translation of microbiome research into targeted therapeutics and personalized nutrition strategies [1].

Lactiplantibacillus plantarum is one of the most extensively studied probiotics, which can mitigate irritable bowel syndrome, diminish pathogenic bacterial burdens, and enhance the prevalence of gut commensals (e.g., *Bifidobacterium*) and butyrogenic bacteria in clinical settings [2]. Comparable to other probiotic bacteria, lactate produced by *L. plantarum* can be employed by cross-feeding microorganisms to make butyrate [3], so indirectly affecting the gut metabolome. Nevertheless, the probiotic efficacy may be limited by the survival of viable cells during gastrointestinal transit, with viability reductions undermining therapeutic results. Short-chain fatty acids (SCFA), especially butyrate, are a multifunctional postbiotic, produced during bacterial fermentation of dietary fibers. Butyrate functions as the primary energy source for colonocytes, improves mucosal barrier integrity by stabilizing hypoxia-inducible factor, and attenuates epithelial permeability [4]. Its immunomodulatory activities encompass the inhibition of histone deacetylases, modification of regulatory T-cell differentiation, and reduction of pro-inflammatory cytokines [5]. Pre-clinical and clinical studies have shown that butyrate supplementation reduces the severity of colitis, facilitates remission of inflammatory bowel disease (IBD), and safeguards against pathogen-induced mucosal injury [6].

Probiotics utilize living microorganisms to provide health benefits through direct interactions with the host and the generation of metabolites, whereas postbiotics, such as butyrate, eliminate the necessity for microbial viability, providing benefits in terms of stability, safety, and uniformity. Comparative research indicates that postbiotics may have more significant microbiome modulatory

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effects in specific situations [7]; nonetheless, comprehensive direct comparisons with probiotics under controlled settings are scarce. Despite comprehensive research on both *L. plantarum* and butyrate, it remains unclear whether the health-promoting effects of probiotic consumption are predominantly attributable to live bacterial activity or metabolites like butyrate. Therefore, we hypothesized that supplementation with *L. plantarum* and butyrate would induce unique but potentially complementary alterations in gut microbiome composition and metabolite profiles using a novel *in vitro* pseudo-colon system. Specifically, we hypothesize that *L. plantarum* will induce extensive alterations in microbial diversity and competitively exclude opportunistic pathogens via live cell interactions and diverse metabolite synthesis. Butyrate supplement, on the other hand, was expected to augment anti-inflammatory metabolomic signatures without substantially modifying living community composition. This direct comparison is expected to elucidate both overlapping and unique impacts, thereby defining the degree to which butyrate facilitates the probiotic activities of *L. plantarum*. Collectively, this direct comparison elucidates the gut-level mechanisms of probiotic-postbiotic action and guide focused therapy options for gut health management.

Materials and methods

Chemicals and reagents

Gifu anaerobic medium and sodium butyrate were purchased from HiMedia, India. Oxgall, sodium hydrogen carbonate, pancreatin, sodium sulphate, and n-hexane were acquired from LOBA Chemie, India. N, O-Bis(trimethylsilyl)trifluoroacetamide was purchased from Sigma-Aldrich, USA. Sodium hydroxide and hydrochloric acid for pH adjustment were purchased from Rankem, India. Distilled water was obtained using the Millipore Milli-Q ultra-pure water (Ω18.2) system.

Functioning of the Artificial Colon Model for Microbiota Research (AMMR)

To replicate the human colonic luminal microenvironment, we have designed and developed a proprietary, fully customizable anaerobic gut microbiome culturing system named the AMMR system (Indian design patent #394577-001; Indian patent application #202311013073; PTC #PTC/IN2023/050834). The benefits of the anaerobic *in vitro* gut microbiome culturing system have been recognized [8-11] and applied for the understanding of microbiota and nutrient reciprocal

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interactions independent of the host influence [12-14]. The AMMR system comprises two stainless-steel modular units, left and right, each with dimensions of 540 mm (L), 215 mm (H). Each unit comprises three independent interconnected anaerobic reactors mimicking the ascending, transverse, and descending colon of the human intestine. The internal architecture of each reactor includes a stainless-steel baffle with a bottom opening space for homogenous mixing of the fecal slurry and mimicking the peristaltic movement of the human gut. The individual reactor vessel has a total volume of 500 mL (400 mL of effective volume and 100 mL of headspace) and contains 5 ports (1/4'') each for pH sensor, headspace gas collection, liquid sample collection, gas purge, and feed addition. The reactor lid with several access ports is connected to the vessel using autoclavable gaskets and clamp-based seals, ensuring airtight closure. When not in use, each port is sealed to maintain sterility and anaerobic conditions.

Both left and right units can be controlled by the common central control unit that continuously monitors and regulates the reactor temperature and pH. Temperature control could be achieved in the range of room temperature (~25 °C) to a maximum of 80 °C using an external water jacket coupled with an integrated thermostat. The system can be used for both batch and fed-batch modes of operation. Each of the six reactors is independently detachable from the adjacent reactors for easy cleaning, sterilization and handling purposes. This configuration allows either for individual operation of isolated reactors (representing a specific region of the intestine), or combined operation of 3 reactors (representing ascending, transverse, and descending colon). A fed-batch and individual operation approach was used for the current study. Adjacent reactors remain connected through sealed connectors, allowing one-way flow of effluent. All the reactor chambers can be independently flushed with N_2 gas to ensure an anaerobic environment for optimal *in vitro* gut microbial growth.

Sample collection

The following experiments were conducted under established rules and regulations. The approval for the experiment was acquired from the Institutional Ethical Committee (TIET/EC/2023-08). Informed consents were obtained from human participants of this study. All 5 donors **(Supplementary Table 1)** were explained the experiment objectives and signed an informed consent form for fecal matter donation, and a questionnaire regarding age, ethnicity, food type,

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medical history, etc., was also obtained at the time of sample collection. The donors had not received any antibiotic treatments or probiotic supplements for at least 3 months before donating the fecal sample [15].

L. plantarum strain acquisition and evaluation of probiotic characteristics

L. plantarum MTCC 2621 (equivalent to ATCC 8014; *Lactiplantibacillus plantarum*) was acquired from the Microbial Type Culture Collection (MTCC), Council of Scientific & Industrial Research – Institute of Microbial Technology (CSIR-IMTech), Chandigarh, India, and was cultured and maintained under standard conditions. These strains were stored as 20% glycerol stock (v/v) in freezing conditions (−80 °C). The glycerol stock was used as the inoculum and was grown in Gifu anaerobic medium, which was then used directly as the inoculum for treatment.

Experimental design

Pooled fecal samples from n=5 individuals were used for the preparation of the stock fecal slurry solution [16-19]. In brief, morning fecal sample was collected individually from the donors in sterile containers, pooled and mixed with Gifu anaerobic medium containing 13.5 g/L digested serum, liver extract (1.2 g/L), 5 g/L soluble starch, L-cysteine hydrochloride (300 mg/L), sodium thioglycolate (300 mg/L), soya peptone (3 g/L), and HM peptone B (2.2 g/L) [20-22]. Samples were homogenized in the media and directly used for inoculation in the AMMR system through the entry port **(Fig. 1)**. Three independent reaction mixtures were prepared to evaluate the comparative probiotic and postbiotic treatments on gut microbiota in terms of gut microbial population diversity and overall metabolome: control group, butyrate (postbiotic) treated group, and *L. plantarum* (probiotic) treated group (n=3 sets for each treatment). The chemical composition of each treatment was uniform, i.e., Gifu anaerobic medium (5.9%), oxgall powder (0.6%), sodium hydrogen carbonate (1.25%), and pancreatin (0.1%) [23]. The pH of each of the reactors was maintained at 7.5 – 8.0, which critically maintained the gut microbial populations [24, 25]. In *L. plantarum* treatment, $1X10^6$ CFU/mL was inoculated, and 20 mM butyrate (corresponding to colonic concentration in healthy individuals) was added for butyrate treatment [26, 27]. After feeding was done at 0-h, the reactors were flushed with N_2 gas and allowed to run for 48-h at 37 °C [28, 29]. Feeding of media was done every 4-h to maintain

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optimal nutrient availability. After completion of the incubation period, samples were collected from chamber 3 under aseptic conditions for metabolomic and metagenomic analysis.

Sample preparation for untargeted metabolomics

Gas Chromatography-Mass Spectrometry (GC-MS)-based untargeted metabolomics was performed to elucidate the metabolic profile under each treatment. The culture media (2 mL) was mixed with 2 mL of n-hexane and 100 µL of N, O-Bis(trimethylsilyl)trifluoroacetamide, vortexed vigorously for 30 min, and incubated at 40˚C for 4-h at 200 rpm. The mixture was allowed to settle down, the upper organic layer was separated, and centrifuged at 10,000 rpm for 15 min. The supernatant obtained was mixed with a few granules of sodium sulfate, vortexed for 10 min, and centrifuged at 10,000 rpm for 15 min. The resultant was filtered through 0.2 µ syringe filter, and finally transferred to autosampler vials, sealed with a polytetrafluoroethylene cap.

GC-MS analysis

The metabolite extract was analyzed using SHS-40 instrument (Massachusetts, USA) equipped with Bruker 436-GC series, SCION-SQ-MS model, and Rxi®-17Sil-ms column (30 m X 0.25 mm X 0.25 µm). The initial temperature of the program was set at 60˚C (solvent delay of 5 min) with a hold of 4 min, followed by a ramp of 5˚C/min, and the end temperature was set at 280˚C with a hold of 10 min. The derivatized samples (1 µL) were injected in splitless mode, with a constant drift of helium gas (1 mL/min). The MS transfer line temperature was kept at 280˚C. Automated Mass Spectral Deconvolution and Identification System (AMDIS) version 2.70 was used to analyze MS data. Identification of major compounds was done based on mass fragmentation patterns (m/z) using reference compounds in MS Interpreter version 2.0. The compounds were also matched with the National Institute Standard and Technology (NIST) with an MS Library V2011. Metabolites identified were filtered out based on a probability score of <60%.

Metabolic pathway impact prediction

Metabolic abundance data from GC-MS analysis were used for identifying metabolic pathway impact using the Metaboanalyst V6 tool [30]. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used as a reference library for pathway analysis and to evaluate how identified

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metabolites significantly impact the general function of metabolic pathways based on the position and connectivity of metabolites within the identified and impacted pathways. Identified compounds were mapped against PubChem and KEGG identifiers.

Metagenomic sequencing

The reaction mixtures were processed for DNA isolation and 16S rRNA amplicon sequencing. In brief, the DNA isolation was done using NucleoSpin Soil, Mini Kit (Cat# 740780, MACHEREY-NAGEL GmbH & Co. KG) as per the manufacturer's instructions. DNA integrity was evaluated using agarose gel electrophoresis and estimation of DNA concentration was done using Qubit Quant-It dsDNA HS Kit on Qubit Fluorometer (Thermo Fisher). The V3-V4 region of the 16S rRNA genes was amplified by polymerase chain reaction with a universal forward primer and unique barcode primer (bakt-341F: CCCTACACGACGCTCTTCCGATCTG-barcode-CCTACGGGNGGCWGCAG; bakt-805R: GACTGGAGTTCCTTGGCACCCGAGAATTCCA-barcode-GACTACHVGGGTATCTAATCC). The first amplification was performed using thermal cycling conditions as below: 94 °C for 5 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 20 s, and elongation at 65 °C for 30 s; 20 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 30 s; and a final extension at 72 °C for 10 min. Illumina bridge PCR compatible primers were introduced in the second amplification as follows: 3 min of denaturation at 95 °C; 5 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 30 s; and a final extension at 72 °C for 10 min. Amplicons were purified using AMPure XP beads, and Amplicon concentration was estimated using a Qubit 3.0 DNA Kit, 10 ng of amplicons from each sample were sequenced using the Illumina MiSeq 2 × 300 bp platform.

16s rRNA sequence data analysis

Sequence data were analyzed using Quantitative Insights into Microbial Ecology (QIIME v2) by excluding primers and spacers from the sequences as previously outlined [31, 32]. The Divisive Amplicon Denoising Algorithm 2 (DADA2) was used for trimming, denoising, merging the forward and reverse reads (paired-end), and removing chimeric sequences. OTU clustering was performed on denoised sequences using the q2-vsearch function in the QIIME2 pipeline. Forward and reverse reads were discarded if their quality was poor (Q<25%). The obtained feature table was

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rarefied using the diversity core-metrics-phylogenetic (q2-diversity) plugin in QIIME2 at a sample depth of 12,000 to calculate microbial diversity. The diversity of microorganisms across different taxa was elucidated by using the readings from the feature table and reference taxonomic annotations from the SILVA database (version 138). Reads were obtained with 99% 16S coverage and raw taxonomy files that were trained using the Naive Bayes classifier. The trained classifier was later used using representative sequences produced by DADA2 to allocate sequences to certain taxa. Sequences constituting 0.5% of low abundance were omitted from all cases. The enrichment of microbial metabolic pathways was calculated using 16S rRNA sequences using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). Operational Taxonomic Units (OTUs) were aligned with the GreenGenes database (gg_13_08) using QIIME2, using a 97% similarity criterion and closed OTU selecting methodology. The abundance of OTUs was calibrated to the 16S rRNA gene copy number obtained from recognized bacterial genomes in Integrated Microbial Genomes. Anticipated functions were aligned using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analysis

GraphPad V8 was used for statistical analysis of data, and quantitative data were interpreted as mean ± SEM. Metaboanalyst V6 was used for biochemical pathway enrichment utilizing a metabolite abundance list obtained from GC-MS analysis [33]. The enrichment ratio (ER) for the subclass was computed using observed hit/expected hit values. Holm-Bonferroni correction for P-value and false discovery rate (FDR) was computed for each enrichment analysis entry. $P<0.05$ was considered significant. A linear correlation between any two independent variables was done using Pearson's method. The KEGG database was used as a reference library for pathway analysis to examine functionally related metabolites and their considerable enrichment. This analysis focused on the identification of metabolites that could prevent the requirement for preselecting metabolites based on arbitrary cut-off thresholds [34]. The identified metabolites were cross-referred with PubChem and KEGG identifiers. Pathway analysis was done using Fisher's exact test, and pathway impact was calculated based on the topological importance of different metabolites in a certain pathway. The metabolome and microbiome data were log-transformed and mean-centered

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normalized for one-factor analysis to get equal variance. The differences in microbial communities and metabolites in different treatments were analyzed using STAMP v2.1.3. For comparison, t-test (equal variance) with a p-value filter (>0.05) was used. Extended error bar plots were made to show statistically significant values, Welch's confidence intervals, and differences in mean proportion effect size.

Results

Microbiome profile

Both butyrate and *L. plantarum* differentially altered the gut microbial architecture at various taxonomic levels compared to control. At the phylum level, five considerable phyla were identified, and the gut microbiota in all three groups were predominantly composed of Firmicutes (43.61%), Bacteroidetes (35.28%), and Proteobacteria (14.31%) (**Fig. 2A**). Although low in abundance, Tenericutes were observed to be uniquely identified in *L. plantarum,* indicating enrichment of specific species associated with the Tenericutes phylum. Compared to the control, we found that butyrate induced a 12.0% reduction in Firmicutes and a 4.3% decrease in Bacteroidetes, accompanied by a 49.6% increase in Proteobacteria. Conversely, *L. plantarum* resulted in a 7.4% decrease in Firmicutes, an 8.9% increase in Bacteroidetes, and a minor 3.9% rise in Proteobacteria, with negligible impacts on other phyla. As a result, the Firmicutes:Bacteroidetes (F:B) ratio demonstrated a trend of reduction due to the treatments *i.e.*, initially 1.34 in control, decreased to 1.23 (P=0.35) following butyrate treatment and to 1.14 (P=0.12) due to *L. plantarum*.

At the order level (**Fig. 2B**), a total of 37 orders were detected. In the control group, the gut microbiota was predominantly composed of Lactobacillales (41.55%), Bacteroidales (34.74%), and Enterobacterales (12.12%). Following butyrate treatment, the microbial community was different, resulting in Bacteroidales (33.18%), Enterobacterales (18.07%), and Clostridiales (15.60%) emerging as the predominant groups, due to butyrate's established ability to influence gut microbial composition through anti-inflammatory short-chain fatty acid signaling. The treatment with *L. plantarum*, in contrast, reinstated a profile similar to the controls - Bacteroidales (37.82%), Lactobacillales (37.63%), and Enterobacterales (12.60%) - demonstrating its capacity for probiotic-

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induced community rearrangement. A direct comparison of the two interventions on the principal orders revealed that butyrate causes suppression of Lactobacillales (4.23% vs. 37.63%), moderately reduced Bacteroidales (33.18% vs. 37.82%), and increased Enterobacterales (18.07% vs. 12.60%) in relation to *L. plantarum*, underscoring the contrasting effects of a postbiotic compared to a live probiotic on essential gut taxa.

In case of genus, 145 genera were identified, of which 75, 108, and 64 absolute genera were identified in control, butyrate, and *L. plantarum* treatments, respectively, indicating an increased microbial diversity in butyrate treatment. In the control group, the three most abundant genera were found to be *Enterococcus* (39.2%), *Bacteroides* (33.7%), and *Klebsiella* (6.9%); in the butyrate treatment, the top three genera were *Bacteroides* (30.1%), *Peptoniphilus* (6.94%) and *Lachnoclostridium* (4.25%), whereas in the *L. plantarum* group the dominant genera were *Enterococcus* (37.3%), *Bacteroides* (36.9%), and *Klebsiella* (7.9%) (**Fig. 2C**). *Pectobacterium* (0.022%), *Flavonifractor* (2.207%), and *Micrococcus* (0.003%) were the most unique genera as identified in control, butyrate, and *L. plantarum* groups, respectively, having the highest abundance among all the unique genera. While increasing *Parabacteroides* (0.008 – 1.55%) and certain butyrate-producers like *Blautia* (0.017 – 0.137%) and *Butyricicoccus* (0 – 0.005%), we observed a slight drop in *Bacteroides* after butyrate supplementation as compared to control as well as notable decreases in classic pathobionts *Enterococcus* (39.2 → 4.0%), *Klebsiella* (6.9 → 0.01%), and *Enterobacter* (0.60 → 0.009%) were also inferred. By comparison, *L. plantarum* treatment showed increased *Bacteroides* and *Klebsiella* but mostly maintained high *Enterococcus* levels and had no impact on *Escherichia* and *Enterobacter*. We deduced that butyrate therapy thereby changed the gut microbiota toward lower possible pathogens and increased SCFA producers; *L. plantarum* strengthened important butyrate-producing taxa but had little effect on pathobiont suppression.

Fifteen and four differential genera were identified between control and butyrate treatment and control and *L. plantarum* treatment, respectively **(Fig. 2D-F)**. Of note, the mean proportion of *Lachnoclostridium* (p<0.05) was seen to be significantly higher in butyrate treatment **(Fig. 2D)** and significantly lower in *L. plantarum* treatment **(Fig. 2E)** when compared to the control. Butyrate treatment showed significant enrichment of *Bacteroides* **(Fig. 2D, F)**, and *L. plantarum* treatment

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showed significant enrichment of *Klebsiella* **(Fig. 2E, F)**. The correlation heatmap of the top 50 genera with the highest abundance across the treatments showed a close association between control and *L. plantarum* treatment at the gut microbiome level. Our data indicates that butyrate treatment is positively correlated with butyrogenic and fiber-degrading genera, i.e., *Intestinimonas, Clostridium, Ruminococcus, Shigella,* etc., while some of them are negatively correlated, like *Roseburia, Eubacterium, Faecalibacterium,* etc*.* **(Fig. 2G)**. In contrast, lactic acid-associated genera like *Lactonifactor, Bacteroides, Lactobacillus,* etc., were observed to be positively correlated with *L. plantarum* treatment, and negatively correlated with butyrate treatment, indicating specific enrichment of lactic acid bacteria in *L. plantarum* treatment.

Microbial metabolic functions

Significant differences in various microbial metabolic functions were computed under butyrate and *L. plantarum* treatment, compared to the control (**Fig. 3**). In our results, butyrate treatment was found to redirected microbial metabolism from antibiotic and fatty acid biosynthesis to amino acid synthesis, energy metabolism, and motility (**Fig. 3A**). For instance, butyrate showed significantly increased processes associated with amino acid synthesis and central energy metabolism. The biosynthesis of valine, leucine, and isoleucine has shown a significant rise in addition to histidine metabolism, alanine, aspartate and glutamate metabolism, and pantothenate and CoA biosynthesis. Energy pathways such as carbon fixation via the citrate cycle (TCA cycle) show minor enrichments. Critical pathways for antibiotic synthesis were seen to be significantly downregulated. The production of vancomycin group antibiotics decreased considerably, as did the biosynthesis of streptomycin. The production of secondary bile acids was depleted under butyrate treatment. *L. plantarum* treatment showed limited alterations in gut microbial metabolic functions which included enrichment of pathways pertaining to ascorbate and aldarate metabolism, and nucleotide excision repair, while a depletion in glycosaminoglycan degradation, histidine metabolism, and taurine and hypotaurine metabolism was noted in *L. plantarum* group compared to control (**Fig. 3B**). Distinct difference in microbial function was also observed between butyrate and *L. plantarum* groups (**Supplementary fig. 1**). A total of 113 common microbial metabolic functions were predicted, out of which 1 and 7 were unique in control and butyrate treatment, respectively **(Fig**

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3C). Association heatmap coupled with sample clustering clearly indicated a close association between control and *L. plantarum* group, whereas butyrate resulted in altered microbial function, which was different from that of control and *L. plantarum* (**Fig. 3D**).

Untargeted metabolomic profile

A total of 44, 39, and 51 metabolites were identified in control, butyrate, and *L. plantarum* groups, respectively **(Fig. 4A) (Supplementary Tables 5-7)**. Although they differed in abundance, octanoic acid and capric acid were the top-abundant metabolites in all groups, followed by indole in control and butyrate, and gln-glu peptide in *L. plantarum*. Seven metabolites were common among the treatments with variable abundance **(Fig. 4B) (Supplementary Table 8)**. These metabolites include indole, homovanillic acid, octanoic acid, capric acid, phenylacetylglycine, D-galactose, and nonanoic acid. Although the abundance of these metabolites differed across treatments, a significant difference in mean proportion was observed only in the case of indole, capric acid, nonanoic acid, and octanoic acid **(Fig. 4C-E)**. The mean proportion of capric acid and octanoic acid (p<0.05) was observed to be significantly less in the butyrate treatment **(Fig. 4C)**, but higher in the *L. plantarum* treatment **(Fig. 4D)** in comparison to the control group. The mean proportion of indole was significantly higher in the butyrate treatment than control and *L. plantarum* treatment **(Fig. 4C, E)**. Unlike the correlation heatmap of genera identified and microbial metabolic functions for various treatments, the correlation heatmap of the top 50 highly abundant metabolites showed that control and butyrate treatment confer a close association in terms of metabolome data **(Fig. 4F)**. It was seen that some of the metabolites showed similar patterns of correlation in both butyrate and *L. plantarum* treatments. Homovanillic acid, nonanoic acid, methyl acetate, methyl propionate, p-cresol, palmitic acid, etc., showed positive correlation, and phenylacetylglycine, and several peptides like asn-asn-lys, trp-glu, etc., showed negative correlation with both the treatments in comparison to the control. Specifically, in *L. plantarum* treatment, 9-octadecenoic acid, acetic acid, indole-3-carboxylic acid, etc., showed positive correlation, and urea, stearic acid, L-saccharopine, indole, etc., showed negative correlation. Similarly, in butyrate treatment, N-acetylputrescine, caproic acid, propionamide, etc., showed positive correlation, and capric acid, octanoic acid, D-galactose, myristic acid, etc., showed negative correlation. The control group exhibited baseline correlations with

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fermentation byproducts (2,3-hexanediol) and higher aromatic metabolites, indicating unmodulated gut metabolism.

Chemical class enrichments

Based on the identified metabolites, respective chemical class enrichments were identified. It was found that amino acid, peptides, and analogues was significantly enriched in control, and fatty acids and conjugates in butyrate and *L. plantarum* treatments **(Fig. 4H-J) (Supplementary Tables 9-11)**, followed by fatty acids and conjugates in control, carbonyl compounds in butyrate and amino acids, peptides and analouges in *L. plantarum* treatment as second significant metabolite class. In terms of enrichment ratio, the control group showed higher enrichment of alloxazines and isoalloxazines, purine nucleosides, and dibenzylbutanediol lignans **(Fig. 4H)**. Similarly, for the butyrate group, phenylacetic acid, carboximidic acids, and cresols have a high enrichment ratio **(Fig. 4I)**; and pyrimidine ribonucleosides, cinnamic acids, and pyridines were highly enriched for the *L. plantarum* group **(Fig. 4J)**. A total of 6 metabolite classes were common across the three groups, while 6, 5, 12 metabolite classes were inferred to be distinctly enriched in control, butyrate, and *L. plantarum* treatment, respectively **(Fig. 4G) (Supplementary Table 12)**.

Metabolic pathway enrichments

The identified metabolites were mapped against the KEGG as a reference library for further identification of impacted pathways. The examination of metabolic pathway enrichment demonstrates unique patterns across the control, butyrate, and *L. plantarum* groups. The pathway impact analysis showed that in control, the top 3 highly impacted pathways include riboflavin metabolism (impact=0.11), D-amino acid metabolism (impact=0.05), and pyrimidine metabolism (Impact=0.04) **(Fig. 5A) (Supplementary Table 13)**. In butyrate group, the top 3 highly impacted pathways comprise phenylalanine, tyrosine and tryptophan biosynthesis (impact=0.04), propanoate metabolism (Impact=0.04), and pentose phosphate pathway (impact=0.02) **(Fig. 5B) (Supplementary Table 14)**. The same in *L. plantarum* group includes D-amino acid metabolism (impact=0.17), valine, leucine and isoleucine degradation (impact=0.15), and taurine and hypotaurine metabolism (Impact=0.14) (**Fig. 5C**) **(Supplementary Table 15)**. Six metabolic

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pathways were found to be common among all treatments. In contrast, butyrate showed specific enrichment of pentose phosphate pathway **(Fig. 5D) (Supplementary Table 16)**.

Discussion

The current study was conducted to infer the differential effects of *L. plantarum* (butyrogenic probiotic) [35] and butyrate (postbiotic) on gut microbial population and diversity, metabolites, and associated metabolic pathways using metagenomics and metabolomics approaches. Our results indicate that *L. plantarum* and butyrate exhibit distinct yet complementary effects on gut microbes, suggesting that both live bacteria and metabolic byproducts of *L. plantarum* can confer health benefits.

The human colon, possessing the greatest microbial density among human-associated microbial communities, offers a stable anaerobic environment for many bacterial populations vital to host physiology [36]. Comprehending colonic biogeography and host-microbe interactions is fundamental to translational microbiome research and clinical treatment advancements, hence improving metabolic resilience. Nevertheless, research on pre-clinical and clinical colonic microbiomes studies exhibits significant variation, which is attributed to individual host characteristics (e.g., genetics, immunological status), variations in housing and feeding, and uncontrolled fluctuations in luminal composition [37, 38]. Variations in transit durations, pH gradients, and mucus layers further induce compositional alterations, complicating comparison analysis among cohorts. Microbial interactions with leftover feed, bile salts, and host-secreted substances create uncontrolled chemical heterogeneity within the lumen [39]. These confounding variables compromise the reproducibility and translational validity of pre-clinical findings. Consequently, rigorously regulated, standardized *in vitro* or gnotobiotic models with entirely chemically specified luminal inputs are crucial to eradicate variability and facilitate reliable, reproducible mechanistic understanding. For strict gut microbial control to eliminate unnecessary variability, various *in vitro* strategies have been developed over the years to emulate the intricacies of the human gastrointestinal tract, addressing the shortcomings of fecal batch cultures and animal models [9, 40, 41]. These multicompartment dynamic systems facilitate standardized luminal conditions and continuous or batch feeding, hence

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minimizing variability arising from host variables, luminal heterogeneity, and environmental variations. Thus, these *in vitro* models provide a consistent mechanistic understanding of microbial metabolism, host-microbe interactions, and the effectiveness of interventions in gut microbial research.

According to 16S rRNA gene sequencing data, *L. plantarum* reshaped the gut microbial community, irrespective of a considerable impact on the overall microbial diversity. This observation aligns with the previous pre-clinical and clinical reports that probiotics, especially belonging to the genus *Lactobacillus* may exert health-beneficial effects without impacting the gut microbial diversity [42, 43]. In fact, an earlier study indicated that supplementation of a single probiotic sp. may not be efficient enough to improve the overall diversity of the gut microbiota [44]. This strict nature of the gut microbiota, especially within the closed AMMR system, could result due to causes such as niche occupation without displacing other community members, transient colonization and impact on the entire microbiota population, resilience nature of the core microbiome preventing displacement and colonization success by *L. plantarum*, and innate compensatory dynamics of the gut microbiota where functionally analogous spp. may bloom upon *L. plantarum*-mediated suppression of specific genera [45, 46].

On the contrary, butyrate treatment tends to increase the gut microbial diversity (108 vs. 75) due to improved energy metabolism and cross-feeding in the gut microbial population [47]. Butyrate is a metabolic end-product of primary fermenters (e.g., *Faecalibacterium*, *Roseburia*), which is then used as an energy source by secondary microbial consumers [48]. This establishes interconnected trophic networks in which butyrate supports auxotrophic organisms that lack the ability for primary fibre breakdown. This cross-feeding enhances niche specialization and stabilizes community structure, hence directly augmenting microbial diversity [49]. It ecologically converts butyrate into a communal resource that supports mutualistic relationships.

Differential alteration at the microbial taxonomic levels was noted by butyrate and *L. plantarum*. The increased abundance of Proteobacteria observed following butyrate treatment remains aligned with previous reports [27]. Although the bloom of Proteobacteria has historically been associated with mucosal inflammation and gut-barrier dysfunction due to the presence of pyrogenic

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endotoxin, recent studies indicate diverse beneficial role of Proteobacteria that includes the production of essential vitamins [50], providing colonization resistance [51], and metabolic signaling [52]. Tenericutes was identified only under *L. plantarum* treatment, and this aligns with previous report that lactic acid bacteria favor the growth of Tenericutes [53]. Our data show a decreased trend of F:B ratio ($P>0.05$) after probiotic and postbiotic treatments. This is important since increased F:B ratio is often associated with obesity [54, 55], and previous studies also showed that probiotic treatment generally decreases F:B [56].

At the order level, the populations of Clostridiales, Veillonellales, and Enterobacterales were distinctly enriched due to butyrate treatment. Various members of the Clostridiales order, especially butyrate-producing species, may directly use butyrate as an energy substrate or a signaling molecule to enhance their growth and activity [57]. Within the AMMR system, where the nutrient availability is tightly controlled and other metabolic substrates are restricted, the direct supply of butyrate might provide a competitive advantage to these organisms, resulting in population growth. Butyrate increased the population of Veillonellales, which generally does not synthesize or utilize butyrate, but uses lactate, which is a prevalent byproduct of carbohydrate fermentation by anaerobes in the gut [58]. If butyrate addition increased the proliferation of primary fermenters that generate lactate, then Veillonellales would subsequently grow owing to increased substrate availability. Butyrate may also affect the community's general metabolic activity, resulting in alterations in fermentation pathways that promote lactate generation. Indeed, our data show an increased population of Bacteroides under butyrate treatment, which is an obligate anaerobe and follows a fermentative mode of energy production [59]. Interestingly, butyrate resulted in suppression of Lactobacillales, a surprising and quite uncommon observation. Lactobacillales are well-studied lactate-producing bacteria and butyrate is typically produced due to lactate fermentation by gut microbes [60]. Consequently, the reduction in lactate-producing microbial orders may result from feedback inhibition caused by butyrate accumulation during feeding or from the acidic pH of the medium created by butyrate (a weak acid), as many lactate-producing species exhibit sensitivity to pH levels. At genus level, control and *L. plantarum* groups showed similar patterns for enriched genera, but butyrate group showed high abundance of *Peptoniphilus* and *Lachnoclostridium* in

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comparison to control and *L. plantarum*. Both are butyrate-producing bacteria and display anti-inflammatory properties [61, 62]. Butyrate supplementation lowers the pH of the medium, favoring the growth of butyrate-producing bacteria [63] and also decreases the abundance of known pathobionts (*Enterococcus*, *Klebsiella*, and *Enterobacter*). The mean proportion of *Bacteroides* and *Klebsiella* was enriched in butyrate and *L. plantarum* treatment, respectively. *Bacteroides* is a known butyrate-producing genus [64, 65], and evidence for increased *Klebsiella* after probiotic treatment is not present in the literature. The possible explanation for this observation could be that probiotic treatment conferred decreased microbial diversity that normally suppresses *Klebsiella*, causing a possibility of niche expansion for it.

Metabolomics data revealed the highest enrichment of octanoic acid and capric acid in all treatments, referring to the healthy metabolome of the gut microbial population [66, 67]. The presence of indole and p-cresol in high concentration in butyrate and *L. plantarum* treatments indicates reduced carbohydrates and increased ammonium levels, potential markers for proteolytic metabolism [68]. This could explain the possible reason for the identification of various peptides, like thr-leu, gln-glu, etc., in both treatments. Capric and octanoic acids are the saturated fatty acids whose mean proportion was found to be higher in *L. plantarum* treatment in comparison to butyrate. Previous studies confirm that fatty acid metabolism and the saturation mechanisms of fatty acids were prominent in *L. plantarum* [69], while butyrate inhibits saturated fatty acids formation and accumulation [70]. *L. plantarum* produces organic acids like lactic acid and acetic acid [71], and in our results, we found a positive correlation between *L. plantarum* treatment and acetic acid. In a previous study utilizing *L. plantarum* for the treatment of high-fat-induced obesity in mice, indole-3-carboxylic acid was found to be one of the significant metabolites associated with *L. plantarum* treatment [72], which confirms a positive association between indole-3-carboxylic acid and *L. plantarum* treatment as found in our study. In case of butyrate treatment, a positive correlation was found with N-acetylputrescine, a polyamine, and caproic acid, a saturated fatty acid. The correlation between butyrate and polyamines is well studied, and in an experiment utilizing mouse colon cancer cells, it was found that butyrate treatment enhances the production of polyamines [73]. Additionally, a study

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by Nzeteu *et al* explains how lactic acid and butyric acid biomass produced in a similar closed anaerobic system can help in increased production of caproic acid [74].

Chemical class enrichment for each treatment revealed the significant enrichment of fatty acids and conjugates in both butyrate and *L. plantarum* treatments. In line, previous study also claimed that probiotic bacteria can produce anti-inflammatory fatty acid conjugates to treat colitis [75]. Butyrate favors the growth of SCFA-producing bacteria [63], and with increased SCFA levels would lead to enrichment of fatty acids. As previously mentioned, identification of indole and p-cresol in butyrate and *L. plantarum* indicates proteolytic activities [68]. This justifies the identification of phenylacetic acid, an intermediate of phenylalanine metabolism, as the highest enriched chemical class and phenylalanine, tyrosine, and tryptophan biosynthesis as highly impacted pathways in butyrate treatment [76]. Also, in *L. plantarum* treatment, the top three impacted pathways are related to protein and amino acid metabolism. Butyrate shows specific enrichment of the pentose phosphate pathway, which aligns with the previous results demonstrating the role of butyrate in higher expression of glucose-6-phosphate dehydrogenase, a key enzyme in the pentose phosphate pathway, for nucleic acid synthesis and reactive oxygen species generation to initiate host defense [77].

The gut microbial metabolic functions based on KEGG functional orthologs were computed using sequence data and further validated using the metabolomic profile. Several commonly enriched microbial pathways were identified using both approaches, while uniquely enriched pathways were identified across treatments. For instance, galactose, lipoic acid, and pyruvate metabolic pathways are universally enriched since they represent fundamental energy and redox modules in gut microorganisms [78, 79]. Galactose catabolism generates glucose-6-phosphate for glycolysis, facilitating ATP production via substrate-level phosphorylation and producing NADH that propels subsequent oxidation [80]. The pyruvate metabolism is crucial since the pyruvate dehydrogenase complex irreversibly decarboxylates pyruvate to acetyl-CoA, connecting glycolysis to the TCA cycle for optimal ATP production by oxidative phosphorylation [81]. These processes

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produce NADH and $FADH_2$, whose reoxidation (via respiration or fermentation) maintains the NAD^+/NADH equilibrium. Under anaerobic conditions, gut bacteria often convert pyruvate to lactate or ethanol to produce NAD^+, hence directly associating these pathways with redox equilibrium [82]. Lipoic acid is an essential cofactor for pyruvate dehydrogenase and other 2-ketoacid dehydrogenases, linking carbon metabolism to electron transport; its redox-active disulfide bond also connects metabolism to oxidative stress responses [83]. These mechanisms together provide energy carriers (ATP), reducing equivalents, and biosynthetic precursors, constituting preserved "housekeeping" capabilities essential for microbial growth and survival. Consequently, all groups exhibit enrichment in these pathways as shown by KEGG annotations. Nevertheless, the elevated enrichment in control and *L. plantarum* groups, relative to butyrate, likely indicates active carbohydrate fermentation within those communities. *L. plantarum* and other fermentative organisms depend on efficient glycolytic and PDH/lipoate mechanisms to extract energy from carbohydrates. In contrast, the butyrate-treated group exhibited reduced enrichment, likely attributable to metabolic feedback and alterations in community dynamics. Exogenous butyrate may undergo β-oxidation to provide acetyl-CoA, which directly energizes the TCA cycle, potentially meeting energy requirements and inhibiting glycolysis/galactose metabolism via end-product feedback mechanisms [84]. Moreover, butyrate may promote SCFA-utilizing taxa to the detriment of sugar-fermenters, hence modifying substrate availability [85]. This feedback control and microbial turnover may reduce the incidence of galactose utilization and PDH-dependent genes.

In the butyrate-treated group, butyrate likely played a central role as a regulatory signal and favored energy substrate for gut microorganisms. Its presence within the closed system could be sensed as excess energy availability, resulting in feedback inhibition. In line, glycolysis and gluconeogenesis diminish since butyrate was the direct energy source (acetyl-CoA), hence lessening the need for glucose catabolism or anabolism [86]. Fatty acid biosynthesis was inhibited because butyrate, a fatty acid derivative, satisfied the requirement for additional fat [87]. The metabolism of lipoic acid, essential as a cofactor in primary energy pathways such as pyruvate dehydrogenase (connecting glycolysis to the TCA cycle), subsequently diminishes when these

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fundamental energy-producing mechanisms become less requisite in the presence of enough butyrate [88]. This signifies a transition towards energy conservation and the exploitation of available butyrate.

However, in the case of *L. plantarum* treated group, the augmentation of certain microbial metabolic pathways may be ascribed to its diverse interactions with the gut microbiota. For instance, *L. plantarum* enhances pyruvate metabolism and glyoxylate/dicarboxylate metabolism likely by acting as a trigger for glycolysis, decomposing available carbohydrates into pyruvate and phosphoenolpyruvate [89]. These intermediates can enter the glyoxylate shunt, circumventing decarboxylation stages in the TCA cycle, hence facilitating efficient carbon utility for biosynthesis under low-energy circumstances. Simultaneously, other carbon fixation pathways are enhanced to facilitate anaplerotic metabolic reactions, restoring oxaloacetate and succinate for the synthesis of amino acids and nucleotides [90]. The enhancement of valine, leucine, isoleucine, and alanine/aspartate/glutamate metabolism under *L. plantarum* can provide essential substrates for microbial growth and colonization [91]. Branched-chain amino acids (BCAAs) act as nitrogen donors for several metabolic pathways [92], while glutamate produces glutathione [93]. Under *in vivo* conditions, the metabolism of arginine and proline can facilitate collagen synthesis and immune cell communication [94], consistent with *L. plantarum*'s established function in improving mucosal integrity [95]. *L. plantarum*-induced enhancement of sulfur metabolism and taurine/hypotaurine metabolism could help alleviate oxidative stress [96]. The reduction of sulfur produces hydrogen sulfide, which, at healthy concentrations, can activate antioxidant mechanisms. Taurine and hypotaurine metabolism, enhanced under *L. plantarum*, can function as an effective ROS quencher. This is crucial in inflammatory environments since *L. plantarum* inhibits pro-oxidant pathogens. Collectively, *L. plantarum* functions as a metabolic coordinator, enhancing cross-feeding networks that maximize energy extraction, redox equilibrium, and structure biosynthesis, thereby explaining the extensive upregulation of several interrelated pathways.

Finally, it is to be noted that the current observations emerged out of an *in vitro* anaerobic culture system. Although the AMMR system is able to strictly control certain biochemical parameters of the human distal intestine, instruments like these, are unable to capture the totality of the intricate and dynamic host-microbe interaction that is influenced by various physiological conditions like gut epithelial response, mucus layer dynamics, hormonal levels, immune cell signalling and metabolomic crosstalk. Additionally, the efficacy of probiotics and postbiotics is inherently connected to their nutritional context and food matrix. Their metabolic fate is influenced by intricate interactions within the specific functional food matrix, which includes various nutrients, fibers, and metabolites present. Consequently, outcomes observed in a specific formulation or a simplified experimental model may not reliably predict their behaviour in a different food or within the human gut. Nevertheless, the current study provides preliminary glimpse of how probiotic and postbiotic differentially impacts the gut microbiota in an host-independent controlled environment. Further, *in vivo* intervention studies are essential to validate these results.

Conclusion

This study elucidates distinct roles of butyrate and *L. plantarum* on gut microbial activities and community composition within an *in vitro* colonic model named AMMR system. Butyrate markedly increased total microbial diversity, likely due to improved cross-feeding, whereby butyrate acts as an energy substrate for secondary fermenters, promoting trophic networks and niche specialization. It inhibited both Firmicutes and Bacteroidetes while enhancing the abundance of Proteobacteria and Actinobacteria. In addition, a reduction in harmful genera (e.g., *Enterococcus*, *Klebsiella*, *Enterobacter*) was observed while selectively fostering beneficial butyrogenic taxa (e.g., *Lachnoclostridium*, *Blautia*). Butyrate functionally switched metabolism from antibiotic and fatty acid biosynthesis to amino acid synthesis (valine, leucine, isoleucine, histidine), energy pathways (propanoate metabolism, pentose phosphate pathway), and motility, while reducing secondary bile acid production (suppression of inflammatory metabolic pathways). The metabolomic profile exhibited a correlation with N-acetylputrescine and caproic acid, indicating modifications in polyamine and fatty acid metabolism. Conversely, *L. plantarum* maintained community diversity

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similar to the control, exerting small influence on the F:B ratio or prospective pathobionts (e.g., *Enterococcus*). It enhanced Bacteroidetes and lactic acid bacteria, and metabolically elevated redox balance pathways (taurine/hypotaurine metabolism, sulfur metabolism) and amino acid breakdown (valine, leucine, isoleucine), while diminishing glycosaminoglycan destruction. The metabolome exhibited a favorable association with acetic acid and indole-3-carboxylic acid, consistent with its lactic acid synthesis. In contrast to butyrate, *L. plantarum* augmented pyruvate and glyoxylate metabolism, facilitating carbon fixation and biosynthesis under low-energy conditions. Collectively, the findings of the study reveal that butyrate primarily functions as a metabolic regulator, influencing community composition and functionality via metabolic feedback and cross-feeding, diminishing pathogens and promoting energy and amino acid metabolism. *L. plantarum,* on the other hand, functioned as a metabolic coordinator, enhancing lactic acid bacteria and stress-response pathways without significantly modifying diversity or pathogen abundance. Therefore, the combined application of both probiotic strain and postbiotic metabolite may provide a promising basis for symbiotic formulations for enhanced gut homeostasis and alleviate gut associated metabolic disorders.

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Conflict of interest: The AMMR system is the proprietary item of Fermibio Solutions Private Limited, with which Priyankar Dey & Prangya Ranjan Rout are affiliated.

Contribution: NB performed the experiments, conducted the analysis and wrote the 1st draft of the manuscript along with PD; PRR edited the manuscript and supervised the project; PD supervised the project, designed the experiments, and performed the statistical analysis. All authors have read and approved the final version of the manuscript.

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Figure legends

Figure 1: Brief experimental design of the study. Fecal sample from healthy individual was used as inoculum for gut bacteria and treated with *L. plantarum* (probiotic) and butyrate (post-biotic) in an individual fermenter of AMMR system. After 48 h of treatment, the sample were collected for subsequent metagenomic and metabolomic analyses.

Figure 2: Shifts in microbiome profile in response to butyrate and *L. plantarum* treatments. (A-C) Bar plots representing relative abundance of taxa at (A) phylum, (B) order, and (C) genus levels across the treatments. (D-F) Differential genus identification using STAMP between (D) control v/s butyrate, (E) control v/s *L. plantarum*, and (F) *L. plantarum* v/s butyrate. (G) Correlation heatmap depicting degree of association between identified genera and the treatments

Figure 3: Functional metagenomic predictions using PICRUSt. Differential prediction of KEGG orthologs (KO) in (A) control v/s butyrate and (B) control v/s *L. plantarum*. (C) Common predicted KO across the treatments. (D) Heatmap showing the relative abundance of KO among the different treatments

Figure 4: Metabolomic profiling of gut microbes under the influence of butyrate and *L. plantarum* treatments. (A) Representative GC-MS graph for all treatments with retention time (min) on x-axis and intensity on y-axis. (B) Venn diagram showing common metabolites identified across the treatments. (C-E) STAMP plots showing the differential abundant metabolites between (C) control v/s butyrate, (D) control v/s *L. plantarum*, and (E) butyrate v/s *L. plantarum*. (F) Correlation heatmap depicting the degree of association between identified metabolites with the treatments. (G) Venn diagram representing the metabolite class common across the treatments. (H-J) Metabolite class enrichment in (H) control, (I) butyrate, and (J) *L. plantarum* treatments. The x-axis represents enrichment ratio value and y-axis depicts the significance value of enrichment.

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Figure 5: Metabolic pathway enrichment in (A) control, (B) butyrate, and (C) *L. plantarum*. The x-axis corresponds to pathway impact value and y-axis depicts significance of impact value. 1. Riboflavin metabolism, 2. D-amino acid metabolism, 3. Pyrimidine metabolism, 4. Phenylalanine, tyrosine and tryptophan biosynthesis, 5. Phenylalanine metabolism, 6. Lipoic acid metabolism, 7. Galactose metabolism, 8. Pyruvate metabolism, 9. Tryptophan metabolism, 10. Purine metabolism, 11. Other carbon fixation pathways, 12. One carbon pool by folate, 13. Propanoate metabolism, 14. Pentose phosphate pathway, 15. Glycolysis or gluconeogenesis, 16. Fatty acid degradation, 17. Fatty acid biosynthesis, 18. Arginine and proline metabolism, 19. Biosynthesis of unsaturated fatty acids, 20. Valine, leucine and isoleucine degradation, 21. Taurine and hypotaurine metabolism, 22. Lysine degradation, 23. Valine, leucine and isoleucine biosynthesis, 24. Methane metabolism, 25. Sulfur metabolism, 26. Alanine, aspartate and glutamate metabolism, 27. Glyoxylate and dicarboxylate metabolism, 28. Selenocompound metabolism, 29. Arginine biosynthesis, 30. Glycine, serine and threonine metabolism, 31. Lysine biosynthesis. (D) Venn diagram representing the pathway common across the treatments.

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Figure 1

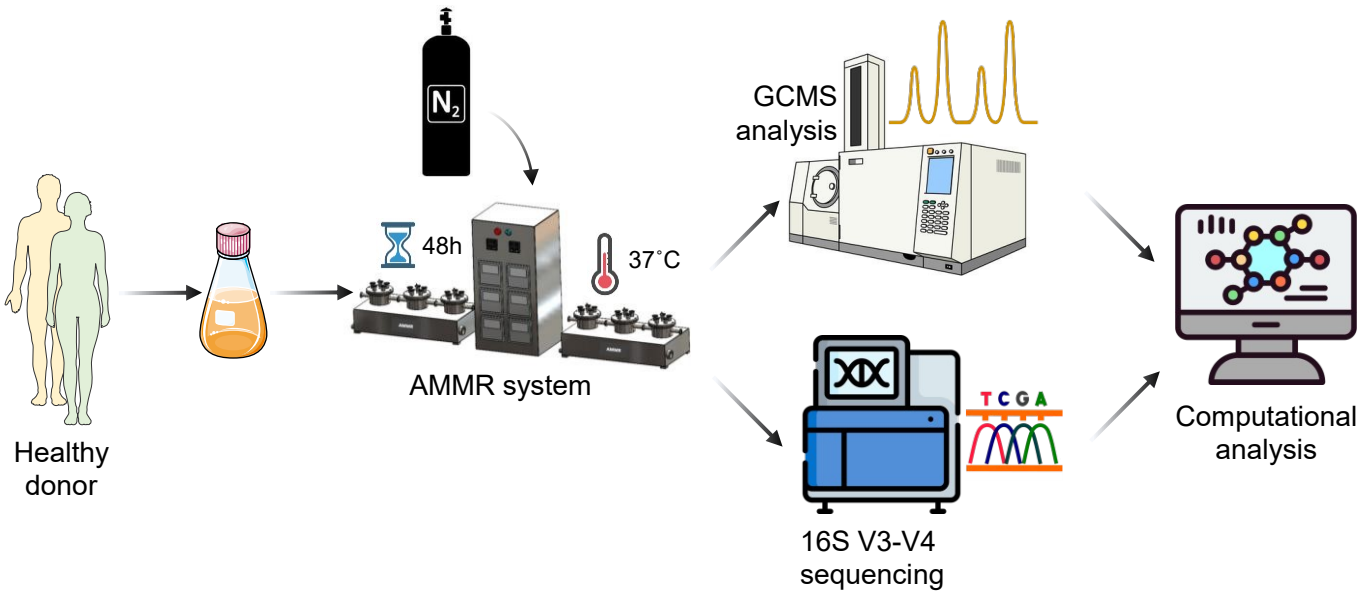

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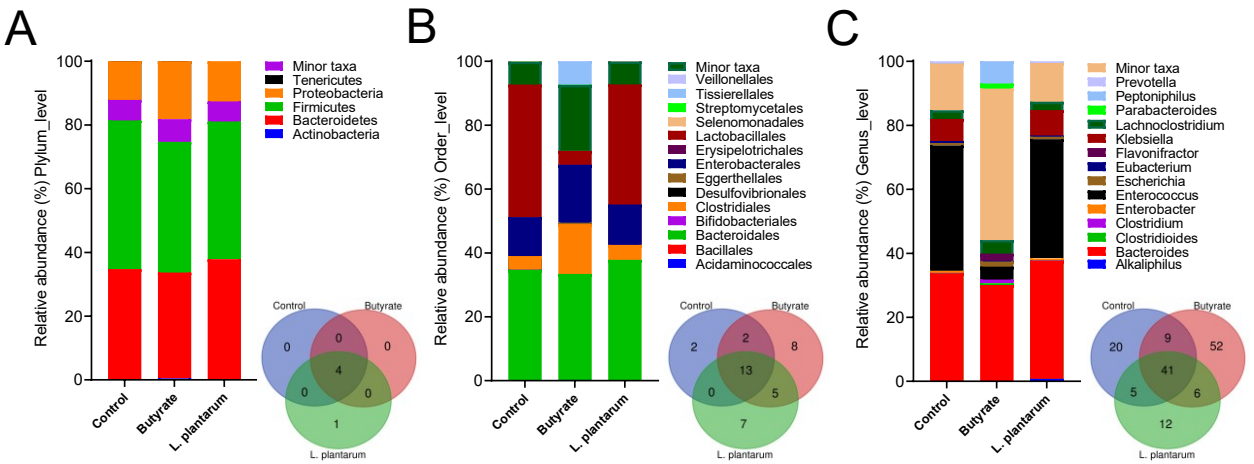
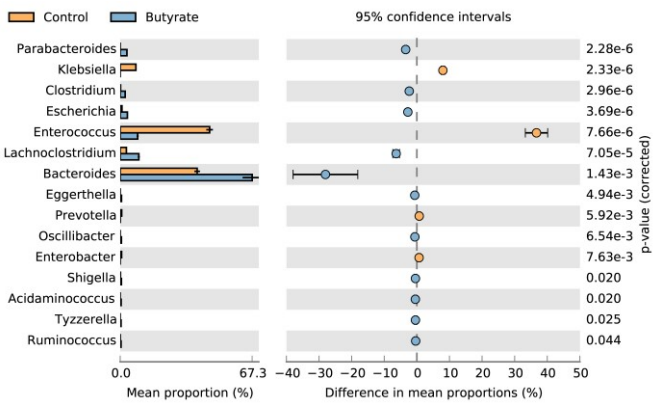
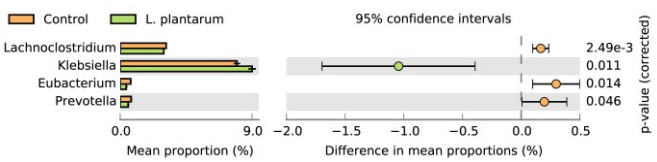
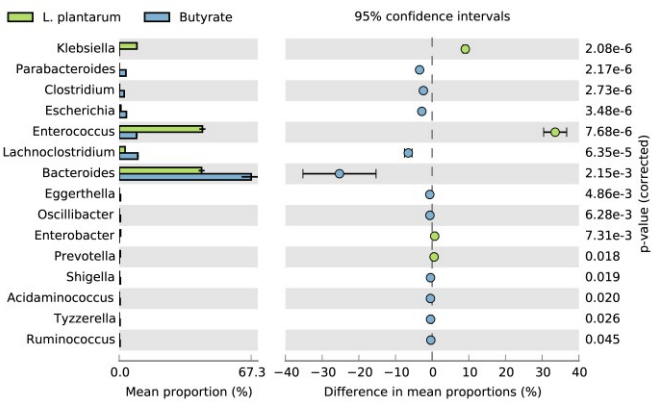
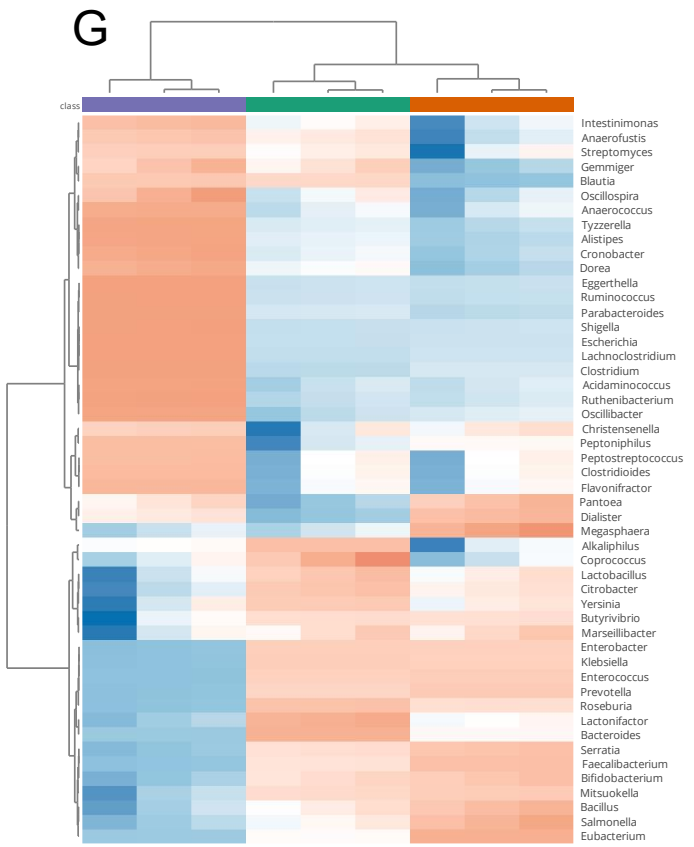
Figure 2











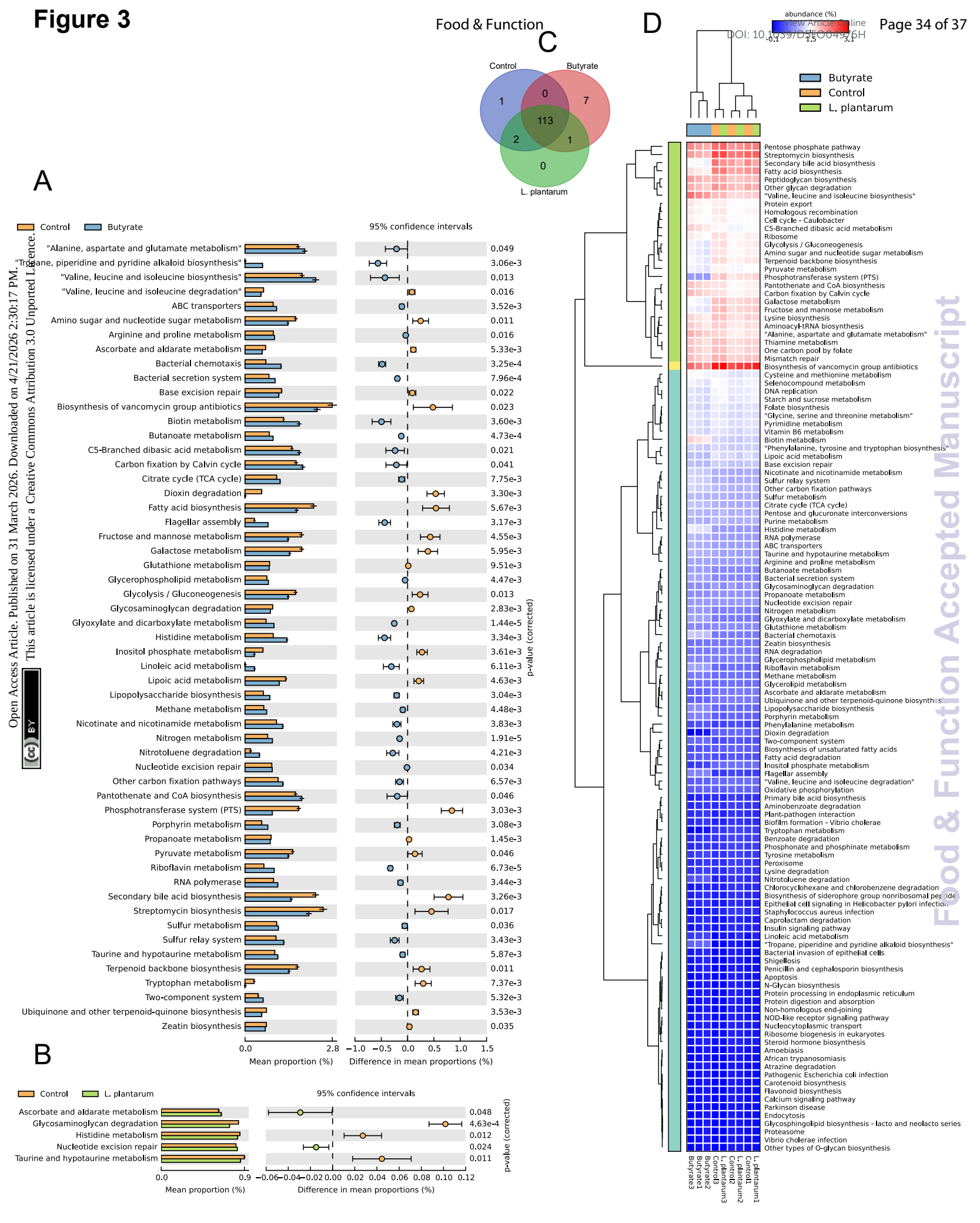


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Figure 4

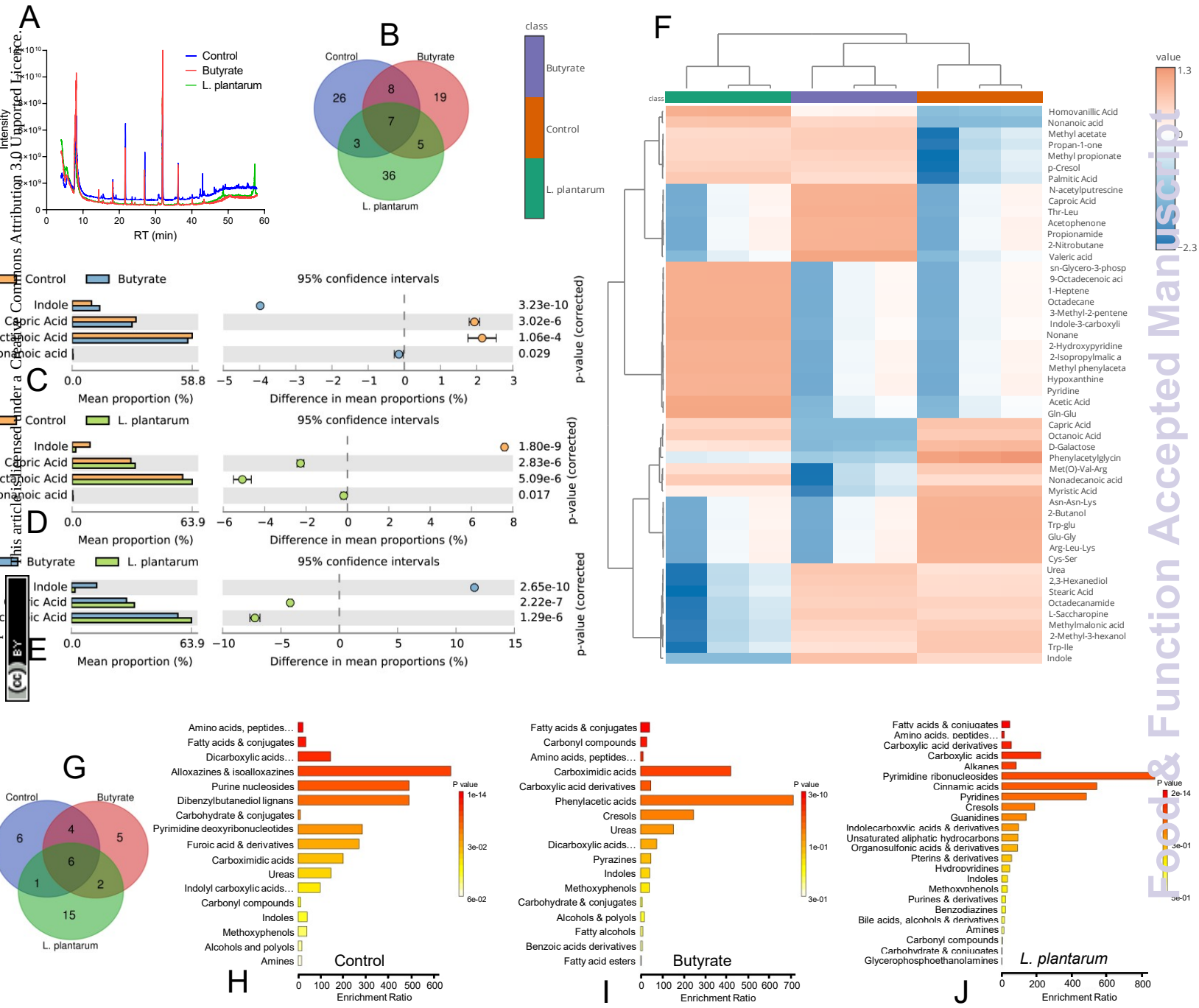

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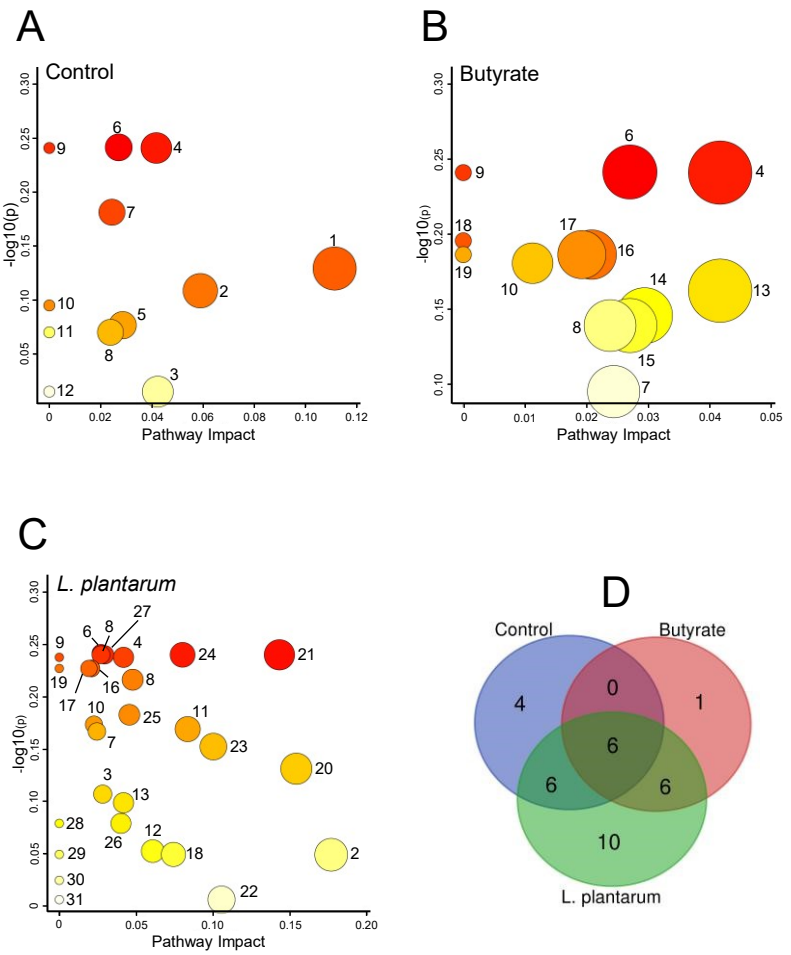
Figure 5



All necessary data are included in the supplementary information.

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