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Inulin-Bentonite Hybrid Microparticles Enhance Orlistat Efficacy While Mitigating Gut Microbiota Disruption in a High-Fat Diet Rat Model

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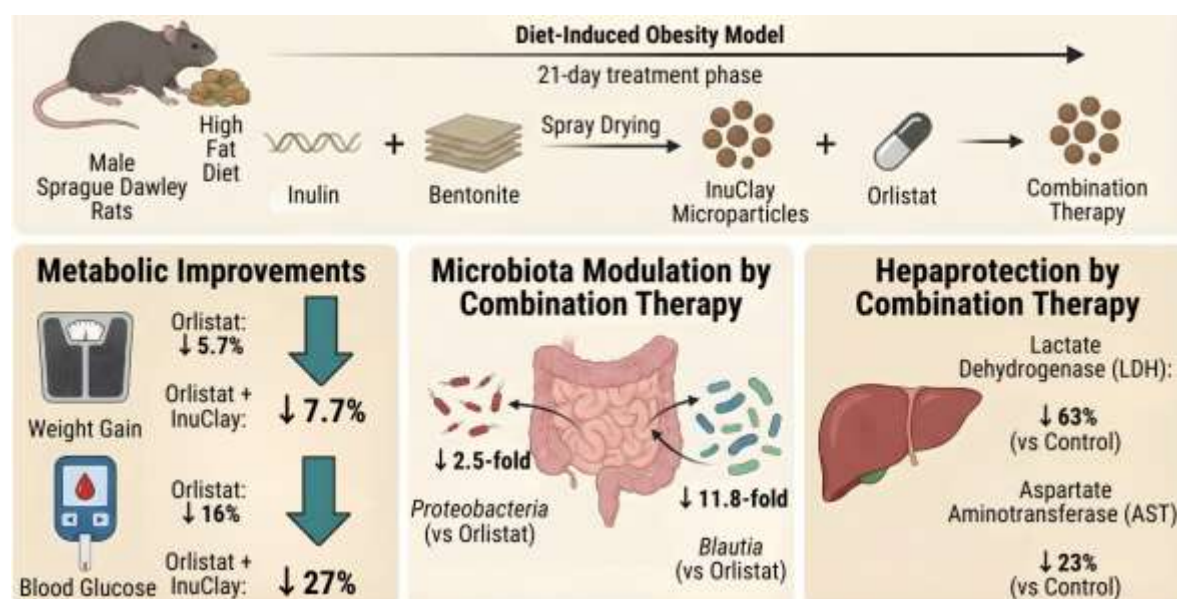
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Graphical Abstract:



Abstract: Orlistat is an established pharmacotherapy for metabolic syndrome (MetS); however, its clinical utility is constrained by gastrointestinal adverse effects and gut microbiota disruption. This study evaluates hybrid inulin (INU)-bentonite (BEN) microparticles (InuClay) as a microbiota-protective adjunct to orlistat therapy. *In vitro* lipolysis under biorelevant fasted-state conditions demonstrated that InuClay significantly reduced free fatty acid liberation relative to untreated controls and INU alone ($p < 0.01$). In a 21-day high-fat diet rat model of MetS, InuClay and orlistat monotherapies reduced cumulative weight gain by 4.5% and 5.7%, respectively. Co-administration achieved a 7.7% reduction that was significantly greater than either monotherapy alone. Combination therapy also produced the greatest reduction in fasting blood glucose (27%) exceeding both InuClay monotherapy (12%) and orlistat monotherapy (16%). At the microbiota level InuClay co-administration prevented orlistat-induced *Proteobacteria* expansion (2.5-fold reduction versus orlistat alone) while selectively enriching butyrate-producing taxa including *Lachnospiraceae* (1.42-fold) and *Blautia* (11.8-fold) without significantly altering alpha diversity. Circulating markers of cellular injury were substantially lower with combination therapy, with lactate dehydrogenase decreased by 63% and aspartate aminotransferase by 23% versus controls. These reductions were absent with orlistat monotherapy. Collectively these findings establish InuClay as a dual-action adjunct that enhances orlistat efficacy while attenuating dysbiotic shifts and systemic markers of tissue stress. This approach represents a promising strategy for improving translational outcomes in lipase inhibitor-based MetS management.

Keywords: orlistat; drug delivery adjunct; obesity; gut microbiota modulation; inulin-bentonite hybrid; metabolic syndrome.

1. Introduction

Metabolic syndrome (MetS) affects approximately one in four adults globally, with prevalence continuing to rise alongside increasing obesity and physical inactivity [231, 2]. MetS is defined by a cluster of metabolic risk factors including abdominal obesity, insulin resistance, hypertension, and dyslipidemia, which collectively increase the likelihood of developing type 2 diabetes and cardiovascular disease [3-5]. While lifestyle interventions remain first-line therapy, pharmacological interventions are frequently required to achieve clinically meaningful outcomes [6, 7].

Orlistat, a gastrointestinal lipase inhibitor, is one of the few FDA-approved long-term pharmacotherapies for obesity and MetS [8]. By irreversibly inhibiting gastric and pancreatic lipases, orlistat reduces dietary fat absorption by approximately 30-35%, leading to modest but clinically significant weight loss of 3.9kg over placebo after 12 months [9-12]. Despite its established efficacy and favorable safety profile compared to systemically-acting anti-obesity drugs, orlistat's clinical utility is substantially limited by steatorrhea, affecting up to 53% of users [13-16]. These gastrointestinal side effects are the leading cause of discontinuation. Meta-analyses and large trials report that 5-8% of orlistat users discontinue due to adverse events, primarily gastrointestinal-related, compared to 0-4% in placebo groups [14, 17, 18]. Moreover, emerging evidence suggests that orlistat alters gut microbiota in obese mouse models, typically reducing overall microbial diversity and shifting the abundance of specific bacterial taxa, typically reducing overall microbial diversity and shifting the abundance of specific bacterial groups [19, 20]. Specifically, orlistat increases fecal fat excretion, resulting in more dietary lipids available to colonic bacteria [21]. This environment can favor lipid-metabolizing taxa, such as *Proteobacteria*, and their metabolites can impair gut barrier integrity [22]. Some studies suggest these shifts may synergize with orlistat's metabolic benefits, but also raise concerns about increased pro-inflammatory bacteria and reduced barrier function [23]. Additionally, orlistat has been associated with rare

but serious hepatic adverse events. Multiple published cases document acute liver failure and severe hepatitis linked to orlistat, sometimes necessitating liver transplantation [24, 25]. These limitations highlight the need for adjunct therapies that can enhance orlistat's lipid-lowering effects while mitigating its adverse impact on gut microbiota and hepatic function.

Addressing these limitations requires therapeutic strategies that can complement orlistat's lipase inhibition while actively supporting beneficial gut microbiota. Food-grade biomaterials represent a promising approach, as they can modulate nutrient absorption and microbiota composition without systemic exposure [26-28]. Among these, hybrid systems combining prebiotic fibers with mineral adsorbents may offer synergistic benefits, such as restricting lipid absorption through physicochemical mechanisms while providing fermentable substrates to maintain or restore beneficial microbial populations. Of these materials, inulin (INU) is a fermentable prebiotic fiber that is selectively broken down by gut microbes expressing enzymes such as exo-inulinase, β -fructosidase, and β -fructofuranosidase [29]. This process depends on the species and strain of bacteria. Certain strains of *Bifidobacterium* and *Lactobacillus* can efficiently ferment INU and produce short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate [30, 31]. Animal studies show that this fermentation increases the release of satiety hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which helps reduce food intake and improve metabolic outcomes [32, 33]. These effects are driven by the activation of FFA receptors 2 and 3 (FFAR2 and FFAR3) on enteroendocrine L-cells by SCFAs, which promotes hormone secretion [34]. Besides weight loss, INU (1 g/kg body weight) has also been demonstrated to reduce systemic glucose levels in HFD rats across 21 days, with long-chain INU (average degree of polymerization = 27) exhibiting the strongest effect [35]. Moreover, INU supplementation has been shown to increase SCFA production and improve gut barrier recovery *in vitro* intestinal models. Building on these established prebiotic effects, our group recently demonstrated that inulin-lipid hybrid systems can target gut microbiota while

controlling drug release, achieving microbiome modulation alongside pharmaceutical delivery [36, 37]. Extending this concept to metabolic interventions, we hypothesized that inulin-based formulations could address orlistat's microbiota-disrupting effects.

However, under high-fat diet (HFD) conditions, the prebiotic benefits of INU can be limited due to pathobiont shifts within gut microbial composition [38]. To improve its metabolic impact in diseased models, recent studies have focused on combining INU with other food-grade materials as novel hybrid or composite materials with improved gut-activity [36, 39]. Complementary to orlistat's enzymatic inhibition, nanostructured clays such as bentonite (BEN) offer a distinct physicochemical mechanism for restricting lipid absorption. These nanostructured clays are proposed to restrict lipid digestion at their platelet edges through hydrogen bonding, rather than by intercalating lipids into its interlayer space [40]. This interaction reduces the availability of FFAs for micelle formation and disrupts the self-assembly of lipid digestion structures, leading to lower lipid absorption during digestion. In an obese animal model, MMT and SBA-15 mesoporous silica were shown to trigger statistically significant reductions in rodent weight gain, compared to a control group that was not exposed to any treatment [41]. Importantly, this non-enzymatic mechanism could provide additive lipid restriction when combined with orlistat, potentially allowing dose reduction while maintaining efficacy. While such studies present promise, the long-term efficacy of these biomaterial-based therapies is dependent on their ability to promote anti-MetS therapeutic effects beyond just weight loss, since targeting weight loss alone has been shown to be ineffective in providing long-term therapeutic effect against MetS [42-45].

Recent findings support the potential of hybrid systems to address this broader therapeutic need [46, 47]. InuClay has been shown to concurrently suppress intestinal lipid digestion and modulate the gut microbiota under HFD conditions [47]. *In vitro* simulated intestinal lipolysis experiments demonstrated that InuClay reduced FFA release approximately four-fold compared with HFD controls, outperforming INU and MMT

administered individually. This enhanced inhibition was attributed to the hybrid's improved clay dispersion, producing smaller, more uniformly distributed particles with superior lipid-binding capacity. *In vivo*, daily oral administration of InuClay (1 g/kg bodyweight) for 21 days significantly attenuated cumulative HFD-induced weight gain by 4.7%, compared with reductions of 2.0% and 1.5% for INU and MMT alone, respectively. Gut microbiota profiling further revealed increased microbial diversity and substantial enrichment of health-associated taxa, including *Peptostreptococcaceae* (8-fold), *Ruminococcaceae* (3.5-fold), *Akkermansiaceae* (2.5-fold), and *Eggerthellaceae* (7.7-fold), alongside a distinct beta-diversity profile relative to HFD and INU groups.

Together, these data indicate that InuClay provides dual mechanistic activity in physicochemical lipid restriction and targeted microbiota modulation - when administered as a standalone therapy in HFD-induced obesity. Despite orlistat's established clinical use, no adjunct therapies currently exist to enhance its weight-loss efficacy while preventing microbiota disruption or limiting gastrointestinal and hepatic side effects. Given the complementary mechanisms of INU and MMT, InuClay represents a candidate biomaterial capable of supporting orlistat therapy through three concurrent pathways: (1) additive restriction of intestinal lipid availability via physicochemical adsorption, (2) mitigation of orlistat-associated microbiota perturbations through selective support of beneficial taxa, and (3) attenuation of pharmacotherapy-associated tissue stress.

In this study, spray dried InuClay hybrid microparticles were evaluated as an adjuvant to orlistat in a HFD rat model of metabolic syndrome. InuClay monotherapy, orlistat monotherapy, and combination therapy of InuClay with orlistat were compared across metabolic, microbiota, and hepatic outcome measures. The findings provide proof-of-concept evidence that microbiota-targeted adjuncts can enhance orlistat therapy while mitigating treatment-limiting adverse effects.

148 2. Materials and Methods

149 2.1. Materials

150 Inulin (INU) from chicory root with a degree of polymerization of 7 (determined by ¹H
151 NMR end-group analysis [48] and sodium bentonite (BEN) were supplied by Pharmako
152 Biotechnologies (Frenchs Forest, Australia). Medium chain triglycerides (MCT; Miglyol
153 812) were obtained from Hamilton Laboratories (Adelaide, Australia).
154 FaSSIF/FeSSIF/FaSSGF powder was purchased from Biorelevant.com Ltd (London, United
155 Kingdom). Lipase from *Candida antarctica* (6000 TBU/mL), starch (soluble), cellulose,
156 sodium hydroxide pellets, glacial acetic acid, sodium chloride, and phosphate buffered saline
157 (PBS) tablets were supplied by Sigma-Aldrich (Castle-Hill, Australia). Porcine pancreatin
158 extract (activity equivalent to 8 × USP specification) was supplied by MP Biomedicals
159 (Seven Hills, Australia). Four-week-old male Sprague-Dawley rats were purchased from the
160 Animal Resources Centre (Perth, Australia). All chemicals and solvents were of analytical
161 grade and used as received. High-purity Milli-Q water was used throughout the study.

162 2.2. Development and Characterisation of Inulin-Bentonite Hybrid (*InuClay*)

163 *Microparticles*

164 2.2.1. InuClay Microparticle Fabrication

165 InuClay microparticles were fabricated by spray drying a hybrid dispersion of INU and
166 BEN at a mass ratio of 1:1. That is, a 2% w/v INU solution was prepared by dissolving INU
167 (20 g) in purified water (1 L) with stirring at room temperature for 30 min. A separate 1.25%
168 w/v BEN dispersion was concurrently formed by adding BEN (20 g) to purified water (1.6
169 L) with rapid stirring at room temperature for 2 h or until the clay platelets were completely
170 delaminated. The INU solution and BEN dispersion were combined and rapidly stirred for
171 an additional 30 min at room temperature. The hybrid dispersion was subsequently spray

dried (Büchi Mini Spray-Dryer B-290, Switzerland) with the following conditions: inlet temperature 200 °C, outlet temperature 105 °C, aspirator setting 100, nozzle cleaning setting 9, and product flow rate 7 mL/min.

2.2.2. Composition Determination using Thermogravimetric Analysis (TGA)

TGA (Discovery TGA, TA Instruments, New Castle, USA) was used to determine the INU-to-BEN concentration within the fabricated InuClay microparticles. TGA thermograms were acquired by heating samples at a scanning rate of 10 °C/min from 20 to 600 °C under a nitrogen gas atmosphere. The INU content decomposed in the temperature range of 250 - 400°C. The weight loss, after correction for water content, was computed using the associated TA Universal Analysis software. A previous study showed that only a minor portion (~ 1%) of BEN decomposes within this temperature range [49]. Accordingly, the calculated weight loss was attributed primarily to the INU fraction within the hybrid microparticles.

2.2.3. Particle Sizing and Morphological Analysis using Scanning Electron Microscopy (SEM)

The primary particle diameter and surface morphology of InuClay microparticles were studied by high-resolution analytical SEM (Zeiss Merlin, Oberkochen, Germany). Samples were mounted on double-faced carbon tape and sputter coated with ~10-20 nm platinum prior to imaging at an accelerating voltage of 1-2 kV. The mean particle diameter was determined from ≥ 100 particles, using ImageJ (Fiji) [50], since InuClay particles were prone to particle aggregation in aqueous media, which precluded the use of conventional scattering-based particle sizing techniques.

2.3. *In Vitro* Gastrointestinal Lipolysis Studies

2.3.1. Preparation of Biorelevant Lipolysis Media

A two-step *in vitro* GI lipolysis model [51] was employed under simulated fasted and fed state conditions to test the inhibitory effect of InuClay microparticles and its precursor materials on lipase-mediated digestion in media that mimic the GI environment before (fasted state) and during (fed state) digestion of a high-fat, high-carbohydrate content meal. FaSSIF/FaSSIF/FaSSGF powder was used to prepare fasted state simulated gastric fluid (FaSSGF; pH 1.6), fasted state simulated intestinal fluid (FaSSIF; pH 6.5), fed state simulated gastric fluid (FeSSGF; pH 5.0) and fed state simulated intestinal fluid (FeSSIF; pH 6.0). Manufacturer's protocol (biorelevant.com) was followed in preparing the gastric and intestinal media, which contained biorelevant concentrations of bile salts and phospholipids, as described previously [41, 52]. Media were freshly prepared before each experiment and used within 48 h, according to manufacturer's recommendations. On the day of the experimental procedure, pancreatin extracts were prepared by stirring 2 g of pancreatin powder in 10 mL of intestinal lipolysis buffer (pH 6.5) for 15 min, followed by centrifugation (2268 ref, 20 min, 4°C). The supernatant was collected and kept refrigerated until use.

2.3.2. Gastrointestinal Lipolysis under Simulated Fasted & Fed State Conditions

Details of the lipolysis media properties/excipients added to mimic the fasted and fed states are provided in Table 1. Firstly, MCT, starch and cellulose were dispersed within FaSSGF or FeSSGF (10 mL) dispersed by continuous stirring at 600 rpm for 10 min in a thermostated glass reaction vessel (37°C). To this dispersion, InuClay microparticles were added at 25% w/w relative to the lipid content. The pH of lipolysis medium was re-adjusted with 0.1 M NaOH or HCl to the pre-set value for FaSSGF or FeSSGF, prior to initiation of simulated gastric lipolysis via addition of lipase from *Candida antarctica*. Gastric lipolysis was monitored for 30 min, where the pH was continuously adjusted with 0.6 M NaOH using a pH-stat titration unit (902 Titrando, Metrohm, Switzerland) to maintain a constant pH within the digestion media. Upon completion of gastric lipolysis, FaSSIF or FeSSIF (20 mL)

was added and the pH was allowed to equilibrate via auto-titration over a 10 min period. Intestinal lipolysis was initiated through the addition of pancreatin extract and was monitored for 60 min, where the degree of free fatty acid liberation was determined by the amount of NaOH titrated to maintain a constant pH. It is important to note that NaOH titration during the gastric phase under fasted state conditions does not accurately correlate to lipolysis since fatty acids are protonated at pH 1.6. Accordingly, gastric lipolysis data under fasted conditions were not reported [51].

Table 1. Lipolysis media compositions used to mimic the fasted and fed state.

Media properties/excipients	Fasted state	Fed state
pH	Gastric: 1.6 Intestinal: 6.5	Gastric: 5.0 Intestinal: 6.0
MCT	200 mg	625 mg
Starch	-	625 mg
Cellulose	-	31 mg
Lipase	Gastric: 100 μ L <i>Candida antarctica</i> (eq. to 600 TBU) Intestinal: 2 mL pancreatin extract (eq. to 2000 TBU)	Gastric: 200 μ L <i>Candida antarctica</i> (eq. to 1200 TBU) Intestinal: 2 mL pancreatin extract (eq. to 2000 TBU)
InuClay microparticles	40 mg	125 mg

2.4. In Vivo Pharmacodynamic Study

2.4.1. Study Design

The *in vivo* pharmacodynamic study was approved by the Animal Ethics Committee of the Adelaide University (approval #U18-17) and adhered to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and ARRIVE guidelines where applicable. The design of this study, including dosing regimen and study length was based on previous studies [35, 37, 41, 53]. Four-week-old male Sprague–Dawley rats were housed two per cage under controlled temperature and humidity with a 12 h light / 12 h dark cycle, acclimatized for one week, then randomized (n = 5/group) to vehicle control (PBS, 10 mL/kg), InuClay microparticles (1 g/kg), orlistat (75 mg/kg), or orlistat plus InuClay (75

mg/kg + 1 g/kg). Treatments in PBS (10 mL/kg) were given once daily by oral gavage between 16:00 and 18:00 for three weeks; body weight was recorded before dosing each day. All rats received a purified high-fat diet SF00-219 (Specialty Feeds; 21.0% fat, 19.0% protein, 4.70% crude fibre; net metabolizable energy 17.8 MJ/kg; digestible energy 19 MJ/kg), which was gamma-irradiated before use; the full batch-level composition is available from Specialty Feeds on request. A normal chow group was not included because grain-based chows differ markedly from purified high-fat diets in ingredients and micronutrients and would confound interpretation; the aim was to test interventions under a single, well-defined obesogenic background.

2.4.2. Sample Collection & Analysis

After 21 days of treatment, rats were fasted for 24 hours with free access to water, then anaesthetised using sodium pentobarbital (200 mg/kg body weight). Blood samples were collected by cardiac puncture and submitted to SA Pathology (Adelaide, Australia) for full blood analysis. After humane euthanasia, the liver and cecum were aseptically removed. Fresh fecal samples were combined with cecal contents and sent to the Australian Genomics Research Facility (Brisbane, Australia) for DNA extraction and 16S ribosomal RNA gene sequencing. Sequencing targeted the V3 to V4 hypervariable regions, and raw reads were clustered into operational taxonomic units (OTUs) at 97 percent similarity using Quantitative Insights Into Microbial Ecology 2 (QIIME 2). Taxonomy was assigned using the Silva reference database (release 138.1). All post-sequencing analysis was completed using the QIAGEN CLC Genomics Workbench (version 23.0.4) and the Microbial Insights Prokaryotic Taxonomy Database (QMI-PTDB).

2.5. Statistical Analysis

All statistical analysis, excluding 16S rRNA sequencing data, was performed using GraphPad Prism version 10.2.0 (GraphPad Software, Boston, Massachusetts). Normality of

data and residuals was assessed using the Shapiro–Wilk test. If data were normally distributed, group comparisons were made using one-way analysis of variance followed by Tukey’s post hoc test for multiple comparisons. If normality was not met, the Kruskal–Wallis test was used followed by Dunn’s post hoc test. Results are reported as mean $\pm$ standard deviation (SD) unless otherwise specified. Line and bar graphs display mean with standard deviation, while box-and-whisker plots represent the full data range (minimum to maximum). Statistical significance was set at $p < 0.05$ and is shown in figures as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, or **** $p \leq 0.0001$. For 16S ribosomal RNA gene sequencing data, beta diversity was visualized using principal coordinate analysis (PCoA) plots generated from Bray-Curtis dissimilarity matrices. Group differences in microbial composition were assessed using permutational multivariate analysis of variance (PERMANOVA) using the Qiagen CLC software.

3. Results & Discussion

3.1. Hybridization enhances InuClay particle uniformity and smooth surface morphology

Spray drying a hybrid aqueous dispersion of INU and BEN at a 1:1 mass ratio produced spherical InuClay microparticles ranging from 1 to 10 μm in diameter. Scanning electron microscopy (SEM) revealed that this process reduced particle size and improved size uniformity compared to the precursor materials. INU formed large, irregular structures with collapsed surfaces and a wide size distribution (**Fig. 1A**), while BEN appeared as fragmented aggregates with rough and jagged morphologies (**Fig. 1B**). In contrast, InuClay microparticles were more spherical and homogeneous in structure (**Fig. 1C & 1D**), indicating that spray drying reorganized the INU and BEN components into a hybrid morphology not observed in either precursor material. This may reflect the ability of soluble INU to migrate toward the droplet surface during drying, forming a surface-enriched shell that smooths the particle exterior. Previous studies report that spray-dried INU often forms collapsed or

wrinkled particles due to uneven water loss across the droplet surface [54]. Similarly, spray drying BEN has been shown to produce granules with layered silicate structures or irregular jagged textures, depending on the swelling properties of the clay [41, 53, 55]. The uniform and smooth morphology observed for InuClay suggests that INU may have filled the interparticle voids between BEN platelets, though further surface and structural analyses would be required to confirm this hypothesis.

TGA determined the INU content of the final InuClay microparticles to be 41.7%, which is lower than the expected 50% based on the input mass ratio. This suggests that INU may have been partially lost during spray drying or unevenly incorporated into the final hybrid particles. INU is sensitive to thermal and shear conditions, and partial breakdown can occur during high-temperature exposure of the spray dryer [54]. However, the reduced content may also reflect preferential localization of INU to specific domains within the hybrid InuClay matrix, rather than uniform incorporation. Further structural investigations using imaging or spectroscopic techniques are needed to confirm whether non-uniform partitioning contributes to the observed INU loss.

The smooth surface morphology observed for InuClay microparticles is not typical of spray-dried BEN, which usually retains its layered platelet structure and forms fractured, jagged surfaces [40, 53]. The absence of these features in the hybrid suggests that INU may play a structural role during spray drying. One proposed mechanism is that dissolved INU migrates toward the droplet surface during solvent evaporation, where it rapidly vitrifies to form a continuous shell [56]. This could account for the smoother surfaces seen in InuClay and the lack of visible clay platelets at the exterior. Spray-dried INU alone has been shown to form smooth-walled capsules when fully solubilized, with surface vitrification contributing to shell formation [57]. Future studies using cross-sectional particle imaging or spectroscopic surface mapping would help confirm whether INU encapsulates the clay phase during particle formation.

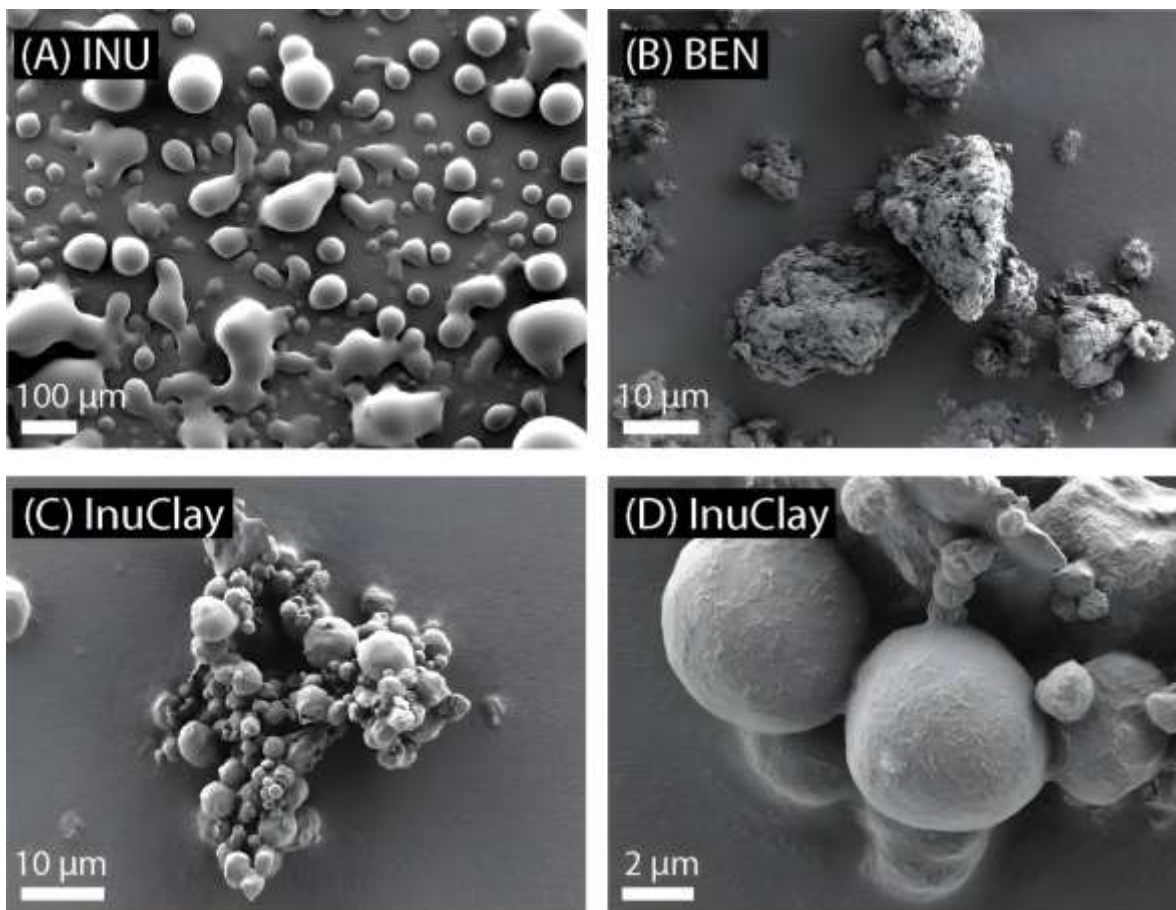


Figure 1. SEM images of spray-dried INU (A), BEN (B), InuClay (C), and higher magnification of InuClay (D) demonstrating that hybridization via spray drying produced spherical microparticles with smooth, uniform surfaces not observed in either precursor material. Scale bars: A = 100 μm, B and C = 10 μm, D = 2 μm.

3.2. *In Vitro* Lipolysis under Simulated Gastrointestinal Conditions

3.2.1. Impact of Combining Inulin & Bentonite on Lipolysis Kinetics

The impact of InuClay on *in vitro* lipolysis under simulated fasted (**Fig. 2A**) and fed state conditions (**Fig. 2B**) was assessed using a two-step GI lipolysis model adapted from prior protocols [41, 58]. This model aims to replicate the sequential digestion processes encountered in the human GI tract. Due to methodological limitations in quantifying gastric lipolysis at pH 1.6 in fasted-state conditions, only data from the intestinal phase are presented. As expected, cumulative FFA release was substantially greater in the fed state ($44.3 \pm 4.10 \mu\text{mol}$) compared to the fasted state ($16.1 \pm 1.20 \mu\text{mol}$), reflecting the physiological upregulation of lipid digestion following food intake. This difference reflects

the higher enzymatic activity and bile salt concentrations characteristic of fed-state digestion, which enhance lipid emulsification and lipase accessibility [59]. In contrast, during fasting, lipase activity is suppressed in adipose tissue and fatty acid metabolism is redirected toward skeletal muscle utilization. Additionally, bile salt concentrations are known to double in the fed state relative to the fasted state, facilitating more efficient emulsification of dietary fats and increasing substrate availability for lipase action [60].

InuClay consistently reduced cumulative FFA release in both nutritional states, though the magnitude and statistical significance of this effect varied between conditions. Under fasted conditions, InuClay significantly suppressed cumulative FFA release compared to untreated controls ($p = 0.0019$, **Fig. 2A inset**). This reduction was also significant when compared to INU alone ($p = 0.0007$), but not to BEN ($p = 0.3595$). Interestingly, BEN and INU exhibited divergent effects, with BEN releasing significantly less FFA than INU ($p = 0.0203$), but not differing significantly from the untreated group. These findings indicate that the hybridization of INU and BEN in InuClay yields a material with enhanced lipolysis-modulating activity under fasted conditions, likely reflecting additive effects not present in the individual components. In the fed state, InuClay did not significantly reduce FFA release compared to the untreated control group ($p = 0.1466$, Fig. 2B inset), nor was it statistically different from either INU ($p = 0.1616$) or BEN ($p = 0.4247$), despite a trend toward lower FFA levels. In contrast, BEN alone significantly suppressed FFA liberation relative to both untreated ($p = 0.0071$) and INU ($p = 0.0080$) groups, reinforcing previous findings that BEN exerts a dominant modulatory effect on lipid digestion under fed-state conditions [40, 41, 49, 53, 61]. INU alone had no measurable impact on lipolysis kinetics, which is consistent with its high aqueous solubility during digestion [62]. Lipolysis is regulated by the accessibility of lipase, an interfacially active enzyme, to the lipid–water interface. Unlike water-insoluble particles that can adsorb at this interface and inhibit enzyme activity, soluble polymers like

INU remain dispersed in the continuous phase and are unlikely to impede lipase-substrate interactions [63].

The lipolytic effects of BEN corroborate mechanistic insights reported in previous studies, which demonstrate that BEN-like smectite clays can reduce FFA liberation during digestion by adsorbing unionized fatty acids via hydrogen bonding at platelet edge sites, achieving up to a 35% reduction in FFA release under simulated intestinal conditions relative to untreated controls [40]. In that study, clay incorporation also disrupted vesicle formation and altered lamellar phase behavior, suggesting interference with lipid self-assembly pathways essential for micelle formation and FFA bioaccessibility. Similarly, the FFA-lowering effects observed with InuClay, particularly under fasted state conditions, support the hypothesis that lipid digestion is attenuated through dual mechanisms: physicochemical adsorption by the BEN phase and stabilization or shielding effects mediated by the INU matrix [64, 65]. This protective effect is particularly relevant under gastric conditions, where low pH promotes the leaching of structural cations (e.g., Al^{3+} , Mg^{2+} , Fe^{2+}) and partial dissolution of silicate layers, leading to degradation of clay integrity [66, 67]. Evidence from X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR) analyses has confirmed such damage, with decreased peak intensities and spectral shifts indicative of clay layer disruption and partial amorphization [66, 68]. As a result, structural degradation of the BEN phase under acidic gastric conditions may compromise its surface area and active adsorption sites, thereby diminishing its capacity to sequester FFAs and attenuate lipid digestion downstream in the intestine. Further mechanistic investigations are warranted to confirm whether InuClay interferes with lipid phase transitions and micellar assembly, as has been observed with MMT. Together, these mechanisms provide a physicochemical basis for additive lipid restriction when InuClay is co-administered with pharmacological lipase inhibition.

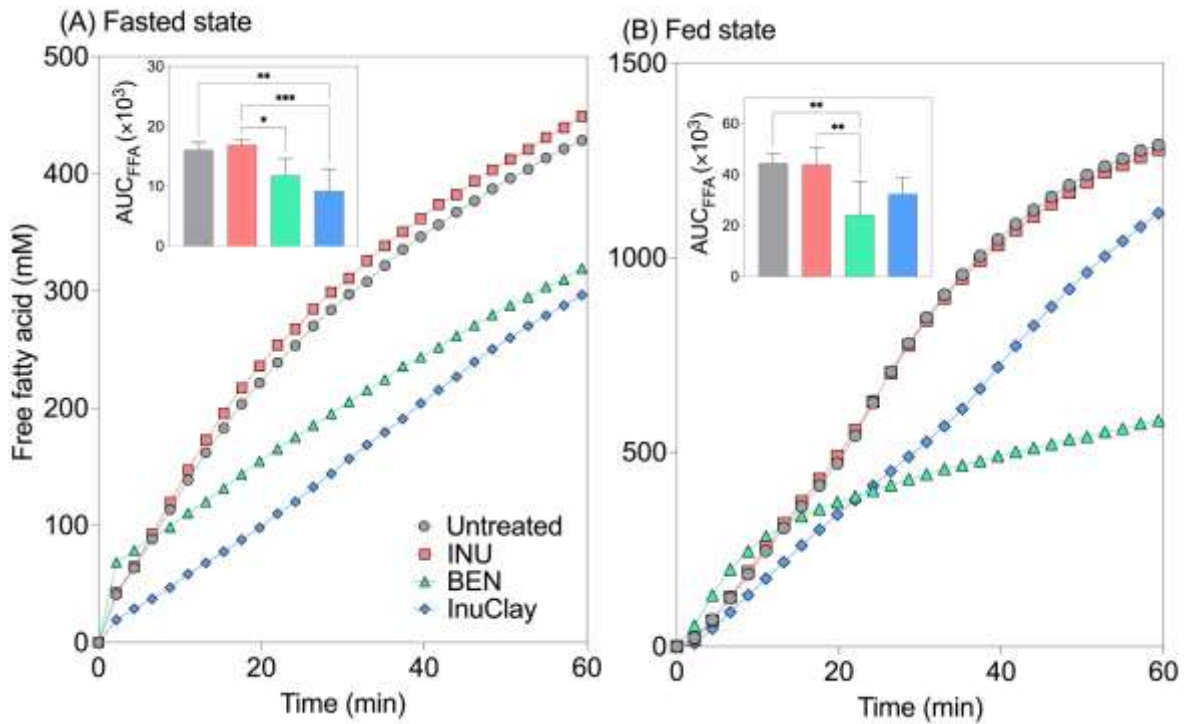


Figure 2. InuClay significantly reduced FFA release under fasted conditions compared to untreated and INU-only groups ($p < 0.01$), while BEN alone significantly reduced FFA release under fed conditions ($p < 0.01$). No significant effect was observed for INU in either state. FFA liberation was monitored over 60 min using a pH-stat model under simulated intestinal conditions replicating (A) fasted and (B) fed states. Untreated control (grey circles), INU (red squares), BEN (green triangles), and InuClay (blue diamonds), each added at 25% w/w relative to lipid content. Insets show cumulative FFA release (AUC_{60min}) as mean $\pm$ S.D. ($n = 3$).

3.3. Impact of InuClay Supplementation on Metabolic Health of Sprague-Dawley Rats

To ascertain the impact of supplementing InuClay on metabolic health, SD rats were fed a HFD and co-administered InuClay via oral gavage for a period extending 21 days. Key focus was placed on investigating the impact of supplementation on rodent weight gain, gut microbial diversity, glycemic levels, and liver health. Findings were directly compared to rats administered the orlistat, which served as a pharmacological control. Additionally, the ability for InuClay to enhance the activity of orlistat was assessed, since it was hypothesized that its multifunctional mechanism of action can promote orlistat action, while the presence of BEN may reduce luminal lipid availability, a mechanism previously associated with improved gastrointestinal tolerability of lipid malabsorption therapies [41, 53].

3.3.1. InuClay mitigates HFD-induced weight gain and enhances orlistat efficacy

Normalized body weight was monitored over the 21-day intervention period to evaluate the effects of daily supplementation with InuClay, orlistat, or their combination (**Fig. 3A**). All treatment groups exhibited reduced weight gain compared to the control group, which followed a consistent upward trajectory throughout the study. Weight gain trajectories for InuClay and orlistat remained closely aligned for the first 9 days. Thereafter, the orlistat group exhibited a lower rate of weight gain; however, the cumulative difference relative to InuClay did not reach statistical significance ($p = 0.0592$). Notably, the group receiving both InuClay and orlistat began to diverge from the other groups after day 9, with the gap widening over time and resulting in the lowest overall body weight gain by day 21.

Statistical analysis of cumulative body weight gain identified significant reductions in all treatment groups compared to the control ($p < 0.0001$). InuClay reduced weight gain by approximately 4.5%, while orlistat achieved a slightly greater reduction of 5.7%. Co-administration of InuClay with orlistat led to the greatest reduction—approximately 7.7% relative to the control group (**Fig. 3B**). This combined treatment group also differed significantly from InuClay alone ($p < 0.0001$) and orlistat alone ($p = 0.0012$), supporting the possibility of an additive effect on weight regulation.

These results support the hypothesis that InuClay enhances the weight-related efficacy of orlistat. Orlistat acts locally in the gastrointestinal tract by inhibiting gastric and pancreatic lipases, reducing triglyceride hydrolysis and increasing fecal fat excretion [11]. In parallel, InuClay may further reduce lipid bioaccessibility by adsorbing liberated lipid digestion products within the intestinal lumen, thereby limiting their availability for absorption [40, 53]. In addition, the INU component may contribute indirectly via microbiota modulation, as inulin-type fructans can promote taxa linked to improved metabolic outcomes, including *Blautia* in some diet-induced obesity models [69-71].

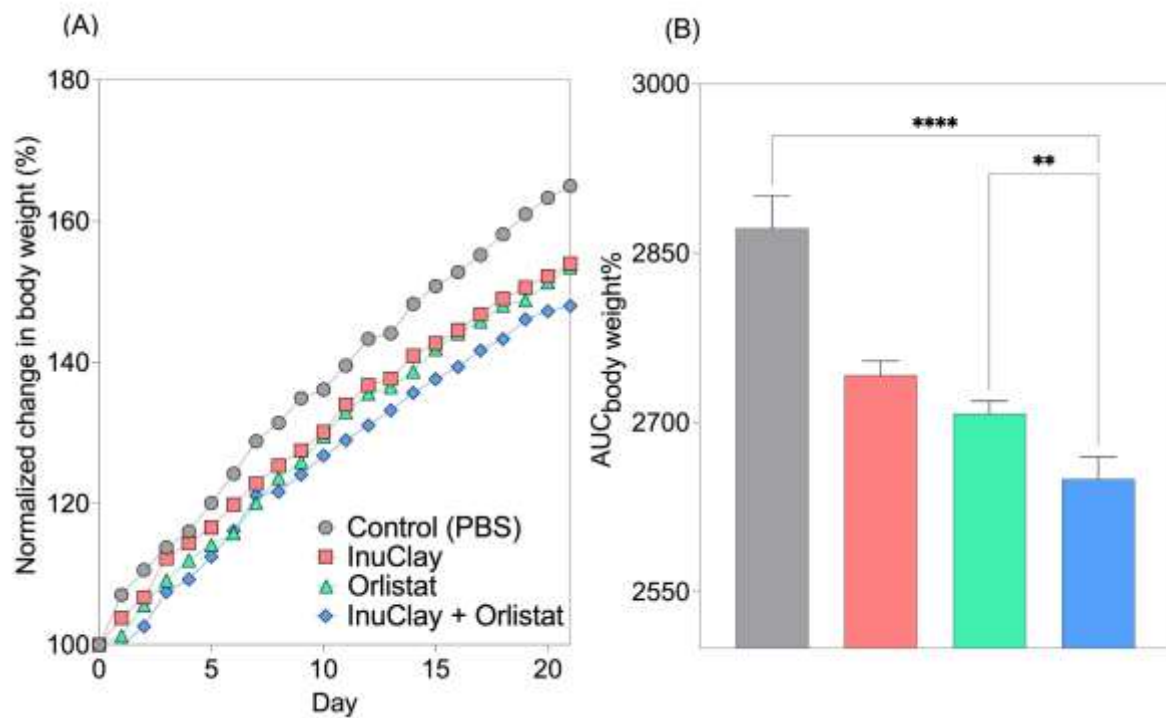


Figure 3. Impact of treatments on body weight gain in high-fat diet-fed rats over 21 days. (A) Normalized cumulative weight gain relative to initial body weight. (B) All treatment groups significantly reduced cumulative weight gain compared to the control group ($p < 0.0001$). The combined InuClay + orlistat group also differed significantly from InuClay alone ($p < 0.0001$) and orlistat alone ($p = 0.0012$), indicating an additive effect. Values represent mean $\pm$ SD, $n = 5$ per group.

3.3.2. InuClay prevents orlistat-induced microbiota disruption while maintaining diversity

Emerging evidence indicates that orlistat treatment induces compositional shifts in gut microbiota that may limit its long-term metabolic benefits [72, 73]. To determine whether InuClay co-administration could prevent orlistat-associated dysbiosis while enhancing therapeutic outcomes, we performed 16S rRNA gene sequencing on fecal samples collected after 21 days of treatment. At the class taxonomic level, relative abundance profiles indicate treatment-associated variation in microbial composition across the different groups (**Fig. 4A**). Alpha diversity was assessed using Shannon's index to evaluate species richness and evenness of these taxa within each group (**Fig. 4B**). No statistically significant differences in alpha diversity were observed across treatment groups ($p > 0.27$). This indicates that supplementation did not substantially alter within-sample diversity during the 21-day

intervention period. These findings align with previous investigations in comparable HFD-induced MetS rat models.

INU supplementation at varying chain lengths (average degree of polymerization: 7, 14, and 27) similarly failed to modulate alpha diversity following 21 days of treatment [35]. However, extended intervention periods appear critical for detecting microbiome shifts. INU supplementation (10% w/v in drinking water) for 49 days significantly increased Chao1 alpha diversity ($p = 0.00014$) in HFD-fed rats [74]. BEN treatment (400 mg/kg BW, administered three times weekly for 70 days) produced no alterations in fecal microbiota alpha diversity in HFD-fed mice [75]. These observations suggest that the absence of alpha diversity changes in the present study may reflect insufficient treatment duration for detectable microbiome restructuring. Moreover, BEN within InuClay may not directly influence microbial community diversity through direct mechanisms, as it is not a fermentable substrate. Further studies with extended treatment durations are warranted to validate whether prolonged INU supplementation modulates alpha diversity and to elucidate the direct effects of BEN on microbiota composition.

PCoA plots based on Bray-Curtis dissimilarity was used to assess beta diversity (**Fig. 4C**). Beta diversity quantifies the compositional dissimilarity between different samples or groups, in contrast to alpha diversity which measures within-sample taxonomic richness and evenness [76]. Modest separation between treatment groups was observed, though these differences were not statistically significant (PERMANOVA $p > 0.05$). The InuClay-orlistat combination group exhibited the greatest divergence from controls along the PCo1 axis, which explained the primary source of variation between samples. In contrast, InuClay and orlistat monotherapy groups displayed substantial overlap with the control group. While not reaching statistical significance, this clustering pattern suggests that combination therapy may induce more pronounced shifts in gut microbial community composition than either treatment alone.

In HFD-fed mice, orlistat-treated groups have consistently clustered separately from both untreated HFD and normal diet groups, indicating distinct microbial community structures [20, 77, 78]. These studies used a longer treatment duration of between 10-20 weeks, which may have contributed to statistical significance reached by the end of the treatment phase. The more pronounced separation observed in the InuClay-orlistat group suggests that prebiotic components actively reshape community structure in beneficial directions, creating a metabolically favorable microbiota profile distinct from orlistat alone. This divergence indicates that InuClay does not simply preserve baseline microbiota, but rather directs orlistat-treated animals toward a microbiota configuration enriched in health-promoting taxa.

Nevertheless, the trend toward enhanced microbiome restructuring with InuClay-orlistat may reflect complementary mechanisms. Orlistat's inhibition of lipid absorption in the small intestine may indirectly result in downstream shifts in the microbiota, while InuClay's prebiotic and adsorptive components may directly exert their effects on the microbiota.

Orlistat inhibits gastrointestinal lipases, leading to increased dietary fat reaching the colon as unabsorbed triglycerides and FFAs [21]. Dietary fats entering the colon increase the activity of bacteria with bile salt hydrolase (BSH) and 7 α -dehydroxylation functions. Gut bacteria initiate bile acid metabolism by deconjugating primary bile acids via BSH, followed by 7 α -dehydroxylation to generate secondary bile acids like deoxycholic acid (DCA) and lithocholic acid (LCA) [79, 80]. Secondary bile acids can inhibit or promote the growth of specific bacterial taxa, often favoring *Firmicutes* over *Bacteroidetes*, and can drive blooms of bile-tolerant or pathobiont species (e.g., *Bilophila wadsworthia*) [81]. These shifts are implicated in MetS, with diet-induced obese rats exhibiting significantly ($p = 0.014$) higher plasma DCA than controls [82]. Concurrently, InuClay's prebiotic component is selectively fermented by bacteria such as *Bifidobacterium*, and *Lactobacillus*, resulting in what is termed a 'bifidogenic' shift in the microbiota [82]. The BEN clay component may also contribute to

a shift in the microbiota through distinct mechanisms. The clay's high cation exchange capacity could allow it to bind bile acids, dietary lipids, and toxins in the gut, reducing their availability and altering the luminal environment [40, 53, 83]. As such, the combination of orlistat-induced substrate shifts and InuClay's multifaceted effects may create a microenvironmental niche that promotes more substantial community restructuring than either treatment alone.

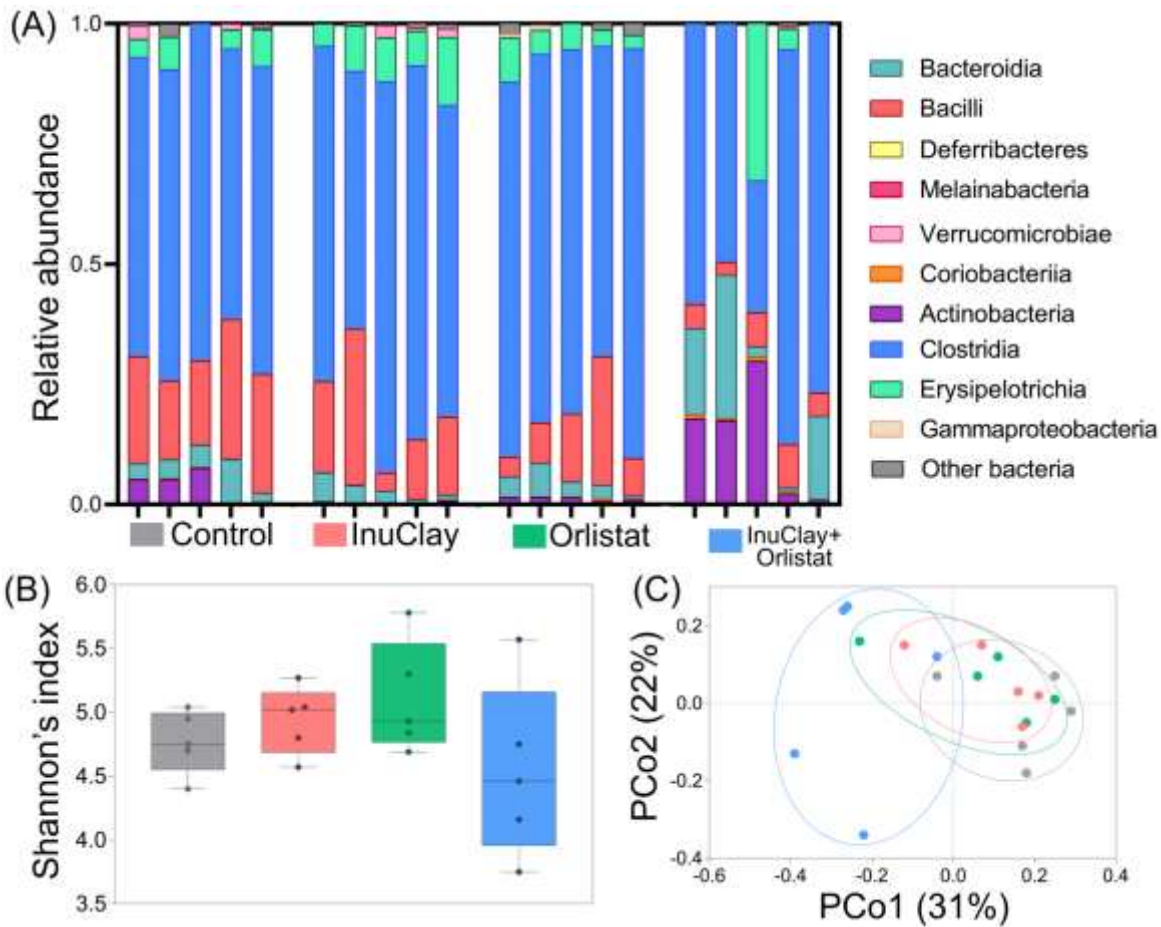


Figure 4. Treatment-associated changes in gut microbial communities after 21 days. (A) Class-level taxonomic profiles in combined fecal and cecal samples. (B) Shannon alpha diversity showed no significant differences between groups ($p > 0.27$). (C) PCoA of Bray-Curtis dissimilarity indicated modest group separation that did not reach statistical significance (PERMANOVA $p > 0.05$).

3.3.3. InuClay prevents orlistat-induced pathobiont expansion while enriching beneficial taxa

InuClay co-administration attenuated the increase in *Proteobacteria* observed with orlistat monotherapy. Orlistat significantly increased *Proteobacteria* relative abundance

511 compared with the InuClay-orlistat combination group ($p = 0.0454$; **Fig. 5A**),
512 corresponding to a 2.5-fold higher abundance in orlistat-treated animals. Differences
513 between orlistat and control did not reach statistical significance ($p = 0.1548$), suggesting
514 that the magnitude of change was modest over 21 days. As *Proteobacteria* include many
515 Gram-negative taxa that contribute lipopolysaccharide (LPS) to the gut lumen,
516 enrichment of this phylum has been associated with metabolic endotoxemia and
517 inflammation relevant to MetS [84]. Critically, InuClay co-administration completely
518 prevented this pathobiont expansion. The combination treatment maintained
519 *Proteobacteria* levels comparable to untreated controls, indicating that InuClay's
520 prebiotic and adsorptive components successfully counteract the dysbiotic selective
521 pressure imposed by orlistat. Potential mechanisms include reduced luminal lipid
522 availability via BEN adsorption, local pH shifts associated with INU fermentation, and
523 SCFA-linked support of epithelial barrier function. This prevention of dysbiosis
524 represents a key advantage of combination therapy and may contribute to the superior
525 metabolic outcomes observed.

526 The InuClay-orlistat combination significantly increased *Lachnospiraceae* abundance
527 compared to controls ($p = 0.0264$; **Fig. 5B**), representing a 42% increase in relative
528 abundance. Monotherapy groups exhibited non-significant increases of 26% for InuClay
529 and 21% for orlistat compared to controls. *Lachnospiraceae* are an important family of
530 butyrate-producing bacteria in the human gut, playing critical roles in maintaining
531 intestinal homeostasis and supporting metabolic health. Butyrate produced by
532 *Lachnospiraceae* supplies the energy needs of healthy colonocytes, supporting epithelial
533 function [85]. The SCFA can also bind and stimulate the aryl hydrocarbon receptor
534 (AhR), GPR41, and GPR109A, further stabilizing the intestinal barrier integrity by

increasing the expression of tight junction proteins (e.g., claudins, occludin, zonula occludens-1) [86].

Within the *Lachnospiraceae* family, *Blautia* demonstrated a highly significant increase in the InuClay-orlistat combination group compared to controls ($p = 0.0087$; **Fig. 5C**), representing an 11.8-fold increase in relative abundance. Near-significant trends were also observed when compared to monotherapy groups ($p = 0.0754$ vs InuClay; $p = 0.0568$ vs orlistat), with the combination group showing 3.3-fold and 3.9-fold higher *Blautia* levels than InuClay and orlistat alone, respectively. This represents a signature benefit of the adjunct approach, as the combination creates a microenvironmental niche that preferentially supports this critical beneficial genus. *Blautia* species, particularly *B. luti* and *B. wexlerae*, reduce pro-inflammatory cytokines (e.g., TNF- α , IFN- γ) and increase anti-inflammatory cytokine ratios *in vitro* and *in vivo*, suggesting a direct role in dampening obesity-related inflammation [71, 87]. The selective enrichment observed exclusively in the combination group provides compelling evidence for additive effects between InuClay and orlistat. Orlistat may increase colonic bile acid flux by inhibiting fat absorption, which, when coupled with INU-derived SCFAs, create conditions uniquely favorable for bile-tolerant, fiber-fermenting *Blautia* species [88, 89]. Additionally, BEN's bile acid binding capacity may modulate secondary bile acid profiles in ways that support *Blautia* over competing taxa [83]. *Blautia* expansion is particularly significant given this genus's established roles in reducing proinflammatory cytokines such as TNF- α and IFN- γ [71, 90, 91].

In contrast, *Bifidobacterium* abundance remained consistently low across all groups with no significant differences detected ($p > 0.15$ for all comparisons; **Fig. 5D**). The InuClay and orlistat combination group exhibited the greatest change, a 3.9-fold increase compared to the control, though this did not reach statistical significance ($p = 0.3218$).

560 This finding was unexpected given INU's well-documented bifidogenic effects [69]. It
561 is possible that the HFD may create a hostile gut environment that suppresses
562 *Bifidobacterium* colonization despite prebiotic availability. Experimental studies show
563 that FFAs and bile salts, both elevated in HFDs, directly inhibit the growth of
564 *Bifidobacterium* species, even when carbohydrates or prebiotics are present [92]. The
565 21-day intervention period may also be insufficient for substantial *Bifidobacterium*
566 expansion when competing against established HFD-adapted microbial communities.

567 A positive correlation was observed between Firmicutes relative abundance and PCo2
568 values from the beta diversity analysis (**Fig. 5E**). This correlation indicates that samples
569 with higher *Firmicutes* abundance tended to have a positively higher PCo2 coordinates,
570 while samples with lower *Firmicutes* abundance had a negatively lower PCo2
571 coordinates. The PCo2 axis captures the variation in community composition across all
572 samples. The correlation reveals that *Firmicutes* abundance varies systematically along
573 the PCo2 axis of compositional variation. Samples are positioned along PCo2 based on
574 their overall taxonomic profiles, and this positioning happens to align with differences
575 in *Firmicutes* proportions. In other words, samples that differ in their PCo2 coordinates
576 also tend to differ in their *Firmicutes* content, suggesting that changes in *Firmicutes*-
577 associated taxa (such as *Lachnospiraceae* and *Blautia*) are a major driver of the
578 compositional differences captured by this axis. This correlation supports the finding
579 that treatment-induced expansion of these butyrate-producing *Firmicutes* taxa contribute
580 substantially to the compositional divergence between treatment and control groups
581 observed in the beta diversity analysis. Extended treatment studies employing whole-
582 genome metagenomics and targeted metabolomic profiling are warranted to confirm
583 whether these compositional shifts translate to enhanced SCFA production, improved
584 bile acid metabolism, and sustained metabolic improvements for MetS. Collectively,

these taxonomic shifts support InuClay as a microbiota-modulating adjunct that complements pharmacological lipase inhibition. By preventing orlistat's pathobiont-promoting effects while selectively enriching health-associated taxa, InuClay addresses a fundamental limitation of lipase inhibitor therapy.

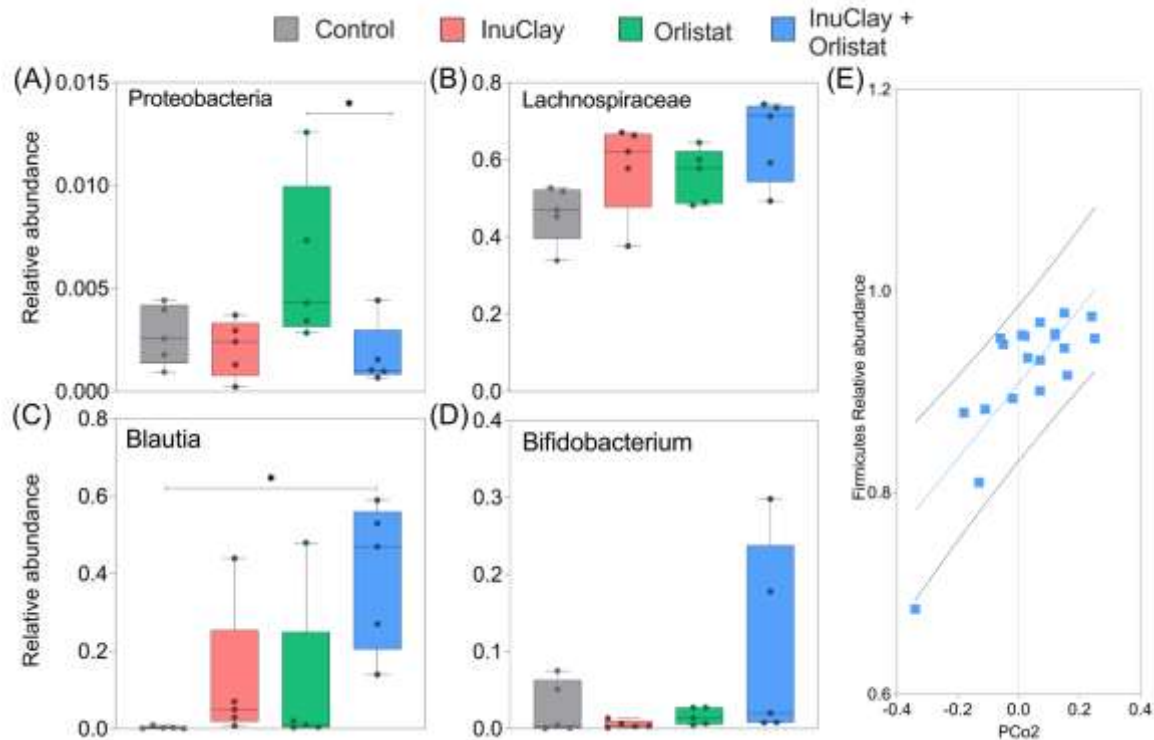


Figure 5. Relative abundance of metabolically relevant taxa after 21-day treatment: (A) *Proteobacteria*, (B) *Lachnospiraceae*, (C) *Blautia*, and (D) *Bifidobacterium*. (E) Correlation between *Firmicutes* abundance and PCo2 scores from Bray-Curtis PCoA. n = 5 per group.

3.3.4. Enhanced glycemic control with InuClay-orlistat combination therapy

Given the central role of hyperglycemia in MetS pathophysiology, we assessed whether InuClay could enhance orlistat's glucose-lowering effects. Fasting blood glucose was measured following the 21-day intervention period (**Fig. 6**). The InuClay-orlistat combination group exhibited significantly reduced blood glucose levels compared to controls ($p = 0.0005$), representing a 27% reduction in glucose concentration. This substantial glucose-lowering effect was significantly greater than InuClay monotherapy ($p = 0.0434$), which exhibited only a non-significant 12% reduction compared to controls ($p = 0.1680$).

602 Orlistat monotherapy also significantly reduced blood glucose compared to controls (p =
603 0.0314), achieving a 16% reduction. However, the combination treatment outperformed
604 orlistat alone by 13%, though this difference did not reach statistical significance (p =
605 0.2199).

606 These findings indicate that combination therapy improved glycemic control versus
607 control and InuClay alone, however, differences versus orlistat monotherapy did not reach
608 statistical significance. Multiple studies in humans and animals exhibit that orlistat improves
609 insulin sensitivity, often beyond what is achieved by weight loss alone [93-95]. This is
610 attributed to reductions in plasma FFAs, improved adipokine profiles (e.g., increased
611 adiponectin, decreased leptin), and decreased hepatic steatosis. The generation of long-chain
612 FFAs from dietary fat is a critical trigger for GLP-1 secretion from L-cells. Orlistat prevents
613 this hydrolysis, thereby reducing GLP-1 release in both healthy individuals and those with
614 type 2 diabetes [96, 97].

615 InuClay's contribution to glycemic improvement may be via distinct but complementary
616 mechanisms. The SCFAs produced via INU fermentation can influence glucose homeostasis
617 through multiple pathways. Propionate stimulates intestinal gluconeogenesis, which
618 produces glucose sensed by the portal vein, activating a gut-brain neural circuit (involving
619 FFAR3 signaling) [98]. Butyrate increases insulin sensitivity by upregulating insulin receptor
620 substrate (IRS1) and glucose transporter 4 (GLUT4) in the muscle, boosting glucose cellular
621 uptake and subsequently reducing blood glucose levels [99]. The significant enrichment of
622 butyrate-producing *Blautia* observed in the combination group (11.8-fold increase) may
623 contribute substantially to these glyceamic benefits. Additionally, SCFA activation of
624 GPR41 and GPR43 on β -cells and enteroendocrine cells lowers blood glucose by enhancing
625 insulin and incretin secretion via G-protein signaling pathways. When activated by SCFAs,
626 these receptors trigger intracellular signaling. GPR43 activation directly stimulates insulin
627 exocytosis [100]. Moreover, SCFA activation of GPR43 expressed on enteroendocrine L-

cells, increases cytosolic Ca^{2+} in L-cells, promoting GLP-1 release. GLP-1 enhances glucose-dependent insulin secretion and suppresses glucagon, further lowering blood glucose [101]. BEN clay may further contribute to glucose regulation indirect mechanisms. The clay's ability to bind bile acids may increase their fecal excretion and therefore, stimulate de novo bile acid synthesis from cholesterol. This process influences glucose metabolism through activation of Takeda G-protein-coupled receptor 5 (TGR5) signaling pathways, which in turn increase GLP-1, and incretin hormone that functions to lower circulating glucose levels [102, 103].

The superior efficacy of combination therapy, particularly when compared to InuClay alone, suggests that orlistat's caloric restriction and lipid-mediated signaling effects complement with InuClay's prebiotic and adsorptive actions. The attenuation of *Proteobacteria* expansion and robust enrichment of *Blautia* in the combination group may translate to reduced systemic inflammation and enhanced SCFA production, creating a metabolic environment more conducive to glucose homeostasis. Extended treatment studies with additional glycemic parameters including insulin levels, are required to comprehensively characterize the insulin-sensitizing effects and determine whether these acute improvements translate to sustained glycemic control and prevention of diabetes progression in MetS.

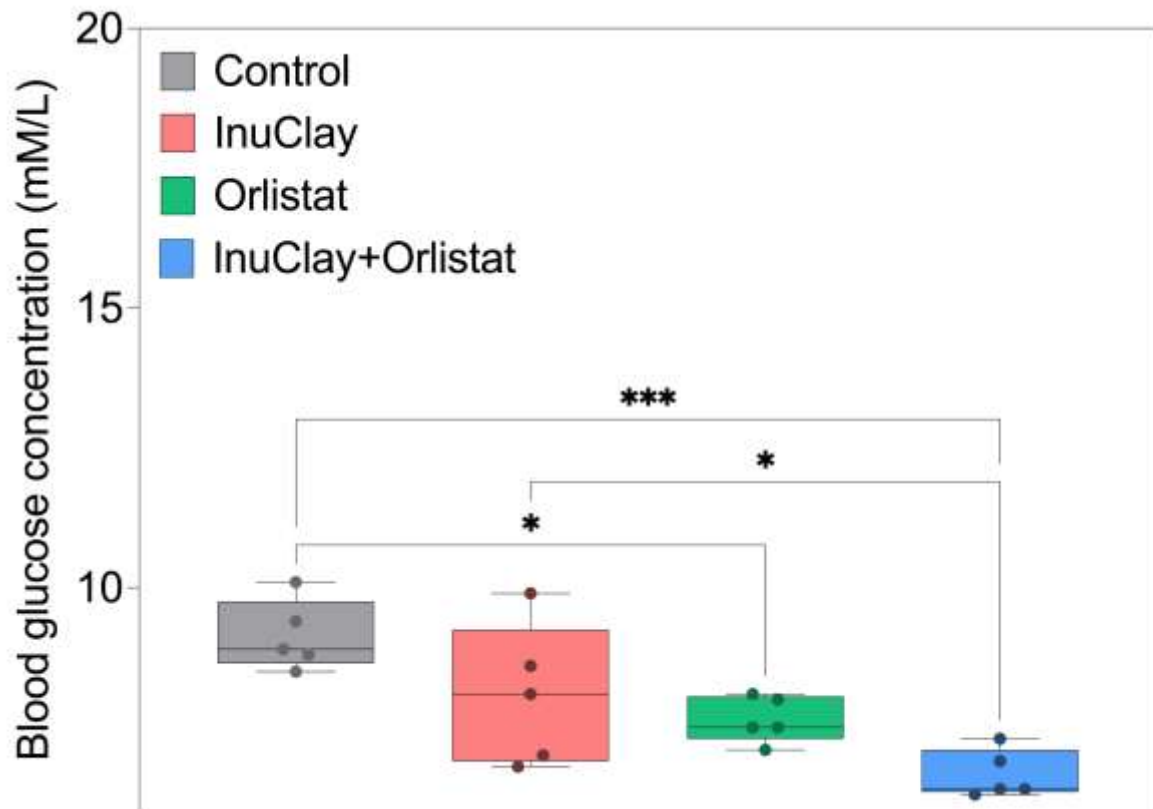


Figure 6. Fasting blood glucose after 21-day treatment. The InuClay–orlistat group showed lower glucose than controls ($p = 0.0005$) and InuClay monotherapy ($p = 0.0434$). Orlistat also reduced glucose versus controls ($p = 0.0314$). The difference between combination therapy and orlistat monotherapy was not significant ($p = 0.2199$). Values represent mean $\pm$ SD; $n = 5$.

3.3.5. InuClay provides hepatoprotection against orlistat-associated liver stress

Given the role of hepatic dysfunction in MetS, we assessed treatment effects on liver weight and circulating enzymes after 21 days (**Fig. 7**). Liver weight normalized to body weight did not differ significantly between groups, although the combination group showed a numerical reduction versus controls (**Fig. 7A**). This is consistent with the relatively short intervention duration, as changes in hepatic lipid remodeling and mass often require longer exposure periods in both clinical and preclinical studies [104-107].

Plasma lactate dehydrogenase (LDH), a non-specific marker of cellular injury, was substantially lower in the combination group, corresponding to a 63% reduction versus controls ($p = 0.0065$) and a 65% reduction versus orlistat ($p = 0.0042$; **Fig. 7B**). LDH is

expressed across multiple tissues (including liver, muscle, and erythrocytes) and therefore should be interpreted as a systemic injury marker rather than a liver-specific endpoint [108, 109]. Alkaline phosphatase (ALP) did not differ significantly between groups (**Fig. 7C**). Similarly, AST was reduced in the combination group versus controls (23%; $p = 0.0080$; **Fig. 7D**), whereas ALT showed non-significant trends toward reduction across treatment groups (**Fig. 7E**). Given that AST is not liver-specific and ALT changes were not significant, these enzyme profiles suggest a modest improvement in circulating markers consistent with reduced tissue injury, but do not alone establish hepatoprotection.

AST:ALT ratios ranged from 1.56 to 1.79 across groups (**Fig. 7F**). While this metric is used clinically in some contexts, interpretation in short-duration rodent HFD models is limited without complementary endpoints such as histology and hepatic lipid quantification. Future studies incorporating liver histopathology (e.g., steatosis and inflammation scoring), hepatic triglycerides, and broader liver function panels are warranted to clarify whether InuClay co-administration confers liver-specific benefit during orlistat therapy.

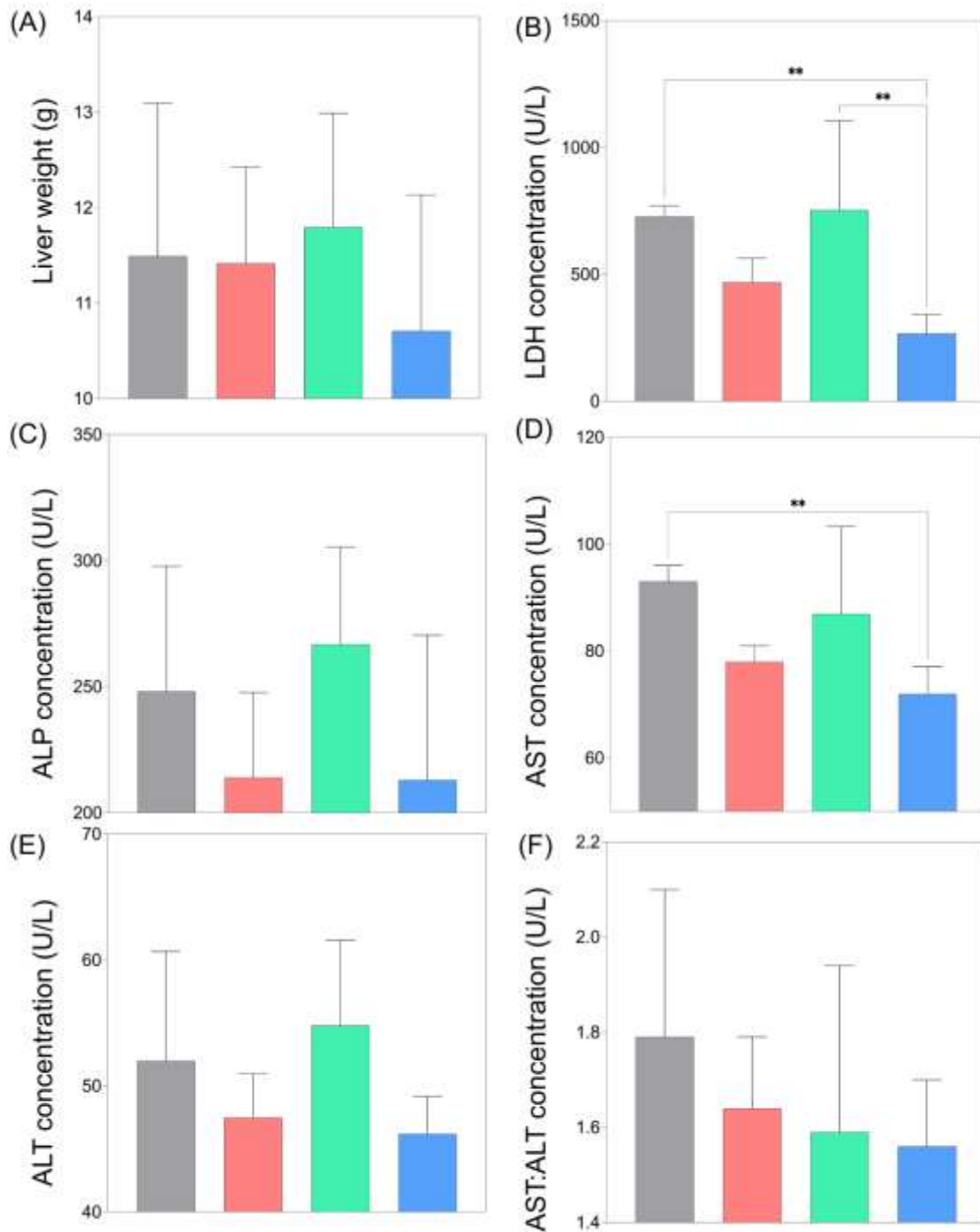


Figure 7. Impact of InuClay and/or orlistat on hepatic-related biomarkers after 21 days: (A) liver weight normalized to body weight; (B) plasma LDH; (C) plasma ALP; (D) plasma AST; (E) plasma ALT; and (F) AST:ALT ratio. Values represent mean $\pm$ SD; n = 5.

4. Conclusions

This study demonstrates that spray-dried InuClay hybrid microparticles function as a microbiota-modulating adjunct to orlistat therapy, enhancing metabolic outcomes while attenuating treatment-associated microbiota. Co-administration with orlistat resulted in the greatest reductions in cumulative weight gain (~7.7%) and fasting blood glucose (~27%) over 21 days, exceeding the effects observed with either monotherapy. At the microbiota level, InuClay co-administration attenuated the increase in *Proteobacteria* observed with orlistat alone and selectively enriched butyrate-associated taxa, most notably *Blautia*, which increased 11.8-fold relative to controls. These compositional shifts occurred without significant changes in alpha diversity, suggesting targeted modulation rather than broad disruption of microbial communities. Combination therapy was also associated with reductions in circulating LDH and AST relative to controls and orlistat monotherapy, consistent with lower systemic markers of cellular injury. While these findings do not establish liver-specific protection, they suggest that InuClay may mitigate treatment-associated physiological stress under HFD conditions. Collectively, these findings support InuClay as a promising adjunct strategy for lipase inhibitor therapy, leveraging complementary physicochemical and microbiota-associated mechanisms to enhance metabolic efficacy while moderating microbiota perturbations. This work extends prior investigations of inulin-clay hybrids as standalone interventions and highlights their potential role as next-generation biomaterial adjuncts in metabolic disease management. Longer-duration studies incorporating histological, metabolomic, and functional microbiome endpoints are warranted to define durability, mechanism, and translational relevance.

5. Statements and Declarations

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5.2. Competing Interests

The authors declare that they have no relevant financial or non-financial interests to disclose.

5.3. Author Contributions

Conceptualization: Amin Ariaee. Methodology: Amin Ariaee, Paul Joyce, Anthony Wignall, Alex Hunter, Aurelia S. Elz, and Tahlia R. Meola. Validation: Amin Ariaee, Paul Joyce, and Clive A. Prestidge. Formal analysis: Amin Ariaee and Hannah R. Wardill. Investigation: Amin Ariaee. Resources: Paul Joyce and Clive A. Prestidge. Data curation: Amin Ariaee. Writing – original draft preparation: Amin Ariaee. Writing – review and editing: Aurelia S. Elz, Hannah R. Wardill, Alex Hunter, Tahlia R. Meola, and Clive A. Prestidge. Visualization: Amin Ariaee. Supervision: Paul Joyce. Project administration: Amin Ariaee. All authors read and approved the final manuscript.

5.4. Data Availability

The 16S rRNA gene sequencing datasets generated and analysed during this study are available from the corresponding author on reasonable request.

5.5. Ethics Approval

All animal procedures were approved by the Animal Ethics Committee of the Adelaide University (approval #U18-17) and conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985) and the ARRIVE guidelines. All institutional and national guidelines for the care and use of laboratory animals were followed.

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