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Self-emulsifying drug delivery systems (SEDDS) disrupt the gut microbiota and trigger an intestinal inflammatory response in rats

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ABSTRACT

Self-emulsifying drug delivery systems (i.e. SEDDS, SMEDDS and SNEDDS) are widely employed as solubility and bioavailability enhancing formulation strategies for poorly water-soluble drugs. Despite the capacity for SEDDS to effectively facilitate oral drug absorption, tolerability concerns exist due to the capacity for high concentrations of surfactants (typically present within SEDDS) to induce gastrointestinal toxicity and mucosal irritation. With new knowledge surrounding the role of the gut microbiota in modulating intestinal inflammation and mucosal injury, there is a clear need to determine the impact of SEDDS on the gut microbiota. The current study is the first of its kind to demonstrate the detrimental impact of SEDDS on the gut microbiota of Sprague-Dawley rats, following daily oral administration (100 mg/kg) for 21 days. SEDDS comprising a lipid phase (i.e. Type I, II and III formulations according to the Lipid Formulation Classification Scheme) induced significant changes to the composition and diversity of the gut microbiota, evidenced through a reduction in operational taxonomic units (OTUs) and alpha diversity (Shannon's index), along with statistically significant shifts in beta diversity (according to PERMANOVA of multi-dimensional Bray-Curtis plots). Key signatures of gut microbiota dysbiosis correlated with the increased expression of pro-inflammatory cytokines within the jejunum, while mucosal injury was characterised by significant reductions in plasma citrulline levels, a validated biomarker of enterocyte mass and mucosal barrier integrity. These findings have potential clinical ramifications for chronically administered drugs that are formulated with SEDDS and stresses the need for further studies that investigate dose-dependent effects of SEDDS on the gastrointestinal microenvironment in a clinical setting.

1. Introduction

The gastrointestinal (GI) tract is colonised by a large number of microorganisms, collectively referred to as the gut microbiota, which are key players in regulating and maintaining essential physiological processes in the body (de Vos et al., 2022; Vijay and Valdes, 2022). In recent years, there has been increasing focus on understanding the bidirectional relationships that exist between the gut microbiota and drug molecules, opening a new realm of knowledge in the field of pharmacomicrobiomics (Weersma et al., 2020; Kamath et al., 2023). Drug-microbiota relationships are complex and multifaceted, given the composition of the microbial community can modulate the pharmacokinetics and pharmacodynamics of orally administered drugs, while conversely, drug administration can significantly alter the abundance and diversity of microbes within the gut (Maier et al., 2018). The commonly prescribed antipsychotic drug, olanzapine, is a clinically relevant example of this complex bidirectional relationship, where oral olanzapine bioavailability has been shown to be 1.8-fold greater in rats with antibiotic depleted microbiota (Cussotto et al., 2021), while the drug itself induces gut microbiota dysbiosis, triggering a cascade of metabolic disorders including weight gain and intestinal inflammation (Morgan et al., 2014). Understanding this bidirectional relationship presents new opportunities for optimising treatment outcomes through interventions that mitigate drug-induced gut dysbiosis (Cross et al., 2024) and control oral pharmacokinetics (Kamath et al., 2023).

To date, pharmacomicrobiomic studies have largely investigated the bidirectional relationship that exists between active drugs and the gut microbiota, ignoring the capacity for excipients (commonly present in

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oral dosage forms) to also modulate the composition and diversity of the GI microenvironment (Subramaniam et al., 2023). As such, a more holistic approach to pharmacomicrobiomic studies is required to elucidate the bidirectional interactions between clinically relevant oral dosage forms and the gut microbiota.

In this context, self-emulsifying drug delivery systems (SEDDS), a subset of lipid-based formulations (LBFs) (Pouton, 2000), have been widely investigated for their capacity to enhance the oral biopharmaceutical performance of poorly water-soluble drugs (Kohli et al., 2010). This has led to the widespread clinical and commercial translation of multiple SEDDS formulations (Betageri, 2019). SEDDS function by mimicking the pharmaceutical food effect where lipophilic drugs are solubilised within the lipid carrier for improved dissolution and absorption across the GI tract (Joyce et al., 2019). However, high concentrations of triglycerides, surfactants (emulsifiers), co-surfactants and/ or co-solvents are required to facilitate enhanced drug solubilisation and spontaneous emulsion self-assembly in the GI tract (Kohli et al., 2010). Several reports have raised tolerability concerns related to the chronic exposure of SEDDS on the gastrointestinal environment (Pouton and Porter, 2008; Tran and Park, 2021; Gursoy and Benita, 2004), specifically due to the capacity for high surfactant doses to cause gastrointestinal irritation by disrupting the integrity of the mucosal barrier and intestinal epithelium (Bu et al., 2016; Sha et al., 2005; Lam et al., 2019; Bu et al., 2017; Palamakula and Khan, 2004; Ujhelyi et al., 2012). However, previous tolerability assessments have centralised around in vitro cytotoxicity and epithelial monolayer permeation assays and have therefore failed to recognize the multifaceted mechanisms that potentiate acute epithelial damage and inflammation in vivo, including the fundamental role of the gut microbiota in regulating intestinal barrier integrity (Natividad and Verdu, 2013).

Recent investigations from the Chassaing Laboratory have revealed that dietary emulsifiers (surfactants), specifically Polysorbate / Tween 80 (P80) and carboxymethylcellulose (CMC), deplete the gut microbiota and metabolome in animal models (Chassaing et al., 2015; Viennois et al., 2017; Viennois et al., 2020) and humans (Chassaing et al., 2022; Chassaing et al., 2017; Naimi et al., 2021), inducing low-grade intestinal inflammation and metabolic syndrome (Bancil et al., 2021). Critically, a wealth of evidence indicates that gut microbiota dysbiosis is the primary driver of surfactant-induced intestinal inflammation. For example, an inflammatory response was not observed in germ-free mice (i.e. mice without a microbiota) treated with dietary surfactants (Chassaing et al., 2015), but was triggered through increased cytokine expression and colitis-like symptoms when microbiota pre-treated with surfactants were transplanted into germ-free mice (Chassaing et al., 2017). These findings, amongst others, indicate that surfactant-induced depletion of microbial richness and increased pathogenic bacteria abundance induces inflammation, leading to metabolic dysfunction and the onset of inflammatory bowel disease (IBD) (Bancil et al., 2021). Given SEDDS typically employ high concentrations of surfactants (i.e. 30 – 60 % w/w), their impact on the gut microbiota and metabolic health clearly warrants investigation.

In light of this, the objective of this study was to elucidate the impact of common SEDDS formulations on the gut microbiota of Sprague-Dawley rats following daily dosing for 21 days. The dosing regimen used in this study aimed to simulate a chronic dosing scenario, given multiple commercial SEDDS are formulated for drugs requiring chronic dosing (e.g. poorly soluble antiretroviral drugs for the treatment of HIV, such as ritonavir) (Betageri, 2019). It was hypothesised that 1) surfactants are the primary SEDDS excipients that drive gut microbiota dysbiosis and intestinal inflammation; 2) SEDDS interact with the gut microbiota in a formulation-dependent manner due to the varying effects of water- and lipid-soluble surfactants in disrupting biological membranes (Aguirre-Ramírez et al., 2021); and, 3) SEDDS-induced gut microbiota dysbiosis correlates with intestinal inflammation and mucosal barrier injury. To test these hypotheses, four SEDDS formulations with varying compositions of lipids, surfactants, co-surfactants and co-solvents were investigated according to the Lipid Formulation Classification System (LFCS) (i.e. Type I, II, III and IV SEDDS) (Pouton and Porter, 2008; Shrestha et al., 2014). Changes in gut microbiota composition were correlated with intestinal inflammatory responses, measured through the abundance of proinflammatory cytokines within the jejunum, to determine if SEDDS-induced inflammation was linked with a dysbiotic state of the gut microenvironment. Given this is the first study to investigate the impact of SEDDS on the gut microbiota, the insights derived have fundamental implications for the future development and optimisation of oral drug delivery systems that improve the biopharmaceutical properties of drug compounds without comprising on tolerability and adverse effects linked with gut microbiota dysbiosis.

2. Materials & methods

2.1. Fabrication and characterization of SEDDS

Self-emulsifying drug delivery systems (SEDDS) were prepared with varying compositions of medium-chain triglycerides (MCT, Miglyol 812 N; Hamilton Laboratories, Australia), lipid-soluble surfactant (lecithin/ phosphatidylcholine; Sigma Aldrich, Australia), water-soluble surfactant (Tween 85; Sigma Aldrich, Australia), water-soluble co-surfactant (Kolliphor RH40; Sigma Aldrich, Australia), and co-solvent (ethanol) (Table 1) to elucidate the impact of lipid-based formulation composition on the gut microbiota. The compositions investigated are representative of common SEDDS formulations utilized by the pharmaceutical industry and are characterized according to the LFCS (Pouton and Porter, 2008), where Type I is comprised of lipid (i.e. MCT) only; Type II is comprised of lipid and lipid soluble surfactants (with HLB < 10); Type III is comprised of triglycerides, water soluble surfactants (with hydrophilic-lipophilic balance, HLB < 10) and a co-solvent; and, Type IV is comprised of a mixture of water-soluble surfactants (with HLB > 10) dispersed within a co-solvent. Each formulation composition has been extensively investigated for their capacity to improve the solubilization of poorly soluble drugs. To prepare the formulations, each excipient was combined at the specified ratio and mixed via rotation for 24 h (400 rpm, 37 °C), as previously described (Hedge et al., 2021). The capacity for each SEDDS concentrate to spontaneously self-emulsify when dispersed in aqueous media was monitored by dispersing 100 mg SEDDS concentrate in 100 mL Milli-Q. The droplet size of the resultant emulsions was quantified through dynamic light scattering using a nanosizer-ZS (Malvern Instruments, Worcestershire, UK).

2.2. In vivo study

Animal studies were reported in accordance with the ARRIVE guidelines for the accurate and reproducible in vivo experimentation and complied with the Principles of Laboratory Animal Care (NIH publication #85–23, revised in 1985) and the National Health and Research Council (Australia) Code of Practice for Animal Care in

Table 1
Composition of SEDDS formulations investigated within this study based on their Lipid Formulation Classification (Pouton and Porter, 2008).

SEDDS sample/ LFCS category	Lipid Miglyol 812 N (wt%)	Lipid-soluble surfactant Lecithin (wt%)	Water-soluble surfactant Tween 85 (wt%)	Water-soluble co-surfactant Kolliphor RH40 (wt %)	Co-solvent Ethanol (wt%)
Type I	100		–	–	–
Type II	55	45	–	–	–
Type III*	55		25	10	10
Type IV			20	70	10

*Type III used in this study is a Type IIIA MCT formulation according to LFCS.

Research and Training (2014). All animal studies were performed on 8-week-old Sprague-Dawley rats sourced from Ozgene (Canning Vale, Australia). Rats were housed in a temperature-, humidity- and pressure-controlled animal holding facility with a 12 h/ 12 h light/ dark cycle. Rats were randomized upon arrival to the animal holding facility. Researchers were not blinded to the group allocations at any stage of the experimentation or analysis.

A longitudinal study was performed, where rats were dosed various SEDDS treatment groups via oral gavage for 21 days. The sample size for each group ($n = 8$) was based on Power calculations for anticipated changes in Shannon's Index (microbiota alpha diversity), using a power level of ≥ 0.8 and significance level of 0.05. An even distribution of male and female rats were used per group (4 males, 4 females per group). The study was approved by the South Australian Animal Ethics Committee under approval number U38-22. Rats were housed in groups of two with *ad libitum* access to a normal chow diet and water throughout. SEDDS concentrates were dosed at 100 mg/kg relative to rat body weight and were dispersed in PBS (1 mL/kg) immediately prior to daily oral administration. The control group was dosed PBS (1 mL/kg) via oral gavage. Evening dosing (between 16:00 and 18:00) was selected to mimic rodents' natural behaviour. Body weights were measured daily, immediately prior to dosing. On Day 22 (i.e. the morning after the final dose), rats were anaesthetized under 5 % isoflurane prior to cardiac puncture followed by terminal cervical dislocation.

2.3. Gut microbiota analysis

Faecal pellets were collected from each individual animal during handling on Day 0 and 21 and immediately stored in sterile tubes at −80 °C to avoid contamination and degradation of the sample. Faecal samples were sent for DNA extraction, PCR amplification and 16S rRNA sequencing at the Australian Genomics Research Facility (Brisbane, Australia). DNA was extracted from one faecal pellet per animal per timepoint to ensure microbiota signatures could be traced back to individual animals and time points. 16S rRNA sequences were processed for the V3-V4 hypervariable regions and raw reads were clustered (97 % similarity) to operational taxonomic units (OTUs) on Quantitative Insights into Microbiology Ecology (QIIME 1.8) using the Silva reference database. OTUs were assigned taxonomy using the Qiagen Microbial Insights – Prokaryotic Taxonomy Database (QMI-PTDB) on Qiagen CLC Genomics Workbench version 23.0.4 (Hilden, Germany). The Qiagen genomics module was used to derive alpha diversity at the genus level using Shannon's Index and beta diversity Principal Coordinate Analyses (PCoAs) based on Bray-Curtis dissimilarity metrics. Permutational multivariate ANOVA (PERMANOVA) of beta diversity plots was used to determine the statistical significance of microbiota dissimilarities between groups.

2.4. Pro-inflammatory cytokines analyses using ELISA

Jejunal tissue was collected and snap frozen in liquid nitrogen for quantification of proinflammatory cytokines, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumour necrosis factor alpha (TNF-α), using enzyme-linked immunosorbent assay (ELISA; Invitrogen). The LOQ for IL-1β, IL-6 and TNF-α was 4 pg/mL, 12 pg/mL, and 11 pg/mL, respectively. Briefly, jejunal tissue (approx. 30 mg) was weighed and homogenized in 500 μL RIPA buffer (ThermoFisher Scientific, Australia), with protease inhibitor cocktail (ThermoFisher Scientific, Australia) added at 1:100 dilution, prior to quantifying protein concentration using a BCA Protein Assay kit (ThermoFisher Scientific, Australia). Normalized protein concentrations (1000 μg/mL) of tissue homogenate were processed according to the manufacturer's instructions with absorbance measured at 450 nm. Cytokine concentration was calculated according to the standard curve.

2.5. Mucosal injury evaluations

Blood samples were collected via saphenous vein puncture on Day 0, 7, 14, and 21 for quantification of plasma citrulline levels, a validated biomarker of mucosal barrier injury and small enterocyte mass (Blijlevens et al., 2004; Van Der Velden et al., 2013). Plasma was isolated via centrifugation (4000 g, 10 min) and citrulline was quantified using a citrulline fluorometric assay kit (Sigma Aldrich, Australia). The limit of quantification (LOQ) for the citrulline fluorometric assay in plasma was 2 μM. Area-under-the-curve (AUC) values were quantified for plasma citrulline profiles, where a decrease in AUC indicates increased mucosal damage (Wardill et al., 2023).

2.6. Statistical analysis

All statistical analyses (excluding 16S sequencing analyses) of experimental data were performed using GraphPad Prism Version 8.0 (GraphPad Software Inc., California). Statistically significant differences were determined using an unpaired *t*-test and one-way ANOVA followed by Tukey's post-test for multiple comparisons. Values are reported as the mean ± standard deviation (SD), and the data were considered statistically significant when $p < 0.05$. Statistical significance is represented in figures by * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ or **** $p < 0.0005$.

3. Results

3.1. SEDDS comprising a lipid component significantly altered the composition and diversity of the gut microbiota

Four self-emulsifying drug delivery systems (i.e. SEDDS, SMEDDS, and SNEDDS) with varying compositions of lipid, water- or lipid-soluble surfactants, and co-solvent were fabricated according to the LFCS (Pouton and Porter, 2008). The formulations are commonly utilized by industry and academia for enhancing the bioavailability of poorly soluble compounds, and comprise Type I (lipid only), Type II (lipid and lipid-soluble surfactant), Type III (lipid, water-soluble surfactant, co-surfactant and co-solvent), and Type IV (water-soluble surfactants and co-solvent). The emulsion droplet size of each system varied upon redispersion in aqueous media, depending on their composition: Type I (6313 ± 890 nm); Type II (181 ± 21 nm); Type III (52 ± 6 nm); Type IV (44 ± 9 nm).

SEDDS formulations were dosed to Sprague-Dawley rats at 100 mg/kg for 21 days to simulate a chronic dosing regimen, given multiple commercial SEDDS are formulated for drugs that are chronically dosed (Betageri, 2019; Salawi, 2022). The selected dose (i.e. 100 mg/kg) was based on multiple factors: 1) a wealth of preclinical studies have dosed SEDDS at or above 100 mg/kg (where the SEDDS dose is calculated according to the relative drug loadings within the formulation) (see recent detailed reviews (Joyce et al., 2019; Salawi, 2022; Maji et al., 2021; Ruiz et al., 2022); 2) drug toxicology studies in preclinical species are typically investigated at high drug doses (i.e. 300 – 500 mg/kg). For lipophilic drugs encapsulated within lipid-based formulations, it is recommended that toxicology studies are undertaken at a drug dose of ≥ 100 mg/kg (Chen et al., 2012). Thus, the SEDDS dose selected is within the typical range of SEDDS dosing for preclinical animal studies and below that recommended for toxicology studies.

Faecal samples were collected on Day 0 (i.e. before the first dose) and Day 21 (i.e. after the final dose), where the relative abundance of microbes (at the family taxonomic level) for each animal is presented in Fig. 1A, and grouped relative abundance data presented in Fig. 1B. Principal component analysis (PCoA) revealed a significant shift in gut microbiota composition for animals administered with Type I, Type II and Type III SEDDS (i.e. those with a lipid phase) between Day 0 and Day 21, highlighted by separation between PCo1 in Fig. 1C. PERMANOVA (Bray-Curtis) of multi-dimensional beta diversity revealed statistically significant ($p < 0.05$) composition shifts for Type I, Type II and

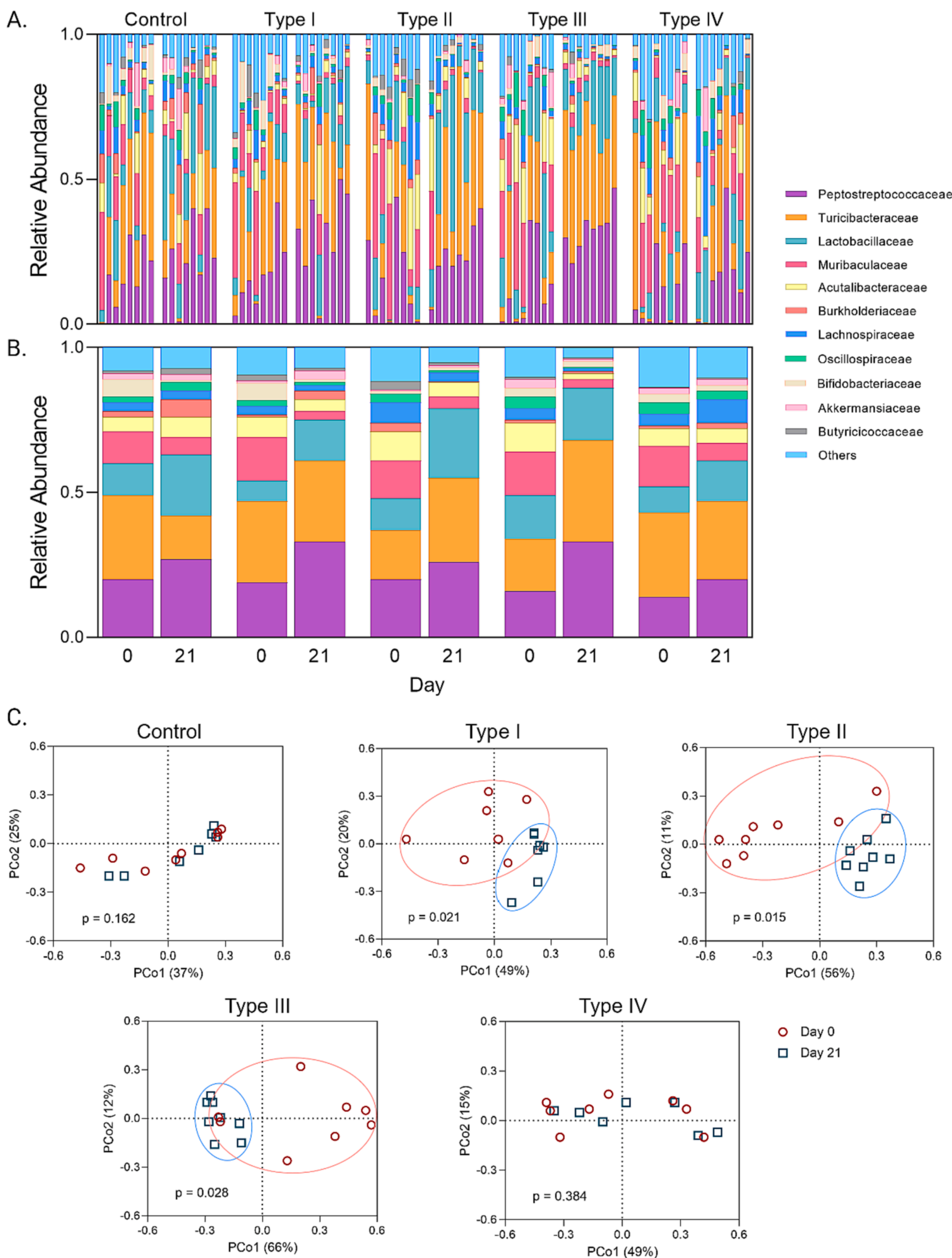

Fig. 1. The composition of the gut microbiota was significantly altered by Type I, Type II, and Type III SEDDS. Panel A contrasts the relative abundance of gut microbes at the family taxonomical level for each animal at Day 0 and Day 21, with Panel B showing the grouped relative abundance for each treatment group. Principle component analyses (Panel C) shows significant shifts in microbiota compositions for Type I, Type II and Type III SEDDS formulations, with no shifts being observed for the control group or Type IV SEDDS.

Type III groups, while no significant shift was observed for the Control or Type IV groups. Signatures of SEDDS-induced gut microbiota dysbiosis were further evident through significant longitudinal reductions in both operational taxonomic units (OTUs) and alpha diversity (Shannon's Index) for SEDDS formulations comprising a lipid phase (Fig. 2). No change in OTUs or alpha diversity was observed for the Control or Type IV group. Animal specific changes in OTUs and alpha diversity are presented in Fig. 2B and 2D, respectively.

Changes in alpha and beta diversity for Type I, Type II and Type III SEDDS were accompanied with an increase in the *Firmicutes*: *Bacteroidetes* (F:B) ratio (Fig. 3A), a common signature of microbiota dysbiosis (note: only Type I and Type III were statistically significant). Key changes in the relative abundance of taxa at the family level were also observed for the various SEDDS treatment groups. *Enterobacteriaceae*, a family comprising several gram-negative pathogens, was enriched by 4 – 7 $\log_2$-fold ($p < 0.05$) by SEDDS comprising a lipid phase (Fig. 3B). In contrast, a minor decrease in *Enterobacteriaceae* abundance was observed for the control group and a −6.11 $\log_2$-fold decrease ($p = 0.002$) was observed for the Type IV SEDDS group. Statistically significant decreases in the abundance of key commensal gram-negative taxa, specifically *Muribaculaceae* (previously S24-7), *Bacteroidaceae* and *Lachnospiraceae*, were observed for SEDDS comprising both a lipid and surfactant phase (i.e. Type II and Type III). Variable effects were observed for the gram-positive commensal family of *Clostridaceae*, where significant enrichment was observed by Type I and Type II SEDDS, whereas Type III and Type IV SEDDS significantly depleted the

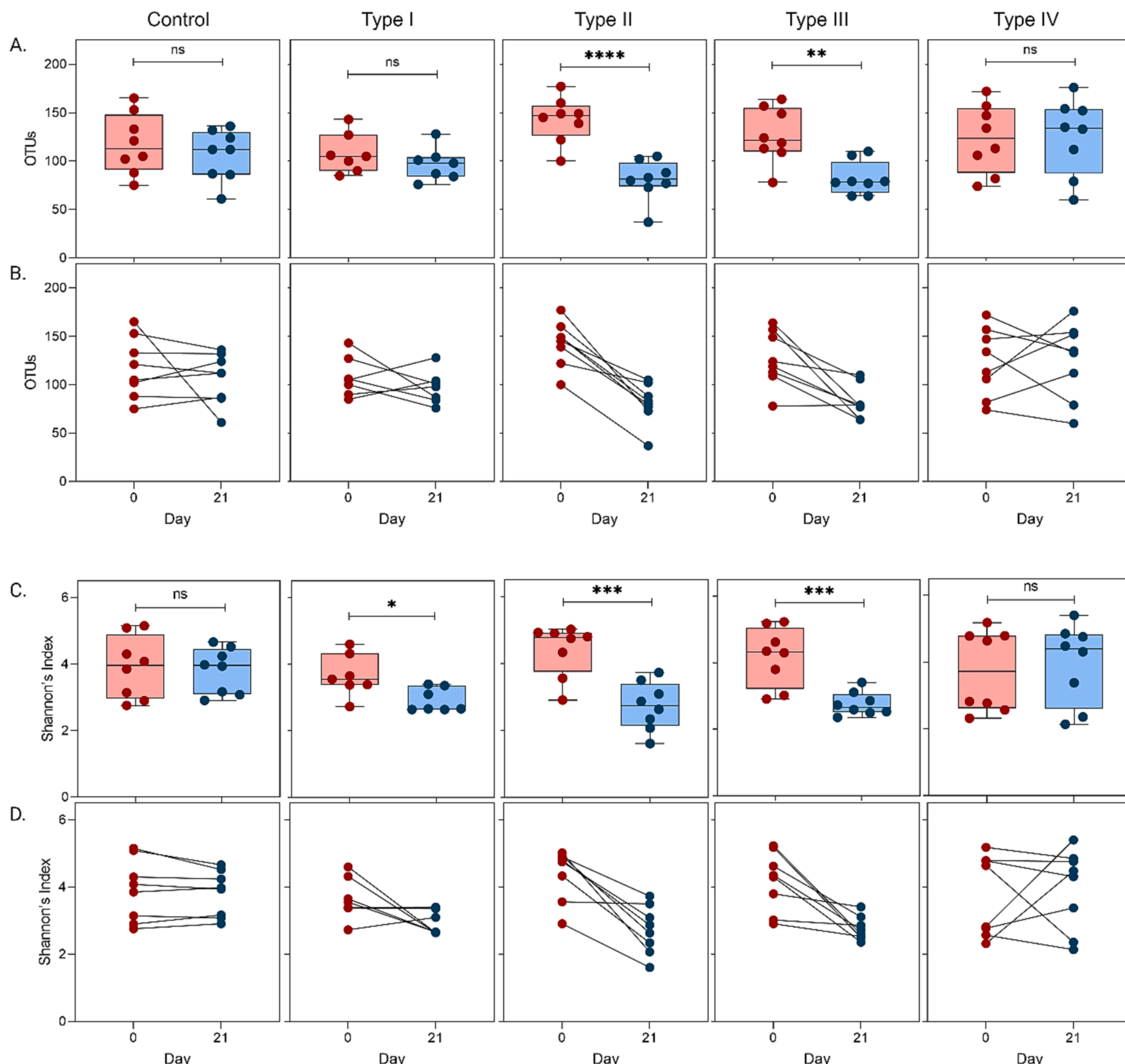

Fig. 2. SEDDS-induced gut microbiome dysbiosis is characterized by reduced OTUs and alpha diversity for formulations with a lipid component. OTUs were significantly reduced following daily dosing of Type II and Type III SEDDS (D), with no significant change being observed for the control group, Type I and Type IV groups. Panel B highlights the change in OTUs for each individual animal between Day 0 and Day 21. Alpha diversity, characterised by Shannon's Index, was significantly reduced following daily dosing of Type I, Type II and Type III SEDDS (C). No change in alpha diversity was observed for the control group or Type IV group. Panel D highlights the change in alpha diversity for each individual animal between Day 0 and Day 21.

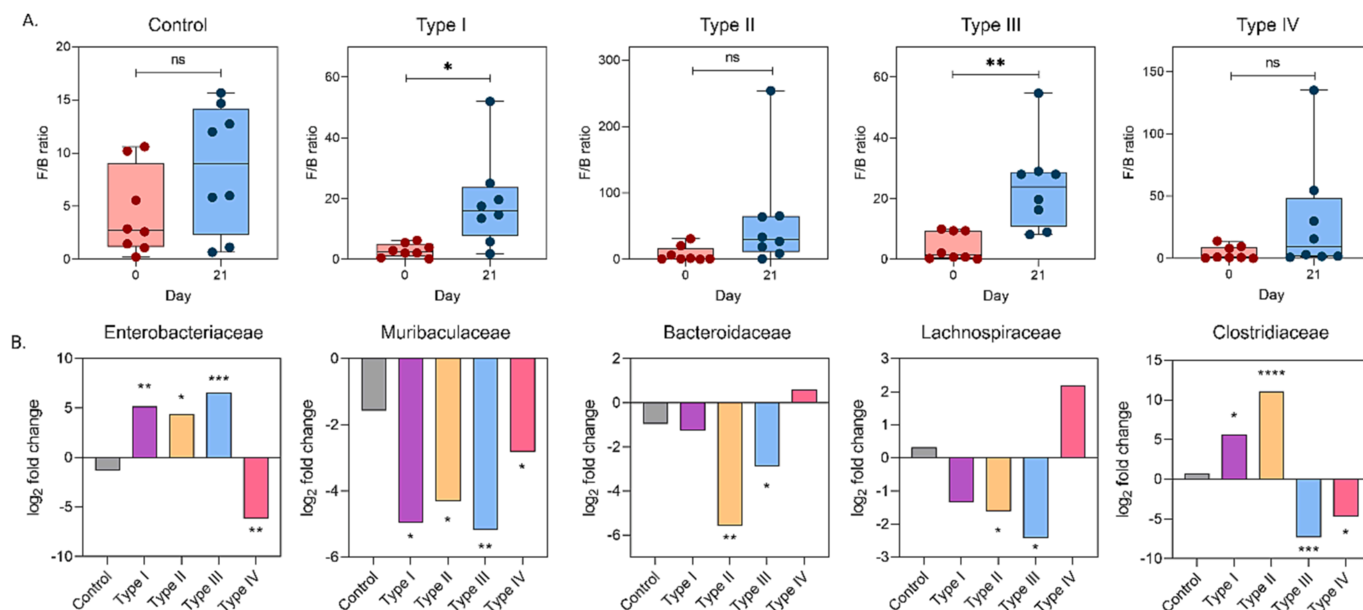

Fig. 3. Evidence of SEDDS-induced gut microbiome dysbiosis through changes in the relative abundance of microbial phyla and families. Type I and Type III significantly increased the *Firmicutes:Bacteroidetes* (F:B) ratio (A), while pathogenic *Enterobacteriaceae* were enriched and commensal microbial families were depleted by various SEDDS treatment groups between Day 0 and Day 21 (B).

Clostridaceae population.

3.2. *SEDDS containing both lipids and surfactants increase the expression of pro-inflammatory cytokines and induce mucosal injury in the small intestine*

The expression of pro-inflammatory cytokines, specifically IL-1β, IL-6, and TNF-α, within jejunal tissue was quantified at the completion of the study to observe the inflammatory response to daily SEDDS dosing. Fig. 4A highlights that SEDDS comprising both lipids and surfactants (Type II and Type III) induced a significant increase in IL-1β and TNF-α expression ($p < 0.05$), with Type II SEDDS also inducing a significant increase in IL-6 expression. Log-linear correlations between cytokine concentrations and OTUs (Fig. 4B) and alpha diversity (Shannon's Index) (Fig. 4C) reveal that both IL-1β and IL-6 expression negatively correlates with microbial abundance and diversity, respectively ($R^2 > 0.5$). That is, a pro-inflammatory response, measured through IL-1β and IL-6 expression, is greatest for animals with a depleted microbiota. In contrast, TNF-α expression poorly correlated ($R^2 < 0.2$) with both OTUs and Shannon's index, suggesting an alternate mechanism drives SEDDS-induced TNF-α expression.

Enterocyte mass and mucosal damage was monitored through changes in plasma concentrations of the validated biomarker, citrulline (Blijlevens et al., 2004; Van Der Velden et al., 2013). Plasma citrulline levels decreased in a time-dependent manner for all SEDDS groups containing a surfactant phase (Fig. 5A), leading to significant decreases in AUC (Fig. 5B). Type I SEDDS, comprising MCT only, caused an initial decrease in plasma citrulline levels to 80.2 ± 4.6 μM after 7 days of dosing, but citrulline concentrations on Day 14 and 21 were restored to be equivalent to the control group. Type II and Type III triggered the greatest reduction in plasma citrulline levels over the 21-day dosing period, with AUCs of 1482 ± 61 μM.d and 1413 ± 59 μM.d, respectively. Despite not inducing a significant pro-inflammatory response, Type IV SEDDS induced a dramatic decrease in plasma citrulline levels through an AUC of 1604 ± 51 μM.d.

Enterocyte and mucosal damage correlated with restricted weight gain for all SEDDS treatment groups (Fig. 5C), relative to the control. Type I and Type IV SEDDS induced equivalent reductions in weight gains, with $AUC_{body\ weight\%}$ of 2205 ± 10 and 2206 ± 7.8, respectively, while $AUC_{body\ weight\%}$ between Type II and Type III SEDDS were also equivalent, with values of 2186 ± 12 and 2183 ± 11, respectively.

4. Discussion

Tolerability and toxicity concerns exist over the chronic use of self-emulsifying drug delivery systems (i.e. SEDDS, SMEDDS, and SNEDDS), due to the high surfactant concentrations required to achieve solubility and bioavailability enhancement (Pouton and Porter, 2008; Thomas et al., 2012). Previous studies have shown that surfactants have the capacity to cause gastrointestinal inflammation, mucosa irritation and acute epithelial damage (Csáki, 2011; Glynn et al., 2017), while dietary emulsifiers potentiate intestinal inflammation by inducing gut microbiota dysbiosis in preclinical and clinical models (Chassaing et al., 2015; Chassaing et al., 2022; Chassaing et al., 2017). Despite this, no studies have investigated the capacity for SEDDS to interact with and disrupt the gut microbiota. Thus, the current study is the first to demonstrate that SEDDS of varying compositions induce significant, longitudinal shifts in the gastrointestinal microenvironment (Fig. 1), which is coupled with intestinal inflammation and mucosal injury (Fig. 4).

SEDDS were shown to exert formulation-dependent changes to the gut microbiota, where SEDDS comprising a lipid component (i.e. Type I, Type II and Type III) depleted the microbiota, as evidenced by decreases in OTUs and alpha diversity (Fig. 2). Type I (i.e. MCT only) triggered statistically significant shifts in beta diversity and an ≈20 % mean reduction in alpha diversity. The digestion products of MCT, specifically medium chain fatty acids and glycerides, have been shown to exert antimicrobial effects both in vitro (Hovorková et al., 2018; Huang et al., 2011; Shilling et al., 2013; Batovska et al., 2009) and in vivo (Yen et al., 2015; Lai et al., 2014; Cui et al., 2020; Tang et al., 2018). Thus, the decrease in microbial richness triggered by Type I SEDDS could feasibly be caused by free fatty acids that transit the gastrointestinal tract without being absorbed and impede the growth of both commensal and pathogenic taxa. However, further studies are necessary to validate this hypothesis and it should be noted that in a diseased or dysbiotic state, MCT has been shown to positively modulate and stabilize the gut microbiota for improved metabolic functioning (Wardill et al., 2023; Rial et al., 2016; Pilla et al., 2020; Yue et al., 2020; Zhou and Wang,

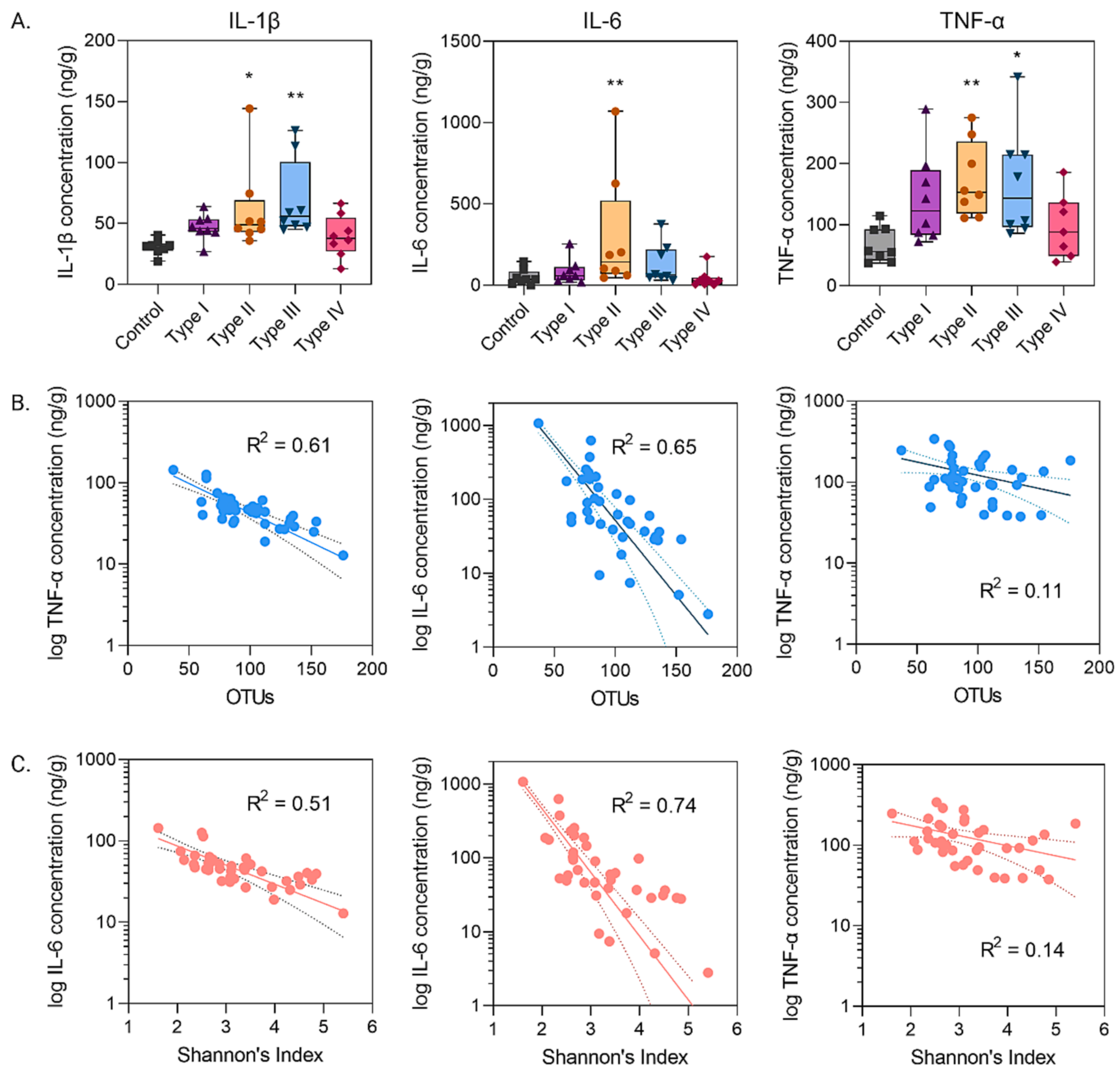

Fig. 4. A pro-inflammatory response in the jejunum is triggered by Type II and Type III SEDDS, evidenced by statistically significant increases in cytokine (IL-1β, IL-6, and TNF-α) expression (A). Log IL-1β and IL-6 tissue concentrations negatively correlated with OTUs (B) and alpha diversity (Shannon's index) (C). Dotted lines represent 95% confidence intervals.

2017). Importantly, Type I SEDDS did not trigger significant intestinal inflammation (Fig. 4) or mucosal damage (Fig. 5) in rats, in line with previous work that has shown MCT (the sole excipient in Type I) retains and restores intestinal epithelial and mucosal integrity (Xu et al., 2018).

Contrasting the gut microbiota changes caused by Type II and Type III SEDDS revealed distinct similarities, as observed through equivalent longitudinal changes in microbial abundance at the family taxa level (Fig. 1) and alpha diversity (Fig. 2). This was not as hypothesised, given Type II and Type III comprise distinctly different surfactant compositions, where Type II incorporates a lipid-soluble surfactant (lecithin) and Type III incorporates water-soluble surfactants (Tween 85, Kolliphor RH40). The mechanisms by which surfactants interact with and disrupt biological membranes differs depending on their polarity and affinity for aqueous- or lipid-rich media (Aguirre-Ramírez et al., 2021). That is, lipid-soluble surfactants penetrate and fluidize biological membranes, while water-soluble surfactants solubilize phospholipids and lipid-soluble membrane components (Pouton and Porter, 2008; Heerklotz, 2008). Based on their different surfactant compositions, it was hypothesized that Type II and Type III SEDDS interact with microbial membranes via different mechanisms leading to different microbiota effects. Despite similarities between complete microbiota analyses (e.g. alpha and beta diversities), Type II and Type III SEDDS induced significant changes to the abundance of the gram-positive commensal family, *Clostridaceae*, where Type II facilitated a > 10-$\log_2$ fold increase and Type III facilitated > 6-$\log_2$ fold decrease in *Clostridaceae* abundance (Fig. 3). This is in accordance with previous studies that have shown water-soluble surfactants, equivalent to those used in Type II, have an enhanced capacity to translocate the peptidoglycan layers of gram-positive bacteria than lipid-soluble surfactants (Sharma et al., 2022), and thus, more readily disrupt the bacterial cell wall of gram-positive strains (Falk, 2019). This indicates that further studies are clearly needed to characterise and contrast the mechanism of antibacterial

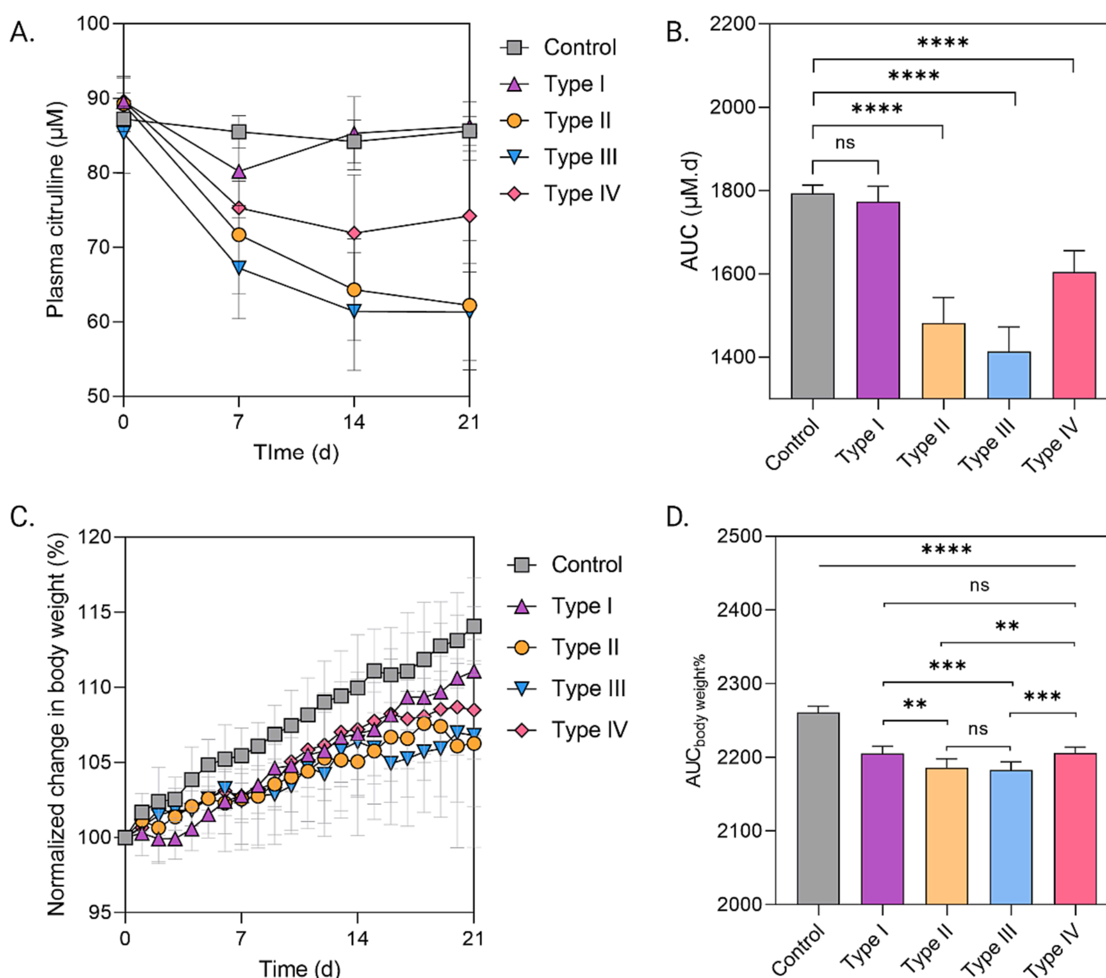

Fig. 5. Epithelial and mucosal injury was evident through reductions in plasma citrulline levels, a proven biomarker for gastrointestinal mucosal injury, and impeded weight gain. Panel A shows plasma citrulline levels significantly reduced in a time-dependent manner for Type II, Type III and Type IV. AUC values are significantly reduced for SEDDS treatment groups comprising a surfactant phase, relative to the control, indicating a surfactant phase is necessary to induce mucosal damage. Panel C shows each SEDDS treatment group triggered a reduction in body weight gain over the study period, corresponding to significantly reduced AUCs for all SEDDS group relative to the control (Panel D).

action between Type II and Type III SEDDS and their individual surfactants.

Surprisingly, Type IV did not induce any significant longitudinal changes to the gut microbiota (Fig. 2). The initial hypothesis was that the high surfactant content of SEDDS was a key driver of gut microbiota dysbiosis. However, given Type IV comprises water-soluble surfactants and a co-solvent, without a lipid phase, this provides further evidence that the lipid phase is necessary to trigger a dysbiotic state, at least for the dosage regimen used for this study. While Type IV did not disrupt the gut microbiota, the surfactant-only formulation did cause mucosal barrier injury, as evidence through decreases in plasma citrulline levels (Fig. 5A, B). This may indicate that SEDDS-induced mucosal injury is not specifically linked with changes to GI microbial richness and that multiple mechanisms are likely present. One potential hypothesis is that lipids and surfactants are differentially absorbed across the gastrointestinal tract into systemic circulation, which may alter the colonic exposure of the various SEDDS. Additional studies in germ-free rodents, with and without pre-treated microbiota transplantation, are recommended to further elucidate the mechanisms and link between microbiota dysbiosis and inflammation (Chassaing et al., 2015; Chassaing et al., 2017).

SEDDS-induced gut microbiota dysbiosis has potentially significant clinical implications, given dysbiosis is implicated with a myriad of detrimental health outcomes, ranging from metabolic syndrome (e.g. obesity, type 2 diabetes, IBD) to mental illnesses (e.g. depression, anxiety) (Jovel et al., 2018). Despite SEDDS componentry being classified as Generally Regarded as Safe (GRAS) by the US Food and Drug Administration (FDA) (Chen, 2008), the capacity for SEDDS to induce gut microbiota dysbiosis and inflammation raises significant tolerability concerns. From a drug delivery perspective, changes to the gut microbiota can trigger changes to every aspect of pharmacokinetics (i.e. absorption, distribution, metabolism, excretion) (Kamath et al., 2023; Saad et al., 2012), raising the question of whether SEDDS-facilitated drug absorption is impacted by changes to the gut microbiota. Subsequently, the current study provides the basis for clinical testing to elucidate whether the current findings in rodents correlate with the impact of SEDDS on the human gut microbiota. A critical consideration when designing future clinical studies will be the selected dosage regimen. The current study employed a dose that exceeds clinical dosing in humans (i.e. 100 mg/kg) and hence, clinical studies should employ a SEDDS dose that is based on currently prescribed and clinically adopted SEDDS formulations to ensure the findings inform the future clinical application of SEDDS.

From a preclinical perspective, the current study demonstrates that rodents' microbiota are altered by oral lipid-based formulations. This should be taken into account for preclinical pharmacokinetic studies, since microbiota changes may drive indirect changes to pharmacokinetics (Cussotto et al., 2021). A key limitation of the current study,

however, is the single SEDDS dose and limited time points of faecal collection, which raises the question of how much SEDDS exposure is required to trigger gut microbiota dysbiosis. Therefore, dose-dependent and multi-time course studies should be performed to determine how much SEDDS exposure is required to cause microbiota dysbiosis and intestinal side effects. Such studies will uncover the need for identifying and engineering solubilisation and bioavailability enhancing oral formulations that do not disrupt the gastrointestinal microenvironment.

Finally, the current study highlights the need for exploring the impact of oral delivery formulations on the gut microbiota. Current focus within the field of pharmacomicrobiomics has been solely focused on understanding the bidirectional interaction between active drugs and the microbiota, in the absence of formulation excipients relevant to each drug. Given previous work has highlighted that pharmaceutical excipients interact with the gut microbiota (Subramaniam et al., 2023) and in light of the current findings that have demonstrated the detrimental impact of SEDDS on the gut microbiome, it is critical that future studies are designed to elucidate the impact of the entire drug formulation on the gut microbiota (Kamath et al., 2023). It is expected that such studies will uncover significant clinical ramifications for the application of oral delivery systems, where microbiota effects can drastically impact efficacy and safety profiles of drugs.

5. Conclusions

This is the first study to provide evidence that self-emulsifying drug delivery systems alter the composition and diversity of the gut microbiota. For the current dosing regimen (100 mg/kg SEDDS, daily for 21 days), it was established that SEDDS comprising a lipid component depleted the gut microbiota of Sprague-Dawley rats through changes to alpha and beta diversity, which correlated well the enhanced expression of pro-inflammatory cytokines within the jejunum and intestinal barrier injury. In contrast, SEDDS comprised of only surfactants did not disrupt the gut microbiota but induces intestinal barrier injury. Thus, the mechanisms driving SEDDS-induced inflammation is multifaceted and formulation-dependent. The insights derived from this study emphasise the need for undertaking pharmacomicrobiomics investigations using commercially and clinically relevant formulations, given oral formulations (e.g. SEDDS) directly interact with and modify the gut microbiota.

CRediT authorship contribution statement

Santhni Subramaniam: Methodology, Investigation, Formal analysis, Writing – original draft. **Aurelia Elz:** Methodology, Investigation, Data curation, Writing – review & editing. **Anthony Wignall:** Methodology, Investigation, Data curation. **Srinivas Kamath:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Amin Ariaee:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Alexander Hunter:** Investigation, Data curation. **Tahlia Newblack:** Investigation, Data curation. **Hannah R. Wardill:** Validation, Formal analysis, Writing – review & editing. **Clive A. Prestidge:** Resources, Writing – review & editing. **Paul Joyce:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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