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Investigating the Impact of Short-Chain Fatty Acids (SCFAs) from the Maternal Microbiome on Pregnancy and Fetal Health of C57BL/6J Mice: A Research Protocol

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Abstract

Short-chain fatty acids (SCFAs), produced by commensal gut bacteria through fibre fermentation, are essential for regulating immune responses during pregnancy, benefiting maternal and fetal health. Propionate, for example, activates G protein-coupled receptors (GPCRs), promoting colonic T regulatory cell (Treg) differentiation that is critical in maintaining inflammatory homeostasis. These molecules are not confined to the gut. They can traverse the placental barrier, directly reaching the fetal compartment, where they may influence placental efficiency, impact pregnancy outcomes, and potentially predispose the child to autoimmunity and allergy. Despite the known association between dysbiosis and adverse pregnancy outcomes, few direct mechanistic studies have investigated the interaction between the maternal microbiome and the developing immune system during pregnancy. To develop a robust protocol, we designed a murine model experiment with differential SCFA exposure before pregnancy using C57BL/6J mice. Separate groups of female mice will be provided with different drinking water treatments before pregnancy: standard diet with untreated drinking water (untreated control) or one supplemented with SCFAs. A standard diet with an antibiotic cocktail in the drinking water will serve as a comparison, the purpose of this approach is to deplete SCFA-producing microbiota and assess the effects of SCFA deficiency. Maternal fecal samples will be collected during the perinatal stage for SCFA analysis using mass spectrometry, and for microbiome profiling via 16S ribosomal RNA gene sequencing. Fecal samples from neonatal mice will be collected shortly after birth for the same analyses. Systemic immune alterations will be examined using maternal and fetal blood samples to quantify key cytokines using a multi-plex ELISA. Flow cytometry will be conducted to compare differences in immune cell composition between different maternal diet groups. Other data including birth weight, litter size, and gestation duration, will also be compared to assess the influence of SCFA on pregnancy outcomes. We hypothesize that SCFA supplementation fosters an anti-inflammatory microbiome, elevating Tregs and anti-inflammatory cytokines while downregulating proinflammatory responses. This may lead to fewer pregnancy complications and improved fetal development. This research will help elucidate the role of SCFAs in maternal-fetal immune crosstalk. It could inform dietary or therapeutic strategies to reduce infant immune-related diseases and support long-term health.

Keywords: gut microbiota; maternal diet; fetal development; immune modulation; pregnancy outcomes; short-chain fatty acids; microbial metabolites; c57bl/6j mice; cytokines; maternal-fetal interaction

Introduction

Recent advances in understanding the gut microbiome have revealed its profound impact on individual health and long-term health sequelae for mother and child [1]. The microbiome consists of bacteria, archaea, lower and higher eukaryotes, their genomes, and the surrounding environmental conditions, contributing to a well-described range of physiological functions [2]. It is well-established that gut microbiota produces bioactive metabolites capable of entering maternal circulation, crossing the placenta through active or passive mechanisms, and modulating bacterial colonization in the gut [3].

Short-chain fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid are primary products of the gut microbes' anaerobic fermentation of indigestible dietary fibres [4]. Evidence suggests that SCFAs can function as immunomodulatory agents, influencing the maternal immune system by promoting anti-inflammatory responses [3, 4]. These metabolites are essential in maintaining gut homeostasis and

regulating immune tolerance, which prevents maternal autoimmune responses during pregnancy [5].

Koren et al. stated that SCFAs exert anti-inflammatory effects by inhibiting the synthesis of pro-inflammatory mediators such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) [6]. This inhibition occurs through interacting SCFAs with specific G-protein-coupled receptors (GPCRs) on immune cells, suppressing inflammatory pathways [6]. Furthermore, SCFAs have been shown to promote the production of anti-inflammatory mediators, including IL-10 and IL-22, thereby contributing to immune homeostasis [6, 7]. SCFAs were also demonstrated to regulate T and B cell gene expression epigenetically, thereby regulating antigen-specific adaptive immunity. For example, they can inhibit histone deacetylases (HDACs) in naïve T cells, which alters the chromatin landscape to favour the expression of key genes (e.g., *Foxp3*) involved in Treg cell differentiation, thus helping to decrease the risk of chronic inflammation and support immune tolerance [6, 7]. While numerous review articles highlight the association between dysbiosis (an imbalance in microbiota composition) and various pregnancy complications or adverse fetal outcomes, a limited number of studies have directly explored the interactions between the gut microbiome and the immune system during pregnancy, and how these interactions impact offspring development [4, 6, 8].

Thus, we aim to establish a protocol using C57BL/6J mice as a model to investigate how SCFAs from the maternal microbiome influence maternal immune function during pregnancy. Furthermore, we will evaluate how SCFAs affect pregnancy outcomes, fetal health, and growth development, focusing on the abovementioned evidence.

Although there is a considerable gap in knowledge regarding maternal–driven immune alterations in offspring, our findings will provide insight into the role of the maternal microbiome in healthy pregnancy from an immunological perspective. They may reveal mechanisms that warrant further investigation.

Methods

Study Type

In vivo study with C57BL/6J mice to investigate the impact of dietary SCFA during pregnancy on maternal immune responses and fetal health.

Phase 1: Initial Purchase and Acclimatization

C57BL/6J mice (n=72), a commonly used strain in immunology and microbiome research, will be obtained from The Jackson Laboratory at 3-4 weeks of age immediately after weaning (21-28 days of age) [9]. Each cohort consists of 24 female and 12 male subjects, with two cohorts in total. Six females in each cohort will be assigned to each of the following groups: the untreated control group (normal microbiome), the SCFA group (SCFA-supplemented to assess SCFA effects independent of the microbiota), and the antibiotic group (antibiotic-treated to deplete SCFA-producing microbiota and assess the effects of SCFA deficiency). An additional 6 females will be non-pregnant controls, maintained on normal water and diet throughout the experiment. The males (n=12 per cohort) will be used in mating trios, with one male and two females per trio. A one-week acclimatization period will be provided for the mice to adapt to the laboratory environment, designated as week 0 (See Figure 1). Both non-pregnant and untreated control groups will be age-matched with the treatment groups. Mice will be housed in a controlled temperature (22–24°C) room, 12:12 hours light/dark cycle, fed ad libitum with a standard diet (5015 - Mouse Diet from lab diet) and water [5, 10].

Phase 2: Pre-Mating Phase

Females will be treated according to their respective groups during the experiment's pre-mating period (weeks 1-3), as illustrated in Figure 1. Without additional treatment, the control groups will receive standard water and diet (5015 - Mouse Diet). The SCFA group will have drinking water supplemented with sodium acetate (67.5 mM), sodium propionate (25 mM), and sodium butyrate (40 mM) from Sigma-Aldrich for three weeks [11]. The antibiotic group will receive a broad-spectrum antibiotic cocktail consisting of vancomycin (0.5 g/L), ampicillin (1 g/L), neomycin (1 g/L), and metronidazole (1 g/L) in drinking water for three consecutive weeks, after which normal drinking water will be provided [12]. The altered microbiome of the treatment groups should persist for a sufficient duration to support the objectives of this experiment [13, 14]. Fresh fecal pellets are collected aseptically from each mouse at two key time points: pre-intervention and post-treatment, immediately frozen on dry ice, then stored at –80 °C [15].

Microbiome Profiling and Fecal SCFA Quantification

Microbial DNA will be extracted from fecal samples using a commercial kit optimized for bacterial DNA recovery (e.g., QIAamp Fast DNA Stool Mini Kit, Qiagen) [15, 16]. The V4 region of the 16S rRNA gene will be amplified using the universal primer pair 515F/806R, which provides broad coverage of bacterial taxa and is widely used in gut microbiome studies [15, 17]. Sequencing will be performed on an Illumina MiSeq platform. Demultiplexing, quality filtering, denoising, and taxonomic classification will be performed using the QIIME2 pipeline (Quantitative Insights into Microbial Ecology; version 2023.2) software package that enables microbial community analysis [18].

To measure SCFAs, approximately 100 mg of fecal material will be homogenized with 900 µL of sterile phosphate-buffered saline (PBS; pH 7.4) in a sterile tube [15]. The mixture will then be centrifuged, and the supernatant will be filtered through a 0.45 µm membrane filter [15]. Concentrations of acetate, propionate, and butyrate (expressed in µmol/g of feces) will be quantified using high-performance liquid chromatography (HPLC) on a Waters 2695 Alliance system (Waters Corporation, Milford, MA,

USA) equipped with a diode array detector (DAD) set at 210 nm [15]. An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) will be used for separation [15].

Phase 3: Mating Phase

At week 4, treated females will be paired with untreated males in a 2:1 female-to-male ratio to form mating trios, as illustrated in Figure 1. Mating success will be confirmed by checking for vaginal plugs daily, with successful pregnancy recorded as day 0 of gestation (GD 0). Once pregnant, dams will be housed individually (1 female per cage).

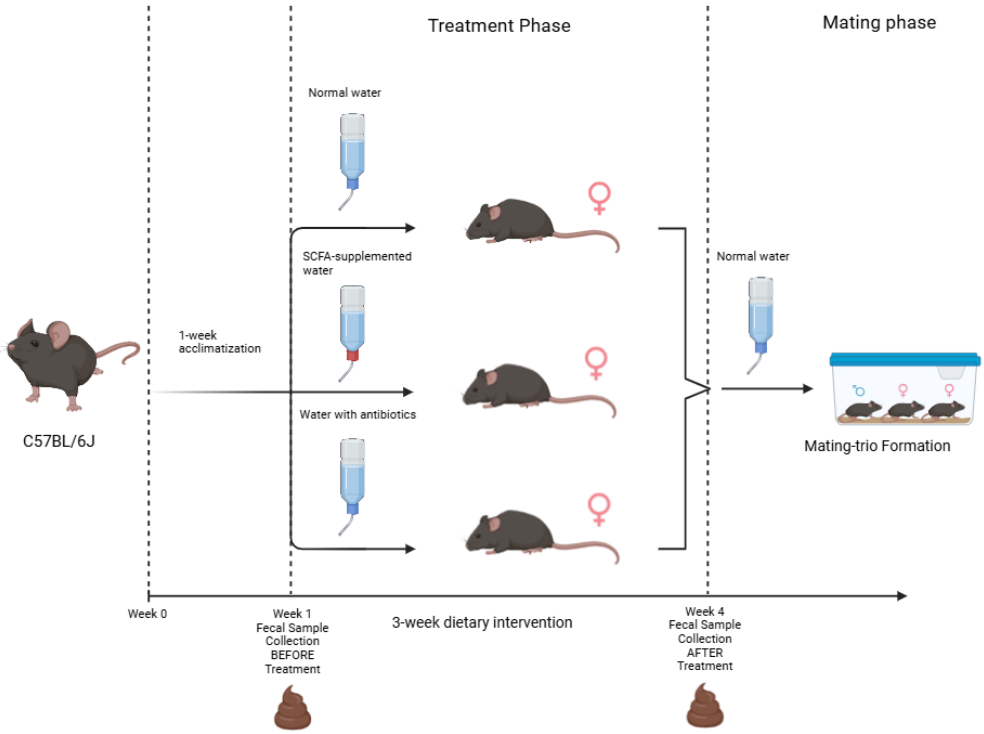

Figure 1. Experimental Design of Phases 1-3 of The Experiment: Different Diet Treatments and Mating-Trio Formation on C57BL/6J Female Mice. Mice will undergo a 1-week acclimatization period followed by a 3-week dietary intervention to receive normal, SCFA-supplemented, or antibiotic water. Fecal samples will be collected before treatment (onset of Week 1) and after treatment (onset of Week 4). All mice will be provided with normal water after the treatment phase and introduced into a mating-trio formation for the mating phase. Figure created using BioRender (https://www.biorender.com).

Phase 4: Gestation and Litter Birth

During weeks 5 to 8, at GD 10, fecal samples will be collected from pregnant mice for SCFA analysis using mass spectrometry. Maternal blood samples (~600 µL) will also be collected in EDTA anticoagulant via cardiac puncture on the same day by sacrificing one cohort of pregnant female mice to ensure collection of sufficient volume for the experiment, as illustrated in Figure 2. Plasma (supernatant) will be isolated by centrifuging whole blood and used for cytokine analysis via multi-plex enzyme-linked immunosorbent assay (ELISA) [20]. The buffy coat, containing white blood cells, will be carefully collected and used for immune cell analysis via flow cytometry. The plasma layer will be removed after centrifugation to isolate the buffy coat, and the thin buffy coat layer will be carefully aspirated. Residual red blood cells (RBCs) will be lysed with 1X ACK lysing buffer. Cytokine levels (IL-6, TNF-α, IL-10, IL-22) in systemic circulation and analysis of TH1, TH2, and Treg populations with subset-specific markers will be measured. Specifically, $CD4^+$ $T\text{-}bet^+$ for TH1, $CD4^+$ $GATA3^+$ for TH2, and $CD4^+$ $Foxp3^+$ for Treg [21]. The body weight of pregnant females will be recorded before blood collection, while the duration of gestation, litter size, and fetal weights will be recorded at birth, as illustrated in Figure 2 and 3.

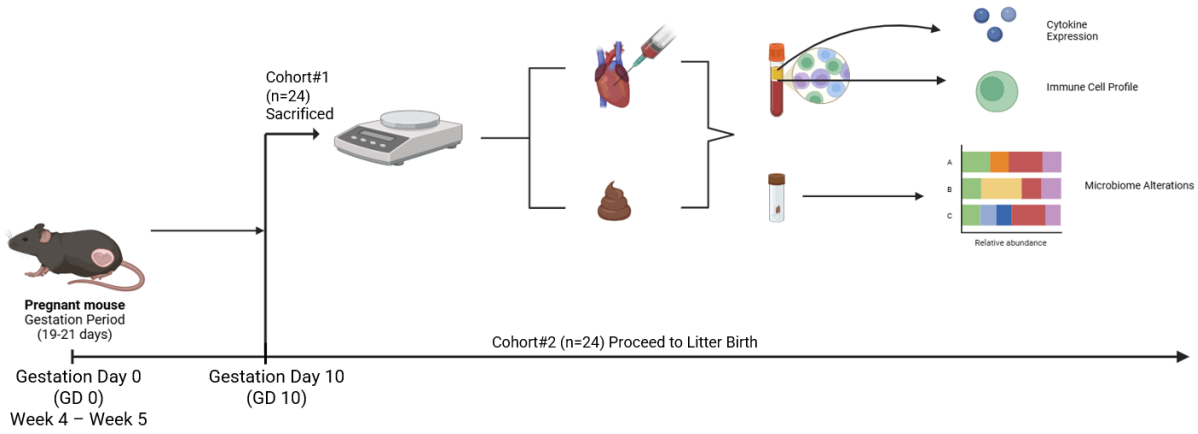

Figure 2. Experimental Design for Phase 4 of The Experiment: Pregnant Mice During Gestation and Sample Collection. Pregnant C57BL/6J mice (gestation period in 19-21 days) will be monitored from GD 0 through either mid-gestation (GD 10) or full-term birth. At GD 10, Cohort 1 (n=24) will be sacrificed to collect cardiac samples for cytokine and immune cell analysis, and fecal samples for microbiome assessment. Cohort 2 (n=24) will proceed to full-term pregnancy and litter birth. Figure created using BioRender (https://www.biorender.com).

Phase 5: Postnatal Monitoring and Offspring Assessment

At postnatal day 10 (PND10), fecal samples will be collected from offspring for SCFA analysis via mass spectrometry. Blood samples (at a volume appropriate for PND10 mice) will also be collected via cardiac puncture to assess cytokine levels (IL-6, TNF-α, IL-10, IL-22) and immune cell populations (TH1, TH2, and Treg) using ELISA and flow cytometry, consistent with the methods described above, as illustrated in Figure 3. By PND10, the immune system in mice is expected to have matured enough to reflect appropriate cytokine profiles and immune cell populations [22]. The body weight of offspring will also be recorded at PND10 before blood collection.

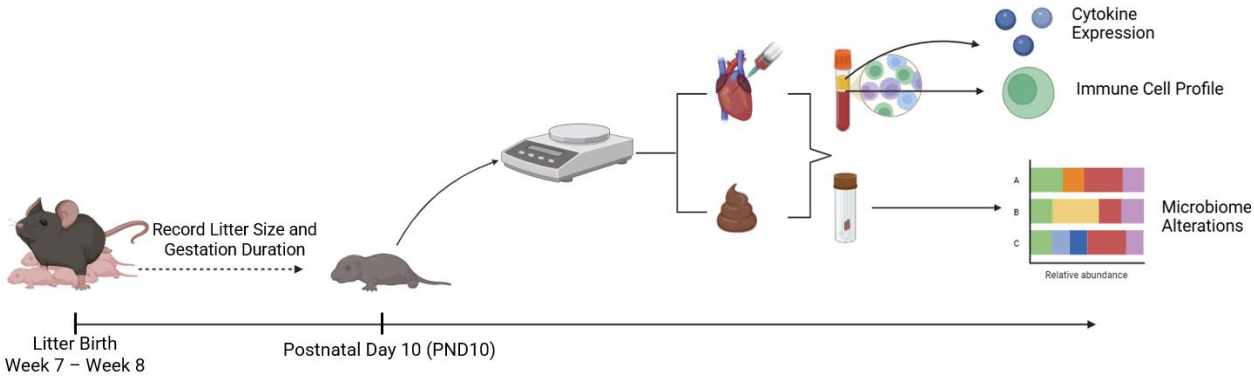

Figure 3. Experimental Design for Phase 5 of the Experiment: Postnatal Analysis. Litter size and gestation duration will be recorded after litter birth in Weeks 7-8. On PND 10, offspring will be sacrificed for sample collection. Cardiac and blood samples will be analyzed for cytokine expression and immune cell profiling, while fecal samples will be collected to assess microbiome alterations. Figure created using BioRender (https://www.biorender.com).

Data Analysis Plan

Use of ANOVA and t-tests to determine if there are significant differences between the groups.

Results

The SCFA supplied C57BL/6J mice group will be expected to have increased concentrations of the three most abundant luminal SCFAs—acetic acid, propionic acid, and butyric acid—compared to the two other experimental groups. On the other hand, the antibiotic-treated group is expected to have the lowest concentrations compared to other experimental groups [23]. Additionally, as suggested in previous literature, 16S rRNA gene sequencing and mass spectrometry are expected to show that SCFA levels positively correlate with gut microbiota diversity, with antibiotic treatment leading to a rapid decline in this diversity

[13]. According to Laubitz et al, microbiota recovery required 20 days following a 10-day antibiotic regimen; thus, the microbiome composition and diversity of the female mice should remain stable after three consecutive weeks of treatment, with any potential changes persisting long enough for this experiment [13].

SCFA supplementation is anticipated to modulate the maternal gut microbiota toward an anti-inflammatory profile, characterized by increased differentiation of Treg cells and elevated levels of anti-inflammatory cytokines such as IL-10 and IL-22, while reducing the production of pro-inflammatory cytokines including IL-6 and TNF-α [5, 7, 23]. Although IL-22 exhibits both tissue-protective and pro-inflammatory properties depending on the physiological context, SCFA-driven IL-22 expression is thought mediate anti-inflammatory and barrier-supporting effects in pregnancy unless in the presence of chronic inflammation, microbial imbalance, or an unhealthy maternal immune environment [24]. Conversely, antibiotic-induced dysbiosis has been shown to impair the development of colonic Treg cells and to predispose the host to dysregulated immune responses, contributing to systemic and intestinal inflammation [25]. In the context of pregnancy, the maternal gut microbiome and its metabolites, particularly SCFAs, are critical for supporting placental growth and vascular development [26]. Therefore, we expect that SCFA depletion resulting from microbiota disruption of antibiotics will negatively impact pregnancy outcomes by disturbing maternal immune homeostasis and impairing placental function. It is expected that the immune response of neonatal mice at PND10 will be influenced by the maternal diet, with newborn mice potentially showing an immune profile that mirrors the immune modulation observed in their mothers. However, the neonatal immune system may exhibit distinct developmental characteristics. Additionally, differences in SCFA levels, body weight, and immunological factors are anticipated between untreated pregnant and non-pregnant control mice.

If these results are proven experimentally, that is, SCFAs can improve maternal metabolism, reduce inflammation, and support placental morphogenesis, these factors likely positively influence the nutrient availability for the fetus, contributing to optimal fetal growth and development. Thus, we also predict that the SCFA-supplemented group will display the highest fetal body weight and the largest litter size, in contrast to the control group, which will show standard body weight and size, and the antibiotic-treated group, which is predicted to have the lowest fetal body weight and smallest litter size.

In terms of dysbiosis-associated pregnancy complications (i.e. preterm birth, low fetal weight), these adverse outcomes are expected to occur predominantly in the antibiotic-treated group. In contrast, they are anticipated to be minimal or absent in the SCFA-supplemented group. ANOVA and t-test will be expected to indicate a significant difference between groups.

Discussion

This study would explore the effects that accompany a changing maternal microbiome. In particular, we examine short-chain fatty acids (SCFA) and their potential impact on maternal immune function during pregnancy, including immunophenotyping, peripheral blood cells, cytokine profiles, and offspring outcomes.

If the results are as expected, they would show that the SCFA supplementation group significantly maintains and improves intestinal microbial diversity, regulates the transformation of the maternal immune system to a tolerogenic state, and thus improves pregnancy outcomes, manifested in increased fetal weight and litter number. These predictions are supported by existing research evidence, suggesting that SCFAs and gut microbiota are intricately linked, with SCFAs being products of the fermentation of dietary fibres by gut bacteria, which can also act as signalling molecules, fostering a stable and diverse microbiota [27]. This microbiome diversity leads to significant alterations in the maternal immune system. Sun et al. state that SCFA can activate GPCRs, which are expressed on virtually all immune cells, including neutrophils, macrophages, and epithelial cells [5]. Specifically, this activation has been shown to inhibit pro-inflammatory cytokine TNF-α production in epithelial cells and neutrophils [5], butyrate has been further shown to modulate the function of intestinal macrophages by inhibiting LPS-induced proinflammatory mediators such as IL-6 [5]. Additionally, a recent study reported that SCFA can enhance the production of immunoregulatory cytokines such as IL-10 in T cells, which is essential for Treg-mediated suppression [23]. They can also promote IL-22 production by CD4+ T cells, supporting intestinal barrier integrity and immune homeostasis [7]. In murine models, SCFA supplementation increases the frequency and function of regulatory T cells in the colonic lamina propria, characterized by elevated *Foxp3* and *IL-10* expression, further reinforcing an anti-inflammatory immune environment [23].

The expansion of this T cell population is consistent with the known immunoregulatory effects of SCFAs, which are thought to promote tolerance and immune homeostasis [27]. This observation could have important implications for maternal health, particularly in preventing inflammation-driven pregnancy complications. SCFA supplementation can help reduce excessive immune activation that might otherwise impair placental function or fetal development by fostering a more anti-inflammatory milieu [5, 7, 23]. This immunomodulatory effect is expected to contribute to a normal gestation period, increased litter sizes due to reduced intrauterine fetal death, and healthier fetal weights. The latter may result from the essential role of SCFAs in supporting placental morphogenesis and vascularization, which enhances nutrient availability to the fetus [26]. Pregnancy alone has been shown to alter the levels of SCFAs accompanied by inflammatory processes and weight gain essential for nurturing and adapting to offspring [4, 8]. Thus,

we expect to see SCFA, weight, and immune response differences between pregnant and non-pregnant control mice. On the other hand, in the antibiotic treatment group, the imbalance of microbiota and decreased SCFA levels will lead to immune dysregulation and increased inflammatory response, which increases the risk of adverse pregnancy outcomes.

SCFAs produced in the mother's gut can also have lasting effects on the offspring after birth. Antibiotic-treated mothers with lowered SCFA levels and disrupted gut microbiota diversity is expected to result in both an increase in proinflammatory effector CD4+ subsets and a decrease in CD8+ T cell populations, alongside a reduction in the proportion of Treg cells in the offspring (similar to reduction of Treg population is shown in germ-free mice [23]). Pups born to mother mice treated with antibiotics during pregnancy have shown increased susceptibility to vaccinia virus infection due to reduced interferon-γ (IFN-γ) production by CD8+ T cells. This is linked to altered activation and T cell receptor (TCR) expression, which is critical for sustained cytokine production [28]. Additionally, CD4+ and CD8+ T cells from mice prenatally exposed to vancomycin expressed significantly higher levels of inflammatory cytokines, such as IL-17, IFN-γ, and TNF-α, which may accelerate T cell-mediated autoimmune diseases like Type 1 diabetes [29]. The reduced number of Tregs can further exacerbate the risk of autoimmune diseases, as Tregs play a key role in lowering IFN-γ production, downregulating chemokine receptor expression required for migration to the islets, and limiting the proportion of T cells that produce TNF-α or IL-17 [30]. Furthermore, SCFA supplementation in a mouse model showed that the fetuses had increased expression of the autoimmune regulator (AIRE) gene, which is necessary for autoimmune regulation and the formation of Treg cells in early life [31]. This is consistent with our prediction that the antibiotic group will display opposite trends compared to the SCFA-supplemented group, with adverse pregnancy outcomes and compromised fetal development of immunological tolerance. As such, offspring in our study will be more likely to develop immune-related disorders, including but not limited to allergies, asthma and other autoimmune diseases [32].

One limitation of our current experimental design is that antibiotic treatment broadly depletes the gut microbiota, which may introduce confounding effects beyond SCFA depletion alone. While the antibiotic group is intended to model SCFA-deficient conditions, antibiotics can also independently affect fetal outcomes, such as may induce stress in the mice, interfere with nutrient absorption, or alter other microbial metabolites. Including a rescue group receiving both antibiotics and SCFA supplementation would help isolate the specific effects of SCFAs in the absence of microbiota. Although this group is not included in the present study, future iterations of the protocol may incorporate it to strengthen causal interpretations.

Should SCFA supplementation result in unexpected outcomes – such as a lack of measurable effect or failure to induce anti-inflammatory responses, a plausible explanation can be that differences in maternal microbiota composition or maternal immune status could influence SCFA responsiveness. This is particularly relevant given that pregnancy is a highly dynamic, individualized and unique physiological state, involving complex and intertwined immunological and metabolic adaptations [33]. If SCFA supplementation proves to be beneficial in promoting maternal-fetal immune tolerance and healthy pregnancy outcomes, it could inform dietary recommendations during pregnancy. Conversely, if antibiotic-induced SCFA depletion is linked to immune or developmental dysregulation, it may support more cautious use of antibiotics in expectant mothers.

Conclusions

In this study, a systematic experimental protocol was established. By setting up the untreated control, SCFA supplement and antibiotic treatment groups in the C57BL/6J mouse model, the study will systematically investigate intestinal microbial diversity, immune cell distribution, cytokine expression and fetal growth indicators at various stages, including pre-copulation, pregnancy and postpartum. Specifically, the effects of short-chain fatty acids (SCFAs) in the maternal microbiome on pregnancy immune regulation, fetal development and overall pregnancy outcomes will be investigated. Clearly outlining experimental methodologies sets the stage for robust and reproducible studies in this critical area of research. Through appropriate data analysis, this experiment will provide insights into the role of maternal microbiome modulation (specifically SCFAs) in optimizing pregnancy outcomes and fetal health, offering the potential to develop microbiome-based therapies to improve maternal immune health during pregnancy. These therapies could reduce the potential immune-mediated disease incidence rate among infants and enhance overall health outcomes. Future studies could explore the broader effects of products from the maternal microbiome (not just SCFAs) on pregnancy and fetal health to reveal a more comprehensive understanding of the microbiome's role in pregnancy. Future work could also incorporate cross-species studies comparing the effects of SCFAs and maternal microbiome composition in different animal models to achieve effective translation from animal models to human clinical practice.

List of Abbreviations Used

16S rRNA: 16S ribosomal ribonucleic acid
ACK: ammonium-chloride-potassium
AIRE: autoimmune regulator
ANOVA: analysis of variance
CD4: cluster of differentiation 4
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
Foxp3: forkhead box P3

GATA3: GATA binding protein 3
GD: gestation day
GPCR: G protein-coupled receptor
HDAC: histone deacetylase
IFN- γ: interferon-γ
IL-10: interleukin-10
IL-17: interleukin-17
IL-22: interleukin-22
IL-6: interleukin-6
LPS: lipopolysaccharide
PND: postnatal day
RBC: red blood cell
SCFA: short-chain fatty acid
T-bet: T-box transcription factor TBX21
TCR: T cell receptor
TH1: T helper cell 1
TH2: T helper cell 2
TNF-α: tumour necrosis factor-alpha
Treg: t regulatory cell

Conflicts of Interest
The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent
This study did not require ethics approval and/or participant consent, as this study is a hypothetical proposal.

Authors' Contributions
NS: made contributions to the design of the study, drafted the manuscript, illustrated the figures, and gave final approval of the version to be published.
SC: made contributions to the design of the study, drafted the manuscript, illustrated the figures, and gave final approval of the version to be published.
PW: made contributions to the design of the study, drafted the manuscript, illustrated the figures, and gave final approval of the version to be published.

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