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THE EFFECT OF PSILOCYBIN AND EUGENOL ON LIPOPOLYSACCHARIDE INDUCED INFLAMMATION IN SMALL AND LARGE INTESTINE OF MICE

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ABSTRACT

Intestinal inflammation is a complex gastrointestinal condition, arising from immune dysfunction, epithelial cell abnormalities, and gut microbiota imbalances. Intestinal inflammation contributes to many pathological conditions, including irritable bowel disease and depression. This study seeks to find the potential anti-inflammatory properties of psilocybin and eugenol in systemic intestinal inflammation induced by lipopolysaccharide (LPS). We evaluated the impact of these compounds on inflammatory cytokine levels in intestinal tissues in pre- and post-treatment with LPS. We found that LPS induces inflammation to a greater degree in the large intestinal tissues as compared to the small intestine. We also found that psilocybin was effective in reducing the inflammation in pre- and post-treatment in large intestine, while only effective in post-treatment in small intestine. Eugenol was only effective in reducing inflammation in post-treatment experiments in both tissues. Finally, in the large intestine, different ratios of psilocybin to eugenol (1:10, 1:20 and 1:50) were shown to be effective in reducing inflammation, while only certain ratios worked in the small intestine and were less effective. Our work demonstrates that small and large intestines respond to LPS-induced inflammation in a different manner and that psilocybin and eugenol are more efficient in reducing inflammation in the large intestine and when applied after the induction of inflammation.

KEYWORDS: lipopolysaccharide; small intestine; large intestine; inflammation; psilocybin; eugenol; cytokines.

INTRODUCTION

Inflammation is the response of the organism to local and systemic threats in the form of pathogen infection, chemical irritants, mechanical damage or abnormal cell growth or changes in cell morphology.[1] It includes activation of variety of cells and molecules, including various cytokines, and is typically short-lived. When the inflammation trigger is not removed or when the positive feedback loops that enhance the inflammatory response are maintained, acute inflammation develops into a chronic inflammation.[2] As a result, multiple cell types are drawn in, activated, and transformed, and pro-inflammatory cytokine signaling is maintained.

Chronic inflammation, which lasts for weeks, months, or even years, is linked to a number of chronic illnesses, including cancer, autoimmune diseases, neurodegenerative diseases, vascular diseases, and metabolic diseases.[3,4] Various pro-inflammatory and anti-inflammatory cytokines are upregulated in human infectious, neoplastic, and inflammatory diseases and are responsible for maintaining the pathological inflammatory state by activating acute-phase proteins such as C-reactive protein.[5] Tumor necrosis factor (TNF-α) and interleukin 1 beta (IL-1β) play a key role in stimulating cellular immune response and IL-6 is involved in humoral immune response.[6] Cyclooxygenase 2 (COX-2) is an important enzyme that plays a role in inflammation progression; it can be stimulated by pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, stress, and lipopolysaccharide (LPS).[7]

Inflammation plays crucial role in the development of various gastrointestinal conditions, including inflammatory bowel diseases (IBDs), a chronic progressive condition that dysregulate mucosal immune system by remitting mucosal inflammation.[8] Intestinal epithelial cells (IECs) play a key role in maintaining intestinal barrier integrity by providing physical barrier between lumen microorganisms and lamina propria and deeper layers of intestine.[9] Moreover, IECs express toll-like receptors which activate in contribution to luminal microbial components such as lipopolysaccharide, leading to the activation of the COX-2 signaling pathway.[10]

Various mediators and regulators have been shown in intestinal inflammation associated with IBDs development. Among those inflammatory mediators, cytokines play a key role in development, exacerbation and recurrence of IBDs. Cytokine pattern specific to IBDs is initiated by T cell (Th1 and Th17 in Crohn disease (CD) and Th2-like in ulcerative colitis (UC)) differentiation pathway.[11] Effector cytokines, such as IL-6, TNF-α and IL-1β expand the upstream and downstream inflammatory mediators of Th1/T17-Th2 spectrum.[12] It has been revealed that IL-1β and TNF-α are two key pro-inflammatory cytokines associated with IBDs pathogenesis in both animal models and humans.[13, 14]

The efficacy of medication treatment is typically evaluated using a chemically-induced IBD model in rodents, as this model is simple to initiate, repeatable, and guarantees a long-lasting and manageable inflammatory response.[15, 16] LPS can be used in animal models for mimicking systemic inflammation; it provides various significant benefits such as its technical simplicity and high reproducibility, particularly with regards to the inflammatory response triggered.[17] Pro-inflammatory cytokine levels in circulating serum can be measured shortly after LPS administration due to the high levels released. Moreover, it was revealed that LPS originating from bacteria are crucial contributors to the inflammation seen in inflammatory bowel disease. An impaired intestinal tight junction barrier stands out as a significant factor in the development of IBD and various other gut-related inflammatory disorders.[18] Previous studies determined the suitable LPS dosage for intraperitoneal injections of mice. It was shown that an intraperitoneal (IP) LPS dose of 0.1 mg/kg results in LPS plasma levels (with a peak of 2.23 ng/mL and a trough of 0.41 ng/mL) achievable in clinical settings.[19] Also, IP injection of mice with 5 mg/kg of LPS induces TNF-α expression in the large intestine, liver and brain 1h after treatment.[20]

Inflammation of intestines, through bidirectional gut-brain axis also plays a crucial role in the development and maintenance of depression. Managing inflammation in the gut may reduce the symptoms of depression.[21] Thus, drugs with psychoactive properties, such as psilocybin, that also have document anti-inflammatory properties, may be useful for curbing gut inflammation and reducing depression symptoms.[22]

In this work, we decided to explore the role of psilocybin and eugenol in regulating LPS-induced inflammation. O-phosphoryl-4-hydroxy-N,N-dimethyl-tryptamine (psilocybin) is an alkaloid derived from tryptophan indole from specific genus of mushrooms.[23] In an *in vivo* study using a rat model, it has been demonstrated that psilocybin undergoes rapid hydrolysis and is dephosphorylated under stomach acidic condition and also within the intestines by alkaline phosphatase resulting in its conversion predominantly, if not entirely, into psilocin before absorption. It is hypothesized that psychedelics are serotonergic drugs that bind to and activate the serotonin 2A receptor to exert their immediate effects on mood and behavior.[24] Serotonin (5-hydroxytryptamine; 5-HT) is a crucial signaling molecule in the gut and the brain, with the majority (95%) of 5-HT production in the GI tract and the remaining 5% in the brain. HTR2A and HTR2B are serotonin receptors that have been implicated in various physiological and pathological processes, including inflammation.[25] Psilocybin was also shown to have direct anti-inflammatory properties.[7] Three different extracts of magic mushrooms strongly suppressed the generation of nitric oxide, PGE2, and IL-1 cytokines induced by lipopolysaccharide in a dose-dependent manner.[7] Moreover, it was shown that psilocybin-containing mushrooms significantly reduced the production of TNF-α, IL-1β, and also decreased the concentration of IL-6 and COX2 in LPS- induced inflammation in human macrophages.[7]

4-allyl-2-methoxyphenol (eugenol) is a phenolic compound found in clove oil that has a substantial anti-inflammatory and anti-oxidant properties.[26] Eugenol significantly reduced the levels of cytokines in peripheral blood mononuclear cells in rheumatoid arthritis patients and also had an effect in neutralizing reactive oxygen/nitrogen species formation.[27] Eugenol (150 mg/kg) suppressed the inflammatory cytokines TNF-α, IL-1, and IL-6 in mice.[28] A study on the IPEC-J2 cell line (porcine intestinal epithelial cells), a low dose of eugenol was found to reduce inflammatory responses and improve selectively permeable barrier function during inflammation induced by LPS. It was shown that pre-treatment with eugenol led to a notable reduction in the level of IL-8 induced by LPS, as well as decreased mRNA levels of *TNF-α*. Furthermore, eugenol restored the mRNA levels of tight junction proteins that were decreased by LPS and helped to maintain barrier integrity.[13]

We originally planned to test whether intestinal inflammation would cause symptoms of depression in mice model. We hypothesized that lipopolysaccharide or dextran are able to induce inflammation in the intestine and systemically in the liver and the brain.[29-31] We

further hypothesized that psilocybin is able to decrease inflammation in all three organs and also reduce symptoms of depression. We tested the ability of psilocybin, a psychoactive agent with the ability to affect the expression of serotonin receptors in the gut, alone, and in combination with strong anti-inflammatory, eugenol, to reduce inflammation in the brain and the liver. Both psilocybin and eugenol penetrate through blood-brain barrier, and thus would be very effective in curbing neuroinflammation. Indeed, our work showed that these compounds individually and in combination effectively reduced markers of inflammation in the brain[29,30] and the liver.[31]

Here, we tested the hypothesis whether psilocybin (Psi) or eugenol (Eug), or their combination are able to relief intestinal inflammation caused by LPS. Inflammation was tested by measuring mRNA level of various inflammatory biomarkers induced by LPS. Psilocybin and eugenol appeared to be more efficient in reducing inflammation in the large intestine when applied after the establishment of inflammation by LPS.

MATERIALS AND METHODS

Animal model

C57BL/6J male mice (Charles River Laboratories, Laval, QC, Canada) were used according to the Guide to Care and Use of Animals of the Canadian Council of Animal and according to the protocol approved by the Animal Welfare Committee at the University of Lethbridge.

Pilot experiments with LPS treatment

First, the pilot experiment was conducted in two major groups of studies aiming to identify the doses of LPS and the time of the analysis of cytokines for the main experiments. For the pilot experiment, LPS or saline were injected and 5 animals per each time point were used to analyze cytokines. A total of 20 mice were used for control and 20 for LPS. Animals were distributed randomly in each group consisting of 0 h, 24 h, and 48 h treatments.

Inflammation was induced by intraperitoneal (IP) injection of LPS. The specific LPS endotoxin was the L-3129, serotype 0127: B8. For IP administration, a dose of 2 mg/kg (0.2 mg/ml) was employed, with the volume adjusted according to the mouse's weight, averaging approximately 20 g. Previous experiments have demonstrated that an IP dose of 0.83 mg/kg induces chronic inflammation that lasts for an extended period, ranging from days to weeks (58). Additionally, injection of mice with 5 mg/kg of LPS results in the induction of TNF-α in the brain for 10 months, in the liver for a week, and in the blood for 9 hours.[20]

Main experiments with LPS inflammation induction

For the main experiment, the aim was to assess the efficacy of psilocybin, eugenol, or a combination of both in terms of either preventing the initiation of inflammation or reducing its severity once it has already developed.

The dosage of Psi and Eug administered to mice was determined by extrapolating from the dosage intended for humans. We planned to administer 5 mg of Psi (CAS No. 520-52-50, Applied Pharmaceutical Innovation, Edmonton, AB, Canada) in different ratios to Eug (CAS No. 97-53-0, Sigma-Aldrich, Saint Louis, MI, USA), specifically 1:10, 1:20, and 1:50, which correspond to 50 mg, 100 mg, or 250 mg of Eug, respectively.

For an average human weighing approximately 70 kg, the equivalent dosage would be 0.071 mg/kg of Psi and 1.43 mg of EUG. To adjust for mice, we applied a coefficient of equivalence of 12.3 as described before.[59] Consequently, the mice received 0.87 mg/kg of Psi, 17.59 mg/kg, or 43.98 mg of Eug, depending on the ratio used. The administration of these compounds was done orally, via gavage. The selected doses of Psi and Eug are likely not cytotoxic, as we tested comparable doses on three different models, 3D human skin tissues[60], human macrophages[61] and mice beta cells[62], and ound them to be non-toxic.

The main treatment consisted of two groups – “pre-treatment” in which treatments with Psi and Eug were used prior to LPS injection, and “post-treatment” in which treatments were utilized following LPS injection. Each group contained negative control or vehicle in the form of saline solution and the treatment with LPS to induce inflammation.

Pre-treatment with Psi, Eug or combinations

Animals were given Psi, Eug or a combination at 48 h and 24 h prior to the LPS administration to assess the preventive effect of the treatments. The mice randomly were assigned to pre-treatment group consisting of control, Psilocybin (Psi), Eugenol (Eug), Psi+Eug, LPS, LPS+Psi, LPS+ Eug, Psi+Eug (1:10)+LPS, Psi+Eug (1:20)+LPS, Psi+Eug (1:50)+LPS. All pretreated animals were sacrificed 24 h after LPS injection. Small and large intestines were removed separately, then cut and washed with sterile phosphate buffer saline (PBS) and stored in sterile micro tubes and saved in liquid nitrogen, and then stored at -80 ºC. Inflammation levels were assessed 24 hours after treatment via qRT-PCR.

Post-treatment with Psi, Eug or combinations

Mice were administered LPS intraperitoneally for the systemic induction of inflammation. Psi and Eug were given to post treatment group 20 h after IP injection with LPS and animals were sacrificed 24 h after LPS stimulation. This was to mimic treatment of IBD symptoms in real life, with effect of the drug (Psi or Eug) to kick-in between 2 and 4 h. Post-treatment group involved control, LPS+Psi, LPS+ Eug, LPS+Psi+ Eug (1:10), LPS+Psi+Eug (1:20), LPS+Psi+Eug (1:50) groups. Small and large intestines were removed, cut and washed with sterile PBS and stored in sterile micro tubes

dipped in liquid nitrogen, and then stored later at -80 ºC. Inflammatory biomarkers levels were assessed 4 hours after treatment via qRT-PCR.

Reverse transcription-polymerase chain reaction (RT-PCR)

TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total cellular RNA according to the manufacturer's instruction. RNA quantification was done using NanoDrop spectrometry (Thermo Fisher Scientific, Wilmington, DE, USA) iScriptTM Select cDNA synthesis kit (Cat# 1708897, BioRad, Hercules, CA, USA) was used to convert RNA to cDNA according to the manufacturer's instruction. Quantitative real-time PCR (qPCR) was performed with SsoFastTM EvaGreen® Supermix (Cat# 1725202, BioRad, Hercules, CA, USA). Specific primers for the target sequences of interest designed throughhttps://www.idtdna.com/Primerquest platform (Table 1). The reference genes (GADPH) were analyzed with GeNorm method, which involves a C1000TMR Thermo Cycler equipped with a CFX96 TouchTM Real-Time PCR Detection System (BioRad, Hercules, CA, USA). A PCR was performed according to SsoFastTm guidelines with the annealing temperatures as specified for the specific primer pairs was used. The BioRad Software (CFX Manager) was used to perform the expression analysis and were based on the ΔΔCt method with the reference genes that stably expresses in the GeNorm Analysis.

Table 1: Primer sequences used in qRT-PCR in order to detect the target genes in intestine tissues.

Target Gene	Accession Number	Primer Sequence
IL-1β	NM_008361.4	Forward (5' → 3'): CAGGCAGGCAGTATCACTCATT Reverse (5' → 3'): AAGAAGGTGCTCATGTCCTCATC
TNFα	NM_001278601.1	Forward (5' → 3'): GCCTCTTCTCATTCCTGCTTGT Reverse (5' → 3'): TGGGAACTTCTCATCCCTTTGG
IL6	NM_001314054.1	Forward (5' → 3'): GACTTCCATCCAGTTGCCTTCT Reverse (5' → 3'): TATCCTCTGTGAAGTCTCCTCTCC
COX2	NM_011198.4	Forward (5' → 3'): CCTTCTCCAACCTCTCCTACTACA Reverse (5' → 3'): AGCTCCTTATTTCCCTTCACACC

Statistical analysis

The data was calculated as the mean of at least three samples per group with standard error of mean (SEM) or 95% confidence interval as indicated. Mean values plus/minus SEM and statistical analyses were calculated and plotted using GraphPad Prism 9. Statistical analysis of data quantification was performed using a one-way ANOVA test and Dunnett's Post Hoc Test, or multiple unpaired Student's t-test with a false discovery rate correction (Q = 5%).

RESULTS

Exposure of animals to LPS – pilot experiment

We first tested the effect of LPS on the large intestine by analyzing the expression of pro-inflammatory cytokines at the mRNA level at 4, 24 and 48 h of exposure. The dose of LPS that was used (2 mg/kg) has been previously found effective in inducing inflammation in the brain of mice (29). We found that LPS induced all tested pro-inflammatory genes (*IL-1β, IL-6*, *COX-2*, *TNF-α*) albeit to a different degree (Figure 1). At 4 h, *IL-6* expression was increased by ~6-fold, and its expression decreased at later time points (Figure 1B). The expression of *TNF-α* was increased by ~5-fold, and its expression also dropped at 24 h and 48 h (Figure 1D). The expression of *IL-1β* was increased by ~2.8 fold (Figure 1A); its expression also decreased at 24 h and 48h but not as much as *TNF-α* and *IL-6*. Finally, *COX-2* expression increased at 4 h but the difference to control was not significant; expression at 24 h was similar to 4h (Figure 1C). In all cases, the increase in the expression of these cytokines was still maintained at 24 h, albeit not at the level of 4 h. We also analyzed the data for individual time points using ANOVA and the analysis is shown in Supplementary Figure 1.

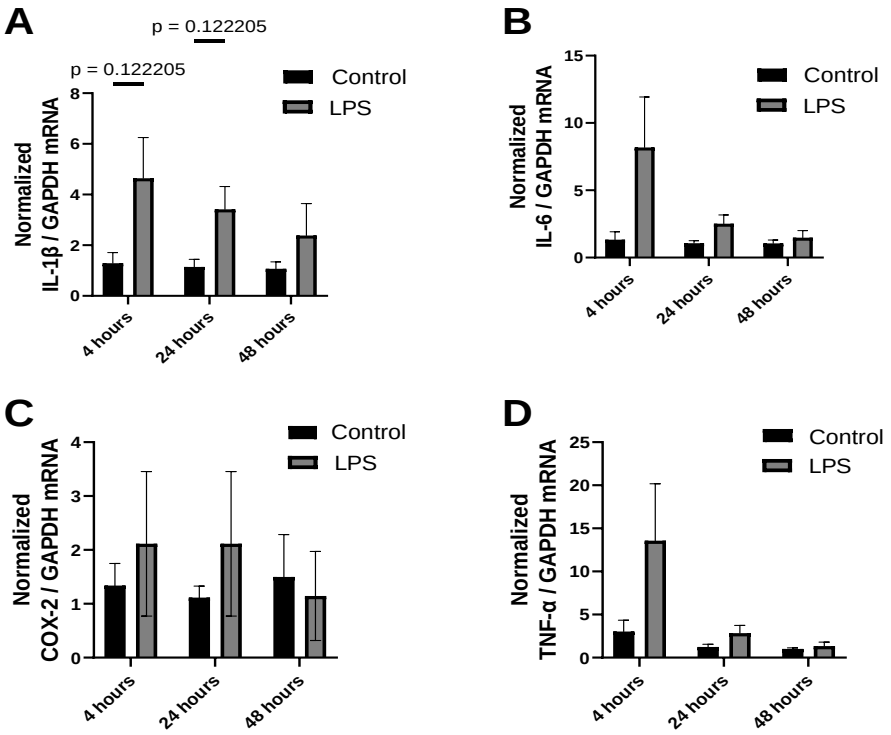

Figure 1: Changes in *IL-1β* (A), *IL-6* (B), *COX-2* (C), *TNF-α* (D) mRNA expression levels in the large intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene *GAPDH*. The data were analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. Bars represent mean ± SEM. We then analyzed the same to find the best time of induction of cytokines by LPS in the small intestine. LPS induced all the tested markers, except IL-6 (Figure 2). All markers except IL-6 remained induced at 48 h, and the increase reached higher levels than at 4 h. It appears that the response to LPS in small intestine was more moderate compared to large intestine, and also somewhat delayed.

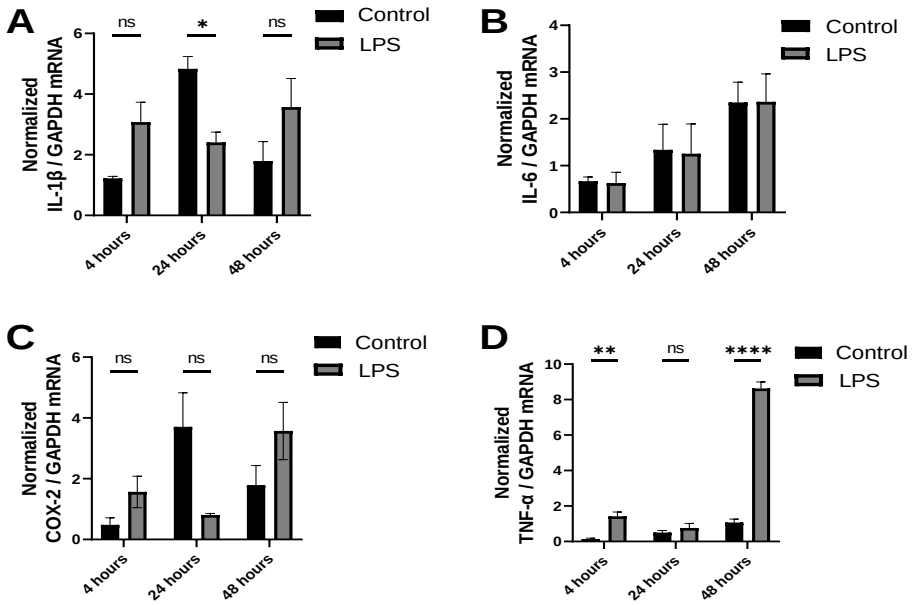

Figure 2: Changes in *IL-1β* (A), *IL-6* (B), *COX-2* (C), *TNF- α* (D) mRNA expression levels in the small intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene *GAPDH*. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. Bars represent mean ± SEM. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. *N* = 3-6. Significance (*p*) is indicated within the figures using the following scale: ns – non-significant; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Treatment of animals with LPS, psilocybin and eugenol – main experiment

For the main experiment, where we analyzed the effect of psilocybin and eugenol on LPS-induced inflammation, we decided to use two different approaches, one where we would pre-treat animals with psilocybin, eugenol or their combination in the ratio of 1:10, 1:20 or 1:50, followed by the injection of LPS, and another where we would use these compounds after the injection with LPS. The specific doses of psilocybin, eugenol and their ratios were selected based on the previous experiments in our lab, where they were effective in reducing LPS-induced inflammation in the brain of LPS-treated mice.[29]

To capture the effect of LPS in pre-treatment experiment, we gave animals Psi, Eug or their combination twice, 48 h and 24 h before the exposure to the LPS, in order to build certain concentration of these compounds in the organism. Based on the preliminary experiment, we decided to analyze cytokines 24 h after injection with LPS, as 4 h and 24 h appeared to be the best time points for the induction of inflammation by LPS in the pilot experiments (Figure 1 and 2 as well as[29,31]).

We first analyzed the data in the pre-treatment group in the large intestine (Figure 3). LPS induced all tested markers (*IL-1β, COX-2, TNF-α*) in comparison to the control group. Pre-treatment with psilocybin effectively reduced the expression of all three cytokines. Eugenol was only effective for the reduction of expression of *IL-1β*. All three combinations of Psi with Eug reduced *IL-1β* expression, while only 1:10 ratio was effective in reducing the expression of *COX-2, TNF-α*. Psi to Eug in the ratio of 1:50 potentiated the increase in expression of *COX-2*, *TNF-α*.

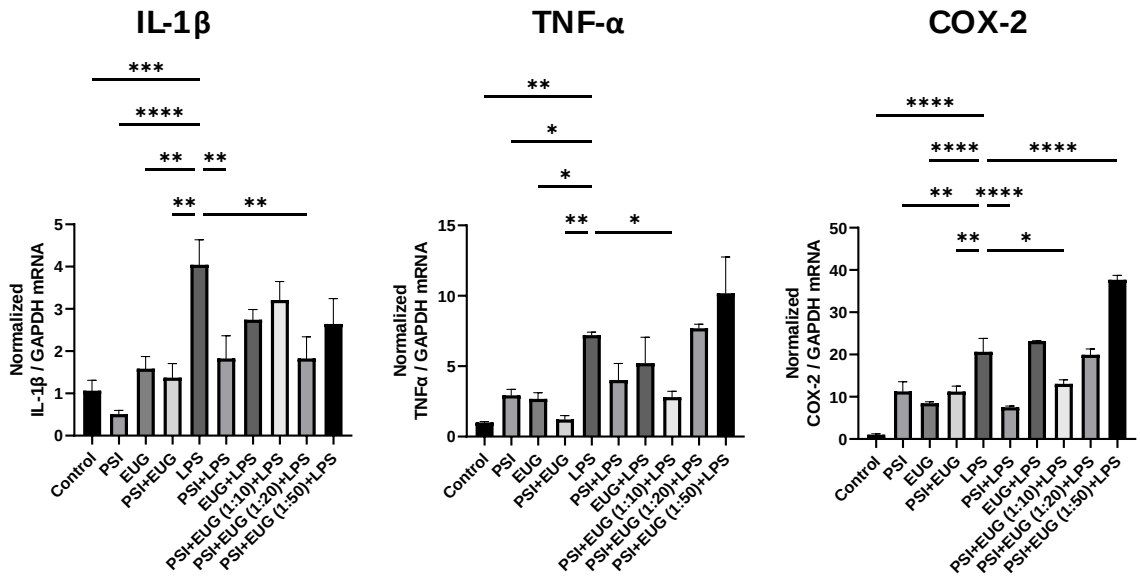

Figure 3: Changes in *IL-1β*, *TNF-α* and *COX-2* mRNA expression level in LPS-induced inflammation in large intestine tissue in pre-treatment with psilocybin, eugenol or their combination (1:10, 1:20 or 1:50). Data were analyzed via qRT-PCR and normalized with GAPDH as a housekeeping gene. Bars represent mean ± SEM. Data were analyzed by ANOVA followed by Dunnett's Post-hoc test. Significance is shown as asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Then, we analyzed the post-treatment group in the large intestine. For the post-treatment group, Psi and Eug were given 16 h after LPS induction LPS increased the expression of all three cytokines (Figure 4). Psi effectively reduced the expression of *TNF-α* and *COX-2* but not *IL-1β*. So did Eug, although not to the same degree as Psi. Psi/Eug combinations were less effective than Psi alone.

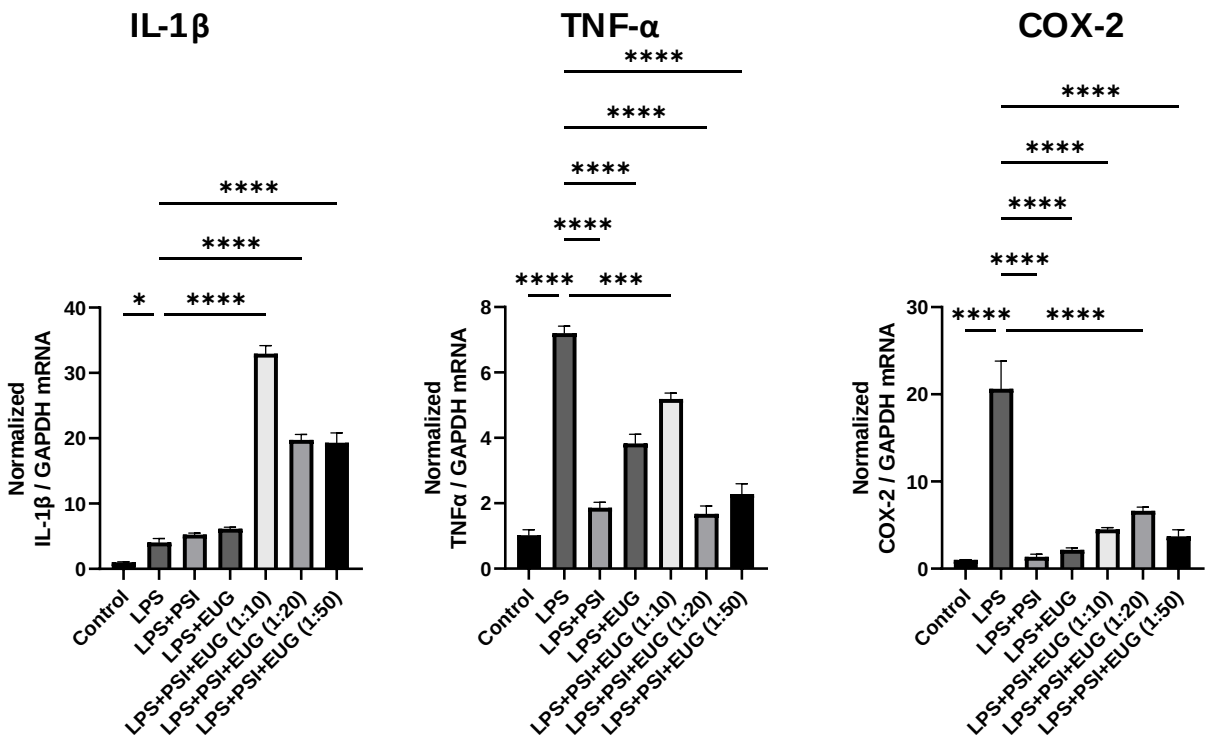

Figure 4: Changes in *IL-1β, TNF-α* and *COX-2* mRNA expression level in LPS-induced inflammation in large intestine tissue post-treatment with psilocybin, eugenol or their combination (1:10, 1:20 or 1:50). Data were analyzed via qRT-PCR and normalized with GAPDH as a housekeeping gene. Bars represent mean ± SEM. Data were analyzed by ANOVA followed by Dunnett's Post-hoc test. Significance is shown as asterisks: *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

We then analyzed the expression of cytokines in the pre-treatment group in the small intestine (Figure 5). LPS induced *IL-1β, IL-6* and *COX-2* but not *TNF-α*. Most of the treatments reduced the expression of the cytokine markers. The largest decrease in *IL-1β* expression was following treatment with eugenol, while Psi to Eug in 1:20 ratio had the highest reduction in *IL-6, TNF-α* and *COX-2* expression level.

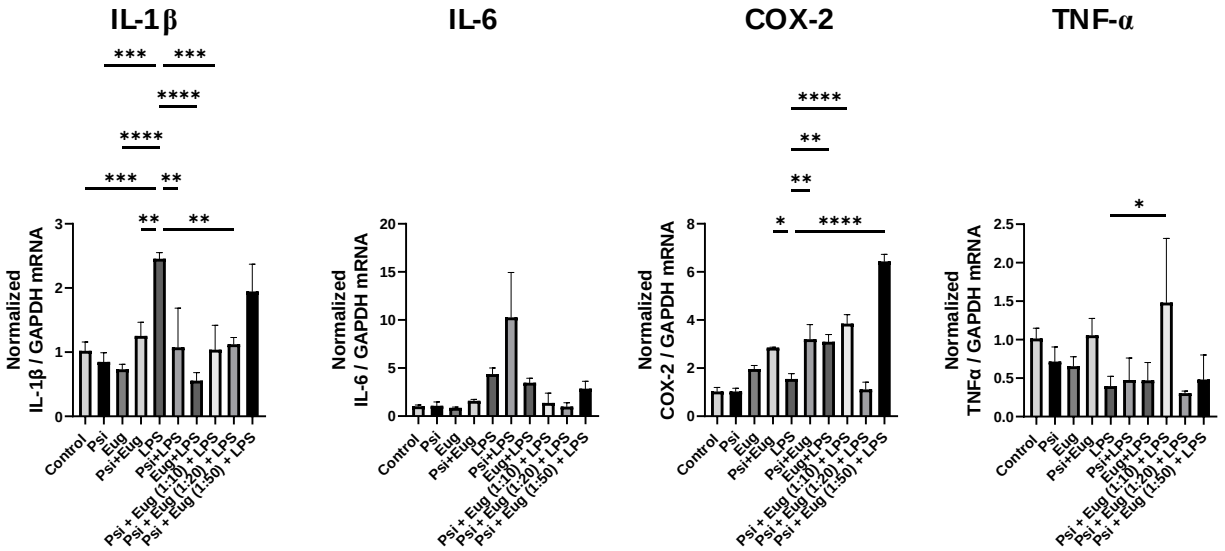

Figure 5: Changes in *IL-1β, IL-6, TNF-α* and *COX-2* mRNA expression level in LPS-induced inflammation in small intestine tissue in pre-treatment with psilocybin, eugenol or their combination (1:10, 1:20 or 1:50). Data were analyzed via qRT-PCR and normalized with *GAPDH* as a housekeeping gene. Bars represent mean ± SEM. Data were analyzed by ANOVA followed by Dunnett's Post-hoc test. Significance is shown as asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

We then examined the post-treatment group in the small intestine (Figure 6). Similarly to the large intestine, LPS induced *IL-1β*, *IL-6* and *COX-2* but not *TNF-α*. It is possible that *TNF-α* plays a minor role in inflammation in the small intestine, or that LPS does not induce this part of inflammation pathway. As with large intestine, Eug was the best in reduction of *IL-1β* and *COX-2* expression level compared to control. Psilocybin to eugenol in 1:50 ratio was the best in *IL-6* downregulation (Figure 6).

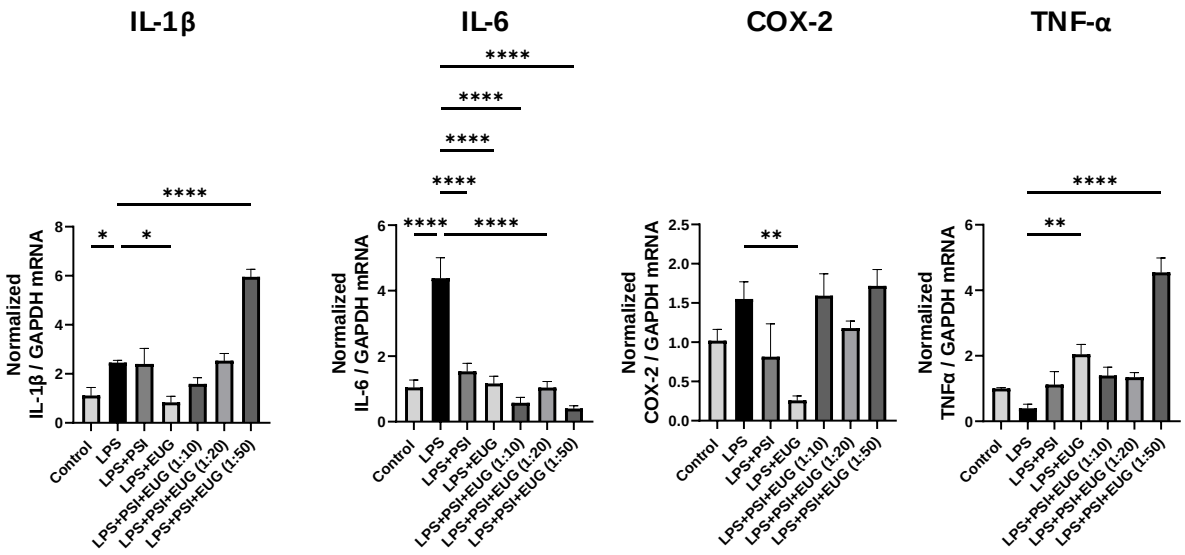

Figure 6: Changes in *IL-1β*, *IL-6*, *TNF-α* and *COX-2* mRNA expression level in LPS-induced inflammation in small intestine tissue in post-treatment with psilocybin, eugenol or their combination (1:10, 1:20 or 1:50). Data were analyzed via qRT-PCR and normalized with *GAPDH* as a housekeeping gene. Bars represent mean ± SEM. Data were analyzed by ANOVA followed by Dunnett's Post-hoc test. Significance is shown as asterisks: *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

DISCUSSION

This study is a continuation of our previous work where we hypothesized that psilocybin and eugenol, alone or in combination, would be able to alleviate inflammation caused by LPS or dextran. In our previous work, we demonstrated that indeed inflammation in the liver and the brain of mice exposed to LPS or DSS is curbed by Psi and Eug[29-31] In this study, we initially induced systemic inflammation in mice intestine using LPS. We then assessed the potential anti-inflammatory effects of psilocybin, eugenol, or their combinations on inflamed small and large intestinal tissues in the pre- and post-treatment groups.

In our pilot study we decided to inject sub-lethal dosage of 2 mg/kg of body weight (BW) LPS IP to induce inflammation at different time frames at 4, 24 and 48h. One study reported that the lethal dose of LPS in mice that leads to 50% of death (LD50) is 10 to 25 mg/kg, depending on the mice strain[32] In our mice strain C57BL/6, LD50 reported to be 10 mg/kg BW (33). Mice model of LPS-induced intestinal inflammation has shown 0.5 mg/kg LPS injected IP induced TNF-α, IL-6, IL-8 and IL-10 expression in ileum at 2 h post injection[34] In another study, injection of LPS at 2 mg/kg dose resulted in the induction of inflammation in spleen, liver and colon[33] These findings support our data where we found that a single IP dose of 2 mg/kg (0.2 mg/ml) LPS induced inflammatory markers (*IL-1β, IL-6*, *COX-2*, *TNF-α*). Similarly, in another study, LPS in the same dosage regimen induced brain and liver inflammation in mice.[29,31]

As far as the timing of the analysis of inflammation caused by LPS is concerned, there are different data in the literature. Analysis of inflammation in mice injected with LPS at 2 mg/kg IP at 2, 4 and 8 h post injection has shown *TNF-α* and *IL-1β* expression elevated at 4 h in colon.[33] Correspondingly in our study, LPS started to induce *IL-1β* and *TNF-α* expression at 4 h in the large intestine, however it did not reach the significances, while in the small intestine, *TNF-α* was significantly induced at 4 h. Radulovic and colleagues also reported that the expression of *IL-1β*, *TNF-α* and *IL-6* reduced to baseline in colon at 8 h, while in our findings, *IL-1β* was elevated significantly at 24 h in the large intestine.[33] Radulovic et al. (2018) reported the reduction of *TNF-α* in spleen, liver and colon to the baseline at 48 h, while in our work *TNF-α* expression in small intestine was still increased significantly at 48 h.[33] Moreover, our analysis performed on large intestine revealed that at 24 h *IL-1β* was the highest post LPS IP injection, indicating the best time for inflammation induction. Likewise, Talley and colleagues reported that 0.5 mg/kg of LPS resulted in strong upregulation of *TNF-α* and *IL-6* in serum and colon 24 h after injection.[35]

We then conducted our main experiment to investigate the potential anti-inflammatory effect of psilocybin and eugenol prior and following inflammation induction with

LPS in large and small intestine. Our finding shows the different expression patterns in the context of inflammation induction method, the treatment time scenarios (pre- or post-treatment) and the tissue type (Table 2).

Table 2: Effects of Psi and Eug on inflammation depend on induction methods, treatment time and tissue types.

Tissue type Animal Model		Large intestine		Small intestine	
		Inflammation Induction	Treatment choice	Inflammation Induction	Treatment choice
LPS	Pre-treatment	IL-1β, TNF-α, COX-2	PSI, PSI+EUG 1:10, 1:20	IL-1β	PSI+EUG 1:20
	Post-treatment	IL-1β, TNF-α, COX-2	PSI, EUG, PSI+EUG 1:10, 1:20, 1:50	IL-1β, IL-6	PSI, EUG, PSI+EUG 1:10

EUG, eugenol; LPS, lipopolysaccharide; PSI, psilocybin.

It appears the induction of inflammation is more prominent in the large intestine than in the small intestine, and that psilocybin was effective in reducing the inflammation in pre- and post-treatment in large intestine, while only effective in post-treatment in small intestine. These observations are aligned with findings by Gil-Cardoso and colleagues where they found LPS notably increased COX-2 activity in the duodenal region but did not have the same effect in the ileum, jejunum, or colon segments.[36]

In our work, IL-1β was activated in both tissue types in both, pre- and post-treatments. TNF-α, COX-2 were induced mostly in the large intestine, and not in small intestine.

Small and large intestines differ from each other by representation of various cells capable of immune response. While large intestine has higher representation of B-cells and $Foxp3^+$ Treg (CD4 T cells), small intestine has higher number of IELs (intraepithelial lymphocytes), ILC3 (group 3 innate lymphoid cell), TCRαβ+ CD8αα+ (type of IELs), eosinophils and mast cells.[37]

In the large intestine, $Foxp3^+CD4^+$ T cells are critical in immune homeostasis, including activation of pro-inflammatory cytokines IFN-γ, IL-17A, and IL-6, and anti-inflammatory cytokines IL-10, TGF-β, and IL-35.[38] B-cells have similar cytokine pattern, but in addition, they produce pro-inflammatory cytokine TNF-α.[39] COX2 is a classical cytokine produced by colon epithelia in response to LPS. And indeed, we found COX2 to be activated in the large intestine, rather than small.[40] Normal ileum epithelial cells do not produce COX2, but cells modified by Crohn's disease do.[41] Thus, COX2 could be a good marker to be used to test the efficiency of psilocybin/eugenol treatment of colitis or other conditions targeting large intestine.

Small intestines produce large number of different cytokines, with pro-inflammatory cytokines predominantly being TNF-α, IL-6 and IL-1β.[42] We tested for all three of these cytokines, and found that the latter two were induced effectively in pre- and post-treatments. As far as TNF-α is concerned, it seems that while it is commonly expressed in various pathologies in the small intestine, our results do not demonstrate consistent activation by LPS (it showed good induction in the pilot experiment, but lacked induction in the main experiment), and show lack of response to psilocybin and eugenol in the small intestine.

IL-1β is a ubiquitous cytokine activated in both small and large intestine, and is one of key regulators recruiting immune cells to the places of infection in duodenum[43] or colon[44] IBD patients often show high level of this cytokine in both parts of the intestine.[45] This cytokine was induced in all experimental settings in our work. Thus, while it is an important cytokine to test for, it is not specific enough to reflect the efficiency of proposed treatment with our formulations.

Eugenol was effective in post-treatment but not in pre-treatment. It is possible that eugenol mainly suppresses the expression of activated cytokines, but can not as effectively prepare cells for inflammation. Similarly, pre-treatment with eugenol in the model of the gastroenteritis induced by virus in piglets did not significantly reduce *IL-1β* expression.[46] The authors suggested that these results might be influenced by factors such as the infection site (duodenum, jejunum and ileum tissues) or the specific mechanisms underlying eugenol's actions.[46] In contrast, the downregulation of IL-6 in the small intestine in the post-treatment with eugenol and psilocybin was also seen in the study on human small intestine cells - combination of 20 µg of psilocybin and 25 µg of eugenol downregulated LPS-induced increase in IL-6 levels.[47]

Among all treatments, the combination of psilocybin and eugenol in 1:10 ratio was the most effective in reducing *COX-2* mRNA level in the large intestine following LPS inflammation induction, but not in the small intestine. In contrast in the study using small intestine epithelial cells, COX-2 protein levels were significantly increased by LPS and reduced upon treatment with the psilocybin and eugenol combination.[47] There is major difference between our study and Robinson et al. (2023a) study – they used cells, while we performed in vivo exposure.[47] It was curious to see the more dramatic effect of LPS and Psi/Eug on large intestine versus small intestine. There

are substantially more bacteria in the large intestine, and it is possible that epithelial cells in the large intestine are primed to respond to LPS, and as a result, inflammation is triggered to a larger degree. While we think that part of the effect of psilocybin on inflammation is due to its interaction with serotonin receptors, it appears serotonin receptors are less common in the large intestine.[48, 49] The predominant location of bacteria in the large intestine may affect the activity of psilocybin through some sort of psilocybin-microbioata-serotonin receptor interaction.

Psilocybin appears to be anti-microbial, although it is known whether this activity is restricted to certain types of bacteria.[50] It is possible that LPS triggers inflammation in the gut and alters microbiota-epithelial cell communication, which leads to additional pro-inflammatory stimulation of the intestine by bacteria. In this case, psilocybin may suppress this effect.

It also appears that psilocybin was more effective in reducing inflammation in the liver and in the brain. Psilocybin is rapidly converted into its active ingredient in the intestine and the liver, with the first round of conversion starting in the small intestine epithelia, followed by the large intestine epithelia, and finally by liver. It is possible that the reason psilocybin was more effective on the large intestine is because by the time it reaches large intestine, a certain amount already converted to psilocin in the small intestine, absorbed into blood stream, and started acting on the large intestinal cell inflammation.[51]

Bacteria have serotonin receptors. *E. coli* have membrane bound histidine sensor kinase CpxA functioning as a bacterial serotonin receptor. Serotonin can bind and dephosphorylate this kinase, resulting in inhibition of expression of virulence genes, reducing bacterial capacity to produce toxins and adhering to intestinal epithelium.[52] Thus, regulation of these receptors can alter bacterial virulence, and thus, inflammation in the large intestine. While no studies demonstrate this, psilocybin can likely bind CpxA receptors in a similar manner, and thus also suppress inflammation more effectively. On the other hand, it was shown that elevated serotonin levels in gut mucosa promote the development of a microbiota associated with colitis, resulting in more severe inflammation and the production of pro-inflammatory cytokines through immune cell activation.[53] Conversely, certain microbiota species like *Clostridium sporogenes* and *Ruminococcus gnavus* in the colon, which are linked to IBD, possess the ability to absorb tryptophan and convert it into tryptamine via decarboxylation.[54] Given that psilocybin resembles l-tryptophan.[23] and can potentially function as a component similar to microbiota metabolites, it becomes crucial to explore the interaction between psilocybin and its metabolites with microbiota populations. So, the interaction between microbiota, serotonin production, psilocybin and intestinal epithelium inflammation is a complex one and needs further research.

One limitation of our study lies in the constraints associated with the duration of pre- and post-treatment intervals in our LPS-induced mouse models. In the post-treatment group, animals were sacrificed after 4 hours following treatment, which primarily captured the immediate effects of treatments on established inflammation. This approach may not fully represent the long-term impact of psilocybin on biomarker expression, especially if chronic inflammation is considered. Additionally, our understanding of the time frame for psilocybin's effects on intestinal gene expression is limited. While some research has demonstrated that psilocin, when orally administered to rats, appears in the urine already at 8 hours after consumption[55], it is not clear what the residual and long-lasting effect of the remaining circulating psilocybin on the intestine. Indeed, it is known that 10-20% of psilocybin can accumulate in tissues, while the majority (80%) being excreted in the urine.[23]

A critical aspect of our study involved extracting proteins and quantifying protein levels from intestine tissue, which can be particularly challenging when working with a limited amount of tissues, and the limited time for harvesting tissues, especially in mice models. In this work, we were unable to obtain proteins of sufficient quality and Western blotting detection of key pro-inflammatory proteins was unsuccessful (data not shown). Future research should consider finding more efficient and rapid techniques for harvesting intestine samples for western blotting or exploring alternative quantification methods, such as ELISA assays.

Another aspect that could enhance the comprehensiveness of our study is the assessment of colitis severity and the presence of clinical symptoms. Evaluating histopathological alterations, such as crypt height, muscle thickness, and gut length, would have provided valuable insights into the physiological changes associated with the induced inflammation.[56]

It is important to consider which section of the intestine tissue is selected for molecular analysis in the future study; in the LPS-induced inflammation in rat, COX-2 was elevated within the duodenal region, yet it did not increase in the ileum, jejunum, or colon segments.[18] Crohn’s disease, which can involve both the small and large intestines, often involves "skip lesions," meaning that inflamed areas are separated by healthy tissue. This can result in a patchy pattern of inflammation.[57] So, it still remains to be demonstrated what sections of intestine need to be analyzed for inflammation in the LPS models.

In the future, it would be also important to understand the effect of psilocybin and eugenol, and their ratio on serotonin receptors and serotonin production in intestinal

tissue. Such a comprehensive analysis should encompass both animal models of IBD and studies involving humans.

Institutional Review Board Statement: This study used C57/BL/6J mice (Charles Rive Laboratories, Laval, QC, Canada) in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care. This study was approved by the Animal Care Services at the University of Lethbridge (Protocol #2113, approved April 25, 2022).

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