THE EFFECT OF PSILOCYBIN AND EUGENOL IN DEXTRAN AND LIPOPOLYSACCHARIDE INDUCED GASTRO-INTESTINAL INFLAMMATION

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The effect of psilocybin and eugenol in dextran and lipopolysaccharide induced gastro-intestinal inflammation

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ABSTRACT

Inflammatory bowel disease (IBD) is a complex gastrointestinal condition, arising from immune dysfunction, epithelial cell abnormalities, and gut microbiota imbalances. This study seeks to find the potential anti-inflammatory properties of psilocybin and eugenol in both local and systemic intestinal inflammation by utilizing a murine model of IBD induced by dextran sulfate sodium (DSS) and lipopolysaccharide (LPS). We evaluated the impact of these compounds on inflammatory cytokine levels in intestinal tissues and also explored changes in serotonin receptor (HTR2A and B) expression, and transient receptor potential (TRP) ion channels (TRP1 and TRPM8), analyzing the influence of these compounds on serotonin signaling pathways.

Our study yielded significant insights into the multifaceted world of inflammation within the gastrointestinal tract. Notably, our findings revealed intriguing disparities in the trends observed pre- and post-treatment, particularly in the context of small and large intestine inflammation induced by LPS and DSS. Additionally, our study unearthed evidence of the anti-inflammatory properties of psilocybin and eugenol, compounds with agonistic effects on serotonin and TRP channels. Most notably, we observed a synergistic effect when these compounds were combined.

More comprehensive studies on the medicinal effects of natural compounds in IBD animal models are pivotal for exploring their implications, specifically the analysis of drug-microbiota-immune system interactions.

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LIST OF ABBREVIATIONS

Inflammatory bowel disease (IBD)

Dextran sulfate sodium (DSS)

Lipopolysaccharide (LPS)

Transient receptor potential (TRP)

Pathogen-associated molecular patterns (PAMPs)

Damage-associated molecular patterns (DAMPs)

Tumor necrosis factor (TNF-α)

Interleukin 1 beta (IL-1β)

Cyclooxygenase 2 (COX-2)

Prostaglandins (PGs)

Crohn's disease (CD)

Ulcerative colitis (UC)

Psilocybin (PSI)

Eugenol (EUG)

Reverse transcription-polymerase chain reaction (RT-PCR)

Gastrointestinal (GI)

Enterochromaffin cells (ECs)

CHAPTER 1: INTRODUCTION

1.1 Inflammation

1.1.1 Acute inflammation

When pathogens invade the body or there is tissue injury, the innate immune system is activated. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are chemicals that set off this reaction (Yao C. & Narumiya S., 2019; Díaz-Muñoz MD. et al., 2010). To remove infection from affected or injured tissues, granulocytes are enlisted, and pro-inflammatory mediators such as cytokines and lipid mediators are also generated. This starts an immediate, short-lived inflammatory response that clears infections and injured tissues (Yao C. & Narumiya S., 2019; Díaz-Muñoz MD. et al., 2010). When PAMPs, DAMPs, pathogens, and injured tissue are removed, granulocyte recruitment stops, and chemokines are down-regulated and scavenged, the acute inflammation is also eliminated (Yao C. & Narumiya S., 2019; Díaz-Muñoz MD. et al., 2010). The development of positive feedback loops that enhance the inflammatory response and the suppression of negative feedback processes that hinder resolution are some of the mechanisms being studied by researchers to understand how an acute inflammation develops into a chronic inflammation. As a result, multiple cell types are drawn in, activated, and transformed, and pro-inflammatory cytokine signaling is maintained. (Díaz-Muñoz MD. et al., 2010).

1.1.2 Chronic inflammation

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. These conditions frequently include an unnatural immune

system activity in which immune system overreacts and aberrantly responds to its own cells and tissues (Haneishi Y. et al., 2023; Yao Ch. & Narumiya Sh., 2019; Díaz-Muñoz MD. et al., 2010).

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 (Lucey DR, et al, 1996). These and other 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 (Lucey DR. et al., 1996). 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) (Nkadimeng SM. et al, 2021). Animal studies conducted in vitro and in vivo have demonstrated that prostaglandins (PGs) play a key role in chronic inflammation. PGs interact with cytokines in chronic inflammation in a variety of ways, including stimulation of the synthesis of cytokines by increasing the expression of cytokine receptors, such as the IL-1 receptor in synoviocytes, which are induced or enhanced by the PGE2-EP2/EP4 receptor signaling (Yao C. & Narumiya S., 2019). Additionally, PGs can work with cytokines like TNF-α to promote NF-κB activation and amplify the signaling of COX-2 induction (Yao C. & Narumiya S., 2019). There are several chronic inflammatory disorders with high level of pro-inflammatory cytokines; for instance, colitis-associated cancer is inflamed with neutrophils, and has high levels of COX-2 and active NF-κB (Yao C. & Narumiya S., 2019). However, the kind of inflammation, the cytokines involved, the cells impacted, the timing of the disease process, and the location of the inflammation, is crucial to note, because it affects how PGs work and how they communicate in chronic inflammation (Yao C. & Narumiya S., 2019).

As mentioned previously, chronic inflammation underlies many immune-mediated diseases such as inflammatory bowel diseases (Haneishi Y. et al., 2023) which are explained below.

1.2. Inflammatory bowel diseases

Inflammatory bowel diseases (IBDs) are chronic progressive gastrointestinal tract conditions that dysregulate mucosal immune system by remitting mucosal inflammation (Seong-Joon K. et al., 2018; Cao R. et al., 2020). IBDs include Crohn's disease (CD) and ulcerative colitis (UC) differ in some clinical symptoms, histopathological alteration and location of the inflammation (Panaccione R., 2013). They are sometimes undistinguishable and mimicking each, and thus are referred to as IBD unclassified (IBDU) (Zhou N. et al., 2011). CD effects any part of gastrointestinal (GI) tract and multiple layers of GI tract may be affected, while UC is restricted to rectum and colon with inflammation present in the mucosal layer of colonic tissue (Panaccione R., 2013). The symptoms include cramps, diarrhea, GI bleeding, and weight loss in both types of IBDs (Panaccione R., 2013). However, CD develops fistula (connection of inflammatory parts within different affected segments or other organs) while UC does not (Fuss, I. et al., 2004). In treatment approaches for UC, surgical removal is the option, while it is not a choice in CD condition, for which only complications and pain reduction like fistula or blockages of bowel are treated (McLeod R., 2003).

There were 163 distinct gene loci associated with IBD development revealed; about 110 out of 163 were also linked to both CD and UC (Meddens CA. et al., 2016). This is noteworthy because CD and UC can present quite differently in individuals. Further exploration within this genetic landscape has unveiled 30 specific regions strongly correlated with CD and an additional 23 genomic regions displaying a specific association with UC (Jostins L. et al., 2012). IBDs, however, is not simply a genetic disease, as lifestyle choices, dietary habits, smoking history,

medication usage, exposure to infections, geographic location, and the impact of stress all significantly contribute to the onset and progression of IBDs (Abraham C. & Cho JH., 2009; Hrncir T., 2022). While the epidemiology of IBD in Canada has reported approximately 0.5% of population suffering from it, the highest incidence prevalence was associated with CD. There is a significant difference in the prevalence of CD in males versus females, with the ratio of 1.31 to 1.00, while no such difference was reported for UC (Bernstein C. N. et al., 2006). IBD can be a risk factor of GI cancer if not treated effectively (Colombel JF. et al., 2011).

1.2.1 Underlying mechanism of IBD

Finding the underlying mechanism of the development of intestinal inflammation has been challenging due to its multicellular complexity (Shin W. & Kim HJ, 2018). The continuous barrage with environmental bacteria and foreign antigens poses a threat to the gut mucosa. As a result, the intestinal barrier is precisely regulated to maintain mucosal immunological homeostasis and diminish excessive inflammation. Data demonstrate that abnormalities in epithelial barrier elements are part of the etiologic factors in IBD (Pastorelli L. et al., 2013; Strober W. et al., 2007). One substantial contributor to the intestinal inflammation in IBD and other gut-related inflammatory disorders is the increasing epithelial permeability due to impaired tight junction barrier, resulting in bacterial translocation from lumen to blood stream (Figure 1.1) (Gil-Cardoso K., 2019). 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 (Figure 1.1) (Ruiz Castro P. A. et al., 2021). Moreover, IECs express toll-like receptors which activate in contribution to luminal microbial component such as lipopolysaccharide, leading to the activation of the COX-2 signaling pathway (Ruiz Castro P. A. et al., 2021; Abreu M. T., 2010). Reports suggest that barrier defect by itself might

be enough for triggering IBD (Ruiz Castro P. A. et al., 2021). However, studies has shown inflammatory cells, including immune and epithelial cells, as well as the gut microbiota, engage in complicated intercellular communication to start intestinal inflammation (Strober W. et al., 2007). Various mediators and regulators have been shown in intestinal inflammation associated with IBDs development. Among those inflammatory mediators, cytokines play key role in development, exacerbation and recurrence of IBDs (Sanchez-Muñoz F. et al., 2008). Study revealed that there are differences and similarities of mucus cytokines profile in IBD patients of CD and UC (Papadakis KA. Et al. 2000). Cytokine pattern specific to IBDs is initiated by T cell (Th1 and Th17 in CD and Th2-like in UC) differentiation pathway. Effector cytokines, such as IL-6, TNF-α and IL-1β expand the upstream and downstream inflammatory mediators of Th1/T17-Th2 spectrum (Strober W. & Fuss I. J., 2011). 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 (Gil-Cardoso K, et al., 2019; Hui Q. et al., 2020; Wang K. et al., 2022)

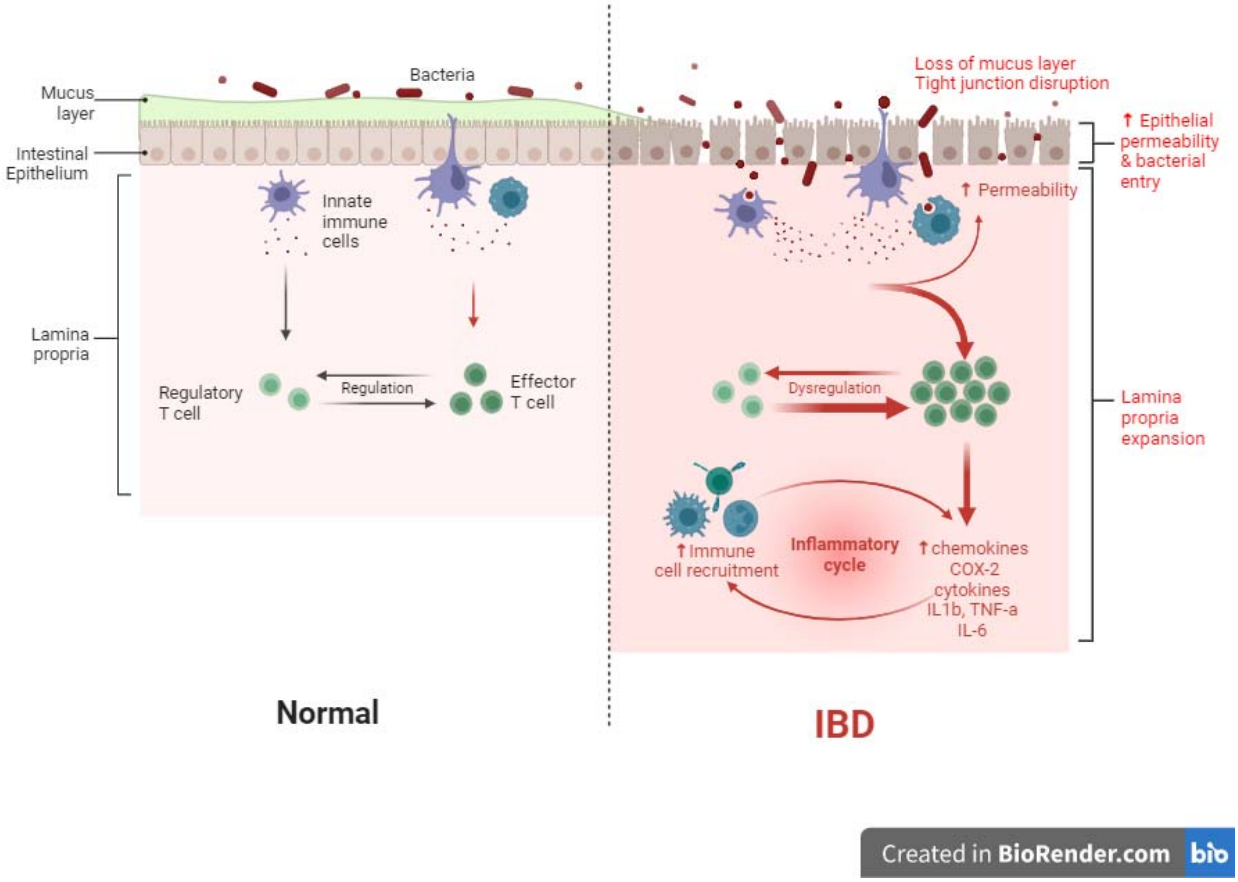

Figure 1.1. IBD mechanism in intestine as compared to the normal state. Normal physiological state, on the left side, represents healthy intestine tissue with mucosal barrier (mucus layer and intestinal epithelium). In a healthy state, barrier separates lamina propria's immune cells from luminal bacteria. There is a regulatory mechanism between regulatory T cells and effector T cells result in a hemostasis. On the right side of the figure, IBD condition is characterized with intestinal barrier defect (loss of mucus layer and tight junction alteration) results in the increasing intestinal barrier permeability causing microbial predisposition and dysregulation in innate immune system. Increased immune cell recruitment leads to chemokines and cytokines upregulation in a positive feedback loop which cause lamina propria expansion.

1.2.2 IBDs epidemiology and comorbidities

IBD incidence in the developed countries has increased in 21st century despite high hygiene along with autoimmune and allergic disorders. It has been suggested that there is a link between microbial diversity reduction and these conditions (Wijmenga C., 2005). Previous studies have shown that there is a link between gut health and brain condition known as gut-brain axis (Kostic

A.D. et al., 2015; Scheperjans F. et al., 2015; Hrncir T., 2022). It has been proven that 90% of body's serotonin (5HT), a neurotransmitter, is originating in the gastrointestinal system from enterochromaffin cells (peripheral 5HT) (Mawe GM. et al., 2013). Furthermore, endogenous serotonin in CNS exerts its effect on improving the mood and anti-depressant effects via serotonin receptor 2A (5HT2A) in the brain (Lopez-Giménez JF. et al., 2018; Vollenweider F. & Kometer M., 2010). Although serotonin cannot pass the blood brain barrier (BBB), its precursor tryptophan can (Chen Y. et al., 2021). However, the mechanism of interaction between peripheral serotonin with CNS serotonin is unclear, natural tryptamine such as psilocybin and DMT (Dimethyltryptamine) as exogenous tryptamine showed promises in psychotropic issues such as depression (Wenwen D. et al., 2023). On the other hand, 5HT was shown as a potent modulator of autoimmune diseases like IBD and MS (Wan M. et al., 2020). Molecules affecting 5HT signaling showed promises in mouse models and clinical trial studies of IBD (Wan M. et al., 2020). Nevertheless, even diseases primarily unrelated to the gastrointestinal system can disrupt gut microbiota, and vice versa, leading to a growing area of research underscoring the pivotal role of the gut microbiota dysbiosis in a spectrum of immune-related, metabolic, and neurological disorders (Kostic A.D. et al., 2015; Scheperjans F. et al., 2015; Hrncir T., 2022). Alterations in gut microbiota have been associated with type I diabetes, Alzheimer's disease, and Parkinson's disease (Kostic A.D. et al., 2015; Scheperjans F. et al., 2015; Chandra S. et al., 2023). There are evidences that demonstrated IBD patients were more prone to depression which resulted in poor IBD prognosis (Potter LE. et al., 2018). It has been suggested that antidepressant (serotonin based) may mitigated IBD (Frolkis AD. et al., 2018). Psychological comorbidities have been reported in patients with IBDs in Spain from 2011 to 2021, encompassing both CD and UC. According to Barreiro's study the prevalence of depression was documented to be up to

20%, while anxiety was reported in up to 11% of IBD patients during this time period. A systemic review of research on adult IBDs patients reported a 20% prevalence rate of anxiety and a 15% prevalence rate of depression (Neuendorf R., et al. 2016).

1.2.3 Approaches for IBDs treatment

The target drug should be involved in mucosal healing by blocking inflammation initiation and maintenance, prevent complication and disease recurrence (Couskun M., 2014). However the conventional treatment consist of aminosalicylates (5-ASA), corticosteroids and immunomodulators may result in advanced therapies approaches based on individual condition (Burisch J. et al., 2023). 5-ASA exact mechanism of action is not known, it has been reported to act on numerous pathways and enzymes related to colon inflammation such as cyclooxygenase, lipoxygenase enzyme inhibition, leukotrienes and prostaglandins production; corticosteroids control pro-inflammatory mediators by the reduction of gene expressions such as pro-inflammatory cytokines like TNF-α, phospholipase A1, COX-2, etc.; immunomodulator drugs contribute to cell-cycle arrest, NF-κB pathway inhibition, T cell apoptosis, pro-inflammatory cytokine expression reduction and many others (Burisch J. et al., 2023). In mild IBDs, the initial therapy is 5-ASA that relieves the symptoms but does not improve the health of inflamed intestinal areas (Colombel JF. et al., 2020; Colombel JF. et al., 2011). It was reported that, within 10 years of diagnosis, 46.9% of patients with Crohn's disease and 15.6% of those with UC get surgical intervention as a result of the disease progression (Ritesh M, et al, 2021). In cases that patients do not react to standard medication, they are subsequently treated with medicines for moderate to severe colitis (advanced therapy), such as: anti-TNF monoclonal antibodies (mAbs), including certolizumab pegol (undergoing Phase 2 clinical study), infliximab (Remicade®, FDA approval in 2006), adalimumab (Humira®, FDA approval in 2012), golimumab (Simponi®,

FDA clearance in 2013), and anti-integrin 4–7 m Vedolizumab (Entyvio®), an anti-IL 12/-23 medication, that received FDA clearance in 2014, Ustekinumab (Stelara®, FDA approval in 2019), tofacitinib (Xeljanz®, FDA approval in 2018), and ozanimod, small molecules that target janus kinase (JAK) signalling, anti-tumor agents (being considered for FDA approval) (Ritesh M. et al, 2021).

All of the conventional and advanced treatments have their own side effects such as the risk for infection, malignancies and cardiovascular conditions (Burisch J. et al., 2023). Researchers are thus interested in identifying a novel treatment that target key factors of IBDs pathology with minimum side effects.

1.2.4 Novel and potential treatment for IBDs

Although the exact etiology and underlying mechanism of IBDs is not easy due to the multicellular complexity of the disease (Shin W. & Kim HJ., 2018), the key factors in IBDs treatment involved mucosal healing, blocking and maintaining intestine inflammation and preventing complication and recurrence of IBDs (Couskun M., 2014; Ritesh M. et al., 2021). Research into the treatment options for IBD continues. It has been reported that metformin hold a promise as a therapy of inflammatory bowel illness since it inhibits NF-κB activation in intestinal epithelial cells, improves murine colitis, and reduces tumors linked to colitis in mice (Seong-Joon K. et al., 2014). The other suggested treatment is bone marrow mesenchymal stem cells which promoted recovery of the intestinal mucosa in small intestine of mice (Lykov A.P. et al., 2018). Pabari and colleagues have shown that infliximab improves colitis clinical signs in moderate and severe dextran (DSS)-induced in vitro murine model. They suggested that the inhibition of TNF-α production locally at its production site was part of the success of the therapy (Pabari R. M. et al., 2021). Although these studies showed promise, there have been

limited research on their efficacy and side effects in a long run. Clinical trials with these agents are also lacking. Thus, it is vital to explore a potential treatment based on natural components which have shown promises for their wide range of properties in all of the key factors of IBD, as well as being cost-effective and having minimum side effects.

1.3 Psilocybin

O- phosphoryl-4-hydroxy-N,N-dimethyl-tryptamine (psilocybin) is an alkaloid derived from tryptophan indole from specific genus of mushrooms (Dinis-Oliveira R. J., 2017). Psilocybin-containing mushrooms, also known as magic mushrooms, have shown promise for treating a variety of mental diseases, including pathological anxiety, mood depressive disorder, and addiction (Coppola M. et al., 2022). 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 (further metabolization of psilocin is by the liver). Moreover, most of psilocin reported to be excreted in urine (65%), bile and feces (15-20%) and the rest (10-20%) remained for longer time (detected in urine after 7 days of administration orally) in the organs in a rat model (Dinis-Oliveira R. J., 2017). 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 (Lopez-Giménez JF, et al., 2018; Vollenweider F. & Kometer M., 2010). In terms of gut function, the 5-HT2A receptor plays a role in the contraction of gut smooth muscle, and is the primary target of psychedelics (Fiorica-Howells E. et al., 2002; O'Mahony S.M. et al., 2015). Serotonergic medications exerts the control over various gastrointestinal functions, including vasodilation, secretion, peristalsis, pain sensation, etc. (Mawe GM. et al., 2013) with selective serotonin reuptake inhibitors being useful

for treating GI issues (Foster JA. et al., 2017). Within the GI tract, boosting serotonin signaling leads to increased growth of intestinal epithelial cells, reducing damage caused by intestinal inflammation. Additionally, activating the serotonin receptor type 4 in enteric neurons promotes the generation of new neurons and provides protection against injury in the context of intestinal damage (Shah P. A. et al., 2021). 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 (Hao Y. et al., 2023).

On the other hand, the serotonin-microbiota axis plays a significant role in gastrointestinal inflammation. Elevated serotonin levels in gut mucosa promote the development of a microbiota associated with colitis, resulting in more severe inflammation in DSS-induced mouse models and the production of pro-inflammatory cytokines through immune cell activation. 5-HT, as a key player in this axis, significantly contributes to the pathogenesis of IBD (Kwon, Y. H., et al. 2019). Furthermore, psychedelic compounds with serotonergic properties such as psilocybin influence the gut microbiota, leading to a transient elevation in 5-HT levels through interactions with enterochromaffin cells within the host (Császár-Nagy N. et al., 2022). 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 (Koopman N. et al., 2021).

Psilocybin was also shown to have anti-inflammatory properties (Nkadimeng SM. et al., 2020). It has revealed that 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 (Nkadimeng SM, et al, 2020). 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 (Nkadimeng SM. et al, 2021).

1.4 Eugenol

4-allyl-2-methoxyphenol (eugenol) is a phenolic compound found in clove oil that has a substantial anti-inflammatory and anti-oxidant properties (Murakami Y. et al., 2003). This compound blocked TNF signaling, which activates NF-κB. In fact, in cultured macrophages activated by LPS, TNF-induced NF-B activation is inhibited by phenolic antioxidants (Murakami Y.et al., 2003; Murakami Y. et al., 2005). Eugenol showed protective properties against cadmium-induced toxicity and enhanced the cellular redox system in rat model (Abhishek K. et al., 2021). Furthermore, 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 (Mateen S. et al., 2019). In LPS-stimulated macrophages, eugenol inhibited production of IL-6 and IL-10 and oxidative stress in a mice model of acute lung injury. In a dose-dependent manner, eugenol successfully inhibited lung functional and histological changes. Eugenol (150 mg/kg) might also suppress the inflammatory cytokines TNF-α, IL-1, and IL-6 in mice (Magalhães C. B. et al., 2019). 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 (Hui Q. et al., 2020). Moreover, in another study of porcine small intestinal epithelial cell line, eugenol was found to mitigate transmissible gastroenteritis virus (TGEV)-induced damage to the IPEC-J2 by upregulating the expression of tight junction proteins such as ZO-1 and Occludin. This effect is potentially associated with the suppression of the NF-κB signaling pathway. In piglets infected with TGEV, eugenol not only raised the concentration of serum immunoglobulin (IgG), but also significantly lowered the concentration of serum inflammatory cytokine (TNF-α). Additionally, eugenol reduced the expression of NF-κB mRNA and the level of phosphorylated NF-κB P65 protein in the jejunum mucosa of TGEV-infected piglets. Eugenol also increased villus height and the villus height to crypt depth ratio in both the jejunum and ileum, while reducing serum D-lactic acid levels. The findings suggest that eugenol could be a viable option for treating intestinal diseases caused by coronaviruses (Wan K. et al., 2022). It has been reported that the intestinal pathophysiological effects of eugenol are unrelated to any modifications in the gut microbiome (Chen S. et al., 2021). Eugenol, besides its substantial anti-inflammatory and anti-oxidant properties also functions as the Transient Receptor Potential (TRP) channel agonist (Vriens J. et al., 2008). TRP-1 and TRPM8 are members of the TRP family of ion channels. TRP channels have been associated with diverse cellular functions, including inflammation and pain sensation (Broad L.M. et al., 2009). Their anti-inflammatory effects were observed in colitis model of mice and in human (Ramachandran R. et al., 2013). It was reported that eugenol activates TRPM8 (Vriens J. et al., 2008). Icilin, a synthetic compound resembling mint and a super agonist of TRPM8, attenuated colitis and improved all clinical scores and showed the anti-inflammatory property of TRPM8 in colitis and IBS (Ramachandran R. et al., 2013; Peiris M. et al., 2021). Nevertheless, it's important to note that eugenol also activates various other TRP channels like TRPA1 and TRPV1 (Behrendt HJ, et

al. 2004; Vriens J. et al., 2008). Additionally, research has shown that eugenol, effectively prevented the development of colitis when administered prior to or concurrently with the dextran solution (Chen Y. et al., 2021).

1.5 Animal models of IBD

1.5.1 Inflammation induction

The efficacy of medication treatment is typically evaluated using a chemically-induced inflammatory bowel disease model in rodents, as this model is simple to initiate, repeatable, and guarantees a long-lasting and manageable inflammatory response (Eichele DD. et al., 2017; Randhawa P. K. et al., 2014; Chassaing B. et al., 2014).

1.5.1.1 Dextran sulfate sodium (DSS)-induced inflammation

One established experimental model for examining inflammatory bowel disease is DSS-induced colitis, which has several advantages over other experimental models, such as the ability to induce acute or chronic disease course using different DSS doses. Additionally, chronic inflammation is commonly linked to intestinal mucosa dysplasia, similar to what was found in ulcerative colitis (Chassaing B. et al., 2014; Lykov A. P. et al., 2018). The DSS contact with epithelium and immune cells elevates the oxidative stress which results in the production of inflammatory cytokines and recruitment of immune cells (Shin W. & Kim HJ., 2018). This triggers local inflammation. A review literature on IBD mice model reported that feeding mice with DSS polymers in their drinking water over several days reliably induces acute colitis with distinct symptoms like bloody diarrhea, ulcerations, and granulocyte infiltrations (Wirtz S. et al., 2007). This is thought to occur because DSS can directly harm the epithelial cells lining the gut, especially in the basal crypts, disrupting the mucosal barrier's integrity. Moreover, the DSS

model is effective for investigating how the gut's epithelial cells repair themselves. In cases where DSS administration is repeated in cycles (e.g., 7 days of DSS followed by 14 days of water), it can lead to chronic colitis, especially when combined with an initial dose of the genotoxic colon carcinogen azoxymethane (AOM). This combination induces inflammation-associated colorectal cancer. Since colonic inflammation is believed to play a crucial role in colorectal cancer linked to IBD, the AOM/DSS model becomes a valuable tool for exploring the mechanisms that connect inflammation to colon carcinogenesis (Wirtz S. & Neurath MF., 2007).

1.5.1.2 LPS (lipopolysaccharide)-induced inflammation

LPS can be used in animal model 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 (Seemann S. et al., 2017). Pro-inflammatory cytokine levels in circulating serum can be measured shortly after LPS administration due to the high levels released (Seemann S. et al., 2017). 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 inflammatory bowel disease and various other gut-related inflammatory disorders (Gil-Cardoso K., 2019). Previous studies determined the suitable LPS dosage for intraperitoneal injections of mice. The findings revealed that an 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) that can be replicated in clinical investigations (Liu C. et al., 2009). In vivo investigations demonstrate the rise in rat intestinal permeability induced by LPS (Gil-Cardoso K., 2019).

1.6 Hypotheses

Taking into consideration the results of pervious work on psilocybin and eugenol, we hypothesized that these two compounds can be used for treatment of IBD. Specifically, we hypothesized that: 1) LPS and DSS induce inflammation in mice in a dose- and time-dependent manner

2) Psilocybin (PSI) and Eugenol (EUG) or the combination of them have anti-inflammatory properties in inflamed intestine tissue induced by LPS

3) PSI and EUG or the combination of them have anti-inflammatory properties in inflamed intestine tissue induced by DSS

To test these hypotheses, we decided to use mice models of intestinal inflammation, where inflammation will be induced with DSS or LPS applications for mimicking local and systemic inflammation, which previously were shown to trigger in IBD in animal models. Furthermore, we investigated the effects of treatments involving PSI or EUG, either individually or in combination, both before and after the induction of inflammation with DSS/LPS. Inflammation was tested by measuring mRNA level of various inflammatory biomarkers reported to be altered in IBD.

CHAPTER 2: METHODOLOGY

2.1 Animal models

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.

2.1.1 Pilot experiments with LPS and DSS treatments

First, the pilot experiment was conducted in two major groups of studies aiming to identify the doses of LPS and DSS and the time of the analysis of cytokines for the main experiments.

2.1.1.1 Pilot experiment with LPS

The best time for cytokine induction by LPS was evaluated. 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 0h, 24h, and 48h treatments.

Inflammation was induced by intraperitoneal (IP) injection of LPS. The specific LPS endotoxin we used was 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 (Wickens RA. et al., 2018). 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 (Qin L. et al., 2007).

2.1.1.2 Pilot experiment with DSS

For the pilot experiment, DSS in two different concentrations 2.5% and 3% or saline was given to animals for 4, 5 or 6 days, each group consisting of 4 animals. Each of the controls, DSS 2.5% and DSS 3% group consist of 12 animals in total.

Initially, we assessed the impact of dextran concentration and treatment duration. Typically, dextran is administered at concentrations ranging from 1.5% to 3%, with lower concentrations resulting in a milder and delayed effect. In terms of treatment duration, dextran is usually given for a period of 5 to 10 days, with a seven-day regimen being the most commonly employed.

For our study, a 2.5% and a 3% dextran solution was obtained from MPBio (Dextran Sodium Sulfate (DSS), Colitis Grade | MP Biomedicals). The animals received the dextran solution for a duration of four to six days. Previous studies have demonstrated that this model is well-tolerated and induces colitis in animals (Eichele DD. et al., 2017).

2.2 Main experiments with LPS and DSS 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 psilocybin (PSI) and eugenol (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 (Nair AB. et al., 2016). 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 main treatment consisted of two groups – "pre-treatment" in which treatments were used prior to LPS injection and "post-treatment" in which treatments were utilized following LPS injection. In both DSS and LPS- inflamed mice model, we used the same treatment groups, shown in chart below. Each group contained negative control or vehicle in the form of saline solution and the treatment with LPS or DSS to induce inflammation.

2.2.1. Main experiments with LPS induction

2.2.1.1. Pre-treatment with PSI, EUG or combinations in LPS-induced model

Animals were given PSI, EUG or a combination at 48h and 24h 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 intestine 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 qRTPCR.

2.2.1.2. Post-treatment with PSI, EUG or combinations in LPS- induced model

Mice were administered LPS intraperitoneally (IP) for the systemic induction of inflammation. Psi and Eug were given to post treatment group 20 h after LPS IP injection and animals were sacrificed 24 h after LPS stimulation. Post-treatment group involved control, LPS+ Psi, DSS/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 qRTPCR.

2.2.2. Main experiments with DSS- induction

2.2.2.1. Pre-treatment with PSI, EUG or combinations in DSS-induced model

Mice were gavaged with psilocybin, eugenol or Psi+Eug, 24h and 1h prior to the DSS exposure. Then animals were given DSS in the drinking water for six days (based on our pilot experiment results). The mice randomly were assigned to the pre-treatment groups consisting of control, Psilocybin (Psi), Eugenol (Eug), Psi+Eug, DSS, DSS+ Psi, DSS+ Eug, Psi+ Eug (1:10) +DSS, Psi+ Eug (1:20) +LPS, Psi+Eug(1:50) +DSS. Mice were sacrificed at the last day of exposure to DSS, and the large and small intestine tissues were collected, washed in PBS, then cut into pieces, frozen in liquid nitrogen and stored at -80ºC for molecular analysis.

2.2.2.2. Post-treatment with PSI, EUG or combinations in DSS-induced model

Animals were first exposed to DSS in their drinking water for 6 consecutive days, and then received Psi, Eug or combination treatments via gavage and sacrificed 4 h after the treatment. Post-treatment groups included control, DSS/LPS+ Psi, DSS/LPS+ Eug, DSS/LPS+ Psi + Eug (1:10), DSS/LPS+ Psi + Eug (1:20), DSS/LPS+ Psi + Eug (1:50). Tissues were collected separately, washed in PBS and cut into pieces. We used liquid nitrogen to freeze them immediately and then stored them in -80ºC for further analysis.

2.3. 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. 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 2.1. Primer sequences used in qRTPCR 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

2.4 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%).

CHAPTER 3: RESULTS

3.1 Exposure of animals to LPS – pilot experiment

We first tested the effect of LPS on 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 (Zanikov T., et al., 2023). We found that LPS induced all tested pro-inflammatory genes (IL-1β, IL-6, COX-2, TNF-α) analyzed through qRT-PCR. The upregulation of tested genes started at 4 h and were elevated at 24 h and remained induced at 48 h (Figures 3.1-3.4). LPS had the lowest effect of induction of COX-2 level at 4 h, compared to control group, (Figure 3.-3).

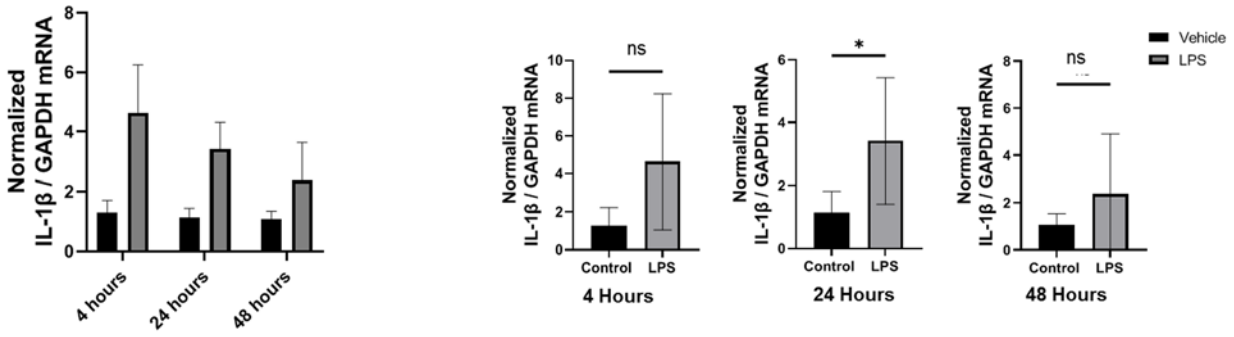

*Figure 3.1. Changes in IL-1β mRNA expression levels in large intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

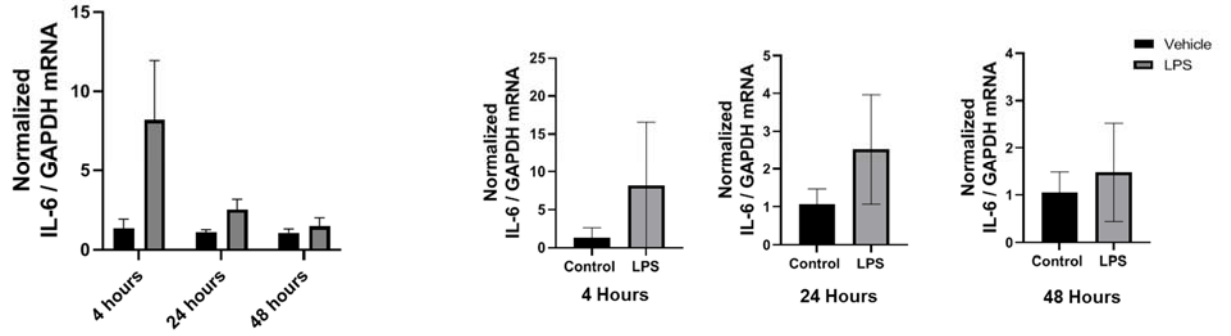

*Figure 3.2. Changes in IL-6 mRNA expression levels in large intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

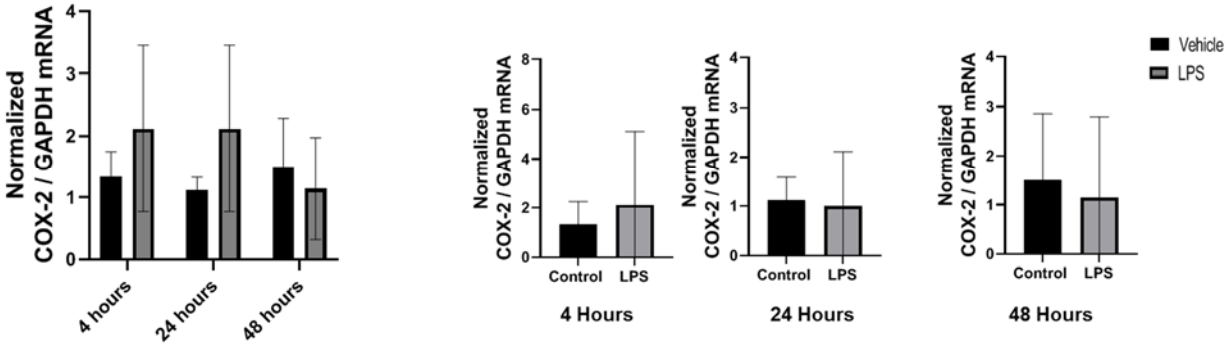

*Figure 3.3. Changes in COX-2 mRNA expression levels in large intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

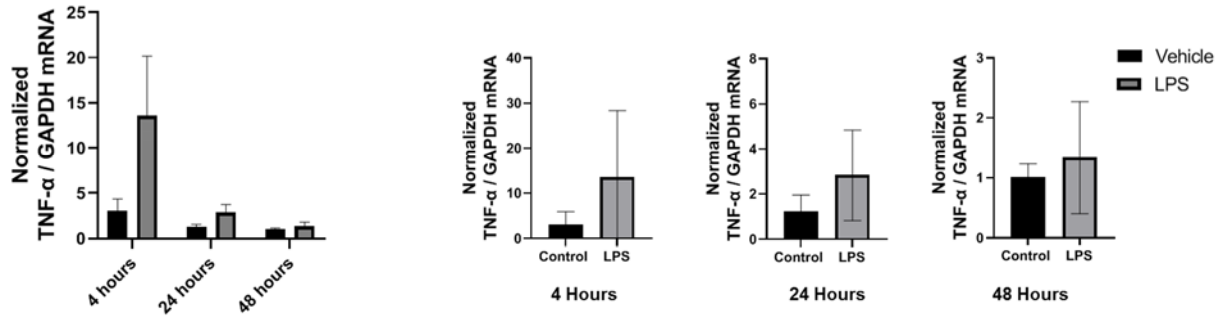

*Figure 3.4. Changes in TNF-α mRNA expression levels in large intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

We then analyzed the same to find the best time and dose for LPS-induction in the small intestine. LPS induced all the tested markers, except IL-6. All markers except IL-6 remained induced at 48h. The expression of COX-2 and IL-1β markers decreased at 24h (Figure 3.5-3.8).

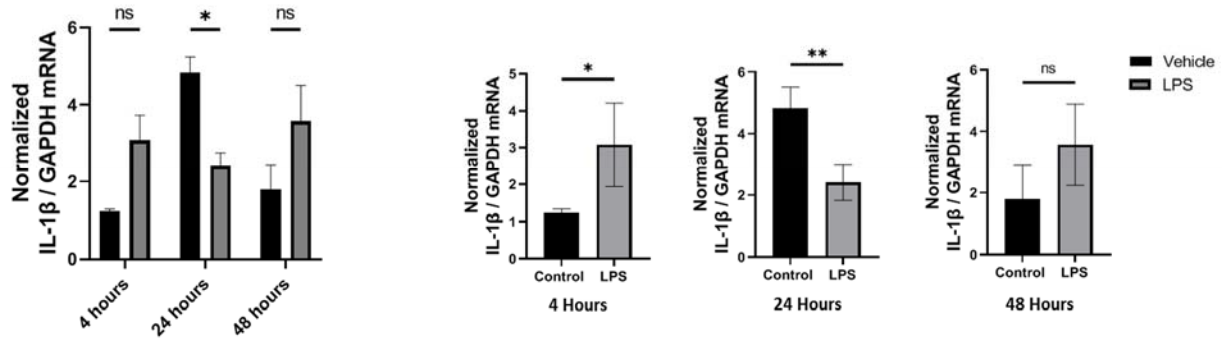

*Figure 3.5. Changes in IL-1β mRNA expression levels in small intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

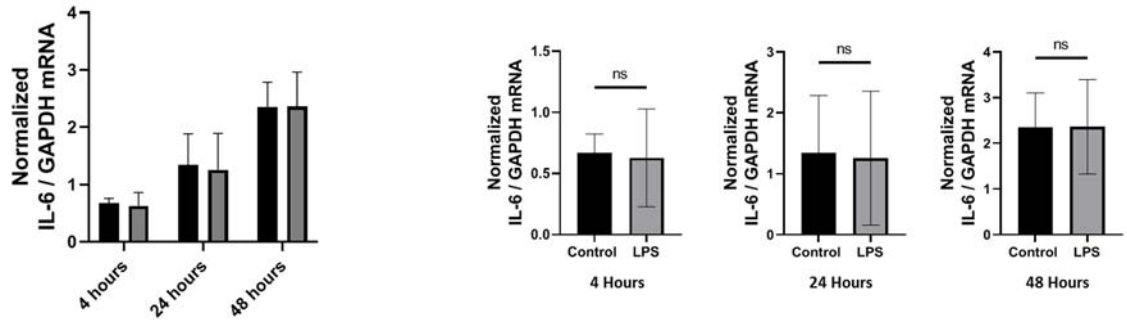

*Figure 3.6. Changes in IL-6 mRNA expression levels in small intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

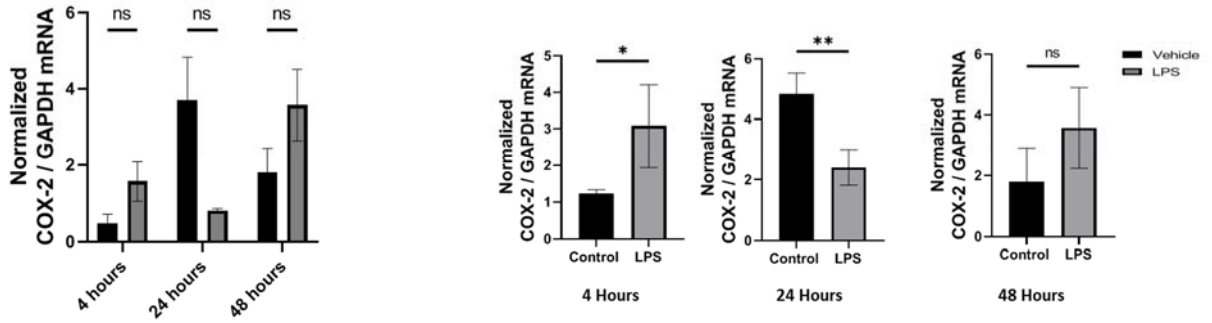

*Figure 3.7. Changes in COX-2 mRNA expression levels in small intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

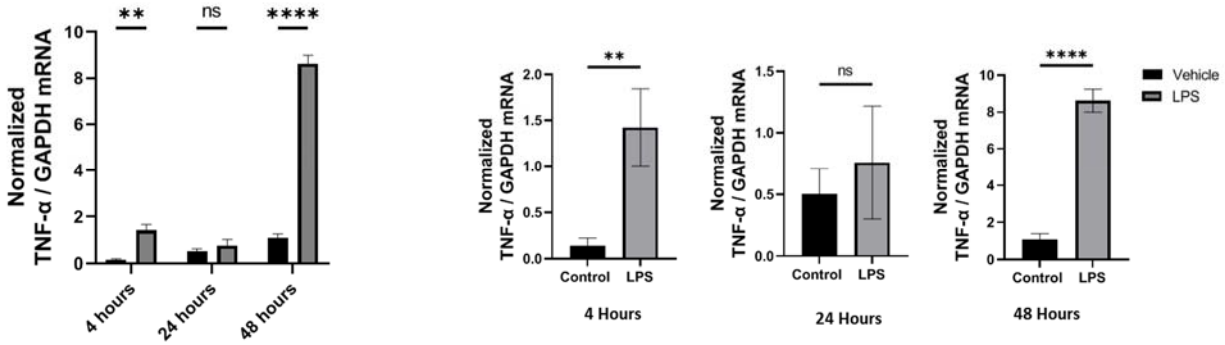

*Figure 3.8. Changes in TNF-α mRNA expression levels in small intestine exposed to LPS at 4, 24, and 48 h. The expression was normalized with housekeeping gene GAPDH. The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. 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.*

3.2 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 liver and the brain of LPS-treated mice.

We first analyzed the data in the pre-treatment group in the large intestine. LPS induced all tested markers (IL-1β, COX-2, TNF-α) in comparison to the control group and psilocybin effectively reduced the expression of IL-1β and COX-2, whereas slightly downregulated TNF-α (Figure 3.9). However, the psilocybin to eugenol 1:10 treatment was the most effective to reduce the TNF-α expression (Figure 3.9).

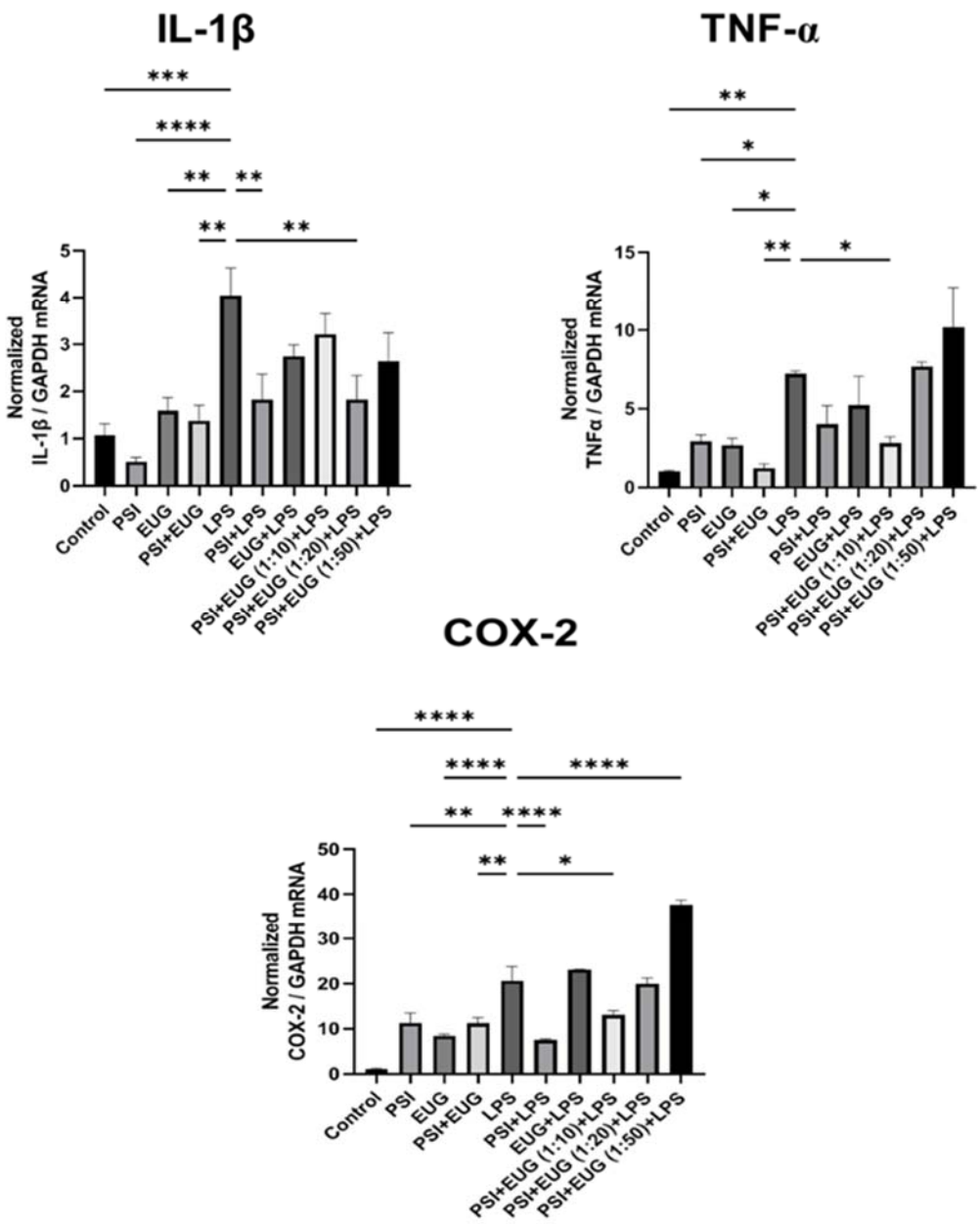

*Figure 3.9. Changes in IL-1β, TNF-α and COX-2 mRNA expression level in LPS-induced inflammation in large intestine tissue in pre-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data analyzed via qRTPCR and normalized with GAPDH as a housekeeping gene. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Then, we analyzed the post-treatment group in the large intestine. LPS induced TNFα and COX-2 but not IL-1β. All treatments with psilocybin, eugenol and combinations reduced TNFα and

COX-2 expression. However, the effect of psilocybin alone was more pronounced in reducing inflammation induced by LPS relative to control (Figure 3.10).

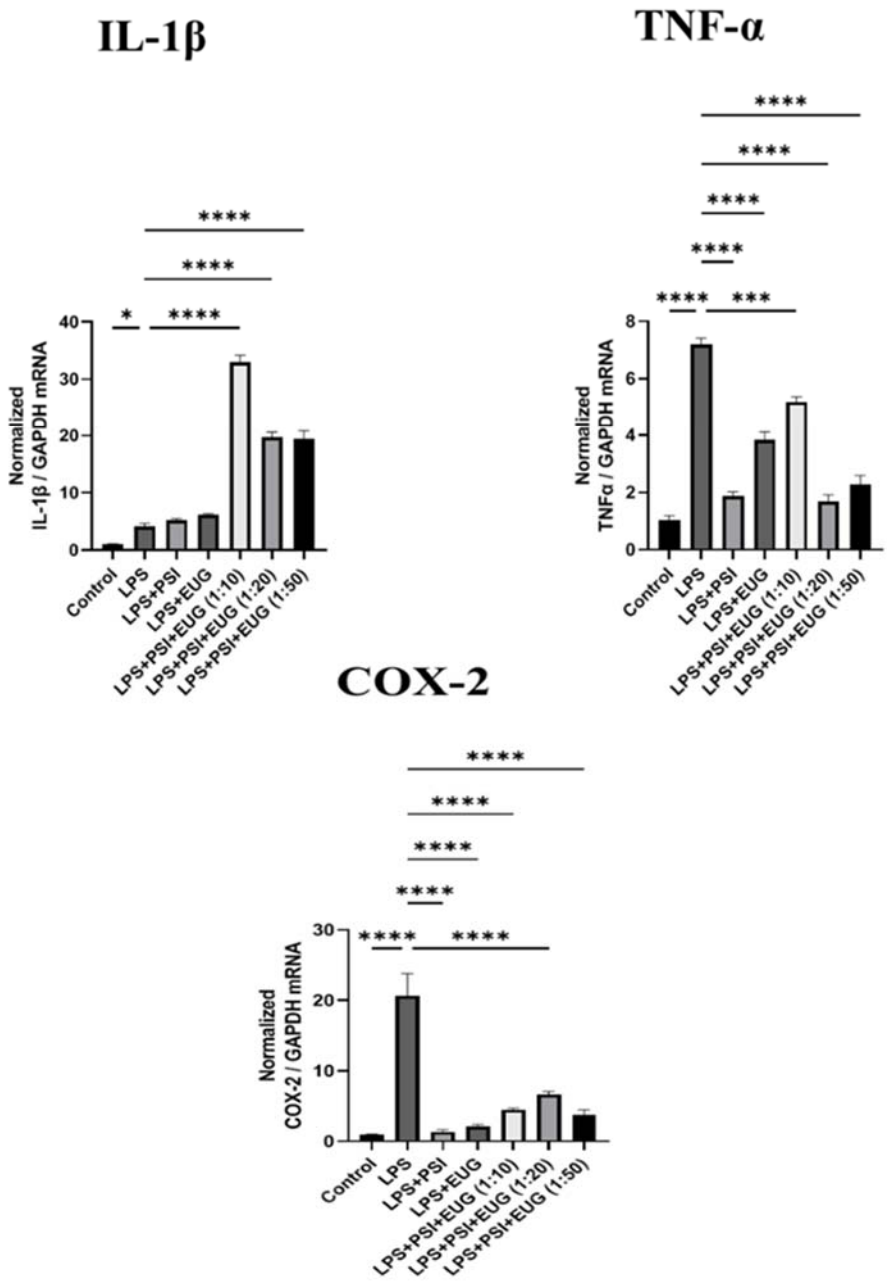

*Figure 3.10. Changes in IL-1β, TNF-α and COX-2 mRNA expression level in LPS-induced inflammation in large intestine tissue in post-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data analyzed via qRTPCR and normalized with GAPDH as a housekeeping gene. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

We also studied the data in the pre-treatment group in the small intestine which showed LPS induced IL-1β, IL-6 and COX-2. Most of the treatments reduced the expression of the cytokine markers. The largest decrease in IL-1β expression was following treatment with eugenol, while psilocybin to eugenol in 1:20 ratio had the highest reduction in IL-6, TNF-α and COX-2 expression level (Figure 3.11).

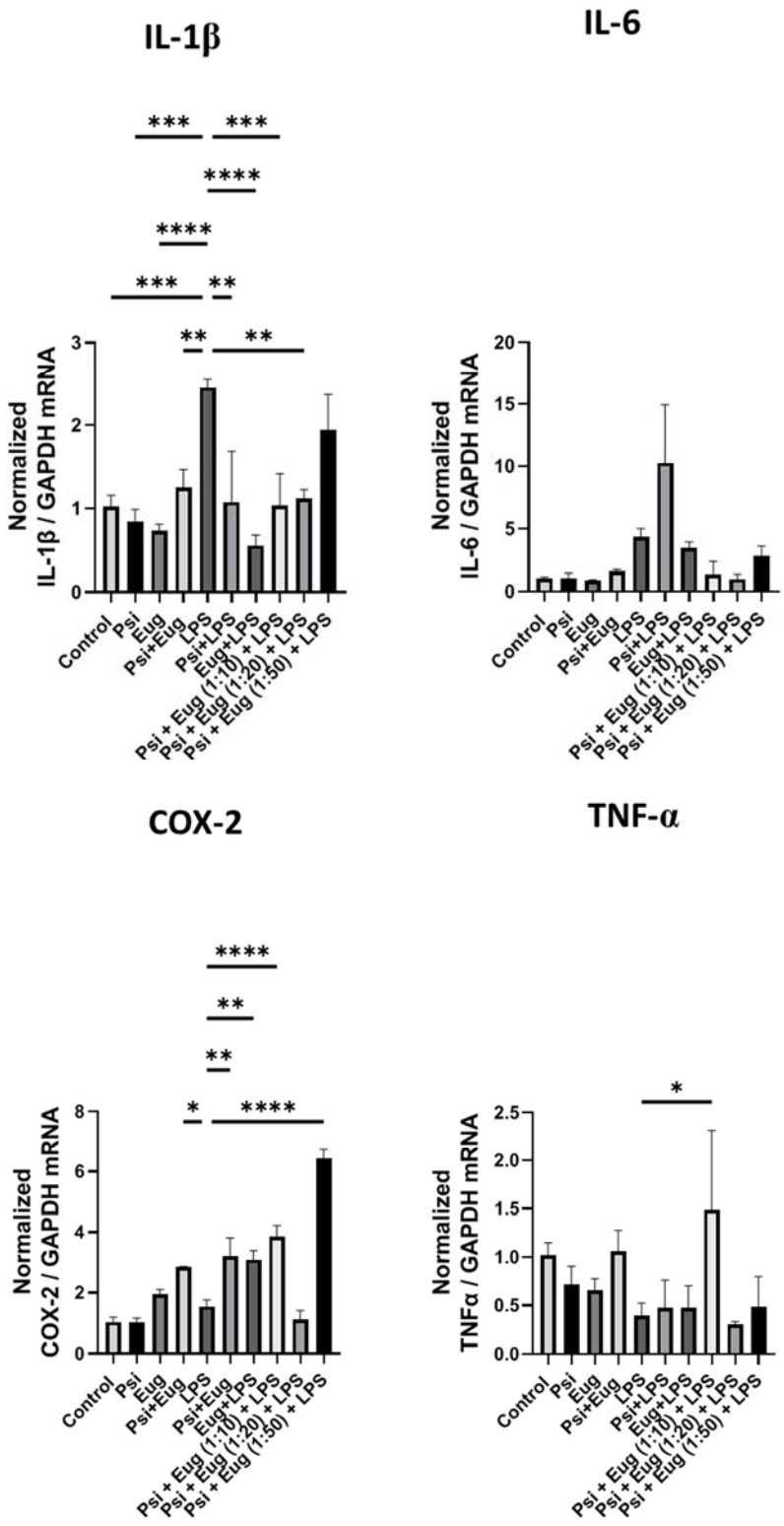

*Figure 3.11. Changes in IL-1β, TNF-α and COX-2 mRNA expression level in LPS-induced inflammation in small intestine tissue in pre-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data analyzed via qRTPCR and normalized with GAPDH as a housekeeping gene. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Then we examined the post-treatment group in the small intestine (Figure 3.12). LPS induced IL-1β, IL-6 and COX-2. Eugenol was the best in reduction of IL-1β and COX-2 expression level compared to control. Psilocybin to eugenol 1:50 was the best in IL-6 downregulation compared to control (Figure 3.12).

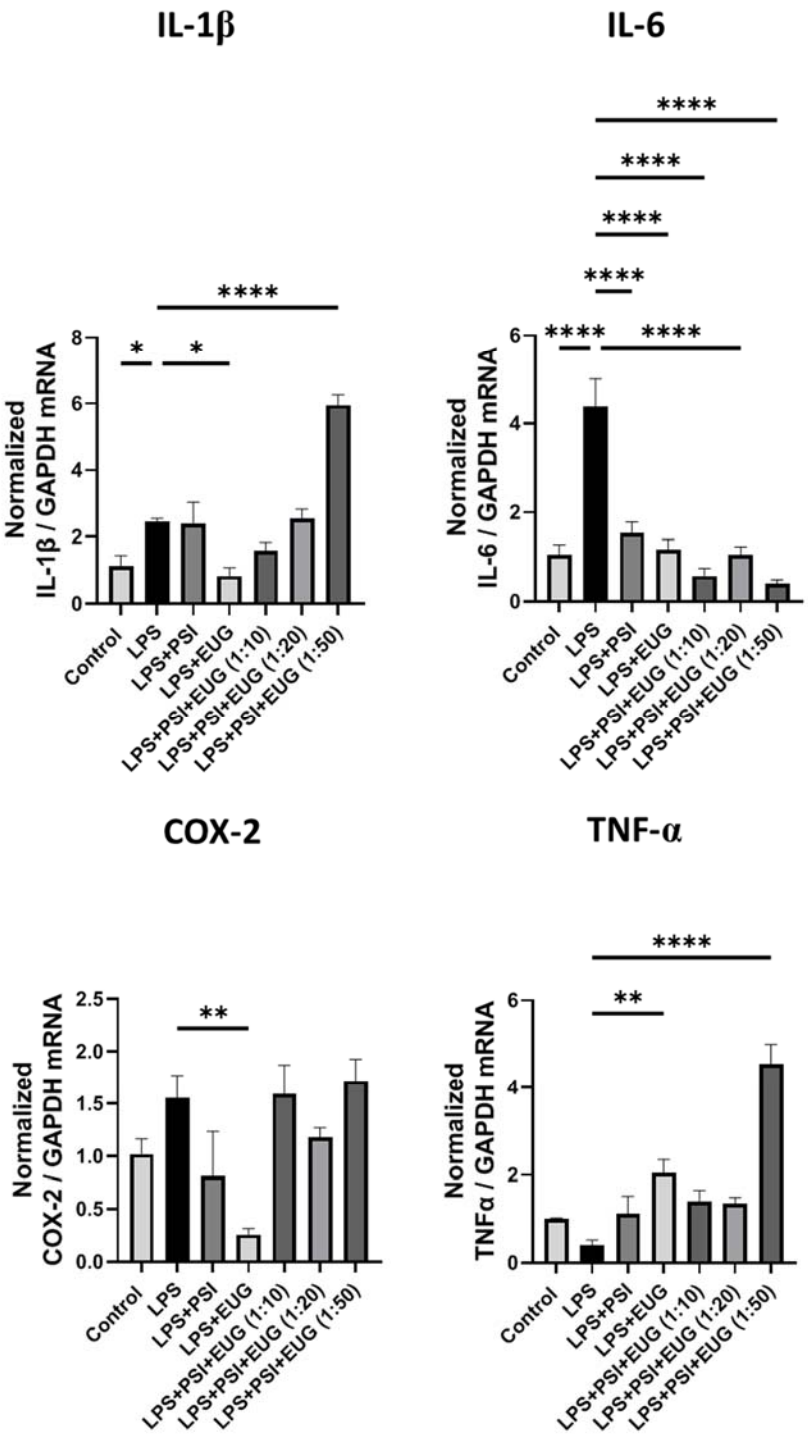

*Figure 3.12. IL-1β, TNF-α and COX-2 mRNA expression level in LPS-induced inflammation in small intestine tissue in post-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data analyzed via qRTPCR and normalized with GAPDH as a housekeeping gene. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.3 Exposure of animals to DSS – pilot experiment

We investigated the effect of DSS in large intestine by assessing the pro-inflammatory cytokines expression over 6 days. We found that the best concentration of DSS which induced IL-1β (Figure 3.13), COX2 (Figure 3.15) and IL-6 (Figure 3.16) was 3% on day 5, except for TNF-α expression induction (Figure 3.14), for which 2.5% was the best concentration on day 6.

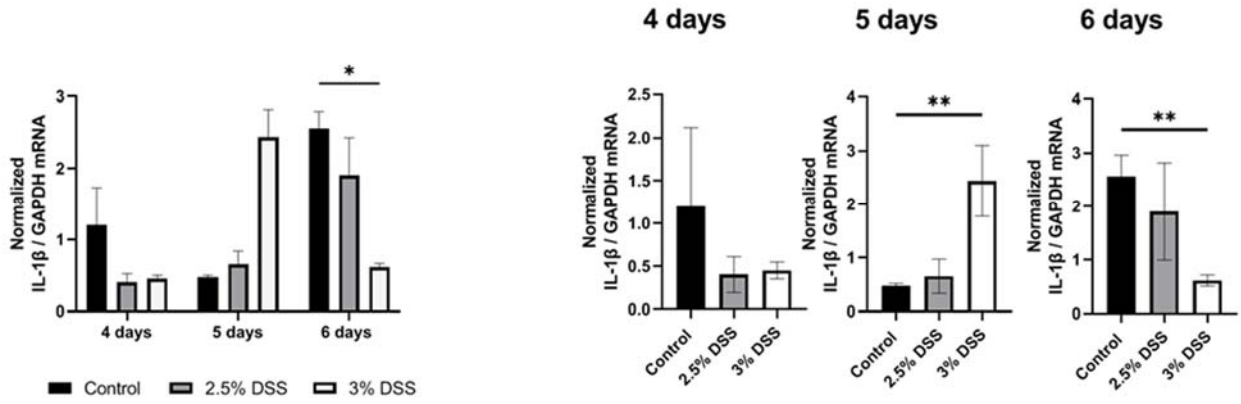

*Figure 3.13. Changes in IL-1β mRNA expression level of DSS-induced inflammation in large intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

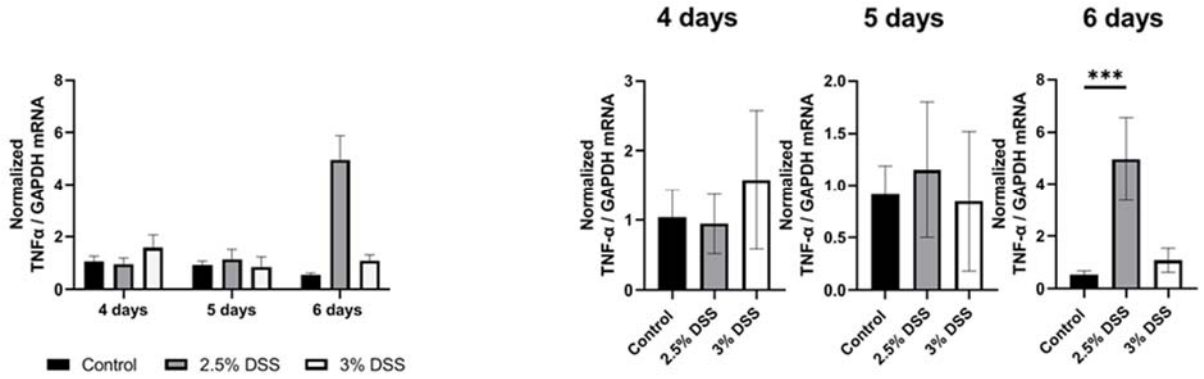

*Figure 3.14. Changes in TNF-α mRNA expression level of DSS-induced inflammation in large intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

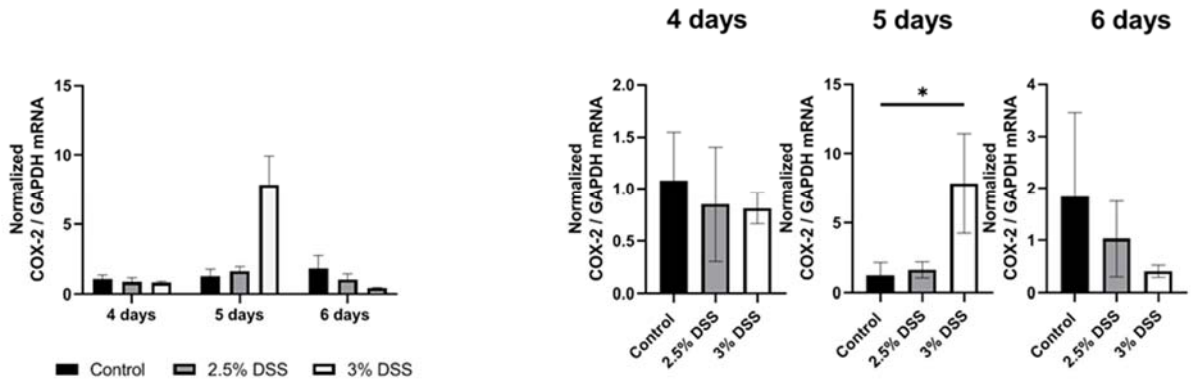

*Figure 3.15. Changes in COX-2 mRNA expression level of DSS-induced inflammation in large intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

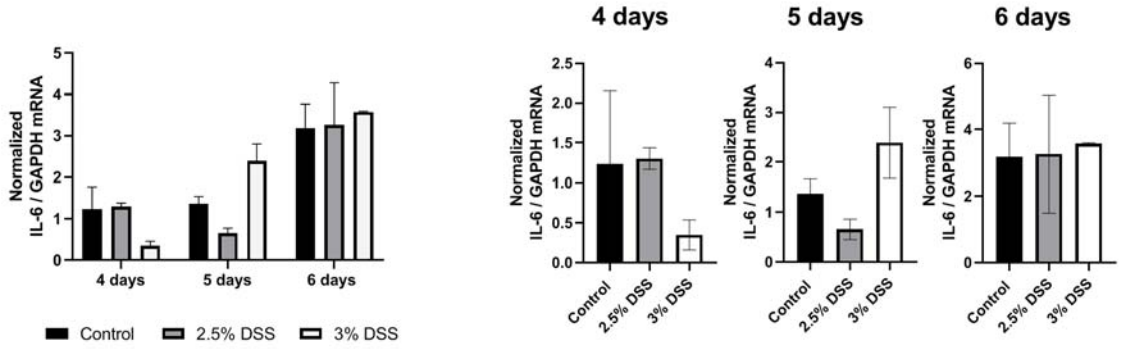

*Figure 3.16. Changes in IL-6 mRNA expression level of DSS-induced inflammation in large intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

We then analyzed the same in the small intestine. According to Figure 3.17, the significant reduction in the expression level of IL-1β in response to DSS was on day 4 ($p<0.05$) and 6 ($p<0.001$). The expression of TNF-α (Figure 3.18), COX2 (Figure 3.19) and IL-6 (Figure 3.20) was induced at day 6 by 3% of DSS concentration. Based on the results, we decided to choose 3% of DSS concentration given over 6 days for dose and time point, respectively.

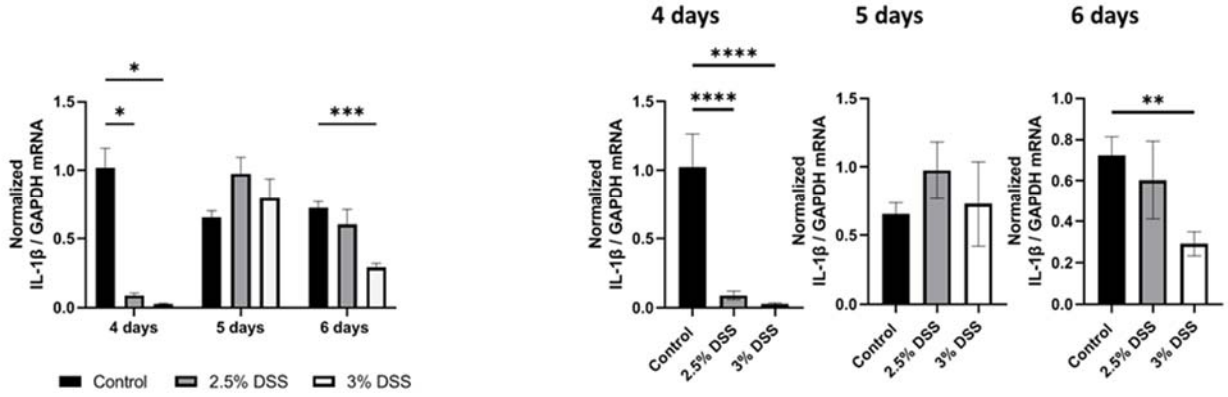

*Figure 3.17. Changes in IL-1β mRNA expression level of DSS-induced inflammation in small intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

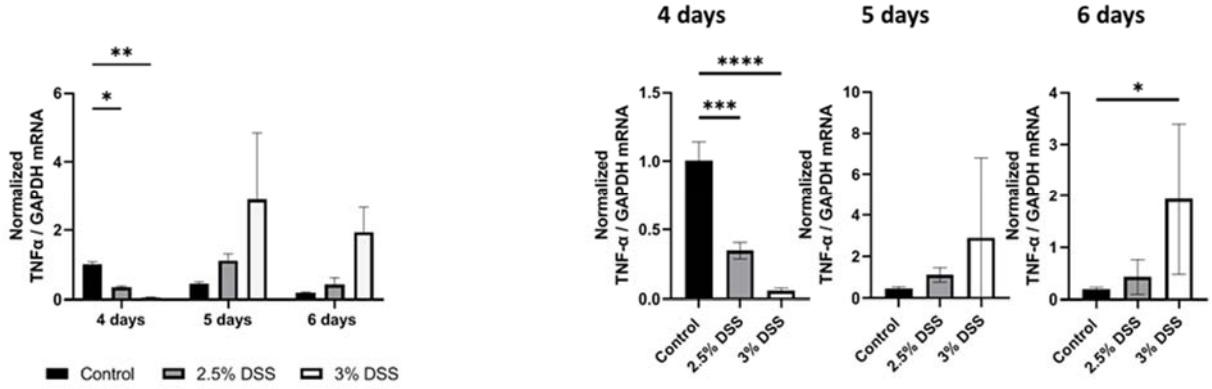

*Figure 3.18. Changes in TNF-α mRNA expression level of DSS-induced inflammation in small intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

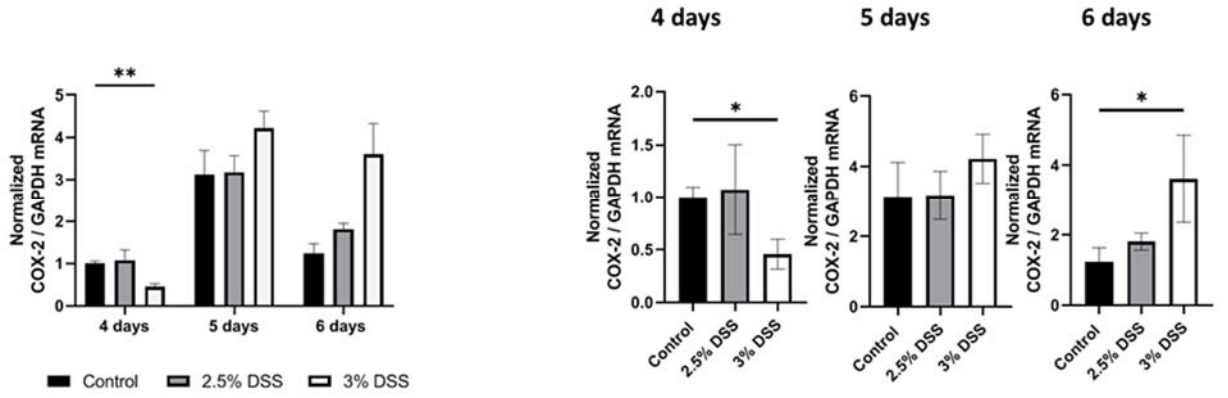

*Figure 3.19. Changes in COX-2 mRNA expression level of DSS-induced inflammation in small intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

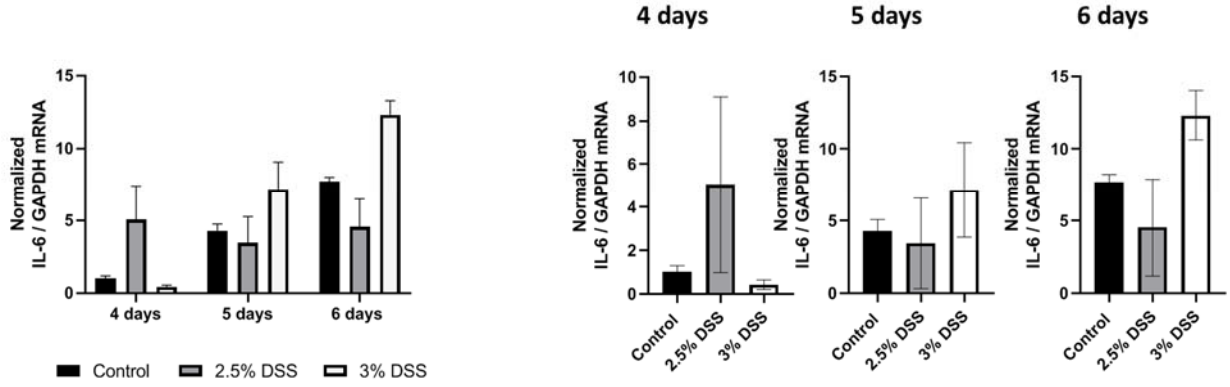

*Figure 3.20. Changes in IL-6 mRNA expression level of DSS-induced inflammation in small intestine over 6 days of exposure. Data analyzed with qRTPCR (with GAPDH used as a control). The panel on the left shows the data analyzed with a Mixed-effects analysis (multiple comparisons), followed by Dunnett's Post-hoc test. The panel on the right shows the data analyzed with an ANOVA, followed by Dunnett's Post-hoc test. Significance is indicated within the figures using the following scale: Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean ± SEM.*

3.4 Treatment of animals with 3% DSS, pre- and post-treatments with psilocybin and eugenol - main experiment

We analyzed the effect of psilocybin and eugenol on DSS-induced inflammation; for this set of experiments, we exposed animals to 3% DSS for 6 days. Similar to the experiment with LPS, we used 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 DSS consumption in the drinking water, and another one where we would use these compounds after animals consumed DSS for 6 days. 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 DSS-induced inflammation in the liver and the brain of DSS-treated mice.

We first analyzed the data in the pre-treatment group in the large intestine. DSS at 3% concentration induced IL-1β and TNF-α (Figure 3.21). Psilocybin downregulated IL-1β, IL-6 and TNFα compared to control. The most effective in reduction of COX2 and TNFα was psilocybin and eugenol in 1:50 ratio. DSS also induced the expression of HTR2A and HTR2B receptors, and reduced TRP1 and TRPM8 expression in large intestine (Figure 3.22). On one hand, psilocybin slightly decreased HTR2A and HTR2B and increased TRPM8, while eugenol increased HTR2A, TRP1 and TRPM8 expression. On the other hand, psilocybin to eugenol in 1:10 ratio increased HTR2B and TRP1 expression level. Moreover, psilocybin to eugenol in 1:50 ratio increased TRP1 expression level.

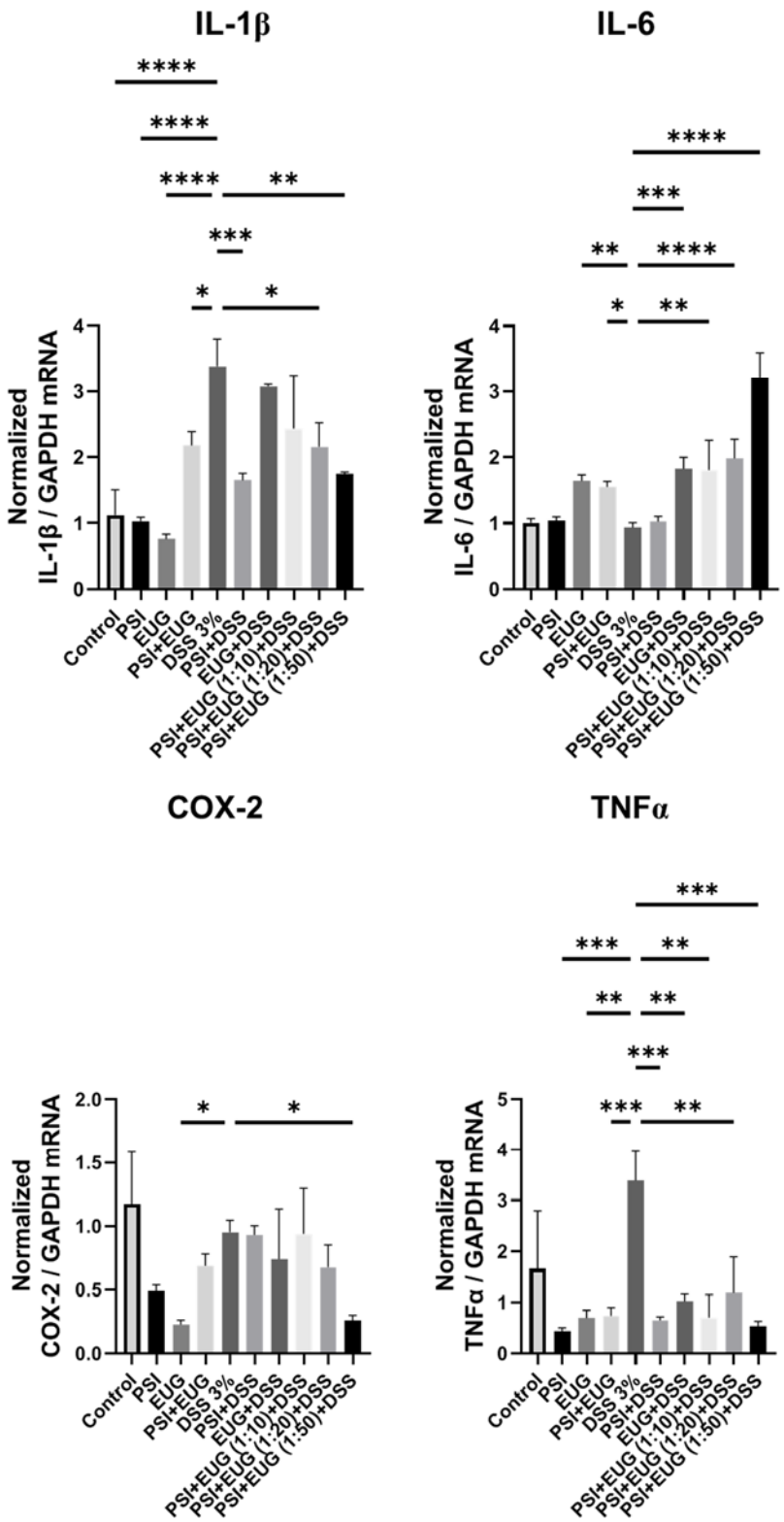

*Figure 3.21.Changes in IL-1β, TNF-α, IL-6 and COX-2 mRNA expression level in DSS-induced inflammation in large intestine tissue in pre-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

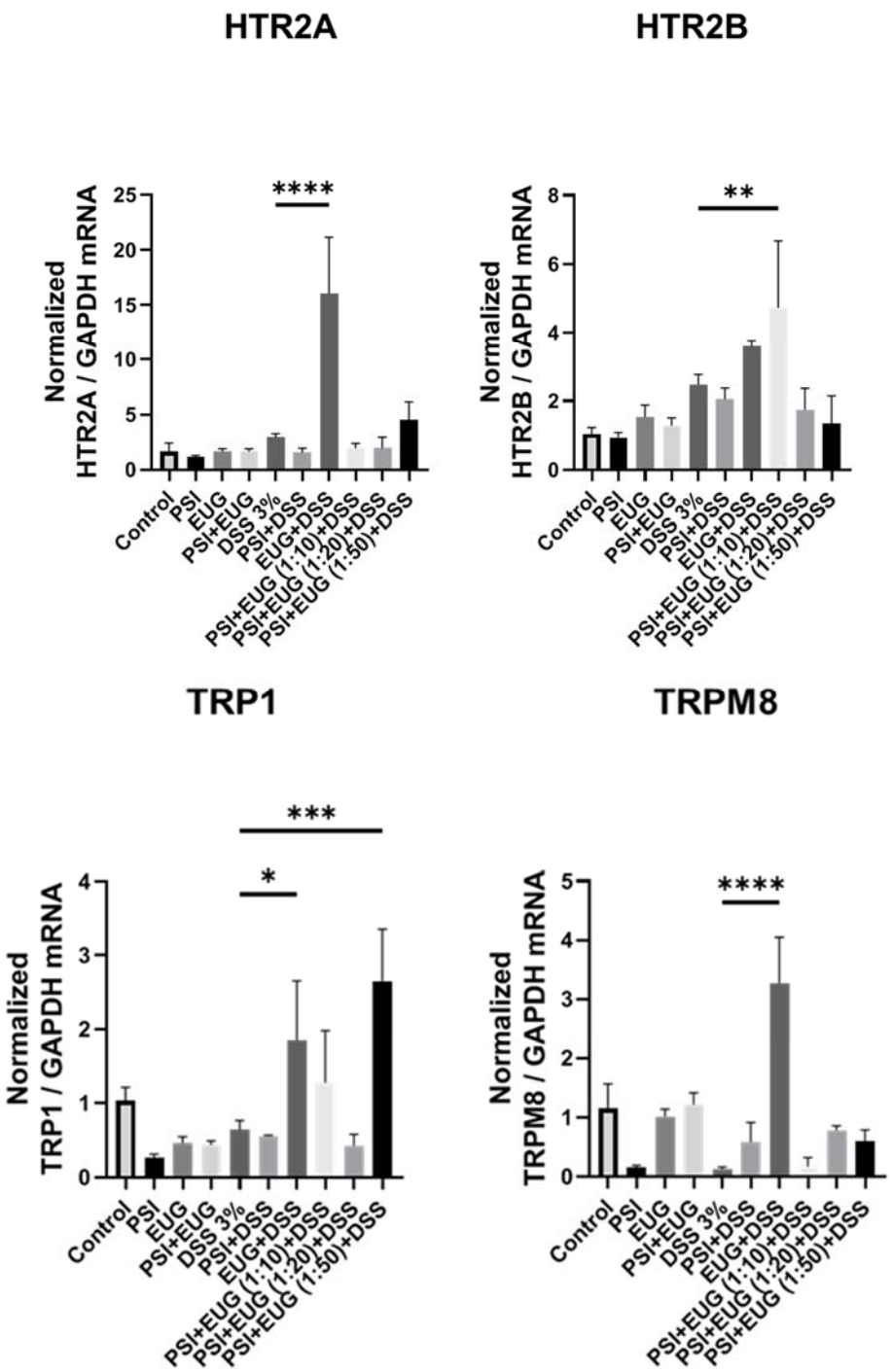

*Figure 3.22. Changes in HTR2A, HTR2B, TRP1 and TRPM8 mRNA expression level in DSS-induced inflammation in large intestine tissue in pre-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

We then tested the post-treatment group in the large intestine. DSS at the concentration of 3% induced the expression of IL-6. Psilocybin decreased IL-1β and IL-6 while increasing TNFα. However, psilocybin to eugenol in 1:10 ratio reduced IL-1β and COX-2 expression level (Figure 3.23). Furthermore, DSS also induced the expression of TRPM8 while reduced HTR2A, HTR2B and TRP1. Psilocybin, however, increased HTR2B and TRP1, while decreasing TRPM8. Eugenol, on the other hand, increased HTR2A, HTR2B and TRP1, while reducing TRPM8 expression. Nevertheless, psilocybin to eugenol in 1:50 ratio was the best in decreasing the expression of HTR2B, TRP1 and TRPM8 (Figure 3.24).

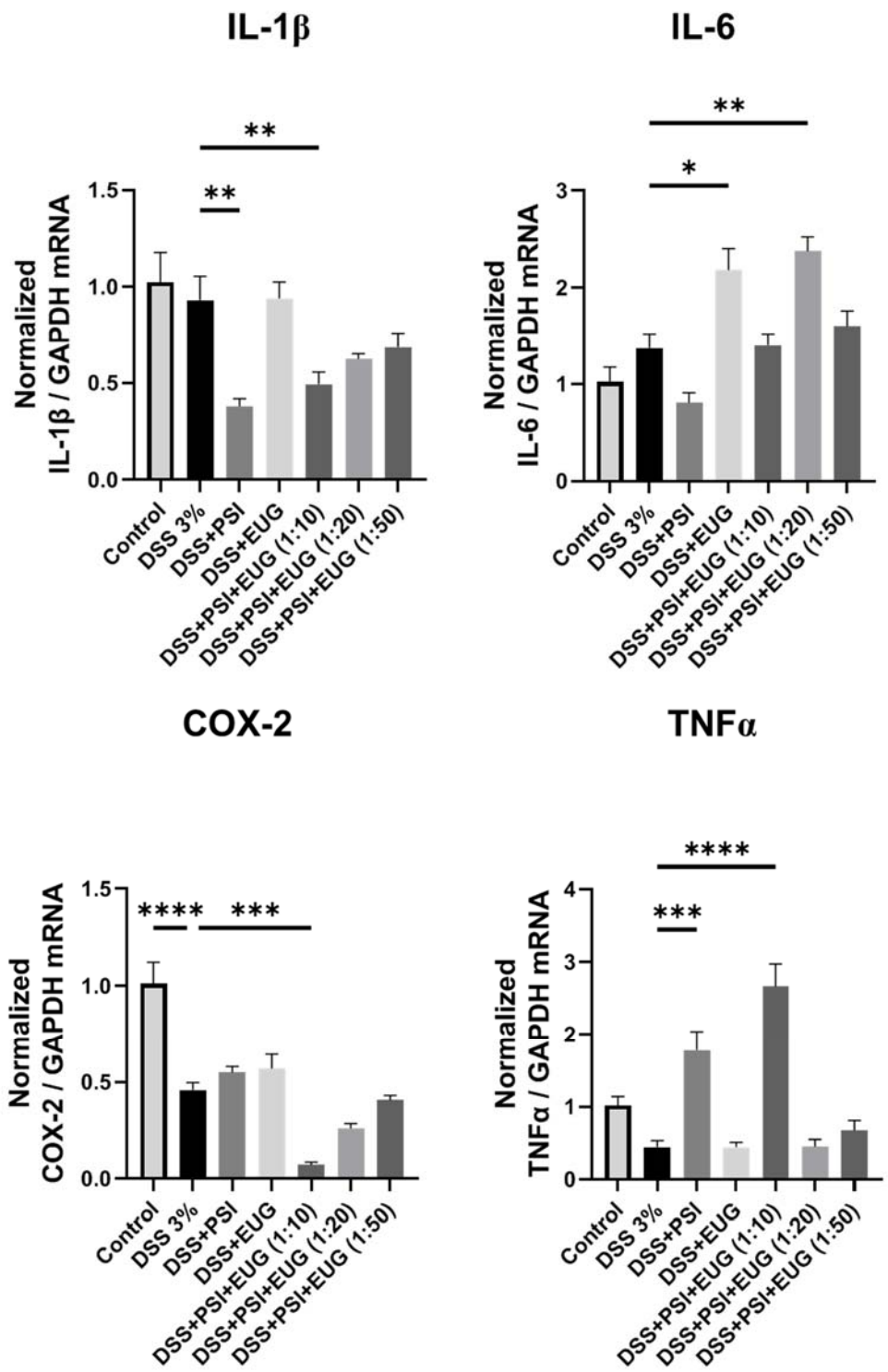

*Figure 3.23. Changes in IL-1β, TNF-α, IL-6 and COX-2 mRNA expression level in DSS-induced inflammation in large intestine tissue in post-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

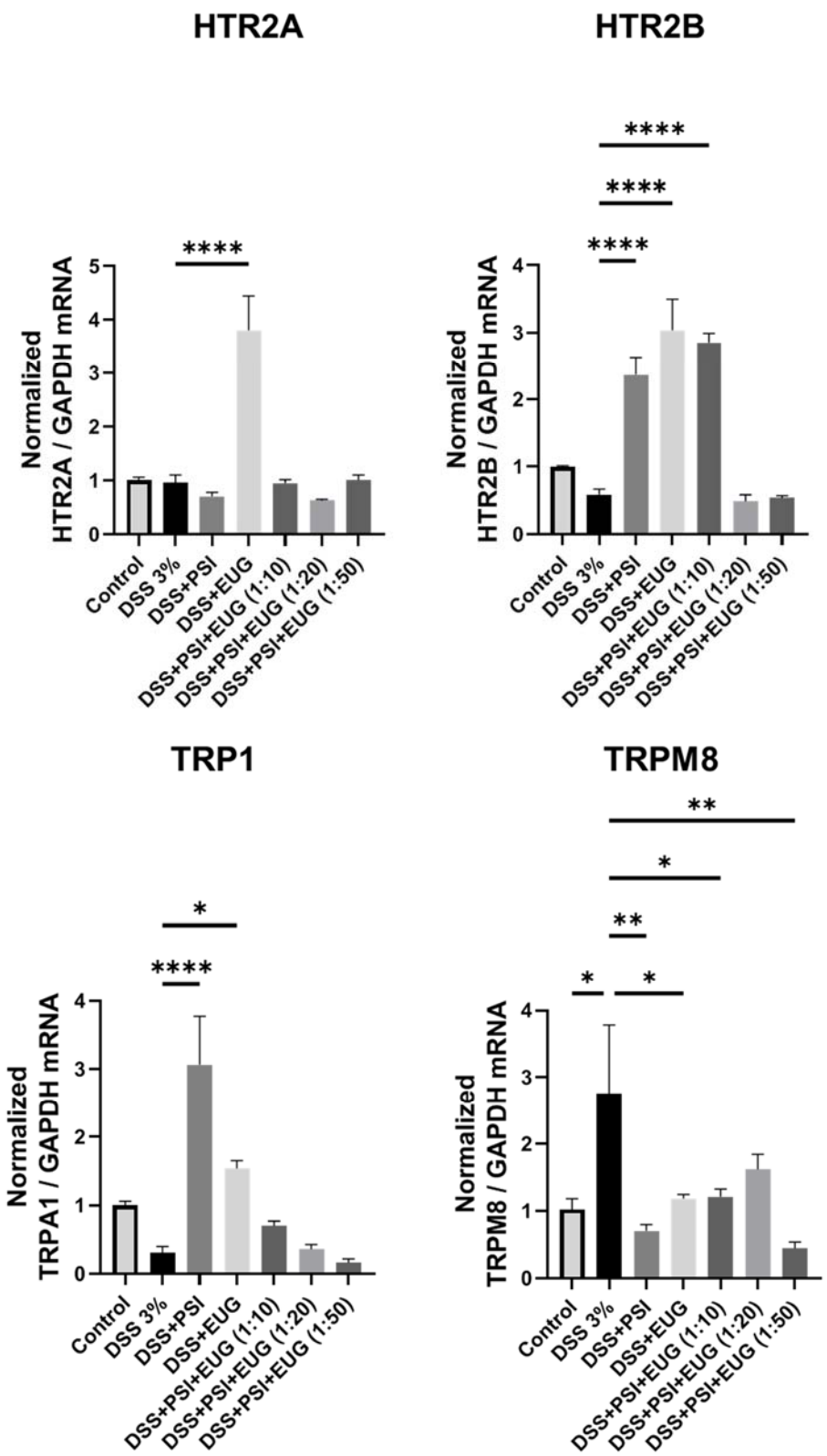

*Figure 3.24. Changes in HTR2A, HTR2B, TRP1 and TRPM8 mRNA expression level in DSS-induced inflammation in large intestine tissue in post-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

We assessed the same for pre-treatment experiment for the small intestine. DSS at the 3% concentration reduced the expression of IL-1β, COX2 while increased TNF-α. However, psilocybin was the best in decreasing TNF-α whereas, eugenol was the best in decreasing IL-1β and IL-6 expression (Figure 3.25).

DSS also reduced the expression of HTR2A, HTR2B and TRPM8. On one hand, psilocybin increased HTR2B and TRP1, while decreasing TRPM8. On the other hand, eugenol was effective in decreasing HTR2A expression (Figure 3.26).

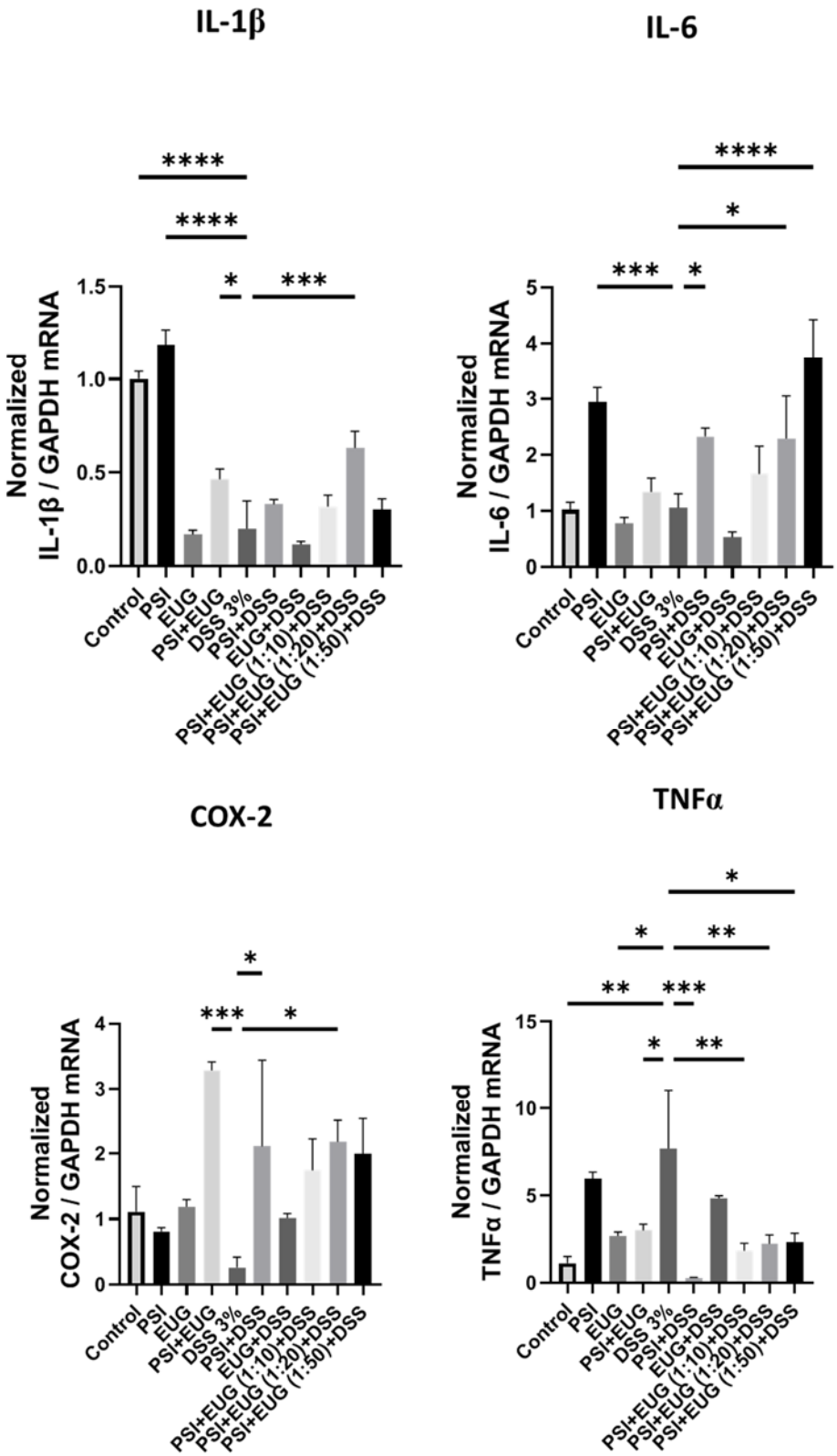

*Figure 3.25.Changes in IL-1β, TNF-α, IL-6 and COX-2 mRNA expression level in DSS-induced inflammation in small intestine tissue in pre-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

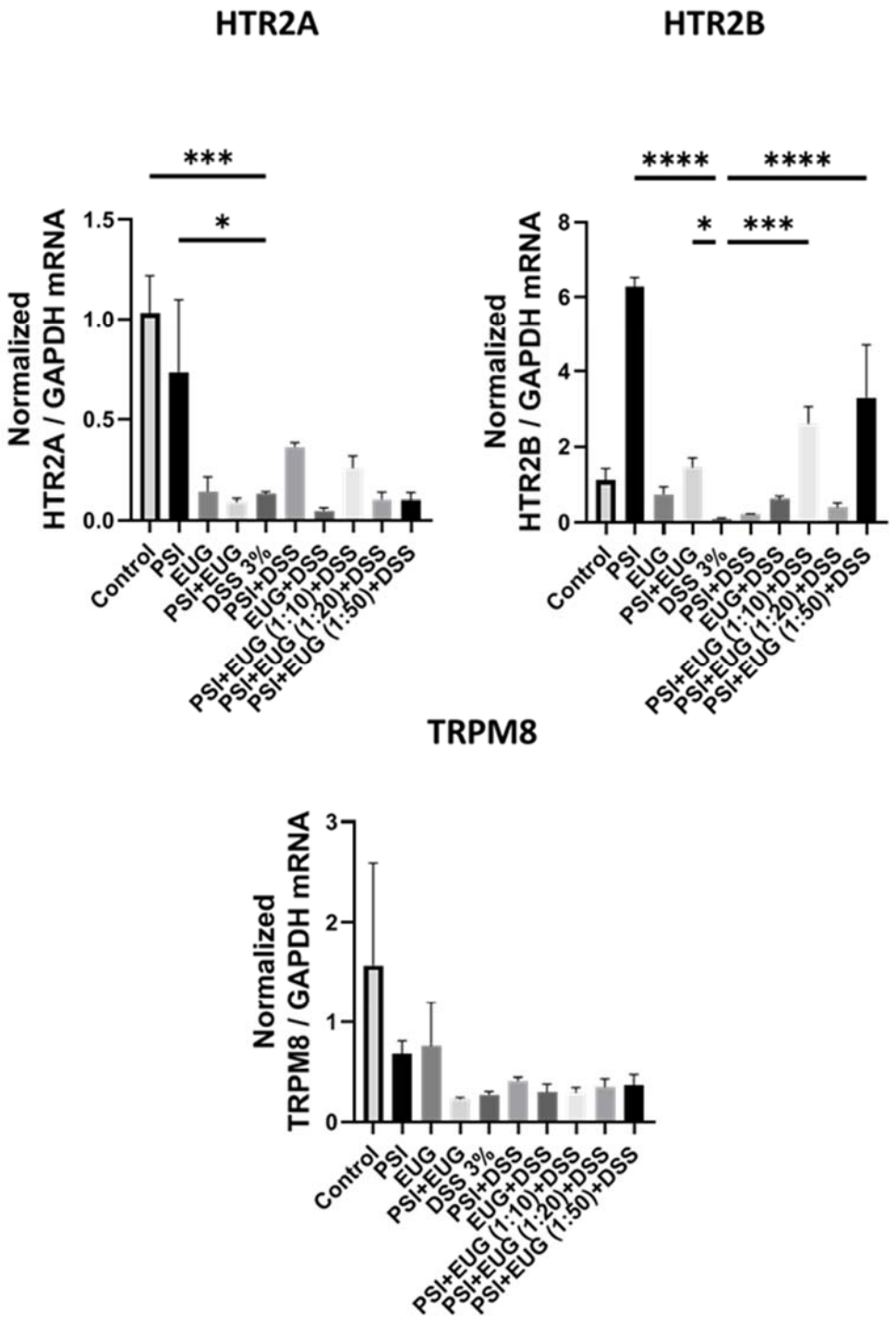

*Figure 3.26. Changes in HTR2A, HTR2B and TRPM8 mRNA expression level in DSS-induced inflammation in small intestine tissue in pre-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

We then analyzed the small intestine in the post-treatment group. DSS at the 3% concentration induced the expression level of COX2 and TNF-α (Figure 3.27). Psilocybin decreased COX2 and TNF-α gene expression. Eugenol, on the other hand, decreased IL-6 expression. Moreover, psilocybin to eugenol in the ratio of 1:20 showed greatest decrease in the expression of IL-6 relative to control (Figure 3.27). DSS also induced the expression of HTR2A, HTR2B and TRPM8. Psilocybin, however, decreased the expression of all three tested receptors. Nevertheless, eugenol decreased HTR2A, whereas increased HTR2B and TRPM8 expression. On the other hand, psilocybin to eugenol in the ratio of 1:10 increased the expression of HTR2B and TRPM8 (Figure 3.28).

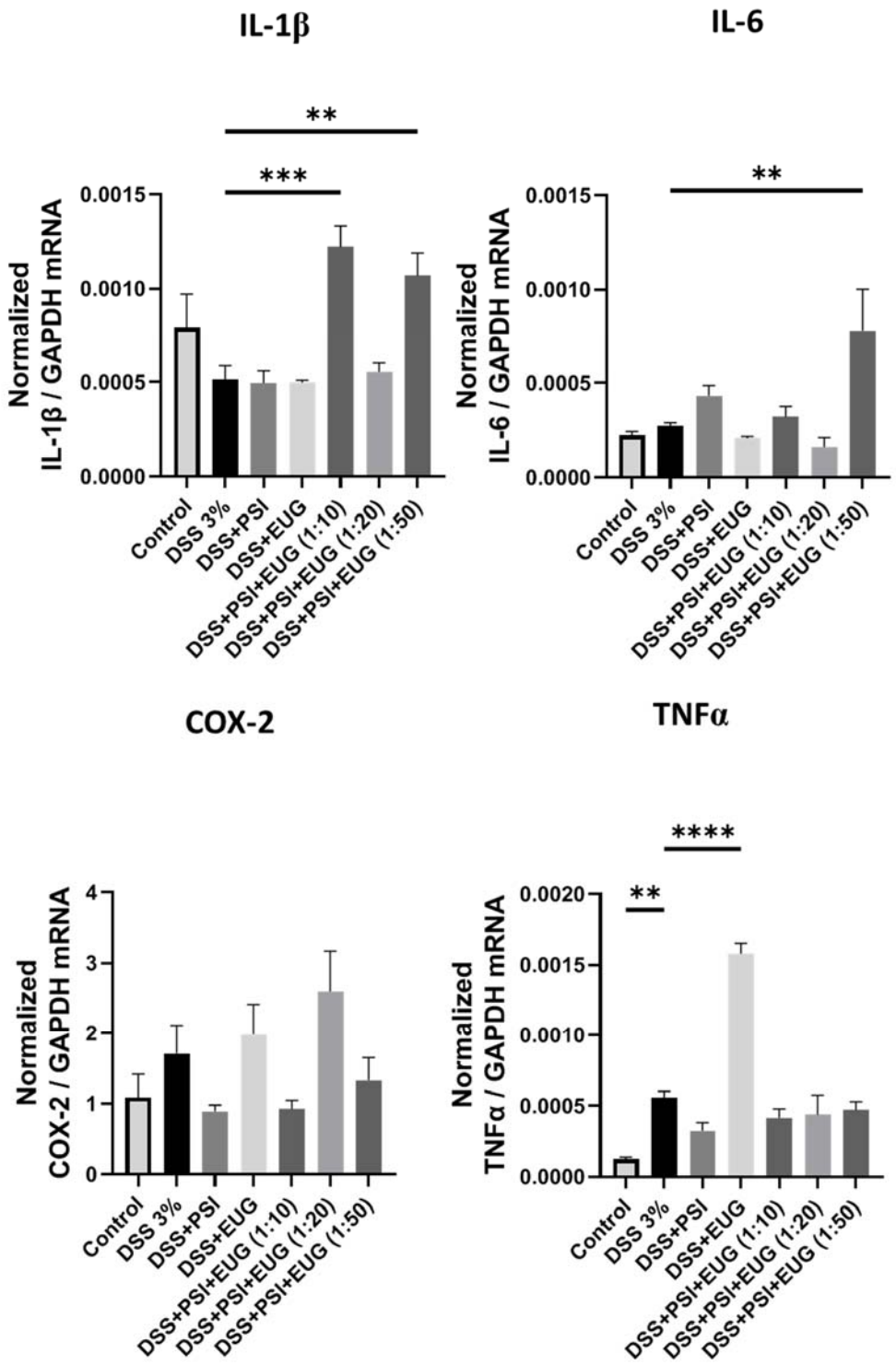

*Figure 3.27. Changes in IL-1β, TNF-α, IL-6 and COX-2 mRNA expression level in DSS-induced inflammation in small intestine tissue in post-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

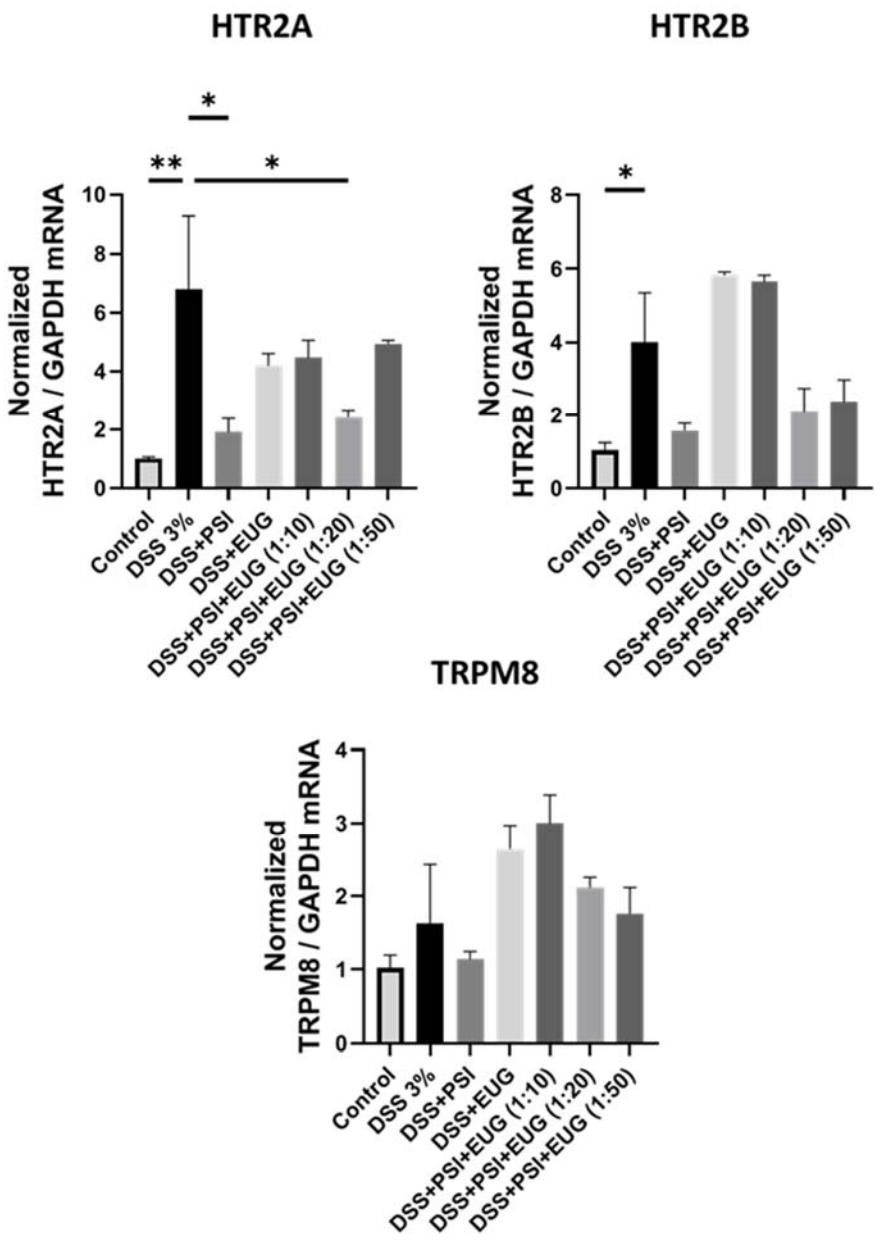

*Figure 3.28. Changes in HTR2A, HTR2B and TRPM8 mRNA expression level in DSS-induced inflammation in small intestine tissue in post-treatment (psilocybin, eugenol or their combination (1:10, 1:20 or 1:50)) group. Data = mean ± SEM. ANOVA followed by Dunnett's Post-hoc test. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

In order to illustrate the differences between various treatment models – systemic (LPS) versus local (DSS), various treatment compounds – Psilocybin versus Eugenol or their combination, treatment protocols – pre- versus post-treatment, and two tissue types –small versus large intestine, we summarized these comparison data in separate tables, attached in the appendix part.

CHAPTER 4: DISCUSSSION

Inflammatory bowel disease (IBD) is a chronic progressive gastrointestinal tract disease related to dysregulation of mucosal immune system and epithelial cells and dysbiosis of the gut microbiota (Pastorelli L. et al., 2013). However, finding the exact etiology and underlying mechanism of IBD is not easy due to the multicellular complexity of the disease (Shin W and Kim HJ., 2018). It is proven that specific factors in host such as genetic background, health condition and environmental factors like lifestyle can alter IBD (Hrncir T., 2022). Therefore, it is important to identify treatments that target the key factors for IBD pathology, including those that improve mucosal healing, blocking and maintaining intestine inflammation and preventing complication and recurrence of IBD (Couskun M., 2014; Ritesh M. et al., 2021). Due to the lack of efficacious treatment and associated side effects of existing treatments, and the importance of IBD that can be a risk factor of GI cancer if not treated effectively (Seril D.N. et al. 2003; Colombel J.F. et al., 2011), it is vital to explore potential treatments based on natural products.

In this study, we initially induced systemic and local inflammation in mice using LPS and DSS, to create a model mimicking IBD (Seemann S. et al., 2017). We then assessed the potential anti-inflammatory effects of psilocybin, eugenol, or their combination on inflamed small and large intestinal tissues in the pre- and post-treatment groups utilizing qRT-PCR to analyze mRNA expression levels of inflammatory cytokines in both systemic and local inflammation.

Synergistic effect of 5HTR2A agonist and TRP receptor agonist revealed that the combination of them reduced inflammation and pain, alleviated digestive issues and enhanced mental and physical health state (Kargbo R.B., 2023). Thus, we also studied the effect of combination of the serotonin (5HTR2A and B) and TRP channel (TRP1 and TRPM8) receptor agonists in local (DSS) and systemic (LPS) models of inflammation through qRT-PCR.

LPS can be used in animal model for mimicking systemic inflammation and local intestinal inflammation, depending on the mode of administration. LPS originating from bacteria are crucial contributors to the inflammation, increasing intestinal permeability and impairing tight junction barrier, significant factors in the development of IBD and other gut-related inflammatory disorders (Gil-Cardoso K., 2019).

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 frame 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 (Fink MP., 2014). In our mice strain C57BL/6, LD50 reported to be 10 mg/kg BW (Radulovic K. et al., 2018).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 (Song W. et al., 2019). In another study, injection of LPS at 2mg/kg dose resulted in the induction of inflammation in spleen, liver and colon (Radulovic K. et al., 2018). 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 inflammation in mice (Zanikov T. et al., 2023).

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 8h has shown TNF-α and IL-1β expression elevated at 4h in colon (Radulovic K. et al., 2018). 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 8h, while in our findings, IL-1β was elevated significantly at 24 h in the large intestine. 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. Moreover, our analysis performed on large intestine revealed that at 24 h IL1-β was the highest at 24 h post LPS, 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 (Talley S. et al., 2021).

DSS has shown a direct detrimental effect on the epithelial cells of the gut, particularly in the basal crypts, leading to a disruption in the integrity of the mucosal barrier. This disruption can subsequently trigger local inflammation characterized by infiltration of granulocytes and distinct colitis symptoms (Wirtz S., 2007; Lykov A. P. et al., 2018; Shin W. & Kim HJ., 2018). Our observations revealed a noticeable increase in various inflammatory biomarkers occurred when we employed 3% DSS over 5 and 6 days, as opposed to the relatively insignificant effects observed after 4 days of treatment. In the case of TNF-α, we observed a significantly higher level on day 6 in response to 2.5% DSS treatment in colon tissue. Our findings are consistent with a study on DSS-induced colitis, which utilized 2% DSS for 5 days and followed by 2 days of water, that detected a significant increase in TNF-α in colon, serum and brain at day 7 (Talley S. et al., 2021).

Similarly, in our investigation of the effects of 3% DSS on the small intestine, we observed that TNF-α, COX-2, and IL-6 were significantly induced on day 6 while IL-1β, which was induced by 2.5% and 3% DSS on day 5 and exhibited a significant decrease on day 6. In small intestine, COX-2 expression was significantly increased on day 5 with 3% DSS treatment, similar to the large intestine.

We then conducted our main experiment to investigate the potential anti-inflammatory effect of psilocybin and eugenol prior and following inflammation induction with LPS and DSS in large and small intestine. Our finding shows the different expression pattern in the context of inflammation induction method, the treatment time scenarios (pre- or post-treatment) and the tissue type (Table 4.1).

Table 4.1 Comparisons of inflammation induction methods, treatment time and tissue types.

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

In LPS- and DSS-induction of inflammation method, there were differences in the expression level of inflammatory cytokines (Table 4.1). Although in pre-treatment group, there was a similar pattern in gene expression between the LPS and DSS models, in post-treatment group there were notable differences between the LPS and DSS models. In the posttreatment, DSS did not trigger substantial changes in inflammation markers and treatments with psilocybin or eugenol did not result in any decrease in these markers. Thus, the differential expression of biomarkers in pre- and post-treatment in small and large intestine in DSS model may be due to the different type of microbiota present in different sections of intestine and the complexity of interaction between microbiota, tissue and DSS. It has been shown that DSS directly impacts the mucosa and intestinal cells in both small and large intestines and causes dysbiosis at the site of exposure (Kim JJ. Et al., 2012; Gil-Cardoso K. et al., 2019). The complexity of microbiota and DSS interaction has been

shown, in the experimental models of induced gut inflammation; the microbiota acts as a double-edge sword. Initially, it offers protection against the development of chemical-induced colitis, but subsequently, it exacerbates the inflammation by increasing pro-inflammatory signaling molecules (Chassaing B. et al., 2014).

On the other hand, comparison of LPS as a systemic inflammation stimulant and DSS as a localized inflammation induction chemical revealed a similar and statistically significant induction pattern of IL-1β and TNF-α in the large intestine in the pre-treatment in DSS model and in both treatment scenarios in LPS model (Table 4.1). Indeed, systemic inflammation with LPS can lead to tight junction impairment and intestinal inflammation (Radulovic K. et al., 2018). Furthermore, IL-6 expression did not increase in the large intestine under 3% of DSS over 6 days regimen in our mice model (Table 4.1), which is in contrast with a previous study of murine model of IBD, where colon tissue exposed to 5% DSS for 5 days exhibited elevated levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α (Kim JJ. et al., 2012). These findings together may support the hypothesis that 3% DSS concentration or 6 days of treatment were a sub-optimal in our study and might need to be revisited.

LPS, on the other hand, induced inflammation more consistently compared to DSS, specifically in the large intestine, by increasing IL-1β, TNF-α and COX-2 expression levels; such difference with DSS could be due to many reasons, including the dose of the compound used, route of administration or even the length of treatment (Gil-Cardoso K. et al., 2019). Previous study of the LPS effect in a rat model of intestine inflammation induction has shown an increase in the expression of 54 genes in duodenum, predominantly linked to an immune response with a pro-inflammatory nature such as interleukins, like IL-1β, and TNF ligands as well as chemokines, like COX-2 stimulation (Gil-Cardoso K. et al., 2019). On one hand, our result similarly demonstrated

an elevation in IL-1β expression in pre-treated as well as in IL-1β and IL-6 in post-treated small intestine exposed to LPS. On the other hand, in contrast to what Gil-Cardoso and colleagues reported, COX-2 expression did not exhibit a consistent trend in our result in small intestine (Table 4.1). Another report validated our findings LPS induces TNF-α expression in the large intestine - treatment of mice with 5 mg/kg of LPS injected IP induced TNF-α in liver and brain 1h after treatment (Qin L. et al., 2007).

It is noteworthy that differences in context of tissue type were seen in both inflammation induction methods we utilized (Table 4.1). Exposure to LPS resulted in a different expression pattern of COX-2 and IL-6 in small and large intestine (Table 4.1). 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 (Gil-Cardoso K. et al., 2019).

While the colon has traditionally been recognized as the primary site of DSS activity, studies have shed light on its effects on the small intestine as well (Takahashi S. et al., 2005). Our observation in DSS model showed TNF-α induction in pre- and post-treated small intestine, while in large intestine, post-treatment they did not change. Treatment of mice with 2% DSS for 7 days resulted in histological changes in ileum and cell proliferation elevation and hyperplasia (Geier MS., 2009). Although we did not perform histopathological assessment, and our investigation differs from previous study in tissue changes assessment our result similarly showed inflammation triggered in small intestine in both pre- and post-treated mice compared to control. Another study demonstrated an increase in the number of T cells in small-intestinal Peyer's patches and described elevated damage in the small intestine compared to normal mice; the changes were less prominent than in the colon, which is in contract with our findings, since we did not observed induction of

inflammation by DSS in the large intestine in post-treated animals; we did, however, observed the increase in the TNF-α expression in small intestine (Takahashi S. et al., 2005). These collective findings signify measurable damage in the distal small intestine following DSS treatment, while having less of an effect on the large intestine, suggesting the importance of the analysis of all sections of the intestine. While inflammation may be more prominently altered in the small intestine by DSS, morphologically, the impact of DSS on colon is typically more prominent than on the small intestine (Geier MS. et al., 2007).

Observed changes in the intestine in response to DSS depend on the time of the analysis. 48h of 3% DSS exposure induced DNA damage but not intestinal inflammation in the ilium and proliferation was not notable compared to control in rat model (Hong MY. et al., 2005). Similar results were observed in the small intestine of our pilot study where 3% DSS exposure for 4 days decreased all the tested inflammatory biomarkers, including COX-2, TNF-α and IL-1β as compared to control.

Our findings also underscore the complexity of the interactions between the administered treatments prior or following inflammation induction and the regulation of inflammatory biomarkers expression in the intestinal tissues (Table 4.1) both in systemic and localized inflammation induction models.

Our findings in the pre-treatment model revealed that DSS induced significant upregulation of IL-1β and TNF-α mRNA expression in large intestine and psilocybin alone or in combination with 1:20 and 1:50 ratio to eugenol reduced both pro-inflammatory cytokines expression. Similarly, in response to 2% DSS over 7 days, a significant upregulation of TNF-α and IL-1β in the serum and brain (systemic inflammation) and the development of colitis (local inflammation) was observed

in mice; treatment with the paquinimod (ABR-215757) as an anti-inflammatory agent was effective in decreasing these biomarkers and reducing colitis severity (Talley S. et al., 2021).

While psilocybin and eugenol were effective in decreasing inflammation in the pretreatment model of DSS exposure, no such thing occurred in the posttreatment model. This is in contrast with Robinson and colleagues' findings where a significant synergistic effect of eugenol and psilocybin on inflamed human small intestine epithelial cells exposed to TNF-α and IFNγ was found (Robinson G. I. et al., 2023).

In contrast to the DSS model, in the LPS model in the large intestine, both pre- and post-treatment displayed inflammation induction and psilocybin and eugenol alone or in combination exhibited anti-inflammatory effect.

While we found pre-treatment with eugenol or its combination with psilocybin in the small intestine to decrease LPS-induced inflammation, in the report where the gastroenteritis was induced by virus in piglets, eugenol pre-treatment did not significantly reduce IL-1β expression (Wang K. et al., 2022). 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 (Wang K. et al., 2022).

In contrast, downregulation of IL-1β and 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 downregulation LPS-induced increase in IL-6 levels (Robinson G. I. et al., 2023).

Unlike in the large intestine, in the small intestine we did not observe upregulation of TNF-α; in contrast, in an in vitro study, which focused on the IPEC-J2 epithelial cells from the jejunum of

neonatal porcine, low-dose pre-treatment with eugenol reduced mRNA levels of TNF-α after LPS induction. However, in their study, neither LPS stimulated IL-8 (attract neutrophils to inflammation site and increases with IL-1) nor did eugenol decrease its expression level (Hui Q. et al., 2020).

Among all treatments, the combination of psilocybin and eugenol in 1:10 ratio was the most effective in reducing COX-2 mRNA level after the LPS in the large intestine, but not in the small intestine. In contrast COX-2 protein levels were significantly increased by LPS and reduced upon treatment with the psilocybin and eugenol combination (Robinson G. I. et al., 2023). There are many differences between our study and Robinson et al. (2023) study – they used cells, while we performed in vivo exposure, and they tested proteins, while we tested mRNA levels.

We then compared the data on the receptors in our model and in the reports by others. Natural compounds like eugenol, in addition to their anti-inflammatory properties, have been found to act as ligands for TRP1 and TRPM8 channels in irritable bowel syndrome (IBS) (Behrendt HJ. et al., 2004; Vriens J. et al., 2008; Peiris M et al., 2021). Moreover, TRP channel receptors have been shown to contribute to the abdominal pain symptoms in the preclinical model of IBD, suggesting they could be a good target for IBD pain treatment (King J. W. et al., 2023). This supports our finding in the pre-treatment group in the large intestine where eugenol and the highest ratio of eugenol to psilocybin significantly increased TRP1 and TRPM8 expression. Besides, it is also aligned with our post-treatment group result in large intestine, where psilocybin and eugenol increased TRP1 expression.

TRPM8 levels were upregulated in the colon of DSS-treated animals and IBD patients (Ramachandran R et al., 2013). Correspondingly, in our study in the post-treated large intestine, DSS upregulated TRPM8. Likewise, in a colon cancer associated to colitis model with the

induction by AOM (Azoxymethane)/DSS for 10 weeks, TRP channel receptors expression was elevated (Kaya M.M. et al., 2023). Unlike in the findings by Kaya and colleagues, TRP1 expression did not change in our models

Furthermore, all the treatments in the post-treatment model in the large intestine decreased TRPM8 expression significantly, while psilocybin, eugenol (known as TRPM8 ligand), and two ratios (10:1 and 50:1) of eugenol and psilocybin significantly upregulated it. This is aligned with a previous study in which TRPM8 was upregulated in the colon of DSS-treated animals in response to icilin in mice. Icilin is a synthetic compound resembling mint and a super agonist of TRPM8 (Ramachandran R et al., 2013); it attenuated colitis and improved all clinical scores and showed the anti-inflammatory property of TRPM8 in colitis and IBS ((Ramachandran R et al., 2013; Peiris M et al., 2021).

DSS alters 5HT receptors leading to hypersensitivity of viscera in mice (Matsumoto K. et al., 2012). Authors reported that 5HTR3 was increased in 3% DSS-induced colitis model on day 7, while expression of 5HTR4 decreased in rectum mucosa of mice (Matsumoto K. et al., 2012). In our experiment, similarly, we observed different expression pattern of 5HTR2A and B exposed to DSS albeit in small intestine, where 5HTR2A decreased in pre- and post-treatment, while 5HTR2B increased.

Interestingly, contrary to the results of prior studies that demonstrated psilocybin to be a serotonergic drug that primarily modulates serotonin receptors like HTR2A (Lopez-Giménez JF. et al., 2018; Vollenweider F. & Kometer M., 2010), our results showed a decrease in HTR2A expression in the pre- and post-treatment group of the small intestine in response psilocybin, as well as 1:20 ratio of it to eugenol. However, eugenol alone significantly increased HTR2A in pre- and post-treated in the large intestine, which was similar to what was found earlier by Halberstadt

et al. (2016), where monoamine oxidase inhibitor (like eugenol) activated 5HT2A receptors (Halberstadt A. L., 2016) .

No selective 5HT2B agonist has yet been discovered; the therapeutic use of potential 5HT2B agonists is prohibited by regulators due to its possible contribution to cardiac valve defects condition (Wsół A., 2023; McIntyre R. S., 2023). We observed the 5HTR2B expression increased in our study in post-treated large intestine in response to single dose of either psilocybin or eugenol or their combination at a 1:10 ratio; the 1:10 ratio exhibited an induction also in pre-treatment in both intestinal tissues. HTR2B expression was also induced psilocybin and its combination with eugenol in the post-treatment. These findings may suggest that psilocybin functions as a serotonin receptor agonist, through interaction with enterochromaffin cells modulated by host microbiome (Császár-Nagy N. et al., 2022). The effect of psilocybin on different sections of the intestine may be in part dependent on the presence of 5HT receptors and in part on the diversity of microbiota in the small versus large intestine. 5HT levels and serotonin biosynthesis in the host can be modulated by microbiota (Jessica M. et al., 2015; Császár-Nagy N. et al., 2022), and it is possible that psilocybin brings another level of complexity into this interaction. Further research is needed on the effect of psilocybin and eugenol on 5HT2B, especially in the prolonged treatment regimens, as there is inconsistent information on cardiovascular safety of psychedelics (Wsół A., 2023; McIntyre R. S., 2023).

Drawbacks

Our study is not without the drawbacks. Here, we highlight some of them.

1. Limited Assessment of Long-Term Effects

One limitation of our study lies in the constraints associated with the duration of pre- and post-treatment intervals in our LPS or DSS-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 in 8 hours after consumption (Kalberer F. et al., 1962), 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 (Dinis-Oliveira R.J., 2017).

2. Use of Alternative Housekeeping Genes

We used GAPDH as the house-keeping gene. While we did not observe major fluctuations in its expression in response to treatments, changes in the expression of house-keepinggenes is a recurring challenge, particularly in the context of inflammation and the dynamic nature of the intestinal environment. It would be beneficial to incorporate multiple house-keeping genes, ideally those known for their stability, in addition to GAPDH (Eissa N. et al., 2017; Wang S. et al., 2018).

3. Incorporation of Microbiome Analysis

Unfortunately, our study lacks data on the microbiome due to challenges we faced in attempting to purify DNA of reliable quality for microbiome analysis. Understanding the interplay between the gut microbiome and our treatment outcomes could provide valuable insights.

4. Broad Scope and Statistical Analysis

In this study exploring the effects of psilocybin and eugenol on DSS and LPS-induced IBD models in mice, we faced the challenge of covering a wide array of comparisons across different models, tissues (large and small intestine), receptors, channels, and inflammatory cytokines. To address this complexity, it may be advantageous to employ more suited statistical analyses such as Multivariate Analysis of Variance (MANOVA), Bayesian analysis, leveraging bioinformatics and artificial intelligence tools.

5. Challenges in Protein Detection

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 sample, and limited time for harvesting tissues, especially in mice models. Western blotting, a common method for protein analysis, often presents difficulties in sample preparation and detection in such tissues (Mishra M. et al., 2017). This was evident in our study where meaningful results from western blots could not be obtained (data not shown). Similar challenges in protein detection were reported in DSS-induced inflamed intestine in mice, rendering the samples unsuitable for cytokine analysis (Heinsbroek S. et al., 2015). Future research should consider finding more efficient and rapid techniques for harvesting intestine samples for western blotting or explore alternative quantification methods, such as ELISA assays. ELISA is a more sensitive technique suitable for quantifying multiple cytokines in a single tissue sample, while

western blotting is highly specific but relies on the availability of specific antibodies (Bass J. et al., 2017).

6. Assessment of Colitis Severity and Clinical Symptoms

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 (Kim JJ. et al., 2012). These measures are essential for understanding the overall impact of treatments on the intestinal tissue and could be considered in future investigations to gain a more holistic view of the research findings.

Future direction

1. 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 (Gil-Cardoso, K, et al. 2019). 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 (Ruiz Castro, P. A., et al. 2021). There are reports that shows DSS impact the small intestine to a lesser degree than the large intestine. So, it still remains to be demonstrated what section(s) of intestine need to be analyzed for inflammation in the DSS or LPS models.

2. The interaction between the gut microbiota and gastrointestinal inflammation is proven. The gut microbiota encompasses a diverse array of microorganisms within the digestive system, including bacteria, fungi, protists, archaea, and viruses (Afridi O.K., et al. 2021). Dysbiosis within the microbiota, influenced by a variety of host-specific factors such as genetics, inflammation, or environmental elements like drugs and chemicals, has the potential to significantly impact the host's immune system through complex mechanisms. These mechanisms encompass inflammasome signaling, Toll-like receptor activation, NOD-like receptor signaling, and the capacity to influence the equilibrium between regulatory and pro-inflammatory immune cells (Chu H., et al. 2019). Interestingly, even diseases primarily unrelated to the gastrointestinal system can disrupt gut microbiota, and vice versa, leading to a growing area of research in immune-related, metabolic, and neurological disorders (Kostic A.D., et al. 2015; Scheperjans F., et al. 2015; Hrncir, T. 2022).

Moreover, dysbiosis can exacerbate its own conditions through a negative feedback loop. For instance, in the cases of liver disease, the regulation between bile acids and the microbiota, alongside various modulatory factors, may become impaired, contributing to the complexity of microbiota-related disorders (Hrncir, T. 2022). Notably, alterations in the gut microbiota have been observed preceding disease onset in specific conditions such as type I diabetes, Alzheimer's disease, and Parkinson's disease, highlighting the role of gut microbiota in the development of immune-mediated, metabolic, and neurological diseases (Kostic A.D., et al. 2015; Scheperjans F., et al. 2015; Chandra, S., et al. 2023).

Additionally, probiotics have shown promise in mitigating conditions such as ulcerative colitis and offer potential benefits when administered orally in the context of metabolic, cardiovascular, or neurological diseases (Mardini H.E., et al. 2014; Hrncir, T. 2022). As such, it becomes crucial

to delve into the pharmaceutical effects of natural products like psilocybin in both animal models and humans, with a focus on the potential interactions between microbiota and drug efficacy.

Understanding these relationships holds promise for the development of targeted therapies for IBD. In summary, while the ability of microbiota to metabolize tryptophan into tryptamine is indeed intriguing, using psilocybin as a treatment for IBD remains a complex and relatively uncharted territory. Our findings suggest promising benefits of psilocybin for gut health and inflammation, yet further research is warranted to fully grasp the implications and therapeutic potential. Moreover, the use of Psi in medical applications necessitates in-depth investigation of its long-term effects, particularly in chronic diseases such as IBD and chronic intestinal inflammatory disorders. The connection between psilocybin and the microbiome remains a subject for further exploration, and our results underscore the multifaceted nature of its impact on gut health and inflammation within the complex landscape of intestinal inflammation.

The serotonin-microbiota axis plays a significant role in gastrointestinal inflammation. Elevated serotonin levels in gut mucosa promote the development of a microbiota associated with colitis, resulting in more severe inflammation in DSS-induced mouse models and the production of pro-inflammatory cytokines through immune cell activation. 5-HT, as a key player in this axis, significantly contributes to the pathogenesis of IBD (Kwon, Y. H., et al. 2019). 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 (Koopman, N., et al. 2021).

Given that psilocybin resembles l-tryptophan (Dinis-Oliveira R.J., 2017) 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. Investigating

how these interactions influence microbiota populations, their biological impact on the host, and drug functionality (Pant A, et al. 2022) holds significant promise.

Furthermore, research focusing on the direct effects of psilocybin and its metabolites, including psilocin, on microbiota, their influence on 5HT receptors' expression, 5HT secretion, and the intricate pathways and cells involved in 5HT signaling will be instrumental in shaping future research directions. Understanding these complex interactions can provide invaluable insights into the modulation of gut microbiota for therapeutic purposes and enhanced comprehension of the mechanisms underlying psychedelic treatments.

3. On one hand, serotonin, originating in the gastrointestinal system from enterochromaffin cells, exerts control over various gastrointestinal functions, including vasodilation, secretion, peristalsis, pain sensation, etc (Mawe GM, et al. 2013). On the other hand, TRPM8 is one of TRP ion channel that play role in pain sensation. Besides, TRPM8 acts as a potential anti-inflammatory modulator in IBS (Peiris M, et al. 2021). To gain a more profound comprehension of psilocybin and eugenol efficacy with the complex pathways and underlying mechanisms that are characteristic of IBD, involving factors like microbiota, epithelial cells, and immune cells, it is strongly recommended to analyze the correlation between TRPM8 and 5HT expression in combination with psilocbyin, a serotonin receptor agonist and eugenole, a TRP ligand (Behrendt HJ et al., 2004; Vriens J et al., 2008; Vollenweider, F. & Kometer, M., 2010; Lopez-Giménez JF, et al. 2018; Császár-Nagy et al., 2022).

In the future, it would be also important to understand the effect of these compounds on serotonin receptors and serotonin production in intestinal tissue. Such a comprehensive analysis should encompass both animal models of IBD and studies involving humans.

Conclusion

In conclusion, comprehensive studies on the use of natural products to mitigate IBD and the development of IBD animal models are essential. This motivated us to test two models of inflammation, local by DSS, and systemic by LPS.

Our primary aim was to unravel how psilocybin and eugenol influence inflammation by studying their impact on the expression of pro-inflammatory cytokines in the intestines of two well-recognized IBD models. Our research demonstrated that psilocybin, eugenol, and their combination exhibit anti-inflammatory effects and modulate TRP channels, as well as serotonin receptors 2A and 2B. However, these effects were found to vary depending on the method of inflammation induction, the timing of treatment administration relative to inflammation stimulation, and the specific intestinal tissue under investigation. In particular, LPS induced intestinal inflammation more consistently and to a larger degree compared to DSS; pre-treatment in DSS model showed more promises compared to post-treatment; and large intestine showed more inflammatory biomarker expression compared to small intestine.

In summary, our approach extends beyond conventional models, striving not only to provide a comprehensive view of inflammation through systemic and localized induction but also to illuminate the potential differences between pre- and post-treatment scenarios in specific intestinal tissues. We anticipate that these methodologies and comparisons will pave the way for innovative approaches to manage gastrointestinal disorders and shed light on the intricate interactions between drugs, the microbiota, and the immune system.

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APPENDIX

Multiple comparisons of the effect of inflammation inductions, pre- and post-treatments with psilocybin (Psi) and eugenol (Eug) on small and large intestine

Table 1. The effect of pre- and post-treatment with PSI, EUG, or1:10, 1:20 and 1:50 ratio of PSI and EUG on mRNA expression level changes of pro-inflammatory cytokines in LPS inflamed large intestine in mice.

Treatment / Target gene	Pre-treatment									Post-treatment					
	LPS	PSI		EUG		PSI+EUG				LPS	LPS^+ PSI	LPS^+ EUG	LPS^+ PSI+EUG,		
		LPS^-	LPS^+	LPS_-	LPS^+	LPS^-	1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↑ P<0.001	↓	↓ P<0.01	↑	↓	↑	↓	↓ P<0.01	↓	↑ P<0.05	↑	=↑	↑ P<0.0001	↑ P<0.0001	↑ P<0.0001
IL-6*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TNF-α	↑ P<0.01	↑	↓	↑	↓	↑	↓ P<0.05	-	↑	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.001	↓ P<0.0001	↓ P<0.0001
COX-2	↑ P<0.0001	↑	↓ P<0.0001	↑	↑		↓ P<0.05	↓	↑	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001

Arrows indicate the increase or the decrease in the expression of the tested gene. * The data was missing.

Table 2. The effect of pre- and post-treatment with PSI, EUG, or1:10, 1:20 and 1:50 ratio of PSI and EUG on mRNA expression level changes of pro-inflammatory cytokines in LPS inflamed small intestine in mice.

Treatment / Target gene	Pre-treatment									Post-treatment					
	LPS	PSI		EUG		PSI+EUG				LPS	LPS^+ PSI	LPS^+ EUG	LPS^+, PSI+EUG		
		LPS^-	LPS^+	LPS_-	LPS^+	LPS^-	1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↑ P<0.001	↓	↓ P<0.01	↓	↓ 0.0001	↑	↓ 0.001	↓ 0.001	↓	↑ P<0.05	↓	↓ P<0.05	↓	-	↑ P<0.0001
IL-6	↑	-	↑	↓	↓	↑	↓	↓	↓	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001
TNF-α	↓	↓	↑	↓	↑	↑	↑ P<0.05	↓	↑	↓	↑	↑ P<0.01	↑	↑	↑ P<0.0001
COX-2	↑	-	↑ P<0.01	↑	↑ P<0.01	↑	↑ P<0.0001	↓	↑ P<0.0001	↑	↓	↓ P<0.001	-	↓	↑

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 3. The effect of pre- and post-treatment with PSI, EUG, or1:10, 1:20 and 1:50 ratio of PSI and EUG on mRNA expression level changes of pro-inflammatory cytokines in DSS-inflamed large intestine in mice.

Treatment / Target gene	Pre-treatment									Post-treatment					
	DSS	PSI		EUG		PSI+EUG				DSS	DSS^+ PSI	DSS^+ EUG	DSS^+, PSI+EUG		
		DSS^-	DSS^+	DSS^-	DSS^+	DSS^-	1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↑ P<0.0001	↓	↓ P<0.001	↓	↓	↑	↓	↓ P<0.05	↓ P<0.01	↓	↓ P<0.01	-	↓	↓	↓
IL-6	-	-	↑	↑	↑ P<0.001	↑	↑ P<0.001	↑ P<0.0001	↑ P<0.0001	↑	↓	↑ P<0.05	-	↑ P<0.01	↑
TNF-α	↑ P<0.001	↓	↓ P<0.001	↓	↓ P<0.001	↓	↓ P<0.01	↓ P<0.01	↓ P<0.001	↓	↑ P<0.001	-	↑ P<0.0001	-	↑
COX-2	↓	↓	-	↓	↓	↓	-	↓	↓ P<0.05	↓ P<0.0001	↑	↑	↓ P<0.001	↓	↓

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 4. The effect of pre- and post-treatment with PSI, EUG, or1:10, 1:20 and 1:50 ratio of PSI and EUG on mRNA expression level changes of serotonin receptor 2A and 2B and TRP ion channel, TRP1 and TRPM8 in DSS-inflamed large intestine in mice.

Treatment / Target gene	Pre-treatment									Post-treatment					
	DSS	PSI		EUG		PSI+EUG				DSS	DSS$^+$ PSI	DSS$^+$ EUG	DSS$^+$, PSI+EUG		
		DSS$^-$	DSS$^+$	DSS$^-$	DSS$^+$	DSS$^-$	1:10	1:20	1:50				1:10	1:20	1:50
HTR2A	↑	↓	↓	-	↑ P<0.001	-	↓	↓	↑	-	↓	↑ P<0.0001	-	↓	-
HTR2B	↑	↓	↓	↑	↑	↑	↑ P<0.01	↓	↓	↓	↑ P<0.0001	↑ P<0.0001	↑ P<0.0001	↓	-
TRP1	↓	↓	↓	↓	↑ P<0.05	↓	↑	↓	↑ P<0.001	↓	↑ P<0.0001	↑ P<0.05	↑	↑	↓
TRPM8	↓	↓	↑	↓	↑ P<0.0001	-	-	↑	↑	↑ P<0.05	↓ P<0.01	↓ P<0.05	↓ P<0.05	↓	↓ P<0.01

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 5. The effect of pre- and post-treatment with PSI, EUG, or1:10, 1:20 and 1:50 ratio of PSI and EUG on mRNA expression level changes of pro-inflammatory cytokines in DSS-inflamed small intestine in mice.

Treatment / Target gene	Pre-treatment									Post-treatment					
	DSS	PSI		EUG		PSI+EUG				DSS	DSS$^+$ PSI	DSS$^+$ EUG	DSS$^+$, PSI+EUG		
		DSS$^-$	DSS$^+$	DSS$^-$	DSS$^+$	DSS$^-$	1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↓ P<0.0001	↑	↑	↓	↓	↓	↑	↑ P<0.001	↑	↓	-	-	↑ P<0.001	↑	↑ P<0.01
IL-6	↑	↑ P<0.001	↑ P<0.05	↓	↓	↑	↑	↑ P<0.05	↑ P<0.0001	↑	↑	↓	↑	↓	↑ P<0.01
TNF-α	↑ P<0.001	↑	↓ P<0.001	↑	↓	↑	↓	↓ P<0.01	↓ P<0.01	↑ P<0.01	↓	↑ P<0.0001	↓	↓	↓
COX-2	↓	↓	↑ P<0.05	↑	↑	↑	↑	↑ P<0.05	↑	↑	↓	↑	↓	↑	↑

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 6. The effect of pre- and post-treatment with PSI, EUG, or1:10, 1:20 and 1:50 ratio of PSI and EUG on mRNA expression level changes of serotonin receptor 2A and 2B and TRP ion channel, TRP1 and TRPM8 in DSS-inflamed small intestine in mice.

Treatment / Target gene	Pre-treatment									Post-treatment					
	DSS	PSI		EUG		PSI+EUG				DSS	DSS$^+$ PSI	DSS$^+$ EUG	DSS$^+$, PSI+EUG,		
		DSS$^-$	DSS$^+$	DSS$^-$	DSS$^+$	DSS$^-$	1:10	1:20	1:50				1:10	1:20	1:50
HTR2A	↓ P<0.001	↓	↑	↓	↓	↓	↑	↓	↓	↑ P<0.01	↓ P<0.05	↓	↓	↓ P<0.05	↓
HTR2B	↓	↑	↑	↓	↑	↑	↑ P<0.001	↑	↑ P<0.0001	↑ P<0.05	↓	↑	↑	↓	↓
TRPM8	↓	↓	↑	↓	↑	↓	↑	↑	↑	↑	↓	↑	↑	↑	↑

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 7. The comparison of the cytokine expression between small and large intestine of animals pre-treated with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG and then exposed to LPS

Treatment	Pre-treatment large intestine									Pre-treatment small intestine								
Target gene	LPS	PSI		EUG		PSI+EUG				LPS	PSI		EUG		PSI+EUG			
		LPS^-	LPS^+	LPS^-	LPS^+	LPS^-	1:10	1:20	1:50		LPS^-	LPS^+	LPS^-	LPS^+	LPS	1:10	1:20	1:50
IL-1β	↑ P<0.001	↓	↓ P<0.01	↑	↓	↑	↓	↓ P<0.01	↓	↑ P<0.001	↓	↓ P<0.01	↓	↓ 0.0001	↑	↓ 0.001	↓ 0.001	↓
IL-6	-	-	-	-	-	-	-	-	-	↑	-	↑	↓	↓	↑	↓	↓	↓
TNF-α	↑ P<0.01	↑	↓	↑	↓	↑	↓ P<0.05	-	↑	↓	↓	↑	↓	↑	↑	↑ P<0.05	↓	↑
COX-2	↑ P<0.0001	↑	↓ P<0.0001	↑	↑		↓ P<0.05	↓	↑	↑	-	↑ P<0.01	↑	↑ P<0.01	↑	↑ P<0.0001	↓	↑ P<0.0001

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 8. The comparison of the cytokine expression between small and large intestine of animals pre-treated with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG and then exposed to DSS

Treatment	Pre-treatment large intestine									Pre-treatment small intestine								
Target gene	DSS	PSI		EUG		PSI+EUG				DSS	PSI		EUG		PSI+EUG			
		DSS^-	DSS^+	DSS^-	DSS^+	DSS^-	1:10	1:20	1:50		DSS^-	DSS^+	DSS^-	DSS^+	DSS^-	1:10	1:20	1:50
IL-1β	↑ P<0.0001	↓	↓ P<0.001	↓	↓	↑	↓	↓ P<0.05	↓ P<0.01	↓ P<0.0001	↑	↑	↓	↓	↓	↑	↑ P<0.001	↑
IL-6	-	-	↑	↑	↑ P<0.001	↑	↑ P<0.001	↑ P<0.0001	↑ P<0.0001	↑	↑ P<0.001	↑ P<0.05	↓	↓	↑	↑	↑ P<0.05	↑ P<0.0001
TNF-α	↑ P<0.001	↓	↓ P<0.001	↓	↓ P<0.001	↓	↓ P<0.01	↓ P<0.01	↓ P<0.001	↑ P<0.001	↑	↓ P<0.001	↑	↓	↑	↓	↓ P<0.01	↓ P<0.01
COX-2	↓	↓	-	↓	↓	↓	-	↓	↓ P<0.05	↓	↓	↑ P<0.05	↑	↑	↑	↑	↑ P<0.05	↑
HTR2A	↑	↓	↓	-	↑ P<0.001	-	↓	↓	↑	↓ P<0.001	↓	↑	↓	↓	↓	↑	↓	↓
HTR2B	↑	↓	↓	↑	↑	↑	↑ P<0.01	↓	↓	↓	↑	↑	↓	↑	↑	↑ P<0.001	↑	↑ P<0.0001
TRP1*	↓	↓	↓	↓	↑ P<0.05	↓	↑	↓	↑ P<0.001	-	-	-	-	-	-	-	-	-
TRPM8	↓	↓	↑	↓	↑ P<0.0001	-	-	↑	↑	↓	↓	↑	↓	↑	↓	↑	↑	↑

Arrows indicate the increase or the decrease in the expression of the tested gene. * The data are missing.

Table 9. The comparison of the cytokine expression between DSS- and LPS-induced inflammation in the large intestine of animals pre-treated with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG

Treatment	Pre-treatment large intestine									Pre-treatment large intestine								
Target gene	DSS	PSI		EUG		PSI+EUG				LPS	PSI		EUG		PSI+EUG			
		DSS^-	DSS^+	DSS^-	DSS^+	DSS^-	1:10	1:20	1:50		LPS^-	LPS^+	LPS^-	LPS^+	LPS^-	1:10	1:20	1:50
IL-1β	↑ P<0.0001	↓	↓ P<0.001	↓	↓	↑	↓	↓ P<0.05	↓ P<0.01	↑ P<0.001	↓	↓ P<0.01	↑	↓	↑	↓	↓ P<0.01	↓
IL-6*	-	-	↑	↑	↑ P<0.001	↑	↑ P<0.001	↑ P<0.0001	↑ P<0.0001	-	-	-	-	-	-	-	-	-
TNF-α	↑ P<0.001	↓	↓ P<0.001	↓	↓ P<0.001	↓	↓ P<0.01	↓ P<0.01	↓ P<0.001	↑ P<0.01	↑	↓	↑	↓	↑	↓ P<0.05	-	↑
COX-2	↓	↓	-	↓	↓	↓	-	↓	↓ P<0.05	↑ P<0.0001	↑	↓ P<0.0001	↑	↑		↓ P<0.05	↓	↑

Arrows indicate the increase or the decrease in the expression of the tested gene. * The data are missing.

Table 10. The comparison of the cytokine expression between DSS- and LPS-induced inflammation in the small intestine of animals pre-treated with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG

Treatment / Target gene	Pre-treatment small intestine									Pre-treatment small intestine								
	DSS	PSI		EUG		PSI+EUG				LPS	PSI		EUG		PSI+EUG			
		DSS^-	DSS^+	DSS^-	DSS^+	DSS^-	1:10	1:20	1:50		LPS^-	LPS^+	LPS^-	LPS^+	LPS^-	1:10	1:20	1:50
IL-1β	↓ P<0.0001	↑	↑	↓	↓	↓	↑	↑ P<0.001	↑	↑ P<0.001	↓	↓ P<0.01	↓	↓ 0.0001	↑	↓ 0.001	↓ 0.001	↓
IL-6	↑	↑ P<0.001	↑ P<0.05	↓	↓	↑	↑	↑ P<0.05	↑ P<0.0001	↑	-	↑	↓	↓	↑	↓	↓	↓
TNF-α	↑ P<0.001	↑	↓ P<0.001	↑	↓	↑	↓	↓ P<0.01	↓ P<0.01	↓	↓	↑	↓	↑	↑	↑ P<0.05	↓	↑
COX-2	↓	↓	↑ P<0.05	↑	↑	↑	↑	↑ P<0.05	↑	↑	-	↑ P<0.01	↑	↑ P<0.01	↑	↑ P<0.0001	↓	↑ P<0.0001

Arrows indicate the increase or the decrease in the expression of the tested gene.

Table 11. The comparison of the cytokine expression in small and large intestine after LPS-induced inflammation and post-treatment with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG

Treatment / Target gene	Post-treatment large intestine						Post-treatment small intestine					
	LPS	LPS^+ PSI	LPS^+ EUG	LPS^+, PSI+EUG			LPS	LPS^+, PSI	LPS^+ EUG	LPS^+, PSI+EUG		
				1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↑ P<0.05	↑	↑	↑ P<0.0001	↑ P<0.0001	↑ P<0.0001	↑ P<0.05	↓	↓ P<0.05	↓	-	↑ P<0.0001
IL-6*	-	-	-	-	-	-	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001
TNF-α	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.001	↓ P<0.0001	↓ P<0.0001	↓	↑	↑ P<0.01	↑	↑	↑ P<0.0001
COX-2	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↑	↓	↓ P<0.001	-	↓	↑

Arrows indicate the increase or the decrease in the expression of the tested gene. * The data are missing.

Table 12. The comparison of the cytokine expression in small and large intestine after DSS-induced inflammation and post-treatment with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG

Treatment / Target gene	Post-treatment large intestine						Post-treatment small intestine					
	DSS	DSS^+ PSI	DSS^+ EUG	DSS^+, PSI+EUG			DSS	DSS^+ PSI	DSS^+ EUG	DSS^+, PSI+EUG		
				1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↓	↓ P<0.01	-	↓	↓	↓	↓	-	-	↑ P<0.001	↑	↑ P<0.01
IL-6	↑	↓	↑ P<0.05	-	↑ P<0.01	↑	↑	↑	↓	↑	↓	↑ P<0.01
TNF-α	↓	↑ P<0.001	-	↑ P<0.0001	-	↑	↑ P<0.01	↓	↑ P<0.0001	↓	↓	↓
COX-2	↓ P<0.0001	↑	↑	↓ P<0.001	↓	↓	↑	↓	↑	↓	↑	↑
HTR2A	-	↓	↑ P<0.0001	-	↓	-	↑ P<0.01	↓ P<0.05	↓	↓	↓ P<0.05	↓
HTR2B	↓	↑ P<0.0001	↑ P<0.0001	↑ P<0.0001	↓	-	↑ P<0.05	↓	↑	↑	↓	↓
TRP1*	↓	↑ P<0.0001	↑ P<0.05	↑	↑	↓	-	-	-	-	-	-
TRPM8	↑ P<0.05	↓ P<0.01	↓ P<0.05	↓ P<0.05	↓	↓ P<0.01	↑	↓	↑	↑	↑	↑

Arrows indicate the increase or the decrease in the expression of the tested gene. * The data are missing.

Table 13. The comparison between post-treatment with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG in DSS vs LPS-induced large intestine inflammation

Treatment	Post-treatment large intestine						Post-treatment large intestine					
Target gene	DSS	DSS^+ PSI	DSS^+ EUG	DSS^+, PSI+EUG			LPS	LPS^+ PSI	LPS^+ EUG	LPS^+, PSI+EUG		
				1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↓	↓ P<0.01	-	↓	↓	↓	↑ P<0.05	↑	=↑	↑ P<0.0001	↑ P<0.0001	↑ P<0.0001
IL-6*	↑	↓	↑ P<0.05	-	↑ P<0.01	↑	-	-	-	-	-	-
TNF-α	↓	↑ P<0.001	-	↑ P<0.0001	-	↑	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.001	↓ P<0.0001	↓ P<0.0001
COX-2	↓ P<0.0001	↑	↑	↓ P<0.001	↓	↓	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001

Arrows indicate the increase or the decrease in the expression of the tested gene. * The data are missing.

Table 14. The comparison between post-treatment with PSI, EUG or 1:10, 1:20 and 1:50 ratio of PSI and EUG in DSS vs LPS-induced small intestine inflammation

Treatment	Post-treatment small intestine						Post-treatment small intestine					
Target gene	DSS	DSS^+ PSI	DSS^+ EUG	DSS^+, PSI+EUG			LPS	LPS^+ PSI	LPS^+ EUG	LPS^+, PSI+EUG		
				1:10	1:20	1:50				1:10	1:20	1:50
IL-1β	↓	-	-	↑ P<0.001	↑	↑ P<0.01	↑ P<0.05	↓	↓ P<0.05	↓	-	↑ P<0.0001
IL-6	↑	↑	↓	↑	↓	↑ P<0.01	↑ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001	↓ P<0.0001
TNF-α	↑ P<0.01	↓	↑ P<0.0001	↓	↓	↓	↓	↑	↑ P<0.01	↑	↑	↑ P<0.0001
COX-2	↑	↓	↑	↓	↑	↑	↑	↓	↓ P<0.001	-	↓	↑

Arrows indicate the increase or the decrease in the expression of the tested gene.