The synergic impact of lignin and *Lactobacillus plantarum* on DSS-induced colitis model via regulating CD44 and miR-199a alliance

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Abstract

Chronic or recurrent immune system activation and inflammation inside the gastrointestinal tract are characterized inflammatory bowel disease (IBD). Due to the lack of safety and efficacy of traditional medications, the use of food supplements for IBD management is on the rise. The numerous studies reported that, certain food supplements have a variety of therapeutic benefits for IBD. In the present study, mouse model of IBD was used to the anti-colitis effects of lignin supplementation with \textit{L. plantarum} on intestinal inflammation. The animal model was treated with DSS, the illness index increased, and colon length and body weight declined, but these effects were reversed when lignin and \textit{L. plantarum} treated groups. In addition, lignin and \textit{L. plantarum} supplementation inhibited the DSS induced increase in levels of cytokines (TNF-\alpha, INF-\gamma, IL-1\beta and TGF-\beta). Gene and protein expression study revealed that Lignin and \textit{L. plantarum} supplementation induced the expression of E-cad and suppressed the expression of STAT3. Lignin and \textit{L. plantarum} supplementation also suppressed CD44 expression by up regulating the expression of miR199a. Our study suggests that \textit{Lactobacillus}, lignin, and their synergistic treatments have protective roles against inflammatory bowel disease through changes in inflammatory cytokines, and miR 199a expression in DSS-induced colitis.

Introduction

Inflammatory bowel disease (IBD) is an inflammatory irritation in bowel of host system. It is caused by a multifactorial disease interaction between factors (Microbial, immunological and genetical) in nature, which is progressed to development of bowel syndrome (Friedrich et al. 2019). IBD is the global autoimmune idiopathic issues of intestine (Blumberg et al. 1999). In IBD there are two major conditions namely Ulcerative colitis (UC) and Crohn's Disease (CD). Under Ulcerative colitis condition, the inflamed mucosal layer of colon leads to chronic inflammation (Baumgart and Sandborn 2007). Whereas, under Crohn's disease condition the whole gastrointestinal tract was affected. Both the inflammatory bowel disease are major bottleneck in healthcare system in developed countries and currently increasing in the developing countries (Baumgart and Sandborn 2007). The intestinal environmental factors, which is complex to the immune response yet not clear in the etiology of IBD (Orel 2014). IBD diagnosis was very challenging in the early period over 60 years before, now both UC and CD are diagnosis with technology of fiberoptic colonoscopy and ileocolonoscopy (Mulder et al. 2014).

Microflora of the human intestine constitute bacteria, fungi and viruses. Bacteria are dominating 96% in the intestine, around 5000 species were found in the human bowel. The enormous amount microorganisms are obtained in the large intestine of human bowel, \(1 \times 10^{12}\) microbial cells indexed in the intestinal bowel. The microbiota of intestinal flora could influence host health through both direct and indirect way by its metabolites. The profile of flora may vary based on host life style, genetic factors and diet (Basso et al. 2019). The beneficial microbes, which positively regulate the host immune system are known as probiotics.

Probiotic is distinct live microorganisms which is help to protect in the host bowel system against inflammations (Borchers et al. 2009). \textit{Lactobacillus} species are well known probiotics, Specific strain of probiotic are having immunoregulatory properties, which stimulate the intestinal immune response to fight against the inflammation in bowel system. Probiotic could be classified as a two groups, such as immunostimulating and anti-inflammatory (Macho Fernandez et al. 2011). These probiotics secrete short chain fatty acids, protease, butyrate, vitamins etc., Few reports revealed that, SCFA inhibit the adherence of pathogenic microbes in host gut. Gut epithelium injured in IBD were restoration by butyrate, which supply the essential nutrient to colonocytes and deed as an agent to inactivating intracellular transcriptional factors pathways to synthesis the cytokines factors (Kanauchi et al. 2005).
In the binding sites of mucus layer the pathogenic microbes were dispatched from luminal mucosal (Veerappan et al. 2012). The probiotic enhance the gut barrier and mucous layer to reduce the deleterious microbes and other antigen (Collado et al. 2007). Probiotics regulates various mRNA transcripts of bowel inflammatory symptoms. MUC3 gene were regulated by lactobacillus strains, in goblet cells of gut mucosa and tend to increased mucus production (Mack 2003). Probiotic help to produce and secrete more cytokines against inflammation by stimulating the lymphocytes. The probiotic products and metabolites regulates inflammatory cytokines such as TNFα, IL1β, TGFβ, etc., (Caballero-Franco et al. 2007). Beneficial microbes many of probiotics, in this case the Lactobacillus sp are plays an important role as a probiotics, genetically engineered species are increased to eradicate the IBD.

L. plantarum is a potential probiotics which improve the host health benefits against bowel inflammation. L. plantarum which inhibit activation of the NF-κB signaling pathway and reduction of gastrointestinal pro-inflammatory factors (Dubuquoy et al. 2002). Lactobacillus sp. are regulating the cytokines factors to reduce the inflammation in the Bowel diseases. L. plantarum which suppressed the pathogens and regulate production of cytokines (TNF-α, (IL)-1β, IL-6, IL-10, IL-12, and IFN-γ) to modulate the balance between Th1 and Th2 of the T-cells (Ahrne and Johansson Hagslatt 2011). It also modulate the T-cell maturation. L. plantarum induced Treg cells suppress the effector T-cells and activate anti inflammatory cytokines such as IL10 and TGFβ. The suppression of effector T-cells also activate hyperplasia of goblet cell population (Mayne & Williams, 2013; Kim et al. 2020). Lactobacillus interfere the progression of macrophages DSS induced IBD mouse. The M1 macrophage reciprocally regulate the inflammatory cytokines in IBD patients as well as IBD mouse (Liew et al. 2010, Khalifa et al. 2022).

Prebiotics are indigestible carbohydrates that improve the health of the host by encouraging the growth and activity of specific bacterial species in the colon (Gibson and Roberfroid 1995; Schrezenmeir and de Vrese 2001). After cellulose, lignin is the second most prevalent natural aromatic polymer in terrestrial ecosystems, accounting for about 30% of organic carbon stored in the biosphere (Ayyachamy et al. 2013). Based on their protective properties against lipid peroxidation caused by oxygen radicals, lignin from various sources have been labeled as antioxidants (Ugartondo et al. 2009). Although lignin's prebiotic effect is modest, the prebiotic nature of lignin fragments may aid in the eradication of intestinal pathogens in cattle products, protecting people from illness. More research is needed to determine the complete prebiotic value of lignin and lignin, as well as the best dosage for health benefits, animal welfare, and product safety (Ayyachamy et al. 2013). But, no studies have looked into whether probiotic bacteria can use lignin as a prebiotic an in vivo animal model to cure colitis.

In this study, the effect of Lactobacillus, lignin and their synergistic effects were investigated against DSS induced C57BL/6J mice. The attenuation of inflammatory markers in IBD mice were evaluated by disease index, body weight, and fecal haem. The microscopic evaluation was done by H&E staining and inflammatory markers were quantified using ELISA, quantitative RT-PCR and Western blot.

**Materials And Methods**

**Effect of Lignin against L. plantarum**

The effect of lignin against L. plantarum was performed by slightly modified method of Etchepare et al. (2016). The overnight culture of L. plantarum was streak in MRS agar media (Himedia, India) and added 10% DMSO as a control and different concentrations of lignin (1 µm, 5 µm, 10 µm, 50 µm and 100 µm) into culture plate and
maintained at 37°C under continuous stirring (150 rpm/min) to a total of 4 hours. The results were expressed as log CFU/ml.

Animals

The experiment was conducted as per CPCSEA guidelines after obtaining approval from Institute of Animal Ethical Committee (018/IAEC/KIMS/2019) from Karpaga Vinayaga Institute of Medical Sciences, Chengalpattu, Tamilnadu, India. Three weeks old male mice (C57BL/6J) was procured from National Institute of Nutrition, Hyderabad, India and maintained at 25°C with 55% humidity and 12 hours cycle Light/Dark in Polypropylene cage at Karpaga Vinayaga Institute of Medical Sciences. The pellet feed containing sufficient nutrition (Carbohydrates-74%, Protein-22% and Fat 4%) were procured from Agrotech LLP, Pune and used to feed the animals ad libitum. The animals were also provided ad libitum access to normal drinking water. Treatment was carried out using oral canula.

Colitis Induction With Treatment:

The mice were segregated as five groups and each group holding 6 mice were used for the experiment. Animals provided with PBS (100 µl kg /BW) - Group I (control), animals provided with 2.5% of Dextran Sodium Sulphate (DSS) - Group II (Induced Colitis control), animals provided with 2.5% DSS and Lignin (100 µl) for treatment - Group III, and animals provided with 2.5% DSS, L. plantarum strain (1 × 10^8 load / 250 µl) and Lignin (5 mg kg / BW) for treatment - Group IV. At the end of study, the experimental mice were euthanized and sacrificed using CO₂ inhalation at the end of experiment. Immediately, the colon tissues that were preserved in 10% buffered formalin for histopathology. While the tissues that were required for other protein related studies and stored at -70°C immediately upon dissection.

Colitis Severity Assessment:

The preserved colon tissues were washed with PBS and used for assessment of inflammation in the mucous tissue. Macroscopic assessed to identify the damage of tissue and given the scoring as follows: 0 (No inflammation), 1 (Local hyperaemia), 2 (ulceration without hyperaemia), 3 (ulceration and inflammation at one site only), 4 (ulceration and inflammation at more sites) and 5 (ulceration more than 2cm).

Histological Studies:

The preserved colon tissue were sectioned in 8 µm thickness and stained with haematoxylin-eosin stain. The colon tissue were scored for crypt distortion: 1 (> 5%), 2 (20–25%), 3 (50–75%) and 4 (> 75% of the field), Inflammation: 1 (one site), 2 (2–5 sites), 3 (6–10 sites) and 4 (at Multifocal MNC infiltration), and Necrosis: 1(one site), 2 (2–5) sites, 3 (6–10 sites) and 4 (> 10 sites necrosis).

Estimation Tnf-α, Il-1β, Ifn-γ And Tgf-βin Colon Tissue:

The colon tissues were homogenized in 1000µl of ice-cold lysis buffer for the estimation of TNF-α, IL-1β, IFN-γ and TGF-β. Lysis buffer containing 0.5% Nonidet P40, 0.5% Triton X-100, 10% (v/v) glycerol, 50 mM Tris (pH 7.5), 5 mM
EDTA, 0.1 M NaCl, 10 mM K$_2$HPO$_4$, 0.5% deoxycholic acid, 20 mMNaF, 0.1 mM PMSF, 20 mM glycerol-2-phosphate, 1 mMNa$_3$VO$_4$, and a protease inhibitor cocktail. The lysate was centrifuged with 15000 RPM at 4°C for 15 mins and the supernatant were collected for the estimation of cytokines to determine the TNF-α, IL-1β, IFN-γ and TGF-β concentration using commercial ELISA kits (e-Bioscience, USA).

**Quantitative Real-time Rt-pcr (Qrt-pcr)**

Total RNA was extracted from tissues using Tri Isolation Reagent (Thermo scientific, MA, USA) according to the standard protocol. The extracted RNA was quantified using Nano Drop® 2000 spectrophotometer (Thermo Scientific) and 100 ng/target was used for cDNA preparation. The upstream forward primers and downstream reverse primers for STAT3, E-cad, CD44, and GAPDH are shown in Table. Quantitative real-time RT-PCR (qRT-PCR) was performed with template cDNA using SYBR master mix method. The expression of miRNA was analysed using stem loop RT-PCR assay using U6snRNA (Table) as internal control as described earlier (Ibrahim et al. 2021). mRNA and miRNA expressions were analyzed using quantitative real-time PCR in VII 7A (Applied Biosystems, Waltham, MA, USA). Results were expressed as relative gene expression using 2(-Delta Delta C(T)) method (Livak and Schmittgen 2001).

**Table List of primer sequences for genes used for qRT-PCR.**

| Gene    | Forward primer                     | Reverse primer                       | Amplicon size (bp) | Reference               |
|---------|------------------------------------|--------------------------------------|--------------------|-------------------------|
| STAT3   | ACCCAACAGCGCGCCGTAG                | CAGACTGGTTGTTCCATTCAGAT              | 192                | (Mechoulam and Pierce 2005) |
| E-cad   | GGGTTTCTACAGCATCACCAG              | GCTTTCCCATTTTGATGACAC                | 162                | (Guan et al. 2014)       |
| CD44    | AGCAGCGGCTCCACCATCGAGA             | TCGGATCCATGAGTCACAGTG                | 185                | (Desai et al. 2010)      |
| GAPDH   | CTCCACCTCTTCCACCTTCG               | GCCTCTCTTGCTCAGTGTCG                | 189                | (Ruiz-Villalba et al. 2017) |
| Mmu-miR-199a-RT | CTCAACTGGTGCTGGAGTCCGGCAATTCAGTTGAGGAACAGGT |                      |                    | This study               |
| Mmu-miR-199a-FP | ACACTCCAGCTGGGCCAGCTGTTG |                      |                    |                        |
| Universal RP | AACTGGTGCTGGAG                  |                      |                    |                        |
| U6-FP   | GCTTCGCCAGCAGCAGTACATACAAAT       |                      |                    |                        |
| U6-RP   | CGCTTCAGAATTTTGCGTGTCAT          |                      |                    |                        |

**Western Blot Analysis**
Tissues were lysed in RIPA lysis buffer (Santa Cruz, Paso Robles, CA, USA) and a 1× protease inhibitor cocktail. The lysate was prepared and preserved at −80°C. Protein concentrations of cell lysate were estimated using a Bradford assay at 630 nm. The equivalent of 50 µg of protein extract was separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (pore size: 0.45 µm, Bio-Rad, Hercules, CA, USA). Membranes were blocked using 5% non-fat dry milk in TBS buffer, followed by probing with the primary antibodies overnight at 4°C, according to the manufacturer's protocol. The primary antibodies STAT3 (rabbit monoclonal antibody 1:1000) (Biorybt-CB4 0WY), E-cad (rabbit polyclonal antibody 1:1000) (Biorybt, CB4 0WY), and β-actin (rabbit polyclonal antibody 1:2000) (Cell Signaling Technology, Beverly, MA, USA, 4967S) were incubated overnight at 4°C, and then washed with TBST. Washed blots were incubated with horseradish peroxidase-conjugated primary specific secondary antibody at room temperature for 1 h. The blots were visualized by an enhanced chemiluminescence (ECL) system (Pierce, Life Technologies, Austin, TX, USA) and scanned using a LICOR detection system and expressed bands were analyzed using Image Quant software and quantified by densitometry using ImageJ software v1.8 (Ibrahim et al. 2021).

**Statistical analysis:**

The data are presented as the mean ± SD of a representative experiment from three different trials that yielded similar outcomes. The statistical significance between the variables was determined using one-way ANOVA and post-doc test was performed between the mean difference of P ≤ 0.05 using SPSS 22.0 ver.

**Results**

**Effect of Lignin against L. plantarum:**

To our knowledge, this is the first study to indicate that dietary lignin and *Lactobacillus* supplementation, either alone or in combination, can reduce DSS-induced colitis in mice. Lignin toxicity was evaluated against *L. plantarum*. Lignin concentration from 1-100µM was tested against *L. plantarum*. The significant bacterial toxicity was observed above 50µM concentration whereas below 50µM was not inhibit the growth of *Lactobacillus* (Fig. 1). Lignin concentration 5 µM and 10µM was not significantly toxic P ≤ 0.05. 10% DMSO used as a control and it showed log 2x10⁻⁷ microbial load was recorded. The non-toxic concentration of lignin was fixed below 10µM concentration. Primarily, the results revealed the lignin as a biologically compatible prebiotic to *Lactobacillus* till 50 µM.

**To determine the impact of lignin mediated L. plantarum against DSS induced colitis:**

The disease severity of treated lignin with / without *L. plantarum* was evaluated using Disease index, body weight and colon length. These parameters were observed from both experimental groups (Figs: 2a & 2b). The lactobacillus group had a significant recovery disease index (Fig: 2a), body weight (Fig: 2b) and colon length (Fig: 2c) than DSS induced group (P ≤ 0.05). Meanwhile lignin with *L. plantarum* treated groups had potent reduction of disease index and improved colon length and body weight (P ≤ 0.05). The individual tested group showed less significant than combined tested groups. Further the Microscopic score and cytokines were evaluated. The disease index was reduced from 4.5 to 2.2 in DSS group and Lignin with *L. plantarum* group prospectively. Interestingly the colon length was observed in DSS group 4.1cm whereas the lignin with *Lactobacillus* group showed increased colon length 7.1cm. The body weight of the DSS induced group was reduced from 22.2 gm to 15gm, whereas the Lignin with *Lactobacillus* group were 24 gm, which showed gaining of the body weight compared with DSS group.

*L. plantarum* retard DSS induced colitis in Mice:
The histology examination showed multiple erosive lesions and extensive inflammatory cellular infiltrations was observed in the colon tissue of DSS mice. The macrophages, lymphocytes, neutrophils and few eosinophils was observed in the infiltrated parts of colon tissue (Figs. 3a and b). The significant reduction of infiltrate results was observed in lignin and Lactobacillus group (Figs. 3c and d). The corresponding histological severity score was potentially reduced in Lignin with L. plantarum mice (Fig. 3e). Even though the colon tissue of lignin DSS mice showed inflammatory lesions but the severity of inflammation was lesser than DSS induced mice (\(P \leq 0.05\)) (Fig. 3f)

**Lignin Controls The Inflammatory Cytokines In Colitis Mice:**

The estimation of cytokines was performed in DSS induced colitis mice. After 14th day of DSS induction the distal colon was excised and homogenated for analyzing inflammatory markers. Lignin with and without the treatment of L. plantarum showed significant decreasing of inflammatory cytokines revealed that the reduction of infiltrated goblet cells in the site of mucus layer of colon. The inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) were reduced significantly \(P \leq 0.05\) in Lignin with L. plantarum mice compared to DSS induced mice. The immuno-modulatory cytokines IFN-\(\gamma\) and TGF-\(\beta\) were negatively augmented in Lignin with and without L. plantarum treated mice compared to DSS induced mice. Lignin with L. plantarum showed potentially significant \(P \leq 0.05\) than Lignin alone group (Figs. 4a – d). The levels of cytokines (TNF-\(\alpha\), INF-\(\gamma\), IL-1\(\beta\) and TGF- \(\beta\)) increased in DSS-treated mice. The levels of all cytokines studied significantly decreased in Lactobacillus, lignin, and synergistic groups.

**Lignin and L. plantarum modulates STAT 3 and E.cad expression in DSS mice:**

The study evaluates the level of STAT3 in lignin treated colitis mice. Lignin treated mice were estimated after 14th day of DSS induction revealed down regulation of STAT 3 mRNA and protein expression in colitis mice (Fig. 5a and b). The mRNA of STAT 3 was not significantly changed by Lignin associated with L. plantarum treatment \(P \geq 0.05\), whereas, the protein expression was significantly decreases (\(P \leq 0.05\)) in the treatment. Followed by this treatment cell adhesion epithelial molecule E.cad was significantly regulated in inflammatory bowel regions.

The expression of E-cad was down regulated in colitis mice the level of E-cad mRNA and protein expression was increased in lignin with and without L. plantarum treated mice. The expression of E-cad gene in Lignin with / without L. plantarum was potentially increased compared to Lignin alone treated mice. These results revealed that the transformation of mesenchymal tissues into normal epithelial cells by reversion of cell adhesion molecule E.cad (Fig. 5c and d).

**Micro Rna 199 Depletes Stem Cell Modification In Dss Induced Mice:**

miRNA is single stranded short nucleotide interferes the mRNA post transcriptional regulation of host. CD-44 is intestinal stem cell marker which is increased in the DSS induced mice, but in lignin with / without L. plantarum, the marker was suppressed their expression due to increase of E.cad expression in the epithelial cells (Fig. 6a, b and c). miR-199 level was decreased in colitis mice, whereas Lignin and L. plantarum treatment showed significant \(P \leq 0.05\) miR-199 progression in colitis mice. These reports conclude that miR-199 involved in expression of stem cell marker CD-44 and showed reciprocal regulation in Lignin with/without L. plantarum treated colitis mice. These results
compiled the action of Lignin activates the *L.plantarum* regulation in DSS induced colitis mice and these treatment controls the inflammatory progression in colitis mice through miR-199.

**Discussion**

The results explored the biocompatibility of Lignin against *L. plantarum* and the result revealed that the lignin as a biologically compatible prebiotic to *L.plantarum* was not toxicity up to 50 µM. According to a recent study, less cytotoxicity of lignin makes them suitable candidates for nanosystem development for cancer medication delivery and therapeutic applications (Imlimthan et al. 2020). In support to this study, (Koh et al. 2013) reported that the prebiotic, tagatose significantly enhanced the growth of probiotic *Lactobacillus*. The result determined disease index and body weight was reduced and colon length was increased in the lignin with *Lactobacillus* group. Which was compared with DSS group.

Host–probiotic interactions improve gut immune homeostasis and regulate inflammatory markers (Hairul et al. 2014). The reduction in IL-1, IL-6 and IL-8 levels in TNF-α-induced cells can be explained by the ability of BA to suppress anti-inflammatory mediators, as further confirmed in an in vivo model. Comparable findings were obtained in many in vitro studies. *L. acidophilus*, *B. coagulans* and *B. subtilis* exhibited potential anti-inflammatory effects on Caco2, dendritic and PBM cells (Khalifa et al. 2022). These results confirm that gut laminal and epithelial cells of mucous play a major role in probiotic interactions via secreting inflammatory mediators such as chemoattractant proteins, interleukins and infiltration signals. Similarly, (Kangwan et al. 2022) reported that *L. pentosus*, which was obtained from fermented tea leaves, improved colon abnormality. Treatment of probiotics significantly protected the colon from being inflamed by mitigating histological and clinical damage traits, enhancing the intestinal barrier integrity, and attenuating inflammation symptoms induced by DSS induction. (Hairul et al. 2011; Khalifa et al. 2022).

The crypt structure was abnormal in the DSS group due to crypt loss, epithelial cell death, and significant inflammatory cell infiltration. Whereas, DSS-induced colon shortening and acute inflammation were reduced by the *Lactobacillus*, lignin, and synergetic therapies. A considerable decrease in colon length as a result of intestinal injury is a frequent hallmark of DSS-induced colitis (Rumi et al. 2004; Mizoguchi and Mizoguchi 2008). Both macroscopic and microscopic observations revealed decrease in severity of DSS induced colitis across the treatment groups. Prebiotic fibres that are soluble and non-viscous were reported to alleviate the symptoms of inflammatory bowel diseases (Hardy et al. 2013). In rats, symbiotic *Lactobacillus* and fructo oligosaccharide supplementation was found to reduce intestinal and systemic inflammation in Crohn's disease (Lindsay 2006; Delcenserie et al. 2008).

Rumen lactobacillus regulates the immune response and acts as an anti-inflammatory strain and balance the cytokine secretion, whereas other two active strains were particular inhibition on anti-inflammatory cytokines (Arokiyaraj et al. 2014). Few studies reported that, the lactobacillus sps and some rumen bacteria stimulate low levels of proinflammatory cytokines (TNF-a, IL-6 and IL-8), while activate high levels of IFN-c and IL-12p70, these two active cytokines to promote cellular immunity and enhance the clearance of pathogens. Probiotic strains might boost host immune status through activation of particular and nonspecific immune pathway. This also can involve modulate of humoral, cellular, and nonspecific immunity (Hairul et al. 2014, Delcenserie et al. 2008).

Several cytokines generated by lactic acid bacteria serve critical roles in immunological modulation, and they are involved in colitis pathophysiology. These proinflammatory cytokines are important in building an immunogenic setting and in the anti-inflammatory response (Kim et al. 2015; Mantovani et al. 1992). Earlier study revealed *Lactobacillus* and prebiotic (tagatose) have inhibited DSS induced proinflammatory cytokines and prevented
initiation of the inflammatory response (Son et al. 2019). In this study, the levels of cytokines (TNF-α, INF-γ, IL-1β and TGF-β) increased in DSS-treated mice. The levels of all cytokines studied significantly decreased in Lactobacillus, lignin, and synergetic groups. STAT3 is involved in a number of autoimmune diseases, including inflammatory bowel disease (IBD) (Sugimoto 2008).

In both Ulcerative colitis (UC) and Crohn's disease (CD), there was an increase in total STAT3 protein relative to non-inflammatory control cells, and total STAT3 corresponded with greater activated pSTAT3 in tissue sections from both UC and CD (Mudter et al. 2005). Lower expression of E-cadherin on gut epithelial and immune cells resulted in more gut inflammation in DSS-induced colitis (Ooi et al. 2013). In this study the mRNA of STAT 3 was not significantly changed by Lignin associated with L.plantarum treatment, where the protein expression was significantly down regulated. In the E.cad mRNA and protein expression in the lignin with or without L.plantarum were increased potentially which was compared to Lignin alone treated group. CD44 has been identified as a potential therapeutic target for inflammatory disorders such as IBD, rheumatoid arthritis, multiple sclerosis, and other autoimmune diseases (Wittig et al. 2002). The expression of CD44, was stronger in the UC-associated lesions of colon carcinoma (Mikami et al. 2000). CD44 was identified as a direct target of miR-199a and contributed in significant role in carcinogenesis (Henry et al. 2010; Gao et al. 2015). In this study revealed that the E.cad expression increased in the epithelial cells. The lignin with L.plantarum group controls the inflammatory progression in the colitis mice through miR-199.

**Conclusion**

In conclusion, we used a DSS-induced colitis mouse model to evaluate the synergic effects of Lignin and L.plantarum against IBD. Our findings suggest that combining Lignin with L.plantarum improves intestinal damage recovery and suppresses recurrence of experimental colitis, indicating that this new protective or preventative strategy for IBD could be used in the food sector.

**Declarations**

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**Author Contribution Statement**

Venugopal Kaliyamoorthy designed, conducted the experiments, analysed and wrote the manuscript. Sivakumar Kandhasamy and Hairul Islam Mohamed Ibrahim Conceptulization, design the work, data analysis and finalized the draft. Justin Packia Jacop and Thirugnanasambantham Krishnaraj oversaw the research and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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**Competing Interests**

The authors have no relevant financial or nonfinancial interests to disclose.

**Ethical approval**

All experimental procedures involving mice were performed in accordance with the CPCSEA guidelines

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**Figures**

**Figure 1**

Microbial toxicity of Lignin against *L. plantarum*.

Values are represented as mean ± SD (n=3)

Different superscripts on column showed significant different (*P* ≤ 0.05).
Figure 2

Disease Index assessment of Lignin against DSS induced colitis mice model.

A) Disease activity index was estimated by the consolidated value of food uptake water uptake, rhythmic movement and fecal load.

B) The colon length of DSS induced colitis mice was estimated by centimeter index.

C) The body weight of mice was calculated in grams.

Values are represented as mean ± SD (n=3)
Different superscripts on column showed significant different ($P \leq 0.05$).

**Figure 3**

Light micrograph of HE-stained colonic section of DSS induced mice groups. Scale bar represents 100µm. Microscopic scoring of DSS induced colon of five groups. (A - E) indicates that microscopic image of colon with and without DSS induced groups.

F) The scoring of histological damage - 0: intact tissue construction with no apparent damage; 1: damage limited to surface epithelium; 2: localized ulcer confined to mucosa; 3: focal, transmural inflammation, and ulceration; 4: extensive transmural ulceration and inflammation adjacent to normal mucosa; and 5: extensive transmural ulceration and inflammation involving the entire section.

Values are represented as mean ± SD (n=3)

Different superscripts on column showed significant different ($P \leq 0.05$).

**Figure 4**

Cytokines were estimated in *L. plantarum* with lignin from DSS induced colitis mice. A) TNF-α B) IFN-γ C) IL1-β and D) TGF β

Values are represented as mean ± SD (n=3)

Different superscripts on column showed significant different ($P \leq 0.05$).

**Figure 5**

Lignin suppresses the STAT 3 and induced the E.cad mRNA and protein in DSS induced colitis mice.

A) mRNA level of STAT 3
B) Protein level of STAT 3
C) mRNA level of E.cad
D) Protein level of E.cad
Values are represented as mean ± SD (n=3)

Different superscripts on column showed significant different ($P \leq 0.05$).

**Figure 6**

CD44 expression and estimation of miR 199a in DSS induced colitis and *L.plantarum* with/without Lignin treated mice.

Values are represented as mean ± SD (n=3)

Different superscripts on column showed significant different ($P \leq 0.05$).