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ORIGINAL ARTICLE

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Akkermansia muciniphila improves chronic colitis-induced enteric neuroinflammation in mice

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Abstract

Background: Inflammatory bowel diseases (IBD) are chronic diseases that are not fully understood. Drugs in use can only be applied for a short time due to their side effects. Therefore, research is needed to develop new treatment approaches. In addition, it has been proven that IBD causes degeneration in the enteric nervous system (ENS). In recent years, it has been discussed that probiotics may have positive effects in the prevention and treatment of inflammatory enteric degeneration. *Akkermansia muciniphila* (*A. muciniphila*) is an anaerobic bacterium found in the mucin layer of the intestinal microbiota. It has been found that the population of *A. muciniphila* decreases in the case of different diseases. In light of this information, the curative effect of *A. muciniphila* application on colitis-induced inflammation and enteric degeneration was investigated.

Methods: In this study, 5 weeks of *A. muciniphila* treatment in Trinitro-benzene-sulfonic acid (TNBS)–induced chronic colitis model was investigated. Colon samples were examined at microscopic, biochemical, and molecular levels. Fecal samples were collected before, during, and after treatment to evaluate the population changes in the microbiota. Specific proteins secreted from the ENS were evaluated, and enteric degeneration was examined.

Results: As a result of the research, the ameliorative effects of *A. muciniphila* were shown in the TNBS colitis model-induced inflammation and ENS damage.

Discussion: In light of these results, *A. muciniphila* can potentially be evaluated as a microbiome-based treatment for IBD with further clinical and experimental studies.

KEYWORDS
Akkermansia muciniphila, chronic diseases, enteric nervous system, gut health, inflammatory bowel diseases

1 | INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract.[1] IBDs were traditionally divided into Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are differentiated by their clinical manifestations and hypothesized pathogenic mechanisms. UC is a relapsing nontransmural chronic inflammatory disease that is restricted to the colon and during disease flares characterized by bloody diarrhea and stomach pain. CD is a chronic, segmental localized granulomatous disease that can affect anywhere in the entire gastrointestinal tract from the mouth to the anus. UC and CD can appear at any age, but most patients are diagnosed in their third decade of life. The prevalence of UC in Northern Europe varies from 35 to 50 and CD from 30 to 100 per 100,000 inhabitants.[2]

The etiology of IBDs is still unknown, though studies refer to several possible causes such as genetics, immunology, nutrition, bacteria, viruses, and other environmental factors. Various drug treatments for inflammatory bowel diseases have been in use for a long time. However, the effectiveness of these drugs is limited to reducing inflammation and related complications.[3] Regular use of these drugs causes side effects such as gastric ulcers, Cushing's syndrome, hyperglycemia, muscle weakness, infection, delayed wound healing, cataract, and lymphoma. It was also shown that used drugs increase the morbidity rate with a decreased quality of life. In recent years, antitumor necrosis factor-alpha (TNF-α), anti-γ-4 integrin, and peroxisome proliferator-activated receptor-γ ligands have been used as a pharmacological approach.[4] However, it has been suggested that commonly used anti-TNF drugs such as infliximab, Adalimumab, and certolizumab may increase the risk of infection and malignancy and may cause non-Hodgkin lymphoma.[5] Heart failure and acute coronary syndrome conditions and acute infection attacks were also reported among the potential side effects of these drugs. Published reports show that 40% of IBD patients choose alternative treatment methods.[6] In recent years, the inspiring results of microbiota-based treatments on IBD led to a higher interest in using specific bacterial strains in therapeutic approaches.[7,8]

A. muciniphila is one of the most abundant bacteria in the gut microbiota (approximately 0.5–5% of the population). It is a gram-negative strict anaerobic bacterium and a member of the Verrucomicrobia family. It degrades mucin and resides in the mucus layer.[7] In recent years, *A. muciniphila* has attracted attention due to its positive effect on human and animal health. In various studies, it has been shown that probiotic treatment with *A. muciniphila* decreased the rate of weight gain after diet-induced obese mice. In addition, the population of *A. muciniphila* increased approximately 100 times in animals that obtained the prebiotic compared to the normal diet.[8] Furthermore, it has been shown that there is a significantly decreased number of *A. muciniphila* in pathological conditions such as obesity and type 2 diabetes, IBD, hypertension, and liver diseases.[9] In 2018, it was shown that metabolites obtained from *A. muciniphila* affect mucosal barrier permeability by regulating tight junction proteins, thus having a protective effect on inflammation.[10] In a striking study published in 2013, it was shown that there was a significant drop in the extracellular vesicles (AMeV) of *A. muciniphila* in fecal samples collected from a dextran sodium sulfate (DSS)-induced ulcerative colitis model in mice, and administration of AMeV was shown to have a protective impact on colitis.[11]

Besides local signs of inflammation in the mucous layer, the intrinsic nervous system of the gut, the so-called Enteric Nervous System (ENS) is also impacted by IBD. The ENS is a collection of plexuses located in the gastrointestinal tract and forming the local control center of the digestive tract.[12] This complex structure of nerve cells intrinsic to the gut is organized into[1] the myenteric (or Auerbach's) plexus, which is specifically concerned with regulating the motility of the gut; and[2] the submucus (or Meissner's) plexus, which is located, just under the mucus membranes of the gut and is concerned with chemical and glandular secretion.[13] Studies on laboratory animal models and human patients show hypertrophy, hyperplasia and axonal damage in nerve fibers, hyperplasia and numerical increase in glial cells, and structural morphology changes in IBD.[14] In a study published in 2005, it was shown that abnormalities in enteric neurons due to inflammation cause deterioration in the intestinal structure.[15] Disruption in the neural control of epithelial secretion increased the excitability of enteric neurons, so changes in synaptic transmission were indicated as the main neural changes in the same study. However, the relationship between ENS and colitis and how this disruption can be treated is far from being completely understood.

Enteric glial cells constitute one of the prominent cellular structures in ENS and have a significant influence on the integrity and health of the mucosal structure.[14] Phenotypically, they resemble

Key points

- Microbiome-based approaches have a high potential for IBD treatment with fewer secondary health problems. *Akkermansia muciniphila* could have a beneficial impact on chronic colitis-induced enteric inflammation and degeneration.
- Our current research showed that *Akkermansia muciniphila* treatment led to a decrease in inflammatory markers as well as parameters of inflamed enteric neurons and glial cells in the gut.
- It is the first research to show a positive effect of a single bacterium on chronic colitis-induced enteric nervous system degeneration and inflammation.
- Further research on *Akkermansia muciniphila* may result in improvements in the management of IBD. It suggests a novel microbiome-based strategy for treating intestinal inflammation brought on by chronic colitis and advancing clinical practice.

astrocytes found in the central nervous system. Mature glial cells are rich in intermediate filament protein (GFAP), and its expression has been shown to increase significantly during inflammation such as IBD.[16] Various studies have shown that glial cells have an essential role in the control of homeostasis and intestinal barrier function.[17] A significant decrease in mucosal inflammation and GFAP expression was shown in the colitis-induced mice model after the antiprotozoal drug administration in 2012.[18] It was found that the anti-inflammatory effect of this drug is targeting glial cells' inflammation and supporting the recovery period.

A study in 2014 on germ-free mice showed that the gut microbiota is essential for the development of ENS in the early postnatal period. The animals growing in a sterile environment and genetically lacking microbiota have been found to have impaired neural crest and reduced cell line count.[19] These germ-free mice have had spontaneous muscle contractions of the jejunum and ileum and decreased sensory neuron excitability. Moreover, it was shown that ENS regulates intestinal microbiota composition and that the loss of ENS cells can cause dysbiosis in the microbiota composition.[20]

In the light of these studies, we hypothesized that *A. muciniphila* might have a therapeutic and anti-inflammatory effect on TNBS-induced chronic colitis and colitis-induced ENS inflammation, which was investigated in the following study.

2 | MATERIALS AND METHODS

2.1 | Animals

Male and female BALB/c mice (8–12 weeks old, $n = 12$/group) were used and kept in constant humidity and temperature conditions in a 12-h light/dark cycle. Mice were fed with a standard pellet and tap water ad libitum. The animals were weighed and followed up daily. All experiments were approved by the Local Ethics Committee of Acıbadem Mehmet Ali Aydınlar University (ACU-HADYEK 2019/28). A total of 36 mice were used by calculating the α error value of 0.5 and the force as 0.85 on power analysis.

2.2 | Induction of Chronic Colitis

Animals were randomly divided into the control group, the colitis group, and *A. muciniphila*-treated colitis group. Chronic colitis was induced as previously described.[21] After overnight fasting, Chronic TNBS colitis was induced by weekly administration of increasing doses of TNBS in 45–50% of ethanol for 8 weeks of the induction period. Mice were lightly anesthetized with isoflurane and then administered TNBS/ethanol per rectum via a 3.5 F catheter equipped with a 1-ml syringe; the catheter was advanced into the rectum until the tip was 4 cm proximal to the anal verge, at which time the TNBS was administered in a total volume of 150 μL. To ensure the distribution of TNBS within the entire colon and cecum, mice were held in a vertical position for 30 s after the intrarectal injection. All the tissue samples were collected from the TNBS-treated 4 cm region of distal colon.

The control group was injected intrarectally with saline instead of TNBS. From the 3rd week of induction, 0.2×10^8 CFU mL^{-1} of *A. muciniphila* suspended in PBS was given daily via oral gavage until the last day of the experiment. Control and colitis groups were treated with phosphate-buffered solution (PBS) instead of the bacterial suspension. Daily food and fluid consumption, diarrhea status, and weekly weight changes were monitored. At the end of the 7th week, the animals were sacrificed by the exsanguination method, and colon tissue, blood, and fecal samples were collected.

2.3 | Bacterial Culture and Treatment

A. muciniphila Derrien et al. (ATCC® BAA-835™) bacterial origin was obtained from the American Type Culture Collection (ATCC). Lyophilized bacteria were opened in an anaerobic biosafety cabinet and reconstituted in thioglycolate broth (Merck-Millipore). It was then inoculated into a BD BACTEC Plus Anerobic/F culture flask and Columbia medium with 5% sheep blood (Becton, Dickinson and Company, USA). The blood culture bottle was placed in the BD BACTEC™ blood culture system, and the growth chart was followed with the signal of the device.

The sample inoculated in Columbia medium with 5% sheep blood (Becton, Dickinson and Company, USA) was placed in the BD GasPak™ EZ pouch system and incubated in a 37°C, CO_2 incubator (Becton, Dickinson and Company, USA) until colonies were observed. Confirmation of bacterial growth was made by obtaining a high score on the MALDI-TOFF device.

The bacterial suspension was prepared using the 0.5 Mcf standard and then diluted with sterile anaerobic phosphate buffer saline solution to 0.2×10^8 CFU mL^{-1}. It was prepared in 2 mL screw-capped tubes for each day at this treatment dose according to the literature and stored at −80°C.

2.4 | Macroscopic evaluation

2.4.1 | Colonoscopy imaging

A Hopkins telescope (Karl Storz Endoscopy, Germany) was used to investigate the macroscopical changes of the mucous layer every 2 weeks at 4 cm proximal to the anal verge.

Hyperemia, bleeding, and ulceration were evaluated according to the Wirtz scoring system.[22]

2.4.2 | Disease index and fecal scoring

The disease index was calculated by scoring the stool consistency, presence of blood, and intestinal adhesion in mice at the 3rd, 5th, and 7th weeks. Fecal consistency was evaluated prior to TNBS

TABLE 1 (A) Disease index scoring criteria. (B) Microscopic damage scoring criteria. (C) Antibodies used for WB and IHC.

(A) Disease index scores			
Score	**Stool consistency**	**Presence of bleeding**	**Intestinal adhesion**
0	Normal	Not observed	Normal
1	Soft-in shape	Minimal	
2	Soft	Average	Average
3	Extremely soft-wet	Visible in stool	
4	Diarrhea	Rectal Bleeding	High

(B) Evaluation criteria of microscopic damage	
Evaluation criteria	**Score**
No leukocyte infiltration	0
Low level of leukocyte infiltration	1
Moderate level of leukocyte infiltration	2
High level of leukocytic infiltration, high vascular density, and thickening of the colon wall	3
Transmural leukocyte infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall.	4

(C) Primary and secondary antibodies for WB and IHC	
Antibodies	
Monoclonal anti-mouse Tubulin III	Sigma T8578
Poly anti-rabbit GFAP	Abcam Ab7260
Poly anti-rabbit NFκB	Santa Cruz sc8008
Anti-mouse Alexa Flour 488	Invitrogen A32723
Anti-rabbit Alexa Flour 555	Invitrogen A32732

administration on the same day of each week. The evaluation scoreboard is shown below (Table 1A).

2.5 | Measurement of cytokines and permeability

Colon samples were collected for 4 cm from the distal colon at the end of the experiment for assessment of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and anti-inflammatory cytokine IL-10. Colon tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. To analyze LPS changes blood samples from the maxillomandibular artery were collected at different time points. The serum samples were stored at −80°C. On the measurement day, tissue and serum samples were quantified according to the manufacturer's instructions and guidelines using the enzyme-linked immunosorbent assay (ELISA) method. All samples were assayed in triplicates and the values were expressed as picograms per milliliter.

2.6 | Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity is frequently used as a marker to estimate neutrophil accumulation in inflamed tissues. MPO activity was assessed by measuring the color change during the H_2O_2-dependent oxidation of o-dianizidine.2HCl. Briefly, colon samples were homogenized in potassium phosphate buffer (K_2HPO_4) and hexadecyltrimethylammonium bromide (HETAB). They were centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatants were removed. Then, pellets were homogenized with a solution of K_2HPO_4, HETAB, and Ethylenediaminetetraacetic acid (EDTA) for 15 s. Then this mixture was reacted with the o-dianisidine and H_2O_2. The reaction is stopped with sodium azide and measured spectrophotometrically according to the reaction-dependent color change. One unit of enzyme activity is the amount of MPO that causes a change in absorbance of 1.0 min^{-1} at 460 nm and 37°C and is expressed in units per g of tissue.

2.7 | Microbiota analyses

Genomic DNA (gDNA) isolation was performed using ZYMO BIOMICS DNA Miniprep Kit according to manufacturer's instructions using the frozen fecal samples. The resulting gDNA was used as a template for the preparation of amplicon sequencing libraries using the Oxford Nanopore 16S024 Kit 16S PCR kit using the kit primers and using the solutions provided by Oxford Nanopore Technologies (ONT) FLO-MIN106D. 16S raw reads were obtained as fast5 files. Initial bioinformatics analyses were performed using ONT guppy version 5.0.11., bbtools 38.91, magicblast 1.6.0, and samtools 1.13 to create the consensus sequences. For statistical analysis, R Statistical Computer Language version 4.0.4 and Rstudio IDE 1.4 were used, with the packages tidyverse, readr, xlsx, and ggplot2. Bonferoni verification method

was applied after a statistical evaluation. The comparisons took in consideration the time frame difference and the differences in groups.

2.8 | Evaluation of microscopic damage score

Colon samples were fixed in 10% formalin and dehydrated with a graded series of ethanol (70%, 90%, 96%, and 100%), cleared with xylene, and incubated in paraffin using an automated tissue processor (Citadel 2000, Thermo Scientific, Germany). Tissues were then embedded and blocked-in paraffin at room temperature using an embedding workstation (Histostar, Thermo Scientific, Germany). About 5-μm-thick paraffin sections were cut and stained with hematoxylin and eosin (H&E). To evaluate microscopic damage score; H&E-stained colon tissue sections were scored semi-quantitatively using a scale ranging from 0 to 4 for each criterion (Table 1B).[23]

2.9 | Ultrastructural examination of myenteric plexus

The colon samples were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) (0.1 M, pH 7.2), then postfixed in 1% osmium tetroxide in PBS (0.1 M, pH 7.2), dehydrated in increasing concentrations of ethyl alcohol series, and embedded in Epon 812 (Fluka, Sigma–Aldrich Chemical, Steinheim, Switzerland). Semi-thin sections were stained with toluidine blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate and evaluated under a transmission electron microscope (Thermo Scientific- Talos L120C).

2.10 | Assessment of epithelial cell morphology

Tissue samples were fixed in 2.5% glutaraldehyde fixative with 0.1 M PBS buffer (pH 7.2). After washing with PBS buffer, tissue samples were postfixed with 1% osmium tetroxide for 1 h. These samples were dehydrated by an ascending series of alcohols (70%, 90%, 96%, and 100%). After dehydration, tissue samples were applied to 3/1, 1/1, and 3/3 alcohol/amyl acetate series, incubated in pure amyl acetate, and then dried in a critical point dryer. Samples were coated with gold and examined under a scanning electron microscope (Thermo Scientific-Quattro SEM).

2.11 | Immunofluorescence analyses of Tubulin III and GFAP in myenteric plexus

Myenteric plexus in colon tissue samples were analyzed by immunohistochemistry using Tubulin III and GFAP antibodies. Tissues were fixed with 4% PFA for 72 hours. After fixation, these samples were dehydrated with an ascending series of ethyl alcohol (70%, 90%, 96%, and 100%), cleared with xylene, and embedded in paraffin. The sections were kept at room temperature in 10% goat and 10% fetal calf serum prepared in phosphate buffer (PBS). Then, the sections were incubated overnight with anti-mouse Tubulin III (Sigma T8578) (1:500) and anti-rabbit GFAP (Abcam Ab7260) (1:200) antibodies prepared in blocking buffer and washed with PBS (3×10 min). The sections were then incubated with anti-rabbit secondary antibody conjugated with Alexa Flour 555 (1:1000) for 1 h at room temperature and then washed with PBS. Then, the anti-mouse secondary antibody conjugated with Alexa Flour 488 (1:400) was applied for 1 h. The sections were incubated with 4′-6-diamidino-2-phenylindole (DAPI) at room temperature in the dark and analyzed with a confocal microscope (Zeiss LSM 700). Intensity in the sections was evaluated using the ImageJ program (1.44 software, National Institutes of Health).

2.12 | Protein extraction and western blot analysis

Colon samples were homogenized in 1 mL of RIPA buffer lysis buffer to extract total protein. The total protein was subjected to electrophoresis on a 10%–12% SDS gel and then transferred to a PVDF membrane. The expression levels of anti-mouse-Tubulin III (Sigma T8578), anti-rabbit GFAP (Abcam Ab7260), anti-rabbit p65 subunit of NFκB (Santa Cruz sc8008), and anti-mouse Beta-actin (In vitrogen MA1-140) were evaluated using corresponding antibodies. Primary and secondary antibodies are listed in Table 1C. After the incubation period for primary and secondary antibodies, membranes were evaluated in the darkroom using ChemiDoc (MP Biosystems, Biorad). Beta-actin was used as housekeeping protein and all the bands were normalized housekeeping protein at statistical analysis.

2.13 | Statistical analysis

Data were expressed as mean ± standard error of the mean. Histological and other parameters were analyzed using one-way or two-way analysis of variance. Post hoc testing was completed with Tukey's multiple comparisons test, and significance of differences was taken at the level of $p < 0.05$. Calculations were performed using instant statistical analysis package (Prism 6.0 GraphPad Software, San Diego, CA, USA).

3 | RESULTS

Chronic TNBS-induced colitis was evaluated via disease score index periodically in the 3rd, 5th, and 7th week. It was found that TNBS application caused a significant increase in the disease index in 5th and 7th weeks ($p < 0.001$ and $p < 0.01$). Moreover, there was a significant decrease in the disease index with treatment compared to the colitis group ($p < 0.01$ and $p < 0.05$) (Figure 1A).

Colonoscopy was performed on the mice periodically for macroscopic evaluation among the experimental groups (Figure 1B).

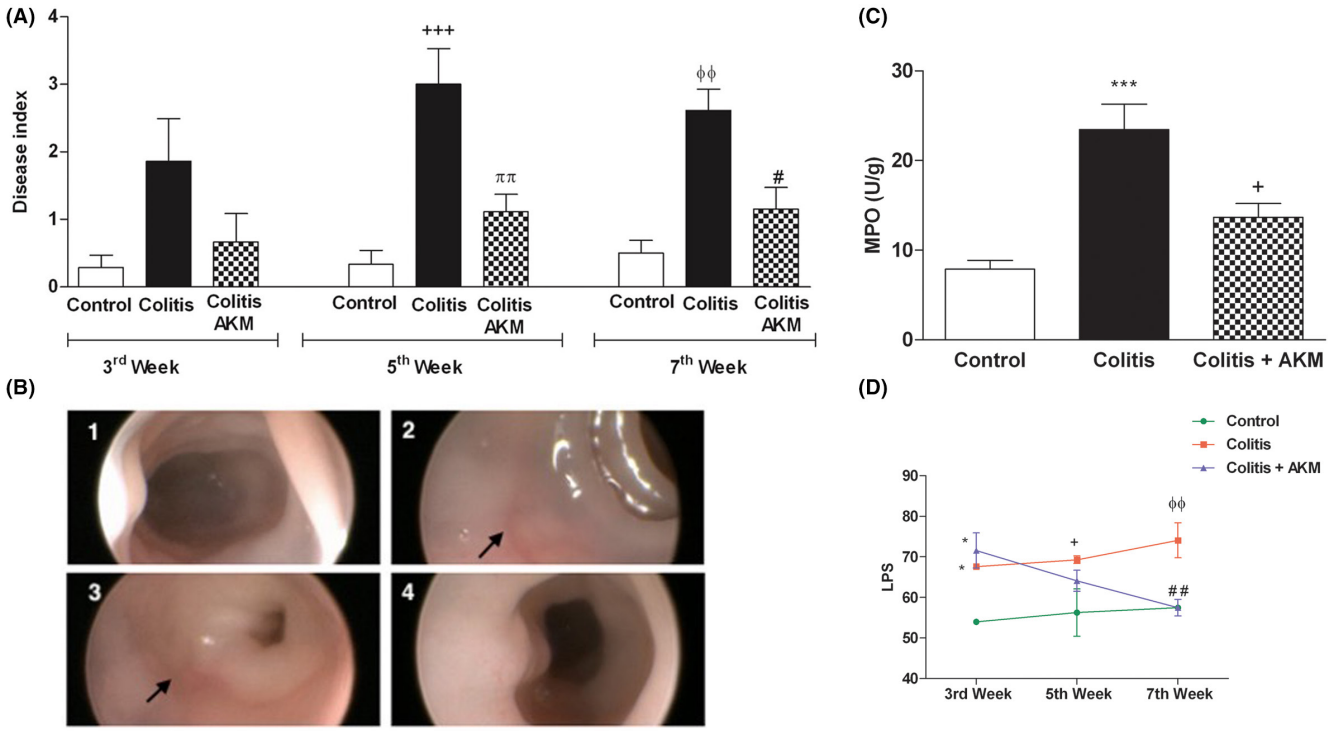

FIGURE 1 Experimental results. (A) Disease Index Scores from 3rd, 5th, and 7th weeks within groups (Control n:7, TNBS, n:7, *A. muciniphila* n:6). (B) Colonoscopy images from 5th week between groups. (B.1) Control Group. (B.2–B.3) Colitis Groups. (B.4) Treatment Group. Black arrow: hyperlipidemia and mild ulcerations. C: Neutrophil infiltration results from dissected colon tissue samples. (D) Permeability assessment within groups from submandibular vein blood samples (Control n:4, TNBS, n:6, *A. muciniphila* n:6). $^{+}p<0.05$ compared with the colitis group. $^{***}p<0.001$ compared with the control group. $^{+++}p<0.001$ compared with the control group within 5th week. $^{\pi\pi}p<0.01$ compared with the colitis group within 5th week. $^{\#}p<0.05$ compared with the 7th week colitis group. $^{\varphi\varphi}p<0.01$ vs 7th week control group.

Although the healthy colon structure of the control group was observed in Figure 1B.1, hyperemia and mild ulceration started to be observed in the colon starting from the 5th week (Figure 1B.2–B.3). While mild hyperemia was observed in the treatment group, ulceration was not observed (Figure 1B.4).

These results showed significant macroscopic damage in the TNBS colitis group. *A. muciniphila* treatment demonstrated its positive effect from the 5th week and continued at the 7th week.

3.1 | Myeloperoxidase activity

MPO activity was evaluated as an indicator of neutrophil infiltration (Figure 1C). While MPO activity increased significantly in the colitis group ($p<0.001$), a significant decrease was observed with treatment compared with the colitis group ($p<0.05$). This result showed that *A. muciniphila* had a significant impact on elevated neutrophil infiltration and supported treatment as a potential approach.

3.2 | Assessment of permeability

To assess intestinal integrity and permeability, lipopolysaccharide (LPS) assessment was performed with an ELISA kit (Figure 1D). Analyzes were performed with serum samples collected periodically at 3rd, 5th, and 7th week. In the 3rd week, the amount of LPS in the colitis and treatment group was higher than in the control group ($p<0.05$ and $p<0.01$). With the increased period of treatment, LPS decreased from 5th week and showed a significant change in the 7th week compared to the colitis group ($p<0.05$). In the 7th week, a significant increase was observed in the colitis group compared with the control group and *A. muciniphila* group ($p<0.01$). Gut permeability changes showed that *A. muciniphila* reversed endotoxemia back to control group levels in the 7th week.

3.3 | Western blot analysis of Tubulin III, GFAP, p65 subunit of NFκB

The expression of glial marker, GFAP was significantly increased in the TNBS group ($p<0.001$). Furthermore, *A. muciniphila* treatment significantly decreased the GFAP expression compared to the colitis group ($p<0.001$). NFκB p65 subunit was evaluated to investigate the molecular pathway of inflammation. There was a significant increase of NFκB p65 in the TNBS-colitis group compared with the control group ($p<0.001$). While *A. muciniphila* treatment led to a decrease in NFκB p65, this decrease did not

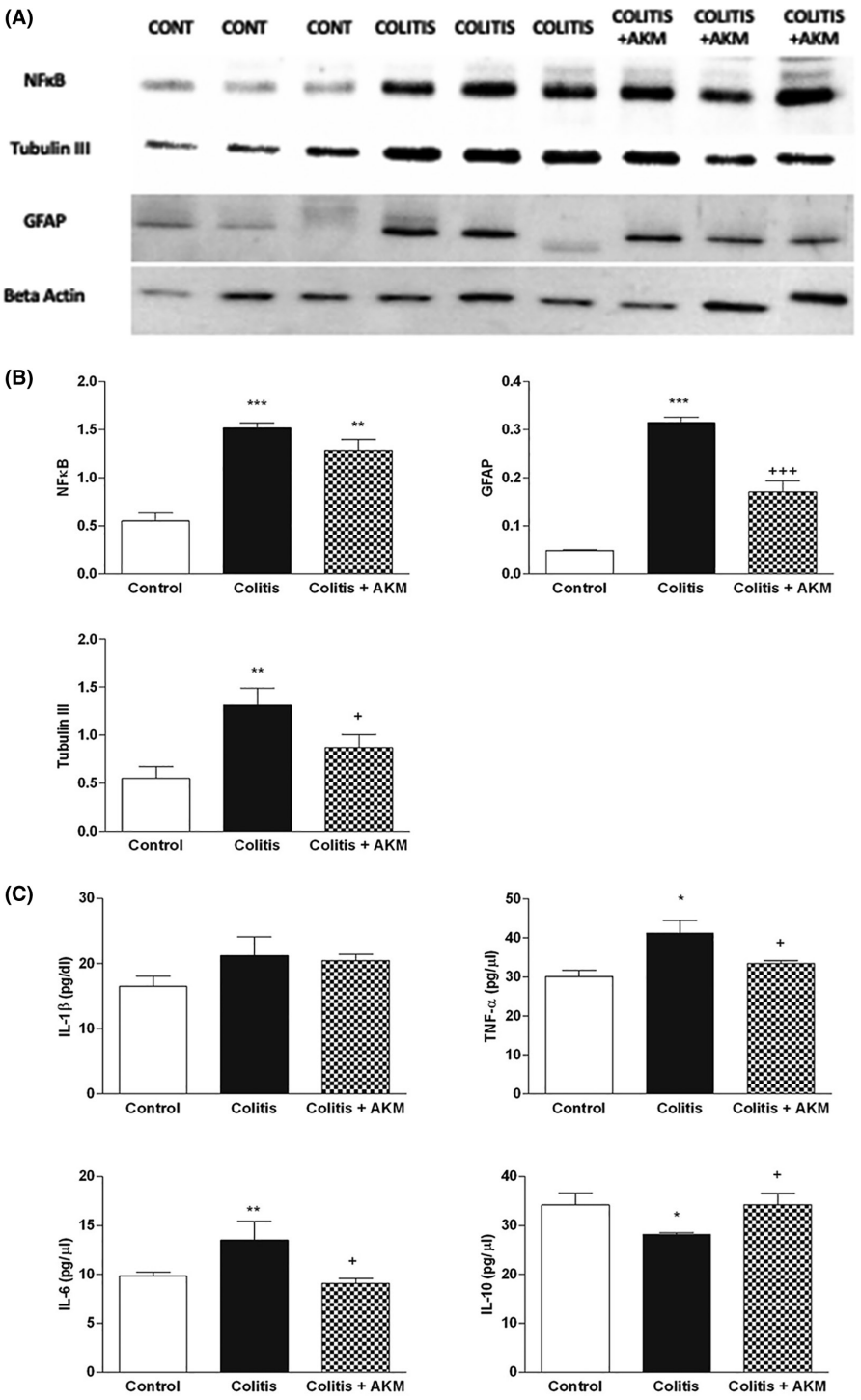

FIGURE 2 Western blot and ELISA results. (A) Bands of western blot for NFκB, Tubulin III, GFAP, and beta-actin within triplet groups. The bands represent control, colitis, and *A. muciniphila* groups, respectively. The last band of beta-actin image was spliced from previous findings. The gel bands for GFAP and beta-actin were displayed on a different background to highlight the weak bands. (B) Statistical evaluation of western blot results normalized with housekeeping protein. (control n:4, TNBS, n:5, *A. muciniphila* n:5). (C) Cytokine results via ELISA. $^{+}p<0.05$ compared with the colitis group. $^{*}p<0.05$ compared with the control group. $^{**}p<0.01$ compared with the colitis group. $^{***}p<0.001$ compared with the control group. $+++p<0.001$ compared with the colitis group. GFAP: Abcam Ab7260. Tubulin III: Sigma T8578. NFκB: Santa Cruz sc8008. (IL-6: Control n:10, TNBS, n:11, *A. muciniphila* n:12; Il-10: Control n:5, TNBS, n:7, *A. muciniphila* n:7; TNF-α: Control n:8, TNBS, n:11, *A. muciniphila* n:12; Il-1β: Control n:9, TNBS, n:12, *A. muciniphila* n:12).

reach significance($p>0.05$). In addition, the expression of neuronal marker, Tubulin III, was significantly increased in the TNBS group ($p<0.01$) while a significant decrease was observed with *A. muciniphila* treatment ($p<0.05$). All the bands and analyses are illustrated in Figure 2A,B. Enteric neurons and glial cells were significantly damaged by TNBS induction. Glial damage marker, GFAP, was significantly dropped with the treatment compared to the colitis group. It demonstrated a significant effect of *A. muciniphila* treatment on gliosis. Additionally, Tubulin III and NFκB p65 were significantly effected supporting the potential anti-inflammatory impact of *A. muciniphila* treatment.

3.4 | Measurement of TNF-α, IL-1β, IL-6, and IL-10 Levels

Pro-inflammatory cytokines TNF-α and IL-6 were significantly increased in the colitis model ($p<0.05$ and $p<0.01$) and significantly

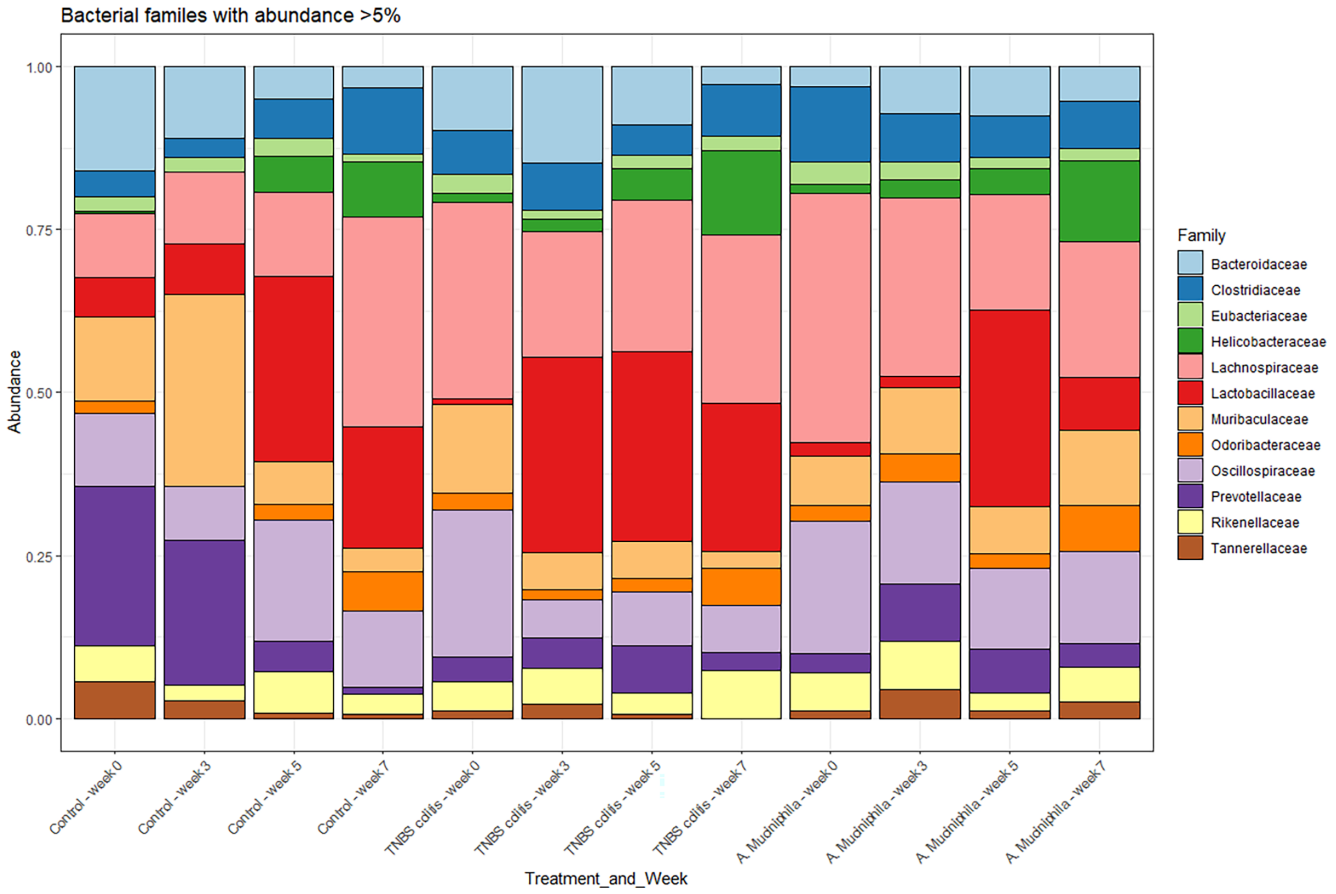

FIGURE 3 16srRNA sequencing microbiome results. Families with at least 5% abundance are represented from pooled fecal samples from different time points.

decreased after treatment ($p < 0.05$). There were no significant differences between the groups in IL-1β results. The anti-inflammatory cytokine IL-10 decreased significantly with colitis ($p < 0.05$) and increased after the treatment ($p < 0.05$) (Figure 2C). These findings showed that pro-inflammatory and anti-inflammatory markers were significantly affected by *A. muciniphila* treatment. The concentration of cytokines had no difference between control and treatment groups which could support the recovery.

3.5 | Microbiome results

The results of the analysis are given in Figure 3. Since *A. muciniphila* was started to be given in the 3rd week, the biggest difference was in the 5th and 7th weeks. Figure 3 shows the overall microbial composition of the most abundant taxa. In addition, Helicobacter pylori was observed on the last week of the study compared with the first week. In addition, the amount of Helicobacter in mice exposed to TNBS was approximately 90% higher, and more than 100-fold in mice fed *A. muciniphila*. We observed in week 7, that there was no significant difference anymore between the *A. muciniphila* and TNBS groups in terms of microbial diversity while only the control group decreased. In the last week of the study, there was no significant difference between the *A. muciniphila* and TNBS groups at the investigated species level.

3.6 | Microscopic damage scores

The histopathologic scores of the colonic tissue samples were statistically evaluated. The histopathologic score was higher in the colitis group ($p < 0.01$) than in the control group. The score was significantly lower in the *A. muciniphila* group ($p < 0.05$) compared with the colitis group (Figure 4E). In the control group, histological organization of the colon was observed in a normal morphology, while in the colitis group detachment and hemorrhage of the colon epithelium, degeneration of the glandular structure, decrease in the number of goblet cells, and vascular congestion were observed. In the *A. muciniphila* group, a slight detachment of the surface epithelium was observed, while the glandular structure showed regular morphology (Figure 4A).

When the structure of the myenteric plexus was analyzed, there was a normal morphology to be found, while in the colitis group, neurons and enlarged axon structures with vacuolization in the structure of the myenteric plexus and partial degenerative findings were observed. In the *A. muciniphila* group, normal histologic organization with minimal expansion of axon structures in the myenteric plexus was observed (Figure 4B).

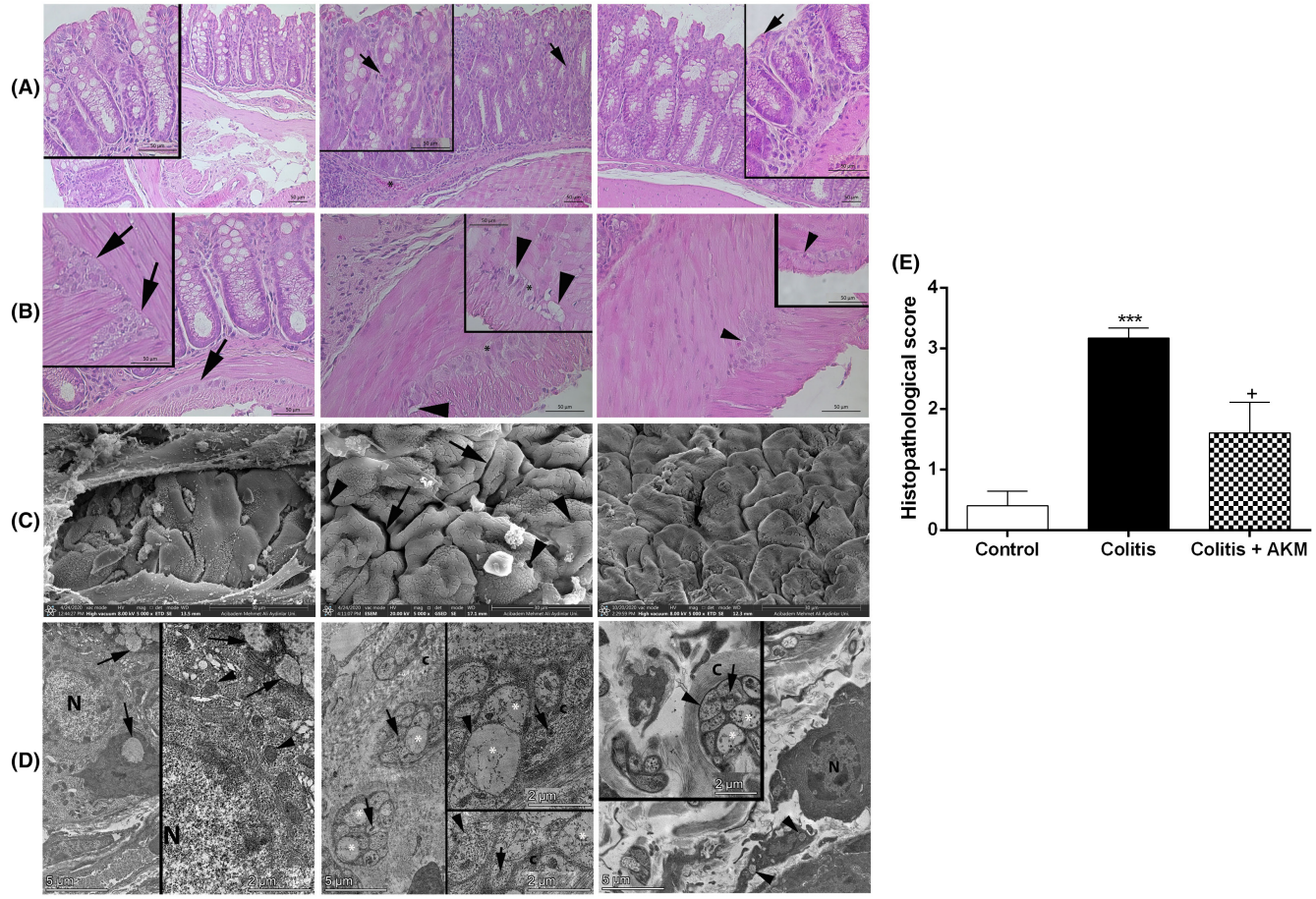

FIGURE 4 Microscopic evaluation results. (A) Representative light micrographs of experimental groups. Degenerated mucosal morphology with disrupted glands, neutrophil infiltration (arrow), and decreased goblet cells were observed in the colitis group. Normal mucosal morphology with some degenerated surface epithelial disruption and neutrophil infiltration and an increased number of goblet cells were observed in the *A. muciniphila* group. (B) Myenteric plexus between circular and longitudinal smooth muscle bundles. Enlarged axons (arrowhead) in the colitis group with vacuolization (asterisk) in the myenteric plexus were observed in the colitis group. Regular histological organization with minimal degeneration (arrowhead) of axon structures in the myenteric plexus in the *A. muciniphila* group. (Paraffin section, H&E staining). (C) Representative scanning electron micrographs of experimental groups. Degenerated surface epithelium (arrow) with prominent pit like degeneration on the cell surfaces was observed in the colitis group, whereas relatively minor degeneration in the surface epithelium was observed after treatment. D: Representative transmission electron micrographs of experimental groups. Neuron nuclei (N), axon bundles (arrow), and mitochondria with normal morphology (arrowhead) of the myenteric plexus in the control group (A), mitochondria with distorted morphology (arrow), degenerated axon bundles (asterisk), and irregular collagen structures (c) were observed in the colitis group. Neurons with normal morphology (arrow) and regular collagen morphology (c) and some degenerated axon bundles (asterisks) were observed in the *A. muciniphila* group. E: The histopathological scores of the experimental groups. $^{***}p < 0.001$ compared with the control group; $^{+}p < 0.05$ compared with the colitis group.

Microscopic findings were consistent and supported the hypothesis. *A. muciniphila* had a positive impact on TNBS colitis-induced pathology and myenteric plexus damage.

3.7 | Epithelial cell morphology

The scanning electron microscopic findings also showed the epithelial morphology of the experimental groups. Normal epithelial morphology was observed in the colon tissue of the control group. In the colitis group, degenerated areas were observed between the surface epithelium, whereas in the *A. muciniphila* group, relatively few degenerated areas were observed between the epithelial cells (Figure 4C).

3.8 | Ultrastructural examination of myenteric plexus

Ultrastructural examination of the structure of the myenteric plexus revealed that the structure of the myenteric plexus in the experimental groups has degenerative findings. In the control group, neurons with normal morphology were observed in the myenteric plexus structure, axon terminals, and mitochondria with normal

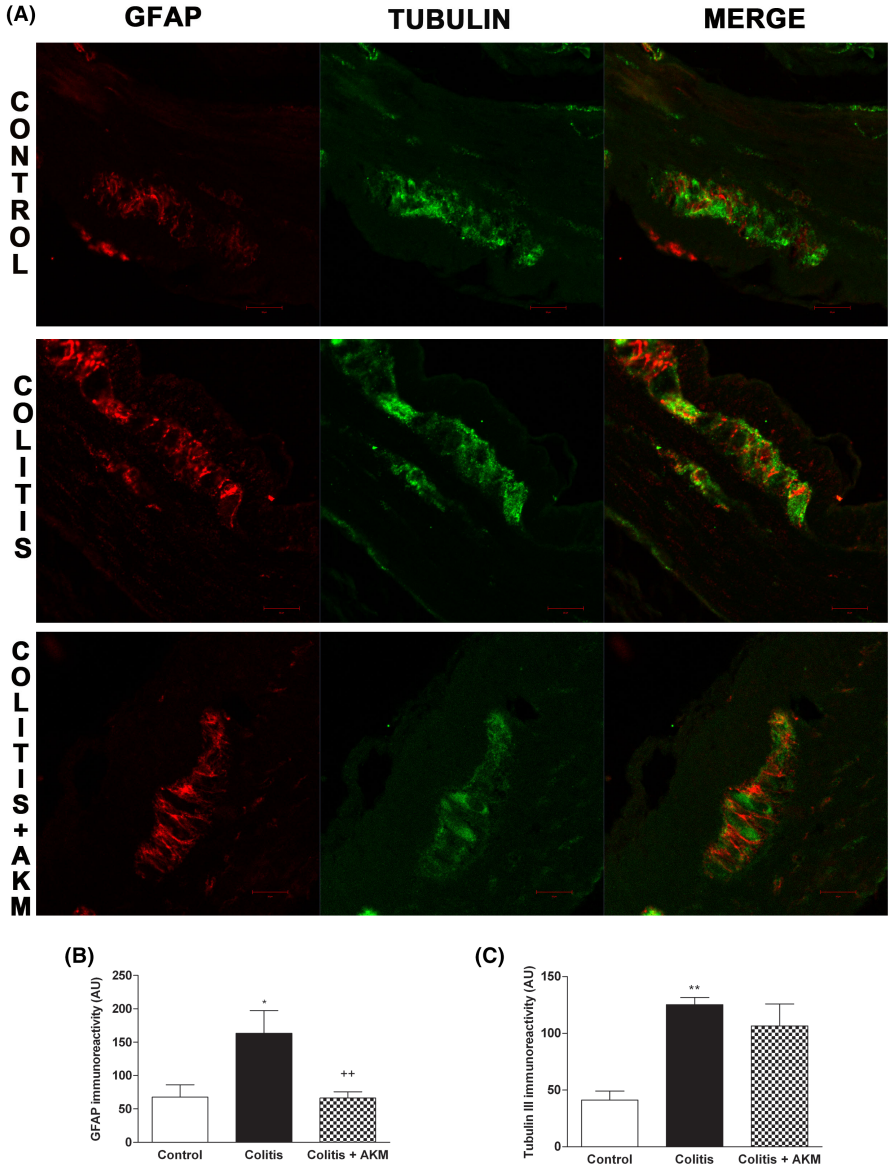

FIGURE 5 Immunofluorescence analyses of tubulin and GFAP in myenteric plexus. (A) Colocalization of GFAP and Tubulin III from colon samples. (B) Statistical evaluation of GFAP immunoreactivity. (C) Statistical evaluation of Tubulin III immunoreactivity. $^{*}p < 0.05$ compared with the control group. $^{**}p < 0.01$ compared with the colitis group. $^{++}p < 0.05$ compared with the colitis group. GFAP: Abcam Ab7260. Tubulin III: Sigma T8578.

morphology. In the colitis group, morphologically distorted mitochondria, degenerated axon bundles, and irregularities in collagen structure in the cytoplasm were observed, while in the *A. muciniphila* group, neurons with normal morphology, collagen structure in regular morphology, and axon terminals, as well as a small number of degenerated axon terminals in some locations were observed (Figure 4D).

3.9 | Immunofluorescence analyses of Tubulin and GFAP in myenteric plexus

Tubulin and GFAP-positive intensity were observed in the myenteric plexus (Figure 5A). The intensity of GFAP and Tubulin III was higher in the colitis group than in the control group. It was observed that both GFAP and Tubulin III intensities were lower in the *A. muciniphila* group than in the colitis group. Statistical evaluation is illustrated in Figure 5B. These results were consistent with WB findings. *A. muciniphila* had a potentially ameliorative impact on TNBS colitis.

4 | DISCUSSION

The study assessed the efficacy of *A. muciniphila* treatment on inflammation and enteric damage caused by chronic colitis. Identifying the inflammatory and protein markers of enteric nervous system damage was the focus. First, to investigate the effectiveness of *A. muciniphila* on inflammatory cytokine levels, MPO activity and the NF-kB signaling pathway-associated proteins were measured. Second, the expression of enteric neurons and glial proteins was measured, and myenteric plexus damage was evaluated. Positive divergences in the protein expression levels of glial cells and enteric neurons were examined in colon tissue samples collected at the end of the experiment. Microscopic examinations with confocal microscopy and electron microscopy corroborated these alterations.

The pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and anti-inflammatory IL-10 were measured. While chronic colitis elevated TNF-α and IL-6 levels, *A. muciniphila* therapy significantly reduced both. In line with the findings, the anti-inflammatory cytokine IL-10 decreased in the colitis group while increasing significantly with *A. muciniphila* therapy. In addition, although IL-1β levels increased in the colitis group and decreased with treatment with treatment toward significance. In 2019, Bian et al showed that *A. muciniphila* has anti-inflammatory effects in DSS colitis model in mice.[24] It has been reported decreased cytokine and chemokine levels in the same study. Additionally, significant alterations in gut microbiota were observed after the treatment. Furthermore, Ottman et al. discovered that pili-like surface proteins of *A. muciniphila* are involved in host immunological homeostasis at the gut mucosa and improvement of gut barrier function.[25] Likewise, Ashrafian et al. recently reported an improvement in gut health by enhancing intestinal integrity and maintaining immune homeostasis.[26] In addition to cytokine release, neutrophil infiltration is a key indication of inflammation.[27] Cytokines and chemokines secreted in response to pathogenic or irritating substances encourage neutrophil accumulation. MPO activity was assessed as a neutrophil infiltration marker in our study. It is released by the colon mucosa during inflammation, and the change in concentration in the tissue indicates neutrophil accumulation in the colon. When compared to the control group, it was shown to be considerably higher in the colitis group. Furthermore, the *A. muciniphila* therapy resulted in a considerable reduction.

Depending on the cytokines and chemokines released after TNBS application, neutrophils migrate to the injury site by infiltrating the vascular wall. A significant decrease in MPO activation was found with treatment-related mucosal healing.[28] In the study published by Lin et al. in 2017, it was shown that the activity of MPO increased significantly in the TNBS colitis model.[29] As a result, the decrease in MPO activation as well as the changes in cytokines after *A. muciniphila* treatment supported these results and indicated its potential therapeutic efficacy.

The inflammatory process begins with increased expression of the NF-kB p65 subunit, which has been demonstrated to have an essential role in the genesis of IBD and to play an important function in the inflammation response.[30] Furthermore, Quaglio et al. discovered that activating the NF-κB signaling pathway resulted in TNF-α, IL-1β, and IL-6 production.[31] *A. muciniphila* supplementation lowered NF-κB activation by boosting NF-kB inhibitory protein expression in research by Zhao et al.[32] Therefore, metabolic inflammation was attenuated. In our study, a substantial rise in protein expression levels of the p65 subunit was seen in the TNBS group and decreased after therapy. These findings are consistent with previous research, indicating that *A. muciniphila* exhibits anti-inflammatory effects through blocking the NF-kB signaling pathway. In the chronic TNBS colitis model, *A. muciniphila* is thought to work by suppressing the NF-κB pathway and decreasing IL-6, TNF-α, and neutrophil infiltration. Furthermore, recent studies demonstrated that LPS generated by the microbiota reaches the bloodstream in IBD patients due to intestinal barrier breakdown and inflammation.[33] As a permeability measurement, LPS analysis was done on serum samples at regular intervals in our investigation. LPS considerably rose in the TNBS group until the seventh week, according to the findings. After the therapy began in the third week, LPS reduced dramatically until the completion of the trial, and the treatment's success was determined. These findings showed that treatment with *A. muciniphila* demonstrates an ameliorate effect on intestinal integrity.

To evaluate microbiome composition changes, microbiome analysis was applied to fecal samples from different time points. According to our data, Resoburia hominis in the TNBS group was not detectable while it was increased 60-fold in mice treated with *A. muciniphila*. The positive effect of this bacteria in the treatment of colitis is also stated in the literature.[34] Furthermore, Prevotella saccharolytica subspecies were measured to increase 30-fold in *Akkermansia*-treated mice. Larsen J M et al. specified in their study that this bacterium induced a Th17 immune response.[35] In addition, in this study, the Oribacterium genus was found to have increased 80 times. Dziarksi et al found that Oribacterium bacteria were reduced in immunocompromised mice in 2016,[36] which shows a trend toward improvement. At week 5, *A. muciniphila* and TNBS had no statistically significant difference in diversity between groups.

Moreover, it was observed that an increase in Helicobacter spp bacteria in the last week of the study compared with the first week within all groups (more than 100-fold). Recently, Franks et al. found that H. pylori bacteria prevented the formation of colitis or caused a decrease in the presence of colitis.[37] This would be one of the pathogenicity features of H. pylori, acting through intense activation of the immune response.[37] In addition, the amount of Helicobacter in mice exposed to TNBS was approximately 90% higher, and more than 100-fold in mice fed *A. muciniphila*. We observed in week 7, that there was no significant difference anymore between the *A. muciniphila* and TNBS groups in terms of microbial diversity while only the control group decreased. At the last week of the study, there was no significant difference between the *A. muciniphila* and TNBS groups as species were investigated. We estimate that the dominance of *H. pylori* on gut microbiota has an overwhelming effect on the less abundant taxa. When combining all these findings, the results were consistent and showed that *A. muciniphila* has a potentially beneficial effect on microbiome composition.

Furthermore, it has been demonstrated that enteric neurons and glial cells show changes in protein expression during inflammation. GFAP, a specific protein released from glial cells, is used as a glial cell inflammatory measure.[38] During the inflammatory process, there is a considerable rise in GFAP in both in vivo and in vitro investigations. At the same time, it was reported that GFAP expression increased considerably in samples collected from IBD patients.[39] Beta-tubulin III is a protein belonging to the tubulin family secreted only by neurons and testicles. Studies have shown that Beta-tubulin III expression was significantly decreased after acute TNBS administration.[40]

According to the assessment of neuroinflammation in this study, GFAP protein expression results increased significantly in

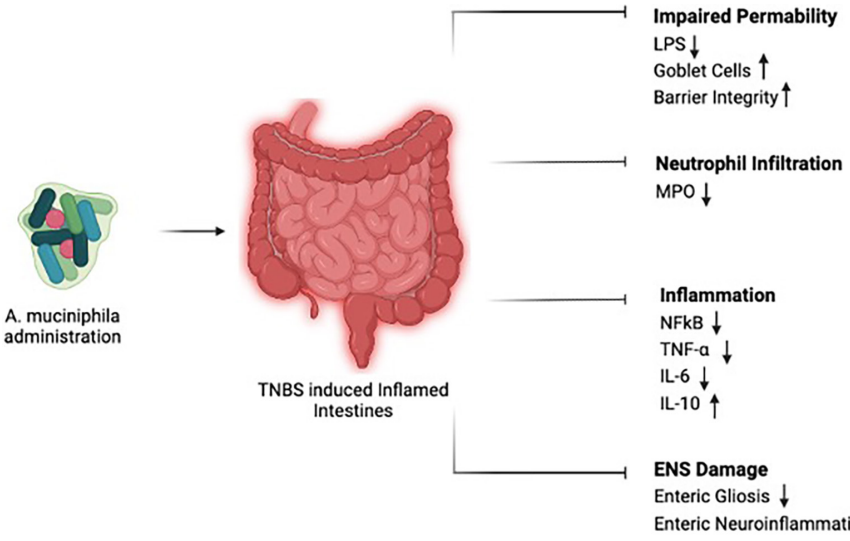

FIGURE 6 Summary of the experiment results.

the TNBS group and decreased statistically with the treatment. In the histological evaluations, the GFAP expression change results were in accordance with the protein analysis. These results indicate increased enteric damage and the development of enteric gliosis after TNBS administration. The data analysis on enteric glial cells within groups are consistent with the literature, and the alterations depending on the efficacy of the treatment were found to be significant. On top of that, it is the first study to show the effectiveness of *A. muciniphila* treatment on enteric gliosis in the literature. Besides, Beta Tubulin III expression in enteric neurons was examined for neuronal damage. Protein expression was statistically higher in the TNBS group and dropped considerably following therapy. Surprisingly, our findings revealed that Beta Tubulin III expression, which was demonstrated to decrease in the acute TNBS model, was not altered in the same way in the chronic form. According to the literature, acutely applied models showed signs of motility problems and neuroplasticity alterations caused by inflammation. In research by Mawe et al., published in 2015, it was demonstrated that enteric neuron expression increased to avoid chronic intestinal motility issues.[41] For these reasons, it is suggested that Beta-tubulin III expression in the chronic TNBS colitis model is not decreased but increased in protein assessment to prevent motility problems related to enteric neuronal damage. In addition, immunofluorescence imaging showed an increase in Tubulin III expression in the TNBS group while there was no change with the treatment. These results did not reach statistical significance. Additionally, these are the first results indicating the potential efficiency of *A. muciniphila* treatment of enteric neuroinflammation and gliosis in the colitis model.

5 | CONCLUSION

Our findings are summarized in Figure 6. Treatment with *A. muciniphila* improves the symptoms of chronic TNBS-induced colitis. It exerts its effects by reducing permeability and blocking the NFkB p65 signaling pathway. These results are further supported by lower MPO activity, a reduction in the number of goblet cells, and cytokine results. These studies also showed ameliorative alterations in neuroinflammation and gliosis using microscopic analysis and protein expression levels. These results suggest that *A. muciniphila* has a promising anti-inflammatory impact, and more study is required to confirm our findings and apply them to clinical research.

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AUTHOR CONTRIBUTIONS

OY, SOO, MC, MAE, SA, YP, and MK contributed to data acquisition and analysis; OY, KHS, and MK contributed to the drafting of the manuscript and the critical revision of the manuscript; OY and MK were responsible for the study design; OY, MC, and MK were involved animal experiments, tissue collection, protein analysis; MAE, OY and SA were involved all histological analysis and interpretation; YP, OY and MK contributed microbiome analysis; KHS and MK were responsible for supervision; OY and MK obtained funding. All authors approved the final version of the manuscript before submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings will be available in Drive link/ Ozgur Yilmaz at https://drive.google.com/drive/folders/1HdUYykrttOTmtvc6SpJ9L37sHxS8FZ8L?usp=drive_link following an embargo from the date of publication to allow for commercialization of research findings.

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