RESEARCH ARTICLE

The effect of plasma-activated water in a mouse model of inflammatory bowel disease

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Funding information
Vedecká Grantová Agentúra MŠVVaŠ SR a SAV; Agentúra na Podporu Výskumu a Vývoja

Abstract
Inflammatory bowel disease (IBD) is a group of disorders with complex pathogenesis, including oxidative stress and microbial dysbiosis. The aim of this study was to analyze the effect of intracolonic administration of plasma-activated water (PAW) on the oxidative status and gut microbiota diversity in the colon of healthy mice and mice with IBD. Interestingly, PAW increased oxidative stress markers in the colon tissue, and this effect was more pronounced in IBD mice. Our results show that PAW increases microbial diversity in a healthy gut and decreases it in an inflamed gut. We conclude that the effect of PAW is bidirectional and depends on the underlying condition. Our findings do not support the proposed therapeutic potential of PAW in IBD.
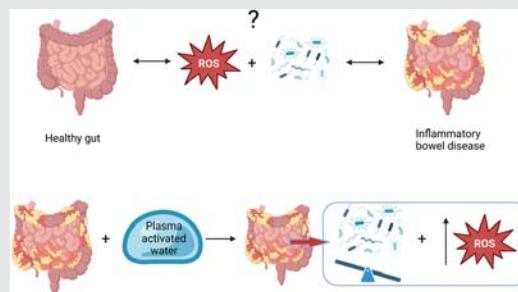
KEYWORDS
inflammatory bowel disease, microbiome diversity, oxidative stress, plasma-activated water

1 | INTRODUCTION

Plasma occurs naturally in the environment as the fourth state of matter; however, in laboratory conditions, plasma can be generated by various sources using pulse or direct discharge.[1] Plasma-activated water (PAW) represents a mixture of ionized gases, positively and negatively charged ions, atoms, and free radicals, such as reactive oxygen/nitrogen species (ROS/RNS) in water. Currently, the application of PAW is being studied in several fields, including biomedicine, where it has been developing rapidly in recent years. It is most widely used

for microbial inactivation and sterilization, but it also found its use in dermatology, dentistry, and oncology.[2–6] The effectiveness of PAW lies in oxidative damage to the cell wall, cytoplasmic leakage, and DNA breakdown.[7] The ROS/RNS cause an increase in redox potential and a decrease in pH to create an antimicrobial environment capable of effectively killing bacteria. This has been confirmed by several studies examining the effect of PAW on Gram-positive and Gram-negative bacteria. In general, gram-positive cells are more resistant to PAW treatment due to a thicker peptidoglycan layer that acts as a protective shield.[8] However, a study by Hozak et al. found that PAW was able to inhibit *Staphylococcus epidermidis* (Gram-positive) more effectively than *Escherichia coli* (Gram-negative), probably due to the presence of unidentified active compounds in PAW.[9]

Effective inactivation of live bacteria largely determines the effectiveness of PAW in wound healing. A recent study showed that PAW treatment effectively treats acute wounds, especially in combination with other treatments.[3] On the other hand, a meta-analysis revealed that low-temperature plasma is not as effective for chronic open wounds.[10] Next, a review by Semmler et al. excellently summarized the understanding of PAW-mediated selective killing of cancer cells via intracellular pathways that induce growth inhibition or cell death.[11] The interaction of ROS/RNS with the interplay of altered expression of aquaporins, cholesterol, or the ability to protect against oxidative stress by the antioxidant system was outlined, on the basis of which ROS/RNS can enter the cell and interfere with intracellular signaling pathways. Yan et al. stress that anticancer plasma treatment is promising, especially through apoptosis induction caused by reactive oxygen species production inside the cells.[12] In addition, the plasma-activated medium has been shown to be effective in tumor treatment in mice in vivo.[13] Therefore, plasma-activated liquids, despite their limited stability, represent a novel tool that could be used as a treatment for diseases, especially in organs and tissues that are difficult to reach and treat with direct plasma.

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is one of the current global chronic diseases with rising incidence and yet unknown pathogenesis.[14] The characteristic feature is uncontrolled inflammation of the intestinal mucosa accompanied by an abnormal immune response to the presence of microbiota in genetically susceptible individuals. The spectrum of currently available therapies is relatively wide; however, most of these are asymptomatic and/or nonspecific and thus provide only limited or temporal effects. A significant proportion of patients do not achieve clinical remission after treatment or lose their

response over time.[15] Therefore, new therapeutic strategies are more than welcome. Overproduction of ROS is part of the pathogenesis of IBD and is associated with the change in the composition of intestinal microbiota in the colon. Association between the oxidative status of the tissue and the composition of intestinal microbiota along the gastrointestinal tract has been shown previously.[16]

Enteric bacteria can regulate the oxidative status of the gut by activating cellular signaling pathways in intestinal epithelial cells.[17] Thus, it is not clear whether oxidative stress in the colon is a factor that contributes to the pathogenesis of the disease or whether it is a compensatory mechanism that allows the intestinal cells to cope with gut microbiota dysbiosis found in IBD. Modulation of gut microbiota through the change in the oxidant status of the intestinal tissue has not been studied before. We hypothesize that in vivo administration of PAW can represent a therapeutic approach to treat IBD, which consists of (1) restoration of the gut microbiota homeostasis by inhibiting the overgrown bacterial species through the direct antibacterial effect of ROS and (2) modulation of immune response by changing the redox status of the colon tissue mediated by ROS. The aim of this study was to analyze the effect of intracolonic administration of PAW on the oxidative status and gut microbiota diversity in the colon of healthy mice and mice with IBD.

2 | MATERIAL AND METHODS

2.1 | Animals

Thirty-eight female C57BL/6 mice (Charles River Laboratories International) at 8 weeks of age were used. Mice were divided into four groups—control group ($n = 5$), control PAW group ($n = 8$), DSS group ($n = 12$), and DSS PAW group ($n = 13$). The animals were housed in conventional cages with ad libitum access to standard chow and water under standard conditions (21–24°C ambient temperature and 55%–65% humidity) and 12 h light/dark cycle. The experiment was approved by the Ethics Committee of the Institute of Molecular Biomedicine, Faculty of Medicine, Bratislava, Slovakia.

2.2 | PAW preparation

PAW was prepared as described previously.[18] Briefly, glow discharge PAW was prepared from phosphate-buffered saline (PBS), which was plasma treated by

operating the discharge on the surface of the liquid in the Petri dish in batch treatment.

The grounded electrode was a stainless-steel wire submerged in PBS at the bottom of the Petri dish. The ballast resistor was set to $R = 1.1\,M\Omega$. The mean current was maintained at $I \approx 7.4\,mA$, and the voltage was constant $U = 2.16\,kV$, resulting in the mean power $P \approx 16\,W$. This discharge is DC (pulseless), and so the current was measured by an analog multimeter (AX-7030; Axiomet). During the batch preparation, PAW was continuously stirred using a magnetic stirrer to ensure the equal spread of reactive species in the water. The volume of PBS treated with plasma was 15 mL, and the treatment time was set at 12 min, so the ratio was 1 mL/min. The treated volume was set to 15 mL because of enhanced evaporation due to a higher temperature of the discharge. The temperature increase in the bulk liquid after treatment was up to 10°C.

A direct-current high-voltage (DC HV) power supply (Imax = 30 mA, Umax = 25 kV) was used. The discharge voltage was measured using a high-voltage probe (P6015A: Tektronix), with $R = 100\,M\Omega$, $C = 3\,pF$, and 1000x voltage attenuation factor. The inner electrode spacing from the high-voltage needle to the grounded electrode was 10 mm. A positive DC HV was applied through the ballast resistor R (1.1 MΩ). The current and voltage signals were processed by a digitizing 200 MHz oscilloscope (TDS 2024, Tektronix).

PAW was stored at −80°C for up to 1 week. Right before the experiment, PAW was thawed at 37°C and applied immediately. Details of PAW preparation and chemical analysis of freeze-thawed PAW were shown in our recent study.[18]

2.3 | Design of the experiment

To induce colitis, DSS and DSS PAW groups received 3% dextran sulfate sodium (DSS; molecular weight 40,000, PanReac AppliChrem) for 7 days ad libitum in drinking water. Control groups received drinking water. Daily intracolonic administration of PBS or PAW (250 µL) was performed using a cannula in isoflurane-anesthetized mice. Body weight, stool consistency, and rectal bleeding were monitored daily throughout the experiment. The stool consistency score was determined on a scale from 0 to 3, representing as follows: 0 = thick, formed stool, 1 = soft stool, 2 = watery stool, and 3 = soft or watery stool with the presence of blood. All animals were killed by cervical dislocation under ketamine/xylazine anesthesia (100/10 mg/kg) on the last day of the experiment. The colon length was measured from ileo-cecal margin to the anus, and its proximal and distal parts were washed with

PBS, collected, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

2.4 | Markers of oxidative stress

Proximal and distal colon tissues were homogenized for 10 min at 20,000 Hz using a Tissue Lyzer (Qiagen) and centrifuged for 10 min at 1600 g. Supernatants were taken and used for measurement of protein concentration using the Bicinchoninic acid kit for protein determination (Sigma Aldrich) and markers of oxidative stress. Thiobarbituric acid reactive substances (TBARS) were measured by pipetting 20 µL of samples or standards (1,1,3,3-tetraethoxypropane), 30 µL of distilled water, 20 µL of 0.67% thiobarbituric acid, and 20 µL of glacial acetic acid into a microtiter plate. The plate was mixed and incubated for 45 min at 95°C. One hundred microliters of n-butanol was added to the samples, and the plate was centrifuged for 10 min at 2000 g and 4°C. After this, 80 µL of the organic phase was taken into a new microtiter plate, and fluorescence was measured at excitation = 515 nm and emission = 535 nm. For advanced oxidation protein products (AOPP) measurement, 200 µL of samples and standards (chloramine T mixed with potassium iodide) were mixed with 20 µL of glacial acetic acid for 2 min. Absorbance was measured at 340 nm. Total antioxidant capacity (TAC) was determined by the difference between the first and second absorbance measurements at 660 nm. The first measurement (blank) was performed after mixing 20 µL of samples with 200 µL of acetate buffer (pH = 5.8). This was followed by the addition of 20 µL of ABTS solution (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to the previous mix, and the absorbance measurement was repeated.

2.5 | Myeloperoxidase

Myeloperoxidase (MPO) levels, as a marker of inflammation (especially of neutrophil origin), were determined in blood plasma samples using the Mouse MPO ELISA Kit (Sigma Aldrich) according to the manufacturer's instructions.

2.6 | Sequencing

Contents of the distal part of the colon were used for the extraction of bacterial DNA (QIAamp DNA Stool Mini Kit). The 16S rDNA was amplified using primer pair sequences for the V3 and V4 of the 16S region. The

primers 16S Amplicon PCR Forward Primer (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) and 16S Amplicon PCR Reverse Primer (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) produced amplicons of approximately 460 bp. Both primers contained Illumina adapter regions. The PCR mixture contained template DNA (approximately 12 ng), 2x KAPA HiFi HotStart ReadyMix (12.5 μL), and Amplicon primers (0.5 μL, 10 pM) in the total reaction volume of 25 μL. Reaction conditions consisted of an initial 95°C, 5 min; 35 cycles of 94°C, 60 s, 60°C, 45 s, and 72°C, 60 s; and a final extension of 72°C, 7 min. After Amplicon PCR, all samples were purified using AMPure XP for PCR Purification (Beckman Coulter) by adding 4.0× volume of reaction volume −100 μL of magnetic beads.

DNA libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina Inc). Index PCR, a step which attaches dual indexes and Illumina sequencing adapters using Nextera XT Index kit (Illumina Inc), was performed following the protocol using 2x KAPA HiFi HotStart ReadyMix (25 μL), Nextera XT Index Primers (5 μL per sample), PCR Grade Water, and 5 μL of template DNA solution in the total reaction volume of 50 μL. PCR Clean-Up 2 was performed as described in the protocol. The final libraries were quantified using the Qubit 3.0 Fluorometer (Life Technologies) and the Qubit dsDNA HS assay kit (Invitrogen) and quality checked using the 2100 Bioanalyzer (Agilent Technologies) with use of the High Sensitivity DNA analysis kit (Agilent Waldbronn). The libraries were normalized to 4 nM concentration, and all samples were pooled together and denatured according to the standard protocol. The final library pool was sequenced on the MiSeq system (Illumina Inc) using the MiSeq Reagent kit v3 with a paired-end of 2 × 300 bp reads.

Adapters and low-quality ends of sequenced reads were removed using Trimmomatic (version 0.36)[19] based on quality control statistics generated by FastQC (version 0.11.5).[20] After trimming, fragments without sufficient length of both reads (>35 bp) were removed from the data set. We also excluded reads mapped to the mouse genome (version GRCm38.p6) using Bowtie 2 (version 2.3.4.3).[21] All trimmed paired reads with sequence overlaps were merged using PEAR (version v0.9.6)[22] and passed to QIIME 2 (version 2019.10.0) for microbial analyses.[23]

The preprocessed fastq files were dereplicated using QIIME 2 built-in tool search. Next, operational taxonomic units (OTUs) with 99% similarity were created with de novo clustering of features. Singletons and OTUs with low abundance were filtered out. Then, chimeras and "borderline chimeras"[24] were removed from the data set using de novo chimera filtering (with parameters: mindiv = 1.0, minh = 0.5, xn = 3.0).

Taxonomy of the resulting OTUs was assigned by QIIME's taxonomy classifier,[25] trained by the Naive–Bayes classifier on Silva 16S database (release 132).[26] Finally, Faith's phylogenetic diversity for all samples was calculated using the faith-pd plugin.[27] All computational analyses were written and executed using the SnakeLines framework (v0.9.2).[28,29] The functionality of the metagenomic content was investigated by PICRUSt (version 2.3.0)[30] based on the abundance of corresponding OTUs. Differences in the abundance of bacterial families or KEGG pathways[31] between groups were analyzed by t-test with the false detection rate correction. Correlations of the bacterial abundance or KEGG pathways with metabolic parameters were examined by Spearman's test. All statistical tests were performed in the Python scipy library (version 1.5.3)[32], and results were visualized using Seaborn library (version 0.11.0).[33]

2.7 | Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.4.1 (GraphPad Software). Data were analyzed using one-way or RM analysis of variance (ANOVA), and differences between the groups were evaluated using Bonferroni multiple comparison post hoc test. Data are shown as mean ± standard deviation (SD).

3 | RESULTS AND DISCUSSION

3.1 | Effect of PAW treatment on the course of colitis

To determine whether the intrarectal PAW administration affects the development of colitis, we used the standard 3% DSS-induced mice model of colitis lasting 7 days, measuring body weight and stool consistency daily. Both DSS groups showed a progressive decrease in body weight. However, the DSS PAW group started losing weight even earlier than DSS alone (Figure 1a). The gradual development of colitis was accompanied by deterioration of the stool consistency with rectal bleeding in both DSS groups with no difference between them (Figure 1b). The severity of colitis is generally indicated by a shortening of the colon due to persistent inflammation. However, there was no difference in colon length between the DSS PAW group and the DSS group

(Figure 1c). On the other hand, DSS mice treated with PAW showed decreased levels of myeloperoxidase (MPO) in blood plasma (Figure 1d).

3.2 | Oxidative stress and antioxidant status

We analyzed the effect of PAW on the tissue of the proximal and distal parts of the colon in the context of their oxidative status. Markers of oxidative damage to lipids (TBARS) and proteins (AOPP) were chosen, along with a marker of response to oxidative damage (TAC). In the proximal colon, all markers were slightly higher in the CTRL PAW group compared with CTRL, although this difference was not statistically significant. Interestingly, TBARS and AOPP levels were significantly higher in the DSS PAW group compared to the DSS and control groups (Figure 2a,b). Antioxidant status represented by the
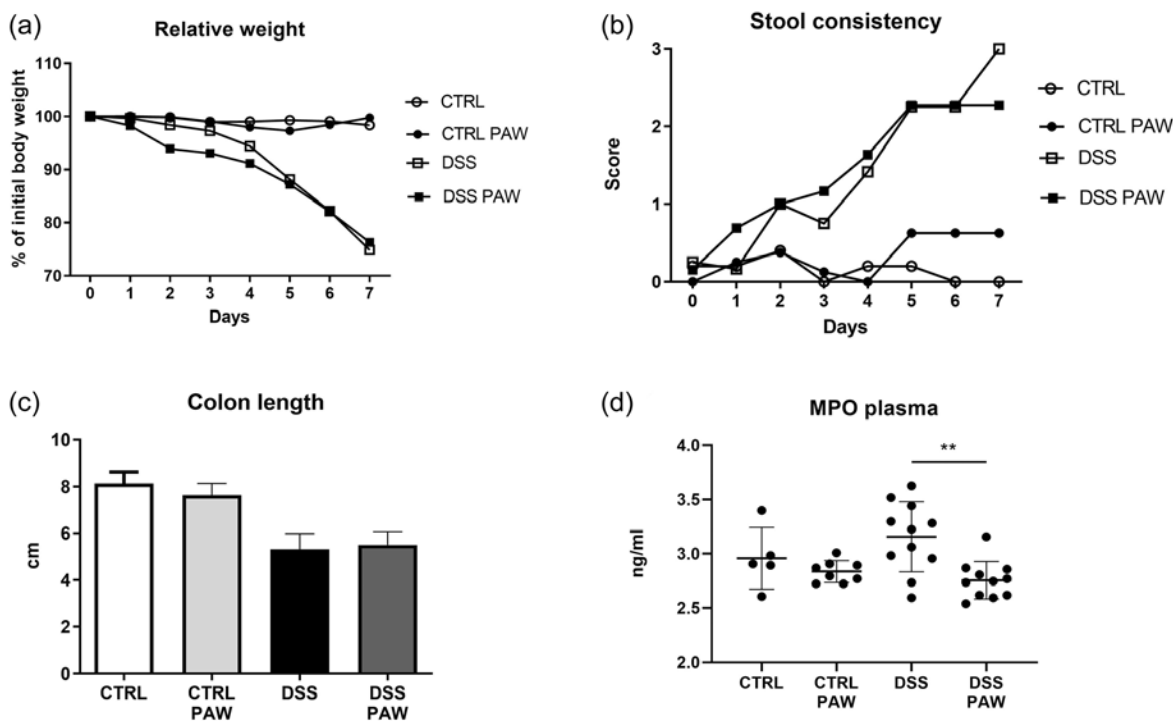

FIGURE 1 Markers of disease activity. Administration of plasma-activated water (PAW) did not lead to amelioration of weight loss (a) and did not improve stool consistency (b) and colon length (c). PAW decreased myeloperoxidase concentration in the blood plasma of inflammatory bowel disease (IBD) mice (d).
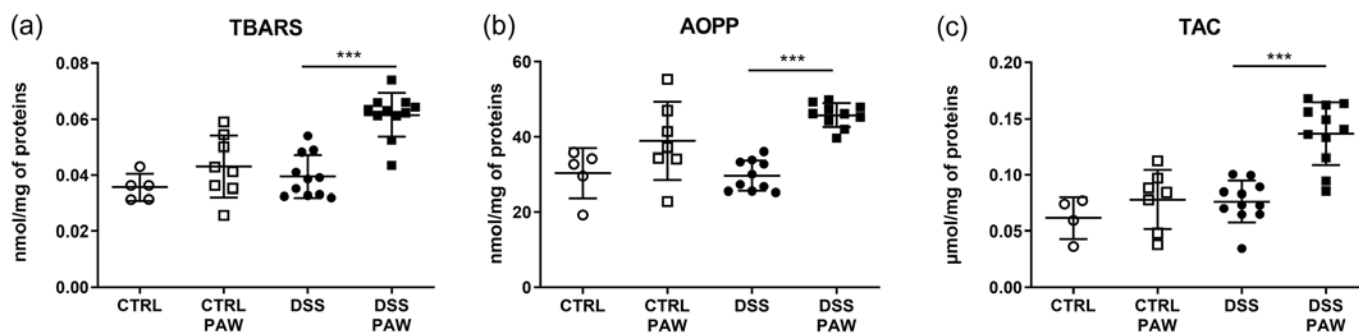

FIGURE 2 The concentration of oxidative stress markers and antioxidant capacity in the proximal colon. Data are presented as mean ± SD. Both markers of oxidative stress (a) thiobarbituric acid reactive substances (TBARS) and (b) advanced oxidation protein products (AOPP) in colitis mice treated by plasma-activated water (PAW) were significantly increased compared to colitis and healthy mice. (c) Total antioxidant capacity (TAC) had a significant increase in the colitis mice treated by PAW compared to colitis and healthy mice ($***p < 0.001$).
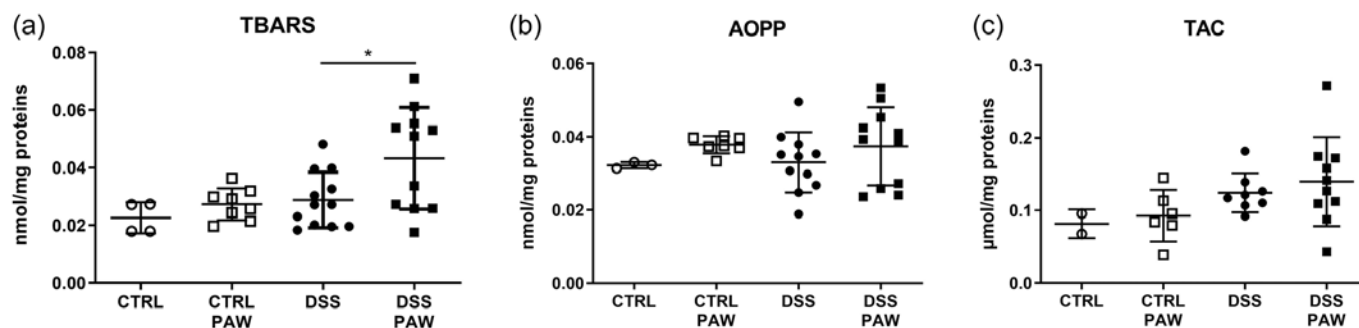
FIGURE 3 The concentration of oxidative stress markers and antioxidant capacity in the distal colon. Data are presented as mean $\pm$ SD. (a) An increase in thiobarbituric acid reactive substances (TBARS) level was noted in the dextran sulfate sodium (DSS) plasma-activated water (PAW) (*$p < 0.05$). (b) No significant differences were observed in the concentration of the second chosen marker of oxidative stress—advanced oxidation protein products (AOPPs) among groups. (c) No significant differences were observed in the concentration of total antioxidant capacity among groups.

TAC level was also higher in the DSS PAW group compared to the DSS and control groups (Figure 2c).

In the distal colon, the concentration of TBARS was significantly higher in the DSS PAW group than DSS and control groups. The concentration of the second marker of oxidative stress, AOPP, was the same in all groups. Regarding antioxidant status, there were no significant differences in TAC levels among all groups (Figure 3).

3.3 | Alpha diversity of the microbiome

To analyze the effect of DSS and PAW treatment on the composition of the intestinal microbiome, we have determined alpha diversity. Four algorithms of alpha diversity were used to determine how diverse the microbiome is within the respective groups. Figure 4 shows lower microbiome diversity in DSS mice treated with PAW compared with untreated DSS mice. On the contrary, administration of PAW in healthy mice leads to higher microbiome diversity compared with untreated healthy mice.

3.4 | Beta diversity of the microbiome

To explore how far are the experimental groups from each other in terms of composition, we have analyzed the beta diversity. Figure 5 shows three indices of sample dissimilarity based on phylogenetic distance. The highest distance can be seen between CTRL PAW and DSS PAW groups. This confirms that PAW changes the microbiome composition in different directions in healthy and diseased mice, respectively.

3.5 | Cladogram and representative species

Analysis of the 16S rRNA gene showed the dominant taxonomic groups in the experimental groups as follows. Compared to the CTRL group, the families Bifidobacteriales and Deferribacteriales dominated the DSS group. In the CTRL PAW group, several families of the phyla Bacteroidetes and Patescibacteria were dominant. The classes Chloroflexa and Clostridiaceae were abundant in the DSS PAW group.

Colitis-associated inflammation is associated with the production of ROS by immune and intestinal cells representing key signaling molecules that contribute to their functions and homeostasis. On the other hand, ROS also show harmful properties, including cellular and molecular damage, perpetuating intestinal inflammation and tissue destruction.[34] Antioxidant therapy for IBD has been proven effective.[35,36] However, understanding of the pathomechanisms that link oxidative stress to IBD is still insufficient. This opens new avenues for the research of redox-targeted approaches to study the pathogenesis and new therapies of IBD. In the present study, we generated glow discharge PAW, which is exceptionally rich for reactive oxygen/nitrogen species, including hydrogen peroxide, nitrates, and nitrites. The aim of this study was to determine how glow discharge PAW modulates the development of DSS-induced colitis in terms of the oxidative status of the colonic tissue and intestinal microbiota composition.

Plasma-activated water has recently emerged as a potent antimicrobial agent. Medical and biotechnological applications of PAW have been previously shown in other settings.[37,38] Administration of PAW in mouse models of disease has not been extensively examined in
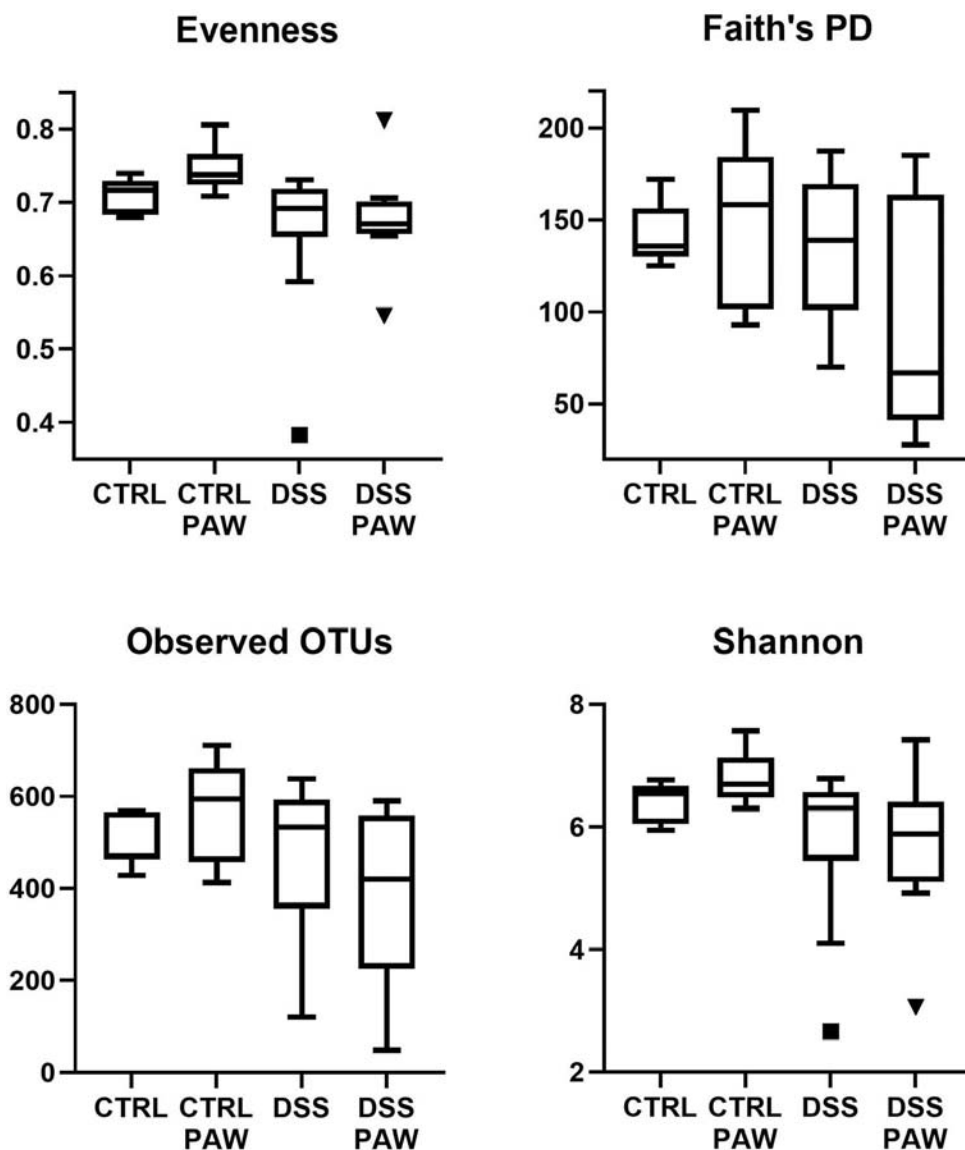
FIGURE 4 Alpha diversity indices. A similar diversity pattern is apparent in all four indices. Treatment with plasma-activated water (PAW) increases the microbiome diversity in healthy mice but decreases the diversity in dextran sulfate sodium (DSS) mice. Evenness represents the distribution of abundances within groups, Faith's PD reflects phylogenetic diversity, Observed operational taxonomic units (OTUs) are the total number of species (richness), and the Shannon index accounts for both abundance and evenness of the species present.
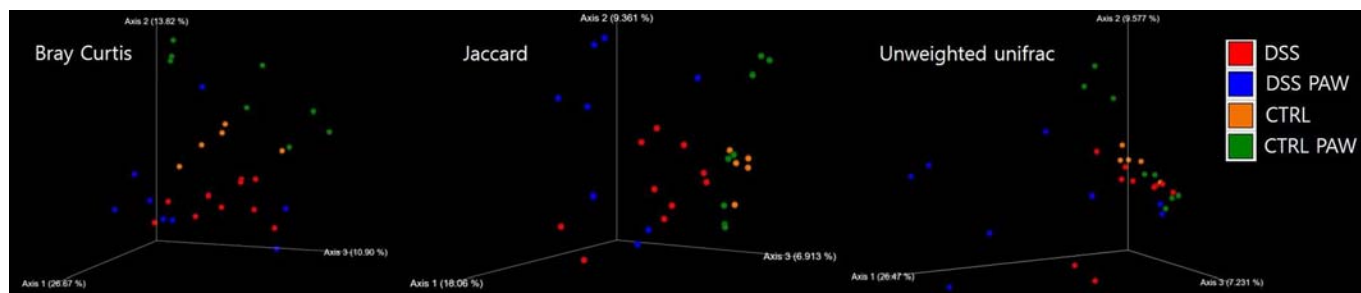

FIGURE 5 Beta diversity metrics. Three beta diversity metrics are shown to visualize the distance between samples in experimental groups. Administration of PAW increased the distance between treated and untreated mice, especially in dextran sulfate sodium (DSS) mice (blue vs. red dots). Bray Curtis dissimilarity examines the microbial abundances shared between two samples and the total number of microbes. In contrast, Jaccard distance is based on the presence/absence of the microbes. Unweighted unifrac incorporates phylogenetic distances between samples.

the past. A recent study has shown the effects of long-term (90 days) watering with PAW on the health of mice.[39] This is the only available study examining the intraoral administration of PAW. The authors conclude that the mice tolerated PAW very well, and PAW had no hematological, biochemical, or immunological effects in the blood and no histopathological effects on tissues throughout the body, including the colon.

Notably, no intrarectal application of PAW has been published so far. However, to target the effect of PAW directly to the colon and to avoid possible deactivation processes during the passage through the gastrointestinal tract, in the current study, we chose intrarectal administration. Thus, PAW was administered on a daily basis throughout the duration of the DSS model of colitis (7 days). This is the first experimental study using PAW as a treatment for IBD and the first time using intrarectal administration of PAW at the same time.

Herein, we show the effect of intragastric administration of PAW on measures of oxidative stress and microbial diversity in healthy versus inflamed mouse colons. Our results show that PAW has no therapeutic effect in a DSS-induced model of IBD. Interestingly, PAW increases oxidative stress markers in the colon tissue, both in healthy and IBD mice. However, this effect is more pronounced in IBD mice (Figures 2 and 3). Interestingly, neither DSS nor PAW administration alone did lead to a strong increase in oxidative stress. The highest levels of oxidative stress markers were shown in the DSS PAW group. Thus, the combination of DSS and PAW seems to have additive pro-oxidative effects. The mechanism underlying this finding is not known. In addition, we have shown a reduced level of systemic inflammatory marker (MPO) in the DSS PAW group compared with DSS mice without PAW. In light of the other results, this finding is unexpected. It shows that on the background of the inflamed colon, local effects of PAW are detrimental, while systemic effects might be beneficial. More analyses are needed to explain this phenomenon.

Further, we analyzed how PAW modulates the diversity of the gut microbiome. It has been previously proposed that oxidative stress in the gastrointestinal tract correlates with the composition of the microbiome.[16] At the same time, it is known that IBD patients, as well as mice with chemically induced colitis, show dysbiosis of gut microbiota characterized by decreased diversity, which might contribute to the pathogenesis of the disease even during remission.[40,41] The effects of PAW on the gut microbiome have not been shown before. Therefore, to explore the therapeutic potential of PAW in IBD, we focused on investigating the effect of PAW on gut microbiome diversity. Our results show that PAW increases microbial diversity in a healthy gut and decreases it in an inflamed gut (Figure 4). Thus, we conclude that the effect of PAW on the microbiome and
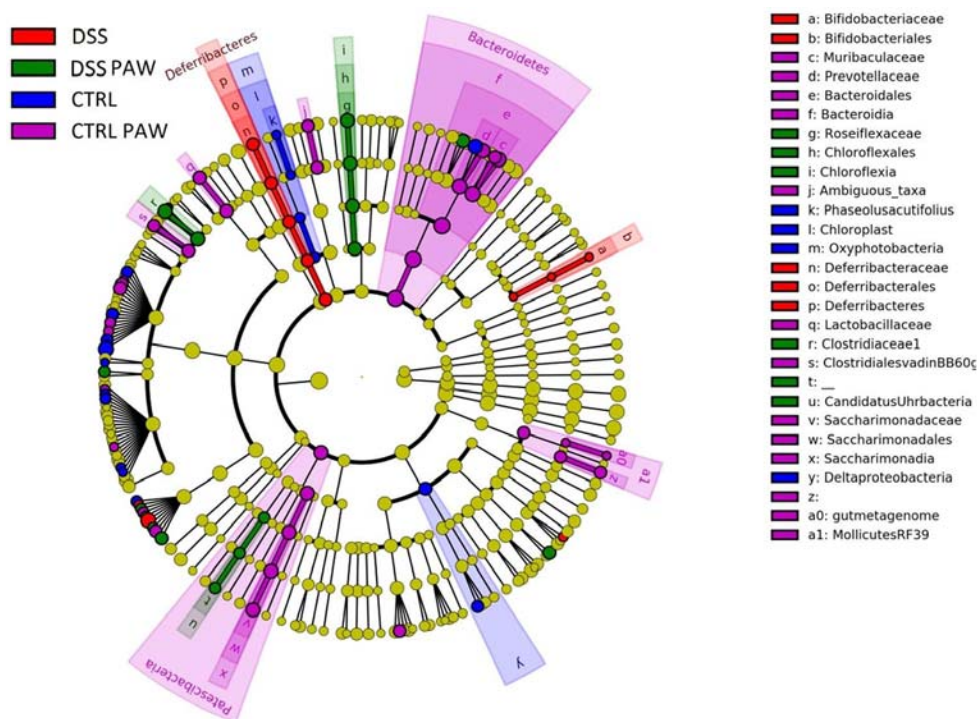

FIGURE 6 Cladogram and representative species. The tree's branches correspond to distinct taxonomic groups. Taxons with differentially abundant changes are denoted by red, green, blue, and violet colors corresponding to experimental groups.

oxidative stress is possibly bidirectional and depends on the underlying condition. This is further supported by analyzing beta diversity—PAW increases the distance between samples in DSS-treated mice more than in healthy mice (Figure 5). It is not clear whether the decrease in microbial diversity is a cause or a consequence of increased oxidative stress found in the colon of IBD mice treated with PAW. Herein, we also show the representative microbiota taxonomic units in the experimental groups. However, comparing single species, families, or higher taxonomic units does not provide the desired informational value. This is partly given by the nature of the 16 S rRNA sequencing, which does not allow deeper taxonomic analysis and, more importantly, does not provide any information on the functional characteristics of the gut microbiota. Therefore, analysis of the diversity might be more valuable in this context since it is known that lower gut microbiota diversity is associated with worse gut health and functionality Figure 6.

4 | CONCLUSION

Overall, PAW did not improve any of the clinical parameters of IBD, including weight loss, stool consistency, and colon length. Moreover, our findings do not support the proposed therapeutic potential of PAW in IBD as far as oxidative stress and microbial diversity are concerned. However, the administration of PAW increased the gut microbial diversity in healthy mice, which might bear some potential for future applications. However, the therapeutic potential of PAW in gastroenterology is unclear. The possible use of PAW during the colonoscopy has to be studied in more detail to draw any conclusion. The effects of PAW on the gut mucosa need to be further explored. Last but not least, local and systemic long-term effects associated with PAW-mediated change in gut microbiome composition are also to be studied.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request. Oxidative stress marker measurement and sequencing data that support the findings of this study are available from the corresponding author upon reasonable request.

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