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Research article

Enterococcus durans 98D alters gut microbial composition and function to improve DSS-induced colitis in mice

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ABSTRACT

Enterococcus durans, is a potential functional strain with the capacity to regulate intestinal health and ameliorate colonic inflammation. However, the strain requires further investigation regarding its safety profile and potential mechanisms of colitis improvement. In this study, the safety of *E. durans* 98D (Ed) as a potential probiotic was studied using *in vitro* methods. Additionally, a dextran sulfate sodium (DSS)-induced murine colitis model was employed to investigate its impact on the intestinal microbiota and colitis. *In vitro* antimicrobial assays revealed Ed sensitivity to common antibiotics and its inhibitory effect on the growth of *Escherichia coli* O157, *Streptococcus pneumoniae* CCUG 37328, and *Staphylococcus aureus* ATCC 25923. To elucidate the functional properties of Ed, 24 weight-matched 6-week-old female C57BL/6J mice were randomly divided into three groups (n = 8): NC group, Con group (DSS), and Ed group (DSS + Ed). Ed administration demonstrated a protective effect on colitis mice, as evidenced by improvements in body weight, colonic length, reduced disease activity index, histological scores, diminished splenomegaly, and decreased goblet cell loss. Furthermore, Ed downregulated the expression of the pro-inflammatory cytokine genes (*IL-6*, *IL-1β*, and *TNF-α*) and upregulated the expression of the anti-inflammatory cytokine gene *IL-10*. The 16S rRNA gene sequencing revealed significant alterations in microbial α-diversity, with principal coordinate analysis indicating distinct differences in microbial composition among the three groups. At the phylum level, the relative abundance of Actinomycetota significantly increased in the Ed-treated group. At the genus level, Ed treatment markedly elevated the relative abundance of *Paraprevotella*, *Rikenellaceae_RC9*, and *Odoribacter* in DSS-induced colitis mice. In conclusion, Ed exhibits potential as a safe and effective therapeutic agent for DSS-induced colitis by reshaping the colonic microbiota.

1. Introduction

The intestinal microbiota represents a complex ecosystem that performs vital biological functions, such as defense against pathogen infection and synthesis of vitamins and essential metabolites [1]. The dynamic interplay of the host and its gut microbiota is instrumental for maintaining the stability of the host immune system [2]. The gut microbiota sustains its functions by modulating the

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host immune response and adapting to the intestinal environment [2]. However, gut microbiota dysbiosis can lead to pathological consequences in the host, including colitis [2], diarrhea [3], and irritable bowel syndrome [4]. Treating intestinal inflammation typically involves the use of anti-inflammatory and immunosuppressive drugs [5]. With increasing study of the relationship between gastrointestinal bacteria and colitis pathogenesis in humans and animals, probiotics have emerged as a potential alternative for treating colitis.

Enterococcus is a common bacterial genus found in the intestines of mammals, as well as in various habitats such as water, soil, air, and plants [6]. Taxonomically, *Enterococcus* belongs to the domain Bacteria, phylum Bacillota, class Bacilli, order Lactobacillales, and family *Enterococcaceae* [7]. *Enterococcus* spp. are characterized by being gram-positive, catalase-negative, facultative anaerobes, possessing fermentative capabilities, and can tolerate certain temperature variations and acidic or alkaline environments [8,9]. Most of the *Enterococcus* spp. can produce enterocins (various types of proteins and peptides) to inhibit the activity of pathogenic bacteria [10]. Among these, the subfamily II.1 is the main subclass of enterocins, characterized by a common protein sequence YGNGV at the N-terminal, which is a prerequisite for anti-pathogenic activity. However, other studies have demonstrated that *E. faecalis* and *E. faecium* secrete adhesins, invasins, and hemolysins that aid infections [11,12]. Moverover, Several studies have demonstrated that *E. durans* promotes the production of butyric acid to alleviate intestinal inflammation, induces the production of regulatory T-cells (Tregs), and reshapes the intestinal microbiota to alleviate dextran sulfate sodium salt (DSS)-induced colitis in mice [13,14]. *E. durans* EP1 is a strong inducer of secretory immunoglobulin A that suppresses inflammation by increasing the abundance of *Faecalibacterium prausnitzii* [15]. *E. durans* A8-1 tolerates the gastrointestinal environment, possesses strong adhesive capabilities, and alleviates inflammatory responses by competing with pathogenic bacteria for ecological niches [16]. These findings suggest that certain *E. durans* strains have probiotic potential. Furthermore, potential probiotics must also demonstrate tolerance to gastric acid, bile salts, and various digestive enzymes and fluids and be capable of stable colonization to exert their long-term beneficial effects on the gastrointestinal tract [17].

In this study, an *in vitro* safety assessment protocol was utilized to evaluate the tolerance of *E. durans* 98D (Ed) to gastric acid, bile salts, and digestive enzymes, and to investigate its sensitivity to common antibiotics and its inhibitory effect on pathogenic bacteria. Additionally, the study explored the synergistic effect of Ed in treating colitis with other gut probiotics in the colon.

2. Results

2.1. Strain morphology and in vitro safety assessment

The *in vitro* culture and microscopic examination of Ed revealed colonies with a creamy white and raised appearance, consisting of bacteria (gram-positive) with an ellipsoidal morphology (Fig. 1A). In addition, Ed exhibited logarithmic growth after a 2-h culture period, achieving stationary growth after approximately 8 h (Fig. 1B). *In vitro* safety assessment demonstrated that Ed survived in an acidic environment (pH = 2; Fig. 1C); however, exposure to bile salts resulted in a low survival rate (Fig. 1D). When the culture environment exceeded 50 °C, the growth of Ed is also inhibited (Fig. 1E). Furthermore, Ed demonstrated tolerance to pepsin and trypsin, with a significant increase in OD_{600} relative to that of the control group following a 4-h culture ($P < 0.05$, Fig. 1F). Among the

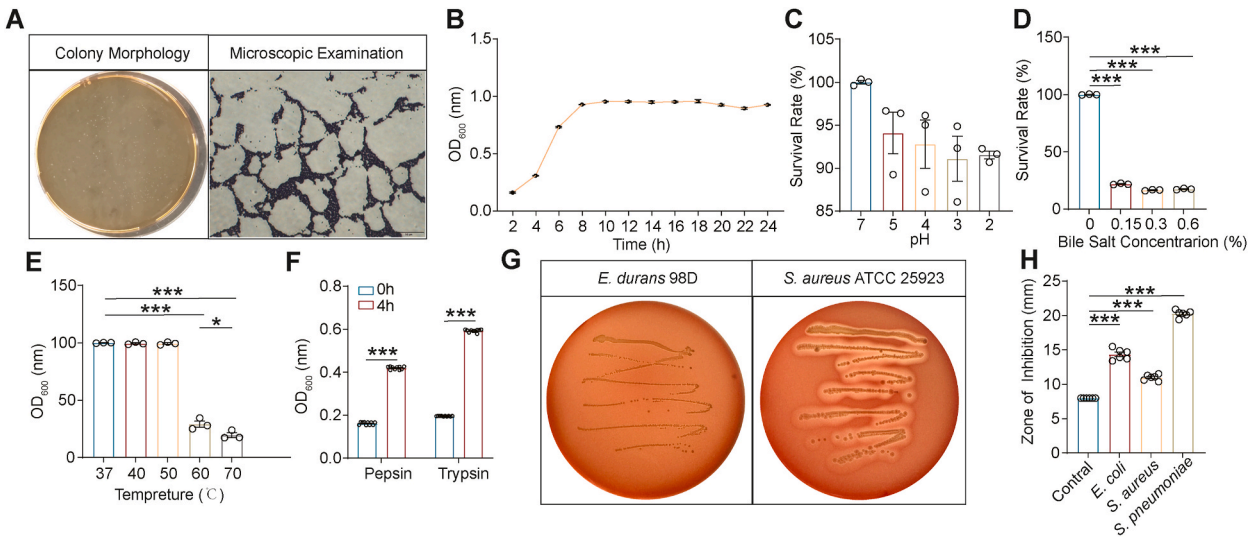

Fig. 1. *In vitro* safety assessment of *Enterococcus durans* 98D. (A) Colony morphology and Gram staining microscopic examination of *E. durans* 98D. (B) Growth time curve of *E. durans* 98D. The tolerance of *E. durans* 98D to (C) acid, (D) bile salt, (E) temperature, and (F) pepsin or trypsin. (G) Hemolytic activity of *E. durans* 98D and positive control strain (*Staphylococcus aureus* ATCC 25923). (H) Inhibition zone diameters of *E. durans* 98D against *Escherichia coli*, *S. aureus*, and *Streptococcus pneumoniae*. Data are expressed as the mean ± SEM, $n = 3$–10. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (unpaired *t*-test or one-way ANOVA with Tukey's post hoc).

23 drugs tested, Ed exhibited moderate sensitivity to aminoglycosides, cephalosporins, and quinolone antibiotics and high sensitivity to all penicillins, amides, and macrolides (Table 1). Furthermore, compared with those of the positive control, the dishes inoculated with Ed exhibited hemolysis instead of β-hemolytic rings (Fig. 1G). Notably, Ed cells exhibited cell surface hydrophobicity (CSH) and autoagglutination (AAg) values of 39.14 ± 2.02% and 66.90 ± 0.72%, respectively (Table 2). *In vitro* antibacterial assays demonstrated varying degrees of Ed-mediated growth inhibition against *Escherichia coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. In particular, the strongest inhibitory effect was observed against *S. pneumoniae*, with an average inhibition zone diameter of 20.37 mm, followed by *E. coli* and *S. aureus*, with average inhibition zone diameters of 14.35 mm and 10.98 mm, respectively ($P < 0.05$, Fig. 1H). This finding indicated that Ed may function as a potential probiotic, thereby necessitating further in-depth exploration of its probiotic capabilities.

2.2. *E. durans 98D ameliorated symptoms of colitis*

To assess the therapeutic efficacy of Ed against colitis, the mice were classified into three distinct groups: negative control (NC), control (Con), and Ed treatment (Ed). Both Con and Ed groups were consistently provided with water containing 2.5% DSS throughout the experiment. From day 15 onwards, mice in the Ed group were orally administered Ed, whereas those in the other two groups received sterile saline over a period of 10 d (Fig. 2A). The results showed that DSS administration notably decreased the body weight and colon length of mice and increased the disease activity index (DAI) score and spleen weight, suggesting the successful induction of colitis. ($P < 0.05$, Fig. 2B–F). Conversely, Ed treatment significantly mitigated the colitis-induced weight loss and spleen enlargement, increased colon length, and reduced the DAI score ($P < 0.05$, Fig. 2B–F). Furthermore, compared with that in the Con group, Ed treatment significantly augmented the thickness of the mucosal layer in the colon and number of goblet cells within the epithelium of the colon ($P < 0.05$, Fig. 2G–I). The aforementioned results implied that Ed treatment alleviated DSS-induced damage to the mechanical barrier, thereby ameliorating colitis.

2.3. *E. durans 98D modulated inflammatory cytokine gene transcripts levels and reinstated colonic barrier integrity*

To decipher the mechanism through which Ed alleviates colitis, the transcript levels of key inflammatory cytokines and tight junction proteins in mice colonic tissues were compared among all three groups. The results showed that Ed treatment significantly downregulated the transcript levels of pro-inflammatory cytokines such as *tumor necrosis factor-α* (*TNF-α*), *interleukin* (*IL*)*-1β*, and *IL-6* and upregulated the expression of the anti-inflammatory cytokine gene *IL-10* in colonic tissues ($P < 0.05$, Fig. 3A–D). Concurrently, the treatment of Ed led to a significantly upregulated the expression of tight junction protein genes, including *ZO-1*, *claudin-1,* and *occludin* in colonic tissues ($P < 0.05$, Fig. 3E–G). Collectively, these results indicated that Ed treatment alleviated the DSS-induced damage in the colonic immune barrier by modulating the expression of both pro- and anti-inflammatory cytokines in colonic tissues and

Table 1
Antibiotic susceptibility test results for *Enterococcus durans* 98D.

Antibiotics	Sensitivity criteria based on diameter (mm)			Measureddiameter (mm)	Antibacterial degree
	Resistant (R)	Moderately sensitive (I)	Highly sensitive (S)		
Penicillin G	≤14	–	≥15	23	S
Carbenicillin	≤19	20–22	≥23	25	S
Oxacillin	≤10	11–12	≥13	14	S
Ampicillin	≤16	–	≥17	22	S
Piperacillin	≤17	–	≥18	21	S
Medimycin	≤13	14–17	≥18	27	S
Doxycycline	≤13	14–22	≥23	27	S
Gentamicin	≤12	13–14	≥15	13	I
Neomycin	≤12	13–16	≥17	13	I
Minocycline	≤14	15–18	≥19	30	S
Tetracycline	≤14	15–18	≥19	29	S
Erythromycin	≤12	13–15	≥16	31	S
Cefoperazone	≤15	16–20	≥21	19	I
Cefuroxime	≤14	15–22	≥23	15	I
Cefradine	≤14	15–17	≥18	20	S
Cefazolin	≤14	15–17	≥18	20	S
Cephalexin	≤14	15–17	≥18	18	S
Vancomycin	≤14	15–16	≥17	26	S
Norfloxacin	≤12	13–16	≥17	16	I
Ofloxacin	≤12	13–15	≥16	20	S
Cycloprofluoric acid	≤15	16–20	≥21	21	S
Dysentrin	≤14	15–16	≥17	24	S
Chloramphenicol	≤12	13–17	≥18	31	S

Note: The inhibition zone diameter was determined by placing the antibiotic-soaked paper in the *Enterococcus durans* 98D culture dish, and the inhibition zone formed was measured using a caliper. The antibacterial degree was then compared with the standard diameters representing resistance, moderate sensitivity, and high sensitivity.

Table 2
Cell surface hydrophobicity and autoagglutination values of *Enterococcus durans* 98D.

Adhesion index	OD_{600} optical density				H (%) and AAg (%)
CSH	H0	0.603	0.621	0.605	39.14 ± 2.02%
	H	0.358	0.408	0.348	
AAg	A0	0.619	0.624	0.615	66.90 ± 0.72%
	A	0.194	0.211	0.210	

Note: CSH and AAg represent the percentage of cell surface hydrophobicity and autoagglutination, respectively. Data are mean ± standard error of the mean (SEM).

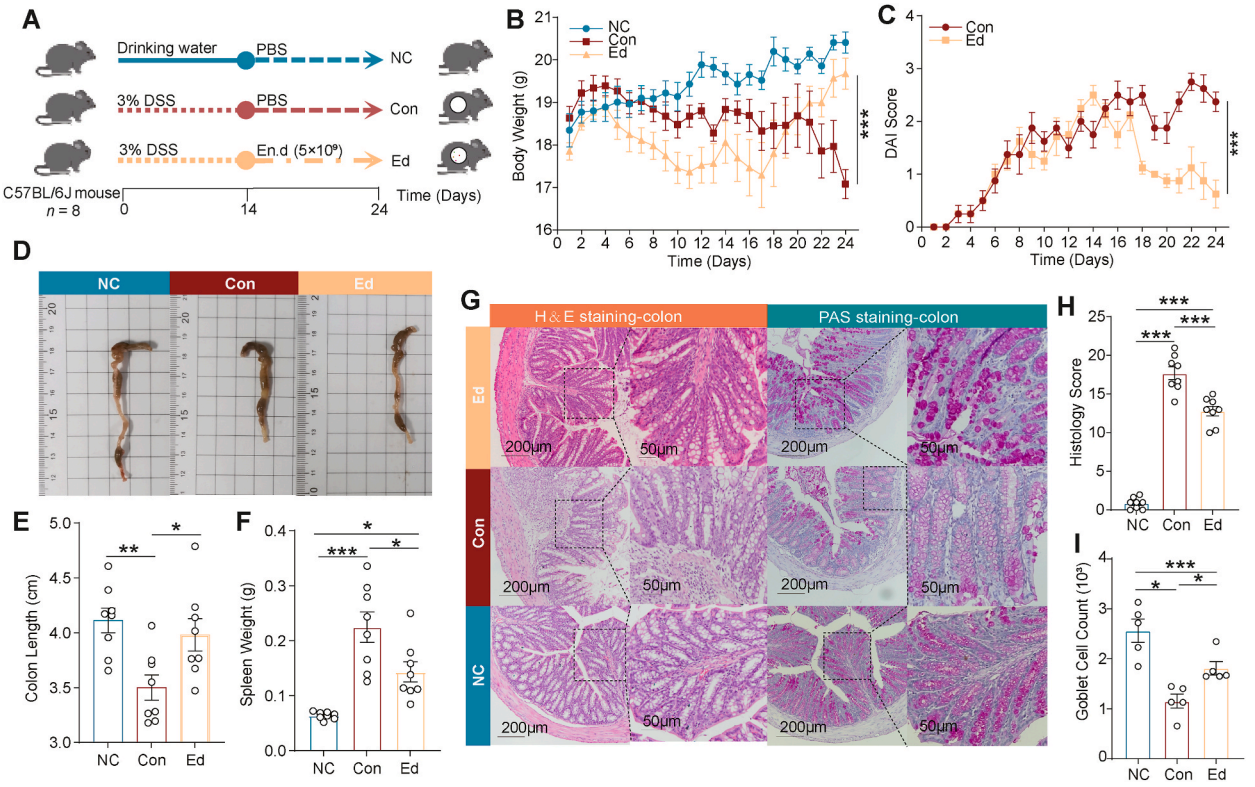

Fig. 2. *Enterococcus durans* 98D gavage ameliorates dextran sulfate sodium-induced colitis. (A) A schematic diagram of the experimental design using a mouse model of DSS-induced colitis. Mice (female) were given 2.5% DSS throughout the experimental period, whereas *E. durans* 98D was administered via gavage from day 15 to day 24. (B) Changes in the body weight of mice during the experiment. (C) Effect of *E. durans* 98D on the DAI score, (D, E) colon length (cm), and (F) spleen weight in mice with DSS-induced colitis. (G) Histological analysis of murine colon tissues following H&E and PAS staining (scale bar = 200 μm). (H) Histological scores and (I) goblet cell count of colon tissues from mice with DSS-induced colitis. Data are expressed as the mean ± SEM, $n = 5$–8. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (unpaired *t*-test or one-way ANOVA with Tukey's post hoc). DSS, dextran sulfate sodium salt; H&E, hematoxylin and eosin; NC, negative control; Con, positive control; Ed, *Enterococcus durans* 98D, DAI, disease activity index; PAS, periodic acid–Schiff; SEM, standard error mean.

augmenting the expression of tight junction protein genes.

2.4. *E. durans 98D reshaped colitis-caused dysbiosis of colonic microbiota in mice*

Colonic microbiota holds a significant role in the onset of colitis [18]. Hence, prior to investigating the influence of Ed on colonic microbiota in mice suffering from DSS-induced colitis, the DSS-induced disturbances in the structure of the colonic microbiota were explored. By conducting 16S rRNA gene sequencing on colonic contents, a notable decrease in colonic microbial diversity and richness in DSS-treated mice was identified, as indicated by the Shannon, Chao, and Sobs indices ($P < 0.01$, Fig. 4A–C). Similarly, principal coordinate analysis (PCoA) revealed significant changes within the community structure of colonic microbiota in DSS-treated mice (ANOSIM, r = 0.8164, $P = 0.001$, Fig. 4D). Further, liner discriminant analysis Effect Size (LEfSe) analysis was performed to identify biomarkers with differential abundance and biological consistency and found different abundant taxa at multiple phylogenetic levels in the NC and Con groups (Fig. 4E). Importantly, DSS treatment resulted in a significant reduction in the abundance of Bacteroidota (*P*

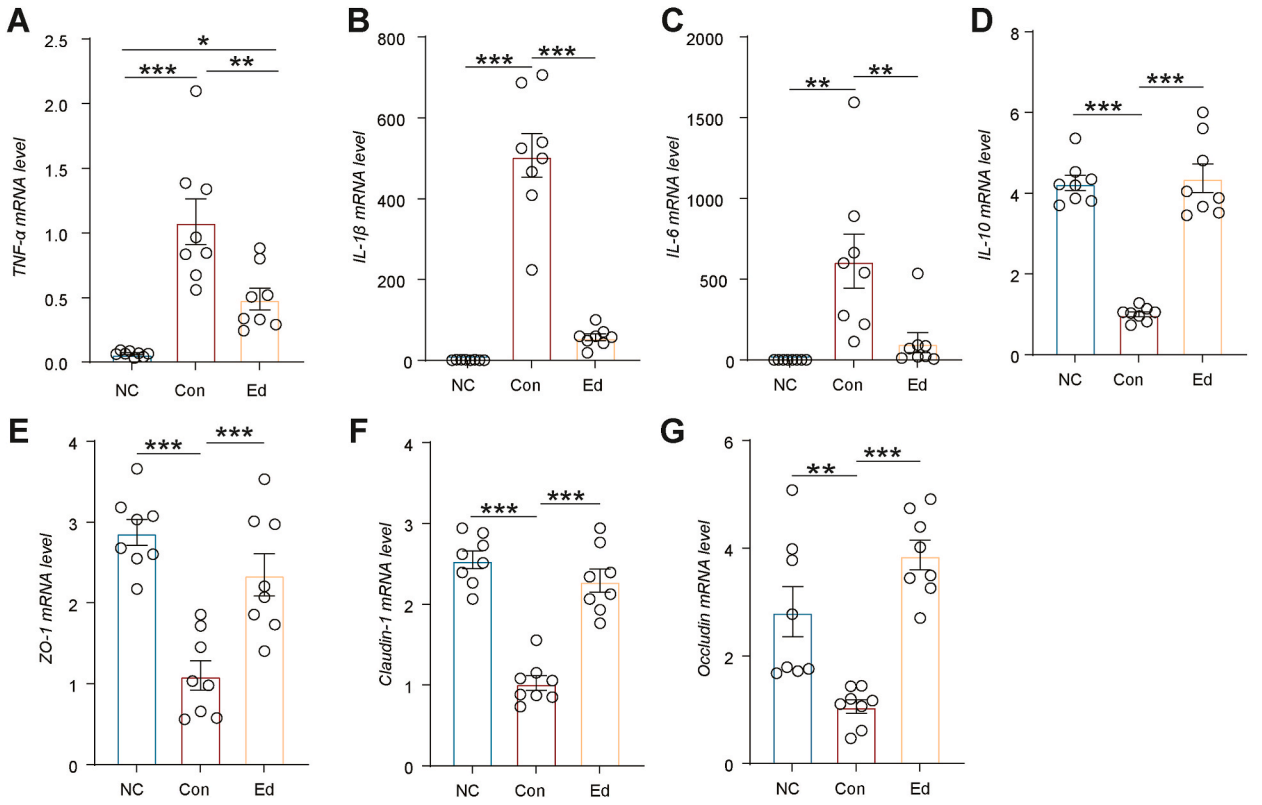

Fig. 3. *Enterococcus durans* 98D regulates inflammatory cytokine gene expression and reinforces the intestinal barrier. The qPCR analysis showing the expression of proinflammatory cytokine genes (A) *TNF-α*, (B) *IL-1β*, (C) *IL-6*, and (D) anti-inflammatory cytokine gene *IL-10* in colonic epithelial tissues. The qPCR analysis showing the expression of the tight junction protein genes (E) *ZO-1*, (F) claudin-1, and (G) occludin in colonic epithelial tissues. Data are expressed as the mean ± SEM, $n = 8$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (one-way ANOVA with Tukey's post hoc). NC, negative control; Con, positive control; Ed, *Enterococcus durans* 98D; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor; IL, interleukin; ZO, zonula occludens; SEM, standard error mean.

< 0.05) at the phylum level and markedly increased that of Pseudomonadota ($P < 0.01$) and Deferribacterota ($P < 0.01$, Fig. 4F), suggesting alterations in the composition of colonic microbiota in mice.

Furthermore, the influence of Ed on the DSS-induced disruption of the colonic microbiota composition was assessed. Continuous administration of Ed reversed the DSS-induced decline in microbiota richness and diversity and significantly altered the microbial community structure (Fig. 4A–D). Subsequently, the differences in microbial community structure at multiple taxonomic levels following Ed treatment were explored, revealing that Actinomycetota was the dominant phylum in the colons of mice with DSS-induced colitis after Ed treatment ($P < 0.01$, Fig. 4F). At the family level, the Con group exhibited a notable increase in the number of *Clostridiaceae*, whereas the abundance of the *Rikenellaceae*, *Prevotellaceae*, *Eggerthellaceae*, *Atopobiaceae*, and *Marinifilaceae* families was significantly increased following Ed treatment. ($P < 0.05$, Fig. 4G). Moreover, compared with that in the Con group, Ed treatment markedly enhanced the content of *Paraprevotella*, *Rikenellaceae_RC9*, and *Odoribacter* at the genus level ($P < 0.05$, Fig. 4H). In addition, Ed treatment significantly upregulated the abundance of potential probiotic amplicon sequence variants (ASV), such as ASV552 (*Paraprevotella*), ASV469 (*Alistipes*), and ASV622 (*Bifidobacterium*), while it downregulated the abundance of pathogenic ASVs such as ASV1502 (*Romboutsia*) ($P < 0.05$, Fig. 4I).

Using PICRUST2, a tool for predicting microbial function, 18 distinct pathways associated with the differentially abundant microbiota between the Con and Ed groups were identified. In the Con group, significant enrichment in pathways linked to diseases were found, such as Huntington's disease, prion disease, and amyotrophic lateral sclerosis ($P < 0.05$, Fig. 5A). In addition, pathways such as mitogen-activated protein kinase (MAPK) signaling pathway-yeast, D-arginine and D-ornithine metabolism, limonene and pinene degradation, and arachidonic acid metabolism were also significantly upregulated in the Con group ($P < 0.05$, Fig. 5A). Conversely, pathways such as the peroxisome proliferator-activated receptor (PPAR) signaling pathway, penicillin and cephalosporin biosynthesis, adipocytokine signaling pathway, Mycobacterium arabinogalactan biosynthesis, and lipoic acid metabolism were significantly enriched after Ed treatment ($P < 0.05$, Fig. 5B). Based on the comprehensive findings, Ed possesses the ability to alter the composition and structure of colonic microbiota in mice afflicted with colitis. In addition, Ed exhibited the ability to alleviate inflammation by regulating the expression of metabolic pathways in the colon. Given that Ed has low tolerance to bile salt concentrations, it may have a transitory rather than a colonizing role in the hindgut.

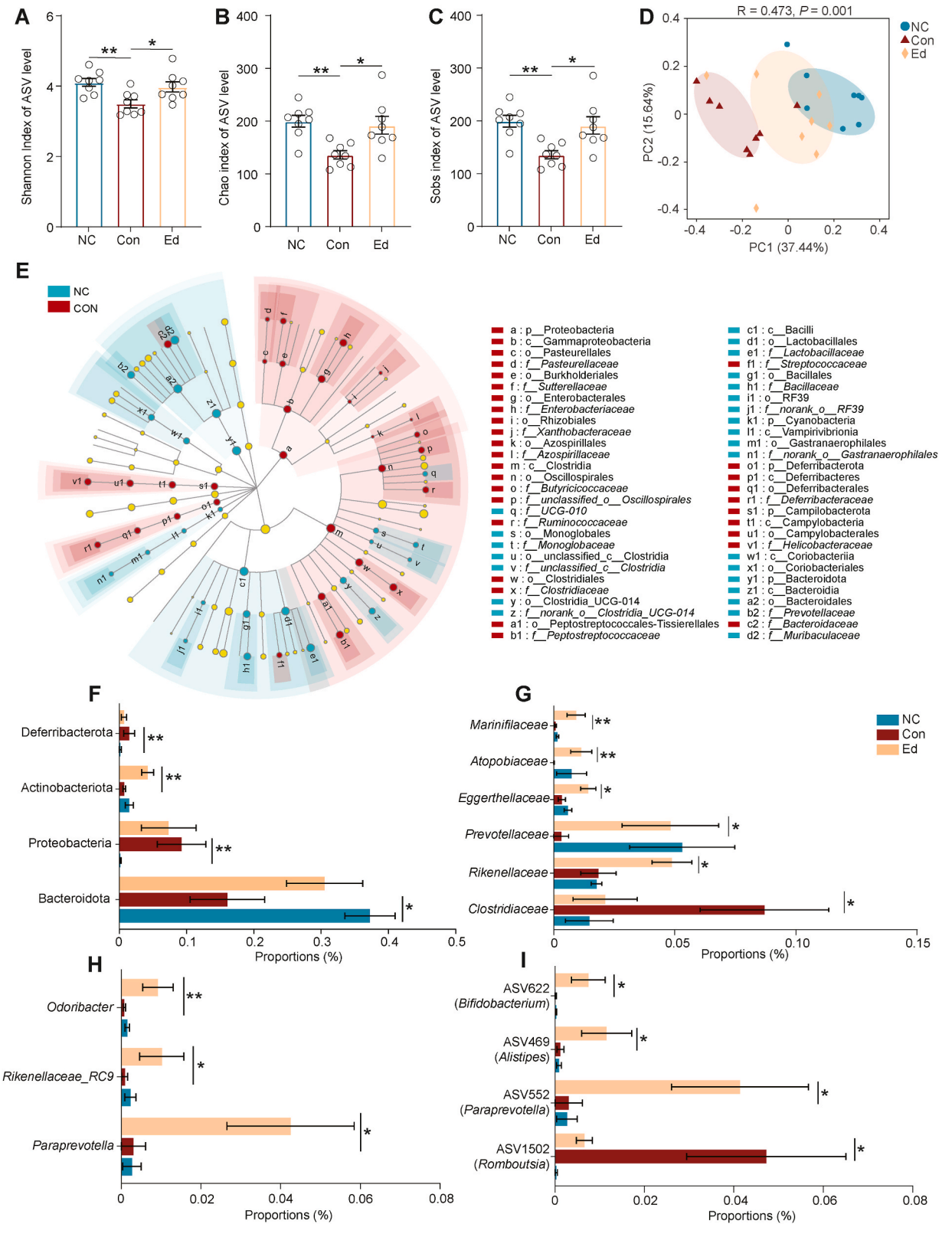

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Fig. 4. *Enterococcus durans* 98D alleviates dextran sulfate sodium-induced colitis in mice by reshaping the gut microbiota and their potential function. The α diversity based on (A) Shannon, (B) Chao, and (C) Sobs indices (female mice). Mice were euthanized after treatment, and colonic content samples were collected for 16S rDNA gene sequencing. Data were analyzed using the Kruskal–Wallis H test with Tukey–Kramer post hoc. (D) The principal coordinate analysis (PCoA) plot on the ASV matrix shows the β-diversity of the gut microbiota determined using ANOSIM. (E) Bacterial taxa identified as differentially abundant between groups according to LEfSe analysis. Comparison of the bacterial taxa from the phylum to family among NC and Con groups. Nodes with different colors represent bacterial taxa that are significantly enriched in the corresponding group and have a significant effect on the differences between groups. Yellow nodes indicate bacterial taxa that are not significantly different among different groups or have no significant effect on the difference between groups. (F) Administration of DSS causes a disturbance in colonic microbiota at the phylum level, whereas treatment with *E. durans* 98D significantly increases the relative abundance of Actinomycetota. Data were analyzed using the Wilcoxon rank-sum test. Significant differences in the relative abundance of representatives of (G) bacterial family, (H) genus, and (I) ASV (parts of relative abundance in the top 60) between the Con and Ed groups, analyzed using the Wilcoxon rank-sum test. Data are expressed as the mean ± SEM, $n = 8$. *$P < 0.05$, **$P < 0.01$. NC, negative control; Con, positive control; Ed, *Enterococcus durans* 98D; DSS, dextran sulfate sodium; ASV, amplicon sequence variants; PC, principal component; LEfSe, Linear discriminant analysis Effect Size; SEM, standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

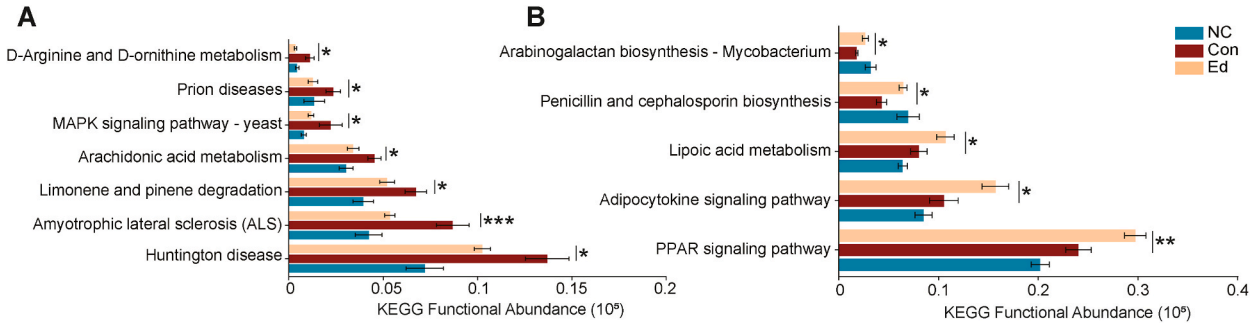

Fig. 5. Prediction of functional pathways in colonic microbiota based on Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis. The potentially upregulated functional pathways of colonic microbiota in the Con (A) and Ed (B) groups, based on PICRUSt2. Data are expressed as the mean ± SEM, $n = 8$. *$P < 0.05$, ***$P < 0.001$. (Wilcoxon rank-sum test). NC, negative control; Con, positive control; Ed, *Enterococcus durans* 98D; PPAR, peroxisome proliferator-activated receptor.

3. Discussion

The intestinal microbiota comprises numerous microbes, many of which can potentially act as probiotics owing to their ability to interact with the host [19]. However, potential probiotics need to adapt to the low pH of the gastric juice, tolerate the high bile salt concentrations, and resist various digestive enzymes [20]. The pH of the gastric juice is approximately 3.0, whereas the quantity of bile salts in the small intestine varies from 0.03 to 0.3% [21]. In this study, Ed tolerated the acidity of the gastric acid; however, its tolerance to bile salts was relatively low, which may have weakened its effect on the hindgut. In addition, digestive enzymes in the stomach, such as pepsin and trypsin, may inhibit the action of probiotics, and high temperatures during utilization may also affect them [22,23]. Nonetheless, Ed tolerated pepsin and trypsin after 4 h of culturing in De Man–Rogosa–Sharpe (MRS) broth, and its high-temperature resistance was in the range of 37–55 °C. Consequently, microencapsulation of Ed could be crucial for ensuring its effectiveness in the hindgut by guaranteeing that a sufficient amount of active Ed reaches the colon [24].

Resistance genes can be transferred horizontally among microbes, potentially accelerating their transmission [25–28]. Therefore, candidate probiotics should be sensitive to common antibiotics. In this study, the sensitivity of Ed to 23 antibiotics was tested and recealed that Ed was moderately sensitive to aminoglycosides, some cephalosporins, and a few quinolones, whereas it remained highly sensitive to other selected antibiotics. Some probiotics trigger endocarditis and hemolysis [29]; however, Ed did not cause hemolysis in this study. The evaluation criteria for bacterial AAg and CSH define high CSH and AAg rates as those over 30% and 50%, respectively [30,31]. Previous studies determined the CSH and AAg of 14 potential probiotic *Bifidobacterium* strains and found that the CSH of 9 *Bifidobacterium* strains was below 40%, with AAg ranging from 20.22% to 67.56% [32]. Additionally, the potential probiotic *E. faecium* strains exhibited CSH ranging from 14 to 16% and AAg below 65% [9]. In our study, Ed demonstrated CSH and AAg of 37.14% and 66.90%, respectively, indicating its strong adhesion and cohesion abilities, which facilitate its colonization in the intestine [33]. In addition, probiotics must modulate the host microbial composition and inhibit the adhesion of pathogenic bacteria [34]. Therefore, the inhibitory effects of Ed against *E. coli*, *S. pneumoniae*, and *S. aureus* were ecaluated. Ed inhibited the growth of all three pathogenic bacteria, with the most significant inhibitory effect observed against *S. pneumoniae*. Several genes related to acid resistance and antioxidant activity have been identified in *E. durans* strains, supporting their potential probiotic effects [7,35]. Moreover, genes encoding two fibronectin-binding proteins and an S-layer protein, surface factors implicated in cell adhesion and intestinal colonization without aggressive virulence, are present in the *E. durans* genome [7]. Furthermore, the presence of a potential type II-A CRISPR and restriction-modification system in *E. durans* ensures the stability of its genome and supports its optimal function as a probiotic [7]. The genome of *E. durans* OSY-EGY contains genes related to acid and bile salt resistance, antioxidation, antibiosis, as well as synthesis

of essential amino acids and vitamins [36]. Thus, considering the findings of previous genomic studies and our *in vitro* safety assessment, *E. durans* should be further evaluated for its probiotic function.

Notably, DSS-induced colonic inflammatory phenotypes are characterized by weight loss and the formation of inflammatory lesions in the colonic epithelium [37]. Butyrate facilitates the interplay between goblet cells and macrophages within the colon and suppresses the expression of hexokinase 2, thereby fostering the repair of the colonic epithelial barrier and mitigating colitis. Therefore, as a potential butyrate-producer, *E. durans* may also play the above roles [38]. In addition, goblet cells in the colonic epithelium secrete mucin [39]. The absence or inability of the colon to secrete mucin can lead to the development of idiopathic colitis because of the direct interaction between commensal bacteria and the colonic epithelium [39]. In this study, the therapeutic potential of Ed in colitis mice was examined. Administration of Ed effectively mitigated the decrease in body weight and reduction in colon length and significantly ameliorated the DAI score and splenomegaly. Furthermore, Ed treatment significantly increased the histopathological score and goblet cell count in mice with colitis. Collectively, Ed significantly alleviated the number of colonic lesions and repaired damage to the colonic mucosa in mice with colitis.

Ed potentially alleviates colitis via various mechanisms, including suppressing the expression of proinflammatory cytokines, enhancing that of anti-inflammatory cytokines, and modulating the function of Tregs [14]. Our study revealed that treatment with Ed effectively normalized proinflammatory cytokine gene expression in the colon, bringing it to levels akin to those seen in the NC group. IL-10, which is primarily produced by Tregs in the mucosal lining of the intestines [40–42], plays a critical role as an immunomodulatory factor by inhibiting the release of proinflammatory cytokines. The present investigation observed a notable increase in *IL*-10 mRNA expression in colonic tissues following Ed treatment compared with that in the Con group. Colitis is the outcome of the disturbance to the mechanical barrier of the colonic epithelium [43]. The maintenance of the colonic mechanical barrier depends on tight junction proteins, such as claudin, occludin, and ZO-1. Disruptions in their expression and distribution significantly contribute to colitis development [44]. Our study revealed a substantial elevation in *ZO-1*, *claudin-1*, and *occludin* transcription levels in the colonic mucosa of colitis-afflicted mice following Ed treatment. In summary, the outcomes of our investigation suggest that Ed possesses the potential to mitigate DSS-induced colitis in mice by regulating the release of inflammatory cytokines and fortifying the mechanical integrity of the intestinal barrier.

The colonic microbiota of the host is vital for colitis pathogenesis and remission; hence, the protective effects of probiotics may be attributed to their effects on microbiota composition and metabolism [18,45]. The intestinal microbial community modulates colitis by concomitantly influencing the nucleotide oligomerization domain-like receptor family pyrin domain containing 6 inflammatory vesicle signaling, the secretion of epithelial IL-18 and downstream antimicrobial peptides such as taurine, histamine, and spermine [46]. Our study suggested that Ed treatment resulted in a significant increase in the proportion of Actinomycetota, along with the operational genera *Paraprevotella*, *Rikenellaceae_RC9*, and *Odoribacter*. Actinomycetota play a role in maintaining colonic environmental balance and are implicated in regulating colonic permeability, immune function, metabolism, and the gut-brain axis [47]. Mice with colitis exhibited a marked reduction in bacteria responsible for the production of short-chain fatty acids in the gut [48]. Prior research has established that *Paraprevotella*, in conjunction with other functional bacteria present in the colon, is capable of producing propionic acid, which, in turn, stimulates the generation of Tregs that effectively control colitis [48]. In addition, *Paraprevotella* prevented the development of colitis by secreting succinic and acetic acid [48]. Similarly, *Rikenellaceae_RC9* protected cells from oxidative stress by selectively neutralizing cytotoxic reactive oxygen species through its hydrogen-producing activity, thereby alleviating colitis symptoms [49]. Moreover, mice deficient in the myeloid intrinsic and acquired immune signaling molecule transforming growth factor-β activated kinase 1and with intestinessignificantly enriched in *Odoribacter*, were shown to be completely resistant to inflammatory bowel disease (IBD) and colon cancer [50]. *Odoribacter* confers resistance to IBD and colon cancer by inducing the generation of T helper 17 cells and production of inflammatory factors [50]. Thus, Ed-mediated inhibition of colitis may be achieved by increasing the relative abundance of synergistic probiotics.

PPARs, including PPARγ, PPARγ, and PPARγ, are a group of ligand-activated nuclear transcription factors [51]. Mice lacking PPARγ are more susceptible to chronic inflammation and insulin resistance than wild-type mice [52]. PPARγ exerts its anti-inflammatory effects by inhibiting the transcriptional activities of nuclear factor kappa B, signal transducer and activator of transcription 1, and activator protein 1, as well as reducing the production of proinflammatory cytokines such as TNF-α and IL-6 [53]. Importantly, synthetic PPARγ agonists also alleviate the symptoms of colitis [46]. The MAPK signaling pathway, comprising extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase 1/2/3, p38-MAPK, and ERK5 cascades, significantly influences intestinal inflammation by regulating the expression of inflammatory cytokines and modulating the activation of inflammatory mediators [54]. In this study, the PPAR signaling pathway was significantly upregulated in Ed-treated mice, whereas the MAPK signaling pathway was activated in Con mice. However, owing to limitations in the sequence length, our study did not investigate specific microbial taxa at the species level. Future studies are needed for evaluating the specific functions of each microbial taxon and their effects on colitis development.

To conclude, our findings collectively highlighted the potential of Ed as a safe and effective probiotic for ameliorating colitis, mediated through its multifaceted actions, including immunomodulation, intestinal barrier restoration, and positive modulation of the gut microbiota. The implications of our study extend to the broader scientific and medical community, facilitating future research on probiotics, gastrointestinal health, and the development of targeted interventions for colitis.

3.1. Limitations

This study has certain limitations. Due to the low tolerance of Ed to bile salts, its abundance may decrease after passing through the small intestine. Therefore, subsequent studies should quantitatively determine the abundance of Ed in the small intestine and hindgut

to establish the effective concentration of the bacterium. In addition, the butyrate production mechanism mediated by Ed and the mechanism by which butyrate inhibits colitis are worth further investigation through whole-genome sequencing and model animals. Finally, considering that this study did not investigate the changes in the colonic microbial metabolome and colonic epithelial transcriptome mediated by Ed in colitis mice, the comprehensive characterization of the mechanisms by which Ed treats colitis was not established. Hence, this should be a focus of future research.

4. Materials and methods

4.1. Isolation of E. durans 98D using improved Brucella broth Base medium

Fecal samples were obtained from healthy goats reared on the livestock farm of Northwest A&F University using the standardized method of abdominal massage close to the anus. After sample collection, the goats were returned to the farm for continued rearing. Fecal samples were swiftly placed into sterile cryopreservation tubes filled with 30% glycerol (Sangon Biotech, Shanghai, China) and instantly frozen using liquid nitrogen and stored at −80 °C. Each fecal sample (1g), was immersed in phosphate-buffered saline (PBS; Sangon Biotech) and diluted by a factor of 10^8. The Brucella Broth Base medium (Solarbio, Beijing, China) was fortified with 0.01‰ heme chloride (Shanghai Yuanye Bio-Technology, Shanghai, China), 0.01‰ vitamin K1 (Sangon Biotech) and 5% defibrinated sheep blood (Solarbio) for enhancing culture conditions. A 50-μL aliquot of every sample was spread onto solid medium and incubated aerobically for 48 h at 37 °C. Individual colonies were subsequently isolated to identify the species, resulting in the acquisition of the desired strains.

4.2. DNA isolation and bacterial identification

Genomic DNA was obtained from isolated strains using the TIANamp Bacterial DNA Kit (DP302-02, Tiangen, Beijing, China). The DNA samples were diluted and thereafter utilized as templates for polymerase chain reaction (PCR) analysis. The 16S rRNA was PCR amplified (TransGen 2 × EasyTaq PCR SuperMix, TransGen Biotech, Beijing, China) using the forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-TACGGYTACCTTGTTACGACTT-3′. The PCR products underwent agarose gel electrophoresis analysis and then sequencing at AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China). Species identification was performed using the BLAST + tool (version: 2.14.0, https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). The strain identified as Ed was enriched in culture and purified. The purified Ed strain was deposited at the China Center for Type Culture Collection (Wuhan, China) under the collection no.: M20221217. Initial identification of the Ed strain was conducted based on Gram staining, morphological examination on Petri dishes, and electron microscopy.

4.3. Preparation and assessment of growth activity of E. durans 98D

The purified Ed was cultured aerobically for 24 h at 37 °C; subsequently, 50 mL of Ed culture was centrifuged for 10 min at 12 000×*g* and 4 °C. The supernatant was discarded and the pellet was rinsed with PBS before being resuspended in normal saline. Subsequently, it was serially diluted to adjust the Ed concentration for safety assessment and intragastric administration. To gauge growth activity, culture inoculates of Ed were introduced into MRS broth (2% w/v) (Solarbio), and the OD_{600} was recorded at 2-h intervals throughout the day.

4.4. Resistance of E. durans 98D to a simulated gastrointestinal environment

The adaptability of Ed within the gastrointestinal tract was gauged by simulating the usual concentrations of acid, bile salts, pepsin, and trypsin found in the gut. For the acid and bile salt resistance assays of Ed, a tailored MRS broth (10% w/v) was utilized. The pH of the broth was set to 2, 3, 4, and 5 using 1.0 M HCL, to which bacterial cultures were introduced and incubated for 4 h at 37 °C. Incubation in MRS broth (10% w/v) at pH 7 served as the control condition. For the bile salt resistance assay, bacterial solutions were combined with MRS broth (10% w/v) containing 0.15%, 0.3%, and 0.6% bile salts (Solarbio), and incubated for 8 h at 37 °C. Incubation in MRS broth (10% w/v) with no bile salts served as the control condition. The optical density of every solution at 600 nm was determined using a spectrophotometer (DengkePray, Beijing, China), and the survival rate was calculated using the formula:

$$\text{Survival rate } (\%) = (\text{OD600 } of \text{ experimental group}) \,/\, (\text{OD600 of control group}) \times 100\%$$

For assessing the tolerance of Ed to trypsin and pepsin, a modified version of a previously described protocol was employed [55]. An overnight culture was centrifuged (8000×*g*, 10 min, 4 °C), and cells were collected and washed twice with PBS. Bacterial concentration was set at 10^{-8} CFU/mL and cells were suspended in either MRS broth (2% w/v) enriched with 1 mg/mL pepsin (1:3000, BIOFROXX, Guangzhou, China) with a pH of 3.0 (adjusted with 1.0 M HCl) or MRS broth (2% w/v) enriched with 1 mg/mL trypsin (1:250, BIOFROXX, Guangzhou, China) with a pH of 8.0 (adjusted with 0.01 M NaOH). The cell suspension was incubated for 4 h at 37 °C, and absorbance at OD_{600} nm was recorded at 0 and 4 h.

4.5. Tolerance to temperature

As previously described, Ed inoculates of overnight cultures were introduced into MRS broth (2% w/v) and incubated at 37, 40, 50, 60, and 70 °C for 20 min; incubation at 37 °C served as the control condition. Following an 8-h incubation at 37 °C, the OD_{600} of each culture was measured.

4.6. Antibiotic susceptibility testing

The disk diffusion approach was employed for assessing the antibiotic susceptibility of isolates [56]. Overnight-grown Ed cultures were spread onto MRS agar plates. In this experiment 23 antibiotic sensitivity disks were tested, namely, penicillin G (10 U), carbenicillin (100 μg), oxacillin (10 μg), ampicillin (10 μg), piperacillin (100 μg), medimycin (30 μg), doxycycline (15 μg), gentamicin (10 μg), neomycin (30 μg), minocycline (30 μg), tetracycline (30 μg), erythromycin (30 μg), cefoperazone (75 μg), cefuroxime (30 μg), cefradine (30 μg), cefazolin (30 μg), cephalexin (30 μg), vancomycin (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), cycloprofluoric acid (5 μg), dysentrin (300 IU), and chloramphenicol (30 μg) (all from Hangzhou Biotechnology Company, Hangzhou, China). The inhibition area was measured following a 24-h incubation at 37 °C.

4.7. Hemolysis activity

Following overnight culturing, the Ed strain was inoculated via streaking on blood agar plates enriched with 5% defibrinated sheep blood. The hemolytic activity of Ed was determined following a 24-h incubation at 37 °C; *S. aureus* was employed as a positive control.

4.8. Surface hydrophobicity and autoagglutination analysis of E. durans 98D

To assess the CSH, a protocol from a previous study was employed with slight modifications [57]. Bacterial cells in the mid-exponential growth stage were rinsed with PBS, and the OD_{600} of the bacterial suspension was set to 0.6 (H0). Subsequently, 2 mL bacterial suspension was mixed with 0.4 mL xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and incubated for 1 h at 24 °C for ascertaining the OD_{600} of the aqueous phase. Surface hydrophobicity (%) was calculated using the following equation:

$$H\ (\%) = 1 - (H\ /\ H0) \times 100\%$$

A method for estimating the percentage of AAg in bacterial cells has been reported previously [57]. For our experiment, cells that were cultured overnight were washed twice with PBS, resuspended in PBS, and adjusted to an OD_{600} value of approximately 0.6. The initial optical density was recorded as A0. The bacterial suspension was kept in a tube at 24 °C for 20 h and the final optical density was recorded as A. The percentage of AAg was calculated using the following formula:

$$AAg\ (\%) = 1 - (A\ /\ A0) \times 100\%$$

In vitro antibacterial assay.

The effectiveness of the Ed strain against pathogenic microorganisms was evaluated using an enhanced agar-well diffusion method [58]. The indicator bacteria included *E. coli* O157 (China General Microbiological Culture Collection Center, Beijing, China) and *S. pneumoniae* CCUG 37328 and *S. aureus* ATCC 25923 from the College of Veterinary Medicine, Northwest A&F University (Yangling, China). Indicator pathogenic bacteria were individually cultured on Luria-Bertani (LB; Solarbio) broth agar plates, and single colonies were subsequently isolated and activated over a 24-h period. The bacterial count within each pathogenic culture was quantified and normalized to 1×10^8 CFU/mL. Thereafter, 100 μL of each pathogenic bacterial culture was uniformly spread onto LB broth medium, and Oxford cups (8 mm in diameter) were positioned within the medium. Each cup was then inoculated with 100 μL of Ed, standardized to a concentration of 1×10^8 CFU/mL (culture conditions: 1% inoculum, 37 °C, incubation for 24 h, 4 °C, 12 000×*g* centrifugation for 15 min). The entire assembly was subsequently incubated for 24 h at 37 °C to facilitate the determination of the inhibition area.

5. Experimental animals

In total, 24 female C57BL/6 mice at an average age of 8 weeks, were provided by Chongqing Tengxin Biotechnology Co., Ltd (Xi'an, China). The mice were kept at the Animal Experiment Center of Northwest A&F University under specific pathogen-free conditions with a 12-h light/12-h dark cycle. The temperature was kept at a steady level of 23 ± 1 °C and humidity at a level of 55 ± 5%. The mice were provided *ad libitum* access to food and water. The investigation was conducted with the appropriate ethical authorization from the Institutional Animal Care and Use Committee of Northwest A&F University (permission number: 2021-06-010).

5.1. Chronic colitis model and treatment strategies

For inducing colitis in mice, a solution of drinking water containing 2.5% (w/v) DSS (molecular weight of 36 000–50,000 Da; MP Biomedicals, Santa Ana, CA, USA) was administered to the animals for a duration of 24 d [59]. Starting from day 15, mice in the Ed group (DSS + Ed) received an oral gavage of 5×10^9 CFU of Ed suspended in 200 μL sterile PBS, whereas each mouse in the NC and Con

(DSS + PBS) groups received an even volume of sterile PBS. This treatment regimen was maintained for 10 d. When the experiment ended, all mice were deeply anesthetized with 3% sodium phenobarbital by experienced researchers and then euthanized via rapid cervical dislocation after confirming the loss of toe pinch reflex. Daily monitoring of body weight, food intake, and diarrhea scores was conducted throughout the experimental duration. The DAI score was calculated using a standardized protocol [60]. The severity of rectal bleeding was assessed using a grading system, where a score of 0 indicated normal feces, 1 indicated brown stool, 2 indicated reddish stool, and 3 indicated bloody stool. The scoring system for diarrhea was as follows: 0 denoted well-formed feces, 1 indicated mildly soft stool, 2 represented quite soft stool, and 3 signified watery stool.

Staining of colonic epithelial tissue with hematoxylin and eosin (H&E) and Alcian blue periodic acid–Schiff (AB-PAS)

The colon tissue samples were conserved in a solution containing 4% paraformaldehyde, subsequently embedded in paraffin, and sectioned for histological assessment. Pathological investigation involved the utilization of H&E staining to assess the tissue samples, whereas AB-PAS staining was employed to quantify the population of goblet cells within the colon. All slides were assessed by two individual investigators, who evaluated various parameters, including inflammatory infiltration, goblet cell loss, crypt density, muscle thickening, submucosal inflammation, crypt abscess, and ulceration. The evaluation used a predetermined scale ranging from 0 to 4 [61]. Quantification of goblet cells was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

5.2. RNA extraction and quantitative real-time PCR (qRT-PCR)

The TRIzol reagent (Cwbio, Taizhou, China) was used to isolate total RNA from mice colonic epithelial tissue samples. Subsequently, total RNA was reverse-transcribed into complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Sunnyvale, CA, USA). The qRT-PCR was conducted on a Light Cycler®96 Real-Time PCR System (Roche cobas 6800; Roche, Basel, Switzerland) using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and using *β-actin* as the reference gene. The primers for our study were created, initially validated using the Oligo 7 software and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and finally synthesized by Zhongke Yutong (Xi'an, China). Primer details are available in Table 3. The comparative cycle approach ($2^{-\Delta\Delta Ct}$) was utilized for assessing the relative mRNA expression levels of target genes.

5.3. DNA isolation, PCR amplification, and 16S rRNA gene sequencing

DNA was isolated from fecal samples using the E.Z.N.A. feces DNA Kit (Omega Bio-tek, Norcross, GA, USA). DNA concentration and purity were determined using the Nanodrop 2000 UV-VI spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA quality was assessed by 1% agarose gel electrophoresis. The V3–V4 16S rDNA region was amplified with the primer pair 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). After purification, amplicons were combined in equimolar ratios for subsequent high-throughput sequencing on the Illumina Miseq platform using 300 paired ends at Majorbio BioTech Co. (Shanghai, China).

5.4. 16S rRNA gene data analysis

Analysis was conducted on the Majorbio Cloud Platform (www.majorbio.com) using recognized bioinformatics methodologies [62]. In summary, the sequencing data underwent multiplexing usingFLASH software (version 1.2.7), and subsequently, the paired-end reads were combined using the FASTP software (version 0.19.6) [63]. Subsequently, all sequences underwent a series of procedures, including quality filtering, denoising, and merging. Chimeras were eliminated using the DADA2 plugin on the QIIME2 platform [64]. To account for the potential influence of sequencing depth on the analysis of alpha and beta diversity, the read count per sample was standardized to a value of 13 981. The obtained data revealed an average coverage of Good's at a rate of 99.98%. The classification of taxonomic ASVs was achieved through the application of naive Bayes consensus classifier on the SILVA 138/16s_bacteria database in QIIME2.

5.5. Statistical analysis

All data are displayed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the Wilcoxon rank-sum, one-way analysis of variance (ANOVA), and Kruskal–Wallis H tests. The α-diversity of microbial communities across two or three groups was tested using the Wilcoxon rank-sum test or Kruskal–Wallis H test, with Benjamini-Hochberg (BH) multiple testing adjustments. PCoA was conducted using the Weighted_Unifrac distance algorithm, and differences between groups were evaluated using ANOSIM with 999 permutations. For comparisons at the phylum, family, genus, and ASV levels across two groups, the Wilcoxon rank-sum test was employed, followed by BH multiple testing adjustments. Multilevel species LEfSe analysis was conducted using a one-against-all multigroup comparison strategy with a linear discriminant analysis threshold set to >4.0. Data analysis was conducted using SPSS (version 26.0, SPSS Inc., Chicago, IL, USA), with group differences evaluated using one-way ANOVA, followed by a post hoc Tukey's multiple comparison test. All findings were expressed as the mean ± SEM [65]. Statistical significance was set at $P < 0.05$, with the level of significance being indicated by asterisks in figures ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Graphs were created using Prism 9.0 (GraphPad, La Jolla, CA, USA) (https://www.graphpad.com/).

Table 3
Primers used in quantitative polymerase chain reaction (qPCR).

Goal	Genes	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product Length
Reference gene	*β-actin*	AGGGAAATCGTGCGTGACAT	GGAAAAGAGCCTCAGGGCAT	172
Pro-inflammatory cytokine genes	*TNF-α*	AGGCACTCCCCCAAAAGATG	TTTGCTACGACGTGGGCTAC	250
	IL-6	AATAGTCCTTCCTACCCCAA	GCTTAGGCATAACGCACT	167
	IL-1β	AACTGCACTACAGGCTCCGAGA	GCCACAGGTATTTTGTCGTTGCTT	163
Anti-inflammatory cytokine gene	*IL-10*	GCCGGGAAGACAATAACTGC	GCCTGGGGCATCACTTCTAC	223
Tight junction protein genes	*ZO-1*	GCCGCTAAGAGCACAGCAA	GCCCTCCTTTTAACACATCAGA	172
	claudin-1	GGCCTTGGCTGTACCTTACC	GGAGCACCTTATCCCCGTTT	109
	occludin	TTGAAAGTCCACCTCCTTACAGA	CCGGATAAAAAGAGTACGCTGG	129

Note: TNF, tumor necrosis factor; IL, interleukin; ZO, zonula occludens.

Ethics approval and consent to participate

All study experimental methods employed in our study were approved by the Institutional Animal Care and Use Committee of Northwest A&F University, with the permit no. 2021-06-010. Moreover, all methods, animal care, selection, collection of data, and data analysis were performed following the guidelines and regulations outlined in the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines). All authors were aware of and agreed to the conduct of all animal experimental procedures.

Consent for publication

Not applicable.

Availability of data and material

The 16S rRNA gene sequencing samples can be accessed on NCBI under the accession no. PRJNA952376.

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CRediT authorship contribution statement

Yu Lei: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Yiting Yan:** Visualization, Resources, Investigation. **Junyu Zhong:** Visualization, Methodology, Investigation. **Yitong Zhao:** Visualization, Methodology, Investigation. **Yangbin Xu:** Visualization, Resources, Investigation. **Ting Zhang:** Resources, Investigation. **Hui Xiong:** Resources, Methodology, Investigation. **Yulin Chen:** Writing – review & editing, Conceptualization. **Xiaolong Wang:** Writing – review & editing. **Ke Zhang:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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