Original Article

Impact of Re-Du-Ning enema treatment on the intestinal microflora in pediatric patients with hand, foot, and mouth disease

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Background: Hand, foot, and mouth disease (HFMD) is a prevalent infectious condition in children. This study aimed to assess the regulatory effects of Re-Du-Ning on the intestinal microflora of pediatric patients with HFMD.

Methods: Fecal samples were collected from children affected by HFMD, who were diagnosed at the traditional Chinese medicine pediatrics outpatient and emergency departments of Liuzhou Women and Children's Healthcare Hospital, as well as from healthy children undergoing physical examinations at the same hospital during the same period. DNA was extracted from these samples and subjected to 16S ribosome DNA amplicon sequencing. The sequenced data were categorized, quantified, and compared. Analyses involved creating relative abundance bar graphs, constructing unweighted pair-group method with arithmetic mean clustering trees, and generating heatmaps of clustering to evaluate the variations in abundance and diversity across different groups. The analysis of molecular variance and *t*-test were used to analyze structural differences in microbial flora between groups, and linear discriminant analysis was used to identify significant differences in microbial genera between the groups.

Results: A total of 67 fecal samples were collected from children with HFMD (13 in the intravenous group, 40 in the enema group) and from healthy children (14 in the healthy group). When compared with the healthy group, the intestinal microflora diversity and similarity were highest after enema treatment, although the microbial structure exhibited significant changes (weighted_unifrac, $P<0.05$). The composition of species relative abundance was comparable between the healthy group and the post-enema group.

Conclusions: Re-Du-Ning enema treatment regulated the intestinal microflora in these children, significantly increasing the abundance of probiotics like *Bifidobacteria* and reducing the abundance of opportunistic pathogens like *Enterobacter*.

Keywords: Children; enema; hand, foot, and mouth disease (HFMD); intestinal microflora; Re-Du-Ning

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Introduction

Hand, foot, and mouth disease (HFMD) is a prevalent infectious condition in children, particularly among preschoolers. It results from acute viral infections like enterovirus 71 (EV71) and coxsackievirus A16 (CA-16), among other enteroviruses (1). The primary site for enterovirus entry and proliferation is the intestinal mucosa. Enteroviruses compromise the intestinal mucosal barrier, impairing the body's immune function and facilitating the onset of related symptoms (2,3). With the rapid advancement of gut microbiota science and the widespread application of high-throughput sequencing technologies,

the relationship between HFMD and intestinal microflora has garnered increasing attention. Current research has demonstrated that children with HFMD exhibit significant dysbiosis in their gut microbiota, characterized by a decrease in overall gut microbial diversity, accompanied by a reduced abundance of butyrate-producing bacterial genera (e.g., *Bifidobacterium*, *Ruminococcus*, and *Roseburia*), and an increased presence of opportunistic pathogenic bacteria (e.g., *Escherichia* and *Enterococcus*) (4-6). With the advancement of traditional Chinese medicine (TCM) in the treatment of digestive tract diseases (7,8), TCM enema therapy, which involves administering medicinal solutions directly into the rectum to apply the absorptive properties of the intestinal mucosa, has emerged as an effective treatment approach (9). Considering that administering TCM injections through the intravenous route has been controversial due to potential side effects, including allergic reactions, it has been found that rectal administration typically results in fewer allergic reactions and is considered safer. The Re-Du-Ning injection, a modern TCM formulation consisting of honeysuckle, artemisia, and gardenia, is recognized for its detoxifying, heat-clearing, and wind-expelling effects (10). In this study, we aimed to assess the regulatory effects of the Re-Du-Ning enema on the intestinal microflora of children with HFMD, providing a foundation for its application in treatment. We present this article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-24-257/rc).

Highlight box

Key findings

- Re-Du-Ning has the effect of regulating intestinal flora in children with hand, foot, and mouth disease (HFMD), with a significant increase in the abundance of probiotics such as *Bifidobacteria* and a significant decrease in the abundance of conditionally pathogenic bacteria such as *Enterobacteriaceae* after treatment.

What is known and what is new?

- The altered intestinal flora in HFMD is mainly characterized by a decrease in the overall diversity of the intestinal flora, accompanied by a decrease in the abundance of butyrate-producing genera and an increase in the abundance of opportunistic pathogenic genera.
- Re-Du-Ning enema treatment regulated the intestinal microflora in these children, significantly increasing the abundance of probiotics like *Bifidobacteria* and reducing the abundance of opportunistic pathogens like *Enterobacter*.

What is the implication, and what should change now?

- Re-Du-Ning enema can be used as a treatment in the children with HFMD to help clinicians implement a more comprehensive treatment.

Methods

General information

Participants

Participants for this study were recruited from the TCM pediatrics outpatient and emergency departments of Liuzhou Women and Children's Healthcare Hospital, where children diagnosed with HFMD and healthy children undergoing physical examinations during the same period were identified. The study initially screened 87 cases, of which 76 were ultimately finalized and randomly allocated into two groups, the Re-Du-Ning enema group and the Re-Du-Ning intravenous group, using a simple randomization method. The enema group comprised of 38 patients with no dropouts, including 27 males and 11 females, aged between 2.1 to 9.3 years, with an average age of 3.956± 1.559 years. The intravenous group consisted of 38 patients with 4 dropouts, including 19 males and 15 females, aged between 2.1 to 9.4 years, with an average age of 4.147± 2.317 years. Statistical analysis revealed no significant difference in age (P=0.18) or gender distribution (P=0.18) between the two groups, indicating comparability. By comparing symptom scores for fever, rash, cough, runny nose, appetite, bowel movements, and tongue/pulse characteristics between the two groups before and after treatment, no significant difference was found between the groups before treatment, indicating comparability (P=0.42).

Due to the challenges associated with collecting fecal samples from children in a clinical setting, samples from all participants could not be obtained. Based on the requirement for three biological samples, a total of 67 fecal samples that passed final quality control were collected for analysis, comprising HFMD-affected children before and after treatment, as well as age-matched healthy children undergoing physical examination during the same period. Specifically, 40 samples were obtained from the enema group, 13 from the intravenous group, and 14 from the healthy group. Following stringent quality control measures, 33 samples from the enema group, 10 from the intravenous group, and 11 from the healthy group were included in the final analysis. All samples were promptly stored at −80 ℃ within one hour of collection to prevent repeated freeze-thaw cycles.

Ethical statement

This study has obtained approval from the ethics committee of Liuzhou Women and Children's Healthcare Hospital (No. 2020-076). Written informed consent was obtained from all legal guardians of the participants. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Diagnostic criteria

Western medicine diagnostic criteria for HFMD

According to the "Integrated Traditional Chinese and Western Medicine Diagnosis and Treatment Guidelines for Hand, Foot, and Mouth Disease", the following criteria must be met for a diagnosis of the typical form of HFMD:

(I) Epidemiology: the patient must present with symptoms during an outbreak in a densely populated area during the epidemic season and have a history of contact with individuals affected by similar diseases. Most affected children were under the age of 5 years.

(II) Acute onset: the illness typically begins acutely, with rashes appearing at the infection site. Associated symptoms mainly include cough and runny nose; however, some patients may only exhibit rash and dermatitis-related symptoms.

(III) Typical rash characteristics: the primary rash is characterized by papules and vesicles, often surrounded by a localized area of inflammatory erythema. Vesicles typically contain minimal fluid and do not present with significant abnormal sensations, healing without crusting. Atypical rashes may appear small, thick, hard, and sparse. Skin lesions caused by CV-A6 and CV-A10 enteroviruses may be more severe, exhibiting large vesicular changes and significant pruritus, with lesions appearing in various locations.

(IV) Laboratory testing: (I) complete blood count and C-reactive protein: most cases show normal white blood cell counts, although a substantial proportion of patients may exhibit markedly elevated C-reactive protein levels. (II) pathogen detection and serology: positive nucleic acid tests or successful isolation of the enterovirus from clinical samples. Positive IgM antibodies against the virus were also indicative. A significant increase in antibodies against CA-16 and EV-71 is noted during the recovery phase.

TCM diagnostic criteria

Based on the "Integrated Traditional Chinese and Western Medicine Diagnosis and Treatment Guidelines for Hand, Foot, and Mouth Disease", the following symptoms indicate a diagnosis of damp-heat toxin accumulation and spleen-lung stagnation during the rash phase:

(I) Symptoms: rash in areas such as the hands, feet, mouth, and buttocks, which may be accompanied by fever or may occur without fever. Other symptoms include fatigue, drooling, sore throat, poor appetite, and constipation. Severe cases may present with large blisters or nail loss.

(II) Tongue and pulse diagnosis: the tongue may appear pale red or red with a greasy coating, while the pulse may be rapid or exhibit purple fingerprints.

Inclusion criteria

(I) Patients must meet the diagnostic criteria for HFMD as defined by both Western medicine and TCM; (II) age range of 2 to 14 years, with a disease duration of less than 48 hours; (III) legal guardians must provide consent and sign an informed consent form for enrollment; (IV) patients must follow a light diet primarily consisting of rice, pork, and vegetables; (V) no use of antibiotics, probiotics, or prebiotics within the past month; (VI) no history of gastrointestinal diseases such as diarrhea, dysentery, or colitis in the past month.

Exclusion criteria

(I) Children exhibiting severe clinical manifestations, such as poor mental state, neurological involvement, or cardiopulmonary failure, which do not meet the criteria for typical clinical diagnosis; (II) children with severe malnutrition or serious cardiovascular, liver, or kidney diseases, accompanied by mental disorders that hinder normal communication; (III) patients with allergies to the study medication, individuals with G-6PD deficiency, or those receiving other drug treatments; (IV) patients with severe diarrhea that affects the efficacy of the treatment; (V) poor compliance and high likelihood of dropout; (VI) disease duration exceeding 48 hours or adult participants.

Experimental procedure

Treatment methods

In the Re-Du-Ning enema group, participants were administered a Re-Du-Ning injection, containing primary

ingredients like artemisia, honeysuckle, and gardenia (Approval No. National Medicine Standard Z20050217), at a dosage of 0.6–0.8 mL/kg/day. The injection was mixed with 10 mL of 0.9% sodium chloride solution, drawn into an injection syringe, and connected to an anal tube. The front end of the tube was lubricated, and the child was positioned on their side. The tube was inserted approximately 15–20 cm into the anus, and the medication was fully administered before the tube was removed.

In the Re-Du-Ning Intravenous Infusion Group, participants were administered with Re-Du-Ning injection at a dosage of 0.6–0.8 mL/kg/day, dissolved in 150 to 250 mL of 5% glucose for intravenous drip. The infusion was administered at a drip rate of 3–5 mL/kg/hour.

The treatment duration for both groups was 3 days, with treatment administered once daily. Participants in both groups received routine care and were advised to drink lots of water, and maintain a light diet. If the body temperature exceeded 38.5 ℃, oral administration of ibuprofen suspension (produced by Shanghai Johnson Pharmaceuticals Co., Ltd., main ingredient: ibuprofen, approval number: National Medicine Standard H10980251) was recommended at a dose of 5–10 mg/kg, to be taken with lukewarm water.

The healthy group comprised age-matched healthy children undergoing physical examination during the same period. Fecal samples obtained from both HFMD-affected children prior to and following treatment, as well as from the healthy group, were subjected to high-throughput 16S ribosome DNA sequencing to assess changes in the intestinal microflora.

Observation indicators

(I) Clinical efficacy indicators: improvement scores for symptoms and signs such as fever, rash, throat redness and pain, cough, runny nose, loss of appetite, and bowel and urinary function; (II) changes in gut microbiota before and after treatment.

Experimental methods

Stool collection and DNA extraction

Fecal samples from all three groups were collected in sterile vessels, approximately 3 g each, and stored in sterile cryotubes at –80 ℃. DNA was extracted using the genomic extraction kit DP304, following the instructions provided by the manufacturer when samples were collected. The cetyltrimethylammonium bromide (CTAB) method was used for DNA extraction. The quality of the extracted DNA samples was assessed using an Agilent Bioanalyzer 2100 and Qubit@ 2.0. Sequencing data was deemed acceptable if it met the following criteria: more than 70,000 tags, Q20 over 98%, and Q30 over 94%.

Amplification primer sequence

By using diluted DNA as a template, the 16S V4 region was amplified with the following primers: forward: CCTAYGGGRBGCASCAG; reverse: GGACTACNNGGGTATCTAAT. Barcoded specific primers and a high-efficiency high-fidelity enzyme were used for polymerase chain reaction (PCR) amplification. The amplified 16S V4 library was then subjected to magnetic bead purification to produce the initial library, which is ready for sequencing.

Mixing and purification of PCR products

Gel electrophoresis was performed to inspect the PCR products. If the results were satisfactory, magnetic bead purification was the next step. The purified products were mixed in equal amounts based on their concentrations, and to accomplish this, enzyme-linked quantification was used. The mixed products were inspected by gel electrophoresis and target bands were recovered.

Library construction and sequencing

The library was constructed with the TruSeq® DNA PCR Kit. Initially, the library was quantified using Qubit 2.0. If it met the requirements, it was then amplified via quantitative PCR (qPCR) to determine its effective concentration and ensure that it met quality standards. Once the library requirements were met, sequencing was performed based on the instructions provided in the manual.

Statistical analysis

Qualitative data were expressed as n (%), and a non-inferiority test was conducted based on the non-inferiority margin. Quantitative data were presented as mean ± standard deviation. After testing for normal distribution, the *t*-test was used for normally distributed data, while the Friedman test was applied for non-normally distributed data. A P value of less than 0.05 indicated a statistically significant difference, while a P value greater than 0.05 indicated no significant difference. Statistical analysis was performed using SPSS 26.0 and SAS 9.3.

Analysis of similarities (ANOSIM) analysis was used to test the differences within and between groups, thereby determining if the grouping met the requirements. The R-value ranged from –1 to 1; a positive R-value indicates that the differences between groups were greater than

Table 1 Intergroup ANOSIM analysis

Group	R-value
VEIN2–Enema1	0.1437
Enema2–Enema1	0.4817
Enema2–VEIN2	0.189
Health–Enema1	0.3701
Health–VEIN2	0.1795
Health–Enema2	0.00001
VEIN1–Enema1	0.2094
VEIN1–VEIN2	–0.1204
VEIN1–Enema2	0.1485
VEIN1–Health	0.1688

Health, the healthy group; Enema1, the enema group before treatment; Enema2, the enema group after treatment; VEIN1, the intravenous group before treatment; VEIN2, the intravenous group after treatment; ANOSIM, analysis of similarities.

those within groups, while an R-value less than 0 indicates that the differences within groups were greater than those between groups. The credibility of the statistical analysis was indicated by the P value, with a P value <0.05 signifying statistical significance. In this study, the R-values for all groups were greater than 0, indicating that inter-group differences were greater than intra-group differences. The groups were represented as follows: 'Health' for the healthy group, 'Enema1' for the enema group before treatment, 'VEIN1' for the intravenous group before treatment, 'Enema2' for the enema group after treatment, and 'VEIN2' for the intravenous group after treatment. The analysis revealed that the intra-group differences before and after intravenous treatment were greater than the inter-group differences, whereas for the other groups, the inter-group differences were greater than the intra-group differences. Refer to *Table 1* for detailed results.

Results

Following sample species annotation, the differences among species between groups were analyzed using several methods, including the relative abundance bar graph, unweighted pair-group method with arithmetic mean (UPGMA) clustering tree, and inter-group difference analysis. The study examines the differences between children with HFMD and healthy children, both before and after enema treatment, as well as before and after intravenous treatment. This analysis assesses the changes in the intestinal microflora of children with HFMD and assesses the impacts of enema and intravenous treatments on the intestinal microflora.

Symptom scores before and after treatment in both groups

Symptom scores, including fever, rash, cough, runny nose, appetite, bowel movements, and tongue/pulse, were assessed in both groups before and after treatment. Before treatment, there was no significant difference in symptom scores between the two groups (P=0.22). After treatment, symptom scores significantly decreased in both the enema group and the intravenous infusion group (P<0.001), but the difference between the two groups was not significant (P=0.61). The mean change in symptom scores for the intravenous group was 21.29±4.92, and the non-inferiority margin was set at –4.2. The difference in the mean change between the two groups was –1.610 [95% confidence interval (CI): –3.957 to 0.737, P=0.02]. Additionally, the non-inferiority margin of –4.2 was not within the 95% CI, indicating that the symptom score improvement in the enema group was not inferior to that of the intravenous group. See *Table 2* for details.

Efficacy in both groups

According to the efficacy evaluation criteria, both groups achieved satisfactory results. The overall effectiveness rate was 97.1% in the intravenous infusion group and 97.4% in the enema group. With a non-inferiority margin of –10%, the difference in overall effectiveness rates between the two groups was 0.003 (95% CI: –0.073 to 0.079), or 0.3% (95% CI: –7.3% to 7.9%, P=0.006). Since the non-inferiority margin of –10% was not within the 95% CI, this indicates that the overall effectiveness rate of the enema group was not inferior to that of the intravenous group. See *Table 3* for details.

Species diversity curve

Rarefaction curve

At a sequencing depth of 50,000 tags, the slope of the corresponding curve gradually decreases, indicating that the sequencing data volume is approaching saturation. Further increases in data will not result in significant changes (Figure S1).

Table 2 Symptom scores before and after treatment in both groups

Groups	Symptom scores (mean ± SD)		MD (95% CI)	P
	Enema group	Intravenous group		
Before treatment	27.41±4.74	25.79±6.13	−1.622 (−4.220 to 0.975)	0.22
After treatment	6.12±4.97	6.11±4.72	−0.012 (−2.298 to 2.274)	0.61
Mean change	21.29±4.92	19.68±5.04	−1.610 (−3.957 to 0.737)	0.02
t	−5.099	−5.390		
P	<0.001	<0.001		

SD, standard deviation; MD, mean deviation; CI, confidence interval.

Table 3 Efficacy in both groups

Groups	Clinically cured	Significant effect	Effective	Ineffective	Overall effectiveness rate
Intravenous group, n (%)	2 (5.9)	23 (67.6)	8 (23.5)	1 (2.9)	33 (97.1)
Enema group, n (%)	5 (2.9)	21 (55.3)	11 (28.9)	1 (2.6)	37 (97.4)
RD (95% CI)			0.003 (−0.073, 0.079)		
Z			2.491		
P value			0.006		

RD, risk difference; CI, confidence interval.

Rank abundance curve

The rank abundance curve is used to describe species richness and evenness. In this study, operational taxonomic units (OTUs) were ranked according to their relative abundance and assigned numbers, with the rank number on the x-axis and relative abundance on the y-axis. From the results of this figure, it can be observed that most samples have OTU sequence ranges between 200 and 330, with no signs of contamination or outlier samples (Figure S2).

Species accumulation boxplot

The species accumulation boxplot helps determine whether the sample size is sufficient. A sharp increase in the boxplot indicates insufficient sample size, suggesting the need for more samples, while a leveling-off suggests that the sample size is adequate for data analysis (Figure S3). In this study, the y-axis represents the number of OTUs after sampling, and the x-axis represents the sample size. With a sample size of 51 and the discovered species exceeding 1,200, the species accumulation curve has leveled off. The results indicate that the sample size is reasonable.

Relative abundance bar graph

For each taxonomic level, species ranking in the top 25 in terms of abundance were selected, and their cumulative abundance bar graphs were plotted. Based on the graphical results, it became visually evident that prior to enema treatment, the microbial flora composition in the relative abundance bar graph revealed significant differences from that of the healthy group. After enema treatment, the microbial flora composition in the relative abundance bar graph appeared similar to that of the healthy group and indicated considerable differences from the pre-treatment structure. See *Figure 1* for a visual representation of these findings.

UPGMA clustering tree

A clustering analysis was conducted on the selected samples, and the results were processed to generate a corresponding clustering tree. During the clustering analysis, both Weighted Unifrac and Unweighted Unifrac distance matrices were used based on the sample characteristics.

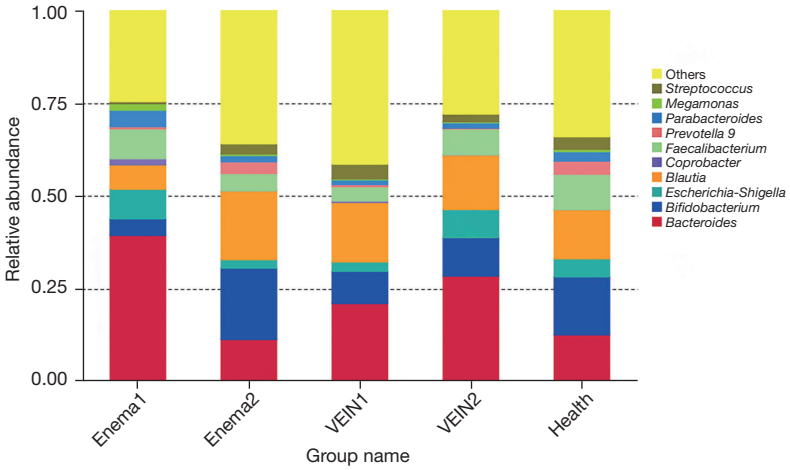

Figure 1 Species-relative abundance bar graph. Health, the healthy group; Enema1, the enema group before treatment; VEIN1, the intravenous group before treatment; Enema2, the enema group after treatment; VEIN2, the intravenous group after treatment.

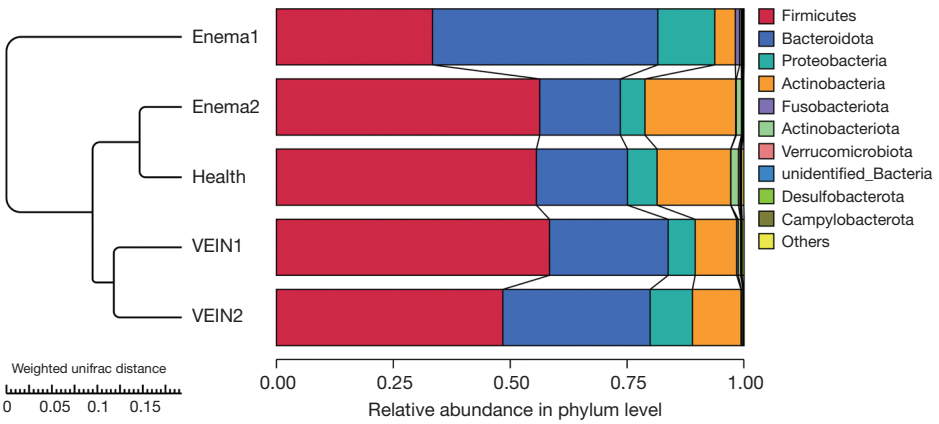

Figure 2 UPGMA clustering tree. Health, the healthy group; Enema1, the enema group before treatment; VEIN1, the intravenous group before treatment; Enema2, the enema group after treatment; VEIN2, the intravenous group after treatment; UPGMA, unweighted pair-group method with arithmetic mean.

When fused with the relative abundance data, the results clearly indicated that the greatest similarity was between the healthy group and the group after enema treatment, while substantial similarity was observed between the groups before and after intravenous treatment (*Figure 2*).

Clustering heatmap

Based on the functional annotations and abundance data from the database for the samples, the top 18 functions and their related abundance information were selected. After processing these results, a heatmap was constructed. The heatmap was based on functional data for clustering, and the plotted results were revealed in *Figure 3*.

From the clustering tree, clustering heatmap, and *Table 4*, it became visually evident that at the Phylum level, the relative abundance composition of species in the healthy group and post-enema group were quite similar. When comparing HFMD-affected children (before enema and before intravenous treatment) with the healthy group, there was an increase in the relative abundance of Bacteroidota, Fusobacteriota, and Campylobacterota, while there was a decrease in Actinobacteria, Actinobacteriota, Verrucomicrobiota, Cyanobacteria, Euryarchaeota, and Deinococcota, indicating relatively significant differences.

Before and after enema treatment, there was an increase

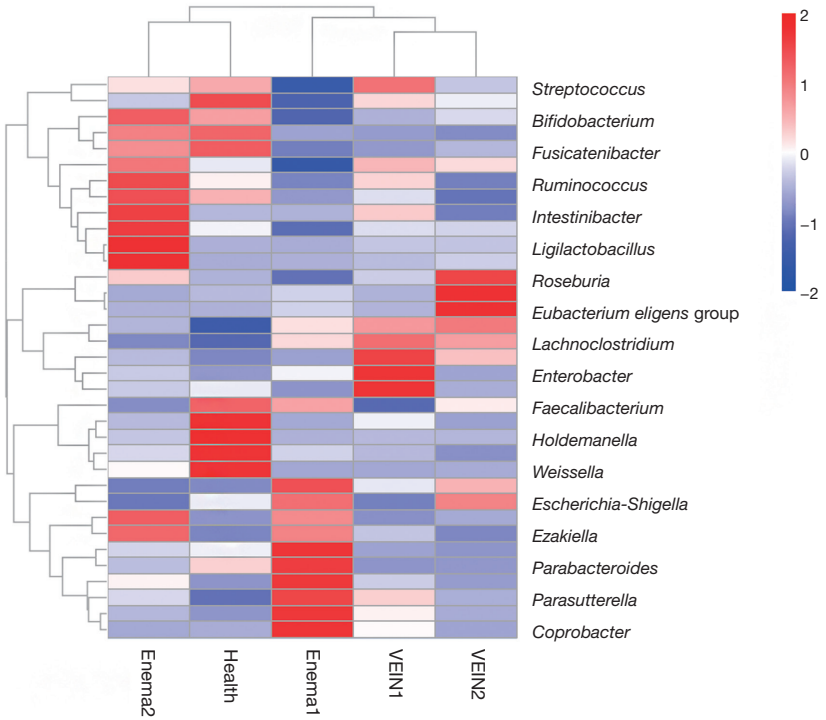

Figure 3 Heatmap of abundance among groups. Health, the healthy group; Enema1, the enema group before treatment; VEIN1, the intravenous group before treatment; Enema2, the enema group after treatment; VEIN2, the intravenous group after treatment.

Table 4 Microbial flora abundance parameters among groups

Taxonomy	Before enema	After enema	Before intravenous	After intravenous	Healthy
Firmicutes	0.334119	0.561674	0.584075	0.484246	0.556008
Bacteroidota	0.481991	0.184864	0.254122	0.315207	0.195142
Proteobacteria	0.121889	0.05044	0.057762	0.090175	0.063032
Actinobacteria	0.043526	0.185666	0.088447	0.104116	0.1578
Fusobacteriota	0.009294	0.001266	0.003309	0.000981	0.000282
Actinobacteriota	0.003361	0.010717	0.00525	0.003044	0.016118
Verrucomicrobiota	0.001534	0.00182	0.001256	0.000445	0.003491
Unidentified bacteria	0.001795	0.001119	0.00077	0.000359	0.001849
Desulfobacterota	0.001061	0.000606	0.000848	0.00029	0.001408
Campylobacterota	0.000466	0.000524	0.000676	0.000023	0.000022
Cyanobacteria	0.000022	0.000027	0.000017	0.000006	0.000182
Euryarchaeota	0	0	0	0	0.000077
Deinococcota	0.000014	0	0	0	0.000022

Table 5 Intergroup difference analysis—AMOVA function table

vs_group	SS (residual value)	Df [residual value]	Fs	P
Enema1–Enema2	1.89228 (3.36774)	1 [38]	21.3516	<0.001*
Enema1–Health	1.4251 (2.89664)	1 [35]	17.2194	<0.001*
Enema2–Health	0.0619634 (2.56917)	1 [29]	0.699424	0.60
VEIN1–VEIN2	0.0335796 (1.02593)	1 [11]	0.36004	0.71

Health, the healthy group; Enema1, the enema group before treatment; VEIN1, the intravenous group before treatment; Enema2, the enema group after treatment; VEIN2, the intravenous group after treatment; vs_group, comparisons between the different groups. *, the difference between two groups was statistically significant. AMOVA, analysis of molecular variance; SS, Stdev square; Df, degree of freedom; Fs, F-statistic.

in the relative abundance of Bacteroidota, Firmicutes, Actinobacteria, Actinobacteriota, Verrucomicrobiota, Campylobacterota, and Cyanobacteria. Conversely, Bacteroidota, Proteobacteria, Fusobacteriota, Euryarchaeota, Deinococcota, Desulfobacterota, and unidentified bacteria decrease in abundance. Notably, Euryarchaeota was only present in the healthy group (*Table 4*).

Inter-group difference analysis—analysis of molecular variance (AMOVA)

Based on Unifrac distances, the AMOVA function in the mothur software was used for inter-group difference analysis. The results indicated significant differences between the pre-enema groups and the healthy group, as well as between the pre-enema and post-enema treatment groups. However, no significant differences were discovered between the groups post-enema and the healthy group, nor between the groups before and after intravenous treatments (*Table 5*).

Inter-group microbial flora difference analysis

An in-depth study was conducted for the described sample groups by statistically analyzing the differences in community structures to identify enriched species across different groups and scrutinize the distinctions.

T-test

In the analysis comparing HFMD-affected children (E1V1) to healthy children (health), significant differences in the relative abundance of species were identified at various taxonomic levels (phylum, class, order, family, genus, species) using inter-group *t*-tests (P<0.05). Specifically, in HFMD-affected children compared to the healthy group: the relative abundance of *Polymorpha*, *Enterobacter genus*, *Alistipes*, *Microbacterium* (*Haemophilus*), *Sutterella*, *Flavobacterium*, and *Verrucomicrobiaceae* was significantly higher. The relative abundance of *Collinsella* and *Romboutsia* was significantly lower. For detailed results, please refer to *Figure 4*.

In the analysis of before and after enema treatment, notable changes in the relative abundance of microbial flora were observed. Specifically, species like Polymorpha, *Faecalibacterium*, *Veillonella*, *Lachnospira*, *Koalabacterium*, and *Sutterella* revealed decreased relative abundance after treatment. Conversely, the relative abundance of *Bifidobacterium*, *Blautia*, *Streptococcus*, *Eubacteria*, *Propionibacterium*, *Subgroup*, *Romboutsia*, *Egersella*, *Butyrobacter*, and *Trichuris* increased compared to before treatment (*Figure 5*).

In contrast, the analysis prior to and following intravenous treatment revealed no significant differences in the relative abundance of microbial flora. Specific results can be found in *Figure 6*.

Linear discriminant analysis (LDA) effect size (LEfSe)

Statistically significant biomarkers between groups were identified using LEfSe, and an LDA threshold of >4.

In children affected by HFMD (E1V1) compared to healthy children, significant biomarkers included *Lachnospiraceae*, *Lachnospira*, Firmicutes, *Fusobacteria*, *Blautia*, *Streptococcus*, *Polymorpha*, *Bacteroides fragilis*, and *Bacteroides ovatus*. Conversely, in the healthy group, significantly abundant taxa comprised Actinobacteria, *Bifidobacterium longum*, *Eubacteria*, and *Propionibacterium*. Detailed results can be observed in *Figure 7*.

Regarding the analysis pre- and post-enema treatment,

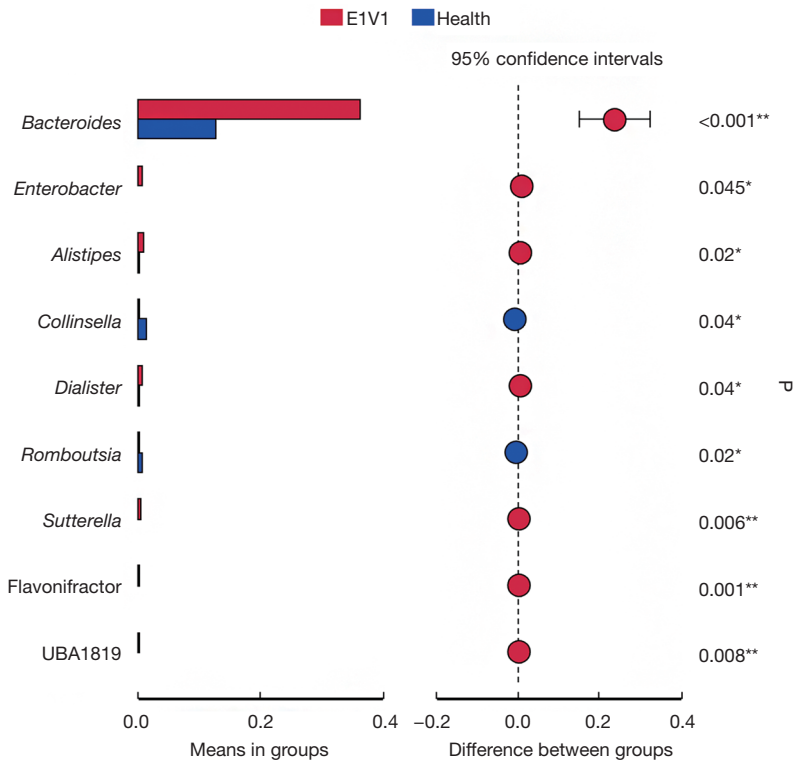

Figure 4 *T*-test graph comparing healthy children (Health) and children with HFMD (E1V1). *, P<0.05; **, P<0.01. Health, the healthy group; VEIN1, the intravenous group before treatment; HFMD, hand, foot, and mouth disease.

significantly abundant taxa observed before enema included Bacteroidota, *Bacteroidaceae*, Polymorpha, *Bacteroides fragilis*, *Proteus*, Proteobacteria, *Enterobacter genus*, and *Enterobacteriaceae*. Post-enema, significantly abundant taxa encompassed Firmicutes, Fusobacteria, Lachnospira, *Lachnospiraceae*, Blautia, Actinobacteria, unclassified Actinobacteria, Bifidobacterium, and *Bifidobacteriaceae* (*Figure 8*).

Summary

- Children affected by HFMD demonstrate dysbiosis, characterized primarily by an increased abundance of opportunistic pathogens like *Enterobacter* and a reduction in bacteria known for their anti-inflammatory properties.
- Enema treatment in HFMD-affected children leads to regulation of intestinal microflora. Post-treatment, there is a notable increase in probiotic abundance, particularly *Bifidobacteria*, along with a significant decrease in opportunistic pathogens like *Enterobacter*.
- Re-Du-Ning intravenous treatment in HFMD-affected children does not exert a significant impact on microbial flora. No significant differences in microbial abundance were observed before and after treatment.

Discussion

The previous study has demonstrated that stressors like trauma or infection can disrupt the balance of intestinal microflora, causing changes in species composition and proportions (11). This disruption typically results in a reduction of beneficial probiotics within the gut, proliferation of pathogenic bacteria like *Enterococci* and *Escherichia coli*, impairment of the intestinal mucosal barrier, and increased gut permeability. Consequently, various inflammatory factors can traverse compromised barriers into the bloodstream, activating and intensifying systemic inflammatory responses. After being infected by enteroviruses, these pathogens extensively propagate within the gut, inducing substantial changes in the structure of intestinal microflora and disrupting its previously stable equilibrium. This viral infection also impacts mucosal barrier function, compromising the resilience of the body

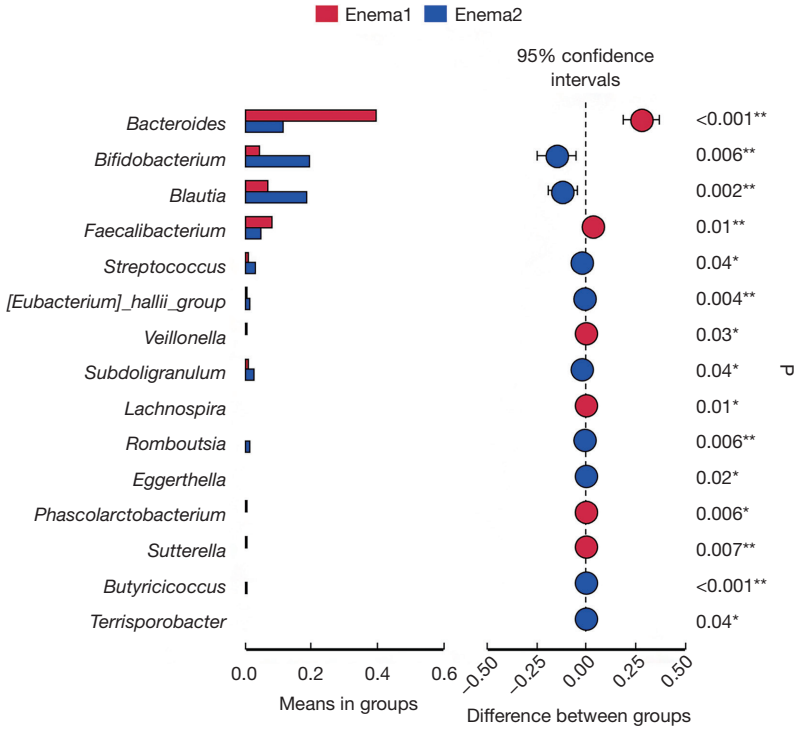

Figure 5 *T*-test graph prior to (Enema1) and following (Enema2) enema treatment. *, P<0.05; **, P<0.01. Enema1, the enema group before treatment; Enema2, the enema group after treatment.

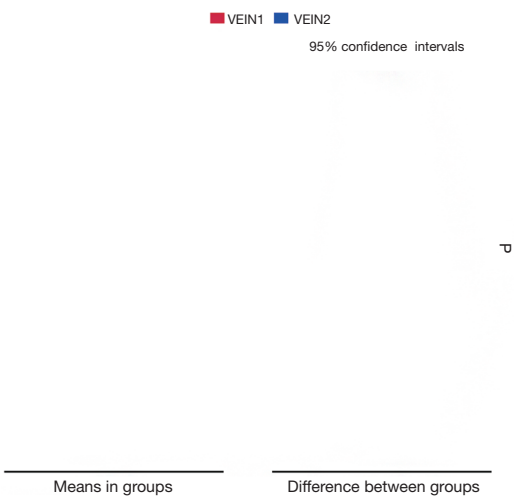

Figure 6 *T*-test graph prior to (VEIN1) and following (VEIN2) intravenous treatment. VEIN1, the intravenous group before treatment; VEIN2, the intravenous group after treatment.

(12,13). Crucial probiotics like *Bifidobacteria* and *Lactobacillus* play key roles in inhibiting the proliferation of pathogenic bacteria and contributing to mucosal barrier integrity, thereby maintaining intestinal microflora balance (13). During HFMD, changes in intestinal microflora primarily manifest as decreased microbial diversity in the gut. This dysbiosis is characterized by reduced populations of butyrate-producing bacteria (e.g., *Bifidobacterium*, *Ruminococcus*, *Roseburia*) and increased abundance of opportunistic pathogens (e.g., *Escherichia*, *Enterococcus*). The findings of our study corroborate previous conclusions, indicating dysbiosis in HFMD-affected children marked by an upsurge in opportunistic pathogens and a decline in inflammation-inhibiting bacteria (14-20).

Comparing the microbial flora of HFMD-affected children before treatment with that of healthy children revealed significant differences. HFMD-affected children

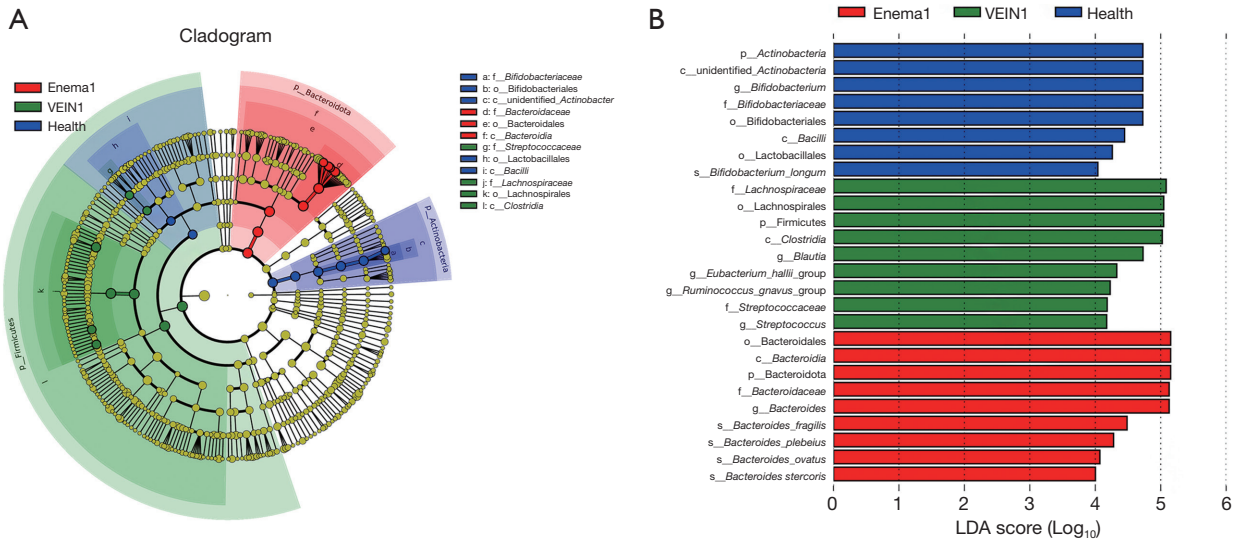

Figure 7 LEfSe graph comparing healthy children (Health) and children with HFMD (E1V1). Health, the healthy group; Enema1, the enema group before treatment; VEIN1, the intravenous group before treatment; HFMD, hand, foot, and mouth disease; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis.

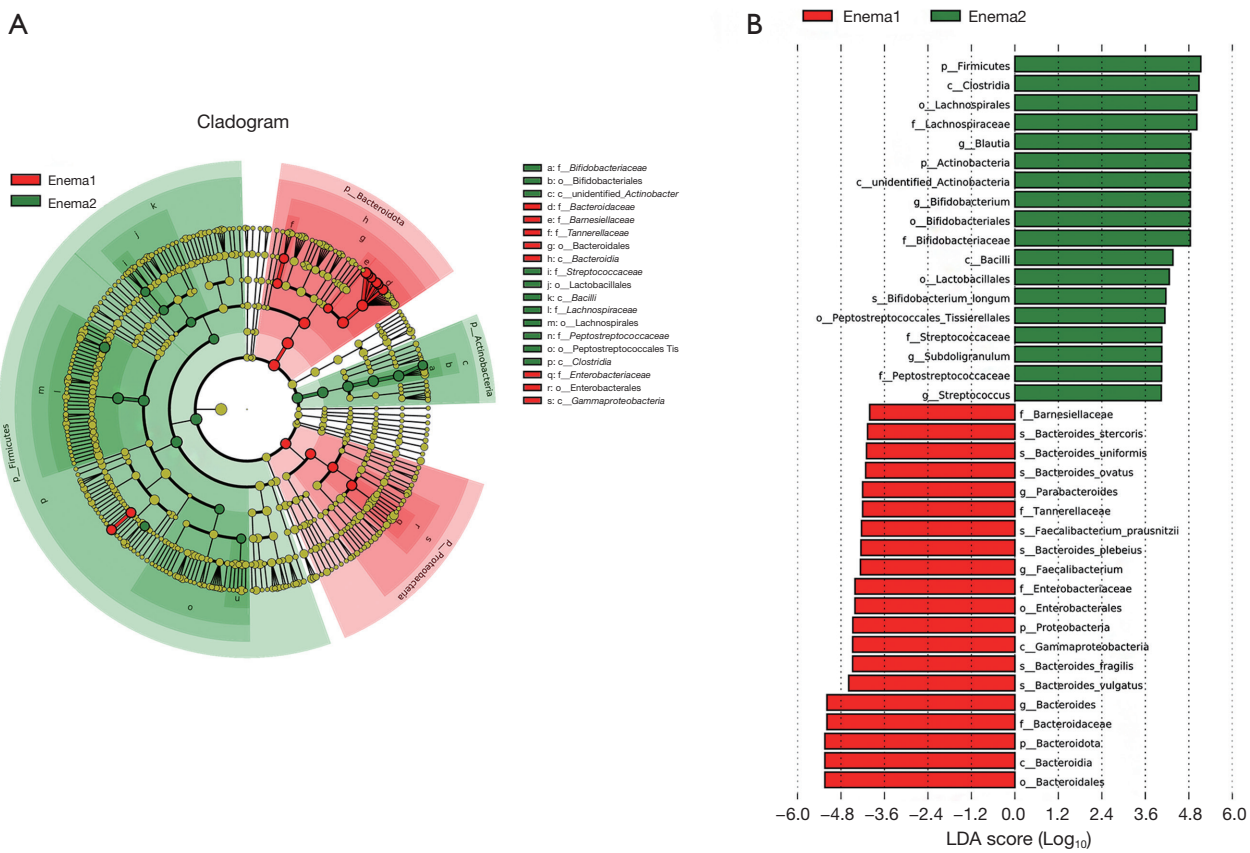

Figure 8 LEfSe graph prior to (Enema1) and following (Enema2) enema treatment. Enema1, the enema group before treatment; Enema2, the enema group after treatment; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis.

exhibited notably higher relative abundances of Polymorpha, *Enterobacter genus*, Anthozoa, *Microbacterium* (*haemophilus*), *Sutterella*, *Flavobacterium*, and *Verrucomicrobiaceae* UBA1819 compared to healthy children. Conversely, the abundance of *Collinsella* and *Romboutsia* was lower in HFMD-affected children than in healthy children. Polymorpha typically inhabits human and animal oral cavities, upper respiratory tracts, intestines, and reproductive tracts, helping in food breakdown and essential nutrient production. Bacteroidota, significant clinical pathogens found in anaerobic infections, particularly *Melaninogenic bacteroides*, have been associated with an increased risk of endogenous infections under conditions of immune dysfunction. While these bacteria can maintain beneficial symbiotic relationships within the intestines, they may trigger related pathologies like bacteremia and inflammation upon escaping this environment (13). Anaerobic bacterium *Bacteroides fragilis*, a member of the *Bacteroides genus*, is known for infections near mucosal surfaces like the oral cavity, sinuses, nasopharynx, chest and abdominal cavities, perianal, and perineum regions, often causing inflammation, abscesses, and deep pus-forming infections. *Enterobacter*, another opportunistic pathogen within the Firmicutes phylum, is among the most prevalent gram-negative bacterial pathogens, associated with skin, gastrointestinal, respiratory, and urinary tract infections (21). Notably pathogenic members of the *Enterobacteriaceae* family, such as *Escherichia coli* and *Salmonella*, are capable of producing enterotoxins under specific conditions, which increase plasma diamine oxidase activity and damage intestinal epithelial cell membranes (22). This process reduces anti-inflammatory cytokine expression levels while elevating inflammatory factor levels (23). Probiotics present in the gut, primarily *Bifidobacteria* and *Lactobacillus*, competitively inhibit harmful bacteria that produce metabolic toxins, thereby preventing these toxins from entering the bloodstream and providing protection (24).

Further experimental research indicates that *Bifidobacteria* produce bacteriocins, which are antibacterial substances that inhibit the growth of opportunistic pathogens and reduce intestinal antigen levels, thereby attenuating inflammatory responses (25,26). Probiotics like *Bifidobacteria* play a crucial role in protecting the intestinal barrier, preserving intestinal wall integrity, and regulating antigen entry into the bloodstream, thereby reducing inflammatory reactions (27,28).

The intestinal microflora harbors genes absent in the human genome, which facilitate the fermentation of complex carbohydrates by the host. These genes metabolize substances into short-chain fatty acids (SCFAs) like n-butyric acid, acetic acid, and propionic acid. SCFAs bind to G protein-coupled receptor 43 (GPR43) receptors in intestinal epithelial cells, providing energy to the host and supporting its growth (29-31). Butyrate possesses anti-inflammatory properties by inhibiting nuclear factor κB (NF-κB) transcription factor activation, suppressing interferon gamma (IFN-γ), and upregulating peroxisome proliferator-activated receptor γ (PPARγ). Consequently, serum levels of inflammatory cytokines such as interleukin (IL)-6, IL-8, IL-12, IFN-γ, and tumor necrosis factor-α (TNF-α), are notably elevated in children with HFMD, decrease (32,33). Common SCFA-producing anaerobic bacteria include *Bifidobacterium*, *Fusobacteria*, Bacteroidota, and *Streptococcus* (34-36). Experimental findings demonstrate that increased abundance of SCFA-producing probiotics correlates with decreased IL-6 levels observed in clinical studies.

Following enema treatment, a comparison of microbial taxa before and after revealed substantial changes. Prior to enema administration, prevalent taxa included Bacteroidota, *Bacteroidaceae*, Polymorpha, *Bacteroides fragilis*, *Proteus*, Proteobacteria, *Enterobacter genus*, *Enterobacteriaceae*, *Faecalibacterium*, *Bacillus*, Danielliaceae, *Prevotella* amylolytica, *Bacteroides ovatus*, *Bacteroides*, and Barnesiellaceae. Post-enema, significantly abundant taxa comprised of Firmicutes, Fusobacteria, *Lachnospira*, *Lachnospiraceae*, *Blautia*, Actinobacteria, unclassified Actinobacteria, Bifidobacterium, *Bifidobacteriales*, *Bifidobacteriaceae*, Bacilli, Bacilliformes, Bifidobacterium longum, Peptostreptococcus tissierellales, *Streptococcus*, a subgroup of gastrostricoccaceae, and *Streptococcus*. It is evident from these findings that post-enema treatment, there was a significant increase in the abundance of probiotics like *Bifidobacteria*, whereas the abundance of opportunistic pathogens like *Enterobacter* decreased. Compared to intravenous therapy, enema therapy has a regulatory effect on the gut microbiota of children with HFMD. Administering Re-Du-Ning via enema is safe, with no significant adverse side effects. Enema treatment with Re-Du-Ning has demonstrated clear efficacy in treating HFMD, with its clinical effectiveness and symptom improvement scores being non-inferior to intravenous administration.

Some potential limitations of this study include the relatively small sample size, which may limit the generalizability of the findings to a broader population. Additionally, the dropout rate during the study could introduce bias, as participants who discontinued may differ in important ways from those who completed the study. These

factors could affect the overall robustness and reliability of the results, and future research with larger and more diverse samples would help validate and expand on these findings.

Conclusions

The clinical efficacy rate and symptom score improvement of Re-Du-Ning enema in treating HFMD are not inferior to intravenous therapy. Enema treatment also shows greater potential in inhibiting inflammation-related indicators. Children with HFMD often experience gut microbiota imbalance. Re-Du-Ning enema has a regulatory effect on gut microbiota, significantly increasing the abundance of beneficial bacteria such as Bifidobacterium, while reducing the abundance of opportunistic pathogens like *Enterobacter*. In contrast, no significant microbiota changes were observed before and after intravenous therapy with Re-Du-Ning treatment. These findings indicate that the Re-Du-Ning enema is as effective as intravenous therapy for treating HFMD while exhibiting a greater ability to inhibit inflammation-related indicators, making it a viable alternative or complementary option in clinical practice. Additionally, the enema's positive impact on gut microbiota highlights its potential role in restoring gut health in affected children and enhancing overall patient outcomes.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-257/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-257/dss

Peer Review File: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-257/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-257/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study has obtained approval from the ethics committee of Liuzhou Women and Children's Healthcare Hospital (No. 2020-076). Written informed consent was obtained from all legal guardians of the participants.

References

1. Sun JL, Zhang J. A review on the advancement of epidemiology on hand-foot-mouth disease. Chinese Journal of Epidemiology 2009;30:973-6.
2. Xu H, Pan Z. Progress of hand, foot and mouth disease caused by enterovirus 71 infection and natural immune cells. Chinese Journal of Applied Clinical Pediatrics 2018;33:1681-6.
3. Xu PF, Deng HL, Wang XY, et al. Different severity of children with hand, foot and mouth disease of intestinal barrier function and researches on the change of S - 100 protein levels. Chin Clin Dr 2020;48:239-41.
4. Li W, Zhu Y, Li Y, et al. The gut microbiota of hand, foot and mouth disease patients demonstrates down-regulated butyrate-producing bacteria and up-regulated inflammation-inducing bacteria. Acta Paediatr 2019;108:1133-9.
5. Quan YL. Enteric flora distribution of hand-foot-and mouth disease infants, and its influence on patients' immunofunction and enteric mucosal permeability. Chinese Journal of Coloproctology 2020;40:26-8.
6. Zhu S, Shen N, Zhou Y, et al. Analysis of the structural

characteristics of intestinal flora in EV71 hand-foot-mouth disease. Journal of Xuzhou Medical University 2022;42:353-7.
7. Zhou JY, Lai YT, Ren L, et al. Effects of acupuncture at Tianshu (ST25) and Shangjuxu (ST37) on the intestinal mucosa and metabolites of local skin tissues at Tianshu (ST25) in 2,4,6-Trinitrobenzene sulfonic acid and ethanol-induced rats. World Journal of Traditional Chinese Medicine 2023;9:131-40.
8. Liu X, Cao JN, Liu T, et al. Effect of herb-partitioned moxibustion on structure and functional prediction of gut microbiota in rats with irritable bowel syndrome with diarrhea. World Journal of Traditional Chinese Medicine 2023;9:141-9.
9. Bi CY, Liu Y, Zhang N, et al. Three kinds of commonly used for injection treatment of hand, foot and mouth disease of TCM and clinical research. Nei Mongol Journal of Traditional Chinese Medicine 2016;35:99-100.
10. Chang XJ, Sun XP, Hu HF, et al. Effect of Reduning Injection on Vero cells and suckling mice infected by EV71 virus and its mechanism. Chinese Traditional and Herbal Drugs 2018;49:2097-102.
11. He Q, Tan WJ, Yang Y. Characteristics of intestinal flora and inflammatory factors in patients with Parkinson disease. South China Journal of Preventive Medicine 2021;47:1558-60.
12. Zhang N, Mou D, Li T, et al. Integrated analysis reveals important differences in the gut and oropharyngeal microbiota between children with mild and severe hand, foot, and mouth disease. Emerg Microbes Infect 2023;12:2192819.
13. Yang L. Helicobacter pylori infection in children with intestinal flora correlation studies. Jinan: Shandong University, 2020.
14. Durack J, Lynch SV. The gut microbiome: Relationships with disease and opportunities for therapy. J Exp Med 2019;216:20-40.
15. Manor O, Dai CL, Kornilov SA, et al. Health and disease markers correlate with gut microbiome composition across thousands of people. Nat Commun 2020;11:5206.
16. Iddrisu I, Monteagudo-Mera A, Poveda C, et al. Malnutrition and Gut Microbiota in Children. Nutrients 2021;13:2727.
17. Zhong H, Penders J, Shi Z, et al. Impact of early events and lifestyle on the gut microbiota and metabolic phenotypes in young school-age children. Microbiome 2019;7:2.
18. Zoghi S, Sadeghpour Heravi F, Nikniaz Z, et al. Gut microbiota and childhood malnutrition: Understanding the link and exploring therapeutic interventions. Eng Life Sci 2024;24:2300070.
19. Lee M, Chang EB. Inflammatory Bowel Diseases (IBD) and the Microbiome-Searching the Crime Scene for Clues. Gastroenterology 2021;160:524-37.
20. Guo L, Wang LS. The research on gut microbiota dysbiosis inliver cirrhosis patients: Current status. Chinese Journal of Microecology 2018;30:104-8.
21. Wang BF. Antibiotic resistance genes in mice intestinal and enterobacter and enterococcus and the effect of lactobacillus. Shandong University; 2020.
22. Zhou Y, Ni X, Duan L. Lactobacillus plantarum BSGP201683 Improves the Intestinal Barrier of Giant Panda Microbiota-Associated Mouse Infected by Enterotoxigenic Escherichia coli K88. Probiotics and Antimicrobial Proteins 2021;13:664-76.
23. Chen Y, Liu L, Yu L, et al. Curcumin Supplementation Improves Growth Performance and Anticoccidial Index by Improving the Antioxidant Capacity, Inhibiting Inflammatory Responses, and Maintaining Intestinal Barrier Function in Eimeria tenella-Infected Broilers. Animals (Basel) 2024;14:1223.
24. Bradford G, Asgari B, Smit B, et al. The Efficacy of Selected Probiotic Strains and Their Combination to Inhibit the Interaction of Adherent-Invasive Escherichia coli (AIEC) with a Co-Culture of Caco-2:HT29-MTX Cells. Microorganisms 2024;12:502.
25. Alba C, Carrera M, Álvarez-Calatayud G, et al. Evaluation of Safety and Beneficial Health Effects of the Human-Milk Strain Bifidobacterium breve DSM32583: An Infant Pilot Trial. Nutrients 2024;16:1134.
26. Sharma D, Gajjar D, Seshadri S. Understanding the role of gut microfloral bifidobacterium in cancer and its potential therapeutic applications. Microbiome Res Rep 2024;3:3.
27. Yoo S, Jung SC, Kwak K, et al. The Role of Prebiotics in Modulating Gut Microbiota: Implications for Human Health. Int J Mol Sci 2024;25:4834.
28. Zhang J, Wang H, Liu Y, et al. Advances in fecal microbiota transplantation for the treatment of diabetes mellitus. Front Cell Infect Microbiol 2024;14:1370999.
29. Guarner F, Malagelada JR. Gut flora in health and disease. Lancet 2003;361:512-9.
30. Chaiyasut C, Sivamaruthi BS, Thangaleela S, et al. Influence of Lactobacillus rhamnosus Supplementation on the Glycaemic Index, Lipid Profile, and Microbiome of Healthy Elderly Subjects: A Preliminary Randomized Clinical Trial. Foods 2024;13:1293.

31. Wang H, Kim R, Wang Y, et al. In vitro co-culture of Clostridium scindens with primary human colonic epithelium protects the epithelium against Staphylococcus aureus. Front Bioeng Biotechnol 2024;12:1382389.
32. Siddiqui MT, Cresci GAM. The Immunomodulatory Functions of Butyrate. J Inflamm Res 2021;14:6025-41.
33. Xie JC, Lin GY, Lu XD, et al. Levels of serum cytokines in children with hand-foot-and-mouth disease and their correlation with disease severity. Chinese Journal of Applied Clinical Pediatrics 2013;28:756-9.
34. LeBlanc JG, Chain F, Martín R, et al. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microb Cell Fact 2017;16:79.
35. Lee YT, Senturk M, Guan Y, et al. Bacteria-organelle communication in physiology and disease. J Cell Biol 2024;223:e202310134.
36. Junaid M, Lu H, Li Y, et al. Novel Synergistic Probiotic Intervention: Transcriptomic and Metabolomic Analysis Reveals Ameliorative Effects on Immunity, Gut Barrier, and Metabolism of Mice during Salmonella typhimurium Infection. Genes (Basel) 2024;15:435.

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