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RESEARCH ON GUT MICROBIOME ALTERATIONS AND EFFICACY OF PROBIOTIC THERAPY FOR PERSISTENT DIARRHEA IN CHILDREN AGED 3-24 MONTHS AT VIETNAM NATIONAL CHILDREN'S HOSPITAL (2022-2023)

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The thesis can be found at:

- The National library
- The library of the National Insitute of Malariology - Parasitology - Entomology

LIST OF RESEARCH WORKS RELATED TO THE THESIS TO BE PUBLISHED

1. Ha Thuy Dang, Dien Minh Tran, Thuy Bich Phung et al. (2024), "Promising clinical and immunological efficacy of *Bacillus clausii* spore probiotics for supportive treatment of persistent diarrhea in children", *Nature Scientific Reports,* 14:6422.
2. Dang Thuy Ha, Tran Minh Dien, Phung Bich Thuỷ. (2024), "Evaluate the safety and effectiveness of *Bacillus* probiotic spores in children with persistent diarrhea". *Vietnam Journal of Science and Technology,* 66(6), tr. 74-80.

INTRODUCTION

Persistent diarrhea (PD) in children under 5 years old remains a significant medical challenge, especially in developing countries. Although PD accounts for only 10% of total diarrhea cases, it is responsible for up to 35% of diarrhea-related deaths. Recent studies have highlighted the crucial relationship between gut microbiota dysbiosis and PD, opening new avenues for diagnosis and treatment.

In Vietnam, research on PD is limited, particularly regarding its pathogenesis and effective treatment methods. This study aims to explore gut microbiota alterations in children with PD and evaluate the efficacy of probiotic therapy using advanced gene sequencing technology. The research results promise to provide a scientific basis for developing effective intervention strategies, contributing to reducing the burden of disease and mortality due to PD in Vietnamese children. Therefore, we conduct: **"Gut Microbiota Alterations and the Efficacy of Probiotics Therapy in Persistent Diarrhea Management among Children Aged 3-24 Months: A Prospective Study at the Vietnam National Children's Hospital (2022-2023) "** with three objectives:

1. *Characterize the clinical and laboratory features of persistent diarrhea in children aged 3-24 months at the Vietnam National Children's Hospital.*
2. *Elucidate the gut microbiota alterations in children with persistent diarrhea using next-generation sequencing techniques.*
3. *Evaluate the therapeutic efficacy of probiotics intervention in the management of persistent diarrhea.*

The urgency of this research is manifested in three main aspects:

- Persistent diarrhea is a serious health issue, especially in children aged 3-24 months, a critical period for the development of gut microbiota and the immune system.
- There is a need for a better understanding of the causes and pathogenesis of PD, particularly its relationship with gut microbiota. This understanding could lead to new, more effective diagnostic and treatment methods.

- Evaluation of the efficacy of probiotic therapy in treating PD. If proven effective, this could provide a new, safe, and effective treatment method for children.

The novel contributions of this thesis include:

- Application of Illumina MiSeq 16S rRNA gene sequencing technique, pioneering research on PD in children in Vietnam.
- We provide in-depth insights into the relationship between gut microbiota and PD in the Vietnamese context.
- Directly compare the efficacy of single-strain and multi-strain probiotics in treating PD.
- Providing a scientific basis for developing more effective PD treatment methods.

Thesis structure:

The thesis consists of 127 pages, including sections on problem statement (2 pages), literature review (31 pages), research subjects and methods (21 pages), research results (34 pages), discussion (36 pages), conclusion (2 pages), and recommendations (1 page). The thesis includes 14 tables, 32 figures, and 175 references.

Chapter 1
LITERATURE REVIEW

1.1. Clinical and laboratory features of pesistent diarrhea

1.1.1. Definition and epidemiology of persistent diarrhea

Persistent diarrhea (PD) is defined by the World Health Organization (WHO) and the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN/ESPID) as diarrhea lasting between 14 and 28 days. This definition excludes causes such as food allergies, congenital intestinal disorders, and celiac disease. PD represents a significant public health concern, with prevalence rates ranging from 1.4% to 28.4% in low and middle-income countries. Globally, approximately 3-5% of infants are affected by this condition. In Vietnam, the rate of acute diarrhea progressing to PD ranges from 2.8% to 5.3%, reflecting the substantial burden of this pathology in the local context.

1.1.2. Etiology of persistent diarrhea

1.1.2.1. Microbial Causes

- Direct pathogenic agents: These include pathogenic strains of *E. coli* (EAEC, EPEC, EHEC/STEC, ETEC, EIEC), Campylobacter jejuni, *Clostridium difficile*, *Shigella* spp., *Cryptosporidium* spp., norovirus, and rotavirus.
- Gut microbiota dysbiosis: Characterized by an increase in the density of harmful bacteria and a decrease in beneficial bacteria, leading to an imbalance in the intestinal microbiome.

1.1.2.2. Non-microbial Causes

- Post-infectious diarrhea (PID): Occurs following episodes of acute intestinal infection, primarily due to rotavirus, norovirus, bacteria, or parasites.
- Antibiotic-associated diarrhea (AAD): Accounts for 5-30% of PD cases in children, reflecting the impact of antibiotics on the gut microbiota.

1.1.3. Risk Factors

Research has identified several key risk factors associated with persistent diarrhea (PD)

- Age: Children under 12 months of age are at the highest risk, reflecting the immaturity of their immune system and gastrointestinal tract.
- Nutritional status: Lack of exclusive breastfeeding increases the risk of PD by 2 to 4 times. Malnutrition doubles the risk. Micronutrient deficiencies, particularly vitamin A, zinc, folic acid, and iron, also increase the risk of PD.
- Medical history: Recent infections or a history of PD increase the risk by 2 to 4 times. Immunodeficiency increases the risk by 1.8 times.
- Environmental and social factors: Inappropriate diarrhea treatment and antibiotic misuse. Poor environmental hygiene and inadequate care. Low rotavirus vaccine coverage.

1.1.4. Clinical and laboratory evaluation

The assessment of PD in children requires a comprehensive approach, combining medical history, clinical evaluation, and laboratory tests.

Clinically, attention should be paid to risk factors, stool characteristics, accompanying symptoms, dehydration status, electrolyte imbalances, and nutritional status. Abdominal and perianal examinations also provide important information.

Regarding laboratory evaluation, blood tests help assess anemia, infection, and electrolyte disorders. Stool examinations, particularly real-time PCR techniques, are crucial in identifying causative agents. Imaging methods such as abdominal X-rays and ultrasounds also aid in differential diagnosis.

1.2. The Gut Microbiome

1.2.1. Characterization of the gut microbiome through 16S rRNA gene sequencing

16S rRNA gene sequencing technology, particularly targeting the V3-V4 region, utilizing the Illumina platform, has emerged as the gold standard in gut microbiome research. The 16S rRNA gene possesses several ideal characteristics for bacterial identification and classification, including ubiquity, evolutionary conservation, optimal length, and distinctive structure. Next-Generation Sequencing (NGS) technology demonstrates superiority over traditional Sanger methods in terms of throughput, accuracy, speed, and cost-effectiveness. This approach has opened new horizons in gut microbiome research, promising significant applications in diagnostics and therapeutics. The method not only provides a detailed and comprehensive view of intestinal bacterial composition but also facilitates the development of novel diagnostic and treatment strategies based on a deeper understanding of human-microbiome interactions.

1.2.2. Composition and functional roles of the gut microbiota

The gut microbiota is a complex ecosystem comprising hundreds of bacterial species from over 50 phyla. The three dominant phyla -

Firmicutes, Bacteroidetes, and Actinobacteria - play crucial roles in carbohydrate metabolism, short-chain fatty acid production, and immune system support. Other phyla, including Proteobacteria, Fusobacteria, and Verrucomicrobia, contribute to the diversity and balance of the gut microbiota. The development of the gut microbiota during the first 1000 days of life is critical for long-term health outcomes. Firmicutes, represented by *Lactobacillus* and *Clostridium*, are involved in lactose metabolism and SCFA production. Bacteroidetes, including Bacteroides and Prevotella, are essential for complex fiber fermentation and immune regulation. Actinobacteria, primarily *Bifidobacterium*, dominate the infant gut microbiome and metabolize HMOs.

1.2.3. Establishment of a healthy gut microbiota

The development of the gut microbiome during the first 1000 days of life encompasses three critical stages: prenatal, postnatal, and the introduction of solid foods. The mode of delivery, breastfeeding, and complementary feeding play pivotal roles in shaping the composition and diversity of the gut microbiota. The transition from *Bifidobacterium* dominance to a more diverse bacterial community reflects adaptation to increasingly complex dietary patterns. Initially, vaginal delivery and breastfeeding promote the establishment of beneficial bacteria, particularly *Bifidobacterium,* which metabolizes human milk oligosaccharides efficiently. The weaning period marks a significant shift, characterized by a decrease in *Bifidobacterium* and an increase in *Bacteroides* and *Clostridium*, along with enhanced short-chain fatty acid production capacity. By the end of the second year, the infant gut microbiome approaches an adult-like composition. Understanding these developmental stages may provide opportunities for early interventions to improve long-term health outcomes in children.

1.2.4. The role of gut microbiota in diarrheal diseases

The gut microbiota plays a crucial role in maintaining gastrointestinal health and immune function. Dysbiosis, a state of

microbial imbalance, can lead to various disorders, including persistent diarrhea.

1.2.4.1. Reduced diversity and altered abundance of bacterial components

Dysbiosis is characterized by a decrease in the diversity and density of beneficial bacteria, coupled with an increase in pathogenic or opportunistic bacteria. Factors such as mode of delivery, early antibiotic use, and dietary patterns can significantly influence the development of gut microbiota in neonates and infants.

The decline of *Bifidobacterium* species (e.g., *B. longum* subsp. *infantis*, *B. bifidum, B. breve*) is particularly significant. These bacteria not only stimulate the developing immune system but also create an acidic and anaerobic environment, inhibiting the growth of pathogenic bacteria. This imbalance can persist up to 12 months of age and have long-term effects on the child's immune, metabolic, and neurological development.

1.2.4.2. Impact of gut microbiota dysbiosis on intestinal immune system

Dysbiosis can lead to intestinal immune dysfunction, including:

- Reduction in Regulatory T cells (Tregs):

Tregs play a crucial role in maintaining immune tolerance and preventing chronic inflammation. They secrete anti-inflammatory cytokines such as TGF-β and IL-10, controlling excessive immune responses. A decrease in Tregs can lead to immune imbalance and increased risk of chronic inflammation [52,58,62].

- Increase in T helper 17 (Th17) cells:

Th17 cells produce IL-17, a key cytokine in activating inflammatory responses. Dysbiosis can increase intestinal permeability, activating Pattern Recognition Receptors (PRRs) on intestinal epithelial cells. PRRs stimulate the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-18, TNF-α), thereby promoting the development and increased density of Th17 cells.

In conclusion, maintaining gut microbiota balance is crucial for ensuring normal immune responses and preventing chronic intestinal

inflammation. A deep understanding of the relationship between dysbiosis and immune dysregulation opens new prospects for developing intervention strategies to prevent and treat PD.

1.3. Probiotics as a treatment for persistent diarrhea

Probiotics are emerging as a promising adjunctive therapy for persistent diarrhea (PD) in children. International studies and guidelines, particularly from ESPGHAN, have demonstrated the efficacy of specific probiotic strains in reducing the duration and severity of diarrhea. However, for PD, evidence remains insufficient for formal recommendations. Key points of consideration:

There is a notable absence of studies evaluating the impact of probiotics on the gut microbiome in children with PD, both in Vietnam and globally. This significant gap in the literature presents an opportunity for groundbreaking research.

In Vietnam, while probiotics have been incorporated into guidelines for acute diarrhea treatment, their application in PD management remains limited. This underscores the urgent need for studies assessing probiotic efficacy within the specific context of Vietnam.

Spore-forming probiotics (SFP), particularly Bacillus strains, are emerging as a promising research direction. Bacillus strains offer several advantages: (i)High survival rates in the harsh gastrointestinal environment; (ii) Beneficial genetic and metabolic characteristics, lacking antibiotic resistance genes; (iii) Safety profiles recognized by authoritative bodies such as the FDA and EFSA; (iv) Capacity to produce extracellular enzymes and compounds beneficial for gut health. However, several challenges remain to be addressed:

Further long-term clinical studies are needed to comprehensively evaluate the efficacy and safety of SFP; The strain-specific nature of probiotic efficacy necessitates individual assessment for each Bacillus strain. Research is required to determine the specific efficacy of SFP in treating pediatric PD, including optimization of dosage and duration of use.

Chapter 2
SUBJECTS AND METHODS

2.1. Study Population

2.1.1. Inclusion Criteria

- Infants and toddlers aged 3-24 months with persistent diarrhea for 2-4 weeks, occurring ≥3 times/day.
- Children who have not responded to previous treatments and require hospitalization.
- Parents/guardians have provided informed consent.

2.1.2. Exclusion Criteria

- Children with severe conditions requiring intensive care.
- Children with chronic diseases, immunodeficiency, or congenital malformations.
- Preterm infants or those with severe malnutrition.
- Children who have previously used probiotics containing the study strains.

2.2. Study Period and Location

- **Study Period:** From 2022 to 2023.
- **Study Location:** Department of Gastroenterology

2.3. Research Methodology

2.3.1. Study design and sample size calculation methods

Objective 1: Cross-sectional Descriptive Study

- **Design:** Cross-sectional descriptive study
- **Sample Size Calculation Method:** Applied formula for determining a single proportion.
- **Purpose**: To estimate the proportion of children with persistent diarrhea exhibiting specific clinical and labatory characteristics.
- **Sample Size:** $n \geq 147$

Objective 2: Comparative Cross-sectional Study

- **Design:** Comparative cross-sectional study
- **Sample Size Calculation Method:** Utilized formula for comparing two independent groups.

- **Purpose**: To evaluate differences in microbiota composition between children with PD and healthy children.
- **Sampling Method:** Stratified random sampling to ensure balance between groups on key factors
- **Sample Size:** PD group: $n \geq 17$; Healthy control group: $n \geq 13$

Objective 3: Randomized Controlled Trial (RCT)

- **Design:** Randomized Controlled Trial
- **Sample Size Calculation Method:** Applied formula for superiority clinical trials
- **Purpose**: To compare treatment efficacy between probiotics and control groups.
- **Sampling Method:** Based on previous studies and assumptions about probiotic treatment efficacy

  Sample Size: Total: $n \geq 147$; Intervention group (probiotics): $n1 = 98$; Control group (placebo): $n2 = 49$

2.3.2. Research Procedure

Children aged 3-24 months with persistent diarrhea at the National Children's Hospital. Minimum target of 147 subjects.

2.3.2.1. Data Collection for Objective 1

Utilization of standardized medical records, clinical assessments, and laboratory tests (blood, stool).

2.3.2.2. Randomization and Sampling for Objective 2

- Randomization: Implementation of GraphPad software.
- Stratified random sampling: Minimum of 17 PD children and a healthy reference group.
- 16S rRNA analysis of stool samples.

2.3.2.3. Intervention and Data Collection for Objective 3

- Three arms: Control (placebo), Clausy (single-strain probiotic), Dia30 (multi-strain probiotic).
- Specific dosage regimen and monitoring protocol.
- Periodic clinical evaluations and laboratory assessments.

2.3.3. Ensuring Double-Blinding

Identical packaging, sample coding, emergency unblinding protocol.

2.3.4. Study Variables

Independent variable (treatment method), dependent variable

2.3.5. Research Indicators

2.3.5.1. Assessment Indicators

- Primary: Proportion of children with resolved persistent diarrhea (PD) at day 5 (clinical and laboratory criteria).
- Secondary: Alterations in gut microbiome and immunological parameters.

2.3.5.2. Evaluation of probiotics efficacy

- Comparative analysis of recovery rates between groups.
- Assessment of changes in gut microbiome and immune response.
- Safety monitoring.

2.3.6. Data Management and Analysis

- Data collection via REDCap, coded according to ICD-10.
- Two-tier data quality assurance process.
- Intention-to-Treat (ITT) analysis.
- Statistical tools: GraphPad Prism and R for analyses.
- Methods: 95% confidence intervals, Shapiro-Wilk test, MaAsLin2.
- Supplementary analyses: Kaplan-Meier analysis for time-to-recovery; Gut microbiome diversity analysis (Alpha and Beta diversity).

2.3.7. Bias and Bias Control

2.3.7.1. Systematic Bias

- Identification: Selection bias, data collection errors, non-compliance with intervention, loss to follow-up, measurement bias, and confounding.
- Control measures: Strict selection criteria application, research team training, compliance monitoring, intention-to-treat analysis, standardized measurement methods, and confounding factor control.

2.3.7.2. Random Error

- Sources: Measurement biological variation, and sampling error.

- Mitigation: Increasing sample size, repeated measurements, protocol standardization, and appropriate statistical analysis.

2.3.8. Research Ethics

Approval by the Ethics Committee of the National Children's Hospital. Adherence to Helsinki Declaration, ICH GCP, and Ministry of Health regulations. Provision of comprehensive information and obtaining informed consent, ensuring the right to withdraw from the study; Personal information confidentiality. Reporting of serious adverse events within 24 hours. Study registration on ClinicalTrials.gov. Results publication following CONSORT guidelines. Declaration and management of potential conflicts of interest.

Chapter 3
RESEARCH RESULTS

This study examined 158 children with PD aged 3 to 24 months.

3.1. Clinical and laboratory characteristics

3.1.1. Epidemiological characteristics and risk factors

Median age was 5 months (IQR: 3 months), with 57.6% of children under 6 months old. Males accounted for 58.2% of cases. Key risk factors: cesarean delivery (72.1%), history of illness (45.6%), antibiotic use (42.4%) within 2 months prior to PD onset. 53.8% of mothers had illnesses during pregnancy, of which 72.9% were COVID.

3.1.2. Clinical manifestations of children with persistent diarrhea

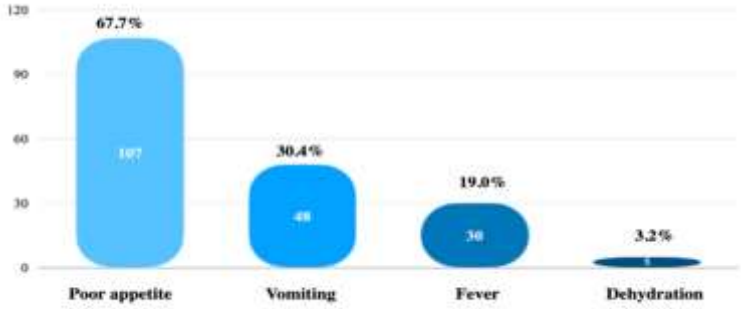


Figure 3.1. Common clinical symptoms in children with persistent diarrhea (n = 158)

Observations:

- Poor appetite was the most common symptom (67.7%).

- Followed by vomiting (30.4%) and fever (19.0%).
- Only 3.2% of children showed signs of dehydration upon admission.

3.1.2.2. Stool Characteristics of children with persistent diarrhea

43.0% of children had 6-7 stools/day, 52.5% had a large amount of mucus in stool, and 25.3% had blood in stool. Stools were mainly type 4 (50.6%) and type 5A (43.7%) according to the Diapered classification. Most children had normal white blood cell counts (88.6%). The rate of decreased neutrophils was 19.6%, and anemia was observed in 32.3% of children, mostly mild. All children had white blood cells in stool, with 69.6% having a density of 3+ or higher; 44.3% had red blood cells in stool; 32.9% had acidic stool pH (≤ 5.5).

Table 3.8. Results of Bacterial Isolation by Stool Culture (n = 158)

Results	Number	Percentage (%)
Negative	150	94.9
Positive	8	5.1
Pathogens		
Escherichia coli	7	87.5
Salmonella enterica	1	12.5

Observations: The positive rate of stool culture was low (5.1%). Among positive cases, *Escherichia coli* was predominant, with one case of *Salmonella enterica*.

Table 3.9. Distribution of Pathogens Detected by PCR (n = 158)

Pathogens	Number	Percentage (%)
Negative	118	74.7
Positive	40	25.3
Bacteria	19	47.5*
Co-infection (bacteria and virus)	12	30.0*
Virus	9	22.5*

Observations: High negative rate of 74.7% (118/158 cases) with no specific pathogens detected by PCR. Positive rate was 25.3% (40/158 cases). Among positive cases, bacteria accounted for the highest

proportion at 47.5% (19/40), followed by co-infection of bacteria and viruses at 30.0% (12/40), and viruses alone at 22.5% (9/40). The most common pathogens were *C. difficile*, Norovirus, *Campylobacter* spp., EAEC, and *Salmonella* spp. Children aged 3-6 months showed the greatest diversity of pathogens.

3.2. Description of gut microbiota changes in children with persistent diarrhea

3.2.1. Diversity of gut microbiota

3.2.1.1. Alpha diversity of gut microbiota

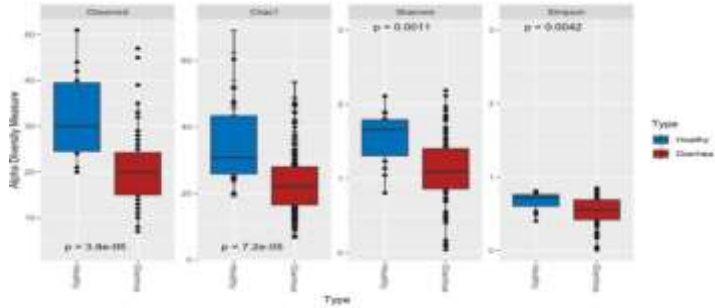


Figure 3.4. Comparison of alpha diversity indices of gut microbiota between healthy children and children with persistent diarrhea

Observations:

Children with persistent diarrhea (PD) have lower species diversity compared to healthy children. The distribution of bacterial species is less even in children with PD. The number of rare species is lower in children with PD.

3.2.1.2. Beta diversity of gut microbiota

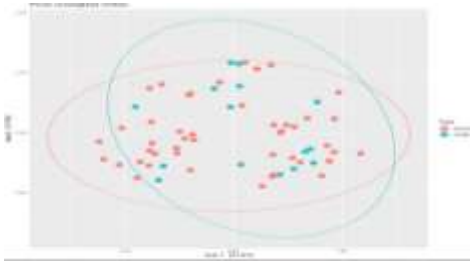


Figure 3.5. Principal Coordinate Analysis of gut microbiota in healthy children and children with persistent diarrhea

Observations: The incomplete separation between healthy and PD groups in PCoA analysis suggests that PD does not cause an abrupt change in the microbiome, but rather a gradual transition process.

3.2.2. Composition of gut microbiota in children with persistent diarrhea

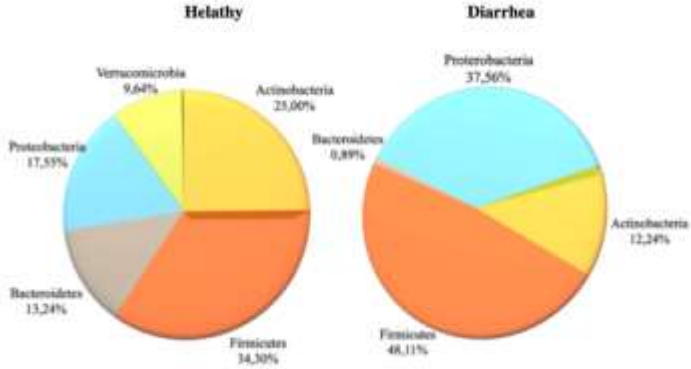
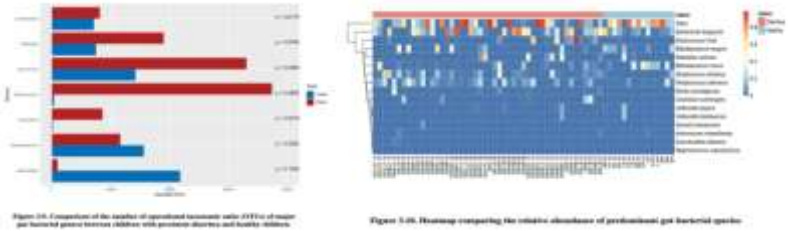


Figure 3.6. Comparison of gut microbial abundance in healthy children and children with persistent diarrhea at the phylum level

Observations: In children with PD, there is a significant increase in the phyla Proteobacteria and Firmicutes, while the phyla Actinobacteria and Bacteroidetes decrease markedly compared to healthy children.



Observations:

Children with PD show compositional changes including a significant decrease in the genus *Bifidobacterium*, and increases in the genera *Escherichia*, *Clostridium*, and *Streptococcus* compared to healthy children. These differences are statistically significant. There is

a decrease in *B. longum* and *B. breve*, along with an increase in *E. fergusonii*, *K. variicola*, and *C. perfringens* in children with PD.

3.3. Evaluation of treatment efficacy for persistent diarrhea with probiotic supplementation

3.3.1. Primary study outcomes

The study demonstrates the superior efficacy of probiotics in treating PD. The Dia30 group (3 probiotic strains) had the highest cure rate (77.4%) and shortest treatment duration (median 2-3 days), followed by the Clausy group (60.8%, 3-4 days), and lowest in the Control group (31.5%, 5-6 days).

The likelihood of cure in the Dia30 and Clausy groups was 7.43 and 3.37 times higher than the Control group, respectively. The differences were statistically significant between groups ($p<0.0001$), with the Dia30 group showing significantly better results compared to the Clausy group.

Adverse events leading to treatment discontinuation: No cases were reported in all three groups (Control, Clausy, Dia30). No cases required treatment discontinuation in all three groups.

3.3.2. Secondary study outcomes on day 5 of intervention

3.3.2.1. Impact on intestinal microbiota

The Control and Clausy groups showed a trend of decreasing alpha diversity indices compared to day 0, with statistically significant differences in Shannon and Simpson indices ($p < 0.05$). The Dia30 group showed a trend of increasing these indices at day 5, higher than the other two groups. Both probiotic preparations (Clausy and Dia30) demonstrated efficacy in reducing the density of potentially pathogenic bacteria such as *Escherichia* and *Clostridium*, with Dia30 showing superior efficacy. Dia30 better maintained the density of *Bifidobacterium*, while both Clausy and Dia30 significantly promoted the increase of *Lacticaseibacillus*.

3.3.3.2. Impact on immune factors

Effect on immune response: Dia30 demonstrated superior efficacy in modulating the inflammatory response, with significant

reductions in several pro-inflammatory cytokines (IL-17, IL-23, IL-6, TNF-α). Clausy also showed positive effects, albeit to a lesser extent, mainly focusing on IL-17 and IL-23. A slight increase in IL-10 (anti-inflammatory cytokine) in the Dia30 group suggests a potential for balancing the immune response. Impact on fecal secretory IgA: Both Dia30 and Clausy significantly reduced the concentration of fecal secretory IgA, with Dia30 showing more pronounced effects. This may reflect an improvement in intestinal barrier integrity and a reduction in inflammation.

Chapter 4
DISCUSSION

4.1. Clinical and labatory characteristics of children with persistent diarrhea

4.1.1. Epidemiological characteristics of children with persistent diarrhea

The most striking finding is the high concentration of persistent diarrhea (PD) in the 3-6 month age group (57.6%), which differs significantly from the traditional view that PD is common in children aged 6-24 months. This suggests changes in environmental and biological factors are affecting the development of the gastrointestinal and immune systems in infants. This phenomenon can be explained by the following hypotheses:

(i) The development of the intestinal microbiome in the first 6 months of life is being influenced by environmental factors such as early antibiotic use and diet;

(ii) Changes in infant feeding practices, especially trends towards early weaning or inappropriate food supplementation;

(iii) Genetic factors may be impacting the maturation process of the gastrointestinal and immune systems in infants.

The study also noted a high proportion of mothers who experienced illness during pregnancy (53.8%), with COVID-19 accounting for up to 72.9%. This reflects the profound impact of the COVID-19 pandemic on maternal and child health, while suggesting a link between SARS-CoV-2 infection during pregnancy and the risk of PD in children. The mechanism may be related to the strong

inflammatory response caused by viral infection, as well as changes in the maternal intestinal microbiome affecting the initial formation of the infant's intestinal microbiome.

The high rate of cesarean delivery (72.1%) in the PD group is also a noteworthy finding, suggesting a link between delivery method and PD risk. This may be due to the lack of exposure to beneficial maternal bacteria during the birthing process, affecting the development of the immune system and gut microbiome in infants.

4.1.2. Clinical and labatory characteristics of children with persistent diarrhea

The study applied standardized assessment tools such as the CoMiSS and Diapered scales to comprehensively evaluate the clinical features of persistent diarrhea (PD). A significant finding in our research was the low rate of dehydration in children with PD. Only 3.2% of children showed signs of dehydration, and no cases of severe dehydration were recorded. This reflects an important characteristic of PD in infants: dehydration often progresses silently and less dramatically than in acute diarrhea. This result aligns with previous studies, such as Pathela et al., which indicated that only 5% of children with PD exhibited clinical signs of dehydration. This phenomenon can be explained by several mechanisms, including reduced digestive enzyme secretion leading to decreased loss of digestive fluids, gradual adaptation of the child's body to slow-onset dehydration, and possible activation of compensatory mechanisms by the endocrine and renal systems to maintain water and electrolyte balance over an extended period. However, despite the low dehydration rate, careful assessment and management remain crucial in PD treatment.

Our study applied the Diapered scale to systematically and comprehensively assess stool characteristics in infants with PD. Results showed: Most children had stool frequency of 5-7 times/day, reflecting intestinal dysmotility and increased digestive fluid secretion. Moreover, the majority of children had mucoid, sticky stools that were looser than normal (type 4 on the Diapered scale). This suggests malabsorption and

increased digestive fluid secretion due to intestinal mucosal inflammation. About a quarter of the children had gross blood in their stools, accompanied by moderate to high levels of mucus. These changes indicate intestinal inflammation and may be related to mucosal damage from prolonged inflammation or infection. Other symptoms such as vomiting may be related to bloating and abdominal distension due to food indigestion and increased intestinal motility. Anorexia was observed in 67.7% of children, similar to Vernacchio's study results (72%). Anorexia directly affects the nutritional status and quality of life of children.

The study combined both traditional methods and modern techniques to comprehensively assess the paraclinical characteristics of PD. Fresh stool microscopy revealed that all children had fecal leukocytes of grade 2 (+) or higher, and nearly half had fecal erythrocytes, significantly higher than previous reports. This reflects the degree of intestinal mucosal damage in PD. Additionally, children had decreased fecal pH $\leq$5.5, possibly due to a deficiency of beneficial *Bifidobacterium*, leading to reduced antibacterial capacity and increased risk of infection. The positive stool culture rate was very low (5.1%), with pathogenic bacteria identified as *E. coli* and *Salmonella*. Our study is the first in Vietnam to apply modern multiplex real-time PCR techniques to detect PD-causing agents in children. Results showed a 25.3% detection rate of PD-causing agents, significantly higher than the 5.1% when using traditional culture methods. However, up to 74.7% of cases remain unidentified, posing a challenge for further in-depth research.

4.2. Gut microbiota in children with persistent diarrhea

One of the most significant findings of the study is the substantial decrease in alpha diversity of the gut microbiota in children with persistent diarrhea (PD) compared to healthy children. This not only reflects a reduction in species richness but also indicates a decline in diversity and balance within the microbial community structure. This finding aligns with previous studies, further supporting the hypothesis

of a strong link between gut microbiota dysbiosis and diarrheal conditions in children. This decrease in alpha diversity may be both a consequence of intestinal inflammation and dysfunction, as well as a contributing factor to the prolongation of diarrhea. Notably, the study highlighted that the 3-6 month age period is a sensitive phase when the infant's gut microbiota is in its initial establishment and development process. Disturbances during this period not only increase the risk of PD but may also lead to increased sensitivity to food antigens and other enteric pathogens. This emphasizes the importance of protecting and maintaining gut microbiota balance in the early months of life.

Beta diversity analysis based on the unweighted UniFrac index has provided a new perspective on the nature of PD in children. Results showed significant overlap between the gut microbial community structures of healthy children and those with PD, rather than a clear separation. This suggests that PD may not be associated with an abrupt and comprehensive change in microbial community structure, but rather a gradual process with the retention of some characteristics of the normal microbiota. This finding has important implications for developing treatment strategies aimed at restoring gut microbiota balance.

At the phylum level, the study noted an increase in the abundance of Proteobacteria and Firmicutes, along with a decrease in Actinobacteria and Bacteroidetes in the PD group. The abnormal increase in Proteobacteria is considered a sign of dysbiosis in the gut microbial community. These changes may result from multiple factors, including maternal diet during pregnancy, maternal-infant microbiota transfer, mode of delivery and antibiotic use, as well as the child's nutritional regimen.

At the family level, the study observed a decrease in the abundance of beneficial bacterial families such as Bifidobacteriaceae and Bacteroidaceae, while potentially pathogenic bacterial families like Enterobacteriaceae, Streptococcaceae, and Clostridiaceae increased in abundance in the PD group. This imbalance may explain the

pathogenesis of PD through two main mechanisms: reduced production of short-chain fatty acids (SCFAs) and increased production of pro-inflammatory mediators.

At the genus level, the study noted a significant decrease in the abundance of the genus *Bifidobacterium*, a predominant beneficial bacteria in infants. Simultaneously, there was an increase in potentially harmful bacterial genera such as *Escherichia, Klebsiella,* and *Clostridium* in the PD group. This imbalance is crucial in understanding the pathogenesis of PD and may explain the trend of earlier onset of PD in children.

At the species level, the study detected the dominance of potentially pathogenic species with a significant increase in the abundance of certain species such as *Klebsiella variicola*, *Escherichia fergusonii*, and *Clostridium perfringens* in the PD group. Concurrently, there was a decrease in beneficial species belonging to the genera *Bacteroides* and *Bifidobacterium*. These changes may lead to disruption of important physiological functions of the gut microbiota, contributing to the pathogenesis of PD.

4.3. Efficacy of probiotics supplementation in the treatment of persistent diarrhea

Clinically, probiotics, especially the multi-strain preparation Dia30, demonstrated superior efficacy in improving symptoms of persistent diarrhea (PD). Specifically, on the fifth day of intervention, the Dia30 group recorded the highest proportion of children with less than 3 stools/day (75.5%), compared to the Clausy group (54.9%) and the Control group (29.6%). Statistical analysis showed that Dia30 increased the likelihood of reducing stool frequency by 7.31 times compared to the Control group. Moreover, Dia30 exhibited superior efficacy in improving stool consistency and characteristics, with 81.1% of children achieving type 2-3 stools according to the Diapered scale, significantly higher than other groups. Notably, probiotics not only improved clinical symptoms but also reduced inflammatory markers in the stool. Both Clausy and Dia30 significantly decreased the presence

of erythrocytes and leukocytes in stool, with Dia30 showing more pronounced effects. This suggests the potential of probiotics in improving intestinal mucus layer integrity and reducing inflammation.

Regarding cure rates and recovery time, Dia30 continued to demonstrate superior advantages. On the fifth day post-intervention, the Dia30 group achieved the highest cure rate (77.4%), surpassing Clausy (60.8%) and the Control group (31.5%). Probiotics also significantly shortened treatment duration, with Dia30 helping children recover 3 days faster than the Control group. Notably, 100% of children using probiotics recovered within 10 days, while the control group required an additional 5 days of treatment. Besides clinical efficacy, the study provided deep insights into the mechanism of action of probiotics through gut microbiota analysis. Results showed that probiotics, especially the multi-strain preparation Dia30, can significantly modulate the structure and composition of gut microbiota in children with PD.

The assessment of alpha diversity of the gut microbiota showed that the Dia30 group was the only group exhibiting an increase in alpha diversity indices compared to pre-intervention and the other two groups. This suggests that multi-strain probiotics have a special ability to protect and even restore the gut microbiota against the impact of antibiotics. Conversely, both the Control and Clausy groups recorded a decrease in alpha diversity indices, reflecting the negative impact of antibiotics on the gut microbiota.

Regarding the composition of the gut microbiota, the study noted significant changes in the relative abundance of bacterial phyla after intervention. Notably, both intervention groups (Clausy and Dia30) showed a strong trend of reducing the proportion of Proteobacteria - a phylum containing many enteric pathogens. At the family level, the abundance of Enterobacteriaceae decreased sharply in the intervention groups, with the most pronounced reduction in the Dia30 group. When analyzing at the genus level, the study observed a particularly significant decrease in the genus *Escherichia* - a common cause of diarrhea. The relative abundance of *Escherichia* decreased 5-7 fold in

the probiotic-receiving groups, with the most prominent effect in the multi-strain Dia30 group. Similarly, the genus *Clostridium*, a group of bacteria including many enteric pathogens, also recorded a significant decline after probiotic intervention.

In addition to controlling pathogenic bacteria, the study also noted the emergence and increase of beneficial bacterial genera. Notably, the appearance of the genera *Bacillus* and *Lacticaseibacillus* belonging to the phylum Firmicutes, which are not native members of the gut microbiota. The presence of *Bacillus* was only observed in the group treated with the multi-strain probiotic Dia30, increasing from 0.21% to 4.66%. Furthermore, there was a significant increase in members of the family Atopobiaceae (phylum Actinobacteria), especially in the probiotic intervention group.

These changes in the gut microbiota structure are closely related to the expression of cytokines and Immunoglobulin A (IgA). The study showed that the group treated with multi-strain probiotics Dia30 had significant improvement in cytokine balance. Specifically, there was a marked decrease in pro-inflammatory cytokines such as IL-6, IL-17, IL-23, TNF-α, along with an increase in the anti-inflammatory factor IL-10 compared to pre-treatment. Notably, the single-strain Clausy group only recorded decreases in IL-23 and IL-17, while the Control group showed no significant changes. The research results also indicated the ability of probiotics to intervene in the IL-23/IL-17 cytokine axis, which is closely related to chronic intestinal inflammation and intestinal barrier damage. The study noted improvements in the secretion levels of mucin-2 (Muc2) and IgA through the intestine, reflecting better function of the mucosal mucus barrier and immune barrier. Particularly, in the group of children treated with Dia30, the mucus secretion time was shorter and the decrease in fecal IgA secretion was stronger compared to other groups.

The synthesis of these analyses has elucidated the complex relationship between gut microbiota, immune response, and inflammatory status in persistent diarrhea (PD). The study not only reinforces existing understanding but also expands knowledge about the

mechanism of action of probiotics in treating PD. In particular, probiotic intervention, especially multi-strain forms, has shown the ability to effectively regulate these factors through effects on the IL-23/IL-17 axis.

Although the study has provided valuable insights, there are still some limitations that need to be considered. The fact that the research was conducted at the National Children's Hospital may lead to bias in sample selection, as cases brought here are often more complex. Additionally, the lack of longitudinal follow-up data limits the ability to assess changes in gut microbiota over time and the causal relationship with PD.

CONCLUSIONS

1. Clinical and labarotory characteristics of children with persistent diarrhea

General characteristics: PD mainly affects children aged 3-6 months (57.6%), with a higher proportion of males. The majority were born by cesarean section (72.1%), and over half of the mothers experienced illness during pregnancy, primarily COVID-19.

Main clinical manifestations include: Anorexia is most common (67.7%), with dehydration being rare (3.2%). Stool frequency is 6-7 times/day (43.0%). Stools are mainly type 4 and 5A according to the Diapered scale, with 52.5% having high mucus content and 25.3% containing blood.

Paraclinical findings: 32.3% of children have anemia, mostly mild. Stool microscopy shows 100% of children have positive fecal leukocytes, 44.3% have positive erythrocytes. Pathogenic microorganism detection rates: 5.1% by stool culture and 25.3% by real-time PCR.

2. Alterations in gut microbiota in children with persistent diarrhea

Reduction in biodiversity: Significant decrease in biodiversity and balance in microbial community structure in children with PD

compared to healthy children. The alteration process occurs gradually over time, not as an abrupt change.

Gut microbiota imbalance manifested by: Increased density of Firmicutes and Proteobacteria phyla along with a decrease in Actinobacteria and Bacteroidetes phyla in PD children compared to healthy children. Significant reduction in the genus *Bifidobacterium*, increase in *Escherichia*, *Clostridium*, and *Streptococcus* in the PD group. Decrease in *B. longum* and *B. breve*, along with an increase in *E. fergusonii, K. variicola*, and *C. perfringens* in PD children.

3. Efficacy of probiotic therapy in treating persistent diarrhea

The primary research indicator shows that the cure rate on day 5 of the Dia30 group (77.4%) was significantly higher than the Clausy group (60.8%) and the control group (31.5%).

Impact on gut microbiota: Strong reduction in pathogenic bacteria density of *Escherichia* and *Clostridium* in probiotic groups, especially Dia30. Significant increase in beneficial bacteria *Lacticaseibacillus* in both Dia30 and Clausy groups.

Impact on immune response: Reduction in inflammatory markers through the IL-23/IL-17 pathway. Increase in anti-inflammatory cytokine IL-10. Decrease in fecal IgA secretion, particularly evident in the Dia30 group.

RECOMMENDATIONS

1. Optimize the diagnosis and treatment protocol for persistent diarrhea in children by combining real-time PCR techniques with traditional culture methods, while integrating high-dose multi-strain probiotic therapy into early treatment regimens;
2. Promote in-depth research on the interaction between gut microbiota and persistent diarrhea through longitudinal studies and the establishment of a multi-center research network.