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The Effect of Vitamin D_3 Supplementation on Diarrhea Frequency, Duration, and Neutrophil-to-Lymphocyte Ratio (NLR) in Children with Diarrhea

Satrio Wibowo [1,*], Kirnia Tri Wulandari [1] and Nurlinah Amalia [2,3]

[1] Division of Gastroenterohepatology, Department of Pediatrics, Saiful Anwar General Hospital, Faculty of Medicine, Universitas Brawijaya, Malang 65122, Indonesia

[2] Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia

[3] Medical Research Center of Indonesia, Surabaya 61257, Indonesia

[*] Correspondence: satrio_wibowo@ub.ac.id; Tel.: +628113030582

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Abstract: Diarrhea remains a major cause of morbidity in children under five, and although current management is mainly supportive, Vitamin D_3 supplementation has emerged as a potential immunomodulatory approach whose clinical efficacy requires further clarification. This study aimed to evaluate the effect of Vitamin D_3 supplementation on clinical outcomes, specifically diarrhea frequency, duration, and neutrophil-to-lymphocyte ratio (NLR) in children with acute diarrhea. This single-blind, randomized controlled trial employed a pre-post test control group design involving 34 pediatric patients with diarrhea. Participants were allocated into two groups: one receiving standard therapy alone (control) and the other receiving standard therapy plus oral Vitamin D_3 supplementation for 14 days. Outcome measures included diarrhea frequency, duration, and neutrophil-to-lymphocyte ratio (NLR), evaluated pre-post intervention. Post-intervention analysis showed significant improvements in the intervention group compared to controls: shortened diarrhea duration ($p = 0.002$), reduced diarrhea frequency ($p = 0.000$), but no significant with NLR reduction ($p = 0.124$); however, the median delta NLR decreased more in the Vitamin D_3 group (-2.57) than in the control group (-0.13) ($p = 0.031$). No significant differences in dehydration status were observed between groups ($p = 1.000$). The intervention group also exhibited a marked increase in serum 25(OH)D levels post-supplementation ($p = 0.000$). However, after adjusting for baseline NLR using ANCOVA, the reduction was no longer directly attributable to the intervention ($p = 0.09$), though a strong trend favoring the Vitamin D group persisted. Vitamin D_3 supplementation may serve as an adjunctive immunotherapy, as it demonstrated beneficial effects on clinical outcomes in pediatric diarrhea, suggesting its potential through anti-inflammatory and immunomodulatory properties.

Keywords: Vitamin D; Diarrhea; Children; Neutrophil-to-Lymphocyte Ratio (NLR)

1. Introduction

Diarrhea remains one of the most common diseases affecting children and continues to be a significant global health concern, including in Indonesia. According to the World Health Organization (WHO) and UNICEF, approximately 1.7 billion cases of diarrhea occur annually, resulting in the deaths of 443,832 children under the age of five and 50,851 children aged 5–9 years worldwide. Seventy-eight percent of these fatalities transpire in poor nations, especially in areas of Africa and Southeast Asia. In low- and middle-income nations, each child under

five years old endures an average of 3 to 4 episodes of diarrhea annually. The 2020 Indonesian Nutrition Status Survey (SSGI) indicated a diarrhea prevalence of 9.8%. Based on the 2022 Indonesia Health Profile, diarrhea accounts for 6.6% of deaths among children aged 29 days to 11 months, and 5.8% of deaths among children aged 12 to 59 months [1,2].

Current therapeutic approaches for diarrhea predominantly emphasize dietary adjustments for etiologies such as osmotic diarrhea from lactose intolerance, alongside urgent rehydration therapy to prevent dehydration-related morbidity and mortality [3]. One of the primary therapeutic approaches to avoid recurrent diarrhea is zinc supplementation, which exerts its benefits partly by modulating the immune system [4]. This aligns with the concept of immunotherapy, where the primary goal is to reestablish the immune system's ability to prevent and combat disease [5]. While this is an important therapeutic principle, the application of immunotherapy-based strategies in pediatric diarrhea remains limited, with zinc being the sole widely implemented option. Given the immunomodulatory potential could provide additional clinical benefits in pediatric diarrhea [6,7].

Vitamin D is a fat-soluble vitamin that plays multiple essential roles in human physiology. Because of its lipophilic nature, its intestinal absorption and systemic bioavailability depend greatly on the formulation and delivery system used. Sustained-release or matrix-based oral formulations have been shown to maintain more stable plasma concentrations and improve therapeutic consistency for lipophilic agents. Such delivery strategies are particularly relevant for vitamin D, as they may optimize absorption and prolong biological activity. Beyond its classical role in calcium and phosphorus homeostasis, Vitamin D also functions as a potent immunomodulator, influencing both innate and adaptive immune responses [8–10]. Low levels of Vitamin D have been associated with increased susceptibility to and severity of acute infections, as well as adverse outcomes in several chronic infections [11,12].

Nevertheless, studies investigating the relationship between Vitamin D levels and diarrhea remain limited [13, 14]. Cojic et al. reported on the use of Vitamin D_3 supplementation to reduce the incidence of diarrhea in children, highlighting the pleiotropic nature of Vitamin D, particularly its ability to modulate immune function by enhancing innate immunity, including the production of intestinal antimicrobial peptides. Given its association with enteric immune function, the role of Vitamin D in diarrhea has become a subject of growing research interest. For instance, several studies have found that low serum levels of 25-hydroxyvitamin D are associated with increased diarrhea severity and poorer outcomes among young children in Bulgaria [15,16]. Conversely, other researchers in Afghanistan reported that Vitamin D supplementation failed to reduce the risk of recurrent diarrhea in their infant study population [17,18]. These mixed findings suggest that Vitamin D may be involved in the pathogenesis of diarrhea.

In diarrhea, dysregulated immune responses can be reflected in biomarkers such as the neutrophil-to-lymphocyte ratio (NLR), where elevated values often indicate inflammation [19]. Azim et al. reported a higher neutrophil percentage and lower lymphocyte percentage in children with diarrhea in Bangladesh [20]. Compared to other biomarkers, the NLR may serve as a diagnostic tool due to its cost-effectiveness, accessibility, and ease of calculation from routine hematological tests. NLR plays a significant role in the diagnosis, differential diagnosis, assessment of disease severity, and prognosis of various infectious diseases, including diarrhea [21]. Vitamin D may influence the NLR in children with diarrhea through its immunomodulatory effects. NLR functions as a marker of systemic inflammation; an increased NLR reflects elevated neutrophil activity and decreased lymphocyte levels, which are commonly associated with infection and inflammation [19].

A large randomized controlled trial by Aluisio et al. (2023) involving 3046 children aged 1 to 11 years investigated the effects of quarterly high-dose Vitamin D_3 supplementation (100,000 IU) on diarrheal outcomes. The study reported no significant reduction in the time to first diarrheal episode or risk of recurrence between intervention and control groups, though a reduction in overall diarrheal burden was observed. These findings highlight the complexity of Vitamin D's role in diarrheal disease and suggest that while Vitamin D supplementation may confer some benefit, its effects on duration and recurrence remain uncertain. This inconsistency underscores the need for further research to clarify the potential therapeutic impact and optimal supplementation strategies of Vitamin D_3 in pediatric diarrhea management [21]. Therefore, the present study was initiated due to the lack of prior research on the role of Vitamin D_3 supplementation in managing diarrhea. This study aims to determine the effect of Vitamin D_3 supplementation on diarrhea frequency, duration, and neutrophil-to-lymphocyte ratio (NLR) in children with diarrhea.

2. Materials and Methods

2.1. Study Design

This study is an experimental, randomized clinical trial with a single-blind, pre- and post-test control group design. The study received approval from the ethics committee of Saiful Anwar General Hospital (Approval No. 400/076/K.3/102.7/2025), ensuring adherence to ethical standards. The study sample was divided into two groups. Group 1 consisted of pediatric patients with diarrhea who did not receive Vitamin D supplementation (standard therapy alone), while Group 2 included pediatric patients with diarrhea who received oral Vitamin D supplementation (standard therapy with Vitamin D). Both groups received standard therapy consisting of oral rehydration solution (ORS), zinc supplementation (20 mg/day), and dietary advice, in accordance with WHO and national guidelines for diarrhea management.

The intervention and observation were conducted over 14 days for each research subject. The sample size for each group (n) was determined using the clinical trial formula [22].

$$n = \left(\frac{[Z_\alpha + Z_\beta]}{X_1 - X_2}\right)^2 Y.$$

Y represents $(1 - p)$ adjustment for data correlation. Based on prior studies and preliminary data, an effect size (Cohen's d) of approximately 0.8 was assumed, representing a large effect. An alpha level of 0.05 and a statistical power (β) of 80% were set. The researchers established a type I error rate of 5% and a type II error rate of 10%, which determined the required sample size for each group. Thus, the required sample size for each group is 16.2 (fixed at 17), resulting in a total sample size of 34 participants.

2.2. Inclusion and Exclusion Criteria

Inclusion criteria for this study were children aged 1 to 18 years diagnosed with infectious diarrhea by a pediatric gastrohepatology consultant at Saiful Anwar General Hospital, Malang. Infectious diarrhea was defined as diarrhea caused by viral, bacterial, or parasitic pathogens and confirmed using laboratory examinations. Confirmation included complete blood count (leukocyte count and neutrophil-to-lymphocyte ratio/NLR) and complete stool analysis, which supported the clinical diagnosis. Eligible participants had not received Vitamin D supplementation for at least one month before the study and had provided informed consent through their parents or guardians. Exclusion criteria included children who had taken Vitamin D or multivitamin supplements containing Vitamin D, received steroid therapy, were diagnosed with non-infectious diarrhea, or had been administered antibiotics (even a single dose) within one month before the study.

Dropout criteria involved children with diarrhea who missed a single dose of Vitamin D during the study and showed poor compliance upon routine monitoring, failed to attend follow-up visits at the pediatric gastrohepatology clinic, or died during the study period.

2.3. Randomisation and Blinding

A total of 34 participants were included in the study and allocated into groups using simple randomization based on a randomization table. Each participant was assigned a sequential number according to the order of enrollment and subsequently included as a research subject.

This study employed a single-blind design, in which the research subjects were unaware of the specific objectives of the data collection process, the manner in which the data were processed, or the nature of the intervention they received. However, the researchers and data analysts were fully informed regarding the intervention being administered. To support this design, informed consent was obtained from participants, and a standardized supplementation protocol was provided and explained to each patient. Participants received daily supplementation according to their assigned group and duration, with daily follow-up conducted to monitor adherence. At the end of the intervention, researchers confirmed whether the supplementation was taken completely and as prescribed.

2.4. Outcome Measure

The outcomes assessed in this study included diarrhea frequency, duration of diarrhea, and the neutrophil-to-lymphocyte ratio (NLR) in pediatric patients with diarrhea who received Vitamin D_3 supplementation as part

of their treatment. Diarrhea frequency and duration were assessed based on parental recall using a structured questionnaire. Diarrhea frequency was defined as the number of bowel movements per day, with diarrhea classified as ≥3 loose or watery stools within 24 hours. The frequency was recorded in units of days. Diarrhea duration was defined as the total number of consecutive days from the onset of diarrhea until the last diarrheal episode, also recorded in units of days.

The data were analyzed in stages, beginning with univariate analysis to summarize categorical variables as frequencies and numerical variables as mean ± SD or median (min–max), based on normality (Shapiro-Wilk test). Levene's test assessed variance homogeneity. Associations between groups were tested using Chi-square, independent t-test, or Mann–Whitney as appropriate based on the results of the normality test. To evaluate the effect of Vitamin D supplementation, paired t-tests or Wilcoxon tests were used within groups, and independent t-tests or Mann–Whitney between groups. Correlations between Vitamin D levels and diarrhea outcomes were assessed using Pearson or Spearman tests, with significance set at $p < 0.05$, as determined by SPSS software, version 26.

2.5. Clinical Trial Procedure

Serum 25(OH) Vitamin D levels were measured using 2 mL of venous blood, with reference values defined as follows: <20 ng/mL (deficiency), 20–29 ng/mL (insufficiency), 30–50 ng/mL (sufficiency), and >50 ng/mL (toxic). According to national pediatric guidelines, Vitamin D_3 supplementation is age-adjusted; however, since our study population consisted exclusively of children and adolescents aged 1–18 years, the regimen applied uniformly was 4000 IU/day for 14 consecutive days. Supplementation was administered in the morning or afternoon with fat-containing foods (e.g., eggs, fish, avocado, or olive oil).

Follow-up was conducted over 2 weeks, with laboratory tests performed at baseline and at week 2, including complete blood count (CBC), serum Vitamin D, neutrophil-to-lymphocyte ratio (NLR), and complete stool examination, including fecal occult blood test (FOBT). Pre- and post-test values were compared to assess changes in Vitamin D levels.

3. Results

3.1. Baseline Characteristics

This study involved 34 pediatric patients diagnosed with acute diarrhea who were hospitalized and followed up at the Pediatric Gastrohepatology Outpatient Clinic of Saiful Anwar General Hospital, Malang. Group 1 consisted of children with diarrhea who did not receive Vitamin D_3 supplementation, while Group 2 received oral Vitamin D_3 supplementation.

Based on **Table 1**, which presents the characteristics of the participants, the Vitamin D_3 supplementation group consisted predominantly of females (70.6%), while the control group had more males (64.7%), indicating a significant difference in sex distribution ($p = 0.039$). Both groups were mostly composed of children under 5 years old, with no significant difference in mean age ($p = 0.178$). Nutritional status was similar between groups, with no significant difference observed ($p = 0.753$). Diarrhea frequency differed significantly ($p = 0.001$): 94.1% of the supplementation group experienced 4–6 episodes per day, whereas 58.8% of the control group had more than 6 episodes per day. Most subjects in both groups had mild to moderate dehydration ($p = 1.000$). Respiratory tract infection was the most common comorbidity in both groups, with no significant difference in diagnostic distribution ($p > 0.05$).

Based on **Table 2**, at baseline, serum Vitamin D_3 levels were similar between groups. After Vitamin D_3 supplementation, there was a notable increase in serum levels in the intervention group, while the control group exhibited a slight decline.

Table 1. Baseline characteristics.

Variable	Intervention (n = 17)	Control (n = 17)	Total (n = 34)	*p*-Value
Sex				
Male	5 (29.4%)	11 (64.7%)	16 (47.1%)	0.039*
Female	12 (70.6%)	6 (35.3%)	18 (52.9%)	
Age (years), mean ± SD	5.76 ± 4.48	5.71 ± 5.11	5.73 ± 4.73	

Table 1. *Cont.*

Variable	Intervention (n = 17)	Control (n = 17)	Total (n = 34)	*p*-Value
Age category (years)				
<5	9 (52.9%)	10 (58.8%)	19 (55.9%)	
5–10	7 (41.2%)	3 (17.6%)	10 (29.4%)	0.178
11–18	1 (5.9%)	4 (23.5%)	5 (14.7%)	
Nutritional status				
Normal	12 (70.6%)	12 (70.6%)	24 (70.6%)	0.753
Mild undernutrition	2 (11.8%)	2 (11.8%)	4 (11.8%)	
Severe undernutrition	3 (17.6%)	2 (11.8%)	5 (14.7%)	
Overweight	0 (0%)	1 (5.9%)	1 (2.9%)	
Diarrhea frequency (times/day)				
<4	1 (5.9%)	0 (0%)	1 (2.9%)	0.001*
4–6	16 (94.1%)	7 (41.2%)	23 (67.6%)	
>6	0 (0%)	10 (58.8%)	10 (29.4%)	
Dehydration status				
None	3 (17.6%)	4 (23.5%)	7 (20.6%)	1.000
Mild - moderate	14 (82.4%)	13 (76.5%)	27 (79.4%)	
Comorbid				
Respiratory tract infection	12 (70.6%)	9 (52.9%)	21 (64.8%)	0.290
Malignancy	2 (11.8%)	2 (11.8%)	4 (11.8%)	1.000
Urinary tract infection	2 (11.8%)	3 (17.6%)	5 (14.7%)	1.000
Nephrotic syndrome	1 (5.9%)	0 (0%)	1 (2.9%)	1.000
Dengue	1 (5.9%)	1 (5.9%)	2 (5.9%)	1.000
Congenital heart disease	0 (0%)	1 (5.9%)	1 (2.9%)	1.000
Epilepsy	1 (5.9%)	1 (5.9%)	2 (5.9%)	1.000
Rotavirus vaccination				
Male	5 (29.4%)	11 (64.7%)	16 (47.1%)	0.039*
Female	12 (70.6%)	6 (35.3%)	18 (52.9%)	

Note: p values marked with * indicate statistical significance at the 5% level ($p < 0.05$).

Table 2. Serum Vitamin D levels in the intervention and control groups.

Timepoint	Group	N	Mean (ng/mL)	SD
Before Intervention	Intervention	17	17.59	6.55
	Control	17	18.36	6.63
After Intervention	Intervention	17	25.31	9.62
	Control	17	17.09	6.42

3.2. Effect of Vitamin D Supplementation on Duration and Frequency of Diarrhea

Figure 1 demonstrates that the duration of diarrhea was shorter in the group receiving Vitamin D_3 supplementation compared to the control group. Statistical analysis indicated that Vitamin D_3 supplementation was associated with a reduction in diarrhea frequency among pediatric patients.

3.3. Effect of Vitamin D_3 Supplementation on Serum 25-Hydroxyvitamin D Levels

Based on **Table 3** and **Figure 1**, the median serum 25-hydroxyvitamin D [25(OH)D] level before Vitamin D_3 supplementation was 15.30 ng/mL, which increased to 21.40 ng/mL after supplementation. Wilcoxon test results showed a statistically significant increase in Vitamin D levels following supplementation ($p = 0.000$). In the control group, the median baseline 25(OH)D level was 16.40 ng/mL, which decreased to 14.80 ng/mL after the observation period. The Wilcoxon test also showed a statistically significant decrease in Vitamin D levels in the control group ($p = 0.000$).

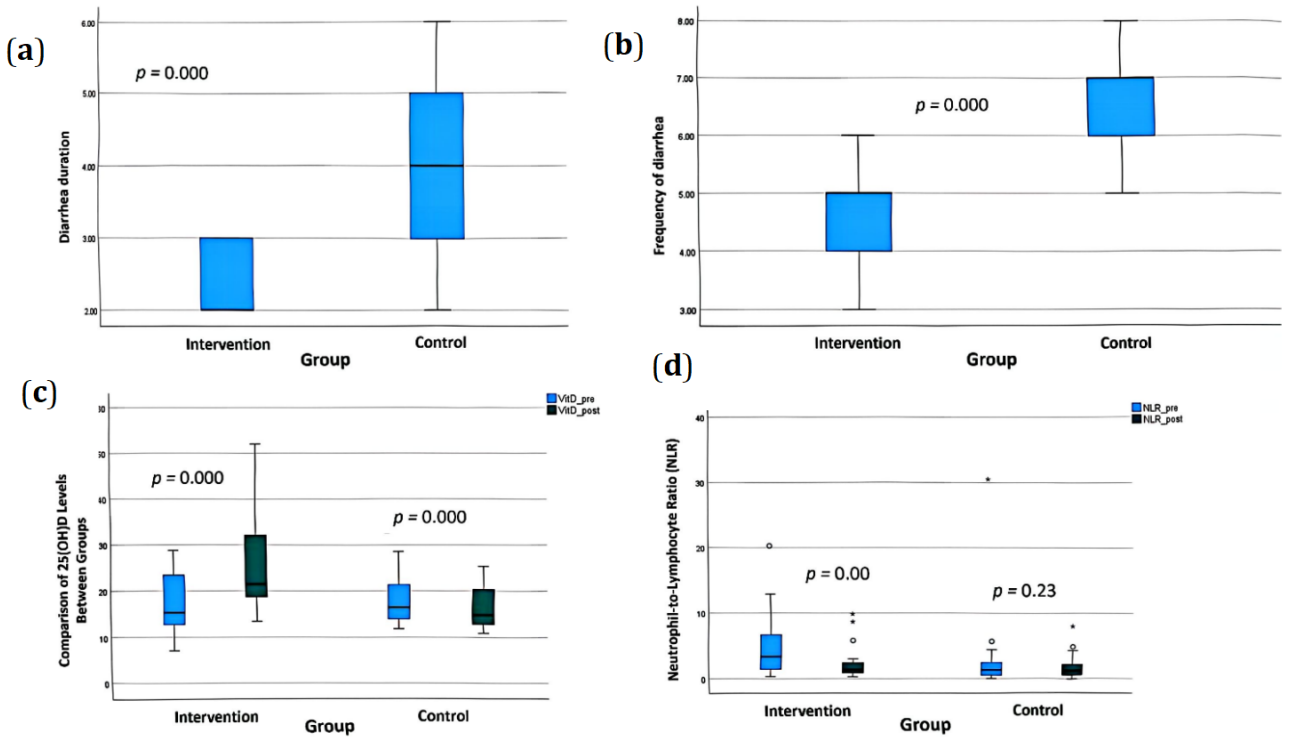

Figure 1. Box plots illustrating the clinical effect of Vitamin D_3 supplementation in children with diarrhea. Panels show (**a**) Duration of diarrhea in days; (**b**) Frequency of diarrhea episodes per day; (**c**) Difference in 25-hydroxyvitamin D levels between groups; (**d**) Neutrophil-to-lymphocyte ratio (NLR) before and after the intervention period. Significant differences between groups are indicated.

Table 3. Comparison of 25(OH) Vitamin D Levels Between Groups (before and after intervention).

Group	25-OH Vitamin D Levels*		Delta*	*p*-Value
	Before	After		
Intervention	15.30 (7.07–28.80)	21.40 (13.40–52.00)	5.9 (3.30–25.50)	0.000*
Control	16.40 (11.80–36.10	14.80 (10.90–35.80)	−0.8 (−3.80–−0.20)	0.000*
Mann-Whitney (p-value)	0.654	0.003*	0.000*	

Note: *Median (min-max).

Intergroup comparison revealed no significant difference in baseline 25(OH)D levels between the control and intervention groups ($p = 0.654$). However, after the intervention, the median final 25(OH)D level was significantly higher in the Vitamin D_3 group compared to the control group ($p = 0.003$). The delta change in 25(OH)D levels was positive in the Vitamin D_3 group (+5.90 ng/mL), indicating an increase, while the control group showed a negative delta (−0.80 ng/mL), indicating a decrease. This difference in delta values between groups was statistically significant ($p = 0.000$).

3.4. Effect of Vitamin D_3 Supplementation on Neutrophil-to-Lymphocyte Ratio (NLR)

Based on **Table 4** and **Figure 1**, the median neutrophil-to-lymphocyte ratio (NLR) prior to Vitamin D_3 supplementation was 3.36 and decreased significantly to 1.36 after supplementation ($p = 0.001$, Wilcoxon test). In the control group, the baseline median NLR was 1.28 and decreased slightly to 1.15, with no statistically significant change ($p = 0.236$). Intergroup comparison showed a significantly higher baseline NLR in the supplementation group compared to the control ($p = 0.034$). Post-treatment, the median NLR between the two groups was comparable with no significant difference ($p = 0.418$). The median delta NLR showed a greater reduction in the Vitamin D_3 group (−2.57) compared to the control group (−0.13), and this difference was statistically significant ($p = 0.031$).

Table 4. Comparison of NLR Levels Between Groups (before and after intervention).

Group	NLR Levels*		Delta*	*p*-Value
	Before	After		
Intervention	3.36 (0.32–20.28)	1.36 (0.27–9.88)	−2.57 (−10.40–2.09)	0.001*
Control	1.28 (0.1–30.52)	1.15 (0.0–9.02)	−0.13 (−22.50–2.66)	0.236
Mann-Whitney (p-value)	0.034*	0.418	0.031*	

Note: *Median (min-max).

3.5. Correlation of Serum Vitamin D Levels with Diarrhea Frequency, Duration, and NLR in Pediatric Patients Before and After Vitamin D_3 Supplementation

Based on **Figure 2**, a statistically significant negative correlation was found between serum 25-hydroxyvitamin D [25(OH)D] levels and the duration of diarrhea following Vitamin D_3 supplementation ($p = 0.002$; $r = -0.522$), indicating a moderate inverse relationship. This suggests that higher serum Vitamin D levels were associated with shorter duration of diarrhea. A very strong and statistically significant negative correlation was observed between diarrhea frequency and serum 25(OH)D levels ($p = 0.000$; $r = -0.801$), indicating that increased Vitamin D levels were associated with fewer episodes of diarrhea. Although the correlation between neutrophil-to-lymphocyte ratio (NLR) and serum 25(OH)D levels was negative ($r = -0.269$), it did not reach statistical significance ($p = 0.124$), and the strength of the relationship was weak.

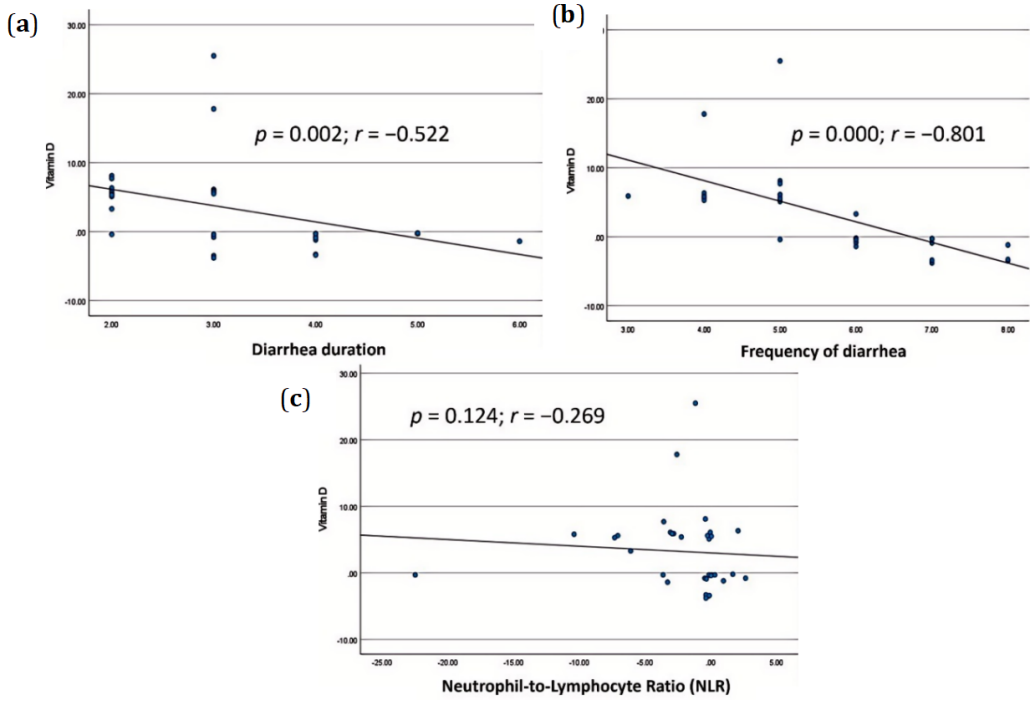

Figure 2. Scatter plots depicting correlations between serum 25-hydroxyvitamin D [25(OH)D] levels and clinical outcomes in pediatric diarrhea patients: (**a**) Correlation with diarrhea duration; (**b**) Correlation with diarrhea frequency; (**c**) Correlation with neutrophil-to-lymphocyte ratio (NLR). Correlation coefficients (r) and p-values are reported for each plot.

In this study, the majority of children in the intervention group experienced mild to moderate diarrhea (82.4%), which was comparable to the control group (76.5%). Statistical analysis revealed no significant association between Vitamin D_3 supplementation and the degree of dehydration ($p = 1.000$).

Multiple linear regression analyses were conducted to evaluate the effect of Vitamin D supplementation on diarrhea outcomes while controlling for potential confounders. Regression results for diarrhea duration showed a significant reduction associated with the intervention ($B = 1.63$, $p = 0.002$), independent of sex and diarrhea

frequency (**Table 5**). Similarly, frequency of diarrhea was significantly lower in the intervention group compared to controls ($B = 2.15$, $p < 0.001$) when adjusting for sex (**Table 6**).

Table 5. Multiple Linear Regression for Duration of Diarrhea.

Predictor	*B*	*p*-Value
Group (Intervention)	1.63	0.002
Sex	0.32	0.29
Frequency	0.004	0.98

Table 6. Multiple Linear Regression for Frequency of Diarrhea.

Predictor	*B*	*p*-Value
Group (Intervention)	2.15	0.000
Sex	0.1	0.75

To further adjust for baseline differences in NLR between groups, an analysis of covariance (ANCOVA) was performed. ANCOVA adjusting for baseline NLR, diarrhea frequency, and sex showed a non-significant trend toward lower post-intervention NLR levels in the Vitamin D group compared to controls ($F = 3.08$, $p = 0.090$) (**Table 7**). Baseline NLR and diarrhea frequency were significantly associated with post-intervention NLR ($p < 0.001$ and $p = 0.018$, respectively). Sex was not significantly associated with NLR changes. These findings suggest that while Vitamin D may reduce systemic inflammation, the effect was not statistically significant after controlling for baseline inflammatory status.

Table 7. Analysis of covariance (ANCOVA) comparing post-intervention neutrophil-to-lymphocyte ratio (NLR) between Vitamin D_3 supplementation and control groups, adjusted for baseline NLR.

Source	F	*p*-Value
Group (Intervention)	3.08	0.090*
NLR pre-intervention	55.33	0.000
Frequency of diarrhea	6.24	0.018
Sex	0.48	0.5*
Group	**Adjusted Mean NLR Post**	
Intervention	1.41	
Control	2.94	

Note: *not significant.

These findings collectively demonstrate the potential benefits of Vitamin D_3 supplementation in reducing both the frequency and duration of diarrhea episodes in pediatric patients. However, the impact on systemic inflammation as reflected by NLR requires further investigation due to a lack of statistical significance after adjusting for baseline values.

4. Discussion

This study has several limitations that warrant cautious interpretation of our findings. First, there were baseline imbalances between the intervention and control groups regarding diarrhea frequency and neutrophil-to-lymphocyte ratio (NLR), with the intervention group exhibiting higher initial severity and NLR values. These differences could potentially confound the observed effects of Vitamin D_3 supplementation. To address this, we performed additional analyses, including multiple linear regression and analysis of covariance (ANCOVA) to adjust for baseline differences, which still demonstrated a consistent trend toward reduced diarrhea frequency, duration, and systemic inflammation markers in the supplementation group. However, the small sample size and baseline disparities limit the generalizability and robustness of the conclusions, emphasizing the need for cautious interpretation and further studies with larger, more balanced populations.

While acknowledging these limitations and the need for cautious interpretation, it is important to consider the broader epidemiological context of diarrhea in pediatric populations. Diarrhea is more commonly observed in children under the age of five due to a combination of biological, behavioral, and environmental factors. This age group is particularly vulnerable to infections that cause diarrhea, thereby increasing morbidity and, in severe cases, leading to mortality [23]. Deficiency of Vitamin D is commonly observed in children with diarrhea, and several studies have suggested that low serum Vitamin D levels may be associated with increased risk and severity of diarrheal illness. A study by Hassam et al. (2019) involving 188 children under five years of age found that 53.7% had Vitamin D deficiency, 34% had insufficiency, and only 12.2% had sufficient levels. There was a significant difference in Vitamin D levels between children with and without diarrhea [24].

Vitamin D deficiency may be both a cause and consequence of diarrhea, especially in children. Diarrhea can exacerbate or contribute to Vitamin D deficiency through several mechanisms: impaired absorption of fat-soluble vitamins, reduced dietary intake, intestinal inflammation, decreased sun exposure, and increased fecal loss of Vitamin D [25–29].

4.1. Effect of Vitamin D_3 Supplementation on Duration and Frequency of Diarrhea

The pharmacological impact of Vitamin D_3 supplementation is also influenced by its formulation and delivery characteristics. As emphasized in drug delivery research, sustained or controlled-release oral formulations can optimize systemic bioavailability and maintain steady plasma concentrations over time, enhancing therapeutic efficacy [10]. Although the present study used a standard oral formulation, these pharmacokinetic considerations are relevant for future clinical optimization. Analysis in this study showed that Vitamin D_3 supplementation was associated with reduced duration of diarrhea in children. Mechanistically, the active metabolite of Vitamin D, 1,25-dihydroxyvitamin D_3 (calcitriol), enhances innate immune responses by stimulating the production of antimicrobial peptides like cathelicidin and β-defensins, which are crucial for defending against enteric pathogens [30].

Similar to phytoconstituents and nutraceutical compounds that exhibit immunomodulatory and antimicrobial properties through modulation of innate immune pathways, Vitamin D_3 exerts pleiotropic effects that reinforce mucosal defense mechanisms [31]. Within the innate immune system, Vitamin D_3 upregulates the transcription of genes encoding antimicrobial peptides, notably cathelicidin (LL-37) and β-defensins, which possess broad-spectrum microbicidal activity against enteric pathogens. These peptides disrupt microbial membranes, neutralize endotoxins, thereby accelerating pathogen clearance and reducing intestinal microbial load [32]. These immunomodulatory effects of Vitamin D_3 are mechanistically analogous to those observed with several bioactive dietary compounds, such as curcumin and resveratrol, which also act through modulation of NF-κB signaling and cytokine suppression to restore mucosal immune homeostasis [33,34].

Furthermore, it exerts a potent immunomodulatory effect by downregulating pro-inflammatory cytokines such as TNF-α and IL-6 while promoting the expression of anti-inflammatory cytokines like IL-10, thereby mitigating the excessive inflammatory response characteristic of infectious diarrhea [35]. In the adaptive immune system, Vitamin D_3 suppresses the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ), while concurrently enhancing the differentiation and expansion of regulatory T cells (Tregs) and promoting a balanced Th1/Th2 response. This immunoregulatory shift mitigates excessive mucosal inflammation, preserves epithelial barrier integrity, and prevents secondary tissue injury [36–39]. This immunomodulation is mediated through the suppression of key signaling pathways, including NF-κB and JAK/STAT, which are often upregulated during enteric infections. Inhibition of these pathways contributes to the reduction of cytokine storms and prevents prolonged diarrhea [40]. Additionally, Vitamin D regulates both innate and adaptive immunity [35,41–43].

Following ingestion or endogenous synthesis, Vitamin D_3 undergoes sequential hydroxylation in the liver and kidneys to yield its biologically active metabolite, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$]. This active form binds with high affinity to the Vitamin D receptor (VDR), a nuclear transcription factor expressed in a wide range of immune cells, including macrophages, dendritic cells, and T lymphocytes. The $1,25(OH)_2D$–VDR complex translocates to the nucleus, where it interacts with Vitamin D response elements (VDREs) in target gene promoters, thereby modulating the transcription of genes involved in antimicrobial peptide synthesis (e.g., cathelicidin and defensins), antigen presentation, cytokine production, and T-cell differentiation. Through these mechanisms, Vitamin D_3 promotes a shift toward an anti-inflammatory immune profile, enhances mucosal barrier integrity, and

suppresses excessive pro-inflammatory responses, collectively contributing to improved clinical outcomes in diarrheal disease [44–46].

Concurrently, Vitamin D is understood to support intestinal epithelial barrier function by upregulating the expression of tight junction proteins, including claudin, occludin, and ZO-1 [30]. This action helps preserve epithelial barrier integrity, reduce intestinal permeability, and limit the translocation of microbes and antigens, which is a key driver of systemic inflammation [47]. An additional indirect mechanism involves the modulation of the gut microbiota; Vitamin D supplementation has been shown to promote the proliferation of beneficial bacteria (e.g., Lactobacillus and Bifidobacterium) while suppressing pathogenic species, contributing to overall gut homeostasis and resilience [21].

It is important to note that the present study did not directly assess markers of mucosal integrity, such as tight junction proteins or fecal markers of intestinal permeability, which are critical for conclusively demonstrating improved gut barrier function. Instead, the systemic inflammatory condition was evaluated using the NLR as an indirect biomarker. Therefore, the observed reduction in diarrhea duration and systemic inflammation (as reflected by NLR) following Vitamin D_3 supplementation is likely mediated through these proposed but not directly measured immunomodulatory and barrier-strengthening pathways.

Future research incorporating direct measurements of mucosal integrity markers, such as tight junction protein expression or circulating gut barrier damage markers (e.g., zonulin, intestinal fatty-acid binding protein), is essential to fully elucidate the mechanistic role of Vitamin D in enhancing gut barrier function and reducing systemic inflammation in pediatric diarrheal diseases.

Comparable to Vitamin D_3, several nutraceuticals and dietary bioactive compounds have been recognized for their immunomodulatory and gut-protective properties. Phytoconstituents such as curcumin, resveratrol, and quercetin have been shown to modulate the gut–immune axis by enhancing intestinal barrier integrity, regulating proinflammatory cytokine expression, and promoting the production of antimicrobial peptides through Vitamin D-dependent or convergent signaling pathways. These nutraceuticals act synergistically with host defense mechanisms to maintain mucosal homeostasis and reduce enteric inflammation. Therefore, the immunonutrient role of Vitamin D_3 can be viewed within a broader spectrum of dietary immunomodulators that target epithelial defense, offering a promising direction for integrative strategies in managing pediatric diarrhea [33,34].

Integrating Vitamin D_3 supplementation within broader dietary and nutraceutical frameworks may therefore represent a promising adjunctive approach in pediatric diarrhea management. Future studies exploring combined nutraceutical–Vitamin D formulations or delivery systems could provide further insights into synergistic immunotherapeutic effects.

Effect of Vitamin D_3 Supplementation on Neutrophil-to-Lymphocyte Ratio (NLR)

In the current study, Vitamin D_3 supplementation did not demonstrate a statistically significant direct effect on reducing NLR levels after adjustment for baseline differences and clinical severity via ANCOVA ($p = 0.09$). However, a strong and consistent trend favoring a reduction in NLR persisted within the intervention group, suggesting the observed attenuation of systemic inflammation may be modulated by initial inflammatory status and clinical severity at baseline rather than attributable solely to the Vitamin D intervention.

However, as is well established, neutrophils are the most abundant white blood cells, play a central role in innate immunity, and typically increase during acute inflammation, bacterial infections, or tissue injury. In contrast, lymphocytes are key components of adaptive immunity and may decrease during immunosuppression, chronic illness, or stress. The neutrophil-to-lymphocyte ratio (NLR), calculated by dividing the absolute neutrophil count by the lymphocyte count, serves as a simple biomarker for systemic inflammation and immune status [48]. Elevated NLR indicates an imbalance favoring inflammation and immune suppression and has been associated with disease severity in conditions such as sepsis, COVID-19, gastrointestinal infections, and cancer [21,49].

Recent clinical studies support Vitamin D's role in modulating inflammatory responses as reflected by NLR. For example, Jeyakumar et al. (2024) demonstrated in a meta-analysis that Vitamin D supplementation significantly lowered NLR and improved inflammatory cytokine profiles in children with infectious diseases [50]. Tabatabaeizadeh et al. (2017) also reported reductions in pro-inflammatory cytokines and NLR following Vitamin D intervention in pediatric inflammatory conditions [51]. However, variations in baseline inflammation and disease severity complicate interpretations of NLR changes, underscoring the need for larger, well-designed trials to clarify Vitamin D's effects

on systemic inflammation and related hematological markers.

Despite the lack of statistical significance, a downward trend in NLR was observed in the Vitamin D-supplemented group compared to controls, consistent with Vitamin D's known immunomodulatory properties that modulate innate and adaptive immune cells, suppress pro-inflammatory cytokines, and enhance regulatory T cell function, all potentially contributing to lowered NLR. Overall, these findings imply that Vitamin D_3 supplementation may contribute to a downward trend in systemic inflammation as measured by NLR, but this pilot study, limited by sample size and baseline imbalances, could not definitively establish a statistically significant anti-inflammatory effect.

Based on an immunotherapy perspective, Vitamin D_3 acts as a pleiotropic biological modulator capable of reestablishing immune competence, thereby enabling the host to more effectively prevent and eliminate infectious agents. Compared to zinc supplementation, which remains the standard immunomodulatory adjunct in pediatric diarrhea and primarily facilitates epithelial regeneration and maintenance of barrier integrity, Vitamin D_3 exerts a broader spectrum of immune regulation. In addition to promoting mucosal healing, Vitamin D_3 orchestrates a coordinated interplay between innate and adaptive immune responses, thus addressing both the underlying cause and the downstream consequences of diarrheal disease. At the cellular level, Vitamin D_3 can stimulate intestinal stem cells within the crypts of Lieberkühn to undergo proliferation, lineage-specific differentiation, and terminal maturation into specialized epithelial cell types, including absorptive enterocytes, goblet cells, and Paneth cells. This process accelerates the restoration of mucosal integrity, enhances the production and secretion of antimicrobial peptides, and optimizes nutrient absorption. Furthermore, by strengthening epithelial–immune cell interactions within the gut-associated lymphoid tissue (GALT), Vitamin D_3 reinforces mucosal immune surveillance and contributes to long-term intestinal homeostasis [30,52–55].

This study reinforces the pivotal role of Vitamin D as an adjunctive immunotherapeutic agent in pediatric diarrhea, functioning not only as a nutritional supplement but also as a regulator of both innate and adaptive immune pathways. This study was conducted using a single-blind design, where investigators were aware of group allocation while participants were blinded. This approach carries an inherent risk of performance and detection bias, as knowledge of intervention status by investigators might influence patient management or outcome assessment, potentially affecting the internal validity of the study. A placebo-controlled, double-blind design would have been methodologically stronger to mitigate these biases. However, practical and ethical considerations constrained our study design choices. We explicitly acknowledge this limitation and recommend that future research employ double-blind protocols to enhance the rigor and reliability of findings regarding Vitamin D_3 supplementation effects in pediatric diarrhea.

5. Conclusions

This pilot study suggests that Vitamin D_3 supplementation may be associated with improvements in diarrhea frequency, duration, and systemic inflammation in children. These findings support the concept of Vitamin D_3 as an adjunctive immunotherapy, leveraging its dual role in enhancing mucosal barrier repair and modulating both innate and adaptive immune pathways. However, due to the small sample size and baseline differences between groups, these results should be interpreted cautiously. Larger, well-controlled studies are needed to confirm these preliminary observations and better define the therapeutic potential of Vitamin D_3 in pediatric diarrhea management.

Author Contributions

S.W. contributed to conceptualization, methodology, and validation; K.T.W. contributed to investigation and writing original draft preparation; and N.A. contributed to writing review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was authorized by Saiful Anwar General Hospital's Institutional Review Board (IRB) (Approval No. 400/076/K.3/102.7/2025) and carried out in compliance with the ethical guidelines specified in the Declaration

of Helsinki. All procedures involving human subjects were guaranteed to comply with the strictest guidelines for patient safety and study integrity, thanks to ethical clearance.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Due to institutional regulations, the datasets created and/or analyzed during this investigation are not publicly available; however, they can be obtained from the corresponding author with the proper institutional approvals and upon reasonable request.

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Conflicts of Interest

The authors declare no conflict of interest.

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