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Pro and Anti-inflammatory Cytokine Variations Arising from *Bifidobacterium* Occurrence in Diarrheal Infants

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Abstract:

The gut microbiota represents a sophisticated ecosystem of microorganisms that inhabit the gastrointestinal tract and play a crucial part in maintaining human health, among which *Bifidobacterium* is a vital member. This study aims to investigate the impact of *Bifidobacterium* incidence on different pro- and anti-inflammatory cytokines in infants suffering from diarrhea. 140 infants with diarrhea and 20 healthy infants below 2 years of age were enrolled in this study. For the isolation of *Bifidobacterium*, fecal samples were cultivated on the selective media under anaerobic conditions. The levels of interleukins (IL-10, IL-4, and 1L-6), tumor necrosis factor alpha (TNF- α) and transforming growth factor beta (TGF-β) were measured using the ELISA technique. IL-4, IL-6 and TNF- α were significantly ($P < 0.05$) higher in diarrheal infants, with medians of 17.549, 3.492, and 23.055 pg/ml, respectively, than in their counterpart healthy ones, 15.057, 2.557, and 1.945 pg/ml, respectively. According to the results, both of IL-10 and TNF-α significantly decreased in diarrheal infants harboring *Bifidobacterium* compared to those without *Bifidobacterium* (4.52 vs. 7.467; 9.87 vs. 30.5 pg/ml, respectively). It has been proven that *Bifidobacterium* enhances the immune system by decreasing TNF-α and IL-10 levels in infants with diarrhea, making it a potentially powerful immunological booster.

Key words: *Bifidobacterium*, Diarrhea, Gut dysbiosis, Interleukins, Inflammation .

التغيرات في الحركيات الخلوية المؤيدة والمضادة للالتهابات الناتجة عن وجود البيفيدوبكتريوم لدى الأطفال المصابين بالإسهال

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الخلاصة:

تمثل ميكروبات الأمعاء نظامًا بيئيًا متطورًا من الكائنات الحية الدقيقة التي تعيش في الجهاز الهضمي وتلعب دورًا حاسمًا في الحفاظ على صحة الإنسان، ومن بينها بكتيريا البيفيدوبكتريوم التي تعد عضوًا حيويًا. تهدف هذه الدراسة الى التحقيق في تأثير وجود البيفيدوبكتريوم على الحركيات الخلوية المؤيدة و المضادة

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للالتهاب المختلفة عند الاطفال الرضع الذين يعانون من الاسهال. تم تضمين 140 رضيعا مصابا بالإسهال و 20 رضيعا سليما بعمر اقل من السنتين في هذه الدراسة. لعزل بكتريا البيفيدوبكتريوم, زرعت عينات البراز على الاوساط الانتخابية تحت ظروف لاهوائية. قُيست مستويات البين ابيضاض (IL−10، IL−4، 1L−6)، وعامل نخر الورم ألفا، وعامل النمو المحول بيتا، باستخدام تقنية ELISA. كانت مستويات IL−4، IL−6، وTNF−α أعلى بشكل ملحوظ (P< 0.05) لدى الرضع المصابين بالإسهال، حيث بلغ متوسطها 17.549، 3.492، و23.055 بيكوغرام/مل على التوالي، مقارنةً بنظرائهم الأصحاء، حيث بلغ متوسطها 15.057، 2.557، و1.945 بيكوغرام/مل على التوالي. ووفقًا لوجود البيفيدوبكتريوم، كلٌّ من IL−10 وTNF−α انخفض بشكل معنوي في الرضع المرضى الحاملين البيفيدوبكتريوم عن الرضع المرضى الغير حاملين (4.52 مقابل 7.467 بيكوغرام/مل؛ 9.87 مقابل 30.5 بيكوغرام/مل، على التوالي. وقد ثبت أن البيفيدوبكتريوم يُعزز جهاز المناعة عن طريق خفض مستويات TNF−α وIL−10 في مصل الرضع المصابين بالإسهال، مما يجعلها مُعززًا قويًا محتملًا للمناعة.

1. Introduction:

The gut microbiota, which represents a sophisticated ecosystem of microorganisms that inhabit the gastrointestinal tract, plays a crucial role in maintaining human health [1]. Since their discovery in the human gut, *Bifidobacteria* have been the focus of many research studies due to the multiple health benefits they offer, including maintaining the intestinal barrier, modulating the gut microbiota, and exerting anti-inflammatory effects. Their characteristics position them as potential therapeutic agents for both gastrointestinal and extra-intestinal diseases [2].

Bifidobacteria are Gram-positive, non-motile, and non-spore forming bacteria that have a curved shape, often branching rods with a V or Y form [3]. *Bifidobacterium* and *Lactobacillus* are the most common bacteria that are being utilized as commercial probiotics [4]. *Bifidobacteria* are equipped with a genetic arsenal that allows them to cope with the harsh conditions of the gastrointestinal tract (GIT) and further establish themselves for long-term colonization[5].

They are among the first bacteria to enter an infant's gut [6]. *Bifidobacteria's* capacity to break down Human Milk Oligosaccharides (HMOs), which are indigestible by infants, contributes to its early colonization [7].

Interleukins, a subset of cytokines, are multifunctional polypeptides produced and secreted by multiple types of body cells. They possess clinical significance in the diagnosis, treatment, and prevention of illness [8]. As previously stated by many authors, *Bifidobacterium* serves a vital role in immune homeostasis. It can diminish intestinal T helper 17 (Th17) and T helper 2 (Th2) programs, up regulate T regulatory (T reg) cells, and modulate the activity of macrophage and dendritic cell (DC). Mechanisms involved in these events include many surface protein components of *Bifidobacterium* and also their metabolites [9]. *In vitro* experimental studies on mice treated with *B. adolescentis* ATCC15703 revealed a reduction in the pro-inflammatory cytokines (IL-6, IL-1β, IL-18, IL-9, IL-22, and TNFα) in the colon homogenate and an increase in the anti-inflammatory ones (IL-10, IL-4, and IL-5) in comparison to control [10]. Another study demonstrated that *Bifidobacterium* influences T-cell responses by inducing regulatory T-cells, enhancing IL-10 and galectin-1 secretion, and promoting cytotoxic T lymphocyte-associated protein 4 (CTLA-4)-dependent inhibition. This helps in modulating excessive immune reactions, especially in infections, contributing to the resolution of harmful immune overreactions[11]. Its effect on T reg is also confirmed by a previous study [12].

Bifidobacterium consumption could be an effective adjunct therapy for children's diarrhea, where Chen *et al.,* [13] revealed that *Bifidobacterium animalis subsp. lactis BLa80* was able to profoundly shorten the duration of diarrhea in the intervention group compared to the control.

Thus, this research aimed to investigate the impact of *Bifidobacteria* incidence on the inflammatory cytokine in newborns with diarrhea.

2. Materials and methods

2.1 Subject

A total of 160 samples were enrolled in this study between November 2023 and March 2024 from all admitted cases to the Children's departments of Fallujah Women and Children Hospitals. Those participants involved 140 newly born and below 2 years old infants with diarrhea of both genders and 20 healthy controls of the same age and gender. Exclusion criteria for this study were: (I) Infants with Inflammatory bowel disease. (II) Various other health conditions. (III) Dysentery and (IV) infants who received probiotics in the four weeks before. Patients' information was recorded from their relatives.

2.2 Sample collection

2.2.1 Bacterial isolation

Fecal samples were collected from infants and cultivated on de Man, Rogosa, and Sharpe (MRS) broth supplemented with 0.05% L-cysteine HCL and incubated for 48 hrs. at 37ºC under anaerobic conditions using a jar and gaspak (Thermo scientific, USA), then the inoculum was streaked on the selective media (Bifidobacterium agar) and incubated at the same conditions. *Bifidobacterium* isolates were identified initially by colony morphology [14] and the Vitek 2 Compact system using (ANC card)[15] .

2.2.2 Immunological analysis

For immunological tests, five milliliters of arm venous blood (median cubital vein) was collected from the infants in a gel tube. The samples were centrifuged at 3000rpm for 10 minutes, and the serum was obtained. Then, serum samples were stored in aliquots in a freezer at -80°C [16]. Serum samples were used for the measurement of inflammatory cytokines IL-10, 1L-6, 1L-4, TNF-α, and TGF-β. The sandwich enzyme-linked immunosorbent assay (ELISA) technique was used for the quantitative determination of cytokines (ELK Biotechnology, China)[17]. The detection procedures were conducted in accordance with the guidelines provided by the manufacturer.

2.3 Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Science (SPSS) program, version 20, for Windows. Results were expressed as median and range. To indicate the statistical significance, P values of < 0.05 and < 0.01 were used. The receiver operating characteristic (ROC) curve was used to indicate the predictive significance of the studied cytokines. The correlation coefficient and the relation between the different variables were also measured

3. Results

3.1 Laboratory identification of Bifidobacterium

Based on colony morphology (convex, circular, milky color, and small colonies on the selective media), bacterial isolates were primarily identified as *Bifidobacterium* isolates. Vitek 2 Compact system using (ANC card) further confirmed the organism.

3.2 Immunological analysis

The serum cytokine levels of IL-10, IL-4, IL-6, TGF-B, and TNF-α were analyzed in patient and control groups using a sandwich ELISA technique. Serum cytokine levels were tested for normality, and it was found that they were not normally distributed by Shapiro – Wilk test (P value > 0.001) for all tested cytokines. Accordingly, they were given as median and range. The present findings revealed that, as illustrated in Table 1, each of IL-4, IL-6, and TNF-α levels was significantly ($P < 0.05$) higher in diarrheal infants with a median of 17.549, 3.492, and 23.055 pg/ml, respectively, than in their counterpart healthy ones, 15.057, 2.557, and 1.945 pg/ml, respectively. However, IL-10 and TGF-β levels showed no significant differences ($P > 0.05$) between the study groups.

Table 1: Median serum level of cytokines distributed according to study groups

Cytokines	Cytokines Median (range) levels pg/ml			*P value*
	Total levels	Patients	Healthy control	
IL-10	6.266 (1.861-26.9)	5.749 (1.861 -26.9)	6.651 (3.43 – 12.367)	0.902
IL-4	16.589 (6.633- 62.214)	17.549 (6.633- 62.214)	15.057 (7.148- 28.201)	0.0313*
IL-6	3.36 (0.648 - 62.202)	3.492 (1.139 -62.202)	2.557 (0.648 -5.805)	0.01*
TGF-β	19.237 (2.071-59.808)	19.346 (2.071- 59.808)	16.025 (9.806 - 51.4)	0.886
TNF-α	19.498 0.321- 98.19)	23.055 (88.64- 98.19)	1.945 (0.321 -10.45)	0.001**

*, ** Indicate significant differences between study groups at $p < 0.05$ and $p < 0.01$ respectively.

Analysis of ROC was used in this study to determine the most accurate, specific, and sensitive parameter in diarrheal infants. ROC curve analysis revealed the predictive significance of IL-6 (AUC= 0.6958; 95%CI= 0.5306 to 0.8611; p= 0.0457; Youden index= 10.8; cut-off value = 1.339-fold; sensitivity= 30.0%; specificity =97.22%) in discriminating between diarrhea group and control Figure 1.

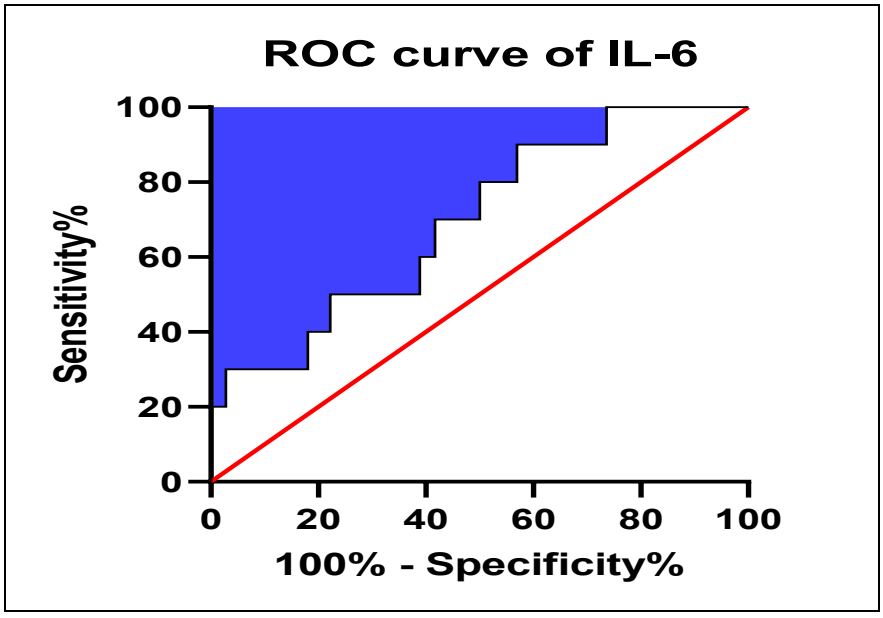

Figure 1: ROC curve plot of IL-6 for predicting diarrhea

Additionally, ROC curve analysis revealed the predictive significance of TNF-α (AUC= 0.9292; 95%CI= 0.8597 to 0.9987; p= <0.0001; Youden index= 43.20; cut-off value = 3.620-fold; sensitivity= 60.0%; specificity =98.61%) in discriminating between the diarrhea group and the control Figure 2.

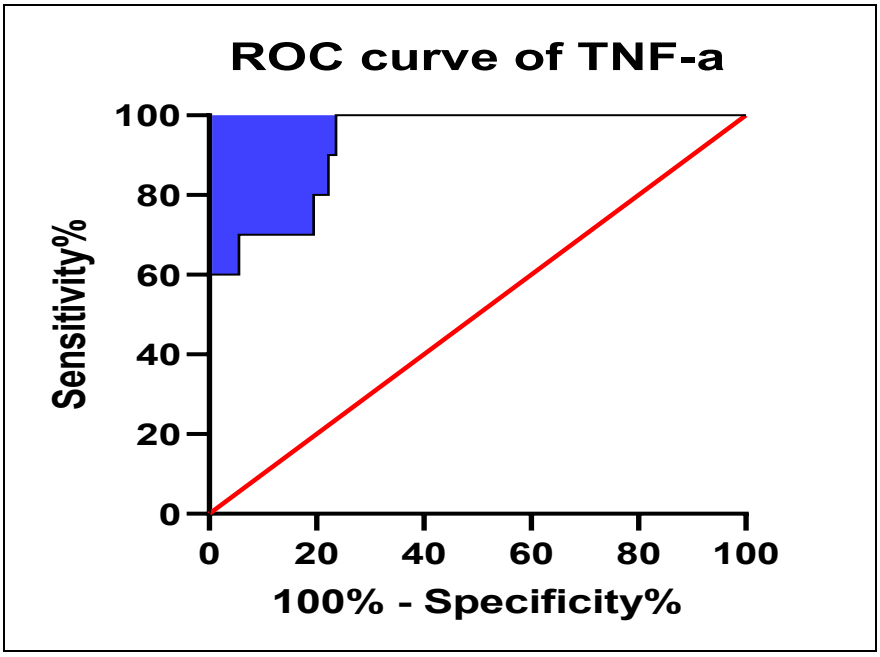

Figure 2: ROC curve plot of TNF- α for predicting diarrhea

However, the IL-10 serum levels cannot serve as a predictor of diarrhea. Given that AUC was 0.567 (95% CI = 0.4067 to 7.266; *p* <0.496). According to the Youden index, the cut-off value was calculated to be 3.8 fold, and the sensitivity and specificity of IL-10 serum levels were 26.39% and 90%, respectively. Moreover, ROC curve analysis revealed the predictive insignificance of IL-4 (AUC= 0.6333; 95%CI= 0.4664 to 0.8002; p= 0.173; Youden index= 3.6; cut-off value = 8.437 fold; sensitivity= 20.0%; specificity =94.44%) in discriminating between cases and control. ROC curve analysis revealed the predictive insignificance of TGF-β (AUC= 0.5097; 95%CI= 0.3142 to 0.7053; p= 0.9210; Youden index= 1.490; cut-off value = 17.34-fold; sensitivity= 60.0%; specificity= 59.72%) in discriminating between cases and control.

According to the existence of *Bifidobacterium*, a significant reduction in the inflammatory cytokines TNF-α and IL-10 was observed in diarrheal infants harboring *Bifidobacterium*. Both IL-10 and TNF-α exhibited significant differences between diarrheal infants with *Bifidobacterium* and diarrheal infants without *Bifidobacterium* (with other bacterial genera) (4.52 vs. 7.467 pg/ml; 9.87 vs. 30.5 pg/ml, respectively). While no significant difference in the other studied cytokines was observed. On the other hand, in the control group, there were no significant differences between infants with *Bifidobacterium* and infants without in terms of any of the studied pro- and anti-inflammatory cytokines, as shown in Table 2.

Table 2: Median serum level of cytokines distributed according to the presence of *Bifidobacterium*

Cytokines	Cytokines Median (range) levels pg/ml		*P* value
	Patients with *Bifidobacterium*	**patients without *Bifidobacterium***	
IL-10	4.52 (1.86 – 10.96)	7.67 (2.51 – 26.9)	>0.001**
IL-4	15.26 (6.63 -50.93)	18.30 (6.66 – 62.21)	0.30
IL-6	2.72 (1.13- 23.31)	3.76 (1.43 – 62.20)	0.133
TBG- β	19.18 (4.56 – 47.47)	19.39 (2.07- 59.80)	0.59
TNF- α	9.87 (3.07 – 48.89)	30.5 (5.18 – 83.79)	>0.001**
	Control with *Bifidobacterium*	**Control without *Bifidobacterium***	
IL-10	5.61 (3.43 – 12.36)	7.39 (5.17 -11.16)	0.55
IL-4	13.14 (8.29 -18.30)	20.91 (7.14 – 28.20)	0.308
IL-6	2.03 (0.648 -5.80)	3.23 (0.94 – 3.98)	0.70
TBG- β	12.98 (9.80 – 51.4)	26.17 (14.26 – 49.62)	0.362
TNF- α	1.63 (0.321 -10.45)	3.70 (1.93 – 8.43)	0.90

*, ** Indicate significant differences between study groups at $p < 0.05$ and $p < 0.01$ respectively.

The correlation coefficient r between the study parameters was calculated. Results showed no significant correlation between any of the studied cytokines, as illustrated in Table 3.

Table 3: Correlation between the studied cytokine in the studied group

	IL-10	**IL-4**	**IL-6**	**TGF-β**
IL-4	0.024			
IL-6	-0.063	0.366		
TGF-β	0.113	0.144	0.069	
TNF-α	0.294	0.404	0.328	0.273

Additionally, the correlation between the studied cytokines within each group of infants according to the presence of *Bifidobacterium* was also calculated. No significant correlation between cytokines was observed except for a significant positive correlation between IL-10 and IL-4 in infants harboring *Bifidobacteria,* with $r = 0.381$ at $P < 0.05$, Table 4.

Table 4: Correlation between the studied cytokine in the studied subgroup

		IL-10	**IL-4**	**IL-6**	**TGF-β**
Infants with *Bifidobacteria*	**IL-4**	0.381*			
	IL-6	0.171	-0.166		
	TGF-β	-0.045	-0.009	0.055	
	TNF-α	-0.071	-0.102	0.118	-0.107
		IL-10	**IL-4**	**IL-6**	**TGF-β**
Infants without *Bifidobacteria*	**IL-4**	0.071			
	IL-6	0.041	0.059		
	TGF-β	0.094	0.046	-0.195	
	TNF-α	0.105	0.195	-0.121	-0.161

* Indicate significant differences between study groups at $p < 0.05$

4. Discussion:

The human gastrointestinal tract (GIT) is habitat to one of the densest populations of bacteria that have been investigated to date, amongst them, *Bifidobacterium* represents a main group. They are considered to be one of the first microbial colonizers of the mammalian gut and a dominant member as well [18]. Notably, *Bifidobacterium* is highly utilized as a probiotic agent, as it is commonly found in the uterine region of pregnant women and the gut of infants, and is assumed to treat and prevent gastrointestinal tract disorders [19]. The antimicrobial resistance of pathogens arising from the inappropriate use of antibiotics has shed light on exploring alternative substances with the same effect. Among these, *Bifidobacterium*, as a probiotic, has been identified as an efficient candidate [20]. The immunomodulatory mechanisms exerted by members of the *Bifidobacterium* genus are variable, including protein elements and surface structural polysaccharides such as pili, exopolysaccharides, which facilitate host-microbe interactions. Additionally, the production of metabolic products that serve as mediators of immune homeostasis and products that facilitate cross-feeding patterns enhances the growth of beneficial organisms [21].

Diarrhea continues to rank among the world's leading causes of death and disability-adjusted life years lost, especially in infants and children [22].

The immunomodulation capacity of *Bifidobacteria* was manifested by its ability to elevate immunoglobulin's levels and further pro- or anti-inflammatory cytokines induction or reduction, respectively [23]. An in vitro animal study revealed that oral gavage of heat-killed *Bifidobacterium* strains to colitic mice resulted in lower levels of IL-1β and TNF-α and higher levels of the anti-inflammatory IL-13, indicating an attenuation of the inflammatory state [24].

Current results, as mentioned above, were partially in agreement with Li. *et al.,* [25], who found significantly higher levels of IL-8, IL-2, IL-10, and TNF-α in infants with diarrhea compared to the control group. In the present study, a significant increase in TNF-α, IL-4, and IL-6 was also noticed in infants with diarrhea. They suggested that this increase is associated with abnormalities in the balance of intestinal flora. Current results, as mentioned above, were also in agreement with Qin. *et al.,* [26], who observed an increase in the pro-inflammatory cytokines IL-6 and TNF-α along with other cytokines (IL-1 and IL-17), in children with non-infectious diarrhea. They proposed that this increase in cytokine levels is correlated with gut dysbiosis. *Bifidobacteria,* along with multiple other strains used as probiotics, have been shown to play a role in the alleviation of gastrointestinal tract-related diseases, such as diarrhea and infection. The immune modulation mechanisms exerted by these organisms activated the production of anti-inflammatory cytokines, such as IL-10, IL-4, and IL-13, and impeded the production of pro-inflammatory cytokines, including IL-6 and TNF-α. Additionally, production of various bioactive compounds was also involved [27]. One of the multiple anti-inflammatory mechanisms of *Bifidobacterium* sp. is suppression of the nuclear factor kappa B (NF-kB) pathway. Previous reports declared that *B.bifidum* metabolites, like short-chain fatty acids (SCFA) and exopolysaccharides (EPS) inhibited TNF-α/NF-κB inflammatory pathway [28].

Within the diarrheal group, the present study revealed a significant reduction in TNF-α and IL-10 in infants with *Bifidobacterium* in their feces compared to infants with other bacterial genera. Cytokine levels in diarrheal infants dominated by *Bifidobacterium* were low, and some were partially converged to their counterparts in the control, which may indicate *Bifidobacterium* ability to attenuate the severity of the disease. Wang *et al*., [29] reported that

combining *Bifidobacterium* with certain substances reduced the levels of the pro-inflammatory IL-6 and TNF-α and improved serological indicators in diarrheal infants, which further reduced the hospitalization time and diarrhea duration. Furthermore, Zhang *et al.,* [30] also reported a significant improvement in IL-7, IL-6, and TNF-α levels in children with chronic diarrhea following the treatment with *Bifidobacterium*.

Different strains were found to be able to alleviate diarrhea caused by *E. coli,* which is one of the most common causes of infants diarrhea. It was demonstrated that *B. breve* reduced IFN- γ, *B. bifidum* improved IL-10, and all isolates dowenregulated TNF-α, thereby restoring the gut microbiota balance [31]. Moreover, another study reported that *B. adolescentis* decreased IL-6, IL-17A, IL-1β, and TNF-α, and increased the anti-inflammatory IL-10 and IL-4 in colitic mice induced with Dextran Sodium Sulfate (DSS)[32].

In the present study, within infants harboring *Bifidobacterium,* a significant positive correlation was observed between the anti-inflammatory cytokines IL-10 and IL-4. IL-10 is a potent anti-inflammatory cytokine that regulates the immune response to infection and has been found, in previous reports, to be associated with disease prognosis and severity [33].

Conclusion

The present results concluded that IL-6, IL-4, and TNF-α were significantly higher in diarrheal infants, and TNF-α could serve as a key indicator of the disease. Additionally, serum TNF- α levels decreased with the presence of *Bifidobacterium*. This study demonstrated that the presence of *Bifidobacterium* in infants is a key factor in supporting the immune system, as evidenced by decreased serum IL-10 and TNF-α levels in diarrheal infants harboring *Bifidobacterium* in their feces compared to diarrheal infants without *Bifidobacterium*, indicating its potential as a powerful immunological booster.

Conflict of interest:

The author declares that they have no conflicts of interest.

Ethical Approval

The Ethical Approval Committee of the University of Anbar in Iraq approved all research methods used in the study
(Approval No. 116, Jul 30, 2024)

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