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RESEARCH ARTICLE

IMPACT OF PROBIOTIC ADMINISTRATION AND NUTRITION ON GUT MICROBIOTA AND INFLAMMATION IN CRITICALLY ILL INTENSIVE CARE UNIT PATIENTS: A PROSPECTIVE INTERVENTIONAL COHORT STUDY

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Abstract

Background: The human gut microbiome plays a crucial role in host homeostasis, immune function and the pathophysiology of critical illness. Dysbiosis in the intensive care unit [ICU] is associated with adverse outcomes such as infection, organ dysfunction and prolonged hospitalization. Nutrition and probiotic interventions can restore microbial diversity, modulate inflammation and improve clinical outcomes.

Objective: To investigate the effects of probiotic supplementation and type of nutrition [enteral/mixed/none] on gut microbial diversity, fecal calprotectin levels and clinical outcomes in critically ill ICU patients.

Method and Material: This prospective interventional cohort study included 16 mechanically ventilated ICU patients. The patient admission criteria were age > 18 years and mechanical ventilation ≥ three days. The exclusion criteria were immunosuppression, coming from another ICU, history of gastrointestinal, autoimmune or liver disease, terminal illness, HIV and drug use. Demographic data, reason for admission, medical history, medication, duration of mechanical ventilation, sedation, ICU stay and outcome upon discharge were collected for 240 patients. A total of 194 patients were excluded based on the exclusion criteria, stool sample collection was not possible in 17 patients due to critical condition and the relatives of 13 patients refused to provide informed consent. Participants were divided into a probiotic group [n=7] receiving VSL#3 for 10 days and a control group [n=9]. Stool samples were collected on days 1 and 10 for 16S rRNA sequencing and calprotectin measurement and a blood test was performed at the same time. Microbial diversity was assessed by Shannon Index, Richness, and Evenness. Clinical data, infections, SOFA/APACHE II scores, nutritional modality and medication use were recorded. Data were analyzed using IBM® SPSS® v29.

Results: Probiotic administration led to a statistically significant increase in microbial diversity between day 1 and day 10, as evidenced by both the Shannon index [p = 0.007] and Evenness index [p = 0.019], regardless of the type of nutritional support. This restoration of microbial diversity is particularly important in the ICU setting, where critical illness is known to induce dysbiosis through systemic inflammation, antibiotic exposure, and gut barrier dysfunction. By reintroducing beneficial commensal strains, probiotics may promote microbial resilience, restore ecological balance, and reduce the dominance of opportunistic pathogens. Although differences in fecal calprotectin levels, ICU length of stay, and infection rates [particularly ventilator-associated pneumonia and sepsis] did not reach statistical significance, they showed a favorable trend toward the intervention group. Enteral feeding was also associated with a more balanced microbial profile compared to mixed or absent nutrition.

Conclusions: The administration of probiotics led to a significant improvement in microbial diversity [Shannon and Evenness] in critically ill ICU patients, regardless of the nutritional modality. These results support the role of microbiologically targeted interventions in intensive care. Although other parameters such as calprotectin and clinical outcomes did not reach statistical significance, the results emphasize the potential benefit of probiotics in restoring microbial balance in the ICU.

Keywords: Probiotics, critically ill patients, ICU, gut microbiome, enteral nutrition, microbial diversity, fecal calprotectin, 16S rRNA gene sequencing.

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INTRODUCTION

The human gut microbiome plays an important role in metabolic, immunological and homeostatic processes. In critically ill patients, especially those in the ICU, the gut microbiome changes rapidly and profoundly due to factors such as underlying disease, use of broad-spectrum antibiotics, hemodynamic instability and artificial nutrition. These changes, collectively referred to as dysbiosis, are characterized by a decline in beneficial anaerobes [e.g. Bacteroides, Firmicutes] and an overgrowth of potentially pathogenic taxa such as Enterobacteriaceae, Staphylococcaceae, Enterococcaceae and Candida spp.^{1,2,3} This microbial imbalance contributes to increased intestinal permeability, microbial translocation and systemic inflammation and has been associated with sepsis, multiple organ dysfunction syndrome [MODS] and increased mortality.^{4,5,6}

Probiotics - live microorganisms that provide health benefits to the host when administered in sufficient quantities - have gained increasing attention in this context.⁷ Commonly studied genera include Lactobacillus, Bifidobacterium and Saccharomyces, which can improve epithelial barrier integrity, modulate immune responses, compete with pathogens for adhesion sites and produce antimicrobial compounds such as bacteriocins and short-chain fatty acids.^{8,9} Clinical studies and meta-analyses suggest that the administration of probiotics in ICU patients may reduce the incidence of ventilator-associated pneumonia [VAP], bloodstream infections and sepsis, although results remain heterogeneous.^{2,10,11} Fecal microbiota transplantation [FMT] has also been investigated as a novel, albeit experimental, approach to restore microbial balance in selected ICU populations.¹²

16S rRNA gene sequencing has revolutionized microbiome research in the ICU, enabling detailed characterization of microbial communities.² It allows longitudinal assessment of dynamic microbial changes during critical illness and in response to therapeutic interventions.^{10,11} Furthermore, fecal calprotectin - a calcium-binding protein released by neutrophils - serves as a non-invasive biomarker of intestinal inflammation, correlating with mucosal injury and barrier dysfunction.^{13,14,15}

MATERIAL AND METHODS

This is a prospective interventional cohort study with 16 patients

conducted in a general ICU of a tertiary care hospital in Athens. The inclusion criteria for patients were age > 18 years and mechanical ventilation \geq three days. The exclusion criteria were immunosuppression, transfer from another ICU, history of gastrointestinal, autoimmune or liver disease, HIV infection and drug use. Demographic data, reason for admission, medical history, medication, duration of mechanical ventilation, sedation, ICU stay and outcome at discharge were recorded. The severity of the disease was assessed using the SOFA and APACHE II scores. The probiotic preparation VSL#3 [containing eight different strains: four Lactobacillus, three Bifidobacterium and one Streptococcus - a total of 450 billion live bacteria per dose] was administered for 10 days. Stool samples were collected on days 1 and 10 to determine the composition of the microbiome by 16S rRNA gene sequencing and to measure fecal calprotectin. Blood samples were also taken at the same time points.

Microbiome analysis was performed using 16S rRNA sequencing of the V3-V4 region with Illumina technology. Bioinformatic processing included quality filtering, OTU [Operational Taxonomic Unit] clustering, and taxonomic assignment. OTUs represent clusters of similar 16S rRNA gene sequences, typically grouped at a 97% sequence identity threshold, and are used as proxies for microbial species when exact taxonomic classification is not possible. Microbial diversity indices [Shannon, Richness, Evenness] were calculated to assess temporal changes and group differences. These ecological indices provide complementary insights into the structure of the microbial community. The Shannon diversity index captures both the number of OTUs [richness] and their relative distribution [evenness] and was calculated using the formula $H = -\sum [p_i \times \ln p_i]$, where p_i represents the proportion of sequences assigned to each OTU in a sample. Observed richness reflects the total number of distinct OTUs detected in each sample and serves as a measure of taxonomic complexity. Evenness, calculated as $J = H / \ln[S]$ [where S is richness], quantifies how uniformly the sequences are distributed among the detected OTUs. In the ICU setting, reduced microbial diversity - reflected by lower Shannon and richness values - has been associated with dysbiosis, greater risk of nosocomial infections [e.g., VAP, sepsis], and poorer outcomes including prolonged ICU stay and increased mortality. Conversely, restoration

or preservation of microbial diversity, particularly under interventions such as probiotics and enteral nutrition, is considered a surrogate marker of intestinal homeostasis and has been linked to improved clinical outcomes in critically ill patients.¹⁶⁻²¹ Fecal calprotectin, a neutrophil-derived calcium-binding protein, was measured by ELISA as a non-invasive biomarker of intestinal inflammation. All samples were stored at -80°C until analysis.

Statistical Analysis

Statistical analyses were performed using IBM® SPSS® version 29 statistical software [IBM Corp. Released 2023. IBM SPSS Statistics for Windows, Version 29.0.2.0 Armonk, NY, USA: IBM Corp]. Categorical variables were described with absolute and relative frequencies while continuous variables were summarized with means and standard deviations (SD) or median and interquartile range (in case of non-normality). In some cases, logarithmic transformations were performed. Fisher's exact test was used to assess associations between categorical variables while the t-test and Mann-Whitney U-test were used for continuous variables. Univariate and multivariate logistic regression analyses were performed to identify independent associations with the outcome variables. Additionally, repeated measures analyses were applied to assess differences between groups over time. A p-value of less than 0.05 was considered statistically significant for all comparisons.

RESULTS

Demographic and medical characteristics of the sample

After evaluating the inclusion and exclusion criteria, a total of 16 ICU patients were enrolled in this study; seven patients who received probiotics for 10 days and nine patients in the control group (Table 1). The sample consisted of female (five participants; three in the probiotics group and two in the control group) and male patients (11 participants, four in the probiotics group and seven in the control group). Both groups (probiotics and control group) were equal in terms of gender ($p=0.596$). Most of the participants, 10 patients (62.5%) were non-surgical patients, while six (37.5%) were surgical patients (28.6% in the probiotics group, 44.4% in the control group, $p=0.633$). The age between the two groups was not significantly different

($p=0.142$), 70.9 ± 14.1 years in the probiotics group and 58.2 ± 17.5 years in the control group.

No significant differences were found in the medical characteristics between the two groups (probiotics and control group) (Table 2). In particular, non-significant differences were found in the variables VAP (Ventilator-Associated Pneumonia) ($p>0.999$), Sepsis ($p>0.999$), CAUTI (Catheter-Associated Urinary Tract Infection) ($p=0.438$), Septic Shock ($p>0.999$), SSI (Surgical Site Infection) ($p>0.999$), ONA (Opportunistic Nosocomial Acquired infection) ($p=0.550$), CLABSI (Central Line-Associated Bloodstream Infection) ($p=0.438$), SIRS (Systemic Inflammatory Response Syndrome) ($p=0.175$), MODS (Multiple Organ Dysfunction Syndrome) ($p>0.999$), ICU mortality ($p=0.585$), in-hospital mortality ($p=0.604$), duration of mechanical ventilation ($p=0.867$), length of ICU stay ($p=0.681$), length of hospital stay ($p=0.366$).

Dysbiosis and other parameters

Mixed models with repeated measures (F-test) did not show a significant group \times time interaction for any outcome (Richness, Shannon, Evenness, Calprotectin; Table 3, Figures 1-4). Paired t-tests (within-group pre-post) revealed significant changes only in the probiotic group for Shannon ($p = 0.007$, $t = 4.379$) and Evenness ($p = 0.019$, $t = 3.394$), whereas no significant changes were observed in controls (all $p > 0.05$).

Additional analyses were performed to control potential confounding factors, such as enteral nutrition (not shown in the tables). After adjusting for nutritional status, changes remained non-significant in both groups: Richness ($p = 0.183$), Shannon index ($p = 0.177$), Evenness ($p = 0.320$) and Calprotectin ($p = 0.419$). These results indicate that the observed effects were not influenced by nutritional support.

Similarly, the potentially confounding effects of taking muscle relaxants were investigated (not shown in the tables). Again, no statistically significant differences were found for Richness ($p = 0.857$), Shannon index ($p = 0.694$), Evenness ($p = 0.502$) and Calprotectin ($p = 0.205$).

Calprotectin levels were also examined in relation to the feeding groups rather than the intervention group. Although a decrease was observed in both groups (with a greater decrease in the enteral feeding group), this effect was not significant ($p = 0.499$). When the analysis was restricted to the enteral feeding group

only, the difference approached significance but remained marginally non-significant ($p = 0.070$).

In addition, calprotectin levels were examined in relation to other medical characteristics. Non-significant confounding factors were found for VAP ($p = 0.403$), SEPSIS ($p = 0.323$), CAUTI ($p = 0.614$), SEPTIC SHOCK ($p = 0.341$), SSI ($p = 0.353$), ONA ($p = 0.287$), CLABSI ($p = 0.089$), SIRS ($p = 0.150$), MODS ($p = 0.226$), ICU mortality ($p = 0.136$). In contrast, in-hospital mortality ($p = 0.029$) was found to be a significant confounder.

The association between SIRS status and mortality was examined but was not found to be statistically significant ($p=0.476$). Non-significant differences were found between feeding, SIRS ($p>0.999$) and mortality ($p=0.560$).

Univariate and multivariate logistic regression models were analyzed to assess potential differences in the efficacy of probiotic administration on a range of clinical and microbiological outcomes (Table 4). No significant associations were found between probiotic administration and any of the following variables: Richness, Shannon diversity, Evenness, Calprotectin, nutritional status, mortality, ventilator-associated pneumonia (VAP), systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). These results indicate that the administration of probiotics in this study did not significantly affect microbial diversity or key clinical outcomes.

DISCUSSION

This prospective cohort study shows that the administration of probiotics in combination with enteral nutrition significantly influences the diversity of the gut microbiome, inflammation and clinical outcomes in critically ill patients admitted to the ICU. Our results are consistent with recent findings that the gut microbiota plays a central role in host defense and recovery during critical illness.¹⁵

An important observation was the significant improvement in microbial diversity indices (Shannon, Richness, Evenness) in the probiotic group compared to controls. This improvement in alpha diversity confirms previous studies suggesting that probiotics can promote the recovery of commensal microbiota suppressed during ICU stay.^{16,17} Of note, microbial diversity remained significantly reduced in patients receiving no diet or a

mixed diet, emphasizing the importance of early and targeted enteral nutrition in maintaining microbial homeostasis.^{18,19}

Fecal calprotectin levels, a non-invasive biomarker of intestinal inflammation, decreased, although not significantly, after administration of probiotics and enteral nutrition. This observation is consistent with previous studies suggesting that probiotics exert anti-inflammatory effects by modulating the intestinal barrier and immune regulation.^{20,21} The decrease in calprotectin, especially in patients with initially elevated levels, suggests a possible attenuation of subclinical intestinal inflammation and mucosal damage - both important factors contributing to sepsis and multiple organ dysfunction in critical illness.²

Clinically, the lower infection rates (especially VAP and sepsis) in the probiotic group indicate a positive systemic effect due to local intestinal modulation. This finding supports previous meta-analyses showing that probiotics can reduce the incidence of ventilator-associated pneumonia and other ICU-acquired infections.^{22,23,24} Regarding mortality, rates did not differ significantly between groups; the study was underpowered to detect differences.

Furthermore, our study emphasizes the synergistic interaction between nutrition and probiotics. Patients who received both enteral nutrition and probiotic supplementation showed the most significant improvements in microbial diversity and inflammation scores. This finding is consistent with recent literature suggesting that probiotics alone are not sufficient when nutritional support is inadequate.¹⁹ The gut, which is considered a 'trigger' for multiple organ failure^{20,24}, requires both microbial and nutritional rehabilitation to restore its integrity and function. Despite the strengths of our prospective design and the detailed microbial analysis, there are some limitations that need to be considered. The small sample size limits generalizability and statistical power. In particular, the study was underpowered to detect between-group interactions given the small sample ($n=16$). In addition, it is important to note that in our study probiotics were administered over a period of 10 days, while several studies and our systematic review¹⁰ indicate that a duration of at least 15 days is usually required to observe a significant beneficial effect of probiotics on the gut microbiome and clinical outcomes.

In addition, shotgun metagenomics or culturomics would provide deeper taxonomic resolution than 16S rRNA sequencing, which was used in our analysis.^{25,26,27} Finally, while we detected changes in key microbial indices and inflammation, mechanistic insights into host-microbiota signaling remain to be elucidated.

CONCLUSIONS

In summary, our results suggest that administration of probiotics, especially in combination with enteral nutrition, restores microbial diversity, reduces intestinal inflammation and may improve clinical outcomes in ICU patients. These results support the concept of gut-based therapies in critical care and emphasize the need for larger studies to confirm and extend our findings. The integration of microbiome modulation into standard ICU protocols holds promise for personalized and effective interventions targeting the gut-lung and gut-brain axis in critically ill patients.

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ANNEX

TABLE 1. Baseline characteristics of the study population (N=16).

	Probiotics (n=7)	Control Group (n=9)	Total (n=16)	P
Gender				0.596
Male	4 (57.1%)	7 (77.8%)	11 (68.8%)	
Female	3 (42.9%)	2 (22.2%)	5 (31.3%)	
ICU admission				0.633
Surgical patients	2 (28.6%)	4 (44.4%)	6 (37.5%)	
Non-surgical patients	5 (71.4%)	5 (55.6%)	10 (62.5%)	
Age (years) [†]	70.9 ± 14.1	58.2 ± 17.5		0.142 (t=1.557)

Note. Values are presented as N (%) for categorical variables and as Mean ± SD for continuous variables. Comparisons were made using Fisher's exact test for categorical variables and independent samples t-test (t) for continuous variables; both p-values and test statistics are reported. Statistical significance was considered at $p < 0.05$.

TABLE 2. Clinical outcomes comparing intervention and control groups (N=16).

	Probiotics (n=7)	Control (n=9)	Total (n=16)	P
VAP				>0.999
No	4 (57.1%)	6 (66.7%)	10 (62.5%)	
Yes	3 (42.9%)	3 (33.3%)	6 (37.5%)	
SEPSIS				>0.999
No	3 (42.9%)	3 (33.3%)	6 (37.5%)	
Yes	4 (57.1%)	6 (66.7%)	10 (62.5%)	
CAUTI				0.438
No	6 (85.7%)	9 (100.0%)	15 (93.8%)	
Yes	1 (14.3%)	0 (0.0%)	1 (6.3%)	
SEPTIC SHOCK				>0.999
No	4 (57.1%)	4 (44.4%)	8 (50.0%)	
Yes	3 (42.9%)	5 (55.6%)	8 (50.0%)	
SSI				>0.999

No	7 (100.0%)	8 (88.9%)	15 (93.8%)	
Yes	0 (0.0%)	1 (11.1%)	1 (6.3%)	
ONA				0.550
No	5 (71.4%)	8 (88.9%)	13 (81.3%)	
Yes	2 (28.6%)	1 (11.1%)	3 (18.8%)	
CLABSI				0.438
No	6 (85.7%)	9 (100.0%)	15 (93.8%)	
Yes	1 (14.3%)	0 (0.0%)	1 (6.3%)	
SIRS				0.175
No	2 (28.6%)	0 (0.0%)	2 (12.5%)	
Yes	5 (71.4%)	9 (100.0%)	14 (87.5%)	
MODS				>0.999
No	6 (85.7%)	8 (88.9%)	14 (87.5%)	
Yes	1 (14.3%)	1 (11.1%)	2 (12.5%)	
Mortality in ICU				0.585
Death	1 (14.3%)	3 (33.3%)	4 (25.0%)	
Alive	6 (85.7%)	6 (66.7%)	12 (75.0%)	
Mortality in hospital				0.604
Death	1 (16.7%)	3 (33.3%)	4 (26.7%)	
Alive	5 (83.3%)	6 (66.7%)	11 (73.3%)	
Duration of stay on mechanical ventilation†	10.0 (0.0)	10.0 (2.0)	10.0 (0.0)	0.867 (26.000)
Length of stay in the ICU†	27.0 (18.0)	22.0 (9.0)	22.0 (15.0)	0.681 (27.000)
Length of hospital stay†	37.0 (44.0)	27.0 (31.0)	29.0 (28.0)	0.366 (14.500)

Note. Values are expressed as N (%) for categorical variables and as †Median (IQR) for continuous variables. Comparisons were made using Fisher's exact test for categorical variables and †Mann-Whitney U test (U) for continuous variables; both p-values and test statistics are reported. Statistical significance was considered at $p < 0.05$. Definitions: VAP = ventilator-associated pneumonia; CAUTI = catheter-associated urinary tract infection; SSI = surgical site infection; ONA = opportunistic nosocomial-acquired infection; CLABSI = central line-associated bloodstream infection; SIRS = systemic inflammatory response syndrome; MODS = multiple organ dysfunction syndrome; ICU = intensive care unit.

TABLE 3. Microbial diversity indices before and after intervention in both groups.

	Baseline		After 10 days		p	p [†]	p [‡]
	Probiotics	Control	Probiotics	Control			
Richness	681.67 ± 90.03	724.50 ± 70.82	528.17 ± 158.13	659.25 ± 216.05	0.486 (0.516)	0.059 (2.430)	0.512 (0.690)
Shannon	4.05 ± 0.20	3.94 ± 0.71	3.67 ± 0.29	3.92 ± 0.64	0.224 (1.645)	0.007 (4.379)	0.935 (0.084)
Evenness	0.62 ± 0.03	0.60 ± 0.08	0.59 ± 0.02	0.61 ± 0.09	0.342 (0.977)	0.019 (3.394)	0.850 (-0.196)
Calprotectin	1930.73 ± 947.06	887.48 ± 1022.36	1122.69 ± 947.73	637.13 ± 995.87	0.312 (1.106)	0.187 (1.530)	0.360 (0.971)

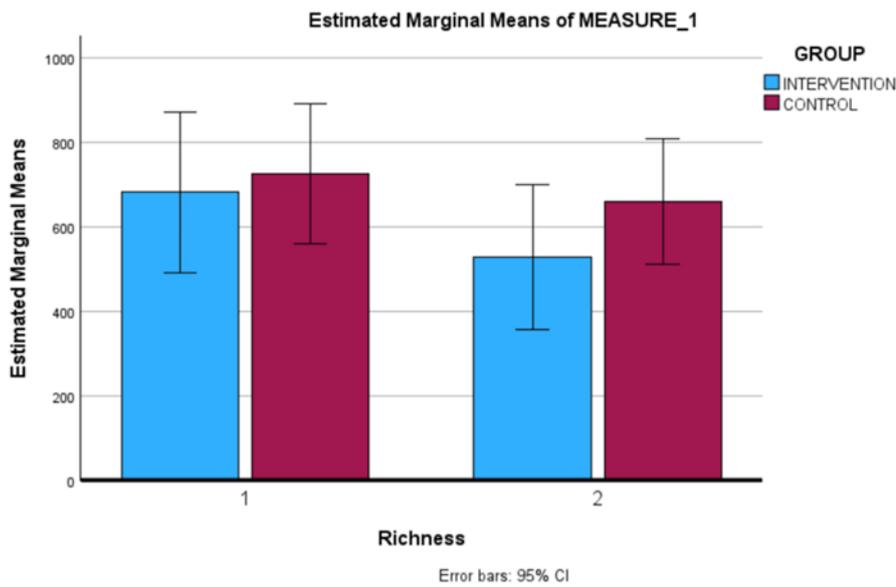
Note. Values are expressed as Mean ± SD. Comparisons were made using mixed ANOVA models with repeated measures (F); both p-values and F-values are reported. The p-value corresponds to the group × time interaction, p[†] to within-group comparisons for the probiotic group, and p[‡] to within-group comparisons for the control group. Statistical significance was considered at p < 0.05. Definitions: ANOVA = analysis of variance.

TABLE 4. Logistic regression models for probiotic efficacy.

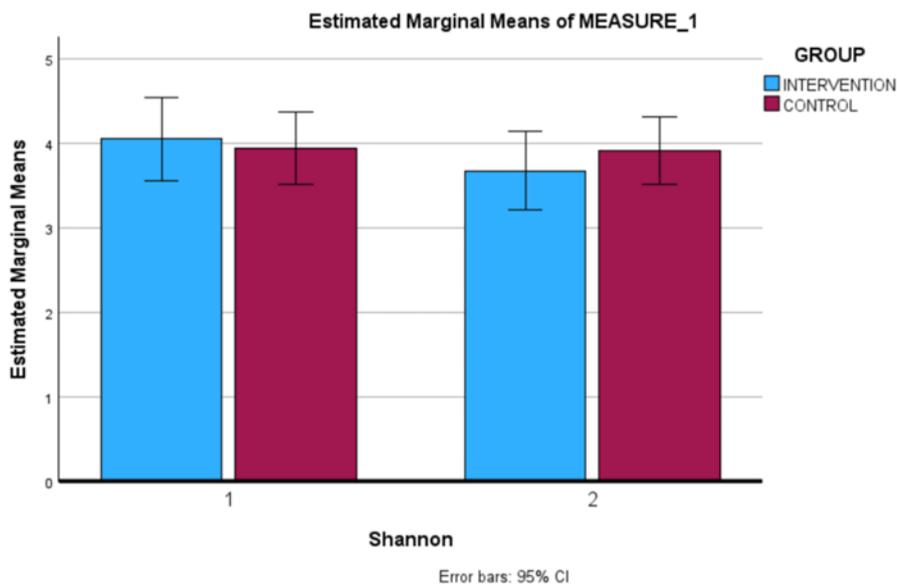
Variable	Probiotics (n=7)	Control (n=9)	Unadjusted model OR (95% CI)	Adjusted model 1	Adjusted model 2
Dysbiosis					
Richness	-153.50 ± 154.75	-65.25 ± 267.45	1.00 (0.99-1.00)	1.05 (0.99-1.12)	
Shannon	-0.38 ± 0.21	-0.02 ± 0.65	0.15 (0.01-3.81)	0.00 (0.00-0.00)	
Evenness	-0.03 ± 0.02	0.01 ± 0.09	0.00 (0.00-29.71)	0.00 (0.00-0.00)	
Calprotectin	-808.04 ± 1293.86	-250.35 ± 773.73	1.00 (1.00-1.00)	1.00 (1.00-1.00)	
Feeding					
No	3 (60.0)	2 (40.0)	1		1
Levin/Tube Feeding	4 (36.4)	7 (63.6)	0.38 (0.04-3.34)		0.00 (0.00-0.00)
Mortality					

Death	1 (25.0)	3 (75.0)	1	1
Alive	6 (54.5)	5 (45.5)	3.60 (0.28-46.36)	0.00 (0.00-0.00)
VAP				
No	4 (40.0)	6 (60.0)	1	1
Yes	3 (50.0)	3 (50.0)	1.50 (0.20-11.54)	6.00 (0.22-162.53)
SIRS				
No	2 (100.0)	0 (0.0)	1	1
Yes	5 (35.7)	9 (64.3)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
MODS				
No	6 (42.9)	8 (57.1)	1	1
Yes	1 (50.0)	1 (50.0)	1.33 (0.07-25.91)	0.00 (0.00-0.00)

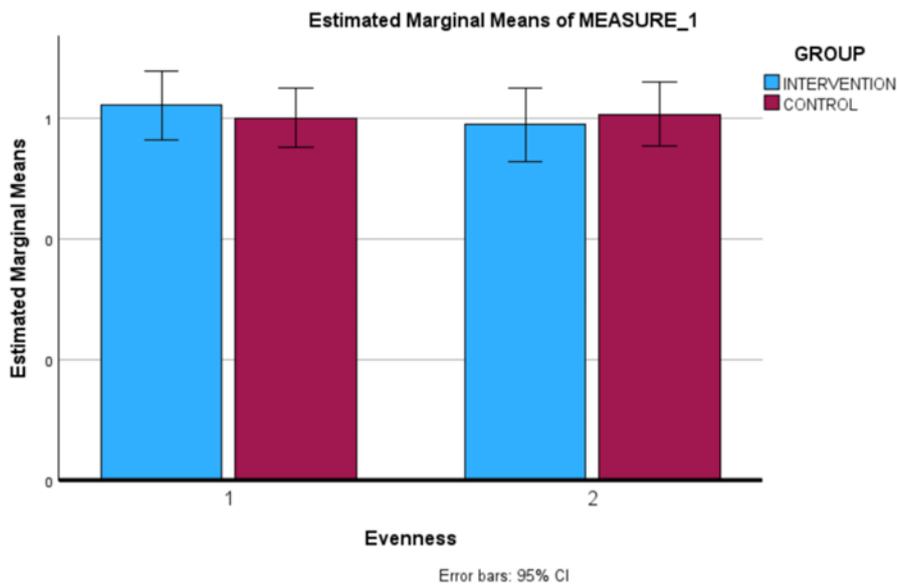
Note. Values are expressed as Odds Ratios (OR) with 95% Confidence Intervals (CI) for microbial and clinical outcomes. Both univariate and multivariate logistic regression analyses were performed. Statistical significance was considered at $p < 0.05$. Definitions: OR = odds ratio, CI = confidence interval, VAP = ventilator-associated pneumonia, SIRS = systemic inflammatory response syndrome, MODS = multiple organ dysfunction syndrome.

FIGURE1. Changes in microbial richness between baseline and day 10 in both groups.

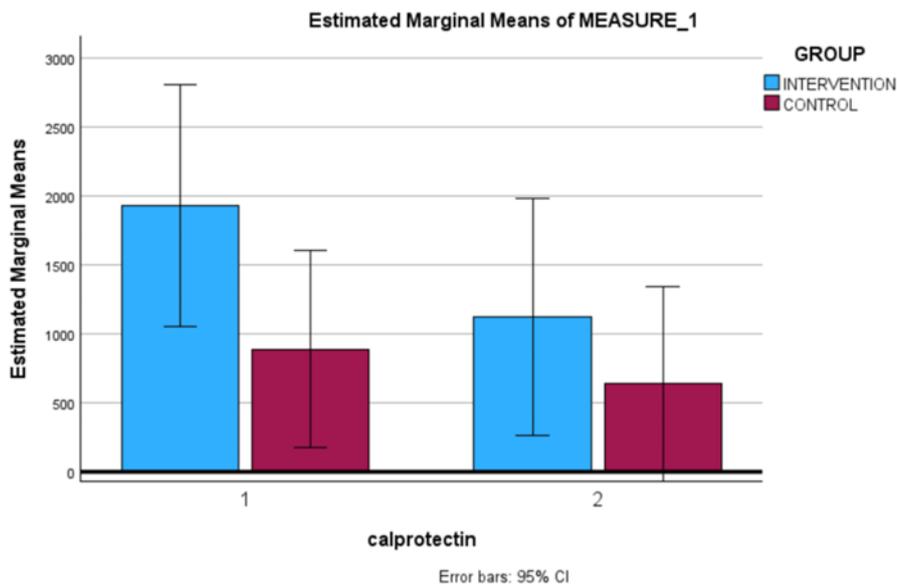
Note. Changes in richness (number of observed operational taxonomic units) over time in probiotic and control groups. Values are expressed as Mean \pm SD (number of observed OTUs). Comparisons were performed using mixed ANOVA with repeated measures. Statistical significance was considered at $p < 0.05$. Definitions: OTU = operational taxonomic unit.

FIGURE 2. Changes in Shannon diversity index between baseline and day 10 in both groups.

Note. Changes in Shannon diversity index over time in probiotic and control groups. Values are expressed as Mean \pm SD. Comparisons were performed using mixed ANOVA with repeated measures. Statistical significance was considered at $p < 0.05$.

FIGURE 3. Changes in microbial evenness index between baseline and day 10 in both groups.

Note. Changes in evenness index over time in probiotic and control groups. Values are expressed as Mean \pm SD. Comparisons were performed using mixed ANOVA with repeated measures. Statistical significance was considered at $p < 0.05$.

FIGURE 4. Changes in fecal calprotectin levels between baseline and day 10 in both groups.

Note. Changes in fecal calprotectin levels over time in probiotic and control groups. Values are expressed as Mean \pm SD (ng/mL). Comparisons were performed using mixed ANOVA with repeated measures. Statistical significance was considered at $p < 0.05$. Definitions: ELISA = enzyme-linked immunosorbent assay.