

Impact of prebiotic (Lacto-N-neotetraose) and probiotic (*B. infantis* EVC001) supplementation on gut inflammation and fecal pH in young infants suffering from severe acute malnutrition: findings from a randomized controlled trial

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Abstract

Background

Severe acute malnutrition (SAM) is associated with high levels of gamma Proteobacteria chronic gut inflammation and poor gut health. In this study, we aimed to assess the effects of probiotic and/or prebiotic supplementation on gut inflammation and fecal pH in young infants with SAM.

Methods

This study was a single-blind RCT where infants aged between 2 and 6 months were randomized to receive either: probiotic (*B. infantis* EVC001), synbiotic (prebiotic, Lacto-N-neotetraose [LNnT] plus probiotic [*B. infantis* EVC001]), or placebo (Lactose) for 28 days followed by 28 days of post-supplementation follow up. Stool samples were collected at baseline and at day 10, day 28 and day 56. Fecal myeloperoxidase (MPO), as an indicator of gut inflammation and fecal pH were measured and the change in the levels of these biochemical parameters between the sample collection time points were calculated and denoted as Δ MPO and Δ pH. Kruskal-Wallis test was done to assess the effect of the supplementations on Δ MPO and Δ pH. Multivariate quantile regression analysis was performed to analyze the association of the supplements with Δ MPO and Δ pH.

Results

In comparison to the placebo group at day 10, a significant decrease in Δ MPO was found for the probiotic group (β -coefficient: -18.44; 95% CI: -31.62 µg/ml, -52.47 µg/ml; $p = 0.007$) and for the synbiotic group (β -coefficient: -17.24; 95% CI: -30.94 µg/ml, -35.36 µg/ml; $p = 0.015$). This reduction in Δ MPO in comparison to the placebo group was sustained only in the synbiotic group at other time points. Decrease in Δ pH was observed only in the synbiotic group at day 10 and day 28, but not at day 56 in comparison to the placebo group.

Conclusion

Our findings demonstrate that the use of this synbiotic supplementation may result in sustained effects in reduction of gut inflammation in young infants with SAM. However, further studies are required to fully evaluate the role of this synbiotic intervention for sustained reduction in fecal pH in this study population.

Trial registration

The trial is registered at ClinicalTrials.gov (NCT0366657). Registered on 12 September, 2018 (<https://clinicaltrials.gov/ct2/show/NCT03666572>)

Background

Severe acute malnutrition (SAM) is the most extreme and visible form of undernutrition, and is commonly defined by a weight-for-length (WLZ) score of less than -3 or by the presence of bilateral pedal edema,

independent of anthropometric value (1). Recent reports suggest that on a global scale about 4 million infants of less than a months of age are affected with SAM (2)-(3).

Analysis of risk factors associated with malnutrition in those infants indicate that breastfeeding support may be an integral component of future management, however there is a desperate need for other nutritional intervention strategies for effective treatment of SAM (4)-(5). Although intervention strategies involving the use of therapeutic foods resulted in reduction of mortality in children with SAM, restricted rehabilitation of healthy growth remains a serious conundrum (5)-(6). Infancy represents a critical window of rapid development of the gastrointestinal system, a process which is accompanied by characteristic age specific changes in the intestinal microbiota (7)-(8)-(9). A study conducted at the Dhaka Hospital of icddr, b in children with SAM demonstrated considerable immaturity of gut microbiota (10). Such alterations in the microbiota, a condition referred to as dysbiosis, have been found to be closely associated with intestinal inflammation (11).

Probiotics are refer to live microbial food ingredients that confer health benefits to the physiology of the host, with the most studied probiotics belonging to the genera *Lactobacilli* and *Bifidobacteria* (12). A recent probiotic supplementation study conducted among breast-fed infants showed that infants supplemented with *B. infantis* EVC001, developed a persistent and stable population in the microbiome of the infant gut (13). Marked changes in the intestinal metabolome of these infants was also observed, with lower fecal pH and higher amounts of fecal lactate and acetate compared to the control group. This was accompanied by a reduction of certain gut pathogens by over 90% (14), indicating a possible therapeutic intervention against early gut dysbiosis. Prebiotics generally refer to oligosaccharide polymers that are not digested by the host system but are consumed by a probiotic bacterium (15). Human milk contains unique prebiotic oligosaccharides, known as human milk oligosaccharides (HMOs) which are not digested and subsequently utilized by most other by both the infant and majority of the bacteria constituting the infant gut microbiome. In fact, only *Bifidobacterium longum* subspecies *infantis* has the genetic and biochemical capability to consume all HMOs (16). The metabolism of HMOs by *B. infantis* results in production of short chain fatty acid molecules such as acetate and lactate, which are important fuel sources for enterocytes and also lower the gut pH to levels that are unfavorable to most opportunistic gut pathogens (16). Since *B. infantis* is able to consume all of the HMOs in human milk, it quickly dominates the microbiome of the breast-fed infant to levels of over 80% of the total gut microbiome once it is introduced (13). Infant formulas are now commercially available with certain HMOs added at very low levels(17). One trial with healthy formula-fed term infants supplemented with two HMOs (2'Fucosyllactose [2'FL] and Lacto-N-neotetraose [LNnT]), demonstrated an increase in Bifidobacteria in the gut microbiome, but not to the levels seen in healthy breast-fed infants who have been provided *B. infantis* (18). In a large, double-blind, placebo-controlled trial conducted among SAM children in Malawi (19) involving a cocktail of four different probiotic lactic acid bacilli (but not containing any *Bifidobacterium* species) and four prebiotic fermentable fibers given in adjunct to the Ready-to-use- Therapeutic Food (RUTF), there was no effect on the recovery in weight for height, despite exhibiting reduced mortality in outpatient settings (19). These observations suggested to us that combinations of *B. infantis* and HMO at levels found in human milk would be the best option for remodeling the gut microbiome of the SAM infant.

Recent reports have shown intestinal inflammation to have a strong association with mortality in children with SAM (20). In addition, intestinal inflammation was found to be with subsequent faltering in linear growth in children (21). Although infant gut dysbiosis has only been correlated with enteric inflammation (22)-(23)-(24) conclusively demonstrated that when *B. infantis* EVC001 was provided to newborn breast-fed infants, the gut microbiome became dominated (> 85%) with *B. infantis* and levels of inflammatory cytokine biomarkers were significantly reduced relative to control infants who exhibited chronic enteric inflammation over the first 60 days from birth. The inflammatory biomarker myeloperoxidase (MPO) has been shown to be unaffected by breast milk intake in malnourished children (25) and has demonstrated positive association with features of subclinical intestinal inflammation. However, levels of *B. infantis* in the gut microbiome of those infants were not determined.

A gradual increase in the infant fecal pH has been observed over the course of the past century, with a marked rise in fecal pH from 5.0 to 6.5 being observed (26). Fecal pH of children and is heavily influenced by the abundance of *Bifidobacterium* which produce high concentrations short chain fatty acids and of acetate and lactate. Thus, a raised fecal pH is representative of a distinct change in the gut microbiome of infants even indicating early gut dysbiosis (26). Recent studies have demonstrated a significant positive association of fecal pH with severe acute malnutrition (27) and with stunted growth in early childhood (28).

The current study is a single-blind, randomized controlled trial (RCT) where our primary objective was to evaluate the effect of three supplementations, namely: placebo (lactose), probiotic (*B. infantis* EVC001) alone, and prebiotic plus probiotic (synbiotic of the HMO LNnT and *B. infantis* EVC001) on the change in gut inflammation and in fecal pH in SAM infants under six months of age. The safety and tolerability of this probiotic strain, *B. infantis* EVC001, has been evaluated elsewhere and this strain was safely consumed and well tolerated among infants of a similar age group (29). As an indicator of intestinal inflammation, we assessed fecal myeloperoxidase (MPO) since it is relatively unaffected by age or breastfeeding status (25)-(30).

Methods

Study Participants, Study Site and Ethics Statement

This short-term, single-blind, randomized controlled trial of prebiotic and probiotic was carried out at the Dhaka Hospital of the International Center for Diarrheal Disease Research, Bangladesh (icddr,b) between September 2018 and June 2019. This study was conducted among infants aged between 2 and 6 months suffering from SAM, who were admitted to the hospital with acute diarrhea and were transferred to the Nutritional Rehabilitation Unit (NRU) following acute phase management and stabilization. Infants suffering from septic shock or very severe pneumonia requiring assisted ventilation, acute kidney injury on hospital admission, jaundice or tuberculosis were not enrolled in the study. Moreover, participants screened with congenital defects interfering with feeding such as cleft palate or chromosomal anomalies were also excluded. Additional exclusion criteria included infants receiving $\geq 75\%$ of nutrition from breast milk, as measured by the test weighing method (31) and a history of ongoing maternal antibiotic usage (for breastfeeding infants only).

All activities were conducted in consonance to the all the outlined ethical principles of the Declaration of Helsinki and in accordance to the guidelines of Good Clinical Practice. Due approval from the Institutional Review Board (IRB) of icddr, b was obtained and the trial is registered at ClinicalTrials.gov (NCT0366657). Written consents from the parents or legal guardians of the study participants were obtained prior to enrolment in the study. Strict adherence to CONSORT guidelines for reporting clinical trials was maintained all throughout the study (CONSORT checklist in Additional file 1).

Trial Arms

Upon transfer to the NRU, a total of 62 infants with SAM were randomized to receive one of the three interventions for 28 days. These interventions included: placebo (lactose, 625mg), *B. infantis* EVC001 (8 billion CFU/daily single serving), or *B. infantis* EVC001 plus HMO prebiotic (Lacto-N-neotetraose [LNnT], 1.6g/ 6 hourly feed at the NRU and 3.2g/day at community). The infants were also stratified by age (2-3.9 months vs. 4-5.9 months) to ensure equal participation of infants by age groups. The study participants were followed up for a further 28 days after the respective interventions, making a total of 56 days of study duration for each participant.

Study Design and Data Collection

A summary of the study design is illustrated in Figure 1.

Infants in each trial arm were assigned to receive the standard of care diet (F-75) during the acute phase management of SAM, F-100 during hospital stay for nutritional rehabilitation, and standardized infant formula upon discharge. The interventions were available in powder format in sachets contained within feeding kits marked with unique code numbers that match the randomization code of each infant. The contents in the sachets were mixed with a teaspoon of infant formula or breast milk prior to oral administration to the infant.

Following enrolment in the study, anthropometric data from all study participants were collected by trained field research assistants (FRAs). Participants were discharged from the hospital upon completion of nutritional rehabilitation and fulfillment of hospital discharge criteria as described elsewhere (32). After discharge from NRU, participants were visited twice weekly by FRAs both during and after the intervention through the entire study period. During these home visits, participants were supplied with the respective interventions and the standardized infant formula for the time period until the next home visit. Proper storage and intake of the supplements and any antibiotic use during these home visits were also documented. Cases of hospital readmissions during the study period following possible serious adverse events were subsequently reported to the IRB of icddr, b. All the clinical record forms were de-identified by coded numbers to maintain the confidentiality of the study participants and to enable tracking throughout the study.

Collection of Fecal Samples and Laboratory Assessments

Stool samples were collected at baseline or prior to the start of supplementation, on day 10, on day 28 and on day 56. Stool samples collected were aliquoted in pre-labeled cryovials and flash frozen in liquid nitrogen

within 20 minutes after defecation, as practiced in other studies (33). Aliquots were stored in -80°C freezers until analysis. Inflammatory marker MPO was measured using commercially available ELISA kits (Alpco, Salem, NH, USA). Fecal pH was measured by using a portable pH meter (Hanna Instruments, USA) and by following a standard operating protocol described elsewhere (28). All the laboratory assays were carried out at icddr,b in Dhaka, Bangladesh.

Statistical Analysis

Categorical variables were represented as frequency and percentage of the total and quantitative variables were represented by median and interquartile range, due to the non-parametric distribution of the data. Baseline indices as well as fecal MPO and fecal pH for each of the study groups at each time interval was compared using Kruskal-Wallis test and resultant p-values of less than 0.05 were considered to be statistically significant.

Change in each of the analyzed biochemical parameters (MPO and pH) between the different time points in the study were expressed by delta (Δ), calculated as:

$$\Delta \text{MPO } 1/\Delta \text{pH } 1 = \text{MPO}/\text{pH at day 10} - \text{MPO}/\text{pH at baseline}$$

$$\Delta \text{MPO } 2/\Delta \text{pH } 2 = \text{MPO}/\text{pH at day 28} - \text{MPO}/\text{pH at baseline}$$

$$\Delta \text{MPO } 3/\Delta \text{pH } 3 = \text{MPO}/\text{pH at day 56} - \text{MPO}/\text{pH at day 28}$$

$$\Delta \text{MPO } 4/\Delta \text{pH } 4 = \text{MPO}/\text{pH at day 56} - \text{MPO}/\text{pH at baseline}$$

Kruskal-Wallis test was done to compare ΔMPO and ΔpH between the three supplementation groups at different time points during the study period. P-values were adjusted using the Bonferroni-Holmes method and considered to be significant if $p<0.05$. If ΔMPO or ΔpH for any of the intervals between the three study groups was found to differ in a statistically significant manner ($p<0.05$), post-hoc Dunn's test was done to assess any possible statistical significant difference for each of the two interventions (probiotic and synbiotic) with respect to placebo.

Multivariate quantile regression was performed to analyze the association of the different supplements on the ΔMPO and ΔpH , where ΔMPO and ΔpH were selected as the final outcomes. Covariates for the multivariable model were selected based on previous reports and literature survey (28)-(34)-(35)-(36) and included: age, sex, gestational age, baseline length, maternal age, presence of edema at baseline, birth order, percentage of breast milk received as proportion of total feed volume, family income, ongoing use or completed recommended dosage of antibiotics between the selected time points and mode of delivery of the infant. β -coefficient values and the corresponding 95% confidence intervals (CI) were used to determine the strength of the association. All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, N.Y., USA) and STATA version 13.0 (college station, Texas).

Results

A total of 62 infants aged between 2 and 6 months of age with SAM at baseline completed the study, including the 28 days of supplementation and a further 28 days of post-supplementation follow up. Among these infants, 56.5% (n = 35) were aged between 4 and 6 months and the rest were between 2 and 4 months of age. Out of these 62 study infants, 56.5% (n = 35) infants were male, 51.6% (n = 32) infants were delivered by C-section, and 67.8% (n = 42) infants had bilateral pedal edema at baseline prior to the start of supplementation. 21 infants received placebo, 20 received only the probiotic (*B. infantis* EVC001) and the remaining 21 infants received the synbiotic supplementation (LNnT + *B. infantis* EVC001).

Table 1 illustrates the baseline socio-demographic information and parameters of nutritional status collected from the study infants upon randomization to the three different supplementation arms. No significant statistical difference was found in the baseline socio-demographic and nutritional status between the study infants, indicating that the baseline characteristics were well-comparable among the study arms. Breast milk intake as represented by percentage of total feed was inadequate for infants in each of the study arms, indicating a distinct lack of breastfeeding practices among all the infants.

Table 1
Socio-demographic and nutritional parameters collected from the study infants randomized to the three different treatment arms.

Baseline Indices	Placebo (n = 21)	Probiotic (n = 20)	Synbiotic (n = 21)	p-value
Male sex (%)	11 (52)	11 (55)	13 (62)	0.816
C-section (%)	11 (52)	13 (65)	8 (38)	0.231
Gestational age (weeks)	37 (36, 40)	37 (36, 38)	38 (35, 39)	0.653
Birth weight (kg)	2.6 (1.9, 3)	2.7 (2.3, 3.2)	3.0 (2.5, 3.5)	0.347
Breast milk percentage (as percentage of total feed)	21.0 (12.6, 28.3)	19.7 (7.9, 28.2)	16.5 (12.3, 27.5)	0.833
Maternal age (years)	23 (18, 28.5)	25 (20, 29.3)	23 (19, 25.5)	0.600
Monthly Family Income (USD)	179 (119, 238)	179 (143, 211)	191 (143, 327)	0.384
Baseline WLZ	-4.1 (-4.3, -3.4)	-3.8 (-3.9, -3.6)	-3.6 (-4.3, -3.4)	0.597
Presence of bilateral pedal edema at baseline	14 (67%)	12 (60%)	16 (76%)	0.542
Footnotes:				
Data represented as median and interquartile range. The p-value shown for each time point is a result of Kruskal-Wallis test comparing each of the baseline indices for each of the study arms.				

Levels of fecal myeloperoxidase and fecal pH at each time interval for each of the three study arms

The median levels of fecal MPO and fecal pH measured at the different time points in infants randomized into the three intervention arms have been shown in Fig. 2 and Fig. 3, respectively. Before supplementation, no significant statistical differences in the fecal MPO and fecal pH were observed among the infants belonging to three arms. Following the supplementations, fecal MPO levels measured on day 10 and day 28 differed significantly ($p < 0.05$) between the infants enrolled in the three study arms (Fig. 2). The lowest MPO levels across each time point were found among infants receiving synbiotic supplementation. Infants who had received only the probiotic supplementation had higher fecal MPO levels during supplementation compared to the infants receiving the synbiotics, while a consistent rise in fecal MPO levels among the placebo group infants was observed. At day 56 of study, markedly lower levels of fecal MPO were observed among infants receiving either probiotic only (12.4 $\mu\text{g}/\text{ml}$) or synbiotic (11.8 $\mu\text{g}/\text{ml}$) and these results were statistically significant ($p < 0.05$) compared to those who had received placebo.

Fecal pH measured on day 10, day 28 and at day 56 among the infants irrespective of supplementation did not differ significantly (Fig. 3). However, infants receiving synbiotic supplementation had consistently lower fecal pH throughout the study post-baseline.

Change in the levels of fecal myeloperoxidase and in fecal pH (ΔMPO and ΔpH) between the different sample collection time points

All three study groups showed a sustained pattern in increased levels of fecal MPO in comparison to levels at baseline (Table 2, Fig. 4) both at day 10 (ΔMPO 1), at day 28 (ΔMPO 2) and at day 56 (ΔMPO 4). These increased levels in ΔMPO 1 and in ΔMPO 2 was the highest among the placebo group and lowest among the group that had received the synbiotic supplementation. This difference in increase in fecal MPO level compared to the baseline level among the three groups was statistically significant at day 10 (ΔMPO 1; $p = 0.04$), but not at day 28 (ΔMPO 2; $p = 0.323$). When post-hoc test was applied to determine any potential significance in difference of ΔMPO 1 for either the probiotic or synbiotic group with respect to placebo group (Table 2), statistically significant difference for ΔMPO 1 was found between synbiotic and placebo group ($p = 0.034$) but not between probiotic and placebo group ($p = 0.539$).

Table 2
Change in fecal MPO levels within the different time points throughout the study.

Interval considered for evaluation	Change in fecal MPO levels ($\mu\text{g/ml}$)					
	Placebo (n = 21)	Probiotic (n = 20)	Synbiotic (n = 21)	p-value for comparison between three groups	p-value for placebo vs probiotic	p-value for placebo vs synbiotic
$\Delta\text{MPO 1}$	20.11 (-1.19, 35.02)	7.95 (-4.36, 20.53)	3.04 (-12.66, 10.12)	0.040	0.539	0.034
$\Delta\text{MPO 2}$	8.28 (-18.12, 27.70)	7.30 (-2.89, 32.15)	0.46 (-7.73, 10.24)	0.323	—	—
$\Delta\text{MPO 3}$	1.24 (-8.39, 13.00)	-8.58 (-27.44, -2.03)	0.33 (-6.48, 5.15)	0.038	0.057	1.00
$\Delta\text{MPO 4}$	17.06 (-13.18, 23.70)	17.35 (-7.58, 14.30)	2.53 (-10.01, 8.63)	0.342	—	—

Footnotes:

Data represented as median and interquartile range.

$\Delta\text{MPO 1} = \text{MPO at day 10} - \text{MPO at baseline}$

$\Delta\text{MPO 2} = \text{MPO at day 28} - \text{MPO at baseline}$

$\Delta\text{MPO 3} = \text{MPO at day 56} - \text{MPO at day 28}$

$\Delta\text{MPO 4} = \text{MPO at day 56} - \text{MPO at day baseline}$

The level of fecal MPO at day 56 of study compared to the level at day 28, i.e.- $\Delta\text{MPO 3}$ (Table 2, Fig. 4), the trend of rise in fecal ΔMPO was found in the placebo group. However, for the infants who had received the probiotic only, a marked decrease in $\Delta\text{MPO 3}$ was observed and this difference was statistically significant among the three study arms ($p = 0.038$). However, upon a subsequent post-hoc analysis of $\Delta\text{MPO 3}$, no statistically significant difference was found (Table 2), either between probiotic and placebo group ($p = 0.057$) or between synbiotic and placebo group ($p = 1.00$). The level of fecal MPO at day 56 compared to the baseline level, $\Delta\text{MPO 4}$, was found to be lower for the synbiotic group in comparison to the groups that had received either the probiotic or the placebo. However this difference in $\Delta\text{MPO 4}$ between the three study arms was not statistically significant ($p = 0.342$).

On the other hand, a persistent decrease in fecal pH both at day 10 and at day 28 compared to the level at baseline, i.e. - $\Delta\text{pH 1}$ and $\Delta\text{pH 2}$, was observed for only the synbiotic group and the placebo group and

(Table 3, Fig. 5), with the sharpest decrease in ΔpH 1 ΔpH 2 being observed in the group that had received the synbiotic supplementation. Although the difference in pH at day 10 compared to that at baseline (ΔpH 1) was not statistically significant ($p = 0.559$), but at day 28 this difference in pH compared to that at baseline (ΔpH 2) was statistically significant between the three study groups ($p = 0.039$). However upon a post-hoc analysis for ΔpH 2, no statistical significance was found (Table 3) either between probiotic and placebo group ($p = 0.160$) or between synbiotic and placebo group ($p = 1.00$).

Table 3
Change in pH within the different time points throughout the study.

Interval considered for evaluation	Change in fecal pH					
	Placebo (n = 21)	Probiotic (n = 20)	Synbiotic (n = 21)	p-value for comparison between three groups	p-value for placebo vs probiotic	p-value for placebo vs synbiotic
ΔpH 1	-0.50 (-0.95, 0.25)	-0.20 (-0.70, 0.10)	-0.60 (-1.10, 0.05)	0.559	—	—
ΔpH 2	-0.70 (-1.30, 0.15)	0.00 (-0.98, 0.38)	-1.10 (-1.20, 0.10)	0.039	0.16	1.00
ΔpH 3	0.20 (-0.30, 0.60)	0.00 (-0.58, 0.38)	0.10 (-0.25, 0.95)	0.649	—	—
ΔpH 4	-0.50 (-0.90, -0.25)	0.00 (-0.65, 0.38)	-0.70 (-1.40, 0.25)	0.208	—	—
Footnotes:						
Data represented as median and interquartile range.						
ΔpH 1 = pH at day 10 – pH at baseline						
ΔpH 2 = pH at day 28 – pH at baseline						
ΔpH 3 = pH at day 56 – pH at day 28						
ΔpH 4 = pH at day 56 – pH at baseline						

A small rise in the pH at day 56 compared to the level at day 28 (ΔpH 3) was observed in the placebo and in the synbiotic groups (Table 3, Fig. 5), although these changes were not statistically significant ($p = 0.649$). For the probiotic group, a decrease in pH from the baseline level was only observed at day 10, i.e. - ΔpH 1. Otherwise for the probiotic group there was no difference in fecal pH at day 28 and 56 compared to the baseline level, i.e. - ΔpH 2 and ΔpH 4, respectively. On the other hand, a decrease in pH at day 56 compared

to the baseline level, i.e. - ΔpH 4 was observed for both the synbiotic and placebo group, although these changes were not statistically significant among the three groups ($p = 0.208$).

Association of the nutritional interventions on change in ΔMPO and in ΔpH

Both the probiotic (*B. infantis* EVC001) and the synbiotic (LNnT + *B. infantis* EVC001) resulted in reduction in the levels of ΔMPO 1 compared to the placebo group (Table 4). For the group that had received only the probiotic, the ΔMPO 1 was found to be lowered by 18.44 $\mu\text{g}/\text{ml}$ compared to the placebo group (95% CI: -31.62 $\mu\text{g}/\text{ml}$, -52.47 $\mu\text{g}/\text{ml}$; $p = 0.007$) while for the group that had received the synbiotic, ΔMPO 1 was reduced by a margin of 17.24 $\mu\text{g}/\text{ml}$ compared to the placebo group (95% CI: -30.94 $\mu\text{g}/\text{ml}$, -35.37 $\mu\text{g}/\text{ml}$; $p = 0.015$).

Table 4

Results of multivariate quantile regression showing association of supplements on $\Delta\text{fecal MPO}$ throughout the study.

Intervention Group	ΔMPO 1 ($\mu\text{g}/\text{ml}$)		ΔMPO 2 ($\mu\text{g}/\text{ml}$)		ΔMPO 3 ($\mu\text{g}/\text{ml}$)		ΔMPO 4 ($\mu\text{g}/\text{ml}$)	
	β -coefficient, 95% CI	p-value						
Probiotic (n = 20)	-18.44 (-31.62, -52.47)	0.007	24.77 (-12.60, 17.55)	0.742	-47.94 (-19.04, 94.54)	0.502	-3.58 (-16.87, 9.81)	0.590
Synbiotic (n = 21)	-17.24 (-30.94, -35.37)	0.015	-15.31 (-30.96, 33.00)	0.055	-24.39 (-16.99, 12.11)	0.737	-12.99 (-27.42, 1.44)	0.077
Footnotes:								
Data for ΔMPO at each interval has been represented as β -coefficient, 95% CI.								
ΔMPO 1 = MPO at day 10 – MPO at baseline								
ΔMPO 2 = MPO at day 28 – MPO at baseline								
ΔMPO 3 = MPO at day 56 – MPO at day 28								
ΔMPO 4 = MPO at day 56 – MPO at day baseline								
Multivariate quantile regression performed adjusting for possible co-variates based on literature survey and previous reports and includes: age, sex, gestational age, baseline length, maternal age, presence of edema at baseline, birth order, percentage of breast milk received as proportion of total feed volume, family income, ongoing use or completed recommended dosage of antibiotics between the selected time points and mode of delivery of the infant.								

However, this trend of reduction in the levels of MPO levels at day 28 compared to the baseline level (ΔMPO 2) was not found with the group that had received only the probiotic supplementation (Table 4), where there was an increase in ΔMPO levels by 24.77 $\mu\text{g}/\text{ml}$ compared to the placebo group (95% CI: -12.60 $\mu\text{g}/\text{ml}$, 17.55 $\mu\text{g}/\text{ml}$; $p = 0.742$). Subsequently, for the group that received the synbiotic supplementation, there was

a sustained reduction in Δ MPO levels at day 28 compared to the baseline level (Δ MPO 2), with a reduction by 15.31 $\mu\text{g}/\text{ml}$ compared to the placebo group (Table 4), although this change was not found to be statistically significant at 95% confidence level (95% CI: -30.96 $\mu\text{g}/\text{ml}$, 33.0 $\mu\text{g}/\text{ml}$; $p = 0.055$). The change in MPO levels at day 56 of study compared to the levels at day 28 (Δ MPO 3) was found to be lowered in both the intervened groups in comparison to the placebo group, with the Δ MPO being lowered by 47.94 $\mu\text{g}/\text{ml}$ in the probiotic group (95% CI: -19.04 $\mu\text{g}/\text{ml}$, 94.55 $\mu\text{g}/\text{ml}$; $p = 0.502$) and by 24.39 $\mu\text{g}/\text{ml}$ in the synbiotic group (95% CI: -16.99 $\mu\text{g}/\text{ml}$, 12.11 $\mu\text{g}/\text{ml}$; $p = 0.737$). Despite an overall reduction in the levels of Δ MPO 3 for both groups receiving the respective nutritional supplementations, the results were not statistically significant in comparison to the levels of Δ MPO 3 observed in the placebo group.

Subsequently, a reduction in the levels of Δ MPO 4 (MPO levels at day 56 compare to the baseline level) was observed for both the probiotic and synbiotic group in comparison to the placebo group, whereby a lowering of Δ MPO 4 by 3.58 $\mu\text{g}/\text{ml}$ was observed in the probiotic group and by 12.99 $\mu\text{g}/\text{ml}$ in the synbiotic group. These decreases in Δ MPO 4 were not statistically significant at 95% confidence level for both the probiotic group (95% CI: -16.87 $\mu\text{g}/\text{ml}$, 9.71 $\mu\text{g}/\text{ml}$; $p = 0.590$) and the synbiotic group (95% CI: -27.42 $\mu\text{g}/\text{ml}$, 1.44 $\mu\text{g}/\text{ml}$; $p = 0.077$).

On the contrary, in comparison to the placebo group there was an increase in the levels Δ pH across all analyzed time points throughout the study for the group that had received only the probiotic supplementation (Table 5). However, these changes in the levels of Δ pH for the probiotic group were not statistically significant in comparison to the placebo group (Table 5). For the group that had received the synbiotic supplementation, there was a decrease in Δ pH 1 and Δ pH 2. In comparison to the placebo group, Δ pH 1 reduced by 0.24 units (95% CI: -1.02, 0.54, $p = 0.535$) and Δ pH 2 reduced by 0.10 units (95% CI: -0.86, 0.66, $p = 0.794$) in the synbiotic group, although these results were not statistically significant (Table 5). On the other hand, despite not being statistically significant, the levels of Δ pH 3 and Δ pH 4 for the synbiotic group was found to be increased by 0.26 units (95% CI: -0.33, 0.86, $p = 0.374$) and 0.30 (95% CI: -0.54, 1.13, $p = 0.476$) units, respectively in comparison to the placebo group (Table 5).

Table 5

Results of multivariate quantile regression showing association of supplements on Δ fecal pH throughout the study.

Intervention	Δ pH 1		Δ pH 2		Δ pH 3		Δ pH 4		
	Group	β -coefficient, 95% CI	p-value	β -coefficient, 95% CI	p-value	β -coefficient, 95% CI	p-value	β -coefficient, 95% CI	p-value
Probiotic (n = 20)		0.15 (-0.60, 0.90)	0.690	0.56 (-0.13, 1.29)	0.131	0.18 (-0.40, 0.76)	0.529	0.47 (-0.30, 1.24)	0.226
Synbiotic (n = 21)		-0.24 (-1.02, 0.54)	0.535	-0.10 (-0.86, 0.66)	0.794	0.26 (-0.33, 0.86)	0.374	0.30 (-0.54, 1.13)	0.476
Footnotes:									
Data for Δ pH at each interval has been represented as β -coefficient, 95% CI.									
Δ pH 1 = pH at day 10 – pH at baseline									
Δ pH 2 = pH at day 28 of – pH at baseline									
Δ pH 3 = pH at day 56– pH at day 28									
Δ pH 4 = pH at day 56– pH at baseline									
Multivariate quantile regression performed adjusting for possible co-variates based on literature survey and previous reports and includes: age, sex, gestational age, baseline length, maternal age, presence of edema at baseline, birth order, percentage of breast milk received as proportion of total feed volume, family income, ongoing use or completed recommended dosage of antibiotics between the selected time points and mode of delivery of the infant.									

Discussion

This study addresses the effects of probiotic supplementation alone or in combination with a prebiotic in the change in status of gut inflammation and in fecal pH among young Bangladeshi infants with SAM. To our knowledge, this is the first study exploiting an RCT to explore the role of such supplementations in the possible amelioration of gut inflammation and in the change in fecal pH among severely malnourished infants, through simple and non-invasive measurement of biochemical parameters.

Previous studies conducted among Bangladeshi children suffering from severe acute malnutrition have reported continual impairment in gut microbiota maturity, implying a definite depletion of the beneficial microorganisms that usually colonize the human gut (10). This phenomenon, also referred to as “dysbiosis” has also been reported to lead to the development of chronic intestinal inflammation (37) and also resulting in a marked elevation of fecal pH (26).

In our study, levels of fecal MPO at baseline for the participants randomized into each of the study arms (Table 2) was markedly higher compared to the level of MPO (< 2 µg /ml) considered to be normal in tropical settings (38), thus indicating raised levels of intestinal inflammation in our study group. Data from the Bangladesh site of the MAL-ED birth cohort study (36) also indicate that among Bangladeshi infants of a similar age group of 2–6 months, similar trends in elevation of fecal MPO levels were found (38), despite these infants not suffering from SAM. In addition, we also report a general increasing trend in the levels of fecal MPO during the course of each of the respective supplementations.

A recently conducted study among breast-fed infants receiving the same probiotic (*B. infantis* EVC001) for 21 days reported a lowering of intestinal inflammation as measured by quantitation of fecal calprotectin and proinflammatory cytokines (39). This was found to positively correlate with increased colonization of *B. infantis* EVC001 and was negatively correlated with *Enterobacteriaceae* abundance. However, in this present analysis, we do not have the extent of colonization seen with healthy breast-fed infants (13). In our multivariate quantile regression analysis after adjusting for the above-mentioned covariates, only ΔMPO_1 and ΔMPO_3 showed a lower β -coefficient value compared to that of the placebo group, indicating potential inconsistency in the sustainability of our probiotic supplementation in our study population. Subsequent regression analysis showed that ΔMPO at each time point was lowered for the prebiotic and probiotic group in comparison to the control group, thus indicating a sustained effect of both supplementations in reducing the intestinal inflammation, when given together in tandem. A continually lowered β -coefficient value for ΔMPO for this group nevertheless may indicate a newer strategy for a larger clinical trial to explore the role of this probiotic and prebiotic supplementation in amelioration of gut inflammation in such patients.

On the other hand, baseline fecal pH values for all the study participants were comparable to the levels reported in previous studies on fecal pH of children with SAM. This finding also corroborates with previous reports of immaturity in gut microbiota implying a certain depletion of short-chain fatty acid producing bacteria, in particular, *Bifidobacterium* (26)-(40) and inadequacy in production of short-chain fatty acids (SCFA) in stool and an elevated fecal pH. However, no sustained trend in change in fecal pH over the sample collection time points (ΔpH) in either of the supplementation arms (synbiotic or probiotic) was observed throughout the study period in comparison to the placebo group. However, our regression analysis illustrated a decrease in the β -coefficient ΔpH_1 and in ΔpH_2 values for the synbiotic group in comparison to the placebo group, implying potential colonization of the probiotic, *B. infantis* EVC001 in the presence of LNNT during the course of the supplementation. An increase in the β -coefficient value for ΔpH_3 and ΔpH_4 in the synbiotic group may otherwise be a possible indication of the lack of sustainability of *B. infantis* EVC001 colonization of the gut. This in turn, implies for the potential implementation of a more extensive prebiotic and probiotic dosing for a sustained lowering of the fecal pH in such infants. However it must also be noted that unlike other studies involving the measurement of fecal pH (28), where measurement of fecal pH was done using fresh stool samples, in this study the measurement of fecal pH was done using thawed samples that were previously flash frozen and stored in -80°C temperature.

B. infantis is involved in the selective degradation of HMO components, in particular, the Core-1 and Core-2 based HMOs (i.e. 80% of all HMOs) and glycoproteins with N-glycan linkages, but not glycoproteins with O-glycan linkages such as mucins (41)-(42). Insufficient breast milk intake by the study participants,

particularly in the probiotic group, may have thus resulted in the inability of *B. infantis* EVC001 to effectively colonize the gut to high levels, possibly leading to reduced acetate and lactate production in stool and minimizing the ΔpH in the probiotic group, in comparison to the placebo group. The reduction in the values of ΔpH 1 and in ΔpH 2 in the placebo group may be indicative of possible natural colonization of the gut by lactic acid producing bacteria.

Limitations Of The Study

The small sample size underpowered the overall study findings, despite some regularly observed positive effects of the interventions being observed at different time points throughout this pilot study. In addition, collection of morbidity data was not conducted in a systematic manner, leading to our inability of adjusting morbidity data of patients during the course of the study in our final analysis. No supporting data delineating possible infections by characterized enteric pathogens was available in our study. Moreover, data regarding the impact of complementary feeding along with the lack of data on levels of acetate and lactate in the stool and on potential *B. infantis* EVC001 colonization during the course of supplementation, fails to endow us with the capability of assembling assumptions regarding the potency of such interventions.

Conclusion

This pilot study provides preliminary insights into the possible roles of the prebiotic, LNnT, and the probiotic, *B. infantis* EVC001, in the improvement of gut inflammation and fecal pH in young infants suffering from severe acute malnutrition. *B. infantis*, when given alone, was seen to result in an inconsistent trend in reduction of enteric inflammation compared to the placebo group, with substantial reduction in enteric inflammation only being observed during the post-supplementation follow up. However, no apparent lowering of fecal pH was observed in this group throughout the study period, possibly indicating the inability of *B. infantis* EVC001 to colonize the gut of these study participants which is consistent with the very low intake of human milk by these SAM infants.

However, when *B. infantis* EVC001 is given in conjunction with LNnT, it shows sustained effects in the reduction of intestinal inflammation throughout the study period. Reduction in fecal pH was only seen during the course of the intervention with *B. infantis* EVC001 plus LNnT and was not sustained after the supplementation. This in turn may imply a potential need for an increased dose of the supplementation or a longer duration of supplementation for a continued effect on the improvement of overall gut health status.

Abbreviations

RCT: randomized controlled trial; SAM:severe acute malnutrition; WLZ:Weight for Length Z score, LNnT:Lacto-N-neotetraose; ELISA:Enzyme Linked Immunosorbent Assay; MPO:myeloperoxidase; CFU:colony forming units.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Review Board (IRB) of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b) on 16 February, 2018 (code: PR-17112). Written, informed consent was obtained from the parents or legal guardians of the prospective study participants prior to enrolment in the study. The declaration of Helsinki and the Good Clinical Practice guidelines were followed throughout the study.

Consent for publication

Not applicable.

Availability of data and materials

This data set contains some personal information of the study patients (such as name, admission date, age, etc.). The policy of our institute (icddr, b) is that we should not make the availability of whole data set in the manuscript, the supplemental files, or a public repository. However, data related to this manuscript are available upon request and for researchers who meet the criteria for access to confidential data may contact with Armana Ahmed (armana@icddrb.org) to the Research Administration of icddr,b (<http://www.icddrb.org/>).

Competing interests

The authors declare that they have no competing interests.

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Author contributions

TA originated the idea for the study and led the protocol design. PP, SN, MRI, MM, MMI, SAS and TA participated in the design of the study. PP, MAG, SN, MRI, MAA, MMI, MM, SAS, RLF, DJK and TA conducted the study and supervised the sample and data collection. PP, MAG, SN, MRI and MM performed and supervised the sample analysis. PP, MAG, SN, MRI, MAA and TA were involved in data analysis. PP, MAG, SN, MM and TA interpreted the results. PP, MAG, SN, MM and TA were involved in manuscript writing. All authors read and approved the final manuscript.

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Figures

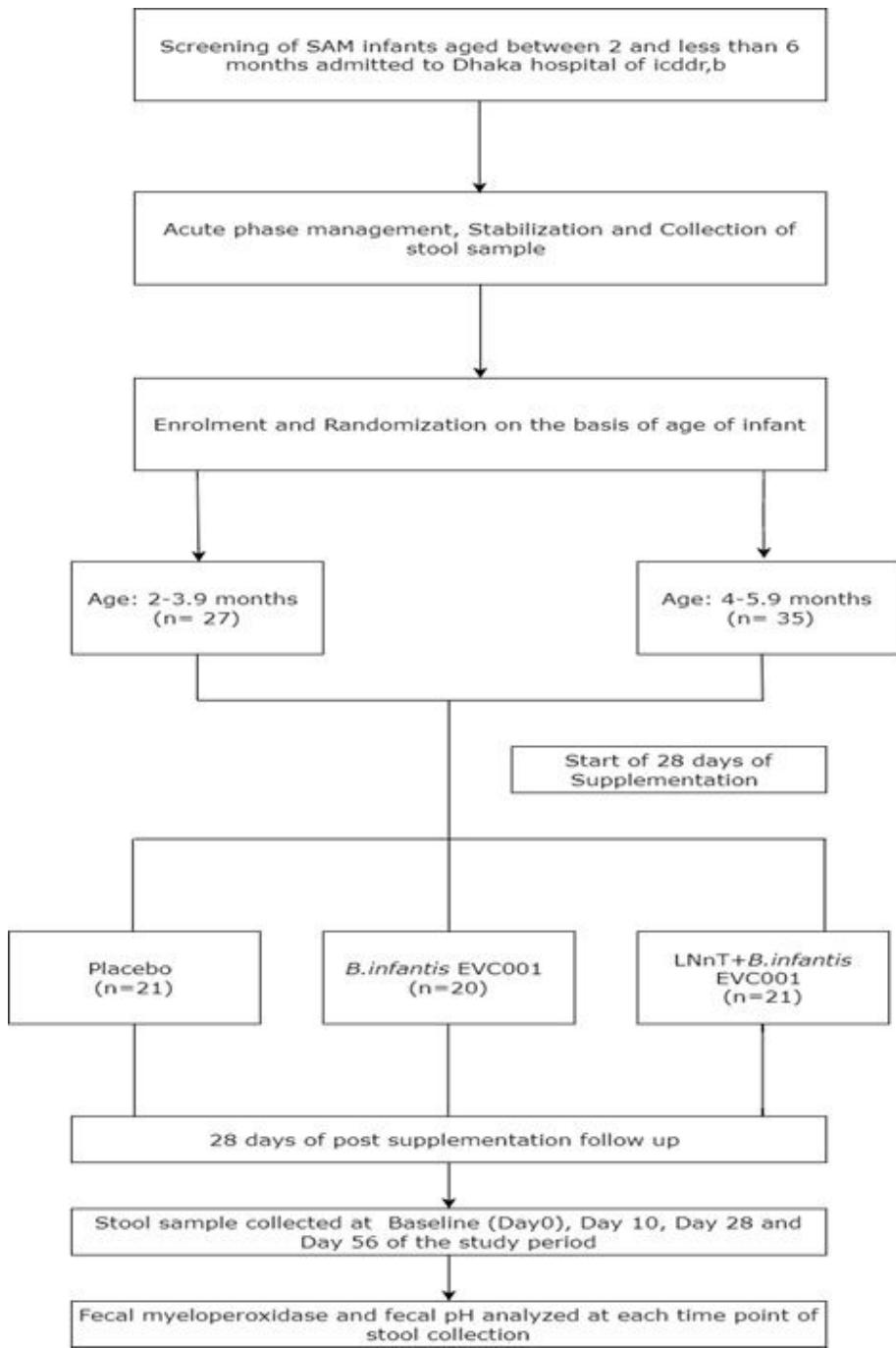


Figure 1

Workflow of the study

Fecal myeloperoxidase at each time interval for each of the study arms

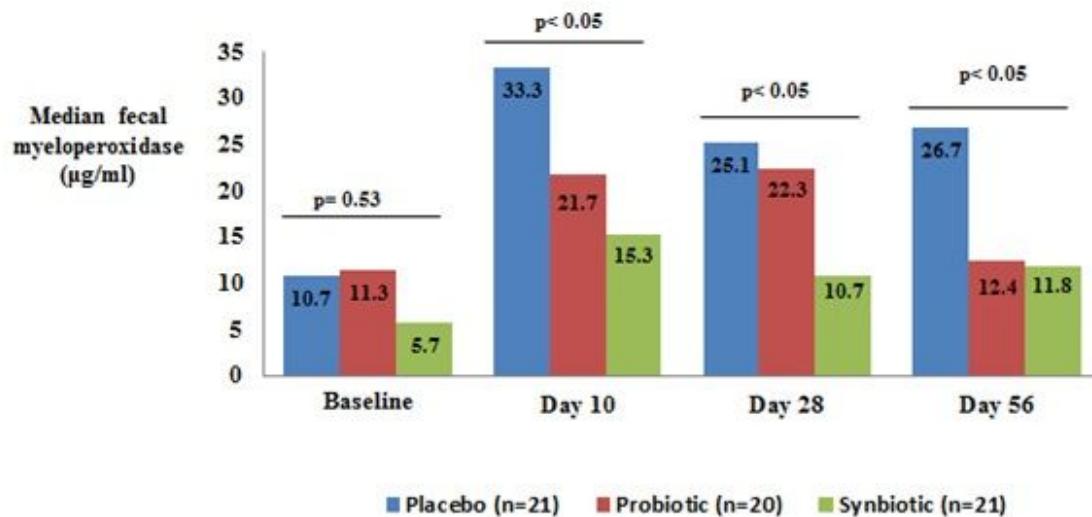


Figure 2

The p-value shown for each time point is a result of Kruskal-Wallis test using the median MPO values for each of the study arms at the respective time points.

Fecal pH at each time interval for each of the study arms

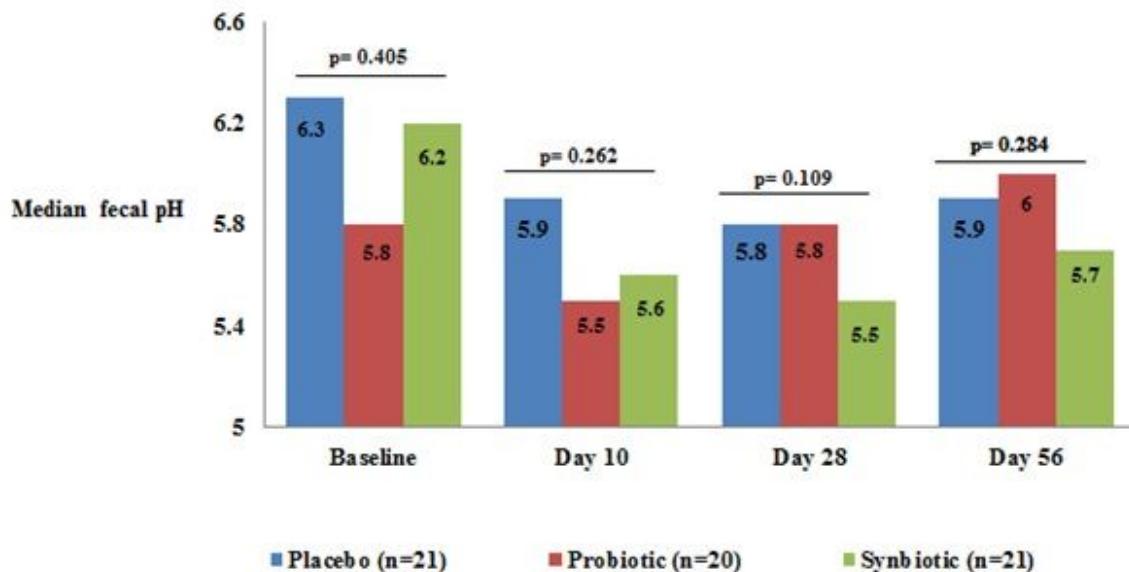


Figure 3

The p-value shown for each time point is a result of Kruskal-Wallis test using the median fecal pH values for each of the study arms at the respective time points.

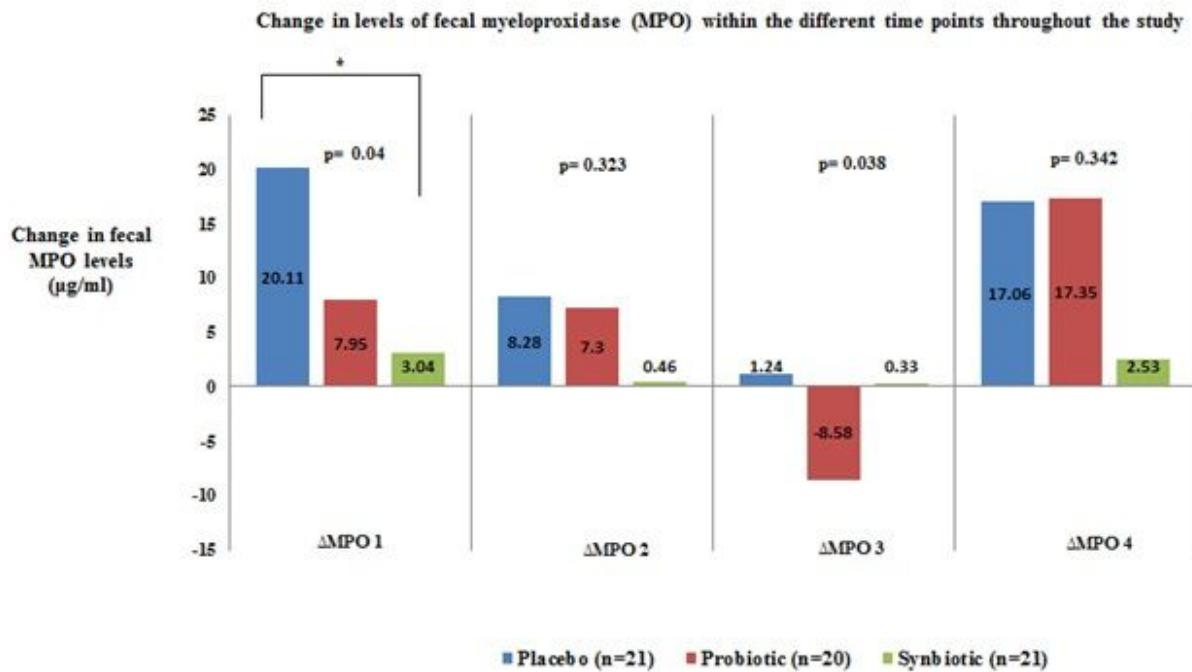


Figure 4

The p-value shown for each time point is a result of Kruskal-Wallis test using the median Δ MPO values for each of the study arms at the respective time points. Asterix symbol (*) represents a statistically significant difference between the study arms, upon post-hoc analysis using Dunn's test. Δ MPO 1 = MPO at day 10 – MPO at baseline Δ MPO 2 = MPO at day 28 – MPO at baseline Δ MPO 3 = MPO at day 56 – MPO at day 28 Δ MPO 4 = MPO at day 56 – MPO at day baseline

Change in levels of fecal pH within the different time points throughout the study

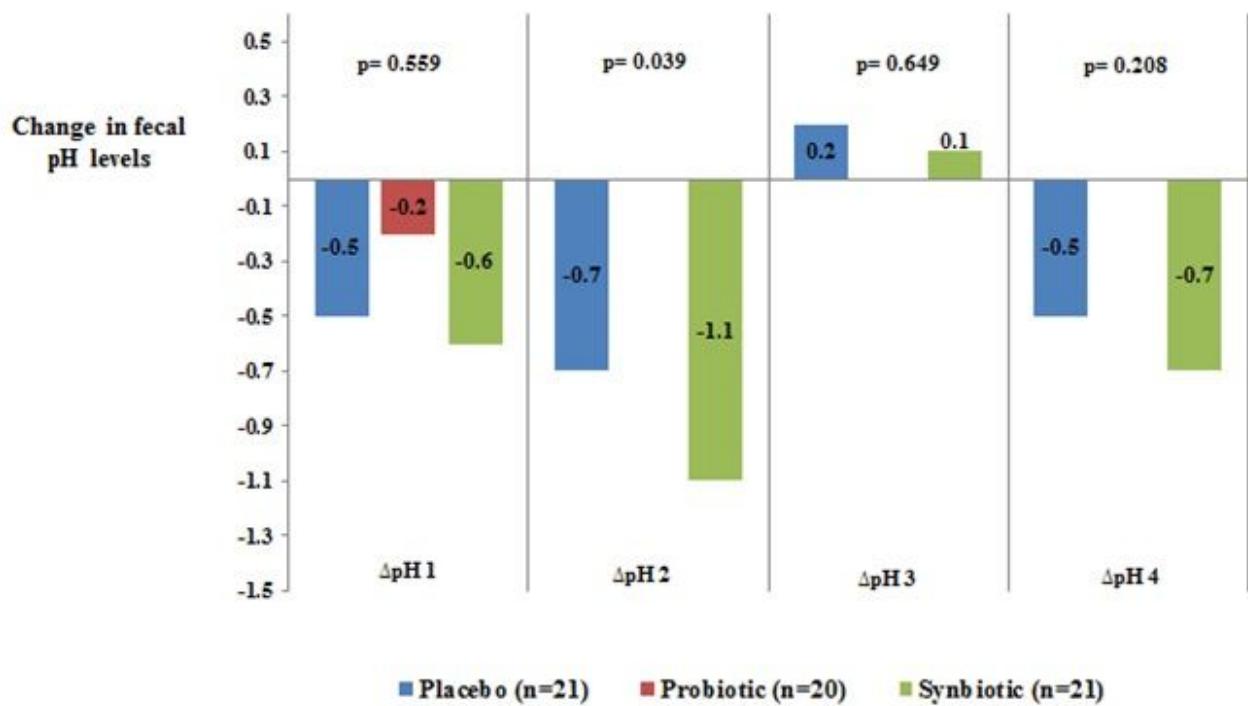


Figure 5

The p-value shown for each time point is a result of Kruskal-Wallis test using the median ΔpH values for each of the study arms at the respective time points. $\Delta\text{pH} 1 = \text{pH at day 10} - \text{pH at baseline}$ $\Delta\text{pH} 2 = \text{pH at day 28} - \text{pH at baseline}$ $\Delta\text{pH} 3 = \text{pH at day 56} - \text{pH at day 28}$ $\Delta\text{pH} 4 = \text{pH at day 56} - \text{pH at baseline}$

Supplementary Files

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- Additionalfile1CONSORTchecklist.doc