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Effects of galacto-oligosaccharide prebiotics in blood profile of severely acute malnourished children

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Abstract: This study assessed the effects of galacto-oligosaccharides (Oligomate) on hematocrit, serum enzymes, total bilirubin levels, and serum electrolytes in controls and severely malnourished infants, with emphasis on gastrointestinal symptoms. Oligomate doses and phases did not affect stools frequency per day, indicating that prebiotic effect on stool may be due to the prebiotic type. The number of vomits per day during phases 2 and 3 were significantly reduced ($p<0.05$) in response to prebiotics, despite the prebiotic dose effect was not significant ($p>0.05$). Moreover, prebiotics administration during phases 2 and 3 markedly improved hemoglobin levels ($p<0.05$), but not the dose. Similarly, hematocrit levels and white blood cells were significantly improved during the last 2 phases, but dose have no effects on blood hematocrit levels. Erythrocyte sedimentation rate significantly decreased ($p<0.05$) in phases 2 and 3 compared to phase 1. No dose-related effect was stated on erythrocytes sedimentation rate. Regarding the serum enzymes, SGPT significantly decreased ($p<0.05$) in phases 2 and 3 compared to phase 1, whereas SGOT significantly decreased only in phase 3. Total bilirubin levels increased significantly ($p<0.05$) in phase 3 when compared to phases 1 or 2. Prebiotics significantly decreased ($p<0.05$) sodium levels in the treated group, while potassium levels did not change in all groups, excepting during phase 2, where it increased significantly. Thus, our results confirm the hypothesis that prebiotic supplementation improves blood parameters and health status, consequently decreasing the infection risk and number of vomit per day in infants.

Key words: Prebiotics; Blood hematology; Serum enzymes; Electrolytes.

Introduction

In developing countries, malnutrition affects 165 million children with less than 5 years (1). It also has been noted a serious health problem in Pakistan's children. About 31.5% of children are underweight, with higher rates in rural areas. Among the South Asian Association for Regional Cooperation (SAARC) countries, Pakistan has the 2nd highest stunting rate (43.7%), while wasting rate is 15.1% (2). It arises in one of the two forms marasmus or kwashiorkor. Children affected with marasmus reveal severe muscle wasting, whereas those with kwashiorkor are characterized mainly by muscle wasting, apathy and generalized edema with hair and skin changes (3). The poor understanding of the basic biology of infants and children severely acute malnourished (SAM) may stalk from the fact that most research studies are cross-sectional, while prospective research studies are generally not furnished owing to ethical reasons. Moreover, these particular patients are awfully vulnerable, so as massive encroachment of understanding is intricate.

Biochemical investigations give the most impartial and quantitative facts on nutritional prestige. Nutritional deficiencies have been urged by the biochemical changes at the onset, after that, cells or organs finally established clinical malnutrition (4). Measuring iron status has been called a powerful tool for evaluating under nutrition. It is documented that nutritional anemia is the most prevalent problem among preschool and school aged children (5). This may be particularly important in the intestine and liver function, because they are key factors for proper digestion and metabolism. Changes in small intestine function and structure and of intes-

tinal flora has increasingly hinted as causal factors of kwashiorkor development. During malnutrition, a sway in the intestinal microbiota may indulge the temptation of an endotoxin serotype more likely to cause edema and liver damage (6).

Serum albumin levels have been signified as vital tool for protein-energy nutritional status evaluation (7). Infant diarrhea is a serious problem in early life stages, mainly treated or managed with extensive antibiotics use, thus leading to the appearance of antibiotic-resistant bacteria and problems regarding antibiotic residuals in the body (8). Now, the alternatives have to be found out to promote growth and stabilize gut health. These may include improved nutrition, such as those containing prebiotics (9).

Briefly, prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of beneficial bacteria in the colon that improve host health (41). They can feed the intestinal microbiota, and their degradation products are short-chain fatty acids that are released into blood circulation, consequently, affecting not only the gastrointestinal (GI) tract, but also other distant organs (42). Galacto-oligosaccharides (GOS) has been termed as non-digestible substances that beneficially affects the host by modulating intestinal flora. GOS, the product of lactose extension, are classified into two subgroups: first the GOS with excess galactose at C3, C4 or C6, and second the GOS manufactured from lactose through enzymatic transglycosylation (43). GOS can greatly stimulate *Bifidobacteria* and *Lactobacilli* spp. Specifically, *Bifidobacteria* spp. in infants have shown high incorporation with GOS. *Enterobacteria*, *Bacteroidetes*, and *Firmicutes* spp. are also stimulated by GOS, but to a lesser extent than *Bifidobacteria*. There are some lactulose-derived GOS (44). They occur naturally as a complex mixture in breast milk and colostrum, as well as in bovine milk. Human milk GOS exhibit a complex and diverse chemical profile of over 130 different compounds (10). Human colostrum may contain higher concentrations of oligosaccharides as 25.6 g/L. In particular, a GOS product that contain only β-linkages, as Oligomate 55N/55NP (di-, tri-oligo- and tetra-oligo-saccharides, 4'-GL, lactose, glucose and galactose) is not digested by endogenous enzymes (8). As a consequence, various GOS products produce physical and physiological effects on GI tract, such as increases in colonic microbiota fermentation, reduces intestinal and fecal pH, changes fecal characteristics and frequency, increases cecal tissue weight, flatulence, borborygmic and abdominal distension (11). The increase in microbiota composition results in β-galactosidases and β-glucosidases increase, in higher concentration of short-chain fatty acids (SCFAs) as well as in a rise in breath-hydrogen concentration (12). Also, GOS ingestion causes a decrease in the numbers of specific pathogens, and of pathogenic bacteria adherence and invasion to host cells. GOS has currently a GRAS (Generally Recognized as Safe) notification as it is especially used in infant formula, including dairy and baked products, acidic beverages (13). To improve bacterial flora, the GOS ingestion recommendation is 2 to 3 g/day. For people with diabetes and high content of blood fat (cholesterol and triglycerides), the recommended amounts range from 8 to 20 g/day. GOS do not have toxicity, and the only known adverse effect is diarrhea when GOS consumed in excess (at doses estimated of 0.3 to 0.4 g/kg b.w.) (45).

Hence, the rationale of this research study is to assess the role of prebiotic-functional foods in the nutritional status of 6–59 months SAM infants and young children by measuring some hematological and biochemical parameters.

Materials and Methods

The current study was done at Nutrition Rehabilitation Unit (NRU), Department of Social and Preventive Pediatrics, Mayo Hospital, Lahore, Pakistan, a Tertiary Care large urban teaching and referral hospital and was approved by University Ethical Committee. This study was conducted according to the Helsinki declaration. GOS (Oligomate™) was procured from Yakult Pharmaceutical Industry, Japan and stored at room temperature (20-25°C) until further study.

Efficacy trial

Thirty SAM in-patients of 6-59 months of age were enrolled, while follow-up treatment was given at the out-patient department. We define SAM as weight-for-height of <70% of the median, nutritional edema (Kwashiorkor), or both, and mid-upper arm circumference (MUAC) <11.5 cm (14). Anthropometry protocols were followed by research standards (15). Proforma regarding demographic characteristics (gender, age, weight, height), general health and disease history, dietary plan, medications, anthropometric measurements and other attributes were recorded before study onset. To children's parents was given a comprehensive presentation on the project, and those who agreed (on behalf of their children) were asked to sign the consent form.

Nutritional status

Children were weighed on digital scales that were calibrated daily. Lengths and MUAC were measured using locally made height boards and MUAC insertion tapes, respectively, procured by UNICEF. Treatment protocol was based on standard international World Health Organization (WHO) and Community-based Management of Acute Malnutrition program (CMAM) guidelines (16). All children were initially fed F-75 (75 kcal/100ml) therapeutic milk (Phase I) and then progressed to F-100 (100 kcal/100ml) (Phase II) and Plumpy'nuts (F-100 in spread form with iron fortification) (Phase III) with follow-up for a period of 48 days. GOS-based prebiotics (Oligomate™) at concentration of 1 g/100 kcal and 1.5 g/100 kcal were given to SAM children admitted to the hospital and their impacts on physical parameters and blood indices were evaluated. The control group received standard therapeutic foods, whereas the intervention group received therapeutic foods plus Oligomate™, fortified in F75 at the rate of 7.5 g/L containing 4.1 g/L GOS for 1 g/100 kcal dose, and at the rate of 11.5 g/L containing 6.1 g/L GOS for 1.5 g/100 kcal dose, while in F100 and Plumpy'nuts it was fortified at the rate of 10 g/L containing 5.5 g/L GOS for 1 g/100 kcal dose, and at the rate of 15 g/L containing 8.25 g/L GOS for 1.5 g/100 kcal.

Biochemical analysis

For hematological and biochemical screening, 5 mL venous blood was taken after 12 h fasting in vacutainers tubes containing ethylene diamine tetra-acetic acid (EDTA). Complete blood count (CBC) was carried out using a hemo-cytometer (17). The mechanism involved is the red blood cells lysis through glacial acetic acid, but not the white blood cells so gentian violet slightly stains the leukocytes nuclei. Blood samples were diluted (1:20) in a WBCs pipette with the diluting liquid. Cells were counted under micro-scope by using counting chamber, and the cells numbers of in undiluted blood were reported per microliter whole blood.

Serum albumin levels were evaluated according to the procedures (18). Bromocresol green (BCG) assay detects serum albumin concentration. The assay is based on the selective interaction between BCG and albumin forming a chromophore that can be detected at 620 nm using a spectrophotometer. The signal is directly proportional to the albumin amount present in the serum. BCG does not react with other abundant plasma proteins, like IgG. The assay can detect as low as 5 µg (0.01 g/dL) of albumin in serum samples.

Albumin + BCG $\longrightarrow$ Absorbance (OD 620 nm)

Sample Albumin Concentration (C) = B/V X D µg/µL; where: B is the albumin amount in the sample well (µg); V is the sample volume added into the reaction well (µl); D is the sample dilution factor.

ESR determination

For ESR determination (19), anti-coagulated blood was allowed to stand in a narrow vertical glass tube, undisturbed for a period of time, the RBCs under the influence of gravity settled out from the plasma. The rate at which they settled was measured as the number of millimeters of clear plasma present at the column top after 1 h (mm/h). Serum samples were analyzed for enzymes such as serum glutamic-pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT) for liver functioning (20).

Determination of alkaline phosphatase

For alkaline phosphatase determination, the following protocol was adopted. Alkaline phosphatase release phenol from *p*-nitrophenylphosphate, the phenol in alkaline medium gives yellow color, which can be estimated with spectrophotometer at 405 nm. The reagents required for this experiment are R1 = diethanolamine (pH 9.8, 1.2 mol/L), magnesium chloride (0.6 mmol/L); R2 = *p*-nitrophenylphosphate (50 mmol/L).

p-nitrophenylphosphate + H_2O $\longrightarrow$ Phosphate + *p*-nirteophenol

The following formula is used to calculate:

With factor: From absorbance reading calculation ΔA/min and multiply by the corresponding factor: ΔA/Min x factor = ALP activity [U/L]

$$\text{ALP}[U/L] = \frac{\Delta A/\text{min sample}}{\Delta A/\text{min calibrator}} \times conc.Calibrator\ [U/L]$$

Conversion factor: ALP [U/L] X 0.0167 = ALP [ukat/L]

SGPT determination

For SGPT (ALT), the following protocol (20) was adopted. ALT is present at high concentrations in liver and to a lesser extent in kidney, heart and skeletal muscle, pancreas, spleen and lung. Usually, ALT levels are lower than AST levels. Increased ALT levels are generally a result of liver disease associated with some degree of hepatic necrosis. ALT is an important indicator of liver disease. The series of reactions involved in the assay system is as follows: L-Alanine + 2- oxoglutarate → Pyruvate + L-Glutamate; Pyruvate + NADH → Lactate+ NAD. Then, the rate of NADH consumption is determined photometrically that is proportional to ALT activity in the sample. The following formula is used to calculate:

With factor: From absorbance reading calculate ΔA/min and multiply by the corresponding factor from table below: ΔA/min x factor = ALAT activity [U/L]

With calibrator: ALAT [U/L] = ΔA min sample / ΔA min Calibrator x Conc. Calibrator [U/L]

SGOT/AST determination

AST is a cellular enzyme (21), and is found in highest concentration in heart muscle, liver and skeletal muscle cells.

L-Aspartate + α-Ketoglutarate $\xrightarrow{\text{AST}}$ Glutamate + Oxalacetate

Oxalacetate + NADH + H+ $\xrightarrow{\text{MDH}}$ Malate + NAD+

The rate of decrease in NADH concentration, measured photometrically at 340 nm is proportional to the catalytic concentration of AST present in the sample, and is calculated using the following formula: ΔA/min × Factor = U/L SGOT(AST); ΔA/min × 1750 = U/L SGOT(AST).

Determination of serum electrolytes (Na, K)

Serum electrolytes, such as Na and K were analyzed by electrolyte analyzer (22). Briefly, 10 mL blood was obtained from each subject and then blood samples were allowed to stand for 1 h to clot. Thereafter, blood samples were centrifuged at 300 rpm, 15 min at room temperature. The supernatant was then separated from the settled bottom blood cells. All serum samples were then analyzed for sodium and potassium ions (22).

Statistical analysis

All data was analyzed through 2 factor ANOVA under complete randomized design (CRD) technique (23) by Cohort-CoStat-2003 (Software version 6.33). Means of three phases were compared with means of the 2 doses (i.e. control, 1 g/100 kcal and 1.5 g/100 kcal) using Duncan's Multiple Range test, and the level of significance was defined as $p<0.05$.

Results

GOS prebiotics (Oligomate™) at different doses orally administered for a period of 48 days in 30 children aged from 6 month to 5 years during 3 phases were carefully addressed.

Prebiotics effect on serum albumin levels

According to the results (Table 1), there was a significant difference ($p<0.05$) regarding the prebiotics effect on serum albumin levels during these phases as well as at distinct doses.

Prebiotics effect on number of stools

According to the results (Table 2), there was no significant differences ($p>0.05$) regarding GOS prebiotics effect during the 3 phases. Similarly, there were no differences ($p>0.05$) regarding the effect of prebiotics doses on stools number.

Prebiotics effect on serum electrolyte sodium levels

There were no significant differences ($p>0.05$) regarding GOS prebiotics effect on serum electrolyte sodium levels during the 3 phases (Table 3), but a dose-related effect was stated ($p<0.05$) throughout the treatments.

Prebiotics effect on serum electrolyte potassium levels

There were significant differences ($p<0.05$) regarding GOS prebiotics effect on serum electrolyte potassium levels during the 3 phases (Table 4), but dose-related effect remained non-significant ($p>0.05$) throughout the treatments.

Prebiotics effect on white blood cells

A marked variation was stated ($p<0.05$) on GOS prebiotics effect in white blood cells over the 3 phases (Table 5), although the dose-related effect remained

Table 1. Effect of probiotics on serum albumin levels (g/dL).

Treatments	Phase 1	Phase 2	Phase 3
Control	$2.69\pm0.65^{a,B}$	$3.1\pm0.64^{a,AB}$	$3.42\pm0.39^{b,A}$
T1 (1 g/100 Kcal)	$3.1\pm0.58^{a,B}$	$3.65\pm0.75^{a,B}$	$4.43\pm0.75^{a,A}$
T2 (1.5 g/100 Kcal)	$3.24\pm0.83^{a,A}$	$3.65\pm0.77^{a,A}$	$3.89\pm0.46^{b,A}$

Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 2. Effect of probiotics on number of stools per day.

Treatments	Phase 1	Phase 2	Phase 3
Control	$4.5\pm0.52^{a,B}$	$5.2\pm0.91^{a,A}$	$4.5\pm0.52^{a,B}$
T1 (1 g/100 Kcal)	$4.5\pm1.50^{a,A}$	$4.4\pm0.84^{b,A}$	$3.8\pm0.91^{a,A}$
T2 (1.5 g/100 Kcal)	$4.9\pm1.19^{a,A}$	$4.8\pm0.78^{ab,A}$	$4.5\pm1.26^{a,A}$

Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 3. Effect of probiotics serum electrolyte sodium levels (mmol/L).

Treatments	Phase 1	Phase 2	Phase 3
Control	$152.5\pm6.27^{a,A}$	$146\pm7.19^{a,A}$	$158.5\pm26.50^{a,A}$
T1 (1 g/100 Kcal)	$137.9\pm18.97^{b,A}$	$132.5\pm7.67^{b,A}$	$133.6\pm9.21^{b,A}$
T2 (1.5 g/100 Kcal)	$138.4\pm15.19^{b,A}$	$141\pm10.44^{a,A}$	$138.4\pm9.69^{b,A}$

Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 4. Effect of probiotics serum electrolyte potassium levels (mmol/L).

Treatments	Phase 1	Phase 2	Phase 3
Control	$4.22\pm0.34^{a,B}$	$4.63\pm0.41^{a,A}$	$4.13\pm0.38^{b,B}$
T1 (1 g/100 Kcal)	$4.17\pm0.24^{a,B}$	$4.79\pm0.44^{a,A}$	$4.66\pm0.51^{a,A}$
T2 (1.5 g/100 Kcal)	$4.26\pm0.33^{a,B}$	$4.64\pm0.36^{a,A}$	$4.29\pm0.36^{ab,B}$

Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 5. Effect of probiotics on white blood cells (thous/µL).

Treatments	Phase 1	Phase 2	Phase 3
Control	$10.17\pm1.69^{a,A}$	$9.79\pm1.03^{a,A}$	$10.89\pm1.16^{a,A}$
T1 (1 g/100 Kcal)	$9.01\pm1.41^{ab,B}$	$9.8\pm0.73^{a,B}$	$11.44\pm1.22^{a,A}$
T2 (1.5 g/100 Kcal)	$8.14\pm1.19^{b,C}$	$9.56\pm0.97^{a,B}$	$11.54\pm0.91^{a,A}$

WBCs= White Blood Cells; Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

non-significant throughout the treatments ($p>0.05$).

Prebiotics effect on erythrocyte sedimentation rate

There was a significant difference ($p<0.05$) regarding GOS probiotics effect on erythrocyte sedimentation rate during the 3 phases (Table 6), but the dose-related effect remained insignificant throughout the treatments ($p>0.05$).

Prebiotics effect on serum glutamic pyruvate transaminase levels

A significant difference ($p<0.05$) was stated regarding GOS prebiotics effect on serum glutamic pyruvate transaminase levels during the 3 phases (Table 7), but the dose-related effect was non-significant throughout the treatments ($p>0.05$).

Prebiotics effect on serum glutamic oxaloacetic transaminase levels

There was a significant difference ($p<0.05$) regarding GOS prebiotics effect on serum glutamic oxaloacetic transaminase levels during the 3 phases (Table 8), but the dose-related effect remained non-significant throughout the treatments ($p>0.05$).

Prebiotics effect on alkaline phosphatase levels

There were non-significant differences ($p<0.05$) regarding GOS prebiotics effect on alkaline phosphatase levels during these phases as well as doses (Table 9).

Discussion

In this study, the distinct GOS doses used in all three phases did not affect the number of stools per day, thus meaning that GOS prebiotics effect on stool might be related to the prebiotic type (specific). Another plausible reason in our case is that children's GI system was adversely disturbed at the time of prebiotic administration as they received antibiotics. The beneficial microbes that have been supposed to use prebiotics in our expe-

Table 6. Effect of probiotics on erythrocyte sedimentation rate.

Treatments	Phase 1	Phase 2	Phase 3
Control	$47.3\pm11.85^{ab,A}$	$43.6\pm10.85^{a,A}$	$30.4\pm6.51^{a,B}$
T1 (1 g/100 Kcal)	$56\pm13.95^{a,A}$	$38.2\pm13.21^{a,B}$	$25.3\pm7.28^{a,C}$
T2 (1.5 g/100 Kcal)	$40.4\pm10.50^{b,A}$	$44.4\pm12.33^{a,A}$	$28.8\pm7.40^{a,B}$

ESR= Erythrocyte Sedimentation Rate; Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 2= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 7. Effect of probiotics on serum glutamic pyruvate transaminase levels.

Treatments	Phase 1	Phase 2	Phase 3
Control	$52.1\pm7.62^{b,A}$	$50.5\pm7.72^{a,A}$	$30.5\pm4.42^{a,B}$
T1 (1 g/100 Kcal)	$50.5\pm7.72^{b,A}$	$46.6\pm8.54^{a,A}$	$33.9\pm5.10^{a,B}$
T2 (1.5 g/100 Kcal)	$63\pm7.13^{a,A}$	$49.8\pm6.72^{a,B}$	$30.3\pm4.34^{a,C}$

SGPT= Serum Glutamic Pyruvate Transaminase; Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 8. Effect of probiotics on serum glutamic oxaloacetic transaminase (SGOT) levels.

Treatments	Phase-I	Phase-II	Phase-III
Control	$55.9\pm8.27^{a,A}$	$49.4\pm6.51^{a,A}$	$39.4\pm7.96^{a,B}$
T1 (1g/100Kcal)	$46.6\pm7.26^{b,A}$	$43\pm6.61^{b,A}$	$39.7\pm8.85^{a,A}$
T2 (1.5g/100Kcal)	$45.1\pm11.36^{b,A}$	$46.6\pm6.16^{ab,A}$	$42.6\pm10.55^{a,A}$

SGOT= Serum Glutamic Oxaloacetic Transaminase; Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 3= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

Table 9. Effect of probiotics on alkaline phosphatase levels.

Treatments	Phase 1	Phase 2	Phase 3
Control	$269\pm54.84^{a,A}$	$233\pm46.25^{a,A}$	$220.1\pm58.61^{a,A}$
T1 (1 g/100 Kcal)	$249.2\pm95.22^{a,A}$	$227.2\pm61.85^{a,A}$	$217.7\pm69.20^{a,A}$
T2 (1.5 g/100 Kcal)	$247.9\pm78.07^{a,A}$	$245.1\pm70.79^{a,A}$	$216.3\pm64.70^{a,A}$

ALP= Alkaline Phosphatase; Phase 1= F75 means 75 kcal/100 mL of formula milk; Phase 2= F100 means 100 kcal/100 mL of formula milk; Phase 2= Plumpy'nuts 100 kcal/100 mL with iron fortification in paste form: Mean values with $P \geq 0.05$ is not significantly different; ANOVA was used to analyze the data under CRD design while DMR test was used to separate the means at probability level 0.005; Different letters in a column indicate the significant differences; Small letters indicate significant differences between doses; Capital letters indicate significant differences between phases.

riment were not effectively used by gut beneficial microbiota and, thus, stool numbers remained unchanged. The GOS existent in various products at different doses have shown to not significantly affect stool frequency (13,24) in adult human subjects. Again, Chaikham (25) indicated that GOS (7 or 15 g/day) does not affect stool composition in adults with normal bowel function. Villares et al (26) reported that infant formula-containing prebiotics changed the GI microbiota that mimics mother milk. Moreover, infants on prebiotics formula have shown better stool consistency and frequency, as was indicated in the study of Bisceglia et al (27), who showed that neonates receiving prebiotics showed a larger stools number compared to placebo. In this sense, in younger children, prebiotics at various levels seems not to affect stools frequency, thus, the hypothesis that prebiotics dose may have different effects on children's stool is rejected based on data obtained in this study. On the other side, the number of vomits per day during the 3 phases was significantly reduced ($p<0.05$) in the GOS prebiotics group when compared to controls; however, again, GOS prebiotic dose effect was not statistically different. The data obtained here are in line with the study conducted on formula milk supplementation with GOS (2.4 g/L), showing that GOS did not increase vomiting in children (28). Likely, Giovannini et al (29) showed that prebiotic-supplemented formula significantly lowered GI issues.

When looking at specific biochemical biomarkers, GOS prebiotics administration during the 3 phases significantly improved hemoglobin levels, although dose-related GOS prebiotics remained unchanged. A randomized controlled study conducted in Kenyan infants revealed that GOS prebiotic reduced iron-related adverse effects on gut microbiome and morbidity (30). As indicated by Hare et al (31), iron affects many biochemical processes, but most importantly, during the critical stages, iron plays a key role in brain development. Thus, during early stages insufficient iron uptake would lead to permanent neurodevelopmental deficits. In addition, prebiotic is reportedly increased iron absorption from a micronutrient formula (30). Therefore, it is suggested to use additional iron sources for infants, such as in the form of food fortification or food supplementation; however, an issue related to iron overdose should also be considered. Indeed, as prebiotics improve the absorption capacity of the small intestine, there is no need for further iron fortification, although prebiotics in powdered milk should be included to increase absorption by the small intestine.

Regarding white blood cells, GOS prebiotics significantly improved erythrocytes levels in last two phases, but the dose-related effect was quite insignificant throughout the treatments. Kruger et al (32) showed that α-GOS feeding to rats (90-day trial) have no adverse effects on blood hematology. In an animal study, Vendrig et al (33) documented that GOS supplementation produced no significant changes in blood parameters. In another study, it was shown that anemia decreased by ≈50% in the Fe and Fe plus GOS groups ($p<0.001$) compared to control group (30). Similarly, both hematocrit and white blood cells levels were significantly improved during the last two phases, although the doses used have no effects on blood hematocrit levels. In our studies these hematological indices have indicated marked beneficial effects of GOS prebiotics in improving blood parameters. Hoseinifar et al (34) in an animal study showed that hemoglobin, leucocyte and lymphocytes levels increased significantly in the 2% oligofructose fed fish than in the 3% oligofructose fed fish. Moreover, hematocrit % increased 2% oligofructose group than in the control group. Moreover, Ahmdifar et al (35) showed that dietary prebiotic inulin given to fish result in a significant increase in white blood cell count in the group treated with 1% inulin compared with the other groups ($p<0.05$). However, some hematologic and biochemical parameters, such as red blood cell count, mean corpuscular hemoglobin (MCH) were not changed.

Concerning to erythrocyte sedimentation rate, it was significantly decreased ($p<0.05$) in phases 2 and 3 compared to phase 1. No dose-related effect ($p>0.05$) was stated on erythrocytes sedimentation rate. Increased erythrocyte sedimentation rate is thus considered an indication of increased infection (36). Arslanoglu et al (37) reported that prebiotics addition to infant formula is related to a decreased incidence of infection. The study also showed that the ESR can be used as a significant indicator in malnourished children with acute or chronic infection. Thus, prebiotics supplementation markedly improves blood hematology and decrease the infection risk in infants.

Liver enzymes, namely SGPT and SGOT, have also been described as indicating the useful effect of prebiotics (Oligomate). In this study, results obtained show that SGPT was significantly decreased in phases 2 and 3 compared to phase 1, while SGOT level was significantly decreased only in phase 3. Dose-related effect was not significant on SGPT levels. In the absence of clinical liver disease, as in our case, we feel that the frequent rise in SGOT and SGPT is primarily owing to tissue breakdown. In case of injury, the transamination process is increased to metabolize amino acids released from the exaggerated tissue breakdown, leading to enhanced SGOT and SGPT activity. Prior studies have also highlighted this fact (38). Increased levels of SGOT and SGPT have been documented, being maximum in Protein Calorie Malnutrition (PCM) grade-I cases, possibly indicating maximum tissue breakdown in the early stages of PCM (4). This may reflect an endeavor on the part of the body in order to sustain homeostasis via protein synthesis from amino acid mobilization as well as tissue breakdown (39). The alkaline phosphatase levels were not affected in all phases at two dose levels. In the study of Ahmdifar et al (35) the dietary prebiotic inulin did not lead to significant differences regarding serum enzymes between the treatment groups ($p>0.05$). Further, with the increase in inulin level, alkaline phosphatase level decreased. On the other side, total bilirubin levels significantly increased ($p<0.05$) in phase 3 of the study when compared to phases 1 or 2. The present results are not in line with another study (27), where it was shown that neonates receiving prebiotics evidenced decreased bilirubin levels. In their study the intervention period was 28 days, which was that less than the present one (48 days); so, the difference may be related to the study duration. Regarding the GOS prebiotics effect in serum albumin levels, they significantly increased in phases 2 and 3 ($p<0.05$) compared to phase

1, as well as the prebiotics dose significantly increased serum albumin levels. Serum albumin levels have relatively long half-life (14-20 days) and a large body pool (4-5 g/kg b.w.) and slowly respond to malnutrition, thus making an early indication of protein reduction (40). In actual, almost 60% of protein is found outside the bloodstream. Meanwhile, the decline in serum concentrations during early malnutrition, which is redundant vascular albumin in the bloodstream, helps to maintain normal serum concentrations. The concentration of serum albumin does not fall in the initial stage, especially children with mild and moderate malnutrition (2). During malnutrition, hypoalbuminemia may be owing to reduced albumin synthesis. The effect of GOS prebiotics on serum electrolyte sodium levels also revealed to be promising. In all phases, sodium levels remain statistically unchanged. Prebiotics significantly decreased ($p<0.05$) sodium levels in the treated group versus control group. Similarly, potassium levels were not changed in all groups, with the exception of phase 2, where they increased significantly.

Prebiotics supplementation was not related with changes in stools frequency, thus indicating this effect may be related to the prebiotic type (specific). Prebiotics supplementation led to a reduction of number of vomits per day in the treated groups, showing prebiotics health benefits versus control groups. Likely, it increases hemoglobin, hematocrit and white blood cells levels in malnourished children, during the last two phases, and erythrocyte sedimentation rate significantly decreased in phases 2 and 3. Serum enzymes, namely SGPT, and sodium levels significantly decreased and total bilirubin levels increased in the last phases. Thus, GOS prebiotics supplementation improved blood hematology, at same time that decreased the risk of infection and the number of vomits per day in severely malnourished infants.

Conflict of interests

The authors declare no conflict of interests.

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