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Original Research

Bio-therapeutics effects of probiotic strain on the gastrointestinal health of severely acute malnourished children

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Abstract: The core objective was to evaluate the effect of probiotic fortification at three phases of formula milk administration in malnourished children. A dose related effect was determined in 30 severely acute malnourished children (6-59 months) in a double-blind, randomized design. According to the results, serum albumin levels, treatment T_2 (6 billion cfu) has significantly increased albumin levels (3.7g/dL) and the effect of phase-III (Plumpy'nut) was found to be better. Results regarding sodium levels showing probiotic-dose have significant effect ($P \leq 0.05$) in phases as well. Moreover, the effect of T_1 i.e. 3 billion cfu of probiotics has significantly reduced sodium levels (141.8mmol/L) *vs.* others and the effect of phase-II was better on reducing sodium levels. which is further confirmed in terms of reduced erythrocyte sedimentation rate levels at phase-III (29.566 *vs.* phase-II, 41.3 and phase-I, 46.533 mm/h). Conclusively, the effect of 6 billion cfu at phase-III was more effective on blood parameters.

Key words: Probiotics; Hematological parameters; Serum enzymes; Malnutrition.

Introduction

Probiotics are generally known as mono- or mixed cultures of live microorganisms, i.e. bacteria that, when given at proper amounts produce useful effects on human health. These useful effects consist of disease prevention, absorption and digestion improvement in the host (1,2,4), although more recently probiotics have emerged as medical therapies for gastrointestinal and non-gastrointestinal diseases, such as diarrhea, constipation, inflammatory bowel disease, irritable bowel syndrome, asthma, atopic dermatitis, peptic ulcer, colon cancer, and coronary heart disease and urinary tract infections (31). Human gastrointestinal (GI) tract is also known as a hidden metabolic organ that contains more than 1500 pathogenic, opportunistic, and healthful microorganisms. Among them, probiotics regulate major biological processes in the human body and have positive therapeutic impacts on some diseases and GI disorders via a broad range of mechanisms of action (32). GI problems are reportedly causing childhood morbidity and mortality throughout the world as absorption and digestion function is adversely affected. Most studies have been conducted in animal models, where the probiotics effect is investigated to a very lesser extent in young children, while specifically the role of functional foods in malnutrition is vague. Young children are more prone to malnutrition, infections and GI issues; so, the use of a specific probiotic strains is highly warranted. Although much progress has been achieved in understanding the pathogenesis of diarrheal ailments, it is still a crucial reason of global childhood deaths. The cause behind the disease is infection with pathogens occupying gut (5). This reduces the efficiency of beneficial microbiota, as gut pathogenic bacteria are in competing with beneficial gut microbiota.

Convincing evidences suggest that probiotics lower the constipation-associated risk which affect nearly ~14% of adults (6). However, there is a gap in research, as both dose-related and strain-related specific effects are not well investigated.

Probiotics are micro-nutritional assistant that beneficially affects the host functioning by modifying mucosal and systemic immunity; additionally, probiotics

enhance growth and microbial stability in the GI zone. *Lactobacillus paracasei ssp. paracasei* are non-invasive, non-pathogenic probiotics bacteria. They have shown to induce numerous epithelial cell reactions by competing with pathogenic bacteria for host hold binding sites, thus improving epithelial cell barrier function (7). They have been increasingly administered to children with the intent of decreasing the risk of acute diseases, as well as chronic diseases of childhood, such as asthma and atopic disease. The mechanisms, by which probiotics decrease inflammation include the decrease in intestinal permeability, altering intestinal microbiota, and influencing metabolism (37). Around 10 trillion microbes of 500-1000 diverse microbial species colonize the GI tract and remain in a complex equilibrium. These include *Bacteroides spp., Lactobacillus spp., Clostridium spp., Fusobacterium spp., Bifidobacterium spp., Eubacterium spp., Peptococcus spp., Peptostreptococcus spp., Escherichia spp. and Veillonella spp.* Colonization of these microbes in the human gut start at birth and eventually get exposed to foreign microbial population and antigens derived from digested foods. Therefore, the intestine acts as an interface between the host and exogenous agents, such as pathogenic bacteria, viruses, allergens. The intestinal mucosa also plays a central role in host microbiota-pathogen interactions (33). Gut microbiota influences human health through an impact on gut defense barrier, immune function, and nutrient utilization and potentially by direct signaling with GI epithelium (34). The interaction between the host microbiota and exogenous agents may disturb/alter the normal microbial balance or their activity in GI tract. Changes in such microflora is implicated in the pathogenesis of various diseases. Enteric diseases are caused by several pathogens, like *Escherichia coli, Salmonella* spp., *Shigella* spp. along with various other foodborne pathogenic strains, such as *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes* and *Vibrio cholera*. They may trigger infections in two steps; during the first step of infection process, the pathogens may attach themselves to the intestinal epithelial cells surfaces through certain adhesive receptors, like glycoproteins and glycolipids and, later on, in second step they cause direct cytotoxic injury, intracellular migration, and finally disrupt the epithelial tight junctions that lead to mucosal infection (35). Probiotics promote the GI homeostasis and stimulate the growth of indigenous beneficial gut microbiota by inhibiting pathogenic or opportunistic microbes' growth. Therefore, probiotics are recommended as alternative biotherapeutic agents for GI pathogenic infections. These may act via several mechanisms, such as producing antimicrobial compounds, competiting for nutrient substrates, competitive exclusion, enhancing intestinal barrier function and even through immunomodulation (35).

In this sense, the present study aimed to evaluate whether probiotics species and dose/levels improve GI issues in protein energy malnutrition.

Materials and Methods

Participants

Severely acute malnourished (SAM) infants aged from 6 to 59 months age (n=30) were enrolled in this study at the Nutrition Rehabilitation Center, Mayo Hospital, Lahore, Pakistan, a tertiary care large urban teaching and referral hospital. This study was approved by human ethics Institutional Review Board (IRB) of the University.

SAM was defined as weight-for-height of less than 70% of the median, nutritional edema (Kwashiorkor), or both, mid-upper arm circumference (MUAC) of less than 11.5 cm (8). Anthropometry protocols were followed by research standards (9). Three or more bowel movements per day were termed as diarrhea, with stool condition having looser than normal consistency and may contain pus, mucus or blood. The condition remains for less than 5 days, but more than 1 day was considered as diarrhea.

Interventions

Infants with diarrhea as discussed above were admitted to the hospital. Moreover, children were assigned to control group and received standard therapeutic foods (F-75, F-100, Plumpy'nuts, n=10) on daily basis for 48 days, or to the intervention group (n=20), where it was given therapeutic foods (F-75, F-100, Plumpy'nuts) in combination with probiotics containing Resiton (*L. paracasei ssp. paracasei*) procured from MakNsons Pharmaceutical Industry, Italy. Briefly, the following intervention groups were made: F-75 plus Resiton at 3 billion colony forming units (cfu)/day and F-75 plus Resiton at 6 billion cfu/day; F-100 plus Resiton at 3 billion cfu/day and F-100 plus Resiton at 6 billion cfu/day; Plumpy'nuts plus Resiton at 3 billion cfu/day and Plumpy'nuts plus Resiton at 6 billion cfu/day.

Nutritional status

Children were weighed on digital scales that were daily calibrated. Lengths and MUAC were measured using locally made height boards and MUAC insertion tapes, respectively. Treatment protocol was based on standard international World Health Organization (WHO) and Community-based Management of Acute Malnutrition program (CMAM) guidelines (10). All children in control group were initially fed F-75 (75 kcal/100 mL) therapeutic milk (Phase 1) and then progressed to F-100 (100 kcal/100 mL) (Phase 2) and Plumpy'nuts (F-100 in spread form with iron fortification) (Phase 3) for 48 days. The intervention group received therapeutic foods plus probiotics containing Resiton as mentioned above. To get better and accurate results, parents of children were advised to fill the children data in diaries on stools number and oral rehydration solution (ORS).

Biochemical analysis

For hematological and biochemical screening, 5 mL venous blood was taken after 12 h fasting in vacutainers tubes containing ethylenediamine tetra-acetic acid (EDTA). Complete blood count (CBC) was carried out using hemocytometer (11). The mechanism involves the lyses of red blood cells through glacial acetic acid, but not of 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. The cells numbers in undiluted blood were reported per microliter whole blood.

Serum albumin levels were evaluated according to the procedures (12). Bromocresol green (BCG) assay detects albumin concentration in serum. The assay is based on the selective interaction between BCG and albumin forming a chromophore that can be detected at 620 nm using spectrophotometer. The signal is directly proportional to the albumin amount present in the serum. BCG does not react with other abundant plasma proteins, like immunoglobulin (Ig)-G. The assay can detect as low as 5 µg (0.01 g/dL) of albumin in serum samples.

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

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

Determination of ESR

For ESR determination, the method of Vennapusa *et al.* (2011) was used. 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 (13). The rate at which they settled was measured as the number of millimeters of clear plasma present at the top of the column after 1 h (mm/h). serum samples were analyzed for enzymes, i.e. serum glutamic-pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT) for liver functioning (14).

Determination of alkaline phosphatase

For alkaline phosphatase determination, the following protocol was adopted. Briefly, alkaline phosphatase release phenol from *p*-nitrophenylphosphate, the phenol in alkaline medium gives yellow color which can be estimated with a 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).

$$p\text{-nitrophenylphosphate} + H_2O \longrightarrow \text{Phosphate} + p\text{-nitrophenol}$$

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]

With calibrator:

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

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

Determination of SGPT

For SGPT (ALT) determination, the protocol of Yatzidis, (1960) was used. ALT is present at high concentration in liver and to a lesser extent in kidney, heart and skeletal muscle, pancreas, spleen and lung; ALT levels are usually lower than AST levels (15). Increased ALT levels are generally a result of liver-associated disease with some degree of hepatic necrosis. ALT is an important indicator of liver disease. The series of reactions involved in the assay system are as follows: **L-Alanine + 2- oxoglutarate → Pyruvate + L-Glutamate; Pyruvate + NADH → Lactate+ NAD.** Then, the NADH consumption rate is determined photometrically, that is proportional to ALT activity in the sample.

Calculation 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]

Determination of SGOT/AST

AST is a cellular enzyme, and Yatzidis, (1960) found it at highest concentration in heart muscle, liver and of skeletal muscle cells (15).

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Glutamate} + \text{Oxalacetate}$$
$$\text{Oxalacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{Malate} + NAD^+$$

The rate of NADH concentration decrease, measured photometrically at 340 nm is proportional to the catalytic AST concentration present in the sample. It 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 sodium (Na^+) and potassium (K^+) were analyzed by electrolyte analyzer (Biolyte 2000; BioCare Corporation, Taiwan). 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 Na^+ and K^+ ions (17).

Statistical analysis

Data was analyzed through 2 factor analysis of variance (ANOVA) under complete randomized design (CRD), Steel (1997) technique by Cohort-CoStat-2003 (Software version 6.33). Means of 3 phases were compared with means of the 2 doses used (i.e. control, 3 billion cfu and 6 billion cfu) through Duncan's Multiple Range (DMR) test and significance level assumed was $p<0.05$ (17).

Results

Probiotics effect on serum albumin levels

The dose-related effect of probiotics at the 3 phases is presented in Table 1. As indicated, both dose levels and phases have a significant impact ($p<0.05$) on albumin levels. In Phase 1, all treatments showed similar albumin levels, and phase 3 findings suggest further improvements in albumin levels particularly for T_2 vs T_1. A similar trend was found in phase 3. In short, T_2 at phase 3 revealed to be better regarding albumin levels.

Effect of probiotics on number of stools

Table 2 describes the probiotics effect at 2 levels (3 and billion *cfu*) on stools number per day. This parameter was not affected by the dose level ($p>0.05$), but the number of stools per day decreased significantly between phases ($p<0.05$). On average, phase 3 has 3.33

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

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$2.69\pm0.65^{a,B}$	$3.1\pm0.64^{b,AB}$	$3.42\pm0.39^{b,A}$
T_1 (3 billion *cfu*)	$2.75\pm0.51^{a,B}$	$2.99\pm0.61^{b,B}$	$3.89\pm0.83^{ab,A}$
T_2 (6 billion *cfu*)	$3.14\pm0.88^{a,B}$	$3.7\pm0.60^{a,AB}$	$4.26\pm0.68^{a,A}$

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 difference between doses; Capital letters indicate significant difference between phases.

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

Treatments	**Phase 1**(F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$4.5\pm0.52^{b,B}$	$5.2\pm0.91^{a,A}$	$4.5\pm0.52^{a,B}$
T_1 (3 billion *cfu*)	$6.7\pm0.94^{a,A}$	$4.5\pm0.52^{b,B}$	$3.3\pm0.94^{b,C}$
T_2 (6 billion *cfu*)	$6.6\pm1.07^{a,A}$	$4.6\pm0.51^{ab,B}$	$2.2\pm1.03^{c,C}$

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 difference between doses; Capital letters indicate significant difference between phases.

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

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$152.5\pm6.27^{b,A}$	$146\pm7.19^{ab,A}$	$158.5\pm26.50^{a,A}$
T_1 (3 billion *cfu*)	$147.2\pm15.08^{b,A}$	$133.5\pm18.14^{b,A}$	$144.7\pm25.67^{ab,A}$
T_2 (6 billion *cfu*)	$169.9\pm28.11^{a,A}$	$150\pm14.41^{a,B}$	$138.4\pm8.77^{b,B}$

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 difference between doses; Capital letters indicate significant difference between phases.

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

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$4.22\pm0.34^{a,B}$	$4.63\pm0.41^{a,A}$	$4.13\pm0.38^{b,B}$
T_1 (3 billion *cfu*)	$4.06\pm0.43^{a,B}$	$4.47\pm0.31^{a,A}$	$4.47\pm0.15^{a,A}$
T_2 (6 billion *cfu*)	$4.22\pm0.38^{a,B}$	$4.6\pm0.54^{a,A}$	$4.31\pm0.26^{ab,AB}$

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 difference between doses; Capital letters indicate significant difference between phases.

stools per day, while phases 2 and 1 have, respectively, 4.77 and 5.93. Overall, the phase 3 effect was more pronounced and effective.

Effect of probiotics serum electrolyte sodium levels

The probiotics effect at 2 levels (3 and 6 billion *cfu*) on Na^+ mmol/L is presented in Table 3. Results indicated that both dose levels and phases have a significant effect ($p<0.05$) on serum Na^+ levels. Although control and T_2, i.e. 6 billion cfu of probiotics, has similar effect on Na^+ levels, control has 152.33 mm/L and T_2 152.66 mm/L, although these values were significantly higher than at T_2, i.e. 3 billion cfu of probiotics (141.8 mmol/L). In conclusion, T_1 was better in reducing Na^+ levels vs other doses. Likely, the 3 phases have significantly different Na^+ levels. Phase 1 has significantly higher Na^+ levels (156.53 mmol/L) than phase 2 (143.17 mmol/L). However, during phase 3, Na^+ level significantly increased (147.2 mmol/L) when compared to phase 2. Overall, the phase 2 effect was better.

Effect of probiotics serum electrolyte potassium levels

The probiotics effect at 2 levels (3 and 6 billion cfu) on K^+ (mmol/L) is presented in Table 4. Results indicated that dose level has no significant effect ($p>0.05$), although a different trend was stated of phase ($p<0.05$) on serum K^+ level. Regarding the phases, phase 1, 2 and 3 have different effects on K^+ levels. Phase 1 has significantly higher K^+ levels (4.17 mm/L) when compared to

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

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$10.17\pm1.69^{b,A}$	$9.79\pm1.03^{a,A}$	$10.79\pm1.24^{a,A}$
T_1 (3 billion *cfu*)	$11.48\pm1.22^{a,A}$	$10.15\pm1.73^{a,AB}$	$9.71\pm1.74^{ab,B}$
T_2 (6 billion *cfu*)	$8.99\pm1.19^{b,A}$	$8.46\pm1.01^{b,A}$	$8.62\pm1.50^{b,A}$

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 difference between doses; Capital letters indicate significant difference between phases.

Table 6. Effect of probiotics on erythrocyte sedimentation rate.

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$47.3\pm11.85^{a,A}$	$43.6\pm10.85^{ab,A}$	$27.4\pm6.63^{b,B}$
T_1 (3 billion *cfu*)	$48.8\pm6.19^{a,A}$	$45.7\pm7.31^{a,A}$	$35.3\pm10.17^{a,B}$
T_2 (6 billion *cfu*)	$43.5\pm10.31^{a,A}$	$34.6\pm12.04^{b,AB}$	$25.6\pm6.13^{b,B}$

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 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 difference between doses; Capital letters indicate significant difference between phases.

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

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$52.1\pm7.62^{b,A}$	$50.5\pm7.72^{a,A}$	$30.5\pm4.42^{b,B}$
T_1 (3 billion *cfu*)	$55.3\pm13.91^{ab,A}$	$52.4\pm12.80^{a,AB}$	$43.6\pm6.55^{a,B}$
T_2 (6 billion *cfu*)	$66.9\pm16.16^{a,A}$	$56.9\pm13.40^{a,A}$	$32.7\pm4.42^{b,B}$

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 difference between doses; Capital letters indicate significant difference between phases.

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

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3** (Plumpy'nuts)
Control	$55.9\pm8.27^{a,A}$	$49.4\pm6.51^{a,A}$	$39.4\pm7.96^{a,B}$
T_1 (3 billion *cfu*)	$51.4\pm16.36^{a,A}$	$54.7\pm15.86^{a,A}$	$37.4\pm8.78^{a,B}$
T_2 (6 billion *cfu*)	$58.4\pm16.16^{a,A}$	$54.8\pm13.31^{a,AB}$	$43.1\pm12.94^{a,B}$

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 difference between doses; Capital letters indicate significant difference between phases.

2 (4.57 mm/L). The lowest K^+ level was found in phase 3 (4.30 mmol/L).

Probiotics Effect on white blood cells

The probiotics effect at 2 levels (3 and 6 billion cfu) on leukocytes is presented in Table 5. Again, dose level significantly affected ($p<0.05$) leukocytes levels, but not phase ($p>0.05$). T_2 i.e. 6 billion probiotics administration significantly reduced leukocytes concentration (8.69 thous/µL) when compared to T_1 (3 billion, with 10.446 thous/µL leukocytes) and control (10.25 thous/µL, leukocytes). In conclusion, T_2 (6 billion cfu) was better than 3 billion cfu dose.

Probiotics effect on erythrocyte sedimentation rate

The probiotics effect at 2 levels (3 and 6 billion cfu) on erythrocyte sedimentation rate (ESR) is presented in Table 6. Both dose level and phase significantly affected ($p<0.05$) ESR (mm/h). T_2 (6 billion cfu) significantly reduced ESR (34.566 mm/h) when compared to T_1 (3 billion) and control, with ESR of, respectively 43.27 and 39.43 mm/h. In conclusion, T_2 (6 billion cfu) was better than 3 billion cfu dose and control. Similarly, phase 3 significantly reduced ESR, where it was found to be 29.57 mm/h versus phases 2 (41.30 mm/h) and 1 (46.53 mm/h). Overall, phase 3 was more effective in reducing ESR versus phases 2 and 1.

Probiotics effect on serum glutamic pyruvate transaminase (SGPT) levels

The probiotics effect at 2 levels (3 and 6 billion cfu) on SGPT (IU/L) is presented in Table 7. Both dose level and phase significantly affected ($p<0.05$) SGPT level. In T_2 (6 billion cfu) and T_1 (3 billion cfu) similar enzyme activity (52.17 and 50.43, respectively) was

Table 9. Effect of probiotics on alkaline phosphatase levels.

Treatments	**Phase 1** (F-75)	**Phase 2** (F-100)	**Phase 3**(Plumpy'nuts)
Control	269±54.84^{a,A}	233±46.25^{a,A}	220.1±58.61^{a,A}
T_1 (3 billion *cfu*)	229.5±59.44^{a,A}	221.6±47.69^{a,A}	238.2±44.06^{a,A}
T_2 (6 billion *cfu*)	244±64.12^{a,A}	217.7±50.38^{a,A}	161.4±58.59^{b,B}

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 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 difference between doses; Capital letters indicate significant difference between phases.

stated, while it was significantly lower in control group (44.37 IU/L). Likely, phase 3 significantly lower SGPT activity (35.60 IU/L) versus phases 2 (53.27 IU/L) and 1 (58.10 IU/L).

Probiotics effect on serum glutamic oxaloacetic transaminase (SGOT) levels

The probiotics effect at 2 levels (3 and 6 billion cfu) on SGOT (IU/L) is presented in Table 8. Though dose level has no significant effect on SGOT level ($p>0.05$), phase has a significant ($p<0.05$) impact. SGOT level was similar in both phase 1 (55.23 IU/L) and 2 (52.97 IU/L), but remained significantly higher than that of phase 3, where SGOT tremendously reduced to 39.97 IU/L.

Probiotics effect on alkaline phosphatase (ALP) levels

The probiotics effect at 2 levels (3 and 6 billion cfu) on ALP (IU/L) is presented in **Table 9**. Though dose level has no significant effect ($p>0.05$), phase has a significant impact ($p<0.05$) on ALP levels. The 3 phases have significantly different levels, with phase 1 of 247.50 IU/L, phase 2 of 224.10 IU/L and phase 3 of 206.57 IU/L. In conclusion, phase 3 revealed better effect when compared to other phases.

Discussion

In this study, among other aspects, the probiotics impact on blood constituents and enzymes activity was assessed. First of all, and looking at blood constituents, albumin is the most abundant protein in human blood and is highly conserved among vertebrates. It plays a pivotal physiological role in plasma osmotic pressure maintenance, vascular permeability, and transport of cholesterol, bile pigments, nitric oxide, metals, and other small molecules in the body. It also acts as a free radical scavenger of reactive oxygen and nitrogen species (ROS/RNS), triggers cell signaling processes, possesses anti-inflammatory and coagulatory effects. Results obtained to albumin levels reveal that both dose level and phase have a significant impact on this parameter, with particularly T_2 significantly increasing the albumin levels (3.7 g/dL). Similarly, the 3 phases have significantly affected the albumin levels (phase 1: 2.86 g/dL; phase 2: 3.263 g/dL and phase 3: 3.856 g/dL). In conclusion phase 3 revealed to be better. Our findings are in line with the earlier study of Reddy (2013), who showed that probiotics added to diet increase albumin levels in the treated group (18).

When looking at the probiotics' effects on stools number per day, our data revealed that it is not affected by the dose level, but decreased significantly among the different phases ($p<0.05$). On average, in phase 3 the number of stools per day was 3.33, while in phases 2 and 1 were, respectively, 4.77 and 5.93. Overall, the phase 3 effect was more pronounced and effective, but less than control. Miller *et al.* (2017) reported that supplementation with probiotics (Lactobacillus or Bifidobacterium spp) increases stool frequency (19). Suares and Ford showed significantly higher stool frequency in response to *Bifidobacterium lactis* supplementation, but not for *L. casei* (6). Thus, our findings do not show improvement in stool frequency, instead of reducing the number of stools per day in phase 3, it is indicative of an intervention strategy for managing diarrhea. Overall, the phase 3 effect was more pronounced and effective. Similarly, group of reserachers, they found that *Bifidobacterium animalis subsp lactis* and *Lactobacillus rhamnosus* had no effect on diarrhea in children with severe acute malnutrition (SAM) during hospitalization, however, probiotics lowered the number of days with diarrhea in outpatient treatment (20, 21). However, our findings are in line that of Horosheva *et al.* (2014), who reported that *Bacillus* spp. supplementation during antibiotic therapy significantly reduced the incidence of antibiotic-associated diarrhea (22).

Regarding Na^+ levels, our data shows that both dose and phase have significant effects on serum levels. Moreover, T_2 led to a significant reduction in Na^+ levels (141.8 mmol/L). Likely, the 3 phases have significantly different Na^+ levels. Overall, the phase 2 effect was better in reducing Na^+ level. These results indicate that probiotics influence ions absorption from the small intestine and that is why during phase 2 and at lower dose (i.e. 3 billion cfu) lower Na^+ levels were stated. Regarding K^+ levels, no significant effects were stated when looking at dose, but phase displayed a significant effect. Regarding phases, phases 1, 2 and 3 had significantly different effects on K^+ levels, with phase 1 revealing the higher levels (4.166 mm/L) when compared to others.

Specifically addressing leukocytes level, the dose level exerted a marked effect on leukocytes (thous/µL) but not phase. T_2 (6 billion cfu) markedly reduced leukocytes concentration (8.69 thous/µL) when compared to T_1 (3 billion cfu, with 10.446 thous/µL) and control (10.25 thous/µL). In conclusion, T_2 was better than T_1. Our findings are in line with that of Dahiya (2012), who reported that probiotics administered fish reduced leukocytes levels when compared to the control group (23). Concerning to erythrocytes levels, both dose and phase significantly affected ESR (mm/h). T_2 significantly reduced ESR (34.566 mm/h) when compared to T_1 (43.266 mm/h) and control (39.433 mm/h). In conclu-

sion, T_2 was better than T_1 dose and control. Similarly, phase 3 significantly reduced ESR (29.57 mm/h) when compared to phases 2 (41.3 mm/h) and 1 (46.533 mm/h). Overall, phase 3 was more effective in reducing ESR than phases 2 and 1. Our results are in line with that of Rahman *et al.* (2013), where they showed that ESR and serum alanine amino transaminase levels decreased significantly ($p<0.01$) in response to probiotics administration in birds (24).

When looking at enzyme levels, both dose level and phase significantly affected SGPT level. In T_2 and T_1 similar enzyme activities were stated (52.17 and 50.43, respectively) when compared to the control group (44.37 IU/L). Likely, phase 3 evidenced significantly lower SGPT activity (35.6 IU/L) than others. With regards to SGOT (IU/L) levels, though dose level had no significant effect on SGOT, phase had a significant impact. SGOT levels in both phase 1 (55.233 IU/L) and 2 (52.966 IU/L) were similar, but remained significantly higher than that of phase 3 (39.97 IU/L). The study conducted by Ahmed *et al.* (2015) reported that SGOT is not affected in response to probiotics administration, but SGPT value increased significantly ($p<0.01$) in probiotic administered chicken (25). Moreover, our findings are in line with that of Liu *et al.* (2015), who reported that probiotics supplementation significantly decreased SGOT and SGPT activities (26). Although an elevated serum AST level is not specific of hepatic disease, it is used mainly to diagnose and verify the disease course with other enzymes, like ALT and ALP. Also, it is used to control patients after myocardial infarction, in case of skeletal muscle disease and others. Finally, to ALP (IU/L), the dose level had no significant effect, but phase exerted a significant impact. The 3 phases revealed significantly different levels, where in phase 3 the ALP levels were significantly lower (206.57 IU/L). Our findings are thus matching with that of Kirpich *et al.* (2008), showing that patients treated with probiotics had significantly lower liver enzyme activity (AST and ALT activity) at the end of treatment, indicating that study duration is an important factor in this regard (27). However, there are conflicting results regarding the probiotics effect on ALP level. Wang and He (2009) investigated the probiotics effect on ALP level in shrimps (28), and showed no significant effects. Abass and Al-Qayim (2014) reported an increase in ALP level in mice whose probiotics were administered, but Khalesi *et al.* (2018) reported no changes in serum ALP levels (29,30).

Probiotics supplementation markedly affected important parameters in SAM infants. Both phase and dose exerted effects on the assessed parameters, although differently. For instance, the dose did not affect the number of stool and vomit, but phase significantly reduced, thus meaning to be a useful intervention strategy for managing diarrhea and vomiting. Regarding hemoglobin, both dose and phase were key factors affecting hemoglobin; a similar trend was also stated in red blood cells. In case of leukocytes, dose level significantly affected the cells number, but not phase, thus indicating the useful effect of probiotics supplementation to prevent infection, aspect that was further confirmed in terms of reduced ESR level. A similar beneficial trend was stated to both serum enzymes and Na^+ and K^+ ions levels. Further studies are needed to deepen knowledge on this field as also to assess the impact of distinct probiotic strains on SAM.

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Conflict of Interest

The authors declare no conflict of interest.

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