EFFECT OF ADMINISTRATION OF COMBINED ENTERAL LACTOFERRIN AND PROBIOTIC ON INVASIVE FUNGAL INFECTIONS IN PRETERM

By

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ABSTRACT

Background: Invasive fungal infections (IFI) are responsible for late-onset sepsis in neonates especially preterm, with significant morbidity and mortality. Oral bovine lactoferrin (LF) alone or with probiotics has several potentially significant health benefits and immunomodulation.

Objectives: to evaluate efficacy and safety of enterally administered bovine LF alone or in combination with probiotics in prevention of IFI in preterm in comparison to placebo.

Patients and Methods: This study is registered in clinicalTrials.gov (ClinicalTrials.gov Identifier: NCT05283278). A prospective double blinded study conducted on 80 preterm, randomly received LF (100 mg/day) (n=20), LF + (LF 100 mg/day plus probiotics 109–1010 colony forming unit) (n=20), or placebo (n=40) for 4 weeks. Blood culture for fungal infection was done at enrollment, days 7, 14, 21 and 28.

Results: There was no significant difference between LF and LF+ vs placebo as regards the initial CBC and CRP. Then, placebo showed significantly more clinical signs of sepsis vs LF and vs LF+ (P = 0.003 and 0.001, respectively). Rate of increase of milk intake (mean ±SD) was higher in both LF (18.75 ± 6.04) and LF+ (19.2 ± 6.6) vs placebo (14.00 ± 4.76) (ml/kg/day), (p = 0.0015 and p= 0.0009, respectively); and time to reach full enteral feeding (days) was shorter in both LF and LF+ vs placebo; (10.95± 4.72) and (7.4 ± 1.98) vs (14.67 ±6.61), (p = 0.03 and p < 0.001 respectively). Both LF and LF+ had less packed red blood cell (PRBC) transfusion than placebo, but only LF vs placebo reached statistical significance (P = 0.05). No reported deaths in either LF or LF+, however, placebo had 25 % deaths (P = 0.017 each). Placebo had the longest hospital stay (29.55 ± 9.87) days vs (23 ± 9.52) and (15.1 ± 2.94) in LF and LF+, respectively (P = 0.0173 and 0,0001 respectively). Placebo had significantly more fungal infection vs LF and LF+ groups (P = 0.0001 each) mainly Candida albicans followed by Candida tropicalis.

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Conclusion: *Prophylactic enteral administration of LF either alone or in combination with probiotic reduces IFI, increased hemoglobin concentration, decrease blood transfusion, LOS, and mortality in preterm.*

Key words: *Fungal infection, neonatal sepsis, lactoferrin, probiotics.*

INTRODUCTION

Invasive fungal infections (IFI) are the third most common cause responsible for late-onset sepsis in preterm in neonatal intensive care units (NICUs) after coagulase-negative staphylococci, followed by Gram-negative bacilli. In the past decade, the prevalence of IFIs has increased dramatically. Rates range 1.6-9% in very low birth weight (VLBW) neonates and 15% in extremely low birth weight (ELBW) neonates **(Hu et al., 2017)**. This can be attributed to several risk factors: immature immunity, VLBW, fungal colonization, multiple antibiotics, prolonged intensive care, central venous catheters, parenteral nutrition, and disorders in gut microecology **(Manzoni et al., 2012) (Tezer et al., 2012)**. Neonatal IFIs are associated with prolonged length of hospital stay, high morbidity and mortality, and neurodevelopmental impairment **(Hu et al., 2017)**.

Infection from different fungi including Candida, Aspergillus, Malassezia, and Blastomyces species have been documented in preterm, however, Candidemia remains the most common infection among all IFIs **(Hundalani and Pammi, 2013)**.

Specific prevention - rather than treatment - should be the optimal strategy. Fluconazole prophylaxis reduces the incidence of IFI in very preterm **(Manzoni et al., 2012) (Tezer et al., 2012)**. In spite of reassuring studies, some concerns still present related to cost, tolerability, long-term safety and modification of the fungal ecology induced by this azole with emergence of resistant strains **(Manzoni et al., 2012) (Hu et al., 2017)**. Alternative options needing more conclusive assessments include use of bovine lactoferrin (LF) or probiotics.

LF is an iron-binding glycoprotein, predominately available in mammalian milk and colostrum, found in mucosal secretions: tears, saliva, vaginal, semen, nasal, gastrointestinal, and urine. It is also found in considerable amounts in secondary neutrophil granules, plasma, and amniotic fluid. Its name was derived from being a major iron-binding protein in milk **(Pandita et al., 2015) (Giansanti et al., 2016)**.

It has been proved that LF was involved in several physiological functions, including iron absorption in the bowel; antimicrobial, anticancer, antinflammatory and antioxidant (**Giansanti et al., 2016**). Noteworthy, bovine, and human LF share a high structural homology (77%) (**Manzoni et al., 2019**).

Probiotics, live microorganisms, provide health benefits to a host when administered at adequate doses (**Hu et al., 2017**). In preterm, they were found to significantly reduce the risk of mortality, and late onset sepsis, and assist feeding tolerance (**Deshpande et al., 2017**).

Studies related to the safety of bovine LF (**Pammi and Suresh, 2020**) or probiotics (**Hu et al, 2017**) in neonates have not discovered evidence of adverse effects or intolerance.

We hypothesized that bovine LF alone or in combination with probiotics (lactobacillus acidophilus) can be used in prevention of IFI in preterm admitted to the NICU. So, this randomized interventional study aimed at evaluation of the efficacy and safety of enterally administered bovine LF alone or in combination with probiotics in prevention of IFI in preterm in comparison to placebo.

Sample size:

Sample size determination assuming a rate of IFIs ranging between 0.7% and 2.0% in placebo group and treated group respectively, sample size of 20 patients in each group is enough to detect such difference if true, at 0.05 alpha error and 0.08 power of the test.

Ethical consideration:

Funding Source: None.

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Conflict of Interest: The authors have no potential conflicts of interest to disclose.

Registration number: ClinicalTrials.gov Identifier: NCT05283278.

Parents of the newborns were provided written consent for the protocol which was approved by the Research Ethical Committee of Ain Shams University hospitals, and in accordance with the Helsinki Declaration of 1975.

Inclusion criteria:

Preterm neonates ($\leq$ 36 weeks' gestation) were included regardless their birth weight.

Exclusion criteria:

- Patients failed to enroll in the 1st 72 hours of age.

- Preterm with conditions necessitating nothing per os such as intestinal obstruction, etc.

- Patients who started prophylaxis with antifungal drugs.

- Patients with clinical congenital anomalies or suspected inborn error of metabolism.

PATIENTS AND METHODS

This was a prospective double blinded study conducted on 80 preterm neonates ($\leq$ 36 weeks' gestation) admitted to NICUs of maternity and children's hospitals, Faculty of Medicine, Ain Shams University, during the period from February 2020 till April 2021.

Study design: All studied preterm were subjected to:

A full history including maternal and perinatal history. Gestational age (GA) was calculated based on the date of last menstrual period and confirmed by using modified Ballard score (**Ballard et al., 1991**). Mode of delivery and Apgar score at 1 and 5 min were recorded. Birth anthropometric parameters were measured. All neonates underwent thorough clinical examination and received routine neonatal care according to our NICU protocol.

The studied preterm were classified into 3 groups using blindness done by aliquots covered with opaque stickers into LF, LF+ and placebo groups.

- **Group I:** Lactoferrin group (LF) consisted of 20 preterm neonates who received Lactoferrin granules in a dose of 100 mg/day (1 sachet) once daily in 2 ml of distilled water with the beginning of feeding by enteral route starting from day of enrolment for 4 weeks.

- **Group II:** LF+ group consisted of 20 preterm neonates who received lactoferrin (100 mg/ day) in combination with the probiotic; lactobacillus acidophilus, the capsule content at daily doses of 109–1010 colony forming unit (CFU) with lactoferrin were dissolved in 2 ml of distilled water, with the beginning of feeding by enteral route starting from day of enrolment for 4 weeks.

- **Group III:** Forty preterm received 2 ml distilled water once per day with the beginning of feeding by enteral route starting from day of enrolment for 4 weeks.

Systematic clinical surveillance for LF and probiotics adverse effects was performed. Regular evaluation for the neonates during admission for occurrence of clinical and laboratory sepsis. IFI was defined as occurring ≥ 4 days after birth and included clinical signs and symptoms consistent with sepsis together with isolation of a fungal causative organism from blood (drawn from peripheral sites) (**Manzoni et al., 2012**). Established diagnosis of sepsis was based on the development of clinical evidence suggestive of sepsis with Tollner's scores ≥10 (**Tollner, 1982**).

Nutritional and feeding policies were stable during the study following our NICU protocol. Administration of expressed maternal milk was encouraged. When needed, feeding supplementations were made with a premature formula. Minimal enteral feeding with small amounts of maternal milk or premature formula (10 mL/kg per day divided every 3 hours) was initiated within the first 2 days of life. Cautious volume advancements were performed by adding 10-30 mL/kg per day. Parenteral nutrition was started at admission and continued until enteral feeding reached 150 mL/kg per day.

Laboratory studies including:

- Complete blood count (CBC), using Sysmex XT-1800i (Sysmex, Kobe, Japan).

- C reactive protein (CRP), by latex agglutination test.

- Bacterial blood cultures and fungal cultures:

For all enrolled neonates blood culture for fungal infection was done at enrollment, days 7, 14, 21 and 28 using Sabouraud agar for detecting Candida species and Hichrome agar to detect other fungal types (primary outcome), we compared length of hospital stay (LOS), use and duration of inotropes, use and duration of mechanical ventilation, rate of increase of enteral feeding, time to reach full enteral intake, signs of feeding intolerance, complete blood count (CBC), C reactive protein (CRP), packed red blood cell transfusion (PRBCs) and mortality in the 3 groups as (secondary outcome).

Under strict aseptic technique we collected blood samples for bacterial blood cultures routinely withdrawn on baby's admission using conventional blood culture technique and done using BD BACTEC PEDS PLUS/F culture vials (Becton, Dickinson and Company Spark, Ireland), and

fungal cultures at enrolment, days 7, 14, 21 and 28.

Blood cultures incubated aerobically at 37°C for 2-3 days for yeast and for 2-3 weeks for fungi. Incubated media is then subcultured on Sabauroud agar (Himedia company, India®) for detecting fungal species and Hichrome candida differential agar (Himedia company, India®) to detect candida species. Sabouroud and Hichrome agars were incubated at 37°C and at room temperature and weren't discarded until 2 weeks.

Statistical analysis:

Data were collected, revised, coded, and entered Statistical Package for Social Science (IBM SPSS), version 23. Parametric quantitative data were presented as mean, standard deviation and range, while non-parametric data were presented as median and inter-quartile ranges (IQR). Qualitative variables were presented as numbers and percentages. We used chi-square test and/or Fisher exact test for comparison of qualitative data within groups. Independent t test was used to compare parametric quantitative data and Mann-Whitney for non-parametric data.

The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, p value was considered significant if <0.05 and highly significant if <0.01.

RESULTS

Our results will be demonstrated in the following tables and figures:

Table (1):   Neonatal demographic data

Demographics	LF (no=20)	LF+ (no=20)	Placebo (no=40)	P	P*
Gender, % of boys	7 (35.0%)	8 (40.0%)	19 (47.5%)	0.357	0.582
Birth weight, g Mean (±SD)	1720±490.1	1361±229.41	1502.75±386.92	0.066	0.137
Gestational age in weeks	33.75±2.00	33.45±1.47	33.05±2.50	0.28	0.512
Caesarian section, %	20 (100.0%)	20 (100.0%)	36 (90.0%)	0.147	0.147

P is LF vs placebo, P* is LF+ vs placebo

There was no significant difference between LF, LF+ and placebo groups regarding all neonatal demographic data; sex distribution, GA, birth weight, and mode of delivery **(Table 1)**.

Table (2):   Feeding characteristics in the 3 studied groups

Variable	LF (no=20)	LF+ (no=20)	Placebo (no=40)	P	P*
Breast	1 (5.0%)	2 (10.0%)	2 (5.0%)	p= 0.854	P = 0.765
Formula	8 (40.0%)	9 (45.0%)	19 (47.5%)		
Mixed	11 (55.0%)	9 (45.0%)	19 (47.5%)		
Rate of increase of milk (ml/kg/day) (mean ±SD)	18.75 ± 6.04	19.2 ± 6.6 10-33	14.00 ± 4.76	P = 0.0015	P = 0.0009
Time of full intake (days) (mean ±SD)	10.95± 4.72	7.4 ± 1.98 4-11	14.67 ±6.61	p = 0.03	P < 0.001

P is LF vs placebo, P* is LF+ vs placebo

There was no significant difference between LF and LF+ vs placebo as regards the type of feeding. However, both LF and LF+ showed highly significant rapid rate of increase of milk intake and significantly were faster to reach full enteral intake than placebo **(Table 2)**.

Table (3):   Neonatal septic score, fate, and length of hospital stay of the studied neonates

	LF (no=20)	LF+ (no=20)	Placebo (no=40)	P	P*
Clinical signs of sepsis (Tollner's scores≥10)	6 (30.0%)	4 (20.0%)	28 (70.0%)	P= 0.003	P= 0.001
Fate (n died, %)	0 (0.0%)	0 (0.0%)	5 (25.0%)	0.017	0.017
Duration of hospital stay (days) Mean ±SD	23.0 ± 9.52	15.10 ± 2.94	29.55 ± 9.87	P= 0.0173	P<0.0001

P is LF vs placebo, P* is LF+ vs placebo

The placebo had significantly more clinical signs of sepsis than LF and LF+ groups. No reported deaths in either LF or LF+, however, placebo had 25% deaths, and the placebo had the longest LOS **(Table 3)**.

Table (4):   Neonatal morbidities

	LF (no=20)	LF+ (no=20)	Placebo (no=40)	P	P*
Necrotizing enterocolitis (positive)	0 (0.0%)	1 (5.0%)	6 (15.0%)	P= 0.255	P= 0.255
Mechanical ventilation (positive)	3 (15.0%)	1 (5.0%)	16 (40.0%)	P= 0.148	P= 0.017
Use of inotropes (positive)	0 (0.0%)	0 (0.0%)	14 (35%)	P= 0.01	P= 0.01
PRBC transfusion (positive)	3 (15.0%)	6 (30%)	16 (40%)	P= 0.05	P= 0.45
Duration of mechanical ventilation (days) Mean ±SD	7 ± 3.25	5 ± 3.8	9 ± 4.4	P= 0.07	P = 0.001

P is LF vs placebo, P* is LF+ vs placebo

The placebo significantly needed more inotropic supports vs either LF or LF+. We also found that both LF and LF+ required mechanical ventilation less than placebo, though it only reached statistically significant in LF+ vs placebo. Both LF and LF+ groups had less necrotizing enterocolitis (NEC) than placebo yet did not reach statistical significance. And though both LF and LF+ groups had less PRBC transfusion than placebo, only LF vs placebo reached statistical significance **(Table 4)**.

Table (5) Fungal culture in the 3 studied groups

| Fungal culture	LF (n=20)	LF+ (n=20)	Placebo (n=40)	P	P*
Candida albicans	0	0	12	X2= 4.9, P= 0.027	X2= 4.9, P= 0.027
Candida Glabrata	0	2	2	X2= 0, P= 1.0	X2= 0.54, P= 0.46
Candida tropicalis	0	0	10	X2= 3.56, P= 0.59	X2= 3.56, P= 0.59
Candida krusei	2	2	4	X2= 0, P= 1.0	X2= 0, P= 1.0
Total positive	2	4	28	X2= 19.2, P=0.0001	X2=13.39, P=0.0001

P is LF vs placebo, P* is LF+ vs placebo

Table (5) shows that placebo had more fungal infection than either LF or LF+. For Candida albicans, there was no infection reported in both LF and LF+, versus 12 cases in placebo. For Candida Glabrata, tropicalis and krusei, they were more reported in placebo than in LF and LF+ however did not reach statistical significance.
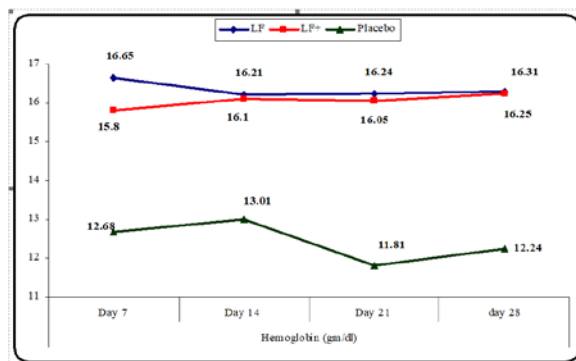
Figures (1): Comparison between the 3 studied groups as regards hemoglobin level on days 7, 14, 21 and 28
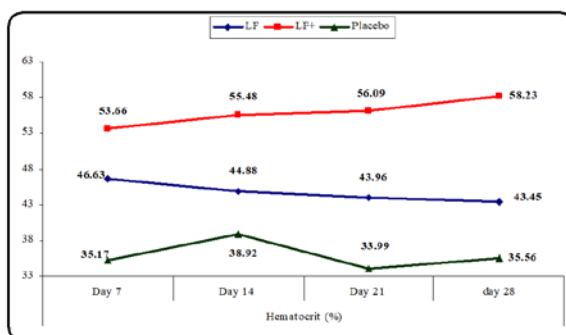
Figures (2): Comparison between the 3 studied groups as regards hematocrit level on days 7, 14, 21 and 28
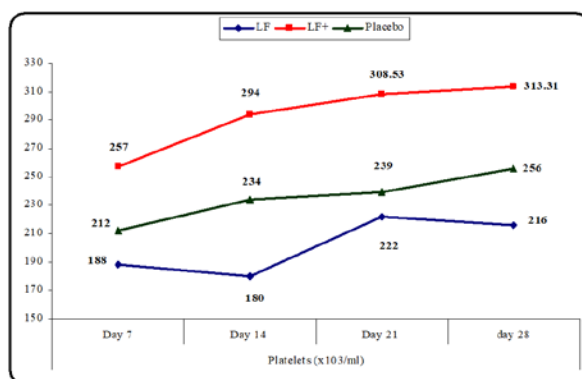
Figures (3): Comparison between the 3 studied groups as regards platelets level on days 7, 14, 21 and 28

There was no significant difference between LF and LF+ vs placebo as regards the initial CBC and CRP, however, as shown in Figures **(1, 2, and 3)** both LF and LF+ had higher hemoglobin and hematocrit levels than placebo, and that LF+ had higher platelets than placebo.

DISCUSSION

IFI is responsible for 12% of late-onset sepsis in VLBW neonates, with mortality approaches 40% and adverse outcomes occur in 35% in survivors. With such terrible results, interventions aimed at primary prevention have been sought **(Lollis and Bradshaw, 2014)**.

LF a human milk glycoprotein has many potentially significant health benefits and immunomodulation **(Manzoni, 2019)**. Probiotics promote gut barrier, immune response, and direct inhibition of gut colonization by pathogens **(Deshpande et al., 2017)**.

In the present study, we demonstrated that there was no significant difference between LF vs placebo nor LF+ vs placebo as regards type of feeding, however, both LF and LF+ showed significantly faster rate of feeding advancement, reached full enteral intake faster, additionally they had less NEC than placebo. **Manzoni et al., 2014** concluded in their study that LF supplementation alone or in combination with probiotics reduced the incidence of death and/or ≥ stage 2 NEC in VLBW neonates compared to placebo. Similarly, **Indrio et al., 2017** showed that newborns receiving probiotics had a significant decrease in the number of days needed to reach full enteral feeding (p < 0.01). Though, this contrasted with the study by **Zielonka et al., 2019** who described that preterm with gestational age of 30-33 weeks showed no advantages to routine use of probiotics as regards time to reach full intake, number of aspirates/emesis, spells /or symptoms of gastroesophageal reflux disease and secondary outcomes include incidence of NEC, bloody stools, and LOS.

Yet in their retrospective cohort study, **Al-Alaiyan et al., 2021** found that the rate of NEC but not the sepsis was less among infants who received probiotics and LF (p = 0.023) compared to controls. Prophylactic therapy with recombinant human LF and probiotic foster defenses against invasive E. coli in the neonatal small intestine in rat pups **(Sherman et al., 2004)**, and they suggested that recombinant human LF and probiotic may reduce NEC and gut-related sepsis in human preterm infants. Similarly, NEC of stage ≥ 2 occurred less frequently with LF plus probiotics vs placebo (P=0.002) but not with LF group **(Manzoni et al., 2009)**.

In the present study we found that both LF and LF+ had higher hemoglobin and hematocrit levels

than placebo and that LF+ had higher platelets than placebo on day 7, 14, 21 and 28 of life. This was comparable to a study by **Ke et al., 2015** who declared that infants aged 4 to 6 months in LF group had significantly higher hemoglobin and serum ferritin, than those infants in control group after 3 months of intervention.

There were no significant differences between both LF and placebo groups as regards serum ferritin, Hb, hematocrit, mean corpuscular volume, red cell distribution width, platelet count, and total leukocytic count on day 7 (**El Barbary et al., 2018**), however, they found that there was statistically significant higher serum ferritin, Hb, hematocrit, and mean corpuscular volume, and lower red cell distribution width and total leukocytic count in the LF group than the placebo group on day 30.

Thrombocytopenia occurs in half of neonates with proven sepsis (**Ree et al., 2017**). Platelets have many receptors where inflammatory molecules can bind. During severe sepsis circulating cytokines will hyperactivate platelets, causing thrombocytopenia (**Kell et al., 2020**).

In the LF group platelet count were significantly increased

(249.8±68.5 vs. 173.1±80.9×1000/mm3) on day 30 of life compared to day 7 (**El Barbary et al., 2018**). Similarly, in COVID-19 infection, LF showed role in not only sequestering iron and inflammatory molecules that severely expand during the cytokine storm, but also in occupying receptors where these inflammatory molecules may bind. Furthermore, it may assist in preventing thrombocytopenia, and hypercoagulation (**Kell et al., 2020**).

The placebo had more clinical signs of sepsis, needed more inotropic supports and mechanical ventilation vs LF and vs LF+ groups. Also, we found that there were no reported deaths in both LF and LF+ groups however, in placebo group there was 5 (25%) deaths, also both LF and LF+ needed shorter LOS than placebo. This was comparable to **Indrio et al., 2017**, as they declared that newborns receiving probiotics had a significant reduction in LOS (p < 0.01), and days of antibiotic treatment (p < 0.01).

In one meta-analysis of 13 randomized controlled trials (RCT) involving 1,969 patients including neonates, pediatrics, adults, and geriatrics, it was found that probiotics significantly decreased risk of ventilator-

associated pneumonia in mechanically ventilated patients (**Weng et al., 2017**). Also, Manzoni et, 2009 found that both LF and LF + probiotic groups had less LOS vs placebo (P=0.002 and P<0.001, respectively), and less reported mortality in the 2 treatment groups vs placebo (P=0.008 and P=0.04, respectively).

However, in contrast to our results, a meta-analysis that included nine RCTs, indicated that enteral LF was not associated with lowering in LOS in all neonates. However, only in subgroup analysis could demonstrate that enteral LF significantly decreased the incidence of LOS in VLBW and ELBW neonates. LF supplementation did not reduce the incidence of NEC stage II or III, bronchopulmonary dysplasia, retinopathy of prematurity, IFI, intraventricular hemorrhage, urinary tract infection, or mortality compared with placebo (**Gao et al., 2020**).

In the present study, we reported that placebo had more fungal infection vs LF and LF+ groups, and that for Candida albicans, there was no infection reported in both LF and LF+, while 12/40 cases were reported in placebo. As regards Candida Glabrata, tropicalis and krusei,

they were more reported in the placebo than in LF and LF+.

Manzoni et al., 2012, in their study showed that neonates with high intensity of colonization (> 3 different sites concomitantly colonized) was lower in LF or LF+ probiotic, but the difference was not significant (P = 0.10). The overall incidence of IFI was lower in both LF and LF plus probiotic groups compared with placebo (P = 0.002 and 0.02, respectively). The rate of progression from colonization to infection was significantly lower in both LF groups (P= 0.001 and P = 0.02 respectively). In LF groups, IFIs were less frequent in infants < 1500 g. Also, comparable to our study, **Manzoni et al., 2012** found that Candida albicans was the most frequent Candida. And due to the small numbers of isolates, only Candida albicans was possible to reveal a significant difference between groups, with 60% and 80% reduction in the treatment groups.

LF has a specific antifungal action through binding to cell wall's receptor and its disturbance (**Lupetti et al., 2003**). It could also modify the growth and development of the nascent enterocytes, promoting gut barrier thus blocking translocation of colonizing fungal colonies from the gut to the blood (**Buccigrossi**

et al., 2007) (Manzoni et al., 2012). Probiotics have been shown to be able to prevent enteric fungal colonization, thus a synergistic activity with LF can enhance such effect and further lower the rate of colonization and possibly infection in the LF + group **(Romeo et al., 2011)**.

No intolerances or adverse effects to bovine LF with/ without probiotics were recorded in the current study.

CONCLUSIONS

We concluded from our study that prophylactic enteral administration of LF either alone or in combination with probiotic has positive impact on reduction of IFI, increased hemoglobin concentration, decrease frequency of blood transfusion, LOS, and mortality rate in preterm.

RECOMMENDATION

Routine supplementation of preterm with LF either alone or in combination with probiotics to reduce IFI. Also, studies on larger patient samples are recommended and should assess whether different types of feeding: breastfeeding, formula or mixed may influence the outcome of preterm patients supplemented by either LF alone or in combination of probiotics vs placebo group.

LIMITATIONS

We did not stratify patients according to the type of feeding, small sample size and difficulty of follow up of studied neonates after discharge for the period of the study.

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