Original Article

Lactobacillus intake for 60 days favors antioxidant status of human breast milk: an RCT

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Background and Objectives: This study aimed to evaluate the effects of lactobacillus supplementation on trends of breast milk antioxidant parameters. **Methods and Study Design:** In an interventional study, 50 lactating women were randomly allocated to receive a daily supplement of lactobacillus (n=25) or a placebo (n=25) for 60 days. Daily dietary intake, anthropometric measures and breast milk antioxidant parameters were determined at the onset, and days 30 and 60 of the study. Repeated-measures ANOVA were performed to assess the change over time in the anthropometric and biochemical parameters between the two groups. The main effect of treatment was compared by using Bonferroni's procedure for CI adjustment. The significance level was set at $p<0.05$. **Results:** There was a significant increase in breast milk total antioxidant capacity (TAC) between onset of study and day 30 ($p=0.01$) and day 60 ($p=0.001$) after lactobacillus supplementation; however, breast milk TAC level did not change significantly between days 30 and 60 ($p=0.7$). In the placebo group, breast milk TAC levels decreased significantly after 60 days ($p=0.001$). Breast milk malondialdehyde (MDA) levels decreased progressively during the study in the lactobacillus group ($p=0.001$); however, there was a significant increase in MDA with time in breast milk samples in the placebo group ($p=0.015$). **Conclusions:** Based on the findings, lactobacillus supplementation for 60 days could significantly increase breast milk TAC and decrease breast milk MDA levels, compared with baseline; however, further studies are needed to confirm these results.

Key Words: lactobacillus, breast milk, total antioxidant capacity (TAC), malondialdehyde (MDA), trend

INTRODUCTION

Human milk has been considered as a package of essential nutrients (vitamins, minerals, essential amino acids and essential fatty acids) and it is commonly known as the best kind of nutrition for neonates and infants for the first six months of life.[1] In addition, human milk has bioactive components that protect newborns from a hyperoxic challenge due to transition of life to an environment far richer in oxygen than intrauterine environment.[2,3] Previous studies showed that breast milk contains hydrophilic as well as lipophilic antioxidants, including certain vitamins and a whole range of antioxidant enzymes.[4]

Similar to other components, the antioxidant status of breast milk seems to be affected by the maternal diet and antioxidant status, which in turn, could influence the antioxidant status of the breast-fed infants.[5,6]

The TAC of breastmilk decreases with the passage of days after birth and it has been proposed that proper nutritional interventions may be helpful to prevent the TAC in breast milk from decreasing with time.[7,8]

The results of previous studies showed that administration of synbiotics or probiotics was beneficial in improving the TAC level of human fluids including breast milk.[9] The results of previous studies raise the question of whether duration of synbiotic or probiotic supplementation may lead to different outcomes in the case of breast milk oxidative status. No studies exist addressing the issue of optimal duration of probiotic supplementation which could affect breast milk anti-oxidant status. So, this study was designed to evaluate the effect of lactobacillus supplementation on trends of breast milk TAC and MDA levels.

METHODS

This double-blind and placebo-controlled trial was conducted in urban areas of Tabriz city in Iran. In this clinical trial, 50 lactating women who were admitted for their infants' vaccination were selected from urban health centers. Mothers were included if they had child who was full term with a normal birth weight between 2500 and

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4000 g and they had exclusively breastfed their infants for 90 days.

The exclusion criteria for mothers and infants were having clinical evidence of chronic illness or gastrointestinal disorders, introducing complementary foods, or if they had received trace elements, antioxidants, probiotic or synbiotic supplements, antibiotics and corticosteroids in the month preceding the recruitment.

The study protocol was approved by the Ethics Committee of Tabriz University of Medical Science and registered in the registration center for clinical trials in Iran (Code: IRCT201110181197N12). The research complied with the principles of the Declaration of Helsinki. All participants were informed about the content and written informed consent provided before the study.

The volunteer mothers were randomly divided into lactobacillus or a placebo (rice starch) group. Each lactobacillus capsule contained (1×10^8 Colony-Forming Unit (CFU)) of freeze-dried *Lactobacillus acidophilus* PXN 35, *Lactobacillus casei* PXN 37, *Lactobacillus bulgaricus* PXN 39, *Lactobacillus rhamnosus* PXN 54 (Protexin, Probiotics International Ltd, Somerset, UK).

During the intervention period, the experimental group was supplemented with two lactobacillus capsules/day, which contained lactobacillus strains (2.0×10^8 CFU), 30 mins after dinner for 60 days.

Participants were also asked to bring the medication containers, and compliance was double checked by counting unused capsules.

Supplements divided between participants in accordance with the allocation code after randomization. Lactating mothers and those involved in enrolling participants, administering interventions, evaluating outcomes, and analyzing data were blind to group assignments.

One mother in the supplemented group and 2 in the placebo group did not complete the study because of having gastrointestinal disturbances, or antibiotic treatment of mothers or infants.

Data collection from mothers and infants

Demographic data (i.e. age), and clinical data (i.e. health status) were obtained through interview at the onset, 30 and 60 days after the intervention. Dietary intakes were evaluated using a 24 h recall method for 2 days and food record questionnaire for 1 day at the onset, and at 30 and 60 days of the study. Dietary intakes were analyzed using the nutritionist 4 software program.

Measurements were conducted using a calibrated weighing scale, wall-mounted stadiometer, and tape measure. The results were recorded to the nearest 0.1 kg, 0.005 m. Maternal and infants' anthropometric indices were measured at the onset and end of the study. The body mass index (BMI) for mothers was calculated as weight (kg) divided by height $(m)^2$.

Breast milk analysis

Breast milk samples (10 mL) were collected at the onset, 30 and 60 days of the study by self-expression before the baby was nursed in the morning and stored at -70°C until analysis.

Before taking measurements, defatted milk was prepared through a double centrifugal process and used for taking the measurements.

Breast milk TAC was measured using a Randox (Crumlin, County Antrim, United Kingdom) total antioxidant status kit. MDA levels were determined by the thiobarbituric acid reaction with acid, which was extracted with n-butanol and measured spectrophotometrically at a wavelength of 523 nm and compared with a standard curve.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0, and the descriptive data were reported as means and standard deviations (SDs). Normal distribution of data was verified with the Kolmogorov-Smirnov test. Repeated-measures ANOVA, with group as a factor, was performed to assess the change over time in the anthropometric and biochemical parameters between the two groups. Repeated-measures ANOVA was then used to assess for significant differences between the various time points in the subjects of both groups independently. Greenhouse-Geisser epsilon values were used in all analyses. The main effect of treatment was compared by using Bonferroni's procedure for CI adjustment. The significance level was set at $p<0.05$.

RESULTS

The mean maternal age and BMI were 27.2±4.3 $kg/(m)^2$ and 28.4±4 $kg/(m)^2$, respectively. The two groups were similar with respect to maternal age, type of delivery, education, gender and birth weight at baseline ($p>0.05$).

Table 1 represents the trends of maternal BMI, daily intake of energy, macronutrients, and micronutrients in lactobacillus and placebo groups. There was no significant difference in the baseline measures between the supplemented and the placebo group.

In contrast to the placebo group, a significant increase was observed in daily mean energy intake, energy intake from lipid, carbohydrate and protein in the lactobacillus group during the study. The repeated-measures ANOVA showed that for the daily mean energy intake, energy intake from lipid, carbohydrate and protein measurements, time × treatment interactions were all significant ($p<0.05$). However, maternal vitamin A, E, and C, zinc, and selenium intake did not change significantly in the two groups ($p>0.05$).

The mean TAC and MDA of the breast milk in all subjects at baseline were 0.3±0.13 mmol/L and 1.68±0.63 μmol/L, respectively, and there were no significant differences in the baseline measures between the two groups. Graphic trends of breast milk TAC concentration for lactobacillus and placebo group at each time are shown in Figure 1. A significant time trend was observed in breast milk TAC levels in the lactobacillus ($p=0.002$) and placebo ($p=0.001$) groups. Also, there was a significant time × treatment interaction in breast milk TAC concentration ($p=0.001$). By applying Bonferroni's multiple comparison test, a statistically significant increase was observed in breast milk TAC between onset of study and 30 ($p=0.01$) and 60 ($p=0.001$) days after lactobacillus supplementation; however, breast milk TAC level did not change significantly between days 30 and 60 ($p=0.7$). In the placebo group, breast milk TAC levels decreased significantly

Table 1. The trends of maternal BMI, daily intake of energy, macronutrients, and micronutrients in lactobacillus and placebo groups.

	Lactobacillus group (mean±SEM)				Placebo group (mean±SEM)			
	Onset	30 days	60 days	*p*-value	Onset	30 days	60 days	*p*-value
BMI (mean±SD)[†]	28.4±4.42 [a]	28.4±4.36 [b]	28.8±4.38[c]	0.02	28.4±3.71	28.4±3.64	28.3±3.68	0.13
Energy (kcal)[‡]	2119.6±80 [a]	2237.3±85[b]	2380.6±93[c]	0.041	2210.9±83	2127±76	2090.8±75	0.3
Carbohydrate (kcal)[§]	1430.2±18.4 [a]	1456±18.4[b]	1490.3±20.5[c]	0.044	1434±14	1410.2±15.4	1399.7±13.7	0.24
Protein (kcal)[¶]	245±15.2[a]	251.4±16.6 [b]	273±19[c]	0.044	249.3±16	238±14.6	233.5±15	0.27
Lipid (kcal)[††]	444±19.9 [a]	523.3±25.8 [b]	617±29[c]	0.033	527.2±24	479±19.6	457.4±22	0.35
Vitamin A (µg)	516±92.5	625.8±102	660.1±110.5	0.53	459.7±88	400±73.6	315±54.9	0.33
Vitamin E (mg)	8.3±1.35	8.25±1.34	8.7±1.9	0.9	9.43±1.6	9.2±1.5	9.15±1.4	0.88
Vitamin C (mg)	100.9±14	128.2±19	135.6±22	0.16	100.6±16	100.9±14.6	96.3±14.1	0.7
Zinc (mg)	7.59±2	8.6±2.1	9.8±2.1	0.08	8.7±2.2	7.8±1.9	7.7±1.9	0.39
Selenium (mg)	0.074±0.006	0.084±0.007	0.093±0.007	0.27	0.078±0.006	0.008±0.008	0.07±0.006	0.44

[‡](Repeated-measures ANOVA), ab (p=0.3), bc (p=0.1), ac (p=0.03).
[§](Repeated-measures ANOVA), ab (p=0.5), bc (p=0.05), ac (p=0.04).
[¶](Repeated-measures ANOVA), ab (p=0.3), bc (p=0.08), ac (p=0.04).
[††](Repeated-measures ANOVA), ab (p=0.1), bc (p=0.07), ac (p=0.03).

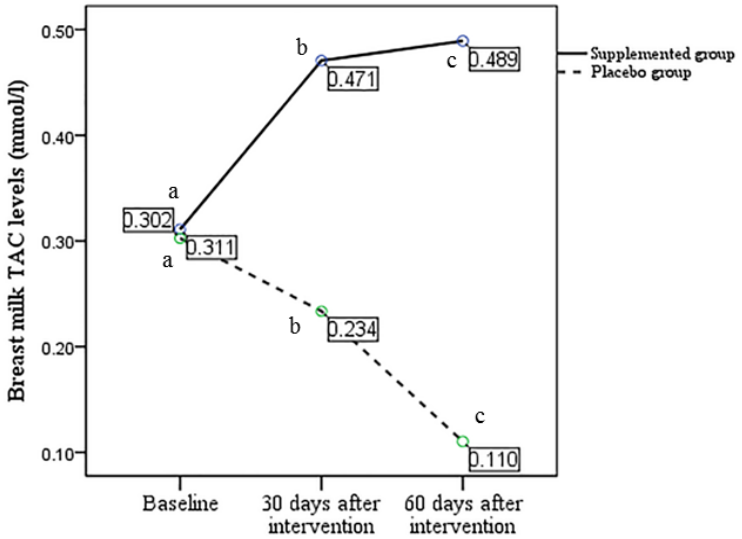

Figure 1. Graphic trends of breast milk TAC concentration for supplemented and placebo groups during the study. Supplemented group (Repeated-measures ANOVA), ab (p=0.01), bc (p=0.7), ac (p=0.001). Placebo group (Repeated-measures ANOVA), ab (p=0.1), bc (p=0.21), ac (p=0.001).

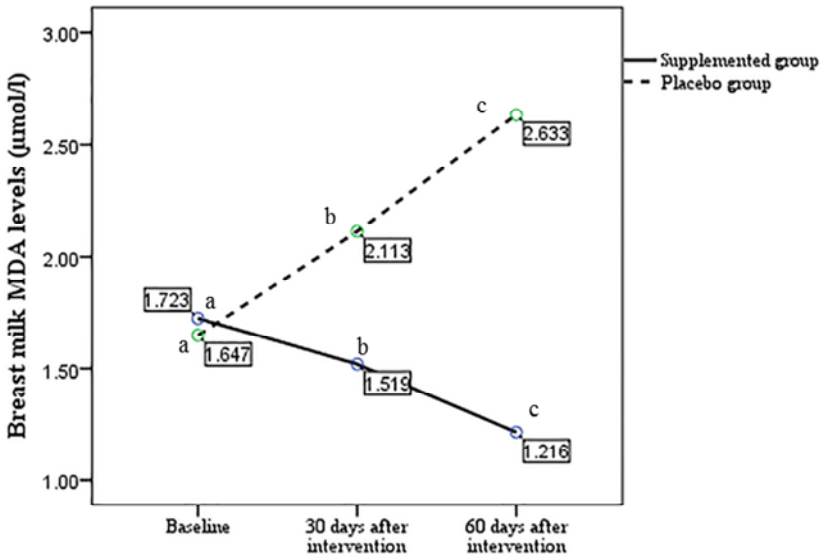

Figure 2. Graphic trends of breast milk MDA concentration for supplemented and placebo groups during the study. Supplemented group (Repeated-measures ANOVA), ab (p=0.16), bc (p=0.01), ac (p=0.001). Placebo group (Repeated-measures ANOVA), ab (p=0.27), bc (p=0.43), ac (p=0.03).

between onset and 60 days after study (p=0.001).

Figure 2 depicts the trends of breast milk MDA concentration for lactobacillus and placebo group at each time. The breast milk MDA levels decreased progressively with time of study in subjects assigned lactobacillus (p=0.001); however, there was a trend toward an increase in MDA with time in breast milk samples in the placebo group (p=0.015). Also, there was a significant time × treatment interaction in breast milk MDA concentration (p=0.001).

Bonferroni's multiple comparison test showed statistically significant decrease in breast milk MDA level between onset and 60 days (p=0.01), and 30 and 60 days (p=0.001) after lactobacillus supplementation; however, breast milk MDA level did not decrease significantly between onset of study and day 30. In the placebo group, breast milk MDA level increased significantly between onset and 60 days after the study (p=0.03).

DISCUSSION

In this study, the mean breast milk TAC level in all subjects at baseline was 0.3±0.13 mmol/L, which was lower than reported values from Iranian (0.314±0.17 mmol/L), Japanese (3.8 mmol/L) and Nigerian (1.1 mmol/L) human milk samples.[7,9,10]

The influence of human milk on oxidative stress intensity in breast-fed neonates and infants is a significant issue. The concentration of antioxidants in milk depends on mother's diet, vitamin supplementation during pregnancy and lactation and geographical area of domicile.[11]

Based on the results of this study, TAC levels of breast milk significantly decreased with the passage of time in the placebo group which can be a natural result of decline in antioxidant storage of the mothers. Similar to the previous study, lactobacillus supplementation resulted in a significant increase in breast milk TAC levels after 30 days of supplementation.[9] On the other hand, in this study, breast milk TAC did not change significantly between 30 and 60 days of supplementation. Since, in this study, daily intake of antioxidant micronutrients did not change over time, for achieving better results, higher dosage of bacterial strains, longer duration of supplementation or accompanying probiotic with other antioxidant supplements may be required.

Wabel et al reported increased glutathione levels after supplementation with synbiotic-fermented milk against lead acetate toxicity in rats.[12] Lin et al also reported that probiotic preparations have a potential to scavenge the free radicals and release their antioxidative constituents.[13] In a study by Songisepp et al, a significant improvement of TAC was seen with the daily consumption of fermented goat milk containing L. fermentum ME-3 in healthy persons after 3 weeks.[14] Kullisaar et al also reported that goat milk fermented by L. fermentum ME-3 increased total antioxidative activity and decreased lipid peroxidation markers in healthy persons.[15]

Improvement of oxidative stress by probiotic bacteria might result from their effects on short chain fatty acid (SCFA) production, in particular butyrate, in the gut. Butyrate leads to NADPH provision for synthesis of glutathione (GSH), apoptosis induction and up-regulation of oxidative pentose pathway activity.[16] Furthermore, the effect of synbiotics on pro-inflammatory cytokines as well as on down-regulation of genes involved in oxidative stress and Toll-like receptor (TLR) pathways might provide some reasons for their effect on circulating GSH levels.[17] In addition, the expression of high levels of antioxidant enzymes by probiotic strains may be another important mechanism.[18]

In the previous study, 30 days of synbiotic supplementation did not affect breast milk MDA level.[9] The results of this study showed that breast milk MDA levels decreased progressively with time of study in subjects assigned lactobacillus (p=0.001) and there was a significant time × treatment interaction in breast milk MDA concentration (p=0.001). Breast milk MDA levels decreased significantly between 30 and 60 days of supplementation which suggests that continuing supplementation had a valuable effect on breast milk MDA level. The results of this study were similar to the results of study conducted

by Abdel-Rahm et al who reported that probiotic supplementation resulted in a significant decrease in MDA levels in broilers.[19] In another study, MDA level decreased significantly by lactobacillus supplementation in rats.[20] Our results may be due to antioxidative activity of probiotic bacteria and their ability to inhibit enzymatic or non-enzymatic lipid peroxidation after 60 days of supplementation.

This study suffered from a number of issues and the results should be interpreted considering the limitations of the study. In this study, bacterial colonization of maternal gut, microbiota of breast milk samples and serum levels of antioxidant factors were not been analyzed due to the limited funds.

Conclusions

As far as we know, this is the first study reporting the effect of duration of lactobacillus supplementation on trends of antioxidant status. This study demonstrated that 60 days of lactobacillus supplementation could significantly increase breast milk TAC and decrease breast milk MDA levels compared with baseline. However, breast milk TAC did not change significantly between 30 and 60 days of supplementation which require further research using higher dosages of bacteria or longer duration of supplementation. In addition, more studies are needed to clarify the effect of different doses of synbiotics or probiotics on levels of TAC and MDA in breast milk and their relationship with maternal blood levels.

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AUTHOR DISCLOSURES

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