Original Article

Decreasing retinol and α -tocopherol concentrations in human milk and infant formula using varied bottle systems

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Abstract

Expressing human milk has become a more common alternative for mothers, as the average work demand has increased. As more mothers must work, bottle feeding trends are increasingly common. The handling and storage of human milk introduce the risk of degradation to expressed human milk and infant formula. In following a 20-minute simulated feeding, Vitamin C has been found to degrade. Vitamin C acts as an anti-oxidant and is responsible for shielding other nutrients from oxidation, such as retinol and alpha-tocopherol. By analyzing a 20-minute simulated feeding, retinol and alpha-tocopherol each displayed decreases over time significantly different than that of the Control, which was milk not exposed to bottle feeding. In human milk, retinol showed as high as a 9.5% decrease compared to the Control. Similar trends were seen with the infant formula samples. The correlation between degradation and bottle feeding systems was dependent upon the formation of bubbles in the milk as the milk was removed from the bottle. The analysis indicated a decrease of up to 12%, as seen in retinol, and 35%, as seen in alpha-tocopherol. These decreases in retinol and alpha-tocopherol should be considered when using a bottle feeding system to deliver either human milk or formula to an infant. More research is necessary to determine the effect of this decrease on the nutritional status of infants, particularly premature infants, who are at higher risk for nutrient deficiencies.

Keywords: vitamin A, vitamin E, breastfeeding, nutrition, breast milk.

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Introduction

The expression of human milk (HM) for later use in infant feeding is on the increase, in part due to the economic activities of lactating mothers. According to the International Comparison of Annual Labor Force Statistics, recent data from developed countries such as the United States of America, Australia, Canada, France, Germany, Italy, Japan, the Netherlands, Sweden and the United Kingdom indicated that an average of 55% of women worked outside the home.

The majority of those working women are of child-bearing age (Bureau of Labor Statistics 2010). In order to provide milk for their infants, lactating women who work outside the home often express their milk, and a bottle system is then used to deliver the milk to their infants (Bins *et al.* 2006). While breastfeeding is consistently recommended as the best choice for infant nutrition, the expression, storage and subsequent provision of HM to infants have not been well studied from a perspective of nutrient delivery.

Retinol (Vitamin A) is required for vision, cell differentiation and immune response. Research reports of children with clinical signs of Vitamin A deficiency indicate that those children have delayed growth and stunting (Wasantwisut 1997). Vitamin A deficiency is also associated with increased morbidity and mortality among infants and children (Lindmark-Mansson & Akesson 2000). α -Tocopherol (Vitamin E) is important for reducing oxidation of lipid membranes during rapid growth (Bell & Filer 1981). α -Tocopherol can interact with lipid-based free radicals to interrupt the peroxidation cycle forming a tocopherol free radical which can be reduced to its antioxidant form by other antioxidants such as ascorbic acid (Turoli *et al.* 2001). Antioxidants – such as ascorbic acid and α -tocopherol – present in milk can minimize nutritional damage caused by oxidation (Aguayo & Baker 2005).

Past recommendations for HM handling and storing have typically been made to prevent bacterial growth (Igumbor *et al.* 2000; Silverstre *et al.* 2006) rather than with concern for nutritional maintenance. Handling and storage of milk (Fenaille *et al.* 2005) and infant formula introduce the possibility of degradation of sensitive nutrients (Rodríguez-Alcalá *et al.* 2007). Some nutrients of interest – retinol (Vitamin A) and α -tocopherol (Vitamin E) – are found in HM and added to infant formula. Vitamin C, or ascorbic acid, is an essential nutrient that protects other nutrients from degradation. Gliguem and Birlouez-Aragon (2005) reported that the oxygen in the headspace of the packaging was associated with the degradation of ascorbic acid in stored milk. Francis *et al.* (2008) reported that ascorbic acid decreased during a simulated feeding, which may also be associated with decreases in retinol and α -tocopherol. Do vitamins A and E decrease in milk drawn from a bottle as that milk is removed and replaced with ambient air?

The purpose of this study was to investigate the effect of the milk delivery system on the concentration of retinol and α -tocopherol in expressed HM and infant formula with commonly used bottles. The objectives of this experiment were: (1) to determine if there was a nutrient loss during a simulated feeding and (2) to determine if there was a difference in nutrient loss between delivery systems.

Materials and methods

Bottles

The Avent® Natural [(AN), Philips, Andover, MA, USA], the BornFree™ [(BF), Boca Raton, FL, USA] the Dr. Brown's Natural Flow® [(DB), Handicraft Company, St. Louis, MO, USA] the Evenflo® Elan [(EL), Evenflo Company Inc., Piqua, OH, USA] Gerber® Classic [(G), Gerber Resource Centre, Fremont, MI, USA] the Playtex Drop-Ins® [(PD), Playtex Consumer Affairs, Neenah, WI, USA] and the Playtex Ventaire™ [(PV), Playtex Consumer Affairs] bottles were used for comparisons. These seven commonly used bottle systems were compared based on type, size and shape. The Control bottle was a glass screw-top bottle of similar shape and size to the test bottles. The bottles varied in size, shape and design. EL and PV had removable screw-off bottoms with a diaphragm designed, according to the manufacturers, to prevent bubbles from forming in the milk. The BF bottle had a small venting system inside the collar of the nipple which, according to the manufacturer, aids in preventing colic. The same or similar types of bottles are available worldwide.

One hundred millilitres of milk was measured in a graduated cylinder and placed in each bottle. The diameter of each bottle was measured in millimetres

Key messages

- The expression of human milk for later use in infant feeding has increased in recent years.
- Past recommendations for human milk handling and storing have typically been based on bacteriology considerations.
- The milk delivery system impacts upon the concentrations of retinol and α -tocopherol in human milk and in artificial baby milk.
- Health problems can be prevented by ensuring adequate nutrient delivery.

at the level of the 100 mL of milk (the Milk-to-Air Interface). The Milk-to-Air Interface surface area was calculated using πr^2 based on half of the diameter measurement. Total volume was calculated by measuring the capacity of each bottle when filled to the top with water, which was measured using a graduated cylinder. Bubble Formation was determined visually with any bubbles forming on the Milk-to-Air Interface and recorded as 1 for Yes and a 0 for No. Headspace was calculated using the Total Volume minus 100 mL of fluid present in the bottle, which was the volume used for testing after the removal of the baseline samples.

Samples

Mature HM donated by anonymous lactating volunteers was used for the expressed HM samples. The milk was pooled and stored at -80°C . Twenty-four hours prior to testing, the pooled milk was thawed in a water bath under dim lighting and mixed using a magnetic stirring rod. Triplicate samples were taken from the centre of the fluid sample using a pipette and aliquotted into amber vials. The samples were analysed for α -tocopherol and retinol concentration. On the day of testing, based on the mean value of retinol and α -tocopherol from the pooled milk samples, the HM was standardized to 400 mcg per litre of retinol acetate and 3 mg of α -tocopherol per litre using standard solutions (Heird 1999). An infant formula with iron (Fe) (Similac with iron, Abbot Nutrition, Columbus, OH, USA) and an infant formula with docosahexaenoic acid (DHA) (Enfamil Lipil, Mead Johnson Nutrition, Glenview, IL, USA) were used for the formula samples, both of which are commercially available. The powdered infant formula was reconstituted as per the manufacturer's instructions using warm de-ionized water (40°C) and mixed for 5 min using a stirring rod to minimize introduction of ambient air into the liquid. One hundred and twenty-five millilitres were placed in the test bottles. Twenty-five millilitres was removed via pipette directly from the bottles and aliquotted for analysis at baseline. After the samples were collected the tubes were flushed with nitrogen, flash frozen, and then stored in the -80°C freezer until analysed. During collection

and testing all samples were held under refrigeration and samples were stored in containers wrapped with foil to protect the samples from light.

After baseline samples were collected, the milk was removed from each bottle system using an electric pump to simulate the infant suckling with a delivery rate of 5 mL min^{-1} . For the Control bottle, the milk was removed carefully via pipette at each time point without being subjected to a simulated feeding, disturbing the milk as little as possible. Each experiment was completed in triplicate. The fluids removed from the bottles included five groups of 25-mL aliquots at baseline, 5, 10, 15 and 20 min. During collection of the milk samples, bubble formation (movement of air through the milk) was observed and recorded as 'yes' or 'no'. Bubble Formation on the surface of the milk marked the movement of air through the milk.

The bottles were tested in alphabetical order, completing the individual triplicate experiments within 4 days. Each collected fraction was then analysed for retinol and α -tocopherol.

High-performance liquid chromatography analysis of vitamins

Retinol and α -tocopherol were evaluated using normal-phase high-performance liquid chromatography (HPLC)–diode array detection (Chavez-Servin *et al.* 2006). Each aliquot was divided into three samples of 1 mL each, which was then transferred into centrifuge tubes. Three millilitres of absolute ethanol was added and the sample was shaken mechanically for 3 min, then 1 mL of hexane was added and the whole shaken for 1 min. The samples were left to stand for 5 min, after which 3 mL of saturated NaCl were added to aid solvent separation. The mixture was then manually shaken by inversion. The tubes were centrifuged for 5 min at 3000 rpm at room temperature. The hexane phase was recovered then directly filtered through a $0.22\text{-}\mu\text{m}$ nylon filter and collected in a 1-mL amber glass vial. The sample vials were stored at -20°C until analysed. Samples were thawed at room temperature in the dark. Twenty microlitres were injected into the HPLC system with a standard repeated as every 36th sample. The amount of retinol and α -tocopherol

Table 1. Characteristics of bottle systems tested: diameter; headspace, milk-to-air interface, total volume and formation of bubbles

Delivery system	Diameter (mm)	Headspace (cc)	Milk-to-air interface (cm ²)	Total volume (cc)	Formation of bubbles
Avent Natural	66	205	34.19	305	Yes
BornFree	65	127	33.17	227	Yes
Control	52	74	20.42	174	No
Dr. Brown's	50	66	19.63	166	No
Evenflo Elan	67	180	35.24	280	Yes
Gerber	52	176	21.23	276	Yes
Playtex Drop-Ins	56	158	24.62	258	Yes
Playtex Ventaire	54	183	22.89	283	Yes

were determined based on the standard curve (standard curves $R^2 = 0.98$). The mean values were used to calculate the amount of retinol and α -tocopherol in milligrams per litre of milk.

Reagents, standards and equipment

All reagents used in the sample preparation were analytical grade. Chromasolv hexane and ethyl acetate were HPLC grade. All standards of retinol and tocopherol were obtained from Sigma-Aldrich (St. Louis, MO, USA). General callipers were used for bottle measurements. A Hewlett-Packard liquid chromatographic system with an HP 1090 pump autosampler with a Pinnacle II silica short narrow-bore column (50 mm $\times$ 2.1 mm ID) with a 3- μ m particle size from Restek (Fuldabruck, Germany) was used in this experiment.

Statistical analysis

All values were entered into a MICROSOFT EXCEL spreadsheet; the mean of each triplicate sample set was calculated using the appropriate EXCEL function. Standard deviations and percentage of baseline were calculated using EXCEL functions.

Statistical analysis was performed using SAS ENTERPRISE GUIDE statistical software (version 4.1, SAS Institute Inc., Cary, NC, USA). A linear mixed model with repeated measures was performed using unstructured covariance structure. A linear mixed model was chosen to account for the similarities to each other that can occur when taking measurements from the same subject (bottle) over time and are no longer

considered independent. A linear mixed model accounts for the lack of independence of the results. A three-level (bottle type, time and replication) model was used, taking into account all of the data rather than using the means. The Akaike Information Criterion was used to determine which model was best.

The independent variables included in the model were Time, Total Volume, Milk-to-Air Interface, Bubble Formation and Milk Type (HM, DHA, Fe). The following variable interactions were also considered: Time $\times$ Bubble Formation, Time $\times$ Volume, Time $\times$ Milk Type, Volume $\times$ Milk Type and Bubble Formation $\times$ Milk Type. Milk Type was included to examine potential differences between HM and infant formula. All of the preceding independent variables and interaction terms were included in the linear mixed model to determine their effect on the outcome variables Retinol Concentration and α -Tocopherol Concentration. Alpha error was set at $P < 0.01$ for all tests to accept or reject.

Results

The diameter of the bottles varied from 50 mm to 67 mm as seen in Table 1. The headspace ranged from a high of 205 mL to a low of 66 mL. Throughout the procedure no bubbles that could be naturally observed formed on the Milk-to-Air Interface of the Control or the DB bottle.

There were no statistically significant differences in α -tocopherol concentrations at baseline for any of the bottle systems. The concentration of α -tocopherol in HM did decrease across all bottle systems as seen in Fig. 1a. Minor decreases occurred in concentration

Fig. 1. (a) Changes in mean α -tocopherol concentration in human milk over 20 min as a percentage of baseline. The human milk was standardized to 3 mg L^{-1} of α -tocopherol at baseline. (b) Changes in mean α -tocopherol concentration in infant formula containing iron over 20 min as a percentage of baseline (Time 0). (c) Changes in mean α -tocopherol concentration in infant formula containing docosahexaenoic acid over 20 min as a percentage of baseline (Time 0). (a–c) are based on the mean triplicate values. The samples were analysed for α -tocopherol using normal-phase high-performance liquid chromatography and compared to a standard curve. The mean concentrations for each triplicate sample were calculated at Time 0, 5, 10, 15, and 20 min. Results represent the mean values of three experiments displayed as a percent of the baseline level. The line in each graph with error bars indicates the trend line from the regression models.

between baseline and the 10-min sampling, with AN and PD decreasing to approximately 90% of baseline. By the 15-min sampling, AN, EL and PD were below 85% of baseline. The greatest difference was seen between the 15- and 20-min samplings, with decreases of 10% for the AN, BF, EL, G and PD bottles. DB and PV maintained concentrations above 80% of baseline and were not statistically different from the Control at the 20-min sampling with $P < 0.01$. Similar trends were seen with the infant formula samples as shown in Fig. 1b,c. The infant formula with DHA did have greater decreases in α -tocopherol concentration than HM, while Fe had the greatest decreases. The concentrations of α -tocopherol are seen in Table 2a.

No statistically significant differences were observed in retinol concentrations at baseline for any of the bottle systems. The concentration of retinol in HM decreased across all bottle systems as shown in Fig. 2a. The percentage of decrease in retinol concentration was less than that observed with α -tocopherol. Similar trends were seen with the infant formula samples as shown in Fig. 2b,c. The concentrations of retinol are seen in Table 2b.

The linear mixed model for α -tocopherol concentration showed similar results as the retinol model

(Table 3a). In the α -tocopherol model, Time, Bubbles and Time $\times$ Bubbles were all significant predictors of α -tocopherol concentration. α -Tocopherol concentration decreased significantly at all measured 5-min intervals. The presence of bubbles in the bottle during the simulated feeding was associated with lower α -tocopherol concentration (Fig. 3a). This effect was seen at times greater than 10 min, as with the retinol model. After 20 min, bottles without visible bubble formation had an average α -tocopherol level that was 91.1% of the baseline level; bottles with bubble formation had an average α -tocopherol level that was 72.4% of the baseline level. Milk type did not have a statistically significant effect on α -tocopherol concentration.

The linear mixed model with retinol as the outcome variable demonstrated that Time, Milk Type, Bubble Formation and Time $\times$ Bubble Formation all had statistically significant effects on retinol concentration (Table 3b). There were differences observed in Milk Type between HM and the infant formulas tested. Headspace was not included in the model as it was found to be highly correlated with other variables. Retinol concentration had a statistically significant decrease at all measured 5-min intervals. Retinol con-

Table 2. (a) Mean α -tocopherol concentrations from triplicate samples at the baseline (Time 0) and 20-min sampling in mg L⁻¹ with standard deviations. (b) Mean retinol concentrations from triplicate samples at the Baseline (Time 0) and 20-min sampling in μ g L⁻¹ with standard deviations

(a)						
	Human milk mg L ⁻¹ (SD)		Formula with additional iron mg L ⁻¹ (SD)		Formula with DHA mg L ⁻¹ (SD)	
	Baseline	20-min	Baseline	20-min	Baseline	20-min
Avent Natural (AN)	3.09 (0.04)	2.18 (0.13)	3.05 (0.14)	2.10 (0.06)	3.02 (0.11)	2.15 (0.06)
BornFree (BF)	3.04 (0.06)	2.35 (0.09)	3.06 (0.18)	2.20 (0.17)	3.04 (0.13)	2.25 (0.12)
Control	3.03 (0.06)	2.92 (0.15)	3.01 (0.17)	2.90 (0.11)	3.03 (0.16)	2.94 (0.08)
Dr. Brown's (DB)	3.03 (0.05)	2.64 (0.21)	2.98 (0.22)	2.60 (0.07)	3.05 (0.13)	2.61 (0.13)
Evenflo Elan (EL)	3.02 (0.05)	2.12 (0.05)	3.02 (0.15)	2.01 (0.14)	3.07 (0.18)	2.08 (0.19)
Gerber (G)	3.06 (0.03)	2.30 (0.15)	3.05 (0.15)	2.13 (0.18)	3.01 (0.13)	2.19 (0.21)
Playtex Drop-Ins (PD)	3.08 (0.03)	2.15 (0.08)	3.04 (0.12)	2.02 (0.08)	3.03 (0.12)	2.09 (0.11)
Playtex Ventaire (PV)	3.00 (0.09)	2.48 (0.11)	3.01 (0.15)	2.36 (0.11)	3.01 (0.17)	2.40 (0.09)

(b)						
	Human milk mg L ⁻¹ (SD)		Formula with additional Iron mg L ⁻¹ (SD)		Formula with DHA mg L ⁻¹ (SD)	
	Baseline	20-min	Baseline	20-min	Baseline	20-min
AN	397.3 (1.15)	374.0 (2.65)	396.7 (1.2)	344.1 (11.5)	398.9 (2.7)	355.3 (13.5)
BF	397.7 (3.06)	368.0 (2.00)	398.0 (3.0)	338.6 (13.6)	399.4 (3.1)	349.6 (9.62)
Control	400.3 (0.58)	393.7 (2.52)	398.7 (2.3)	362.2 (10.5)	399.9 (0.8)	374.0 (11.2)
DB	396.3 (1.53)	381.7 (2.89)	396.0 (1.0)	351.1 (15.3)	397.9 (2.7)	362.6 (12.8)
EL	399.7 (3.06)	361.3 (3.21)	399.3 (3.2)	332.4 (11.6)	398.4 (2.1)	343.3 (11.5)
G	395.7 (1.53)	373.3 (2.08)	397.3 (4.2)	343.5 (15.3)	396.1 (0.8)	354.7 (17.3)
PD	398.3 (2.52)	363.3 (2.89)	396.7 (1.2)	334.3 (12.2)	399.2 (1.5)	345.2 (15.3)
PV	399.0 (4.00)	370.0 (2.00)	400.0 (4.4)	340.4 (14.5)	399.2 (0.6)	351.5 (13.1)

DHA, infant formula with docosahexaenoic acid.

Fig. 2. (a) Changes in mean retinol concentration in human milk over 20 min as a percentage of baseline (Time 0). (b) Changes in mean retinol concentration in infant formula containing iron over 20 min as a percentage of baseline (Time 0). (c) Changes in mean retinol concentration in infant formula containing docosahexaenoic acid over 20 min as a percentage of baseline. (a–c) are based on the mean values from triplicate samples of infant formula with docosahexaenoic were collected. The samples were analysed for retinol using normal-phase high-performance liquid chromatography and compared to a standard curve. The mean concentrations for each triplicate sample were calculated at Time 0, 5, 10, 15, and 20 min. Results represent the concentration of retinol displayed as a percent of the baseline level. The line in each graph with error bars indicates the trend line from the regression models.

Table 3. (a) Linear mixed model for α -tocopherol. (b) Linear mixed model for retinol

(a)								
Type 3 tests of fixed effects								
Outcome variable	Predictor variables	Numerator DF		Denominator DF		F value	Probability > F	
α -Tocopherol	Time	4		67		161.13	<0.0001	
	Bubbles	1		70		43.73	<0.0001	
	Time $\times$ Bubbles	4		67		45.25	<0.0001	
Differences of least square means								
Effect	Difference	Estimate	Standard error	DF	t value	Probability > t	Adjustment	Adjusted P
Time (min)	0 and 5	0.05389	0.007562	70	7.13	<0.0001	Tukey–Kramer	<0.0001
	0 and 10	0.1951	0.01315	70	14.84	<0.0001	Tukey–Kramer	<0.0001
	0 and 15	0.3581	0.01548	70	23.12	<0.0001	Tukey–Kramer	<0.0001
	0 and 20	0.5555	0.02371	70	23.43	<0.0001	Tukey–Kramer	<0.0001
	5 and 10	0.1412	0.01004	70	14.06	<0.0001	Tukey–Kramer	<0.0001
	5 and 15	0.3042	0.01368	70	22.23	<0.0001	Tukey–Kramer	<0.0001
	5 and 20	0.5016	0.02266	70	22.14	<0.0001	Tukey–Kramer	<0.0001
	10 and 15	0.1630	0.01411	70	11.55	<0.0001	Tukey–Kramer	<0.0001
	10 and 20	0.3604	0.02246	70	16.04	<0.0001	Tukey–Kramer	<0.0001
	15 and 20	0.1974	0.01603	70	12.31	<0.0001	Tukey–Kramer	<0.0001
Bubbles	Yes and No	0.2101	0.03177	70	6.61	<0.0001	Tukey–Kramer	<0.0001
Time $\times$ Bubbles	Time = 0; Bubbles Yes and No	−0.01648	0.02983	70	−0.55	0.5823	Tukey–Kramer	0.9999
	Time = 5; Bubbles Yes and No	0.007963	0.02571	70	0.31	0.7577	Tukey–Kramer	1.0000
	Time = 10; Bubbles Yes and No	0.1726	0.03322	70	5.20	<0.0001	Tukey–Kramer	<0.0001
	Time = 15; Bubbles Yes and No	0.3274	0.04283	70	7.64	<0.0001	Tukey–Kramer	<0.0001
	Time = 20; Bubbles Yes and No	0.5589	0.05285	70	10.58	<0.0001	Tukey–Kramer	<0.0001

(b)								
Type 3 tests of fixed effects								
Outcome variable	Predictor variables	Numerator DF		Denominator DF		F value	Probability > F	
Retinol	Milk Type	2		68		11.70	<0.0001	
	Time	4		67		177.33	<0.0001	
	Bubbles	1		45.7		132.72	<0.0001	
	Time $\times$ Bubbles	4		67		29.84	<0.0001	
Differences of least square means								
Effect	Difference	Estimate	Standard error	DF	t value	Probability > t	Adjustment	Adjusted P
Milk type	DHA and Fe	2.0602	0.5579	68	3.69	0.0004	Tukey–Kramer	0.0013
	DHA and HM	−0.4792	0.5579	68	−0.86	0.3934	Tukey–Kramer	0.6678
	Fe and HM	−2.5394	0.5579	68	−4.55	<0.0001	Tukey–Kramer	<0.0001
Time (min)	0 and 5	6.3857	0.5481	70	11.65	<0.0001	Tukey–Kramer	<0.0001
	0 and 10	9.6836	0.5924	70	16.35	<0.0001	Tukey–Kramer	<0.0001
	0 and 15	18.3342	0.8363	70	21.92	<0.0001	Tukey–Kramer	<0.0001
	0 and 20	25.5965	0.9543	70	26.82	<0.0001	Tukey–Kramer	<0.0001
	5 and 10	3.2979	0.5651	70	5.84	<0.0001	Tukey–Kramer	<0.0001
	5 and 15	11.9485	0.6941	70	17.21	<0.0001	Tukey–Kramer	<0.0001
	5 and 20	19.2109	0.8059	70	23.84	<0.0001	Tukey–Kramer	<0.0001
	10 and 15	8.6506	0.6969	70	12.41	<0.0001	Tukey–Kramer	<0.0001
	10 and 20	15.9130	0.7891	70	20.17	<0.0001	Tukey–Kramer	<0.0001
	15 and 20	7.2623	0.4794	70	15.15	<0.0001	Tukey–Kramer	<0.0001
Bubbles	Yes and No	9.1171	0.7914	45.7	11.52	<0.0001	Tukey–Kramer	<0.0001
Time $\times$ Bubbles	Time = 0; Bubbles Yes and No	0.03086	0.7914	53	0.04	0.9658	Tukey–Kramer	1.0000
	Time = 5; Bubbles Yes and No	2.5500	0.9364	50.5	2.72	0.0089	Tukey–Kramer	0.1858
	Time = 10; Bubbles Yes and No	7.9299	0.8748	68.3	9.07	<0.0001	Tukey–Kramer	<0.0001
	Time = 15; Bubbles Yes and No	16.0190	1.4292	56.8	11.21	<0.0001	Tukey–Kramer	<0.0001
	Time = 20; Bubbles Yes and No	19.0558	1.7243	63.2	11.05	<0.0001	Tukey–Kramer	<0.0001

DHA, infant formula with docosahexaenoic acid; HM, human milk; Fe, infant formula with iron; DF, degrees of freedom.

Fig. 3. (a) Decrease in mean α -tocopherol concentration in bottles with bubble formation versus bottles without bubble formation reported as mg L^{-1} . (b) Decrease in mean retinol concentration in bottles with bubble formation versus bottles without bubble formation reported as $\mu\text{g L}^{-1}$.

centration was lower in infant formula containing iron compared to HM and infant formula with DHA. This difference was small in magnitude; however, it was statistically significant. Bottles where bubble formation was visible during the simulated feeding had lower levels of retinol compared to bottles that did not have any bubble formation (Control and DB). Additionally, the interaction of time and bubbles demonstrates that the effect of bubble formation on retinol concentration changes with time (Fig. 3b). At the beginning of the experiment and at 5 min, there was no statistically significant difference in retinol levels in bottles with bubble formation compared to bottles without bubble formation. However, at 10 min, the results indicated that a difference did exist. After 20 min, bottles without visible bubble formation had an average retinol level that was 96.0% of the baseline level; bottles with bubble formation had an average retinol level that was 91.2% of the baseline level.

For this experiment, only the HM was standardized as to α -tocopherol and retinol. The formulas were used directly from the containers and mixed using manufacturer's directions. Milk Type was included initially in the regression models but was not statistically significant for α -tocopherol and was dropped from that model. Milk Type was statistically significant for retinol concentration when comparing infant

formula with DHA and Fe or when comparing infant formula with Fe and HM as seen in Table 3b.

Conclusion

There was significant loss of retinol and α -tocopherol during the simulated feedings. In the Control and DB bottles, bubbles did not form, and these bottles showed the least decreases in retinol and α -tocopherol concentrations. Bottles with bubble formation showed the greatest decreases in the concentrations of these two nutrients. Bubbles rapidly formed on the Milk-to-Air Interface in the G, PV, EL, BF, PD, and AN bottles as the milk was removed. The Control had neither air moving through the milk nor bubbles forming on the surface of the milk, with small decreases in vitamin concentrations observed.

There were greater decreases in retinol concentration in the high iron infant formula. The differences may be due to greater oxidation of iron in the presence of oxygen as it moved through the milk, causing a Fenton reaction. In this reaction the presence of iron is catalytic and hydrogen peroxide is converted into hydroxyl radicals. As seen in Table 2, the high-iron infant formula samples consistently had less retinol and α -tocopherol than the HM samples and the DHA-containing infant formula, indicating a

greater decrease in the concentration of these two vitamins in infant formula compared to HM.

Considering that vitamins A and E are essential nutrients that become especially important during infancy due to rapid physical growth, any decrease in the intake of these nutrients would be of concern. A recent report states that the concentrations of retinol and α -tocopherol in infant formula decreased during storage at room temperature in open boxes. The decrease in concentration of these nutrients was accompanied by an increase in pro-oxidants and a decrease in antioxidants in all the formulas studied (Shilina *et al.* 2007). Additionally, a recent study reported the loss of ascorbic acid from HM and infant formula, which could also lead to an increase in pro-oxidant activity (Francis *et al.* 2008).

Antioxidants in HM provide defence mechanisms for infants (Ledo *et al.* 2009). Loss of these defence mechanisms could impact the health of infants. The importance of adequate amounts of nutrients in infant nutrition cannot be overstated. Additional research is necessary to understand the clinical impact of these decreases and to evaluate the nutritional impact on infants feeding from bottles. The loss of these nutrients from HM or infant formula could increase an infant's risk of morbidity.

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Conflict of interest

None of the authors own stock or shares in any organization that could benefit from this study. None of the authors hold or are applying for any patents relating to the content of this manuscript.

Authors' contributions

JF conceived of the study, performed some of the statistical analyses, participated in the study implementation and helped to draft the manuscript. KR carried out the extractions, participated in the sample processing and helped draft the manuscript. DD par-

ticipated in the sample collection and processing, inputted data, performed statistical analysis and helped draft the manuscript. RT performed statistical analysis and helped draft the manuscript. RP participated in the study design, implementation/coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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