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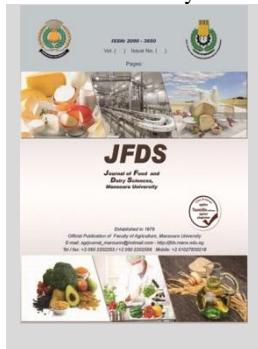
Effect of camel's milk processing on Orotic acid stability

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ABSTRACT

The objective of this study was to evaluate orotic acid stability throughout the manufacture and storage of camel milk products. The results observed that, freezing camel milk for 3 months at -18°C led to stability of orotic acid, pH and acidity did not change during storage. Thermal treatment carried out on camel milk at three treatments ($63^{\circ}\text{C}/30$ min, $72^{\circ}\text{C}/15$ s and $80^{\circ}\text{C}/20$ s). The results showed that, the treatment at 80°C caused less change in microbial load, pH, acidity and a 16.74% decrease in orotic acid during storage than that of other treatments. Then we were advised to make fermented camel milk (heated at $80^{\circ}\text{C}/20$ s) with a traditional starter and another commercial probiotic starter. The results showed that, probiotic fermented camel milk significantly affect the stability of orotic acid compared to the traditional starter during fermentation and storage at 5°C .

Keywords: freezing, heat treatment, fermentation

INTRODUCTION

Camel milk's nutritional and therapeutic properties have been employed to promote human health (Gader & Alhaider, 2016), because it has higher concentrations of essential fatty acids, immunoglobulins, lactoferrin, lactoperoxidase and other antimicrobial components as well as insulin-like protein than milk from other animals (Yadav *et al.*, 2015). Although the exact mechanism underlying camel milk's hypocholesterolaemic effects is still unknown, several suggestions have been put out, such as the reduction of cholesterol caused by interactions between bioactive peptides produced from camel milk proteins and cholesterol (Li and Papadopoulos, 1998; Seelig and Seelig, 1996), and the presence of orotic acid in camel milk, which is believed to be responsible for lowering cholesterol in humans (Mohammadabadi, 2020; Swelum *et al.*, 2021; Seifu, 2022). Orotic acid is the main component of acid-soluble nucleotides in camel milk (Haddadin *et al.*, 2008), followed by lower concentrations in cow milk but much lower concentrations in goat and sheep milk (Gil and Sanchez-Medina, 1981; Karatas *et al.*, 2008). Orotic acid also induced hepatic steatosis in rats (Chen and Larson, 1971; Durschlag and Robinson, 1980; Jesse *et al.*, 2010) and lowered serum cholesterol in humans (Robinson and Dombrowski, 1983). The more direct nutritional value in humans may be due to the high amount of orotic acid (Korycka-Dahl *et al.*, 1979), and the reduced risk of cardiovascular disease (Hadj *et al.*, 2003). These compounds are used in medicine as biostimulators of exchange processes in organisms. Orotic acid (OA), a metabolic intermediate of pyrimidines, exerts beneficial cardiovascular effects by ameliorating cardiac ischemia (Pôrto *et al.*, 2012). Orotic acid is also crucial for maintaining cell function and reducing cell death, making it a potential therapeutic method for the prevention and treatment of diabetes in the future. The thermal stability of orotic acid in cow's milk during processing may be of practical importance, although little information has been published on the effects of heat processing in their industrial

milk heat production experiments (Fushimura *et al.*, 2021). The orotic acid concentration of milk is not significantly affected by heating temperature, but rather by the duration of heating time. As recorded by (Deeth and Tamime 1981 and Alm 1982), orotic acid is metabolized by yogurt starter cultures, probably *L. delbrueckii* subsp. *bulgaricus*, during yogurt processing and its concentration in milk is reduced by up to 50 percent (Saidi and Warthesen, 1989; Navder *et al.*, 1990). Additionally, as per (Larson and Hegarty 1979 and Suzuki *et al.* 1986), the amount of soluble whey solids and the degree of fermentation influence the amount of orotic acid in cultured dairy products. It is crucial to measure this amount as well as, it can serve as a reliable gauge of a cow's quality (Ferreira, 2003; Kavaz and Bakirci, 2014). This study investigated the effect of freezing, different heat treatments and storage, fermentation time and storage of fermented camel milk on orotic acid content and stability.

MATERIALS AND METHODS

Materials

Camel milk (total solids 11.0%, protein 3.2%, fat 3.4%, acidity 0.15% and pH 6.71) were obtained from Matrouh station, Desert research center Egypt., was used for preparing fermented camel milk.

Direct vat set (DVS) classic (traditional) yogurt culture (*L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* YoFlex® Premium 4.0, 1:1).

The bacterial strains contained 7.7 and 7.4 Log CFU/g of freeze-dried culture.

ABT-5 culture (*L. acidophilus* La-5, *Bifidobacterium* spp. and *S. thermophilus* TH-4, 1:1:1). The bacterial strains contained 8.2, 9.5 and 7.3 Log CFU/g of freeze-dried culture; respectively, was purchased from Chr. Hansen, Hoersholm, Denmark, by Misr Food Additives (MIFAD), Egypt.

The study was performed in three stages:

First study: comparable estimate orotic acid in local animals' milk (goat, sheep, cow and camel milk).

Stage 1: The samples of raw camel milk were freezing at -18°C during 3-month of storage and analysis stability of orotic acid during 1, 2 and 3 months.

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Stage 2: The samples were heated in glass vials in a water bath. The heat treatment consists of three sets of treatments: 63°C for 30 min, 72°C for 15 s and 80°C for 20 s. It was immediately cooled to 5°C and stored for 15 days for analysis, and then determined number of bacteria, pH, acidity and orotic acid after 1, 5, 10 and 15 days.

Stage 3: Fermented milk was prepared by the best heat treatment results. Camel milk was divided to two portions, the first portion, with traditional yogurt culture and second portion was ABT-5 culture. Raw camel milk was heated to 80°C for 20 s, cooled to 37°C, and then inoculated with a fermented milk culture and 0.02% (w/v) probiotic bacteria (ABT-5) and set in a plastic container until pH 4.7±0.05. Fermented milk samples were stored at 5±2°C and measured during fermentation (2, 4, 6, 8 and 10 h) and after cooling for total bacterial count, stability of orotic acid, acidity and pH during storage period (1,7,15 and 21days).

Methods of analyses

Camel milk samples were chemically analyzed according to (AOAC 2019) for protein, fat, total solid contents, acidity (as lactic acid %) and pH value.

Orotic acid by HPLC methods:

Extraction of Orotic acids:

Orotic acid in camel milk and fermented camel milk was extracted using 0.13 N H₂SO₄ and the milk samples were centrifuged at 7000 ×g for 7 min at 5°C. The top layer is Whatman No. 1 after analysis using filter paper; the filtrate was passed through a 0.45 µm Millex PVDF Millipore (Billerica, MA, USA) syringe filter. High performance liquid chromatography equipment was used to perform the separation.

HPLC conditions:

An Agilent 1260 HPLC series was used for the study. The separation was done with Kromasil 60-5-Hilik-D (4.6 mm x 100 mm). 30% sodium phosphate (pH = 6.7 with phosphoric acid) + ACN (3:7) is the mobile part and the flow rate is 1 ml/min. An isovolumetric linear regression method was used to sequentially lyse the mobile phase. Monitoring at 214 nm is the DAD detector. An injection volume of 2 µl was used for all sample solutions. The column temperature was maintained at 35°C. Calibration curve data points were subjected to least squares regression analysis (Güler, 2014).

Microbiological analyses:

Viable cell counts were performed by the standard pour-plate method after serial dilutions in saline solution (0.85% w/v) according to American Public Health Association (APHA, 1978). The results were expressed as

Table 2. Effect of freezing raw camel milk on pH, acidity and orotic acid during storage.

Component	fresh	1-month	2-month	3-month
orotic acid(µg/ml)	98.357±0.008 ^a	98.341±0.01 ^a	98.293±0.013 ^a	98.376±0.007 ^a
pH	6.66±0.01 ^a	6.65±0.04 ^a	6.66±0.005 ^a	6.64±0.014 ^a
Acidity (%)	0.15±0.024 ^a	0.15±0.046 ^a	0.15±0.034 ^a	0.15±0.011 ^a

^aMeans followed by different letters in the same row are significantly different (P≤0.05).

2. Effect of heat treatments and storage time:

The effect of heat treatment on the content of orotic acid is shown in Table 3. Average values for heated milk were; 96.97±0.07, 96.69±0.01 and 97.10±0.04 µg/ml for 1 day (65°C/30 min, (72°C/15 s and (80°C/20 s; respectively. The results showed that the heat treatment has no significant

Log cfu/g when fresh and after 5, 10, 15 and 21 day of storage for fermented milk and after 1, 5, 10 and 15 days for heated camel milk.

Statistical analysis:

Experimental data was analyzed using analysis of variance (ANOVA) and significant differences among means from a triplicate analysis at (P≤0.05) were analyzed by Duncan's multiple range test (DMRT) using the SPSS (2012) software.

RESULTS AND DISCUSSION

Orotic acid content in various milk species:

The orotic acid content of various milk species samples presented in Table 1. It could be noticed that, camel's milk recorded the highest value for orotic acid content (98.357±0.9 µg/ml), followed by cow's milk, sheep's milk and goat's milk (83.456±0.7, 37.285±1.2 and 19.548±1.4); respectively. These results agreement with previous researcher (Larson and Hegarty, 1979; Akalin and Gonc 1996), who reported that mid lactation cow, sheep and goat's milk contained 82.43±9.64; 30.59±1.71 and 23.87±1.36mg/l orotic acid, respectively. Variations in orotic acid content of the studied milk samples are due to several factors account for this variation including breed of animal, nutrition's stage of lactation, milking times, method of determination and finally could be due to the process of milk secretion (Ahmed, *et al.*, 1978; Motyl *et al.*, 1991).

Table 1. The average of orotic acid content in milk of various species.

Species	Orotic acid (µg/ml)
camel	98.357±0.9 ^a
goat	19.548±1.4 ^d
sheep	37.285±1.2 ^c
cows	83.456±0.7 ^b

^{a,b,c,d} Means followed by different letters in the same column are significantly different (P≤0.05).

Effect of processing camel's milk on orotic acid stability:

1. Effect of freezing:

Average concentrations of orotic acids during fresh and after 1, 2 and 3 months of frozen storage in Table 2. Concentrations of orotic acids during storage were not statistically significant (P≤0.05, pH and acidity) for acids, indicating that frozen storage significantly changed the levels of these acids. Mean differences for other organic acids did not differ by large standard deviations. Freezing also caused a significant (P ≤ 0.05) increase in formic and uric acid and reduction of tartaric acid in soft cheese (Izco *et al.*, 2002). However, significant effects that freezing can have on the organic acid content of cow's milk Mozzarella cheese due to differences in milk source (cow versus goat) or cheese type and composition (Califano and Bevilacqua, 1999).

(p≤0.05) effect on orotic acid content in milk. These results are consistent with previous findings by (Gil and Sanchez-Medina, 1982; Saidi and Wartsen, 1989; Salem *et al.*, 2002), who stated that orotic acid is very stable to normal pasteurization treatments. The results showed the effect of storage time on the stability of orotic acid in heated camel milk

at different treatments (65°C/30 min, 72°C/15 s and 80°C/15 s) with different significant ($p \leq 0.05$) degrees depending on the heat temperature. This is explained by the development of acidity and the microbial load that consumes orotic acid during storage, leading to a significant increase in acidity and a

decrease in orotic acid content. The maximum reduction of orotic acid showed in Fig 1 during storage (15 days) at (65°C/30 min, 72°C/15 s and 80°C/20 s) were (27.59, 20.71 and 16.74%); respectively.

Table 3. Effect of heat treatments on pH, acidity TVBC and orotic acid content in camel's milk during storage:

Treatments	Storage period	Orotic acid ($\mu\text{g/ml}$)	pH	Acidity (%)	TVBC (\log_{10} CFU/ml)
(63°C)-30min	1-day	96.97±0.07 ^a	6.69±0.02 ^a	0.15±0.01 ^a	7.97±0.011 ^a
	5-days	89.75±0.01 ^d	6.62±0.05 ^b	0.17±0.01 ^b	8.34±0.21 ^b
	10-days	80.90±0.05 ^f	6.56±0.01 ^c	0.18±0.02 ^b	8.56±0.007 ^{ab}
	15-days	69.59±0.07 ^h	6.19±0.12 ^d	0.20±0.04 ^c	8.62±0.004 ^{ab}
(72°C)-15sec	1-day	96.69±0.01 ^a	6.68±0.02 ^a	0.16±0.03 ^b	7.88±0.009 ^a
	5-days	94.27±0.01 ^b	6.65±0.03 ^b	0.17±0.02 ^b	8.08±0.021 ^a
	10-days	87.13±0.03 ^c	6.60±0.16 ^c	0.17±0.01 ^b	8.15±0.060 ^a
	15-days	76.35±0.08 ^e	6.25±0.07 ^d	0.19±0.03 ^c	8.30±0.049 ^b
(80°C)-20 sec	1-day	97.10±0.04 ^a	6.68±0.01 ^a	0.16±0.02 ^a	7.74±0.122 ^a
	5-days	96.03±0.01 ^{ab}	6.59±0.01 ^b	0.17±0.02 ^b	7.85±0.164 ^a
	10-days	91.86±0.70 ^c	6.47±0.01 ^c	0.18±0.05 ^b	8.08±0.088 ^a
	15-days	80.26±0.22 ^f	6.36±0.02 ^d	0.185±0.03 ^b	8.15±0.154 ^b

Means followed by different letters in the same column are significantly different ($P \leq 0.05$). TVBC=Total viable bacterial Counts.

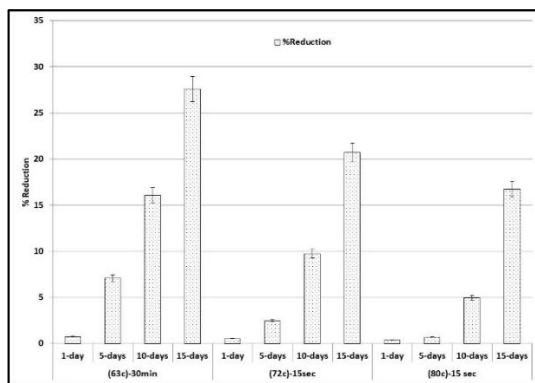


Fig. 1. The effect of heat treatments of camel milk during cold storage on the reduction of orotic acid.

3. Effect of fermentation time and cold storage:

Acidification profiles, acid production capacity has a significant impact on processing and shelf life (Zare *et al.*, 2012; Nguyen *et al.*, 2014). As shown in Figs 2 & 3, the acid production capacity of probiotic fermented camel milk (PFCM) and traditional fermented camel milk (TFCM), pH and acidity were monitored every 2 h during fermentation and after 1, 7, 15 and 21 days in a refrigerator temperature (5°C). Acidification profiles could provide insight into the effects of probiotics and traditional strains on metabolism, similar trends in pH decrease were observed for all samples. The rate of pH (4.60) decrease occurred 8-10 h for TFCM and PFCM; respectively (Fig 2). Fermented camel milk has a longer clotting time compared to other milks; this is due to differences in the size and properties of casein particles (Shuiep *et al.*, 2013). Compared to cow milk coagulation, camel milk coagulation consistently contains larger casein micelles (Farah and Ruegg, 1989). Thus, the addition of probiotic strains (PFCM) can slightly reduce the fermentation time. Clearly, the pH of the PFCM-fermented samples were significantly lower than that of the TFCM samples during the storage period ($P \leq 0.05$), the results agree with (Masud *et al.* 1991; Kneifel *et al.*, 1992; Eissa *et al.* 2010; Eissa *et al.* 2011). In Fig 4, showed the second hour of fermentation, lactic acid was actively produced, and orotic acids were consumed. In Fig 3, orotic acid showed a maximum reduction at 10 h of fermentation, approximately 33.42 & 44.16% for TFCM & PFCM, and from 54.99 & 58.53% for TFCM & PFCM

during 21 days of cold storage; respectively, because orotic acid is an intermediate in the synthesis of nucleotides and free pyrimidine and forms orotidylate in the presence of orotate phosphoribosyl transferase and growth factor for probiotic bacteria (Güzel-Seydim *et al.*, 2000). This reduction was also reported for buttermilk (Marsili *et al.*, 1981) and yogurt (Saidi and Warthesen, 1989; Fernandez-Garcia and McGregor, 1994). (Alm, 1982) reported a 45-48% reduction of orotic acid in products made from fresh milk, however, orotic acid and citric acid were moderately consumed during fermentation, and the slight decrease in orotic acid continued until the 7 day of cold storage. On the other hand, the obtained results showed that, the fermented camel milk during fermentation contained a total bacterial count of 6.214 and 6.738 log cfu/g viable bacterial cells in TFCM and PFCM; respectively. The initial bacterial count increased significantly ($P \leq 0.05$) during storage of both types of yogurt, and peaked on day 10 and then decreased significantly ($P < 0.05$) to 7.924 and 8.394 log cfu/g for TFCM and PFCM; respectively, and then began to decline.

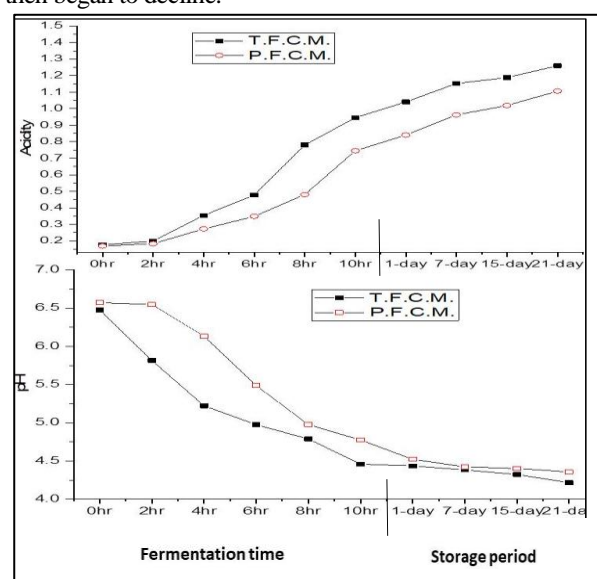


Fig. 2. The effect of fermentation of camel milk and cold storage on pH and acidity
 TFCM= Traditional fermented camel milk;
 PFCM=Probiotic fermented camel milk

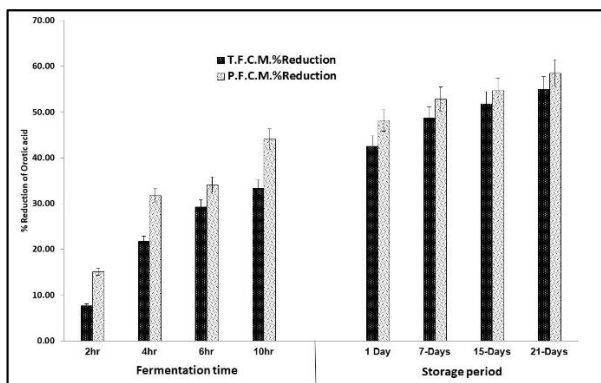


Fig. 3. The effect of fermentation of camel milk and cold storage on the reduction of orotic acid.

TFCM= Traditional fermented camel milk;
PFCM=Probiotic fermented camel milk

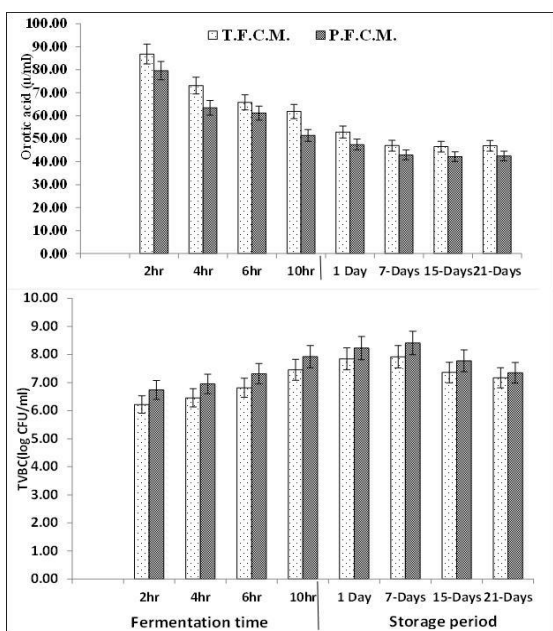


Fig. 4. Total viable bacterial count and orotic acid during fermentation and cold storage

TFCM= Traditional fermented camel milk;
PFCM=Probiotic fermented camel milk

CONCLUSION

The present study concluded that, freezing treatments have no significant effect on orotic acid stability during storage; however, heat treatments improve the microbial quality of camel milk, pH, acidity and orotic acid stability. The best heat treatment was found to be the camel milk at 80°C/20 s, with less reduction of orotic acid and stability during storage. Orotic acid improves the growth of starter cultures (lactic acid bacteria and probiotics), decreases orotic acid concentration and increases reduction by probiotic bacteria.

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تأثير المعاملات التصنيعية على ثبات حمض الأوروتيك في حليب الإبل

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المخلص

كان الهدف من هذه الدراسة هو تقييم ثبات حمض الأوروتيك خلال تصنيع وتخزين منتجات حليب الإبل، وكانت النتائج كالتالي: ثبات حمض الأوروتيك والرقم الهيدروجيني والحموضة لم تتغير أثناء التخزين عند تجميد حليب الإبل لمدة 3 أشهر عند درجة 18- درجة مئوية، أما تأثير المعاملة الحرارية التي أجريت على حليب الإبل عند ثلاث معاملات (63°م، 72°م، 80°م)، فقد أظهرت النتائج أن المعاملة الحرارية عند 80 درجة مئوية لمدة 20 ثانية أحدثت تغيراً أقل في الحمض الميكروبي والرقم الهيدروجيني والحموضة، وانخفاضاً بنسبة 16,74% في حمض الأوروتيك أثناء تخزين الحليب الميسر مقارنة بالمعاملات الأخرى، ثم اختير أفضل معاملة حرارية لتحضير حليب الإبل المخمر (80°م، 20ث) مع بدئ تقليدي وبدئ بروبيوتيك تجاري آخر، وأظهرت النتائج أن حليب الإبل المخمر ببدئ البروبيوتيك يؤثر بشكل كبير على ثبات حمض الأوروتيك مقارنة بالبدئ التقليدي أثناء التخزين عند درجة حرارة 5 درجات مئوية.