DOI : 10.5958/2277-8934.2020.00008.9 *Vol 27 No 1, p 55-60*

ANALYSIS OF ENDOTOXIN LEVEL IN CAMEL MILK SAMPLES COLLECTED FROM VENDING OUTLETS IN SAUDI ARABIA

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ABSTRACT

The present study was done to analyse the endotoxin limits of 20 camel milk samples collected from common vending outlets, i.e. desert farms, shops etc. The milk samples were divided into 4 types (raw milk, refrigerated, boiled, stored at – 20°C) and processed for endotoxin analysis by Limulous Amoebocyte Lysate (LAL) Gel clot method. The microbial count was examined for raw milk sample as per standard protocols. Endotoxin limits observed in the raw milk ranged from 3 to more than 1200 EU/ml. Elevated levels of endotoxin were observed in refrigerated samples. There is no significant change of endotoxin found between raw and deep freezed, boiled samples. Endotoxin value of processed milk from shops were in the range of 3 – 300 EU/mL. Among 20 samples, the total microbial count ranged from 1 to 7.39 log CFU/ml and coliform count was 0 to 3.58 log coliforms CFU/ml. The findings of this report on the endotoxin limits of camel milk showed the levels to be between 3 to 9 EU/ml. However, the samples showed high endotoxin values (>1200 EU/mL) if improperly stored. Presence of high level of microbial and endotoxin in camel milk is unsafe for human consumption.

Key words: Camel milk, endotoxin limit, gel clot method, LAL test, microbial count

People of Kingdom of Saudi Arabia (KSA) consume camel's milk in relatively high quantities during festivals and celebrations (Faye *et al*,2014). Unpasteurised milk is rich in Gram-negative bacteria (GNB) and endotoxins (Kilewein, 1994). Endotoxin contamination in food products and indoor exposure are an increasing medical problem that contribute to the development and severity of asthma and other respiratory symptoms (Loss *et al*, 2011; Sipka *et al*, 2015; Kulhankova *et al*, 2016).

In the PASTURE study, endotoxin concentrations were found to be significant in cow milk samples from non-farming families compared with farming families (Gehring *et al*, 2008) and detected endotoxin levels of shop milk and farm milk samples. Another study by GABRIELA group reported that the elevated endotoxin load in farm milk may involve asthma and atopy protective effect (Loss *et al*, 2011).

There have been no studies available worldwide to analyse the endotoxin levels in camel milk to assess the hygienic quality of raw and processed camel milk.

Hence, the aim of the present work was to analyse the endotoxin levels in camel milk samples collected from vending outlets.

Materials and Methods

Camel milk samples

Random sampling of raw camel milk was done from the desert farms of Zulfi, Majmaah region, Saudi Arabia and stored milk from milking vessels, shops and directly collected from the udder of the camel between September 2018 to May 2019. About 200 ml of fresh whole milk samples were collected from each sampling point using sterile and depyrogenated screw capped bottles. All samples were tightly capped, labelled and immediately transported in an ice-cold condition to the laboratory for analysis.

These samples were classified as 4 categories (Table 1). A total of 20 camel milk samples were collected from the desert farms, among them 4 each were collected from milking container (category I) and storage container (category II). Five samples of bottled camel milk were procured from the shops (category III). Seven samples were collected directly from the camels udder (category IV) following a strict aseptic collection method.

Collected samples were split into 4 parts; the first part was considered raw milk and was immediately processed for microbiological analysis and endotoxin analysis as per standard protocol.

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The second and third parts were given different cold treatment such as 2 – 8°C and –20°C for 24 and 48 hours, respectively. The fourth part was taken for heat treatment (boiling for 10 mins) and the endotoxin limit was checked as per standard protocol.

Microbiological analysis

Total microbial count and coliform count tests were performed for raw milk samples following the international standard for examination of dairy products (EC 2001; EU 2004; ISO 14461-1:2005). Briefly, for standard plate count for total microbial count enumeration, one millilitre of the milk sample was serially diluted in 9 ml of peptone water up to six dilutions. One ml of diluted sample was poured on a sterile Petri dish and then sterile molten media (Plate Count Agar) was poured. The sample and the agar were gently mixed and left for 30 minutes. The plates were sealed with parafilm, incubated at 37°C for 2 days. Duplicates were performed for each sample and the colonies were counted using a colony counter.

For Coliform count, serially diluted samples were poured on sterile Petri dish and then sterile Violet Red Bile Agar was poured. The sample and the agar were gently mixed and left solidified for 30 minutes. Two plates were inoculated with each dilution. The plates were incubated at 37°C for 24 hours. Typical dark red colonies were considered as coliform colonies and number of colonies were recorded and tabulated.

Detection of endotoxin level

Detection of endotoxin levels in camel milk samples was done by gel-clot Limulus Amoebocyte lysate (LAL) assay method (Endosafe, Charles River, USA). All types of samples including raw, cold and heat treated samples were at room temperature and diluted with endotoxin free water or LAL water (EndoSafe, Charles River, USA). Firstly, Endotoxin test was standardised by performing 'inhibition / enhancement test' and adjusting the pH of the milk samples. All the samples were processed at a pH range between 6.0 and 8.0. The testing methodology were followed as per method outlined by the United States Pharmacopoeial Convention chapter <85> Bacterial Endotoxin Test (USP, 2012).

The test endotoxin limit was calculated based on formula

$$MVD = \text{Endotoxin limit (EU/ml)} / \lambda$$

Samples were always tested in the presence of both positive and negative controls. A quarte replicate was performed for each dilution. The minimum sample with LAL water dilution was 1:16, the maximum dilution was 1:76,800. At first, a 10-fold dilution series was prepared, followed by a two-fold dilution series. The amount of endotoxin was expressed in endotoxin units EU/ml. The lysate sensitivity of minimum endotoxin detection limit was 0.03125 EU/ml. A standard curve test was performed whenever a new lot of CSE and LAL reagent was received. Inhibition and enhancement tests were performed to detect the non interfering dilution as per standard protocol USP<85>.

Statistical methods

The significance of differences in endotoxin levels, among the various sampling categories, were analysed using Student's 't' test, a p value less than 0.05 was considered as statistically significant. Microbiological counts were approximately normally distributed after natural log (ln) transformation. Mean value of microbial counts and endotoxin levels were calculated.

Results

Results of bacterial enumeration of raw camel milk samples and endotoxin levels observed in each category of milk samples are shown in Table 2. Among the 20 samples that were tested, the total aerobic flora ranged from 1 to 7.39 log CFU/ml and coliform count was 0 to 3.58 log coliforms CFU/ml. There was no coliforms observed in category IV sample.

Category I of raw farm milk showed an endotoxin limit of more than 300 EU/ml, elevated levels of endotoxin value observed in refrigerated samples (p value 0.0412), and there was no difference observed between raw and deep freezed sample (p

Table 1. Camel milk sample details.

Type	Sample details	Time lapse between milking and sample collection
Category I	Collected from milking container	30 – 60 minutes
Category II	Collected from stored milk in milking vessel	2 – 4 hours
Category III	Processed shop milk	-
Category IV	Directly milking from udder	Not applicable

value =1). The mean total bacterial count was 4.61 and coliform count was 2.52 log CFU/ml (Table 2, 3).

In category II the sample showed a high level of endotoxin throughout all samples, mean endotoxin value of raw milk was 863 EU/ml. The microbial count (5.98 Log CFU/mL) and coliform count (3.23 Log CFU/ml) exceeded the EU reference values. An inconsistent endotoxin value in the range of 6 to 1200 EU/mL was observed in four samples of processed shop milk. There was a slight increase observed between raw and refrigerated samples, i.e. total microbial count and coliform count 4.76, 2.89 log CFU/ml, respectively. The very minimum level of endotoxin observed in category IV sample was 3 to 6 EU/ml. There was no significant difference between raw milk, cold or hot treated samples.

Overall, a range of endotoxin values of raw milk from milking vessels, storage containers, shop milk, and udder milk were 300-600 EU/ml, 1200 EU/mL, 600 – 1200 EU/ml and 3 – 6 EU/ml, respectively. One or two fold increase of endotoxin levels were observed in refrigerated samples than raw milk. There was no significant increase or decrease in endotoxin load in raw and samples which were stored at – 20°C (p value =1).

Discussion

Recent studies show that endotoxin in milk samples can have protective effects against the development of asthma and allergy (Gehring *et al,* 2002 and 2008; Loss *et al,* 2011; Illi *et al,* 2012) but such studies with camel milk are lacking. In this study, camel milk was examined using controlled individual sample collection, storage and heat and cold treatment to find out concentration of endotoxin.

Endotoxin level of raw camel's milk (category I) collected from the milking container was <600 EU/ml, however, there was a slight increase observed in refrigerated samples, this might be due to multiplication of psycrophillic Gram negative organisms which may be present in the samples. These findings match with Sipka *et al* (2016) which reports a similar suggestion with cow milk. There is no significant difference of endotoxin levels observed between raw, boiled or deep freezed milk.

Category II samples that were collected and stored for more than 6 hours showed one to two fold of endotoxin levels when compared to category I samples. This time difference was taken due to difficulties in sample collection such as milking done later in the evening and distance from the testing facility to collection place at desert farms. So these samples were considered as worst case and had higher levels of endotoxin (>1200 EU/ mL, mean 863 EU/ml) than category I. This is evident by Gehring *et al* (2008) who saw similar observations in cow's milk, and accordingly endotoxin levels in farm milk were positively associated with time duration between milking and packing.

Regarding category III milk samples, among the 4 samples analysed there was no consistent levels of endotoxin observed, two samples were < 30 and another two samples were > 600 EU/ml, this might be due to their manufacturing conditions and processing methods. Comparatively, there is no difference of endotoxin level observed in category I of raw milk and III of shop milk, this is very similar to a report in cow's milk (Gehring *et al,* 2008, Sipka *et al,* 2016). However, endotoxin levels of camel's milk collected directly from the udder (category IV) shows < 6 EU/ml, mean value of endotoxin was 4 EU/ml which is 100 to 200 times lower than other category samples. This clearly shows the true value of endotoxin levels in camel milk.

No previous published studies have analysed the endotoxin levels of camel milk. Therefore, comparison of present study results with other reports prove difficult. Although there are still very few studies available on cow's milk but recently Sipka *et al* (2016) analysed cows milk by LAL method and reported that the median value of farm cow milk was 60 EU/ml and shop milk was 102.5 EU/ml. In the PASTURE study conducted in European countries they reported that the geometric mean endotoxin value was 476, 17, 163, 459, 169 EU/ml in Austria, Finland, France, Germany and Switzerland, respectively (Gehring *et al,* 2008). The variations of endotoxin levels obtained by the available reports might be due to the nature of the milk samples, source of samplings, and the test methods employed.

Endotoxin limits were directly associated with total microbial load in particular GNB organisms, hence in the present study this was analysed and compared with total microbial and coliform count. There is no standard microbial limits for camel milk, thus the present study results were compared with European union (EU) microbiological limits (EU Regulation, 2004). Total bacterial flora was not more than 1 X 10^5 CFU/ml (5 log CFU/mL) and Coliform count <10^2 CFU/ml (2 log CFU/ml) for raw milk for human consumption. By comparing this, the mean value of TBC of category I, II and IV were in the range of EU acceptable limits for raw milk. However,

Table 2. Endotoxin level and microbial count of each sample.

Sample number	Sample types	Endotoxin level (EU/ml)				Microbiological analysis	
		Raw milk	Refrigerated#	Deep freezed*	Boiled milk$	Total count (log CFU/ml)	Lactose fermenting coliforms (log coliforms CFU/ml)
1	Category I	<600	600-1200	<600	<600	6.38	3.58
2		<300	>450	<300	<450	3.69	2.12
3		<300	300 – 600	<300	<300	3.84	1.98
4		300 -600	600	300 -600	>600	4.53	2.41
5	Category II	>1200	>1200	>1200	>1200	7.08	3.8
6		>1200	>1200	>1200	>1200	7.39	3.56
7		<600	<600	<600	>600	4.62	2.11
8		300 -600	600	300 -600	>600	4.82	2.66
9	Category III	600 – 1200	>2400	600 – 1200	>600	6.34	2.89
10		<600	<600	<600	<600	4.53	1.78
11		<300	<300	<300	<450	4.84	1.83
12		<30	>60	<30	<30	3.87	0.85
13		<6	6 to 12	<6	<6	4.07	1.95
14	Category IV	< 6	<12	< 6	< 3	2.38	0.55
15		< 3	<8	< 3	< 3	1	0
16		< 3	<6	< 3	< 3	3.65	0.12
17		< 3	<8	< 3	< 3	4.02	0
18		< 3	<6	< 3	< 3	3.99	0
19		<6	6 to 12	<6	<6	4.18	0
20		< 3	<9	< 3	< 3	4.10	0

Temperature 2 – 8°C for 24 hours * stored at – 20°C for 48 hours $ Boiled for 10 mins

Table 3. Comparison of mean microbial count and endotoxin levels of raw camel milk.

Sample Type	Total aerobic flora (Log CFU/mL)	Total coliforms (Log coliforms CFU/mL)	Endotoxin (EU/ml)
Category I	4.61	2.52	413
Category II	5.98	3.23	863
Category III	4.76	2.89	368
Category IV	3.33	0.09	3.8

samples (ID 1,5,6,9) showed a higher value than the reference limit.

The results for total count in this study were in agreement with other report from Saudi Arabia (5.0 log CFU/ml), Ethiopia (5.6 log CFU/ml) and UAE (5.22 log CFU/ml) for tests on camel milk samples (El-Ziney and Alturki, 2007; Semereab and Molla, 2001; Omer and Eltinay, 2008). The variations of total count might be due to the differences in initial microbial contamination originating from the udder surface, quality of water used for cleaning and disinfection of milking utensils and the time lapse from production to marketing. Category IV samples of milk collected directly from the udder were found to have relatively better bacteriological quality than other samples of category I, II and III. This reaffirms the microbial contamination originating from external sources.

Next moving to coliform count, the mean coliform count obtained in the present study was higher than the EU reference value 2 log coliform CFU/ml in category I, II and III. However, this is very similar to the report 2.83 log coliform CFU/ml for camel milk samples collected in UAE (Younan, 2004). In contrast, reports from Ethiopia (Abera *et al,* 2016), Morocco (Benkerroum *et al,* 2003), Algeria (Benyagoub *et al,* 2013) reported 4.03, 6.85, 6.75 log coliform CFU/ml, respectively. A mean coliform count of category IV milk was 0.09 log coliform CFU/ml, which indicates a relative increase in coliform count from udder to milking vessels to

market. This might be due to milk contamination at various levels while milk was passing through different stages of production. A high coliform count may be the reason of improper udder cleaning, preparation in pre-milking, poor hand washing practice of milker and poor quality of milking containers. The presence of high number of coliforms and other Gram-negative organisms in the milk is directly proportional to high endotoxin levels. This could evident why low levels of endotoxin were found in category IV samples.

Moving to the concern about endotoxin exposure, there are very limited studies explaining endotoxin exposure and association with asthma. Presence of elevated levels of endotoxin in farm milk may explain the asthma and atopy protective effect of farm milk noted in study reported by various researchers (Riedler *et al,* 2000 and 2001; Loss *et al* 2011; Illi *et al,* 2012). Apart from the consumption of endotoxin contaminated milk, there are various studies reported that exposure to airborne endotoxin such as home dust, workplace settings (mainly lab animal handling), waste management, and fibreglass manufacturing considered as a major risk factor for asthma, chronic rhinitis and wheezing (Gioffrè *et al,* 2012; Basinas *et al,* 2013; Salonen *et al,* 2013; Barraza *et al,* 2016). It is not clear, whether the ingestion of endotoxin has an effect on the development of asthma and allergies.

Conclusion

Endotoxin levels in camel milk were elevated in refrigerated samples compared to raw milk. Milk sample aseptically collected directly from udder was free of coliforms and had the lowest endotoxin load. However, both the concentration of endotoxin could be influenced by storage time, cleanliness of milking vessels and storage temperature. Lastly, the data indicates the consumption of raw milk might have all the risks and health hazards associated with the unpasteurised, unprocessed state. In addition, milkers keeping dairy camels have to be more aware of the importance of good hygienic conditions for the quality of milk. Finally, the base data of endotoxin in camel milk would support further research on the endotoxins role in asthma and allergy prevention.

Acknowledgement

This research work was funded by the Basic Science Research Unit, Deanship of Scientific Research at Majmaah University, Kingdom of Saudi Arabia (Project number 38/72).

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