# FERMENTED HORSE MILK EXHIBITS ANTIOXIDANT ACTIVITY *IN VITRO*

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Abstract: Oxidative stress has been implicated in the pathogenesis of chronic and degenerative diseases such as coronary heart disease, diabetes and Alzheimer's disease. There is increasing research interest on the therapeutic potential of milk and milk-derived products. This study investigates antioxidant activity of horse milk (fermented, pasteurized and raw) fractions by 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) assays. The fermented milk had the highest radical scavenging activity and ferric reducing power of 63.04±6.85% and 0.822±0.10 respectively, followed by the pasteurized milk (61.35±0.15% and 0.631±0.27), while raw milk (55.28±0.20% and 0.461±0.02) shows least antioxidant activity. The total reducing power and radical scavenging activity was found to be concentration dependent with the least antioxidant activity observed at the lowest concentration of all the three milk samples. The study further demonstrated a time-dependent decrease in both DPPH radical scavenging and ferric ion reducing power of the milk fractions, when stored at 4ºC up to three weeks. These results indicate that fermented horse milk may serves as functional food and used as a supplement in the management of oxidative stress-related diseases.

**Keywords:** Horse milk, fermentation, pasteurization, bioactive peptides, antioxidant, functional food

## Introduction

Milk is a whitish liquid produced by mammary tissue of lactating female animals, and serves as complete food to infants, as it contains all the necessary nutrients required for growth and development. Milk and milk-derived products also serve as important source of nutrition to adults, been part of human diets for thousands of years (Hussein *et al*., 2015). Milk used for human consumption can be obtained from a number of domesticated animals including sheep, goat, buffalo and cow (Santos and Lies, 2015). Besides its nutritional values, biologically active compounds in milk such as casein and whey proteins have been found to be increasingly important for physiological and biochemical functions that have vital impact on human's health (Schanbacher *et al*., 1998; Aliyu *et al.*, 2015). The digestion, processing and fermentation of foods can result in the liberation of bioactive peptides from the native protein by digestive proteases *in vivo* or proteolytic enzymes secreted by microorganisms during fermentation (Atanasova and Ivanova, 2010; Shori and Baba, 2015). Bioactive peptides are health promoting fragment(s) of a particular protein that can be obtain from plant or animal source as well as microorganisms. Thus, they have significant physiological effects on human body systems such as cardiovascular, digestive, immune and endocrine systems depending on their composition and amino acid sequence (Atanasova and Ivanova, 2010; Shori and Baba, 2015).

Several studies have shown that the composition of milk changes between breeds and species, due to influence of environment and composition of the feed (Santos and Lies, 2015). Milk obtained from a mare (female horse) has nutritional values like other sources of milk, hence closely related to that of human due to their similarity on the content of crude protein and lactose (Pieszka *et al*., 2016). Recently, more attention is given to horse's (Mare) milk in Western Europe and the United States (USA) as it is used for children and adults that are allergic to cow milk (Drogoul *et al*., 1992; Businco *et al*., 2000; Curadi *et al*., 2000; Wszolek *et al*., 2007; Dankow *et al*., 2012; Pieszka *et al*., 2016). Similarly, horse milk has been considered medicine in Bashkortostan, Kazakhstan, Uzbekistan and Ukraine (Dankow *et al*., 2009). This may be attributed to its high content of vitamins, minerals, better digestibility, and lower content of fat compared to cow milk (Sheng and Fang, 2009). The milk contains many important bioactive compounds for the mammalian's body growth ranging from proteins (casein and whey), fat (fatty acids, triacylglycerol, phospholipids), carnitine, vitamins (A, C, D, E, and K), to carbohydrates (lactose) (Salimei and Fantuz, 2012; Pieszka and Łuszczyński, 2013). Several studies have demonstrated the potential of bioactive peptides from dietary sources to influence human health by managing or lowering the risk of chronic diseases as well as improving immune system (Shori and Baba, 2015).

Dairy products such as milk consist of a variety of antioxidant compounds that help protects human beings from oxidative stress−related diseases. These antioxidant compounds may also prevent lipid peroxidation and also contribute to nutritional quality of the milk (Castillo *et al*., 2013). For instance, Mare's milk is rich in vitamin C and this vitamin has a high nutritional value due to its resistance to oxidation and inflammatory properties. Hence, the high content of vitamin C in mare's milk influences its antioxidant value (Pieszka *et al*., 2016). Therefore, the amount of antioxidants in milk has significant correlation with the nutritional status of the dairy animal (Castillo *et al*., 2013). However, this antioxidant property can be affected by storage condition and duration as well as processing methods. Thus, we hypothesized that storage time and treatment of horse milk by fermentation and pasteurization do not affect its antioxidant property. In view of the above, the present study investigates antioxidant property of mare milk using DPPH (2, 2-diphenyl-2-picrylhydrazyl radical scavenging) and ferric reducing antioxidant power (FRAP) assays.

## Materials and Methods

### *Sample collection*

Mare (Horse) milk was collected from local farmers in Bakori town of Katsina state, Nigeria. The Milk samples were transported in ice pack to the laboratory, Department of Biochemistry, Ahmadu Bello University, Zaria-Kaduna State, Nigeria. The milk sample was divided into three equal portions and stored in refrigerator at 4ºC.

### *Collection of culture microorganisms*

Lyophilized microbial mixture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Yoflex) were obtained from San Yoghurt Nigeria LTD Zaria, Kaduna state. The microbial mixture was transported in Freeze and dried form to the laboratory, Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria for analysis.

***Preparation of milk sample***

The milk samples were mixed and then divided into three portions of 20 ml each. The first twenty (20) ml of milk fraction was pasteurized at 71ºC for 15 seconds. The second portion was fermented by introducing a mix starter culture of *Lactobacillus bulgaricus* and *Streptococcus thermophiles* (0.2 g/L) and allowed to stand for 3 h at steady temperature of 43ºC. The third portion of the milk (20 ml) fraction was left unprocessed (raw). All the milk fractions were stored in the refrigerator at 4ºC prior to analysis.

***Proximate analysis***

Methods described by AOAC (2006) were used to determine the moisture, ash, crude protein, crude fiber, fat and carbohydrate contents of the horse milk.

***Determination of total antioxidant capacity of the milk fractions***

***Preparation of whey fraction***

Whey protein fraction was obtained from pasteurized, raw and fermented milk by centrifugation at 3000 rpm for 20 min at room temperature. The supernatants were recovered as the whey fractions and filtered through a 0.45 µm syringe filter. The resulting solution was diluted by adding different volume of distilled water to prepare 2, 1.6, 1.2 and 0.4 mg/ml of whey fractions. The solutions were stored at 4ºC until use.

***DPPH radical scavenging activity assay***

The free radical scavenging activity of milk samples were measured by DPPH radical scavenging activity as describes previously (Son and Lewis, 2002) with slight modification. DPPH radical solution (0.002% w/v) was prepared using 95% methanol. One hundred microliter (100 µL) of each whey fractions was added to 2 ml of DPPH in methanol solution and incubated for 30 min at room temperature. Thereafter, the absorbance each whey fraction was recorded at 517 nm using spectrophotometer (company name). The percentage inhibition was calculated using the formula below;

$$\% \text{ inhibition} = \left\{\frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control}\right\} \times 100\%$$

***Ferric ion reducing antioxidant power (FRAP) assay***

The reducing power of various whey fractions was determined according to the method described previously (Saeed *et al*., 2012). Exactly 100 µl of different (0.4 – 2 mg/ml) were added to 2 ml of phosphate buffer (0.2M, pH 6.6) and then 2 ml of 10 mg/ml of potassium ferric cyanide was added to the mixture and homogenized using vortex machine. This mixture was incubated at 50ºC for 20 min in a water bath, followed by addition of 2 ml of trichloroacetic acid (100 mg/L). The resulting mixture was centrifuged at 3000 rpm for 10 min and supernatant was then recovered. Thereafter, 2 ml of distilled water was added to the supernatant recovered, then 0.4 ml of 0.1% (w/v) of ferric chloride solution was added. The absorbance of the reaction mixture was measured at 700 nm using spectrophotometer. Increased absorbance indicates increase in reducing power and vice versa.

**Results and Discussion**

Data of the proximate analysis of horse (mare) milk (shown in Table 1) indicated that the moisture content was 81.97±3.85%. The fat, protein and ash contents were found to be 13.70±1.60%, 2.63±0.32% and 1.60±0.15%, respectively. However, no crude fiber was found in the milk sample. This result indicated that the fat content is significantly higher than that reported by Wells *et al*. (2012) while the protein content is similar. Furthermore, the moisture, protein and ash contents are lower, similar and higher respectively than that reported by Holmes *et al*. (1947).

**Table 1: Proximate Composition of Horse (Mare) Raw Milk**

| Components of milk | Quantity (%) |
|---|---|
| Moisture | 81.97±3.85 |
| Fat | 13.70±1.60 |
| Protein | 2.63±0.32 |
| Carbohydrate | 0.10±0.01 |
| Fiber | ND |
| Ash | 1.60±0.15 |

ND = Not detected

The use of dietary approach for prevention and management of diseases is receiving more attention in recent years, due to minimal side effects and wide acceptability (Shori and Baba, 2015). In this present study, we have investigated the effects of fermentation, pasteurization and storage time on *in vitro* antioxidant property of horse milk. From the results obtained, fermented milk had the highest ferric reducing power and radical scavenging activity, followed by the pasteurized milk, while the raw milk sample showed the least antioxidant activity. This may be due to heating and fermentation of milk which may results in the activation and release of bioactive peptides with antioxidant property, which may serve as electron and proton donors. Our results are consistent with previous studies that demonstrate fermentation improves antioxidant activity of milk (Fitzgerald and Murray, 2006; Moslehishad *et al*., 2013a; Soleymanzadeh *et al*., 2016). However, the higher antioxidant activity of pasteurized horse milk sample in comparison to raw sample observed in this study is contrary to the results obtained by Al-yaqoubi *et al*. (2014) who reported that pasteurization treatment significantly decrease the antioxidant activity of goat milk.

The result of DPPH radical scavenging activity of horse milk and ascorbic acid were shown in Figs. 1 – 3. The free radical scavenging activity of milk samples (fresh, pasteurized, and fermented) at different concentrations (2, 1.6, 1.2 and 0.4 mg/ml) were evaluated for the period of three weeks by 2, 2-diphenyl-2-picrylhydrazyl radical scavenging in comparison with ascorbic acid. Whey fraction of fermented milk had highest DPPH radical scavenging activity (63.04±6.85% and 54.44±1.98%), followed by pasteurized milk with 61.34±0.15% and 40.36±0.21% scavenging activity for the first and second weeks of the study, respectively. The whey fraction of raw milk has the lowest DPPH radical scavenging activity (55.28±0.21% and 49.48±0.15%) compared to fermented and pasteurized milk samples. Ascorbic acid which was used as a standard has the highest DPPH radical scavenging activity of 94.5±0.14%, 91.5±0.71% and 89.75±0.50% for the first, second and third week of the study, respectively, when compared to the milk samples (fresh, pasteurized and fermented). All the milk samples and the standard (Ascorbic acid) significantly scavenged the DPPH radical with increasing concentrations.

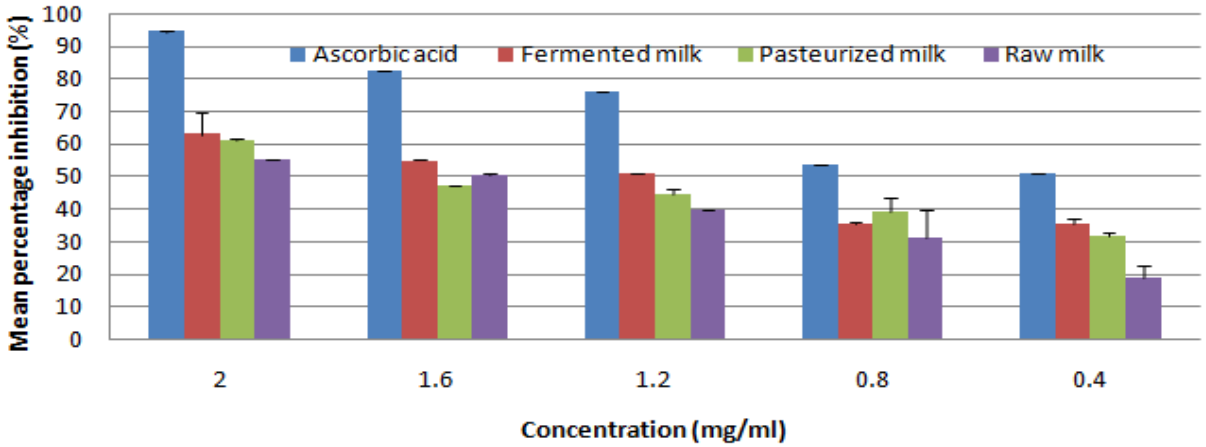


**Fig. 1: DPPH radical scavenging activity of whey fractions of fermented, pasteurized and raw Horse (mare) milk samples in comparison with ascorbic acid at first week.** Values are mean±SD of triplicate determination.

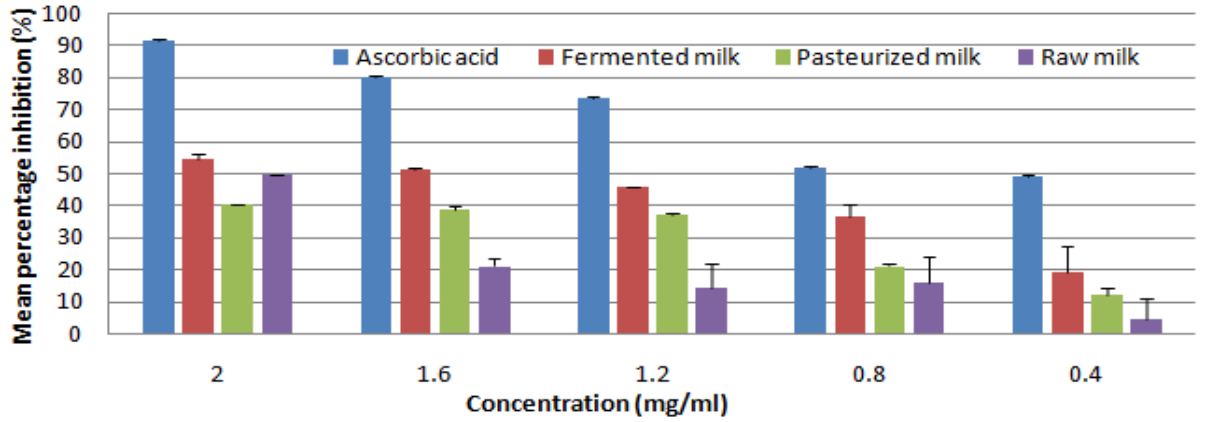


**Fig. 2: DPPH radical scavenging activity of whey fractions of fermented, pasteurized and raw Horse (mare) milk samples in comparison with ascorbic acid at second week.** Values are mean±SD of triplicate determination.

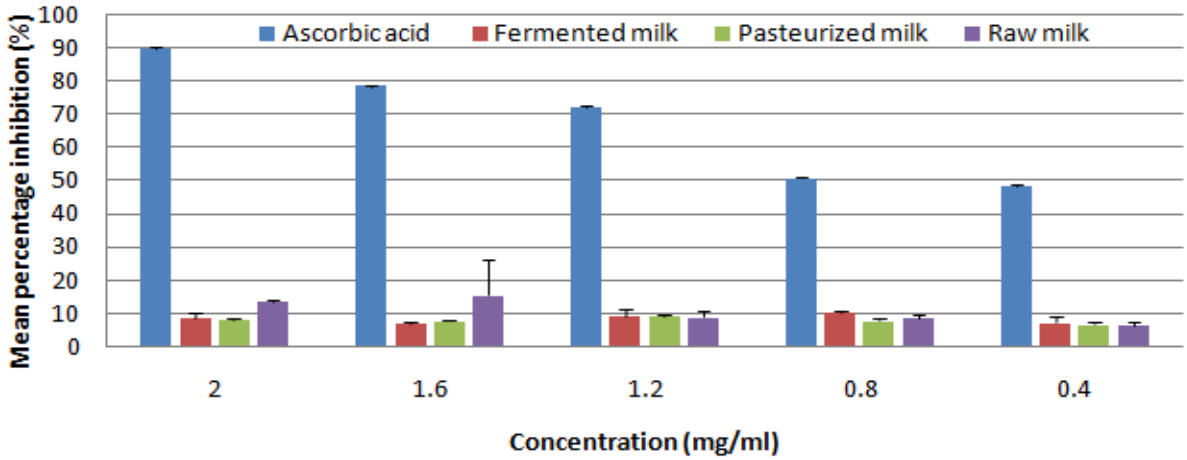


**Fig. 3: DPPH radical scavenging activity of whey fractions of fermented, pasteurized and raw Horse (mare) milk samples in comparison with ascorbic acid at third week.** Values are mean±SD of triplicate determination.

Thus, the present findings indicated that the milk sample fermented with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* has higher antioxidant activity (DPPH radical scavenging activity) than fresh and pasteurised samples. This is in agreement with findings of Namdari and Nejati (2016), which showed that peptides with great hydrophobicity released by fermentation with *L. helveticus* isolates can exhibit stronger DPPH scavenging activity. The higher antioxidant activity of fermented milk samples observed in this study may be due to the release of bioactive peptides and hydrophobic amino acids in the milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Mohanty *et al.*, 2016; Zou *et al.*, 2016). Previous studies reported that fermentation process makes proteins more easily digestible

and accessible by bacteria, thereby releases the peptide fragments that are biologically active (Lv *et al*., 2009; Pieszka *et al*., 2016). This is also in agreement with previous study by Fardet and Rock (2017), who reported that probiotic yoghurt and fermented milk have higher antioxidant activity than conventional yoghurt and milk due to proteolysis by probiotics.

The results of ferric ion reducing capacity of horse milk (fermented, pasteurize and raw) samples in comparison with ascorbic acid are presented in Figs. 4 – 6. Fermentation and pasteurization of horse milk resulted in increased antioxidant capacity when compared to raw milk with highest antioxidant activity observed in fermented milk sample. Whey fraction of fermented milk had highest ferric ion reducing antioxidant activity (0.822±0.10, 0.637±0.01 and 0.486±0.04) for the first, second and third week, respectively, followed by pasteurized milk sample (0.631±0.27, 0.500±0.02 and 0.442±0.10) for the first, second and third week of the study, respectively. The whey fraction of the raw milk sample has the lowest reducing power of 0.461±0.02, 0.458±0.03 and 0.413±0.31 for the first, second and third week of the study, respectively.

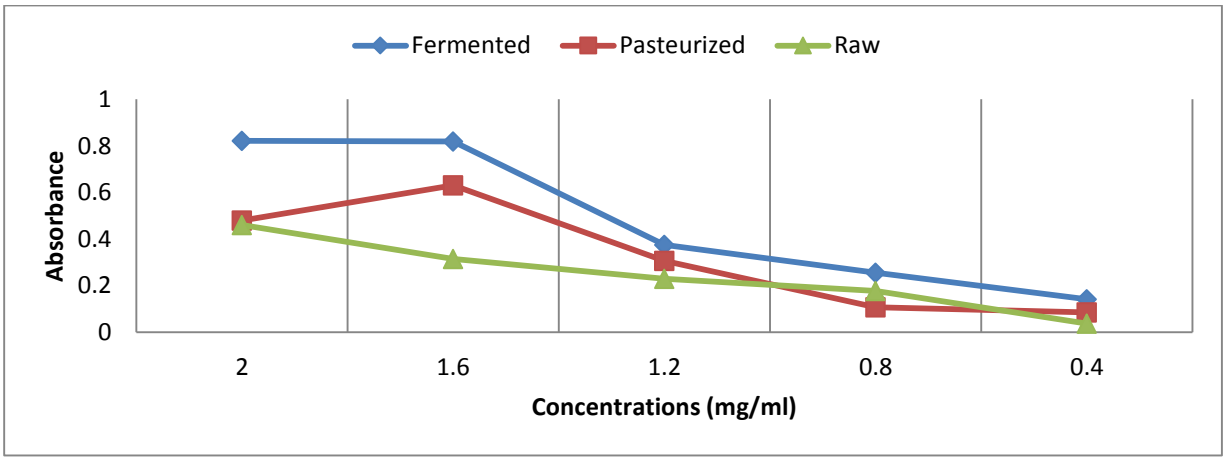


**Fig. 4: Ferric ion reducing capacity of fermented, pasteurized and raw horse milk at first week. Values are mean±SD of triplicate determination.** Values are mean±SD of triplicate determination.

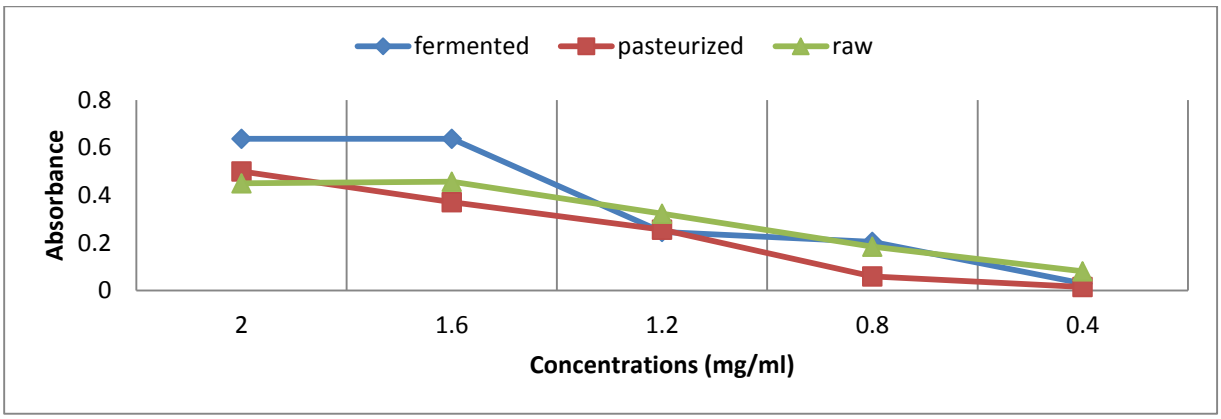


**Fig. 5: Ferric ion reducing capacity of fermented, pasteurized and raw horse milk at second week.** Values are mean±SD of triplicate determination.

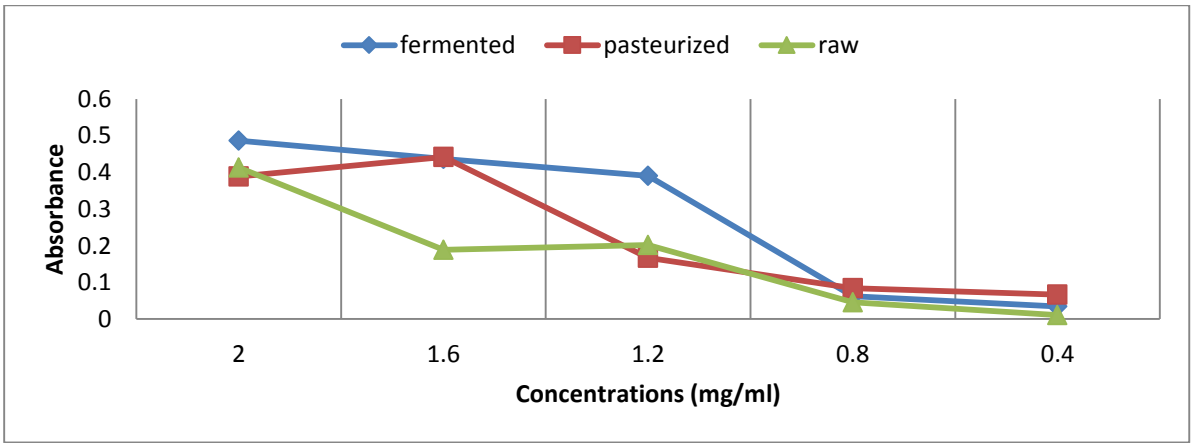


**Fig. 6: Ferric ion reducing capacity of fermented, pasteurized and raw horse milk at third week.** Values are mean±SD of triplicate determination.

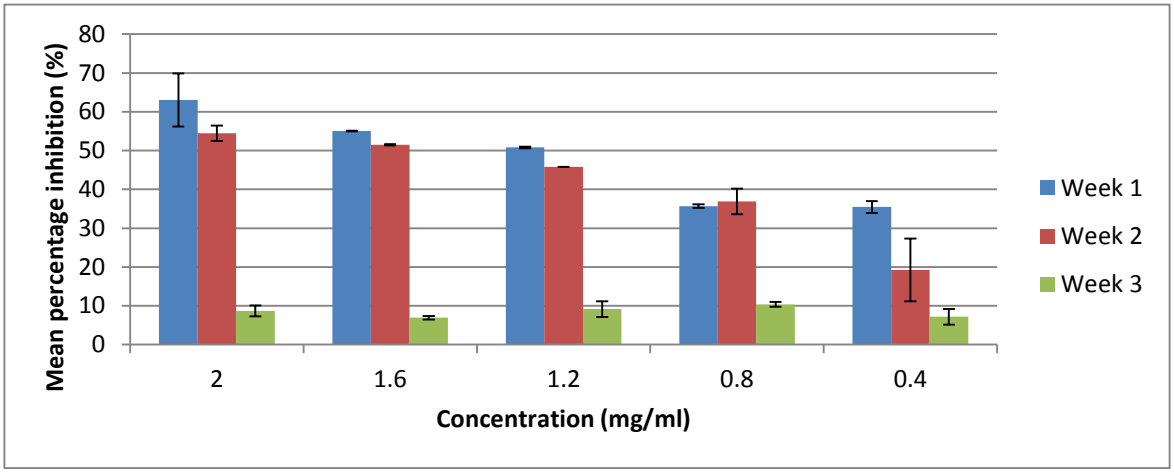


**Fig. 7: Time-dependent DPPH radical scavenging activity of Fermented milk at different weeks.** Values are mean±SD of triplicate determination.

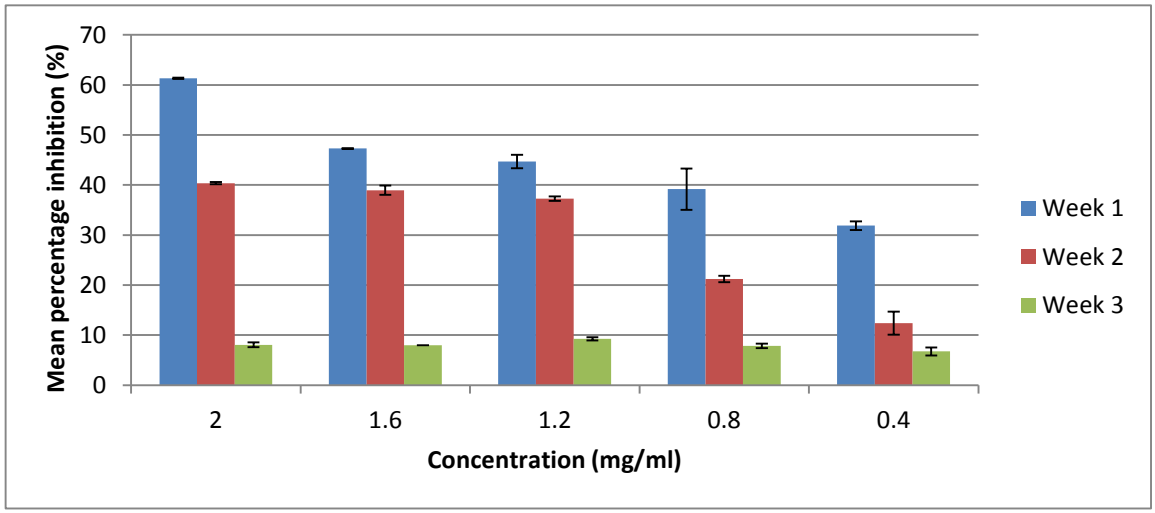


**Fig. 8: Time-dependent DPPH radical scavenging activity of Pasteurized milk at different weeks.** Values are mean±SD of triplicate determination.

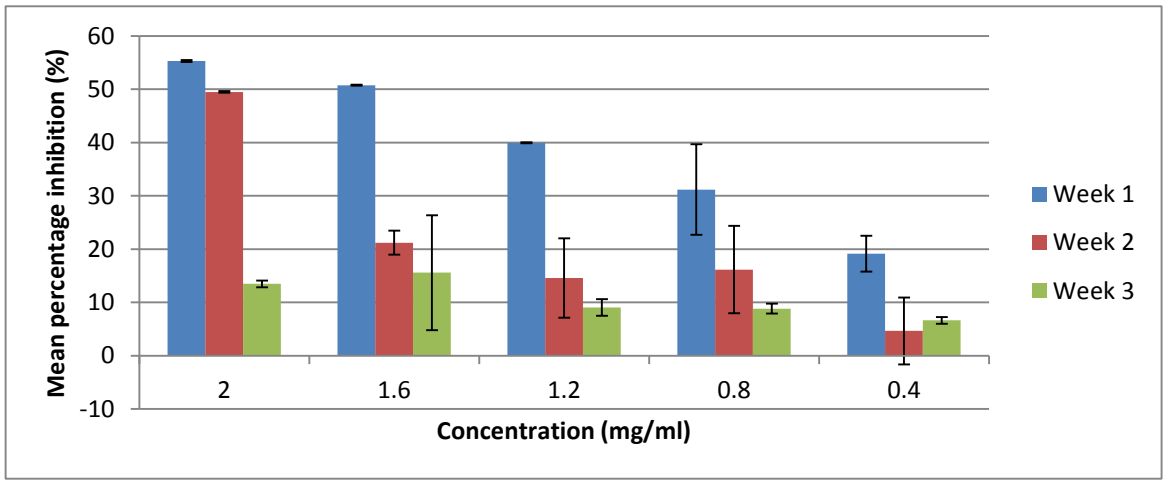


**Fig. 9: Time-dependent DPPH radical scavenging activity of Raw milk at different weeks.** Values are mean±SD of triplicate determination.

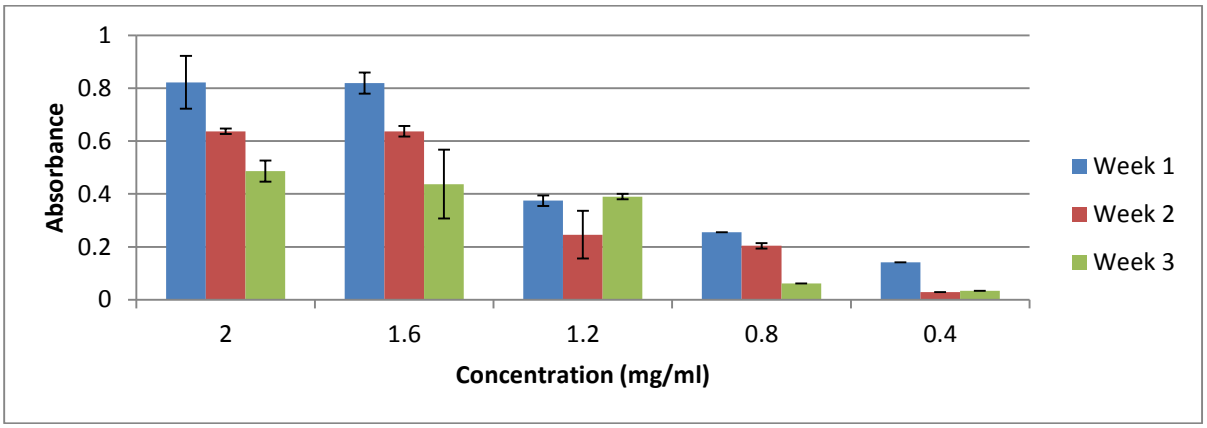


**Fig. 10: Time-dependent Ferric ion reducing power of Fermented milk at different weeks.** Values are mean±SD of triplicate determination.

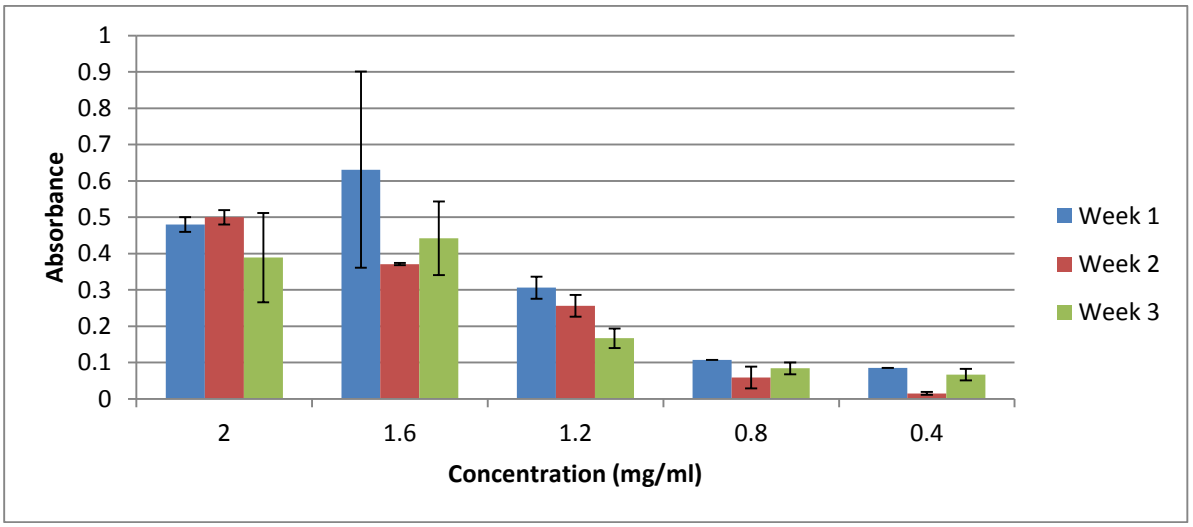


**Fig. 11: Time-dependent Ferric ion reducing power of Pasteurized milk at different weeks.** Values are mean±SD of triplicate determination.

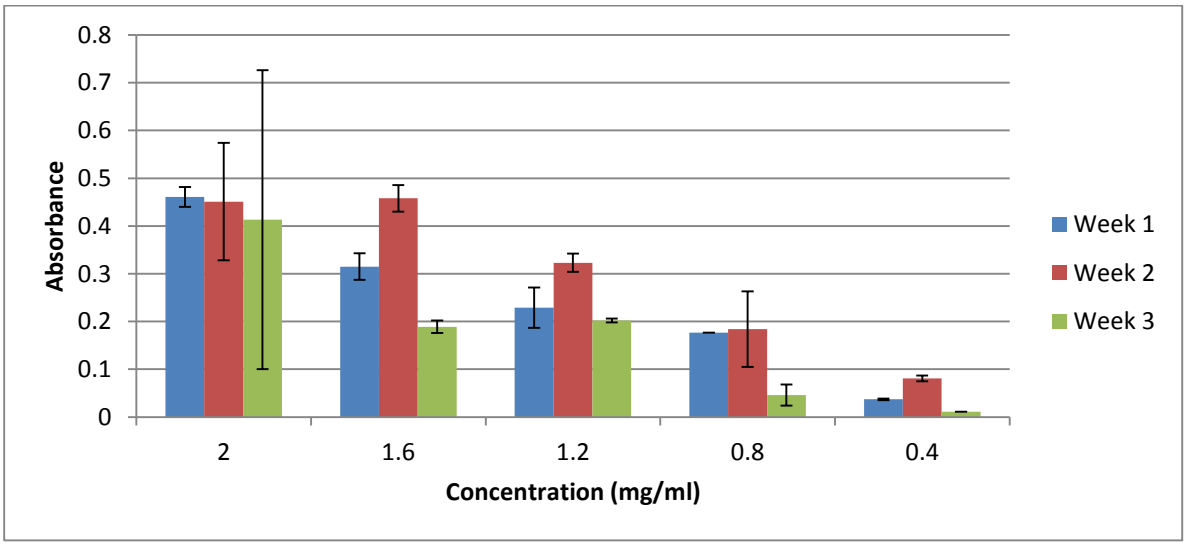


**Fig. 12: Time-dependent Ferric ion reducing power of Raw milk at different weeks.** Values are mean±SD of triplicate determination.

The present study also showed a time-dependent decrease in both DPPH radical scavenging (Figs. 7 – 9) and ferric reducing power (Figs. 10 – 12) of the milk samples, when stored at 4ºC up to three weeks, with highest antioxidant activity observed at the first week. This may be attributed to decrease or loss of constituents of the milk and inactivation of the bioactive peptides. The reduction in the antioxidant activity of horse milk with increase in the duration of storage observed in our study, is in agreement with previous study by Hanna *et al*. (2004) who reported that antioxidant activity of human milk decreases with storage time (Hanna *et al*., 2004). The result in this study is also consistent with previous study

by Citta *et al*. (2017), who reported various types of yogurts demonstrated higher basal antioxidant activity but gradually decreased with increase in the duration of storage.

**Conclusion**

Altogether, the results of this present study demonstrated that fermented and pasteurized horse milk exhibit antioxidant activity proportional to concentration and inversely related to duration of storage. This indicates that antioxidant activity of fermented and pasteurized horse milk is concentration and time dependent and thus, can be a potential functional food in prevention or amelioration of oxidative stress-related diseases. However, to preserve the antioxidant activity, storage time should not exceed one week.

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**Conflict of Interest**

The authors declare that there is no conflict of interest reported in this work.

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